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Post-translational modifications of HOX proteins, an underestimated issue

AMANDINE DRAIME[1,#], LAURE BRIDOUX[1,#], YACINE GRABA*[,2] and RENÉ REZSOHAZY*[,1]

[1]Animal Molecular and Cellular Biology, Louvain Institute of Biomolecular Science and Technology (LIBST), Université Catholique de Louvain, Louvain-la-Neuve, Belgium and [2]Aix Marseille Université, CNRS, IBDM, Marseille, France

ABSTRACT Post-translational modifications (PTMs) are important determinants which contribute to modulating the turn-over, intracellular localisation, molecular interactions and activity of most eukaryotic proteins. Such modifications and their consequences have been extensively examined for some proteins or classes of proteins. This is not the case for the HOX transcription factors which are crucial regulators of animal development. In this review, we provide a survey of the literature and data repositories pertaining to HOX-associated PTMs. This highlights that HOX proteins are also likely widely post-translationally modified, and defines HOX PTMs as an under-valued facet of their biology.

KEY WORDS: *HOX protein, post-translational modification, protein-protein interactions*

Introduction

Post-translational modifications (PTMs) of proteins are plethoric and modify the activity, localisation, stability, molecular interactions of virtually all proteins in eukaryotic systems. This includes transcription factors (Niklas *et al.*, 2015). Consequently, among the best studied transcription factors, we find proteins for which PTMs have been deeply investigated and revealed to be crucial for their specific and contextual activities. The tumor suppressor p53, for example, is a protein that plays multiple roles in the cell, from cell-cycle control, to the DNA damage response, apoptosis, autophagy, metabolism (Green and Kroemer, 2009, Maiuri *et al.*, 2010) etc. p53 is primarily known as being a transcription factor (Riley *et al.*, 2008). Nonetheless, like many transcription factors, p53 also fulfills non-transcriptional functions, like by directly interacting with apoptosis regulators at the mitochondrion or by modulating cell-signaling (Speidel, 2010). More than 60 amino acid residues over 393 (thus more than 15%) have been identified to be the target of post-translational adducts (Gu and Zhu, 2012, Nguyen *et al.*, 2014), including mono- and di-methylation, phosphorylation, mono- and poly-ubiquitination, acetylation, SUMOylation, Neddylation, O-GlcNAcylation or poly(ADP-ribosyl)ation. These PTMs contribute to modulate p53 stability, intracellular distribution, DNA binding ability, transcriptional activity or protein-protein interactions. Importantly, although individual PTMs have been associated to such changes in p53 abundance or activity, it now appears that these PTMs define a network of modifications which act in combination to influence in a context-specific manner the behavior of p53 (Gu and Zhu, 2012, Uversky, 2016). NFκB defines a family of transcription factors acting as heterodimers (Oeckinghaus *et al.*, 2011, Perkins, 2007), also controlled through PTMs. The so-called "canonical" NFκB dimer is composed of the p65(RelA)/p50 subunits. p65 PTMs have in particular been well-documented. Phosphorylations, ubiquitination, nitrosylation, mono- and di-methylation of p65 have been reported, contributing, again in modulating the localisation, stability or degradation, molecular interactions or activity of NFκB (Lu and Stark, 2015, Won *et al.*, 2016). There are numerous other examples of transcription factors which have been well described to be abundantly modified (pRb, E2F-1, ERα, etc. see reviews: (Anbalagan *et al.*, 2012, Biswas and Johnson, 2012, Kolupaeva and Janssens, 2013, Munro *et al.*, 2012, Poppy Roworth *et al.*, 2015, Sengupta and Henry, 2015)).

HOX proteins are also well-studied transcription factors, however their PTMs have not been intensely investigated. This is intriguing considering that the functional specificity of HOX proteins remains a major issue for HOX biologists. HOX proteins have initially been identified for their remarkable functions during animal develop-

Abbreviations used in this paper: HD, homeodomain; PTM, post-translational modification.

***Address correspondence to:** Yacine Graba. IBDM, Campus de Luminy Case 907, 13288 Marseille Cedex 9 France. Tel: +33 (0)4 91 26 96 13. E-mail: yacine.graba@univ-amu.fr - Web: http://www.ibdm.univ-mrs.fr - https://orcid.org/0000-0003-1324-1308 or René Rezsohazy. Animal Molecular and Cellular Biology, Louvain Institute of Biomolecular Science and Technology, 5 (L7.07.10) place Croix du Sud, 1348 Louvain-la-Neuve, Belgium. Tel: +32(0)10 47 37 01. E-mail: rene.rezsohazy@uclouvain.be - Web: https://uclouvain.be/en/research-institutes/libst/rene-rezsohazy.html - https://orcid.org/0000-0003-4253-7431 - **#Note:** AD and LB contributed equally to this work.

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ment, in particular in patterning the main body axis of bilaterian embryos (Alexander *et al.*, 2009, Duboule, 2007, Wellik, 2009). *Hox* gene mutation typically leads to homeotic transformations in the embryo, i.e. specific segments of the developing body adopt, in part or totally, the fate of other ones. This eventually provokes appendages mislocation in *Drosophila* (two pairs of wings instead of one pair of wings and one pair of halters), misshaping of vertebrae in the mouse (several atlas-like vertebrae in the neck or additional ribs in the thoracic cage), altered identity of cranio-facial bones and cartilages (duplicated and missing middle ear ossicles), etc. Later, it appeared that HOX proteins are involved in lot more processes than initially thought, being active until adulthood in controlling cell differentiation, cell stemness or proliferation for example (Rezsohazy *et al.*, 2015). In addition HOX proteins have also been associated to non-transcriptional activities as it is now well admitted for numerous other recognised transcription factors as well (Biswas and Johnson, 2012, Rezsohazy, 2014, Speidel, 2010).

Structurally, all HOX proteins share a very well-conserved DNA binding domain, the homeodomain. Strikingly, the amino-acid residues establishing the interface between HOX proteins and DNA bases are extremely well-conserved. Nonetheless, HOX proteins fulfill very specific functions. The conservation of the homeodomain sequence and functional specificity of the HOX proteins raised what has been called the "HOX paradox" (Bobola and Merabet, 2017, Merabet and Mann, 2016, Rezsohazy *et al.*, 2015). The resolution of this paradox has been and remains quested. The main lead to solve the HOX paradox was to study specific protein-protein interactions, mainly with the PBC class cofactors PBX and Exd, another class of homeodomain containing protein. These interactions have been shown to unmask latent DNA binding specificity of HOX proteins (Slattery *et al.*, 2011), increase HOX DNA binding specificity (Crocker *et al.*, 2015, Merabet and Mann, 2016), or modify the activity of HOX proteins, switching them from repressing to activating transcription (Li *et al.*, 1999). While initially thought to rely on a single and unique interaction mode implying the conserved HOX hexapeptide motif, HOX-PBC interaction was more recently shown to rely on additional protein regions, that bear characteristics of Short Linear Motives (SLIMs) located in intrinsically disordered protein regions (Baeza *et al.*, 2015, Dard *et al.*, 2018, Hsiao *et al.*, 2014, Niklas *et al.*, 2015, Tompa *et al.*, 2014)(see also Rinaldi *et al.*, this issue). In that respect, it is worth noting that the non- or less-conserved regions of HOX proteins have been largely predicted to be disordered (Hsiao *et al.*, 2014, Liu *et al.*, 2008), providing the frame for multiple and diverse context-specific and versatile interactions to HOX proteins (Baeza *et al.*, 2015).

PTMs have been shown to provide regulatory action on protein domain folding, localisation, stability and protein-protein interactions, suggesting they likely may also, through such processes, control HOX protein activity and functional specificity in a context specific manner. PTMs could thus define an additional lead to resolve the HOX paradox (Niklas *et al.*, 2015). In addition, unravelling which PTMs take place on HOX proteins and identifying the enzymes involved will uncover regulatory networks within which to anchor HOX proteins to other cellular processes. In this review, we provide a survey of PTMs potentially associated to HOX proteins, relying on available databases and large scale interactomic screens, before extracting from the literature examples that support roles for PTMs in controlling several aspects of HOX protein biology (Figs. 1,2,3).

