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INO80 Dependent Promoter Access Facilitates Activation of Pluripotency Genes in Embryonic Stem Cell Self-Renewal, Reprogramming, and Blastocyst Development

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Summary

The master transcription factors play integral roles in the pluripotency transcription circuitry of embryonic stem cells (ESCs). How they selectively activate expression of the pluripotency network while simultaneously repressing genes involved in differentiation is not fully understood. Here we define a requirement for the INO80 complex, a SWI/SNF family chromatin remodeler, in ESC self-renewal, somatic cell reprogramming, and blastocyst development. We show that Ino80, the chromatin remodeling ATPase, co-occupies pluripotency gene promoters with the master transcription factors, and its occupancy is dependent on Oct4 and Wdr5. At the pluripotency genes, Ino80 maintains open chromatin architecture and licenses recruitment of Mediator and RNA Polymerase II for gene activation. Our data reveal an essential role for INO80 in the expression of the pluripotency network, and illustrate the coordination among chromatin remodeler, transcription factor, and histone modifying enzyme in the regulation of the pluripotent state.

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Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of blastocyst stage embryos. They have two defining features: the ability to become any other cell type, known as pluripotency, and the ability to proliferate indefinitely while maintaining the pluripotent state, known as self-renewal. At the transcriptional level, ESC self-renewal and pluripotency is maintained by a highly orchestrated gene expression program (Dejosez and Zwaka, 2012; Jaenisch and Young, 2008; Ng and Surani, 2011; Young, 2011). The regulators of this program can activate, repress, or establish a poised state for gene expression. Master ESC transcription factors Oct4, Nanog, and Sox2 form the core of the pluripotency transcription network. They form a self-regulatory loop, and activate pluripotency genes while repressing those involved in differentiation (Boyer et al., 2005). Many other pluripotency factors further modulate and refine the core circuitry and augment the function of Oct4, Nanog, and Sox2 (Chen et al., 2008; Kim et al., 2008). How the master transcription factors act differently to activate ESC and repress differentiation genes is not clear.

In addition to transcription factors, recent studies indicate that the chromatin of ESCs has a unique open conformation, which likely contributes to self-renewal and pluripotency by providing an appropriate environment for gene expression (Mattout and Meshorer, 2010). Indeed, ESCs are highly sensitive to reduced levels of chromatin architectural proteins such as Cohesin and Mediator (Kagey et al., 2010). They are also sensitive to the depletion of chromatin regulators such as histone modifying enzymes and chromatin remodelers (Fazzio et al., 2008; Gaspar-Maia et al., 2009; Ho et al., 2011; Kidder et al., 2009; Landry et al., 2008; Schaniel et al., 2009; Singhal et al., 2010). While the complex interplay of the master ESC transcription factors with chromatin regulators is a focus of intense investigation, much remains to be learned.

Of the chromatin regulators, the ATP-dependent chromatin-remodeling complexes can move, eject, or restructure nucleosome composition and dynamics (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011). As a result, they play a central role in gene regulation by controlling the packing and unpacking of the chromatin to provide regulated DNA accessibility. Several chromatin remodelers, including Chd1, Chd7, esBAF, and Tip60-p400, have been studied in ESCs (Fazzio et al., 2008; Gaspar-Maia et al., 2009; Ho et al., 2011; Kidder et al., 2009; Schnetz et al., 2010), providing insights into how these factors contribute to ESC biology. However, little is known regarding how the master ESC transcription factors themselves utilize chromatin remodeling factors to selectively activate the genes required for self-renewal and pluripotency.

We and others have previously identified the INO80 complex as a novel self-renewal factor in RNAi screens (Chia et al., 2010; Hu et al., 2009). INO80 belongs to the INO80 subfamily of the SWI/SNF chromatin remodeling complexes. Although it is known to function in a variety of nuclear transactions, including transcription regulation, DNA repair, and DNA replication (Conaway and Conaway, 2009; Morrison and Shen, 2009; Watanabe and Peterson, 2011), its role in ESCs remains undefined. Here we show that the INO80 chromatin remodeling complex is required for ESC self-renewal, as well as for reprogramming and embryonic development. INO80 selectively occupies promoters of core

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pluripotency genes bound by the master ESC transcription factors, but not those involved in differentiation, and its occupancy is dependent on Oct4 and Wdr5. Importantly, INO80 maintains accessible chromatin architecture and facilitates recruitment of Mediator and RNA polymerase II at these genes, promoting their expression. These results define an essential role for the INO80 complex in the establishment and maintenance of the pluripotent state through its specific action on a core network of pluripotency genes.

Results

INO80 is important for ESC self-renewal

To define the role of INO80 in ESCs, we silenced individual subunits of INO80 with siRNAs. Using the Oct4GiP reporter ESCs (Ying and Smith, 2003; Zheng and Hu, 2012), in which the expression of the enhanced green fluorescent protein (EGFP) is driven by the ESC-specific Oct4 promoter, we found that silencing of all the tested subunits (Figure S1A) resulted in ESC differentiation as evidenced by the loss of EGFP expression (Figure 1A). Furthermore, INO80 silencing also led to the loss of typical ESC morphology (Figure 1B), suggesting that it plays an essential role in ESC maintenance.

As Ino80 is the SWI/SNF ATPase in the complex and its knockdown (KD) led to the most pronounced phenotype (Figure 1A, 1B), we chose to focus on this specific subunit to elucidate the role of the INO80 complex in ESCs. Consistent with the above results, Ino80 KD led to the loss of alkaline phosphatase (an ESC marker) staining (Figure S1B). Furthermore, it led to decreased expression of key pluripotency factors including Oct4, Nanog, Sox2, Klf4 and Esrrb, as well as increased expression of lineage markers such as Cdx2, Fgf5, Nestin and Pax3 (Figure 1C and 1D). To minimize the possibility of the off-target effect, we used 2 siRNAs and 3 shRNAs for Ino80 silencing. In all cases, Ino80 silencing led to similar changes in pluripotency marker expression (Fig. S1C, S1D). Thus, Ino80 is required for ESC self-renewal.

In agreement with its role in self-renewal, Ino80 is down-regulated during ESC differentiation, similar to the ESC marker Oct4 (Figure 1E). In addition, it is expressed at higher levels in ESCs compared to other cell types, such as trophoblast stem cells (TSCs) and mouse embryonic fibroblasts (MEFs) (Figure S2). Interestingly, analysis of published chromatin immunoprecipitation (ChIP) datasets showed that key pluripotency factors such as Oct4, Nanog, Sox2, Klf4, and Esrrb co-occupy regions near the Ino80 transcription start site (TSS) (Figure 1F). Moreover, Oct4 or Sox2 KD resulted in significant down-regulation of Ino80 within 48 hours (Figure 1G). Collectively, these results strongly suggest that Ino80 expression correlates with the ESC state and is tightly regulated by pluripotency factors.

INO80 is required for pluripotency gene expression

To understand how the INO80 complex regulates self-renewal and pluripotency, we carried out microarray analysis upon silencing of individual subunits in ESCs. Ino80, Ino80b, Ino80c and Ino80e KD induced expression changes of 2406, 1845, 1810 and 1276 genes, respectively. Overlap analysis indicated that 440 genes had altered expression patterns no matter which subunit was silenced (Figure 2A). Surprisingly, the overwhelming majority of

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these genes (407 of the 440) were down-regulated following depletion of INO80 subunits, suggesting that the INO80 complex may function to sustain their expression. Furthermore, this gene set included key pluripotency genes such as Oct4, Nanog, Klf4, Esrrb, Tcl1, Tbx3 and Foxd3 (Figure S3A–B), suggesting that INO80 is required for the expression of pluripotency factors in ESCs.

To monitor the dynamic gene expression changes upon Ino80 KD, we performed microarray analysis at days 2, 4 and 6 after silencing the ATPase Ino80 with a lentiviral-based shRNA. Consistent with the above results using siRNAs, Ino80 KD by shRNA induced down-regulation of almost all the well-characterized pluripotency factors over time, including Oct4, Nanog, Sox2, Klf4, Esrrb, Tcl1, Tbx3, Nr0b1, Nr5a2, Foxd3, Zfp42, and Tet1 (Figure 2B, S3C). Furthermore, Ingenuity pathway analysis (IPA) indicated that Ino80-regulated genes are highly enriched for pluripotency factors (Figure S3D), and gene set enrichment analysis (GSEA) showed that genes down-regulated after Ino80 KD are highly enriched for genes that are down-regulated during ESC differentiation (Figure 2C). In contrast, the vast majority of housekeeping genes were not down-regulated after Ino80 silencing (Figure S3E–F).

To understand how Ino80 fits into the pluripotency transcription network, we next compared the gene expression changes caused by Ino80 KD with those caused by the depletion of other pluripotency factors. Ino80 KD clustered with Oct4, Sox2, and Nanog (Figure 2D), suggesting that similar to those master transcription factors Ino80 plays a critical role in maintaining the gene expression program in ESCs. Together, these results support the notion that the INO80 complex is an important component of the pluripotency transcription circuitry and is required for the expression of pluripotency factors.

Ino80 occupies pluripotency gene promoter proximal regions

The fact that key pluripotency factors such as Oct4, Nanog, Sox2, Klf4, and Esrrb were quickly down-regulated after INO80 silencing (Figure 2B, S3A–C) suggested that INO80 may directly regulate their expression. To test this hypothesis, we performed chromatin immunoprecipitation followed by high through-put sequencing (ChIP-seq) and identified 12,749 genomic regions occupied by Ino80 with high confidence (FDR = 10^{-6}). The Ino80 peaks were enriched in gene-rich chromosomal regions where they tended to co-localize with transcription start sites (TSS) (Figure 2E, S4A) as has been observed in yeast (Yen, 2013). Interestingly, Ino80 peaks co-localized with active histone marks including H3K4me3 and H3K27ac, but not inactive chromatin marks such as H3K27me3. They also closely co-localized with the master ESC transcription factors Oct4, Nanog, and Sox2 (Figure 2F, S4B). Inspection of individual gene tracks and ChIP followed by quantitative PCR (ChIP-qPCR) showed that Ino80 occupied genomic regions near well-characterized pluripotency genes, including the master ESC transcription factors (Figure 2G). Of the 2126 genes that showed differential expression after Ino80 KD, 678 had Ino80 occupancy nearby and were likely its direct targets (Figure 2H). These Ino80 target genes included nearly all the key pluripotency factors and were strongly enriched for genes involved in ESC self-renewal and pluripotency (Figure 2H, 2I).

