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A Genome-Scale RNAi Screen for Oct4 Modulators Defines a Role of the Paf1 Complex for Embryonic Stem Cell Identity

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SUMMARY

Pluripotent embryonic stem cells (ESCs) maintain self-renewal while ensuring a rapid response to differentiation cues. The identification of genes maintaining ESC identity is important to develop these cells for their potential therapeutic use. Here we report a genome-scale RNAi screen for a global survey of genes affecting ESC identity via alteration of Oct4 expression. Factors with the strongest effect on Oct4 expression included components of the Paf1 complex, a protein complex associated with RNA polymerase II. Using a combination of proteomics, expression profiling, and chromatin immunoprecipitation, we demonstrate that the Paf1C binds to promoters of key pluripotency genes, where it is required to maintain a transcriptionally active chromatin structure. The Paf1C is developmentally regulated and blocks ESC differentiation upon overexpression, and the knockdown in ESCs causes expression changes similar to Oct4 or Nanog depletions. We propose that the Paf1C plays an important role in maintaining ESC identity.

INTRODUCTION

Embryonic stem cells (ESCs) have unlimited capacity for self-renewal and can be kept undifferentiated for many passages under appropriate conditions while maintaining the competence to generate a wide range of cell types upon differentiation (Chambers and Smith, 2004). Because of these distinctive properties, ESCs are widely used for studies of developmental processes (O'Shea, 2004). The potential to differentiate into many cell types also makes ESCs a starting point for potential cell-based therapies (Keller, 2005). However, a systematic molecular understanding of self-renewal and differentiation is required to harness the full potential of ESCs.

Several transcription factors that contribute to the regulation of self-renewal and differentiation of ESCs have been identified. Oct4, Nanog, and Sox2 form a transcriptional core unit upon which ESC pluripotency is critically dependent (Silva and Smith, 2008). Recently, an RNAi analysis of 70 candidate transcription factors identified roles for additional genes, including Tbx3, Esrrb, Tcl1, and Dppa4, in the maintenance of ESC pluripotency (Ivanova et al., 2006). Depletion of these genes negatively affected self-renewal, and induced ESC differentiation.

Several signaling pathways including the LIF/Stat3, PI3K, Wnt, and Bmp/Smad pathways also contribute to the regulation of self-renewal and differentiation of ESCs. For example, the PI3K pathway regulates multiple cascades including Ras/MAPK and mTOR pathways, which are essential for proliferation of mouse ESCs (Takahashi et al., 2005).

In addition to transcription factors and signaling pathways, a defined epigenetic state has been shown to be essential to maintain ESC identity (Pietersen and van Lohuizen, 2008). Accordingly, many promoters of key developmental genes including Sox, Hox, Pax, and Pou gene family members display both an activating (H3K4me) and a repressive (H3K27me) histone mark on the nucleosomes at their promoters in ESCs (Bernstein et al., 2006). This "bivalent" histone code silences lineage-control gene expression due to the dominant effect of H3K27me over H3K4me, while preserving their potential to be rapidly activated upon differentiation stimuli via the removal of the H3K27me mark. It is therefore not surprising that proteins, which regulate chromatin structure, are important for ESC identity, as recently shown by a focused RNAi screen of ~1000 chromatin proteins (Fazzio et al., 2008).

The work performed on transcription factors, signaling, and chromatin has substantially improved our understanding of ESCs. However, our knowledge of how these processes are connected is still limited. A global survey of genes essential for ESC self-renewal and identity would not only advance our

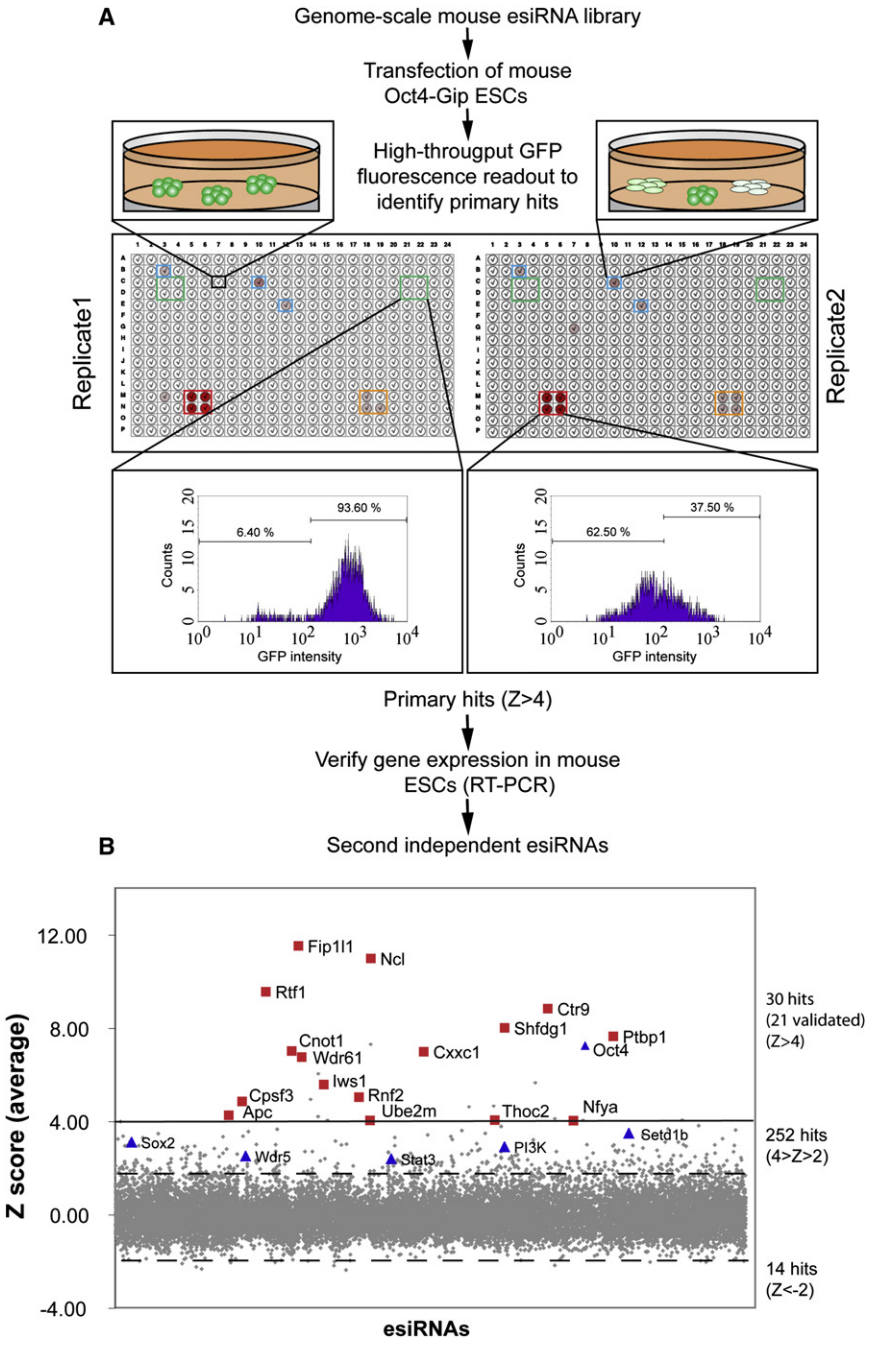

Figure 1. Genome-Scale esiRNA Screen

(A) Flow diagram of the screening strategy to identify genes essential for mouse ESC identity. Eight negative controls (boxed in green), eight positive controls (four GFP esiRNA, red; and four Sox2 esiRNA, orange), three primary hits (blue), and one random well without a phenotypic consequence (black) are highlighted. The red color intensity reflects the strength of phenotypes observed in each well, which is illustrated by FACS readouts for two exemplified wells (boxes below the plates).

(B) Dot plot of the primary screen results. The average Z scores of the GFP readouts are shown. The dotted lines indicate Z score > 2 or < −2. The solid line marks Z scores > 4. Validated genes with Z scores > 3 with a second, independent esiRNA are shown as red squares. Selected pluripotency genes that scored with a Z score > 2 are shown as blue triangles.

understanding of this fundamental biological process but should also help to develop better protocols for directed differentiation of ESCs for their potential therapeutic use.

To obtain a more systematic understanding of the genes associated with ESC identity, we performed a genome-scale RNAi screen in mouse ESCs using an Oct4 reporter assay as a surrogate for ESC identity. We identified many novel genes that affected Oct4 expression and therefore ESC identity and analyzed one group of genes, which form the Paf1 complex (Paf1C), in more detail. Our work therefore expands the inventory of genes required to maintain ESC identity and defines a role of the Paf1C in maintaining active transcription of pluripotency genes in ESCs.

RESULTS

Primary Screen for Oct4 Modulators

To perform an RNAi screen in mouse ESCs, we generated a genome-scale mouse endoribonuclease prepared (e)siRNA library containing 25057 esiRNAs (see Table S1 available online) using the established esiRNA synthesis protocol (Kittler et al., 2005a). EsiRNAs have proven efficacy and specificity in human cells (Kittler et al., 2004, 2007a, 2007b), suggesting that this resource should be useful for RNAi screening in mouse cells. To identify genes essential for the maintenance of ESC identity, we developed a rapid and robust assay amenable to high-throughput detection of differentiation triggered by RNAi (Figure 1A). Oct4 expression is a hallmark of ESC identity, and constant expression levels of Oct4 are required for self-renewal of ESCs (Niwa et al., 2000). Depletion of Oct4 in ESCs via knockout or RNAi leads to exit from self-renewal and to differentiation (Carpenter and Zernicka-Goetz, 2004; Nichols et al., 1998). In turn, the expression of Oct4 is rapidly switched off in differentiating cells during embryonic development (Pesce and Scholer, 2001). Hence, Oct4 expression levels can be used to monitor the differentiation status of ESCs.