Survey of PTM databases and systematic interactomic screenings: probably just the tip of the iceberg

To appreciate to what extent HOX proteins are post-translationally modified, we started by exploring databases devoted to list PTMs detected from high-throughput analyses. This reveals that HOX modifications have been detected in several studies. Most studies refer to human HOX proteins, but strikingly examples where the mouse HOX proteins (and rat orthologues) have been identified to carry equivalent modifications as the human homologue are numerous (Table 1). This underlines that PTMs are, at least in some cases, evolutionary conserved, suggesting that they may be functionally important. This mainly applies to phosphorylation which has been the foremost detected modification,

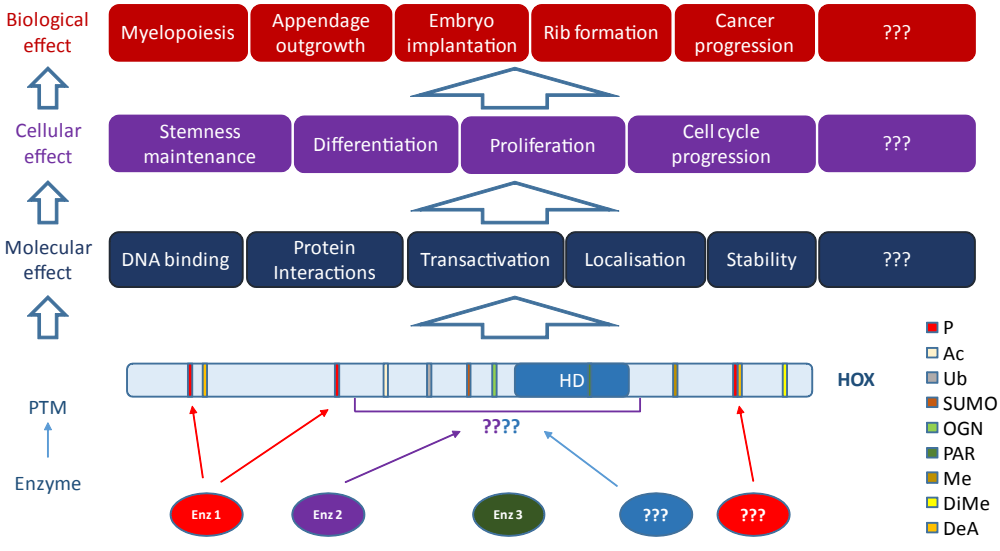

Fig. 1. Distribution and effects of HOX-associated post-translational modifications (PTMs). *Distribution and effects of HOX-associated PTMs. Distinct adducts have been identified to modify HOX proteins all along their sequence, including within their homeodomain (HD). HOX PTMs have been identified to modify their molecular properties and in some instances their influence on cell physiology. The* in vivo *biological response to HOX PTMs has been characterised in a very few instances and awaits in depth characterisation to make the link between HOX structural biology, HOX mode of action and HOX functional specificity. Abbreviations: Ac, acetylation; DeA, deamidation; DiMe, demethylation; Enz, enzyme; Me, methylation; OGN, O-GlcNAcylation; P, phosphorylation; PAR, poly(ADP-ribosyl)ation; SUMO, SUMOylation; Ub, ubiquitination.*

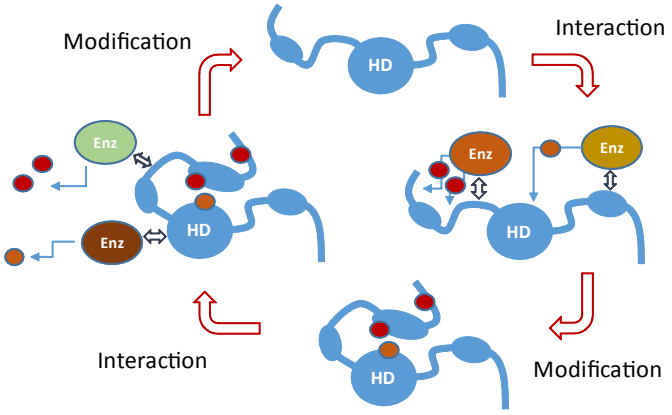

Fig. 2. Interplay of protein interaction and modification. *HOX post-translational modification (PTM) is a reversible process involving interactors with enzymatic activities and addition or removal of molecular adducts. As depicted in Tables 1 and 2, in numerous instances HOX interactors with enzymatic activity have been identified, but neither the target residues nor the molecular consequences of these interactions have been identified. Conversely, an important number of PTMs have been identified on HOX proteins, but neither the enzyme involved nor the molecular consequences of these PTMs have been identified. Small red and orange circles represent PTMs added onto HOX proteins.*

but a few examples for other modifications also exist. Obviously the functional consequences of these PTMs have not been addressed, but it is expected that they could influence protein lifetime, localisation or activity (Figs. 1,3). While most PTMs actually take place in protein regions predicted to be disordered and/or to define SLiMs (enter a HOX protein name to search on http://elm.eu.org/; or https://www.phosphosite.org/), it is noteworthy that multiple modifications have been detected in the DNA binding homeodomain. PTMs in the homeodomain have been detected in 20 human or mouse HOX proteins to date (Table1). It can be hypothesised, as it has been addressed in a handful of cases (see below), that adding phosphate, methyl groups or even ubiquitin to the homeodomain (like in HOXA10 or HOXA13, for example) will modify the behavior of the protein. In particular, in case the ubiquitination of a homeodomain would not change the localisation of the HOX protein (nuclear exit or even degradation), it can be postulated that grafting a 76 amino acid adduct to a HOX homeodomain will modify its DNA binding (the involvement of monoubiquitin in DNA binding has been evidenced for p53 for example (Landre *et al.*, 2017)). This clearly needs to be appraised while considering the HOX paradox and the rules governing HOX protein specificity, i.e. DNA binding site selection and functional outcome. Most of the PTMs quoted in databases have been detected in the context of non-directed, large-scale proteomic analyses.

A more indirect way to approach the extent of HOX-associated PTMs is to infer possible PTMs from HOX protein-protein interactions involving enzymes mediating PTMs by promoting the covalent addition of functional groups. Only two systematic interactomic screens have been reported so far for HOX proteins, namely for HOXA1 and Ultrabithorax (Ubx) (Bondos *et al.*, 2006, Lambert *et al.*, 2012). Additional HOX-mediated interactions have been detected in distinct large screens identifying enzymes which might be candidates for mediating PTMs onto HOX. These datasets define kinases, phosphatases, ubiquitin ligases, methyl-, acyl-, palmitoyl-, glycosyl-transferases as candidate interactors of HOX proteins (Table 2), and suggest that the related PTMs may target HOX proteins (Fig. 2). Before reaching such a conclusion, these interactions first need to be validated by orthogonal assays, and the issue of whether the PTM mediated by these enzymes occurs on HOX proteins need to be investigated. The interaction might alternatively modulate the activity of the enzyme (Bergiers *et al.*, 2013, Bridoux *et al.*, 2015b) or bring it to a third partner defining the genuine target for PTM, as is the case for histone-modifying enzymes recruited by transcription factors to ultimately modify histones. Whether the interaction between a HOX protein and an enzyme leads to HOX PTMs will straightforwardly be determined by proteomic approaches. Functional assays, based on mutational approaches, also need to be developed to establish the existence of the inferred PTM as well as its biological outcome. These mutation-based approaches are quite challenging for several reasons. First, interaction interfaces are most probably used for multiple protein-protein interactions. As an example, it appears

