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Regulation of Embryonic and Induced Pluripotency by Aurora Kinase-p53 Signaling

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SUMMARY

Many signals must be integrated to maintain self-renewal and pluripotency in embryonic stem cells (ESCs) and to enable induced pluripotent stem cell (iPSC) reprogramming. However, the exact molecular regulatory mechanisms remain elusive. To unravel the essential internal and external signals required for sustaining the ESC state, we conducted a short hairpin (sh) RNA screen of 104 ESC-associated phosphoregulators. Depletion of one such molecule, aurora kinase A (Aurka), resulted in compromised self-renewal and consequent differentiation. By integrating global gene expression and computational analyses, we discovered that loss of Aurka leads to upregulated p53 activity that triggers ESC differentiation. Specifically, Aurka regulates pluripotency through phosphorylation-mediated inhibition of p53-directed ectodermal and mesodermal gene expression. Phosphorylation of p53 not only impairs p53-induced ESC differentiation but also p53-mediated suppression of iPSC reprogramming. Our studies demonstrate an essential role for Aurka-p53 signaling in the regulation of self-renewal, differentiation, and somatic cell reprogramming.

INTRODUCTION

Self-renewal and pluripotency of ESCs is maintained by the integration of multiple internal and external signaling pathways that converge on well-characterized transcription factors (TFs) and chromatin modifiers. The TFs Oct4, Sox2, Nanog, Esrrb, Tbx3, Tcl1, Foxo1, and Foxo3a play essential roles in controlling the ESC state by assembling a core regulatory network (Ivanova et al., 2006; Zhang et al., 2011b). Auto- and cross-regulatory network interactions maintain self-renewal by activating pluripotency genes and suppressing lineage determinant genes (Ivanova et al., 2006). Chromatin-remodeling complexes and other epigenetic modifiers have been shown to regulate self-renewal and differentiation by interacting with the core TF circuitry (Ang et al., 2011a, 2011b; Schaniel et al., 2009). In contrast to the intensive analyses of TFs and epigenetic regulators, only a few cell signaling pathways, such as Lif, BMP, and Wnt, have been shown to be important for mouse (m) ESC self-renewal (Sato et al., 2004; Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). It remains unclear whether other signaling molecules/pathways are required for ESC self-renewal and how the range of signaling events is integrated to sustain a transcriptional output that balances self-renewal and differentiation. In order to identify the complete range of signaling requirements in ESC fate control, we have applied a systematic shRNA loss-of-function screening strategy. We focused on protein kinases and phosphatases (PKases and PPases) because these phosphoregulators play central roles in signal transduction. Our results identify the Aurka-p53 signaling pathway as a critical cell fate regulator and establish an important link between a specific phosphorylation event and p53 function in the maintenance and reacquisition of pluripotency.

RESULTS

Loss-of-Function shRNA Screen Identifies Protein Kinases and Phosphatases Required for ESC Self-Renewal and Pluripotency

PKase- and PPase-mediated phosphorylation and dephosphorylation events control a wide range of biological processes. To

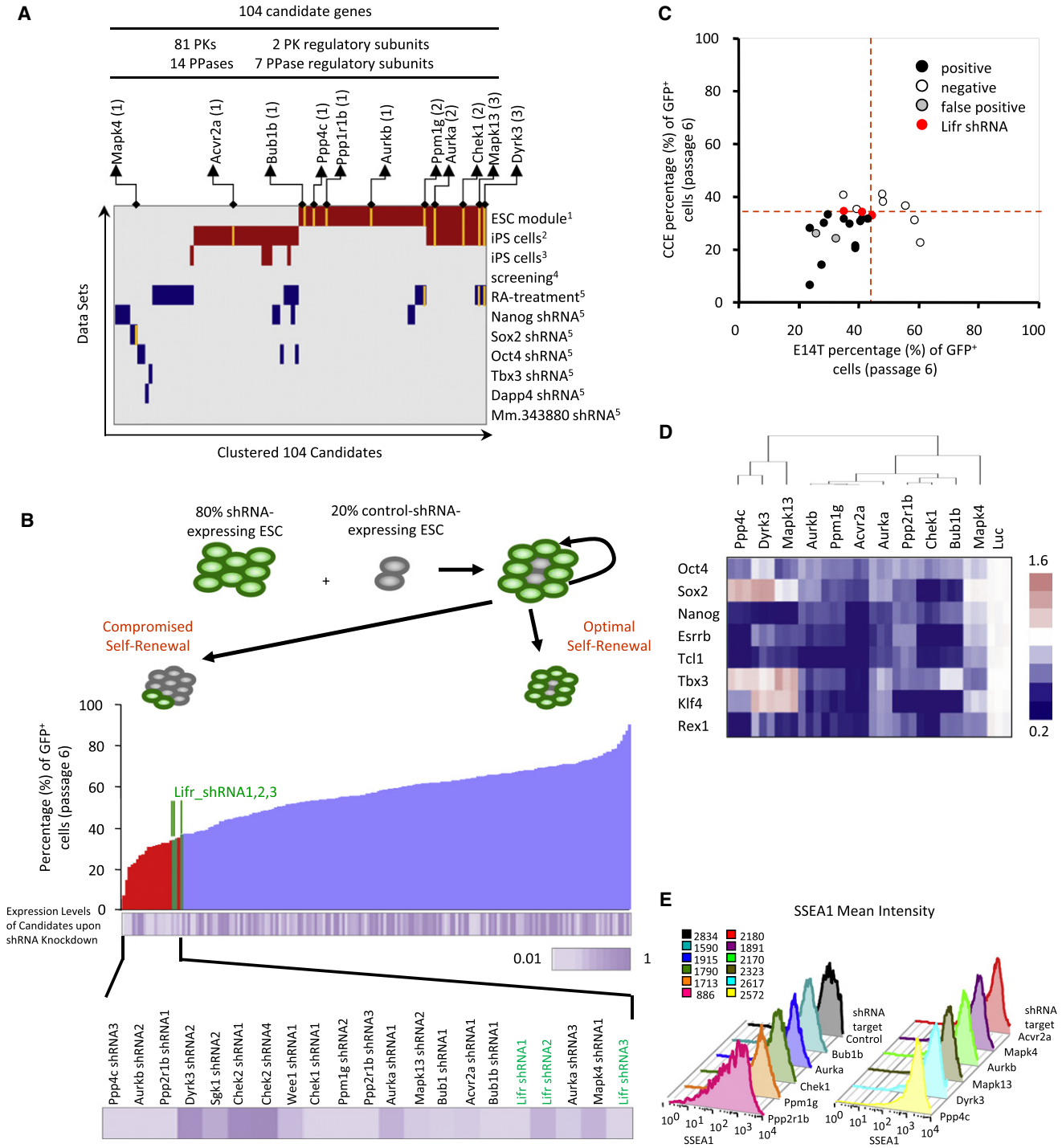

Figure 1. Identifying PKases and PPases Involved in ESC Self-Renewal

(A) Selection of 104 gene products for functional analyses. Heat map showing 104 candidates chosen based on several different criteria including high expression levels in ESCs, iPSCs, and cancer stem cells (red), downregulated expression upon RA treatment, and decreased expression upon the knockdown of ESC self-renewal-associated transcription factors (TFs) (blue). Genes with no identified effects in the individual published studies are shown in gray. The *x* axis represents the clustered 104 candidates identified in the individual studies. The *y* axis represents the individual data sets extracted from Wong et al. (2008); Mikkelsen et al. (2008); Takahashi and Yamanaka (2006); Pritsker et al. (2006), and Ivanova et al. (2006). The 11 molecules functionally identified in the initial stage of the screen are labeled in orange. The total numbers of times the gene products occur in the selection criteria studies are indicated in parentheses above their names.

(B) A competition strategy is used to identify the effects of PKase and PPase knockdowns on mESC self-renewal. A total of 181 shRNAs (1–4 per gene depending on their knockdown efficiencies) were designed targeting the 104 candidate genes. Knockdowns of 15 out of 104 genes show reduced propagation in comparison to knockdown of Lifr in CCE ESCs.

elucidate the phosphoregulatory proteins essential for mESC self-renewal, we selected 104 candidates (81 PKases, 14 PPases, 2 PKase regulatory subunits, and 7 PPase regulatory/structural subunits) (Table S1 available online). Selection criteria included enriched expression in pluripotent cells and/or downregulation after differentiation induced by exposure to retinoic acid (RA) or depletion of specific pluripotency TFs (Figure 1A; Ivanova et al., 2006; Mikkelsen et al., 2008; Pritsker et al., 2006; Takahashi and Yamanaka, 2006; Wong et al., 2008). A total of 181 shRNAs targeting these gene products were evaluated with a competition assay (Figure S1A; Ivanova et al., 2006; Lee et al., 2012). This fluorescence- and proliferation-based assay reveals compromised self-renewal by decreasing GFP^+/GFP^- ratios during coculture of GFP^+ shRNA-transduced CCE ESCs and GFP^- control cells. Downregulation of the Lif receptor (Lifr) was used to set an emperical threshold for impaired self-renewal. Depletion of 15 candidate gene products suggested compromised self-renewal to an extent greater than downregulation of Lifr (Figure 1B). To rule out cell line-specific effects, we utilized a second line (E14T) and confirmed decreased GFP^+/GFP^- ratios after depletion of 11 candidates (Figure 1C). These include Acvr2a, Aurka, Aurkb, Bub1b, Chek1, Dyrk3, Mapk4, Mapk13, Ppp4c, Ppm1g, and Ppp2r1b. Because shRNA-transduced cells are first expanded in puromycin, severe impairments to cell survival and proliferation would not be detected in the screen. Indeed, cells expanded after depletion of known apoptosis- and cell cycle-associated gene products have lower knockdown efficiencies (~70%) (Figure 1B). This suggests that mild depletion of such molecules is permissive for normal viability and proliferation. Apoptosis- and cell cycle-associated gene-product silencing has diverse effects in the competition assay (Figures S1B and S1C). This argues against the possibility that decreasing GFP^+/GFP^- ratios are due to compromised general cell cycle mechanisms or increased apoptosis. To confirm impaired self-renewal, we measured mRNAs encoding the essential pluripotency TFs Oct4, Sox2, Nanog, Esrrb, Tbx3, Tcl1, Klf4, and Rex1 as well as surface expression of SSEA1. Depletion of all 11 candidates led to reduced expression of pluripotency markers, directly demonstrating compromised self-renewal and pluripotency (Figures 1D, 1E, and S1D).

