RESEARCH ARTICLE

Chd1 is essential for the high transcriptional output and rapid growth of the mouse epiblast

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ABSTRACT
The pluripotent mammalian epiblast undergoes unusually fast cell proliferation. This rapid growth is expected to generate a high transcriptional demand, but the underlying mechanisms remain unknown. We show here that the chromatin remodeler Chd1 is required for transcriptional output and development of the mouse epiblast. Chd1−/− embryos exhibit proliferation defects and increased apoptosis, are smaller than controls by E5.5 and fail to grow, to become patterned or to gastrulate. Removal of p53 allows progression of Chd1−/− mutants only to E7.0-8.0, highlighting the crucial requirement for Chd1 during early post-implantation development. Chd1−/− embryonic stem cells (ESCs) have a self-renewal defect and a genome-wide reduction in transcriptional output at both known mRNAs and intergenic transcripts. These transcriptional defects were only uncovered when cell number-normalized approaches were used, and correlate with a lower engagement of RNAP II with transcription in several species (Sims et al., 2005; Stokes et al., 2008) that act as readers of activating histone marks, such as H3K4me3, that Chd1 mutants (Gkikopoulos et al., 2011) and chromatin factors essential for ESC self-renewal (Ang et al., 2011). Chromatin factors might therefore be important regulators of the transcriptional output of pluripotent cells.

INTRODUCTION
Embryonic development involves a sophisticated coordination of cell proliferation and differentiation. Mammalian embryos display the fastest cell proliferation rate in the pluripotent epiblast soon after implantation, with doubling times of 2-8 h at embryonic day (E) 5.5-6.5 (Snow, 1977). This growth is essential to establish the cell population that will undergo gastrulation and give rise to the embryo proper (Lewis and Rossant, 1982; Power and Tam, 1993; Snow, 1977). Unlike the early embryos of other model organisms, such as Drosophila or Xenopus, the mammalian post-implantation epiblast has minimal maternal supplies of mRNA and/or protein left over from the zygote stage. Therefore, the fast proliferation and growth of the epiblast may generate a high demand for transcriptional output by the RNA polymerases (RNAPs) and for ribosome biogenesis. However, how the transcriptional output of rapidly expanding pluripotent cells is regulated remains unknown.

Pluripotent cells have a decondensed chromatin organization that is associated with genome-wide elevated levels of transcription, including at intergenic regions (Ahmed et al., 2010; Efroni et al., 2008; Meshorer and Misteli, 2006). Moreover, embryonic stem cells (ESCs) have higher levels of the activating histone mark H3K4me3 than differentiated cells, and Wdr5, a core component of the Trithorax complexes responsible for trimethylation of H3K4, is essential for ESC self-renewal (Ang et al., 2011). Chromatin factors that act as readers of activating histone marks, such as H3K4me3, might therefore be important regulators of the transcriptional output of pluripotent cells.

Chromodomain helicase DNA-binding protein 1 (Chd1) is a conserved protein associated with decondensed chromatin and transcription in several species (Sims et al., 2005; Stokes et al., 1996; Woodage et al., 1997). Chd1 binds with high specificity to H3K4me2/3 (Flanagan et al., 2005; Sims et al., 2007) and enhances transcription in several species (Sims et al., 2005; Stokes et al., 1996), including at intergenic regions (Ahmed et al., 2010; Efroni et al., 2008). However, how the transcriptional output of rapidly expanding pluripotent cells is regulated remains unknown.

We previously reported that Chd1 binding in the ESC genome is highly correlated with H3K4me3 and RNAP II, and that Chd1 RNAi mouse ESCs can be expanded in the undifferentiated state but show self-renewal defects and a propensity to accumulate heterochromatin (Gaspar-Maia et al., 2009). These findings raise the question of which role Chd1 might play in pluripotent cells in the context of the developing embryo. Studies in other organisms argue against an essential role for Chd1: yeast Chd1 mutants are viable (Tsukiyama et al., 1999; Woodage et al., 1997), and Drosophila Chd1 mutants are
also viable, although they have wing abnormalities and are infertile (Konev et al., 2007; McDaniel et al., 2008). Morpholino-mediated knockdown of the gene amplified in liver cancer (ALC1), also known as Chd1l, in early mouse embryos prevents development to the blastocyst stage (Snider et al., 2013). However, the name Chd1l might be confusing, because ALC1/Chd1l is only distantly related to Chd1 (or indeed to other Chd family members): ALC1/Chd1l lacks the H3K4me3-binding chromo-domains and the DNA-binding domain of Chd1, and instead has a macro-domain (Flaus et al., 2006).

Here, we report that genetic deletion of mouse Chd1 results in arrest of epiblast development at E5.5-6.5, prior to the onset of gastrulation. We further show that Chd1 is required for the maintenance of optimal transcriptional output by RNAP I and II in ES and epiblast cells. These results indicate that Chd1 promotes a globally elevated transcriptional output that underlies the rapid growth of the pluripotent epiblast.