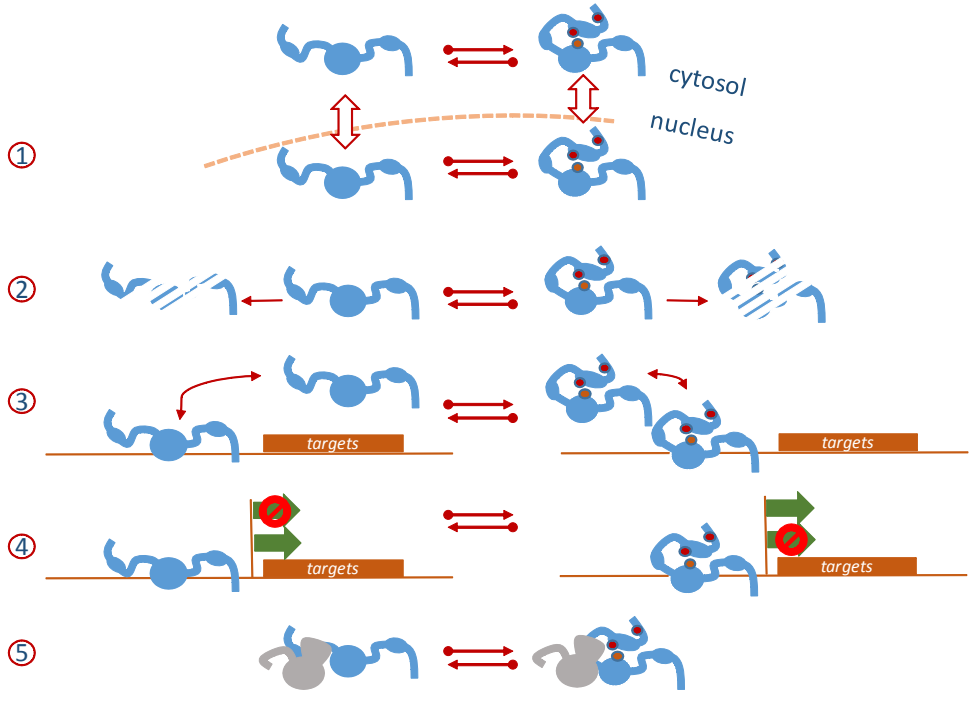

Fig. 3. Molecular and cellular impact of HOX-associated post-translational modifications (PTMs). *The literature supports that HOX PTMs can modify their* **(1)** *intracellular distribution,* **(2)** *stability,* **(3)** *interaction with DNA sites,* **(4)** *ability to activate or repress transcription, as well as* **(5)** *interaction with other proteins. Small red and orange circles represent PTMs added onto HOX proteins.*

TABLE 1

POST-TRANSLATIONAL MODIFICATIONS OF HUMAN, MOUSE AND RAT HOX PROTEINS

HOX Protein	Phosphorylation	Acetylation	Ubiquitination	SUMOylation	Methylation	OGN
HOXA1	Y28; S30; *T154 or S157*; T255; S296; T299; *T308*; S334					T154
HOXA2	S139; S143	K130; K197; K205				
HOXA3	S143; S148*; *S149**; S184; S263					
HOXA4	S109; *S169*, Y210; Y239; *S266*; S286; S288; S290					
HOXA5	Y45; Y49; S55; S59; S121					
HOXA6						
HOXA7	T195; S206; T210		K103; K147			
HOXA9	S120; S126; *S160*; *S183**; S184*; *S204**; S205*; *T205*; *Y212* and/or Y213; *Y229* and/or Y230		K209; K215		R140(Me2)	
HOXA10	S19; S93; S272; T277; S335; Y343; T348; Y360	K338; K339	K339; K345	K164	R193; R378	
HOXA11	S84; S98; T119	*K297*	K72			
HOXA13	Y171; Y210; S356; T358; S362		K303; K335			
HOXB1		K277				
HOXB2	T52; S55; S119; S124; T148; T151; T153; S274					
HOXB3	T82; S103; S104; *T149*; S185					
HOXB4	S46; *S89**; S90*; S130; Y142					
HOXB5	S55; S186*; *S186**; S257; S259		K211			
HOXB6	S136*; S136*; S140; S209*; S209*; S211; S214*; *S214**; S214*					
HOXB7	Y6; *S116*; S132; S133; T203; T204		K191; K193			
HOXB8	S125	K243				
HOXB9	S30; Y33; S35; S36; T133; S137	K27; K117; K159; K167; K202; K239; K241; K242	K130	K117	R93; R96; R183	
HOXB13	T8; S202; S203; S250; S254	K270; K277				
HOXC4	S68; *T70*; T239; T242; T251				R79	
HOXC5						
HOXC6	S131; Y137; *S200*; *T203*	K198; K221	K198			
HOXC8	Y23					
HOXC9	S145; S159*; *S159**; S180	K119				
HOXC10	T8; S60; S63; S78; S115; S122; S152; S154; S156; T161; S189; S197; T201; S204*; S204*; S206*; S206*; T208*; T208*; S210; S219; T216; S226*; S226*; S228*; S228*; S230; S337; T342		K55; K271; K277	K106; K195; K215; K254		
HOXC11	S12; S60; S88; T206			K82; K116; K178		
HOXC12	*S41*; S71; S192; T240				R109	
HOXC13	S234; S294; T296; S300					
HOXD1	Y223; Y229; S230					
HOXD3	T37; T38; S188; S194; S261*; S261*; S266					
HOXD4	S123; T125; Y134; Y178; S233					
HOXD8	Y10; S11; Y13					
HOXD9	S146; S150; S194; S199; S202; S203					
HOXD10	S87; T95; *S125*; *S130*; *S191*; *S206*; *T211*; S218; S238*; *S238**; S239*; *S239**	K59	K269; K275			
HOXD11	*Y62*; S203; S223; T229; S241		K256		R76	
HOXD12	Y101; Y137; Y139				R114(Me2)	
HOXD13	Y127; T313; S316		K162; K289			

PTMs as recorded in databases and reported in the literature. Amino acids (single letter code) correspond to the human reference proteins, mouse reference proteins (italics) or rat reference proteins (underlined). An asterisk indicates that a modified human protein residue shows the corresponding modification in the mouse or rat protein as well. Homeodomain residues are highlighted in red. OGN, O-GlcNAcylation; Me2, demethylation. Sources: https://www.phosphosite.org; http://cplm.biocuckoo.org; https://proteomescout.wustl.edu; http://www.uniprot.org; references cited in the text.

clear now that the short hexapeptide involved in the HOX-PBX interaction has a wider functional importance than initially considered (Baeza *et al.*, 2015). Second, effects of PTMs may vary according to combinations of PTMs, which is at present hardly predictable as very little is known about how such combinations assemble into a functional code (Benayoun and Veitia, 2009, Van Roey and Davey, 2015). Third, simply mutating the target residue of a modification might have functional consequences distinct from that caused by the modification. Indeed, mutating a target residue like changing a serine or a threonine into an alanine implies changing the identity of the amino acid in addition to losing its possible modification. In sum, while the interactomic data appears as a rich resource to identify potential PTMs associated to HOX proteins, a substantial amount of investigation is still required.

Overall, the PTMs data sets and interactomic data collectively suggest that our current appreciation of HOX associated PTMs define the tip of the iceberg of the HOX PTMs wealth. Once the existence and cellular outcome of these modifications are established, a main challenge will be to appraise their role in biologically relevant situations, i.e. ultimately *in vivo* (Figs. 1,2,3).

HOX PTMs and their consequences, inventory from the literature

As discussed in the introduction, PTMs have been shown in many instances to control several aspects of proteins “cellular journey” and molecular properties. In what follows we inventory the current grasp of how PTMs affect HOX proteins.

Post-translational modifications of HOX proteins: modulation of protein stability

The HOXA9 protein interacts with the cullin protein CUL-4A, a scaffold protein of E3 ubiquitin ligase complexes (Zhang *et al.*, 2003). This interaction results in the ubiquitination and subsequent drastic proteosomal degradation of HOXA9. HOXA9 has been shown to have a half-life of 26 hours in HeLa cells, which was reduced to 3 hours in the presence of CUL-4A. CUL-1, another member of the cullin family, has no impact on the half-life of HOXA9, suggesting that the effect is specific to CUL-4A. The so-called degradation motif or degron, i.e. the sequence required for targeting the protein to degradation, has been mapped in the first helix of the homeodomain (Zhang *et al.*, 2003). In another study, the same group similarly reported that CUL-4A can interact with HOXB4 in yeast two-hybrid assays, and that this interaction provokes HOXB4 proteosomal degradation via ubiquitination (Lee *et al.*, 2013). A short tetrapeptide motif, LExE, at the first helix of the homeodomain seems required for the CUL-4A-induced HOXB4 degradation. Substituting the first three residues of this motif extended the HOXB4 half-life compared to the wildtype in the presence of CUL-4A. However it is not clear if this motif is involved in the CUL-4A complex interaction or is required for the addition of ubiquitin by the E3 ligase activity. Answering this question will be interesting since this LExE motif is conserved in several other HOX homeodomains. In addition, the half-life of HOXA1, HOXA2, HOXB7, HOXB8, HOXA11 and HOXA13 has also been shown to be altered by an increasing concentration of CUL-4A. For these proteins, however, the hypothetical ubiquination by CUL-4A was not characterised further (Lee *et al.*, 2013). Further investigation of the role of CUL-4A on all HOX proteins would be interesting in order to determine if it is a common way to regulate their abundance or duration of activity, or alternatively, if different HOX proteins may display distinct sensitivity to CUL-4A-mediated control.

