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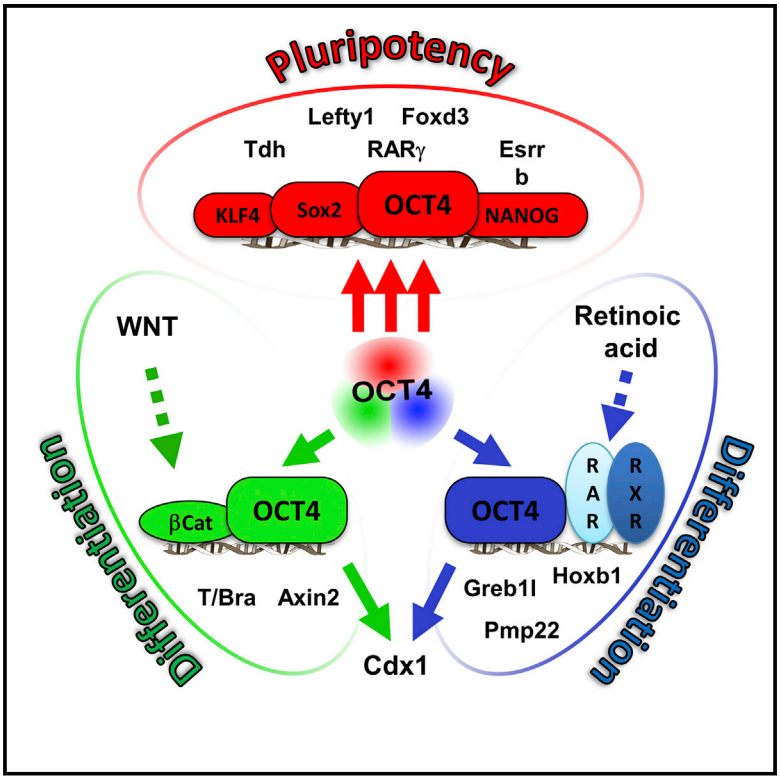
OCT4 Acts as an Integrator of Pluripotency and Signal-Induced Differentiation

Graphical Abstract



Authors

Zoltan Simandi, Attila Horvath, Lyndsey C. Wright, ..., Lorraine J. Gudas, Shaun M. Cowley, Laszlo Nagy

Correspondence

lnagy@sbpdiscovery.org

In Brief

Simandi et al. demonstrate that, beyond maintaining pluripotency, OCT4 plays a role in integrating responses to signals differentiating embryonic stem cells. OCT4 primes a large set of low-accessible genomic regions and is required for their proper activation upon retinoic acid or WNT signal exposure.

Highlights

- OCT4 occupies differentiation-related and low-accessible genomic regions in ESCs
- OCT4 positively controls the level of RARγ
- Loss of OCT4 causes dysregulation of tissue-specific RA and WNT/β-catenin response
- Overexpression of OCT4 is sufficient to reprogram a cell type-specific signal response

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OCT4 Acts as an Integrator of Pluripotency and Signal-Induced Differentiation

Zoltan Simandi,[1,2,10] Attila Horvath,[2,10] Lyndsey C. Wright,[3] Ixchelt Cuaranta-Monroy,[2] Isabella De Luca,[1] Katalin Karolyi,[1] Sascha Sauer,[4,5,6] Jean-Francois Deleuze,[7] Lorraine J. Gudas,[8] Shaun M. Cowley,[3] and Laszlo Nagy[1,2,9,*]
[1]Sanford Burnham Prebys Medical Discovery Institute, Orlando, FL 32827, USA
[2]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, 4032 Debrecen, Hungary
[3]Department of Molecular and Cell Biology, University of Leicester, Leicester LE1 9HN, UK
[4]Otto Warburg Laboratory, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany
[5]CU Systems Medicine, University of Würzburg, 97070 Würzburg, Germany
[6]Max Delbrück Center for Molecular Medicine (BISMB and BIH), 13125 Berlin, Germany
[7]Centre National de Génotypage, Institut de Génomique, CEA, 91000 Evry, France
[8]Department of Pharmacology, Weill Cornell Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, USA
[9]MTA-DE "Lendulet" Immunogenomics Research Group, University of Debrecen, 4032 Debrecen, Hungary
[10]Co-first author
*Correspondence: lnagy@sbpdiscovery.org
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SUMMARY

Cell type specification relies on the capacity of undifferentiated cells to properly respond to specific differentiation-inducing signals. Using genomic approaches along with loss- and gain-of-function genetic models, we identified OCT4-dependent mechanisms that provide embryonic stem cells with the means to customize their response to external cues. OCT4 binds a large set of low-accessible genomic regions. At these sites, OCT4 is required for proper enhancer and gene activation by recruiting co-regulators and RAR:RXR or β-catenin, suggesting an unexpected collaboration between the lineage-determining transcription factor and these differentiation-initiating, signal-dependent transcription factors. As a proof of concept, we demonstrate that overexpression of OCT4 in a kidney cell line is sufficient for signal-dependent activation of otherwise unresponsive genes in these cells. Our results uncover OCT4 as an integral and necessary component of signal-regulated transcriptional processes required for tissue-specific responses.

INTRODUCTION

One of the major challenges of developmental cell biology is to understand the mechanisms that underlie cell type-specific differential gene expression. Embryonic stem cells (ESCs) represent an excellent model system for studying how the transcriptional output of a given cell type is controlled by distinct combinations of transcription factors under the control of extrinsic signals. ESCs can be maintained in a "naive pluripotent state" (serum + leukemia-inhibiting factor [LIF]) or in a "ground state of pluripotency" (2 inhibitor [2i]) (Nichols and Smith, 2009), conditions under which these cells have a unique gene expression program and regulatory chromatin landscape that allows them to self-renew yet remain capable of differentiating into any cell type (Guo et al., 2016a, 2016b; Kolodziejczyk et al., 2015; Kumar et al., 2014; Nichols and Smith, 2009; Young, 2011). The POU family homeodomain transcription factor OCT4 (also known as POU5F1) is an indispensable component of this regulatory circuitry (Hay et al., 2004; Ivanova et al., 2006; Loh et al., 2006; Nichols et al., 1998; Niwa et al., 2000; Okamoto et al., 1990).

To understand the transcriptional regulatory network in ESCs, genome-wide studies were conducted to determine the OCT4 cistrome (the genomic binding sites of OCT4) (Boyer et al., 2005, 2006; Chen et al., 2008; Kim et al., 2008; Lee et al., 2006; Liang et al., 2008; Loh et al., 2006; Morey et al., 2015). Recent studies revealed a substantial reorganization of OCT4 occupancy between "naive" or "ground state" (pre-implantation) versus "primed" (post-implantation) stem cell states, suggesting that the engagement of OCT4 with the genome is dynamic and context-dependent in early differentiation (Buecker et al., 2014; Factor et al., 2014; Yang et al., 2014). These results, along with additional evidence, suggest that OCT4 is likely involved in early cell fate determination as well (Frum et al., 2013; Funa et al., 2015; Niwa et al., 2000; Radzisheuskaya et al., 2013; Yang et al., 2014). However, it is not known how it might contribute to signal-specific differentiation events, if at all.

Depending on the signals provided, ESCs can differentiate into a variety of cell types (Wobus and Boheler, 2005). LIF/STAT3, WNT/β-catenin, and bone morphogenetic protein (BMP)/SMAD have been suggested as context-dependent regulators of both ESC self-renewal and differentiation (Chatterjee et al., 2015; Funa et al., 2015; Itoh et al., 2014; Niwa et al., 1998; Zhang et al., 2013). In contrast, retinoic acid (RA) is known as an efficient inducer of ESC differentiation (Gudas and Wagner, 2011; Rochette-Egly, 2015; Simandi et al., 2010).

