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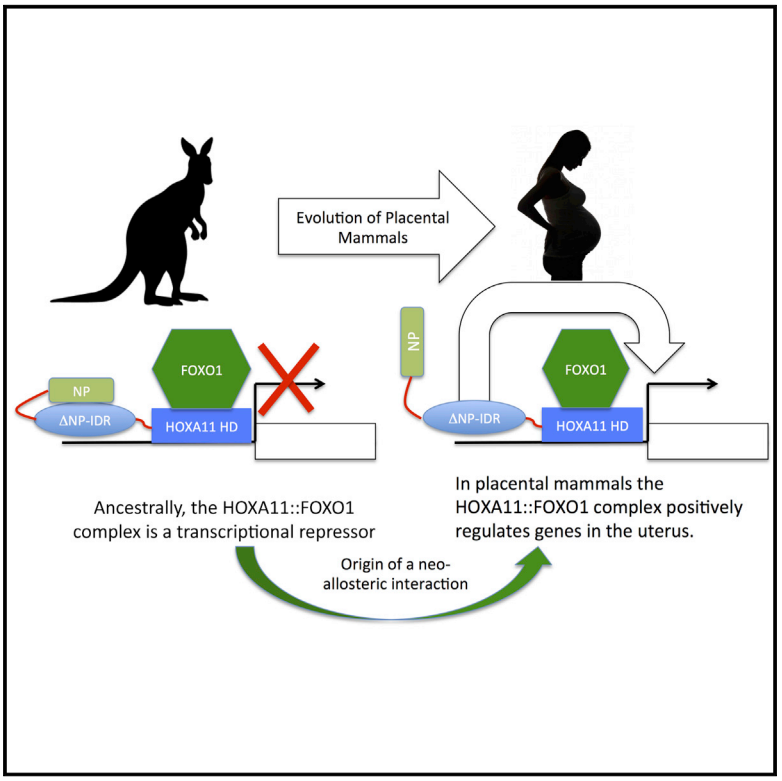
Report

A Derived Allosteric Switch Underlies the Evolution of Conditional Cooperativity between HOXA11 and FOXO1

Graphical Abstract



Highlights

- The HOXA11 protein has a regulatory motif that masks its activation domain
- In placental mammals, upon binding to FOXO1, HOXA11 unmasks its activation domain
- In the ancestral HOXA11, binding to FOXO1 does not unmask the activation domain
- In placental mammals the HOXA11::FOXO1 complex evolved a neo-allosteric switch

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In Brief

TFs are assumed to be evolutionarily constrained because of their multiple biological roles. In eutherian mammals HOXA11 evolved a derived function regulating genes in the uterus. This function is caused by a neo-allosteric switch in response to interaction with FOXO1, thereby circumventing constraints associated with pleiotropy.

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A Derived Allosteric Switch Underlies the Evolution of Conditional Cooperativity between HOXA11 and FOXO1

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SUMMARY

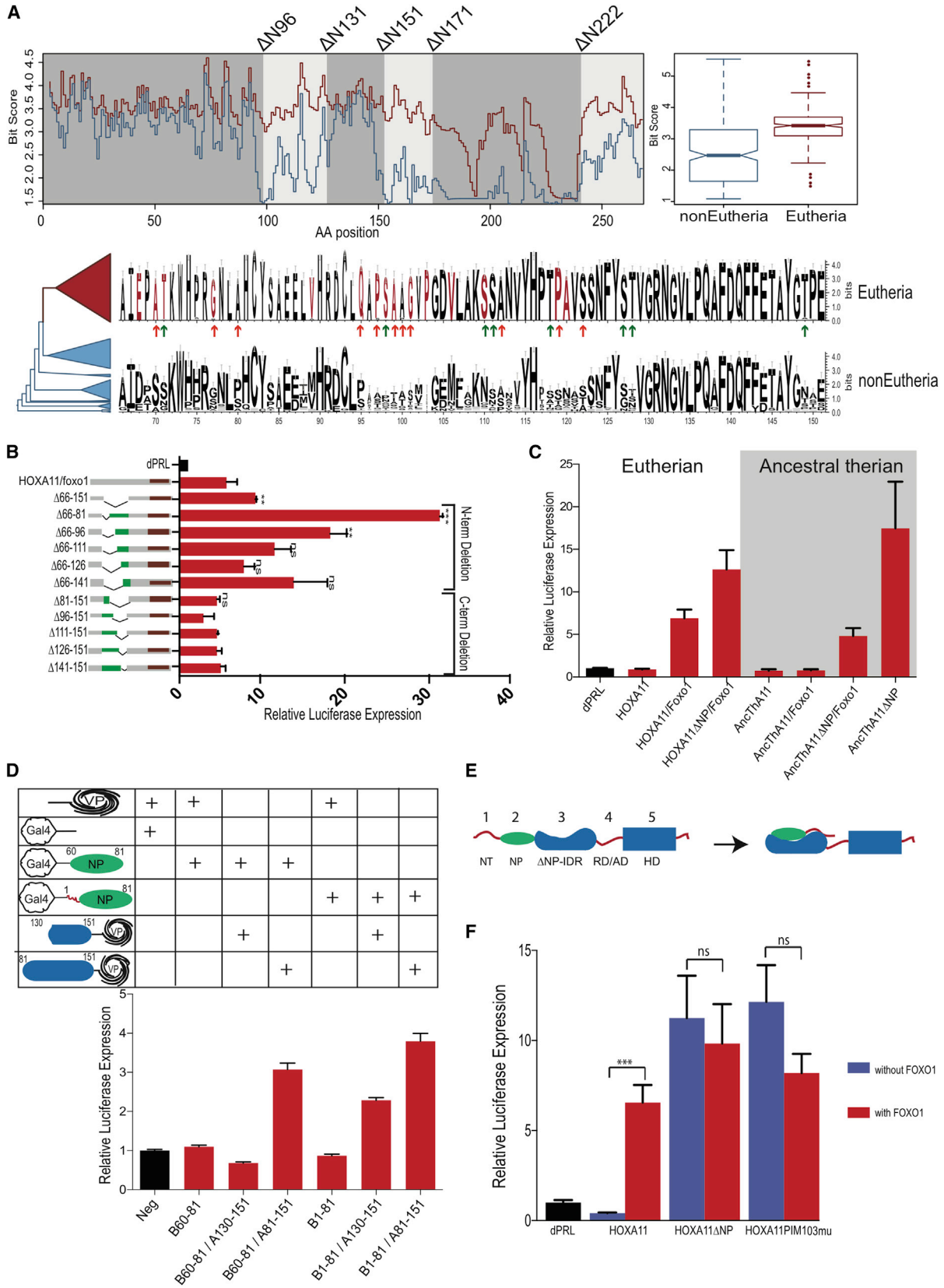
Transcription factors (TFs) play multiple roles in development. Given this multifunctionality, it has been assumed that TFs are evolutionarily highly constrained. Here, we investigate the molecular mechanisms for the origin of a derived functional interaction between two TFs, HOXA11 and FOXO1. We have previously shown that the regulatory role of HOXA11 in mammalian endometrial stromal cells requires interaction with FOXO1, and that the physical interaction between these proteins evolved before their functional cooperativity. Here, we demonstrate that the derived functional cooperativity between HOXA11 and FOXO1 is due to derived allosteric regulation of HOXA11 by FOXO1. This study shows that TF function can evolve through changes affecting the functional output of a pre-existing protein complex.

INTRODUCTION

Changes in *cis*-regulatory elements and transcription factors both contribute to the evolution of gene regulation. The dominant paradigm of regulatory evolution excludes transcription factor evolution as a source of regulatory variation. The major arguments are (1) that most transcription factors play multiple roles (Hu and Gallo, 2010) and (2) that transcription factors from different species are functionally equivalent with respect to certain functions (Carroll, 2008; Davidson, 2006; Grens et al., 1995; Halder et al., 1995; McGinnis et al., 1990). In contrast, *cis*-regulatory elements tend to be modular and evolve rapidly (Kapusta et al., 2013; Lynch et al., 2015). Thus, functional changes in transcription factors are assumed to be constrained due to pleiotropic effects, while changes in *cis*-regulatory elements seem to be favored as a source of regulatory variation. Here, we investigate the molecular mechanisms of transcription factor evolution, i.e., the changes of HOXA11 during the evolution of mammals. We show that the evolution of a neo-allosteric regulation within the HOXA11 protein resulted in a novel functional output from a pre-existing protein complex.

