Enhancer priming by H3K4 methyltransferase MLL4 controls cell fate transition

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Transcriptional enhancers control cell-type–specific gene expression. Primed enhancers are marked by histone H3 lysine 4 (H3K4) mono/di-methylation (H3K4me1/2). Active enhancers are further marked by H3K27 acetylation (H3K27ac). Mixed-lineage leukemia 4 (MLL4/KMT2D) is a major enhancer H3K4me1/2 methyltransferase with functional redundancy with MLL3 (KMT2C). However, its role in cell fate maintenance and transition is poorly understood. Here, we show in mouse embryonic stem cells (ESCs) that MLL4 associates with, but is surprisingly dispensable for the maintenance of, active enhancers of cell-identity genes. As a result, MLL4 is dispensable for cell-identity gene expression and self-renewal in ESCs. In contrast, MLL4 is required for enhancer-binding of H3K27 acetyltransferase p300, enhancer activation, and induction of cell-identity genes during ESC differentiation. MLL4 protein, rather than MLL4-mediated H3K4 methylation, controls p300 recruitment to enhancers. We also show that, in somatic cells, MLL4 is dispensable for maintaining cell identity but essential for reprogramming into induced pluripotent stem cells. These results indicate that, although enhancer priming by MLL4 is dispensable for cell-identity maintenance, it controls cell fate transition by orchestrating p300-mediated enhancer activation.

enhancer | MLL4/KMT2D | H3K4 methyltransferase | cell fate transition | p300

In mammalian cells, enhancers coordinate with promoters to precisely control cell-type–specific gene transcription, which determines the cell identity (1, 2). Comprehensive genome-wide studies have provided insights into the chromatin signatures of enhancers. Primed enhancers are marked by H3K4me1/2. Active enhancers (AEs) are further marked by the histone acetyltransferases CBP/p300-mediated H3K27ac (3). Recent studies classify AEs into typical enhancers and superenhancers. Superenhancers are clusters of AEs bound by lineage-determining transcription factors (TFs). Compared with typical enhancers, superenhancers are more cell-lineage–specific and control cell identity (4). Enhancer activation is orchestrated through a regulatory network involving lineage-determining TFs and chromatin-modifying complexes (2). However, how chromatin-modifying complexes regulate enhancer activation and cell fate transition is poorly understood.

Embryonic stem cells (ESCs) derived from blastocysts are capable of unlimited replication in vitro, a property known as ESC self-renewal. ESC identity is maintained during self-renewal. ESC self-renewal is controlled by a core circuitry of TFs including Oct4, Sox2, and Nanog (4). ESCs can rapidly respond to environmental cues and differentiate into three germ layers—ectoderm, endoderm, and mesoderm—which consist of all cell lineages within days (5). Differentiated somatic cells can also be converted back to the pluripotent stage by ectopic expression of Oct4 and Sox2 together with Klf4 and c-Myc, a process known as somatic cell reprogramming into induced pluripotent stem cells (iPSCs) (6). The dramatic changes of cell identity that accompany ESC differentiation and somatic cell reprogramming make these two processes

excellent models for studying cell fate transition and the underlying molecular mechanism.

MLL4 is a major enhancer H3K4 mono- and di-methyltransferase with partial functional redundancy with MLL3 in mammalian cells (7, 8). MLL4 colocalizes with lineage-determining TFs on AEs during adipogenesis and myogenesis. Furthermore, MLL4 is required for enhancer activation, cell-type–specific gene expression, and cell differentiation during adipogenesis and myogenesis (8). These observations prompted us to investigate whether MLL4 plays a more general role in the control of cell fate transition and how MLL4 regulates enhancer activation. In this study, we use mainly ESC differentiation and somatic cell reprogramming as model systems to explore functions and mechanisms of MLL4 in enhancer activation, cell-identity maintenance, and cell fate transition.

Results

MLL4 Associates with Active Enhancers on Cell-Identity Genes in ESCs. MLL4 is essential for early embryonic development in mice whereas MLL3 is dispensable. In cultured cells, MLL3 partially compensates for the loss of MLL4 (8). To investigate the role of MLL4 in ESC self-renewal and differentiation, we derived *Mll3* KO and *Mll4* conditional KO (*Mll3$^{-/-}$Mll4$^{f/f}$*, hereafter referred to as f/f) ESCs from blastocysts. By transfecting these cells with a Cre-expressing plasmid to delete the *Mll4* gene, we generated

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Significance

Transcriptional enhancers control cell-identity gene expression and thus determine cell identity. Enhancers are primed by histone H3K4 mono-/di-methyltransferase MLL4 before they are activated by histone H3K27 acetyltransferase p300. Here, we show that MLL4 is dispensable for cell-identity maintenance but essential for cell fate transition using several model systems including embryonic stem cell (ESC) differentiation toward somatic cells and somatic cell reprogramming into ESC-like cells. Mechanistically, MLL4 is dispensable for maintaining p300 binding on active enhancers of cell-identity genes but is required for p300 binding on enhancers activated during cell fate transition. These results indicate that, although enhancer priming by MLL4 is dispensable for cell-identity maintenance, it controls cell fate transition by orchestrating p300-mediated enhancer activation.

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Mll3/Mll4 double KO (DKO) ESCs. Deletion of *Mll4* from the f/f ESCs resulted in an approximately twofold decrease of global H3K4me1 and ~30% decreases of global H3K4me2 and H3K27ac with little effect on H3K4me3 and H3K9ac levels (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). Thus, MLL3 and MLL4 are H3K4me1/2 methyltransferases in ESCs.

