# Protein interactions of the transcription factor Hoxa1

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<table>
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<tr>
<th>Citation</th>
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</table>

(Article begins on next page)
Protein interactions of the transcription factor Hoxa1

Barbara Lambert1, Julie Vandeputte1, Sophie Remacle1, Isabelle Bergiers1, Nicolas Simonis2, Jean-Claude Twizere3, Marc Vidal4,5 and René Rezsohazy1*

Abstract

Background: Hox proteins are transcription factors involved in crucial processes during animal development. Their mode of action remains scantily documented. While other families of transcription factors, like Smad or Stat, are known cell signaling transducers, such a function has never been squarely addressed for Hox proteins.

Results: To investigate the mode of action of mammalian Hoxa1, we characterized its interactome by a systematic yeast two-hybrid screening against ~12,200 ORF-derived polypeptides. Fifty nine interactors were identified of which 45 could be confirmed by affinity co-purification in animal cell lines. Many Hoxa1 interactors are proteins involved in cell-signaling transduction, cell adhesion and vesicular trafficking. Forty-one interactions were detectable in live cells by Bimolecular Fluorescence Complementation which revealed distinctive intracellular patterns for these interactions consistent with the selective recruitment of Hoxa1 by subgroups of partner proteins at vesicular, cytoplasmic or nuclear compartments.

Conclusions: The characterization of the Hoxa1 interactome presented here suggests unexplored roles for Hox proteins in cell-to-cell communication and cell physiology.

Keywords: Hox, Hoxa1, ORFeome, Interactome

Background

The conserved family of homeodomain Hox transcription factors is critically involved in patterning the body plan of bilaterian embryos by controlling multiple morphogenetic and organogenetic processes during animal development [1-4]. Modifications in Hox protein expression and activity have likely contributed to the evolutionary diversification of animal forms [5,6]. Misregulation or mutation of several Hox proteins has been associated with pathologies like cancer or neuropathies [7,8].

Hox proteins are transcription factors which regulate expression of target genes and chromatin remodeling [9]. A handful of proteins that interact with Hox proteins have been identified so far, and these are almost exclusively transcription factors, like the well-characterized Three Amino acid Loop Extension (TALE) homeodomain proteins Pbx/Exd and Prep/Meis/Hth [10], TFIIEβ [11], TATA Binding Protein (TBP) [12], Gli3 [13], Maf [14], Smad [15,16], High Mobility Group protein 1 (HMG1) [17], or transcriptional coregulators like CREB Binding Protein (CBP)/p300 [18-20]. Hox proteins may also form complexes with the translation initiation factor eIF4E to control the translation of target mRNAs [21]. Some Hox-like homeodomain proteins can be secreted into the extracellular compartment and translocate through the cell membrane to gain access to the cytosol and nucleus of neighboring cells, so it has been proposed that Hox proteins could display a paracrine transcriptional activity [22,23].

Numerous transcription factors, involved in critical developmental processes, like Smad, STAT, β-catenin or NFκB, are primarily signal transducers. Though primarily cytoplasmic, upon activation these can translocate to the nucleus, where they convey signaling by affecting gene regulation. As signal transducers these transcription factors can interact with enzymatically active membrane receptors, adaptor proteins, signal transducing kinases, or ubiquitin ligases. Possibly, Hox transcription factors could similarly fulfill pivotal roles at the heart of...
developmental processes, acting at the crossroads between cell-to-cell communication and cell fate determination. To our knowledge no exhaustive interaction screen has been performed to detect functional connections for a Hox protein.

Here, we conducted a proteome-wide screening for candidate interactors of Hoxa1. Hoxa1 is one of the earliest Hox genes to be expressed during embryonic development. It is involved in hindbrain segmentation and patterning [1,24,25]. Hoxa1 misregulation has been associated with mammary carcinogenesis [26]. We used a stringent high-throughput yeast two-hybrid (Y2H) approach to systematically test pairwise combinations, using Hoxa1 both as a bait and as a prey against the human ORFeome v3.1 resource, which contains 12,212 ORFs representing 10,214 genes [27]. Of the 59 Hoxa1 interactions identified, 45 could be validated by in vivo affinity binding assays in co-transfected animal cells. A striking subset of the validated interactors are not proteins involved in gene regulation. Rather, these interactors are adaptor proteins or modulators of the Bone Morphogenetic Proteins (BMP)/Tumor Growth Factor (TGF) β, Tumor Necrosis Factor (TNF), Receptor-Tyrosine Kinases (RTK) and integrins signal transduction pathways. Other interactors participate in cell adhesion or endosomal trafficking. We detected 41 interactions in live cells by Bimolecular Fluorescence Complementation (BiFC). Depending on the different proteins identified, interactions either take place in the cytoplasm, in the nucleus, in association with vesicles or show a variable pattern from cell to cell, underscoring a dynamic interplay with Hoxa1. Numerous identified Hoxa1 partners reported to interact with each other within known pathways share similar intracellular patterns of Hoxa1 interaction by BiFC. We conclude that Hoxa1 can contact several subunits of multi-molecular functional platforms involved in cell signaling, cell-adhesion, or cell shape regulation.