HOXA11 is involved in development of limb, kidney, reproductive organs, cloaca, and hindgut and in T and B cells (Davis et al., 1995; Hsieh-Li et al., 1995; Schwab et al., 2006; Speleman et al., 2005; Wellik and Capecchi, 2003; Yokouchi et al., 1995). Given its many roles, evolutionary changes to HOXA11 might be expected to disrupt at least one of its functions. However, HOXA11 evolved a new functional interaction with FOXO1 in the stem lineage of eutherian ("placental") mammals (Brayer et al., 2011; Lynch et al., 2008). Similar evolutionary changes to transcription factors have been documented in *Tinman/Nkx2.5* (Ranganayakulu et al., 1998; Schwartz and Olson, 1999), *Ubx* (Galant and Carroll, 2002; Grenier and Carroll, 2000; Ronshaugen et al., 2002), flower development regulators (Bartlett and Whipple, 2013; Lamb and Irish, 2003), and *HOM/Ftz* (Löhr et al., 2001) among others (see reviews by Cheatle Jarvela and Hinman, 2015; Hsia and McGinnis, 2003; Sivanantharajah and Percival-Smith, 2015; Wagner and Lynch, 2008). Among the barriers to a model of transcription factor evolution is a lack of detailed mechanistic studies showing how transcription factors evolve new functions (Baëza et al., 2015; Sivanantharajah and Percival-Smith, 2015).

Here, we investigate how a new functional interaction evolved between HOXA11 and FOXO1. We found that the intrinsic activation function of HOXA11 protein is repressed through an intra-molecular interaction between a CBP/P300 binding domain and a regulatory domain. The physical interaction between FOXO1 and HOXA11 unmasks the HOXA11 activation domain. Evolutionary changes in the eutherian HOXA11 protein generated a neo-allosteric switch in response to binding FOXO1. This allosteric switch likely minimized the pleiotropic



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consequences of the derived HOXA11 function because it is only triggered in cells where FOXO1 is expressed.

RESULTS

The N Terminus of HOXA11 Contains Transactivation and Internal Regulatory Domains

We have previously shown that the resurrected ancestral therian HOXA11 (AncThHOXA11) protein was expressed, localized to the nucleus, and appropriately regulated target genes; and it was unable to functionally cooperate with FOXO1 to activate gene expression from the dPRL reporter (Brayer et al., 2011; Lynch et al., 2008). These results indicate that amino acid changes in the eutherian stem lineage generated a new functional interaction between HOXA11 and FOXO1, but the mechanisms that underlie this interaction are unknown.

To identify the functional domains of HOXA11, we designed eight N-terminal truncation mutants based on patterns of conservation in HOXA11 (Figure 1A) (Roth et al., 2005) and tested their ability to activate gene expression from the dPRL promoter (Figure S1B). Co-transfection of wild-type HOXA11 and FOXO1 upregulated gene expression (>3-fold, $p < 0.001$) (Lynch et al., 2008, 2009). Truncation up to amino acid 150 (ΔN150) had a significant negative effect on transactivation. Further truncations restored gene expression. These results indicate that HOXA11 contains a dominant activation domain between amino acids (aa) 96–150, a repressive domain between aa 150–222, and a secondary activation domain in the homeodomain.

To dissect the dominant activation domain (96–150), we generated five internal deletions from the N terminus (ΔN66–81, ΔN66–96, ΔN66–111, ΔN66–126, ΔN66–141) and five internal deletions from the C terminus of the activation domain (ΔC141–151, ΔC126–151, ΔC111–151, ΔC96–151, ΔC81–151). We found that the Δ66–81 construct strongly enhanced (>6-fold, $p < 0.001$) reporter gene expression relative to wild-type HOXA11 (Figure 1B). More progressive deletions from the N terminus diminished the enhanced luciferase expression, whereas C-terminal deletions of the activation domain generally reduced gene expression.

These data suggest that within amino acids 66–81 is a negative regulatory peptide (NP) that masks a N-terminal activation domain. We hypothesized that the interaction between HOXA11 and FOXO1 relieves the repressive effect of NP, unmasking an activation domain. This model predicts that deletion of NP should lead to FOXO1-independent activation by HOXA11. Indeed, we found that the HOXA11ΔNP construct transactivated luciferase expression independently of FOXO1 (Figure 1C). We then tested whether ancestral HOXA11 protein also has a NP that suppresses an activation domain. We produced a NP deletion construct of AncThHOXA11 (i.e., Δ66–81, AncThA11ΔNP), and this deletion also leads to FOXO1-independent activation (Figure 1C). We concluded that the key innovation in the eutherian HOXA11 protein was the FOXO1-dependent disruption of the intramolecular suppression of an activation domain.

NP Is an Intramolecular Regulatory Domain

Our data suggest that NP masks the function of the activation domain in HOXA11. To directly test this hypothesis, we used a mammalian-two-hybrid system (M2H) to detect physical interactions between NP (60–81) and an extended-NP (1–82) with residues 81–151 and 130–151 of HOXA11. We found that co-transfection of NP construct with the 81–151 construct led to a significant increase in luciferase expression. In contrast, co-transfection of extended-NP construct and either the 81–151 or 130–151 constructs lead to an increase in luciferase expression (Figure 1D). These data suggest that NP physically interacts with residues 81–130 of the activation domain, whereas residues 1–60 interact with residues 130–151 of the activation domain (Figure 1E).

Derived Substitutions Are Required for Cooperativity between HOXA11 and FOXO1

To infer which amino acid substitutions are responsible for the derived functional interaction between HOXA11 and FOXO1, we computationally identified substitutions that are predicted to have altered short linear interaction motifs (SLiMs). We identified several derived phosphorylation sites (Figure 1A, bottom), as well as a eutherian-specific proline insertion at site 103 (ins103P) and a methionine to valine substitution at site 106 (M106V) (Figure S1C; Table S2).

To test whether these changes are responsible for the derived functional interaction between HOXA11 and FOXO1, we back mutated the eutherian-specific changes in mouse HOXA11 (AncEuHOXA11-103mu) and forward mutated these sites in the ancestral reconstructed HOXA11 (AncThHOXA11-103 dr) proteins, and tested their ability to cooperate with FOXO1. We found that the AncEuHOXA11-103mu mutant transactivated gene expression independent of FOXO1, similar to ΔNP construct (Figure 1F). The AncThHOXA11-103 dr forward mutant, however, was unable to induce cooperativity with FOXO1 (Figure S1E). We conclude that the derived ins103P and M106V substitutions were necessary but not sufficient for the evolution of functional cooperativity with FOXO1.

Figure 1. Characterization of the Intrinsically Disordered Region and Intra-Molecular Regulatory Domain of HOXA11

(A) Conservation plot of Eutherian (n = 82, red line) and non-Eutherian (n = 55, blue line) HOXA-11 proteins.

(B) Sequence conservation of N-terminal amino acids of HOXA11. Derived Eutherian amino acid changes are shown in red. Green and red arrows indicate potential gain and loss in phosphorylatable amino acids, respectively.

(C) Reconstructed ancestral therian (AncThA11) ΔNP mutant co-transfected with FOXO1 in HESC cell lines (shaded area).

(D) Mammalian two-hybrid (M2H) assay identifies interaction between the NP and IDR (prey). Luciferase values are shown as fold changes (mean ± SEM., n = 6) relative to background measured using CheckMate Negative Control Vectors: pBIND and pACT.

(E) Illustration of the intramolecular interaction between the IDR and N-terminal region.

(F) Both ΔNP and backward mutation of a derived PIM103 site in the eutherian HOXA11 are FOXO1-independent transactivators.

See also Figures S1, S2, and S5.