We used an Oct4 reporter cell line (Oct4-Gip) in which the expression of GFP is controlled by Oct4 regulatory elements to establish an assay for analyses of ESC identity (Ying et al., 2002). Quantification of GFP fluorescence faithfully reflects the self-renewal and differentiation status in individual cells and

was thus used as a rapid and accurate readout to identify genes required for ESC identity (Figure 1A). To test the reliability of this assay, we transfected the Oct4-Gip cells with control esiRNA (Luc) and esiRNAs directed against known pluripotency genes, including Sox2, Stat3, and Oct4. The ratio of GFP-positive versus GFP-negative cells was determined by microscopy and by flow cytometry 96 hr posttransfection. In both cases, a loss of GFP expression was readily detected upon Sox2, Stat3, and Oct4 depletion, which coincided with differentiation of these cells (Figure S1). In contrast, no loss of GFP expression or differentiation was observed when the cells were transfected with the control esiRNA. To rule out the possibility that essential genes that are not required for ESC self-renewal, but are required for general cell growth and viability, would score in this assay, we transfected the Oct4-Gip cells with esiRNAs targeting genes with housekeeping functions, such as ribosomal, proteasomal, mitochondrial, and Pol II subunits. As expected, depletion of housekeeping genes strongly affected cell viability (Figure S1). However, none of these esiRNAs caused a significant loss of GFP-positive cells, demonstrating that this assay is highly specific to identify genes affecting ESC identity.

The screen was carried out in duplicate using a high-throughput FACS-based readout. EsiRNAs that reproducibly scored were identified using statistical methods (see the Supplemental Data for details). We nominated 296 esiRNAs, which significantly regulated GFP expression (Z score > 2, or Z score < −2) from the primary screen (Figure 1B, Table S1). GO term analysis indicated that transcription factors (25 genes) and gene expression regulators (51 genes) were significantly enriched in the primary hit list ($p < 0.05$), suggesting that this subset of genes can be a rich starting point to dissect the regulation of self-renewal and early differentiation of mouse ESCs.

The Screen Identified Known and Discovered Novel Candidate Pluripotency Genes

Many known pluripotency-controlling genes including Oct4, Sox2, Stat3, PI3K, Set1b, and Wdr5 scored with significant Z scores, demonstrating the effectiveness of the screen. We also identified many additional genes that have been linked to ESC biology, such as direct Oct4 and Nanog target genes (Cxxc1, Vti1a, Nfix, Etv1, Rad21, Ina, Cdh4, Spred2, Bach2, Myh9, Map3k7, Tcf12) (Fouse et al., 2008) and genes that are regulated by Oct4 and Nanog (Rnf2, Ncl, Ina, Spred2, Thbs3, Ell2, Tmem4, Etv1, Foxp1) (Loh et al., 2006). Some known pluripotency genes, including Esrrb, Tbx3, and Klf4, did not score significantly in the primary screen, possibly reflecting insufficient knockdown, redundancy, or that their roles in ESC identity are not reflected in Oct4 expression levels.

For this study, we concentrated on the genes with the strongest phenotypic scores (Z score > 4) and validated the phenotype of these candidates with a second, independent esiRNA. Twenty-nine independent esiRNAs were successfully synthesized (Table S2) and transfected into the Oct4-Gip ESCs. Twenty-one of these caused a reduction of GFP expression greater than two times the standard deviation, validating their role in maintaining Oct4-driven GFP expression (Table S2). Sixteen hits with Z scores above three were further analyzed. Gene ontology analyses placed these genes into different functional classes, including transcription regulation (Nfya, Ptbp1, Ctr9, Rtf1, Wdr61, Cpsf3, Fip1l1, Iws1, Thoc2), chromatin modulation (Rnf2, Cxxc1, Cnot1, Rtf1, Ctr9, Wdr61, Ncl), signaling (Apc), and protein degradation/DNA repair (Ube2m, Shfdg1).

Self-Renewal Assays

To substantiate a direct role of the 16 candidate genes in ESC identity, we performed three additional, independent self-renewal assays. First, we evaluated changes in cell morphology and alkaline phosphatase (AP) staining after esiRNA transfection. For the negative control (Luc)-transfected ESCs, nearly all of the colonies showed an undifferentiated morphology and highly positive AP staining (Figure 2A). In contrast, knockdown of most candidates resulted in reduced AP staining and obvious morphological changes in the Oct4-Gip cells, demonstrating the loss of pluripotency along with differentiation (Figure 2A, Figure S2). To exclude the possibility that these effects are Oct4-Gip cell-type specific, we performed the same experiments in R1/E ESCs and obtained identical results (Figure S2 and data not shown).

Second, we analyzed changes in cell-cycle profiles upon RNAi. Transfection of all tested esiRNAs lead to a reduction of cells in S phase (Figure 2B), demonstrating a change of cell-cycle regulation and consistent with an exit of these cells from self-renewal.

Third, we quantified endogenous Oct4 and Nanog transcript levels by qRT-PCR after esiRNA transfections. The transfection of all esiRNAs led to a reduction of Oct4 expression with 12 knockdowns, resulting in a significant ($p < 0.05$) or highly significant ($p < 0.01$) decrease of Oct4 transcript levels (Figure 2C), demonstrating that the Oct4-driven GFP expression closely mimicked the endogenous Oct4 expression. Many candidates also reduced Nanog expression (Figure 2D), albeit to a lower extent, possibly reflecting that some knockdowns primarily affect Oct4 expression. To test whether knockdown of the candidate genes induced ESC differentiation by interfering with the LIF/Stat3 cascade, we analyzed Stat3 and phosphorylated-Stat3 (Stat3-P) levels. We observed no obvious change of Stat3 and Stat3-P protein levels for the knockdowns, indicating that the loss of ESC identify was not mediated through perturbing the LIF/Stat3 pathway (Figure 2E). Collectively, these results suggest that candidate genes directly affect ESC identity through reduction of Oct4 levels.

The Paf1C Is Essential for ESC Identity

Among the validated knockdowns strongly reducing Oct4 levels in ESCs were the genes Rtf1 and Ctr9 (Figure 1B), two components of the Pol II-associating factor 1 complex (Paf1C). The Paf1C, minimally composed of Paf1, Ctr9, Cdc73, Rtf1, and Leo1, has been implicated in multiple processes such as transcription initiation and elongation, transcript start site selection, and RNA processing (Costa and Arndt, 2000; Penheiter et al., 2005; Stolinski et al., 1997). In addition, the Paf1C has been linked to histone modifications in different organisms (Adelman et al., 2006; Krogan et al., 2003) through a stimulation of H2B ubiquitination (Ng et al., 2003a), coupling Pol II elongation with SET1 and SET2 activities (Carrozza et al., 2005; Krogan et al., 2003; Ng et al., 2003b). Because of its potential role for the regulation of ESC chromatin and because two independent components of this protein complex were among the top hits, we selected the Paf1C for further analysis.

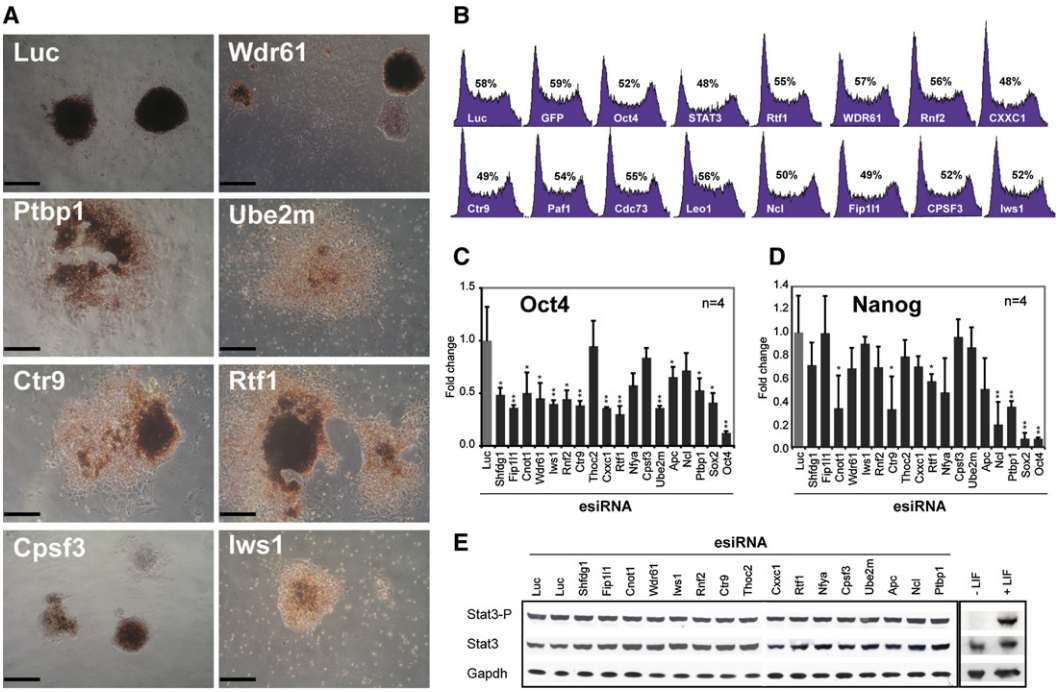

Figure 2. Functional Analysis of Validated Hits

(A) AP staining of ESCs 4 days after treatment with indicated esiRNAs. Note the loss of staining and the morphological changes of cells transfected with esiRNAs against Wdr61, Ptbp1, Ube2m, Ctr9, Rtf1, Cpsf3, and Iws1. Scale bars, 200 μm.

(B) Cell-cycle analysis of ESCs after gene knockdown. Cell profiles recorded 4 days after transfection with indicated esiRNAs are shown. The percentage of cells in S phase is indicated above each graph.