RA acts through binding to the heterodimer of retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Chambon, 1996;

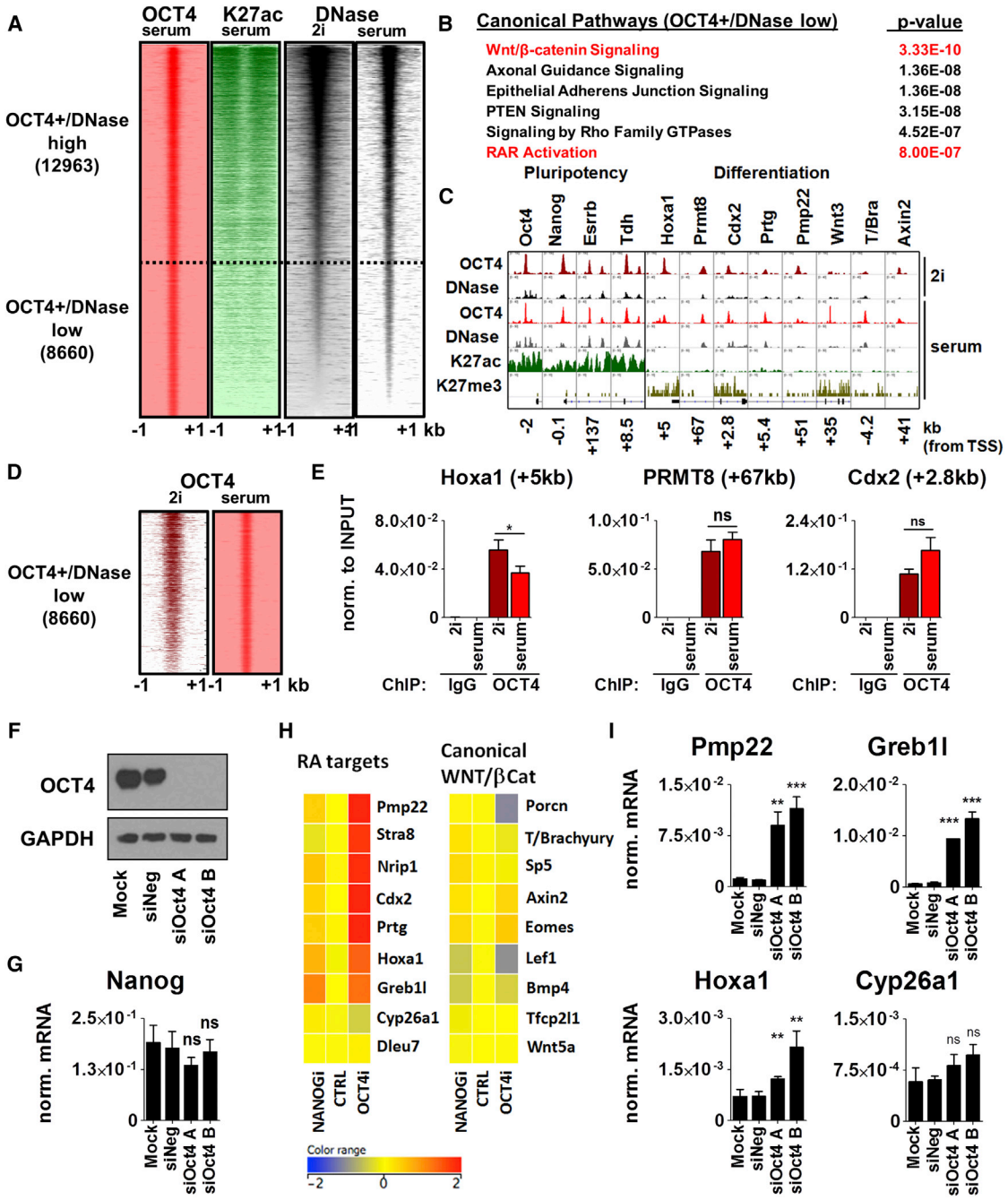

Figure 1. OCT4 Occupies Low-Accessible Genomic Regions

(A) Read distribution heatmap of OCT4 and H3K27ac ChIP-seq and DNase-seq reads (±1 kb, relative to the summits of the detected OCT4 peaks). The black dotted line marks the detection threshold of highly accessible regions predicted from DNase-seq. See also Table S1.

(B) Canonical upstream regulators in the activation of OCT4+/DNase low regions as predicted by Ingenuity.

(C) Integrative Genomics Viewer (IGV) snapshot of enhancers in the proximity of pluripotency-related (*Oct4*, *Nanog*, and *Esrrb*) or differentiation-related genes. Distances of the enhancers from the TSS of the indicated genes are shown.

(D) Read distribution heatmap of OCT4 ChIP-seq obtained in cells grown under 2i + LIF (ground state) versus serum + LIF (naive) culture conditions. OCT4+/DNase low sites are compared (see A).

(E) ChIP-qPCR analysis of OCT4 binding at the indicated enhancers.

(F) Western blot assay showing expression of OCT4 in total extracts from untransfected cells (Mock) or cells transfected with non-specific (siNeg) or *Oct4*-specific siRNAs (siOct4 A and siOct4 B). Samples were collected 24 hr following transfection.

(G) qRT-PCR analysis of *Nanog* at the mRNA level 24 hr after RNAi transfection.

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Giguere et al., 1987). These nuclear receptors bind to *cis*-acting retinoic acid response elements (RAREs) in the regulatory region of their target genes (Chatagnon et al., 2015; Cunningham and Duester, 2015). According to canonical models, RAR:RXR receptors are bound by the nuclear receptor co-repressor silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and histone deacetylase 3 (HDAC3) in the absence of ligand and keep the target genes repressed (Nagy et al., 1999). Ligand binding induces a structural change that will initiate the exchange of these co-repressors for co-activators, such as the histone acetyltransferase P300.

WNT/β-catenin signaling is a highly conserved signaling pathway that supports the pluripotency of ESCs but also promotes early developmental processes (Lindsley et al., 2006; ten Berge et al., 2011; Zhang et al., 2013). In the absence of WNT, β-catenin is phosphorylated by GSK-3, which tags it for proteosomal degradation. Activation of WNT signaling with the GSK-3 inhibitor CHIR99021 has been shown to have pleiotropic effects, promoting non-neural differentiation, suppressing neural differentiation, and enhancing growth capacity (Merrill, 2012; Nichols et al., 2009; Ying et al., 2008).

Here we performed an unbiased and comprehensive analysis of OCT4 functions in signal-induced ESC differentiation. Analysis of genome-wide OCT4 occupancy in the context of the chromatin state revealed binding of OCT4 to regions with low DNA accessibility. We demonstrate that such OCT4-mediated binding events are priming a distinct set of enhancers to respond to external cues, such as retinoic acid and WNT. Mechanistically, RAR:RXR shares a subset of OCT4-occupied enhancers, and OCT4 also occupies enhancers that are activated by the canonical WNT/β-catenin signal. Consequently, removal of OCT4 dysregulates the induction of RA or WNT target genes and affects subsequent lineage specifications.

This study provides insights into the transcriptional regulation of cell fate by highlighting the direct links, interdependence, and multi-level collaborative connections between lineage-determining (OCT4) and signal-dependent (RAR:RXR or β-catenin) transcription factors.

RESULTS

OCT4 Occupies Low-Accessible Genomic Regions in Undifferentiated ESCs

Previous studies revealed the existence of active and poised enhancers in various model systems, suggesting that enhancers contain information not only about the current state of the cell but its future developmental potential as well (Boyer et al., 2006; Creyghton et al., 2010; Factor et al., 2014; Heinz et al., 2015; Rada-Iglesias et al., 2011). To determine whether OCT4 contributes to subsequent developmental stages or responds to external cues by marking or priming lineage-specific enhancers, we first delineated the OCT4 cistrome by chromatin immunoprecipitation sequencing (ChIP-seq) in mouse ESCs. We identified ~21,000 genomic regions enriched for OCT4 in naive ESCs (Figure 1A). Motifs found in these loci were a perfect match to the previously identified elements in stem cells, including the OCT4-SOX2 composite element, NANOG, and SOX2 binding sites (Figure S1A). To assess whether all OCT4 binding sites overlap with active, highly accessible genomic regions, we generated chromatin state maps for H3K27ac (histone 3 lysine 27 acetylation) enrichment and also analyzed DNase hypersensitive sites. A major fraction of OCT4 binding (>40%) was associated with regions characterized by H3K27ac-negative chromatin and lower DNase accessibility (termed OCT4+/DNase low) (Figure 1A). Similarly, OCT4 also occupied low-accessibility sites in ground state pluripotency (Figure S1B). Motif analysis revealed that while the "OCT4+/DNase high" regions determined in Figure 1A were enriched for the binding motif of NANOG and KLF4 in addition to OCT4 and SOX2 elements, the "OCT4+/DNase low" regions were only significantly enriched for OCT4 (Figure S1C), suggesting that the low-accessible regions are regulated independently of NANOG and KLF4. Pathway analysis of genes linked to the OCT4+/DNase low regions revealed that many of these sites are targets for WNT/β-catenin and RA signaling (Figure 1B). In line with this, targets of WNT/β-catenin (e.g., *Wnt3* and *T/Brachyury*) and RAR:RXR (e.g., *Hoxa1*, *Prmt8*, *Cdx2*, and *Pmp22*) showed OCT4 enrichment but low chromatin accessibility in their proximity (Figure 1C). These sites were enriched for the repressive histone mark H3K27me3 (histone 3 lysine 27 trimethylation) but not for H3K27ac. OCT4 binding was generally weaker at these sites (Figure S1D). Comparison of OCT4 binding in naive cells (serum + LIF) versus cells grown under defined culture conditions (2i + LIF) revealed that Oct4 is already present on these sites in ground state pluripotency (Figures 1C–1E).