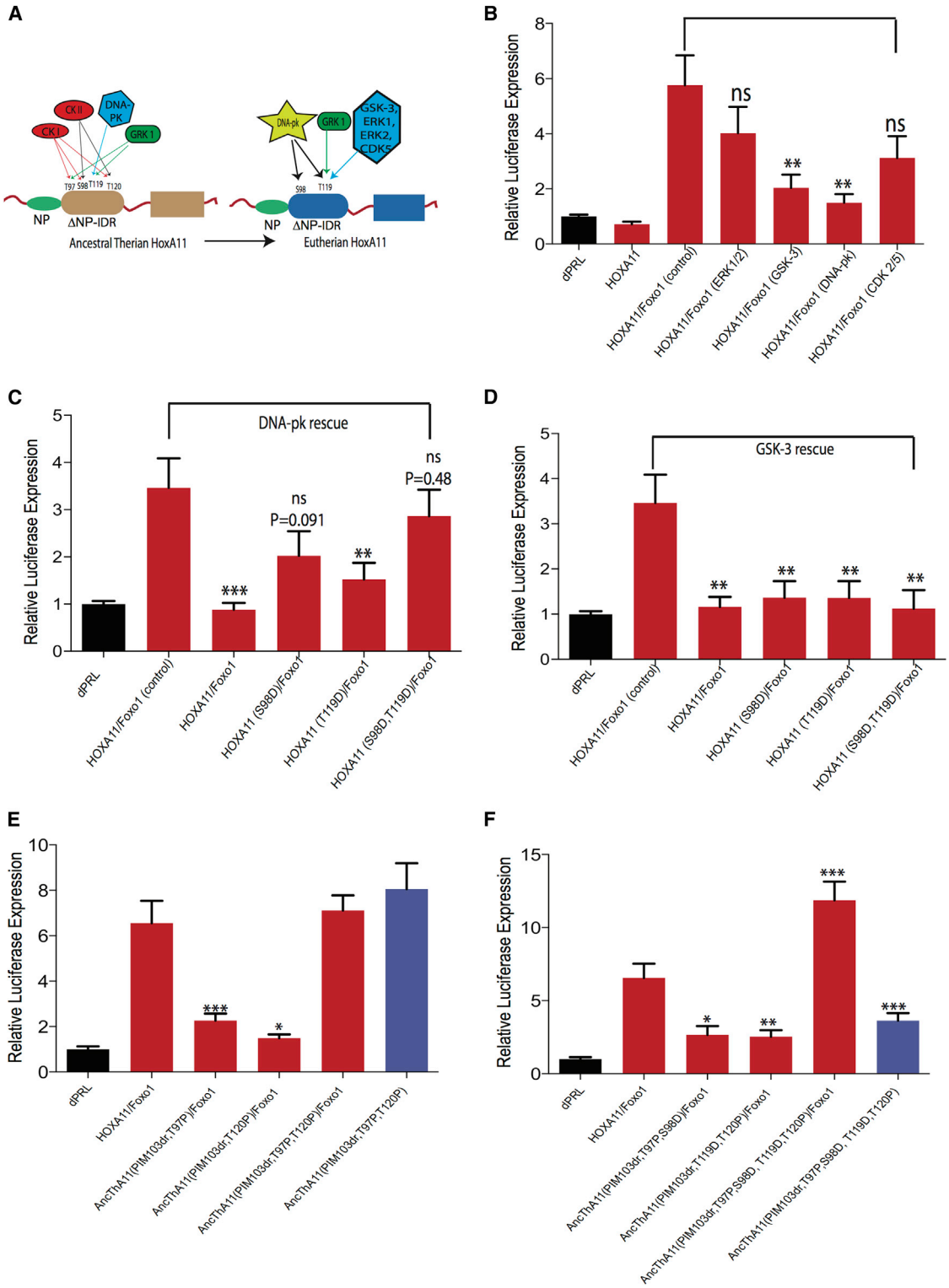

Figure 2. Gain in Function of HOXA11 Is Mediated by DNA-PK Kinase Activity

(A) Illustration of the gain and loss of Kinase motifs between ancestral therian (left image) and eutherian HOXA11 (right image) at derived phosphorylation sites S98 and T119.

(B) Effects on the cooperative transactivation activity of HOXA11 and FOXO1 in HESCs treated with ERK1/2, GSK-3β, DNA-pk, and CDK 2/5 kinase inhibitors.

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HOXA11 Evolved DNA-pk Kinase Phosphorylation Sites Necessary for Transactivation

Among the amino acid substitutions in the eutherian stem lineage, several are predicted to generate new phosphorylation sites (Figure 1A; Table S4). To experimentally identify phosphorylated sites in HOXA11, we overexpressed AncEuHOXA11 in hormone-treated and -untreated HeLa cells and immunoprecipitated the protein to identify posttranslational modifications. Mass spectroscopy results identified two phosphorylation sites S98 and T119 that evolved in eutherian mammals (Figure S2; Table S3). Computational analysis identified six kinases predicted to phosphorylate these sites (Figure 2A; Table S4). We performed reporter assays with AncEuHOXA11 and FOXO1, but blocked kinase activity with ERK1 Inhibitor II, GS-3 Inhibitor XIII, DNA-pk inhibitor III, or CDK 2/5 kinase inhibitors. We found that blocking GSK-3 or DNA-pk inhibited transactivation (Figure 2B).

To infer whether phosphorylation at the derived sites mediates transactivation by AncEuHOXA11, we simulated phosphorylation by substituting S98 and T119 with aspartic acid (Pearlman et al., 2011; Thorsness and Koshland, 1987) and tested whether these mutants could rescue the effect of kinase inhibition. We found that substitutions of either S98D or T119D resulted in a significant recovery of transactivation in the DNA-pk kinase inhibition assay. The double substitution completely recovered transactivation activity (Figure 2C). In contrast phospho-mimicking substitutions did not rescue inhibition of the kinase GSK-3 (Figure 2D). These results suggest that phosphorylation of S98 and T119 is mediated by kinase DNA-pk.

Changes Sufficient for Cooperativity of Derived HOXA11-FOXO1

The previous experiments suggest that two evolutionary changes are responsible for the cooperative interaction between HOXA11 and FOXO1: (1) mutations that lead to the derived Protein Interaction Motif (PIM103, at aa 103–107), and (2) the derived proline residues associated with amino acids S98 and T119 that lead to derived phosphorylation sites. We introduced these mutations into the AncThHOXA11 protein and tested their effects in luciferase reporter assays to determine whether these mutations are causal for the derived HOXA11-FOXO1 cooperativity. Introducing the derived PIM103 and the proline residues at P97 and P120 were sufficient to impart the AncThHOXA11 protein with activation abilities (Figure 2E). Interestingly, the forward mutated AncThHOXA11 protein is a FOXO1-independent activator. Only after introducing phospho-mimicking S98D and T119D mutations did we observe FOXO1-dependent activation (Figure 2F). We conclude that the evolution of the derived motif at aa 103–107 together with DNA-pk-dependent phosphorylation at S98 and T119 is sufficient to cause FOXO1-dependent activation at the dPRL promoter.

HOXA11 N Terminus Is Disordered with a Tendency to Form α Helices

Our functional data suggest that FOXO1 induces an allosteric change in the N terminus of HOXA11. To explore the structural basis for this effect, we generated secondary structure predictions of the HOXA11 N terminus using PSIPRED (Buchan et al., 2010; Jones, 1999) and JUFO (Leman et al., 2013). Both methods identified regions with tendencies to form α helices (aa 85–93, 104–107, and 140–148) or β strands (around aa 14, 20, 44, and 60) (Figure 3A), suggesting these segments might form when interacting with other proteins (Buchan et al., 2010; Dyson and Wright, 2005; Fuxreiter et al., 2004; Jensen et al., 2009; Jones, 1999; Leman et al., 2013; Tompa, 2005; Vucetic et al., 2005).

To test whether these predictions are plausible, we utilized de novo structure prediction algorithm Rosetta (Das et al., 2009; Fleishman et al., 2011; Simons et al., 1997). We folded residues 1–150, 58–155, and 85–157 in three experiments. In all cases, Rosetta modeled α helices and β strands in the regions predicted to have a tendency to form secondary structure. Furthermore, Rosetta sometimes folded regions 58–154 and 85–157 into helical bundles consisting of three to four α helices (Figures S3A and S3B). To experimentally test these predictions, we collected circular dichroism spectra (MacDonald et al., 1964) for two constructs (aa 64–152 and 80–152). The spectra were similar for both constructs and exhibited characteristics of intrinsically disordered proteins. Addition of 2,2,2-trifluoroethanol (TFE) caused a rapid increase in α helicity from around 8% to 35% (Figures S3C and S3D). We conclude that α helices can be readily induced in the HOXA11 N terminus.

