

Ncoa3 functions as an essential Esrrb coactivator to sustain embryonic stem cell self-renewal and reprogramming

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Embryonic stem cell (ESC) pluripotency depends on a well-characterized gene regulatory network centered on Oct4, Sox2, and Nanog. In contrast, little is known about the identity of the key coregulators and the mechanisms by which they may potentiate transcription in ESCs. Alongside core transcription factors, the orphan nuclear receptor Esrrb (estrogen-related receptor β) is vital for the maintenance of ESC identity and furthermore is uniquely associated with the basal transcription machinery. Here, we show that Ncoa3, an essential coactivator, is required to mediate Esrrb function in ESCs. Ncoa3 interacts with Esrrb via its ligand-binding domain and bridges Esrrb to RNA polymerase II complexes. Functionally, Ncoa3 is critical for both the induction and maintenance of pluripotency. Through chromatin immunoprecipitation (ChIP) sequencing and microarray experiments, we further demonstrate that Ncoa3 shares overlapping gene regulatory functions with Esrrb and cooperates genome-wide with the Oct4–Sox2–Nanog circuitry at active enhancers to up-regulate genes involved in self-renewal and pluripotency. We propose an integrated model of transcriptional and coactivator control, mediated by Ncoa3, for the maintenance of ESC self-renewal and somatic cell reprogramming.

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Embryonic stem cells (ESCs) are derived from the inner cell mass of early embryos and can be propagated for extended periods of time in culture while retaining their pluripotency; i.e., the capacity for multilineage specification and differentiation (Smith 2001). Coordinating with essential components of the LIF and BMP signaling pathways (Niwa et al. 1998; Matsuda et al. 1999; Ying et al. 2003), intrinsic transcription factors are crucial for specifying the undifferentiated state of mouse ESCs. Oct4 is essential for establishing and maintaining pluripotency (Nichols et al. 1998; Niwa et al. 2000; Boiani et al. 2005) and interacts with Sox2 (Avilion et al. 2003; Chew et al. 2005) in ESCs, where both factors cotarget multiple genes through cooperative binding at Oct–Sox sequence elements (Boyer et al. 2005; Loh et al. 2006). Moreover, Oct4 and Sox2, along with Klf4 and cMyc, can mediate repro-

gramming of mouse embryonic fibroblasts (MEFs), generating ESC-like induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). A third well-characterized core pluripotency factor is Nanog, which is notably capable of promoting ESC self-renewal even in the absence of LIF (Chambers et al. 2003; Mitsui et al. 2003).

Recent work has begun to identify new components of the ESC core transcriptional network. Along with Oct4, Sox2, and Nanog (OSN), these factors participate in auto- and cross-regulation to activate each other's expression as well as downstream self-renewal regulators while simultaneously repressing genes that promote differentiation (Chambers and Tomlinson 2009). Perturbation of these factors collapses the self-renewal circuitry and triggers specific or mixed-lineage differentiation (Ivanova et al. 2006; Loh et al. 2006). Whole-genome mapping of DNA-binding sites further revealed that the OSN triad colocalizes with a variable yet overlapping set of pluripotency-associated transcription factors at many promoters and enhancers (Chen et al. 2008). Clusters with a relatively high number of factors correlate with expression of nearby genes

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(Kim et al. 2008). It remains less clear, however, how this well-established network functionally interacts with basal transcription machinery complexes to activate transcription and, most importantly, which factors mediate such interactions. While the list of ESC-specific transcription factors is rapidly expanding, relatively little is known about the identity of essential coregulators (Fong et al. 2011) and the mechanisms by which they may potentiate transcription.

To gain greater insight into how transcription factors function mechanistically in ESCs, we focused here on estrogen-related receptor β (Esrrb). This orphan nuclear receptor binds many target sites that are co-occupied by OSN and Klf4 in ESCs and up-regulates the expression of these factors themselves (Chen et al. 2008). Levels of *Esrrb* decline rapidly upon differentiation (Xie et al. 2009), and its depletion results in a loss of ESC characteristics (Ivanova et al. 2006; Loh et al. 2006). Overexpression of *Esrrb* inhibits lineage commitment toward the meso- and neuro-ectodermal paths and allows robust self-renewal in the absence of LIF (Zhang et al. 2008). In addition, Esrrb can substitute for Klf4 in somatic cell reprogramming (Feng et al. 2009), further highlighting a pivotal role in both maintaining and inducing pluripotency. In somatic cells, the activity of estrogen-related receptors (ERRs) is known to be regulated through the recruitment of coactivators to the AF-2 portion of their ligand-binding domain (LBD) (Giguere 2008); for example, PGC-1 in metabolic tissues (Lin et al. 2005; Charest-Marcotte et al. 2010). These coactivators in turn act as scaffolds for other protein complexes required to bring about transcription (Rosenfeld et al. 2006). Although well studied within the context of adult somatic systems, little is known about the mechanisms by which Esrrb function is conferred in ESCs.

In this study, we show that Esrrb transcriptional and self-renewal activity is absolutely dependent on protein-protein interactions mediated via its LBD/AF-2 domain. Despite this, we found that the coactivators *PGC-1 α* and *PGC-1 β* commonly required for ERR activity are not expressed in ESCs and instead identify Ncoa3 (also known as SRC-3/AIB1) as an essential coactivator of Esrrb. We show that *Ncoa3* expression positively correlates with the undifferentiated ESC state and itself is required for both the induction and maintenance of pluripotency. Ncoa3 binds to Esrrb specifically via its AF-2 region in ESCs and thus is corecruited to *Esrrb*, *Klf4*, *Nanog*, and *Sox2* enhancer regions as a critical step to trigger Esrrb-dependent gene activation. Through chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq) and transcriptome analysis in *Ncoa3* knockdown ESCs, we further establish that Ncoa3 and Esrrb have overlapping gene regulatory functions and cooperate genome-wide with the Oct4–Sox2–Nanog circuitry at active enhancers to up-regulate genes involved in self-renewal and pluripotency. Finally, we demonstrate mechanistically that Ncoa3's presence links Esrrb to RNA polymerase II (RNAPol2) complexes, identifying a crucial role for the Ncoa3–Esrrb partnership in the ESC core transcriptional network and shedding new light on how pluripotency transcription factors may be bridged to the general transcription machinery to activate transcription.

Results

The LBD and AF-2 regions are essential for Esrrb transcriptional activity in ESCs

How the assembly of transcription factor clusters leads to active transcription and which essential mediating components contribute to this process are yet to be fully investigated in ESCs. Combining mutagenesis and luciferase reporter assays, we here asked whether Esrrb activity is conferred in ESCs through specific protein recruitment. Flag-tagged Esrrb mutants lacking either the entire LBD (Δ LBD) or N-terminal AF-1 (Δ AF-1) domain—regions key for mediating nuclear receptor protein-protein interactions—were generated (Supplemental Fig. S1A), and their expression was verified alongside wild-type Esrrb in ESCs (Supplemental Fig. S1B). To test these deletion mutants, we initially took advantage of previously described constructs that carry luciferase downstream from a minimal *Oct4* promoter, together with ERR response element (ERRE)-containing regulatory fragments found at the *Esrrb*, *Klf4*, or *Sox2* loci (Fig. 1A; Feng et al. 2009), and an additional Esrrb-dependent *Nanog*-Luc reporter (Rodda et al. 2005; van den Berg et al. 2008). These Enhancer-Luc vectors all show a loss of luciferase activity following knockdown of endogenous *Esrrb* in ESCs, an activity readily regained by coexpressing RNAi-resistant wild-type *Esrrb* cDNA (Supplemental Fig. S1C). As opposed to wild-type control, we found that the Δ LBD mutant failed to restore transcription from *Esrrb*, *Klf4*, *Nanog*, and *Sox2* reporters. In contrast, deleting the AF-1 domain had no such suppressive effect on Esrrb activity (Supplemental Fig. S1C). Unlike other nuclear receptors, ERR activity is not thought to be ligand-dependent but nevertheless involves the recruitment of essential coactivators to the AF-2 region, a domain of 12 helices forming a unique protein-binding surface within the LBD (Wurtz et al. 1996). Accordingly, we repeated these experiments using Esrrb point mutants in either helix 3 (AF-2 mut1) or helix 12 (AF-2 mut2)—two analogous mutations previously found to inactivate the AF-2 region of the estrogen receptor (Danielian et al. 1992; Henttu et al. 1997)—along with a DNA-binding domain (DBD) mutant unable to bind DNA generated as a control (Fig. 1B). Despite equal expression to wild-type Esrrb in ESCs (Fig. 1C), all three point mutants were similarly unable to rescue the luciferase activity of *Esrrb*, *Klf4*, *Nanog*, and *Sox2* reporters (Fig. 1D), unequivocally demonstrating that Esrrb in ESCs requires an active LBD/AF-2 domain to trigger transcription.