Next we performed ChIP-Seq (chromatin immunoprecipitation coupled with DNA sequencing) of MLL4 in both f/f and DKO ESCs. After filtering out nonspecific signals observed in the DKO cells (8), 12,383 high-confidence MLL4 binding sites were identified in f/f ESCs, the vast majority of which were marked by H3K4me1 and/or H3K4me2 (Fig. 1*B*). Consistent with our previous findings in adipocytes and myocytes (8), ChIP-Seq revealed that MLL4 was preferentially enriched on AEs in ESCs (Fig. 1*C*).
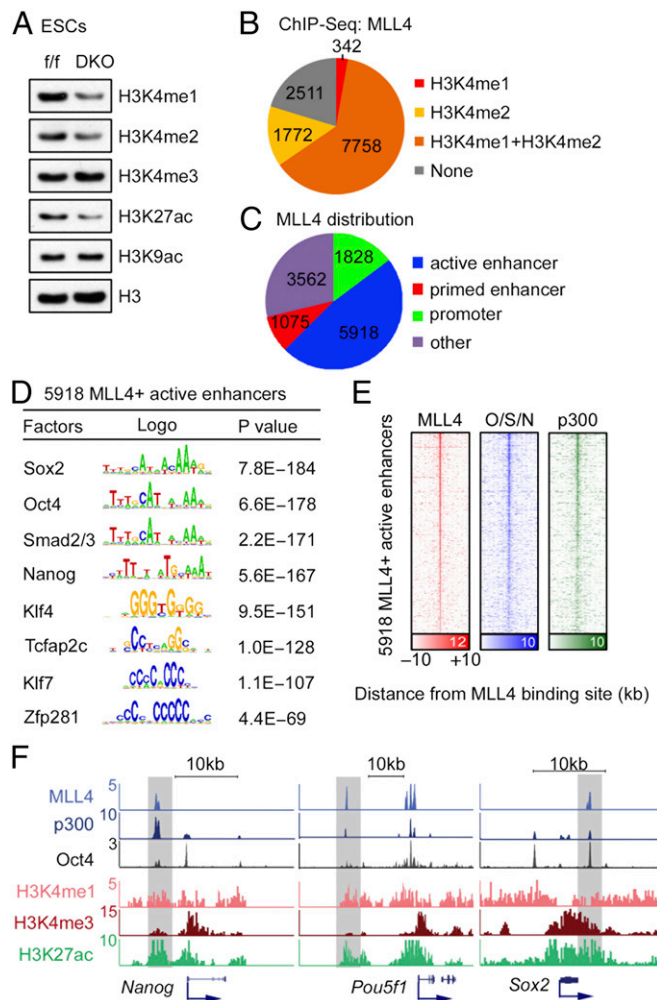

Fig. 1. MLL4 associates with active enhancers on cell-identity genes in ESCs. (*A*) KO of the *Mll4* gene in f/f (*Mll3*⁻/⁻;*Mll4*^flox/flox) ESCs decreases global levels of H3K4me1/2. Histone extracts from f/f and *Mll3/4* DKO ESCs were analyzed with immunoblotting using indicated antibodies. (*B*) Pie chart illustrating that MLL4⁺-binding sites are marked by H3K4me1 and H3K4me2 in ESCs. (*C*) MLL4 is enriched on AE regions in ESCs. Promoter: ±1 kb of transcription start site (TSS). AE: H3K4me1⁺;H3K27ac⁺ promoter-distal region. Primed enhancer: H3K4me1⁺;H3K27ac⁻ promoter-distal region. (*D*) Motif analysis of 5,918 MLL4-positive (MLL4⁺) AEs in ESCs. (*E*) MLL4 colocalizes with pluripotent TFs O/S/N (Oct4, Sox2, and Nanog) and H3K27 acetyltransferase p300 on AEs in ESCs. Density maps are shown for ChIP-Seq data at MLL4⁺ AEs. Color scale indicates ChIP-Seq signal in reads per million. (*F*) ChIP-Seq binding profiles (reads per million) of MLL4, p300, Oct4, and histone modifications on the ESC identity genes *Nanog*, *Oct4* (*Pou5f1*), and *Sox2*. MLL4⁺ AEs are shaded in gray.

Motif analysis showed that MLL4⁺ AEs were enriched with motifs of major ESC TFs including Sox2, Oct4, Smad2/3, Nanog, and Klf4 (Fig. 1*D*). By comparing the genomic localization of MLL4 with those of Oct4, Sox2, and Nanog (O/S/N) and the H3K27 acetyltransferase p300 that marks AEs (4, 9), we found that MLL4 colocalized with O/S/N and p300 on AEs (Fig. 1*E*). A physical interaction between MLL4 and Oct4 was also observed in ESCs (*SI Appendix*, Fig. S1*B*), consistent with the previous report that the MLL4 complex physically interacts with pluripotency TFs (10). Thus, MLL4 colocalizes with pluripotency TFs on AEs in ESCs.

