The chromatin remodeling factor Chd1l is required in the preimplantation embryo

Alyssa C. Snider[1], Denise Leong[2], Q. Tian Wang[1,3], Joanna Wysocka[1,4], Mylene W. M. Yao[2] and Matthew P. Scott[1,5,*]

[1]Departments of Developmental Biology, Genetics, and Bioengineering, University School of Medicine, Stanford, CA 94305-5101, USA
[2]Department of Obstetrics and Gynecology, University School of Medicine, Stanford, CA 94305-5101, USA
[3]Department of Biological Sciences, University of Illinois, Chicago, IL 60607, USA
[4]Department of Chemical and Systems Biology, University School of Medicine, Stanford, CA 94305-5101, USA
[5]Howard Hughes Medical Institute, Clark Center West W252, 318 Campus Drive, Stanford University School of Medicine, Stanford, CA 94305-5439, USA

*Author for correspondence (mscott@stanford.edu)

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Summary

During preimplantation development, the embryo must establish totipotency and enact the earliest differentiation choices, processes that involve extensive chromatin modification. To identify novel developmental regulators, we screened for genes that are preferentially transcribed in the pluripotent inner cell mass (ICM) of the mouse blastocyst. Genes that encode chromatin remodeling factors were prominently represented in the ICM, including *Chd1l*, a member of the *Snf2* gene family. *Chd1l* is developmentally regulated and expressed in embryonic stem (ES) cells, but its role in development has not been investigated. Here we show that inhibiting Chd1l protein production by microinjection of antisense morpholinos causes arrest prior to the blastocyst stage. Despite this important function *in vivo*, Chd1l is non-essential for cultured ES cell survival, pluripotency, or differentiation, suggesting that Chd1l is vital for events in embryos that are distinct from events in ES cells. Our data reveal a novel role for the chromatin remodeling factor Chd1l in the earliest cell divisions of mammalian development.

Key words: *Chd1l*, *ALC1*, Preimplantation development, ICM, ES cells, Chromatin remodeling

Introduction

The first differentiation decision in the mammalian embryo is made prior to the blastocyst stage, when blastomeres must commit to becoming either part of the trophectoderm (TE) or the inner cell mass (ICM) (Rossant and Tam, 2009). Cells of the ICM possess the property of pluripotency and will contribute to the many tissues of the embryo, whereas the TE will give rise to extra-embryonic material (Rossant and Tam, 2009). We reasoned that factors compartmentalized in the ICM could be novel developmental regulators of pluripotency or early differentiation. To identify candidate preimplantation regulators, we performed an expression analysis on purified ICM and whole blastocysts and identified genes enriched in the ICM. Gene ontology clustering revealed a large group of chromatin regulatory enzymes.

The high degree of chromatin organization within the nucleus is oppressive to transcription and other processes that require DNA accessibility (Knezetic and Luse, 1986; Lorch et al., 1987). Chromatin remodeling factors (CRFs) utilize energy to alter nucleosome positioning and contain a core SNF2-like ATPase/helicase domain responsible for enzymatic activity (Hirschhorn et al., 1992; Flaus et al., 2006). CRFs participate in key chromatin-dependent processes including transcriptional activation and repression, histone exchange, cell cycling, DNA repair, and many others (Hirschhorn et al., 1992; Cairns, 2005; Morrison et al., 2004; Fyodorov and Kadonaga, 2001). CRFs assemble into multi-subunit complexes, and their functions depend in part on the composition of the complexes (Flaus and Owen-Hughes, 2004; Wang et al., 1996a; Wang et al., 1996b).

During the development of the zygote and the preimplantation embryo, chromatin undergoes profound changes that allow the parental genomes to achieve a state of totipotency and that are necessary for normal development. Despite successful reprogramming of somatic cells, reprogramming in the embryo remains largely enigmatic (Takahashi and Yamanaka, 2006; Okita et al., 2007; Wernig et al., 2007; Yu et al., 2007; Niemann et al., 2008). Relatively few factors involved in preimplantation development have been identified because early phenotypes of homozygous mutants are often masked by maternally provided transcripts and proteins. Therefore, techniques aimed at early development will likely be fruitful in discovering additional chromatin modifiers that are essential in the preimplantation embryo.

Among the genes identified in our screen was *Chd1l*, encoding a largely unexplored CRF of the Snf2-like family. Its compartmentalization in the ICM, expression in ES cells, and temporal regulation prior to the blastocyst stage (Wang et al., 2004) led us to hypothesize that Chd1l is a chromatin enzyme

critical for early development. The protein has a Snf2-like ATPase domain but lacks any of the signature domains of the four classic Snf2 subclasses. Instead, Chd1l contains a unique C-terminal "macro" domain and therefore defines a distinct subclass (Yan et al., 2002; Mohrmann and Verrijzer, 2005). The macro domain binds poly(ADP-ribose), or PAR, a post-translational modification added to nuclear acceptor proteins. The nucleosome remodeling activity of Chd1l is dependent on PAR synthesis, indicating that the PAR-binding macro domain is central to its function as a chromatin remodeler (Ahel et al., 2009; Gottschalk et al., 2009).

Chd1l is involved in the DNA damage response. Chd1l localization to sites of induced DNA damage is dependent on a functional macro domain, and dissociation from sites of damage is dependent on a functional ATPase domain (Ahel et al., 2009; Gottschalk et al., 2009). Recent studies have also implicated Chd1l as an oncogene. The majority of hepatocellular carcinomas in humans are associated with genomic amplification of a region that includes *Chd1l*, and its overexpression in liver cell lines and mouse models is tumorigenic (Chen et al., 2009; Ma et al., 2008). While evidence is accumulating for a role for Chd1l as an oncogene and in DNA repair, its importance during development has not been examined.

We find that *Chd1l* is expressed in cultured embryonic stem (ES) cells, which are derived from the ICM and share the ability to differentiate into the three major germ layers. Our data show that Chd1l is not required for ES cell viability, pluripotency or differentiation. Using a morpholino (MO) knockdown approach in the zygote-stage embryo, we show that Chd1l is required for the very earliest stages of development.

Results

Chromatin remodeling factors are compartmentalized in the blastocyst

The decision to become inner cell mass (ICM) or trophectoderm (TE) is the first lineage commitment a totipotent blastomere must make. The ICM retains pluripotency, the ability to give rise to the three primary germ layers, whereas the TE will give rise to extra-embryonic tissue. We reasoned that mRNAs enriched in the ICM would encode proteins that contribute to the development of the blastocyst and/or the establishment of pluripotency. An alternative model would be that repression of these mRNAs in the TE marks an important step in the differentiation of TE and that continued expression in the ICM restricts TE differentiation. To screen for ICM-enriched mRNAs, we purified the ICM by immunosurgery (Solter and Knowles, 1975), taking advantage of the structural organization of the blastocyst (Fig. 1A). Outer TE cells of the blastocyst were labeled with IgG by incubation with rabbit anti-mouse serum and specifically lysed by the complement cascade, leaving behind purified ICMs. RNA