To investigate the biological relevance of OCT4 binding to these low-accessible genomic regions in the proximity of RAR:RXR and WNT/β-catenin target genes, we examined the expression changes of RA and WNT targets upon OCT4 depletion. Small interfering RNA (siRNA) knockdown of OCT4 did not affect most of the studied differentiation markers, including the WNT/β-catenin targets (Figures 1F–1H; Figure S1E). In contrast, a subset of early retinoid target genes showed elevated basal mRNA expression (Figures 1H and 1I; Figures S1F and S1G). The depletion of OCT4 had no effect on the expression levels of some retinoid target genes (e.g., *Cyp26a1* and *Dleu7*) (Figures 1H and 1I), suggesting that OCT4 selectively contributes to the regulation of retinoid-regulated genes. Importantly, loss of NANOG did not influence the expression of the OCT4-dependent RA target genes (Figure 1H), confirming that this regulation is independent of other members of the canonical pluripotent network.

(H) Heatmap displays of microarray data (re-analyzed from Loh et al., 2006) showing expression changes of RA- or WNT-regulated genes in NANOG- or OCT4-depleted cells versus the parental controls.

(I) qRT-PCR analysis of mRNA levels of selected retinoid target genes in OCT4-depleted cells 24 hr after transfection. Gene expression data are expressed as a ratio of the indicated genes' transcript relative to *36b4*.

See also Figure S1. ns, non-significant. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

OCT4 Maintains *Rarg* Expression in Pluripotent Cells, and a Subset of Retinoid Target Genes Are Co-occupied by OCT4/RAR:RXR

To understand how OCT4 controls RA signaling in ESCs, we compared the expression levels of the RA pathway components in control versus OCT4-depleted cells. Immunostaining confirmed the presence of RAR in ESCs (Figure S2A). OCT4 knockdown did not affect *Aldh1a1-3*, *Stra6*, and *Crabp2* levels (Figure 2A), suggesting that OCT4 does not control RA transport or synthesis per se. However, the major RAR isoform in ESCs, *Rarg*, was significantly reduced upon OCT4 depletion, suggesting that OCT4 positively controls its level in pluripotent cells (Figures 2A and 2B). We identified OCT4 binding on a canonical motif in a conserved, highly acetylated intronic region of *Rarg* (Figures 2C and 2D; Figure S2B). The activity of this enhancer was similar in naive versus ground state pluripotent cells (Figure 2E), but we could detect a reduced level of enhancer transcript in the absence of OCT4 (Figure 2F), further supporting the notion that OCT4 positively controls *Rarg* expression through this enhancer. As a consequence of OCT4-depletion and decreased RAR expression, downstream target genes of *Rarg* also showed decreased expression (Figure 2G; Al Tanoury et al., 2014).

Next we studied whether OCT4 modulates RA signaling at the enhancer level as well. We used ChIP-seq to monitor the genome-wide binding profiles of OCT4 and RXR, the obligate heterodimeric partner of RAR (Kliewer et al., 1992). This analysis revealed that more than 1,200 genomic regions were co-occupied by both OCT4 and RXR (termed "common") (Figure 2H). Conversely, a large fraction of the RXR-bound sites lacked OCT4 ("RXR only"), suggesting the existence of both OCT4-associated and OCT4-independent RXR-bound enhancers. Transcription factor binding motifs present among the genomic loci shared by OCT4 and RXR revealed enrichment for both OCT4 and the nuclear receptor response elements (nuclear receptor [NR] half) (Figure S2C), indicating that these transcription factors might cooperate in DNA binding. Indeed, OCT4 (but not NANOG, KLF4, or SOX2) and RAR:RXR were found to be overlapping in close proximity of several retinoid-regulated genes both in the ground state and naive state (Figure 2I; Figures S2D and S2E). ChIP-chromatin re-immunoprecipitation (reChIP) experiments confirmed that RAR:RXR and OCT4 are binding simultaneously to these sites in the same cells (Figure 2J; Figure S2F). OCT4 binding could not be detected in the proximity of retinoic acid target genes that were unaffected at the mRNA level in OCT4-depleted cells (e.g., *Cyp26a1* and *Dleu7*), suggesting that these genes are regulated by OCT4-independent, RAR:RXR-bound enhancers (Figure 2I; Figure S2D).

To address whether OCT4 binding to the RAR:RXR-occupied enhancers in the proximity of retinoic acid target genes is required for their repression, we measured enhancer transcripts at these sites in OCT4-depleted cells. Enhancers co-occupied by OCT4 and RAR:RXR showed increased enhancer RNA (eRNA) production, whereas loss of OCT4 had no effect on the *Cyp26a1* enhancer (Figure 2K; Figure S2G). Taken together, these results indicated a gene- and enhancer-specific negative regulation of retinoid signaling by OCT4 both in naive and ground state ESCs.

Co-regulators Are Selectively Recruited to OCT4-Containing RXR Sites

OCT4-mediated repression of retinoid signaling raises the question of whether this repressive function is mediated through interactions with known nuclear receptor co-repressors such as SMRT and HDAC3 (Cunningham and Duester, 2015; Jepsen et al., 2007; Urvalek and Gudas, 2014). Genome-wide analysis of the SMRT and HDAC3 cistrome by ChIP-seq highlighted several binding motifs for pluripotency-associated transcription factors, but no canonical binding sites for nuclear receptors were found (Figure S3A). The co-activator P300 also showed similar motif enrichment (Figure S3B), suggesting that primarily OCT4, rather than RAR:RXR, recruits these co-regulators in stem cells. Further interrogation of the genome-wide distribution of P300, SMRT, and HDAC3 in ESCs demonstrated that only sites co-occupied by OCT4/RAR:RXR are bound by these co-factors, and none of these co-regulators were detected at RXR only sites (e.g., *Cyp26a1* −1.8 kb) (Figures 3A and 3B).

To establish whether OCT4 is indeed required for co-factor recruitment, we depleted OCT4 and examined RAR:RXR, P300, SMRT, and HDAC3 binding at candidate loci representative of the OCT4-RXR co-occupied (common) regions (the regions are shown in detail in Figure S3C). Depletion of OCT4 resulted in decreased co-regulator occupancy (Figure 3C) without affecting their total protein levels (Figure 3D).

Co-occupancy of co-repressors and co-activators at the same genomic regions was somewhat surprising. ChIP-reChIP experiments confirmed that P300 and HDAC3 are present on the same genomic regions in the same cells (Figure 3E; Figure S3D). Loss of HDAC3 selectively enhanced H3K27ac and mRNA levels of OCT4/RAR:RXR targets but did not change the acetylation status of the control *Cyp26a1* enhancer or its mRNA expression (Figure 3F; Figure S3E). These findings support the co-existence of acetyltransferases and deacetylases at the OCT4/RAR:RXR co-occupied sites and their function in the maintenance of chromatin accessibility.

OCT4 Binding Shifts in Response to RA Exposure

We next focused on characterizing OCT4's involvement in the early events of RA-induced ESC differentiation. *Oct4* mRNA was substantially repressed by RA 24 hr post-treatment (Figure 4A). However, at the protein level, OCT4 was more stable and could still be detected 72 hr after RA addition (Figure 4B). As Figure 4C indicates, approximately half of the sites displayed OCT4 binding only in untreated ESCs ("ES-specific"). A large number of sites showed similar enrichment levels in both untreated and RA-treated cells ("constitutive"). Strikingly, we could also identify ~2,000 sites occupied by OCT4 only in the presence of RA ("RA-induced"). Genes previously linked to pluripotency (e.g., *Otx2*, *Tdh*, and *Foxd3*) (Shyh-Chang et al., 2013) showed a loss of OCT4 binding, whereas retinoid target genes typically showed constitutive or RA-induced OCT4 binding (Figure 4D).

RA signaling is known to play an important role in determining the anterior boundary of *Hox* gene expression in the neural tube during embryogenesis (Mallo et al., 2010). Based on our H3K27ac, RXR, and OCT4 ChIP-seq data, we found that the RARE located +5 kb from the transcription start site (TSS) of *Hoxa1* (Kashyap et al., 2011; Langston and Gudas, 1992) and