Results
A proteome-wide yeast two-hybrid screening for Hoxa1 interactors

The yeast two-hybrid (Y2H) is a powerful approach for large-scale screenings to identify binary protein-protein interactions [28,29]. DB-Hoxa1 was tested pairwise against 12,212 open reading frame (ORF)-derived proteins from the human ORFeome version 3.1 [27] fused to the Gal4 activation domain (AD). In this configuration, we detected 40 distinct interactions (Table 1). We also screened in the other configuration, Hoxa1 as a prey (AD-Hoxa1) against the full hORFeome in fusion with the Gal4 DB. In the second configuration we detected 28 interactions, of which 8 were also detected in the DB-Hoxa1/AD-ORFs configuration (Table 2). A total of 59 candidate Hoxa1 interactors were identified. We found the Hoxa1 homodimerization interaction and 8 out of the 9 Hoxa1 interactions, previously described in the literature [28,30] (Table 1 and 2).

Co-purification from animal cells validate forty-five Hoxa1 interactors

To validate the 59 interactions identified by the Y2H screen by an orthogonal assay we turned to affinity co-purification of a FLAG-Hoxa1 fusion protein co-expressed with glutathione S-transferase (GST)-tagged candidate interactors in transfected COS7 or HEK293T cells. In absence of GST-partners, there was no or very weak background binding of FLAG-Hoxa1 onto the glutathione-agarose beads (Figure 1). As positive controls we measured Hoxa1 dimer formation [30,31] and the reproducible interaction between Hoxa1 and Pbx1a [32] (Figure 1). In total, affinity co-purification from co-transfected cells confirmed 45 out of the 59 Y2H interactors (Table 1 and 2), in the presence of which a detectable amount of FLAG-Hoxa1 remained associated to the GST-fusion/glutathione-agarose beads and could be detected on western blots. It should be noted however that some interactions could not be confirmed because the corresponding GST-ORF fusion was expressed at an undetectable level, if at all (data not shown).

Bioinformatics functional analysis

To determine if Hoxa1 preferentially targets particular biological functions or pathways, we tested for statistical enrichment in regards to the Gene Ontology GO [33]), Kyoto Encyclopedia of Genes and Genomes KEGG: [34]) and Pathway Commons databases (www. pathwaycommons.org).

We observed that six GO terms were significantly overrepresented (Table 3). These enriched annotations are consistent with known functions of Hoxa1, linking our set of interactors to developmental and transcription factor function. There were several additional enriched, though not statistically so, GO terms linked to development and transcription factors (Table 3).

The immediate interactors of Hoxa1 were not enriched for annotated pathways, which could be due to incomplete coverage or relative sensitivity of the Y2H assay [35], or be intrinsic to the way Hoxa1 interacts with pathways, needing only one or few direct contacts. To account for the latter possibility, we also analyzed second-degree interactors, proteins that interact with Hoxa1 targets. Proteins associated with 21 pathways are overrepresented compared to random expectation (Table 4), showing that Hoxa1 could play a role in various processes other than gene regulation, such as focal adhesion, axon guidance or several signaling cascades.
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* Previously reported by Rual et al., 2005.
§ Revealed by both AD-ORF and DB-ORF screening.
Y = yes; N = no.
Table 2 Interaction partners for Hoxa1 revealed by Y2H screening using AD-Hoxa1

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<td>Q9BQ66</td>
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<td>Cytoplasmic</td>
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</tr>
<tr>
<td>KRTAP5-9</td>
<td>keratin associated protein 5-9</td>
<td>3846</td>
<td>P26371</td>
<td>Keratin associated</td>
<td>Vesicular and cytoplasmic</td>
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<tr>
<td>LIMS1 (PINCH1)</td>
<td>LIM and senescent cell antigen-like domains 1</td>
<td>3987</td>
<td>P48059</td>
<td>Cytoskeleton and signaling regulator (focal adhesion, integrins, receptor tyrosine kinases)</td>
<td>Nuclear</td>
<td>Y</td>
</tr>
<tr>
<td>MDF1* (I-mfa)</td>
<td>MyoD family inhibitor</td>
<td>4188</td>
<td>Q97500</td>
<td>Signaling regulator (channels, Wnt, JNK pathways) - Transcription factor (I-mfa domain),</td>
<td>Nuclear, vesicular, cytoplasmic</td>
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<tr>
<td>PCSK5§</td>
<td>proprotein convertase subtilisin/kexin type 5</td>
<td>5125</td>
<td>Q92824</td>
<td>Pro-protein convertase</td>
<td>/</td>
<td>N</td>
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<tr>
<td>PDCD6IP (Alix)</td>
<td>programmed cell death 6 interacting protein</td>
<td>10015</td>
<td>Q8WUM4</td>
<td>Endosome formation and vesicular trafficking, cytoskeleton and signaling regulator (Focal adhesion, TNFR pathway, EGFR, PDGFR)</td>
<td>Vesicular and cytoplasmic</td>
<td>Y</td>
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<tr>
<td>PFKM</td>
<td>phosphofructokinase, muscle</td>
<td>5213</td>
<td>P08237</td>
<td>Glycolysis</td>
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Table 2 Interaction partners for Hoxa1 revealed by Y2H screening using AD-Hoxa1 (Continued)