RESULTS

Mouse Chd1 is required in the epiblast for post-implantation development

To understand the role of mammalian Chd1 in vivo, we generated mice and ESCs carrying either null or conditional (floxed) alleles of Chd1 (supplementary material Fig. S1A). The Chd1 null allele lacks exon 16, which codes for a conserved fragment of the helicase domain. The null allele also includes an IRES-lacZ construct to report on endogenous Chd1 expression (supplementary material Fig. S1A). Cre-mediated recombination of the conditional allele produces the same exon 16 deletion and frame-shift. Both approaches to delete Chd1 were validated by Southern blotting, western blotting and qRT-PCR (supplementary material Fig. S1B,C). Western blotting and qRT-PCR (supplementary material Fig. S1B,C) approaches to delete Chd1 were validated by Southern blotting, report on endogenous Chd1 expression (supplementary material Fig. S1A). The Chd1 mice and ESCs carrying either null or conditional (floxed) alleles of Chd1 can be derived from them at normal Mendelian distributions (Table 1).

Table 1. Intercrosses of Chd1+/− mice produce E3.5 and E6.5 embryos at the expected Mendelian distribution, but no Chd1+/−/+ pups are observed at birth

<table>
<thead>
<tr>
<th></th>
<th>Total n</th>
<th>Chd1+/−/−</th>
<th>Chd1+/−/−</th>
<th>Chd1+/−/+</th>
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<tbody>
<tr>
<td>E3.5</td>
<td>40</td>
<td>10 (25%)</td>
<td>21 (52.5%)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>(ES cells)</td>
<td>(7 litters)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6.5</td>
<td>244</td>
<td>70 (28.7%)</td>
<td>123 (50.4%)</td>
<td>42+9* (20.9%)</td>
</tr>
<tr>
<td>(ES cells)</td>
<td>(28 litters)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At birth</td>
<td>51</td>
<td>19 (37%)</td>
<td>32 (63%)</td>
<td>0</td>
</tr>
<tr>
<td>(ES cells)</td>
<td>(8 litters)</td>
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(*) Nine very small embryos collected at E6.5 could not be genotyped and were presumed to be Chd1+−/−.

In order to better define the developmental phenotype of Chd1−/− embryos, we next analyzed the expression of Oct4 (also known as Pou5f1), Nodal and Fgf5. These genes are specifically expressed throughout the epiblast at E5.5 (Hébert et al., 1991; Mesnard et al., 2006; Rosner et al., 1990) and are important markers of pluripotency. We observed that Oct4, Nodal and Fgf5 are expressed in the Chd1−/− epiblast at E5.5 (Fig. 2A). However, by E6.5, the mutant epiblast expresses greatly reduced levels of Oct4 and Fgf5 relative to littermate controls (Fig. 2B). These data indicate that Chd1 is not required for induction of the epiblast cell fate, but rather for maintaining epiblast identity.

Fig. S2). We were not able to perform immunofluorescence (IF) for Chd1 in post-implantation embryos using available commercial antibodies, but the Chd1lacZ reporter allele is preferentially expressed in the E6.5 epiblast (Fig. 1D). We carried out deletion of Chd1 specifically in the epiblast using the conditional allele and the Sox2-Cre deleter strain (Hayashi et al., 2002). Epiblast-specific deletion of Chd1 results in an embryo resorption phenotype by E9.5 that is very similar to that of the full Chd1 mutants (Fig. 1B). These data do not exclude a potential additional role for Chd1 in the extra-embryonic tissues, but document that Chd1 is required in the post-implantation epiblast for its subsequent development.

Chd1−/− embryos do not sustain epiblast gene expression, establish the anterior-posterior (A/P) axis or gastrulate

Developmental defects at peri-implantation can be due to abnormalities in the embryo proper, extra-embryonic tissues or in both. We therefore aimed to test whether Chd1 is required for development of the embryo proper. Chd1 is expressed maternally and throughout pre-implantation development (supplementary material Fig. S2). We were not able to perform immunofluorescence (IF) for Chd1 in post-implantation embryos using available commercial antibodies, but the Chd1lacZ reporter allele is preferentially expressed in the E6.5 epiblast (Fig. 1D). We carried out deletion of Chd1 specifically in the epiblast using the conditional allele and the Sox2-Cre deleter strain (Hayashi et al., 2002). Epiblast-specific deletion of Chd1 results in an embryo resorption phenotype by E9.5 that is very similar to that of the full Chd1 mutants (Fig. 1B). These data do not exclude a potential additional role for Chd1 in the extra-embryonic tissues, but document that Chd1 is required in the post-implantation epiblast for its subsequent development.

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for its maintenance. This is reinforced by analysis of gene expression during differentiation of embryoid bodies (EBs) in vitro. We observed that Chd1−/− cells are capable of robustly inducing markers of all three germ layers upon differentiation (supplementary material Fig. S3A).