AF-2 region inactivation results in a loss of self-renewal in ESCs

Overexpressing *Esrrb* is sufficient to allow robust self-renewal in ESCs, negating the need for LIF to prevent differentiation (Zhang et al. 2008). To link the AF-2 dependence of Esrrb transcriptional activity with its self-renewal function, we next generated stable ESC lines that constitutively express Flag-tagged wild-type, AF-2 mut1, or AF-2 mut2 Esrrb (Fig. 1E). Cells were plated at low density and cultured for 5 d in the absence of LIF, then

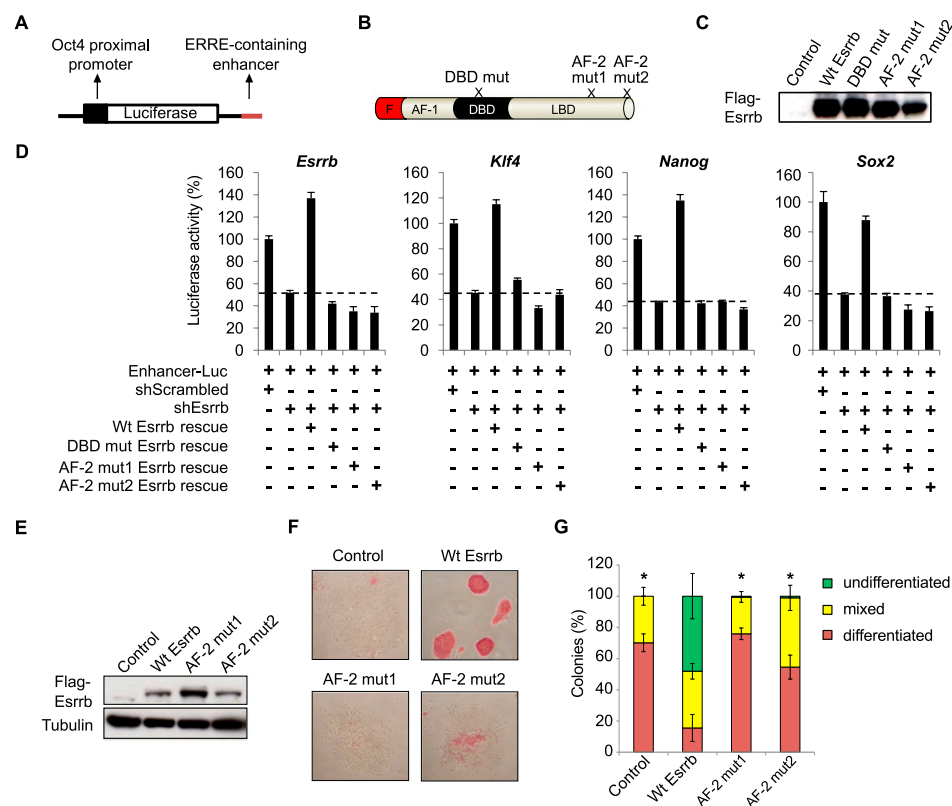


Figure 1. The AF-2 region is essential for *Esrrb* activity in ESCs. (A) Schematic of *Esrrb*, *Klf4*, and *Sox2* reporters used in luciferase assays. Enhancer fragments containing ERREs from the indicated genes are inserted downstream from luciferase (Feng et al. 2009). An additional *Nanog*-Luc reporter was also used (Rodda et al. 2005). (B) Diagram indicating Flag-tagged wild-type (Wt) and *Esrrb* point mutants. (DBD mut) C120G/C123G; (AF-2 mut1) K259A; (AF-2 mut2) L424A/F425A. (C) Expression of Flag-tagged wild-type *Esrrb* or point mutants in ESCs. (D) Luciferase assays for *Esrrb*-bound enhancers. ESCs were transfected with the indicated reporter plus either control (shScrambled) or shRNA to endogenous *Esrrb* (shEsrrb) and with or without RNAi-resistant *Esrrb* rescue constructs as depicted in B. Data are the mean \pm SEM of three transfections. Three experiments were performed with similar results. (E) Levels of CAG-Flag-*Esrrb* construct expression in ESCs following stable transfection with wild-type, AF-2 mut1, or AF-2 mut2 *Esrrb* forms. (F,G) Ability of wild-type or *Esrrb* point mutants to confer LIF independence in stably transfected ESCs. Each cell line was plated at low density in normal ESC medium and, the following day, cultured in medium minus LIF for 5 d. Colonies were fixed and stained for AP and scored as undifferentiated, mixed, or differentiated. Data are the mean \pm SEM of three independent experiments. Student's *t*-test was used to compare numbers of undifferentiated colonies in wild-type and either AF-2 mutant ESCs or empty vector control; (*) *P*-value < 0.05.

stained for alkaline phosphatase (AP) to score the number of undifferentiated, mixed, and differentiated colonies formed under these conditions (Supplemental Fig. S1D). Strikingly, and in contrast to overexpressing wild-type *Esrrb* cells, we observed a near-complete absence of compact, AP-positive colonies in both AF-2 mut1 and AF-2 mut2 ESC cultures (Fig. 1F,G), indicating a loss in the ability of *Esrrb* point mutants to confer LIF independence. To support this, we also examined the effect of treating ESCs with an ERR-specific AF-2 antagonist, diethylstilbestrol (DES). As previously reported, this compound interacts with all three ERR isoforms to block coactivator binding and thereby prevent their transcriptional activity (Supplemental Fig. S1E; Tremblay et al. 2001; Greschik et al. 2004). We demonstrated that DES treatment consistently inhibited transcription from *Esrrb*-dependent reporters in ESCs (Supplemental Fig. S1F) and significantly impaired the self-renewal ability of these cells in a dose-dependent fashion (Supplemental Fig. S1G,H). Taken together, these

findings reveal the importance of the AF-2 region to potentiate *Esrrb* function and imply a crucial role for a cognate *Esrrb* coactivator in ESCs.

Ncoa3 is highly expressed in undifferentiated ESCs

In somatic cells, the best-characterized ERR coactivators belong to the PGC-1 family. PGC-1 α and PGC-1 β both potentiate ERR activity in various adult cell types to activate genes involved in processes such as cellular energy metabolism and homeostasis (Huss et al. 2002; Kamei et al. 2003; Charest-Marcotte et al. 2010). Most recently, a third member of the PGC-1 family, PRC, was identified as a regulator of NRF-1 and mitochondrial function (Vercauteren et al. 2009). Among all three PGC-1 members, only PRC was expressed in ESCs (Supplemental Fig. S2A), and thus its role as a putative *Esrrb* coactivator was further investigated. Knockdown of PRC in ESCs did not, however, compromise *Esrrb* transcriptional activity or ESC

self-renewal, as seen upon depletion of *Esrrb* itself (Supplemental Fig. S2B–E). We next turned to the SRC/p160 family that comprises Ncoa1, Ncoa2, and Ncoa3, three alternative coactivator proteins also capable of interacting with the ERR family in mammalian cell models (Hong et al. 1999; Zhang and Teng 2000; van den Berg et al. 2010). *Ncoa3* notably emerged as an interesting candidate, being highly expressed in self-renewing ESC cultures, in contrast to *Ncoa1* and *Ncoa2* (Fig. 2A), and promptly down-regulated alongside *Esrrb* and other pluripotency factors during differentiation, as assessed upon addition of retinoic acid and/or LIF withdrawal (Fig. 2B,C). A sharp decline in *Ncoa3* transcript levels was also confirmed upon embryoid body (EB)-mediated differentiation, further pointing to a close association between *Ncoa3* expression and the undifferentiated ESC state (Fig. 2D).

