KPC2 relocalizes HOXA2 in the cytoplasm and decreases its transcriptional activity

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Abstract

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Abbreviations

BiFC, Bimolecular Fluorescence Complementation; DB DNA Binding domain; AD, Activation Domain; ORF, Open Reading Frame; GST, Glutathione S-Transferase.

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Introduction

_Hox_ genes define a class of homeobox genes coding for evolutionary conserved transcription factors. In mammals, there are 39 _Hox_ genes organized into four complexes (HoxA-D) located on separate chromosomes. Based on sequence similarity and relative position along the clusters, _Hox_ genes have been subdivided into 13 parologue groups. _Hox_ genes have been characterized as master regulatory genes in development as they were initially found to play key roles in determining the identity of body segments along the main body and appendicular axes of bilaterian embryos. Accordingly, in many cases, _Hox_ mutations lead to homeotic transformations meaning that developing structures in the embryo take on the identity of distinct, usually adjacent ones along the rostro-caudal axis (reviewed in [1]). For example, knockout mice for _Hoxa2_ display cranial transformations caused by the replacement of second branchial arch derivatives by skeletal elements typical of the first branchial arch in a mirror image configuration [2,3]. In addition to their function as homeotic genes in patterning embryonic territories, _Hox_ genes have also been reported to control multiple events in later organogenesis and differentiation sequences up to adulthood (reviewed in [4]).

As transcription factors, HOX proteins are known not to act alone. They contact partner proteins which modulate their DNA binding specificity [5,6] and transcription activity [7,8] or, conversely, which are affected by the Hox interaction [9]. Nonetheless, data about Hox partner proteins remain scarce, the only well-documented interactions being those involving members of the PBC, MEIS or PREP family (reviewed in [10]). Additional levels of regulation of transcription factor activity by post-translational modifications are well-known but are again poorly characterized for HOX proteins. Among the few known instances, both in drosophila and mammals, phosphorylation of several HOX proteins (ANTP, UBX, HOXA9, and HOXB7) by casein kinase II has been shown to be essential to their activities [11-14]. The PKC-mediated phosphorylation of HOXA9 decreases its DNA binding and its ability to form cooperative DNA binding complexes with PBX [14]. Conversely, tyrosine dephosphorylation of HOXA10 by SHP1-2 increases its DNA binding affinity and consequently affects transcriptional regulation of its target genes [15,16]. Upon binding to PARP-1, HOXB7 and HOXA7 are poly(ADP-ribosyl-)ated which results in the reduction of both DNA-binding and transcriptional activity [17]. HOXA9 methylation is mediated by PRMT5 leading to an induction of HOXA9 targets [18]. The PCAF enzyme acetylates, decreases the activity and destabilizes HOXA10 [19].

Ubiquitination is a versatile post-translational modification involved in several cell processes such as proteasomal protein degradation, cell signalling, membrane trafficking or DNA damage response (reviewed in [20]). The ubiquitin polypeptide addition to target proteins is carried out by a three-step enzymatic cascade: ubiquitin is first activated by an E1 ubiquitin-activating enzyme, transferred to an E2 ubiquitin-conjugating enzyme, and then bound to the substrate by a specific E3 ubiquitin ligase. Multiple HOX proteins have been reported to be post-translationally ubiquitinated by the CUL4 ubiquitin ligase promoting their proteasome-dependent degradation [21,22]. Similarly, HOXC10 might be regulated by the anaphase promoting complex (APC) in an ubiquitin-dependent and proteasomal manner and its abundance was shown to oscillate during the cell cycle [23].
In total, data concerning the regulation of HOX protein relating to their transcriptional activity, subcellular localization, stability or degradation remain surprisingly sparse. In recent years, proteome-wide interactomic approaches have enabled investigation of protein-protein interactions and two large screenings centred on HOX proteins have been reported to date, about UBX in drosophila and Hoxa1 in mammals. These analyses highlighted a number of candidate interactors which could be involved in the regulation of HOX activity [24,25]. Within 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, but surprisingly this interaction does not seem to lead to Hoxa2 activity modulation or degradation but instead impacts RCHY1 stability itself [26].

Here, we report a novel interaction involving Hoxa2 and a subunit of the KPC complex, KPC2. The KPC (kip1 ubiquitination promoting complex) complex consists in 2 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 poly-ubiquitinated proteins and the proteasome [27,28]. The KPC complex influences cell cycle via p27Kip1 regulation [27], a cyclin-dependent kinase inhibitor promoting the G1/S transition. KPC was shown to interact with the cytoplasmic form of p27Kip1 (pp27ser10), mediating its ubiquitination and leading to its proteasomal degradation [27-30]. Our data indicate that contrarily to its negative impact on p27Kip1 stability, the KPC complex does not seem to negatively regulate HOXA2 stability but instead impacts on 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 Hoxa2 expression.
Material and Methods

Plasmid constructs

Gateway® expression vectors for AD-Hoxa2, DB-Hoxa2 and FLAG-Hoxa2 [26], construction of expression vectors for Hoxa2 [31], pCMVlacZ [32], pCMV-PBX1a [33], pCS2-Prep1 [34], Hoxa2 r4 HRE enhancer [35] and the pKS-Hoxa2 [36] plasmids have been described elsewhere. Gateway® entry vectors (pEnt) for human KPC2 (refers as UBAC1) was obtained from the hORFeome v3.1 (http://horfdb.dfci.harvard.edu) [37] and HOX1A, HOX2A, HOX3A, HOX4C, HOX5B, HOXD10 and HOXC11 genes 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), that for p27Kip1 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, p27Kip1 and KPC1, were PCR-amplified using the primers listed in Table 1 and previously described templates. The resulting PCR products were inserted into the pDON223 vector using the Gateway® Technology from Invitrogen to generate the corresponding pEnt vectors. The resulting 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 and GST-KPC2 (pDest-GST N-terminal [39]), VN_{173}KPC1, VN_{173}p27Kip1, VN_{173}human-HOX and VN_{173}Hoxa2-deletion derivatives (pDest-VN_{173} [24]), and VC_{155}KPC2 (pDest-VC_{155} [24]).