<table>
<thead>
<tr>
<th>Interaction Partner</th>
<th>Gene ID</th>
<th>Protein ID</th>
<th>Description</th>
<th>Localization</th>
<th>Interaction</th>
<th>Note</th>
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<tr>
<td>PITX2</td>
<td>5308</td>
<td>Q99697</td>
<td>Transcription factor (homeodomain)</td>
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<tr>
<td>PLSCR4§</td>
<td>57088</td>
<td>Q9NRQ2</td>
<td>Phospholipid scramblase, transcription factor</td>
<td>Nuclear</td>
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<tr>
<td>RAB33A</td>
<td>9363</td>
<td>Q14088</td>
<td>Small GTPase, vesicular trafficking (Ras pathway)</td>
<td>Nuclear</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>SMOC1</td>
<td>64093</td>
<td>Q9H4F8</td>
<td>Extracellular matrix protein, signaling, migration and differentiation modulator</td>
<td>n.d.</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>TRAPPC6A*</td>
<td>79090</td>
<td>Q75865</td>
<td>Vesicular trafficking</td>
<td>Nuclear</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>ZZZ3</td>
<td>26009</td>
<td>Q8IYH5</td>
<td>Transcription factor (zinc finger)</td>
<td>Nuclear</td>
<td>Y</td>
<td></td>
</tr>
</tbody>
</table>

* Previously reported by Rual et al., 2005.
§ Revealed by both AD-ORF and DB-ORF screening.
* Y = yes; N = no.
Figure 1 Validation of 45 out of the 59 interactions revealed for Hoxa1 by affinity co-purification on glutathione-agarose beads. Candidate interactors were fused with a GST-tag and co-expressed in transfected cells with a FLAG-Hoxa1 fusion protein. Western blots were run to detect FLAG-Hoxa1 from cell extracts before (Input) or after (Co-P) purification. The Hoxa1-Hoxa1 or PBX1A-Hoxa1 interactions were used as positive controls (see lanes with arrowheads). Negative control corresponds to transfected cells with the only FLAG-Hoxa1 fusion protein (C, lanes with blue arrows). Some interactors which could not be confirmed by co-purification are also shown (red asterisks).
Hoxa1-mediated interactions take place in distinct cell compartments

We tested the 45 validated Hoxa1 interacting proteins by Bimolecular Fluorescence Complementation (BiFC) assay, which not only tests for protein interactions but can also visualize where the distinct interactions occur in live cells. For BiFC, the ORF corresponding to each interactor was fused C-terminally to the N-terminal 173 amino acids of the Venus fluorescent protein (VN173), while the Hoxa1 ORF was fused downstream of the C-terminal moiety of Venus (amino acids 155 to 243; VC155). Detectable fluorescence in cells transfected for the complementary VN173 and VC155 fusion proteins means that a functional Venus has been reconstituted, indicating that the partner proteins interact. As a preliminary control, BiFC was assayed for the well-established Hoxa1-PBX1A interaction (Figure 2). The VN173-PBX1A and VC155-Hoxa1 fusion proteins provided fluorescence complementation (Figure 2A), whereas the VN173-PBX1A/VC155 and VN173/VC155-Hoxa1 combinations did not (Figure 2B, C). This therefore supported that the N- and C-terminal Venus fragments did not reassociate if not fused to interacting proteins. In addition, the immunocytolocalization of Venus consistently revealed that the VN173- and VC155-containing fusion proteins displayed a broad intracellular distribution that completely encompassed the narrower BiFC signal. In agreement with these controls, like the VN173-PBX1A fusion (Figure 2B), none of the VN173-interactor fusions provided fluorescence alone or in the presence of the VC155 Venus fragment alone (data not shown). For 41 out of the 45 interactors tested specific fluorescence was observed upon addition of the VC155-Hoxa1 fusion protein. Distinct patterns of intracellular interactions were observed (see Table 1 and 2, Figure 3). For 31 proteins, interactions took place in the nucleus (Figure 3A and C). Of these, 16 proteins appeared to contact Hoxa1 exclusively in the nucleus, while 15 also displayed other patterns of subcellular fluorescence complementation. Among the proteins found to bind Hoxa1 in the nucleus, some were known transcription factors (Table 5) or were known to have nuclear functions, but other were not (e.g. LGALS13, LIMS1, LNX2, MGAT5B, RBPMS, RAB33A, RGS20, TSCR1). A set of proteins shared a similar interaction pattern characterized by a diffuse, finely-punctuated cytoplasmic signal without nuclear staining (Figure 3B). This subcellular localization pattern was observed for different proteins reported to participate in a common signaling pathway. Examples are TRAF, TRIP or PDCD6IP (also known as Alix) which are found associated with the TNFR family of receptors [36-41], SPRY1 and PDCD6IP modulating RTK downstream signaling [42-46], PDLIM7 (alias LMP1) and RBPMS (also known as Hermes) which are involved in the BMP/TGFβ signaling regulation [47,48] and LPXN, PDCD6IP and TRIP6 known to associate with focal adhesion sites and related signal transduction [49-53]. As a control, in cells co-expressing GST-TRAF1 fusion and wildtype Hoxa1, proteins displayed an overlapping intracellular distribution.