Aurka Is Enriched in Pluripotent and Embryonic Cells

Aurka was selected for in-depth analyses for several reasons. First, high Aurka mRNA levels are characteristic of embryonic tissues, oocytes, and fertilized eggs but not differentiated cells and tissues (Figure 2A). Second, analyses in conditional TF "rescue" (R) ESCs showed decreases in Aurka mRNA and protein upon differentiation triggered by removal of doxycycline (Dox) in Nanog_R and Sox2_R (Figures 2B and 2C) but not in control (Ctrl_R) (Figure 2D) cells. Downregulated Aurka expression was also detected in Oct4-repressible ESCs upon addition of Dox (Figure 2E). Furthermore, transient RNAi-mediated knockdowns of the pluripotency factors Nanog, Oct4, and Wdr5 confirm decreasing Aurka levels as ESCs lose their pluripotent state (Figure 2F). Third, decreasing Aurka mRNA and protein levels accompany embryoid body (EB) and RA-mediated differentiation (Figures 2G). Taken together, the correlations between Aurka expression and the undifferentiated state strongly suggest a role in ESC pluripotency.

Loss of Aurka Impairs Self-Renewal and Triggers Differentiation in ESCs

To exclude potential off-target effects, we used a complementation strategy where depletion of endogenous Aurka is "rescued" by a Dox-inducible shRNA-"immune" version (Figure 3A; Ang et al., 2011b; Ivanova et al., 2006; Lee et al., 2012). Lentiviral cassettes were transduced into reverse tetracycline transactivator (rtTA)-expressing ESCs (Ainv15) (Kyba et al., 2002). In Aurka rescue (Aurka_R) cells cultured for 5 days without Dox, low levels of Nanog, Oct4, Sox2, Esrrb, Tbx3, Tcl1, Klf4, and Rex1 mRNAs were observed relative to cultures with Dox (Figure 3B). We also observed reduced pluripotency TF protein levels (Nanog, Oct4, and Esrrb) upon removal of Dox from Aurka_R cells (Figures 3C and 3D). In contrast, no significant changes in TF levels were observed in Ctrl_R cells cultured without Dox (Figure 3C, left). In the presence of Dox, Aurka_R cells maintained an undifferentiated morphology, high alkaline phosphatase (AP) activity, and SSEA1 expression (Figures 3E and S2A). Moreover, treatment with the Aurka-specific chemical inhibitor MLN8237 suppressed pluripotency TF expression and decreased fluorescence in the NG4 Nanog-GFP reporter line (Figures S2B and S2C; Schaniel et al., 2006, 2009). After Dox removal, mRNA and protein levels of early mesodermal markers (Brachyury/T and Mixl1) and ectodermal markers (Cxcl12 and Fgf5), but not trophectodermal or endodermal markers, were increased (Figures 3F and 3G). Furthermore, suppression of Aurka in Brachyury/T-GFP reporter ESCs (Fehling et al., 2003) led to increased fluorescence, supporting induction of mesodermal differentiation (Figure S2D).

We also measured cell cycle, proliferative, and apoptotic parameters in Aurka_R cells and did not observe any significant differences after Aurka depletion (Figures S3A–S3C). This suggests that the residual Aurka levels (30%) are sufficient for normal proliferative and survival functions. Although the passage-dependent increase in the G2/M population upon Aurka knockdown might have some effect in the competition assay (Figure S3A), depletion of two other major cell cycle regulators (Cdc2a and Cdk2) causing similar G2/M effects did not lead to significant defects in ESC self-renewal (Figures S3C, S3D, and S1C). These observations strongly argue that the decreased percentage of GFP^+ cells after Aurka knockdown is not simply a consequence of generic cell cycle perturbation. In addition, expression levels of mRNAs encoding apoptosis-associated proteins showed only marginal changes, in contrast

(C) Knockdown effects on self-renewal of 15 candidates are validated in E14T ESCs. Depletions of 11 out of 15 genes in both CCE and E14T cells lead to a more severe competitive defect in comparison to threshold-defining knockdown of Lifr. Chek2 shRNAs (gray circles) show a significant effect on ESC self-renewal but have no effect on Chek2 mRNA expression and are likely to represent nonspecific, off-target effects.
(D) Heat map depiction of decreased pluripotency TF expression after 6 cell passages after knockdown of the 11 PKases and PPases.
(E) Knockdown of 11 identified genes correlates with the lower staining intensity of ESC surface marker SSEA1 in comparison to a Luc control shRNA.
See also Figure S1.

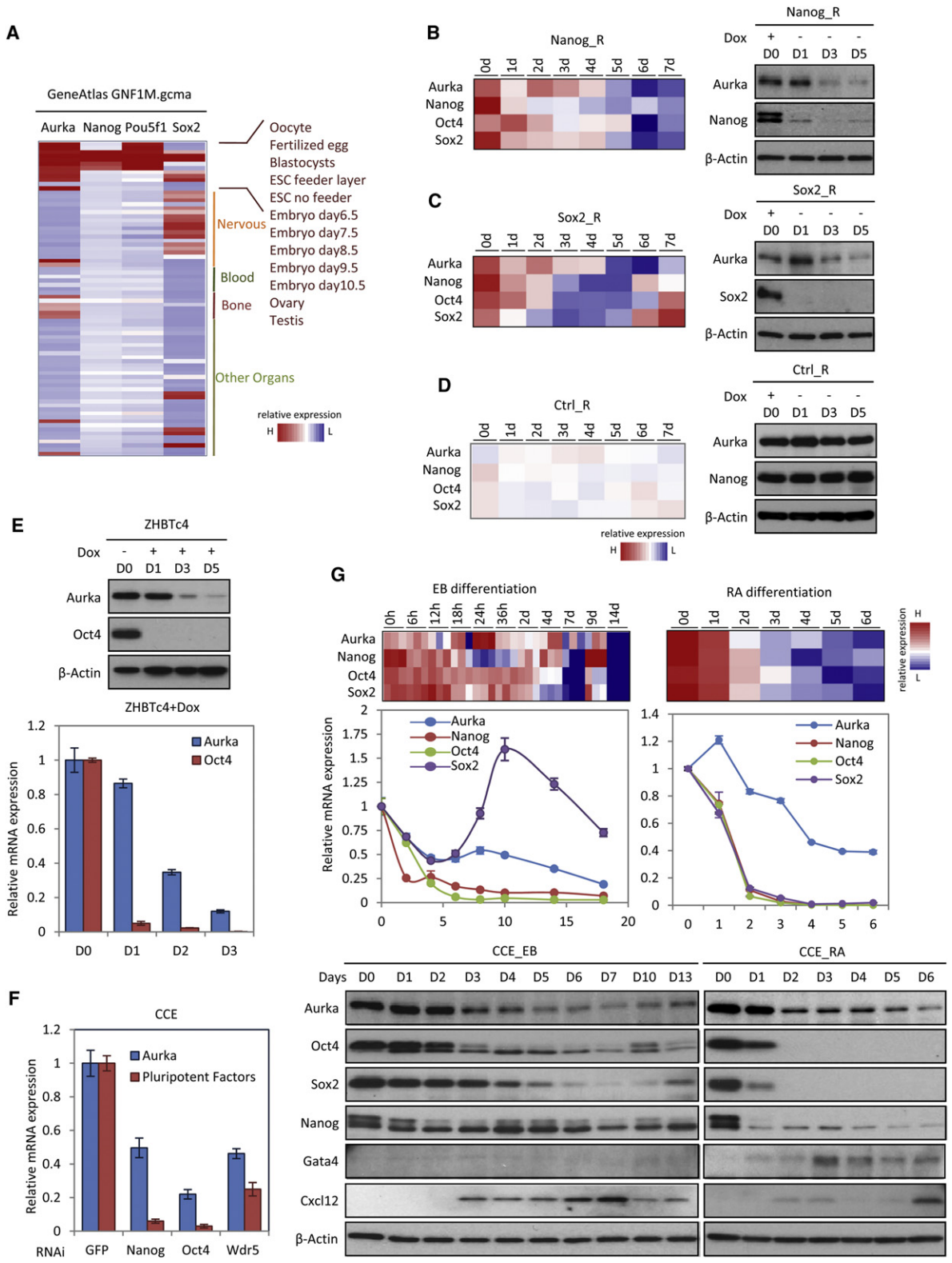

Figure 2. ESC Pluripotent State Is Impaired upon Downregulation of Aurka Expression

(A) Heat maps showing enriched expression of Aurka in preimplantation tissues, embryonic tissues, and ESCs. Gene expression data from different tissues, including embryonic stage, nervous system, blood system, bone tissue, and other organs, are obtained from BioGPS with the mouse GeneAtlas GNF1M database and further analyzed by Cluster and displayed by Treeview. High and low expression levels of indicated genes in different tissues are represented in red and blue colors, respectively.

to extensive increases of lineage-specific gene expression levels (Figure S3F). Therefore, milder knockdown of Aurka in mESCs appears to preserve its basic proliferative and viability functions but directly impairs pluripotency and promotes differentiation. Collectively, our results strongly support a direct role of Aurka in maintaining pluripotency.

