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KPC2 relocalizes HOXA2 to the cytoplasm and decreases its transcriptional activity☆

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ABSTRACT

Regulation of transcription factor activity relies on molecular interactions or enzymatic modifications which influence their interaction with DNA cis-regulatory sequences, their transcriptional activation or repression, and stability or intracellular distribution of these proteins. Regarding the well-conserved Hox protein family, a restricted number of activity regulators have been highlighted thus far. In the framework of a proteome-wide screening aiming at identifying proteins interacting with Hoxa2, KPC2, an adapter protein constitutive of the KPC ubiquitin-ligase complex, was identified. In this work, KPC2 was confirmed as being a genuine interactor of Hoxa2 by co-precipitation and bimolecular fluorescence complementation assays. At functional level, KPC2 diminishes the transcriptional activity and induces the nuclear exit of Hoxa2. Gene expression analyses revealed that *Kpc2* is active in restricted areas of the developing mouse embryo which overlap with the *Hoxa2* expression domain. Together, our data support that KPC2 regulates Hoxa2 by promoting its relocation to the cytoplasm.

1. Introduction

Hox genes define a class of homeobox genes coding for evolutionarily conserved transcription factors. In mammals, there are 39 *Hox* genes organized into four complexes (HoxA–D) located on separate chromosomes. Based on their sequence similarity and relative positions along the clusters, *Hox* genes have been subdivided into 13 paralogue groups. *Hox* genes have been characterized as master regulators in development as they were initially found to play key roles in determining the identity of body segments along the main and appendicular axes of bilaterian embryos. Accordingly, in many cases, *Hox* mutations lead to homeotic transformations that result from the modification of the fate of developing structures which adopt the identity of distinct, usually adjacent structures along the rostro-caudal axis in the embryo (reviewed in [1]). For example, knockout mice for *Hoxa2* display cranial transformations caused by the replacement of second branchial arch derivatives by skeletal elements similar to those from the first branchial arch in a mirror image configuration [2,3]. In addition to their function in patterning embryonic territories, *Hox* genes have also been reported to control multiple events in later organogenesis and differentiation up to adulthood (reviewed in [4]).

Hox proteins have been well-documented to interact with members of the PBC, MEIS or PREP family of transcription factors (reviewed in [5]) which modulate their DNA-binding specificity [6,7] and transcription activity [8,9] or, conversely, which are affected by the Hox interaction [10]. Besides these thoroughly studied interactions, data about Hox partner proteins remain surprisingly scarce. Similarly, while modulation of transcription factor activity, subcellular localization, stability or degradation is known to occur, notably by post-translational modifications, such regulatory pathways are poorly characterized for Hox proteins. Phosphorylation of a few HOX proteins by casein kinase II [11–14] or PKC [14] and dephosphorylation by SHP1–2 [15,16] have been reported to control their DNA-binding affinity, transcriptional activity or capacity to form cooperative DNA-binding complexes with PBX. Moreover, poly(ADP-ribosyl-)ation, methylation or acetylation have also been shown to modify the DNA-binding or transcriptional activity of four human HOX proteins [17–19]. Finally, some HOX proteins have been reported to be ubiquitinated by the CUL4 and Anaphase Promoting Complex (APC) ubiquitin ligases promoting their proteasome-dependent degradation [20–22]. But overall, known instances of regulation of HOX protein activity remain limited.

In recent years, proteome-wide interactomic approaches have enabled investigation of protein–protein interactions and two large

Abbreviations: BiFC, bimolecular fluorescence complementation; CHX, cycloheximide; GST, glutathione S-transferase; HD, homeodomain; HOX, homeobox; ISH, in situ hybridization; KPC2, Kip1 ubiquitination-promoting complex 2; LMB, leptomycin B; ORF, open reading frame; r, rhombomere.

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screenings focusing on HOX proteins, UBX in drosophila and Hoxa1 in mammals, have been reported thus far. These analyses highlighted a number of candidate interactors which could be involved in the regulation of HOX activity [23,24]. Using a similar framework, we recently conducted a yeast two-hybrid screening for candidate interactors of Hoxa2. We thereby identified RCHY1, an E3 ubiquitin ligase targeting apoptosis and cell cycle regulators (reviewed in [25]). However, this interaction does not seem to lead to Hoxa2 activity modulation or degradation but instead appears to impact RCHY1 stability itself [26].