The abundance of human HOXC10 (hHOXC10) oscillates during the cell cycle: it is low during G1 but increases from mid-G1 to G2 phases and is undetectable during mitosis (Gabellini *et al.*, 2003). In contrast, the level of *HOXC10* transcripts remains constant throughout the cell cycle, suggesting that hHOXC10 is affected by mitotic proteolysis. In fact, hHOXC10 has been shown to be poly-ubiquitinated and next degraded by the 26S proteasome. A well-known E3 ubiquitin ligase acting during mitotic proteolysis is the anaphase-promoting-complex (APC). A common characteristic of all APC target proteins known to date is the presence of a KEN box and/or D-box as degron. hHOXC10 contains two D-boxes (aa 177-185 and aa 320-328) which are involved in the mitotic proteolysis. Substitution of D-box residues (R177, L180, R320 and L323) by alanines leads to a decrease in hHOXC10-ubiquitin conjugates and stabilisation of hHOXC10. Moreover, hHOXC10 co-immunoprecipitates with CDC27, a core subunit of APC, and less hHOXC10-ubiquitin conjugates are observed in the presence of a dominant negative mutant of UBCH10, an E2 ubiquitin-conjugating enzyme working with APC. Finally, proteolysis of hHOXC10 is delayed by a high concentration of cyclin A, a well-known substrate of APC. All these results suggest that hHOXC10 is a substrate of APC for its mitotic proteolysis. The biological relevance of the mitotic proteolysis of hHOXC10 has further been investigated. The transcriptional activity and the DNA binding of the D-box mutant proteins are similar to those of the wildtype hHOXC10. However, the cell cycle is affected by the mutant hHOXC10 and a delay in mitotic progression is observed, suggesting that hHOXC10 has a role in regulating events in mitosis. In contrast, it appears that the abundance of hHOXD10 and hHOXC13 proteins does not oscillate during the cell cycle, suggesting that the involvement of hHOXC10 in controlling molecular events during mitosis is specific and is not shared by close relatives, even from the same paralog group, despite the fact that at least one of the D-boxes is present in hHOXD10 (Gabellini *et al.*, 2003). The sequence of both D-boxes is conserved in the murine homologue mHOXC10 suggesting the mitotic function of HOXC10 might be conserved in mammals.

The HOXA10 protein is important for implantation of the mammalian embryo. In that context it regulates downstream genes like *ITGB3* that are involved in endometrial receptivity. By yeast-two-hybrid and co-precipitation assays, it was shown that hHOXA10 can interact with the p300/CREB-binding protein-associated factor (PCAF, also known as KAT2B), an acetyltransferase also expressed in the endometrium (Zhu *et al.*, 2013). The hHOXA10-PCAF interaction takes place in the nucleus. It requires the C-terminal moiety of hHOXA10 (aa 318-410) which comprises the homeodomain. The HAT (histone acetyltransferase) domain of PCAF is not necessary for the interaction. PCAF acetylates hHOXA10 in a dose-dependent way at lysines K338 and K339 residing in the homeodomain. This decreases hHOXA10 half-life and, in turn, induces a decrease in *ITGB3* expression. To determine the effect of the hHOXA10-PCAF interaction on embryo adhesion, Zhu *et al.,* developed a choriocarcinoma cell attachment assay (Zhu *et al.*, 2013). These cells attach to co-cultured cells expressing hHOXA10 whereas they do not with cells co-expressing PCAF and hHOXA10, since PCAF acetylates hHOXA10, provokes its decay and the downregulation of *ITGB3*. The two target residues K338 and K339 are conserved in mHOXA10 as well as in HOXC10 and HOXD10 in both human and mouse, however whether these proteins also interact with and are regulated by PCAF remains to be addressed.

In addition of being acetylated, hHOXA10 can also be modified by small ubiquitin-like modifier 1 (SUMO1) in the context of its role in uterus receptivity. hHOXA10 SUMOylation has indeed been recently shown to impair the process of embryo implantation (Jiang *et al.*, 2017). The major site of hHOXA10 SUMOylation in an *in vitro* cell model of endometrial epithelium was identified to be an evolutionary conserved lysine residue, K164, residing in a well-conserved protein motif of unknown function. The impact of the SUMO adduct has been identified to primarily decrease the hHOXA10 half-life, which has been correlated to enhanced ubiquitination. The intracellular distribution of hHOXA10 appeared however unchanged upon SUMOylation or K164R amino acid substitution. Next, hHOXA10 and SUMO1 coexpression also caused lower retrieval of hHOXA10 upon DNA pull-down assays, supporting that hHOXA10 SUMOylation could also impair its ability to bind DNA. Consistently with the decreased half-life and possible lower DNA binding capacity, expression of SUMO1 also decreased the ability of hHOXA10 to stimulate the activation of a reporter target gene. Finally, although hHOXA10 SUMOylation and acetylation target distinct lysine residues, these modifications apparently cross-talk with abolishment of hHOXA10 SUMOylation ($hHOXA10^{K164R}$) promoting acetylation. The modulation of hHOXA10 activity by SUMOylation appears to be related to estradiol and progesterone exposure which inhibits hHOXA10 SUMOylation in relationship with increased expression of the SUMO-deconjugating enzymes SENP1 and SENP2. These findings seem relevant *in vivo*: aber-