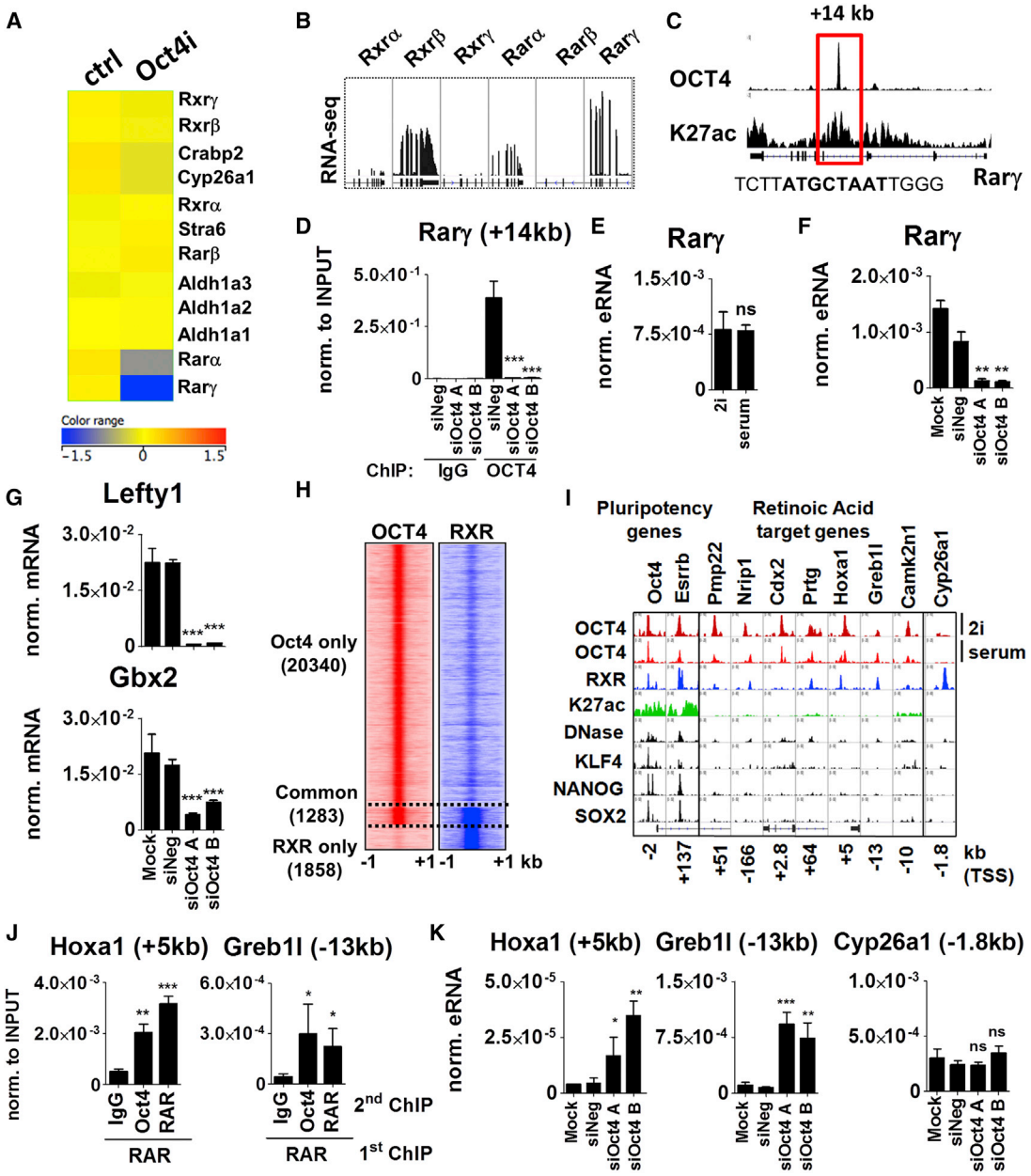

Figure 2. OCT4 Regulates *Rarg* Expression

(A) Heatmap display of microarray data showing expression changes of RA signaling-related genes affected by OCT4 depletion. The microarray data are from Loh et al. (2006). ctrl, control.

(B) IGV snapshot of RNA-seq data showing expression levels of RXR and RAR receptor isoforms in undifferentiated ESCs.

(C) IGV snapshot of the *Rarg* locus showing the OCT4 and H3K27ac ChIP-seq profiles.

(D) ChIP-qPCR analysis of OCT4 enrichment at the enhancer depicted in (C). IgG, immunoglobulin G.

(E) qRT-PCR analysis of *Rarg* +14 kb enhancer activity (eRNA production) from cells grown under 2i + LIF versus serum + LIF conditions.

(F) eRNA production of *Rarg* +14 kb enhancer 24 hr following OCT4-depletion.

(G) qRT-PCR analysis of *Rarg* downstream target genes following OCT4 depletion.

(H) Read distribution heatmap of OCT4 and RXR ChIP-seq reads (serum + LIF) relative to the midpoint of their enrichments. See also Table S2.

(I) IGV snapshot of ChIP-seq data.

(J) ChIP-reChIP analysis of OCT4 and RAR co-occupancy. The first ChIP was carried out using anti-RAR antibody. Antibodies against IgG, OCT4, or RAR were used for the reChIP.

(K) qRT-PCR analysis of eRNA levels in OCT4-depleted cells. Cells were collected for RNA isolation 24 hr after RNAi transfections. Data are expressed as a ratio of the indicated enhancer's transcript relative to *36b4*.

See also Figure S2. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

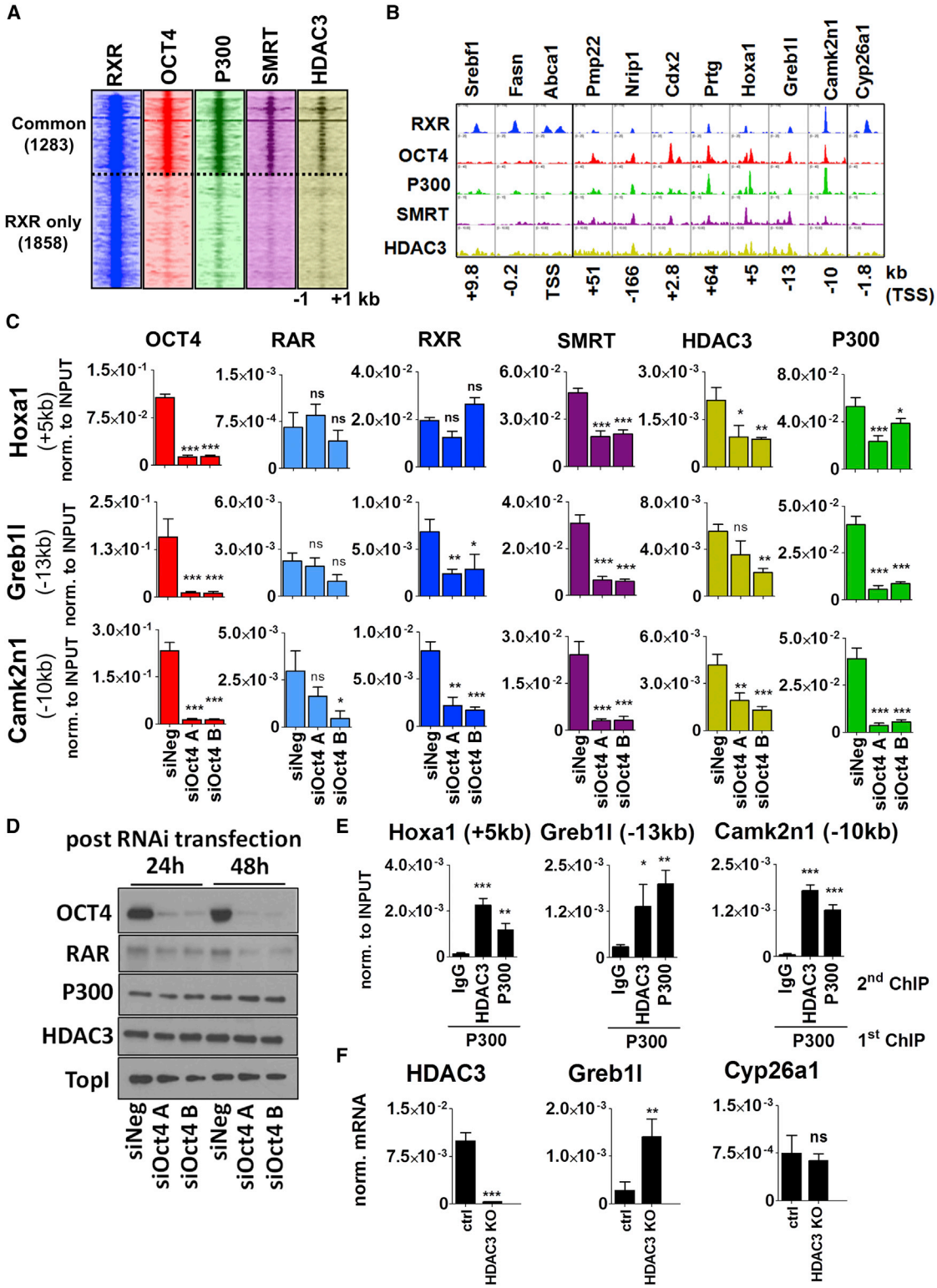

Figure 3. OCT4-Dependent Co-regulator Enrichment on RAR:RXR-Bound Enhancers

(A) Density of RXR, OCT4, P300, SMRT, and HDAC3 ChIP-seq reads relative to the summit of the RXR peaks (±1 kb).

(B) IGV snapshot of the indicated ChIP-seq data at enhancers driven by RXR heterodimers (LXR:RXR: *Srebf1*, *Fasn*, *Abca1*; RAR:RXR: *Pmp22-Cyp26a1*).

(legend continued on next page)

two additional enhancers in close proximity of *Hoxa1* (+51 kb and + 79kb from the TSS) are prototypical examples of constitutive OCT4-bound enhancers (Figure 4E). Strikingly, P300 was occupied at these sites prior to RA induction, further supporting the concept that these sites are primed for signal-dependent activation (Figure 4E). This P300 binding was completely diminished in OCT4-depleted cells (Figure 4F; Figure S4A). Increased H3K27ac and enhancer RNA production confirmed that all of these enhancers are indeed RA-inducible (Figures 4E and 4G).