Superenhancers (SEs), densely occupied by master TFs, are lineage-specific and associate with highly expressed cell-identity genes (4, 11). We found that ~90% of ESC SEs were occupied by MLL4. In contrast, only ~30% of typical enhancers (TEs) were MLL4⁺ (*SI Appendix*, Fig. S1*C*). SEs had higher levels of MLL4-binding density comparing to TEs in ESCs (*SI Appendix*, Fig. S1*D*). Moreover, genes associated with MLL4⁺ SEs were expressed at significantly higher levels than those with TEs (*SI Appendix*, Fig. S1*E*). Consistently, gene ontology (GO) analysis of genes associated with the 5,918 MLL4⁺ AEs identified stem cell development, maintenance, and differentiation as the top functional categories (*SI Appendix*, Fig. S1*F*). For example, MLL4 colocalized with Oct4 and p300 on AEs of the ESC identity genes *Nanog*, *Oct4* (*Pou5f1*), and *Sox2* (Fig. 1*F*). Together, these results indicate that H3K4me1/2 methyltransferase MLL4 associates with AEs and cell-identity genes in ESCs.

MLL4 Is Dispensable for Maintaining ESC Identity Gene Expression and ESC Identity. To investigate the direct role of MLL4 in regulating ESC identity genes, we performed RNA-Seq in both f/f and DKO cells and analyzed expression of MLL4⁺ AE-associated genes. Surprisingly, only a small fraction of MLL4⁺ AE-associated genes including *Left-right determination factor 1* (*Lefty1*) and *Alkaline phosphatase* (*Alpl*) showed over twofold down-regulation in DKO cells compared with f/f cells. Expression of the majority of MLL4⁺ AE-associated genes, which included ESC identity genes such as *Oct4*, *Sox2*, *Nanog*, *Sall4*, *Klf4*, and *Esrrb*, was generally unaffected (Fig. 2*A*). Expression of MLL4⁺ SE-associated genes was also generally unaffected by *Mll4* deletion (Fig. 2*B*).

Consistent with the gene expression data, deletion of *Mll4* from the f/f ESCs had little effect on the population doubling time (growth rate) and colony formation ability (Fig. 2*C* and *SI Appendix*, S2*A*). Although the DKO cells showed defects in alkaline phosphatase (AP) staining because of the attenuated *Alpl* expression, they could form normal ESC colonies (Fig. 2*D*). Similar results were obtained when cells were cultured in 2i (Mek and GSK inhibitors)-containing medium, suggesting that regulation of ESC gene expression by MLL4 is independent of the naive/primed state (*SI Appendix*, Fig. S2 *B–D*) (13, 14). Thus, MLL3 and MLL4 are dispensable for ESC self-renewal. These results indicate that, although MLL4 associates with AEs on cell-identity genes in ESCs, it is dispensable for maintaining ESC identity gene expression and ESC identity.