(C) Quantification of endogenous Oct4 transcript levels. qRT-PCR analysis performed 4 days after esiRNA transfection with indicated esiRNAs are shown.

(D) Quantification of Nanog transcript levels. qRT-PCR analysis performed 4 days after esiRNA transfection with indicated esiRNAs are shown.

(E) Analysis of Stat3 and Stat3-P protein levels. Protein extracts prepared from ESCs 4 days after transfection with indicated esiRNAs, and analyzed by immunoblotting with anti-Stat3, anti-Stat3-P, and anti-Gapdh (loading control) antibodies are presented. Cells grown for 4 days without LIF (−LIF) are shown as a positive control. (C and D) All values are means ± SD from at least triplicate experiments. * indicates significant ($p < 0.05$) and ** highly significant ($p < 0.01$) results based on Student's t test analyses.

To test whether the whole Paf1C is required for ESC identity, we knocked down the expression of the remaining known Paf1C components (Paf1, Leo1, and Cdc73) in ESCs with two independent esiRNAs and found differentiation phenotypes and decreased Oct4 expression levels similar to those observed upon Ctr9 and Rtf1 knockdown (Figures 3A and 3B and data not shown), demonstrating that the whole Paf1C is required to maintain ESC identity.

To further validate the role of the Paf1C for ESC identity, we performed cross-species RNAi rescue experiments (Kittler et al., 2005b) for three of the Paf1C components (Figures 3C–3E). Stable expression at physiological levels of the human Ctr9, Rtf1, and Leo1 genes in mouse ESCs rendered these cell lines resistant to the corresponding esiRNAs, but not to the esiRNAs targeting the other Paf1C components. Therefore, the human Ctr9, Rtf1, and Leo1 genes function in mouse ESCs and can substitute their mouse orthologs. More importantly, these results manifest an essential role of the Paf1C for ESC identity.

Paf1C Affects the Expression of Pluripotency and Lineage-Marker Genes

Next, we analyzed global transcript changes upon Paf1C depletion. For this purpose, we transfected ESCs with esiRNAs targeting Ctr9, a core component of the Paf1C (Adelman et al., 2006), and control esiRNA (Luc) and analyzed transcript changes using microarrays. This analysis identified a total of 1139 genes whose expression was perturbed significantly ($p < 0.0001$) after treatment with Ctr9 esiRNA (Table S3), with 529 and 610 genes downregulated or upregulated, respectively, suggesting that the Paf1C both activates and represses target genes. GO term analysis of these genes indicated that genes implicated in biological processes relevant to embryonic development such as cell morphology and motility, cell-cycle control, cell proliferation and differentiation, oncogenesis, and ectoderm and mesoderm formation were highly enriched (Table S4). Intriguingly, most known key regulators of pluripotency in ESCs, such as Nanog, Oct4, Tbx3, Esrrb Bmp4, Tcl1, Klf4, and Klf5 were downregulated upon Ctr9 depletion. In contrast, many lineage-control genes were strongly upregulated (Figure 3F), suggesting that the Ctr9 knockdown induced extensive differentiation. For example, ectoderm marker Fst increased 2-fold, and mesoderm markers Lef1 and Mest were upregulated 2.6-fold and 1.8-fold, respectively, indicating differentiation of the cells toward these lineages. Differentiation of the cells was also confirmed by monitoring Fgf5 protein levels in cells transfected with Ctr9 esiRNA, as measured by immunostaining with an Fgf5 antibody and

quantified utilizing an Fgf5 reporter cell line (Figure S3). Interestingly, we did not observe an upregulation of any gene implicated in endoderm development, suggesting lineage-restricted differentiation upon Ctr9 depletion.

To determine whether depletion of other Paf1C components results in similar expression changes, we knocked down Paf1, Rtf1, Leo1, and Cdc73 in ESCs and determined the expression changes of selected pluripotency and lineage-marker genes by qRT-PCR. Consistent with the Ctr9 microarray data, the knockdown of all other Paf1C components induced similar expression changes (Figure 3B) and hence validated the downregulation of pluripotency genes and upregulation of specific lineage-control genes upon Paf1C depletion. The induced expression of trophectoderm, ectoderm, and mesoderm, but not endoderm markers, suggests that regulation of the Paf1C in the early mouse embryo may contribute to lineage specification. To test whether the observed lineage-restricted differentiation is Paf1C specific, we tested the regulation of lineage-control genes upon knockdown of three other candidate genes (Shfdg1, Cnot1, and Ube2m) identified in the primary screen. These three knockdowns induced the expression of lineage markers, including endoderm markers Gata6 and Sox17 (Figure 3G), indicating that lineage-restricted differentiation is not generally observed upon knockdown of Oct4 modulators. In fact, the knockdown of Ube2m showed an upregulation of Gata6 and Sox17 only, indicating that the knockdown of Ube2m leads to endoderm-specific differentiation.

Depletion of the Paf1C by RNAi resulted in downregulation of both Oct4 and Nanog, suggesting that the Paf1C may be part of the Oct4-Nanog transcription circuit. Therefore, we compared the gene expression profiles upon Ctr9 knockdown to those obtained for Oct4 and Nanog knockdown (Loh et al., 2006). We compared all genes regulated with a stringent cutoff ($p < 0.0001$) and found a marked overlap between the Ctr9, Oct4, and Nanog knockdown profiles (Figure 3H; Table S6), with Pearson test correlation coefficients of 0.45 (Ctr9 versus Oct4), 0.50 (Ctr9 versus Nanog), and 0.58 (Oct4 versus Nanog). One hundred thirty genes were significantly regulated for all three knockdowns (Table S6). Importantly, genes that were significantly downregulated included the pluripotency genes Nanog, Esrrb, Tcl1, Bmp4, and Klf4.

Paf1C Binds to Promoters of Pluripotency Genes

To further study the function of Paf1C in mouse ESCs, we generated an ES line stably expressing a *l*ocation and *a*ffinity *p*urification (Cheeseman and Desai, 2005) (LAP)-tagged Ctr9 fusion protein using the *b*acterial *a*rtificial *c*hromosome (BAC)-based TransgeneOmics approach (Kittler et al., 2005b; Poser et al., 2008). Fluorescence microscopy analysis of cell clones expressing Ctr9-LAP showed a punctate, nuclear localization (Figure S4), consistent with a role of Ctr9 in regulating transcription/chromatin. To investigate whether pluripotency and lineage-control genes differentially regulated upon Paf1C depletion are direct targets of the Paf1C, we analyzed the binding of the Ctr9-LAP fusion protein by ChIP-chip and identified 2175 promoter regions that were bound by Ctr9 (Table S5). GO term analysis indicated that genes bound by Ctr9 were enriched for processes relevant for ESC biology, such as cell cycle, apoptosis, development, and chromatin packaging and remodeling (Figure 4A). Notably, many genes that are highly expressed in ESCs were not bound by the Paf1C (e.g., Ubiquitin B, α-tubulin 2, Enolase 1, Hexokinase 1, Calmodulin 2, etc.), suggesting that the Paf1C is not present at promoters of all actively transcribed genes. A closer inspection of genes falling into the developmental classification indicated that promoters of many pluripotency genes, such as Oct4, Nanog, and Sox2 were bound by the Paf1C (Figure 4B). The comparison of genes that were downregulated upon Ctr9 RNAi (see Figure 3 and Table S3) with genes that are bound by Ctr9 (Table S5) revealed a highly significant overlap (p value, 3.03e-07) and included pluripotency genes such as Oct4 and Nanog. Therefore, the Paf1C directly influences the expression of important pluripotency genes.

To further investigate the binding of the Paf1C to pluripotency and lineage commitment genes, we performed chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) of selected genes. Marked Ctr9-LAP binding was observed 5′ proximal to the transcription start sites and coding regions of seven out of eight tested pluripotency genes (Figures 4C and 4D). This finding suggests that the Paf1C binds close to the transcription initiation site of pluripotency genes in ESC and likely travels alongside Pol II during elongation. In contrast, weak or no signals were detected for most lineage-marker and housekeeping genes. An exception was the gene Gata6, a marker for endoderm development (Hay et al., 2004). We measured strong Ctr9 binding at the Gata6 promoter, but no signal in the coding region, indicating that the Paf1C occupies this promoter before the gene is expressed. Collectively, these results suggest that the Paf1C is a specific regulator of transcription for a subset of genes, which in ESCs include many pluripotency genes.