We found that, by E6.5, Chd1−/− embryos are highly reduced in size compared with controls (Fig. 2). Moreover, expression of Lefty1, a marker of the anterior visceral endoderm (AVE) and a regulator of future anterior structures (Perea-Gomez et al., 2002), is absent in the mutants (Fig. 2B). In addition, the mutants are unable to undergo gastrulation, as highlighted by the lack of expression of the nascent mesoderm markers brachyury (T) and Wnt3 (Liu et al., 1999) at the posterior end of the embryo (Fig. 2B). The expression of markers of the extra-embryonic ectoderm, such as Cdx2 and Bmp4, is also absent at E6.5 in Chd1−/− embryos (supplementary material Fig. S4), probably due to the reciprocal molecular interactions between the epiblast and the extraembryonic ectoderm that occur at the onset of gastrulation (Guzman-Ayala et al., 2004). Thus, Chd1−/− embryos arrest in the transition between E5.5 and E6.5, prior to anterior-posterior (A/P) patterning and initiation of gastrulation.

**The E5.5 Chd1−/− epiblast does not grow due to increased apoptosis and defective cell cycle**

A minimum number of cells are thought to be required for the epiblast to undergo patterning and gastrulation (Lewis and Rossant, 1982; Power and Tam, 1993). Notably, the number of epiblast cells is already significantly reduced in the mutants by E5.5 (Fig. 3A), that is, prior to detectable differences in the expression of epiblast markers (Fig. 2). These data are supported by cell counts in vitro: Chd1−/− ESCs, although viable, display a self-renewal deficit both in colony formation assays and in bulk expansion, in agreement with our previous findings using RNAi (Gaspar-Maia et al., 2009) (supplementary material Fig. S3B,C; and data not shown).

A reduction in epiblast cell number could be due to increased apoptosis and/or a defective or delayed cell cycle, and we found evidence for both in Chd1−/− embryos. Control E5.5 embryos are essentially devoid of apoptotic cells marked by cleaved Parp (no cleaved Parp cells in 16/20 embryos and 1 or 2 cleaved Parp cells in 4/20 embryos, see Fig. 3B), in agreement with recently reported findings (Bedzhov and Zernicka-Goetz, 2014). By contrast, cleaved Parp is consistently detected in E5.5 Chd1−/− embryos (more than five cleaved Parp cells in 7/7 embryos). To analyze the cell cycle state of the mutant epiblast, we assayed E5.5 embryos for 5-Ethynyl-2'-deoxyuridine (EdU) incorporation (S phase) and phospho-Histone 3 staining (pH3, M phase). Labeled epiblast cells were counted and presented as a percentage of the epiblast cell number, referred to as the S phase index for EdU or mitotic index for pH3. We found no statistically significant difference in the S phase index between mutant and control embryos (Fig. 3C). Interestingly, the mitotic index in the Chd1−/− epiblast was about 40% higher than in controls (Fig. 3D). This accumulation in mitosis without changes in the proportion of cells in S phase suggests that mutant cells undergo a delay in the completion of mitosis. Alternatively, the increased mitotic index in the mutants could simply be due to a developmental delay. In this case, we would expect earlier stage embryos to have higher mitotic indices. However, we did not observe any correlation between mitotic index and the size of the epiblast (defined by the number of cells) in control embryos (Fig. 3E).

To independently explore the potential mitotic defects of Chd1−/− embryos, we quantified the number of foci of H3 variant CENP-A (Cenpa–Mouse Genome Informatics) in each epiblast cell. Proper incorporation of CENP-A at centromeric regions is considered indicative of a successful mitotic exit (Jansen et al., 2007). Chd1 depletion has been previously reported to lead to reduced CENP-A incorporation in cultured avian cells (Okada et al., 2009). In agreement, we found that the number of CENP-A foci in the Chd1−/− epiblast was significantly reduced (Fig. 3F). Taken together, these data indicate that the smaller Chd1−/− epiblast at E5.5 is due to an increase in apoptotic cell death and a delay in progression through mitosis.

Several mutations in essential growth or DNA repair genes that lead to peri-implantation lethality similar to the case of Chd1−/− embryos can be rescued to mid-gestation (~E9.5-12.5) upon removal of p53. Therefore, we explored the development of Chd1−/− embryos in a p53−/− background. Surprisingly, we found that removal of p53 from the Chd1 mutant background only allows development to ~E7.0-8.0. When recovered at E8.5, Chd1−/−:p53−/− embryos are severely delayed and arrested (Fig. S5). Thus, Chd1 is critically required for post-implantation development, even in the absence of p53.