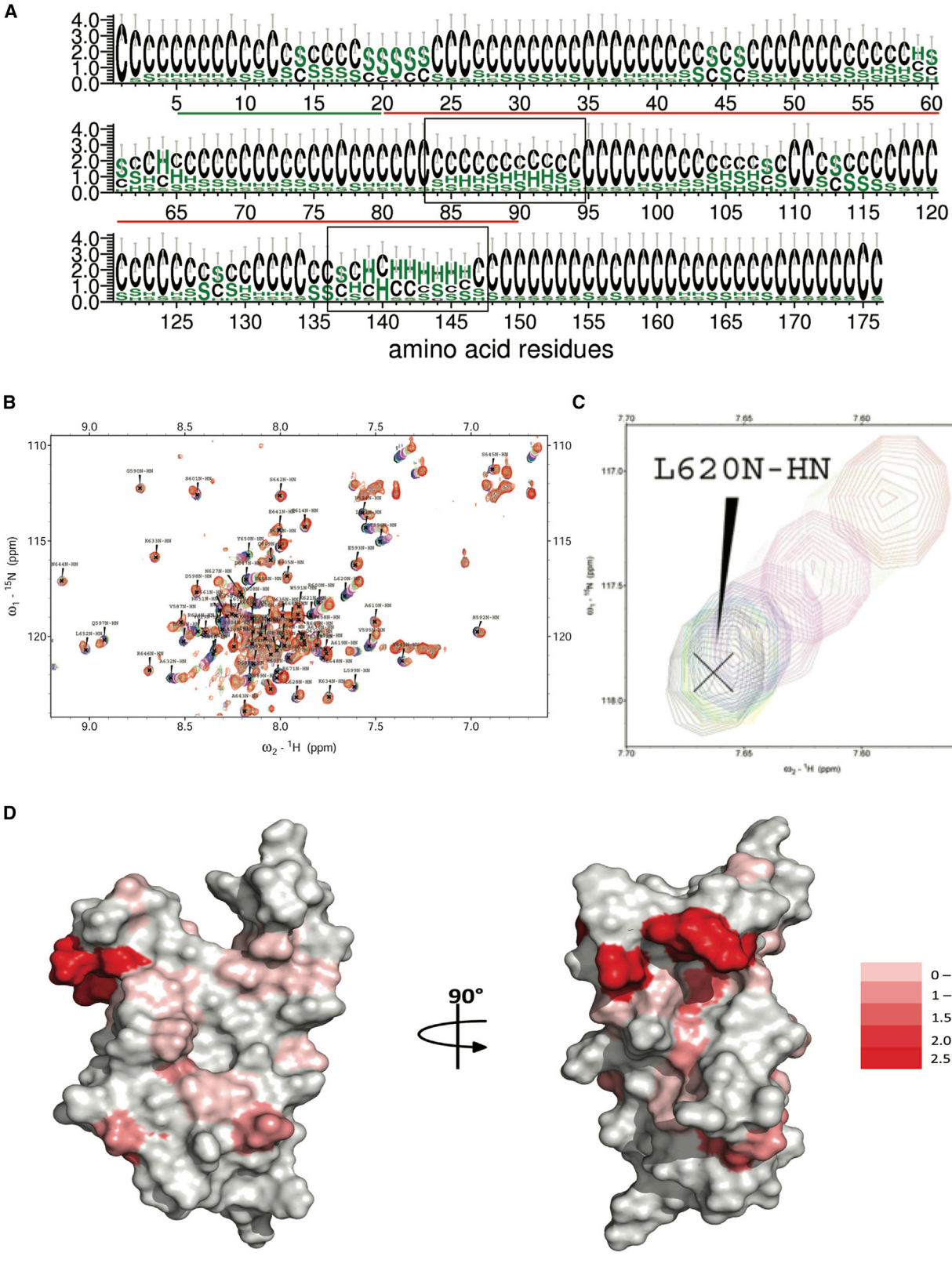
CBP Contributes to Cooperative Transcriptional Regulation

The histone acetyltransferase CREB-binding protein (CBP) is an activating cofactor for many HOX proteins (Bei et al., 2007; Chariot et al., 1999; Choe et al., 2009), suggesting the KIX binding domains (KBD) φ-x-x-φ-φ motifs in HOXA11 could mediate interactions with the CBP KIX domain (Bannister et al., 1995; Campbell and Lumb, 2002; Dai et al., 1996; Ernst et al., 2001; Giordano and Avantaggiati, 1999; Goodman and Smolik, 2000; Vo and Goodman, 2001). To determine whether CBP participates in cooperative transactivation by HOXA11 and FOXO1, we used RNAi to knock down (KD) *CBP* and *P300* in decidualized human cells. We observed strong downregulation of *PRL* in a KD of both *CBP* and *P300* (Figure S1H). However, no change was observed with *CBP* or *P300* KD individually (Figures S1F and S1G). We conclude that the HOXA11/FOXO1 complex interacts with CBP/P300 to mediate transactivation.

(C and D) Rescue of inhibition on transactivation activity by kinase inhibitors in (C) DNA-pk and (D) GSK-3β by phospho-simulation at amino acids S98D and T119D. Luciferase values are shown as fold changes (mean ± SEM., n = 6) relative to the reporter control (dPRL); p values are relative to the WT HOXA11 – FOXO1 *p < 0.05, **p < 0.01, ***p < 0.001, ns p > 0.05. Changes are sufficient for the derived HOXA11-FOXO1 cooperativity.

(E) Conversion of ancestral therian HOXA11 to a transactivator that is FOXO1 independent.

(F) Conversion of ancestral therian HOXA11 to a FOXO1-dependent activator of luciferase gene expression by mimicking phosphorylation at S98 and T119. Luciferase values are shown as fold changes (mean ± SEM., n = 6) relative to the reporter control (dPRL). *p < 0.05, **p < 0.01, ***p < 0.001, ns p > 0.05. See also Figures S2 and S5.



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KBD140–144 of ΔNP-IDR Binds with KIX Domain of CBP

Our functional data suggest that CBP mediates gene activation through a φ-x-x-φ-φ motif at aa 140–144 of HOXA11 (Figures 3A and S1C) (Mészáros et al., 2009). In order to test this model, we investigated the physical interactions between HOXA11 aa 80–152 (ΔNP-IDR) and the KIX domain of mouse CBP (Figures 3B and 3C). We observed fast chemical exchange between the bound and unbound states of KIX, with significant chemical shifts (≥0.04 PPM) for F612, T614, L620, K621, M625, E626, and N627 in the MLL binding site of KIX (Goto et al., 2002), and L607, Y650, H651, I660, and E665 in the cMyb binding site of KIX (Zor et al., 2002) (Figures 3D and S3E). The binding affinity of KIX was quite low (~0.5 ± 0.02 mM), indicating a weak interaction common for disordered proteins (Wang et al., 2012). Further analysis of the two KIX binding pockets revealed that the binding affinity at the mixed lineage leukemia (MLL) site (K_d ~0.33 ± 0.02 mM) was two times higher than at the cMyb site (Figure S3F).

Next, we reverse titrated KIX with ^{15}N labeled ΔNP-IDR (Figure S4). ^{1}H-^{15}N HSQC spectrum of ΔNP-IDR suggests an intrinsically disordered state even when bound to KIX (Figures S4A and S4B). Significant changes in chemical shifts were observed for at least eleven ΔNP-IDR residues. The peaks exhibiting significant chemical shifts were assigned to the perfect φ-x-x-φ-φ motif at aa 140–144 and a few residues flanking the motif.

To further test whether residues 140–144 of HOXA11 are necessary for the interaction with the KIX domain, we mutated the hydrophobic residues at 140–144 (ΔNP-IDR 140–144) (FDQFF to ADQAA). These mutations resulted in complete loss of binding, showing the importance of these aa for the binding of the KIX domain (Figure S4C). We conclude that the HOXA11-FOXO1 complex drives target gene expression, at least in part, through an interaction between residues 140–144 of HOXA11 with the KIX domain of CBP and its paralog P300.

The NP Interferes with Recruitment of CBP

Our functional data suggest that NP interferes with the binding of CBP to the φ-x-x-φ-φ motif at aa 140–145. In order to test this model, we compared the combined chemical shift changes from nuclear magnetic resonance (NMR) spectra between the KIX domain with ΔNP-IDR (80–152) and the KIX domain with intrinsically disordered region (IDR) (64–152, which contains the NP). Titration of IDR caused significant perturbation of residues at the N terminus of KIX that were not observed with ΔNP-IDR construct. Furthermore, titration of the IDR resulted in very little correlation to the chemical shifts observed when titrating ΔNP-IDR (80–152) (r^2 = 0.25) (Figure 4A). These results imply that the binding of IDR and ΔNP-IDR to KIX are not equivalent and that NP alters the interaction of HOXA11 with CBP.

Our M2H results indicate that NP could interact within aa 81–130, most likely through the derived PIM at aa 103–107. We mutated aa 103–107 from PGDVL to its ancestral sequence: GDML (IDR 103–107). The combined chemical shift change between the interactions of IDR 103–107 with KIX correlates poorly with that of wild-type IDR and KIX (r^2 = 0.5) (Figure 4B) and much better to that of ΔNP-IDR and KIX (r^2 = 0.82) (Figure 4C). This suggests that NP is interfering with KIX binding by interacting with PIM at residues 103–107. These results are consistent with our M2H results and with the observation that mutating both NP and derived aa 103–107 results in FOXO1-independent transactivation (Figure 1F). Titration of ^{15}N-labeled IDR to unlabeled KIX produced identical chemical shift changes for the same peaks observed during ^{15}N-labeled ΔNP-IDR titration (Figures S4D and S4E).