Ncoa3 acts as an *Esrrb* coactivator in pluripotent ESCs

To establish a functional link between Ncoa3 and *Esrrb*, we first checked whether these two protein factors could

indeed interact. Coimmunoprecipitation (co-IP) experiments were performed in COS-1 cells coexpressing Ncoa3 and Flag-*Esrrb*, readily demonstrating an Ncoa3–*Esrrb* interaction (Fig. 2E). As expected, this interaction was mediated via the *Esrrb* AF-2 region, as AF-2 mutants failed to bind to Ncoa3 (Supplemental Fig. S2F). Co-IPs were next performed using ESC extracts and, importantly, confirmed that endogenous Ncoa3 and *Esrrb* proteins interact in ESCs (Supplemental Fig. S2G). Moreover, Ncoa3 binding to *Esrrb* was abrogated in the presence of DES (Fig. 2F), a result that reflects the decreased self-renewal ability of DES-treated ESCs (Supplemental Fig. S1). To test the requirement for Ncoa3 on *Esrrb*-dependent enhancers, ESCs were cotransfected with a pool of siRNAs targeting *Ncoa3* together with *Esrrb*, *Klf4*, *Nanog*, and *Sox2* luciferase vectors. *Ncoa3* loss closely phenocopied the suppressive effect of *Esrrb* depletion itself (Fig. 2G; Supplemental Fig. S2H). Furthermore, restoring *Esrrb* expression in depleted ESCs failed to rescue luciferase reporter activity in the absence of *Ncoa3*

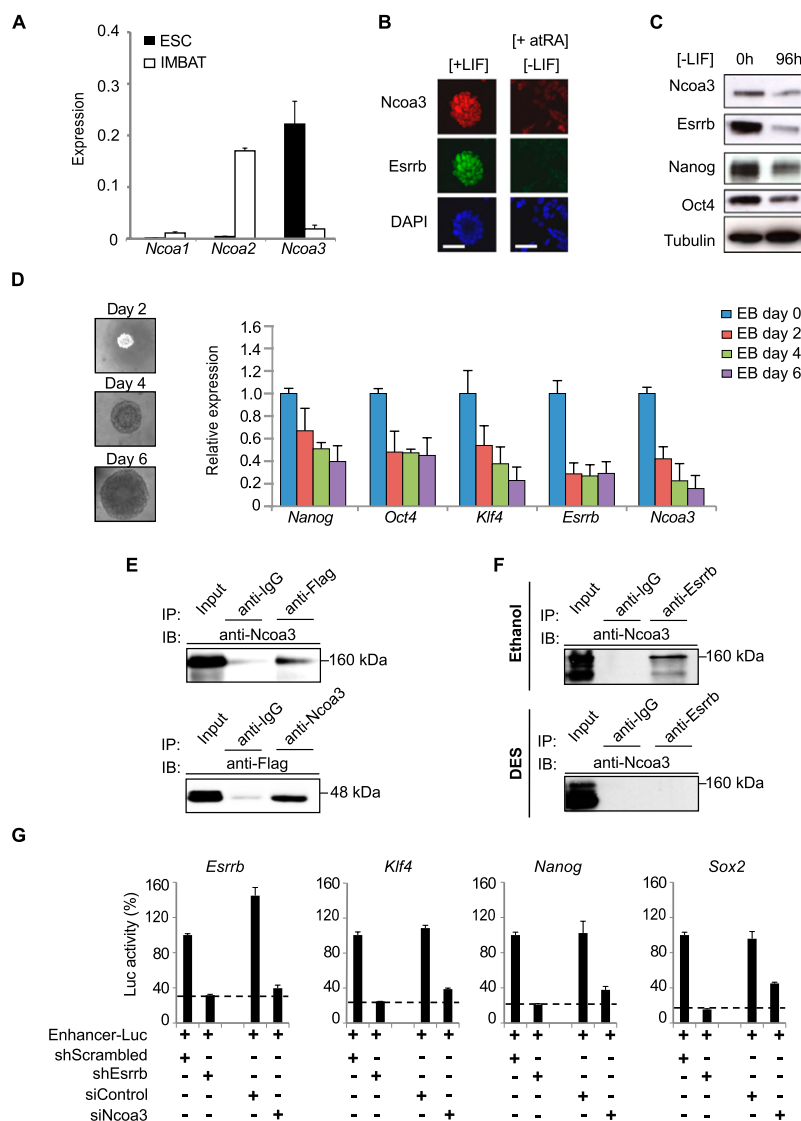


Figure 2. Ncoa3 acts as *Esrrb* coactivator in ESCs. (A) Expression profiling of *Ncoa1*, *Ncoa2*, and *Ncoa3* transcripts in ESCs as assessed by qRT-PCR and normalized to two housekeeping genes. Expression levels in immortalized brown adipose tissue (IMBAT) cells are shown as a control. Data are the mean \pm SEM of three biological replicates. (B) Immunofluorescence staining of ESCs maintained in proliferative conditions or induced to differentiate via addition of 1 μ M retinoic acid (atRA) and removal of LIF for 96 h. Bars, 50 μ m. (C) Western blotting showing Ncoa3 down-regulation along with *Esrrb*, *Nanog*, and *Oct4* upon LIF withdrawal. Tubulin is shown as a loading control. (D) Relative expression levels of *Ncoa3* and other pluripotency factors during EB-mediated differentiation. Data are normalized to housekeeping genes and expressed relative to EB day 0 as the mean \pm SEM of three independent experiments. (E) Co-IP experiments performed in COS-1 cells cotransfected with Flag-*Esrrb* and Ncoa3. (F) Interaction of endogenous *Esrrb* and Ncoa3 proteins in ESC extracts following 24 h of treatment with ethanol or 12.5 μ M DES. At least two experiments were performed with similar results. (G) Loss of ESC-specific luciferase reporter activity 48 h after depletion of endogenous *Esrrb* or *Ncoa3* in ESCs. Data are the mean \pm SEM of three transfections. Three experiments were performed with similar results.

(Supplemental Fig. S2I), indicating that Nco3 is indeed essential for the activation of these ESC-specific enhancers by Esrrb. We next demonstrated that Nco3 is recruited by Esrrb to genomic fragments containing ERREs using DNA pull-down experiments with biotinylated wild-type or ERRE mutated probes incubated together with Nco3 and Flag-Esrrb (Fig. 3A). These proteins both readily purified with *Esrrb*, *Klf4*, *Sox2*, and *Nanog* DNA fragments. Furthermore, point mutations in ERRE motifs that abrogated Esrrb binding also reduced Nco3 detection (Fig. 3A), confirming that Nco3 associates via Esrrb at ERREs in the vicinity of target genes. Similarly, Nco3 and Esrrb were corecruited to endogenous *Esrrb*, *Klf4*, *Nanog*, and *Sox2* target genes in ESCs, as assessed by ChIP and quantitative PCR (qPCR) (Fig. 3B). Nco3 recruitment was also verified here to be Esrrb-dependent, as transient depletion of Esrrb protein reduced Nco3 enrichment at these sites (Fig. 3C). Importantly, both Nco3 and Oct4 protein levels remained unaltered at this time point (Fig. 3D), excluding the possibility that the loss of Nco3 binding is due to ESC differentiation. Given the co-occupancy of many ERRE-containing enhancers with the core pluripotency factors (Chen et al. 2008), we next examined the Oct4 and Nanog dependency of Nco3–Esrrb recruitment. As previously reported for the *Nanog* locus (van den Berg et al. 2008), removing Oct4 protein prevented the detection of Esrrb at all four ESC candidate loci, and this was strictly mirrored by a loss of Nco3 recruitment (Supple-

mental Fig. S3A,B), a trend also observed to a lesser extent upon conditional *Nanog* deletion (Supplemental Fig. S3C,D). However, while Nco3 and Esrrb readily interact (Fig. 2; Supplemental Fig. S2), we could find no evidence for an interaction between Nco3 and Oct4/Nanog themselves (Supplemental Fig. S3E–H), thus highlighting the specificity of the Nco3–Esrrb association at these sites. In line with this, DNA pull-down assays further demonstrated that although Oct4 and Sox2 bind to an Oct–Sox site also contained within the *Nanog* probe, they were unable alone to recruit Nco3 (Fig. 3E). Collectively, these results identify Nco3 as an important Esrrb coactivator in ESCs and point toward a critical role for the Nco3–Esrrb partnership in regulating pluripotency.