Impact of Aurka Downregulation on the p53-Associated Transcriptome

Aurka functions as a mitotic kinase whose dysregulation results in centrosomal abnormalities, chromosome segregation defects, and aneuploidy (Badano et al., 2005). Accordingly, we asked whether ESC identity is mediated through Aurka binding partners or substrates with established functions in chromosome biology (Barr and Gergely, 2007). Depletion of Aurka mitotic substrates mBora, Jub, Tpx2, and Plk1 did not cause significant defects in self-renewal (Figure S4), indicating mitosis-independent mechanisms. In order to elucidate such mechanisms, we analyzed global gene expression changes after Aurka downregulation. Global transcriptome changes in Aurka_R cells were measured with Illumina Beadchip microarrays 5 days after Dox removal (Table S2). As expected, levels of numerous pluripotency factors were downregulated whereas levels of differentiation markers were increased (Figure S5A). Gene set enrichment analyses (GSEA) showed enrichment of an ESC-associated signature only in the presence of Aurka (Figure S5B). Gene ontology (GO) analyses by the PANTHER classification system revealed that Aurka depletion affects biological processes mainly involved in cell motility, immune system process, cell adhesion and, as expected, ectodermal and mesodermal development (Figures S5C–S5E).

In order to identify TFs responsible for the gene expression changes after Aurka depletion, we considered those with known binding motifs in promoter regions (ranging from −2 kb to +2 kb of the transcription start site) or previously shown to interact with promoters by chromatin immunoprecipitation (ChIP) (Lee et al., 2010; Marson et al., 2008). We performed GSEA analyses on target gene sets of these TFs weighted by their measured expression levels. We examined 623 sets and indeed found an enrichment of pluripotency TF targets in the rescued Aurka (+Dox) transcript set and, in contrast, an enrichment of ectodermal and mesodermal TF targets in the Aurka (−Dox) knockdown sets (Figure 4A, left and middle). TFs with downstream targets that were positively enriched upon Aurka downregulation were consistently upregulated during EB differentiation, whereas TFs with negatively enriched targets showed decreasing expression during differentiation (Figure 4A, right). Interestingly, although p53 mRNA expression decreased during EB differentiation, an increased expression of p53 targets, including Mdm2 and $p21^{Cip1}$, was detected upon loss-of-Aurka-induced differentiation (Figures 4A and 4B). These observations strongly suggest posttranscriptional/translational regulation of p53 function in the presence of Aurka. Quantitative (q) RT-PCR confirmed the upregulation of several p53 target genes upon Aurka knockdown (Figure S5F). Increased expression of p53 targets was detected after depletion of Aurka but not the pluripotency TFs Nanog and Esrrb (Ivanova et al., 2006) or the epigenetic modifier Wdr5 (Figure 4C; Ang et al., 2011b). Repression of Aurka kinase activity by different inhibitors also led to increased Mdm2 and $p21^{Cip1}$ levels (Figure S5G). Importantly, simultaneous shRNA-mediated depletion of Aurka and p53 restored pluripotency TF expression levels (Figure 4D). These results strongly suggest that regulation of ESC identity by Aurka is mediated, at least in part, by negative regulation of p53 activity.

Aurka Phosphorylates and Suppresses p53 Activity in mESCs

Our finding that depletion of Aurka results in activation of p53 signaling led us to investigate the underlying mechanism by which Aurka suppresses p53. We noticed two evolutionarily conserved putative Aurka phosphorylation sites on p53 (Ser212 and Ser312) (Figure 5A), implying that p53 is an Aurka substrate. To investigate the interaction between Aurka and p53, we performed coimmunoprecipitation (co-IP) and showed that exogenous Aurka physically associates with p53 (Figure 5B). We confirmed an interaction in mESCs by using antibodies to p53 (Figure 5C). Given the Aurka-p53 interaction and the putative phosphorylation sites, we next asked whether p53 is an Aurka substrate. In vitro kinase assays demonstrated that p53 was strongly phosphorylated by Aurka, whereas mutation of either Ser residue to Ala decreased [p53(S212A) or p53(S312A)] or abolished [double mutant p53(SSAA)] phosphorylation (Figure 5D). These results suggest that Aurka can directly phosphorylate both Ser212 and Ser312 in vitro.

To ask whether these phosphorylation events occur in vivo, we raised mouse polyclonal antibodies to p53 phosphorylation at Ser212 [p-p53(S212)] or Ser312 [p-p53(S312)]. These antibodies

(B and C) Time-course experiments demonstrate decreased expression of both Aurka mRNA and protein upon Dox withdrawal-mediated depletion of ESC pluripotency factors Nanog and Sox2 in Nanog_R and Sox2_R cells, respectively. High and low expression levels of indicated genes in Nanog_R and Sox2_R cells are represented in red and blue, respectively.

(D) Withdrawal of Dox in Ctrl_R cells does not alter Aurka expression. High and low expression levels of indicated genes in Ctrl_R cells are represented in red and blue, respectively.

(E) Depletion of Oct4 leads to diminished Aurka mRNA and protein expression in the Oct4-repressing ESC line ZHBTc4. qRT-PCR data are represented as mean ± SEM; n = 3.

(F) Transient knockdown of Nanog, Oct4, or Wdr5 by RNAi results in reduced Aurka expression in CCE cells. qRT-PCR data are represented as mean ± SEM; n = 3.

(G) Aurka expression decreases in parallel with pluripotency markers Nanog, Oct4, and Sox2 during EB formation and RA-induced differentiation, whereas differentiation markers Gata4 and Cxcl12 increase. In EB-mediated differentiation, the upper panel represents microarray transcriptome analyses during EB differentiation of J1 ESCs adapted from GSE3749, whereas the middle and lower panels represent qRT-PCR results and immnuoblotting data, respectively, during EB differentiation of CCE ESCs. In RA-induced differentiation, the upper panel represents microarray analyses (GSE4679) and the middle and lower panels represent qRT-PCR and immunoblotting results, respectively, from RA-treated CCE ESCs. qRT-PCR data are represented as mean ± SEM; n = 3. High and low expression levels of indicated genes are represented in red and blue, respectively.

