



# Dynamic regulation of Nanog and stem cell-signaling pathways by Hoxa1 during early neuro-ectodermal differentiation of ES cells

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**Homeobox a1 (*Hoxa1*) is one of the most rapidly induced genes in ES cell differentiation and it is the earliest expressed Hox gene in the mouse embryo. In this study, we used genomic approaches to identify Hoxa1-bound regions during early stages of ES cell differentiation into the neuro-ectoderm. Within 2 h of retinoic acid treatment, Hoxa1 is rapidly recruited to target sites that are associated with genes involved in regulation of pluripotency, and these genes display early changes in expression. The pattern of occupancy of Hoxa1 is dynamic and changes over time. At 12 h of differentiation, many sites bound at 2 h are lost and a new cohort of bound regions appears. At both time points the genome-wide mapping reveals that there is significant co-occupancy of Nanog (Nanog homeobox) and Hoxa1 on many common target sites, and these are linked to genes in the pluripotential regulatory network. In addition to shared target genes, Hoxa1 binds to regulatory regions of *Nanog*, and conversely Nanog binds to a 3' enhancer of *Hoxa1*. This finding provides evidence for direct cross-regulatory feedback between *Hoxa1* and *Nanog* through a mechanism of mutual repression. Hoxa1 also binds to regulatory regions of *Sox2* (sex-determining region Y box 2), *Esr1b* (estrogen-related receptor beta), and *Myc*, which underscores its key input into core components of the pluripotential regulatory network. We propose a model whereby direct inputs of Nanog and Hoxa1 on shared targets and mutual repression between Hoxa1 and the core pluripotency network provides a molecular mechanism that modulates the fine balance between the alternate states of pluripotency and differentiation.**

gene regulation | Hox genes | pluripotency | Nanog | regulatory networks

Pluripotency and differentiation are two key opposing states integral to the processes of cellular homeostasis. Regulating the proper progression and balance of these two states is not only important in early embryos and embryonic stem (ES) cells, but also critical during organogenesis and morphogenesis for controlling the formation of differentiated cell populations from multipotential progenitors. A variety of studies have defined a core pluripotency gene regulatory network (GRN) that consists of *Nanog* (Nanog homeobox), *Oct4* (octamer-binding transcription factor 4), and *Sox* (sex-determining region Y box)2, with *Klf4* (Kruppel-like factor 4), *c-Myc* (v-Myc avian myelocytomatosis viral oncogene homolog), *Sall4* (Spalt-like transcription factor 4), *Esr1b* (estrogen-related receptor beta), *Utf1* (undifferentiated embryonic cell transcription factor 1), *Tet2* (Tet methylcytosine dioxygenase 2), and *Glis1* (GLIS family zinc finger 1) representing additional key components of this network (1–4). The core GRN factors actively maintain the pluripotential state by positively modulating the expression of diverse pathways essential for this state, but they also interact with many repressors, like the NuRD (nucleosome remodeling deacetylase) complex, REST (RE1 silencing transcription factor) and co-REST (REST corepressor 1), to inhibit differentiation pathways and maintain the pluripotent state (5–7). Major signaling pathways, such as Wnt, bone morphogenetic protein (BMP)4, and TGF- $\beta$ ,

also feed into this GRN to maintain cells in a pluripotency state by modulating expression of core network components (8–11). The expression of the core pluripotency network involves the extensive deployment of auto- and cross-regulatory feedback interactions. For example, Nanog is known to directly activate *Oct4* and *Sox2*, whereas they in turn positively cross-regulate *Nanog*. *Oct4* and *Sox2* positively feedback to maintain their own expression via direct autoregulation, whereas *Nanog* modulates its level of gene expression by negative autoregulation mediated by interactions with Zfp281 (zinc finger protein 281), which recruits the NuRD repressor complex (12–17).

In embryonic stem cells and developing embryos the processes of differentiation and morphogenesis are initiated through the differential response of cells to overlapping and opposing signaling gradients, such as retinoic acid (RA), Fgfs (fibroblast growth factors), and Wnts (18–21). These signaling pathways in turn induce and modulate the expression of master regulators of cellular fate, such as homeobox (*Hox*) genes, in a spatiotemporal manner. *Hox* genes play highly conserved roles in modulating regional identity and programs of cellular differentiation in a temporally and spatially restricted manner (21). During RA-induced differentiation of murine ES cells, *Hoxa1* is a direct target of RA signaling and is one of the most rapidly induced genes through mechanisms involving control of elongation of paused Pol II (RNA polymerase II) (22–24). This finding is consistent with it being an important early determinant in ES cell differentiation. In murine embryogenesis, *Hoxa1* is the earliest the expressed Hox gene (25, 26) and along with *Hoxb1*, its group 1 paralog, is a direct target of RA signaling in neural and mesodermal tissues through the presence of multiple RA response elements (RAREs) in their flanking *cis*-regulatory regions (27–30). Loss-of-function and lineage studies have implicated *Hoxa1* in development of the inner ear, heart, neural crest specification, and hindbrain patterning (31–33). Growing evidence suggests roles for *Hoxa1* in etiology of various cancers through