TABLE 2

HOX INTERACTING PROTEINS WITH POST-TRANSLATIONAL MODIFICATION ACTIVITIES

HOX	Interactor	Description	HOX	Interactor	Description
HOXA1	AGPAT1	Acyl transferase		PRKCA/B	Protein kinase C
	ALG13	UDP-N-acetylglucosamine transferase		SAT1	Acetyl-transferase
	COQ8B	Hypothetical kinase	HOXB7	CREBBP	Acetyl tran
	DUSP22	Protein phosphatase		CSNK2A1	Casein kinase
	KDM1A	Lysine demethylase		EP300	Acetyl transferase
	KDM5B	Lysine demethylase		IRAK3	IL-R associated Kinase
	LNX2	E3 Ubiquitin ligase		PARP1	Poly(ADP-ribosyl)transferase
	LPXN	Focal adhesion kinase regulation		PRKDC	DNA-activated protein kinase
	MGAT5B	N-acetylglucosamine transferase		UBR3	E3 Ubiquin ligase component
	OGT	O-linked N-acetylglucosamine transferase	HOXB8	EYA3	Tyrosine phosphatase
	PCSK5	Proprotein convertase	HOXB9	ARIH1	E3 Ubiquitin ligase
	PFKM	Phosphofructokinase		BTG1	Arginine methyl transferase
	PIK3R1	PI3 kinase regulatory subunit		BTG2	Arginine methyl transferase
	PRMT6	Arginine methyl transferase		CREBBP	Acetyl transferase
	RBCK1	E3 Ubiquitin ligase		EP300	Acetyl transferase
	RCHY1	E3 Ubiquitin ligase		KAT2B	Acetyl transferase
	SUV39H1	Lysine methyl transferase		MAPK14	Mitogen-activated protein kinase
	TRAF2	E3 Ubiquitin ligase		MID2	E3 Ubiquitin ligase
	TRIM23	E3 Ubiquitin ligase		NAGK	N-acetyl glucosamine kinase
	UBAC1	E3 Ubiquitin ligase complex component		SAT1	Acetyl-transferase
HOXA2	DMPK	Serine/Threonine kinase		SIRT1	Deacetylase
	RCHY1	E3 Ubiquitin ligase	HOXB13	EED	Lysine methyl transferase complex
	UBAC1	E3 Ubiquitin ligase complex component	HOXC4	LYN	Tyrosine kinase
HOXA3	RCHY1	E3 Ubiquitin ligase		NSD3	Lysine methyl transferase
	UBAC1	E3 Ubiquitin ligase complex component		PRKDC	DNA-activated protein kinase
	ZDHHC17	Palmytoyl-transferase		PRMT5	Arginine methyl transferase
HOXA5	MAPK14	Mitogen-activated protein kinase		PRMT6	Arginine methyl transferase
	PARP1	Poly(ADP-ribosyl)transferase		RCHY1	E3 Ubiquitin ligase
	PRMT6	Arginine methyl transferase		SUV39H1	Lysine methyl transferase
HOXA7	PARP1	Poly(ADP-ribosyl)transferase		UBAC1	E3 Ubiquitin ligase complex component
HOXA9	BCR	Serine/Threonine kinase	HOXC6	CHEK2	Checkpoint kinase
	CREBBP	Acetyl transferase		FGFR2	Receptor tyrosine kinase
	CUL4A	E3 Ubiquitin ligase		IGF1R	Receptor tyrosine kinase
	HDAC1	Deacetylase		PARP1	Poly(ADP-ribosyl)transferase
	PRKCA	Protein kinase C		PTPRJ	Tyrosine phosphatase
	PRMT5	Arginine methyl transferase	HOXC8	ABL1	Tyrosine kinase
	SUZ12	Lysine methyl transferase		BTG2	Arginine methyl transferase
	TRIM25	E3 Ubiquitin ligase		FYN	Tyrosine kinase
HOXA10	ARIH1	E3 Ubiquitin ligase		PARP1	Poly(ADP-ribosyl)transferase
	CREBBP	Acetyl transferase		SRC	Tyrosine kinase
	EP300	Acetyl transferase	HOXC9	ARIH1	E3 Ubiquitin ligase
	KAT2B	Acetyl transferase		MAPK14	Mitogen-activated protein kinase
	MAPK14	Mitogen-activated protein kinase		PCGF2	Polycomb like, regulator of ubiquitination and sumoylation
	PTPN6	Protein tyrosine phosphatase		PIGT	Glycosylphosphatidylinositol (GPI)-transamidase
	PTPN11	Protein tyrosine phosphatase	HOXC10	CDC27	APC Ubiquitin-ligase complex, core subunit
	SIRT2	Deacetylase		EGFR	Receptor tyrosine kinase
HOXA11	CREBBP	Acetyl transferase		MAPK9	Mitogen-activated protein kinase
	PRKDC	Serine/Threonine kinase		MAPK14	Mitogen-activated protein kinase
	HDAC1	Deacetylase		TRIM25	E3 Ubiquitin ligase
	HDAC2	Deacetylase	HOXC11	HDAC5	Deacetylase
HOXB1	RCHY1	E3 Ubiquitin ligase		RCHY1	E3 Ubiquitin ligase
	CREBBP	Acetyl transferase		UBAC1	E3 Ubiquitin ligase complex component
	EP300	Acetyl transferase	HOXC12	JADE1	Acetyl transferase complex
	Sirt1	Deacetylase	HOXD4	CREBBP	Acetyl transferase
	UBAC1	E3 Ubiquitin ligase complex component		EP300	Acetyl transferase
HOXB2	CREBBP	Acetyl transferase		HIPK1	Homeodomain interacting protein kinase
	EP300	Acetyl transferase	HOXD10	CREBBP	Acetyl transferase
	RCHY1	E3 Ubiquitin ligase		EP300	Acetyl transferase
	TNKS2	Poly(ADP-ribosyl)transferase		RCHY1	E3 Ubiquitin ligase
	UBAC1	E3 Ubiquitin ligase complex component		UBAC1	E3 Ubiquitin ligase complex component
HOXB3	CREBBP	Acetyl transferase	HOXD12	CREBBP	Acetyl transferase
	EP300	Acetyl transferase		WWP1	E3 Ubiquitin ligase
HOXB4	Crebbp	Acetyl transferase	HOXD13	A4GNT	N-acetyl glucosamine transferase
	CUL4A	E3 Ubiquitin ligase		CREBBP	Acetyl transferase
	DDB1	CUL4 adaptor	Dfd	Cdk2	Cyclin dependent kinase
	EP300	Acetyl transferase		Cdk4	Cyclin dependent kinase
	RBX1	E3 Ubiquitin ligase		Cdk5	Cyclin dependent kinase
HOXB5	CSNK1G2	Casein kinase		Cks85A	Cyclin dependent kinase subunit
	FYN	Src family tyrosine kinase		Cul-3	E3 Ubiquitin ligase complex
	LYN	Src family tyrosine kinase		KP78b	AMPK-like kinase

Post-translational modifications (PTMs) as recorded in databases and reported in the literature. Source: https://www.ncbi.nlm.nih.gov/gene/, for each interaction listed under each gene name in NCBI, a PubMed link allows retrieving the original article in which the interaction was reported; additional references are cited in the text.

rantly elevated levels of SUMOylated hHOXA10 could indeed be retrieved from endometrium of women experiencing recurrent implantation failure.

Post-translational modifications of HOX proteins: impact on DNA binding

hHOXA10 is phosphorylated during myeloid blood cell lineage differentiation induced by IFNγ. This phosphorylation decreases the binding of hHOXA10 to cis-regulatory elements of the *CYBB* and *NCF2* genes encoding respiratory burst oxidase proteins activated during late myeloid differentiation. At the undifferentiated state, hHOXA10 binds to sequences obeying the HOX/PBX binding site consensus in the vicinity of *CYBB* and *NCF2*. This results in transcriptional repression (Eklund *et al.*, 2000, Lindsey *et al.*, 2005). While myeloid differentiation proceeds, two tyrosines of the hHOXA10 homeodomain are phosphorylated (Y343 and Y360 according to the Uniprot entry P31260). These modifications decrease hHOXA10 DNA binding affinity, which presumably relieves gene repression. Accordingly, the hHOXA10 mutant protein (Y343F/Y360F) which cannot be phosphorylated shows increased DNA binding and represses the *CYBB* and *NCF2* genes (Eklund *et al.*, 2000, Lindsey *et al.*, 2005). Mechanistically, the hHOXA10-mediated repression of *CYBB* involves the recruitment of HDAC2, a histone deacetylase, and is independent on PBX1 (Lu *et al.*, 2003).

hHOXA10 phosphorylation has been attributed to the Janus tyrosine kinase 2 (JAK2). Upon IFNγ exposure, the interferon transmembrane receptors dimerise and stimulate the autophosphorylation of JAK2. Kakar *et al.,* (Kakar *et al.*, 2005) have shown that overexpression of JAK2 increases hHOXA10 tyrosine phosphorylation, decreases its *CYBB* binding and increases *CYBB* expression almost as well as the IFNγ treatment. On the opposite, a dominant negative JAK2 kinase blocks the effect of IFNγ treatment on hHOXA10 and *CYBB* expression. Although JAK2 is actually a kinase, whether it can interact with and directly phosphorylate hHOXA10 remains to be demonstrated. It cannot be excluded that the actual kinase modifying hHOXA10 is a downstream effector modulated by the cascade triggered by JAK2 and not JAK2 itself (Kakar *et al.*, 2005). Eklund and collaborators have also discovered two phosphatases able to remove the phosphoryl groups attached to these tyrosine residues: SHP1-protein tyrosine phosphatase (SHP1-PTP, also known as PTPN6) and SHP2-PTP (also known as PTPN11) (Eklund *et al.*, 2002, Lindsey *et al.*, 2007). These enzymes dephosphorylate hHOXA10 in undifferentiated cells to maintain the repression of *CYBB* and *NCF2*. Consistently, these phosphatase activities towards hHOXA10 are abolished during differentiation according to an unknown mechanism (Lindsey *et al.*, 2007).