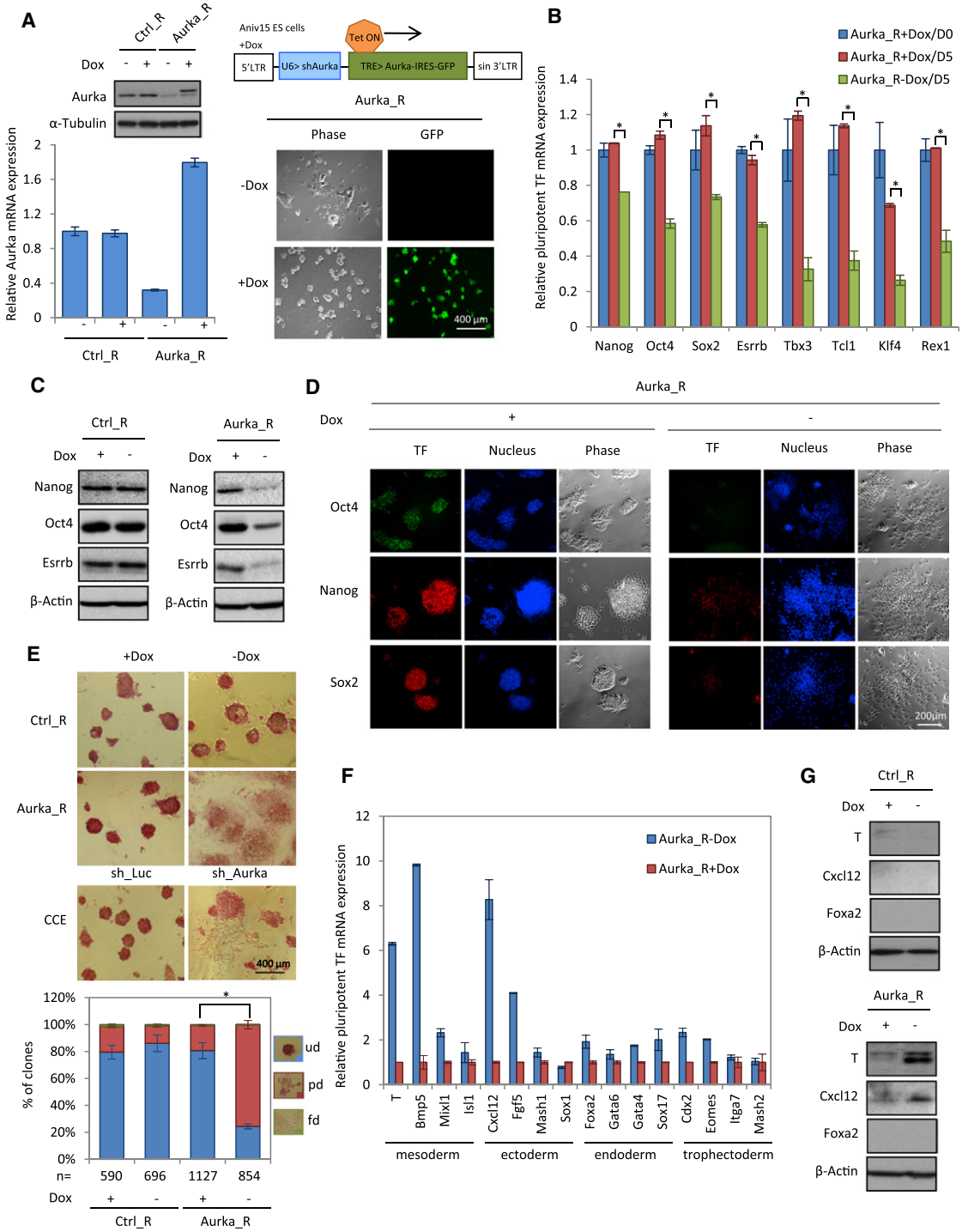

Figure 3. Aurka Depletion Results in Loss of Self-Renewal and Differentiation in ESCs

(A) The lentivirus-based rescue cassette contains a human U6 promoter-driven Aurka shRNA targeting the 3′UTR and a tetracycline response element (TRE) promoter driving the exogenous Aurka and GFP, expressed via an IRES element. In the absence of Dox, both Aurka and GFP are not expressed. In the presence of Dox, the exogenous Aurka is expressed and relieves the knockdown effect caused by Aurka shRNA. GFP is expressed as well.

(B) Defective expression of self-renewal genes *Nanog, Oct4, Sox2, Esrrb, Tbx3, Tcl1, Klf4*, and *Rex1* is restored in Aurka_R cells cultured for 5 days in Dox. qRT-PCR data are represented as mean ± SEM; n = 3. *$p < 0.05$ by Student's t test.

(C) Immunoblotting shows downregulation of Nanog, Oct4, and Esrrb 5 days after removal of Dox in Aurka_R but not Ctrl_R cells.

(D) Immunostaining 5 days after Dox removal from Aurka_R cells shows decreased expression of self-renewal TFs Oct4, Nanog, and Sox2.

(E) ESC differentiation is apparent from the morphologies of Aurka_R cells maintained for 5 days without Dox. In contrast, Ctrl_R cells do not show any differentiation phenotype without Dox. This is consistent with shRNA-mediated knockdown results in CCE cells. The number of undifferentiated (ud), partially

specifically recognize the phosphorylated peptides (Figure 5E). Levels of p-p53(S212) and p-p53(S312) were increased after cotransfection of p53 and Aurka (Figure 5F). Moreover, the levels of p-p53(S212) and p-p53(S312) were decreased in Aurka_R cells cultured without Dox (Figure 5G). Mass spectrometry analysis further showed that depletion of Aurka results in complete absence of p-p53(S212) in Aurka_R cells (Figure 5H), indicating that Aurka is the major, if not the only, p53 Ser212 kinase in mESCs. Given our observations that depletion of Aurka results in increased p53 transcriptional activity (Figures 4A and 4B) and that p53 is an Aurka phsophorylation target (Figures 5D and 5F–5H), we asked whether this modification inactivates p53. We generated phosphomimic mutants of p53 [p53(S212D), p53(S312D), and p53(SSDD) (double mutant)] and transfected these into ESCs together with a p53 transcriptional reporter. Measurement of p53 activity showed that p53(WT) and p53(S312D) but not p53(S212D) and p53(SSDD) activate a p53 reporter (Figure 5I). These findings demonstrate that Aurka-mediated phosphorylation of p53(S212) but not p53(S312) impairs p53 transcriptional activity in mESCs.

Increased Expression of p53-Associated Developmental Genes after Depletion of Aurka

Although p53 has been shown to induce ESC differentiation by suppressing Nanog expression (Lin et al., 2005), downregulation of Nanog results in primitive endodermal differentiation (Ivanova et al., 2006; Mitsui et al., 2003). In contrast, knockdown of Aurka promotes mesodermal and ectodermal differentiation (Figures 3F, 3G, and S5C). Although p53 is clearly involved in this process, these results are not consistent with a Nanog-suppression mechanism. We suggest an unexplored ESC function for p53 beyond suppressing Nanog. To address this we analyzed the direct targets of p53 by using a published genomic localization data set (Lee et al., 2010). Expression levels of p53 target genes are increased in the absence of Aurka (Figure 6A, top) and the majority of these are involved in developmental processes, especially in ectodermal and mesodermal development (Figure 6A, bottom). This is consistent with the biological processes affected by loss of Aurka (Figure S5C). Grid analysis of time-series expression (GATE) of EB differentiation-associated gene expression profiles also showed upregulation of p53 targets (group I) (Figure 6B; MacArthur et al., 2010). Conversely, the biological functions and expression profiles of Nanog targets (group II) are directly correlated with a pluripotent state (Figure 6B). GO analyses revealed that p53 target genes are significantly involved in developmental processes (ectodermal and mesodermal differentiation) and are not enriched in cell cycle or apoptosis categories (Figures 6A and 6C). In agreement with these findings, depletion of Aurka promoted increased expression of p53-occupied genes (e.g., ectodermal and mesodermal development-associated genes) and decreased expression of Nanog targets (e.g., pluripotency TFs) in Aurka_R cells (Figure 6D). Collectively, our observations suggest that a major function of p53 in ESCs is the positive regulation of differentiation-associated genes. Furthermore, studying the effects of Aurka-mediated p53 phosphorylation on ESC pluripotency via wild-type p53 and phosphomimic mutants showed that p53(WT) and p53(S312D) but not p53(S212D) or p53(SSDD) reduced the expression of pluripotency TFs Nanog and Tcl1 and increased the expression of ectodermal and mesodermal genes (Figure 6E). These results were confirmed in NG4 reporter cells, in which enforced expression of p53(WT) and p53(S312D), but not p53(S212D) and p53(SSDD), reduced Nanog reporter levels (Figure 6F). Aurka-mediated phosphorylation of both Ser212 and Ser312 impaired function in somatic cells (Katayama et al., 2004; Liu et al., 2004); however, only the S312D phosphomimic mutant compromised pluripotency and promoted differentiation. These results suggest that Aurka-mediated phosphorylation of Ser212 plays the major role in negatively regulating p53 function in ESCs and further emphasize its distinct regulation in undifferentiated versus differentiated cells. In the absence of Aurka and this single phosphorylation event, p53 directly activates mesodermal and ectodermal differentiation programs.

The Role of Aurka-p53 Signaling in Somatic Cell Reprogramming

Given the essential role of Aurka in maintaining ESC identity, we asked whether it functions during iPSC reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). We observed upregulation of Aurka during mouse iPSC generation by Oct4, Sox2, Klf4, and c-Myc (OSKM) (Figures S6A and S6B). In addition, analyses of data sets from iPSCs generated by different factors and methods and in various species, including humans (Carvajal-Vergara et al., 2010; Ebert et al., 2009; Feng et al., 2009; Ku et al., 2010; Soldner et al., 2009; Zhang et al., 2011a), consistently revealed upregulation of Aurka levels (Figures S6C–S6H). Aurka upregulation is therefore a general phenomenon in reprogramming. Knockdown of Aurka in Dox-inducible reprogrammable Oct4-GFP mouse embryonic fibroblasts (MEFs) markedly reduced the number of AP-positive iPSC colonies (Figure 7A; Stadtfeld et al., 2010). Although Aurka downregulation decreased MEF proliferation, no increases in iPSC colonies were observed even with prolonged culture times (Figure 7A). These results suggest that decreased iPSC generation is not due to adverse effects on proliferative capacity at any stage of reprogramming. Residual iPSC colonies originating from Aurka shRNA-transduced MEF cells did not contain the shRNA expression cassette (Figure S6I, left) nor did they express GFP (Figure S6I, right), and they showed no significant changes in Aurka levels (Figure S6J).

differentiated (pd), and fully differentiated (fd) ESC colonies are represented in the histograms. AP colony data are represented as mean ± SEM; n = 3; *p < 0.01 by Student's t test.

(F) Aurka knockdown induces the expression of Brachyury/T, Bmp5, Mixl1 (mesodermal lineage), Cxcl12, and Fgf5 (ectodermal lineage). The Aurka_R cells are maintained without feeder cells in either Dox or Dox-free conditions for 5 days.