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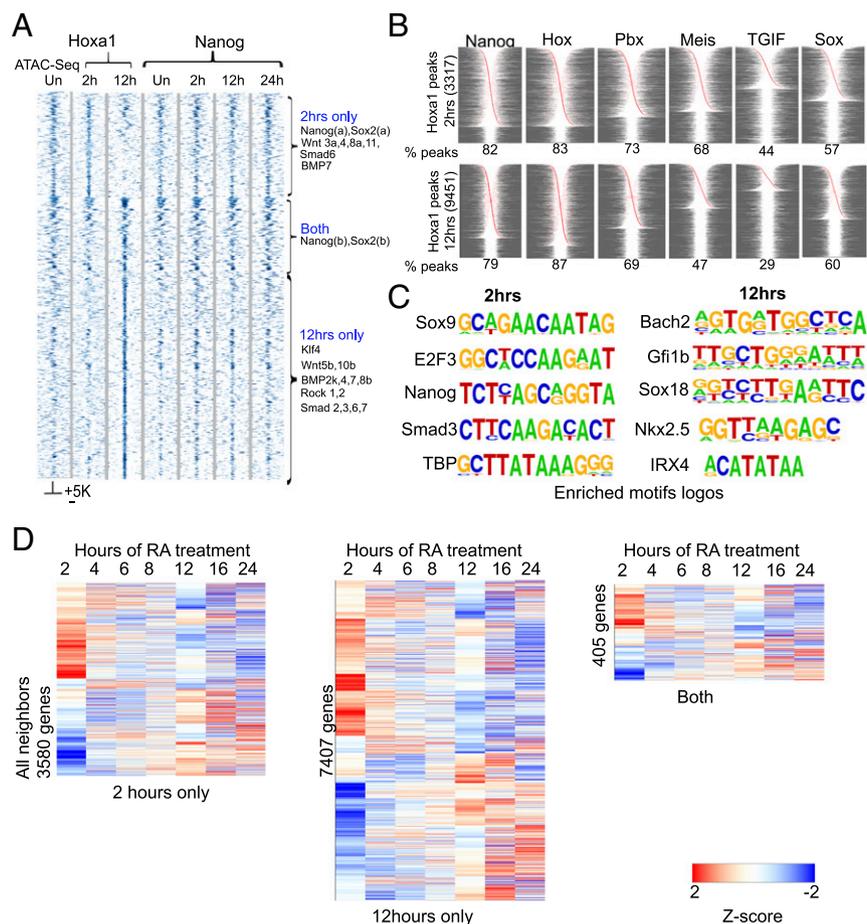
modulation of cell proliferation, invasion, and metastasis (34–37). These diverse functional roles for *Hoxa1* appear to be at least partially related to its ability to influence key signaling pathways in differentiating cells (35).

Despite expanding data characterizing the nature of both the pluripotential regulatory network and Hox-dependent differentiation and developmental programs, there is a lack of understanding of how these two distinct yet interrelated programs for controlling cell states interact with each other to maintain an appropriate balance. In this study, we use genome-wide binding analyses of *Hoxa1* and *Nanog* over very early stages of programmed differentiation of murine ES cells. We find evidence that suggests *Hoxa1* and *Nanog* reciprocally regulate a common set of downstream target genes of the pluripotential regulatory network in early stages of differentiation and are involved in direct mutual repression of each other's expression. This finding suggests a model for *Hoxa1*–*Nanog* regulatory interactions that provides insight into how these two independent GRNs may coordinate modulation of the fine balance between the state of pluripotency and differentiation in ES cells.

## Results

### Dynamic Genome-Wide Occupancy of *Hoxa1* and Co-Occupancy of *Nanog* in *Hoxa1*-Bound Regions in Early Differentiating ES Cells.

*Hox* genes are not expressed in ES cells. During RA-induced neuro-ectodermal differentiation of murine ES cells, *Hoxa1* is one of the most rapidly induced genes (22, 24). In this RA-induced differentiation paradigm, monitoring events at the level of single cells using methods for single-molecule RNA FISH, we found a robust and relatively uniform RA response by the most rapidly induced genes, including *Hoxa1* (22). Following initial activation of *Hoxa1*, we investigated its early downstream target genes after 2 and 12 h of RA-induced differentiation. We performed chromatin immunoprecipitation and deep sequencing (ChIP-seq) using KH2 ES cells carrying an inducible epitope-tagged version of *Hoxa1* (3XFLAG-MYC) (Fig. 1A). At 2 h after RA treatment triggering the initial induction of *Hoxa1* expression, there is evidence for genome-wide occupancy of the *Hoxa1* protein. In duplicate experiments, we identified 3,317 reproducibly bound regions (Dataset S1). These peaks include occupancy near many genes that play important roles in the regulation of pluripotency [e.g.,



**Fig. 1.** Dynamic shifts in occupancy of *Hoxa1* and co-occupancy of *Hoxa1* and *Nanog* during RA-induced differentiation. (A) Heatmap showing occupancy of *Hoxa1* on genomic targets after 2 and 12 h of RA treatment. ATAC-seq for open chromatin states in uninduced ES cells and a time series of *Nanog* occupancy during differentiation on these same targets is also shown. A  $\pm 5$ -kb region around core binding peaks are shown in the heatmap. Three distinct binding classes designated 2-h only, both, and 12-h only plus examples of genes in each class are indicated at the right. (B) The panel shows the fraction and relative position of select enriched motifs for *Nanog*, *Hox*, *Pbx*, *Meis*, *TGIF*, and *Sox* in *Hoxa1*-bound regions at 2 and 12 h of RA treatment. Red dots indicate relative position of the respective associated motif. Peaks are centered on the midpoint of the *Hoxa1*-bound region and arranged according to distance from center and left to right from center. In cases with the presence of multiple motifs of the same sequence, these are collapsed to a single site. The percentage of *Hoxa1* peaks enriched with each motif is shown at the bottom of each graph. (C) Examples of enriched motif logos in regions bound by *Hoxa1* after 2 and 12 h of RA treatment. (D) Heatmaps showing differential expression of genes neighboring *Hoxa1*-bound regions at 2 h, 12 h, or both time points. Values shown are z-scores of  $\log_2$  expression values from Affymetrix expression profiling over a time course of differentiation compared with levels in uninduced ES cells (22). Hierarchical clustering was done with Euclidean distance, average linkage was thresholded on  $[-2, 2]$ .