The phosphorylated tyrosines of hHOXA10 are also present in the homeodomain of HOXA9 and HOXA11 proteins, suggesting that phosphorylation could also be a way to regulate their DNA binding properties (Eklund *et al.*, 2002). Surprisingly, phosphorylation of HOXA9 results in opposite effects when compared to HOXA10: upon differentiation induction by IFNγ, HOXA9 is phosphorylated at the two tyrosines in the homeodomain, but these phosphorylations lead to an increase in DNA binding of HOXA9 to the HOX/PBX cis-regulatory element of *CYBB*. In turn HOXA9 leads to an increase in *CYBB* expression during differentiation (Bei *et al.*, 2005). To conclude, HOXA9 and HOXA10 act in opposite ways on a common target during myeloid differentiation: after phosphorylation in response to IFNγ, HOXA10 and HOXA9 respectively show reduced or increased binding to *CYBB*, which collectively converge in promoting gene expression (Bei *et al.*, 2005, Eklund *et al.*, 2002). This combined control by HOXA10 and HOXA9 tyrosine phosphorylation is most probably more complex than described because these two proteins seem to have additional sites of phosphorylation, i.e. additional and distinctive ways to have their activity regulated. Moreover, HOXA9 and HOXA10 also differently act on additional target genes depending on the context. Indeed, *CDX4* for example is activated by HOXA10 in progenitor cells and repressed by HOXA9 during myelopoiesis (Bei *et al.*, 2014). Phosphatase activity in undifferenciated cells promotes the unphosphorylated form of HOXA10 to bind and activate the *CDX4* gene. Upon phosphorylation, HOXA10 DNA binding is impaired, while the phosphorylated HOXA9 binds *CDX4*, although on a distinct cis-regulatory element, and thereby represses its expression. Next, *ARIH2* codes for the ubiquitin ligase TRIAD1 required to stop emergency granulopoiesis and involved in myelopoiesis. It is also a shared target gene of both HOXA9 and HOXA10 (Wang *et al.*, 2011, Wang *et al.*, 2015, Wang *et al.*, 2018). Here, *ARIH2* has been shown to be activated by hHOXA10 and repressed by hHOXA9, while homeodomain tyrosine phosphorylation decreased hHOXA9

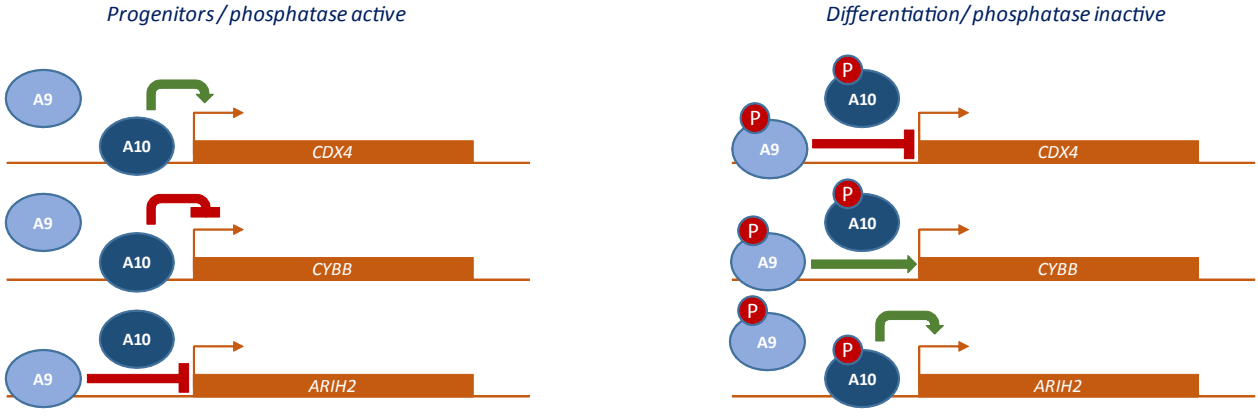

Fig. 4. Differentiated effects of phosphorylation on HOXA9 and HOXA10. *The phosphorylation status of HOXA9 and HOXA10 distinctively influences their ability to bind cis-regulatory sequences and to regulate target genes during myelopoiesis (see text for details). A9, HOXA9; A10, HOXA10. Small red circles (with "P") represent phosphorylations added onto HOX proteins.*

but increased hHOXA10 binding. Together, three distinct situations have been depicted for the coordinated regulation of target genes by HOXA9 and HOXA10 in myelopoiesis, with distinct effects of their phosphorylated status with regard to their DNA binding but also their ability to activate target genes (Fig. 4): unphosphorylated HOXA10 binds to and activates *CDX4*, but represses *CYBB*, while phosphorylated HOXA10 activates *ARIH2*; on the contrary the unphosphorylated HOXA9 binds to and represses *ARIH2*, while phosphorylated HOXA9 binds to and activates *CYBB*, but represses *CDX4*. This convincingly illustrates that the effect of phosphorylation depends on the intrinsic HOX protein context, as well as on the target gene regulatory environment.

Moreover, the Largman group has shown that the murine orthologue mHOXA9 is phosphorylated mainly at the S204 and probably also at the T205 residues by the Protein Kinase Cα (PKCα) during myeloid cell lineage differentiation (Vijapurkar *et al.*, 2004). These residues belong to a STRK tetrapeptide located between a tryptophan-containing PBX interaction motif and the homeodomain. Quite strikingly, this tetrapeptide is conserved among human, mouse, chicken, pig and horn shark HOXA9 sequences and similar putative PKCα sites could be identified in 21 of the 39 mammalian HOX proteins. The consequence of these PTMs is a decrease in the ability of mHOXA9 to bind DNA and a stimulation of cell differentiation (Vijapurkar *et al.*, 2004).

hHOXB9 has been shown to be acetylated on five distinct lysines spread along the protein sequence, including one within the homeodomain (K202). The acetyltransferase p300/CREB Associated Factor (PCAF) was identified to interact with hHOXB9 and to mediate its acetylation on K27 (Wan *et al.*, 2016). The K27 residue of hHOXB9 is conserved among orthologues and multiple mouse tissues appeared to consistently contain K27-acetylated HOXB9 proteins. This PTM is reversed by the class II sirtuin family member SIRT1 which also can interact with hHOXB9. At the molecular level, analysis of wildtype and mutant hHOXB9 (hHOXB9^{K27R} or the acetylo-mimetic hHOXB9^{K27Q} mutations) activities and DNA binding support that acetylation exerts a negative effect on hHOXB9 DNA binding, the acetylation deficient hHOXB9^{K27R} generating stronger ChIP-mediated DNA recovery of target sequences. hHOXB9 has been identified to promote lung cancer progression, however, analyses from patients support that elevated K27 acetylation of HOXB9 defines a positive survival prediction bio-marker.

poly(ADP-ribosyl)ation has been identified as a modification of distinct HOX proteins. Initially, it has been shown that the hHOXB7 protein interacts with the Poly(ADP-ribose) polymerase -1 (PARP-1) (Rubin *et al.*, 2007) and is poly(ADP-ribosyl)ated (Wu *et al.*, 2012). This interaction takes place between the homeodomain of hHOXB7 and the first zinc finger domain of PARP1. The poly(ADP-ribosyl)ation modifies the glutamate-rich C-terminal tail of hHOXB7 and leads to a decrease in the DNA binding affinity of hHOXB7. The hypothesis is that poly(ADP-ribosyl)ation introduces negative charges leading to repulsion from the DNA. hHOXA5, hHOXB6, hHOXC6, hHOXA7, and hHOXC8 all bind to PARP-1 and can be poly(ADP-ribosyl)ated, but a subsequent decrease in their DNA binding ability has only been reported for hHOXA7 and hHOXB7 (Wu *et al.*, 2012). The impact of poly(ADP-ribosyl)ation for the other HOX proteins is not known.