**Chd1−/− ESCs have lower transcriptional output per cell**

In order to better understand the mechanism of action of Chd1 in pluripotent cells, we generated ROSA26CreER;Chd1−/− ESCs, in which Chd1 deletion can be induced by exposure to tamoxifen.
In view of the role of Chd1 in facilitating RNAP II release into productive elongation (Skene et al., 2014) and the self-renewal deficit of Chd1-deficient ESCs (supplementary material Fig. S3; and data not shown), we reasoned that distinct transcriptional changes might be evident in Chd1−/− ESCs. Additionally, in line with previous observations in Chd1−/− RNAi ESCs (Gaspar-Maia et al., 2009), Chd1−/− cells exhibit a global accumulation of Hp1a (Cbx5− Mouse Genome Informatics), suggestive of an increase in heterochromatin (supplementary material Fig. S6). However, similar to Chd1-null strains of Saccharomyces cerevisiae and Schizosaccharomyces pombe (Hennig et al., 2012; Pointer et al., 2012), initial microarray analyses showed very few genes changing in expression in Chd1−/− ESCs (data not shown). Although we had previously reported that Chd1 RNAi ESCs upregulated genes of the neural lineage (Gaspar-Maia et al., 2009), we subsequently found that this could be suppressed by high doses of commercially available leukemia inhibitory factor (LIF) (data not shown), the conditions we currently use (see Materials and Methods). Thus, Chd1−/− ESCs appeared to have a largely unchanged relative distribution of expression levels across genes. However, we considered the possibility that Chd1 might globally regulate absolute transcription levels, given its ubiquitous, genome-wide association with RNAP II (Gaspar-Maia et al., 2009). Standard techniques used to measure relative changes in gene expression, such as qRT-PCR, microarrays or RNA-seq, rely on normalization for RNA amount and cannot detect a global shift in expression, as has been recently described for the case of CMye overexpression (Lin et al., 2012; Lovén et al., 2012; Nie et al., 2012). In order to overcome this obstacle, we performed cell number-normalized RNA-seq by isolating identical numbers of two pairs of control and mutant ESCs and adding a set of spike-in RNAs for downstream normalization (see Materials and Methods). Remarkably, this analysis revealed that the transcriptional output of Chd1−/− ESCs is significantly reduced genome-wide relative to control ESCs (Fig. 4A,B), with a median reduction of 24% across all genes (Fig. 4C, Wilcoxon P<2.2E-16). Whereas these data are considerably more variable for intergenic transcripts, their expression in the mutant cells follows the trend of annotated mRNAs (Fig. 4B,C). The reduction is consistently observed across gene expression levels (Fig. 4C). Chd1−/− ESCs have similar viability and cell cycle stage distribution than controls (supplementary material Fig. S3C), and show no upregulation of differentiation genes, indicating that the reduced RNA output per cell is not due to changes in cell state. Of note, this global shift in transcriptional output would be largely masked if the standard normalization for RNA amount, rather than cell number, had been carried out, explaining our initial confounding results with microarrays.

In order to validate the RNA-seq data, we carried out cell number-normalized qRT-PCR on independent samples for a group of genes covering a range of expression levels. In agreement with the RNA-seq data, every mRNA examined was found to be expressed at lower levels in Chd1−/− ESCs (supplementary material Fig. S7). This includes markers associated with pluripotency, such as Oct4, Nanog and Klf2, and several highly expressed genes associated with ribosome biogenesis and translation, such as Rpl3, Rps9, Taf1d and Elf4a2. Furthermore, the gene downregulation in Chd1−/− ESCs is already evident at primary mRNAs, prior to splicing (pre-mRNA, supplementary material Fig. S7A), suggesting that these defects are not due to altered splicing efficiency or mRNA stability but rather to a defect in RNAP II activity. In agreement with the recent results of Skene et al. in mouse embryonic fibroblasts (MEFs) (Skene et al., 2014), we observed that RNAP II S2p is also reduced in Chd1−/− ESCs in the gene body (GB) at a set of highly expressed genes tested using ChIP-qPCR (Fig. 4D), including Gapdh, Klf2, Rpl3, Rpl9 and Elf4a2.
minor satellite repeats, which are also transcribed by RNAP II (Lu and Gilbert, 2007). Interestingly, we found that total RNAP II is decreased at both the transcriptional start site (TSS) and GB in mutant ESCs (Fig. 4E). Taken together, these results suggest that Chd1 facilitates recruitment or retention of RNAP II to promote a globally elevated transcriptional output in ESCs.
Chd1 directly targets rDNA and regulates pre-rRNA transcription

The global role of Chd1 in RNAP II-mediated transcription led us to explore potential roles for Chd1 in ribosomal RNA (rRNA) transcription, which is carried out by RNAP I. Yeast Chd1 was previously found among several epigenetic factors that physically interact with ribosomal DNA (rDNA) (Hontz et al., 2009). ChIP-qPCR for Chd1-Flag, using the knock-in ES cell line (supplementary material Fig. S8), revealed a strong enrichment for Chd1 at the enhancer, promoter and gene body of rDNA, comparable to the levels observed at protein-coding genes (Fig. 5A). Moreover, cell number-normalized qRT-PCR using primers for nascent, pre-processed rRNA (pre-rRNA) showed a ~25% reduction upon Chd1 deletion (Fig. 5B). In agreement with these observations, Chd1−/− epiblast cells express lower levels of pre-rRNA per cell (Fig. 5C). Immunofluorescence (IF) for nucleolin in E5.5 epiblast cells in vivo and ESCs in vitro reveal that the nucleoli are more elongated and smaller in Chd1−/− cells than in control cells. Graphs represent quantification of this analysis of two independently derived ES cell lines. Circularity: control=0.75±0.17 and Chd1−/−=0.60±0.21. 1.0 represents a perfect circle. Size: control=13.91±10.89 and Chd1−/−=9.51±8.3. All results are mean±s.d.