*Nanog*, *Sox2*, *Smad6* (SMAD family member 6), *BMP7*, and *Wnts*] (Fig. 1A). Surprisingly, by 12 h after RA treatment there is a dramatic shift in the genome-wide binding profile compared with 2 h. There is a loss of occupancy on many of the early target regions bound at 2 h and an expansion of total Hoxa1-binding peaks (9,451), primarily through gain of new sites (Fig. 1A, Fig. S1A, and Dataset S2). Many of these new sites are also adjacent to genes involved in the control of pluripotency (e.g., *Klf4*, *Wnts*, and *TGF- $\beta$*  pathway members). Only 301 targets are occupied at both the 2- and 12-h time points (Fig. 1A and Fig. S1), but this cohort includes the core pluripotency genes *Nanog* and *Sox2*. In light of the relatively homogenous early differentiation of the ES cells (22), these shifts in binding distribution likely reflect differences in the progressive differentiation states between 2 and 12 h, rather than heterogeneity in the population.

Analysis of sequences within the Hoxa1-bound regions reveals that there are both unique and overlapping cohorts of enriched consensus binding motifs associated with occupancy of Hoxa1 at 2 and 12 h (Fig. 1B and C and Datasets S3 and S4). Consistent with occupancy of Hoxa1, analysis using TransFac revealed that 83% of the peaks at 2 h and 87% of the peaks at 12 h are enriched for consensus Hox binding motifs positioned near the center of Hoxa1 occupancy (Fig. 1B). In addition to these Hox motifs, we also observed enrichments for a set of other known consensus binding motifs [e.g., *Nanog*, *Pbx* (Pre-B-cell leukemia homeobox), *Meis* (myeloid ecotropic viral integration site 1 homolog), *TGIF*, and *Sox*] shared at both of these time points. This finding reveals that there are common motif features in many of the Hoxa1-bound regions and these include consensus sites for Hox, cofactors, and pluripotential transcription factors (TFs) (Fig. 1B). However, in accord with the dynamic differences in occupancy and increased number of peaks bound at 12 h vs. 2 h, de novo motif discovery using Homer analysis revealed differences in both the scope and nature of enriched motifs at these time points (Fig. 1C and Datasets S3 and S4). At 2 h, there is more enrichment for TF consensus binding sites of many core pluripotency factors [e.g., *Sox9*, *Nanog*, *Smad3*, *TBP* (TATA-binding protein), and *E2F3* (E2F transcription factor 3)] (Fig. 1C and Dataset S3). At 12 h we observe a greater degree of enrichment for other motifs. This finding is illustrated by a larger number of peaks with consensus bipartite Hox-Pbx motifs and enrichment for motifs of TFs associated with differentiation [*Bach2* (BTB domain and CNC homolog 2), *Gfi1b* (growth-factor-independent 1B transcriptional repressor), *Sox18*, *Nkx2.5* (NK2 homeobox 5), and *Irx4* (Iroquois homeobox 4)] (Fig. 1C and Dataset S4).

In light of the finding that a large number of Hoxa1-bound peaks are enriched for *Nanog* motifs (82% at 2 h and 79% at 12 h) (Fig. 1B), we investigated physical occupancy of *Nanog* on these regions by ChIP-seq in a time course of ES cell differentiation (Fig. 1A). There is clear evidence for co-occupancy of *Nanog* on many of the early and later Hoxa1-bound regions. Unlike Hoxa1, occupancy of *Nanog* shows little dynamic variation over a 24-h time course of RA treatment (Fig. 1A). This finding suggests that functionally relevant *Nanog* motifs are coassociated with many Hoxa1-bound regions in early differentiating ES cells, implying that they may share many common downstream target genes. Furthermore, to explore whether Hoxa1 is remodeling chromatin as a pioneer factor to facilitate accessibility for binding, we used assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) to monitor the openness of chromatin on future Hoxa1 targets in uninduced ES cells. We found that the majority of Hoxa1-bound regions observed at 2 and 12 h are already accessible or open in uninduced cells (Fig. 1A). Consistent with this idea, *Nanog* is already bound to most of these same regions in uninduced ES cells, which suggests that Hoxa1 is binding to regions that are already open and occupied by other TFs and not acting primarily as a pioneer factor to mediate opening of chromatin.

To examine how occupancy of Hoxa1 correlates with the expression, we analyzed changes in the expression of nearest-neighbor genes over a time course of differentiation (Fig. 1D and Fig. S1B). Over the 24-h period of differentiation, a large number of these genes show dynamic and differential expression compared with uninduced ES cells. It is striking that many of the genes adjacent to Hoxa1-bound regions at 2 h, 12 h, or both time points display large increases or decreases in levels of expression at 2 h (Fig. 1D). Other changes appear over the first 8 h of differentiation, but there is another major shift in the expression profiles that appears at 12 h and continues to dynamically change in later time points. The near adjacent genes cobound by Hoxa1 and *Nanog* at 2 h also show significant changes in levels of expression over the initial 4- to 6-h period of RA treatment compared with uninduced ES cells (Fig. S1B). Up-regulated genes in this cobound cohort correlate with differentiation pathways [e.g., *Meis2*, *Pax6* (paired box homeotic gene 6), and *Msx2* (Msh homeobox 2)] whereas down-regulated genes [e.g., *Sox2*, *Esrrb*, and *Lifr* (leukemia inhibitory factor receptor alpha)] are enriched for components of the pluripotency GRN (Fig. S1B). This finding is in agreement with the idea of dynamic and reciprocal inputs of Hoxa1 and *Nanog* into common targets. Genes associated with peaks bound by Hoxa1 independent of *Nanog* also display differential gene expression. This finding suggests additional, *Nanog*-independent inputs of Hoxa1 into the pluripotential and differentiation GRNs. The genome-wide data indicate that both differentiation and pluripotential genes appear to be downstream targets of Hoxa1 and *Nanog*, providing a potential regulatory link between the pluripotency and differentiation pathways.