Nco3 contributes to the maintenance of pluripotency in ESCs

To explore the functional requirement for Nco3 in ESCs, we next knocked down *Nco3* using two independent puromycin-selectable shRNA vectors. The extent of the knockdown was validated by qRT–PCR (Fig. 4A) and Western blot (Fig. 4B) and, strikingly, resulted in a clear and rapid loss of ESC characteristics. By day 4 post-selection, colonies displayed a flattened or spread morphology and stained negative for AP (Fig. 4C), and no stable *Nco3* knockdown ESC clones could be established. *Nco3* depletion was promptly accompanied by a decrease

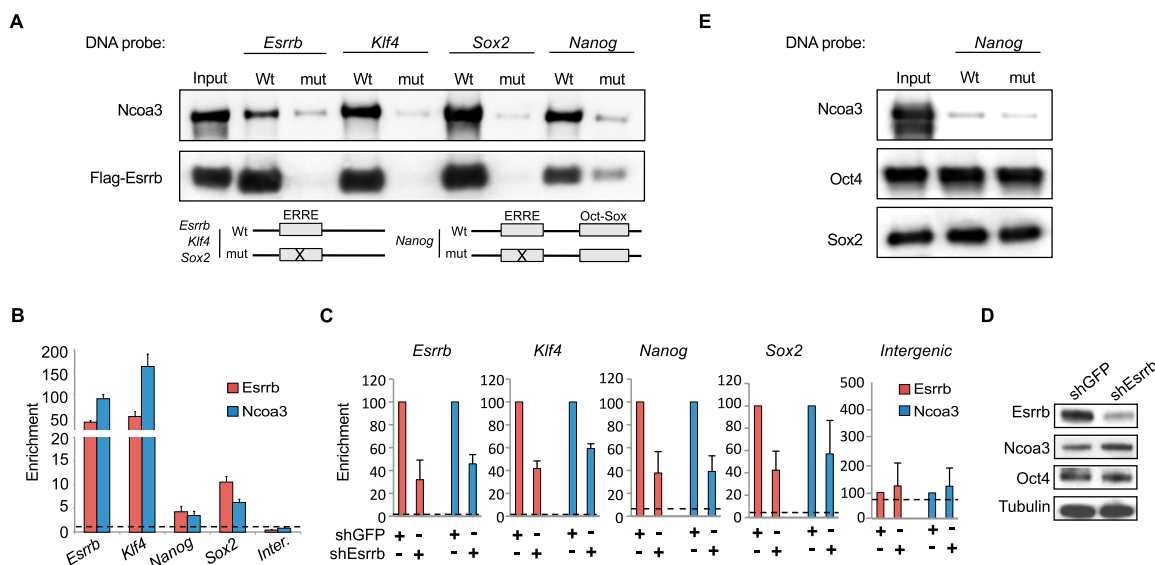


Figure 3. Nco3 is recruited via Esrrb to ERREs at target loci. (A) DNA pull-down assays using previously described wild-type (Wt) or ERRE mutated sequences from *Esrrb*, *Klf4*, *Sox2* (Feng et al. 2009), or *Nanog* (van den Berg et al. 2008). Biotinylated probes (40–50 base pairs [bp]) were incubated with extracts from COS-1 cells transfected with Flag-Esrrb and Nco3 and recovered on streptavidin beads, and DNA-associated proteins were visualized by Western blotting. (B) Esrrb and Nco3 enrichment levels at *Esrrb*, *Klf4*, *Nanog*, and *Sox2* ERREs and an intergenic (Inter.) control region in ESCs as assessed by ChIP and qPCR and expressed relative to input. Data are the mean \pm SEM of three biological replicates. (C) Nco3 and Esrrb enrichment levels at candidate loci following *Esrrb* depletion in ESCs, expressed relative to input. Enrichment for shGFP transfected cells is set at 100% in each individual experiment. Data are the mean \pm SD of at least two independent experiments. Dotted lines in B and C indicate background enrichment by control IgGs (Santa Cruz Biotechnology). (D) Western blotting showing specific Esrrb protein depletion 48 h after transfection with shEsrrb. Note that Nco3 and Oct4 levels are unchanged at this time. (E) DNA pull-down assays using *Nanog* wild-type or ERRE mutated probe, which also contains a neighboring Oct–Sox site (van den Berg et al. 2008), and cell extracts from COS-1 transfected with Nco3, Oct4, and Sox2.