Fig. 5. Chd1 directly targets rDNA and regulates pre-rRNA transcription. (A) ChIP-qPCR shows Chd1 enrichment at the enhancer, promoter and body of the rDNA transcription unit. A schematic representation of the ribosomal DNA repeat and primers used is shown below the graph. ETS, external transcribed spacer; ITS, internal transcribed spacer; IGS, intergenic spacer. Wild-type (WT) cells without the Chd1-Flag knock-in were used as negative control. Significance assessed using unpaired t-test, *P<0.05, **P<0.01. (B) Chd1−/− ESCs express lower levels of pre-rRNA per cell. Cell number-normalized qRT-PCR was carried out in two independently derived ESC cell lines. (C) Chd1−/− epiblast cells express lower levels of pre-rRNA per cell. Images are a reconstitution of four different planes of representative embryos processed for pre-rRNA FISH, with higher magnification images of representative single epiblast cells on the right. (D) IF for nucleolin in E5.5 epiblast cells in vivo and ESCs in vitro reveal that the nucleoli are more elongated and smaller in Chd1−/− cells than in control cells. Graphs represent quantification of this analysis of two independently derived ESC cell lines. Circularity: control=0.75±0.17 and Chd1−/−=0.60±0.21. 1.0 represents a perfect circle. Size: control=13.91±10.89 and Chd1−/−=9.51±8.3. All results are mean±s.d.
findings in cultured ESCs, quantitative fluorescent in situ hybridization (FISH) (Raj et al., 2008) showed a significant reduction in pre-rRNA in mutant E5.5 epiblast cells in vivo (Fig. 5C). The RNA specificity of the pre-rRNA FISH was validated by loss of signal upon RNase I (but not DNase I) treatment in control embryos (supplementary material Fig. S9). Thus, Chd1 is required for the optimal output of rRNA both in vitro and in vivo.

Highly proliferative cells tend to have larger and rounder nucleoli, in which high levels of rRNA synthesis and ribosome assembly occur (Derenzini et al., 2000). Interestingly, we found that Chd1−/− cells, both in the E5.5 epiblast in vivo and in ESCs in vitro, have an altered nucleolar morphology, characterized by a decreased average size and circularity (Fig. 5D). These nucleolar abnormalities are observed throughout the mutant epiblast and in ESCs. Taken together, the data above indicate that Chd1 regulates the transcriptional output of rRNA, indirectly affecting nucleolar morphology of rapidly proliferating cells.

**DISCUSSION**

Our results indicate that a coordinately elevated global transcription by multiple RNAPs, regulated by factors such as Chd1, is essential for mouse embryonic development by sustaining the very rapid growth of the E5.5-6.5 epiblast (Snow, 1977). Data on the transcription factor cMyc lend some support to this model. cMyc directly promotes the activity of RNAP I, II and III (Oskarsson and Trumpp, 2005) and acts as a global amplifier of transcription (Lin et al., 2012; Nie et al., 2012). In mouse ESCs, the genome-wide location of Myc is highly correlated with H3K4me3, RNAP II and gene expression levels (Nie et al., 2012), very similar to that of Chd1 (Gaspar-Maia et al., 2009). In contrast to Chd1, however, cMycc−/+embryos have normal epiblast development and only arrest at mid-gestation (Davis et al., 1993), possibly due to redundancy with N-Myc (Mycn – Mouse Genome Informatics) and L-Myc (Mycl – Mouse Genome Informatics). Interestingly, mouse embryos mutant for Max, an obligate partner to Myc proteins, arrest in development at E5.5-6.5 (Shen-Li et al., 2000). Furthermore, Chd1 RNAi was identified as synthetic lethal with cMyc overexpression in cultured human mammary epithelial cells (Kessler et al., 2012), indicating that Chd1 is required to support a Myc-driven hyper-proliferative state. In further support of this model, Chd1 directly interacts with Ssrp1 (Kelley et al., 1999), a component of the Facilitates chromatin transcription (FACT) complex, and Ssrp1−/−embryos are capable of implanting but arrest by E5.5 (Cao et al., 2003). Moreover, increased transcriptional rates in the epiblast must not only be coordinated between different RNAPs, but they must also be intricately connected to regulation of the cell cycle. Indeed, there is evidence that ribosome biogenesis at the nucleolus, a process involving all three RNAPs, is a crucial sensor of cellular fitness that directly links transcriptional output to regulation of cell cycle progression (Kornberg, 1999; Rudra and Warner, 2004), something that deserves further exploration in the context of epiblast development.