Paf1C Is Required to Maintain the Chromatin Structure of Pluripotency Genes in ESCs

A potential mechanism for Paf1C action on the promoters of pluripotency genes may be the modulation of the local chromatin structure. In yeast, the Paf1C has been implicated in multiple aspects of histone methylation via the recruitment of methyltransferase complexes to Pol II (Krogan et al., 2003). To investigate a potential role of the Paf1C for histone methylation in mouse ESCs, we analyzed the effects of Ctr9 depletion on histone modifications associated with actively transcribed (H3K4 trimethylation or H3K4me3) and repressed (H3K27 trimethylation or H3K27me3) chromatin for promoter regions of selected genes. ChIP-qPCR analyses indicated that H3K4me3 levels on promoters of pluripotency genes strongly decreased upon Ctr9 depletion, suggesting that the Paf1C is required for the maintenance of H3K4me3. For the lineage-control genes, the H3K4me3 levels remained essentially unchanged, whereas the H3K27me3 levels for the ectoderm and the mesoderm specification genes decreased markedly (Figures 4E and 4F). To substantiate this finding, we performed Paf1C double knockdowns with the trithorax group-like, Set1 complex subunit gene, Cxxc1 (required for H3K4me3), and the polycomb group Pc1 complex subunit gene, Rnf2/Ring1b (required for H3K27me3), both of which individually affect ESC pluripotency (Fazzio et al., 2008; Lee and Skalnik, 2005; Figure 1B). We observed a robust enhancement of the phenotype when Ctr9 or Rtf1 was cosilenced together with Cxxc1, but no effect when the same Paf1C subunits were cosilenced together with

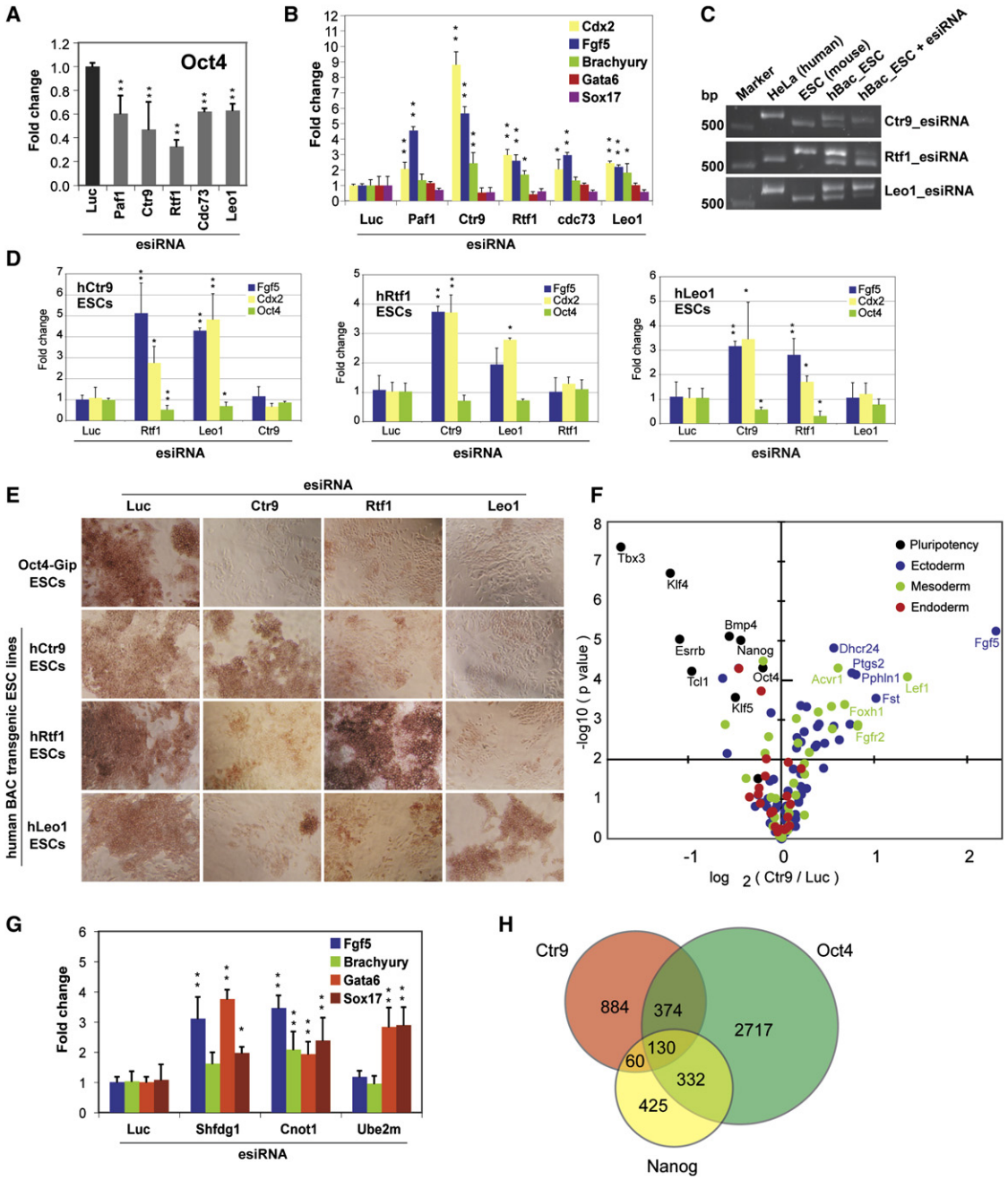

Figure 3. Paf1C Affects the Expression of Pluripotency and Lineage-Marker Genes

(A) RNAi of all Paf1C components leads to downregulation of endogenous Oct4 expression. qRT-PCR quantifications of Oct4 expression levels 4 days posttransfection of indicated esiRNAs are presented.

(B) RNAi of all Paf1C components induces similar lineage-restricted differentiation. qRT-PCR quantifications of indicated ectoderm (blue), mesoderm (green), and endoderm (light and dark red) markers after transfection with indicated esiRNAs are shown.

(C) RNAi in human BAC-transgenic ESC lines specifically depletes the mouse transcripts. PCR fragments digested with a restriction enzyme discriminating between the human and mouse transcripts are shown for human cells (HeLa), WT ESC (ESC), human BAC-transgenic ESCs (hBAC_ESC), and human BAC-transgenic ESCs transfected with indicated esiRNAs (hBAC_ESC + esiRNA), respectively. Note the reduced band intensities of the mouse product after esiRNA transfections in the human BAC-transgenic ESCs. The 500 base pair (bp) band of the marker is shown on the left.

(D) Oct4 and lineage-marker expression analyses in human BAC-transgenic ESCs. qRT-PCR analysis of Oct4 (green), Cdx2 (yellow), and Fgf5 (blue) after transfection of indicated esiRNAs in the indicated human BAC-transgenic ESC lines are shown. Note the rescue of the phenotypes in cell lines transfected with the corresponding esiRNAs.

(E) Expression of the human gene in the mouse ESCs rescues the phenotypes. AP stainings of indicated ESC lines with indicated esiRNAs are shown.

(F) Expression changes of selected genes after Ctr9 depletion. Pluripotency genes (black), lineage-marker genes, ectoderm (blue), mesoderm (green), and endoderm (red) are plotted as $\log_2$(Ctr9/Luc) versus $-\log_{10}$ (p value).

Rnf2 (Figure 4G and Figure S5A). Inspection of Oct4 expression levels and changes in the expression of the differentiation markers Cdx2, Fgf5, and Brachyury indicated that the simultaneous depletion of Paf1C with Cxxc1 enhanced the expression of these differentiation markers (Figure S5B). Together, these results indicate that the Paf1C synergizes with the Set1 complex to maintain ESC pluripotency and support a direct role of the Paf1C in maintaining H3K4me3 at promoter regions of pluripotency genes.

Proteomic Analyses of Paf1C in ESCs

The Paf1C is composed of at least five subunits, Paf1, Ctr9, Rtf1, Cdc73, and Leo1. Recently, the human Paf1C complex has been shown to interact with hSki8, a component of the SKI complex, which together with the exosome mediates 3′-5′ mRNA degradation (Zhu et al., 2005). This interaction suggests a possible link of the human Paf1C to RNA quality control and extends the proteins that comprise the human Paf1C. Interestingly, the mouse ortholog of hSki8, Wdr61 was among the 16 validated strongest hits in our primary screen (Figure 1B), suggesting a potential role of this gene in mouse ESCs as a component of the Paf1C.

To identify potential interaction partners of the Paf1C in ESCs, we performed a proteomic analysis using the Ctr9-LAP and a Leo1-LAP-tagged ESC line. Both lines express similar levels of the LAP-tagged and the endogenous Paf1C transcripts (Figure S4). After affinity purification with an anti-GFP antibody, prey proteins were eluted and analyzed by mass spectrometry. This analysis revealed most of the known Paf1C components (Table 1). In addition, several Pol II-associated and chromatin-modifying proteins were immunoprecipitated, including Pabc1, Ruvbl1, Ruvbl2, Sfpq, Tceb3, Polr2a, Polr2e, CoREST, and Smarce1, implying a complex interacting network of Paf1C with other transcriptional and chromatin regulators. Importantly, Wdr61 was identified as a Paf1C interaction partner by both Ctr9-LAP and Leo1-LAP pulldowns, authenticating the interaction of this protein with the Paf1C in mouse ESCs (Table 1). To validate the interaction of Wdr61 with Paf1C, we generated a Wdr61-LAP ESC line and analyzed its interaction partners via mass spectrometry. The Wdr61-LAP pulldown identified two Paf1C components, Cdc73 and Leo1, and the Pol II-associated proteins Ruvbl1 and Ruvbl2, further validating the interaction between Wdr61, the Paf1C, and several other transcriptional regulators (Table 1). Similar knockdown phenotypes and protein-protein interactions strongly suggest that Wdr61 functions together with Paf1C to maintain ESC identity.

Paf1C Is Downregulated during Embryoid Body Formation

To analyze possible expression changes of the Paf1C during early development, we analyzed the expression of Paf1C subunits during ESC differentiation in embryoid bodies (EBs). For all Paf1C subunits, downregulated expression was observed during the formation of EBs (Figure 5A), indicating that the Paf1C is downregulated during early embryonic development. To test whether the reduction in Paf1C subunit expression levels is just part of a general decrease in the expression of Pol II-associated protein complexes during differentiation, we analyzed the expression levels of subunits of the Pol II-associated mediator complex in EBs (Myers and Kornberg, 2000). In contrast to the Paf1C, transcript levels of the investigated mediator complex subunits did not change significantly during EB formation (Figure 5A). Hence, the downregulation of the Paf1C may be important for proper embryonic development. Support for a role of the Paf1C during development comes from observations in *Drosophila* and *Zebrafish*, in which a variety of embryonic developmental defects have been described for Paf1C component depletions (Akanuma et al., 2007; Tenney et al., 2006), and from Cdc73 knockout mice, which develop normally up to E3.5 but then die either at hatching or implantation (Wang et al., 2008).