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

Two-hybrid screening

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

Cell culture, transfection and treatments

Cultured cells were maintained at 37°C, in a humidified atmosphere with 5% CO₂. 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 (#15140-122, GibCO). 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 (#474970, Calbiochem) or with DMSO as control for periods of 7-15 h. For half-life measurements, 24 h after transfection, the proteasome was inhibited for 4 h as previously described then treated with 200 µg/ml of cycloheximide (#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 in DMSO (#L2913, Sigma), or with DMSO as control, for a period of 16 h.

Protein abundance analysis and Western Blot

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 pDestGST. Cells were lysed for 20 minutes 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 were centrifuged for 5 minutes at 1000 g at 4°C. Supernatants were recovered, equal amounts of proteins were boiled 5 min at 95°C in Laemmli loading buffer (10 % SDS, 30 % glycerol, 350 mM Tris-Cl pH 6.8, 600 mM DTT, 0.1 % bromophenol blue) and loaded on SDS-PAGE for electrophoresis. Proteins were then transferred onto a nitrocellulose membrane (#10600002, Amersham Biosciences). Membranes were blocked in 10 % low-fat milk. Anti-FLAG primary antibody (M2) (#F1804, Sigma) and anti-GST primary antibody (GST-2) (#G1160, Sigma) were used at 1:5000 dilution. Goat anti-mouse secondary antibody was HRP-conjugated and used at a 1 : 10 000 dilution (#sc-2005, Santa Cruz). For beta-actin detection, HRP conjugated anti-β-actin was used at a 1 : 20 000 dilution (#A3854, Sigma). Finally, membranes were treated with a chemiluminescence detection system (#NEL104001EA, PerkinElmer) and exposed to photographic films.

Relative protein quantification was carried out using ImageJ software.

Protein co-precipitation

HEK293T cells were transfected with 500 ng of FLAG-HOXA2 and 500 ng of GST alone or GST-KPC2 vectors. Forty-eight hours after transfection, cells were lysed for 20 minutes at 4 °C in ice-cold IPLS lysis buffer including protease inhibitor cocktail (#11873580001, Roche). Cells lysates were centrifuged for 5 min at 1000 g at 4°C. Supernatants were recovered and samples were incubated overnight on a rotating wheel at 4°C with glutathione-agarose beads (#G4510, Sigma) pre-washed three times with ice-cold IPLS lysis buffer. Beads were washed three times with ice-cold IPLS. Beads were supplemented with Laemmili loading buffer for SDS-PAGE and boiled 5 minutes at 95 °C. Samples were centrifuged and analyzed by Western blotting. As controls, in parallel to protein co-precipitation, expression of fusion proteins was confirmed by Western blotting.

Bimolecular Fluorescence Complementation assay (BiFC)

COS-7 cells were cultured on glass coverslips and transtected 24 h after plating with distinct combinations of pExpVN173 and pExpVC155 vectors for the fusion proteins to be tested and/or pDestVN173 and pDestVC155 empty controls, each at 500 ng. Twenty-four hours after transfection, cells were rinsed in PBS solution and fixed for 20 minutes with 4% paraformaldehyde (PFA) in PBS, then rinsed twice in TBS (50mM Tris, 155 mM NaCl, pH 7.5) containing 0.1% Triton X100. Coverslips were then used for immunofluorescence detection of proteins or rinsed once in TB (50mM Tris pH 7.5) prior to mounting.
**Immunocytofluorescence**

Transfected COS-7 cells were cultured on glass coverslips, rinsed in PBS and fixed for 20 minutes with 4 % PFA in PBS. Cells were further blocked with 10 % low-fat milk in TBS-0.1 % Triton X100 solution for 30-45 minutes at RT, followed by overnight incubation in TBS-0.1% Triton X100-1% low-fat milk solution at 4°C, with mouse anti-FLAG (M2) (#F1804, Sigma), rabbit anti-GST (#G7781, Sigma) or rabbit anti-HOXA2 (#HPA029774, Sigma) used at 1:100. Cells were rinsed three times for 10 minutes in TBS-0.1% Triton X100 and incubated for 45 minutes at RT with Alexa Fluor® 488 Donkey Anti-Rabbit or Alexa Fluor®555 Anti-rabbit and/or Alexa Fluor®555 Anti-mouse (A-21206, #4413S, #4409S, Life technologies) in TBS-0.1% Triton X100 solution. Cells were rinsed twice with TBS-0.1 % Triton X100, once with TB and glass coverslips were mounted in Vectashield®-DAPI medium (Vector laboratories).

**Imaging**

Glass coverslips were mounted in Vectashield®-DAPI medium (Vector laboratories). Slides were then analyzed by epifluorescence (Axioskop 2, Zeiss) or confocal microscopy (LSM710, Zeiss, Jena, Germany). Fluorescence signals were quantified using ImageJ software. BiFC fluorescence from the test and the control conditions were quantified and the interaction was considered as positive when the tested interaction emitted at least 3 times more fluorescence than the 3 control conditions.

**β- Galactosidase and Luciferase assays.**

HEK293T cells were transfected with 250 ng of reporter plasmid, 50 ng of PREP, 50 ng of PBX and 50 ng of Hoxa2 and/or 75 ng of GST-KPC2 vectors. To avoid experimental variations due to transfection efficiency, an internal standard reporter corresponding to the lacZ gene under the control of a constitutive CMV promoter (pCMVlacZ, [21]) was also added in cotransfection experiments (25 ng). Cells were harvested 48 h after transfection for enzymatic assays. Lysis and enzymatic activity dosages were performed with the β-gal reporter gene assay kit (#11758241001, Roche) and the luciferase reporter gene assay kit (#11 669893001, Roche), respectively. Luciferase activity was then normalized to that of β-galactosidase.

**In situ Hybridization (ISH), RNA extraction and RT-PCR from mouse embryos.**

Experimental procedures on animals were performed in accordance with the guidelines of the Animal Experimentation Ethics Committee of the Université catholique de Louvain and in agreement with the European directive 2010/63/UE. Mice were maintained and fed under standard conditions on a 14 h light/10 h dark cycle. All the experiments were carried out on adult CD1 female mice mated overnight with adult CD1 males. When plugs were detected, pregnant mice were killed 10.5 or 11.5 days post-coitus by gas inhalation and embryos were rapidly dissected while kept on ice. Embryos for ISH were rinsed in PBS, fixed overnight with 4 % PFA in PBS at 4 °C, rinsed three times 20 minutes in PBS. For cryopreservation, embryos were incubated 2h in 10 % sucrose/PBS and overnight in 20 % sucrose/PBS, then embedded in OCT medium (Shandon CryomatrixTM, Thermo Electron, France), frozen on dry ice and stored at -80 °C.
Seven sets of 20 µm serial transversal or sagittal cryosections per embryos were cut on a Leica CM 3050S cryostat. Gene expression was detected using digoxigenin-labelled RNA probes as previously described by Hutlet et al. 2014 [40].