To test whether the phosphorylation at S98 and T119 affects HOXA11-KIX binding, we performed NMR titration substituting S98D and T119D in ΔNP-IDR (ΔNP-IDR S98D/T119D). Interestingly, the binding affinity of the phospho-mimic mutant was almost two times higher (0.19 ± 0.01 mM) than the original ΔNP-IDR (Figure 4D) at the MLL binding site. This result suggests phosphorylation at S98 and T119 sites increases the affinity of HOXA11 to CBP.

DISCUSSION

At least two TFs, HOXA11 and CEBPB, have experienced evolutionary changes coincidental with the origin of decidual cells (Lynch et al., 2008, 2011). In this paper, we investigate the derived activity of HOXA11 in response to FOXO1, which evolved in the stem lineage of eutherian mammals. We show that the derived functional cooperativity between HOXA11 and FOXO1 is due to a "neo-allosteric" regulation of HOXA11 activity by FOXO1 and phosphorylation (summarized in Figure 5). This work provides a mechanistic model of how TFs can evolve new context-specific functions, while maintaining ancestral activities.

Ancestral Features Exploited in the HOXA11/FOXO1 Cooperativity

Several factors necessary for cooperative regulation by HOXA11 and FOXO1 were in place prior to the evolution of functional cooperativity. The physical interaction between HOXA11 and FOXO1 evolved prior to the origin of mammals (Brayer et al., 2011). In contrast, the functional cooperativity evolved in the stem lineage of placental mammals (Lynch et al., 2008). We

Figure 3. HOXA11 Secondary Structure Prediction

(A) Weblogo representation of *HOXA11* IDR secondary structure prediction. The secondary structure for *HOXA11* residues 1–175 was predicted using jufo. Vertical axis denotes the probability of a Coil (C), Helix (H), or Strand (S) to occur for a given residue. Prominent helix structures are shown in the black box. Regions for the NP and ΔNP-IDR are indicated with red and green overhead lines, respectively.

(B) Overlaid ^{1}H-^{15}N HSQC spectra of ^{15}N labeled KIX (40 μM) titrated with up to 20-M equivalent of unlabeled ΔNP-IDR.

(C) Expanded region of the NMR spectra showing the chemical shift changes of residue L620 of KIX with increasing ΔNP-IDR concentration.

(D) Normalized backbone chemical shift changes of KIX up on titration of 20-M equivalent of ΔNP-IDR mapped on the solution structure of KIX-MLL-cMyb ternary complex (PDB: 2AGH). Residues marked with different shades of red indicate SD from the average chemical shift change of 0.04 ppm. The sites corresponding to cMyb (left) and MLL (right) are presented through a 90° rotation along the vertical axes.

See also Figures S1, S3, and S4.

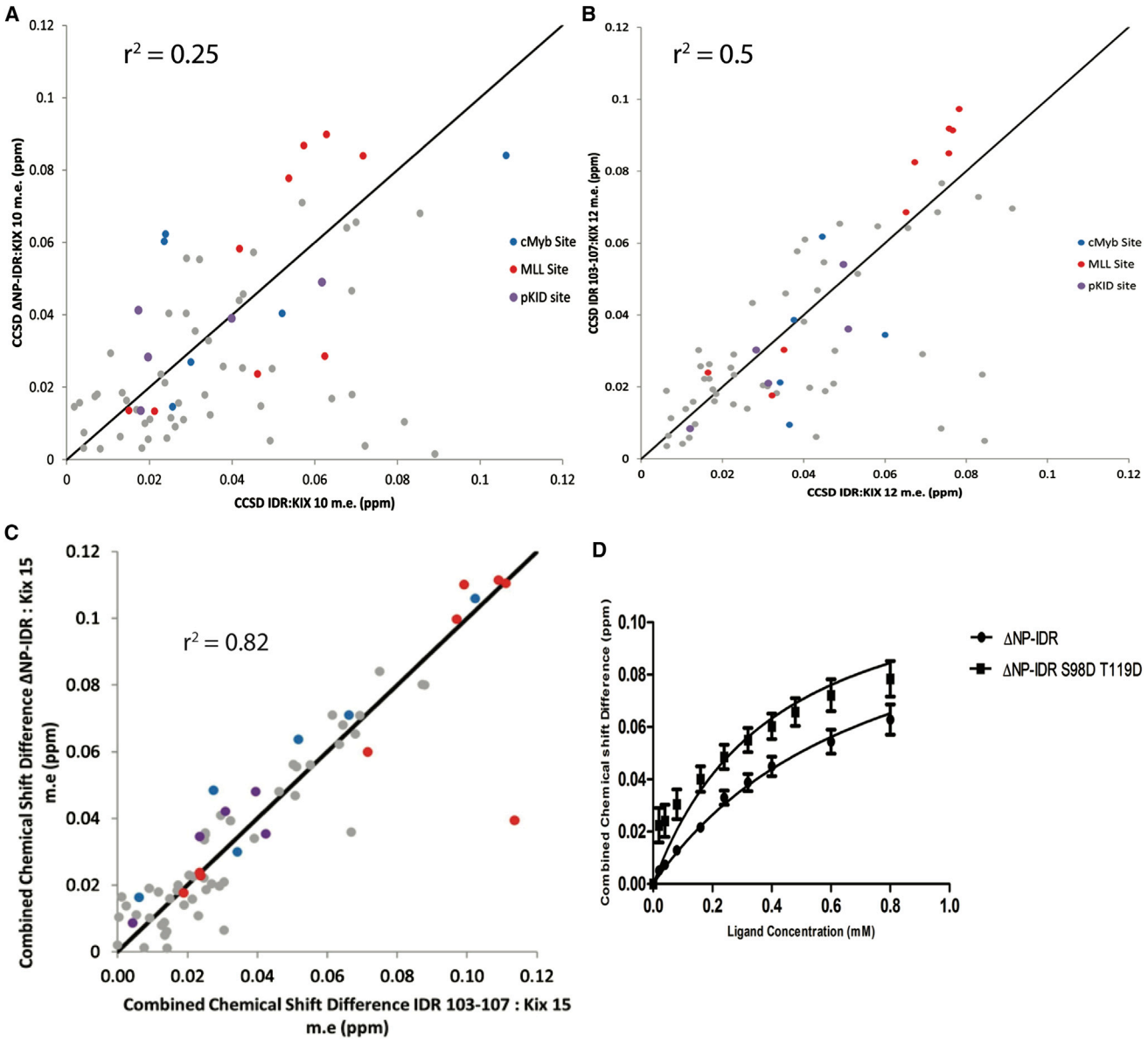

Figure 4. Correlation Plot of Combined Chemical Shift Assays

(A) Correlation plot between chemical shift changes of KIX when titrated to IDR and ΔNP-IDR at 10-M equivalents.

(B) Combined chemical shift difference correlation of IDR 103–107 with IDR.

(C) Correlation plot of chemical shift changes of KIX when titrated to IDR 103–107 and ΔNP-IDR at 15-M equivalents.

(D) Binding curves of ΔNP-IDR and double phosphor-mimic mutant ΔNP-IDR S98D T119D titrated to ^{15}N-labeled KIX measured from ^{1}H-^{15}N HSQC spectra of the MLL binding pocket. The x axis represents increasing concentration of IDR or IDR S98D T119D, combined chemical shift differences of KIX residue in the y axis. See also Figure S4.

suggest that the evolution of a new TF function can arise from a modification of a pre-existing TF complex.

HOXA11 proteins are intrinsic repressors if tested in the absence of FOXO1. *Trans*-activation is masked by the repressive NP domain. Deleting NP from the ancestral HOXA11 causes FOXO1-independent gene activation (Figure 1C). Only HOXA11 from placental mammals is capable of functionally responding to FOXO1 by unmasking its activation domain. This suggests that, in the derived state, FOXO1 is relieving the repression of a pre-existing activation domain. It is likely that HOXA11 is able to interact with other TFs in other cells to unmask its activation domain. If this is the case, the evolutionary event we describe here is an expansion of the set of TFs that can cause transcriptional activation by HOXA11.