MLL4 Is Dispensable for Maintaining AEs on ESC Identity Genes. To understand how MLL4 regulates ESC gene expression, we examined histone modification changes on MLL4⁺ AEs in f/f and DKO cells. H3K4me1 and H3K27ac were overall reduced whereas H3K27me3 showed little change on MLL4⁺ AEs (*SI Appendix*, Fig. S3*A*). Among the 5,918 MLL4⁺ AEs, only 1,700 (~29%) exhibited significant decreases of both H3K4me1 and H3K27ac in DKO cells compared with f/f cells. These 1,700 enhancers were therefore defined as MLL4-dependent AEs (Fig. 2*E*). The remaining 4,218 MLL4⁺ AEs exhibited little change of H3K27ac in DKO cells and were therefore defined as MLL4-independent AEs. ESC master regulator genes *Oct4*, *Sox2*, *Nanog*, *Sall4*, *Klf4*, and *Esrrb* associated with MLL4-independent AEs (Fig. 2*E*). In accordance with the H3K27ac behavior, MLL4 was required for
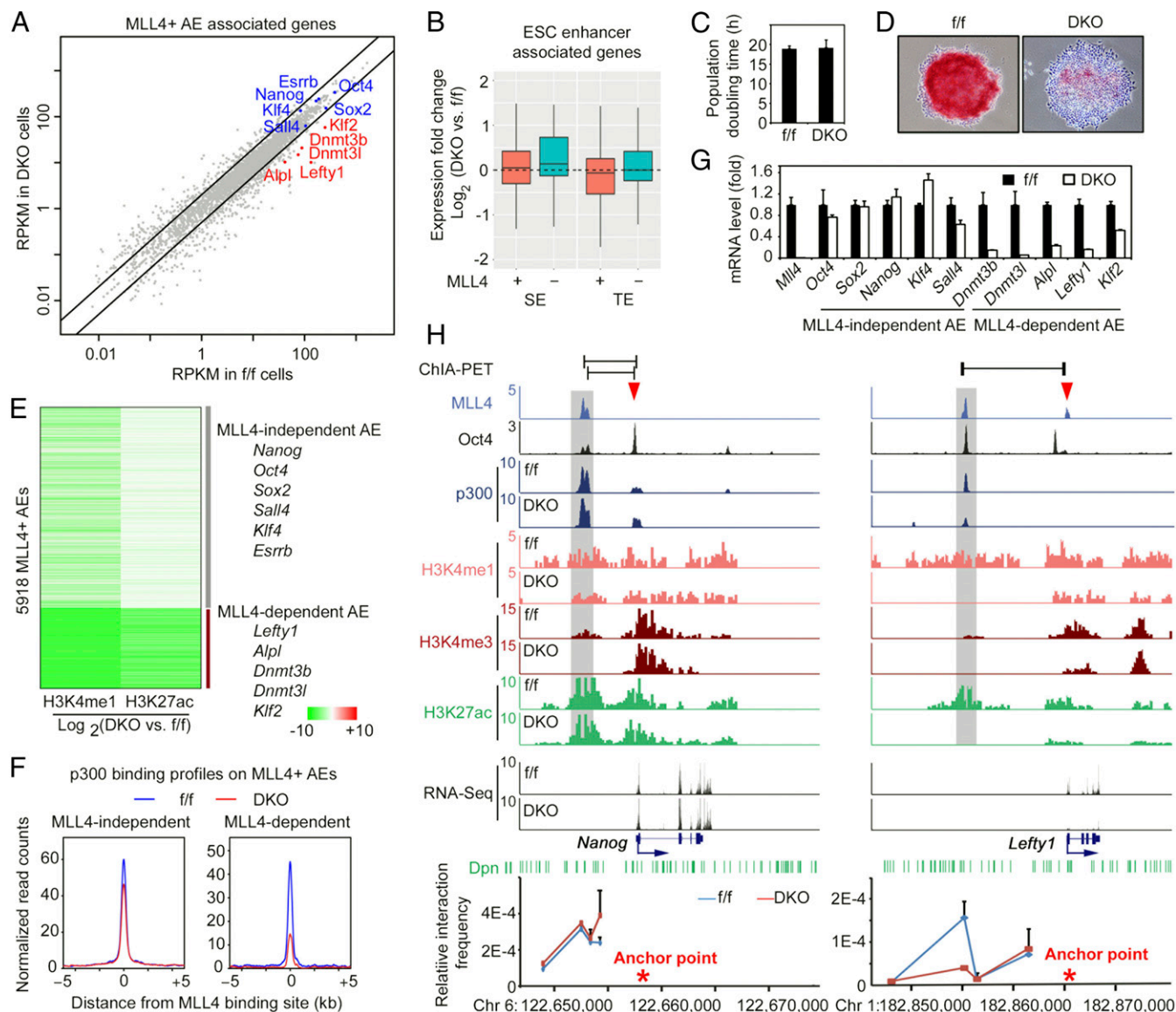
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Fig. 2. MLL4 is dispensable for maintaining ESC identity. (*A* and *B*) MLL4 is dispensable for maintaining ESC identity gene expression. (*A*) RNA-Seq was done in f/f and DKO ESCs. Expression levels of MLL4+ AE-associated genes are shown in the scatter plot. Twofold change threshold lines are indicated. Each dot represents one gene. MLL4-dependent and -independent genes are highlighted in red and blue, respectively. Note that MLL4 is required for *Alpl* expression in ESCs. (*B*) Expression fold changes of genes associated with MLL4+ and MLL4− TEs and SEs, respectively, were obtained by comparing DKO with f/f cells. (*C* and *D*) MLL4 is dispensable for ESC self-renewal. (*C*) Population doubling time of f/f and DKO cells. (*D*) Representative images of AP staining of ESC colonies. (*E* and *F*) MLL4 is dispensable for maintaining AEs on ESC identity genes. (*E*) Heat maps depicting fold changes of H3K4me1 and H3K27ac on MLL4+ AEs were obtained by comparing DKO with f/f cells. Enhancers, which show over threefold decreases of both H3K4me1 and H3K27ac levels in DKO cells, are defined as MLL4-dependent AEs. The rest are defined as MLL4-independent AEs. Selected genes associated with each group are shown on the right. (*F*) Average profiles of p300 around the center of MLL4-independent and -dependent AEs. The reduction of p300 binding on MLL4-dependent AEs is significantly larger than that on MLL4-independent ones (*P* value = 1.8E-19) (*SI Appendix, SI Materials and Methods*). (*G*) qRT-PCR analysis of expression of genes associated with MLL4-independent or MLL4-dependent AEs in f/f and DKO ESCs. (*H*) MLL4-independent and MLL4-dependent AEs on *Nanog* and *Lefty1* gene loci, respectively, are shown. (*Upper*) ChIP-Seq binding profiles (reads per million) for MLL4, p300, Oct4, histone modifications, and mRNA-Seq signals in f/f and DKO cells. MLL4+ AEs are shaded in gray. (*Lower*) Interactions between MLL4+ AEs and promoters were determined by 3C assays in f/f and DKO ESCs. Enhancer–promoter interactions detected by Smc1 ChIA-PET were obtained from GSE57911 (12) and are shown above ChIP-Seq tracks. TSSs are indicated by red arrowheads.

the recruitment of H3K27 acetyltransferase p300 to MLL4-dependent but not MLL4-independent AEs (Fig. 2*F*). Consistently, MLL4 was required for expression of genes associated with MLL4-dependent, but not MLL4-independent, AEs (Fig. 2*G*). Moreover, the dependence of gene expression on MLL4 correlated positively with the number of associated MLL4-dependent AEs (*SI Appendix,* Fig. S3*B*). Although motif analysis failed to distinguish MLL4-dependent and -independent AEs, GO analysis revealed that only genes associated with MLL4-independent

AEs were functionally related to stem cell maintenance (*SI Appendix,* Fig. S3 *C* and *D*). These data indicate that MLL4 is dispensable for maintaining AEs on ESC identity genes, which provides an explanation for the dispensable role of MLL4 in maintaining ESC identity gene expression.