For probe synthesis, the pKS-Hoxa2 and the pCR2.1-TOPO-Kpc2 plasmid were linearized with EcoRI and SpeI, respectively, and the probes were transcribed with the T3 and the T7 polymerases, respectively. Hybridized sections were analyzed on a Leica DM2500 microscope, and pictures were captured with a Leica DFC420C camera.

For the immunochemistry on the hybridized sections, the slides were processed as previously described for the immunocytofluorescence. The anti-islet1/2 antibody was used at a 1/500 dilution (#39.4D5, DSHB).

Total RNA was extracted with the High Pure RNA Isolation Kit (Roche) according to the manufacturer’s instructions. RNA was reverse transcribed using a reaction mix containing 200 ng random hexamer primers (#SO142, Life technologies), 1 mM dNTP (#R0191, Life technologies), 10 U riboLock RNase inhibitor (#EO0381, Life technologies), 100 U RevertAid Reverse transcriptase and the provided buffer (#EP0441, Life technologies). The mixture was incubated 10 minutes at 25°C, 1 h at 42°C and 10 minutes at 80°C. Specific intron-spanning primers were designed based on NCBI database sequences listed in Table 2. PCR reaction mix contained 1.25 U Taq DNA Polymerase (#EP0402, Life technologies) with the provided buffer supplemented with 1.9 mM MgCl₂, 250 µM dNTP (#R0191, Life technologies) and 1.25 mM of each primer. The amplification program started with an activation step at 95°C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, hybridization at primers specific temperatures (Table 2) for 15 seconds and elongation at 72 °C for 45 seconds. The last cycle was completed by a final elongation step at 72 °C for 7 minutes. For Hoxa2 amplification, PCR reaction mix contained 1 U Expand Long Template (Roche) with the provided buffer (n°1), 400 µM dNTP (#R0191, Life technologies) and 250 nM of each primer. The amplification program started with an activation step at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, hybridization at 55 °C for 15 seconds and elongation at 68°C for 45 seconds. The last cycle was completed by a final elongation step at 68°C for 7 minutes.

Statistical analysis

All statistical analyses were performed with JMP11 software. Luciferase activation and HOXA2 subcellular distribution were analyzed using a mixed model using the experiment as a random parameter, KPC2 presence as a fix parameter and Log(Luciferase/β-Gal) or fluorescence relative intensity as the responses, respectively. A Kruskal-Wallis test was used to compare the BiFC subcellular signal under LMB treatment.
Results

Identification of KPC2 as a Hoxa2 interactor

In an effort to identify novel Hoxa2 regulators, we performed a yeast two-hybrid screen with the human ORFeome v3.1, an extensive set of cloned human open reading frames. The complete ORFeome was tested against Hoxa2, and KPC2 was identified as a candidate Hoxa2 interactor. To validate the Hoxa2-KPC2 interaction, co-precipitation of proteins was assayed from transiently transfected HEK293T cells. A FLAG-Hoxa2 fusion protein was co-expressed with glutathione S-transferase (GST)-tagged KPC2, which was specifically precipitated using glutathione-agarose beads. In the presence of GST-KPC2, a band corresponding to FLAG-Hoxa2 was detected by western blotting demonstrating that the two proteins can form a complex in HEK293T cells (Figure 1). As a negative control, when an unfused GST polypeptide was expressed in combination with FLAG-Hoxa2, Western blotting detection for the FLAG epitope showed little or no signal.

Different isoforms of Kpc2 are expressed during mouse embryonic development

The data above indicate that HOXA2 and KPC2, once co-expressed in cells, are able to interact. Consequently, it was of interest to investigate whether this interaction could take place in a physiological context, i.e. to determine if both genes show overlapping expression. As Hoxa2 expression and functions have essentially been described during mouse embryogenesis and, more specifically, after gastrulation, we investigated whether Kpc2 is also expressed between E8.5 to E12.5. First, to provide a qualitative response as to the expression of Kpc2 gene in mouse embryos, RT-PCRs were performed on different pools of embryonic cDNA using primers designed on the 7th and the 10th exons of Kpc2 (Figure 2A-B). The PCR amplification provided 3 DNA fragments that could correspond to different isoforms (Figure 2A-B). To experimentally verify that these fragments correspond to Kpc2, the PCR products were sequenced (Supplemental Figure 1). The upper band corresponded to the validated mRNA (NM_133835.2). The middle band lacked the sequence coding the exon 9 and matched the isoform X1 (XM_006498464.1). Finally, the lower band was devoid of the exon 8 and 9 compared to the full length mRNA and matched the isoform X2 (XM_006498465.1). In conclusion, Kpc2 was shown to be expressed during mouse embryogenesis and the presence of different transcripts was highlighted.

Kpc2 presents a restricted expression pattern in E10.5 and E11.5 mouse embryos which partially overlaps with that for Hoxa2

As Kcp2 is expressed at different embryonic stages in mouse, we were interested in further examining the Kpc2 transcript distribution and whether Hoxa2 and Kpc2 patterns overlap. We therefore analyzed the expression of Kpc2 mRNA on sagittal and transversal sections in E10.5 and E11.5 mouse embryos. Kpc2 expression showed a similar restricted profile at both E10.5 (Figure 2C, K, M, O, Q) and E11.5 (Figure 2D, E, G, I, S, U). Abundant Kpc2 expression was consistently detected in the ventral neural tube (Figure 2H/J/R/V). Caudally, Kpc2 mRNA was also detected in dorsal root ganglia (Figure 2E, G, Q, U). Moreover, its expression was observed on either sides of the otic vesicle (OT) corresponding to the
facio-acoustic (VII-VIII) ganglion complex and the superior and inferior glossopharyngeal (IX) ganglia (Figure 2C, O, D, S).