Detection of OCT4 occupancy at different time points following RA treatment revealed a stable OCT4 enrichment up to 48 hr (Figure 4H; Figure S4B), suggesting that OCT4 binds concurrently with activated RAR:RXR.

Gene ontology analysis of genes linked to enhancers showing constitutive or RA-induced OCT4 binding revealed that these sites are essential for normal embryonic and nervous system development (Figure S4C). To understand whether a newly produced protein is responsible for the OCT4 redistribution (Figure 4C), we carried out de novo motif analysis on these RA-induced sites. Over 20% of RA-induced OCT4 regions showed enrichment for Pbx-Hox-like motifs (Figure 4I). Analysis of the RA response in *Hoxa1* KO cells (Martinez-Ceballos et al., 2005) revealed that HOXA1 is indeed involved in the regulation of these sites (Figures S4D–S4F).

Together, these data demonstrate that at a subset of regions of Hoxa1 promotes OCT4 recruitment to new sites and regulates enhancer activity.

OCT4 Directly Regulates Retinoic Acid and WNT/β-Catenin Signaling and Downstream Differentiation Pathways

The prolonged presence (48 hr following the signal-dependent activation) of OCT4 at differentiation-related enhancers suggests that OCT4 might also play a role in the on-time activation of these sites and the induction of corresponding regulated genes. To explore this possibility, we characterized RA and WNT/β-catenin signaling pathways, predicted in Figure 1B as putative activators of the low-accessible OCT4-occupied sites. Cistromic analysis of RXR upon activation by RA and β-catenin binding following treatment with the GSK-3 inhibitor CHIR99021 revealed the enrichment of these signal-dependent transcription factors at a subset of OCT4+/DNase low sites (Figures 5A and 5B). To understand how OCT4 contributes to signal-dependent activation of these enhancers, we compared eRNA production at OCT4/RAR:RXR and OCT4/β-catenin co-occupied regions upon signal-specific activation. Activity of the identified RA-driven *Hoxa1* enhancers and the WNT/β-catenin-activated *T/Bra* or *Tfcp2l1* enhancers showed absolute OCT4-dependence (Figure 5C; Figure S5A). However, we also identified enhancers that showed an increased response in OCT4-depleted cells (e.g., *Greb1l* and *Axin2*) (Figure 5C), suggesting that OCT4 can either negatively or positively control signal-dependent enhancer activation.

To better understand how OCT4 regulates the activity of these enhancers, first we confirmed, by enhancer reporter assays, that the *Hoxa1* +51 kb (activated by OCT4) and *Greb1l* −13 kb (repressed by OCT4) regions are directly bound and regulated by RAR:RXR (Figure S5B). ChIP-qPCR analysis revealed a dynamic recruitment of RAR and increased acetylation of the *Hoxa1* enhancer in control cells that was blocked by OCT4 depletion (Figure S5C). In contrast, the *Greb1l* −10 kb enhancer showed increased H3K27ac in the OCT4-depleted cells, which is in line with the observed higher eRNA production. These results confirmed the enhancer-dependent effect of OCT4 in the regulation of the signal response. RAR binding and acetylation of sites independent of OCT4 (e.g., *Cyp26a1* −1.8 kb) were not altered by OCT4 depletion (Figure S5D). OCT4 depletion also blocked the CHIR99021-induced enhancer acetylation at the WNT/β-catenin targets (Figure S5E).

To further dissect mechanistically how OCT4 and RAR:RXR collaborate with each other, we cloned the *Hoxa1* +51 kb enhancer containing binding sites of both transcription factors. As shown in Figure 5D, mutation of the OCT4 binding site resulted in decreased RA response, strongly indicating the direct requirement of both OCT4 and RAR:RXR for temporal (on-time) and proper (the required amplitude) enhancer activation in early differentiation. Indeed, we found that transcripts of various early lineage markers (e.g., *Cxcr4*, *Gbx2*, *Sox17*, *Gata4*, *Stra8*, *Pmp22*, etc.) showed improper signal-dependent induction in OCT4-depleted cells (Figures 5E and 5F; Figure S5F). However, RA-mediated induction of the ubiquitously inducible *Cyp26a1* proved to be OCT4-independent (Figure S5F).

Strikingly, we identified OCT4-occupied regions that were co-occupied by both RAR:RXR and β-catenin upon activation of WNT/β-catenin signaling. We chose *Cdx1*, a target for both RA and WNT/β-catenin signaling pathways (Pilon et al., 2007), for further analysis. In undifferentiated ESCs, *Cdx1* showed overlapping OCT4 and RXR binding in close proximity to the *Cdx1* TSS and recruitment of co-regulators (Figure 5G). Control cells showed recruitment of β-catenin to the same OCT4/RAR:RXR-occupied region, suggesting a to date uncovered functional interaction between the two signal-dependent transcription factors and OCT4 in the transcriptional regulation of *Cdx1*. Loss of OCT4 inhibited both the RA- and CHIR99021-dependent activation of this region (Figure 5H), indicating that OCT4 is required for both the RA- and CHIR99021-dependent induction of *Cdx1*. Co-treatment with RA and CHIR99021 resulted in robust induction of *Cdx1* gene expression (Figure 5I), suggesting a synergistic effect of the two pathways in the regulation of *Cdx1*.

(C) ChIP-qPCR analysis of RXR, OCT4, SMRT, HDAC3, and P300 binding at *Greb1l* −13 kb, *Hoxa1* +5 kb, and *Camk2n1* −10 kb enhancers in control parental (siNeg) or OCT4-depleted (siOct4 A and siOct4 B) cells 24 hr following OCT4 depletion.

(D) Western blot assay showing expression of co-regulators in nuclear extracts 24 and 48 hr following the RNAi transfections.

(E) ChIP-reChIP analysis of P300 and HDAC3 co-occupancy. The first ChIP was carried out using anti-P300 antibody. Antibodies against IgG, HDAC3, or P300 were used for the reChIP.

(F) mRNA expression of the indicated genes in control versus *Hdac3* KO cells as determined by qRT-PCR.