### Table 3 Gene Ontology (GO) enrichment analysis

<table>
<thead>
<tr>
<th>GO term</th>
<th>Obs*</th>
<th>Odds ratio</th>
<th>P-value</th>
<th>Corr P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>keratin filament</td>
<td>6</td>
<td>102,352</td>
<td>3.62292E-10</td>
<td>1.19194E-07</td>
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<td>pattern specification process</td>
<td>6</td>
<td>12,3435</td>
<td>2.01548E-05</td>
<td>0.00331547</td>
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<td>regionalization</td>
<td>5</td>
<td>14,3981</td>
<td>0.000048482</td>
<td>0.00531685</td>
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<tr>
<td>cranial nerve morphogenesis</td>
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<td>116,248</td>
<td>0.000331888</td>
<td>0.0272978</td>
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<tr>
<td>kidney development</td>
<td>3</td>
<td>21,403</td>
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<td>0.0306933</td>
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<tr>
<td>zinc ion binding</td>
<td>16</td>
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<td>embryonic development</td>
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<td>5,58735</td>
<td>0.00120012</td>
<td>0.0564059</td>
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<td>receptor signaling protein serine/threonine kinase activity</td>
<td>4</td>
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<td>developmental process</td>
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<td>2,38882</td>
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<td>negative regulation of MAP kinase activity</td>
<td>2</td>
<td>19,4953</td>
<td>0.00596027</td>
<td>0.0676182</td>
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<td>regulation of transcription factor import into nucleus</td>
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<td>31,8952</td>
<td>0.00251195</td>
<td>0.0688964</td>
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<td>cation binding</td>
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<td>2,14904</td>
<td>0.00649842</td>
<td>0.071266</td>
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<tr>
<td>anterior/posterior pattern formation</td>
<td>3</td>
<td>13,3725</td>
<td>0.00198155</td>
<td>0.0724365</td>
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<td>cytoskeletal part</td>
<td>8</td>
<td>3,92527</td>
<td>0.00191605</td>
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<td>inner ear morphogenesis</td>
<td>2</td>
<td>27,0115</td>
<td>0.00335617</td>
<td>0.0788701</td>
</tr>
</tbody>
</table>

* number of Hoxa1 interactors annotated with the corresponding GO term.
  1 the odds ratio represents the enrichment of the corresponding GO term in the set of Hoxa1 interaction partners, an odds ratio of 10 meaning that the considered GO term is observed 10 times more than expected at random.
  2 probability to see at least the number of proteins corresponding to the GO term at random.
  3P-value including a correction for multiple testing.

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<table>
<thead>
<tr>
<th>Pathway name</th>
<th>ID*</th>
<th>Obs$</th>
<th>Odds ratio£</th>
<th>FDR§</th>
<th>Corr FDR¶</th>
<th>Source</th>
<th>Gene symbols</th>
<th>Entrez gene IDs</th>
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<tbody>
<tr>
<td>RXR and RAR heterodimerization with other nuclear receptor</td>
<td>pc926</td>
<td>2</td>
<td>24,83</td>
<td>2,00E-05</td>
<td>2,40E-03</td>
<td>NCI-Nature</td>
<td>FAM120B,NR1H2</td>
<td>84498,7376</td>
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<tr>
<td>Cell adhesion molecules (CAMs) - Homo sapiens (human)</td>
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<td>19,70</td>
<td>9,00E-05</td>
<td>1,35E-02</td>
<td>KEGG</td>
<td>CLDN2,PVRL2</td>
<td>9075,5819</td>
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<tr>
<td>Gap junction - Homo sapiens (human)</td>
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<td>16,59</td>
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<td>1,20E-02</td>
<td>KEGG</td>
<td>GNA12,PDGFRB</td>
<td>2771,5159</td>
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<tr>
<td>Signaling events mediated by PTP1B</td>
<td>pc948</td>
<td>4</td>
<td>12,72</td>
<td>1,00E-05</td>
<td>1,20E-03</td>
<td>NCI-Nature</td>
<td>CRK,SPRY2,TRPV6,PDGFRB</td>
<td>1398,10253,5503,5159</td>
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<tr>
<td>Retinoid acid receptors-mediated signaling</td>
<td>pc960</td>
<td>3</td>
<td>10,19</td>
<td>2,10E-04</td>
<td>2,52E-02</td>
<td>NCI-Nature</td>
<td>NR1H2,NRIP1,FAM120B</td>
<td>7376,8204,84498</td>
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<tr>
<td>Integrins in angiogenesis</td>
<td>pc989</td>
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<td>10,04</td>
<td>3,40E-04</td>
<td>4,08E-02</td>
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<td>CDKN1B,SP1,VCL</td>
<td>1027,6696,7414</td>
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<tr>
<td>Down-stream signal transduction</td>
<td>pc690</td>
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<td>8,28</td>
<td>5,00E-05</td>
<td>3,14E-02</td>
<td>Reactome</td>
<td>PDGFRB,NCK2</td>
<td>5159,8440</td>
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<tr>
<td>Signaling by PDGF</td>
<td>pc876</td>
<td>2</td>
<td>8,28</td>
<td>5,00E-05</td>
<td>3,14E-02</td>
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<td>PDGFRB,NCK2</td>
<td>5159,8440</td>
</tr>
<tr>
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<tr>
<td>Homologous recombination - Homo sapiens (human)</td>
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<td>TOP3B,RAD54B</td>
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<tr>
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<td>SP1,VCL,VASP,CRK,PDGFRB,BIRC2,CCND3,PAK7</td>
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<tr>
<td>TCR signaling in naive CD4+ T cells</td>
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<tr>
<td>Axon guidance - Homo sapiens (human)</td>
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<td>4</td>
<td>6,34</td>
<td>3,80E-04</td>
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<td>p75(NTR)-mediated signaling</td>
<td>pc978</td>
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<td>2,60E-04</td>
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<td>2,64E-02</td>
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<td>BSG,HGS,CRK,SPRY2,SP1</td>
<td>682,9146,1398,10253,6696</td>
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<td>1,92E-02</td>
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<td>BSG,CRK,HGS</td>
<td>682,1398,9146</td>
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<td>3,60E-04</td>
<td>4,32E-02</td>
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<td>CRK,SLC9A1,JRF4,NCK2,YWHA,TRAF6</td>
<td>1398,6548,3662,8440,7531,7189</td>
</tr>
</tbody>
</table>