ESCs exist in a state of hyper-transcription that correlates with a decondensed chromatin landscape (Efroni et al., 2008). Our data indicate that Chd1 is required to maintain this global hyper-transcription state. We note that a propensity for increased heterochromatin levels, initially reported in Chd1 RNAi ESCs (Gaspar-Maia et al., 2009), can still be observed in Chd1−/−ESCs, as assessed by HP1a accumulation (supplementary material Fig. S6). This defect is less pronounced than that observed with RNAi, and appears to correlate with the growth rate deficit of the cells. Chd1 is associated with transcribed genes (Gaspar-Maia et al., 2009, and this study) and is depleted from heterochromatin (supplementary material Fig. S8C). Taken together, our results suggest that the primary role of Chd1 is to maintain high levels of RNAP I and II engagement in the transcribed proportion of the genome, including at protein-coding genes and rDNA, and this state of elevated transcription might in turn inhibit the formation or expansion of heterochromatin.

Our data show that Chd1 is not required for transcription per se, but rather potentiates it, and this role might be particularly crucial in very rapidly proliferating epiblast cells. A recent study on MEFs showed that Chd1 increases the turnover of promoter-proximal nucleosomes at expressed genes to facilitate release of RNAP II into productive elongation (Skene et al., 2014). That study also reported an increase in RNAP II paused at TSSs and a decrease in RNAP II elongating through GBs in MEFs transfected with a dominant-negative (DN) version of Chd1. In agreement with their work, we detect lower levels of elongating RNAP II at all GBs tested in Chd1−/− ESCs (Fig. 4). In contrast to the data of Skene and colleagues (Skene et al., 2014), we found significantly lower levels of total RNAP II at TSSs (Fig. 4E). Thus, in ESCs, Chd1 appears to promote recruitment or retention of RNAP II at the TSS, prior to elongation. The differences between the two studies might be due to the use of different approaches to inhibit Chd1 function: in our genetic deletion model, a functional Chd1 protein is not synthesized, whereas in the DN approach the full length protein is made, can still be recruited to promoters and bind H3K4me3, but is unable to remodel nucleosomes. This defect in nucleosome remodeling probably underlies the RNAP II elongation defect described by Skene et al. (2014). Thus, it is possible that Chd1 regulates several steps of RNAP II engagement with transcription. The difference between the two studies might alternatively or additively be rooted in the difference in cell types, with RNAP II being more crucially dependent on Chd1 in rapidly growing pluripotent stem cells such as ESCs than in MEFs. The identification of proteins that interact with Chd1 in ESCs should help to elucidate further the biochemical regulation of the hyper-transcription state of pluripotent cells.

We had previously found Chd1 expression to be elevated in precursor cell populations, including somatic progenitor cells and the embryonic germline (Grskovic et al., 2007; Ramalho-Santos et al., 2002). It has recently been reported that the level of rRNA output regulates self-renewal versus differentiation in Drosophila germline stem cells (Zhang et al., 2014). Moreover, imbalances between constituents of the ribosome can lead to growth retardation, neural crest defects, anemia or cancer (Teng et al., 2013). It will be of interest to determine whether Chd1 regulates the transcriptional output of other expanding stem/progenitor cells during development as well as in cancer models.

**MATERIALS AND METHODS**

**ESC targeting**

A construct targeting exon 16 of Chd1, schematized in supplementary material Fig. S1A, was obtained from the knockout mouse project (KOMP, www.komp.org). This is a modular construct that allows for the generation of a mutant allele carrying a lacZ reporter driven by the endogenous Chd1 promoter or a conditional (floxed) allele. The plasmid was linearized using AsI Sl digestion and introduced into E14 ESCs by electroporation. Cells were selected using 250 µg/ml G418 (Sigma) and clones were identified based on lacZ expression and long-range PCR genotyping. To generate Chd1−/− or Chd1Δ alleles, pGK-NLS-Cre or pGK-FIPLo plasmids, respectively, were introduced into targeted ESCs by electroporation. Clones were identified based on lacZ expression and PCR genotyping. Southern blotting was used
to confirm the correct integration and recombination of the construct (supplementary material Fig. S1B).

**Generation of Chd1 mutant mice**

Correctly targeted and recombined ESCs were injected into C57BL/6 blastocysts and transferred to pseudo-pregnant females. Chimeras were crossed to C57BL/6 females and germline transmission was confirmed by PCR genotyping. Chd1<sup>−/−</sup> or Chd1<sup>+/−</sup> mice were backcrossed in parallel to 129 and C57BL/6 strains. Chd1<sup>−/−</sup> were intercrossed to obtain Chd1<sup>−/−</sup> embryos. The Sox2-Cre line (Hayashi et al., 2002) was used to delete the floxed allele of Chd1 specifically in the epiblast. The ROSA26-CreER line (Ventura et al., 2007) was used to delete the floxed allele of Chd1 after derivation of ESCs (see below). Care and use of mice were in accordance with the guidelines of the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC).