MLL4 Is Required for p300 Binding and Enhancer-Promoter Looping on MLL4-Dependent AEs. Next we used *Nanog* and *Lefty1* loci, which associated with MLL4-independent and MLL4-dependent

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AEs, respectively, as examples to further investigate how MLL4 regulates transcription in ESCs (Fig. 2*H*). Recent studies have demonstrated that AEs physically interact with promoters to form enhancer–promoter loops (15). In addition, RNA polymerase II (Pol II) binds to AEs and transcribes enhancer DNAs into enhancer RNAs (eRNAs) (16). Consistent with the Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET) data obtained from a previous study (12), Chromatin Conformation Capture (3C) assays confirmed that MLL4$^+$ AEs formed loops with promoters on *Nanog* and *Lefty1* loci. *Mll4* deletion had little impact on the interaction between MLL4-independent AE and promoter on *Nanog* locus but significantly attenuated the interaction between MLL4-dependent AE and promoter on *Lefty1* locus (Fig. 2*H*). *Mll4* deletion also decreased p300, H3K27ac, and Pol II levels as well as eRNA expression on the MLL4-dependent AE on *Lefty1* locus but not on the MLL4-independent AE on *Nanog* locus (Fig. 2*H* and *SI Appendix*, Fig. S3*E*). These results indicate that MLL4 is required for enhancer binding of p300, enhancer–promoter interaction, and eRNA production on MLL4-dependent AEs.

MLL4 Is Required for ESC Differentiation. Because MLL4 is dispensable for ESC self-renewal, we next asked if MLL4 is required for ESC differentiation. Following well-established protocols (17–19), we cultured ESCs without feeder cells and LIF (leukemia inhibitory factor) for 12 days to form embryoid bodies (EBs) consisting of all three germ layers (5). The f/f ESCs formed cystic EBs during differentiation whereas the DKO ESCs failed to do so (Fig. 3*A*). Consistent with the phenotypes, expression of the ESC identity genes *Nanog* and *Oct4* decreased whereas expression of the mesoderm markers *Brachyury* and *Wnt3* and the endoderm markers *Gata4* and *Gata6* increased during differentiation of f/f ESCs. In contrast, DKO EBs retained expression of *Nanog* and *Oct4* and failed to induce mesoderm and endoderm markers during differentiation (Fig. 3*B*).

To investigate the underlying mechanism, we examined gene expression profiles in f/f and DKO cells at day 0 (D0) and day 4 (D4) of differentiation. From D0 to D4, 643 and 717 genes were down- and up-regulated over 2.5-fold in f/f ESCs, respectively. Among the 717 up-regulated genes, 286 were induced in a

MLL4-dependent manner (*SI Appendix*, Fig. S4). Interestingly, GO analysis showed that only MLL4-dependent up-regulated genes were strongly associated with the development of various embryonic lineages such as heart, blood vessel, and vasculature (*SI Appendix*, Fig. S4), suggesting that MLL4 selectively controls induction of early embryonic development genes during ESC differentiation. To verify the differentiation defects of DKO ESCs in vivo, we performed teratoma assays. The f/f ESCs developed into typical teratomas consisting of three germ layers—ectoderm, endoderm, and mesoderm. In contrast, only poorly differentiated primitive cells were detected in the DKO tumors (Fig. 3*C*). Together, these results reveal an essential role of MLL3 and MLL4 in ESC differentiation and induction of early developmental genes.

MLL4 Is Required for p300-Mediated Enhancer Activation During ESC Differentiation. To investigate the mechanism by which MLL4 controls the induction of early developmental genes during ESC differentiation, we performed ChIP-Seq of H3K4me1, H3K27ac, MLL4, and p300 in EBs at day 4, when the vast majority of cells in the population should have differentiated into premesodermal and downstream lineages (20). MLL4 was preferentially enriched on AEs in D4 EBs, with 16,238 (57.9%) MLL4-binding regions located on AEs (Fig. 4*A*). Among these MLL4$^+$ AEs, 9,704 (59.8%) were premarked with H3K27ac in ESCs whereas the remaining 6,534 (40.2%) were de novo AEs that were H3K27ac-positive in D4 EBs but not in ESCs (Fig. 4*B*). GO analysis indicated that only de novo AEs were strongly associated with genes involved in early embryonic development and ESC differentiation (*SI Appendix*, Fig. S5*A*), suggesting that MLL4$^+$ de novo AEs regulate ESC differentiation.

Deletion of *Mll4* led to a moderate decrease of H3K27ac and p300 levels on H3K27ac-premarked MLL4$^+$ AEs. In contrast, deletion of *Mll4* prevented p300 recruitment and the dramatic induction of H3K27ac on MLL4$^+$ de novo AEs from ESC to D4 EB stage (Fig. 4*C*). Among the 6,534 MLL4$^+$ de novo AEs, 3,531 were premarked with H3K4me1 in ESCs and were thus primed enhancers whereas the remaining 3,003 were marked with H3K4me1 only in D4 EBs and were thus H3K4me1-emergent (*SI Appendix*, Fig. S5*B*). MLL4 and p300 were barely detected on H3K4me1-primed enhancers before ESC differentiation, suggesting that H3K4me1 alone is insufficient for p300 recruitment on those enhancers. During ESC differentiation MLL4 bound to both H3K4me1-primed and -emergent enhancers and was required for p300 binding and H3K27ac deposition on both types of enhancers (*SI Appendix*, Fig. S5*B*). These results indicate that MLL4 is essential for the activation of de novo AEs and that MLL4, rather than H3K4me1, is important for p300 recruitment.