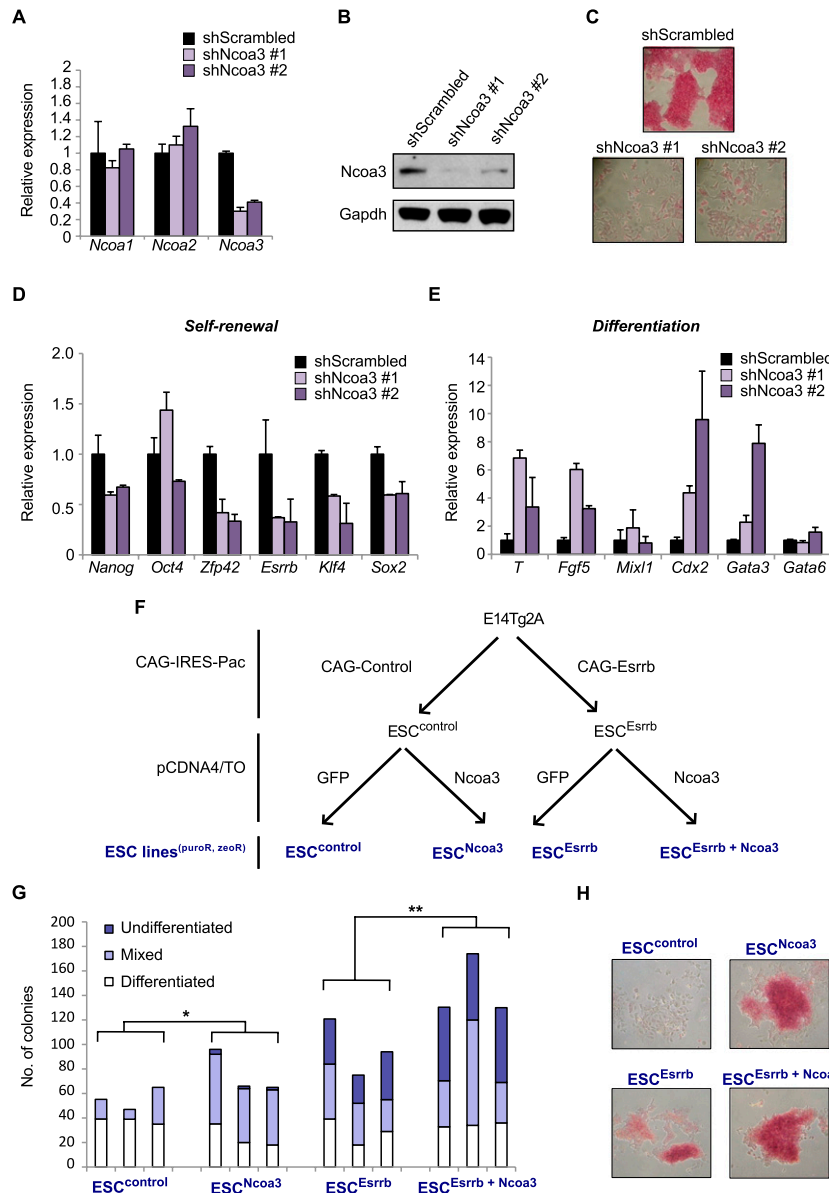


Figure 4. Ncoa3 plays critical roles in the maintenance of ESC self-renewal. (A,B) Specific knockdown of *Ncoa3* in ESCs. ESCs were transfected with two independent puromycin-selectable shRNA vectors targeting *Ncoa3*, and, 48 h after transfection, *Ncoa3* expression was quantified by qRT-PCR (A) and Western blotting (B). (C) AP staining following *Ncoa3* knockdown. ESCs were transfected with shScrambled or shNcoa3 vectors, and, 24 h later, puromycin was added. Selection was maintained for 4 d, then cells were fixed and stained for AP. (D,E) Quantitative gene expression analysis of cells 4 d post-selection for *Ncoa3* depletion. Data are the mean \pm SD of at least two independent experiments. (F) Generation of stable ESC lines overexpressing combinations of *Esrrb* and *Ncoa3*. E14Tg2A (E14) ESCs were first transfected with linearized CAG-Control or CAG-Esrrb vectors and selected with puromycin. Pooled clones for each stable cell line were then transfected with linearized control vector, pCDNA4/TO-GFP, or pCDNA4/TO-Ncoa3, and overexpressing cells were selected using Zeocin. (G) ESC lines generated in F were plated at low density and cultured for 5 d without LIF. Colonies were fixed and stained for AP; shown are three independent experiments. The statistical significance of the difference in undifferentiated colonies between the indicated cell lines was calculated using Student's *t*-test; (*) *P*-value < 0.05, (**) *P*-value < 0.01. (H) Representative colony morphology for each cell line from G.

in self-renewal gene expression (Fig. 4D) and concomitant derepression of lineage-associated markers (Fig. 4E), supporting an effect of *Ncoa3* on differentiation. Conversely, we asked whether, through the potentiation of *Esrrb* activity, elevated levels of *Ncoa3* could enhance resistance to differentiation in ESCs. For this, we established a series of stable ESC lines that overexpress either *Esrrb* (ESC^{Esrrb}), *Ncoa3* (ESC^{Ncoa3}), or both factors together (ESC^{Esrrb+Ncoa3}) alongside control cells (ESC^{control}) through two sequential rounds of transfection and selection, as depicted in Figure 4F. To compare the behavior of these lines, cells were plated at low density and cultured minus LIF for 5 d as previously performed (Fig. 1). The ESC^{Esrrb+Ncoa3} line showed a significant increase in the number of undifferentiated colonies relative to ESC^{Esrrb} cells (Fig. 4G), demonstrating the additive effect conferred by overexpressing *Esrrb* and *Ncoa3* together. Interestingly, a consistent

increase in the number of undifferentiated and mixed colonies was also observed in ESC^{Ncoa3} as compared with control cells (Fig. 4G,H), suggesting that overexpressing *Ncoa3* alone could support the self-renewal capacity of ESCs. This was confirmed in ESCs stably expressing *Ncoa3* using an alternative (pPyCAGIP) episomal vector (Supplemental Fig. S4A; Chambers et al. 2003). These cells similarly exhibited lower levels of differentiation when cultured in the absence of LIF as well as under self-renewing conditions—an effect associated with increased expression of *Esrrb* targets *Esrrb* and *Klf4* (Supplemental Fig. S4B; Feng et al. 2009). Following several passages, however, *Ncoa3*-overexpressing ESCs cultured minus LIF eventually differentiated due to the down-regulation of endogenous *Esrrb* (data not shown). Furthermore, these cells were capable of EB-mediated differentiation (Supplemental Fig. S4C). Collectively, these results show that

Ncoa3 plays critical roles in maintaining an intact ESC state and a self-renewal phenotype but, consistent with its role as a coactivator, is insufficient alone to maintain *Esrrb* levels and block differentiation.

Ncoa3 is required for somatic cell reprogramming

Interestingly, *Ncoa3* was found to be up-regulated during iPSC generation alongside *Esrrb* and *Nanog* (Supplemental Fig. S5A; Mikkelsen et al. 2008), suggesting that *Ncoa3* may also be important for the induction of pluripotency by reprogramming. Detection of high levels of *Ncoa3* protein was confirmed in two independently derived iPSC clones (Fig. 5A) from Oct4GFP MEFs. These MEFs carry a GFP transgene downstream from the *Oct4* distal enhancer, which becomes selectively activated during successful somatic cell reprogramming (Fig. 5B; Supplemental Fig. S5B; Yeom et al. 1996). To test the functional importance of *Ncoa3* in this process, we specifically depleted *Ncoa3* in Oct4GFP MEFs by RNAi (Fig. 5C; Supplemental Fig. S5C) prior to transduction with Oct4, Sox2, Klf4, and cMyc (OSKM) reprogramming retroviruses (Supplemental Fig. S5D). Both scrambled and *Ncoa3* knockdown MEFs showed no difference in cell proliferation (Supplemental Fig. S5E) or apoptosis (Supplemental Fig. S5F) at the time of reprogramming and infected equally highly with retroviruses (Fig. 5D). In contrast, *Ncoa3* depletion induced a

drastic reduction in reprogramming efficiency, as judged by the significantly lower number of GFP-positive (Fig. 5E–F) and AP-positive (Fig. 5G) colonies generated. We next asked whether *Ncoa3* knockdown might inhibit reprogramming simply by inducing MEF senescence, a known impediment of reprogramming. For this, experiments were repeated in MEFs lacking *p53*, which allows enhanced speed and efficiency of reprogramming without cMyc (Banito et al. 2009; Kawamura et al. 2009; Marion et al. 2009). *p53*^{−/−} MEFs transduced with OSK, however, showed similarly striking reductions in the number of AP-positive colonies following *Ncoa3* depletion (Fig. 5H), indicating that the observed reprogramming defect was not due to p53-mediated senescence. Taken together, these findings demonstrate that as well as being critical for the maintenance of ESCs, *Ncoa3* is furthermore required for the induction of pluripotency upon reprogramming.

Ncoa3 participates genome-wide with Esrrb and OSN at active ESC-specific enhancers

To gain greater understanding of how *Ncoa3* functions in ESCs, *Ncoa3* DNA-binding sites were next mapped by ChIP-seq. We first verified that *Ncoa3* (this study) and *Esrrb* (Chen et al. 2008) data sets showed strong overlap at *Esrrb*, *Klf4*, *Nanog*, and *Sox2* target genes and local binding profiles (Supplemental Fig. S6A). Focusing on ESC-