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Notably, while many Ino80-occupied genes were co-occupied by master ESC transcription factors Oct4, Nanog, and Sox2, the converse statement was not true. A significantly smaller fraction of the Oct4, Nanog, or Sox2-bound genes were co-occupied by Ino80 (Figure 3A and S4B). GSEA indicated that Ino80-occupied genes were mostly down-regulated during ESC differentiation (Figure 3A, 3B), consistent with the idea that INO80 facilitates expression at these loci and may function as an activator (Figure 2A). In contrast, Oct4, Nanog, and Sox2-bound genes were either up-regulated or down-regulated during differentiation (Figure 3A, S4C). As Oct4, Nanog, and Sox2 are known to maintain self-renewal by activating ESC genes and repressing differentiation genes, our data suggest that Ino80 is required for the master transcription factors to activate pluripotency genes integral to the ESC state. In agreement with this, genes co-occupied by Ino80, Oct4, Nanog, and Sox2 are highly enriched for pluripotency genes and tend to be down-regulated during differentiation (Figure 3C, S4D–E, Table S5). Genes co-occupied by Oct4, Nanog, Sox2 but not Ino80 are enriched for developmental and differentiation-associated genes and are more likely to be up-regulated during differentiation (Figure 3C, S4D–E, Table S5).

To understand how Ino80 is recruited to pluripotency genes promoters, we searched for factors that are required for its genomic occupancy. As chromatin regulators are often recruited by pioneer factors, we hypothesized that Ino80 may be recruited by master ESC transcription factors. Indeed, it has been reported that Oct4 interacts with Ino80 (Pardo et al., 2010), and we confirmed the interaction by immunoprecipitation (Figure 3D). To test our hypothesis, we used the ZHBTc4 cells in which Oct4 expression can be repressed by Doxycycline treatment (Niwa et al., 2000). We found that Oct4 depletion by 48 hrs of Doxycycline treatment led to a profound reduction in Ino80 occupancy near pluripotency gene promoters, with only a modest decrease in Ino80 expression (Figure 3E, 3F). This result suggested that Oct4 is important for Ino80 recruitment. However, we reasoned that there may be additional factor(s) involved, as Ino80 occupancy was not abolished in Oct4 depleted cells and Ino80 only occupies a small subset of genes that are occupied by Oct4. To search for the additional factor(s), we carried out Ino80 immunoprecipitation (IP) followed by Mass-spectrometry, and found that Ino80 interacts with another known pluripotency protein Wdr5 (data not shown). We confirmed this interaction by IP-western (Figure 3D, S4F). Wdr5 is a key component of the H3K4 methyltransferase complex. It occupies actively transcribed genes in ESCs and is required for self-renewal (Ang et al., 2011). To test its role in Ino80 recruitment, we silenced Wdr5 with shRNAs. At 48 hrs, Wdr5 silencing significantly reduced Ino80 genomic occupancy without affecting its expression (Figure 3G, 3H). Thus, Ino80 occupancy is dependent on both Oct4 and Wdr5. As Oct4 and Wdr5 occupy developmental genes and actively transcribed genes, respectively, they may cooperatively facilitate the recruitment of Ino80 to pluripotency gene promoters. Consistent with this notion, there is a significant overlap between genes co-occupied by Oct4 and Wdr5 and those occupied by Ino80 (Figure 3I). However, there are likely other factors involved in the recruitment of Ino80, as Oct4 and Wdr5 occupy both promoter and enhancer regions while Ino80 preferentially occupy promoter regions.

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INO80 promotes Mediator and Pol II recruitment

Our results indicate that INO80 is required for pluripotency gene expression. We therefore hypothesize that INO80 may activate pluripotency genes by recruiting factors necessary for their transcription. Consistent with this idea, we found that Ino80 occupancy at promoter proximal regions strongly correlates with occupancy of factors important in ESC gene expression and self-renewal, such as Oct4, Nanog, Sox2, Esrrb, Klf4, and Med1 (Figure 4A, 4B, S5A–B). To test whether INO80 occupancy affects the recruitment of these factors, we carried out ChIP-seq for Oct4, Klf4, and Med1 48 hours after Ino80 shRNA virus transduction. At this time point, Ino80 silencing led to significant down-regulation of Ino80 protein, but only subtle decreases in pluripotency mRNA and protein levels and a slight up-regulation of Med1 level (Figure S5C, S5D). However, Ino80 silencing led to an obvious reduction in Med1 binding at Ino80 and Med1 co-occupied TSS regions (Figure 4C, 4D). Furthermore, Med1 binding was reduced near genes that were down-regulated after Ino80 silencing (Figure 4E, 4F). In contrast, Ino80 silencing did not cause a similar decrease in Oct4 or Klf4 binding (Figure 4C, 4D). Oct4 binding was even slightly increased, consistent with a recent report that slightly reduced Oct4 level increases its occupancy (Karwacki-Neisius et al., 2013). Thus, Ino80 does not appear to directly regulate the recruitment of pluripotency transcription factors. Examination of individual gene tracks and ChIP-qPCR confirmed that Ino80 silencing caused significant reduction of Mediator binding near key pluripotency gene promoter regions (Figure 4G, 4H). As Mediator is essential for transcription initiation (Ansari and Morse, 2013), our results suggest that INO80 may normally promote pluripotency gene expression through the recruitment of Mediator.

Because Mediator loss-of-function has been found to compromise RNA polymerase II association with promoters of active genes in other cell types (Ansari and Morse, 2013), we next examined whether Ino80 silencing and impaired Med1 recruitment has an impact on Pol II occupancy. Based on ChIP-seq, Ino80 silencing led to decreased binding of Pol II near promoters occupied by Ino80 (Figure 5A, 5B), as well as promoters of genes down-regulated after Ino80 silencing (Figure 5C, 5D). This result was confirmed by ChIP-qPCR at the Oct4, Nanog, Sox2, and Klf4 promoters (Figure 5E). Importantly, Ino80, Med1, and Pol II co-occupied genes were highly enriched for those that are down-regulated during ESC differentiation into EBs (Figure 5F), and they were also mostly down-regulated during differentiation (Figure 5G). Together, our data suggest that INO80 activates pluripotency genes by facilitating the recruitment of Mediator and Pol II at their promoters.

INO80 maintains an open chromatin structure

Recent work in yeast has demonstrated a striking association of INO80-family chromatin remodelers with nucleosome depleted regions around transcription start sites where it participates in H2A/H2A.Z exchange and nucleosome turnover (Ranjan et al., 2013; Yen et al., 2013). We hypothesized that physical occupancy of these regions by INO80 might preclude stable nucleosome formation in these regions at bound loci, leaving DNA in and around the TSS more accessible to the general transcription machinery, Mediator, and RNA pol II. Thus, we evaluated the nucleosome occupancy in the presence or absence of Ino80 by micrococcal nuclease (MNase) digestion and PCR at selected regions. Whereas no effect was observed on genomic regions that are only occupied by ESC master transcription

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factors, nucleosome occupancy at regions co-occupied by Ino80 and master transcription factors was greatly increased at 48 hrs of Ino80 KD (Figure 6A). Consistent with that observation, Ino80 binding sites resided in nucleosome-depleted regions in ESCs (Figure 6B). In contrast, the overall Oct4 occupied regions were not associated with nucleosome depletion (Figure 6B), but those that were co-occupied by Ino80 showed some depletion (Figure S5E). To determine how Ino80-mediated nucleosome depletion affects genome accessibility, we carried out DNase I sensitivity assay for selected regions in the presence or absence of Ino80. Ino80 depletion led to reduced DNase I sensitivity at regions co-occupied by Ino80 and ESC master transcription factors, but had no effect on those only occupied by master transcription factors (Figure 6C). In agreement with that, almost all Ino80 bound regions (12470 out of 12749, 97.81%) overlapped with DNase I hypersensitive sites (Figure 6D), and Ino80 occupancy positively correlated with DNase I hypersensitivity (Figure 6E). These data suggest that INO80 maintains an open chromatin structure near pluripotency genes.

To test whether Ino80-mediated open chromatin structure facilitates the activation of the neighboring gene, we cloned DNA fragments occupied by Ino80 in ESCs into a luciferase reporter construct (Whyte et al., 2013) and transfected the reporter constructs into ESCs. DNA fragments corresponding to Ino80 peaks in ESCs strongly enhanced the reporter expression (Figure 6F). More importantly, the reporter expression was markedly suppressed by Ino80 KD (Figure 6G). In comparison, the activities of genomic regions occupied by Oct4, Nanog, Sox2 but not Ino80 were not inhibited by Ino80 KD in the same test (Figure 6G). Collectively, our data suggest that the INO80 complex may regulate pluripotency gene expression by maintaining an open chromatin structure.

INO80 is required for reprogramming and blastocyst formation

During the course of reprogramming, pluripotency is established through the activation of the ESC gene expression program, as well as the establishment of ESC-specific chromatin structure (Stadtfeld and Hochedlinger, 2010). Given the essential role of the INO80 complex in ESC maintenance, we asked whether it also has a role in the establishment of pluripotency. We first tested the function of Ino80 in the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). Ino80 expression quickly increased during the course of reprogramming and reached its highest level on day 6, coinciding with the time when the endogenous pluripotency genes start to become activated (Polo et al., 2012) (Figure 7A). More importantly, Ino80 silencing by shRNA dramatically reduced the number of alkaline phosphatase-positive iPSC colonies formed after reprogramming (Figure 7B, S6A). In comparison, Ino80 KD did not reduce the expression of housekeeping genes (Figure S6B) and only modestly affected the growth of MEFs (Figure S6C). To confirm the above results, we carried out the reprogramming assay in MEFs that harbor a reporter EGFP gene under the control of the Oct4 promoter. Ino80 silencing did not impair the expression of the reprogramming factors (Figure S6D), but caused a significant reduction in the number of EGFP-positive iPSCs (Figure 7C, 7D). Furthermore, Ino80 silencing resulted in reduced expression of early markers of reprogramming at day 6 (Figure 7E). It also resulted in a more closed chromatin structure near pluripotency gene promoters, as evidenced by increased nucleosome occupancy (Figure S7A) as well as decreased H3K4me3 and

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increased H3K27me3 occupancy (Figure S7B–C). Therefore, Ino80 is required for efficient somatic cell reprogramming *in vitro*, possibly by activating pluripotency gene expression.