Evolutionary Changes in the Derived HOXA11

Two kinds of evolutionary changes were identified to be sufficient for converting the ancestral HOXA11 into a context-specific

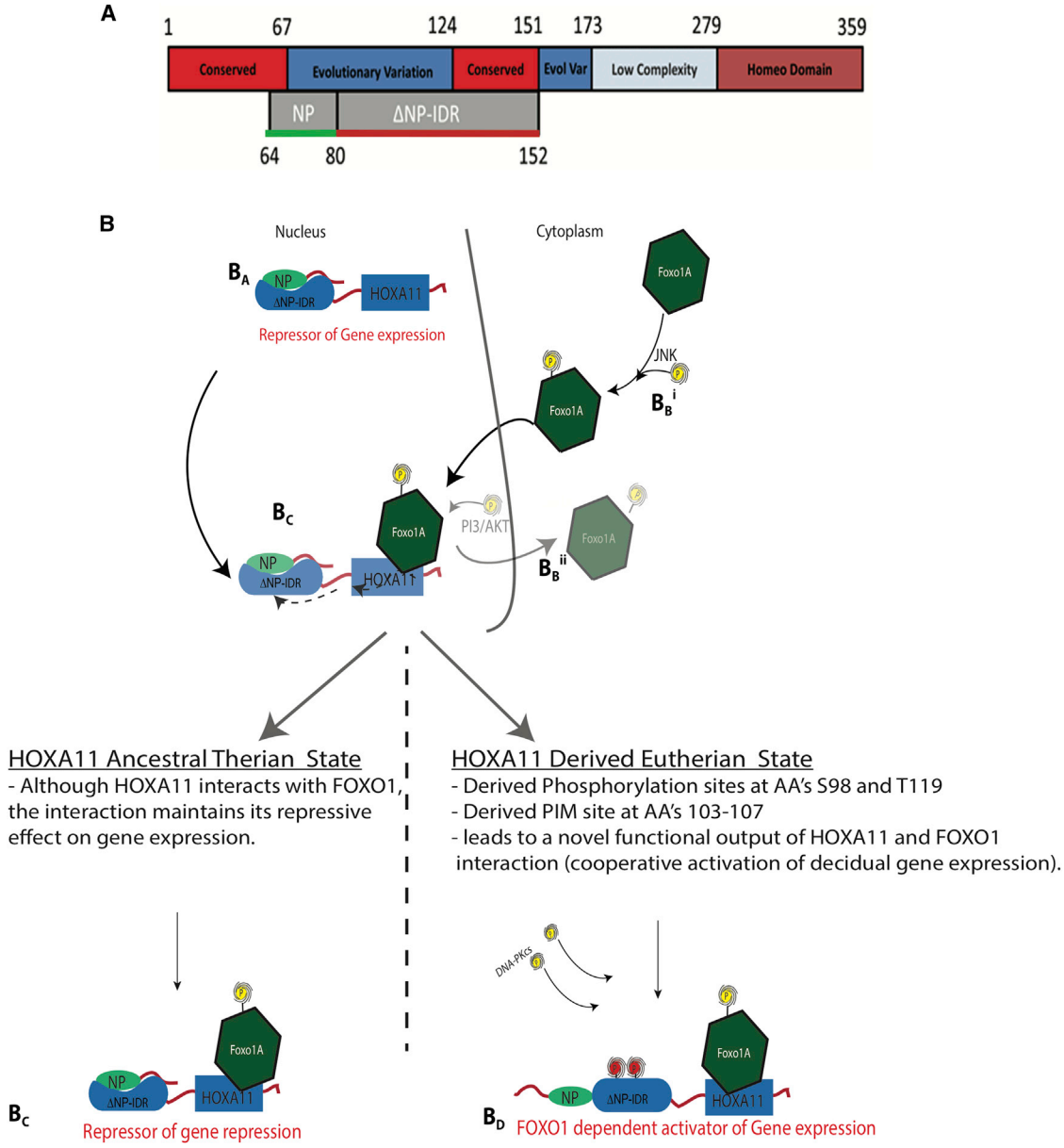

Figure 5. Schematic Representation of HOXA11

(A) Schematic representation of HOXA11. The boxes in gray indicate the two regulatory domains between regions 64–152 (IDR) identified during this study; negative regulatory domain (NP, green underlined), and the disordered regulatory region (ΔNP-IDR, red underlined)

(B) Graphical abstract illustrating the cooperative regulation of dPRL in decidualized HESCs. (B_A) Native HOXA11 is localized in the nucleus and acts as a native transcriptional repressor. (B_B) During HESCs decidualization, phosphorylation events to the FOXO1 protein are one mechanism, among others, that allow a net increase in nuclear FOXO1. (B_c) Cooperativity between Ancestral HOXA11 and FOXO1 does not relieve gene repression. (B_D) The derived phosphorylation and PIM sites is a mechanism that allows for cooperativity between Eutherian HOXA11 and FOXO1 resulting in a novel functional output in regulating decidual specific genes such as *PRL*.

transcriptional activator: (1) a derived intramolecular interaction site and (2) two derived proline substitutions and the phosphorylation of their associated phosphorylation sites.

The first site identified to have a significant impact on the cooperative transactivation was the derived Protein Interaction Motif (PIM, aa 103–107). This site alone, however, could not convert the ancestral HOXA11 into a FOXO1-dependent activator (Figure S1E). A back mutation of this site in the eutherian HOXA11 resulted in a FOXO1-independent transaction (Figure 1F). M2H studies suggest that the derived PIM functions as an interaction site for the repressive NP region in the eutherian HOXA11 (Figure 1D).

The second evolutionary change identified were two proline substitutions at positions T97P and T120P. These sites, in

combination with the derived PIM site, had a significant effect on gene regulation (Figures 2E and 2F). However, only when both proline substitutions were introduced together with the derived PIM site were we able to obtain transactivation at similar levels to the eutherian HOXA11 (Figures 2E and 2F).

Further support for the allosteric regulation of transcriptional activation is provided by NMR data. Comparison of chemical shift patterns between ΔNP-IDR and IDR with the KIX domain of CBP suggested that the NP is affecting KIX binding. Finally the mutations at the PIM103–107, leads to a chemical shift pattern very similar to that of ΔNP-IDR. From these experiments, we conclude that the evolution of the derived cooperativity involves at least four mutations. The two threonine → proline substitutions (T97P and T120P) only require single nucleotide substitutions. G103P needs a minimum of two nucleotide substitutions.

Disordered regions can allosterically regulate TF function. A pertinent example is Ubx, where multiple disordered regions control DNA interactions (Liu et al., 2008). Other examples are the intramolecular activator/repressor regions in *Drosophila* AbdA protein (Merabet et al., 2003) and the striking cooperativity switch in the E1A-CBP-pRb complex found by Ferreon et al. (2013). Intramolecular regulation of protein-protein interactions may be a general mechanism to confer functional specificity to Hox proteins (Baëza et al., 2015).

Conclusions and Perspective

The evolution of TF proteins has been considered unlikely (Carroll, 2005; Prud'homme et al., 2007; Wray, 2007). The rationale was that amino acid substitutions affecting TF functions would lead to many negative pleiotropic effects. However, TFs are capable of evolving new functions (Wagner and Lynch, 2008). Nevertheless, the mechanisms of how TF function can change are not well understood. In this study, we provide a model of TF evolution where a few amino acid substitutions led to a change of the *functional output* of a protein complex. The derived transactivation of the HOXA11::FOXO1 complex evolved through the disruption of an intramolecular repression of an activation domain in HOXA11. The derived mechanism has the potential to be context sensitive; it may only happen in cells that also express FOXO1. Whether this mode of TF evolution leads to changes in gene regulation limited to certain cell types is an important open question.

EXPERIMENTAL PROCEDURES

Characterization of the Intrinsically Disordered Region

Intrinsically disordered regions of HOXA11 proteins for eutherians (n = 82, red line) and non-eutherians (n = 55, blue line) were characterized by conservation plot. Conservation is shown as a moving average (window size = 5 amino acids and step size = 1 aa) of bit scores. The locations of N-terminal deletion constructs are shown above. Boxplot shows aa bit scores from eutherian and non-eutherian HOXA11 proteins. Additional details on disorder prediction and binding probabilities can be found in the Supplemental Experimental Procedures.

HoxA11 Expression Vector Construction

Full-length mouse (*Mus musculus*) *HOXA11* was previously generated (Roth et al., 2005). Amino-terminal flag tag and restriction sites were added to the HOXA11 peptide by PCR mutagenesis using the primer pair FpCDNAFLC (5′-TTTTTTGGTACCATGGACTACAAAGACGATGACGACAAGGATATCGAATTCGGATCCATGATGGATTTTGATGAGCGTGGT-3′), and RpCDNAFLC (5′-TTTTTTTCATTATGCTCGAGCGGCCGCTCTAGAAGCTTCTTAGGTGCGTTGGCC-3′). Truncation and deletion constructs were generated by PCR mutagenesis while site directed mutagenesis were performed using the QuickChange site directed mutagenesis kit (Table S1).

