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

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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.

Highlights
- The HOXA11 protein has a regulatory motif that masks its activation domain
- In placental mammals, upon binding to FOXO1, HOXA11 unmask 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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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., 1999). 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 Capecechi, 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; Ronschagen et al., 2002), flower development regulators (Bartlett and Whipple, 2013; Lamb and Irish, 2003), and Homer/Ftz (Lörh et al., 2001) among others (see reviews by Cheattle 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 (Báezza 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. Evolutionary changes in the eutherian HOXA11 protein generated a neo-allosteric switch in response to binding FOXO1. This allosteric switch likely minimized the pleiotropic
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 (∆N66–81, ∆N66–96, ∆N66–111, ∆N66–126, ∆N66–141) and five internal deletions from the C terminus of the activation domain (∆C141–151, ∆C126–151, ∆C111–151, ∆C96–151, ∆C81–151). We found that the ∆66–81 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 HOXA11-NP 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., ∆66–81, 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 ∆NP 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 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 therian 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 spectrometry results identified two phosphorylation sites S98 and T119 that evolved in therian 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 substituting S98 and T119 with aspartic acid (Pearlman et al., 2011; Thorsness and Koshland, 1987) and tested whether these mutations 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 phosphomimicking substitutions did not inhibit phosphorylation 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-x-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 phosho-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 promoter. 

* 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 values are relative to the WT HOXA11 – FOXO1 promoter. 

* p < 0.05, ** p < 0.01, *** p < 0.001, ns p > 0.05.
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 S1) (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 (Kd ~0.33 ± 0.02 mM) was two times higher than at the cMyb site (Figure S3F).

Next, we reverse titrated KIX with 15N labeled ΔNP-IDR (Figure S4). 1H-15N 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) (r2 = 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 PGDV 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 (r2 = 0.5) (Figure 4B) and much better to that of ΔNP-IDR and KIX (r2 = 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 15N-labeled IDR to unlabelled KIX produced identical chemical shift changes for the same peaks observed during 13N-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 juf. 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 1H-15N HSQC spectra of 15N 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 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.
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
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 ∆NP-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 ∆NP-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 Ferreol 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 were able to obtain transactivation at similar levels to the eutherian HOXA11 (Figures 2E and 2F).

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 a 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 15N-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 1H-15N heteronuclear single quantum coherence (HSCQ) spectra. Additional details can be found in the Supplemental Experimental Procedures.

### 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'-TTTTTTGATCATGACTGACAAAAAGCGTACAGGAGGATATCGAATTGGATCAGTATGATGAGTTGGTCGCGCTCTAGAAGCTTCTTAGGTGCGTTGGC-3'), and RpCDNAFLC (5'-TTTTTCTTATGTCGCGGCGCCCTCTAGAAAGCTTCTTATGAGCAGGCGTACTTGGCC-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).

**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 a 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 15N-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 1H-15N heteronuclear single quantum coherence (HSCQ) 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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