**Embryo genotyping**

Genomic DNA from embryos was extracted using the Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich). ESCs were genotyped by PCR with the following primers: for the Chd1<sup>−</sup> allele, common forward 5′-ATTCG-ATTTAGGAGGACAGC-3′; wild-type reverse 5′-GCCGGGACCATACCGTCT-3′ and mutant reverse 5′-GGCAAAGACATAAAGTGACC-3′; for the Chd1<sup>−</sup> allele, forward 5′-GACATTAGGAGGACAGCGTGC-3′ and reverse 5′-ACCACGTACCAGGCTTCTGGATGTG-3′.

**β-galactosidase whole-mount staining**

Embryos were fixed by 30 min on ice in 1% formaldehyde and 0.2% glutaraldehyde in X-gal buffer (5 mM EGTA, 2 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.02% Triton X-100 and 0.01% deoxycholate in PBS) and then washed three times in X-gal buffer. Staining was carried out for 12.15 h at 37°C in staining solution [5 mM K<sub>2</sub>Fe(CN)<sub>6</sub>(Fe<sub>3</sub>N<sub>2</sub>·5H<sub>2</sub>O, 5 mM K<sub>4</sub>Fe(C<sub>3</sub>N<sub>2</sub>·3H<sub>2</sub>O, 0.5 mg/ml X-gal in X-gal buffer]. Finally, embryos were post-fixed in 4% paraformaldehyde for 60 min on ice and washed three times in PBS.

**RNA WISH and IF**

Standard WISH and IF protocols were used with minor modifications (Guzman-Ayala et al., 2004; Ralston & Rossant, 2008). Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C. For WISH, embryos were dehydrated and rehydrated in a MeOH series before pre-hybridization for 20 min at 65°C and hybridization in the presence of 50-100 ng of digoxigenin (DIG)-labeled probes. Probes used for WISH were as described (Brennan et al., 2001). For IF, embryos or cells were permeabilized with 0.5% Triton X-100 for 20 min at room temperature and blocked in 0.5% BSA in PBS. Antibodies used for this study were: anti-Chd1 (Bethyl, A301-218A; 1:100 for ESC staining; and Santa Cruz, sc-49813; 1:50 for embryo staining); anti-Flag (Sigma, F1804; 1:1000). Two-color blots were false-colored to black and white using the manufacturer’s software (LiCor).

**Mouse cell culture**

Mouse ESCs were routinely passaged in gelatin-coated six-well plates every other day. Cells counts were obtained using a Vi-CELL analyzer (Beckman Coulter) and either 200,000 or 600,000 cells were seeded per well for serum/LIF or 2i/LIF media, respectively. Serum/LIF medium consists of 15% FBS, 1× penicillin/streptomycin, 1× non-essential amino acids, 0.057 mM β-mercaptoethanol and 1000 U/ml LIF (ESGro, Millipore) in high-glucose DMEM, supplemented with GlutaMAX and pyruvate (Gibco, 10569-10). 2i/LIF medium consists of 1× penicillin/streptomycin, 1× N2 supplement (Gibco, 17502048), 1× B27 supplement (Gibco, 17504-044), 50 µg/ml BSA Fraction V, 1000 µM LIF (ESGro, Millipore), 1 µM Mek inhibitor (PD0325901), and 3 µM Gsk3 inhibitor (CHIR99021) in a basal medium consisting of a 1:1 mix of DMEM-F12 (Gibco, 10565-018) and Neurobasal TM (Gibco, 21103049). ROSA26CreER;Chd1<sup>+/−</sup> ESCs were derived in a medium described (Guo et al., 2009). The medium for differentiation of epiblast-like cells was the same as the 2i/LIF medium with the following changes: LIF, Mek inhibitor and Gsk3 inhibitor were removed; activin A (Inhbα – Mouse Genome Informatics) (20 ng/ml) and Fgf2 (12 ng/ml) were added.

**Western blotting**

Two-color western blotting was performed according to the manufacturer’s instructions (LiCor). Samples were prepared using standard RIPA buffer. The soluble fraction was isolated by centrifugation and loaded onto a denaturing gradient SDS acrylamide gel. Transfer to a PVDF membrane (Millipore) was accomplished at 30 V overnight at 4°C. Primary antibodies used were: anti-Chd1 (Santa Cruz, SC-49813; 1:1000); anti-TopoI (Top1 – Mouse Genome Informatics) (Abcam, ab85038; 1:1000); anti-Flag (Sigma, F1804; 1:1000). Two-color blots were false-colored to black and white using the manufacturer’s software (LiCor).

**Viability and cell cycle analyses in ESCs**

Control and Chd1<sup>−/−</sup> ESCs were derived from two independent lines of ROSA26CreER;Chd1<sup>+/−</sup> ESCs were analyzed. Viability was calculated by
analysis of Trypan Blue exclusion with a Beckman Coulter Vi-CELL analyzer. Cell cycle analysis was performed by flow cytometry for EdU (S phase) and FxCycle Violet (DNA content) in an LSR II (BD Biosciences).