Observation of Kpc2-positive cells in the ventral area (Figure 2G, I, Q, U) and on either sides of the neural tube (Figure 2E, G, Q, U) could correspond to expression in motor neurons and dorsal root ganglia, respectively. To test this possibility, we performed immunofluorescence staining of E11.5 embryo with anti-Islet-1/2 antibody, a marker of the motor neurons and the dorsal root ganglia [41,42] and confirmed that Kpc2-stained cells were also positive for Islet-1/2 (Figure 2F, H, J). It is important to notice that a few cells were autofluorescent (to discriminate autofluorescent cells from Islet-1/2 stained cells, see Supplemental Figure 2).

To determine whether Hoxa2 and Kpc2 expression overlap at E10.5 and E11.5, in situ hybridizations were conducted on serial sections of the same embryo. At the boundary between the rhombomere (r) 1 and 2, corresponding to the anterior Hoxa2 expression limit (Figure 2L), staining associated to Kpc2 was also observed in the ventral neural tube (Figure 2K). As shown in Figure 2M-N, both Hoxa2 and Kpc2 are co-expressed at the level of the anterior hindbrain and similar results were observed more posteriorly, in the spinal cord (2Q-R, U-V). However, we could notice that in the ventral part of the neural tube, where Kpc2 staining is strong, Hoxa2 expression although distinguishable, is relatively weak (Figure 2M-N, Q-R). Finally, the facio-acoustic neural crest complex also showed a signal associated to both genes of interest (Figure 2O-P, S-T).

In conclusion, these results highlight that the expression of Kpc2 and Hoxa2 overlap during mouse embryogenesis between E8.5 and E12.5. The Kpc2 expression pattern is restricted and in particular pertains to the motor neurons, the dorsal root ganglia, the facio-acoustic (VII-VIII) ganglion complex and the superior and inferior glossopharyngeal nerve (IX).

**HOXA2 has a short half-life and is targeted for proteasomal degradation independently of the KPC complex.**

Since KPC2 interacts with HOXA2, it appeared reasonable to hypothesize that HOXA2 ubiquitination state and/or stability might be regulated by KPC2. To precisely look into the HOXA2 stability, we started by quantifying HOXA2 half-life. HEK293T cells transiently transfected with FLAG-HOXA2 were treated with cycloheximide (CHX) (200µg/ml) to inhibit de novo protein synthesis. Cells were collected at different timepoints (0, 1.5, 3 and 4.5h) and protein lysates were analyzed by Western blotting. As shown in Figure 3A, the level of HOXA2 quickly decreased within 3h of CHX treatment. Densitometry analysis normalized to β-actin levels indicated that HOXA2 half-life was about 3.7 ± 0.42h demonstrating that HOXA2 is a short-lived protein in this cell context.

To further address whether the proteasome contributes to the HOXA2 decay and therefore conditions its short half-life, HOXA2 levels were assessed in the presence of proteasome inhibition. HEK293T cells were pre-treated with the proteasome inhibitor MG132 for 4h before adding CHX, and harvested at different timepoints. As a consequence, the quick decrease in HOXA2 was no longer observed, the protein still appearing abundant after 4.5 h, a time at which HOXA2 became barely detectable in the
absence of proteasome inhibition (Figure 3A). These data strongly support that HOXA2 is a target for proteasomal degradation.

It is of note that three distinct forms of FLAG-HOXA2 protein could be detected and that a shift in relative band intensity was observed between the examined timepoints. Indeed, while comparing band patterns between 0 and 1.5h timepoints, the lowest band, which is the most abundant at first, becomes weaker while the upper band becomes more intense. At later timepoints, the upper band remains predominant while the lower ones progressively fade down to become hardly detected. The heaviest form is the last to disappear between 3h and 4.5h of CHX treatment. Together these observations are suggestive of a conversion of the smaller Hoxa2 forms into the heavier one prior to proteasome-mediated degradation. This also suggests that the stability versus decay of Hoxa2 is regulated by post-translational modifications.

Next, we tested whether the interaction with KPC2 induces HOXA2 destabilization. To this end, we transfected HEK293T cells with HOXA2 in combination with GST alone or GST-KPC2 and monitored HOXA2 half-life. As shown in figure 3B, a fast degradation of HOXA2 was observed as previously shown. However, HOXA2 did not seem to be destabilized faster in the presence of KPC2. As a complement, we investigated whether HOXA2 post-translational modifications, and specifically the ubiquitination status of HOXA2 could be modulated by KPC2. Cells were treated with the proteasomal inhibitor MG132 for 15h before harvest. Several higher molecular weight bands associated to the FLAG tag were detected, presumably corresponding to ubiquitine-conjugated forms of HOXA2. In an attempt to confirm the molecular identity of these bands, His-tagged ubiquitin was overexpressed together with HOXA2. Nickel-purified, ubiquitin-conjugated proteins were analyzed by Western blotting with the FLAG antibody, but unfortunately, the ubiquitinated forms of HOXA2 were barely retrieved after purification (data not shown).

KPC2 works together with KPC1 to form the KPC ubiquitin ligase complex. KPC1 is known to function as the catalytic subunit of the complex whereas KPC2 was shown to stabilize KPC1, to recruit polyubiquitinated proteins and to interact with the 26S proteasome, i.e. KPC2 acts as an adapter in the KPC complex. Whether KPC1 and KPC2 together were involved in mediating HOXA2 decay was then addressed. Again, the HOXA2 abundance did not appear to be diminished upon expression of both KPC subunits. Rather, we observed a strong increase in HOXA2 abundance upon KPC1 and KPC1/2 overexpression (Figure 3C). In conclusion, it seems that the KPC complex does not target HOXA2 for its proteasomal degradation but instead increases its stability.

KPC2 decreases Hoxa2 transcriptional activity.

Since HOXA2 appears to be slightly stabilized by KPC2, we addressed the influence KPC2 might exert on HOXA2 transcriptional activity. Hoxa2 transcriptional activity was tested by a luciferase reporter assay in HEK293T cells. A reporter construct containing a Hox responsive element was transfected in combination with vectors coding for Hoxa2 and GST-KPC2. Moreover, expression vectors for the Hoxa2 cofactors, PBX1a and PREP1, were added to provide a full activation of the reporter [35]. As a result, we observed a significant activation induced by Hoxa2. Moreover, we observed that the activity of Hoxa2
was lowered in presence of KPC2. Indeed, a significant decrease of 37% ± 4% in Hoxa2 transcriptional activity was measured (Figure 4). As a control, KPC2 alone did not affect the expression of the reporter construct.