* pathway identifier.  
$ number of Hoxa1 interactors belonging to the corresponding pathway.  
£ the odds ratio represents the enrichment of the corresponding pathway, an odds ratio of 10 meaning that the considered pathway is observed 10 times more than expected at random.  
§ corrected false discovery rate accounting for multiple testing.
consistent with the BiFC signal observed with VN173-TRAF1/VC155-Hoxa1 (Figure 4). Fourteen interactors tested displayed variable interaction patterns, showing mostly nuclear to nuclear and cytoplasmic or nuclear and vesicular BiFC signal (Figure 3A and C). This heterogeneous distribution suggests a coordinated shuttling between cell compartments for Hoxa1 and some partners (e.g. MDFI, OGT, PITX2, PRDM14, RBCK1, RBPMS, SPRY1, ZBTB16). The specific associations between Hoxa1 and 41 interactors detected by BiFC shows that Hoxa1 can associate dynamically with distinct categories of proteins in distinct intracellular domains.

Discussion
By a high-throughput Y2H screen we identified 59 Hoxa1 interacting proteins among which 45 were confirmed by co-precipitation from animal cells. The intracellular localization of 41 interactions was further detected by a BiFC approach. This is the first exhaustive screen and analysis for interactors of a Hox protein. Our data support the conclusion that Hox proteins, and Hoxa1 in particular, known as crucial transcription factors controlling developmental processes can fulfill unexplored roles in cell signaling, cell adhesion, or vesicular trafficking.

Hoxa1 appears to interact with several proteins found to be part of molecular platforms associated with a few signaling pathways (TNFR superfamily, RTK, BMP/TGFβ, Focal adhesion, ...), membrane dynamics and vesicular trafficking (Table 5). These platforms contact activated receptors at the plasma membrane and can positively or negatively modulate the downstream signaling or subsequent internalization in the endosomal compartment. By interacting with these proteins Hoxa1 could either act as a modulator or an effector of these signaling pathways. The BiFC assay revealed that most of the interactors involved in signaling pathways display a similar pattern of Hoxa1 interaction in culture cells. LPXN, PDLIM7, PDCD6IP, RBPMS, SPRY1, TRAF1, TRAF2 and TRIP6, for example, showed a BiFC signal...
Figure 3 (See legend on next page.)
in the cytoplasm, with fine punctuated staining probably related to vesicular compartments (Figure 2B). Although further experiments are required to identify these compartments, our data suggest that Hoxa1 interacts with distinct modulators of a given pathway at the level of shared molecular platforms. Finally, some interactors such as MDFI, OGT, RBCK1, RBPMS or SPRY1 display various patterns of Hoxa1 interaction from cell to cell, possibly indicating dynamic partnerships depending on cell physiological state (Figure 3A and C).

Some links might be drawn between the molecular, cellular and developmental processes involving Hoxa1 and its interactors. LIMS1 for example is expressed in neural crest cells and plays an important role in neural crest development through TGFβ signaling [54]; in mouse, a downregulation of SPRY1 inhibits the rhombomere4-derived neural crest cells to colonize the 2nd branchial arch [55]; RBPMS is expressed in the outflow tract of the developing heart [56], a territory colonized by Hoxa1 positive cells [57]. An important group of interactors consists in transcription factors. Some of them are known to be involved in embryonic patterning or cell fate decision (HOXD3, MDFI, PITX2 for example). In that regard, ZBTB16 (better known as Plzf) is a particularly relevant Hoxa1 interactor. It is expressed during hindbrain development at rhombomere boundaries and, like Hoxa1, has been proposed to control hindbrain segmentation [58]. Transcriptional coregulators, like the SET-domain histone methyl-transferase PRDM14 or the O-linked-N-acetyl glucosamine (GlcNac) transferase OGT, have also been identified as Hoxa1 interactors which may contribute to Hoxa1-mediated gene regulation. Most significantly, OGT has recently been shown to be the homologue of the Drosophila Super sex combs (Sxc) protein. Sxc is associated to Polycomb complexes and is required for their ability to repress gene expression, including Hox genes [59].