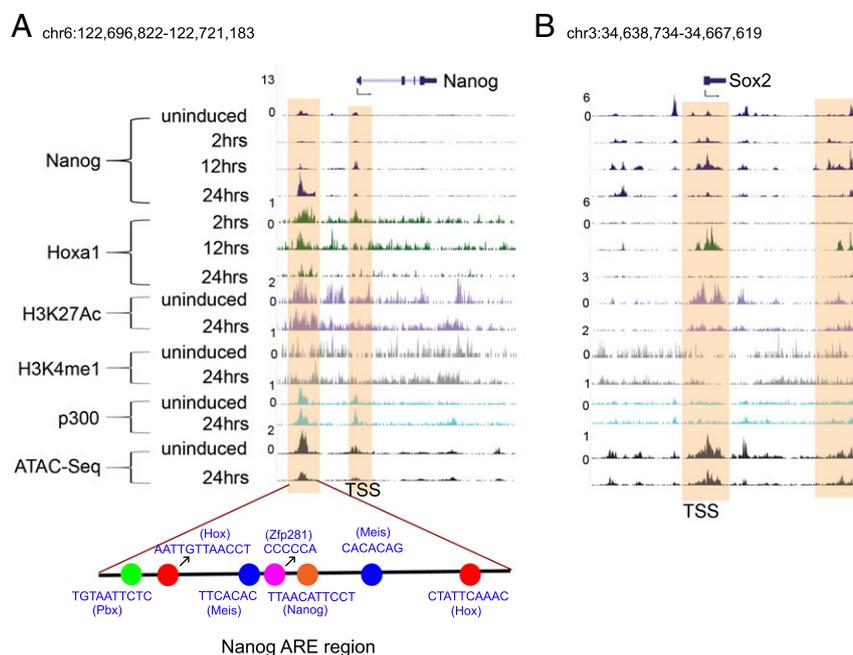
#### Stem Cell-Signaling Pathways Are Enriched Downstream Targets of Hoxa1.

At both the 2- and 12-h time points, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the near adjacent genes bound by Hoxa1 reveals that signaling pathways regulating pluripotency of stem cells and processes of neural differentiation are highly enriched (Figs. S1 and S2 and Dataset S5). Fig. S2 illustrates that many genes associated with both the naive and primed stem cell-signaling pathways are directly bound by Hoxa1. In accord with the changes in genome-wide binding observed between 2 and 12 h, there are dynamic changes in the occupancy of Hoxa1 near genes in these pathways, as evidenced by the gain and loss of multiple targets (Figs. S1 and S2).

Even though it is a narrow and early window into the neuronal differentiation program, comparing the putative target genes using KEGG pathway analysis reveals that there are some major differences in the enriched pathways between the two time points (Fig. S1). This finding most likely reflects rapid and progressive changes in the state of differentiation and cellular identity, as illustrated by the dominance of pathways associated more with neuronal differentiation at 12 h (Fig. S1). This analysis emphasizes the wide repertoire and temporal dynamics of Hoxa1 downstream target genes and pathways in one context.

#### *Nanog* and *Sox2* Are Direct Targets of Hoxa1.

In light of the roles for *Nanog* and *Sox2* as key components of the core pluripotency GRN (6), we looked in more detail at the binding of Hoxa1 to these loci. Fig. 2 shows browser shots of ChIP-seq analyses comparing the binding of Hoxa1, *Nanog*, p300 (E1A binding protein p300), and epigenetic states as marked by H3K27Ac, H3K4me1, and ATAC-seq. It is interesting that Hoxa1 shows occupancy on both the *Nanog* transcription start site (TSS) and a 4-kb region upstream (Fig. 2A), which corresponds to a previously identified autoregulatory enhancer (ARE) of *Nanog* (12). Binding of *Nanog* and *Zfp281* to adjacent sites recruits the NuRD complex and mediates autorepression through this ARE in stem cells to maintain the appropriate levels of *Nanog* (16, 17). The published work correlates well with the detection of active enhancer marks, p300, and open chromatin over the ARE (Fig. 2A). Multiple Hox and Hox



**Fig. 2.** Regulatory regions of the *Nanog* and *Sox2* core pluripotency genes are bound by *Hoxa1*. (A) *Nanog* and (B) *Sox2* genomic loci are indicated at the top and below are displayed ChIP-seq and ATAC-seq results for the position of regions bound by a variety of factors, epigenetic marks and open chromatin states in uninduced and differentiated ES cells. The respective time points, factors, and marks are indicated at the left. For *Nanog*, the distribution of key *cis*-motifs in a known *Nanog* ARE enhancer region is shown at the bottom. The shaded regions indicate the multiple domains of *Hoxa1* binding in regulatory regions of *Nanog* and *Sox2*, such as TSS and distal enhancers. There is evidence for rapid recruitment of *Hoxa1* to these regions.

cofactor (Pbx and Meis) sites are located adjacent to the core *Nanog* motif in the ARE (Fig. 2A). *Hoxa1* is recruited to both the TSS and ARE at 2 h and is progressively reduced over a 24-h time course of differentiation. This finding suggests that *Hoxa1* directly contributes to the repression and regulation of *Nanog* during early ES cell differentiation.

In an analogous manner, *Hoxa1* displays transient and early occupancy at both the TSS and a downstream region of *Sox2* (Fig. 2B). These sites of *Hoxa1* binding are coassociated with *Nanog* binding and correlate with known regulatory inputs of *Nanog* into *Sox2* regulation (13). A similar pattern of co-occupancy between *Nanog* and *Hoxa1* is also observed on the *Myc* and *Essrb* genes (Fig. S3). Together, these data are consistent with the idea that *Hoxa1* is rapidly recruited to *cis*-regulatory regions of major core regulators of the pluripotential GRN and plays a role in both modulating their expression and on common downstream target genes as a part of its function in promoting differentiation.