**KPC2 relocalizes HOXA2 out of the nucleus**

Since KPC2 expression negatively impacts on the HOXA2 activity but does not stimulate its decay, we next examined the cellular localization of the both proteins as well as of their interaction. Whereas HOXA2 mainly exhibited a diffuse nuclear distribution, KPC2 was mainly shown to be present in the cytoplasm (Figure 5A-B).

The subcellular localization of the KPC2-HOXA2 interaction was then addressed by Bimolecular Fluorescence Complementation (BiFC) assay. BiFC relies on the ability of N- and C-terminal parts of the Venus protein to emit detectable fluorescence once they are brought in close proximity when fused to interacting proteins. The first interactor is fused downstream of the N-terminal portion of the Venus, C-terminally to the 173173 amino acid (VN173), while the second is C-terminally fused to the C-terminal moiety of Venus (amino acids 155 to 243; VC155). Fluorescence emission not only validate the direct interaction between two partner proteins but also indicate where they actually interact in live cells or in vivo.

As a preliminary control, BiFC was assayed for the well-established interactions between KPC2 and KPC1 or p27Kip1. The KPC1 ORF and p27Kip1 ORF were fused downstream VN173 (VN173-KPC1, VN173-p27Kip1), while the KPC2 ORF was C-terminally fused to VC155 (VC155-KPC2). Several controls supported that the N- and C-terminal Venus fragments did not reassociate if not fused to interacting proteins. KPC1 and KPC2 were previously shown to extensively colocalize in the cytoplasm [27] and, as expected, the VN173-KPC1 and VC155-KPC2 fusion proteins provided a diffuse or punctuated fluorescence in the cytoplasm (Figure 5C). Surprisingly, for the VN173-p27Kip1-VC155-KPC2 combination, the vast majority of the emitted signal was localized in the nucleus (Figure 5C). It was previously suggested that p27Kip1, once exported from the nucleus, interacts and is ubiquitinated by the KPC complex [27,28]. However, our results suggest that the KPC2-p27Kip1 interaction already takes place in the nucleus.

The HOXA2 ORF was fused downstream VN173 (VN173-HOXA2), and tested in BiFC with VC155-KPC2. In this case, the emitted signal appeared distributed both in the nucleus and in the cytoplasm (Figure 5C). Again as negative controls, the VN173-HOXA2/VC155, VN173/VC155-KPC2 and VN173/VC155 combinations showed no or very weak fluorescence compared to the corresponding test condition VN173-HOXA2/VC155-KPC2 (Supplemental Figure 3). As mentioned above, HOXA2 was mainly nuclear when expressed alone, but its interaction with KPC2 showed a positive cytoplasmic signal in BiFC. This opened the possibility that HOXA2 could be relocalized upon KPC2 interaction. The HOXA2 localization was examined by immunofluorescence in the COS-7 cell line, in the absence or the presence of KPC2. VN173-HOXA2 was transiently transfected in combination with VC155-KPC2 or not. As shown in Figure 6A, when expressed alone VN173-HOXA2 exhibited a diffuse or punctuated nuclear distribution. However, transfection of VC155-KPC2 produced a striking redistribution of VN173-HOXA2 towards the cytoplasm (Figure 6A). Quantification of the HOXA2 distribution measured using imageJ software confirmed a
significant redistribution of HOXA2 in the cytoplasm upon KPC2 expression (Figure 6B). These results suggest that KPC2 can modulate HOXA2 subcellular localization and sequester HOXA2 in the cytoplasm.

Whether HOXA2 is sequestered in the cytoplasm by KPC2 right after its translation or shuttled back to the cytoplasm after entering the nucleus was the next issue. To answer this question, we investigated where the interaction first takes place, i.e. we determined (i) if HOXA2 first interacts with KPC2 in the cytoplasm, the interactors being then relocated to the nucleus or (ii) if KPC2 first interacts with HOXA2 in the nucleus, the partners being then shuttled to the cytoplasm. To investigate in what compartment the HOXA2-KPC2 interaction initially takes place, we inhibited protein nuclear export with leptomycin B (LMB). LMB is a drug known to prevent nuclear export of many proteins by inhibiting the function of CRM1, a receptor of nuclear export signals. Whereas immunofluorescence revealed that HOXA2 is mainly nuclear, a small fraction of the signal was also detected in the cytoplasm. A 16h incubation with LMB abolished this staining corresponding to the cytoplasmic HOXA2 suggesting that the cytoplasmic fraction of HOXA2 translocates from the nucleus (Figure 6A). Moreover, LMB treatment did not prevent the KPC2-HOXA2 interaction to take place, but the signal now appeared to be massively nuclear. Therefore, LMB prevented the interaction from translocating to the cytoplasm (Figure 6A). These results were quantified as the relative nucleus/cytoplasm BiFC fluorescence ratio. As shown in Figure 6C, LMB treatment clearly reduced the cytoplasmic interaction of KPC2 and HOXA2. This together supports that the HOXA2 and KPC2 proteins first associate in the nucleus before being shuttled back to the cytoplasm. This also excludes that HOXA2 is trapped by KPC2 right after its translation and that KPC2 impairs Hoxa2 nuclear entry.

The molecular determinants for KPC2 binding are spread in severable Hoxa2 regions

To reveal which regions in the Hoxa2 protein are involved in KPC2 interaction, we designed four Hoxa2 deletion variants where residues from the N-terminal (Hoxa2ΔN [138-372]), C-terminal (Hoxa2ΔC [1-198]), both terminal domains (Hoxa2ΔHD [139-198]) or the homeodomain (Hoxa2ΔHD Δ[Δ139-198]) were removed, respectively (Figure 7A).

We then assayed the variants for their interaction with KPC2 by BiFC. As carried out previously, Hoxa2 and the deletion mutants were fused to VN173, while KPC2 was fused to VC155. Emitted fluorescence was observed with Hoxa2ΔHD and KPC2 suggesting that the homeodomain alone is sufficient to mediate the interaction. In accordance with this observation, Hoxa2ΔN and Hoxa2ΔC, both containing the homeodomain, also interact with KPC2. Moreover, Hoxa2ΔHD was also shown to mediate KPC2 interaction suggesting that a domain different from the HD contributes to KPC2 binding.