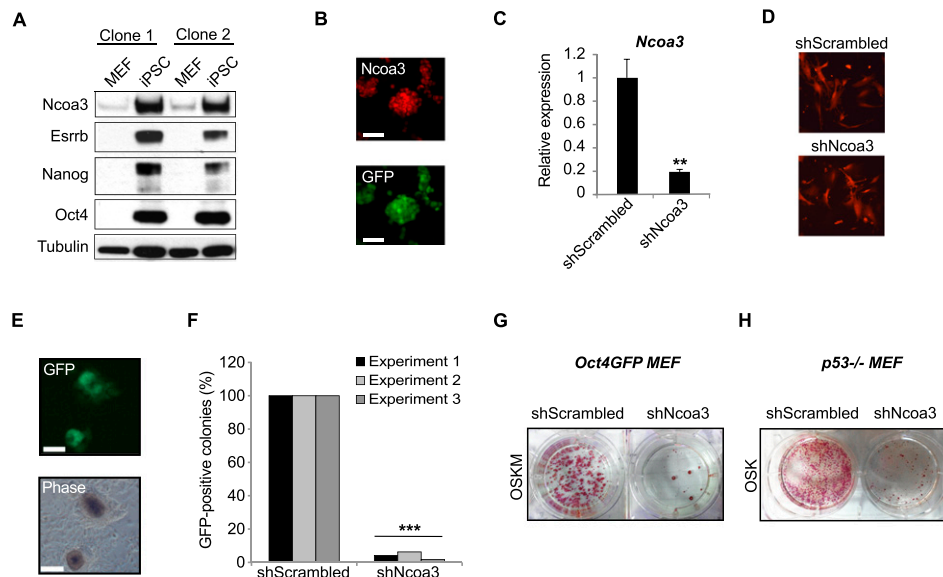


Figure 5. Essential requirement for *Ncoa3* in somatic cell reprogramming. (A) *Ncoa3* protein is up-regulated in fully reprogrammed iPSC lines. Western blotting depicts two independently generated iPSC clones from two different Oct4GFP genetic backgrounds (clone 1: 129/Ola; clone 2: MF1/B6) (Yeom et al. 1996) in comparison with uninfected proliferative MEFs. (B) Immunofluorescence confirming *Ncoa3* and GFP expression in iPSC clone 1. Bars, 50 μ m. (C) Efficient *Ncoa3* knockdown in MEFs analyzed 3 d after infection with lentiviral particles expressing shScrambled or sh*Ncoa3* shRNA. Data are the mean \pm SEM of three independent experiments. (D) Equal retroviral mCherry infection of scrambled or *Ncoa3* knockdown MEFs. (E) Typical GFP-positive colonies arising from transduction of shScrambled-treated Oct4GFP MEFs with OSKM reprogramming factors. Bars, 100 μ m. (F) Inhibition of reprogramming following depletion of *Ncoa3*, analyzed by quantifying GFP-positive colonies 12–14 d after infection with OSKM vectors. The number of colonies produced from shScrambled-infected MEFs is set as 100% for each individual experiment. Three experiments were performed using different batches of MEFs and viruses. (**) *P*-value < 0.01, (***) *P*-value < 0.001, Student's *t*-test. (G) AP staining of iPSC colonies 14 d after infection with OSKM reprogramming factors. (H) AP staining of iPSC colonies generated in *p53*^{−/−} shScrambled or sh*Ncoa3* MEFs using OSK reprogramming factors.

relevant loci, we next compared all Ncoa3–Oct4–Sox2 cobound sites with *Esrrb*–Oct4–Sox2 and found significant overlaps between these groups (P -value = 1.11×10^{-16}) (Fig. 6A). In contrast, the insulator-binding transcription factor CTCF did not show any significant overlap (P -value = 0.84). Using a de novo motif discovery

algorithm (Heng et al. 2010), we identified, as expected, a canonical ERRE motif among significantly overrepresented binding sequences found at Ncoa3 peaks (Fig. 6B; Supplemental Fig. S7). Oct–Sox elements were also detected at these sites, reflecting the frequent colocalization of *Esrrb* with Oct4 and Sox2. GREAT gene ontology

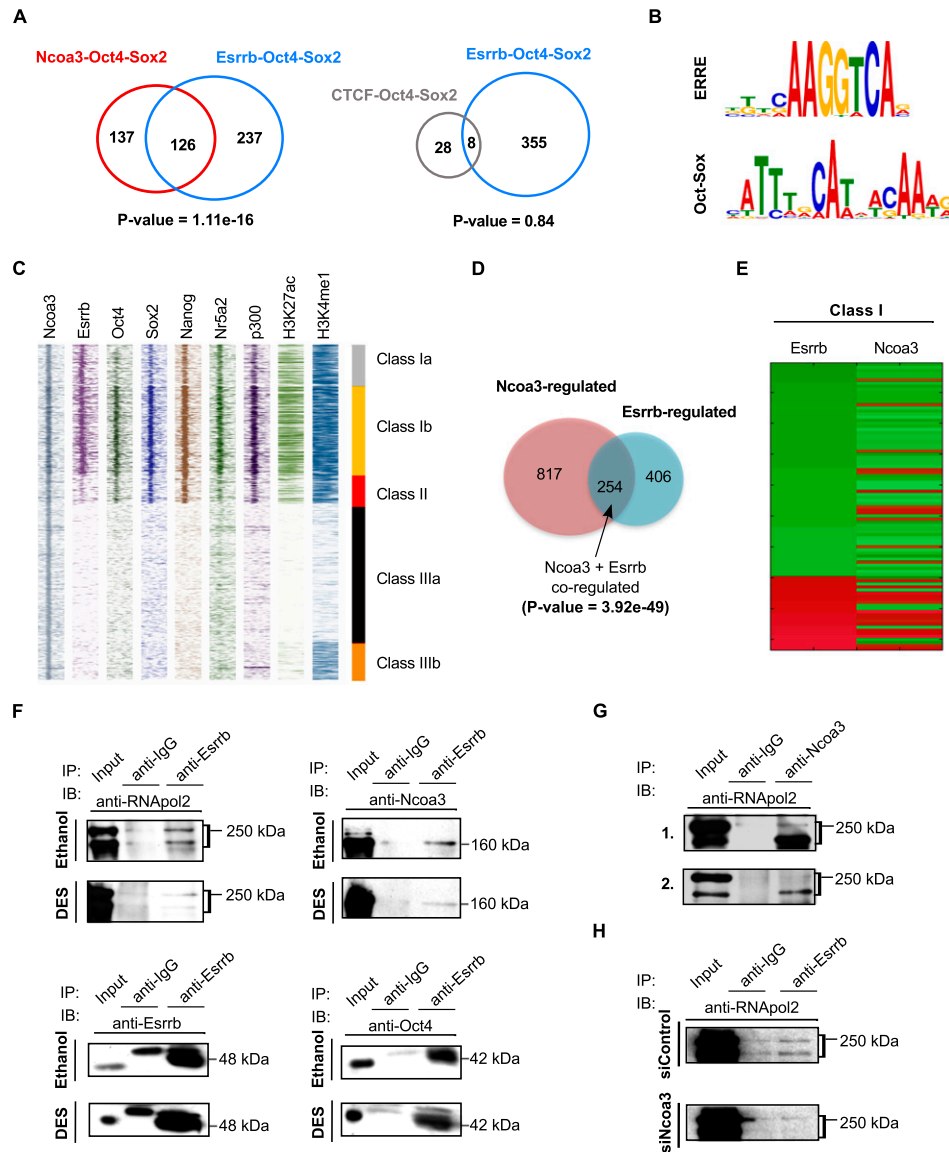


Figure 6. Ncoa3 is an integrated component of the ESC transcriptional network. (A) Significant overlap between *Esrrb* and Ncoa3 peaks within 200 bp at Oct4–Sox2-bound sites. The intersection with CTCF is shown for comparison. (B) MEME de novo motif discovery analysis of top Ncoa3-bound sequences. (C) Spatial heat map depicting binding peak intensity of indicated factors, centered on the peak of maximum Ncoa3 binding for each site. The two-dimensional matrix was provided as input for the K-mean clustering program in Matlab to determine the classes of each position. (D) Venn diagram showing the overlap of genes that are significantly regulated by Ncoa3 and *Esrrb*, as determined by microarray following *Ncoa3* (this study) and *Esrrb* (Feng et al. 2009) knockdown in ESCs (fold change >1.5). The Fisher's exact test P -value for the correlation of fold change is indicated. See also Supplemental Table 5. (E) Heat map of class I genes bound by Ncoa3 and *Esrrb*. First, all of the genes regulated by *Esrrb* within 100 kb of the class I region were taken, then their fold change upon *Esrrb* (left) or *Ncoa3* (right) knockdown was plotted according to *Esrrb* knockdown. Shades of green and red indicate down-regulation and up-regulation, respectively. Note that the majority of genes is down-regulated. (F) Co-IP of *Esrrb* in ESCs treated for 24 h with ethanol or 12.5 μ M DES, immunoblotted for the indicated proteins. (G) Co-IP in ESCs showing the interaction of endogenous Ncoa3 and RNApol2. Two representative blots are shown. (H) Co-IP of *Esrrb* immunoblotted for RNApol2 in ESCs following 48 h of transfection with siControl or siNcoa3 oligos. The square brackets in F–H indicate bands corresponding to RNApol2. All co-IPs were performed at least twice with similar results.