Here, we report a novel interaction involving Hoxa2 and KPC2, a subunit of the Kip1 ubiquitination promoting complex (KPC), also known as UBAC1. The KPC complex consists of two subunits: KPC1 and KPC2 [27]. KPC1, a ring finger domain-containing protein, functions as the catalytic E3 ubiquitin ligase subunit of the complex [27]. KPC2 is considered to be the adapter subunit of the complex as it stabilizes KPC1, and interacts both with polyubiquitinated proteins and the proteasome [27,28]. The KPC complex influences cell cycle by regulating $p27^{Kip1}$ [27], a cyclin-dependent kinase inhibitor, thereby promoting the G1/S transition. KPC was shown to interact with the cytoplasmic form of $p27^{Kip1}$ (pp27ser10) and to mediate its ubiquitination leading to proteasomal degradation [27–30]. Our data indicate that contrarily to its negative impact on $p27^{Kip1}$ stability, KPC2 does not seem to negatively regulate Hoxa2 stability but instead alters its cellular distribution and transcriptional activity. We also provide data showing that *Kpc2* is expressed during mouse embryogenesis following a restricted pattern which partially but significantly overlaps with the *Hoxa2* expression profile.

2. Material and methods

2.1. Plasmid constructs

Gateway® expression vectors (pExp) for AD-Hoxa2 and DB-Hoxa2 [26], construction of expression vectors for Hoxa2 [31], pCMVlacZ [32], pCMV-PBX1a [33], pCS2-Prep1 [34], the Hoxa2 r4-enhancer luciferase reporter [35] and the pKS-Hoxa2 [36] plasmids have been described elsewhere. Gateway® entry vector (pEnt) for human KPC2 (refers as UBAC1) was obtained from the hORFeome v3.1 (http://horfdb.dfci.harvard.edu) [37] and HOXA1, HOXA2, HOXA3, HOXC4, HOXB5, HOXD10 and HOXC11 open reading frames (ORF) were obtained from the hORFeome v7.1 (http://horfdb.dfci.harvard.edu/hv7/). Plasmids for HOXB1 and HOXB2 templates were kindly provided by F. Rijli (Friedrich Miescher Institute, Switzerland), and those for $p27^{Kip1}$ and KPC1 templates were received from L. Nguyen (University of Liege, Belgium) and I. Nakayama (Kyushu University, Japan), respectively. Sequences coding for Hoxa2 deletion derivatives as well as for HOXB1, HOXB2, $p27^{Kip1}$ and KPC1, were PCR-amplified using the primers listed in Table 1 and the templates mentioned above. The resulting PCR products were inserted into the pDON223 vector using the Gateway® Technology from Invitrogen to generate the corresponding pEnt vectors. The pEnt plasmids were confirmed by DNA sequencing and used to generate yeast expression vectors for AD-KPC2 and DB-KPC2 (pDest-AD and pDest-DB destination vectors, Gateway®, Invitrogen); mammalian expression vectors for FLAG–HOXA2 (v1899 destination vector [38]); GST–KPC1, GST–KPC2 and GST–Hoxa2-deletion derivatives (pDest-GST N-terminal [39]), VN^{173}KPC1, $VN^{173}p27^{Kip1}$, VN^{173}human-HOX and VN^{173}Hoxa2-deletion derivatives (pDest-VN^{173} [23]), and VC^{155}KPC2 (pDest-VC^{155} [23]).

The DNA sequence corresponding to the *Kpc2* ISH probe was PCR-amplified from genomic DNA using the following primers, AATCCGCT TAACAGCACCCA and TGCTCTGGGCAGAGACAATG. This 582 bp *Kpc2* fragment was cloned into pCR2.1 TOPO plasmid using the TOPO® TA Cloning® Technology (Invitrogen).

2.2. Two-hybrid screening

The yeast two-hybrid screening was performed as previously described [26].