The lab of W. Gehring has shown by yeast-two-hybrid that the Sex Combs Reduced (Scr) protein interacts with a regulatory subunit of the phosphatase PP2A. The N-terminal arm of the homeodomain of Scr is essential to the interaction. Indeed, switching the N-terminal arm of the Scr homeodomain with that of Antennapedia (Antp) differing by only four amino acids resulted in a loss of interaction (Berry and Gehring, 2000). Phospho-labelling experiments in COS-1 cells overexpressing Scr revealed that it can be phosphorylated by PKA at threonine 329 and that PP2A was active to remove a phosphate group from serine 330. The kinase active on S330 however couldn't be identified. Flies ectopically expressing the wildtype Scr or mutated versions with the two serine residues either substituted by alanines (Scr^{AA}) or aspartates (Scr^{DD}), revealed that Scr^{WT} and Scr^{AA} showed similar activities, i.e. they induced similar phenotypes whereas flies expressing Scr^{DD} were similar as wildtype, suggesting the aspartate mutations abolish Scr activity. Scr^{WT} and mutant proteins were all localised at the nucleus and showed similar half-lives but the aspartate mutant lost its ability to bind DNA at a Scr recognition site even in the presence of the PBC-class cofactor Extradenticle (Exd). In contrast the alanine mutant was binding DNA better than the wildtype in conjunction with Exd. These results together indicate that phosphorylation of Scr leads to its inactivation and dephosphorylation to its activation. Interestingly the T329 and S330 residues are both conserved in Scr homologues from other species. Finally, downregulating PP2A using RNAi induced a loss of salivary glands, a phenotype observed in Scr loss-of-function mutant embryos, suggesting that the phosphatase is essential for the activity of Scr (Berry and Gehring, 2000). This however remains controversial because a fly deleted for the *PP2AB*' gene could not highlight any phenotype similar to the RNA-mediated Scr inactivation, i.e. on the formation of larval salivary glands, adult sex comb bristles, adult pseudo-tracheal rows and ectopic sex comb bristles (Moazzen *et al.*, 2009).

In vitro studies on Scr highlighted that Asn deamidation can take place with time and specifically convert an Asn residing between the homeodomain and the hexapeptide motif that mediates interaction with the PBC cofactors PBX/Exd (O'Connell *et al.*, 2015). Asparagine deamidation is a spontaneous, non-enzymatic change affecting asparagine which then is converted in aspartate or isoaspartate. This deamidation modifies both the protein backbone conformation and the local charge in the vicinity of the DNA minor groove where the N-terminal arm of the homeodomain establishes contacts. Consistently, the modified Scr protein displayed lower DNA binding affinity. Whether Asn deamidation of Scr or of other HOX proteins has any relevance *in vivo* remains to be addressed. Considering that the Asn-to-Asp/iso-Asp transition is spontaneous and takes place with time, one important parameter which could be determinant in that respect is the turn-over rate and stability of the HOX proteins considered. It is worth noticing that the half-life of HOX proteins can differ importantly (Bridoux *et al.*, 2015a, Draime *et al.*, 2018b), and is most probably influenced by contextual circumstances (Jiang *et al.*, 2017, Lee *et al.*, 2013, Zhang *et al.*, 2003, Zhu *et al.*, 2013).

Post-translational modifications of HOX proteins: impact on TALE protein interaction?

The *Drosophila* Antp interacts with the Ser/Thr casein kinase CKII and is phosphorylated *in vitro*. *In vivo*, flies ubiquitously expressing an Antp variant mutated for the 4 putative CKII target residues (T35A, S84A, T358A and S364A) showed a strong phenotype, distinct from that obtained with the ubiquitous expression of the wildtype protein, supporting that the mutated protein displays neomorphic

activity (Jaffe *et al.*, 1997). In sharp contrast, phosphomimetic amino acid substitutions (T35E, S84E, T358E and S364E) rendered the protein hypoactive and the embryos expressing this Antp variant showed very weak phenotypes. All the mutants remained able to bind cognate Antp binding sites on DNA, but the phospho-mimetic glutamate substitution mutant was unable to form complexes with Exd, suggesting that the Antp phosphorylation status modulates the ability of Antp to interact with Exd. The lack of effect on DNA binding or Exd interaction of the Antp alanine mutant which appeared to be a neomorph further suggests that phosphorylation must impact on other aspects of Antp activity.

Post-translational modifications of HOX proteins: effect on the transcriptional activation capacity?

Bandyopadhyay and colleagues have shown that hHOXA9 interacts with PRMT5, an arginine methyl transferase protein involved in many cellular pathways as a transcription cofactor (Bandyopadhyay *et al.*, 2012). This interaction enhances the expression of E-selectin as compared to the level of expression induced by each partner alone, underscoring a synergistic functional interaction. The integrity of a hHOXA9 DNA binding site at the E-selectin (*SELE*) gene is necessary for its expression and data support that hHOXA9 binds the *SELE* promoter before the recruitment of PRMT5. Most strikingly, the hHOXA9-PRMT5 interaction leads to the dimethylation of hHOXA9 at the R140 position, an arginine residue present in the transcriptional activation domain of hHOXA9. Considering this PTM is not required for the binding of hHOXA9 to DNA, but is critical for *SELE* induction, it therefore seems involved in hHOXA9 transcriptional activity *per se* (Bandyopadhyay *et al.*, 2012). Whether this modification is required for additional interactions eventually leading to PolII recruitment and activation needs to be investigated further. The R^{140}RGD sequence targeted by PRMT5 is only present in HOXA9 among the paralog group 9 proteins. The PRMT5-mediated regulation of the transcription activation capacity is therefore most probably specific to HOXA9 proteins.

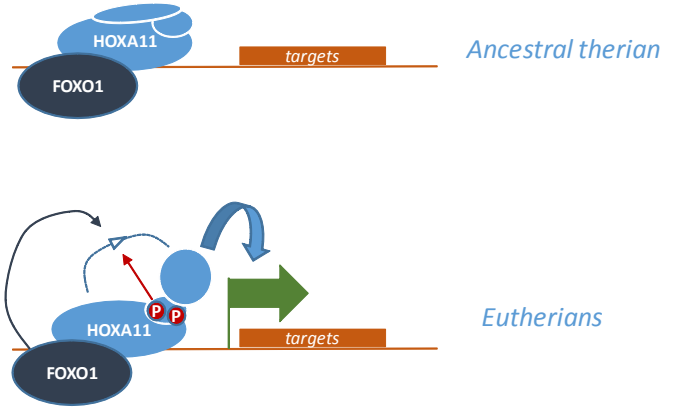

Fig. 5. Phosphorylation-mediated control of the functional output of HOX protein interaction. *The mammalian HOXA11 protein interacts with FOXO1, but the presence of target residues for phosphorylation in eutherian mammals modifies the outcome of the HOXA11-FOXO1 interaction. In placental mammals this interaction unmasks a transactivation domain of HOXA11 which implies that the HOXA11-FOXO1 interaction will induce target gene activation, while this interaction remains unproductive in inferred ancestral mammals. Considering the regulatory role of HOXA11 in endometrial cells, the HOXA11-FOXO1 cooperation might have contributed to the evolution of placentation (Nnamani* et al.*, 2016). Small red circles (with "P") represent phosphorylations added onto HOX proteins.*

The mammalian HOXA11 protein interacts with the FOXO1 transcription factor. However, while the HOXA11-FOXO1 complex has been shown to activate target genes in the uterus of placental mammals, the ancestral complex reconstituted by inference is repressive (Brayer *et al.*, 2011, Nnamani *et al.*, 2016). An evolutionary functional transition therefore occurred, modifying the outcome of the HOXA11-FOXO1 interaction. This transition has recently been investigated by identifying the changes that took place during HOXA11 evolution (Fig. 5) (Nnamani *et al.*, 2016). A handful of amino-acid changes define derived traits in the placental protein that led to regulatory and intrinsic activity changes. The placental protein displays a negative intramolecular interaction that blocks the transcription activation domain of the protein. Two derived serine and threonine residues (S98 and T119) in a central region of HOXA11 predicted to be intrinsically disordered have been identified to be modified by the DNA- protein kinase (DNA-PK). These PTMs in turn allow FOXO1-dependent relief of the inhibitory intramolecular interaction and subsequent binding to the CREB Binding Protein (CBP, also known as CREBBP) acetyl-transferase. The affinity of the HOXA11/FOXO1 complex for CBP is enhanced by S98 and T119 phosphorylation, which allows transcription activation. In ancestral therian mammals, the HOXA11/FOXO1 interaction could take place but would not allow unmasking the transactivation domain of HOXA11. Thus discrete sequence changes in the HOXA11 sequence in mammalian evolution have permitted a new allosteric regulation of the protein involving the pre-existing HOXA11/FOXO1 interaction but derived characteristics allowing PTM- and FOXO1-mediated conformational changes. The way HOXA11 thereby acquired new activities might have contributed to the onset of placentation in mammals.

