A nontranscriptional role for Oct4 in the regulation of mitotic entry

Rui Zhao[a,b,1,2], Richard W. Deibler[c,2], Paul H. Lerou[d], Andrea Ballabeni[c], Garrett C. Heffner[a,b], Patrick Cahan[a,b], Juli J. Unternaehrer[a,b,3], Marc W. Kirschner[c,4], and George Q. Daley[a,b,4]

[a]Stem Cell Transplantation Program, Division of Pediatric Hematology/Oncology, Manton Center for Orphan Disease Research, Boston Children's Hospital, Dana–Farber Cancer Institute, Department of Biological Chemistry and Molecular Pharmacology, Harvard Stem Cell Institute, and [b]Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115; [c]Department of Systems Biology, Harvard Medical School, Boston, MA 02115; and [d]Department of Newborn Medicine and Department of Medicine, Division of Genetics, Brigham & Women's Hospital, Department of Medicine, Division of Newborn Medicine, Boston Children's Hospital, and Harvard Stem Cell Institute, Harvard Medical School, Boston, MA 02115

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Rapid progression through the cell cycle and a very short G1 phase are defining characteristics of embryonic stem cells. This distinct cell cycle is driven by a positive feedback loop involving Rb inactivation and reduced oscillations of cyclins and cyclin-dependent kinase (Cdk) activity. In this setting, we inquired how ES cells avoid the potentially deleterious consequences of premature mitotic entry. We found that the pluripotency transcription factor Oct4 (octamer-binding transcription factor 4) plays an unappreciated role in the ES cell cycle by forming a complex with cyclin–Cdk1 and inhibiting Cdk1 activation. Ectopic expression of Oct4 or a mutant lacking transcriptional activity recapitulated delayed mitotic entry in HeLa cells. Reduction of Oct4 levels in ES cells accelerated G2 progression, which led to increased chromosomal missegregation and apoptosis. Our data demonstrate an unexpected nontranscriptional function of Oct4 in the regulation of mitotic entry.

pluripotent stem cells | mitotic entry | Oct4 | Cdk1 | CDC25

Embryonic stem (ES) cells are derived from preimplantation embryos and can be propagated long term in vitro while maintaining the capacity to form tissues from all three germ layers. Compared with differentiated cells, ES cells and the pluripotent epiblast cells that form the embryo proper undergo an accelerated cell cycle (1–4).

Cell cycle progression is driven by the activation and inactivation of cyclin-dependent kinases (Cdks). The G1-to-S phase transition is regulated by cyclins E and A, both of which activate Cdk2 (5–8). The retinoblastoma protein (Rb) maintains the G1 phase by inhibiting the E2F transcription factors. In somatic cells, increased Cdk activity at the end of G1 leads to phosphorylation and inactivation of Rb and increased expression of E2F targets, including cyclins E and A2 (hereafter A) (9, 10). This feedback, in which rising cyclin levels inactivate Rb to promote further increases in cyclins E and A, mediates the transition to S phase. The G2 to mitosis (M) phase transition is governed by activation of Cdk1, primarily through cyclins A and B1 (hereafter B). Cells synthesize cyclin B in interphase, but cyclin B-bound Cdk1 remains inactive due to phosphorylation on residues T14 and Y15 by Myt1/Wee1 kinases (11–13). Once cyclin B reaches a threshold concentration, the Myt1/Wee1 kinases are inhibited, and the CDC25 phosphatases are activated (14), which leads to cyclin B-bound Cdk1 activation and mitotic entry. Cyclin A promotes the early stage of mitosis in particular, and a critical part of this role is activating cyclin B-Cdk1 by weakening the Wee1-mediated inhibition of cyclin B-Cdk1 and promoting the nuclear localization of cyclin B (7, 15–20).

Cell cycle regulation is noticeably altered in ES cells to allow for more rapid cell proliferation. In contrast to somatic cells, Rb is inactive throughout the ES cell cycle due to constitutive phosphorylation (3), which allows for increased expression of cyclins E and A and extended cyclin E/A-Cdk2 activity (4, 21, 22). Moreover, cyclin-dependent kinase inhibitors (CKIs) such as p16, p21, and p27 are absent or expressed at low abundance in ES cells, which further unleash Cdk activity (4, 22–24). The dampened oscillation of Cdk activity is at least partially responsible for the rapid cell cycle progression by reducing the time needed for Cdk activation.