Consistently, only genes associated with MLL4$^+$ de novo AEs were significantly induced during ESC differentiation in a MLL4-dependent manner (Fig. 4*D*). In fact, 43% of MLL4-dependent genes were associated with MLL4$^+$ de novo AEs (*SI Appendix*, Fig. S5*C*). On gene loci encoding the endoderm regulator Gata4 and the mesoderm regulator Wnt3 (Fig. 4*E* and *SI Appendix*, S5*D*), MLL4 was responsible for not only the p300 binding and activation of MLL4$^+$ de novo AEs but also the induction of both genes. Interestingly, we also observed emergent MLL4 binding on *Wnt3* and *Gata4* promoters in differentiated cells, suggesting that MLL4 may be involved in the establishment of de novo enhancer–promoter looping (Fig. 4*E* and *SI Appendix*, S5*D*). Together, these results suggest that MLL4 regulates induction of early developmental genes during ESC differentiation by controlling p300-mediated enhancer activation.

MLL4 Is Dispensable for Maintaining Somatic Cell Identity but Required for Somatic Cell Reprogramming into iPSCs. The essential role of MLL4 in cell differentiation and the associated induction of cell-type–specific genes suggest that MLL4 is required for
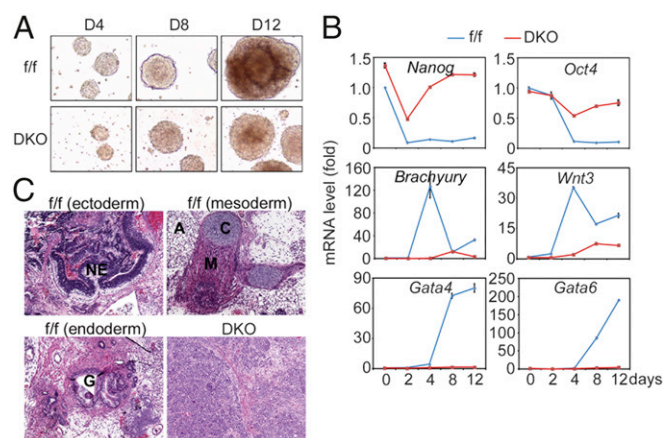

Fig. 3. MLL4 is required for ESC differentiation. ESCs were induced to differentiate into EBs for 12 d. (*A*) Representative microscopic images of EBs at indicated time points. (*B*) qRT-PCR analysis of ESC markers (*Nanog*, *Oct4*), mesoderm markers (*Brachyury*, *Wnt3*), and endoderm markers (*Gata4*, *Gata6*) at indicated time points. (*C*) Representative histological images of teratomas derived from f/f and DKO ESCs. Three germ layers—ectoderm, mesoderm and endoderm—were observed in f/f teratomas, whereas cells in DKO teratomas remained at the primitive stage. A, adipose; C, cartilage; G, gland-like; M, muscle; NE, neural epithelium.
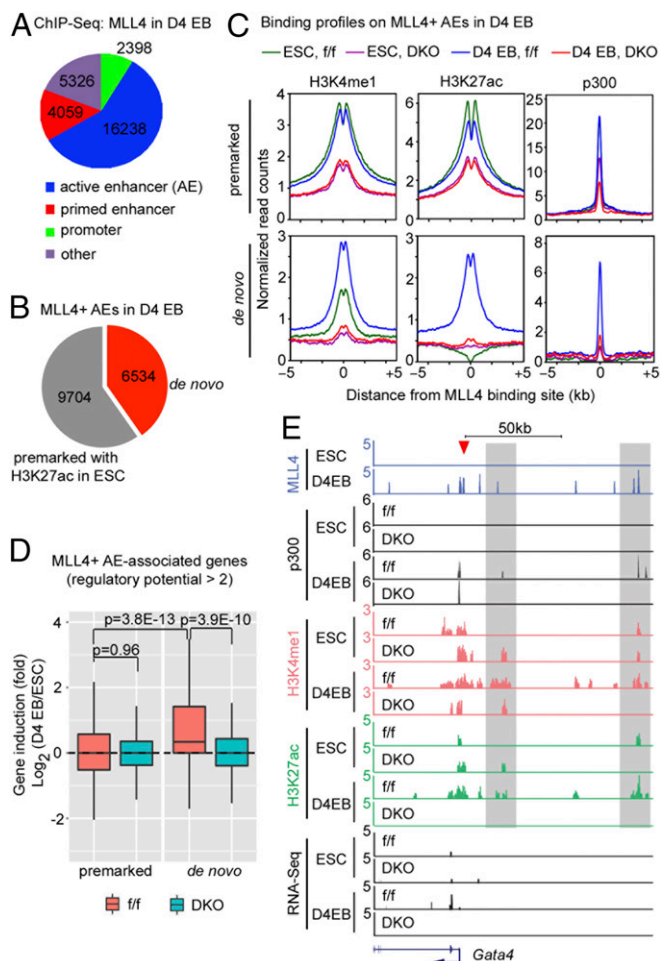
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Fig. 4. MLL4 is required for p300-mediated enhancer activation during ESC differentiation. f/f and DKO ESCs were induced to differentiate into EBs for 4 d, followed by ChIP-Seq of MLL4, p300, H3K4me1, H3K27ac, and RNA-Seq analyses. (*A*) Genomic distribution of MLL4-binding regions in D4 EBs. (*B*) Pie chart depicting that among MLL4$^+$ AEs in D4 EBs derived from f/f ESCs. A total of 6,534 are de novo, whereas 9,704 are premarked with H3K27ac in ESCs. (*C*) Deletion of *Mll4* in f/f ESCs prevents H3K27ac deposition and p300 recruitment on de novo AEs. Average profiles of H3K4me1, H3K27ac, and p300 around the center of premarked or de novo AEs are shown. The reduction of H3K27ac and p300 in DKO EBs on de novo AEs is significantly greater than that on premarked ones (*P* value = 4.4E-253 and 5.2E-97, respectively) (*SI Appendix, SI Materials and Methods*). (*D*) Box plot depicting the induction fold change of genes associated with premarked or de novo AEs. Only genes with a regulatory potential score over 2 are shown. *P* value was calculated using a *t* test. (*E*) ChIP-Seq binding profiles (reads per million) for MLL4, p300, H3K4me1, H3K27ac, and RNA-Seq signals on the *Gata4* gene locus in ESCs and D4 EBs. MLL4$^+$ de novo AEs are shaded in gray. TSS is indicated by a red arrowhead.