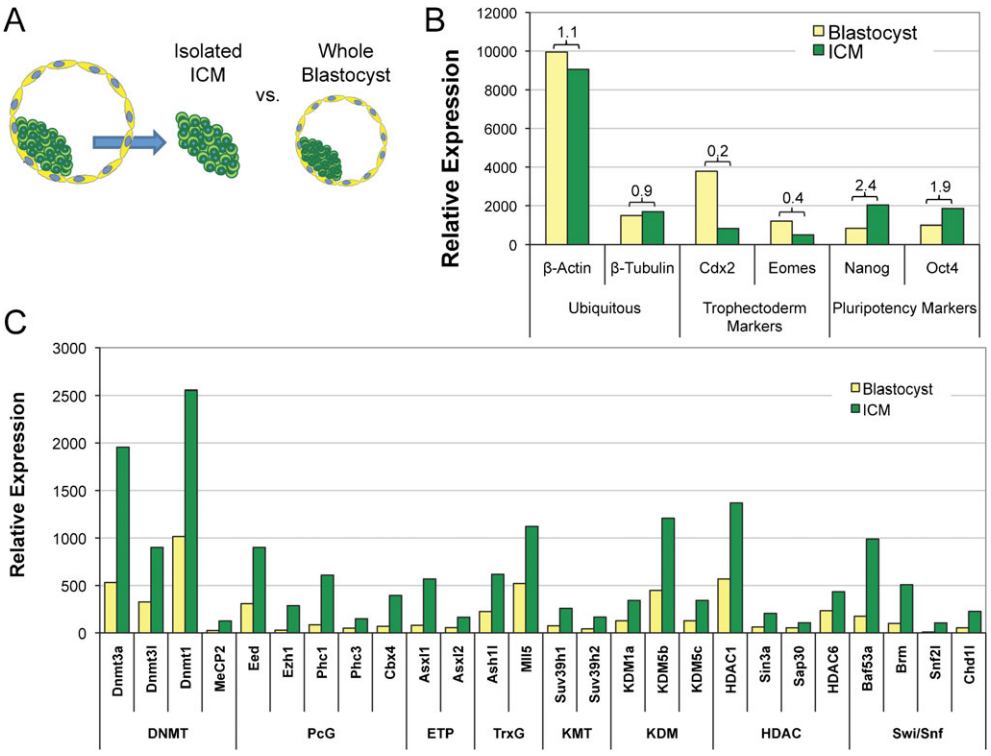

Fig. 1. Chromatin remodeling factors are enriched in the ICM. (**A**) Schematic of immunosurgery followed by whole-genome expression analysis. Inner cell masses (ICMs) were separated by lysing outer trophectoderm (TE) cells. Expression levels of transcripts in purified ICMs were compared to transcripts from whole blastocysts. (**B**) Compartmentalization of mRNAs encoding known ICM and TE factors and mRNAs of ubiquitously expressed genes. The number above the bars represents the ratio of expression in ICM relative to the whole blastocyst. Transcripts for ubiquitously expressed genes are represented with approximately equivalent levels in ICM and whole blastocyst. Relative expression levels of selected markers of pluripotency are higher in the ICM (green) than in the whole blastocyst (yellow); relative expression levels for selected markers of the differentiated TE are higher in the whole blastocysts than in the purified ICM. (**C**) Enrichment of selected classes of chromatin factors in the ICM. Relative expression levels of selected transcripts encoding chromatin factors in the ICM (green) and whole blastocyst (yellow) are shown. TF: transcription factor; DNMT: DNA methyltransferase; PcG: Polycomb group; ETP: Enhancers of Trithorax and Polycomb; TrxG: Trithorax group; KMT: lysine (histone) methyltransferase; KDM: lysine (histone) demethylase; HDAC: histone deacetylase.

extracted from the ICM was compared with total blastocyst RNA using genome-wide expression analysis.

Transcripts encoding Oct4 and Nanog, factors known to be critical for pluripotency, were enriched in the ICM 1.9- and 2.4-fold, respectively, providing proof of sound methodology (Fig. 1B). In addition, mRNAs encoding Cdx2 and Eomes, markers of extra-embryonic material, were under-represented 4.5-fold and 2.4-fold, respectively, in ICM compared to the whole blastocyst (Fig. 1B). Ubiquitously expressed transcripts encoding β-actin and β-tubulin demonstrate roughly equivalent levels in ICM and whole blastocyst (Fig. 1B). Clustering of genes whose transcripts are enriched in ICM revealed three major GO-term classes: cell signaling molecules, transcription factors, and chromatin-modifying enzymes. Some of the chromatin factors identified have known enzymatic activity and/or developmental roles, including the DNA methyltransferases, the polycomb group proteins, and the Snf2 family of chromatin remodeling enzymes (Fig. 1C).

Chromatin remodeling factors are often found in large, multi-subunit complexes (Wang et al., 1996a). Subtle changes in the composition of a complex can have dramatic effects on its function, and on the differentiation status of a cell (Ho and Crabtree, 2010; Lessard et al., 2007; Ho et al., 2009). Enrichment (or repression) of one or more subunits of a complex is one way in which the composition of a complex can be regulated (Peng et al., 2009). In general, our data support a model in which, compared to the trophectoderm, the ICM is characterized as having a chromatin state with tight transcriptional control and an abundance of chromatin proteins that mediate transcription and differentiation.

Chd1l expression patterns suggest a developmental role

Among the Snf2 family of chromatin enzymes whose mRNAs were enriched in the ICM was the CRF called Chd1l. Its enrichment score of 4.28-fold was higher than that of the "master regulator" of pluripotency, Oct4 (1.8-fold) (Fig. 2A). The Snf2 family of chromatin remodeling factors has powerful and diverse roles in development and transcriptional regulation (Ho and Crabtree, 2010; Eisen et al., 1995), and Chd1l is a member of this family by virtue of the split DNA-dependent ATPase/helicase domain (Flaus et al., 2006). Chd1l is the only member of the Snf2 family that contains a poly(ADP-ribosyl)ation binding macro domain (Fig. 2B) (Yan et al., 2002). Chd1l protein expression was confirmed in ES cells using a Chd1l-specific antibody (Fig. 3B).

Our lab previously reported genome-wide gene expression profiles during preimplantation development from the zygote through the blastocyst stage (Wang et al., 2004). In these studies, *Chd1l* expression was found to increase through the first several cell divisions of development, peaking at the late morula stage (Fig. 2C). Upon formation of the blastocyst, total *Chd1l* expression decreases slightly; our ICM data indicate it then becomes preferentially expressed in the ICM. Compartmentalization in the ICM, expression in ES cells, and developmental regulation support a potential role for Chd1l in pluripotency and during early embryogenesis. We chose to investigate the developmental function of this novel CRF of the Snf2 family (Fig. 2D).

Chd1l is dispensable for ES cell pluripotency and proliferation

Mouse ES cells are derived from the ICM of blastocyst stage embryos and maintain the property of pluripotency indefinitely. Because *Chd1l* mRNA is enriched in the ICM and abundant in ES cells, we asked whether Chd1l is essential for ES cell survival and pluripotency. To knock down Chd1l in ES cells, we introduced shRNA-encoding sequence into the EBRTcH3 ES cell line (Masui et al., 2005). These cells are engineered to allow stable, Cre-mediated integration and inducible transgene expression under the control of a CMV promoter (Tet-Off) (Fig. 3A). First, we created a control ES cell line, NS-shRNA EBRTcH3, by integrating DNA encoding shRNA that does not target any transcript in the mouse genome ("Non-Silencing").

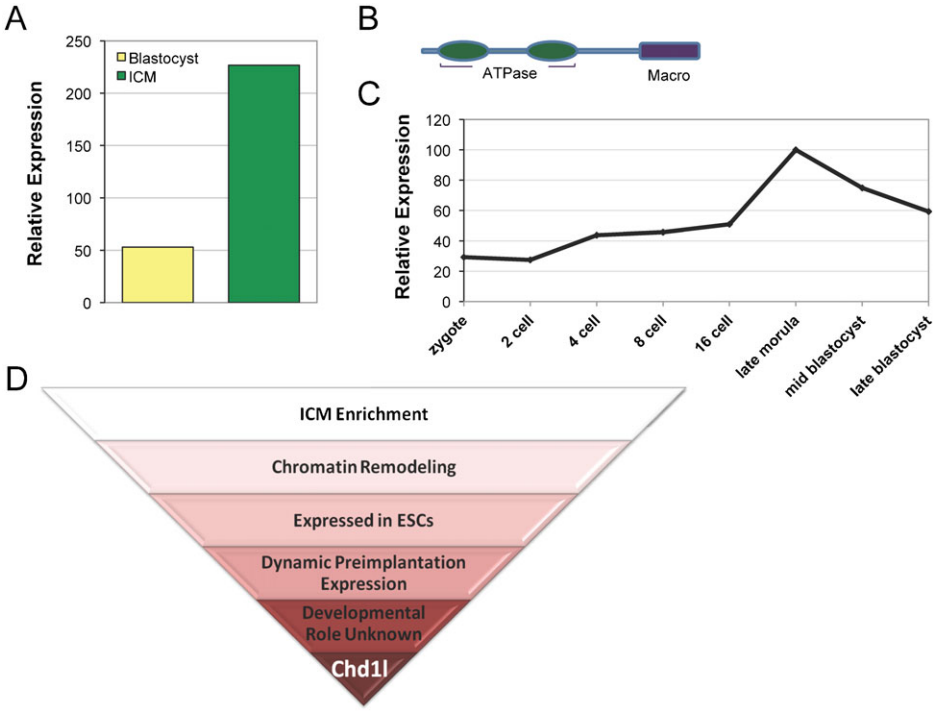

Fig. 2. Chd1l is a candidate developmental regulator. (**A**) *Chd1l* mRNA expression. *Chd1l* mRNA is enriched in the ICM (yellow) compared to the whole blastocyst (green). (**B**) Chd1l protein structure. Chd1l is a SNF2 chromatin remodeling enzyme containing a split ATPase/helicase and a macro domain. (**C**) *Chd1l* expression during preimplantation development (Wang et al., 2004). *Chd1l* expression peaks at the late morula stage before it becomes compartmentalized in the inner cell mass of the blastocyst. (**D**) Decision tree for choosing Chd1l. Chd1l was chosen as a candidate developmental regulator because of its compartmentalization in the ICM and peak expression at the late morula state and its predicted role as a regulator of chromatin.