See also Figure S3. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001,

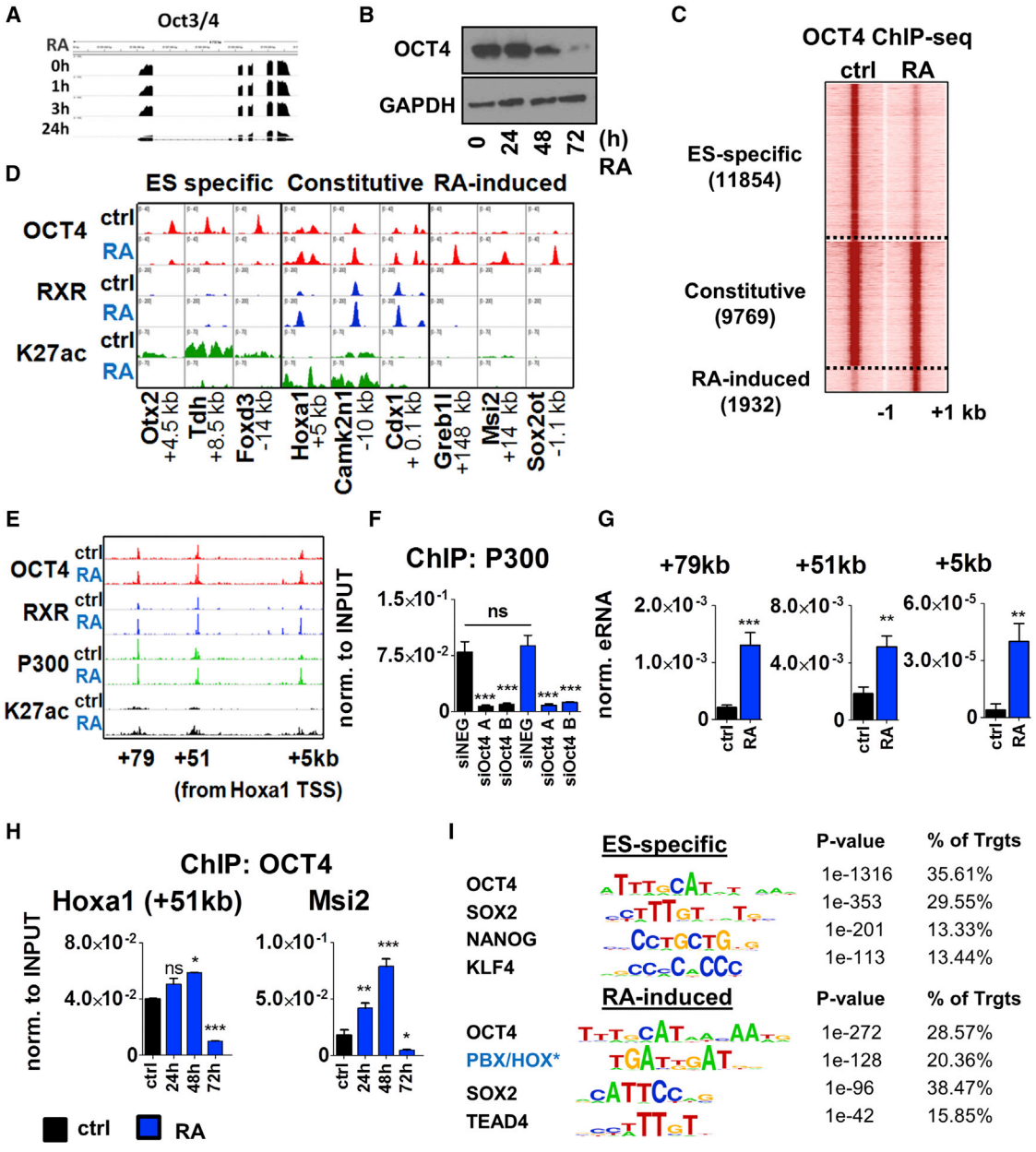

Figure 4. Signal-Dependent Redistribution of the OCT4 Cistrome

(A) IGV snapshot of RNA-seq data showing the *Oct4* coding region. Cells were treated with RA for the indicated time points in 1 μM final concentration.

(B) Western blot analysis of OCT4.

(C) Density of OCT4 ChIP-seq reads relative to the midpoint (±1 kb). Cells were treated with 1 μM RA for 24 hr prior to the ChIP experiment. See also Table S3.

(D) IGV snapshot of enhancers representing ES-specific, constitutive, or RA-induced OCT4 binding.

(E) IGV snapshot of enhancers in the proximity of the *Hoxa* cluster.

(F) ChIP-qPCR detection of P300 enrichment at the *Hoxa1* enhancer (+51 kb from the *Hoxa1* TSS).

(G) qRT-PCR analysis of RA-induced eRNA production.

(H) ChIP-qPCR detection of OCT4 enrichment.

(I) De novo identification of motifs under ES-specific and RA-induced OCT4 sites using Homer (C). % target refers to the ratio of peaks having the given motif. See also Figure S4.

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Hoxb1 Expression Is Controlled by OCT4

The results shown in Figure 3 suggested an interesting mechanistic model; namely, that the pluripotency-specific transcription factor OCT4 drives co-regulator recruitment at lineage-specific genes. *Hoxb1* represents a unique example because binding sites of OCT4 and RAR:RXR in its proximity are separated by

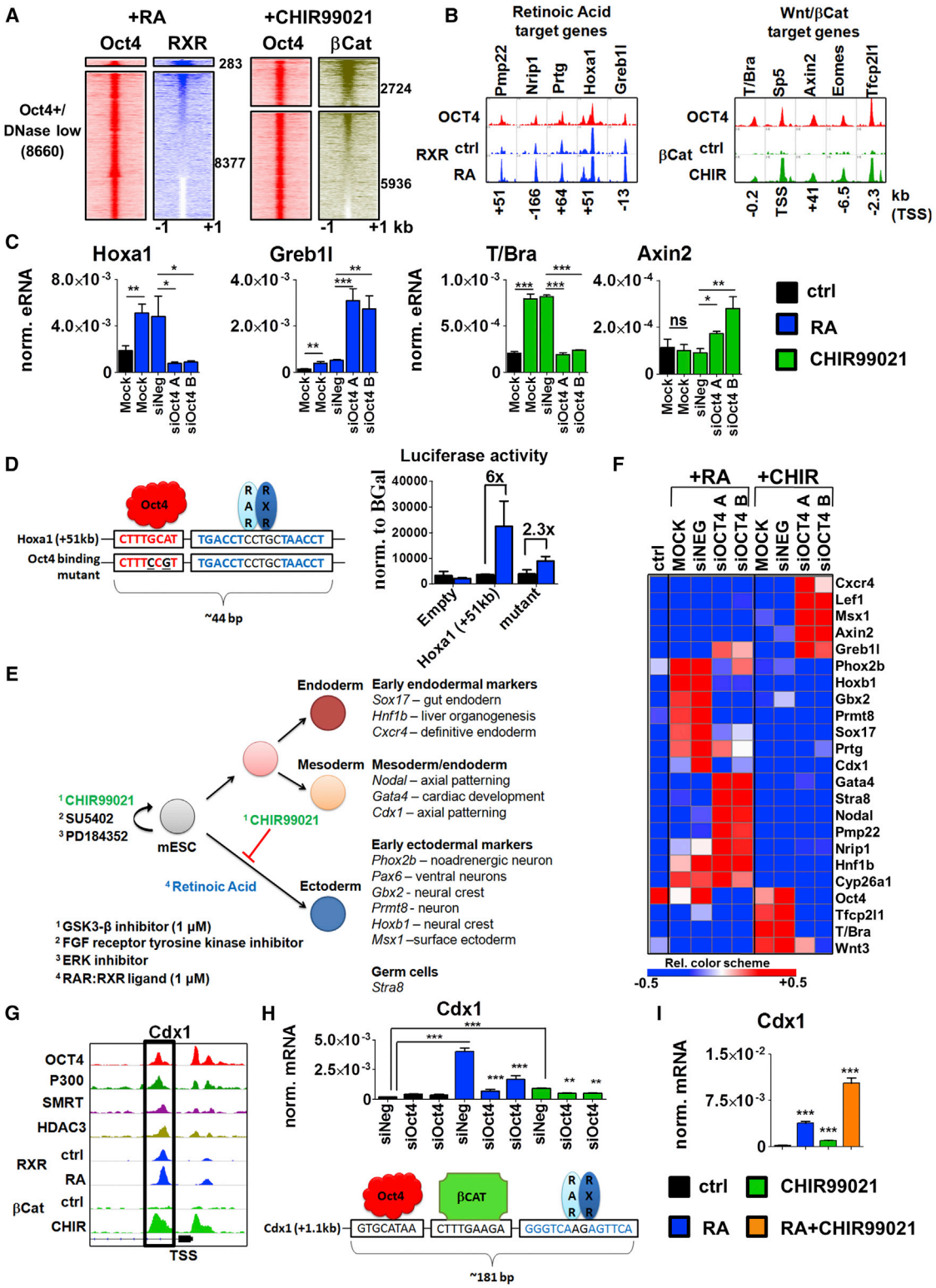

Figure 5. Dysregulated Signal Response in OCT4-Depleted Cells

(A) Read distribution heatmap of RXR or β-catenin ChIP-seq reads relative to the summits of the OCT4 peaks in the OCT4+/DNase low cluster (±1 kb). 283 OCT4+/DNase low genomic sites were occupied by RXR upon RA treatment, and 2724 sites were bound by β-catenin upon WNT activation. See also Table S4.

(B) IGV snapshot of enhancers representing RA or WNT/β-catenin target genes.

(legend continued on next page)

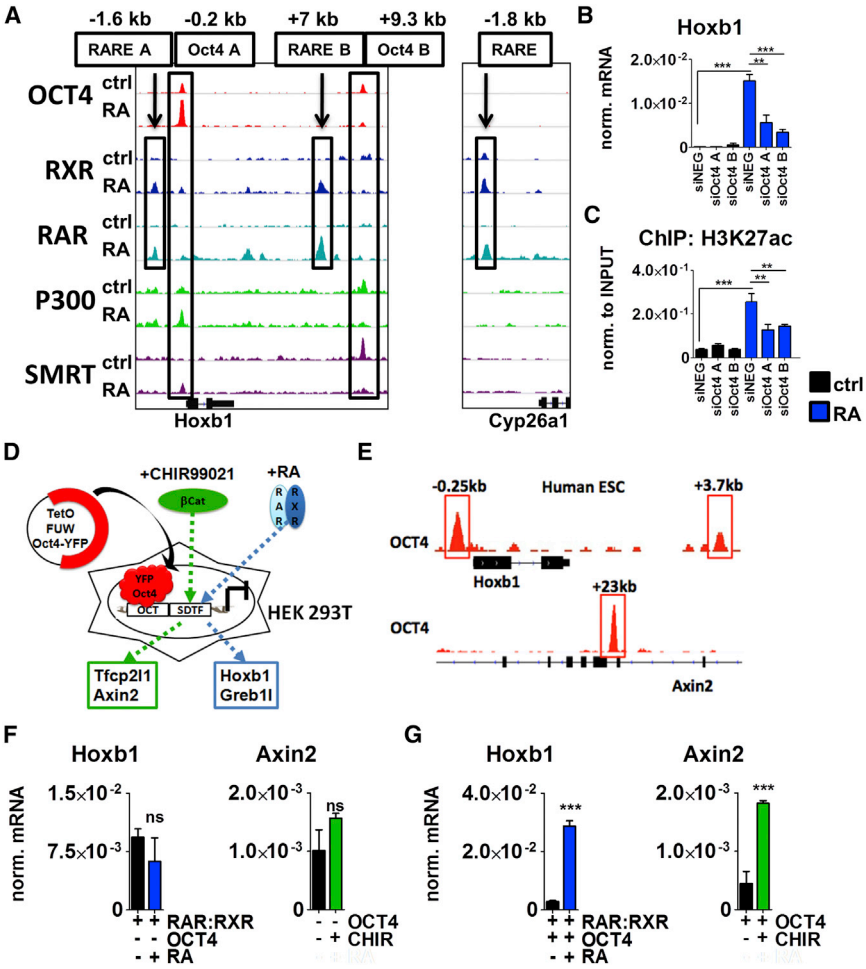

Figure 6. OCT4-Dependent Co-regulator Exchange on the *Hoxb1* Enhancers

(A) IGV snapshot of the *Hoxb1*- and *Cyp26a1*-coding genomic region.