Conclusions

We presented here the first large-scale Hox interactome characterized so far. Although only a handful of interactors are known for other Hox proteins, some interactors identified here for Hoxa1 are shared with other Hox proteins [28]. PLSCR1 has been shown to contact HOXA9 and HOXB6, and HOXA9 is also contacted by TRIP6. RBPMS is able to interact with HOXA9 and HOXB9. These interactions, as well as other described here, underline that Hox proteins should be viewed not only as gene regulators, but also as components of signal transduction and modulation of cell-to-cell communication, cell adhesion and vesicular trafficking.

Methods

Yeast two-hybrid screening

The mouse Hoxa1 coding sequence was amplified from the pGHI327 expression plasmid[60] and cloned into pDONR-223 by Gateway BP recombinational reaction (attB1.1 primer: GGGGACAACCTTTGTACAAAAAAGTTGGGTA GTCATGAACCTTTGTACGGG; attB2.1 primer: GGG GACAACCTTTGTACAAAAAAGTTGGGTA GTGATCGAGTTG; Invitrogen). By Gateway LR recombinational cloning, Hoxa1 was then transferred into pDEST-DB and pDEST-AD-CYH2 centromeric destination vectors [29] to code for Gal4 DNA binding domain (DB)-Hoxa1 and Gal4 activation domain (AD)-Hoxa1 fusion proteins, respectively.

\[\text{MAT} a Y8930\] and \[\text{MAT} a Y8800\] yeast strains (genotype: \(\text{trp1-901}; \text{leu2-3, 112}; \text{ura3-52}; \text{his3-200}; \text{gal4Δ}; \text{gal80Δ}; \text{GAL2-ADE2}; \text{LYS2-GAL1-HIS3}; \text{met2-GAL7-lacZ}; \text{cyh2}^{\Delta}\)) were used for yeast two-hybrid (Y2H) screens. The DB-Hoxa1 coding construct was first tested for auto-activation by transforming it into the \[\text{MAT} a Y8930\] yeast strain and testing for expression of the HIS3 reporter gene in the absence of any AD-hORF fusion protein, on a solid synthetic complete medium lacking leucine and histidine (Sc-L-H) and supplemented with 1mM 3-amino-triazol (3AT) [29]. The DB-Hoxa1 construct did not auto-activate.

High-throughput Y2H screens were essentially performed as described [29]. Briefly, DB-Hoxa1 and AD-Hoxa1 vectors were transformed into \[\text{MAT} a Y8930\] or \[\text{MAT} a Y8800\] yeast strains, respectively. The DB-Hoxa1 construct in \[\text{MAT} a Y8930\] was mated with \[\text{MAT} a Y8800\] containing the AD-hORF library [27], and for the other configuration DB-hORFs library in \[\text{MAT} a Y8930\] were mated with AD-Hoxa1 in \[\text{MAT} a Y8800\]. After overnight growth at 30°C, diploid yeast cells were transferred to plates lacking histidine, leucine and tryptophan, supplemented with 1mM 3AT (Sc-L-T-H+3AT), to select for those with elevated expression of the \(\text{GAL1-HIS3}\) reporter gene.
Table 5 Functional classification of Hoxa1 interactors

<table>
<thead>
<tr>
<th>Function</th>
<th>Interactor</th>
<th>Function</th>
<th>Interactor</th>
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</thead>
<tbody>
<tr>
<td>Cell shape and migration</td>
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<td>Signal transduction (continued)</td>
<td>MDFI</td>
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<td>Focal adhesion associated</td>
<td>LPXN</td>
<td>Other</td>
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<td>TRIP6</td>
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<td>Cytoskeleton binding</td>
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<td>Vesicular trafficking</td>
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<td>TRAPPC6A</td>
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http://www.biomedcentral.com/1471-213X/12/29
Positive colonies were picked, grown on Sc-L-T plates, and retested on Sc-L-T-H, as well as on medium lacking Adenine (Sc-L-T-A) and Sc-L-T-H-A+3AT, to select for colonies with high GAL1-HIS3 and GAL2-ADE2 reporter gene activity. To detect any spontaneous auto-activators arising in the course of the screen, positive colonies were transferred in parallel onto cycloheximide containing media (Sc-H+CHX). Candidate colonies that grew on Sc-H+CHX were discarded.

The identities of candidate interacting pairs was determined by sequencing PCR products amplified directly from yeast cells using primers specific to Gal4DB and Gal4AD (DB primers: GGCTTCAGTGGAGACTGATA TGCCTC, GGAGACTTGACCAAACCTC TGGCG; AD primers: CGCGTTTGGAATCACTACAGGG, GGAGACTTGACCAAACCTC TGGCG). PCR products were purified (Qiagen kit # 28104) and sequenced.

The protein interactions from this publication have been submitted to the IMEx (http://www.imexconsortion.org) consortium through IntAct [pmid: 19850723] and assigned the identifier IM-15418.

**Co-precipitation assays**

The Hoxa1 coding sequence was transferred from the pDONR-223 Gateway® vector to pDEST-FLAG mammalian expression vector by Gateway® LR recombination reaction. Open reading frames coding for interactors from the hORFeome were cloned into a pDEST-GST mammalian expression vector by the same procedure.