Interestingly, although all Hoxa2 variants but Hoxa2ΔHD lacking the homeodomain showed a nuclear localization (Supplemental Figure 4), the BiFC signal emitted upon interaction with KPC2 displayed distinct intracellular distribution according to the Hoxa2 variant. Indeed, whereas Hoxa2ΔC provided both nuclear and cytoplasmic BiFC fluorescence, Hoxa2ΔN and Hoxa2ΔHD presented exclusively nuclear interaction signals. This suggests that a region located in the N-terminal part of Hoxa2 is necessary for its KPC2-dependent nuclear export. For what concerns Hoxa2ΔHD, this protein most likely lacks a
homeodomain-located nuclear localization signal (NLS) [43]. It consistently displayed a cytoplasmic BiFC pattern.

In conclusion, the molecular determinants contributing to the KPC2-Hoxa2 interaction are not included in a short discrete protein motive but are rather spread among at least two severable Hoxa2 protein regions. In addition, since all Hoxa2 deletion variants tested conserved the ability to interact with KPC2, one can conclude that distinct subsets of molecular contacts are sufficient to support the interaction with KPC2 (Figure 7). Finally, the N-terminal region of Hoxa2 is required for its KPC2-mediated nuclear export.

**KPC2 binding is common to HOX proteins**

Based on the finding that the homeodomain of Hoxa2 contributes to the interaction with KPC2, and considering HOX proteins share important sequence identity in their homeodomain, we addressed whether HOX proteins other than HOXA2 were capable of interacting with KPC2. Different HOX proteins from distinct paralogue groups were tested for their KPC2 binding properties by BiFC. All the tested proteins (HOXA1, HOXB1, HOXB2, HOXA3, HOXC4, HOXB5, HOXD10 and HOXC11) showed fluorescent signal (Figure 8). However, the pattern of BiFC signal differed according to the HOX involved. While HOXA1, HOXB1 HOXA3, HOXC4 and HOXB5 mainly showed a clear nuclear interaction, HOXB2, HOXD10 and HOXC11 like HOXA2, mainly interacted with KPC2 in the cytoplasm (Figure 8). These distinctive patterns may underline that although KPC2 binding is a general property of HOX proteins not all of them might be relocated to the cytoplasm upon KPC2 interaction.
Discussion

Hox proteins play important roles in mammalian development as well as organ homeostasis and cell differentiation at adulthood. As transcription factors, their transcriptional activity should be finely tuned to properly control the vast range of processes under their control. In fact, Hox proteins have been shown to display versatile activities according to the multiple cellular contexts they are involved in [4] and subtle deregulation of Hox expression can alter development processes or cell fates and lead to malformations or pathologies [44]. At a protein level, intra-cellular localization or stabilization versus degradation are ways to control transcription factor activities which have barely been addressed for Hox proteins. In this paper, we identified the ubiquitin-ligase adapter protein KPC2 as interacting with HOXA2 and influencing its transcriptional activity and intracellular localization.

While looking for Hoxa2-interacting proteins susceptible to modulate Hoxa2 activity, a large-scale yeast-two hybrid screen allowed identifying candidate interactors among which the KPC2 protein, a KPC-ubiquitin-ligase complex component. The KPC2-HOXA2 interaction was further confirmed by two complementary methods, namely co-precipitation and BiFC, to occur in mammalian cells. As being involved in an ubiquitin-ligase complex, KPC2 was hypothesized to negatively influence HOXA2 stability. The ubiquitine-proteasome machinery indeed controls transcription factors by fine-tuning their steady state and inducing their degradation when their function is no longer appropriate and we showed here that HOXA2 is a short-lived protein that is indeed regulated by the proteasome. However, the short half-life and rapid decay of HOXA2 seems independent of the KPC complex since, KPC2 or KPC1-KPC2 were not shown to induce the degradation of HOXA2. Nonetheless, we further showed that KPC2 expression caused a drop in Hoxa2 transcriptional activity which could be associated with its escape from the nucleus and redistribution in the cytoplasm. In addition, while inhibiting the nuclear export protein pathway mediated by CRM1, the KPC2-HOXA2 interaction is almost exclusively detected in the nucleus, whereas it shows both a nuclear and cytoplasmic pattern in the absence of inhibition. Together this supports (i) that the newly identified HOXA2-KPC2 interaction first takes place in the nucleus, (ii) that KPC2 promotes the export of HOXA2 out of the nucleus and (iii) that this relocalization correlates with a decrease in HOXA2 activity.

Currently, except the proteasomal degradation of p27Kip1, no other role has been described for the KPC complex. The KPC2-p27Kip1 interaction was suggested to take place in the cytoplasm where p27Kip1 proteolysis takes place [27-29]. Surprisingly, the BiFC assay presented here highlights that the interaction mediated between KPC2 and p27Kip1 was mainly nuclear. On the other hand, HOXA2, known to be mainly nuclear, was notably shown to interact with KPC2 in the cytoplasm. For both p27Kip1 and HOXA2, their interaction with KPC2 localized in unexpected subcellular compartments. We suggest that KPC2 might be a shuttling protein involved in the redistribution of its partners. In this model, KPC2 would enter the nucleus, bind p27Kip1 and/or HOXA2 and participate to their cytoplasmic export.

In that context, ubiquitine ligases and the subsequent ubiquitination process were reported to lead to substrate regulation independently of their proteasomal proteolysis. For example, ubiquitination can
regulate the recruitment of partners, induce nuclear entry or modify transcription factor activity by regulating their DNA binding both in positive or negative way (reviewed in [45]). In particular, functions unrelated to the proteasome-mediated degradation have already been reported for UBA-UbL proteins family. Proteins of this family, which includes KPC2 [28], display UBA and UbL domains able to interact with ubiquitinated substrates and subunits of the proteasome, respectively. UBA-UbL proteins have been proposed to act as shuttle-factors involved in the substrate degradation while delivering them to the proteasome. However, in addition to their role in proteasome-mediated degradation of their substrates, functions in autophagy, endocytosis, exocytosis, nucleotide excision repair, spindle pole body duplication or cell growth have been highlighted for UBA-UbL proteins (reviewed in [46]). More precisely, Plic-1 belonging to this family, was shown be involved in the aggresome formation [47], to interact with polyubiquitinated TDP-43 causing its redistribution and increasing its presence in TDP-43 aggregates [48]. Consequently, UBA-UbL proteins previously reported as shuttle-factors between substrate proteins and the proteasome, could be more largely involved in the intracellular relocalization of binding partners, independently of proteasomal targeting, as it is the case for Plic1 and TDP-43 or KPC2 and HOXA2.