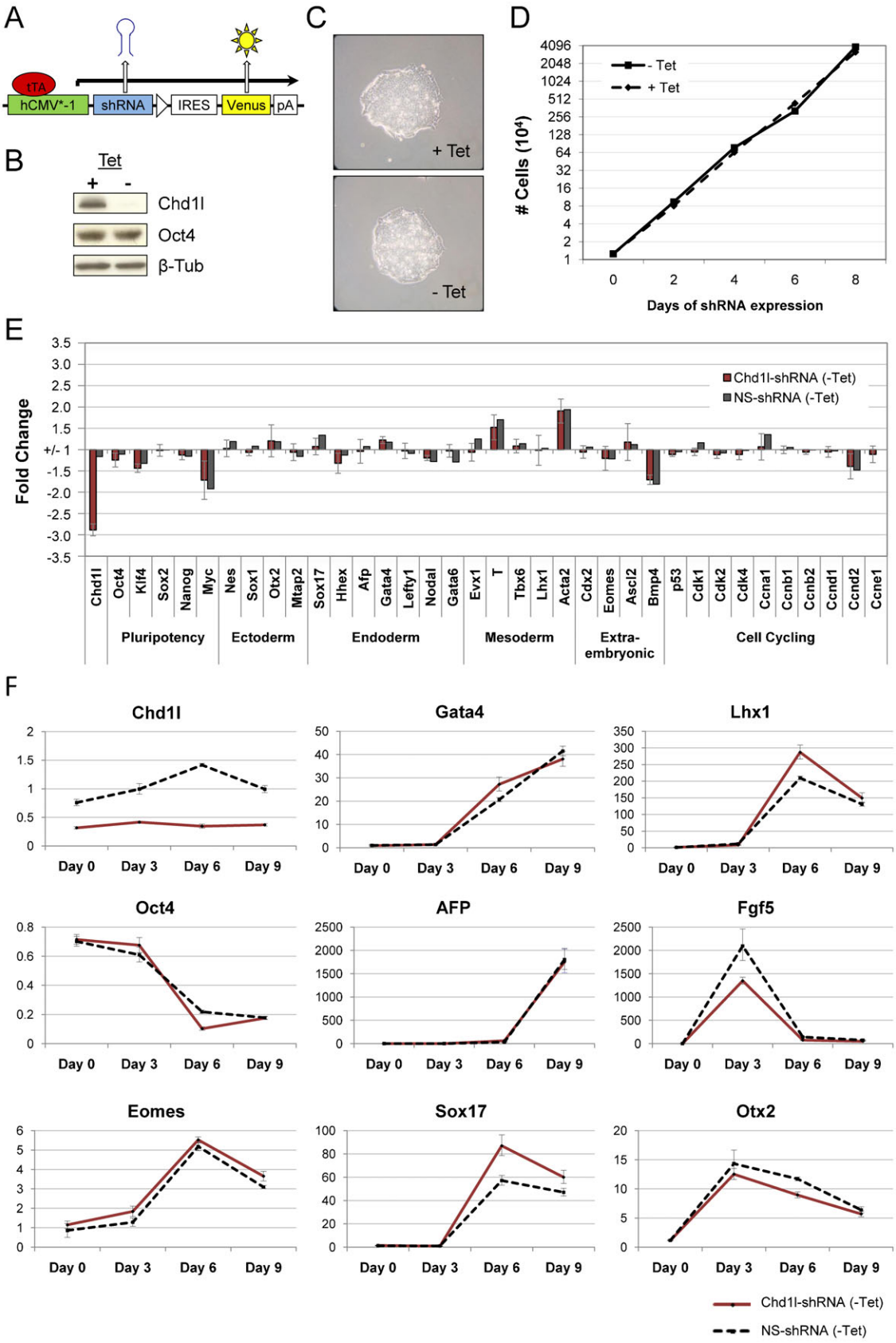

Fig. 3. See next page for legend.

Transcription of the shRNA from the CMV promoter was confirmed by observing robust Venus reporter gene expression 24 hours after inducing expression by Tetracycline withdrawal ("Tet-Off" induction). We created the *Chd1l*-shRNA EBRTcH3 ES cell line by integration of a sequence encoding shRNA that targets the *Chd1l* transcript. To assess knockdown efficiency of Chd1l protein, we blotted ES cell lysates using an α-Chd1l antibody generated in our laboratory, which detects a band at ~100 kD corresponding to Chd1l. Chd1l protein was consistently and reproducibly reduced to nearly undetectable levels in *Chd1l*-shRNA EBRTcH3 cells 48 hours after tetracycline withdrawal (Fig. 3B). In contrast, NS-shRNA ES cells induced to express NS-shRNA for 48 hours had normal levels of Chd1l. These data confirm that induction of *Chd1l* shRNA by removal of Tetracycline from the *Chd1l*-shRNA EBRTcH3 ES cell line is a robust system in which to knock down Chd1l. *Chd1l*-shRNA ES cells with reduced Chd1l had normal levels of Oct4 expression (Fig. 3B), no obvious abnormalities in ES cell morphology or colony formation (Fig. 3C), and normal proliferation over a period of eight days, or ~10 doublings (Fig. 3D). Our results are consistent with a recent RNAi screen performed in ES cells in which Chd1l was included among the chromatin factors screened, but was not identified as necessary for ES cell proliferation or for expression of a pluripotency reporter gene (Fazzio et al., 2008). However, no validation of *Chd1l* knockdown was provided in that study, and no further investigation of more inconspicuous phenotypes was attempted. We therefore proceeded to investigate gene expression profiles and differentiation in ES cells in which *Chd1l* has been knocked down.

Chd1l does not regulate gene expression in ES cells

A primary function of the SNF2 family of DNA-dependent ATPases is transcriptional regulation (Fry and Peterson, 2001; Flaus and Owen-Hughes, 2001). The four major subfamilies, SWI/SNF, CHD, ISWI, and INO80 all regulate gene expression during development (Ho and Crabtree, 2010). Chd1l contains a seven-motif, DNA-dependent ATPase module that defines the SNF2 family of chromatin remodeling factors as well as a macro domain that recognizes PAR-modified nuclear proteins, including PAR-modified histones. Chd1l might also regulate transcription, and ES cells lacking Chd1l could have transcription changes even in the absence of obvious morphological changes. We took a whole-genome approach and obtained the expression profiles of induced (−Tet) EBRTcH3 ES cells expressing *Chd1l*-shRNA or NS-shRNA and uninduced (+Tet) ES cells that did not express shRNA. Expression indices confirm the reduction of *Chd1l* in ES cells expressing *Chd1l*-shRNA (Fig. 3E). Only a small number of other transcripts that changed more than 1.4-fold between induced ES cells expressing *Chd1l*-shRNA and uninduced ES cells (~30), and these transcripts were also differentially expressed between induced ES cells expressing NS-shRNA and uninduced ES cells, indicating the expression changes were a byproduct of inducing shRNA expression. We found no statistically significant changes in expression of pluripotency markers, differentiation markers, or cell cycling genes (Fig. 3E). Our data suggest that Chd1l does not regulate transcription in ES cells.

Chd1l is not required for differentiation of ES cells

Like the ICM, ES cells are capable of differentiating into the three germ layers. While ES cells maintain this property indefinitely *in vitro*, the ICM is only transiently pluripotent as cells rapidly differentiate during embryogenesis. In the absence of the pluripotency cytokine LIF, ES cells can be grown into embryoid bodies (EBs), differentiating cellular aggregates that mimic *in vivo* post-implantation development. We reasoned that Chd1l may regulate gene expression in differentiating cells, when new gene expression patterns are being established. To ask whether Chd1l is required for the formation of the germ layers, Chd1l was reduced in *Chd1l*-shRNA ES cells, which were then differentiated into EBs. We measured the expression of a panel of lineage markers by q-rtPCR over time. For comparison, we measured gene expression in EBs made from induced and uninduced NS-shRNA ES cells. Quantitative rt-PCR confirmed knockdown of *Chd1l* mRNA in induced *Chd1l*-shRNA EBs but not in induced NS-shRNA EBs over nine days of differentiation (Fig. 3F). The lineage markers included genes associated with the establishment of endoderm (*Sox17*, *AFP*, *Gata4*), mesoderm (*Lhx1*), and ectoderm (*Fgf5*, *Otx2*), as well as pluripotency (*Oct4*) and extra-embryonic (*Eomes*) tissues. Under differentiating conditions, EBs expressing *Chd1l*-shRNA reduced *Oct4* expression in a manner similar to EBs expressing NT-shRNA (Fig. 3F). Expression of markers for all three germ layers was induced in a temporally appropriate manner (Fig. 3F). Our results indicate Chd1l does not control gene expression in pluripotent ES cells or in differentiating embryoid bodies under normal culture conditions.