COS7 and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) low glucose or high glucose respectively (Gibco/Invitrogen) supplemented with Glutamine, 10% fetal bovine serum (Gibco/Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco/Invitrogen). Cell lines were maintained at 37°C in a humidified, 5% CO₂ atmosphere. For transient transfection, 1.4 × 10⁵ (COS7) or 4 × 10⁶ (HEK293T) cells were plated into six-well plates. Twenty-four hours

<table>
<thead>
<tr>
<th>DAPI</th>
<th>GST-TRAF1</th>
<th>Hoxa1</th>
<th>merge</th>
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<tr>
<td>anti-Hoxa1</td>
<td>anti-Hoxa1</td>
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**Figure 4 Hoxa1 and TRAF1 intracellular distributions overlap.** MCF10A cells were transfected with GST-TRAF1 and Hoxa1 expression vectors. The immunolocalization of GST-TRAF1 and Hoxa1 (anti-GST, anti-TRAF1 and anti-Hoxa1 immunocytofluorescence) reveals that the partner proteins display partially overlapping intracellular distribution.
after plating, cells were transfected with TransFectin™ reagent (BioRad). One and a half µg of pDEST-FLAG-Hoxa1 expression vector and 3µg of pDEST-GST-hORF were mixed with 250µl of serum-free medium and added to a mix of 1 µl of TransFectin™ and 250µl of serum-free medium. Forty-eight hours after transfection, cells were lysed with Tris–HCl pH7.5 20mM, NaCl 120mM, EDTA 0.5mM, NP40 0.5%, glycerol 10% and Complete™ protease inhibitor (Roche).

Cell lysates were cleared by centrifugation for 5 minutes at 13,000 g. Cleared lysates were incubated overnight on glutathione-agarose beads (Sigma # G4510). Beads were cleared 3 times with the lysis buffer. Beads and third wash samples were then loaded on SDS-PAGE, transferred on nitrocellulose membrane and processed for detection of FLAG tagged proteins with an anti-FLAG M2 antibody (Sigma # F1804).

**Bimolecular Fluorescence Complementation assay (BiFC)**

pDEST-VN173 and pDEST-VC155 plasmids were obtained by cloning sequences encoding N-terminal residues 1–173 and C-terminal residues 155–243 of the yellow fluorescent protein VENUS, respectively, within the pDEST-v1899-FLAG vector instead of the 5′ [KpnI/HindIII] 3xFLAG-fragment (VN173F primers : GAGGTACCATGGTGAGCAAGGGCGAGGAGC, GGAGAAGCTTCTCGATGTTGTCGCGGATC, VC155 primers: AAGGTACCATGGGCGACAAAGCAGAGAAGCGGCAC, GGAAAAGCTTCTCGTGACCGGTCCGTAGACGC).

The Hoxa1 coding sequence was transferred from the pDONR-223 Gateway™ vector to pDEST-VC155 mammalian expression vector by Gateway™ LR recombination reaction. Open reading frames coding for interactors from the hORFome were cloned into the pDEST-VN173 mammalian expression vector by the same procedure.

MCF10A cells were maintained at 37°C in a humidified 5% CO₂ atmosphere, in DMEM-F12+L-glutamine medium (Gibco/Invitrogen) supplemented with 5% horse serum (Gibco/Invitrogen), 100 IU/ml penicillin (Gibco/Invitrogen), 100 µg/ml streptomycin (Gibco/Invitrogen), 100 µg/ml of cholaera toxin (Gentaur), 20 µg/ml of human Epidermal Growth Factor (hEGF; Sigma), 500 ng/ml hydrocortisone (Sigma) and 10 µg/ml insulin (Sigma). For transfection, 3 × 10⁵ cells were seeded on coverslips and transfected as described above. Twenty four hours after transfection, cells were fixed with 4% formaldehyde for 30 minutes. Cells were further blocked with 10% low-fat milk in TBS-0.1% Triton X100 solution for 45 min at room temperature, followed by overnight incubation in TBS-0.1% Triton X100 solution at 4°C, with a rabbit polyclonal anti-GFP (Invitrogen A11122, diluted 1/200), a mouse anti-GST (Sigma G1160, diluted 1/50), a mouse monoclonal anti-TRAF1 (Santa Cruz, sc-6253, diluted 1/50), or a rabbit polyclonal anti-Hoxa1 (Abcam ab64941, diluted 1/50), as primary antibodies. Cells were rinsed three times for 30 min in TBS-0.1% Triton X100 solution and incubated for 45 min at room temperature with a goat anti-rabbit IgG-AF555 (Molecular Probes 4413, diluted 1/750), a goat anti-mouse IgG-FITC (Santa Cruz sc-3699, diluted 1/100), or a bovine anti-rabbit IgG-TRITC (Santa Cruz sc-2367, diluted 1/100), as secondary antibodies. Cells were rinsed three times and glass cover slips were mounted in Vectashield™-DAPI medium (Vector laboratories). Slides were then analysed by confocal microscopy (LSM710, Zeiss, Jena, Germany; Plan-Apochromat 63x/1.40 Oil DIC M27 objective; Oil refraction index 1.5 imaging medium; PMT camera). Images were acquired by using the ZEN 2010 software, and subsequently processed with ZEN 2008 Light Edition.