**Cross-Regulatory Interactions Between *Nanog* and *Hoxa1*.** To examine whether the binding of *Hoxa1* to the *Nanog* locus affects its transcriptional activity, we used single-molecule RNA FISH to quantify expression levels of *Nanog* and *Hoxa1* in individual cells. The advantage of this approach is that it permits the quantification of both total steady-state levels of RNA and nascent transcriptional activity per cell. We find that the total steady-state levels of *Nanog* remain relatively stable over the early time course of RA-induced differentiation as measured by RNA-seq, quantitative PCR, and FISH (22). In contrast, there is a progressive decline in the levels of nascent transcripts from 2 h onward, with fewer transcripts per cell and fewer cells with nascent transcripts (Fig. 3). These data suggest there is an early and rapid decrease in transcriptional activity of *Nanog*. The timing of this decrease reciprocally correlates with the rapid induction of *Hoxa1* for both steady state and nascent transcripts (Fig. 3) (22). This finding suggests a model whereby *Hoxa1* is rapidly induced during differentiation and, through binding to

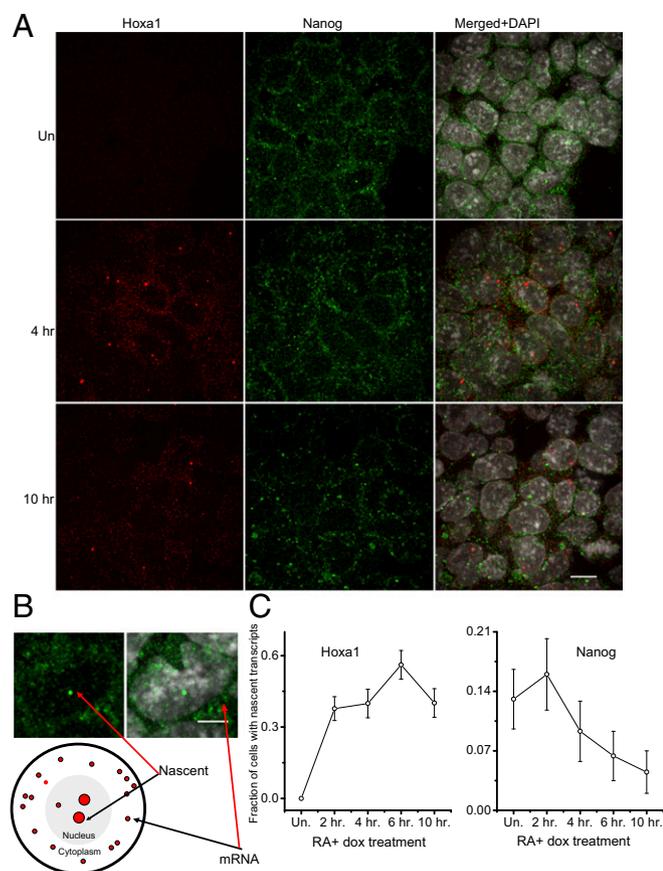
the TSS and ARE of *Nanog*, contributes to the down-regulation of its transcriptional activity.

In the *Nanog* ChIP-seq experiments during the time course of ES cell differentiation (Fig. 1A), we noted that there are two binding peaks in a region located 2 kb 3' of *Hoxa1* (Fig. 4A). One of these peaks contains a *Nanog* binding site located near two conserved elements (CE1 and CE2) and a RARE (Fig. 4A). CE2 and the RARE have been previously shown to have regulatory activity in assays where large genomic fragments from the *Hoxa1* locus were tested in transgenic mouse reporter assays (29, 38). In addition, the RARE has been mutated in the endogenous *Hoxa1* locus and shown to be required for early expression (27). To test the activity of this smaller region with the two *Nanog* binding peaks, we linked a 1.8-kb fragment to a GFP reporter vector and scored for regulatory activity in F<sub>0</sub> transgenic zebrafish embryos (Fig. 4B). Reporter expression is detected in mesodermal and neuro-ectodermal cells in a manner similar to mouse *Hoxa1* expression, suggesting that this is a functional core of the enhancer.

*Nanog* binding is highest in uninduced ES cells and is rapidly lost by 2 h. In uninduced ES cells the region is relatively closed based on ATAC-seq and low occupancy of p300 (Fig. 4A). By 24 h, the region is open, occupied by p300, and contains active enhancer marks. This result suggests that the *Hoxa1* enhancer is not active in uninduced ES cells and that *Nanog* may be involved in repressing its activity and hence the expression of *Hoxa1*. The loss of *Nanog* occupancy on these sites by 2 h correlates with the rapid induction of *Hoxa1*. Together, these experiments suggest that *Hoxa1* and *Nanog* are involved in direct cross-regulatory interactions and mutually repress their expression to modulate the balance between pluripotency and differentiation.

## Discussion

**Temporal Dynamics of *Hoxa1* Binding.** In this study we have uncovered a dynamic nature in occupancy of *Hoxa1* on genome-wide targets during the RA programmed differentiation of mouse ES cells. *Hoxa1* is rapidly induced and binds a large number of targets



**Fig. 3.** Analysis of transcriptional dynamics *Hoxa1* and *Nanog* using single-molecule RNA FISH. (A) Expression of *Hoxa1* and *Nanog* in differentiating ES cells. *Hoxa1* is shown in red channel and *Nanog* is seen in green channel. Nuclei are stained with DAPI. (B) The one-to-two bright foci per cell represent nascent nuclear transcripts, whereas transcripts outside nuclei are steady-state stable mRNAs. (C) Quantification of nascent transcripts of *Nanog* and *Hoxa1*. *Hoxa1* shows rapid up-regulation, whereas *Nanog* undergoes a progressive decrease after 2 h of RA treatment. (Scale bars: A, 10  $\mu$ m; B, 5  $\mu$ m.)

by 2 h of differentiation (Fig. 1A). Many of the genes adjacent to these sites are associated with stem cell-signaling pathways and show rapid up- or down-regulation compared with uninduced ES cells (Fig. 1 and Fig. S1C). By 12 h of differentiation, the genome-wide profile is very different, with a loss of most of the targets observed at 2 h and the appearance of new binding sites (Fig. 1A). Despite this shift in occupancy, the sites bound at 12 h also correlate with genes associated with stem cell and other signaling pathways, but there is a change to include pathways associated with differentiation processes (Fig. S1C). The striking shift in binding-site distribution of *Hoxa1* correlates with temporal changes in expression of markers for cell lineage and differentiation processes that reveal different cellular states exist at 2 and 12 h (22). Interestingly, a recent study on single-cell transcriptome analysis of mouse ES cells differentiated with RA over a 96-h time course also showed similar progressive changes in differentiation and that 12 h was an important transition point for exit from pluripotency (39). Transient dynamics of *Hoxa1* binding on downstream targets implies that its regulatory influence varies with the temporal progression of the differentiation state of the cell.