Next, we assessed the role of INO80 in blastocyst development, as the inner cell mass (ICM) specification during blastocyst formation also requires the establishment of pluripotency (Nichols and Smith, 2012). By RT-qPCR, we found that Ino80 expression gradually increased during early embryonic development and was highest at the blastocyst stage (Figure 7F). Immunofluorescence staining confirmed that Ino80 was expressed in ICM and localized in the nucleus, consistent with its function in ESCs (Figure 7G). We tested the antibody specificity toward Ino80 by carrying out staining in ESCs as well as in embryos treated with control- or Ino80-siRNAs, and confirmed that the antibody specifically recognizes Ino80 (Figure S7D, S7E). To test the role of INO80 during blastocyst development, we injected Ino80 siRNA into one-cell embryos, and cultured the embryos *ex vivo* to allow development to proceed. Visual inspection of the embryos indicated that Ino80 KD significantly inhibited blastocyst formation (Figure 7H, 7I). RT-qPCR confirmed that siRNA injection effectively silenced Ino80 (Figure 7J). Furthermore, in the few blastocysts formed from the Ino80-siRNA injected embryos, the expression of Oct4, Nanog, Sox2, and Klf4 was dramatically reduced (Figure 7K). Therefore, Ino80 is required for blastocyst development *ex vivo*, in part by maintaining the proper expression of pluripotency genes. Together with our results in ESCs and iPSCs, we propose that Ino80 is required for the establishment of pluripotency both *in vitro* and *in vivo*.

Discussion

In this study, we show that the INO80 complex is required for both the establishment and maintenance of pluripotency and is a key component in the core pluripotency transcription circuitry. Our data support the model that INO80 occupies pluripotency gene promoter proximal regions and activates their expression by maintaining an open chromatin structure and facilitating the recruitment of Mediator and Pol II.

Although several chromatin remodeling complexes were previously implicated in ESC biology, their roles in the core ESC transcription circuitry have not been clearly defined. For example, CHD1 regulates open chromatin and the differentiation capacity of ESCs (Gaspar-Maia et al., 2009). Tip60-p400 represses differentiation genes to maintain ESC identity (Fazzio et al., 2008). esBAF facilitates LIF/STAT3 signaling and regulates polycomb function (Ho et al.). NuRD represses pluripotency genes to promote transcriptional heterogeneity and lineage commitment (Hu and Wade, 2012; Reynolds et al., 2012). In contrast, we show that INO80 forms an auto-regulatory loop with the master ESC transcription factors Oct4, Nanog, and Sox2. More importantly, it specifically activates pluripotency genes with the master transcription factors to maintain the ESC state. Consistent with this notion, of all the chromatin remodelers, only INO80 was identified as a self-renewal regulator in the Oct4-reporter based RNAi screens (Chia et al., 2010; Ding et al., 2009; Fazzio et al., 2008; Hu et al., 2009), possibly because of its role in the direct regulation of the core pluripotency circuitry.

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Mechanistically, it has been proposed that INO80 can regulate transcription via the ATP-dependent mobilization of nucleosomes (Conaway and Conaway, 2009; Shen et al., 2000; Watanabe and Peterson, 2011). In agreement with this model, our data indicate that INO80 is required for the maintenance of nucleosome depleted regions and open chromatin structure at the pluripotency gene promoters. Alternative models posit that INO80 regulates the distribution of the histone variant H2A.Z and promotes H2A.Z eviction from promoters (Papamichos-Chronakis et al., 2011). Indeed, INO80 occupies nucleosome-free regions around transcription start sites in yeast, where it promotes H2A/H2A.Z exchange and nucleosome turnover (Ranjan et al., 2013; Yen et al., 2013). In ESCs, H2A.Z is highly enriched at active and bivalent promoters (Hu et al., 2013; Ku et al., 2012). It is post-translationally modified and distinct modification states enables H2A.Z to regulate different class of genes, such as active and poised genes (Ku et al., 2012). Thus, it is conceivable that INO80 may be required for pluripotency gene expression by promoting the cycling of H2A.Z and/or modified H2A.Z at their promoters, facilitating the maintenance of the nucleosome-depleted regions and the accessibility of the general transcription factors.

Consistent with this model, our data demonstrate that INO80 occupancy enhances the recruitment of Mediator and Pol II, and thus activates target gene expression. Mediator interacts with Pol II and the general transcription machinery, coordinating the assembly of the general initiation factors and chromatin machinery into functional pre-initiation complexes (Ansari and Morse, 2013), (Chen et al., 2012). In addition, Mediator is required for the maintenance of ESC identity (Kagey et al., 2010) by forming dense clusters near pluripotency genes to regulate their expression (Whyte et al., 2013). It promotes long-range chromatin interactions at these genes and plays an important role in genome organization in ESCs and reprogramming (Apostolou et al., 2013; Denholtz et al., 2013; Wei et al., 2013; Zhang et al., 2013). However, it is not fully understood how Mediator recognizes pluripotency genes. Our findings suggest that INO80 may act upstream of Mediator for its recruitment to ESC genes and regulate the unique chromatin organization in ESCs.

Finally, the master transcription factors occupy genomic regions near both ESC and differentiation genes (Dejosez and Zwaka, 2012; Jaenisch and Young, 2008; Ng and Surani, 2011; Young, 2011), and it is not clear how they selectively activate ESC genes to maintain the ESC state. Our results suggest that INO80 preferentially occupies ESC genes promoters, and its occupancy is dependent on both the master transcription factor Oct4 and the H3K4 methyltransferase complex component Wdr5. As Oct4 regulates developmental genes while Wdr5 regulates actively transcribed genes in ESCs, we propose that INO80 integrates the input from Oct4 and Wdr5 to specifically regulate pluripotency gene expression. It therefore acts as a deterministic factor in the transcriptional outcome for genes regulated by the master transcription factors. Thus, our findings elucidate a previously unrecognized coordination between INO80, the master transcription factors, and a histone modifying enzyme in controlling the pluripotent state, and shed light on how chromatin remodelers can orchestrate sophisticated transcriptional regulation in cell fate decision with other factors.

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Experimental Procedures

Mouse ESC Culture, Differentiation, and Transfection

J1 ESCs were obtained from the American Type Culture Collection. ZHBTc4 cells are kindly provided by Dr. Hitoshi Niwa. They were cultured on gelatin-coated plates in the ESGRO complete plus clonal grade medium (for maintenance) or the M15 medium (for experiments) as described before (Zheng et al., 2012). M15 medium contains DMEM supplemented with 15% FBS, 10 μM 2-mercaptoethanol, 1 mM nonessential amino acids, 1 × EmbryoMax nucleosides, 1000 U/ml of LIF (Millipore). ESC differentiation and transfections were carried out similarly as described previously (Wang et al., 2013).

Oct4GiP Reporter Assay

Oct4GiP ESCs were kindly provided by Dr. Austin Smith. Oct4GiP reporter assay was carried out as described previously (Zheng and Hu, 2012).

TSC culture

Mouse TSCs were kindly provided by Dr. Janet Rossant, and were cultured based on the published protocol without feeders (Rossant, 2006).

Immunofluorescence Staining

Cells were stained with primary antibodies against Ino80 (1:100, Proteintech), Cdx2 (1:100, Cell Signaling) or Oct4 (1:100, Santa Cruz), and cell nuclei were counterstained with DAPI (Invitrogen). Confocal images were taken on the Zeiss LSM 710 microscope.

Mouse blastocyst stage embryos were collected at E3.5, and incubated with primary antibody against Ino80 (1:300) and then secondary antibody (Alexa Fluor® 594, goat anti-rabbit IgG, 1:1000, Life Technologies). The embryos were stained with DAPI to identify cell nuclei. Confocal images were taken on the Zeiss LSM 710 microscope.

RNA Isolation, Reverse Transcription and Real-time PCR

Total RNA was isolated from cells using the GeneJet RNA purification kit (Thermo Scientific), and 0.5 μg total RNA was reverse transcribed to generate cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. qPCRs were performed using the SsoFast™ EvaGreen® Supermix (Bio-Rad) on the Bio-Rad CFX-384 or CFX-96 Real-Time PCR System. Actin was used for normalization. Primers used in the study were listed in Table S1.

ChIP-qPCR and ChIP-Seq Sample Preparation

Ino80 ChIP was performed as described previously (Whyte et al., 2013). J1 cells were fixed using 1% formaldehyde for 10 min, and followed by 0.125M glycine 5 min to stop the fixation. Then the cells were harvested, and DNA was fragmented to 300–500 bp by sonication with a microtip attached to Misonix 3000 sonicator. Immunoprecipitation was performed with 3 μg Dynabeads protein G (Life Technology) conjugated-rabbit polyclonal anti-Ino80 (Proteintech) antibody overnight at 4 °C. Afterwards, beads were washed, eluted

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and reverse cross-linked. DNA was extracted by phenol/chloroform and precipitated. The resulting DNA was analyzed by qPCR and data were presented as the percentage of input using indicated primers (Table S1). For Oct4, Klf4, Med1 and Pol II ChIP, J1 cells were infected with non-targeting (NT) or Ino80 shRNA. 48 hours after infection, cells were harvested for ChIP against Oct4 (Santa Cruz), Klf4 (R&D), Med1 (Bethyl) and Pol II (Santa Cruz) based on the protocol described above. For ChIP-seq, 1 ng precipitated DNA or input was used to generate DNA library by use of Nextera XT DNA sample preparation Kit (Illumina) according to the manufacturer's instruction. The resulting libraries were used for sequencing by MiSeq (Illumina). Two biological replicates were performed here, and combined reads were used for further analysis.

MNase qPCR

Nucleosome occupancy in ESCs was determined according to a published protocol (Wei et al., 2012) with modifications. Nuclei were isolated from J1 ESCs. Native chromatin without crosslinking was resuspended in digest buffer (15 mM Tris pH 8, 60 mM KCl, 15 mM NaCl, 1 mM CaCl2, 0.25 M sucrose, 0.5 mM DTT) and incubated with 40 units MNase for 5 minutes at room temperature. Digestion was stopped by adding stop buffer (0.5 ml 10% SDS, 0.5 ml 1M NaBicarbonate, 0.2 ml 0.5 M EDTA, 3.8 ml H2O). Chromatin was precipitated, and isolated using 1.2% DNA gel. Mononucleosomal DNA was collected and extracted using a gel extraction kit (Qiagen). Nucleosome occupancy was evaluated by qPCR and presented as percentage of input. Primers used in this assay are listed in Table S1.