Correlatively to its interaction with KPC2 and its nuclear export, we observed a decrease in Hoxa2 transcriptional activity. Indeed, in the presence of KPC2, a significant reduction in Hoxa2 transcriptional activity was measured. Other reports show that transcription factor activities can be influenced by the regulation of their subcellular distribution. This typically involves the nuclear entry of activated transcription factors as a consequence of cell-signalling events like for STATs, Smads, Gli or β-catenin. OGT-mediated transcription factor glycosylation has also been reported to modulate the intracellular distribution and thereby activity of transcription factors [49,50]. Export from the nucleus has also been reported to modulate transcription factor activity. For example, Snail functions have been shown to be controlled by its intracellular localization. Indeed, phosphorylation on a serine rich sequence adjacent to its NES exports Snail from the nucleus by a CRM1-dependent mechanism. This shift out of the nucleus consequently blocks Snail access to the target promoters [51]. The nuclear export of the homeodomain transcription factor Engrailed has been associated to its atypical secretion out of the cell [52]. To our knowledge, the regulated nuclear export of a Hox protein has never been reported. However, the nucleocytoplasmic shuttling of TALE proteins which regulate Hox protein activity and DNA binding specificity has been identified as a way to modulate Hox function in a context-specific manner [53]. Conversely, Hox proteins can also be viewed as context-specific modulators of more pleiotropic transcription factors. Recently, Hoxa2 was shown to influence the fate of branchial arches by regulating Meis DNA binding to specific sites [9]. The nucleocytoplasmic shuttling of Hoxa2 could contribute to such context-specific modulation of TALE protein activity. Consistently, we showed that KPC2 is expressed with a restricted pattern during mouse embryogenesis. Indeed, motor-neurons, dorsal root ganglia and ganglion (VII-IX) were reproducibly stained in ISH, a pattern in accordance with results provided in the Edinburgh Mouse Atlas of Gene Expression (EMAGE, http://www.emouseatlas.org/emage/home.php). Indeed, at E14.5, Kpc2 showed a strong staining for dorsal root ganglia and a moderate signal in different ganglia (V-X)[54]. The Kpc2 expression pattern...
overlaps with that of Hoxa2 which supports that Kpc2 could regulate Hoxa2 transcriptional activity in specific contexts in vivo.

To test whether HOXA2 redistribution induced by KPC2 is a consequence of the direct interaction between HOXA2 and KPC2, we constructed several Hoxa2 deletions derivatives which lack the homeodomain or the C and/or N terminal region of the homedomain. Since all the tested deletion derivatives still interact with KPC2, our data indicate that the KPC2-Hoxa2 interaction require motives spread among at least 2 regions: the homeodomain and the C- or/and N-terminal domain. Moreover, our data support that the N-terminal part of Hoxa2 is actually required for its KPC2-dependent nuclear export.

Since the homeodomain is involved in the interaction with KPC2 and is conserved in HOX proteins, the question arose whether KPC2 could interact with all HOX proteins. Nine HOX proteins were tested which all interact with KPC2. However, we found that these interactions, contrarily to what was observed for Hoxa2, do not always take place in both the nucleus and the cytoplasm. Rather, for some HOX proteins, the interaction appears to mostly if not exclusively occur in the nucleus, which thus supports that although multiple if not all HOX proteins share the capacity to interact with KPC2, only a subset among them are consequently relocated into the cytoplasm and impaired in their transcriptional activity.

In summary, we identified KPC2 as a new interactor of Hoxa2 and provided evidence that KPC2 could contribute to regulate HOXA2 activity, KPC2 expression inducing a nuclear-to-cytoplasm protein redistribution correlated to a diminution in Hoxa2 transcriptional activity.
Author Contributions

The experimental plan was conceived by LB, IB, JCT and RR. Most experiments were performed by LB. The two hybrid screen was performed by IB. MH and ND obtained some of the plasmid constructs. The co-precipitation assay was performed by AD. LB and RR wrote the paper.
Legends to the figures

Figure 1. Hoxa2 interacts with KPC2. HEK293T cells were co-transfected with expression vectors for FLAG-Hoxa2 and GST-KPC2 or GST alone. Forty-eight hours after transfection, cell lysates were subjected to western blotting analyses to detect protein expression (“Input”, β-actin used as a protein load control). Protein interactions were then verified by co-precipitation on glutathione beads directed toward the GST tag. Eluted proteins were analyzed by western blotting to detect the presence of FLAG-Hoxa2 (“Co-precipitation”).

Figure 2. Kpc2 and Hoxa2 expression patterns overlap during mouse embryogenesis. (A) Schematic representation of Kpc2 mRNA and localization of the PCR primers used to amplify sequences corresponding to the three mRNA isoforms (X1, X2 and X3). Numbered boxes (1 to 10) correspond to the Kpc2 exons. “Start” and “Stop” indicate the relative position of the translation initiation and termination codons, respectively. (B) Detection of Hoxa2, Kpc2 and β-Actin transcripts from E8.5 to E12.5 embryos by RT-PCR. Arrowheads indicate the amplicons corresponding to the X1, X2 and X3 mRNA isoforms. (C-D) In situ hybridizations of Kpc2 on sagittal cryosection of E10.5 and E11.5 mouse embryos. (E-J) Sagittal and transversal cryosections of E11.5 mouse embryos hybridized for Kpc2 (E, G, I) and immunolabeled for Iselt-1/2 (red) (F, H, J). (K-R) In situ hybridization of Kpc2 (K,M,O, Q) and Hoxa2 (L, N, P, R) mRNAs on sagittal and transversal cryosections of E10.5. (S-V) In situ hybridization of Kpc2 (S, U) and Hoxa2 (T, V) mRNAs on sagittal and transversal cryosections of E11.5. Black arrows show facio-acoustic (VII-VIII) neural crest complex; red arrows show the superior and inferior glossopharyngeal nerve (IX); green arrows show the dorsal root ganglia and an orange arrow indicates the boundary between the r1 and r2 rhombomeres. Spinal cord transverse sections (G, H, Q, R, U, V) are surrounded by dashed line. OV, otic vesicle; RP, Rathke’s pouch. Scale bar = 100 μm

Figure 3. HOXA2 is a short-lived protein regulated by the proteasome independently of the KPC complex. (A-C) HEK293T cells were co-transfected with expression vectors coding for FLAG-HOXA2 and GST-KPC2, GST-KPC1 or GST. Cell lysates were then subjected to immunoblot analysis with antibodies against FLAG, GST and β-ACTIN. (A-B) To determine the HOXA2 half-life, cells were treated with cycloheximide (CHX) for the indicated time. The involvement of the proteasome in the RCHY1 degradation was assayed by treating cells with proteasome inhibitors (MG132) for 4 hours prior to inhibiting protein translation with CHX.