Paf1C Overexpression Blocks ESC Differentiation

Many pluripotency genes block differentiation when overexpressed (Turksen, 2001; Chambers et al., 2003). To test whether overexpression of a Paf1C component can block ESC differentiation, we transfected a Sox1-GFP reporter cell line (Aubert et al., 2003) with tetracycline-inducible Ctr9 and control constructs (Figures 5B and 5C). Sox1 is not expressed in undifferentiated ESCs but is strongly induced during neuronal differentiation (Aubert et al., 2003) and during cultivation in a neuronal growth and differentiation medium (Diogo et al., 2008). Because we observed a prominent activation of ectodermal genes upon Paf1C RNAi, potentially overexpression of a Paf1C component will block differentiation, as reported by the acquisition of Sox1-GFP expression. Indeed, a marked reduction of GFP-positive cells upon transfection of the Ctr9 construct was only detected when tetracycline was present in the culture medium (Figures 5D and 5E). A block of differentiation was further supported by analyses of the differentiation markers Fgf5 and Nestin and the pluripotency factor Oct4 in these cells (Figure 5F). These data demonstrate that an excess of a Paf1C component can block the differentiation of ESCs.

DISCUSSION

A detailed molecular understanding on how genetic factors influence the balance between pluripotency and differentiation of mammalian cells is crucial to develop ESCs for their potential therapeutic use. This understanding is becoming increasingly important because it is now possible to generate ESC-like cells from somatic cells via direct reprogramming (reviewed in Jaenisch and Young, 2008), avoiding many ethical issues. In the future it seems feasible to bank personalized iPS cells that may be used to generate differentiated cells to replace damaged tissue in the body when needed. Obviously, this scenario

(G) Knockdown of Shfdg1, Cnot1, and Ube2m induces the expression of lineage markers, including endoderm. qRT-PCR quantifications of indicated genes with indicated esiRNAs are shown.
(H) Venn diagram of the number of genes regulated by Ctr9, Oct4, and Nanog. The diagram shows the overlap of genes affected by Ctr9 (red), Oct4 (green), and Nanog (yellow) knockdowns. (A, B, D, and G) All values are means ± SD from at least triplicate experiments. * indicates significant ($p < 0.05$) and ** highly significant ($p < 0.01$) results based on Student's t test analyses.

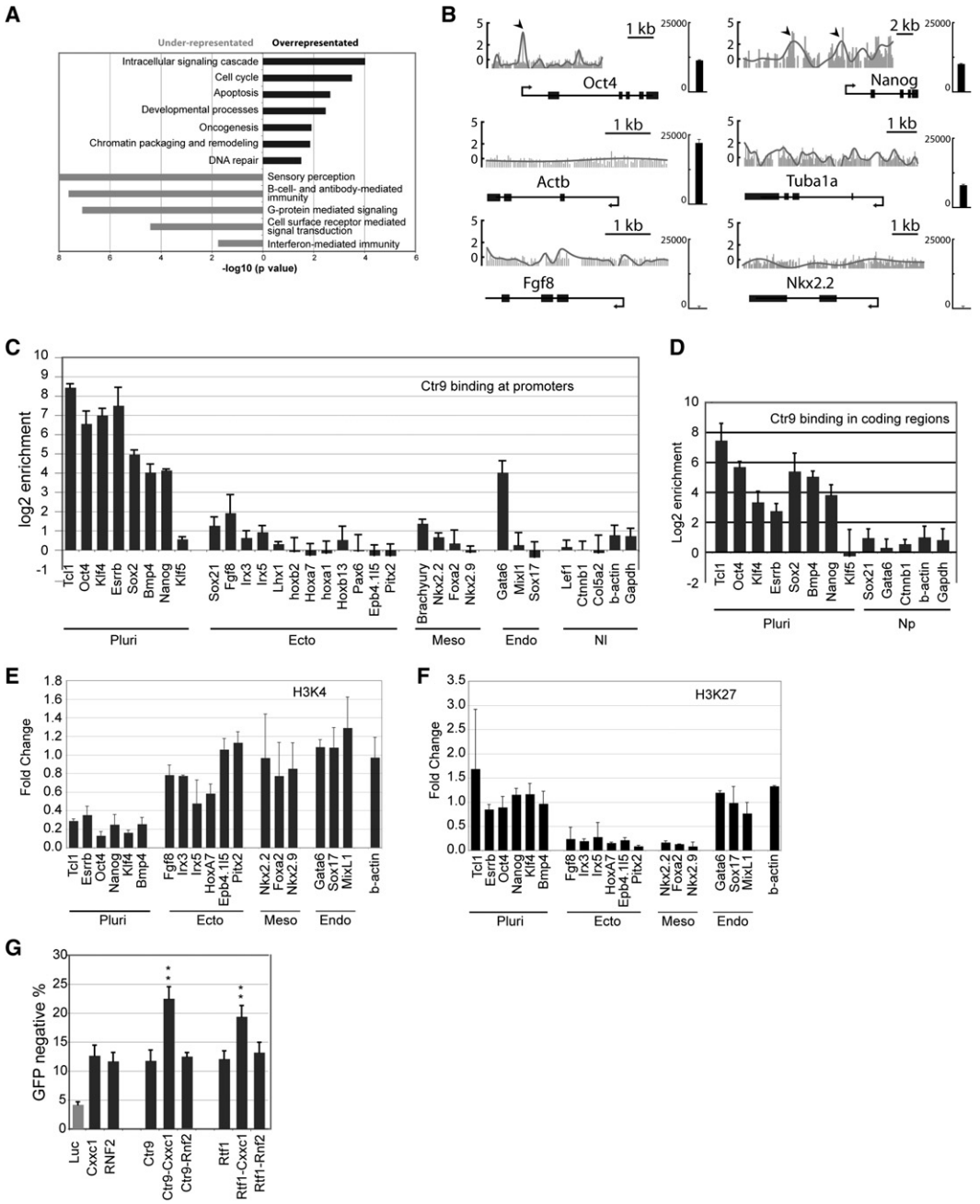

Figure 4. Ctr9 Binds to Promoters and Coding Regions of Pluripotency Genes and Is Required to Maintain the Chromatin Structure in ESCs

(A) ChIP-chip analysis of Ctr9 target genes. GO term enrichment analysis of selected overrepresented and underrepresented categories are presented.

(B) Ctr9-binding profiles of selected genes, extracted from the ChIP-chip experiments. The binding profiles of the pluripotency genes Oct4 and Nanog, the housekeeping genes β-actin (Actb) and α-tubulin (Tuba1a), and the differentiation genes Fgf8 and Nkx2.2 are shown. Arrows indicate significant enrichment peaks for Ctr9 binding. The genomic structure of the genes is shown below the profiles with an arrow indicating the predicted transcriptional start site. The relative expression of the genes in ESCs is shown to the right of each profile.

(C) Ctr9-ChIP-qPCR analysis at the promoter regions of indicated genes grouped for pluripotency (pluri), ectoderm (ecto), mesoderm (meso), endoderm (endo), and nonlineage (nl) control genes. Log2 enrichment represents the abundance of enriched DNA fragments over mock controls.

(D) Ctr9-ChIP-qPCR analysis at the coding regions of indicated genes. Np; nonpluripotency genes.

(E) Depletion of Ctr9 results in a decrease of H3K4me3 on the promoters of pluripotency genes. ChIP assays for indicated genes are shown. Fold enrichment represents the abundance of enriched DNA fragments over mock controls.

(F) Depletion of Ctr9 results in a decrease of H3K27me3 on the promoter of some lineage-control genes. ChIP assays for indicated genes are shown.

(G) Synthetic analysis of Paf1C components with Cxxc1 and Rnf2. FACS quantification of GFP-negative cells 4 days after transfection of Oct4-Gip ESCs with indicated esiRNAs are shown. (C, D, E, F, and G) All values are means ± SD from at least triplicate experiments. * indicates significant ($p < 0.05$) and ** highly significant ($p < 0.01$) results based on Student's t test analyses.

Table 1. Proteomic Analyses of the Paf1C in ESCs

Baits	Name	Accession Number	Mass	Score	Matches	PEP_UNIQ	SEQ_COV
Ctr9	Ctr9	IPI00477468	133,420	2,177	55	43	44%
Ctr9	Paf1	IPI00331654	60,481	1,140	28	23	54%
Ctr9	Cdc73	IPI00170345	60,539	1,116	28	25	52%
Ctr9	Leo1	IPI00474486	76,912	817	17	13	23%
Ctr9	Wdr61	IPI00112320	33,778	640	21	13	59%
Ctr9	Pabpc1	IPI00124287	70,626	414	8	8	17%
Ctr9	RuvB-like 1	IPI00133985	50,182	272	6	6	17%
Ctr9	COREST	IPI00226581	53,272	201	4	4	12%
Ctr9	RuvB-like 2	IPI00123557	50,949	182	4	4	9%
Ctr9	SFPQ	IPI00129430	75,394	148	3	3	7%
Leo1	Leo1	IPI00103090	75,359	798	25	17	22%
Leo1	Ctr9	IPI00120919	133,420	960	26	24	17%
Leo1	Cdc73	IPI00170345	60,539	905	27	20	35%
Leo1	Paf1	IPI00331654	60,481	788	18	14	26%
Leo1	Wdr61	IPI00112320	33,752	472	14	10	27%
Leo1	Tceb3	IPI00317167	87,124	378	11	10	13%
Leo1	Polr2a	IPI00136207	217,039	153	5	5	2%
Leo1	Smarce1	IPI00119892	46,610	116	2	2	5%
Leo1	RuvB-like 2	IPI00123557	51,081	111	2	2	4%
Leo1	Polr2e	IPI00337955	24,555	107	3	3	14%
Wdr61	Wdr61	IPI00019269	33,557	806	19	13	51%
Wdr61	Cdc73	IPI00170345	60,539	208	5	5	13%
Wdr61	Leo1	IPI00474486	75,596	116	2	2	3%
Wdr61	SF3B4	IPI00154082	44,327	196	3	3	10%
Wdr61	RuvB-like 1	IPI00133985	50,182	175	6	6	18%
Wdr61	RuvB-like 2	IPI00123557	51,081	101	3	3	8%

Abbreviations: Baits, Bait used for IP; Name, Name of the gene; ACC_NO, accession number in International Protein Index; Mass, molecular weight of predicted protein; Score, probability-based MOWSE score of MASCOT software; Matches, number of total matched peptides via MASCOT; PEP_UNIQ, number of unique peptide sequences identified via MASCOT; SEQ_COV, percentage of predicted protein sequence covered by matched peptides via MASCOT.
Detailed information on the mass spectrometry analyses is provided in Table S8.

requires a detailed and systematic understanding of ESC/iPS self-renewal and differentiation to develop protocols for safe tissue replacement therapies.