2.3. Cell culture, transfection and treatments

Cultured cells were maintained at 37 °C, in a humidified atmosphere with 5% CO_2. HEK293T cell line was grown in Dulbecco's Modified Eagle Medium (D-MEM) with Gultamax-I (#61965, GIBCO) supplemented with 10% fetal bovine serum (#10270-106, Invitrogen), 100 U/ml of penicillin–streptomycin (#15140-122, GIBCO) and 1 mM sodium pyruvate (11360-070, GIBCO). COS-7 cells were maintained in Dulbecco's Modified Eagle Medium (D-MEM) (#31885-023, GIBCO) supplemented with 10% fetal bovine serum and 100 U/ml of penicillin–streptomycin. Plasmid constructs were transfected with jetPRIME transfection reagent (#114-07, Polyplus-transfection) according to the manufacturer's instructions. For proteasome inhibition, 24 h after transfection, cells were treated with 5–10 μM MG132 dissolved in DMSO (#474790, Calbiochem) or with DMSO alone as control for periods of 7–15 h. For half-life measurements, 24 h after transfection, the proteasome was inhibited for 4 h then treated with 200 μg/ml of cycloheximide (CHX) (#01810, Sigma) dissolved in DMSO following different exposure times. For nuclear export inhibition, 8 h after transfection, cells were treated with 10 ng/ml leptomycine B (LMB) dissolved in DMSO (#L2913, Sigma), or with DMSO alone as control, for a period of 16 h.

2.4. Protein abundance analysis and western blotting

HEK293T were transfected with distinct combinations of expression vectors, at 500 ng each. To keep the amount of transfected DNA constant, the total amount of DNA was adjusted with the pDest-GST. Cells were lysed for 20 min at 4 °C in ice-cold IPLS lysis buffer (0.5% NP-40, 20 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 120 mM NaCl, 10% glycerol) containing protease inhibitor cocktail (#11873580001, Roche). Cells lysates

Table 1
Primers used to generate pEnt plasmids.

Plasmid	Forward primer (5′-3′)	Reverse primer (5′-3′)
pEntHoxa2$^{\Delta N}$	GGGGACAACTTTGTACAAAAAAGTTGGCTCCAGGCGTCTGAGAACCGCG	GGGGACAACTTTGTACAAGAAAGTTGGGTATTAGTAATTCAGATGCTGTAGG
pEntHoxa2$^{\Delta C}$	GGGGACAACTTTGTACAAAAAAGTTGGCATGAATTACGAATTTGAGCG	GGGGACAACTTTGTACAAGAAAGTTGGGTATTAGGTTTGCC
pEntHoxa2$^{\Delta HD}$[a]	GGGGACAACTTTGTACAAAAAAGTTGGCATGAATTACGAATTTGAGCG GCTGCTGCTGCTGCTCAGTGCAAGGAGAACCAAAAC	AGCAGCAGCAGCAGCAGCTCCCCCGCCGCTGCCATCA GGGGACAACTTTGTACAAGAAAGTTGGGTATTAGTAATTCAGATGCTGTAGG
pEntHoxa2^{HD}	GGGGACAACTTTGTACAAAAAAGTTGGCTCCAGGCGTCTGAGAACCGCG	GGGGACAACTTTGTACAAGAAAGTTGGGTATTAGGTTTGCC
pEntHOXB1	GGGGACAACTTTGTACAAAAAAGTTGGCATGGACTATAATAGGATGAACT	GGGGACAACTTTGTACAAGAAAGTTGGGCAGGAGGTGACAGAGCTG
pEntHOXB2	GGGGACAACTTTGTACAAAAAAGTTGGCATGAATTTTGAATTTGAGAGGGA	GGGGACAACTTTGTACAAGAAAGTTGGGGGAAACTGCAGGTCGATGG
pEntp27^{Kip1}	GGGACAACTTTGTACAAAAAAGTTGGCATGTCAAACGTGCGAGTGTC	GGGGACAACTTTGTACAAGAAAGTTGGGTACGTTTGACGTCTTCTGAGGC
pEntKPC1	GGGGACAACTTTGTACAAAAAAGTTGGCATGGCATCCAAGGGGGCC	GGGGACAACTTTGTACAAGAAAGTTGGGTAGGCAGCTGAGGAGGTAG

[a] The Hoxa2$^{\Delta HD}$ PCR product was obtained in 2 steps involving two separate PCR amplifications followed by an overlapping PCR combining the two first amplicons.