(B) qRT-PCR analysis of *Hoxb1* expression in control parental and OCT4-depleted cells.

(C) ChIP-qPCR detection of H3K27ac enrichment at the *Hoxb1* −0.2 kb enhancer depicted in (A).

(D) RA- or CHIR99021-dependent induction of the target genes in doxycycline-inducible OCT4-overexpressing HEK293T cells.

(E) IGV snapshot of OCT4 enrichment in the proximity of *HOXB1* (top) and *AXIN2* (bottom) in human ESCs. ChIP-seq data were used from GEO: GSE21916.

(F and G) qRT-qPCR analysis of *HOXB1* and *AXIN2* mRNA expression. Signal-dependent induction of *HOXB1* and *AXIN2* was determined in control or Dox-treated (inducible OCT4 expression) HEK293T cells. Cells were co-transfected with RAR:RXR prior to RA addition.

See also Figure S6. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

>1 kb. *Hoxb1* has two retinoic acid response elements: RARE A (located −1.6 kb from the TSS) and RARE B (+7 kb from the TSS of the gene) (Marshall et al., 1994; Studer et al., 1994). These enhancers were bound by RAR:RXR upon RA stimulation but were not co-occupied by OCT4 or any co-regulator at any time point investigated (Figure 6A). However, we identified two additional enhancers in the proximity of *Hoxb1* that were occupied by OCT4, termed "Oct4 A" (−200 bp from the TSS) and "Oct4 B" (+9.3 kb from the TSS). Interestingly, the Oct4 B site showed decreased OCT4 occupancy, whereas OCT4 was enriched at Oct4 A following RA addition (Figure 6A). Remarkably, these sites were also enriched for P300 and SMRT, and the co-regulator occupancy correlated with the OCT4 movement (Figure 6A). *Cyp26a1*, which showed OCT4-independent regulation, was only enriched for RAR:RXR (Figure 6A, right). Importantly, as a consequence of OCT4 depletion, RA-dependent induction of *Hoxb1* was completely abolished (Figures 6B and 6C). As shown in Figures S6A and S6B, loss of OCT4 resulted in decreased occupancy of SMRT and HDAC3 at the Oct4 B enhancer in untreated cells, whereas P300 recruitment was also abolished at the Oct4 A enhancer in RA-treated, OCT4-depleted cells. Collectively, these results reinforce the notion that OCT4 is required for co-factor exchange.

To examine the biological relevance of co-repressor recruitment during gene activation, we used HDAC3 knockout (KO) cells. Loss of HDAC3 selectively enhanced the induction of *Hoxb1* upon RA addition but did not change the response of *Cyp26a1* (Figure S6C). This suggests that recruitment of HDAC3 has a negative compensatory function upon gene activation and limits the amplitude of the signal response.

(C) qRT-PCR analysis of RA-induced (e.g., *Hoxa1* +51 kb and *Greb1l* −13 kb) and CHIR99021 (e.g., *T/Bra* −0.2 kb and *Axin2* +41 kb) eRNA production in control parental and OCT4-depleted cells. Twenty-four hours following RNAi transfection, cells were treated with RA (blue), CHIR99021 (green), or DMSO (black) for 3 hr.

(D) Enhancer reporter analysis of the *Hoxa1* (+51 kb) enhancer. The 300-bp-long genomic region was cloned into a thymidine kinase (TK)-luciferase reporter vector. Site-directed mutagenesis was carried out to modify the OCT4-binding site as shown. Retinoid-induced activity of these elements was compared by transfecting these constructs into ESCs.

(E) Summary of lineage markers used in (F).

(F) Heatmap visualization of qRT-PCR analysis of lineage-specific marker genes by GENE-E (Broad Institute). 24 hr following RNAi transfections, control parental and OCT4-depleted cells were treated with 1 μM RA or CHIR99021 for 24 hr.

(G) IGV snapshot of OCT4, RXR, β-catenin, and the indicated co-regulator's occupancy at the *Cdx1* enhancer.

(H) qRT-PCR analysis of *Cdx1* expression in control parental and OCT4-depleted cells 24 hr after RA (blue) or CHIR99021 (green) addition.

(I) qRT-PCR analysis of *Cdx1* mRNA expression 24 hr after RA, CHIR99021, or RA + CHIR99021 addition.

See also Figure S5. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

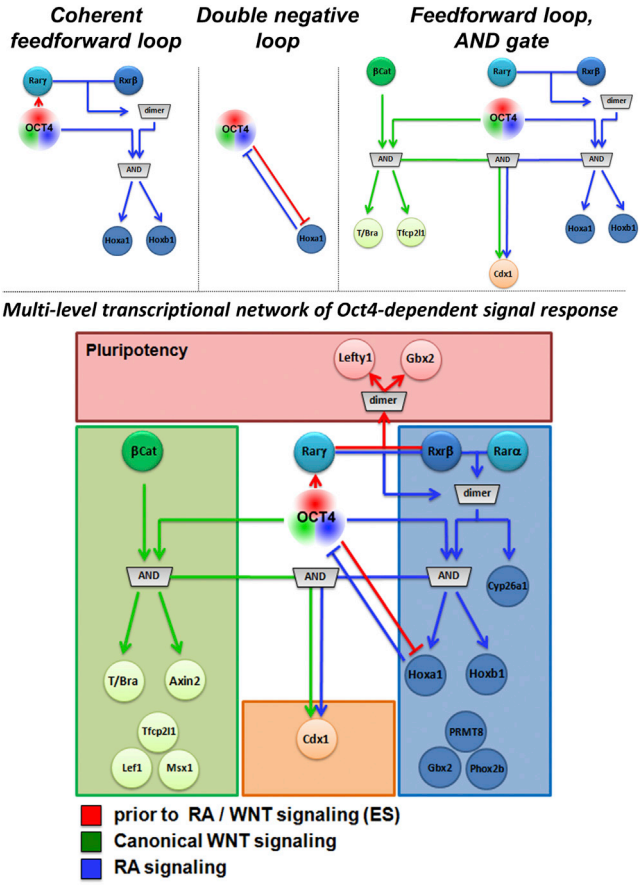

Figure 7. Model of the Multi-level Cooperativity of OCT4, RAR:RXR, and WNT/β-Catenin Signaling in Embryonic Stem Cells

Shown are identified recurring regulation patterns (Alon, 2007) in the OCT4-controlled, RA-induced ESC program. In the pluripotent state, OCT4 contributes to *Rarg* expression, and this receptor, along with RXRβ, maintains the expression of *Lefty1* and *Gbx2*, forming a coherent feedforward loop. OCT4 also works as a repressor of lineage-specific genes (e.g., *Hoxa1*), but RA-induced expression of HOXA1 feeds back on OCT4 levels (Martinez-Ceballos et al., 2005), creating a double-negative loop in which the two factors repress each other. Upon RA exposure, both OCT4 and the RAR:RXR dimer are needed to activate the *Hox* cluster, suggesting that OCT4 and RAR:RXR are integrated (AND gate) and contribute together to a graded retinoid response. Similarly, activation of the canonical WNT signaling (e.g., *T/Brachyury*, *Axin2*) also requires the presence of OCT4. *Cdx1* represents an additional level of cooperativity, integrating the RA and WNT/β-catenin signaling. Long-term RA signal exposure leads to the induction of neurogenesis-related genes (e.g., *Gbx2*, *Phox2b*, *Prmt8*) (Chen et al., 2010; Dubreuil et al., 2000; Simandi et al., 2015), whereas activation of the WNT signal induces primitive streak commitment, represented by the induction of *Axin2*, *Lef1*, and *T/Bra* (Funa et al., 2015; Rivera-Pérez and Magnuson, 2005).