(G) Immunoblotting demonstrates increased Brachyury/T (mesodermal marker) and Cxcl12 (ectodermal marker) but not Foxa2 (endodermal marker) in Aurka_R cells cultured without Dox. Expression of Brachyury/T, Cxcl12, and Foxa2 is not altered upon withdrawal of Dox in the Ctrl_R cells.

See also Figures S2 and S3.

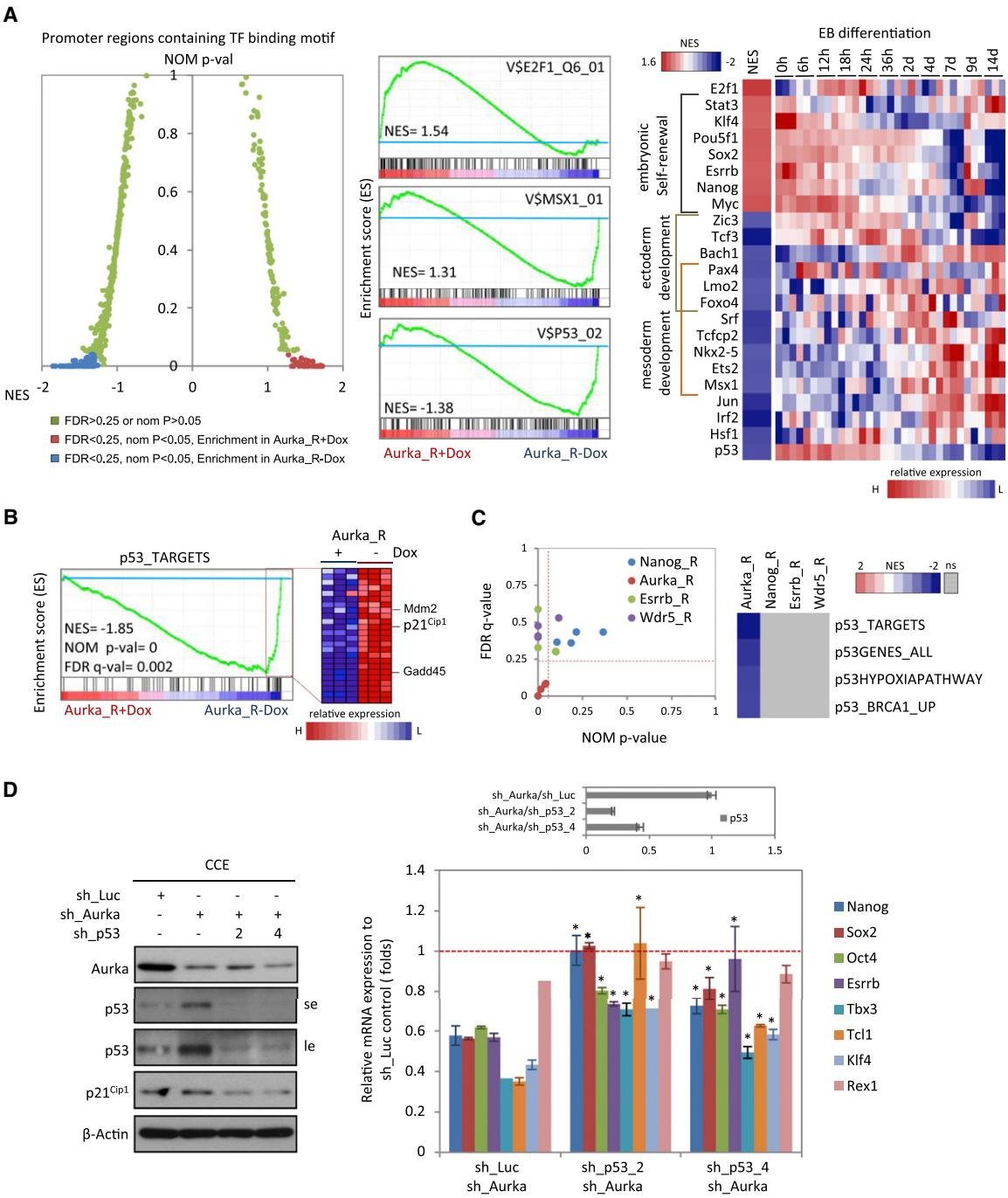

Figure 4. Upregulation of p53 Signaling after Aurka Knockdown

(A) GSEA analyses identify enriched TF targets expressed either in Aurka-expressing ESCs or after Aurka depletion and associated differentiation. A total of 623 TF binding motif or ChIP target gene sets are used (left). Normalized enrichment scores (NES) are examined and correlated with gene expression during EB formation by using a public data set (GSE3749). A positive NES (red) represents targets of selected TFs that are enriched in the transcriptome of ESCs expressing Aurka. A negative NES (blue) represents the targets of selected TFs that are enriched in the transcriptome of ESCs after Aurka depletion. Enriched gene sets are selected based on statistical significance (FDR q value < 0.25 and normalized p value < 0.05). Three examples are shown in the middle panels for E2F1 (top), Msx1 (middle), and p53 (bottom). High and low expression of indicated genes in EB samples (right panel) are represented in red and blue, respectively.

(B) Expression of p53 target genes is enriched in Aurka-knockdown ESCs. High and low expression levels of indicated genes in Aurka_R cells cultured with and without Dox are represented in red and blue, respectively.

(C) FDR q values and normalized p values (left) and heat map depiction (right) showing the enrichment of multiple p53 target gene sets specifically in the Aurka-associated transcript set but not in the Nanog-, Esrrb-, or Wdr5-associated sets. Each data point represents the NES associated with loss of expression of Aurka. GSEA data set significance is calculated at a FDR q value < 0.25 and normalized p value < 0.05 (dashed red lines in left panel). Nonsignificance (ns) is indicated in gray.

Collectively, our results indicate that depletion of Aurka completely blocks OSKM-mediated reprogramming.

To further exclude the possibility that reprogramming defects were due to impaired proliferative ability in the absence of Aurka, we utilized a heterokaryon technique that requires neither genome duplication nor cell division (Pereira et al., 2008). Suppression of reprogramming in this system by inhibition of Aurka supported its essential role in reprogramming (Figure 7B). A positive role in reprogramming was further supported by Aurka overexpression with resultant increases in iPSC colony numbers (Figure S6K). Although overexpression of Aurka promoted MEF proliferation, the noticeable increases in iPSC colonies exceeded these minor proliferative increases.

Because depletion of Aurka leads to increased p53 activity, we asked whether simultaneous depletion of p53 is sufficient to rescue iPSC reprogramming. Indeed, depletion of p53 or Arf, an Mdm2 antagonist and thus an inhibitor of p53 degradation, rescued reprogramming in Aurka-depleted cells (Figures 7C and S7A). Importantly, the p53(S212D) and p53(SSDD) but not the p53(S312D) phosphomimic mutants lost their ability to counteract the reprogramming process after transduction into *p53*$^{-/-}$ MEFs (Figure 7D and S7B). In agreement with the impairment of p53 transcriptional activity via Ser212 phosphorylation, ectopic expression of p53(WT) and p53(S312D) but not p53(S212D) or p53(SSDD) activated p21^{Cip1} and Mdm2 expression even with much lower p53(WT) and p53(S312D) than p53(S212D) and p53(SSDD) protein levels (Figure S7C). Reductions in iPSC colony numbers were observed even after prolonged culture times (Figure 7D). Consistent results were also obtained with reprogrammable Oct4-GFP MEFs (Figure S7D). Taken together, our findings demonstrate that an essential aspect of iPSC reprogramming is Aurka-mediated inhibition of p53 by phosphorylation of a single residue, Ser212. Moreover, out data suggest that the negative effects of p53 on reprogramming also depend, at least in part, on the activities of upstream pathway components such as Arf.

DISCUSSION

Pluripotent stem cells hold great promise for regenerative medicine (Murry and Keller, 2008). In order to realize this potential, an in-depth understanding of the mechanisms controlling self-renewal, pluripotency, and transitions in cell fate is necessary. With the recent ability to derive patient-specific iPSCs, dissection of these regulatory mechanisms may provide more effective and safer avenues for iPSC reprogramming as well as better methodologies to maintain them in a pluripotent state and direct them toward specific differentiated cell fates. To investigate the signaling cascades required for ESC self-renewal, we conducted a loss-of-function screen targeting PKases and PPases and identified the PKase Aurka to be required for pluripotency. Further studies demonstrated that Aurka-mediated phosphorylation of p53 is essential for maintaining ESC self-renewal and pluripotency.

The p53 protein is a stress-response TF that controls the expression of genes involved in DNA repair, apoptosis, cell cycle, and senescence (Ko and Prives, 1996; Riley et al., 2008). Intensive research on p53 has focused on its functions in differentiated cells; however, its role in ESCs remains largely unexplored. Recent studies suggest a role for p53 in ESC self-renewal and pluripotency as well as in somatic cell reprogramming (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Sarig et al., 2010; Utikal et al., 2009). Ectopic expression and/or activation of p53 have been suggested to promote ESC differentiation by suppression of Nanog (Han et al., 2008; Lin et al., 2005). A genome-wide study of p53 binding targets in mESCs has implicated upregulation of the Wnt pathway in preventing differentiation (Lee et al., 2010). Collectively, these findings indicate that p53 must be tightly regulated to ensure ESC identity and proper differentiation decisions.