Chd1l transcripts are abrogated in MO-injected embryos

Next, we addressed the question of whether Chd1l plays a role in development prior to differentiation of the ICM. The preimplantation embryo can be cultured *in vitro* through the blastocyst and hatching stages. We took a rapid knockdown approach, utilizing synthetic antisense oligos called morpholinos (MOs) that inhibit translation and splicing machinery. This approach has been used extensively to study the early development of diverse organisms (Gore et al., 2005; Imai et al., 2006; Sumanas and Larson, 2002; Yamada et al., 2003). In the mouse preimplantation embryo, MOs have been employed to show that *Oct4* has a critical role prior to the blastocyst stage (Foygel et al., 2008). MOs are stable oligos and effectively reduce production of specific proteins in the preimplantation

Fig. 3. Chd1l is non-essential in ES cells. (**A**) Strategy for knocking down Chd1l in EBRTcH3 ES cells. The tetracycline transactivator (tTA) is expressed from the endogenous Rosa26 locus and is bound in the inactive form in the presence of tetracycline. In the absence of tetracycline, the tTA activates the CMV promoter and induces expression of the shRNA-IRES-Venus transcript. (**B**) Efficiency of knocking down Chd1l in uninduced (+Tet) and induced (−Tet) Chd1l-shRNA EBRTcH3 cells. Chd1l protein (~100 kD) levels are nearly undetectable in shRNA-expressing cells 48 hours after Tetracycline withdrawal. Oct4 levels do not change upon knockdown of Chd1l. (**C**) Colony morphology of *Chd1l*-shRNA EBRTcH3 cells with induced (+Tet) or uninduced (+Tet) shRNA expression. Expression of shRNA was induced 24 hours prior to plating cells at clonal density and allowing colonies to grow for six days. (**D**) Proliferation curve of *Chd1l*-shRNA ES cells expressing *Chd1l*-shRNA (−Tet) or uninduced (+Tet). (**E**) Expression changes of selected genes from microarray expression analysis from ES cells expressing Chd1l-shRNA or NS-shRNA. (**F**) Expression of lineage markers during embryoid body (EB) differentiation by quantitative-rtPCR. All values are normalized to uninduced undifferentiated (Day 0) samples from each respective cell line. Similar to EBs expressing NS-shRNA, EBs expressing *Chd1l*-shRNA are able to differentiate and form the three germ layers as evidenced by markers for pluripotency (*Oct4*), extra-embryonic (*Eomes*), endoderm (*Gata4*, *AFP*, *Sox17*), mesoderm (*Lhx1*), and ectoderm (*Fgf5*, *Otx2*).

embryo, with minimal toxicity or off-target effects because they function through steric hindrance rather than through activation of the RNAi pathway (Foygel et al., 2008).

Splice-blocking MOs were designed to target *Chd1l* pre-mRNA. The predicted splice mutants produce truncated proteins due to stop codons within the intron (Fig. 4A). Chd1l MO-1 was microinjected into the cytoplasm of one-cell stage mouse embryos collected from superovulated and mated females. To confirm that MO-1 was functioning as predicted, we used microfluidic q-rtPCR on RNA collected from single MO-injected and control embryos. We used a TaqMan primer-probe assay that targeted the junction between exons 2 and 3 (ex2–3). This junction would be present in the wild-type *Chd1l* transcript but absent if the MO blocks its targeted splicing event. Microfluidic qPCR confirmed abrogation of the wild-type transcript. Ct values showed that amplification of the ex2–3 splice junction was efficient in control embryos but nearly absent in injected embryos (a difference in Ct values of ~20, reflecting >99% reduction of transcripts containing the normal splice junction in injected embryos) (Fig. 4B,C). Wild-type transcripts were similarly abrogated by injection of a second splice-blocking MO (MO-2), which targeted the ex4–5 splice junction (a difference in Ct values of ~10, reflecting a >99% reduction of normal transcripts) (Fig. 4C). Changes in Oct4 gene expression in embryos injected with Chd1l MO were not statistically significant (α=0.05).

Using either MO, the altered splicing would lead to the introduction of a stop codon within the intron. The effect of impaired splicing on the transcript as a whole is unknown, and whether or not nonsense mediated decay (NMD) will be initiated cannot be predicted. The *Chd1l* transcript is not significantly reduced in embryos injected with MO-1 compared to uninjected embryos, as demonstrated by the lack of any significant change in amplification of the 3′ sequences (ex20–21). This suggests that the transcript is stable despite abrogation of splicing between exons 2 and 3. On the other hand, the *Chd1l* transcript (ex20–21) is somewhat reduced in embryos injected with MO-2, indicating that the transcript resulting from the abrogation of splicing between exons 4 and 5 may be more unstable or may trigger NMD. Regardless of transcript stability, the only protein products translated would be truncated near the N-terminus and devoid of any of the known functional protein domains.

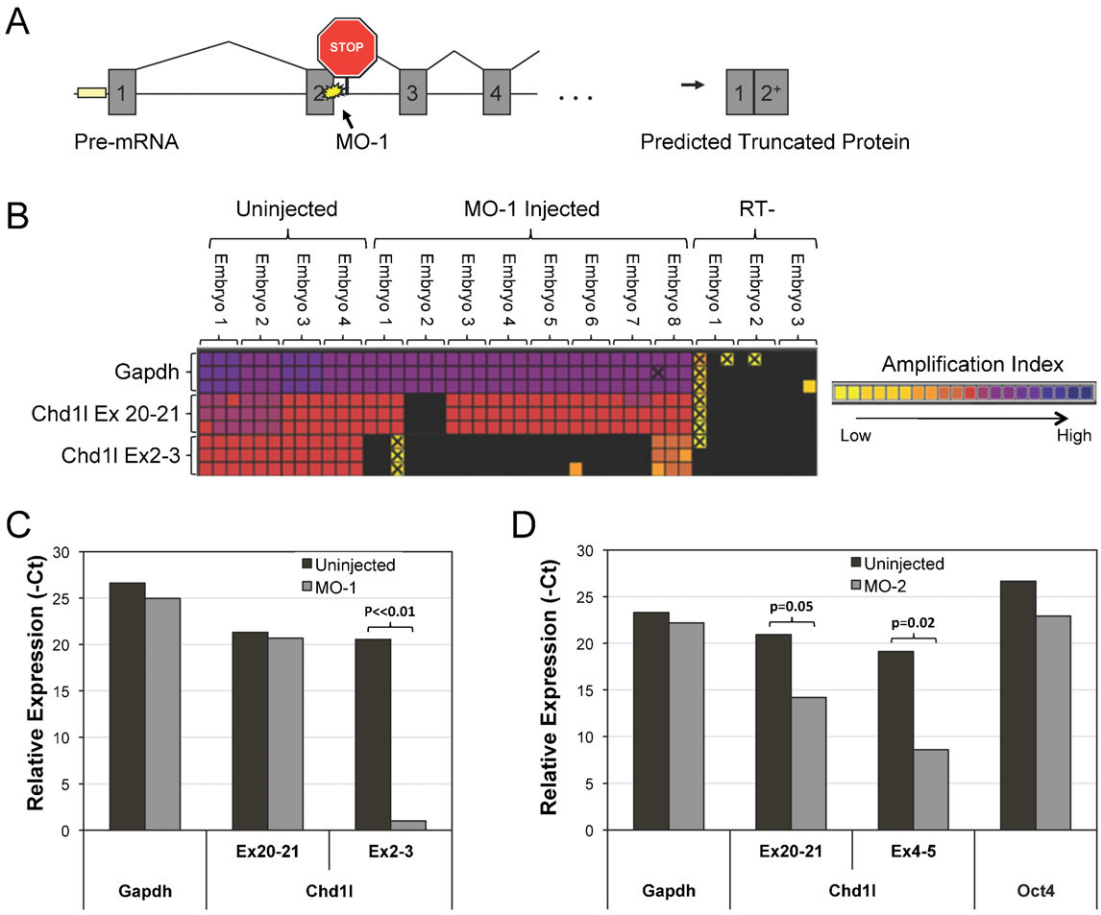

Fig. 4. Efficiency of Chd1l MO knockdown. (**A**) Mechanism of splice-blocking morpholinos (MOs). Splice-blocking MOs were designed targeting exon–intron boundaries. The schematic depicts "MO-1" disrupting the junction between exon 2 and exon 3. Disruption of this junction is predicted to produce a mutant protein truncated prior to the ATPase domain, thus lacking any functional activity. "MO-2" is designed in a similar manner, except that the disrupted splice junction is between exon 4 and exon 5. (**B–D**). Validation of Chd1l MO activity. Heat map (B) and quantitation (C,D) of microfluidic q-rtPCR of *Chd1l* transcripts. The "Ex20–21" probe targets the 3′ end of the Chd1l transcript and will amplify any *Chd1l* transcripts, regardless of splicing aberrations. The "Ex2–3" and "Ex4–5" probes target exon–exon junctions and will only amplify if that splicing event has occurred. MO-1 disrupts exon2–exon3 splicing (B,C). MO-2 disrupts exon4–exon5 splicing (D). Each embryo sample was run nine times. Black represents no amplification above the threshold and an "x" indicates a reaction automatically excluded from Ct value calculations. Ct values for each PCR reaction were subtracted from a value of 40 to reflect a positive correlation with expression levels. P-values were calculated using one-tailed, heteroscedastic Student's *t*-test to compare Ct values of embryos injected with morpholino to Ct values of control embryos.