(GO) analysis at Ncoa3–Esrrb cobound sites revealed significant enrichment for developmental processes and embryonic phenotypes (Supplemental Fig. S6B). In addition, abnormal fertility and organ size were also enriched, agreeing with published mouse knockout models for *Ncoa3* and/or *Esrrb* (Luo et al. 1997; Mitsunaga et al. 2004; Chen et al. 2010). Taken together, these data suggest a high degree of similarity between Ncoa3 and Esrrb transcriptional networks in ESCs. To look more widely at the different types of transcription factor clusters associated with Ncoa3 peaks, we next used spatial heat map analysis (Fig. 6C). This distinguished three main classes of Ncoa3 targets based on their co-occupancies by Esrrb and/or OSN as well as Nr5a2, p300, H3K27ac, and H3K4me1. Here we confirmed that ~40% of all Ncoa3 peaks colocalized with Esrrb at active enhancers, as denoted by the presence of p300, H3K27ac, and H3K4me1 (Fig. 6C; Heintzman et al. 2007; Creyghton et al. 2010). Sites bound by both Esrrb/Ncoa3 and OSN (class Ib) represented key self-renewal regulators that are actively transcribed in ESCs, such as *Esrrb*, *Klf2/4/5*, *Zfp42*, *Tcfcp2l1*, *Oct4*, and *Sox2*, and included GO processes involving stem cells and blastocyst development (Supplemental Table S1). In contrast, sites containing Esrrb and Ncoa3 but with reduced binding for OSN (class Ia) (Fig. 6C) were uniquely associated with developmental phenotypes involving germ cell integrity and fertility (Supplemental Table S2), including *Prdm14*, a recently described factor critical for germ cell lineage (Yamaji et al. 2008; Gillich et al. 2012). To closely investigate transcriptional regulation by Ncoa3 and Esrrb, microarray analysis was performed on ESCs following *Ncoa3* knockdown and compared with *Esrrb* knockdown at the same time point (Feng et al. 2009). Notably, 62.5% of the misregulated genes in *Esrrb*-depleted ESCs were also misregulated in *Ncoa3* knockdown ESCs (Fig. 6D), most often in the same direction (Fig. 6E), showing that gene regulation by Ncoa3 and Esrrb is significantly correlated (P -value = 3.92×10^{-49}). Among them, many target genes with (class Ib) or without (class Ia) high OSN enrichment were significantly down-regulated upon *Ncoa3* depletion, as validated by qRT–PCR (Supplemental Fig. S8A), confirming that Ncoa3 is strictly required for Esrrb-dependent gene activation. Interestingly, at some sites, Ncoa3 also colocalized with Nr5a2—another orphan nuclear receptor previously involved in stem cells and reprogramming (Gu et al. 2005; Heng et al. 2010)—together with (class I) and without (class II) Esrrb and/or OSN (Fig. 6C). GO analysis of class II genes, however, consisted of targets associated with extra-embryonic development (Supplemental Table S3), indicating that although Nr5a2 could potentially recruit Ncoa3 to a set of genes, these targets may be less relevant in ESCs. Finally, the last class identified (class III) (Supplemental Table S4) included Ncoa3-bound sites that were not co-occupied by Esrrb, Nr5a2, or OSN (Fig. 6C), suggesting that Ncoa3 may also be recruited by other factors. While marks of active enhancers—p300, H3K27ac, and H3K4me1—were absent at the majority of these sites (class IIIa), a small subset (class IIIb) colocalized with H3K4me1 alone (Fig. 6C), potentially delineating inactive/poised Ncoa3-bound

enhancers in ESCs (Creyghton et al. 2010). Interestingly, microarray analysis of class IIIb revealed that out of the genes that are misregulated upon *Ncoa3* knockdown, all were up-regulated (Supplemental Fig. S8B).

Ncoa3 bridges Esrrb to the general transcription machinery

Recently, Esrrb, among other pluripotency factors, was identified as being uniquely associated with RNAPol2 complexes (van den Berg et al. 2010), suggesting a critical function for Esrrb, distinct from OSN, in activating transcription. Thus, we investigated the importance of the Esrrb AF-2 domain and Ncoa3 for this function. We first demonstrated by co-IP that RNAPol2 does indeed interact with endogenous Esrrb in ESCs (Fig. 6F, top left). Strikingly, however, upon short treatment with DES, we observed a reduction of the Esrrb–RNAPol2 interaction (Fig. 6F, top left). This appeared to be specifically associated with loss of Ncoa3 binding (Fig. 6F, top right), as neither the interaction between Esrrb and Oct4 (van den Berg et al. 2008) nor the efficiency of anti-Esrrb immunoprecipitation itself was affected (Fig. 6F, bottom). These results suggested that Ncoa3 could mediate the interaction between Esrrb and RNAPol2. To test this further, we performed co-IPs between RNAPol2 and Ncoa3 itself and demonstrated an interaction between these proteins in ESCs (Fig. 6G). Finally, to confirm that Ncoa3 is required for the association between Esrrb and RNAPol2, co-IPs were repeated in cells treated with siControl or siNcoa3 oligos to deplete endogenous Ncoa3 protein. Transfection with siNcoa3 specifically reduced the interaction between endogenous Esrrb and RNAPol2 (Fig. 6H; Supplemental Fig. S8C–E). Taken together, these findings reveal Ncoa3 as a key limiting factor that bridges Esrrb to the general transcription machinery in ESCs.

Discussion

Here, we present the first detailed characterization of a nuclear receptor coactivator in the maintenance of ESC pluripotency. Although it is not a tissue-specific factor, we observed that elevated Ncoa3 expression is a defining property and essential requirement of ESCs and iPSCs. Numerous transcription factors have been well characterized in this context. In contrast, there exist few matching studies for critical coactivators. Our work notably complements recent findings by Fong et al. (2011) identifying a novel Oct4/Sox2 coactivator, the XPC nucleotide excision repair complex, which is essential to activate *Nanog* and support the ESC state while also safeguarding genome integrity.

In this study, we provide significant data to suggest that Ncoa3 action in ESCs is primarily mediated through Esrrb function. We show that Ncoa3 binds to Esrrb specifically via its AF-2 domain and that Ncoa3 recruitment is strictly required to potentiate Esrrb activity in ESCs. AF-2 domain inactivation in ESCs or depletion of *Ncoa3* itself both suppress transcription from Esrrb-dependent gene reporters and result in a loss of ESC self-renewal. Conversely,

overexpressing *Ncoa3* in ESCs enhances Esrrb-mediated resistance to differentiation but is not sufficient alone to block differentiation, in agreement with *Ncoa3* acting as an Esrrb coactivator and not a transcription factor. Through genome-wide ChIP-seq analysis, we demonstrate the presence of *Ncoa3* at a large number of active Esrrb-bound enhancers that are shared with the OSN triad and enriched for p300, H3K27ac, and H3K4me1 (Heintzman et al. 2007; Creighton et al. 2010). GO analysis at this set of targets shows enrichment for key self-renewal regulators whose expression is significantly reduced in *Ncoa3* knockdown ESCs, as determined by microarray and qRT-PCR, identifying *Ncoa3* as an integrated component of the ESC transcriptional network.

A distinct group of *Ncoa3*- and Esrrb-bound loci showing reduced OSN occupancies is exclusively associated with germ cell and fertility-related phenotypes. This corroborates with a primordial germ cell (PGC)-specific defect observed in vivo in the absence of *Esrrb* (Mitsunaga et al. 2004) and an infertility phenotype in *Ncoa3* knock-out mice (Xu et al. 2000). It furthermore suggests that Esrrb and *Ncoa3* might also act in synergy during PGC reprogramming. Consistently, we found that germ cell-relevant targets such as *Prdm14* are significantly down-regulated upon *Ncoa3* knockdown (Supplemental Fig. S8A), supporting a putative role for the *Ncoa3*–Esrrb partnership in this process. Aside from Esrrb, it is still possible that other nuclear receptors or transcription factors may also recruit *Ncoa3* to some genes. Nr5a2, for example, maps in ESCs to many Esrrb-, *Ncoa3*-, and OSN-bound targets (Heng et al. 2010; this study) and could potentially interact with *Ncoa3* via its LBD, although this remains to be formally demonstrated. In contrast, we could find no evidence to support a direct role for OSN in recruiting *Ncoa3* at these sites, although these core factors most likely stabilize the Esrrb–*Ncoa3* complex through protein–protein interactions with Esrrb at multitranscription factor clusters (Supplemental Fig. S3; van den Berg et al. 2008).