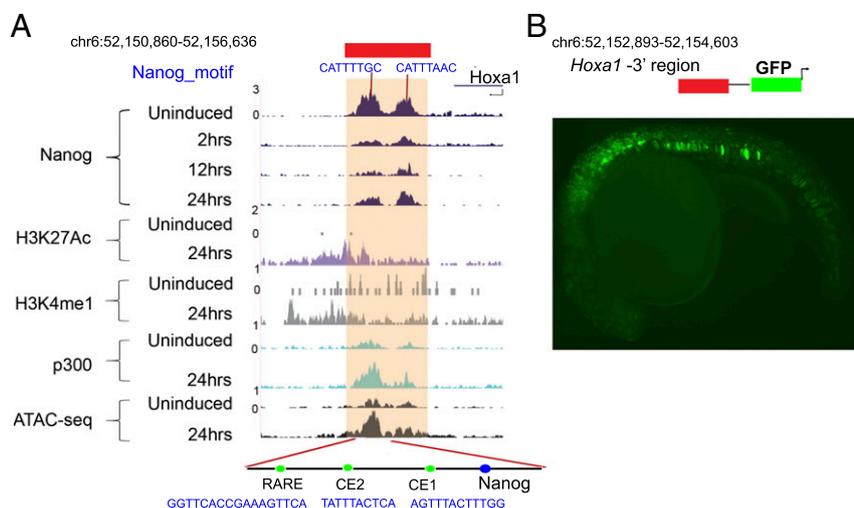
At the mechanistic level, this temporal shift in binding-site distribution may be related to differences in the nature of enriched motifs found in bound regions (Datasets S3 and S4) and the presence of Hox cofactors (e.g., Pbx and Meis). There are both unique and overlapping cohorts of enriched binding motifs

associated with occupancy of *Hoxa1* at 2 and 12 h (Fig. 1B and C). This finding is illustrated by enrichment at 12 h compared with 2 h for bipartite Hox-Pbx motifs, which serve as elements that integrate combinatorial binding of Hox, Pbx, and Meis (40). It is worth noting that Pbx1 is expressed at low levels in uninduced ES cells but Pbx, Meis, and other TALE (three amino acid loop extension proteins) transcription activator-like effector cofactors, known to partner with Hox proteins, are not generally available early in the differentiation process. Hence, associated with the differences in the state of differentiation at 2 h versus 12 h, there is greater availability of *Hoxa1* and the TALE cofactors at the later time point. This finding suggests that there are likely to be temporal differences in the utilization of cofactors that impact site selection and binding specificity/affinity. Therefore, the shifts in binding profiles and sequence preferences may be mediated through temporal differences in the levels of expression or activity of *Hoxa1* itself, and through variation of Hox cofactors, such as TALE proteins, chromatin modifiers, or other TFs that modulate *Hoxa1* binding (40).

**Pluripotency Network as a Target of *Hoxa1*.** The core pluripotential GRN controls many genes and pathways that actively maintain the pluripotential state and at the same time represses developmental and differentiation pathways, such as those dependent upon Hox genes (6, 7, 41, 42). This result can be achieved through direct activation of downstream genes, such as *Esrrb*, which in turn play important positive roles in controlling the pluripotent state (1, 6). Repression is mediated through direct recruitment of repressor complexes, such as NuRD (16, 17), or through maintenance of repressed chromatin states, such as those mediated by Polycomb complexes (7). A large number of the putative *Hoxa1* downstream target genes in our analyses are part of the pluripotential GRN, including *Nanog*, *Sox2*, and *Myc*, and we found that they are cobound by *Nanog* (Figs. 1 and 2 and Fig. S1). Furthermore, these common targets show differential expression upon the early induction of *Hoxa1* (Fig. S1). This raises the possibility that in a reciprocal manner compared with *Nanog* and the core pluripotential network, *Hoxa1* may repress genes that maintain pluripotency and promote expression of those that facilitate differentiation. The mechanistic basis through which *Hoxa1* works as an activator or repressor of transcription on different targets is poorly understood. However, Hox cofactors may dictate functional outcomes in a context-dependent manner and have been suggested to be involved in repression, as well as activation of target genes in *Drosophila* (43–45). Our data suggest a model whereby direct input of *Nanog* and *Hoxa1* on shared targets regulates the fine balance between the pluripotent and differentiation states (Fig. 5A).

**Cross-Regulatory Interactions Between *Hoxa1* and *Nanog*.** In addition to shared target genes, there is another layer of regulatory input between *Hoxa1* and *Nanog* through mutual repression. *Hoxa1* binds to an autoregulatory repressor region and the TSS of *Nanog* (Fig. 2A), whereas *Nanog* binds to a 3' enhancer of *Hoxa1* (Fig. 4A). We have also found that *Hoxa1* binds to regulatory regions of *Sox2* and *Myc* (Fig. 2B and Fig. S3), which shows extensive input of *Hoxa1* into core components of the pluripotential regulatory networks. Integrating this in the model for cross-talk between *Nanog* and *Hoxa1*, in undifferentiated ES cells, the *Hoxa1*-mediated repression of pluripotential gene expression is released by direct repression of *Hoxa1* by *Nanog* (Fig. 5B). In terms of *cis*-regulatory architecture, this type of subcircuit is termed a “double-negative gate,” which is a commonly used efficient mechanism using active repression as a means to spatially or temporally restrict programs of gene expression (46).