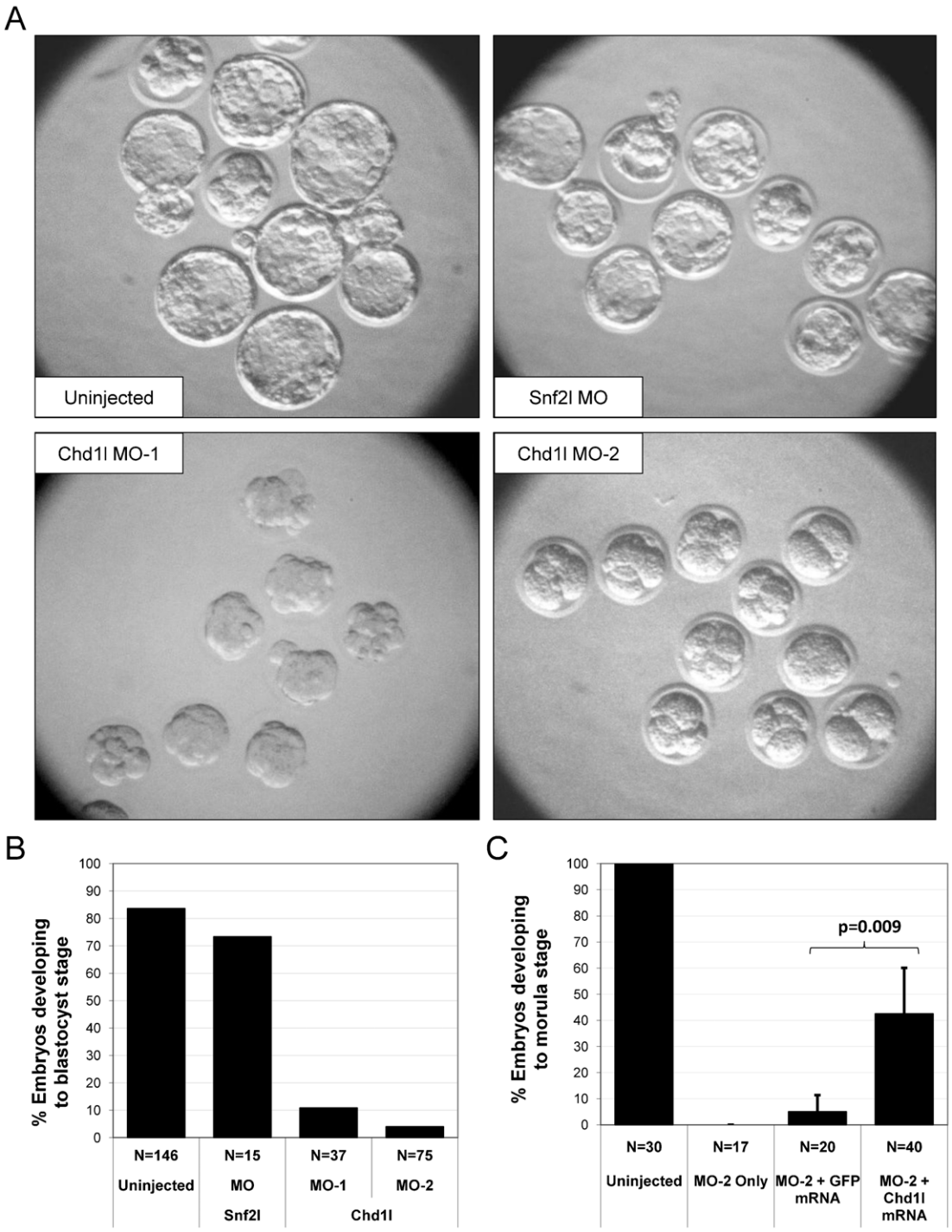

Fig. 5. Chd1l knockdown results in developmental arrest in early embryos. (**A**) Uninjected embryos and embryos injected with no MOs (uninjected), Snf2l MO (negative control), Chd1l MO-1 and Chd1l MO-2, at 4 days after microinjection. (**B**) Quantification of development to blastocyst stage in uninjected embryos and embryos injected with different MOs. (**C**) Partial rescue of developmental arrest phenotype with co-injection of *Chd1l* mRNA. Error bars were calculated using weighted standard deviations. The difference between embryos injected with MO-2 and *Chd1l* mRNA and embryos injected with MO-2 and a control mRNA was significant at the $\alpha=0.01$ level ($P=0.009$) when analyzed using a one-tailed, heteroscedastic Student's *t*-test.

Embryos injected with *Chd1l*-targeting MOs arrest prior to blastocyst stage

To ask whether Chd1l is required during early development we microinjected the zygote-stage embryo with MO-1 targeting *Chd1l* and observed embryos for a period of four days. MO-injected embryos did not reach the blastocyst stage and instead arrested at the compaction stage (Fig. 5A,B). Cells of arrested embryos do not fragment and instead appear morphologically normal. An arrest prior to blastocyst formation is consistent with the peak in *Chd1l* expression at the late morula stage and enrichment in the ICM. In contrast, the majority of embryos microinjected with MO targeting the *Snf2l* transcript, encoding

another Snf2-like chromatin remodeling factor, reached the blastocyst stage (Fig. 5A,B). This result demonstrates that embryonic arrest is not a general effect of microinjection of a CRF MO. To further test our finding we microinjected MO-2 targeting ex4–5 splice junction. These embryos also arrested prior to the blastocyst stage (Fig. 5A,B). The precise timing of the arrest varied between the MOs, perhaps due to different binding affinities of the MO sequences.

The Chd1l phenotype is partially rescued by co-injection of *Chd1l* mRNA

To confirm that the embryonic arrest phenotype is a result of disrupting Chd1l protein production, mRNA encoding Chd1l was co-injected along with Chd1l MO. We reasoned that embryos arrested at an earlier stage would be more able to progress to later developmental stages with addition of mRNA than embryos arrested at later stages, so we used MO-2 for co-injection. MO-2 targets a splicing junction and therefore could not target the injected mRNA, which was synthesized from cDNA lacking intron sequences. Embryos injected with MO-2 alone did not progress to the morula stage, nor did embryos co-injected with MO-2 plus GFP mRNA. About 50% of the embryos co-injected with MO-2 plus *Chd1l* mRNA progressed to the morula stage or further (Fig. 5C). Mitigation of the developmental arrest phenotype by *Chd1l* mRNA confirmed that loss of Chd1l was responsible for the embryonic arrest.

Discussion

Chromatin remodeling activities are abundant in preimplantation embryos and in ES cells, and many of these activities are geared toward initiating pluripotent transcriptional competence and ensuring that differentiation programs are locked in epigenetically (Corry et al., 2009; Albert and Peters, 2009). Although Chd1l is part of the Snf2 family of DNA-dependent ATPases (Flaus et al., 2006), many of which are potent transcriptional regulators, Chd1l itself does not seem to regulate gene expression, at least in ES cells. It remains a formal possibility that Chd1l regulates gene expression in the preimplantation embryo, where a prominent developmental arrest phenotype was observed, but subsequently ceases to have gene regulation activity in ES cells. Despite being abundantly expressed, Chd1l is not required for normal proliferation, pluripotency, or differentiation of ES cells. Chd1l might have no function at all in ES cells, its function may be masked by the function of a redundant ES cell protein, its function may only become apparent when some trigger or insult presents itself, or the ES cell protein may represent a store of protein that will be important later in development. Chd1l is likely function differently in the transiently pluripotent ICM than in ES cells that have undergone artificial epigenetic changes to adapt to immortal growth in culture.