DNase I Sensitivity Assay

DNase I sensitivity assay was performed as described previously (Ho et al., 2011) with modifications. We added equal amount of *Drosophila* genomic DNA to each reactions as an internal control to minimize the variations caused by phenol-chloroform extraction. The same genomic regions assayed in the MNase qPCRs were examined for DNase I sensitivity by qPCRs, and a region near the *Drosophila* Rps49 gene was used as internal control to calculate the fold changes.

Luciferase Reporter Assay

Ino80 occupied regions were generated by PCR from E14 wide type genomic DNA. The regions were cloned into pGL3-Promoter containing the minimal Oct4 promoter as described previously (Whyte et al., 2013). Primers used for the cloning were listed in Table S1.

Mouse Embryo Collection and Microinjection

CF-1 female mice were superovulated and mated to B6D2F1/J males, and one cell embryos were collected. Microinjections were performed using a Leica DMI 6000B inverted microscope equipped with a XenoWorks Micromanipulator system and a PrimeTech PMM-150FU Piezo drill (Sutter Instruments). Five to ten picoliter of the 20 μM non-targeting, or Ino80 siRNA was injected into the cytoplasm of one cell embryos. After injection, the embryos were cultured and inspected at indicated time points to determine

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developmental progress. Each experiment consisted of three separate replicates, and approximately 30 embryos were injected in each replicate.

Reprogramming

MEFs were plated in 12-well plate at 2×10^5 cells / well (day-0), and transduced with the non-targeting or Ino80 pLKO.1 shRNA viruses the next day (day-1). 2 days after transduction, cells were re-plated in 12-well plate at 80 k / well (day-3), and were transduced with the STEMCCA viruses (Sommer et al., 2009) encoding the four Yamanaka factors the next day (day-4). Cells were re-plated in gelatin-coated 12-well plates at 80 k / well at day-5. On day-6, culture medium was switched from MEF medium to M15, and medium was changed every day until colony number was counted by AP staining on day-14.

Microarray Analysis

J1 ESCs were transfected with siRNAs as described above in duplicates, and were collected 96 hrs after transfection. Gene expression analysis was carried out on the Agilent Whole Mouse Genome 4 × 44k arrays following the Agilent 1-color microarray-based gene expression analysis protocol. Data was processed in R-3.0.0 by first reading the Agilent files using Bioconductor package Agi4x44PreProcess version 1.20.0. Data was reviewed using MA-plots, and the “G” platform signal intensities were normalized so the median log2 fold change was zero.

For the time course experiment, Ino80 shRNA sample groups were compared to non-targeting shRNA control at each respective time point. For the subunit experiment, each Ino80 subunit siRNA sample group was compared to the non-targeting siRNA sample group. A statistical model was fit using the Bioconductor limma package version 3.16.1, using the “duplicateeCorrelation” function as described in the Limma User Guide for probes with technical replicates. Statistical comparisons were performed using a moderated t-test, P-values adjusted using the Benjamini-Hochberg adjustment. Statistical hits were defined as having adjusted P-value <= 0.05 and absolute fold change >= 1.5.

Statistical results were combined per-gene using the following logic: 1) For each gene associated with only one probe, its results were used without modification; 2) For genes associated with multiple microarray probes, results were combined using only the statistically significant probes; 3) If multiple probes for a given gene were significant and the fold changes were in opposing directions, the probes with the same direction as the most significant probe were used; 4) If no probes were statistically significant, all probes for the gene were used.

ChIP-Seq Data Analysis

Ino80 ChIP-seq and Input libraries were sequenced using MiSeq technologies at the NIEHS DNA Sequencing Core. Standard Illumina CASAVA 1.8 utilities were used to generate .fastq output files. All libraries were sequenced as single end 36mers. ChIP-Seq datasets profiling the genomic occupancy of H3K4me3, H3K27me3, H3K27ac, Oct4, Sox2, Nanog, Klf4, Med1, DNase I hypersensitivity, and nucleosome occupancy in mouse ESCs were obtained from previous publications (Table S2).

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Sequenced reads from ChIP-seq and Input libraries were combined for replicate samples and filtered as stated in text. Filtered reads were then aligned to the mouse reference genome (NCBI build 37, mm9) using the Bowtie short-read alignment program (v0.12.8 employing parameters -v2, -m1) to retain reads mapped to unique genomic locations with at most 2 mismatches. Only non-duplicate reads were used in subsequent peak calling analyses and the generation of coverage tracks. To make the coverage tracks, aligned reads were extended at the 3′ end to a length of 300 bases (the expected genomic fragment size) for Ino80 and 200 bases for other factors, and bigWig files were generated to visualize aggregate genomic coverage. ChIP-seq peaks for each cell type were called using SICER (Zang et al., 2009) with a FDR threshold of 1e-6 or 1e-20 and the following parameters (Ino80: redundancy threshold=1, window size=200, gap size=600, fragment size=300; Other factors: Ino80: redundancy threshold=1, window size=200, gap size=600, fragment size=200). Sequenced reads from matched supernatant Inputs were used as controls for each cell type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Ang YS, Tsai SY, Lee DF, Monk J, Su J, Ratnakumar K, Ding J, Ge Y, Darr H, Chang B, et al. Wdr5 mediates self-renewal and reprogramming via the embryonic stem cell core transcriptional network. Cell. 2011; 145:183–197. [PubMed: 21477851]

Ansari SA, Morse RH. Mechanisms of Mediator complex action in transcriptional activation. Cellular and molecular life sciences: CMLS. 2013; 70:2743–2756. [PubMed: 23361037]

Apostolou E, Ferrari F, Walsh RM, Bar-Nur O, Stadtfeld M, Cheloufi S, Stuart HT, Polo JM, Ohsumi TK, Borowsky ML, et al. Genome-wide chromatin interactions of the Nanog locus in pluripotency, differentiation, and reprogramming. Cell Stem Cell. 2013; 12:699–712. [PubMed: 23665121]

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122:947–956. [PubMed: 16153702]

Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J, et al. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell. 2008; 133:1106–1117. [PubMed: 18555785]

Chen XF, Lehmann L, Lin JJ, Vashisht A, Schmidt R, Ferrari R, Huang C, McKee R, Mosley A, Plath K, et al. Mediator and SAGA have distinct roles in Pol II preinitiation complex assembly and function. Cell reports. 2012; 2:1061–1067. [PubMed: 23177621]

Chia NY, Chan YS, Feng B, Lu X, Orlov YL, Moreau D, Kumar P, Yang L, Jiang J, Lau MS, et al. A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. Nature. 2010; 468:316–320. [PubMed: 20953172]

Clapier CR, Cairns BR. The biology of chromatin remodeling complexes. Annu Rev Biochem. 2009; 78:273–304. [PubMed: 19355820]

Conaway RC, Conaway JW. The INO80 chromatin remodeling complex in transcription, replication and repair. Trends Biochem Sci. 2009; 34:71–77. [PubMed: 19062292]

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Dejosez M, Zwaka TP. Pluripotency and nuclear reprogramming. Annu Rev Biochem. 2012; 81:737–765. [PubMed: 22443931]

Denholtz M, Bonora G, Chronis C, Splinter E, de Laat W, Ernst J, Pellegrini M, Plath K. Long-range chromatin contacts in embryonic stem cells reveal a role for pluripotency factors and polycomb proteins in genome organization. Cell Stem Cell. 2013; 13:602–616. [PubMed: 24035354]

Ding L, Paszkowski-Rogacz M, Nitzsche A, Slabicki MM, Heninger AK, de Vries I, Kittler R, Junqueira M, Shevchenko A, Schulz H, et al. A genome-scale RNAi screen for Oct4 modulators defines a role of the Paf1 complex for embryonic stem cell identity. Cell Stem Cell. 2009; 4:403–415. [PubMed: 19345177]

Fazzio TG, Huff JT, Panning B. An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell. 2008; 134:162–174. [PubMed: 18614019]

Gaspar-Maia A, Alajem A, Polesso F, Sridharan R, Mason MJ, Heidersbach A, Ramalho-Santos J, McManus MT, Plath K, Meshorer E, et al. Chd1 regulates open chromatin and pluripotency of embryonic stem cells. Nature. 2009; 460:863–868. [PubMed: 19587682]

Hargreaves DC, Crabtree GR. ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell research. 2011; 21:396–420. [PubMed: 21358755]

Ho L, Miller EL, Ronan JL, Ho WQ, Jothi R, Crabtree GR. esBAF facilitates pluripotency by conditioning the genome for LIF/STAT3 signalling and by regulating polycomb function. Nat Cell Biol. 2011; 13:903–913. [PubMed: 21785422]

Hu G, Cui K, Northrup D, Liu C, Wang C, Tang Q, Ge K, Levens D, Crane-Robinson C, Zhao K. H2A.Z facilitates access of active and repressive complexes to chromatin in embryonic stem cell self-renewal and differentiation. Cell Stem Cell. 2013; 12:180–192. [PubMed: 23260488]

Hu G, Kim J, Xu Q, Leng Y, Orkin SH, Elledge SJ. A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. Genes Dev. 2009; 23:837–848. [PubMed: 19339689]

Hu G, Wade PA. NuRD and pluripotency: a complex balancing act. Cell Stem Cell. 2012; 10:497–503. [PubMed: 22560073]

Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell. 2008; 132:567–582. [PubMed: 18295576]

Kagey MH, Newman JJ, Bilodeau S, Zhan Y, Orlando DA, van Berkum NL, Ebmeier CC, Goossens J, Rahl PB, Levine SS, et al. Mediator and cohesin connect gene expression and chromatin architecture. Nature. 2010; 467:430–435. [PubMed: 20720539]

Karwacki-Neisius V, Goke J, Osorno R, Halbritter F, Ng JH, Weisse AY, Wong FC, Gagliardi A, Mullin NP, Festuccia N, et al. Reduced Oct4 expression directs a robust pluripotent state with distinct signaling activity and increased enhancer occupancy by Oct4 and Nanog. Cell Stem Cell. 2013; 12:531–545. [PubMed: 23642364]

Kidder BL, Palmer S, Knott JG. SWI/SNF-Brg1 regulates self-renewal and occupies core pluripotency-related genes in embryonic stem cells. Stem Cells. 2009; 27:317–328. [PubMed: 19056910]

Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. Cell. 2008; 132:1049–1061. [PubMed: 18358816]

Ku M, Jaffe JD, Koche RP, Rheinbay E, Endoh M, Koseki H, Carr SA, Bernstein BE. H2A.Z landscapes and dual modifications in pluripotent and multipotent stem cells underlie complex genome regulatory functions. Genome biology. 2012; 13:R85. [PubMed: 23034477]

Landry J, Sharov AA, Piao Y, Sharova LV, Xiao H, Southon E, Matta J, Tessarollo L, Zhang YE, Ko MS, et al. Essential role of chromatin remodeling protein Bptf in early mouse embryos and embryonic stem cells. PLoS Genet. 2008; 4:e1000241. [PubMed: 18974875]

Mattout A, Meshorer E. Chromatin plasticity and genome organization in pluripotent embryonic stem cells. Current opinion in cell biology. 2010; 22:334–341. [PubMed: 20226651]

Morrison AJ, Shen X. Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. Nat Rev Mol Cell Biol. 2009; 10:373–384. [PubMed: 19424290]

Ng HH, Surani MA. The transcriptional and signalling networks of pluripotency. Nat Cell Biol. 2011; 13:490–496. [PubMed: 21540844]

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Nichols J, Smith A. Pluripotency in the embryo and in culture. Cold Spring Harb Perspect Biol. 2012; 4:a008128. [PubMed: 22855723]

Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nature genetics. 2000; 24:372–376. [PubMed: 10742100]

Papamichos-Chronakis M, Watanabe S, Rando OJ, Peterson CL. Global regulation of H2A.Z localization by the INO80 chromatin-remodeling enzyme is essential for genome integrity. Cell. 2011; 144:200–213. [PubMed: 21241891]

Pardo M, Lang B, Yu L, Prosser H, Bradley A, Babu MM, Choudhary J. An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. Cell Stem Cell. 2010; 6:382–395. [PubMed: 20362542]

Polo JM, Anderssen E, Walsh RM, Schwarz BA, Nefzger CM, Lim SM, Borkent M, Apostolou E, Alaei S, Cloutier J, et al. A molecular roadmap of reprogramming somatic cells into iPS cells. Cell. 2012; 151:1617–1632. [PubMed: 23260147]

Ranjan A, Mizuguchi G, Fitzgerald PC, Wei D, Wang F, Huang Y, Luk E, Woodcock CL, Wu C. Nucleosome-free Region Dominates Histone Acetylation in Targeting SWR1 to Promoters for H2A.Z Replacement. Cell. 2013; 154:1232–1245. [PubMed: 24034247]

Reynolds N, Latos P, Hynes-Allen A, Loos R, Leaford D, O'Shaughnessy A, Mosaku O, Signolet J, Brennecke P, Kalkan T, et al. NuRD suppresses pluripotency gene expression to promote transcriptional heterogeneity and lineage commitment. Cell Stem Cell. 2012; 10:583–594. [PubMed: 22560079]

Rossant J. Culturing Trophoblast Stem (TS) Cell Lines. CSH protocols. 2006; 2006

Schaniel C, Ang YS, Ratnakumar K, Cormier C, James T, Bernstein E, Lemischka IR, Paddison PJ. Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. Stem Cells. 2009; 27:2979–2991. [PubMed: 19785031]

Schnetz MP, Handoko L, Akhtar-Zaidi B, Bartels CF, Pereira CF, Fisher AG, Adams DJ, Flicek P, Crawford GE, Laframboise T, et al. CHD7 targets active gene enhancer elements to modulate ES cell-specific gene expression. PLoS Genet. 2010; 6:e1001023. [PubMed: 20657823]

Shen X, Mizuguchi G, Hamiche A, Wu C. A chromatin remodelling complex involved in transcription and DNA processing. Nature. 2000; 406:541–544. [PubMed: 10952318]

Singhal N, Graumann J, Wu G, Arauzo-Bravo MJ, Han DW, Greber B, Gentile L, Mann M, Scholer HR. Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. Cell. 2010; 141:943–955. [PubMed: 20550931]

Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem Cells. 2009; 27:543–549. [PubMed: 19096035]

Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. Genes Dev. 2010; 24:2239–2263. [PubMed: 20952534]

Wang L, Miao YL, Zheng X, Lackford B, Zhou B, Han L, Yao C, Ward JM, Burkholder A, Lipchina I, et al. The THO complex regulates pluripotency gene mRNA export and controls embryonic stem cell self-renewal and somatic cell reprogramming. Cell Stem Cell. 2013; 13:676–690. [PubMed: 24315442]

Watanabe S, Peterson CL. The INO80 family of chromatin-remodeling enzymes: regulators of histone variant dynamics. Cold Spring Harb Symp Quant Biol. 2011; 75:35–42. [PubMed: 21502417]

Wei G, Hu G, Cui K, Zhao K. Genome-wide mapping of nucleosome occupancy, histone modifications, and gene expression using next-generation sequencing technology. Methods Enzymol. 2012; 513:297–313. [PubMed: 22929775]

Wei Z, Gao F, Kim S, Yang H, Lyu J, An W, Wang K, Lu W. Klf4 organizes long-range chromosomal interactions with the oct4 locus in reprogramming and pluripotency. Cell Stem Cell. 2013; 13:36–47. [PubMed: 23747203]

Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, Rahl PB, Lee TI, Young RA. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell. 2013; 153:307–319. [PubMed: 23582322]

Yen K, Vinayachandran V, Pugh BF. SWR-C and INO80 Chromatin Remodelers Recognize Nucleosome-free Regions Near +1 Nucleosomes. Cell. 2013; 154:1246–1256. [PubMed: 24034248]

Yen KVV, Pugh BF. SWR-C and INO80 Chromatin Remodelers Recognize Nucleosome-free Regions Near +1 Nucleosomes. Cell. 2013; 154:10.

Ying QL, Smith AG. Defined conditions for neural commitment and differentiation. Methods Enzymol. 2003; 365:327–341. [PubMed: 14696356]

Young RA. Control of the embryonic stem cell state. Cell. 2011; 144:940–954. [PubMed: 21414485]

Zang C, Schones DE, Zeng C, Cui K, Zhao K, Peng W. A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics. 2009; 25:1952–1958. [PubMed: 19505939]

Zhang H, Jiao W, Sun L, Fan J, Chen M, Wang H, Xu X, Shen A, Li T, Niu B, et al. Intrachromosomal looping is required for activation of endogenous pluripotency genes during reprogramming. Cell Stem Cell. 2013; 13:30–35. [PubMed: 23747202]

Zheng X, Dumitru R, Lackford BL, Freudenberg JM, Singh AP, Archer TK, Jothi R, Hu G. Cnot1, Cnot2, and Cnot3 maintain mouse and human ESC identity and inhibit extraembryonic differentiation. Stem Cells. 2012; 30:910–922. [PubMed: 22367759]

Zheng X, Hu G. Oct4GiP reporter assay to study genes that regulate mouse embryonic stem cell maintenance and self-renewal. J Vis Exp. 2012

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Highlights

- INO80 is required for ESC self-renewal, reprogramming, and blastocyst development
- INO80 occupies pluripotency gene promoters, which is dependent on Wdr5 and Oct4
- INO80 maintains an open chromatin structure and recruits Mediator and Pol II
- INO80 binding distinguishes active genes from those repressed by master TFs

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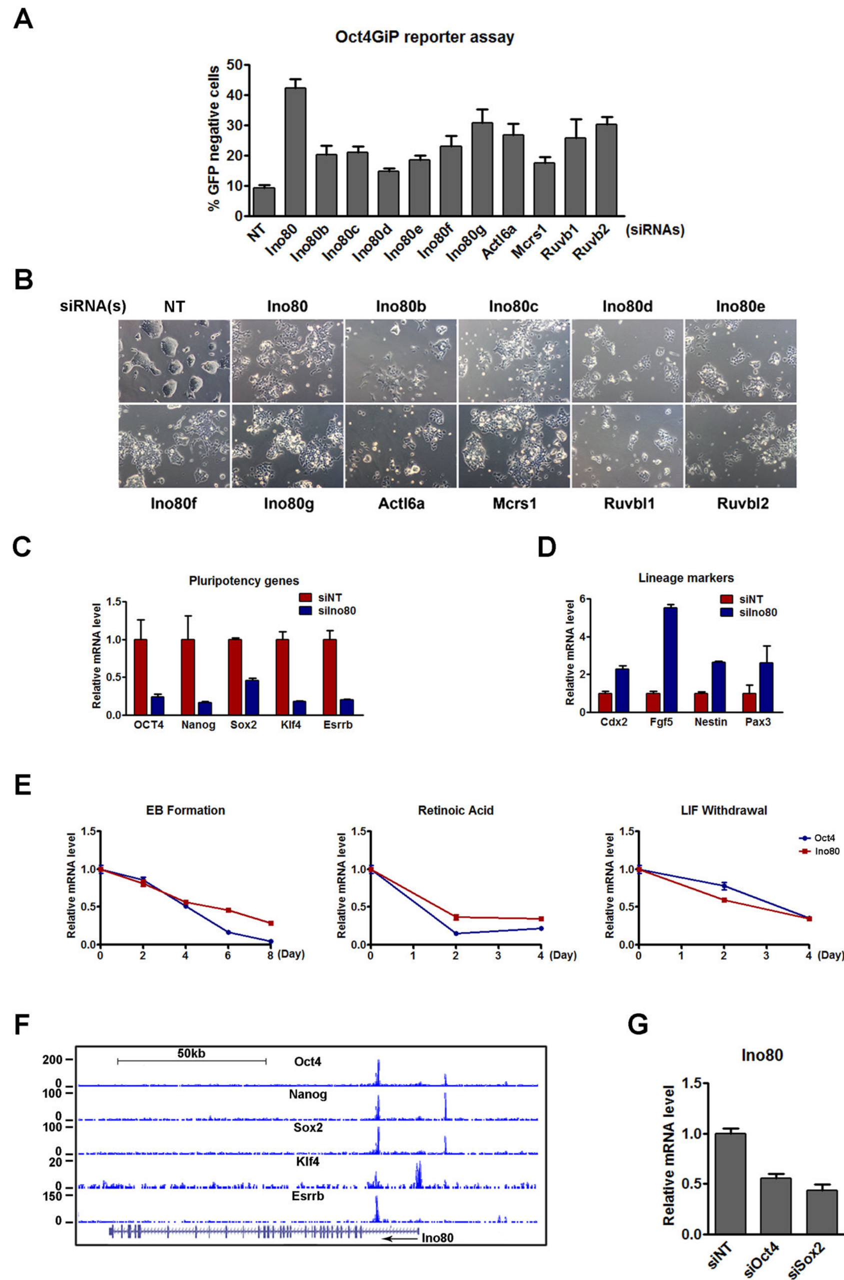

Figure 1. INO80 is Required for ESC Self-renewal

(A) The Oct4GiP reporter assay after silencing different INO80 subunits. NT: non-targeting siRNA as negative control. % Differentiation was plotted as mean ± SEM. (B) ESC morphology after silencing INO80 subunits. (C–D) Pluripotency gene and lineage marker expression 96 hrs after Ino80 KD. Values were plotted as mean ± SEM (E) Ino80 expression during ESC differentiation induced by LIF-withdrawal, retinoic acid (RA) treatment, or embryoid body (EB) formation. Expression was normalized to day-0 by β-actin and plotted as mean ± SEM. (F) Oct4, Nanog, Sox2, Klf4 and Esrrb occupancy near the Ino80 gene locus based on published datasets. (G) Ino80 expression 48 hrs after Oct4 or Sox2 silencing. Expression was normalized to NT and plotted as mean ± SEM.