In contrast to ESCs and iPSCs, adult cells express low levels of p53 (Kawamura et al., 2009; Sabapathy et al., 1997; Solozobova and Blattner, 2010). Differentiated cells show strong responses to modest increases in p53 levels, whereas ESCs are extremely resistant to stress-induced (e.g., DNA damage) p53-mediated signals (Qin et al., 2007; Sabapathy et al., 1997). This suggests that in ESCs the activity of p53 must be restricted. Transcriptional, translational, and posttranslational mechanisms have been shown to modulate p53 levels and activities (Bode and Dong, 2004; Brooks and Gu, 2003). Two key regulators of p53 stability are its transcriptional target the E3 ubiquitin ligase Mdm2 and Arf, an Mdm2 antagonist. Mdm2 regulates p53 by direct binding and ubiquitination resulting in proteasome-mediated degradation. Arf antagonizes Mdm2 by sequestering it in the nucleolus (Weber et al., 1999), thus preventing p53 degradation. This regulation has been shown to play an essential role in p53-mediated processes, including the inhibition of iPSC reprogramming (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Sarig et al., 2010; Utikal et al., 2009). The most common posttranslational modification of p53 is phosphorylation. Phosphorylation events influence the stability and transcriptional activity of p53 as well as its ability to bind to a variety of protein partners (Bode and Dong, 2004). How cell signaling controls p53 function via phosphorylation has been extensively analyzed in adult cells. However, the analogous cellular signals that function in ESCs remain largely unknown.

Aurka is an evolutionarily conserved serine/threonine kinase with key mitotic regulatory functions (Barr and Gergely, 2007). Specifically, Aurka phosphorylates and modulates the activities of multiple mitosis-associated proteins (e.g., Tacc and Ndel1) (Barros et al., 2005; Mori et al., 2007), thereby enabling and orchestrating centrosome maturation, spindle assembly, and mitotic entry. Aurka is considered as the gatekeeper of mitosis;

(D) Decreased expression of pluripotency genes after loss of Aurka is rescued by knockdown of p53 by two distinct shRNAs when examined by immunoblotting (left) and qRT-PCR (right). Expression of p21^{Cip1} is an indicator of functional p53. Relative mRNA expression is normalized to levels in the Luc shRNA control. All data are represented as mean ± SEM; n = 3. *p < 0.05 by Student's t test for p53 shRNA2 and p53 shRNA4 versus Luc shRNA. se, shorter exposure; le, longer exposure.
See also Figures S4 and S5.

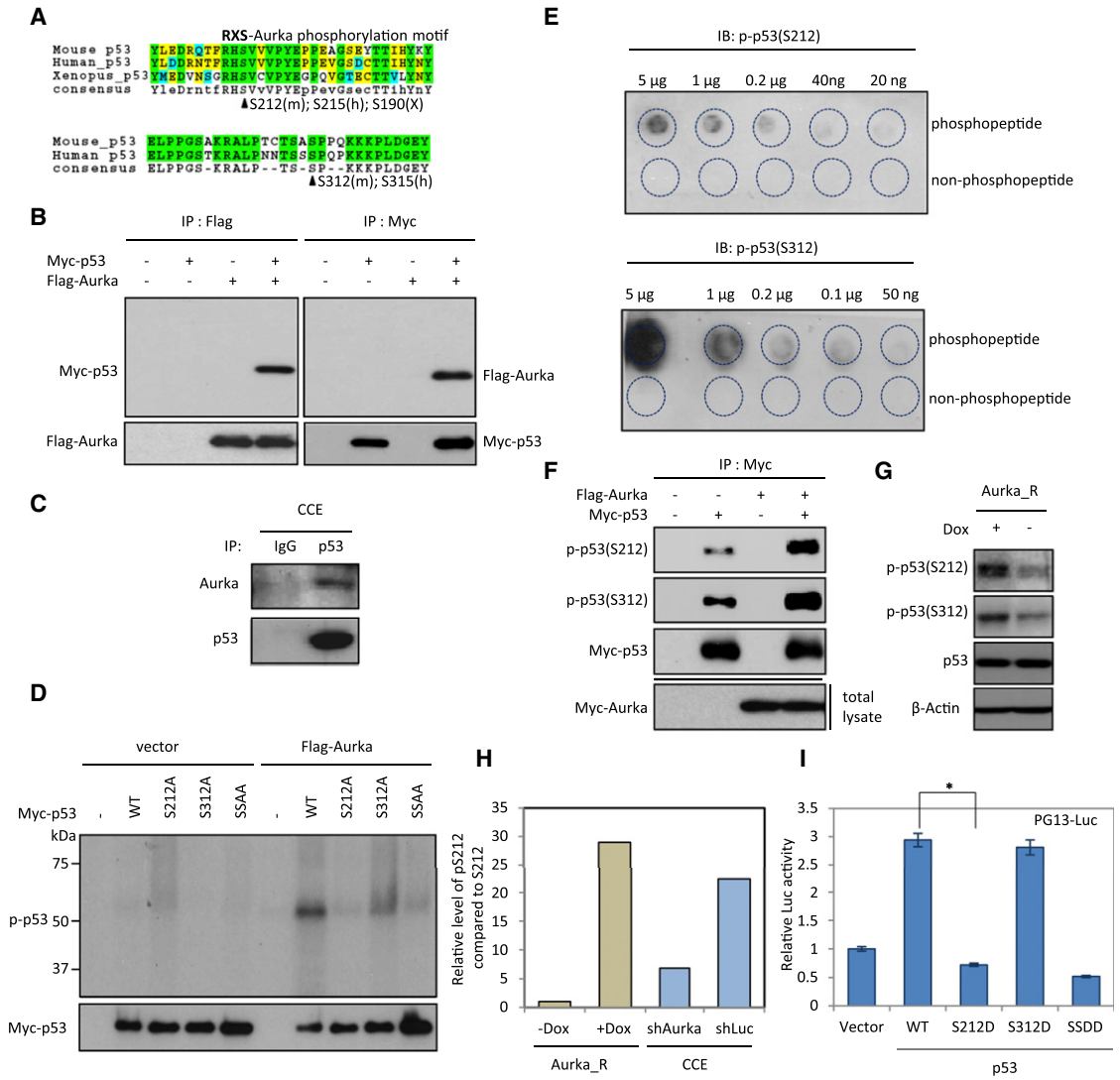

Figure 5. Aurka Interacts and Phosphorylates p53

(A) Two putative Aurka phosphorylation sites, Ser212 and Ser312, are found in p53. Ser212 is a classical (RXS) Aurka phosphorylation site that is evolutionarily conserved among human, mouse, and *Xenopus*. Ser312 is conserved only in human and mouse. R, arginine; X, any amino acid; S, serine.

(B) Interaction between exogenously expressed p53 and Aurka. Lysates of HEK293T cells cotransfected with Myc-tagged p53 and Flag-tagged Aurka are analyzed by reciprocal co-IP and immunoblotting using tag antibodies.

(C) Endogenous p53 interacts with Aurka. Lysates of mouse CCE cells are analyzed by co-IP using anti-p53 (FL-393) or control IgG antibodies and immunoblotting using anti-Aurka and anti-p53 (FL-393) antibodies.

(D) Detection of the Aurka-mediated phosphorylation on p53 Ser212 and Ser312 by in vitro kinase assay. Aurka immunocomplexes are pulled-down from HEK293T cells and incubated with the indicated Myc-tagged p53 proteins.

(E) Characterization of antibodies to p-p53(S212) and p-p53(S312). Dot blot shows that both antibodies specifically recognize their respective phosphopeptides but not nonphosphopeptides.

(F) Aurka phosphorylates p53 at Ser212 and Ser312 in vivo. Myc-tagged p53 and Flag-tagged Aurka are cotransfected into ESCs, immunoprecipitated, and analyzed with antibodies to p-p53(S212) and p-p53(S312).

(G) Depletion of Aurka 2 days after Dox withdrawal decreases p-p53(S212) and p-p53(S312) levels in Aurka_R cells.

(H) Phosphorylation of p53 (Ser212) is assessed with quantitative mass spectrometry with isotopomeric standards of the appropriate tryptic peptides (HSVVVPYEPPEAGSEYTTIHYK and H[pS]VVVPYEPPEAGSEYTTIHYK). Selected reaction monitoring is performed at the appropriate elution time for these peptides in biological samples spiked with the isotopomeric standards. Using this approach, ratiometric values for the degree of phosphorylation in the samples are determined.

(I) Cell lysates from wild-type and mutant p53 transfected together with PG13-Luc, containing wild-type p53 binding sites, and control TK-RLuc into CCE cells for 2 days are assayed for p53 activity. Luminescence ratios of PG13-Luc to TK-Rluc were calculated and normalized to vector-transfected cells. All data are represented as mean ± SEM; n = 3. *p < 0.01 by Student's t test.