The critical developmental role of Chd1l in preimplantation embryos may stem from the ADP-ribose-binding macro module that distinguishes Chd1l from the other members of its family (Ahel et al., 2009; Gottschalk et al., 2009; Karras et al., 2005). ADP-ribose is a post-translational modification that is added to acceptor proteins by several enzymes including the PARPs, which catalyze poly-ADP-ribosylation, and the sirtuins, which catalyze mono-ADP-ribosylation in addition to histone deacetylation (Landry et al., 2000; Imai et al., 2000; Frye, 1999). Chd1l may bind to ADP-ribose catalyzed by these enzymes and act as an effector protein for sirtuin- and/or PARP-mediated processes. The sirtuins have diverse cellular roles, including gene regulation and DNA repair, and have been shown to be critical during embryogenesis (Wang et al., 2006; Rine and Herskowitz, 1987; McBurney et al., 2003; Mao et al., 2011). However, mono-ADP-ribosylation by the sirtuins appears to be weak compared to histone deacetylation, and the physiological significance this activity remains somewhat controversial (Du et al., 2009).

Poly-ADP-ribose (PAR) is synthesized by the PARP family of PAR polymerases and has important roles in diverse chromatin-dependent processes. Evidence is accumulating for the importance of PAR regulation in the embryo. Double knockout of PAR polymerases *Parp-1* and the partially redundant *Parp-2* in mice is embryonic lethal at the onset of gastrulation (Ménissier de Murcia et al., 2003), whereas knockout of the non-redundant PAR depolymerase PARG is lethal at E3.5 (Koh et al., 2004). These data suggest that PAR levels are tightly regulated in the embryo and that fluctuations are highly deleterious. Chd1l contains a macro module responsible for binding PAR. Thus Chd1l could contribute to PAR regulation, and the Chd1l embryonic arrest phenotype may be due to aberrant PAR levels or downstream PAR signaling.

Two independent groups recently demonstrated the ability of Chd1l to respond to DNA damage by interacting with PAR (Ahel et al., 2009; Gottschalk et al., 2009). DNA damage is one of the most prominent triggers of PAR modification and signaling. Parp-1 is activated by DNA damage and synthesizes PAR onto itself in an auto-modification reaction, which initiates DNA repair mechanisms (Berger, 1985; Benjamin and Gill, 1980). Blocking Parp-1 activity with specific inhibitors, or through null mutations, results in cellular hypersensitivity to DNA damaging agents and defects in DNA repair (Herceg and Wang, 2001). Repair of damaged DNA is critical in the early embryo. Damage to DNA occurs frequently as a result of normal cellular metabolism, and the repair of resulting errors is critical in the early embryo as it must maintain genomic integrity for the future organism. Consistent with this requirement, genes involved in all of the major DNA repair pathways are expressed in the preimplantation embryo (Jaroudi and SenGupta, 2007). Therefore, because Chd1l responds to DNA damage through its interaction with PAR, and because DNA repair is crucial during embryogenesis, defects in DNA repair could underlie the Chd1l early embryonic arrest.

Large number of double-stranded break repair proteins are embryonic lethal when deleted (Hakem, 2008). Double-stranded breaks are the most toxic form of DNA damage and can be repaired through either non-homologous end-joining (NHEJ) or homologous recombination (HR). NHEJ can function throughout the cell cycle, whereas HR is restricted to S/G2 phase (Rothkamm et al., 2003). In the zygote, repair of the paternal genome is especially crucial because double-stranded breaks and other errors are introduced during spermatogenesis, and the extreme chromatin compaction of the sperm is inhibitory to repair (Generoso et al., 1979; Matsuda et al., 1985). The zygote spends ~20 hours in G1 prior to the first cell division, and much of the paternal DNA is repaired through NHEJ (Fiorenza et al., 2001; Hagmann et al., 1996; Hagmann et al., 1998; Lee et al., 1997). The ability of Chd1l to function in NHEJ is suggested by its PARP-dependent association with a major NHEJ component, DNA-PKcs, upon induced DNA damage (Ahel et al., 2009).

An intriguing question is why is Chd1l essential in the earliest stages of embryogenesis but not in ES cells? In contrast to the zygote, ES cells have rapid cell cycles with abbreviated G1 and G2 phases and rely heavily on HR to repair lesions during S phase (Savatier et al., 2002). Therefore, one explanation for why reduced Chd1l causes preimplantation arrest but is dispensable for ES cells is that Chd1l plays a role in NHEJ, which is acutely essential during early embryogenesis but not in ES cells.

In summary, numerous mRNAs encoding chromatin remodeling enzymes are enriched in the ICM, which will differentiate into all the cellular lineages of the adult organism. Chd1l, a candidate regulator of pluripotency studied here, is essential for preimplantation embryonic development even prior to the formation of the ICM. Despite its requirement for the earliest cell divisions in the embryo and its expression in ES cells, Chd1l appears to be dispensable for ES cell viability, pluripotency, differentiation, and gene expression. The function of Chd1l could be in PAR signaling via the PAR-binding macro domain and in downstream DNA repair. Recent studies have demonstrated a role for Chd1l as a DNA damage response protein that interacts with members of the NHEJ pathway in a PARP-dependent manner. Impaired NHEJ repair could explain why Chd1l deficiency results in developmental arrest of preimplantation embryos that rely heavily on NHEJ but causes no detectable abnormalities in ES cells. The differential requirement of Chd1l in the preimplantation embryo and in ES cells exemplifies the limitations of extrapolating conclusions from experiments using *in vitro*-derived ES cells and highlights the importance of studying early development directly in mouse embryos.

Materials and Methods

Immunosurgery and expression profiling

E3.5 blastocysts were collected from timed-pregnant mothers and washed in M2 medium. The zona pellucida was removed by incubation in Acid Tyrode solution for 3 minutes. Outer TE cells were labeled with IgGs by incubation with 10% rabbit anti-mouse serum for 60 minutes. Embryos were washed three times in M2 medium, and then TE cells were lysed through the complement cascade by incubation with 30% guinea pig complement for 15–30 minutes, or until lysis was visible. Remaining ICMs were washed three times in M2 medium with a fine pipette to remove residual TE cells. Total RNA was extracted from purified ICMs and whole blastocysts with Trizol reagent. Purified RNA was amplified using an Affymetrix kit, labeled, and hybridized to one Affymetrix mouse 430 2.0 Expression Array per sample. Chip analyses were performed with Dchip, a model-based method for expression analysis (http://www.dchip.org). Normalization of data was performed by the Invariant Set Normalization method (Li and Wong, 2001).

ES cell lines

The EBRTcH3 cell line contains a cassette acceptor utilizing *loxP* and *loxPV* sites at the Rosa locus to allow efficient and directional integration of a transgene by *Cre*-mediated recombination. ShRNA-mir cDNAs were subcloned from pGIPZ vectors (OpenBiosystems, *Chd1l* shRNA Oligo ID: V2LMM_18041 and "non-silencing" shRNA-mir) into the pPthC exchange vector for recombination into the EBRTcH3 ES cell line. The parental EBRTcH3 ES cells and the pPthC exchange vector were gifts from the lab of Dr Hitoshi Niwa of Japan.

The exchange vector containing the shRNA-mir sequence was cotransfected with a Cre expression plasmid using lipofectamine. Transfected cells were plated at single-cell density and cultured with Puromycin (1.5 μg/ml) to select for successful recombinants and with Tetracycline (1.0 μg/ml) to repress transgene expression. Clones were confirmed by PCR genotyping of the 5′ and 3′ recombination sites. To induce shRNA expression, the derived ES cell lines were cultured without Tetracycline but with high Puromycin (7.5 μg/ml). Control, uninduced ES cells were cultured in high Tetracycline (1.5 μg/ml) and high Puromycin (7.5 μg/ml).

ES cell expression profiling

Total RNA was extracted from *Chd1l*-shRNA and NS-shRNA ES cells three days after inducing the expression of *Chd1l*-shRNA or NS-shRNA by Tetracycline removal (7.5 μg/ml Puromycin), and from uninduced *Chd1l*-shRNA and NS-shRNA ES cells that do not express shRNA (7.5 μg/ml Puromycin, 1.5 μg/ml Tetracycline). Three different *Chd1l*-shRNA EBRTcH3 clones and one NS-shRNA EBRTcH3 clone were used. RNA was amplified from the eight samples, labeled using an Affymetrix kit, and hybridized to mouse 430 2.0 Expression Arrays. Fold changes in expression indices were calculated for shRNA-induced ES cells *versus* shRNA-uninduced ES cells. The statistical significance of fold-changes between Chd1-shRNA induced and uninduced samples was determined using a paired *t*-test, a minimum fold change of 1.4, and a delta value of 1.9 (SAM Analysis) (Tusher et al., 2001).