Reporter Assays, Kinase Inhibition, and RNA Interference

Transfection and luciferase reporter assay was carried out as previously described (Lynch et al., 2008) with minor adjustments. Cell-culture media was changed 24 hr after transfection and luciferase expression was assayed 48 hr after transfection using the Dual Luciferase Reporter System (Promega). Each experiment was repeated four times with eight replicates per experiment. Various constructs were assayed by western blot to determine variability of vector protein expression. Kinase inhibition assays were carried under growth conditions as described here and were repeated four times with eight replicates per experiment. For RNAi assays, small interfering RNAs (siRNAs) targeting *CBP* and *P300* (Ambion, Life Technologies) were transfected in triplicate per the manufacturer's protocol and subsequently assayed by qPCR for their effect on mRNA abundance. Additional details on these procedures including specific reagents utilized for transfection, kinase inhibition, western blotting, and RNAi can be found in the Supplemental Experimental Procedures.

Mammalian Two-Hybrid Assay

Cells were grown and transiently transfected with the CheckMate/Flexi Vector Mammalian Two-hybrid system (Promega) following manufacturers suggestions. Reporter activity was measured 48 hr after transfection. Positive interactions were scored as those that showed at least a 2-fold increase over background. For additional details, see the Supplemental Experimental Procedures.

Mass Spectrometry Analysis of *HOXA11*

HeLa cells were grown in undifferentiated media for a least three passes and then transfected with HOXA11 flag-tag. Growth media was changed 24 hr after transfection and were grown for 48 hr in either untreated or treated media (described above). Immunoprecipitation was performed with anti-flag M2 magnetic beads (Sigma-Aldrich, catalog #A2220) following manufacturer's recommendations. Mass spectrometry analysis was performed by the MS & Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory (see the Supplemental Experimental Procedures).

CD Spectroscopy

CD spectra were acquired on a Jasco J-810 spectropolarimeter equipped with Peltier temperature controller. Additional details can be found in the Supplemental Experimental Procedures.

NMR Spectroscopy

All NMR spectra were acquired at 25°C on 500 and 600 MHz Bruker spectrometers equipped with cryoprobes. Unlabeled Hox proteins were titrated into ^{15}N-labeled KIX (40 μM) to a final 20- to 30-fold molar excess concentration. The processed spectra were analyzed by Sparky (https://www.cgl.ucsf.edu/home/sparky/). Interactions between the two proteins were monitored by chemical shift changes of KIX residues in two-dimensional ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra. Additional details can be found in the Supplemental Experimental Procedures.

Statistical Procedures

Effects in the molecular biology experiments were assessed by t test as implemented in Prism software (http://www.graphpad.com/scientific-software/prism/).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.088.

AUTHOR CONTRIBUTIONS

M.C.N. designed experiments and analyzed the data for gene expression assays, M2H assay, mass spectrometry, kinase inhibition assay, conducted mutation experiments, and co-wrote the paper with G.P.W. S.G. expressed NMR proteins and performed and analyzed NMR studies, E.M.E. performed and analyzed siRNA knockdown experiments and western blotting, V.J.L. contributed the conservation analysis for the HOXA11 protein, L.S.M. and H.L.D. cloned and expressed proteins for NMR study, Y.T. assisted in cloning and sequencing of mutant constructs, M.F. performed disorder predictions, J.M. performed computational structure predictions, and G.P.W. conceived the study, contributed to data analysis, and co-wrote the paper with M.C.N. and S.G.

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REFERENCES

Baëza, M., Viala, S., Heim, M., Dard, A., Hudry, B., Duffraisse, M., Rogulja-Ortmann, A., Brun, C., and Merabet, S. (2015). Inhibitory activities of short linear motifs underlie Hox interactome specificity in vivo. eLife *4*, 4.

Bannister, A.J., Oehler, T., Wilhelm, D., Angel, P., and Kouzarides, T. (1995). Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. Oncogene *11*, 2509–2514.

Bartlett, M.E., and Whipple, C.J. (2013). Protein change in plant evolution: tracing one thread connecting molecular and phenotypic diversity. Front. Plant Sci. *4*, 382.

Bei, L., Lu, Y., Bellis, S.L., Zhou, W., Horvath, E., and Eklund, E.A. (2007). Identification of a HoxA10 activation domain necessary for transcription of the gene encoding beta3 integrin during myeloid differentiation. J. Biol. Chem. *282*, 16846–16859.

Brayer, K.J., Lynch, V.J., and Wagner, G.P. (2011). Evolution of a derived protein-protein interaction between HoxA11 and Foxo1a in mammals caused by changes in intramolecular regulation. Proc. Natl. Acad. Sci. USA *108*, E414–E420.

Buchan, D.W., Ward, S.M., Lobley, A.E., Nugent, T.C., Bryson, K., and Jones, D.T. (2010). Protein annotation and modelling servers at University College London. Nucleic Acids Res. *38*, W563–W568.

Campbell, K.M., and Lumb, K.J. (2002). Structurally distinct modes of recognition of the KIX domain of CBP by Jun and CREB. Biochemistry *41*, 13956–13964.

Carroll, S.B. (2005). Evolution at two levels: on genes and form. PLoS Biol. *3*, e245.

Carroll, S.B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell *134*, 25–36.

Chariot, A., Gielen, J., Merville, M.-P., and Bours, V. (1999). The homeodomain-containing proteins: an update on their interacting partners. Biochem. Pharmacol. *58*, 1851–1857.

Cheatle Jarvela, A.M., and Hinman, V.F. (2015). Evolution of transcription factor function as a mechanism for changing metazoan developmental gene regulatory networks. Evodevo *6*, 3.

Choe, S.K., Lu, P., Nakamura, M., Lee, J., and Sagerström, C.G. (2009). Meis cofactors control HDAC and CBP accessibility at Hox-regulated promoters during zebrafish embryogenesis. Dev. Cell *17*, 561–567.

Dai, P., Akimaru, H., Tanaka, Y., Hou, D.X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T., and Ishii, S. (1996). CBP as a transcriptional coactivator of c-Myb. Genes Dev. *10*, 528–540.

Das, R., André, I., Shen, Y., Wu, Y., Lemak, A., Bansal, S., Arrowsmith, C.H., Szyperski, T., and Baker, D. (2009). Simultaneous prediction of protein folding and docking at high resolution. Proc. Natl. Acad. Sci. USA *106*, 18978–18983.

Davidson, E.H. (2006). The Regulatory Genome: Gene Regulatory Networks in Development and Evolution (Academic Press).

Davis, A.P., Witte, D.P., Hsieh-Li, H.M., Potter, S.S., and Capecchi, M.R. (1995). Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. Nature *375*, 791–795.

Dyson, H.J., and Wright, P.E. (2005). Intrinsically unstructured proteins and their functions. Nat. Rev. Mol. Cell Biol. *6*, 197–208.

Ernst, P., Wang, J., Huang, M., Goodman, R.H., and Korsmeyer, S.J. (2001). MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein. Mol. Cell. Biol. *21*, 2249–2258.

Ferreon, A.C., Ferreon, J.C., Wright, P.E., and Deniz, A.A. (2013). Modulation of allostery by protein intrinsic disorder. Nature *498*, 390–394.

Fleishman, S.J., Leaver-Fay, A., Corn, J.E., Strauch, E.M., Khare, S.D., Koga, N., Ashworth, J., Murphy, P., Richter, F., Lemmon, G., et al. (2011). RosettaScripts: a scripting language interface to the Rosetta macromolecular modeling suite. PLoS ONE *6*, e20161.

Fuxreiter, M., Simon, I., Friedrich, P., and Tompa, P. (2004). Preformed structural elements feature in partner recognition by intrinsically unstructured proteins. J. Mol. Biol. *338*, 1015–1026.

Galant, R., and Carroll, S.B. (2002). Evolution of a transcriptional repression domain in an insect Hox protein. Nature *415*, 910–913.

Giordano, A., and Avantaggiati, M.L. (1999). p300 and CBP: partners for life and death. J. Cell. Physiol. *181*, 218–230.

Goodman, R.H., and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553–1577.

Goto, N.K., Zor, T., Martinez-Yamout, M., Dyson, H.J., and Wright, P.E. (2002). Cooperativity in transcription factor binding to the coactivator CREB-binding protein (CBP). The mixed lineage leukemia protein (MLL) activation domain binds to an allosteric site on the KIX domain. J. Biol. Chem. *277*, 43168–43174.

Grenier, J.K., and Carroll, S.B. (2000). Functional evolution of the Ultrabithorax protein. Proc. Natl. Acad. Sci. USA *97*, 704–709.