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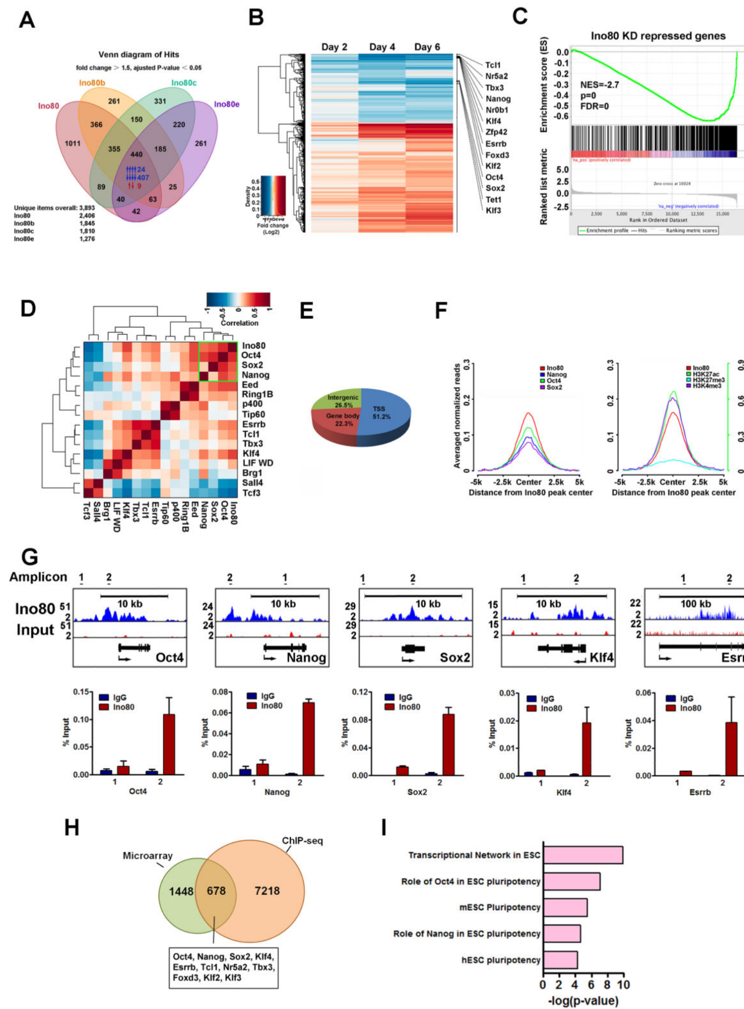

Figure 2. INO80 is Required for Key Pluripotency Gene Expression

(A) Venn diagram showing gene expression changes 96 hrs after INO80 subunits KD. Blue arrows: genes up- or down-regulated by Ino80, Ino80b, Ino80c and Ino80e silencing; red arrows: genes inconsistently affected by Ino80, Ino80b, Ino80c or Ino80e silencing. (B) Gene expression changes upon shRNA-mediated Ino80 silencing at the indicated time points. Selected pluripotency genes that are significantly down-regulated in at least 2 time points are listed. (C) GSEA showing that the Ino80 KD down-regulated genes were enriched for genes down-regulated during ESC differentiation into EBs. (D) Hierarchical clustering of pluripotency factors based on the gene expression changes caused by their KD. See methods for detailed description on GSEA and the hierarchical clustering analysis. (E) Ino80 peak distribution in the genome. (F) Average ChIP-seq read density of Ino80 and other factors near Ino80 peak center. Oct4, Nanog, Sox2, H3K4me3, H3K27ac, and H3K27me3 occupancy was based on published data. (G) Genome browser tracks to show Ino80 occupancy near Oct4, Nanog, Sox2, Klf4, Esrrb. Black bars indicate the regions (1 and 2) selected for ChIP-qPCR verification. (H) Venn diagram showing the overlap between genes differentially expressed after Ino80 KD and those occupied by Ino80. (I) IPA of the 678

Ino80 target genes. Selected top categories were shown and see Table S4 for the complete list of enriched categories.

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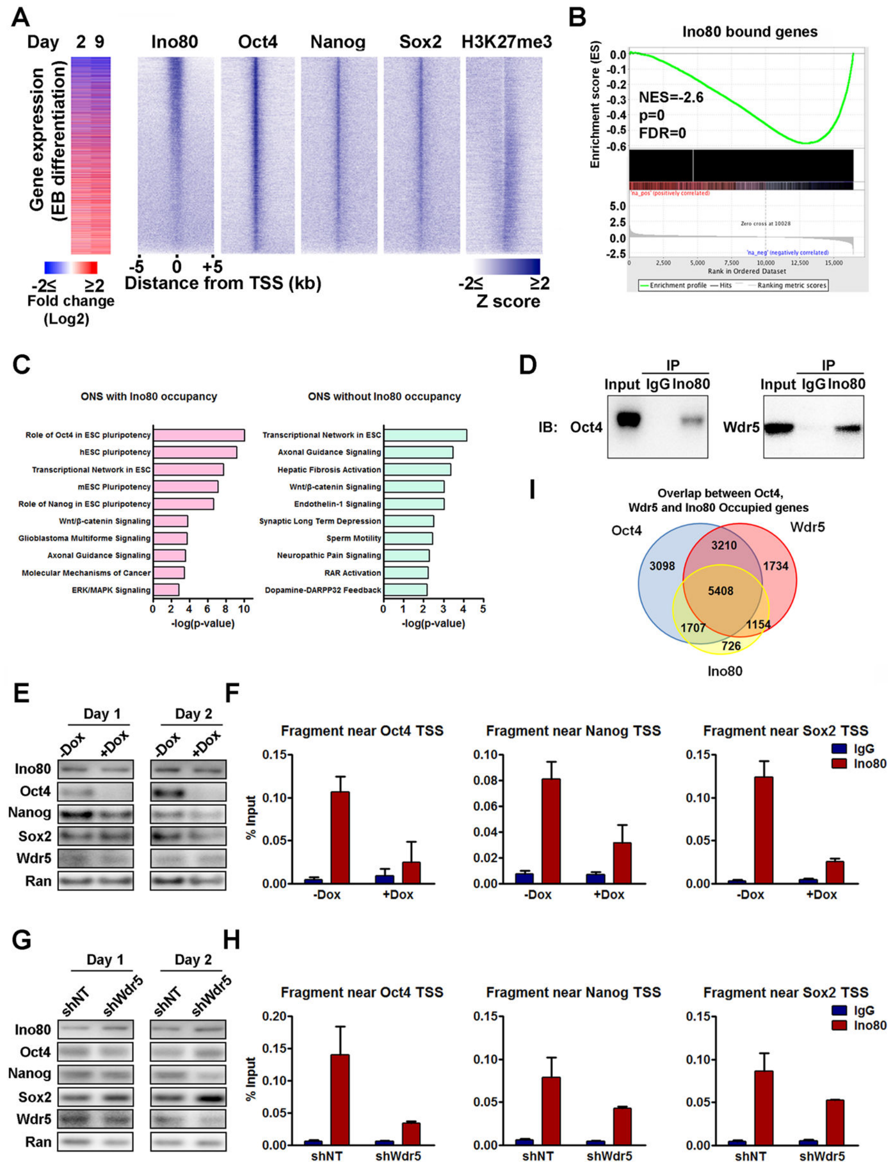

Figure 3. Ino80 Occupies Genomic Regions Near Pluripotency Genes

(A) Factor occupancy near genes that are expressed during ESC differentiation. Left: Heat map showing gene expression fold-changes during EB formation at day-2 and day-9; Right: Heat maps showing Ino80, Oct4, Nanog, Sox2, and H3K27me3 occupancy. Genes are sorted based on Ino80 occupancy. EB differentiation, Oct4, Nanog, Sox2, and H3K27me3 ChIP-seq data were downloaded from the GEO database. (B) GSEA for genes associated with Ino80 occupancy. (C) Ingenuity analysis of genes co-occupied by Ino80 and the master transcription factors (ONS) and those co-occupied by the master transcription factors but not Ino80. (D) Interaction between Ino80 and Oct4, Wdr5. ESC lysates were sonicated and incubated with IgG or Ino80 antibody in the presence of Benzonase to remove nucleic acid contamination, and the presence of Oct4 and Wdr5 in the co-purified proteins were detected by western blot. (E) Western blot showing Oct4 depletion in ZHBTc4 cells at 24 and 48 hrs after Dox treatment. (F) Impact of Oct4 depletion on Ino80 occupancy at Oct4, Nanog, Sox2

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promoter regions. ZHBTc4 cells were treated with Dox for 48 hrs to induce Oct4 depletion, and Ino80 occupancy was determined by ChIP-qPCRs. (G) Western blot showing Wdr5 depletion at 24 and 48 hrs after Wdr5 shRNA lentivirus transduction. (H) Impact of Wdr5 depletion on Ino80 occupancy. ESCs were transduced with Wdr5 shRNA lentivirus, and Ino80 occupancy was determined by ChIP-qPCRs 48 hrs after transduction. (I) Overlap between genes occupied by Ino80, Oct4, or Wdr5. Oct4 and Wdr5 occupancy was based on published data.