This model for extensive cross-talk between *Hoxa1* and the pluripotency network may have relevance beyond ES cells. Recent studies in *Xenopus* have shown that many features of the regulatory



**Fig. 4.** Cross-regulation of *Hoxa1* by Nanog. (A) Occupancy of Nanog on a 3' enhancer of *Hoxa1*. The *Hoxa1* genomic locus is indicated at the top and below are displayed ChIP-seq and ATAC-seq results for the position of regions bound by a variety of factors, epigenetic marks and open chromatin states in uninduced and differentiated ES cells. The respective time points, factors, and marks are indicated at the left. The shaded regions indicate the two adjacent peaks of Nanog binding, which correlate with the presence of Nanog consensus sites, near *Hoxa1*. These Nanog sites fall within a region which harbors two functionally important elements, CE2 and a RARE, as indicated at the bottom. (B) Regulatory analysis of the Nanog-bound region downstream of *Hoxa1* using a transient transgenic assay in zebrafish. The diagram at the top indicates that a 1.8-kb region containing two Nanog sites were linked to a GFP reporter vector. Below is a lateral view of a 20 h postfertilization (hpf) embryo displaying mesodermal and neuro-ectodermal reporter expression. Image dimensions are 1.3 mm  $\times$  0.9 mm.

program that controls pluripotency in the blastula are also present in neural crest cells (47). Because Hox genes, including *Hoxa1*, are known to play important roles in formation and patterning of neural crest cells (48, 49), they may have a similar cross-regulatory interaction with the pluripotential network in this in vivo context. The findings in this study underscore the dynamic and diverse inputs of *Hoxa1* in modulating gene regulatory networks associated with signaling pathways and pluripotency.

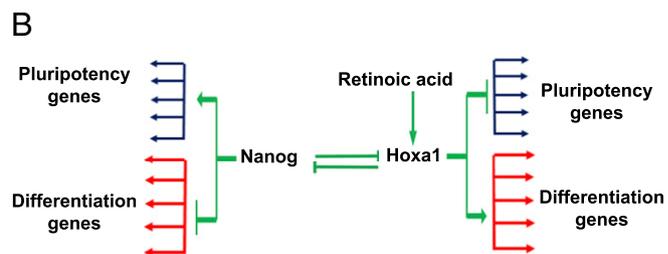
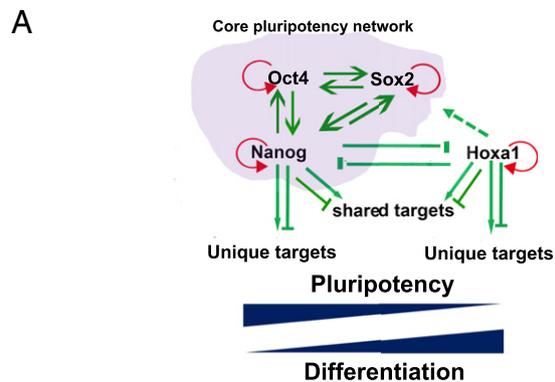
## Methods

**ES Cell Culture.** KH2 ES cells (50) and KH2 ES cells with epitope-tagged *Hoxa1* (3XFLAG-MYC) were grown in feeder-free conditions using N2B27+2i media supplemented with 2,000 U/mL of ESGRO (Millipore). N2B27+2i media consist of neurobasal media (21103-049, Invitrogen), DMEM/F12 media (10565-018, Invitrogen), 0.5 $\times$  N2 (17502-048, Invitrogen), 1 $\times$  B27 (17504044, Invitrogen), 1 $\times$   $\beta$ -mercaptoethanol (ES-007-E, Millipore), 1 $\times$  Glutamax (10378-016), 1 $\times$  NEAA (07600, SCT), 3  $\mu$ M CHIR99021 (4423, Tocris), PD0325901 (72184, SCT), 0.033% BSA (15260037) Thermo Fisher Scientific) (Qi-Long, 2008). Cells were seeded on a gelatinized plate without a feeder layer. After 48 h the media was changed to differentiation media [DMEM + 10% (vol/vol) Serum + NEAA + 0.03  $\mu$ M RA] for a requisite length of time. Uninduced ES cells were grown in N2B27+2i up to 80–90% confluency.

**ATAC, ChIP-seq.** ATAC-seq protocol was done as described by Buenrostro et al. (51). ChIP-seq was done according to the Upstate protocol with certain modifications (22, 52). Cells were fixed by adding formaldehyde to media at a final concentration of 1% followed by incubation at 37  $^{\circ}$ C for 11 min. Immunoprecipitation (IP) experiments were done using monoclonal  $\alpha$ -FLAG M2, (F1804, Sigma-Aldrich). Libraries were prepared using the KAPA HTP Library Prep Kit for Illumina and Bio Scientific NEXTflex DNA barcodes. The resulting libraries were purified using the Agencourt AMPure XP system (Beckman Coulter), then quantified using a Bioanalyzer (Agilent Technologies) and a Qubit fluorometer (Life Technologies). Postamplification size selection was performed on all libraries using a Pippin Prep (Sage Science). Libraries were requantified, normalized, pooled, and sequenced on an Illumina HiSeq. 2500 instrument as 50-bp single read. Following sequencing, Illumina Real Time Analysis v1.18.64 and CASAVA v1.8.2 were run to demultiplex reads and generate FASTQ files.

**Data Analysis.** Raw reads were aligned to the University of California, Santa Cruz (UCSC) mm10 mouse genome with bowtie2.2.0 (53). Primary reads from each bam were converted to bigWig tracks, normalized to reads-per-million, and visualized at

the UCSC genome browser (<https://genome.ucsc.edu>) (54). Peaks were called with MACS2 2.1.0.20140616 [3], parameters “-p 0.25 -m 5 50.” The top 100,000 peaks by *P* value, for each replicate, were compared with inconsistency discovery rate (IDR) 1.7.0 (<https://sites.google.com/site/anshulkundaje/projects/idr>)



**Fig. 5.** Model showing regulatory interactions between the pluripotency and *Hoxa1*-dependent differentiation GRNs. (A) Summary of extensive regulatory interactions between the core pluripotency GRN and *Hoxa1*. Shared targets of Nanog and *Hoxa1* illustrate novel regulatory cross-talk between the pluripotency and differentiation pathways. (B) Mutual direct repression of *Hoxa1* and *Nanog* provide regulatory subcircuits for modulating two alternate states. This *cis*-element subcircuitry represents double-negative gates used to define alternate states of pluripotency and differentiation.