establishing new cell identities during cell fate transitions. To further test this hypothesis, we investigated the role of MLL4 in somatic cell reprogramming into iPSCs. Immortalized f/f (*Mll3*$^{-/-}$*Mll4*$^{f/f}$) mouse embryonic fibroblasts (MEFs) were infected with adenoviral Cre to generate DKO cells. The f/f and DKO cells showed similar growth rates (*SI Appendix*, Fig. S6). f/f and DKO MEFs were then infected with lentiviral vectors expressing the four reprogramming TFs Oct4, Sox2, Klf4, and c-Myc (OSKM) (21), followed by induction of reprogramming for 20 days. As shown in Fig. 5 *A–C*, AP staining positive, ESC-like cell colonies showed up from the f/f cells. Deletion of *Mll4* from f/f MEFs dramatically reduced the reprogramming efficiency and prevented the induction of pluripotency markers such as *Nanog*, *Sall4*, *Fbxo15*,

and *Dppa5a*. Similar results were obtained when we induced reprogramming of preadipocytes into iPSCs (*SI Appendix*, Fig. S7). A recent study on reprogramming identified "ESC-like genes," which are genes specifically expressed in ESC-like cells during reprogramming (22). By RNA-Seq analysis before and after reprogramming, we found that the transcriptome of "ESC-like genes" in f/f cells after reprogramming correlated well with the reported ones in early iPSCs and iPSCs, suggesting that f/f cells were obtaining pluripotency. In contrast, DKO cells failed to express "ESC-like genes" after reprogramming (*SI Appendix*, Fig. S8*A*). ChIP-Seq analyses in MEFs and iPSCs demonstrated that MLL4 bound to de novo AEs on gene loci of the pluripotent TFs Oct4, Sox2, and Esrrb after reprogramming, implying a role of MLL4 in activating de novo AEs during reprogramming (*SI Appendix*, Fig. S9). Together, these results indicate a critical role of MLL3 and MLL4 in establishing pluripotent cell identity during somatic cell reprogramming into iPSCs (Fig. 5*D*).

Interestingly, expression of MEF identity genes (defined by SE-associated genes in MEFs) (*Materials and Methods*) was not altered upon MLL4 loss, indicating that MLL4 is dispensable for maintaining MEF cell identity (*SI Appendix*, Fig. S8*B*). We reported previously that MLL4 is essential for adipocyte differentiation in vitro and in vivo (8). However, deletion of *Mll4* in adult mouse brown adipose tissue did not affect the tissue weight or the expression of cell-identity genes such as *PPARg*, *Cebpa*, *Fabp4*, *Prdm16*, and *Ucp1*, indicating that MLL4 is dispensable for the maintenance of differentiated adipocytes in vivo (*SI Appendix*, Fig. S10). Together with the observation that MLL4 is dispensable for the maintenance of ESC identity (Fig. 2), these
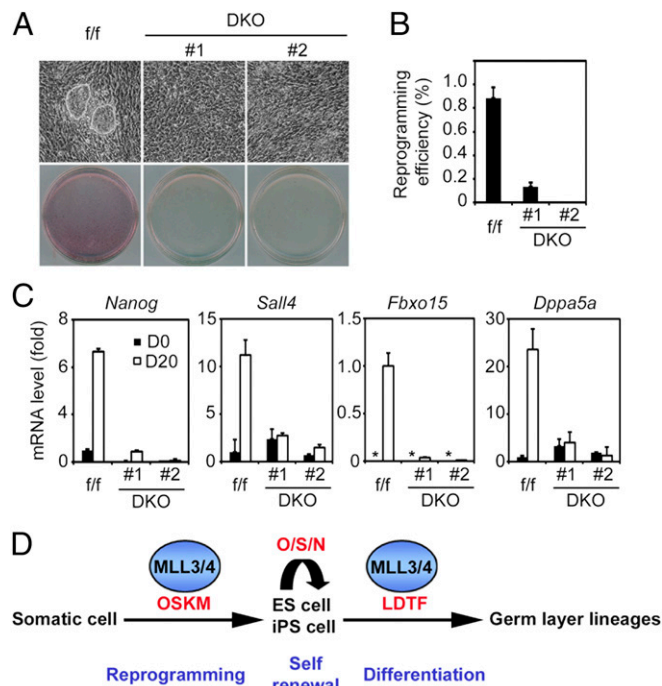

Fig. 5. MLL4 is required for somatic cell reprogramming to iPSCs. Immortalized f/f and DKO MEFs were induced to reprogram for 20 d by infection with lentiviral vectors expressing TFs Oct4, Sox2, Klf4, and c-Myc (OSKM). (*A*) Representative images of reprogrammed cells at day 20. (*Upper*) Cell morphologies under microscope. (*Lower*) AP staining of dishes. (*B*) Reprogramming efficiencies of f/f MEFs and two DKO MEF cell lines. (*C*) qRT-PCR analysis of indicated genes in f/f and DKO cells before and after 20 d of reprogramming. (*D*) A model depicting the essential role of MLL3/MLL4 in ESC differentiation and self-renewal and somatic cell reprogramming. LDTFs, lineage-determining TFs.