We performed a genome-scale RNAi screen to initiate a global survey of genes required to maintain mouse ESC identity. This screen identified 296 candidate genes with numerous functions that may influence Oct4 expression levels, suggesting a complex interplay of several biological processes required to maintain pluripotency. This data set should present a useful resource to characterize factors that influence ESC self-renewal and ultimately may also be useful to improve the reprogramming protocols of somatic cells. Oct4 is one of the factors used to reprogram somatic cells into iPS cells (reviewed in Jaenisch and Young, 2008). Thus, identifying the genes that regulate the expression of Oct4 should be instrumental for understanding the reprogramming process. Understanding the endogenous regulation of Oct4 may reveal alternative ways to activate Oct4 expression in somatic cells. As such, the genes that alter the expression of Oct4 upon RNAi present a good starting point to unravel the network of Oct4 regulation.

Most of the strongest hits identified in our screen are transcription factors and/or chromatin modifiers that regulate transcriptional processes. Modulation of transcription activity requires the interaction of transcription factors, the Pol II basal transcription machinery, and factors regulating chromatin structure. The enrichment of transcription factors and chromatin modifiers on the one hand highlights the crucial role of transcription regulation on ESC fate decision and, on the other hand, offers an opportunity to unravel the interactions between transcription factors, chromatin modifiers, and their target DNA for the maintenance of ESC identity. Here we focused on the Paf1C because we had two independent, highly significant, starting hits, and also we were excited by the potential discovery of a novel regulatory mechanism.

The Paf1C was originally identified in yeast as a specific Pol II-associated protein complex biochemically distinct from the Srb/Med-containing Pol II holoenzyme (Shi et al., 1997). Further genetic and biochemical studies showed that the Paf1C is implicated in transcript start site selection (Stolinski et al., 1997), initiation and elongation (Costa and Arndt, 2000; Mueller and Jaehning, 2002), poly(A) site utilization (Penheiter et al., 2005), and

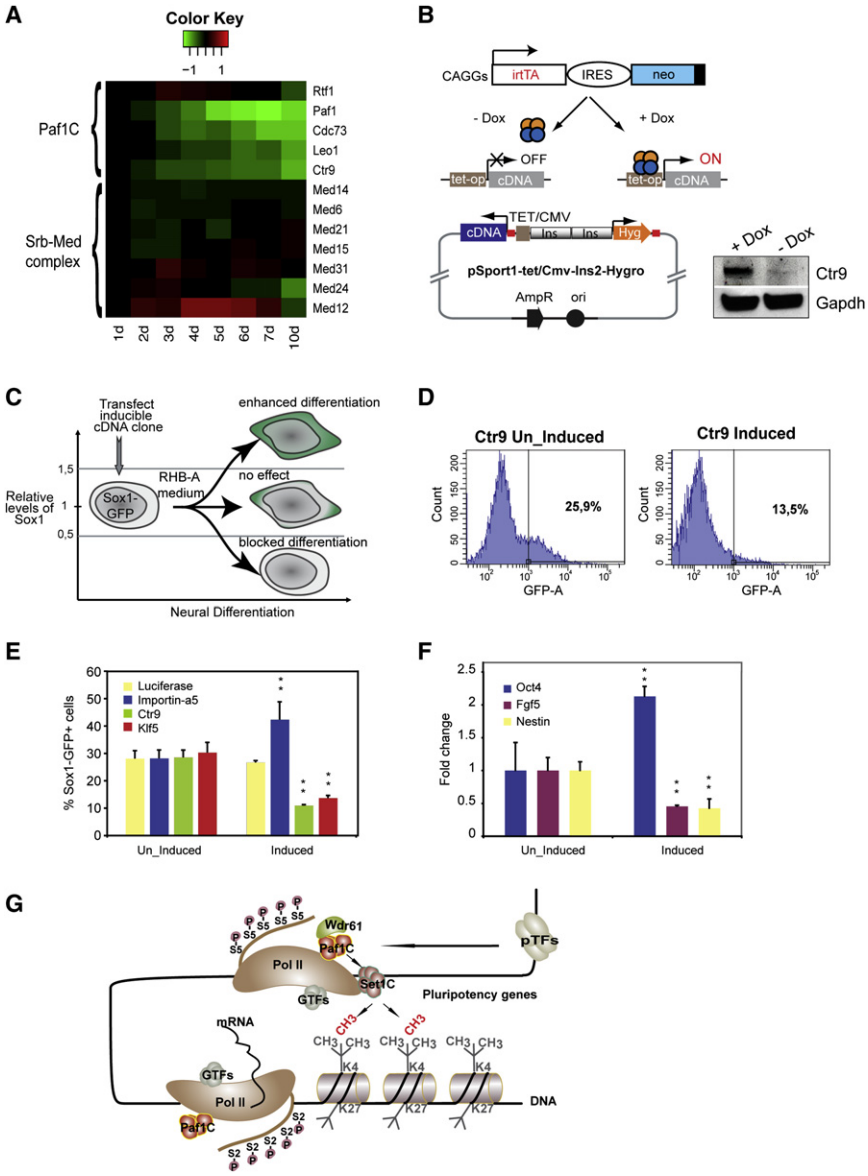

Figure 5. Paf1C Is Downregulated during Embryoid Body Formation, and Overexpression Blocks ESC Differentiation

(A) Expression changes of indicated genes from the Paf1C and the mediator complex (Srb-Med) during EB formation are shown. The heat map shows data (log2 values) extracted from gene expression arrays of RNA hybridized from day 1 to day 10 (1d–10d).

(B) Shown are constructs used to transfect the Sox1-GFP ESCs. Relevant elements and a scheme of the function of the inducibility of the expression constructs by doxycycline (−Dox, +Dox) is presented. A western blot analysis shows the induced expression of Ctr9 after doxycycline treatment. CAGGs, chicken β-actin promoter coupled to a CMV early enhancer; irtTA, reverse-tetracycline repressor; IRES, internal ribosome entry site; neo, neomycin resistance gene; Ins, chicken β-globin insulator; tet-op, tetracycline operator-binding sites; Hyg, hygromycine resistance gene; AmpR, ampiciline resistance gene; ori, ColE1 origion of replication.

(C) Scheme of the differentiation assay. Arrows show possible outcomes after induced expression of cDNA constructs. The variable amount of GFP expression in the cells is indicated by different intensities of green.

(D) FACS profiles and the percentage of GFP-positive Sox1-GFP cells without (Ctr9 Un_Induced) and with (Ctr9 Induced) Ctr9 overexpression are presented.

(E) Comparison of different genes blocking, or enhancing, differentiation in the Sox-GFP assay. The percentage of GFP-positive cells after transfection of cDNA constructs of indicated genes, with and without doxycyline treatment, are presented.

(F) Expression changes of indicated genes upon overexpression of Ctr9 (induced), or without Ctr9 overexpression (Un_induced), are shown.

(G) Model for Paf1C function in ESCs. The model depicts the concerted action of pluripotency transcription factors (pTFs), the Paf1C, and the Set1C to maintain active transcription (H3K4me3) of pluripotency genes. GTFs, general transcription factors; S5-P, phosphorylated serine 5 of the CTD tail of Pol II; S2-P, phosphorylated serine 2 of the CTD tail of Pol II. Values in (E) and (F) are means ± SD from experiments done in triplicate. ** indicates highly significant ($p < 0.01$) results based on Student's t test analyses.

histone methylation (Krogan et al., 2003), suggesting a complex role of the Paf1C for gene regulation in yeast. In metazoans, the Paf1C has additionally been implicated in Notch and Wnt signaling, indicating that this protein complex participates in biological processes such as oncogenesis and embryogenesis (Akanuma et al., 2007; Mosimann et al., 2006; Tenney et al., 2006).

In this manuscript, we demonstrate an important role of the Paf1C for the maintenance of mouse ESC identity. Knockdown of all known complex subunits led to a decreased expression of Oct4 and other pluripotency markers, accompanied by a loss of ESC self-renewal and subsequent differentiation. These phenotypic consequences could be rescued by physiological expression of the human Paf1C components. Global gene expression analysis after Paf1C depletion revealed a change in the expression of specific genes, with most known pluripotency genes being downregulated. This observation was substantiated by ChIP studies, where the Paf1C was found to directly bind to the promoters of many pluripotency genes. Hence, the Paf1C is important for the sustained expression of pluripotency genes in mouse ESCs. The large overlap of regulated genes upon Oct4, Nanog, and Ctr9 knockdown argues that the Paf1C is an integral part of the Oct4-Nanog regulatory circuit. Because the Paf1C interacts with the Pol II machinery, we hypothesize that the Paf1C integrates signals from the pluripotency transcription factors to establish a specialized Pol II complex that maintains

active transcription of these genes (Figure 5G). The observed downregulation of Paf1C components during EB formation and the block of differentiation upon overexpression further supports this notion.