**Immunocytolocalization**

COS7 and MCF10A cells were maintained, seeded on coverslips and transfected as described here above. Twenty four hours after transfection, cells were fixed with 4% formaldehyde for 30 minutes. Cells were further blocked with 10% low-fat milk in TBS-0.1% Triton X100 solution for 45 min at room temperature, followed by overnight incubation in TBS-0.1% Triton X100 solution at 4°C, with a rabbit polyclonal anti-GFP (Invitrogen A11122, diluted 1/200), a mouse anti-GST (Sigma G1160, diluted 1/50), a mouse monoclonal anti-TRAF1 (Santa Cruz, sc-6253, diluted 1/50), or a rabbit polyclonal anti-Hoxa1 (Abcam ab64941, diluted 1/50), as primary antibodies. Cells were rinsed three times for 30 min in TBS-0.1% Triton X100 solution and incubated for 45 min at room temperature with a goat anti-rabbit IgG-AF555 (Molecular Probes 4413, diluted 1/750), a goat anti-mouse IgG-FITC (Santa Cruz sc-3699, diluted 1/100), or a bovine anti-rabbit IgG-TRITC (Santa Cruz sc-2367, diluted 1/100), as secondary antibodies. Cells were rinsed three times and glass cover slips were mounted in Vectashield™-DAPI medium (Vector laboratories). Slides were then analysed by confocal microscopy (LSM710, Zeiss, Jena, Germany; Plan-Apochromat 63x/1.40 Oil DIC M27 objective; Oil refraction index 1.5 imaging medium; PMT camera). Images were acquired by using the ZEN 2010 software, and subsequently processed with ZEN 2008 Light Edition.

**Gene Ontology annotation and pathway analysis**

Gene Ontology (GO) annotations were downloaded from Entrez Gene (September 2009), pathway data from KEGG (September 2008) and Pathway Commons (September 2008) databases. From Pathway Commons, we analyzed the pathways originally annotated in NCI-Nature[pid.nci.nih.gov] and Reactome [61].

Fisher’s Exact Test was used to determine GO annotation and pathway enrichment of Hoxa1 direct targets, using the space of human proteins that have been tested
in our Y2H experiment, the human ORFeome v3.1 [27]. The corrected p-value was computed using the Benjamini-Hochberg multiple testing correction. We limited our results to GO annotations and pathways for which at least two Hoxa1 targets were annotated for.

To estimate the significance of indirect targets enrichment we ran 100,000 simulations for which the identity of the direct targets was randomized. The interactors of these targets were identified in an unbiased protein-protein interaction network [28], to avoid study bias inherent to literature curation. Interactors belonging to each pathway were counted, and the resulting distribution compared to the observed counts. An empirical False Discovery Rate (FDR) determined the significance of the enrichment, with the FDR computed as the proportion of random trials giving at least the observed number of indirect targets in the analyzed pathway. The FDR was corrected for multiple testing using the Bonferroni correction. Pathways with a corrected FDR < 0.05 and at least two observed proteins were considered significant.

Abbreviations

TALE: Three Amino acid Loop Extension; TBP: TATA Binding Protein; HMIG: High Mobility Group; CBP: CREB Binding Protein; Y2H: Yeast two-hybrid; BMP: Bone Morphogenetic Protein; TGF: Tumor Growth Factor; TNF: Tumor Necrosis Factor; RTK: Receptor Tyrosine Kinase; BiFC: Bimolecular Fluorescence Complementation; DB DNA: Binding domain; AD: Activation Domain; 3AT: 3-Amino-Triazol; FDR: False Discovery Rate; GO: Gene Ontology; Fluorescence Complementation; HMG: High Mobility Group; CBP: CREB Binding Protein; Y2H: Yeast two-hybrid; BMP: Bone Morphogenetic Protein; TGF: Tumor Growth Factor; TNF: Tumor Necrosis Factor; RTK: Receptor Tyrosine Kinase; BiFC: Bimolecular Fluorescence Complementation; DB DNA: Binding domain; AD: Activation Domain; 3AT: 3-Amino-Triazol; GO: Gene Ontology; FDR: False Discovery Rate; ORF: Open Reading Frame; GST: Glutathione S-Transferase; NCoR: Nuclear receptor Co-Repressor; SMRT: Silencing Mediator of Retinoid acid and Thyroid hormone receptor; HDAC: Histone Deacetylase; KAP: Keratin Associated Protein.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

BL carried out most of the molecular biology, yeast two-hybrid and cell biology experiments, made a substantial contribution to data analysis and drafted the manuscript. LV contributed to the co-precipitation experiments and substantially contributed to the BiFC assay and immunocytofluorescent detection of proteins. SR and IB set up the BiFC assay and the BiFC controls. NS carried out the bioinformatics analyses. ICT helped in the yeast two-hybrid screening and data interpretation. MV conceived and provided the materials required for the high-throughput yeast two-hybrid assay. RR conceived the study, significantly contributed to data interpretation and helped in drafting and revising the manuscript. All authors read and approved the final manuscript.

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