Grens, A., Mason, E., Marsh, J.L., and Bode, H.R. (1995). Evolutionary conservation of a cell fate specification gene: the Hydra achaete-scute homolog has proneural activity in Drosophila. Development *121*, 4027–4035.

Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. Science *267*, 1788–1792.

Hsia, C.C., and McGinnis, W. (2003). Evolution of transcription factor function. Curr. Opin. Genet. Dev. *13*, 199–206.

Hsieh-Li, H.M., Witte, D.P., Weinstein, M., Branford, W., Li, H., Small, K., and Potter, S.S. (1995). Hoxa 11 structure, extensive antisense transcription, and function in male and female fertility. Development *121*, 1373–1385.

Hu, Z., and Gallo, S.M. (2010). Identification of interacting transcription factors regulating tissue gene expression in human. BMC Genomics *11*, 49.

Jensen, M.R., Markwick, P.R., Meier, S., Griesinger, C., Zweckstetter, M., Grzesiek, S., Bernadó, P., and Blackledge, M. (2009). Quantitative determination of the conformational properties of partially folded and intrinsically disordered proteins using NMR dipolar couplings. Structure *17*, 1169–1185.

Jones, D.T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. *292*, 195–202.

Kapusta, A., Kronenberg, Z., Lynch, V.J., Zhuo, X., Ramsay, L., Bourque, G., Yandell, M., and Feschotte, C. (2013). Transposable elements are major contributors to the origin, diversification, and regulation of vertebrate long noncoding RNAs. PLoS Genet. *9*, e1003470.

Lamb, R.S., and Irish, V.F. (2003). Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. Proc. Natl. Acad. Sci. USA *100*, 6558–6563.

Leman, J.K., Mueller, R., Karakas, M., Woetzel, N., and Meiler, J. (2013). Simultaneous prediction of protein secondary structure and transmembrane spans. Proteins *81*, 1127–1140.

Liu, Y., Matthews, K.S., and Bondos, S.E. (2008). Multiple intrinsically disordered sequences alter DNA binding by the homeodomain of the Drosophila hox protein ultrabithorax. J. Biol. Chem. *283*, 20874–20887.

Löhr, U., Yussa, M., and Pick, L. (2001). Drosophila fushi tarazu. a gene on the border of homeotic function. Curr. Biol. *11*, 1403–1412.

Lynch, V.J., Tanzer, A., Wang, Y., Leung, F.C., Gellersen, B., Emera, D., and Wagner, G.P. (2008). Adaptive changes in the transcription factor HoxA-11 are essential for the evolution of pregnancy in mammals. Proc. Natl. Acad. Sci. USA *105*, 14928–14933.

Lynch, V.J., Brayer, K., Gellersen, B., and Wagner, G.P. (2009). HoxA-11 and FOXO1A cooperate to regulate decidual prolactin expression: towards inferring the core transcriptional regulators of decidual genes. PLoS ONE *4*, e6845.

Lynch, V.J., May, G., and Wagner, G.P. (2011). Regulatory evolution through divergence of a phosphoswitch in the transcription factor CEBPB. Nature *480*, 383–386.

Lynch, V.J., Nnamani, M.C., Kapusta, A., Brayer, K., Plaza, S.L., Mazur, E.C., Emera, D., Sheikh, S.Z., Grützner, F., Bauersachs, S., et al. (2015). Ancient transposable elements transformed the uterine regulatory landscape and transcriptome during the evolution of mammalian pregnancy. Cell Rep. *10*, 551–561.

MacDonald, N.S., Ibsen, K.H., and Urist, M.R. (1964). Effect of Tetracycline on Retention of Calcium and Strontium in Rodents. Proc. Soc. Exp. Biol. Med. *115*, 1125–1128.

McGinnis, N., Kuziora, M.A., and McGinnis, W. (1990). Human Hox-4.2 and Drosophila deformed encode similar regulatory specificities in Drosophila embryos and larvae. Cell *63*, 969–976.

Merabet, S., Kambris, Z., Capovilla, M., Bérenger, H., Pradel, J., and Graba, Y. (2003). The hexapeptide and linker regions of the AbdA Hox protein regulate its activating and repressive functions. Dev. Cell *4*, 761–768.

Mészáros, B., Simon, I., and Dosztányi, Z. (2009). Prediction of protein binding regions in disordered proteins. PLoS Comput. Biol. *5*, e1000376.

Pearlman, S.M., Serber, Z., and Ferrell, J.E., Jr. (2011). A mechanism for the evolution of phosphorylation sites. Cell *147*, 934–946.

Prud'homme, B., Gompel, N., and Carroll, S.B. (2007). Emerging principles of regulatory evolution. Proc. Natl. Acad. Sci. USA *104* (*Suppl 1*), 8605–8612.

Ranganayakulu, G., Elliott, D.A., Harvey, R.P., and Olson, E.N. (1998). Divergent roles for NK-2 class homeobox genes in cardiogenesis in flies and mice. Development *125*, 3037–3048.

Ronshaugen, M., McGinnis, N., and McGinnis, W. (2002). Hox protein mutation and macroevolution of the insect body plan. Nature *415*, 914–917.

Roth, J.J., Breitenbach, M., and Wagner, G.P. (2005). Repressor domain and nuclear localization signal of the murine Hoxa-11 protein are located in the homeodomain: no evidence for role of poly alanine stretches in transcriptional repression. J. Exp. Zoolog. B Mol. Dev. Evol. *304*, 468–475.

Schwab, K., Hartman, H.A., Liang, H.C., Aronow, B.J., Patterson, L.T., and Potter, S.S. (2006). Comprehensive microarray analysis of Hoxa11/Hoxd11 mutant kidney development. Dev. Biol. *293*, 540–554.

Schwartz, R.J., and Olson, E.N. (1999). Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. Development *126*, 4187–4192.

Simons, K.T., Kooperberg, C., Huang, E., and Baker, D. (1997). Assembly of protein tertiary structures from fragments with similar local sequences using simulated annealing and Bayesian scoring functions. J. Mol. Biol. *268*, 209–225.

Sivanantharajah, L., and Percival-Smith, A. (2015). Differential pleiotropy and HOX functional organization. Dev. Biol. *398*, 1–10.

Speleman, F., Cauwelier, B., Dastugue, N., Cools, J., Verhasselt, B., Poppe, B., Van Roy, N., Vandesompele, J., Graux, C., Uyttebroeck, A., et al. (2005). A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of HOXA10 and HOXA11 in a subset of T-cell acute lymphoblastic leukemias. Leukemia *19*, 358–366.

Thorsness, P.E., and Koshland, D.E., Jr. (1987). Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. J. Biol. Chem. *262*, 10422–10425.

Tompa, P. (2005). The interplay between structure and function in intrinsically unstructured proteins. FEBS Lett. *579*, 3346–3354.

Vo, N., and Goodman, R.H. (2001). CREB-binding protein and p300 in transcriptional regulation. J. Biol. Chem. *276*, 13505–13508.

Vucetic, S., Obradovic, Z., Vacic, V., Radivojac, P., Peng, K., Iakoucheva, L.M., Cortese, M.S., Lawson, J.D., Brown, C.J., Sikes, J.G., et al. (2005). DisProt: a database of protein disorder. Bioinformatics *21*, 137–140.

Wagner, G.P., and Lynch, V.J. (2008). The gene regulatory logic of transcription factor evolution. Trends Ecol. Evol. *23*, 377–385.

Wang, F., Marshall, C.B., Yamamoto, K., Li, G.Y., Gasmi-Seabrook, G.M., Okada, H., Mak, T.W., and Ikura, M. (2012). Structures of KIX domain of CBP in complex with two FOXO3a transactivation domains reveal promiscuity and plasticity in coactivator recruitment. Proc. Natl. Acad. Sci. USA *109*, 6078–6083.

Wellik, D.M., and Capecchi, M.R. (2003). Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science *301*, 363–367.

Wray, G.A. (2007). The evolutionary significance of cis-regulatory mutations. Nat. Rev. Genet. *8*, 206–216.

Yokouchi, Y., Sakiyama, J., and Kuroiwa, A. (1995). Coordinated expression of Abd-B subfamily genes of the HoxA cluster in the developing digestive tract of chick embryo. Dev. Biol. *169*, 76–89.

Zor, T., Mayr, B.M., Dyson, H.J., Montminy, M.R., and Wright, P.E. (2002). Roles of phosphorylation and helix propensity in the binding of the KIX domain of CREB-binding protein by constitutive (c-Myb) and inducible (CREB) activators. J. Biol. Chem. *277*, 42241–42248.