The recent identification of "bivalent" promoters carrying both H3K4me3 and H3K27me3 in ESCs identified a subset of genes crucial to lineage commitment decisions. To this subset, we now add another important subset of genes regulated by the Paf1C, which are crucial to self-renewal. Furthermore, our study identifies genes whose downregulation may be essential to initiate differentiation into specific lineages in the developing embryo.

Because the role of the Paf1C is multifaceted, its role in maintaining pluripotency may also be complex. Here we show that the Paf1C is required for the maintenance of H3K4me3 on pluripotency genes. The selective binding of the Paf1C to the promoters of pluripotency genes combined with the synergistic effect of Paf1C knockdown with a key subunit of the Set1 H3K4 methyltransferase complex indicates a direct role of the Paf1C in the modulation of chromatin structure at pluripotency genes in ESCs. In yeast, the Paf1C is required for recruitment of H3K4 methyltransferase activity to Pol II (Krogan et al., 2003; Ng et al., 2003b). Our data suggest a similar role of the Paf1C at pluripotency genes in ESCs. According to this model (Figure 5G), depletion of the Paf1C would result in a decrease of the Set1 complex at promoter regions of pluripotency genes, which consequently would result in the loss of H3K4 trimethylation. As a result, expression levels of pluripotency genes would diminish, initiating the differentiation process.

The proteomic analyses of the Paf1C components support an intricate role of this protein complex in ESCs. Whereas both Ctr9 and Leo1 pulldowns identified most known Paf1C components, they also revealed specific interactions with other Pol II-associated factors. Interestingly, some of these specific interactors have been implicated in ESC pluripotency. For instance, Tceb3, a Leo1-interacting protein, has been shown to regulate transcription of a subset of genes linked to cell-cycle progression in mouse ESCs (Yamazaki et al., 2003).

Lineage-restricted differentiation upon Paf1C depletion was unexpected. Instead of uniformly exiting from self-renewal into all four alternatives (trophectoderm, endoderm, ectoderm, mesoderm), Paf1C-depleted ESCs appear impaired to differentiate toward endoderm. Furthermore, we note that the promoter region of the endoderm control gene Gata6 was occupied by the Paf1C before the gene was expressed. Consequently, we speculate that key endoderm control genes require the Paf1C for the induction of their expression and that Paf1C plays a specific role in early endodermal commitment.

In practical terms, the lineage-restricted ESC differentiation upon Paf1C and Ube2m knockdowns may be useful for the directed generation of specific cell types from ESCs. To date, strategies to accomplish this goal mainly rely on overexpression of lineage commitment genes and the use of certain growth-factor-enriched media (Turksen, 2001). Our study suggests that enhanced ESC differentiation protocols can be developed by combining these protocols with the depletion of certain genes by RNAi. These experimental modifications should improve the efficiency for generating specific cell types for experimental or therapeutic transplantation.

In summary, our work provides a global view of ESC pluripotency, revealing that an integrated analysis of transcription, chromatin structure, signaling, and possibly other biological processes is needed to fully understand ESC pluripotency. By analyzing one of the protein complexes in more detail, we have begun to uncover specific connections between these processes, in which interplay of transcription factors, Pol II-associated factors, and chromatin regulation is needed for maintaining ESC identity. A picture emerges in which specific transcription factor combinations work in concert with specialized Pol II complexes assembled on particularly marked chromatin structures to control the expression of pluripotency genes.

EXPERIMENTAL PROCEDURES

Mouse esiRNA Library

The templates for the esiRNA synthesis were generated using the cDNA library Mouse Unigene Set, RZPD 2.1 (Imagenes, http://www.imagenes-bio.de/products/sets_libraries/non_redundant_sets). EsiRNAs were synthesized as described previously (Kittler et al., 2005a) and normalized to 100 ng/μl in 384-well plates for the genome-scale screen (Table S1).

Cell Culture and High-Throughput Screen

ESCs (E14TG2a, R1/E, Oct4-Gip [Ying et al., 2002], and BAC-transgenic ESC lines) were cultured on gelatin-coated plates in Glasgow Minimum Essential Medium (Sigma) supplemented with 10% FBS (Pan biotech), 2.2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 1× NEAA (Invitrogen), and LIF (generated in house) as previously described (Bernstein et al., 2006). ESCs were trypsinized and split every 2 days, and the medium was changed daily. For the genome-scale screen, reverse transfections were performed using mixtures of 20 ng esiRNA and 0.075 μl Lipofectamine 2000 (Invitrogen) in 10 μl optimum medium (Invitrogen). ESCs were plated in 384-well plates with a density of 900 cells per well in 60 μl ESC medium. On each plate, eight negative controls (Luciferase esiRNA) and eight positive controls (four GFP esiRNAs, four Sox2 esiRNAs) were placed to monitor the transfection efficiency. GFP fluorescence and cell numbers were measured 96 hr posttransfection using a FACS Calibur (BD Biosciences) equipped with an HTS loader for high-throughput readout.

Alkaline Phosphatase Staining

ESCs (2×10^3) were reverse transfected with 50 ng esiRNAs and 0.2 μl Lipofectamine 2000 in 96-well plates. Four days posttransfection, ESCs were fixed in 4% paraformaldehyde (Sigma) for 5 min at room temperature. After two times rinsing with PBS, ESCs were stained using the Alkaline Phosphatase Red Microwell Substrate (Sigma).

Western Blotting

Oct4-Gip cells (8×10^4) were reverse transfected with 800 ng esiRNAs and 2 μl Lipofectamine 2000 in 6-well plates. Four days posttransfection, ESCs were harvested, and 10 μg of protein extracts were separated using NuPAGE 4%–12% Bis-Tris protein gels (Invitrogen) and blotted to nitrocellulose membrane (Millipore). The membranes were probed with the primary antibodies against Stat3 (H-190; sc-7179, Santa Cruz), phospho-Stat3 (Tyr705; 44-380G, Invitrogen), and Gapdh (NB300-221, Novus Biologicals).

RT-qPCR

Total RNA was isolated by using the RNeasy Mini kit (QIAGEN), and 1 μg RNA was reverse transcribed with SuperScript III Reverse transcriptase (Invitrogen) utilizing an oligo$(dT)_{18}$ primer. qPCRs were performed with the SYBR Green qPCR kit (Abgene) on an MX P3000 qPCR machine (Stratagene). Measured transcript levels were normalized to Gapdh. Samples were run in triplicate. Primers used are listed in Table S7.

Gene Expression Analyses

Oct4-Gip cells (8×10^4) were reverse transfected with 800 ng esiRNAs and 2 μl Lipofectamine 2000 in 6-well plates. Four days posttransfection, RNA was

prepared using the RNeasy Mini kit (QIAGEN) and labeled with the One-Cycle Target Labeling and Control Reagent Package (Affymetrix), as described in the manufacturer's instructions. Extracts from four biological replicated were hybridized to Mouse Genome 430 2.0 arrays (Affymetrix), and the data were analyzed by hierarchical clustering using Cluster 3.0 software. Affymetrix data are accessible through the GEO series under accession number GSE12078.

Cell-Cycle Profiling

Oct4-Gip cells were transfected as for the gene expression analyses. After 96 hr, cells were trypsinized, washed with PBS, and fixed overnight with ice-cold 70% ethanol. After washing with PBS, cells were stained with PI solution (25 μg/ml PI, 20 μg/ml RNaseA, 0.02% Triton X-100) for 30 min in the dark, and the cell-cycle profiles were acquired by FACS.

Cross-Species RNAi Rescue

Cross-species RNAi rescue experiments were performed as previously described (Kittler et al., 2005b). Briefly, Oct4-Gip and human BAC-transgenic ESC lines were generated and transfected with esiRNAs, which only target the mouse transcripts. Mouse-specific esiRNAs were generated by using primers listed in Table S7. Cells were transfected as for the gene expression analyses. Four days posttransfection, cells were stained with alkaline phosphstase, and RNAs from esiRNA-transfected ESCs were prepared by using the RNeasy Mini kit (QIAGEN) and reverse transcribed with SuperScript III Reverse transcriptase (Invitrogen). qPCRs were performed as described before to quantify the expression of Cdx2, Fgf5, and Oct4. Samples were run in triplicate, and measured transcript levels were normalized to Gapdh. To evaluate the specificity and efficiency of knockdowns, cDNA fragments from HeLa cells, WT mouse ESCs, human BAC-transgenic ESCs, and human BAC-transgenic ESCs transfected with esiRNAs were PCR amplified with primers that perfectly match the human and mouse transcripts (for primers, see Table S7). To discriminate the human and mouse transcripts, PCR products for Ctr9, Rtf1, and Leo1 were digested with XhoI, XbaI, and PstI, respectively, and separated on a 2% agarose gel.

Chromatin Immunoprecipitation and Protein Identification by Mass Spectrometry

ChIP assays of mouse ESCs were essentially performed as previously described by Bernstein et al. (2006) using antibodies against H3K4 (Ab8580, Abcam) and H3K27 (07-449, Upstate). LAP tag-based ChIP assays were performed with BAC-transgenic ESC lines as previously described using a polyclonal goat anti-GFP antibody (Poser et al., 2008).

BAC-transgenic ESC lines Ctr9-LAP, Leo1-LAP, Wdr61-LAP, and Rtf1-LAP were generated by tagging the BACs RP11-77G5, RP11-56B16, RP11-1132M10, and RP11-16O9, respectively.

Enrichments of target genes were quantified by qPCR with primers listed in Table S7. Genome-wide location analysis was carried out using mouse promoter array 1.0R array (Affymetrix). The data are accessible through the GEO series accession number GSE14654.

GFP-tagged protein complexes were isolated by immunoaffinity chromatography as previously described (Poser et al., 2008) and analyzed by mass spectrometry. See the Supplemental Data for details.