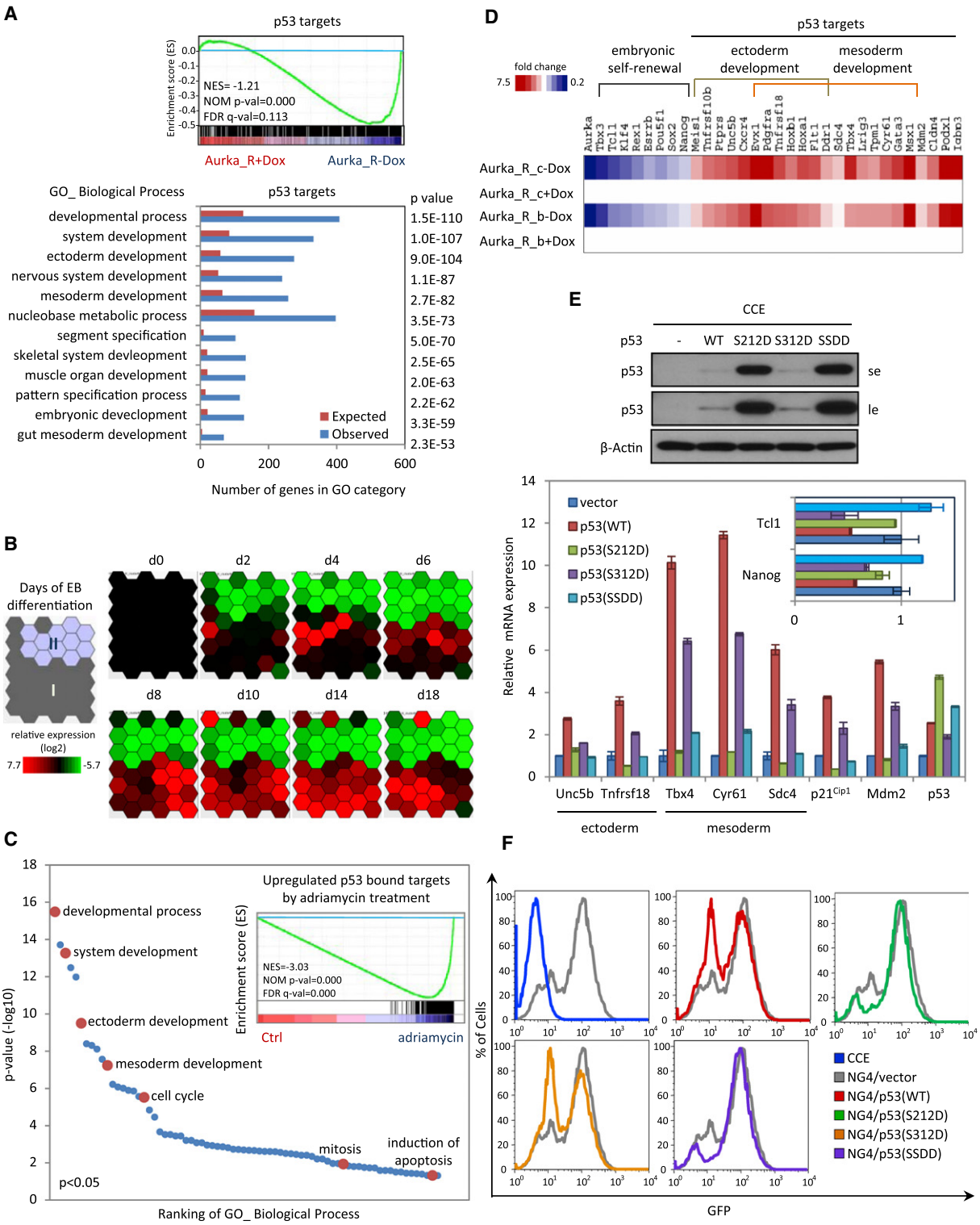

Figure 6. Loss of Aurka Results in p53-Mediated Developmental Gene Activation

(A) GSEA analysis indicates enriched expression of direct p53 transcriptional targets upon knockdown of Aurka and GO biological process analyses of p53 ChIP targets by Panther Classification System.

(B) Diverse regulation of p53 targets (group I) and Nanog targets (group II) during EB differentiation. Gene expression is normalized to day 0. Time series expression data are analyzed by qRT-PCR and visualized by GATE. Red and green represent up- and downregulation of gene expression, respectively.

(C) Functional categorization of upregulated p53-bound targets after Adriamycin treatment is analyzed for GO biological processes via Panther Classification System.

however, growing evidence suggests additional roles in various cellular processes. For example, Aurka regulates the protein translation machinery through phosphorylation of Cpeb (Mendez et al., 2000a, 2000b). In addition, gene amplification and overexpression of Aurka are linked to tumorigenesis via mechanisms that include phosphorylation of Gsk3β (Dar et al., 2009) and p53 (Katayama et al., 2004; Liu et al., 2004; Pascreau et al., 2009). Although the functions of Aurka in adult cells have been extensively explored, information about its roles in ESCs is very limited because homozygous mutant embryos do not survive past the 16-cell stage (Cowley et al., 2009; Lu et al., 2008; Sasai et al., 2008).

Our functional screen identifies a role for Aurka in maintaining ESC self-renewal and pluripotency that is consistent with Aurka's functions in embryogenesis. Systematic integrated analyses were performed to elucidate the putative downstream molecules and pathways involved in this activity. We demonstrate that increased p53 signaling is responsible for the differentiation triggered by loss of Aurka. Interestingly, p53 has been identified as a physiological Aurka substrate with phosphorylation sites Ser215 and Ser315 in human (mouse Ser212 and Ser312) (Katayama et al., 2004; Liu et al., 2004; Pascreau et al., 2009). Phosphorylation of Ser215 impairs DNA binding and transcriptional activity (Liu et al., 2004), whereas phosphorylation of Ser315 facilitates Mdm2-induced degradation (Katayama et al., 2004). By using phosphomimic mutants of p53, we demonstrate that Ser212 phosphorylation plays the major role in Aurka-mediated p53 inactivation in both the maintenance and reacquisition of pluripotency. Without inactivation, p53 promotes differentiation largely by directly inducing mesodermal and ectodermal genes. Although Ser312 phosphorylation might not have an essential role in ESC regulation, we do not rule out the possibility of its involvement in other p53-mediated processes, particularly in various physiological stress responses (Katayama et al., 2004). The unusually high Aurka levels in ESCs or during iPSC reprogramming would also provide a substantial survival advantage by inhibiting p53-mediated cell cycle arrest, apoptosis, senescence, and differentiation.

Interestingly, a significant proportion of p53 targets in ESCs are involved in developmental processes, rather than in cell cycle or apoptosis. Targets upregulated after treatment with Adriamycin are also involved in developmental processes (Figure 6C). We suggest that the primary function of p53 in ESCs may be to balance pluripotency and differentiation. During iPSC reprogramming, p53 levels are increased and activate cell cycle-, apoptosis-, and senescence-related programs, thereby limiting this process. Because expression of specific factors is dependent on cell type and p53 activity is regulated by a variety of mechanisms, we expect that in undifferentiated and differentiated cells its phosphorylation by Aurka may result in a range of functionally diverse p53-containing complexes. Identification of p53 protein-protein interaction networks in the presence and absence of Aurka will provide additional insights into ESC/iPSC self-renewal as well as the variety of mechanisms that cells employ to regulate their fates.

In summary, we demonstrate that in the absence of Aurka, increased p53 signaling promotes ESC differentiation. A single Aurka-mediated phosphorylation event is largely responsible for inactivating p53. We further show that suppression of p53 activity by Aurka is essential during iPSC reprogramming (Figure 7E). The central roles of the Aurka-p53 signaling axis in maintaining and reacquiring a pluripotent state provide important new starting points for uncovering novel signaling mechanisms and developing avenues to control cell fates. Further investigation of the other phosphoregulators identified in our screen will shed additional light on the identities, functions, and the overall complexity of signaling networks in ESCs/iPSCs.

EXPERIMENTAL PROCEDURES

Competition Assay

The competition assay was performed as described previously (Ivanova et al., 2006; Lee et al., 2012). In brief, cells were mixed at a ratio of 80% shRNA-transduced ESCs (GFP^+) to 20% control (Luc) shRNA-transduced cells (GFP^-) and cultured in gelatin-coated 6-well plates. Culture media was replaced daily and cells were trypsinized and replated every 2 days. At the sixth passage, the proportions of GFP^+/GFP^- cells were measured by a BD LSR II flow cytometer (BD Biosciences).

Derivation of Ainv15 Rescue Clones and Differentiation Assay

Generation of Aurka and control rescue clones and differentiation assay were performed as previously described (Ivanova et al., 2006; Lee et al., 2012).

Panther and GSEA Analyses

Gene ontology analysis was performed by Panther Classification System (http://www.pantherdb.org/) with all NCBI *Mus musculus* genes as a reference list. Gene ontology biological process with Bonferroni correction was applied. GSEA analysis was performed by using GSEA software with the enrichment statistic equal to weighted and the metric for ranking genes equal to signal-to-noise. GSEA for Figures 4A–4C, 6A, and 6C was performed with ChIP-Chip data from previous studies (Lee et al., 2010; Marson et al., 2008). The lists of genes with promoter regions [−2 kb, +2 kb] containing TF binding motifs were found in the GSEA website gene sets database c3.tft.v2.5.symbols [motif]. GSEA for Figure S5B was performed with the ESC signature gene set using the embryonic stem cell-like gene expression signature genes from previous studies (Ben-Porath et al., 2008). Global ESC gene expression data upon UV or Adriamycin treatment were obtained from a published database (GSE16428) (Lee et al., 2010). GSEA results were considered significant when the false discovery rate (FDR) q value was less than 0.25 and nominal (NOM) p value is less than 0.05.