Figure 4. KPC2 reduces Hoxa2 transcriptional activity. HEK293T cells were transfected with a luciferase (Luc) reporter construct containing a Hoxa2-responsive element and a constitutive LacZ reporter as an internal control. Expression vectors for Hoxa2, GST-KPC2 or GST were added in combination. Expression vectors for the Hox cofactors Pbx1a and Prep1 were engaged in all transfections to promote a maximal Hoxa2-mediated reporter activation. The relative activity of the Luc reporter was quantified by luminometric assays for the luciferase (Luc) and galactosidase (Gal) enzymes and is presented as a Luc/Gal activity ratio. The Luc/Gal relative activity corresponding to the “GST alone” condition was set as the reference of “1”. Bars indicate the standard deviation (N=4, n=12-15). Asterisks indicate a significant impact of GST-KPC2 on the Hoxa2 activity (NS, non significant, *** p < 0.0001).
**Figure 5.** Bimolecular Fluorescence Complementation (BiFC) reveals KPC2 and HOXA2 interact in the nucleus and the cytoplasm. COS-7 cells were transfected with FLAG-HOXA2 (A) or GST-KPC2 (B) and subjected to immunocytochemistry with anti-FLAG and anti-GST respectively. (C) Bimolecular Fluorescence Complementation assay reveals the interactions in culture cells. COS-7 cells were transfected with VN\textsuperscript{173}KPC1, VN\textsuperscript{173}p27kip1 or VN\textsuperscript{173}HOXa2 and VC\textsuperscript{155}KPC2 coding vectors, as indicated. Upon interaction between the partner proteins, the VN\textsuperscript{173} and VC\textsuperscript{155} moieties of the Venus fluorescent protein brought together provide a fluorescent signal. Nuclei were stained with DAPI (blue).

**Figure 6.** KPC2-HOXA2 interaction initially takes place in the nucleus and is relocalized in the cytoplasm. (A) COS-7 cells were transfected with VN\textsuperscript{173}HOXA2 and VC\textsuperscript{155}KPC2 or VC\textsuperscript{155}, treated with LMB for 16h when indicated and subjected to immunocytochemistry with anti-HOXA2 antibody. Nuclei were stained with DAPI (blue). (B) Signal intensities from the nucleus and the cytoplasm were quantified and plotted. Asterisks indicates a significant impact of KPC2 on HOXA2 relative subcellular distribution (Mixt model, * p < 0.05 ). BiFC signal intensities from the nucleus and the cytoplasm were quantified and plotted. Asterisks indicate a significant impact of LMB on HOXA2-KPC2 interaction localization (Kruskal-Wallis, * p < 0.05 ).

**Figure 7.** Mapping of Hoxa2 domains involved in the KPC2 interaction. (A) Schematic representation of Hoxa2 deletion derivatives. Deletions were generated in the Hoxa2 sequence to remove amino-acids (aa) 1 to 137 at the N-terminal side [Hoxa2\textsuperscript{SN} (138-372)], aa 199 to 372 at the C-terminal side [Hoxa2\textsuperscript{SC} (1-198)], the homeodomain [Hoxa2\textsuperscript{SC}H (Δ139-198)], or all but the homeodomain [Hoxa2\textsuperscript{SH} (139-198)] (HD, homeodomain; HX, hexapeptide). (B) COS-7 cells were transfected with different combination of vectors coding for VN\textsuperscript{173}Hoxa2 or VN\textsuperscript{173}Hoxa2 deletion mutants and VC\textsuperscript{155}KPC2. Nuclei were stained with DAPI (blue).

**Figure 8.** HOX proteins share the capacity to interact with KPC2. COS-7 cells were transfected with different combination of vectors coding for VN\textsuperscript{173}HOX and VC\textsuperscript{155}KPC2 for BiFC analysis. Nuclei were stained with DAPI (blue).

Supplemental Figure 1. Different Kpc2 isoforms are expressed during mouse embryogenesis. RT-PCR were performed on different cDNA pool of embryos between E8.5 and E12.5. PCR products were isolated, purified and sequenced.

Supplemental Figure 2. Distinction between Iselt-1/2 stained and autofluorescent cells. Sagittal cryotsections of E11.5 mouse embryos were immunolabeled with anti-Iselt-1/2 primary antibody and an Alexa Fluor®555 anti-mouse IgG secondary antibody. The Iselt-1/2 fluorescent signal was observed in the red channel. Autofluorescent cells were detected in both red (left pictures) and green channels (right pictures).

Supplemental Figure 3. KPC2 and HOXA2 interact both in the cytoplasm and in the nucleus. COS-7 cells were transfected with vectors coding for VN\textsuperscript{173}HOXA2 and VC\textsuperscript{155}KPC2; VN\textsuperscript{173} and VC\textsuperscript{155}KPC2; VN\textsuperscript{173}HOXA2 and VC\textsuperscript{155}; VN\textsuperscript{173} and VC\textsuperscript{155}. Only the VN\textsuperscript{173}HOXA2 and VC\textsuperscript{155}KPC2 combination provides a BiFC signal. Nuclei were stained with DAPI (blue).
Supplemental Figure 4. Subcellular localization of Hoxa2 deletion derivatives. HEK293T cells were transfected with GST-Hoxa2 or GST-Hoxa2 deletion constructs and subjected to immunocytochemistry with anti-GST. Nuclei were stained with DAPI (blue).
Tables

Table 1

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*The Hoxa2<sup>δN</sup> PCR product was obtained in 2 steps involving two separate PCR amplifications followed by an overlapping PCR combining the two first amplicons.*

Table 2

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Bibliography