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observations indicate that, although MLL4 is dispensable for maintaining cell identity in somatic cells and ESCs, it is required for establishing new cell identity during cell fate transition.

Discussion

MLL4, redundant with MLL3, is a major enhancer H3K4me1/2 methyltransferase important for fat- and muscle-cell differentiation (8). However, whether MLL4 plays a much broader role in cell fate transition is unclear. Furthermore, the role of MLL4 in cell fate maintenance was completely unknown. MLL4 is required for the enrichment of H3K27ac on enhancers activated during fat- and muscle-cell differentiation (8). However, it remained to be determined whether MLL4 regulates the recruitment or the enzymatic activity of H3K27 acetyltransferase p300 on enhancers.

We show that MLL4 is dispensable for cell fate maintenance but essential for cell fate transition using several model systems: ESC self-renewal and differentiation, fibroblast maintenance and reprogramming to iPSCs, and adipocyte differentiation and maintenance. MLL4 is dispensable for the maintenance, but essential for the induction, of cell-identity gene expression. This is achieved by MLL4's dichotomous role in enhancer regulation: MLL4 is dispensable for maintaining AEs, but essential for activating enhancers, of cell-identity genes. Mechanistically, MLL4 is dispensable for maintaining binding of p300 on AEs of cell-identity genes but is required for p300 binding on enhancers activated during cell differentiation. MLL4 protein, rather than MLL4-mediated H3K4 methylation, controls p300 binding to enhancers. Our data also suggest that MLL4 functions as a general epigenomic regulator of cell fate transition and that MLL3/MLL4 control cell fate transition by orchestrating p300-mediated enhancer activation.

In ESCs, MLL4 associates with AEs of cell-identity genes such as *Nanog*, *Oct4*, and *Sox2*. However, these MLL4+ AEs belong to the category of MLL4-independent AEs. H3K27ac levels on these AEs are not affected by MLL4 depletion (Fig. 2*E*). Interestingly, reanalysis of a published ESC ChIP-Seq dataset on MLL2 (KMT2B) (23) revealed that over 50% of MLL4-independent AEs are also occupied by MLL2. In contrast, only ~25% of MLL4-dependent AEs are occupied by MLL2 in ESCs (*SI Appendix*, Fig. S3*F*). It is thus possible that MLL2 or other H3K4 methyltransferases may compensate for the loss of MLL4 on MLL4-independent AEs in the DKO ESCs. Notably, H3K4me1 but not H3K27ac was dependent on MLL4 on a cluster of MLL4-independent enhancers (cluster II in *SI Appendix*, Fig. S3*G*), suggesting that once AEs and AE–promoter interactions are established, MLL4 and H3K4me1 may no longer be needed for maintaining AEs. On ESC genes associated with MLL4-dependent AEs, we observed markedly decreased levels of H3K27ac, eRNA, and enhancer–promoter interaction, suggesting that MLL4 is required for establishing the looping between AEs and the corresponding promoters (Fig. 2*H* and *SI Appendix*, Fig. S3*E*). Future work will be needed to clarify why in ESCs some MLL4+ AEs are MLL4-depedent whereas others are not.

MLL4 and its homolog MLL3 have been shown to function as tumor supressors and are frequently mutated in many types of cancers and developmental diseases (24, 25). Our findings suggest that loss-of-function mutations in MLL3/MLL4 would prevent the activation of de novo enhancers and the induction of cell-type–specific genes, leading to defects in cell fate transition. Such a mechanism may explain, at least in part, the frequent loss-of-function mutations of MLL3/MLL4 in cancers and developmental diseases.

Materials and Methods

ESC Culture, Differentiation, Teratoma Formation Assay, and iPSC Reprogramming. ESCs were maintained on MEF feeder cells and differentiated into EBs as described previously (18). ESCs were split off feeder cells and cultured on gelatin-coated dishes for growth assay, colony formation assay, and AP staining as decribed (18). For ChIP-Seq and RNA-Seq analyses, ESCs were split off feeder cells and harvested. Trypsinized ESCs were injected into immunocompromised mice for teratoma formation as described in *SI Appendix*. The 3T3-immortalized *Mll3⁻/⁻ Mll4^(f/f)* MEFs were infected with adenoviral Cre to generate *Mll3/4* DKO MEFs. Immortalized *Mll3⁻/⁻ Mll4^(f/f)* and DKO brown preadipocytes were described (8). MEFs and preadipocytes were reprogrammed with lentiviral infection of OSKM as described (21). Animal experiments in this study were approved by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Animal Care and Use Committee.

ChIP-Seq, RNA-Seq, Computational Analysis, 3C Assay, and eRNA Expression. ChIP-Seq, RNA-Seq, and downstream analyses were performed as described with the use of Illumina HiSEq. 2000 (8). Data were deposited in the Gene Expression Omnibus database (accession no. GSE50534). Data analysis, 3C assays, and examination of eRNA expression are described in *SI Appendix*.

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