Critically, we shed new light on how the *Ncoa3*–Esrrb self-renewal function is conferred in ESCs. A recent study reported the Oct4 interactome of 166 proteins, including many transcription factors and chromatin-modifying complexes not previously known to associate with the ESC network (van den Berg et al. 2010). Among these Oct4 partners, Esrrb was uniquely found to be associated with the basal transcription machinery. An association between *Ncoa3* and Esrrb was also observed there. Our mechanistic work on *Ncoa3* thus elucidates the functional importance of this interaction and further highlights the relevance of Oct4's broad range of interaction partners. Here, we confirm that endogenous Esrrb and RNAPol2 are physically associated in ESCs and demonstrate that *Ncoa3* mediates this association. We propose that *Ncoa3* functionally bridges Esrrb and RNAPol2 at loci co-occupied by Oct4 as well as Sox2, Nanog, and additional pluripotency factors (see Fig. 7), highlighting a distinct role for the *Ncoa3*–Esrrb partnership to the core ESC transcription factors. *Ncoa3* is also established in this study as being required for efficient somatic cell

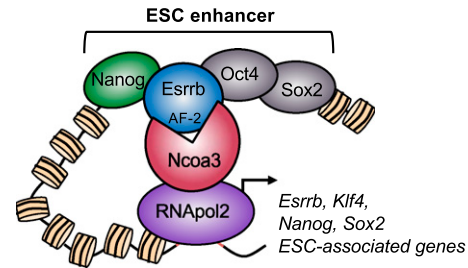


Figure 7. Proposed model. Esrrb interacts with Nanog and Oct4/Sox2 at ESC enhancers and recruits *Ncoa3* via its AF-2 region. *Ncoa3* interacts with RNAPol2 and bridges Esrrb at enhancers to the general transcriptional machinery. These factors would thus act, together with other factors such as chromatin proteins and Mediator (Kagey et al. 2010), to bring about transcription of ESC-associated genes.

reprogramming—a property shared with other Oct4/Esrrb partners, including core members of Trithorax/MLL complexes (Ang et al. 2011). These chromatin-modifying complexes are essential for open chromatin and pluripotency and may therefore open the path to the reprogramming process by facilitating Oct4 occupancy at genomic loci encoding self-renewal genes. Esrrb–*Ncoa3*–RNAPol2 bridging would in turn perpetuate strong transcriptional activation at Oct4/Esrrb-bound sites, allowing for the full establishment of iPSCs or the maintenance of ESC self-renewal.

In summary, our work elucidates a previously unrecognized functional link between the core ESC transcriptional network and a nuclear receptor coactivator and, importantly, provides novel mechanistic insights into the role of *Ncoa3*–Esrrb in sustaining both ESC self-renewal and reprogramming. It would be of interest in future studies to elucidate whether coactivator recruitment is indeed a general requirement for many ESC pluripotency factors. Moreover, acting as “sensors,” these cofactors could also be capable of integrating stimuli from multiple signaling pathways, thus adding another layer of control to the complex network governing pluripotency and differentiation.

Materials and methods

Cell culture and RNAi experiments

Mouse E14Tg2A (E14) ESCs were cultured on 0.1% gelatin as previously described (Azuara et al. 2006). COS-1 and 293T were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine. MEFs and iPSCs were cultured as previously described (Feng et al. 2009). RNAi experiments followed by puromycin selection were performed using pLKO.1 or pSuper vectors. Transfection of ESCs was performed with Lipofectamine 2000 according to the manufacturer's protocols. See Supplemental Table S6 for shRNA sequences.

Antibodies

The primary antibodies used in this study for immunofluorescence, Western blotting, and ChIP were as follows: anti-Esrrb (R&D Systems, H6705 and H6707), anti-Nanog (Cosmo,

RCAB0002P), anti-Ncoa3 (Santa Cruz Biotechnology, M-397; BD Biosciences, #611105), anti-Oct4 (Santa Cruz Biotechnology, C-10), anti-Sox2 (Abcam, ab97959), anti-Flag (Sigma, M2), anti-RNApol2 (Santa Cruz Biotechnology, N-20), anti-Gapdh (Millipore), anti-Tubulin (Sigma), anti-cleaved Caspase-3 (Cell Signaling, #9664), and anti-BrdU (Abcam, ab6326).

Co-IP and ChIP

For co-IP experiments performed with ectopically expressed proteins, COS-1 cells in 10-cm dishes were transfected with 3–5 μ g of each expression vector using Fugene HD (Roche), according to the manufacturer's guidelines. Cells were collected 48 h later and lysed in RIPA buffer. Eight hundred micrograms of protein extract was precleared with protein A Sepharose beads (Sigma, P3391) for 2 h at 4°C, then incubated overnight at 4°C with the indicated antibodies. Protein A beads were added for 2 h, then washed with RIPA and TSE buffer (2mM EDTA, 20 mM Tris at pH 8, 150 mM NaCl) three times and loaded on 7% acrylamide gels. Detection of endogenous interactions was performed using 1- μ g extracts similarly prepared from untransfected E14 ESCs. Anti-Ncoa3 (#611105; mouse monoclonal) was used to coimmunoprecipitate Ncoa3 expressed in COS-1 cells when examining interactions with Nanog (rabbit polyclonal); all other co-IP, immunofluorescence, and ChIP experiments were performed with M-397. ChIP experiments were carried out with chromatin prepared from E14 cells as previously described (Stock et al. 2007) using 8–10 μ g of primary antibody and 600 μ g of precleared chromatin per immunoprecipitation. Immunoprecipitated DNA fragments were purified and analyzed via SYBR Green qPCR.

DNA pull-down assays

COS-1 cells were transfected with Ncoa3 together with Flag-Esrrb or Oct4 and Sox2 expression vectors. Forty-eight hours after transfection, cells were lysed in HKMG buffer (10 mM HEPES at pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.5% NP-40, 1 mM DTT, protease inhibitors [Complete EDTA-free; Roche]) and lysates precleared for 1 h with pre-equilibrated streptavidin-coupled Dynabeads (Invitrogen, M-280). Pairs of complementary DNA oligonucleotides with the sense oligonucleotide biotinylated at the 5' end (Sigma) were annealed and incubated for 2 h at 4°C with precleared cell lysates. DNA-bound proteins were collected by incubation for 1 h at 4°C with streptavidin-coupled Dynabeads (Invitrogen), washed four times in HKMG buffer, and separated by SDS-PAGE. Ncoa3, Flag-Esrrb, Oct4, and Sox2 were detected by Western blot. See Supplemental Table S6 for probe sequences.

Luciferase assays

ESCs were plated on gelatin-coated 96-well plates at a density of 2×10^4 cells per well and, 5 h later, transfected with the indicated luciferase reporters and expression constructs using Lipofectamine 2000. For knockdown experiments, either 30 ng of shRNA vector or 2–3 pmol of siRNA was cotransfected per well. pCmv.Renilla or pCDNA4/TO.eGFP was used to normalize luciferase values in each well. Firefly and Renilla luciferase activities were measured 48 h post-transfection using the Steady-Lite Plus kit (PerkinElmer).

Retroviral production

For reprogramming experiments, PlatE cells were used to produce pMXs-based retroviruses containing the cDNA for *Pou5f1*/

Oct4, *Sox2*, *Klf4*, or *cMyc*, as previously described (Feng et al. 2009). PlatE-generated mCherry retrovirus was used to monitor MEF infection efficiency in each experiment.

iPSC generation

Embryonic day 13.5 (E13.5) Oct4-GFP MEFs were derived from embryos produced via the intercross between wild-type MF1 females and homozygous Oct4-GFP B6 males (Yeom et al. 1996). For iPSC induction, 2×10^4 Oct4-GFP MEFs were seeded per well of a 24-well plate and, 5 h later, infections were performed using equal amounts of each indicated retrovirus in the presence of 8 μ g/mL polybrene. Medium was exchanged for MEF medium 24 h post-infection, and, 48 h after infection, MEFs were collected and reseeded onto inactivated feeders in iPSC medium. Emerging iPSC colonies were monitored until day 12–14 when cells were harvested or individual colonies were picked for further analysis.

ChIP-seq and microarray analysis

ChIP-seq and microarray analysis were performed on mouse ESCs as previously described (Chen et al. 2008; Feng et al. 2009). Detection of Ncoa3 sequenced peaks was carried out using MACS with a *P*-value cut-off of 1.0×10^{-5} .

Accession numbers

ChIP-seq and microarray data are accessible at the Gene Expression Omnibus database under accession number GSE40193.

Detailed Materials and Methods, antibodies, and sequences are available in the Supplemental Material.

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