Somatic Cell Programming

Mouse $p53^{-/-}$ MEFs were seeded at a density of 50,000 cells in 6-well plates and infected with pMXs-based OSKM (Oct4, Sox2, Klf4, and c-Myc) together with either p53 wild-type or mutant retrovirus for 24 hr. Infected cells were split

(D) Upregulation of p53/Nanog co-occupied target genes upon Aurka knockdown (−Dox) in two independent Aurka_R clones.
(E) Ectopic expression of p53(WT) or p53(S312D) but not p53(S212D) or p53(SSDD) impairs ESC pluripotency and promotes mesodermal and ectodermal differentiation. Lentiviruses encoding wild-type and mutant p53 are transduced into CCE cells for 3 days followed by 2 days of puromycin selection. The expression of p53 is measured by immunoblotting (top) and qRT-PCR (bottom). All qRT-PCR data are represented as mean ± SEM; n = 3. se, shorter exposure; le, longer exposure.
(F) Ectopic expression of p53(WT) or p53(S312D) but not p53(S212D) or p53(SSDD) downregulates Nanog promoter activity. Lentiviruses encoding wild-type and mutant p53 are transduced into NG4 cells followed by 2 days of puromycin selection. Selected cells are analyzed by flow cytometry with CCE cells as a negative control.

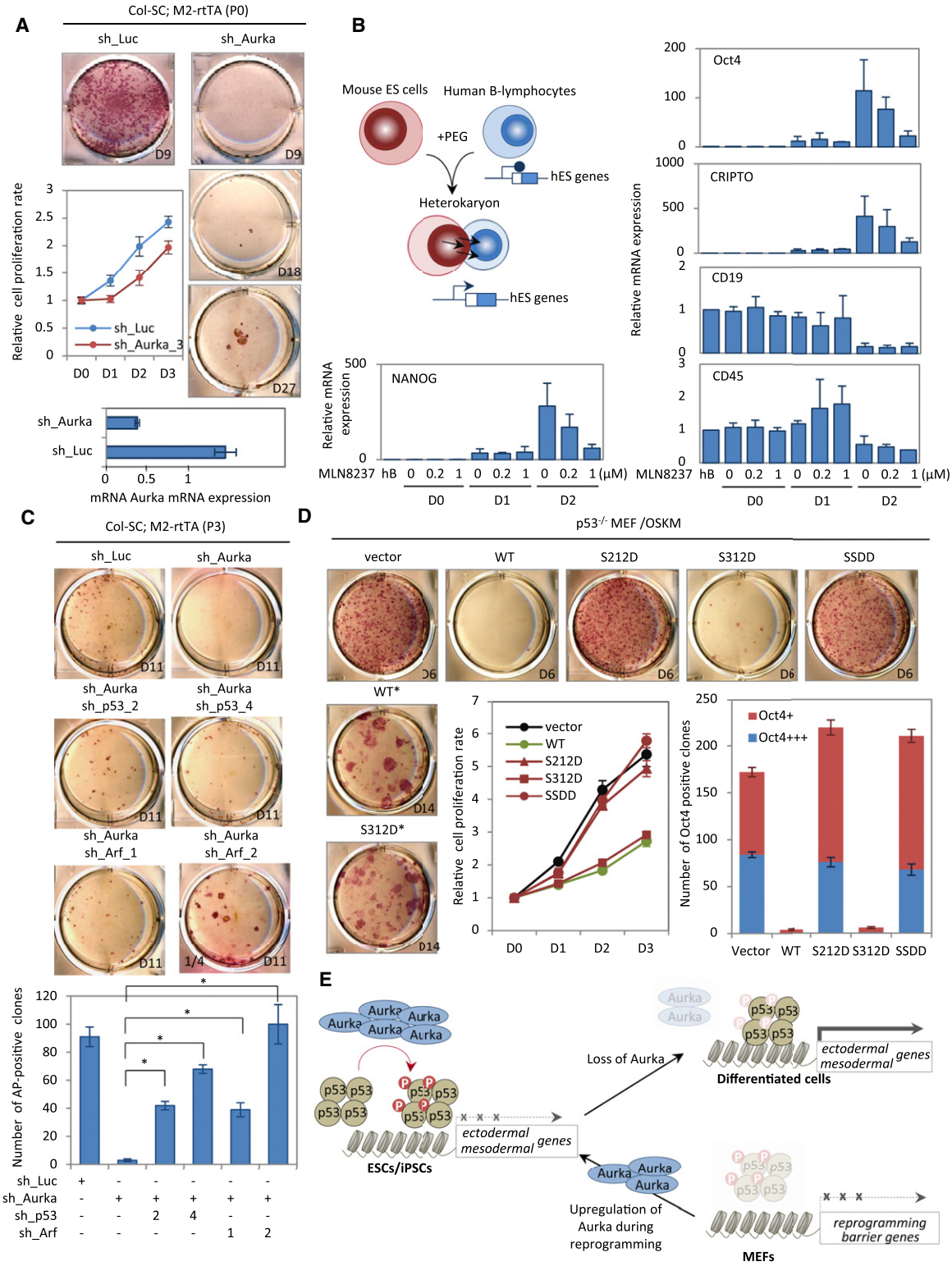

Figure 7. Suppression of p53 by Aurka Is Required for Somatic Cell Reprogramming

(A) Inhibition of the reprogramming process in reprogrammable MEFs by Aurka depletion. Reprogrammed iPSC colonies after Aurka and Luc knockdown are identified by AP staining at day 9. MEF proliferation upon Aurka knockdown is determined by MTT assay. All values shown are mean ± SEM for n = 3.

(B) Inhibiting Aurka activity by MLN8237 suppresses nuclear reprogramming in heterokaryons. Interspecies heterokaryons are generated by PEG-mediated fusion of human B lymphocytes and mouse E14T ESCs. Induction of human embryonic genes (*OCT4*, *NANOG*, *CRIPTO*) and decrease of human B lymphocytic genes (*CD19* and *CD45*) are measured to evaluate the reprogramming efficiency.

into 6-well plates at a density of 5,000 cells per well and maintained in iPSC culture media. Media was changed every day. Simultaneously, 1,000 infected cells were seeded onto a 96-well plate for the MTT assay as described previously (Kuo et al., 2010). After 6 days, iPSC clones were examined by both AP staining and Oct4 immunostaining. The expression of p53 was determined by qRT-PCR 4 days postinfection. For the Dox-induced reprogramming approach, Col-SC, M2-rtTA, and Oct4-GFP MEFs (Stadtfeld et al., 2010) were seeded at a density of 50,000 cells in 6-well plates and infected for 24 hr with shRNA lentiviruses or retroviruses expressing wild-type or mutant p53. Infected cells were split into 6-well plates at a density of 20,000 cells per well and subsequently maintained in iPSC culture media containing 2 μg/ml Dox. After 9 days, iPSC clones were detected by AP staining and GFP fluorescence. All animal housing and procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Animal Welfare Act.

Heterokaryon-Based Reprogramming

Heterokaryons were generated by fusing human B-lymphocytes and MLN8237-pretreated mouse ESCs using polyethylene glycol (pH 7.4) (PEG 1500; Roche Diagnostics) as described previously (Pereira et al., 2008).

ACCESSION NUMBERS

All microarray data are deposited in NCBI-Gene Expression Omnibus database under accession number GSE23541.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.05.020.

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(C) AP staining shows that knockdown of p53 or the Mdm2 antagonist Arf rescues the loss of Aurka-associated decreases in reprogramming efficiency in reprogrammable MEFs. Because of the rapid growth observed in Arf shRNA2-transduced MEFs, only one fourth as many cells were used compared to the other experiments. AP-positive colonies are counted in three independent experiments. All values shown are mean ± SEM for n = 3. *p < 0.01 by Student's t test.
(D) p53(S212D) but not p53(S312D) loses the ability to suppress iPSC reprogramming. Various p53 phosphorylation mutants [p53(S212D), p53(S312D), and p53(SSDD)] are coinfected with OSKM factors into $p53^{-/-}$ MEFs. iPSC colonies are identified by AP staining (top). $p53^{-/-}$ MEF proliferation after transduction of various p53 mutant is measured by MTT assay (bottom, left). High and low Oct4-positive clones are identified with Oct4 antibodies and scored based on expression levels (bottom, right). Oct4-positive colonies are counted in three independent experiments. All values shown are mean ± SEM for n = 3.
(E) Model for the Aurka-p53 signaling axis in ESC self-renewal and iPSC reprogramming. In our proposed model, Aurka-mediated phosphorylation and consequent suppression of p53 activity impairs differentiation in ESCs and facilitates reprogramming of somatic cells.
See also Figures S6 and S7.

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