Differentiation of embryoid bodies

Expression of shRNA was induced by Tetracycline withdrawal in *Chd1l*-shRNA and NS-shRNA EBRTcH3 ES cells for three days prior to differentiation into embryoid bodies (EBs) to ensure complete Chd1l knockdown. RNA was collected at Day 0 of differentiation from induced and uninduced *Chd1l*-shRNA and NS-shRNA EBRTcH3 ES cells. ES cells were suspended at a density of 2×10^4 cells/ml of medium without LIF, and EBs were made using hanging droplets of 500 cells in 25 μl. After two days, embryoid bodies were collected into 10-cm Ultralow Attachment plates (Corning) and cultured for an additional seven days in the absence of LIF. RNA was collected every three days after LIF removal. cDNA was synthesized from each sample and subjected to qPCR. Relative quantities for each cell line were calculated using *Gapdh* as the internal control and shRNA-uninduced, Day 0 samples as references.

TaqMan gene expression assays used in these experiments were predesigned and ordered from Applied Biosystems: Gapdh: Mm99999915_g1; Oct4: Mm00658129_gH; Gata4: Mm00484689_m1; Eomes: Mm01351988_m1; Sox17: Mm00488363_m1; Fgf5: Mm00438919_m1; AFP: Mm01351348_g1; LhX1: Mm00521776_m1; Otx1: Mm00446859_m1; Chd1l: Mm00471579_m1.

Embryo culture and microinjection

Three to five-week-old wild-type F1 (C57BL6×DBA/2) females (Charles Rivers) were superovulated by intraperitoneal injections of 5 IU of pregnant mare's serum gonadotropin (Sigma) followed by 5 IU of human chorion gonadotropin (Sigma) 48 hours later and mated with wild-type males. Mice were sacrificed by cervical dislocation 17 hours after hCG injection, and 1-cell embryos were dissected and released from oviducts. Cumulus cells were removed using hyaluronidase digestion, and single-cell zygotes at the two-pronuclei stage were recovered and immediately micro-injected cytoplasmicly with 5–10 pL of 0.6 mM antisense morpholino. Prior to injection, the MO was heated at 65° for 15 minutes to remove any secondary structure.

Preimplantation embryos were cultured *in vitro* in 20 μl droplets of Quinn's Advantage Cleavage Medium (Sage) supplemented with 10% SPS serum and covered with mineral oil. Dishes were placed in a desiccator filled with mixed gas (90% nitrogen, 5% oxygen, 5% carbon dioxide) in a 37° incubator. Embryos were observed every 24 hours for a period of four days, about the time of hatching.

Morpholinos were obtained from GeneTools: Chd1l MO-1: tcattccacagcaga tacCTGGCAG (in2-EX2); Chd1l MO-2: ttggagagaagcagagggctaCCTC (in4-EX4); Snf2l: tgctgtttaccaccttacCAAGGGC (in2-EX2).

Microfluidic qPCR

Single embryos were collected 48 hours after injection and lysed by one freeze thaw cycle. cDNA was synthesized using the CellsDirect One-Step rtPCR kit (Invitrogen) and subjected to 18 rounds of gene-specific amplification using TaqMan primer/probe assays (Applied Biosystems). TaqMan primer/probe assays and cDNA from single embryos were loaded onto a Fluidigm 48.48 microfluidic array for qPCR analysis using a Biomark thermalcycler.

TaqMan gene expression assays used in these experiments were predesigned and ordered from Applied Biosystems: Gapdh: Mm99999915_g1; Chd1l Ex2–3: Mm00471561_m1; Chd1l Ex4–5: Mm01257091_m1; Chd1l Ex20–21: Mm00471578_m1.

α-Chd1l antibody

A hydrophilic sequence of 122 aa corresponding to amino acid numbers 557–678 of the Chd1l protein was selected for the antigenic region. The antigen was produced as a TrpE fusion protein from the pATH11 vector in BL21 *E. coli*, solubilized, and subjected to SDS-PAGE. The gel slice was excised and submitted to Josman, LLC for injection into two rabbits and two rats. The antiserum was affinity purified using a GST-Chd1l-bound Sepharose column and eluted with low pH buffer. Both the rabbit and the rat antibodies recognize a ~100 kD band on Western blot. Rabbit α-Chd1l antibody was used in all experiments reported here.

Statement regarding animal use

The animal experiments were performed in the labs of M.W.M.Y. and M.P.S. under ethical protocols approved by the Stanford Institutional Animal Care and Use Committee.

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Competing Interests

The authors have no competing interests to declare.

References

Ahel, D., Horejsí, Z., Wiechens, N., Polo, S. E., Garcia-Wilson, E., Ahel, I., Flynn, H., Skehel, M., West, S. C., Jackson, S. P. et al. (2009). Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1. *Science* **325**, 1240-1243.

Albert, M. and Peters, A. H. (2009). Genetic and epigenetic control of early mouse development. *Curr. Opin. Genet. Dev.* **19**, 113-121.

Benjamin, R. C. and Gill, D. M. (1980). ADP-ribosylation in mammalian cell ghosts. Dependence of poly(ADP-ribose) synthesis on strand breakage in DNA. *J. Biol. Chem.* **255**, 10493-10501.

Berger, N. A. (1985). Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat. Res.* **101**, 4-15.

Cairns, B. R. (2005). Chromatin remodeling complexes: strength in diversity, precision through specialization. *Curr. Opin. Genet. Dev.* **15**, 185-190.

Chen, M., Huang, J. D., Hu, L., Zheng, B. J., Chen, L., Tsang, S. L. and Guan, X. Y. (2009). Transgenic CHD_1L expression in mouse induces spontaneous tumors. *PLoS ONE* **4**, e6727.

Corry, G. N., Tanasijevic, B., Barry, E. R., Krueger, W. and Rasmussen, T. P. (2009). Epigenetic regulatory mechanisms during preimplantation development. *Birth Defects Res. C Embryo Today* **87**, 297-313.

Du, J., Jiang, H. and Lin, H. (2009). Investigating the ADP-ribosyltransferase activity of sirtuins with NAD analogues and ^{32}P-NAD. *Biochemistry* **48**, 2878-2890.

Eisen, J. A., Sweder, K. S. and Hanawalt, P. C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. *Nucleic Acids Res.* **23**, 2715-2723.

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

Fiorenza, M. T., Bevilacqua, A., Bevilacqua, S. and Mangia, F. (2001). Growing dictyate oocytes, but not early preimplantation embryos, of the mouse display high levels of DNA homologous recombination by single-strand annealing and lack DNA nonhomologous end joining. *Dev. Biol.* **233**, 214-224.

Flaus, A. and Owen-Hughes, T. (2001). Mechanisms for ATP-dependent chromatin remodelling. *Curr. Opin. Genet. Dev.* **11**, 148-154.

Flaus, A. and Owen-Hughes, T. (2004). Mechanisms for ATP-dependent chromatin remodelling: farewell to the tuna-can octamer? *Curr. Opin. Genet. Dev.* **14**, 165-173.

Flaus, A., Martin, D. M., Barton, G. J. and Owen-Hughes, T. (2006). Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. *Nucleic Acids Res.* **34**, 2887-2905.

Foygel, K., Choi, B., Jun, S., Leong, D. E., Lee, A., Wong, C. C., Zuo, E., Eckart, M., Reijo Pera, R. A., Wong, W. H. et al. (2008). A novel and critical role for Oct4 as a regulator of the maternal-embryonic transition. *PLoS ONE* **3**, e4109.

Fry, C. J. and Peterson, C. L. (2001). Chromatin remodeling enzymes: who's on first? *Curr. Biol.* **11**, R185-R197.

Frye, R. A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochem. Biophys. Res. Commun.* **260**, 273-279.

Fyodorov, D. V. and Kadonaga, J. T. (2001). The many faces of chromatin remodeling: SWItching beyond transcription. *Cell* **106**, 523-525.

Generoso, W. M., Cain, K. T., Krishna, M. and Huff, S. W. (1979). Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. *Proc. Natl. Acad. Sci. USA* **76**, 435-437.

Gore, A. V., Maegawa, S., Cheong, A., Gilligan, P. C., Weinberg, E. S. and Sampath, K. (2005). The zebrafish dorsal axis is apparent at the four-cell stage. *Nature* **438**, 1030-1035.