and valid pairs with IDR  $P \leq 0.01$  were taken as the sample peak list. Nanog samples produced such an abundance of peaks that the IDR  $P$ -value threshold was lowered to 0.0001. IP/input RPM  $\log_2$  fold-change signal across peak coordinates was visualized with the CoverageView package in R (<https://www.bioconductor.org/packages/release/bioc/html/CoverageView.html>) using windows  $\pm 5$  kb from peak midpoints. Nanog binding was essentially the same across time, so peaks from all four time points were collapsed into a single Nanog peak list of 17,651 peaks. ATAC-seq data has no input, so instead of using IP/input  $\log_2$  fold-change, we transformed the coverage matrix to percent-of-maximum, so that signal would be within the same range as  $\log_2$  fold-change. Motif content of peaks was analyzed using the vertebrate motif set from TransFac v2016.2 (55), searched with the FIMO program (56), and also a list of motifs of interest, searched with grep. Each peak set was given a background of 10 random coordinate sets, each with the same length and chromosomal distribution as the original peaks. Motif enrichment in peaks versus background was assessed with Fisher's Exact Test, using BH  $P$ -value correction. For each peak set, all Ensembl 80 protein-coding nearest-neighbor genes upstream and downstream were identified and analyzed for functional enrichments. Briefly, KEGG (57) pathway terms ([www.genome.jp/kegg/](http://www.genome.jp/kegg/)) and Gene Ontology (GO) (58) terms ([geneontology.org](http://geneontology.org/)) downloaded May 2016 were compared between gene lists versus the rest of the genome. Terms overenriched in neighbor genes by Fisher Exact Test, BH-adjusted  $P \leq 0.05$ , with at least three genes, were accepted. Raw data for RNA-seq in uninduced, 4- and 6-h RA time points were taken from National Center for Biotechnology Information (NCBI) Gene Expression Omnibus accession no. GSE61590 (22).

**Zebrafish Transgenic Reporter Assay.** For generating constructs, two oligos 5'-ATAACAGGGTAATGAGGGCCGTAGCCCAAGAGTTTCTTC-3' and 5'-CCTCGAGGATATCGAGCTCGGGAGCTGAGTCTTCATCTTC-3' were used as forward and reverse primers to amplify a 1,711-bp region 3' of *Hoxa1* from mouse genomic DNA. PCR-purified putative enhancer elements were cloned into the HLC vector (59) using the Gibson Assembly Master Mix kit (New England Biolabs). Correct inserts were confirmed by sequencing. Constructs for injection were diluted to 125-ng/ $\mu$ L concentration. Tol2-mediated transgenesis was performed by microinjection into fertilized Slusarski AB (wild-type) zebrafish eggs, as described previously (60). At least 100 embryos were injected for each reporter construct. Embryos were screened for GFP fluorescence using a Leica M205FA microscope and imaged with a Leica DFC360FX camera and LAS AF imaging software. Images were cropped and adjusted for brightness and contrast using Adobe Photoshop CS5.1. All experiments involving zebrafish were approved by the Institutional Animal Care and Use Committee of the Stowers Institute for Medical Research (Krumlauf Protocol No. 2015-0149).

**RNA FISH.** RNA FISH samples were prepared, and data were obtained essentially as previously described, using the Stellaris, multiprobe approach

(22). The *Hoxa1* probe-set sequences are as published previously (22) and the Nanog probe-set sequence is the DesignReady version offered by Stellaris. For probe labeling, unlabeled probe sets carrying a C-term TEG-Amino tag, were purchased from Biosearch Technologies. Probes (4 nmol) were fluorescently labeled overnight in 0.1 M sodium tetraborate pH 9 at 4 °C. *Hoxa1* was labeled with AF647 and *Nanog* with AF555. Two units of amine reactive succinimidyl ester Decapacks (ThermoFisher) were used for each reaction and following quenching labeled probes were purified using Reverse-Phase HPLC. Probes were separated using an Ettan LC (GE Healthcare) using a 4.6  $\times$  250-mm, 5- $\mu$ m, C18-EMS end-capped Kinetex column (Phenomenex). Mobile phase A was 0.1 M ammonium acetate (EMD) pH7 and mobile phase B was acetonitrile (Millipore). A linear gradient of 5% B to 100% (vol/vol) B was run over the course of 20 min at 1 mL/min. Peaks were monitored at 280 nm for probe and either 555 or 647 nm, depending on the dye. Dual positive peaks were collected and concentrated by spin vac.

For quantification of the fraction of cells with nascent transcripts, nuclei were manually segmented using the Dapi channel. After blurring with a Gaussian blur of radius 1 pixel, the pixel of maximum intensity over the manually segmented area in 3D was recorded, over a total z dimension of 11.4  $\mu$ m. For *Hoxa1*, the histogram of maximum pixel intensity per cell over all data was clearly biphasic. A cut-off was set based on the distribution, and any nuclei with a pixel intensity over the cut-off was considered to have a nascent transcript. For the *Nanog* data, although nascent transcripts were apparent, the distribution of maximum intensity per nuclei was not sufficient to set a cut-off. Cells with nascent transcripts were counted by manual inspection of the signal in 3D. For determination of total signal per cell, the region of the nuclear segmentation was dilated 10 pixels in each region, and after thresholding of the background signal, the total integrated intensity per cell was recorded over the dilated region in 3D, over a total z dimension of 11.4  $\mu$ m.

**Data Submission.** All raw sequencing data are submitted to the NCBI as Sequence Read Archive accession no. SRP079975. The raw and processed data from a previous study were submitted to the NCBI Gene Expression Omnibus under accession no. GSE61590 were used for transcriptional profiling. All other original source data not available through a public repository have been deposited in the Stowers Institute Original Data Repository and are available online at [www.stowers.org/research/publications/odr](http://www.stowers.org/research/publications/odr).

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