ChIP-qPCR
ChIP-qPCR was performed as described (Sachs et al., 2013), using 2-2.4 µg of antibody per IP. In the case of Chd1-Flag IP, 1×10^7 cells were used, and cells were crosslinked in 1% formaldehyde for 30 min. The following primary antibodies used were: anti-RNA II P2p (Abcam, ab5095); anti-total RNA II (Diagenode, C15200004); anti-Flag (Sigma, F1804); anti-rabbit IgG (Abcam, ab46540). Quantification was performed using the KAPA SYBR FAST qPCR kit (KK4604) on an ABI 7900HT device. Primers used are listed in supplementary material Table S1.

Cell number-normalized RNA-seq
Two independently derived lines of ROSA26CreER;Chd1fl/fl ES cells were either control-(EtOH) or tamoxifen-treated as described above. Cells were counted twice at different dilutions on a Vi-CELL analyzer and total RNA was isolated from equal numbers of viable cells. RNA was isolated using the RNeasy kit (QiAGEN) with on-column DNase I digestion. Total RNA was quantified using a Qubit fluorometer (Invitrogen). Equal elution volumes of RNA corresponding to ~1 µg of total RNA were mixed with 1 µl of a 1:10 dilution of Mx3000P External RNA Controls Consortium (ERCC) RNA spike-in transcripts, containing 92 polyadenylated transcripts over a range of concentrations (Life Technologies, 4456740). RNA depletion was performed using the Ribo-Zero RNA removal kit (Epicentre, MRZ1H16). Strand-specific libraries were generated using the Illumina TrueSeq Stranded mRNA kit (RS-122-2101). Libraries were prepared using barcoded adaptors, pooled and sequenced over two lanes of an Illumina HiSeq 2500 in rapid mode for paired-end reads of 100 bases at the UC Davis Genome Center. RNA-Seq reads were first trimmed of Illumina adapter sequence. Any pairs with reads containing less than 40 bases of insert sequence were discarded. The remaining trimmed reads were aligned by TopHat (v2.0.10) with a custom genome containing the mm9 mouse assembly, with the ERCC spike-in sequences added. The TopHat alignment was guided by a custom reference gene set, consisting of RefSeq genes >150 bases (downloaded 30 December 2013 from the UCSC Genome Browser, http://genome.ucsc.edu/, mm9) as well as the ERCC spike-in sequences. Only properly paired reads mapping uniquely to the combined mm9+ERCC Genome Center. RNA-Seq reads were retained for further analysis. To obtain raw fragments per kilobase of transcript per million mapped reads (FPKM) values in the paired mutant and control libraries, reads were first processed through Cufflinks (v2.2.0), using the custom gene set as a guide but allowing assembly of novel transcripts. Throughout the mapping and quantification procedures, the ‘fr-secondstrand’ option was invoked whenever possible to indicate the strand-specificity of the sequencing libraries. The custom reference genome was also given to Cufflinks and Cuffdiff for bias correction, and the strand-specificity of the sequencing libraries. The custom reference genome was also given to Cufflinks and Cuffdiff for bias correction, and the ‘max-bundle-fractions’ option was set to 100,000,000 to enable quantification of highly expressed transcripts. Local regression (loess) normalization of the paired mutant and control FPKM values obtained from Cuffdiff was performed as described previously (Lovén et al., 2012). The loess-normalized FPKM values were used in all subsequent analyses. For the fold change distribution plots, only genes with expression of at least 0.1 FPKM in the control sample were considered for each pair. Data have been deposited in GEO (accession number: GSE57699).

Cell number-normalized qRT-PCR
Total RNA from equal numbers of cells from two independently derived lines of ROSA26CreER;Chd1fl/fl ES cells treated with either control (EtOH) or tamoxifen was isolated as described above. Equal elution volumes of RNA were used to generate cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, 4368814). Quantification was performed using the KAPA SYBR FAST qPCR kit (KK4604) on an ABI 7900HT instrument. The 2^ΔΔCT method (Applied Biosystems) was used to calculate the expression level of each gene in mutant cells relative to the level in matched control cells, which was set as 1. Primers used are listed in supplementary material Table S2.

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Competing interests
The authors declare no competing financial interests.

Author contributions
M.R.-S. directed the project. M.G.-A. performed all of the experiments in embryos, with technical assistance from P.W. M.G.-A. also analyzed heterochromatin levels and nucleolar structure in ESCs. M.S. carried out analyses of self-renewal, differentiation, transcriptional output and 2p2p RNAIP in ESCs. He also developed the Chd1-Flag ESCs, with assistance from R.N. F.M.K. carried out gene targeting. Southern blotting and cell cycle analyses in ESCs. C.O. analyzed the RNA-seq data with aid and supervision from J.S.S. and M.R.-S. A.-K. performed Chd1-Flag and total RNAIP II ChIP. C.-J.L. and M.S. derived and genotyped ESCs. P.W., F.M.K., and M.G.-A. managed the mouse colony. P.W. contributed to the culture of ESCs under the supervision of M.G.-A. and M.S. M.G.-A., M.S. and M.R.-S. designed experiments, interpreted the results and wrote the manuscript. M.G.-A. led the revisions of the manuscript.

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Supplementary material
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