Gottschalk, A. J., Timinszky, G., Kong, S. E., Jin, J., Cai, Y., Swanson, S. K., Washburn, M. P., Florens, L., Ladurner, A. G., Conaway, J. W. et al. (2009). Poly(ADP-ribosyl)ation directs recruitment and activation of an ATP-dependent chromatin remodeler. *Proc. Natl. Acad. Sci. USA* **106**, 13770-13774.

Hagmann, M., Adlkofer, K., Pfeiffer, P., Bruggmann, R., Georgiev, O., Rungger, D. and Schaffner, W. (1996). Dramatic changes in the ratio of homologous recombination to nonhomologous DNA-end joining in oocytes and early embryos of *Xenopus laevis*. *Biol. Chem. Hoppe Seyler* **377**, 239-250.

Hagmann, M., Bruggmann, R., Xue, L., Georgiev, O., Schaffner, W., Rungger, D., Spaniol, P. and Gerster, T. (1998). Homologous recombination and DNA-end joining reactions in zygotes and early embryos of zebrafish (*Danio rerio*) and *Drosophila melanogaster*. *Biol. Chem.* **379**, 673-681.

Hakem, R. (2008). DNA-damage repair; the good, the bad, and the ugly. *EMBO J.* **27**, 589-605.

Herceg, Z. and Wang, Z. Q. (2001). Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat. Res.* **477**, 97-110.

Hirschhorn, J. N., Brown, S. A., Clark, C. D. and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.* **6**, 2288-2298.

Ho, L. and Crabtree, G. R. (2010). Chromatin remodelling during development. *Nature* **463**, 474-484.

Ho, L., Ronan, J. L., Wu, J., Staahl, B. T., Chen, L., Kuo, A., Lessard, J., Nesvizhskii, A. I., Ranish, J. and Crabtree, G. R. (2009). An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency. *Proc. Natl. Acad. Sci. USA* **106**, 5181-5186.

Imai, K. S., Levine, M., Satoh, N. and Satou, Y. (2006). Regulatory blueprint for a chordate embryo. *Science* **312**, 1183-1187.

Imai, S., Armstrong, C. M., Kaeberlein, M. and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795-800.

Jaroudi, S. and SenGupta, S. (2007). DNA repair in mammalian embryos. *Mutat. Res.* **635**, 53-77.

Karras, G. I., Kustatscher, G., Buhecha, H. R., Allen, M. D., Pugieux, C., Sait, F., Bycroft, M. and Ladurner, A. G. (2005). The macro domain is an ADP-ribose binding module. *EMBO J.* **24**, 1911-1920.

Knezetic, J. A. and Luse, D. S. (1986). The presence of nucleosomes on a DNA template prevents initiation by RNA polymerase II in vitro. *Cell* **45**, 95-104.

Koh, D. W., Lawler, A. M., Poitras, M. F., Sasaki, M., Wattler, S., Nehls, M. C., Stöger, T., Poirier, G. G., Dawson, V. L. and Dawson, T. M. (2004). Failure to degrade poly(ADP-ribose) causes increased sensitivity to cytotoxicity and early embryonic lethality. *Proc. Natl. Acad. Sci. USA* **101**, 17699-17704.

Landry, J., Sutton, A., Tafrov, S. T., Heller, R. C., Stebbins, J., Pillus, L. and Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc. Natl. Acad. Sci. USA* **97**, 5807-5811.

Lee, S. E., Mitchell, R. A., Cheng, A. and Hendrickson, E. A. (1997). Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle. *Mol. Cell. Biol.* **17**, 1425-1433.

Lessard, J., Wu, J. I., Ranish, J. A., Wan, M., Winslow, M. M., Staahl, B. T., Wu, H., Aebersold, R., Graef, I. A. and Crabtree, G. R. (2007). An essential switch in subunit composition of a chromatin remodeling complex during neural development. *Neuron* **55**, 201-215.

Li, C. and Wong, W. H. (2001). Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol.* **2**, research0032.1-research0032.11.

Lorch, Y., LaPointe, J. W. and Kornberg, R. D. (1987). Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones. *Cell* **49**, 203-210.

Ma, N. F., Hu, L., Fung, J. M., Xie, D., Zheng, B. J., Chen, L., Tang, D. J., Fu, L., Wu, Z., Chen, M. et al. (2008). Isolation and characterization of a novel oncogene, amplified in liver cancer 1, within a commonly amplified region at 1q21 in hepatocellular carcinoma. *Hepatology* **47**, 503-510.

Mao, Z., Hine, C., Tian, X., Van Meter, M., Au, M., Vaidya, A., Seluanov, A. and Gorbunova, V. (2011). SIRT6 promotes DNA repair under stress by activating PARP1. *Science* **332**, 1443-1446.

Masui, S., Shimosato, D., Toyooka, Y., Yagi, R., Takahashi, K. and Niwa, H. (2005). An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit. *Nucleic Acids Res.* **33**, e43.

Matsuda, Y., Yamada, T. and Tobari, I. (1985). Studies on chromosome aberrations in the eggs of mice fertilized in vitro after irradiation. I. Chromosome aberrations induced in sperm after X-irradiation. *Mutat. Res.* **148**, 113-117.

McBurney, M. W., Yang, X., Jardine, K., Hixon, M., Boekelheide, K., Webb, J. R., Lansdorp, P. M. and Lemieux, M. (2003). The mammalian SIR2α protein has a role in embryogenesis and gametogenesis. *Mol. Cell. Biol.* **23**, 38-54.

Ménissier de Murcia, J., Ricoul, M., Tartier, L., Niedergang, C., Huber, A., Dantzer, F., Schreiber, V., Amé, J. C., Dierich, A., LeMeur, M. et al. (2003). Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. *EMBO J.* **22**, 2255-2263.

Mohrmann, L. and Verrijzer, C. P. (2005). Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. *Biochim. Biophys. Acta* **1681**, 59-73.

Morrison, A. J., Highland, J., Krogan, N. J., Arbel-Eden, A., Greenblatt, J. F., Haber, J. E. and Shen, X. (2004). INO80 and γ-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. *Cell* **119**, 767-775.

Niemann, H., Tian, X. C., King, W. A. and Lee, R. S. (2008). Epigenetic reprogramming in embryonic and foetal development upon somatic cell nuclear transfer cloning. *Reproduction* **135**, 151-163.

Okita, K., Ichisaka, T. and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313-317.

Peng, J. C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A. and Wysocka, J. (2009). Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. *Cell* **139**, 1290-1302.

Rine, J. and Herskowitz, I. (1987). Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. *Genetics* **116**, 9-22.

Rossant, J. and Tam, P. P. (2009). Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. *Development* **136**, 701-713.

Rothkamm, K., Krüger, I., Thompson, L. H. and Löbrich, M. (2003). Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol. Cell. Biol.* **23**, 5706-5715.

Savatier, P., Lapillonne, H., Jirmanova, L., Vitelli, L. and Samarut, J. (2002). Analysis of the cell cycle in mouse embryonic stem cells. *Methods Mol. Biol.* **185**, 27-33.

Solter, D. and Knowles, B. B. (1975). Immunosurgery of mouse blastocyst. *Proc. Natl. Acad. Sci. USA* **72**, 5099-5102.

Sumanas, S. and Larson, J. D. (2002). Morpholino phosphorodiamidate oligonucleotides in zebrafish: a recipe for functional genomics? *Brief. Funct. Genomic. Proteomic.* **1**, 239-256.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.

Tusher, V. G., Tibshirani, R. and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**, 5116-5121.

Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress, W. D. et al. (2006). Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. *Nat. Cell Biol.* **8**, 1025-1031.

Wang, Q. T., Piotrowska, K., Ciemerych, M. A., Milenkovic, L., Scott, M. P., Davis, R. W. and Zernicka-Goetz, M. (2004). A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. *Dev. Cell* **6**, 133-144.

Wang, W., Côté, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M. et al. (1996a). Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *EMBO J.* **15**, 5370-5382.

Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B. R. and Crabtree, G. R. (1996b). Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.* **10**, 2117-2130.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E. and Jaenisch, R. (2007). *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**, 318-324.

Yamada, L., Shoguchi, E., Wada, S., Kobayashi, K., Mochizuki, Y., Satou, Y. and Satoh, N. (2003). Morpholino-based gene knockdown screen of novel genes with developmental function in *Ciona intestinalis*. *Development* **130**, 6485-6495.

Yan, L., Echenique, V., Busso, C., SanMiguel, P., Ramakrishna, W., Bennetzen, J. L., Harrington, S. and Dubcovsky, J. (2002). Cereal genes similar to Snf2 define a new subfamily that includes human and mouse genes. *Mol. Genet. Genomics* **268**, 488-499.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R. et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920.