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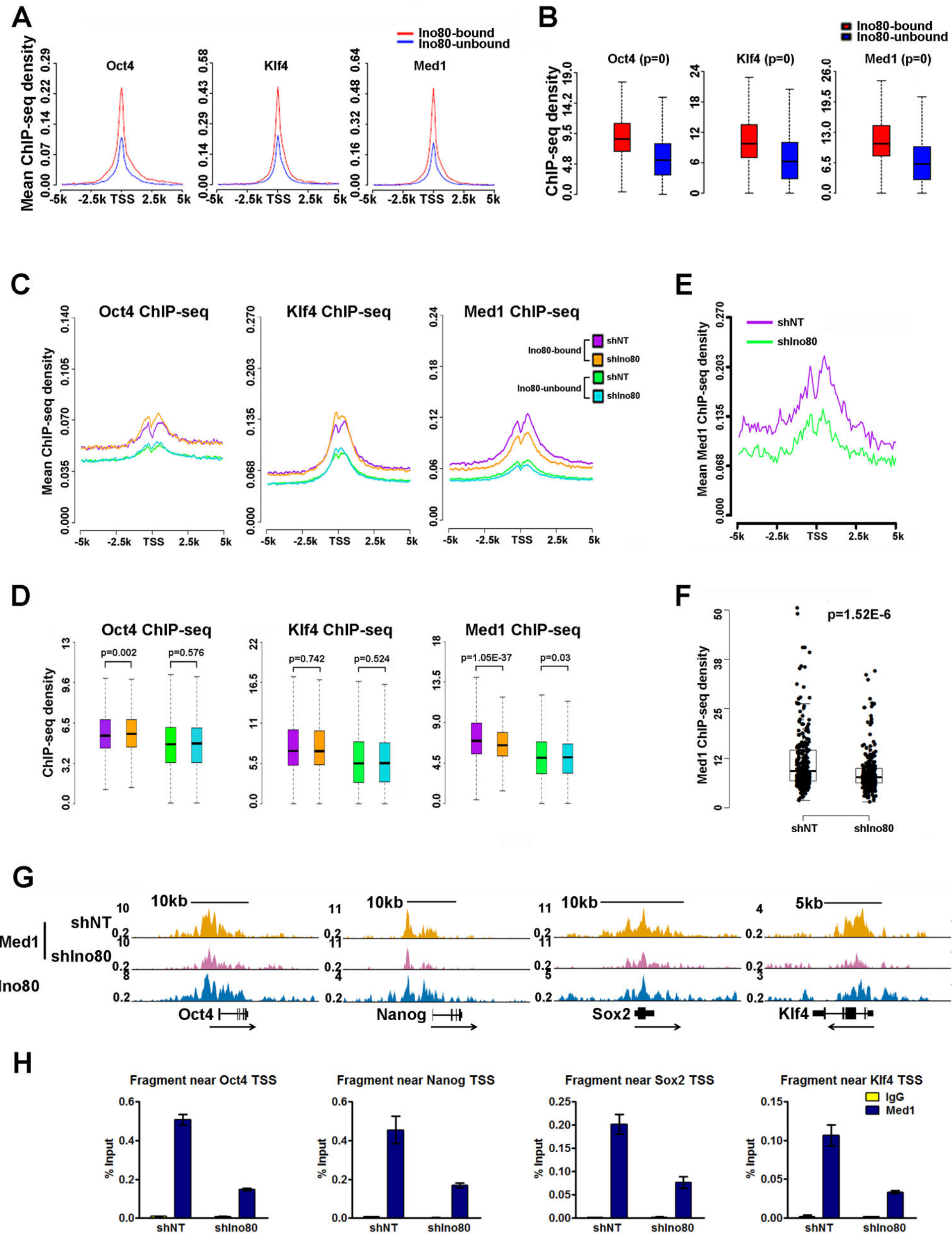

Figure 4. INO80 Promotes Mediator Recruitment at Pluripotency Gene Promoters

(A–B) Oct4, Klf4, and Med1 occupancy near TSS at Ino80-bound and Ino80-unbound genes. Oct4, Klf4, and Med1 occupancy was based on published data. (A) Average ChIP-seq read density. (B) Box plot of ChIP-seq read density. (C–H) Impact of Ino80 silencing on factor occupancy. ESCs were transduced with NT- (non-targeting) or Ino80-shRNA virus (shIno80), and factor occupancy was determined by ChIP-Seq or ChIP-qPCR 48 hrs after transduction. (C) Average ChIP-seq read density of Oct4, Klf4, and Med1 near TSS at Ino80-bound and Ino80-unbound genes in NT- or Ino80-shRNA virus transduced ESCs. (D) Box plot of Oct4, Klf4, and Med1 ChIP-seq read density near TSS. p-values were calculated between Ino80-bound and Ino80-unbound genes by Wilcoxon Rank-Sum test. (E) Average

ChIP-seq read density of Med1 near TSS at Ino80-KD down-regulated genes. p-values were calculated by Wilcoxon Rank-Sum test. (F) Box plot of Med1 ChIP-seq read density near TSS at Ino80-KD down-regulated genes. p-values were calculated by Wilcoxon Rank-sum test. (G) Genome browser tracks to show Med1 occupancy near Oct4, Nanog, Sox2, and Klf4 in NT-or Ino80-shRNA virus transduced ESCs. Ino80 occupancy in ESCs was shown for comparison. (H) ChIP-qPCR to show Med1 occupancy near Oct4, Nanog, Sox2, and Klf4 TSS.

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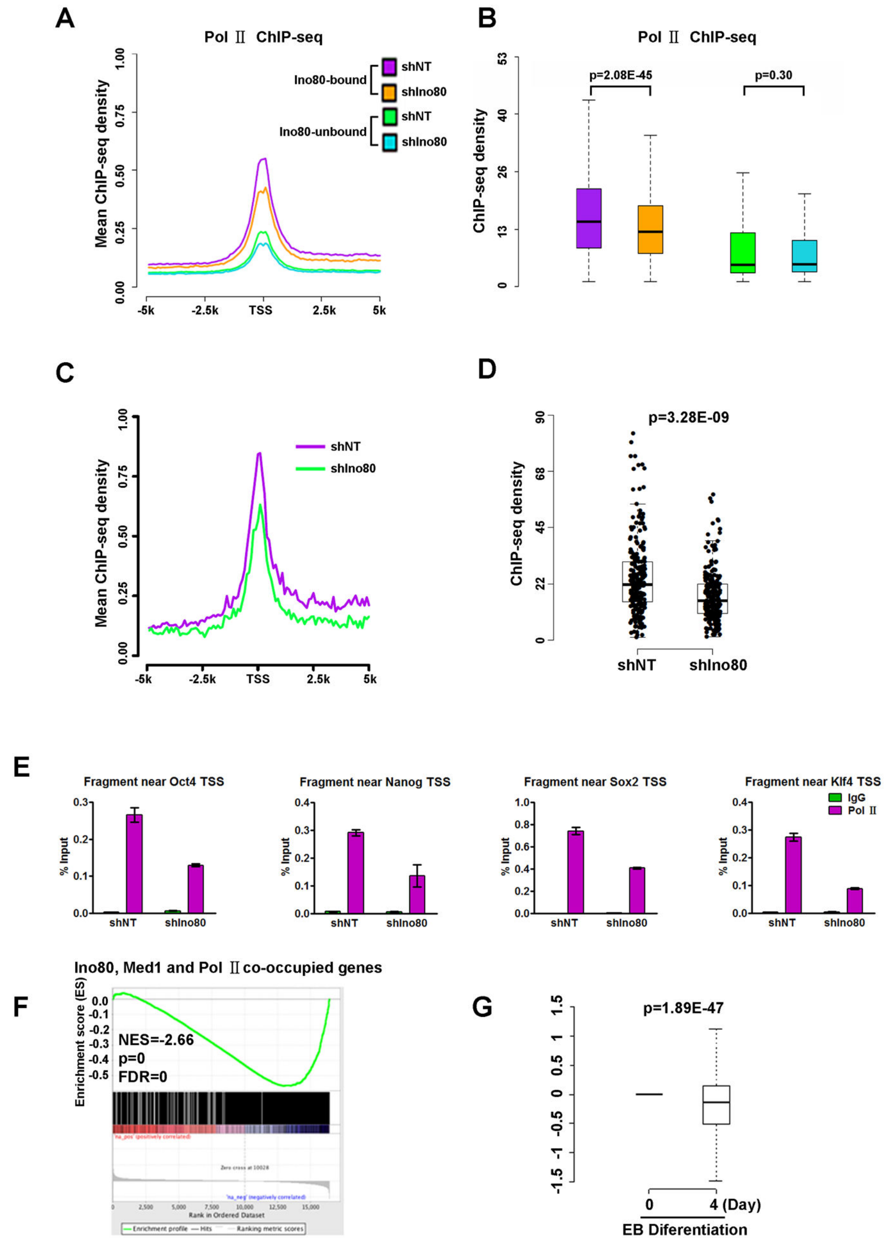

Figure 5. INO80 Promotes Pol II Recruitment at Pluripotency Gene Promoters

(A–E) Impact of Ino80 silencing on Pol II occupancy. ESCs were transduced with NT-(non-targeting) or Ino80-shRNA virus (shIno80), and Pol II occupancy was determined by ChIP-Seq or ChIP-qPCR 48 hrs after transduction. (A) Average ChIP-seq read density of Pol II near TSS at Ino80-bound and Ino80-unbound genes. p-values were calculated between Ino80-bound and Ino80-unbound genes by Wilcoxon Rank-Sum test. (B) Box plot of Pol II ChIP-seq read density near TSS at Ino80-bound and Ino80-unbound genes. p-values were calculated by Wilcoxon Rank-Sum test. (C) Average ChIP-seq read density of Pol II near TSS at Ino80-KD down-regulated genes. p-values were calculated by Wilcoxon Rank-Sum test. (D) Box plot of Pol II ChIP-seq read density near TSS at Ino80-KD down-regulated genes. p-values were calculated by Wilcoxon Rank-sum test. (E) ChIP-qPCR to show Pol II

occupancy near Oct4, Nanog, Sox2, and Klf4 TSS. (F) GSEA for genes co-occupied by Ino80, Med1, and Pol II during ESC differentiation into EBs. (G) Expression of Ino80, Med1, and Pol II co-occupied genes during ESC differentiation into EBs. EB differentiation, Med1, Pol II ChIP-seq datasets were downloaded from the GEO database.