Overexpression Studies in Sox1-GFP ESCs

The pSport1-tet/Cmv-Ins-Hygro expression constructs contain full-length cDNAs (Luciferase, Importin-a5, Ctr9, and Klf5) under the control of a tetracycline-inducible CMV-minimal promoter. The Sox1-GFP (46C) ESC line (Ying et al., 2003), stably expressing a codon-optimized tetracycline activator (irtTA; Anastassiadis et al., 2002) from the CAGGs promoter, was grown in a chemically defined medium (ESGRO complete, Millipore) with BMP4 and LIF. The cells were then trypsinized and plated in RHB-A medium (Stem Cell Sciences) on gelatin-coated 6-well plates in duplicates and retrotransfected at day 0 with the tetracycline-inducible plasmids (1 μg each) plus the normalization plasmid ptet-Gaussia Luciferase (200 ng) in the presence or absence of doxycyline (1 μg/ml). RHB-A medium was changed daily. At day one, the supernatant was collected, and the Gaussia luciferase activity was measured to normalize for transfection efficiencies. At day three, cells were collected, fixed in 2% paraformaldehyde, and analyzed by FACS. At the same time, RNA was extracted for expression analyses by qRTPCR.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, five figures, and eight tables and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00110-6.

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REFERENCES

Adelman, K., Wei, W., Ardehali, M.B., Werner, J., Zhu, B., Reinberg, D., and Lis, J.T. (2006). Drosophila Paf1 modulates chromatin structure at actively transcribed genes. Mol. Cell. Biol. *26*, 250–260.

Akanuma, T., Koshida, S., Kawamura, A., Kishimoto, Y., and Takada, S. (2007). Paf1 complex homologues are required for Notch-regulated transcription during somite segmentation. EMBO Rep. *8*, 858–863.

Anastassiadis, K., Kim, J., Daigle, N., Sprengel, R., Scholer, H.R., and Stewart, A.F. (2002). A predictable ligand regulated expression strategy for stably integrated transgenes in mammalian cells in culture. Gene *298*, 159–172.

Aubert, J., Stavridis, M.P., Tweedie, S., O'Reilly, M., Vierlinger, K., Li, M., Ghazal, P., Pratt, T., Mason, J.O., Roy, D., et al. (2003). Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice. Proc. Natl. Acad. Sci. USA *100* (*Suppl 1*), 11836–11841.

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell *125*, 315–326.

Carpenter, L., and Zernicka-Goetz, M. (2004). Directing pluripotent cell differentiation using "diced RNA" in transient transfection. Genesis *40*, 157–163.

Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W.J., Anderson, S., Yates, J., Washburn, M.P., et al. (2005). Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell *123*, 581–592.

Chambers, I., and Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene *23*, 7150–7160.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643–655.

Cheeseman, I.M., and Desai, A. (2005). A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. Sci. STKE *2005*, pl1. 10.1126/stke.2662005pl1.

Costa, P.J., and Arndt, K.M. (2000). Synthetic lethal interactions suggest a role for the Saccharomyces cerevisiae Rtf1 protein in transcription elongation. Genetics *156*, 535–547.

Diogo, M.M., Henrique, D., and Cabral, J.M. (2008). Optimization and integration of expansion and neural commitment of mouse embryonic stem cells. Biotechnol. Appl. Biochem. *49*, 105–112.

Fazzio, T.G., Huff, J.T., and Panning, B. (2008). An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell *134*, 162–174.

Fouse, S.D., Shen, Y., Pellegrini, M., Cole, S., Meissner, A., Van Neste, L., Jaenisch, R., and Fan, G. (2008). Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell Stem Cell *2*, 160–169.

Hay, D.C., Sutherland, L., Clark, J., and Burdon, T. (2004). Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. Stem Cells *22*, 225–235.

Ivanova, N., Dobrin, R., Lu, R., Kotenko, I., Levorse, J., DeCoste, C., Schafer, X., Lun, Y., and Lemischka, I.R. (2006). Dissecting self-renewal in stem cells with RNA interference. Nature *442*, 533–538.

Jaenisch, R., and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell *132*, 567–582.

Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev. *19*, 1129–1155.

Kittler, R., Putz, G., Pelletier, L., Poser, I., Heninger, A.K., Drechsel, D., Fischer, S., Konstantinova, I., Habermann, B., Grabner, H., et al. (2004). An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. Nature *432*, 1036–1040.

Kittler, R., Heninger, A.K., Franke, K., Habermann, B., and Buchholz, F. (2005a). Production of endoribonuclease-prepared short interfering RNAs for gene silencing in mammalian cells. Nat. Methods *2*, 779–784.

Kittler, R., Pelletier, L., Ma, C., Poser, I., Fischer, S., Hyman, A.A., and Buchholz, F. (2005b). RNA interference rescue by bacterial artificial chromosome transgenesis in mammalian tissue culture cells. Proc. Natl. Acad. Sci. USA *102*, 2396–2401.

Kittler, R., Pelletier, L., Heninger, A.K., Slabicki, M., Theis, M., Miroslaw, L., Poser, I., Lawo, S., Grabner, H., Kozak, K., et al. (2007a). Genome-scale RNAi profiling of cell division in human tissue culture cells. Nat. Cell Biol. *9*, 1401–1412.

Kittler, R., Surendranath, V., Heninger, A.K., Slabicki, M., Theis, M., Putz, G., Franke, K., Caldarelli, A., Grabner, H., Kozak, K., et al. (2007b). Genome-wide resources of endoribonuclease-prepared short interfering RNAs for specific loss-of-function studies. Nat. Methods *4*, 337–344.

Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M., et al. (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol. Cell *11*, 721–729.

Lee, J.H., and Skalnik, D.G. (2005). CpG-binding protein (CXXC finger protein 1) is a component of the mammalian Set1 histone H3-Lys4 methyltransferase complex, the analogue of the yeast Set1/COMPASS complex. J. Biol. Chem. *280*, 41725–41731.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat. Genet. *38*, 431–440.

Mosimann, C., Hausmann, G., and Basler, K. (2006). Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. Cell *125*, 327–341.

Mueller, C.L., and Jaehning, J.A. (2002). Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex. Mol. Cell. Biol. *22*, 1971–1980.

Myers, L.C., and Kornberg, R.D. (2000). Mediator of transcriptional regulation. Annu. Rev. Biochem. *69*, 729–749.

Ng, H.H., Dole, S., and Struhl, K. (2003a). The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. J. Biol. Chem. *278*, 33625–33628.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003b). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol. Cell *11*, 709–719.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379–391.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372–376.

O'Shea, K.S. (2004). Self-renewal vs. differentiation of mouse embryonic stem cells. Biol. Reprod. *71*, 1755–1765.

Penheiter, K.L., Washburn, T.M., Porter, S.E., Hoffman, M.G., and Jaehning, J.A. (2005). A posttranscriptional role for the yeast Paf1-RNA polymerase II complex is revealed by identification of primary targets. Mol. Cell *20*, 213–223.

Pesce, M., and Scholer, H.R. (2001). Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells *19*, 271–278.

Pietersen, A.M., and van Lohuizen, M. (2008). Stem cell regulation by polycomb repressors: postponing commitment. Curr. Opin. Cell Biol. *20*, 201–207.

Poser, I., Sarov, M., Hutchins, J.R., Heriche, J.K., Toyoda, Y., Pozniakovsky, A., Weigl, D., Nitzsche, A., Hegemann, B., Bird, A.W., et al. (2008). BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. Nat. Methods *5*, 409–415.

Shi, X., Chang, M., Wolf, A.J., Chang, C.H., Frazer-Abel, A.A., Wade, P.A., Burton, Z.F., and Jaehning, J.A. (1997). Cdc73p and Paf1p are found in a novel RNA polymerase II-containing complex distinct from the Srbp-containing holoenzyme. Mol. Cell. Biol. *17*, 1160–1169.

Silva, J., and Smith, A. (2008). Capturing pluripotency. Cell *132*, 532–536.

Stolinski, L.A., Eisenmann, D.M., and Arndt, K.M. (1997). Identification of RTF1, a novel gene important for TATA site selection by TATA box-binding protein in Saccharomyces cerevisiae. Mol. Cell. Biol. *17*, 4490–4500.

Takahashi, K., Murakami, M., and Yamanaka, S. (2005). Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. Biochem. Soc. Trans. *33*, 1522–1525.

Tenney, K., Gerber, M., Ilvarsonn, A., Schneider, J., Gause, M., Dorsett, D., Eissenberg, J.C., and Shilatifard, A. (2006). Drosophila Rtf1 functions in histone methylation, gene expression, and Notch signaling. Proc. Natl. Acad. Sci. USA *103*, 11970–11974.

Turksen, K. (2001). Embryonic Stem Cells: Methods and Protocols, First Edition (Totowa, NJ: Humana Press).

Wang, P., Bowl, M.R., Bender, S., Peng, J., Farber, L., Chen, J., Ali, A., Zhang, Z., Alberts, A.S., Thakker, R.V., et al. (2008). Parafibromin, a component of the human PAF complex, regulates growth factors and is required for embryonic development and survival in adult mice. Mol. Cell. Biol. *28*, 2930–2940.

Yamazaki, K., Aso, T., Ohnishi, Y., Ohno, M., Tamura, K., Shuin, T., Kitajima, S., and Nakabeppu, Y. (2003). Mammalian elongin A is not essential for cell viability but is required for proper cell cycle progression with limited alteration of gene expression. J. Biol. Chem. *278*, 13585–13589.

Ying, Q.L., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545–548.

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat. Biotechnol. *21*, 183–186.

Zhu, B., Mandal, S.S., Pham, A.D., Zheng, Y., Erdjument-Bromage, H., Batra, S.K., Tempst, P., and Reinberg, D. (2005). The human PAF complex coordinates transcription with events downstream of RNA synthesis. Genes Dev. *19*, 1668–1673.