Post-translational modifications of HOX proteins with yet unidentified cellular impact

In addition to studies reporting the PTM of HOX proteins along with their impact on HOX protein activity, several reports revealed additional modifications for which the impact on HOX action is unknown. mHOXA1 (Draime *et al.*, 2018a), m- and hHOXA2 (Deneyer, Bridoux and Rezsohazy – unpublished), hHOXD4 (Corsetti *et al.*, 1992), mHOXA5 (Odenwald *et al.*, 1989), hHOXC6 (Corsetti *et al.*, 1992), Scr (Zhai *et al.*, 2008) and Ubx (Gavis and Hogness, 1991, Zhai *et al.*, 2008) for example have all been shown to be phosphorylated but neither the modified residues nor the kinase involved are known.

hHOXB7 is able to inhibit granulocytic differentiation. Yaron *et al.,* used this property to test the importance of different domains or peptidic motives of hHOXB7 on its activity (Yaron *et al.*, 2001). The hHOXB7 sequence harbours short motives obeying the consensus target sequences for CKII, S/TXXD/E, with putative phosphorylated residues S133 and T204, flanking the homeodomain. However a serine and a threonine were also found just adjacent at positions S132 and T203, respectively. Therefore, the authors mutated both serines or threonines in alanines (hHOXB7$^{S132A, S133A}$ and hHOXB7$^{T203A, T204A}$). Cells expressing these mutants displayed an enhancement of differentiation after 1, 2 and 3 days of induction by cytokines, suggesting that these residues are involved in the ability of hHOXB7 to inhibit differentiation. An *in vitro* ATPγ^{-32} phosphorylation assay involving CKII revealed that hHOXB7^{WT}

can be phosphorylated as well as the hHOXB7$^{S132A,\ S133A}$ and hHOXB7$^{T203A,\ T204A}$ double mutants, but not the quadruple mutant, suggesting that CKII phosphorylates both motives. hHOXB7$^{S132A,\ S133A}$ and hHOXB7$^{T203A,\ T204A}$ mutants were located in the nucleus like hHOXB7^{WT}, suggesting that mislocalisation could not be the cause of their inability to repress granulocytic differentiation. Whether these residues could be involved in the DNA binding or the transcriptional activity of hHOXB7 has not been determined. The CKII target motives are conserved in mHOXB7 and mHOXA7 but not in hHOXA7 (Yaron *et al.*, 2001).

Fienberg *et al.,* have shown that mHOXB6 is phosphorylated *in vitro* as well as during embryogenesis in mouse spinal cords at 12.5 day of development. mHOXB6 residue S214, located just after the homeodomain, has indeed been shown to be phosphorylated by the CKII kinase *in vitro* (Fienberg *et al.*, 1999). The CKII target sequence found in mHOXB6 is perfectly conserved in hHOXB6. Fienberg *et al.,* also highlighted two additional mHOXB6 phosphorylated peptides. One is the substrate of the PKA kinase but they could not determine the target residue and the other phosphopeptide has not been characterised (Fienberg *et al.*, 1999). In addition, Shen *et al.,* showed that hHOXB6 is also phosphorylated by PKC and CKI (Shen *et al.*, 2004). A hHOXB6 mutant generated to forbid phosphorylation at S214 (hHOXB6^{S214A}) or to mimic phosphorylation (hHOXB6^{S214E}) did not show DNA binding, transcriptional repression activity or subcellular localisation alterations with respect to the wildtype hHOXB6 (Shen *et al.*, 2004). This lack of effect may result from only one residue being targeted, leaving multiple possible phosphorylable residues intact. Thus, it seems that HOXB6 has multiple sites of phosphorylation and according to the combination of these modifications, the effect could vary. The discovery of the other amino residues phosphorylated within HOXB6 would therefore be required to unravel the roles of the combined modifications.

Ronshaugen *et al.,* (2002) showed that the evolutionary differences in the body plans of *Drosophila* and Artemia could notably be explained by the phosphorylation of Ubx. Indeed, while *Drosophila* Ubx ectopic expression is able to repress thoracic embryonic limb formation, Artemia Ubx is not. Comparing the protein sequences led to the identification of five serines and two threonines in the Artemia Ubx C-terminal portion which when mutated confer a strong embryonic limb repressing activity in *Drosophila* (Ronshaugen *et al.*, 2002). The C-terminus of Artemia Ubx protein predicted to be a good target for CKII was indeed confirmed *in vitro* as being a potential substrate for the enzyme, while the *Drosophila* orthologue was not (Hsia *et al.*, 2010, Taghli-Lamallem *et al.*, 2008). Mutating the CKII target serine residue however did not confer limb repressing activity to the Artemia Ubx protein (Hsia *et al.*, 2010). Nonetheless, it seems that the C-terminal serine/threonine residues of Ubx, and their potential phosphorylation, could play a key role in permitting or repressing limb formation in arthropods and, thereby, have defined an important evolutionary change in HOX protein activity and their ability to shape body segments.

Hox genes of the paralog group 10 share the common characteristic to block rib formation (Wellik and Capecchi, 2003). By comparing their sequence, Guerreiro *et al.,* were able to reveal a motif, NWLTAKS, located N-terminally to the homeodomain of mouse and human HOX10 proteins, contributing to their rib formation repressing activity. Indeed, upon deletion of this motif, the mHOXA10$^{\Delta NWLTAKS}$ protein loses its capacity to repress rib formation when ectopically expressed in mice. The authors then hypothesised that inside the NLWTAKS motif, the threonine and serine residues could be the target of phosphorylation. Mutating either the threonine or serine alone led to transgenic mice with rib cage anomalies while the double mutant (HOXA10$^{TS\text{-}AA}$) gave rise to mice without any detected skeletal abnormalities. These results support that these residues are key to confer the NWLTAKS motif the ability of HOXA10 to repress rib formation. Considering the NWLTAKS motif resides just upstream of the homeodomain, the authors tested by gel shift assays whether it could participate to modulate HOXA10 DNA binding, but the mHOXA10$^{\Delta NWLTAKS}$ protein appeared to bind DNA as well as the wildtype protein (Guerreiro *et al.*, 2012).The functional importance of the NLWTAKS peptide in HOX10 protein activity and the possible modification at the threonine and serine residues both remain to be addressed.

By way of opening

The abundance of HOX entries in PTM databases as well the numerous candidate or established HOX interactors displaying enzymatic PTM activities strikingly contrast with the limited number of studies reporting HOX modification and their consequences. There are examples for which HOX protein stability, DNA binding, protein-interaction or activity changes have been proven to be subsequent to various PTMs, namely phosphorylations, acetylation, methylation, poly(ADP-ribosyl)ation, deamidation, ubiquitination, and SUMOylation. But these examples most probably represent a tiny proportion of the actual modifications taking place at the heart of the HOX protein biology which covers a vast panoply of specific contextual activities. HOX PTMs therefore need to be tackled with more care, in a functional and mechanistic perspective, to determine to what extent they contribute to target gene and protein interaction selectivity and thereby defining functional specificity.

A handful of examples, similar to the ones evoked about p53 while introducing this review paper, further suggest that PTMs modify HOX protein behavior in a combinatorial way. This is supported by the possible modification of the HOX10 rib repression capacity, the additive effect of serine and threonine residue mutations in hHOXB7 as well as the multiple phosphorylations taking place on mHOXB6 and acetylation, SUMOylation, ubiquitination and phosphorylations highlighted for HOXA10 for example. This should likely unveil additional regulatory mechanisms beyond the control of HOX protein activity.

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