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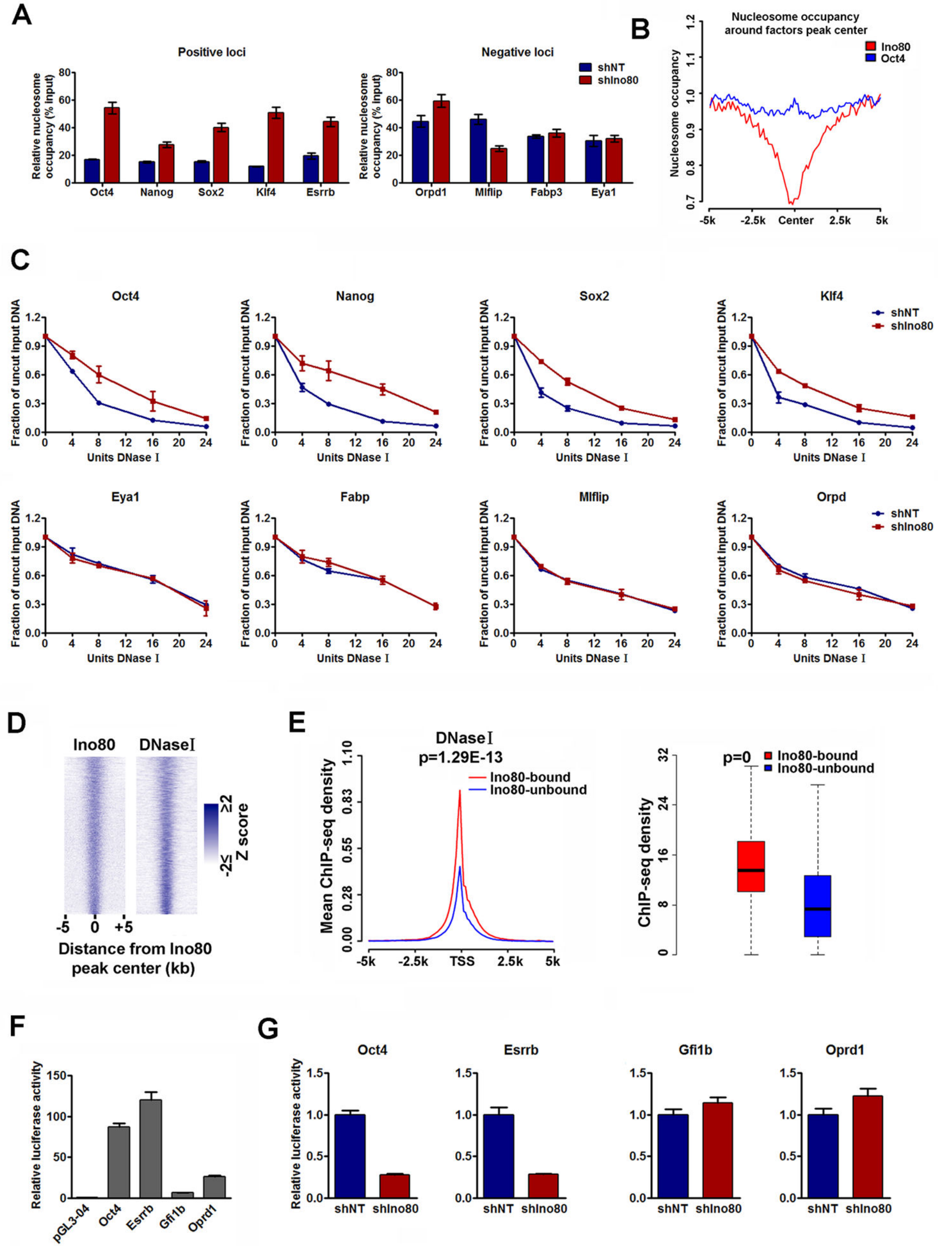

Figure 6. INO80 Maintains an Open Chromatin Structure

(A) ESCs were transduced with non-targeting (NT-) or Ino80-shRNA viruses and nucleosome occupancy was determined 48 hrs after transduction by MNase-qPCR. Relative occupancy was normalized to input and plotted as mean ± SEM. (B) Nucleosome occupancies (based on published data) around Ino80 (red) or Oct4 (blue) peak center in ESCs. (C) DNase I sensitivity assay. ESCs were transduced with non-targeting (NT) or Ino80-shRNA lentivirus. 48 hrs after transduction, cell nuclei were isolated and treated with the indicated amount of DNase I. The amount of uncut DNA fragments near the promoter regions of Ino80-bound (top panel) and unbound genes (bottom panel) was determined by qPCRs. Data were plotted as mean ± SEM. (D) Ino80 and DNase I occupancy (based on

published data) around Ino80 peaks. Peaks are sorted based on Ino80 occupancy. (E) Left: Average DNase I hypersensitivity ChIP-seq read density at Ino80-occupied (red) or non-occupied (blue) regions near TSS. Right: Box plot of the average DNase I read density. (F) Activity of DNA fragments bound (Oct4 and Esrrb) or unbound (Gfi1b and Oprd1) by Ino80 in the luciferase reporter assay. Values were normalized to the vector alone (pGL3-04) and plotted as mean ± SEM. (G) Activity of DNA fragments bound or unbound by Ino80 in NT- or Ino80- shRNA transduced ESCs 48 hrs after transduction. Values were normalized to NT and plotted as mean ± SEM.

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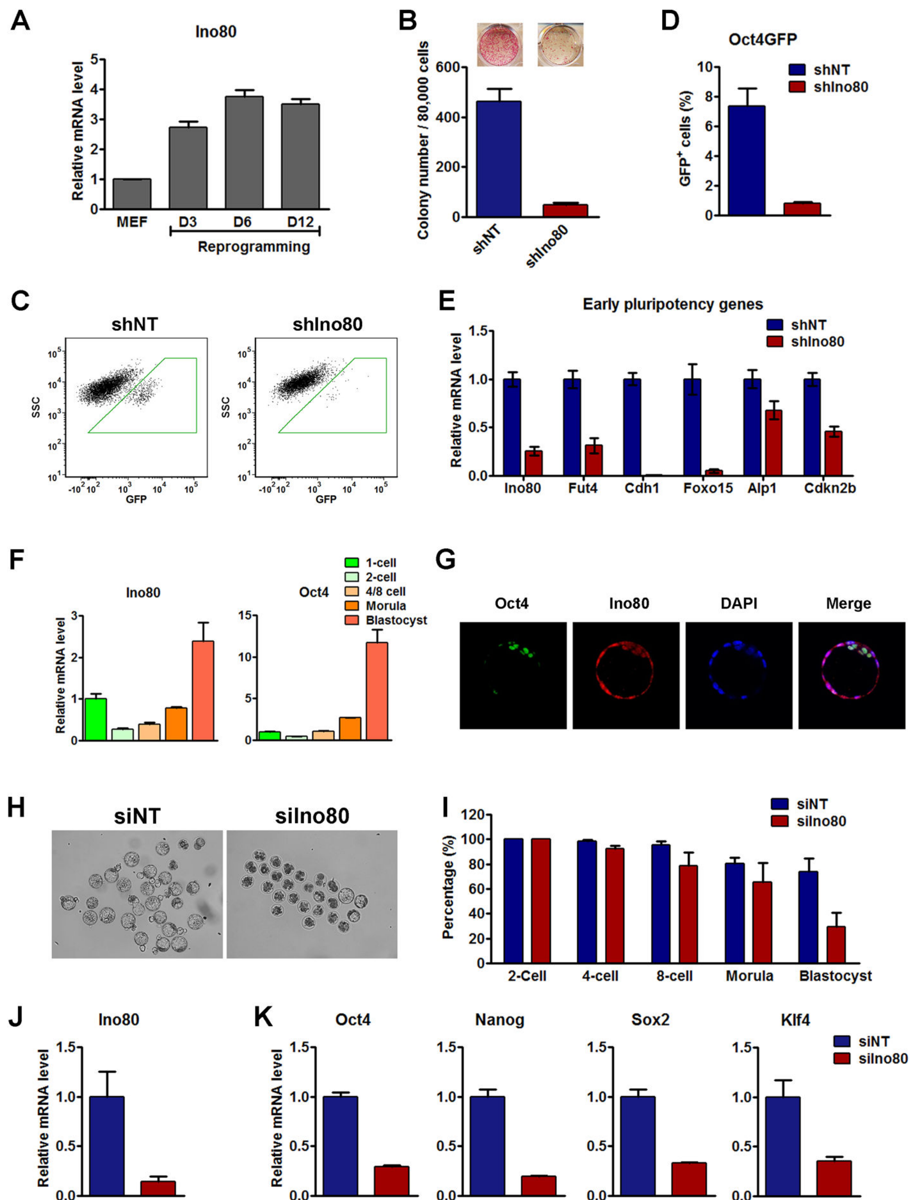

Figure 7. INO80 is Required for Reprogramming and Blastocyst Formation

(A) Ino80 expression during somatic cell reprogramming based on RT-qPCR. Expression was normalized by β-actin and to day-0, and plotted as mean ± SEM. (B–E) Effect of Ino80 KD on reprogramming. (B) Number of AP-positive colonies formed after reprogramming were plotted as mean ± SEM. (C–D) Percentage of Oct4GFP-positive cells formed after reprogramming was determined by FACS and plotted as mean ± SEM. (E) Expression of early reprogramming markers in NT- or Ino80-shRNA transduced MEFs at day-6 of reprogramming. (F) Ino80 expression during early embryonic development *in vivo* based on RT-qPCR. Expression was normalized to 1-cell embryo and plotted as mean ± SEM. (G) Immunofluorescence staining of Ino80 (red) and Oct4 (green) in E3.5 blastocysts. Cell nuclei were counter stained with DAPI (blue). (H–I) Effect of Ino80 KD on blastocyst development. (H) Morphology of embryos 4 days after siRNA injection. (I) Percentage of

normal embryosat each developmental stage. Values were normalized to 2-cell stage and plotted as mean ± SEM from three independent experiments. (J–K) Gene expression analysis upon Ino80 silencing determined by RT-qPCR in the injected embryos. Expression of normalized by β-actin and to NT siRNA injected embryos, and was plotted as mean ± SEM.

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