The identified collaboration between OCT4 and the signal-dependent transcription factors RAR:RXR or β-catenin led us to ask whether cell type-specific signal response is determined by the lineage-determining factors (e.g., *Hoxb1* gene induction by RA is dependent on the presence of OCT4 in ESCs). To address this, we introduced OCT4 ectopically into a human embryonic kidney cell line (Figure 6D; Figure S6D). OCT4 could bind to those sites in kidney cells in the proximity of *HOXB1* that are also OCT4-bound in human ESCs (Figure 6E; Figure S6E). Strikingly, WNT/β-catenin and RA target genes, otherwise unresponsive to these signals in kidney cells, showed induction following overexpression of OCT4 (Figure 6F and 6G; Figure S6F and S6G). *CYP26A1* induction was OCT4-independent in this cell type as well (Figure S6H). RA-mediated induction of *HOXB1* eRNA in OCT4-overexpressing cells confirmed the OCT4-dependent enhancer activation (Figure S6I). This set of experiments provides a proof of concept that OCT4 is sufficient for lineage-specific, signal-dependent transcription.

DISCUSSION

A key unanswered question in developmental cell biology is how a cell type-specific enhancer set is selected during transition from one cellular stage to another. This study addresses this question, demonstrating the existence of an OCT4-dependent priming step at signal-dependent enhancers prior to activation of these loci. Based on our findings and previous work, we constructed a comprehensive model by combining recognizable and stereotypical network motifs (Figure 7; Alon, 2007). This model integrates several molecular connections clarified in this study. First, it points out the OCT4-dependent positive regulation of *Rarg*, a retinoic acid receptor isoform that has been shown to be important in early retinoic acid response in ESCs (Al Tanoury et al., 2014; Su and Gudas, 2008). Recent studies showed that *RARγ* has a critical role in cellular reprogramming (Wang et al., 2011), and its genomic locus frequently interacts with the *OCT4* locus in human ESCs (Gao et al., 2013). Thus, accumulating lines of evidence suggest that unliganded RARγ is a regulator of the pluripotent state and that its OCT4-controlled expression in the early stage of the cellular differentiation is essential for retinoic acid-induced chromatin remodeling (Kashyap et al., 2013). This conclusion is further supported by observations in in vivo models showing very high expression of *Rarg* in the caudal progenitor domain of late gastrulation stage mouse embryos, where progenitor cells generate the posterior neural tube (hindbrain and spinal cord) under the influence of RA synthesis, which begins at embryonic day (E) 7.5 in presomitic mesoderm (Cunningham and Duester, 2015).

Second, our proposed model posits that RAR:RXR shares a subset of OCT4-occupied enhancers in the proximity of differentiation-related genes. Previous studies already noted that OCT4 can bind at genes encoding developmental regulators (Boyer et al., 2006; Loh et al., 2006; Yang et al., 2014). Our analyses extended these results and demonstrate that an unexpectedly large set of OCT4-occupied sites are located in low DNase accessible chromatin regions and independent of the canonical pluripotent network, potentially priming various cell fate-related enhancers. The fact that loss of OCT4 causes deregulation of RA and WNT immediate early genes suggests that OCT4 binding at these sites has a mechanistic role in signal-regulated gene expression and is not only an "innocent bystander." Moreover, the collaboration between OCT4 and RAR:RXR or OCT4 and β-catenin is likely to be relevant in vivo at the very earliest stages of embryonic development (E7.5–E8.5 in the mouse) when *Oct4* is still expressed in the caudal progenitor domain (Osorno et al., 2012). Indeed, *Oct4* and *Hoxa1* expression are co-localized in

E7.25–E9.25 embryos (Dupé et al., 1997; Perea-Gómez et al., 1999). The collaboration between OCT4 and RAR:RXR or OCT4 and β-catenin is likely to require the presence of composite regulatory elements that contain two or more closely situated binding sites for OCT4 and RAR:RXR/β-catenin and that provide a mechanism for crosstalk between the different regulatory pathways at the DNA-binding level. We present here the enhancers of *Hoxa1* +51 kb and *Cdx1* +1.1 kb as examples of such signal integrating regions in ESCs.

A key question is the gene specificity of the interaction between the lineage-determining and signal-dependent transcription factors (LDTFs/SDTFs). In other words, do all signal-regulated genes require LDTF priming and direct binding to their enhancers, or is it only restricted to a certain set? The latter appears to be the case. We show that, strikingly, the ubiquitously responsive RA-degrading enzyme *Cyp26a1* is under OCT4-independent control and is likely regulated by a distinct co-regulator set independent of OCT4. This suggests that enhancer regulation complexity was previously underestimated and that the co-regulator complex composition can be enhancer-selective (Krebs et al., 2011). In line with this, our previous study demonstrated that cell type-specific transcription factors can contribute to selective co-regulator recruitment (Simandi et al., 2015) and that this recruitment allows the cells to selectively limit the effect of external cues on target genes. This is important because it forms the molecular basis of a tissue-selective retinoid response. By this mechanism, the signal-specific regulation of developmentally important genes, such as *Hox* genes, is uncoupled from ubiquitously retinoid-regulated transcriptional events occurring in almost every cell type, such as *Cyp26a1* (Ozpolat et al., 2005).

Simultaneous or co-binding enables OCT4 to act as a key "gatekeeper" of differentiation pathways providing cell type specificity to the signal response. Re-appearance of OCT4 in cells under pathophysiological conditions (e.g., cancer [stem] cells) (Karoubi et al., 2009; Kumar et al., 2012; Wang et al., 2013) may result in unwanted transcriptional events. Comparison of OCT4 ChIP-seq data in various cell types and manipulation of OCT4 in these cells will be required to understand in more detail how OCT4 changes the epigenomic status of cancer stem cells or during cellular reprogramming.

In summary, our study highlights that OCT4 has multiple roles and uncovers the mechanisms by which OCT4 plays essential functions both in maintaining the ESC state and also in directing subsequent differentiation by priming cell identity genes at the level of enhancers and regulating co-factor binding. These mechanisms might be applicable to other transitions during cellular differentiation.

EXPERIMENTAL PROCEDURES

Additional methods, further details of the methods listed below, and related citations can be found in the Supplemental Experimental Procedures.

Cell Culture

Mouse E14 ESCs (termed "serum" or "'naive" in the text) were cultured on primary mouse embryonic fibroblast (PMEF) feeder cells (5% CO_2 at 37°C). ESC medium was prepared by supplementing DMEM Glutamax (Gibco) with 15% FBS (Hyclone), 1,000 U of LIF, penicillin/streptomycin, non-essential amino acids, and 2-mercaptoethanol. 2i ESCs were adapted for a minimum of five passages to grow in serum-free N2B27-based medium supplemented with LIF, PD0326901 (1 μM), and CHIR99021 (3 μM). *Hoxa1* KO and appropriate control cells were provided by the L.J.G. laboratory. HDAC3 KO cells were supplied by the S.M.C. laboratory.

Ligands and Treatment

Cells were treated with vehicle (DMSO) or with the following ligands: all-trans RA (Sigma, 1 mM stock in DMSO, 1/1,000 dilution) and CHIR99021 (Sigma, 3 mM stock in DMSO, 1/1,000 dilution).

siRNA Knockdown

Oct4 and control siRNAs were obtained from Thermo Fisher. Mouse E14 cells were plated on gelatinized plates 12 hr before transfection. siRNA transfection was carried out with Lipofectamine 3000 (Invitrogen). Transfected cells were cultured in embryonic stem (ES) medium for 24 hr prior to experiments.

Primer Sequences

Sequences of primers used for cloning, mutagenesis, ChIP, and eRNA and mRNA detection are available in the Supplemental Experimental Procedures.

ChIP-Seq, DNase-Seq, and ATAC-Seq Analyses

Primary analysis of the ChIP-seq raw reads was carried out using the ChIP-seq analyze command line pipeline (Barta, 2011). Human OCT4 (GEO: GSM545202), mouse OCT4 (2i + LIF) (Galonska et al., 2015), mouse NANOG, KLF4, SSOX2 (GEO: GSE11431) (Chen et al., 2008), and β-catenin (GEO: GSE43597) (Zhang et al., 2013) ChIP-seq data, assay for transposase accessible chromatin sequencing (ATAC-seq) (GEO: GSM1563569) (Kearns et al., 2015), and DNase sequencing (DNase-seq) data (GEO: GSM1014154 and GSM1657364) (Consortium, 2012; Domcke et al., 2015) were downloaded from the NCBI GEO database and re-analyzed.

ACCESSION NUMBERS

The accession number for the RNA-seq and ChIP-seq data reported in this paper is SRA: PRJNA302640.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.06.039.

AUTHOR CONTRIBUTIONS

Conceptualization, Z.S. and L.N.; Methodology, Z.S., A.H., S.S., L.J.G., J.F.D., and L.N.; Software, Z.S. and A.H.; Validation, Z.S.; Formal Analysis, Z.S., A.H., and L.N.; Investigation, Z.S., L.C.W., I.C.M., K.K., and I.D.L.; Resources, L.J.G., S.M.C., and L.N.; Writing – Original Draft, Z.S. and L.N.; Writing – Review and Editing, Z.S., A.H., L.J.G., S.S., J.F.D., S.M.C., and L.N.; Visualization, Z.S. and A.H.; Funding Acquisition, L.N.; Supervision, L.N.

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