Although the features producing a shortened cell cycle are physiologically normal in ES cells, they are frequently observed in transformed cells and lead to mitotic defects and genomic instability (25–28). In particular, reduced oscillations of cyclin A (4, 21) and cyclin B (22) result in nearly mitotic levels in interphase and a potential risk of entering mitosis prematurely, should cyclin concentration increase. The risk for genomic instability of ES cells has raised safety concerns for potential therapeutic applications (29).

Oct4 is known as a pluripotency-associated transcription factor expressed in blastomeres, the inner cell mass of blastocysts, and germ cells of postgastrulation embryos (30, 31). Deletion of *Oct4* leads to developmental arrest at the blastocyst stage and an inability to isolate ES cells (32). Forced Oct4 expression, together with Sox2, Klf4, and c-Myc, enables generation of induced pluripotent stem (iPS) cells from terminally differentiated cells (33–36). The activities of Oct4 have been attributed to its canonical function as a transcription factor whereas a nontranscriptional role of Oct4 has not been considered.

Noting previous reports of interactions between Oct4 and Cdk1 (37–39), we hypothesized that ES cells might use Oct4 to

Significance

Embryonic stem cells and induced pluripotent stem cells have abbreviated cell cycles. To achieve this rapid proliferation, several molecular safeguards that normally distinguish healthy from transformed cells are altered. Understanding how these pluripotent stem cells balance the demands of their unique cell cycles against the need to maintain a stable genome is critical to unlocking their great promise for regenerative medicine. Here, we demonstrate that Oct4 (octamer-binding transcription factor 4), a transcription factor required to maintain pluripotency, inhibits the activation of cyclin-dependent kinase (Cdk) 1, the master regulator of mitosis, and delays mitotic entry in a nontranscriptional manner. To our knowledge, our study is the first demonstration of a nontranscriptional function of the pluripotency regulator Oct4.

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[1]Present address: Department of Biochemistry and Molecular Genetics and UAB Stem Cell Institute, University of Alabama at Birmingham, Birmingham, AL 35294.

[2]R.Z. and R.W.D. contributed equally to this work.

[3]Present address: Division of Biochemistry, Loma Linda University, Loma Linda, CA 92354.

[4]To whom correspondence may be addressed. Email: marc@hms.harvard.edu or george.daley@childrens.harvard.edu.

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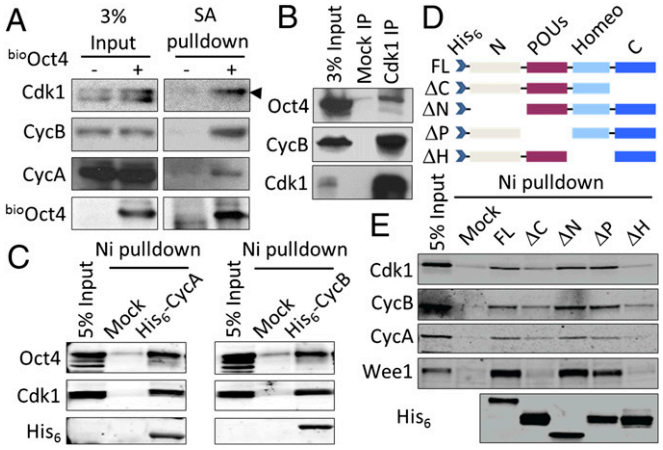

Fig. 1. Oct4 forms a complex with Cdk1. (*A*) Coprecipitation of Cdk1 and cyclin B and A by streptavidin-agarose (SA) bead pulldown from the BirA-^{bio}Oct4 cell extracts. BirA cell extracts were used as controls. Arrowhead, phosphorylated Cdk1. (*B*) Coprecipitation of Oct4 by anti-Cdk1 antibody. Mock immunoprecipitation (IP) was performed using total IgG. (*C*) Coprecipitation of Oct4 and Cdk1 by Ni-NTA bead pulldown. Recombinant His_6-cyclin A (*Left*) or His_6-cyclin B (*Right*) was bound to Ni-NTA beads and incubated with ES-cell extracts. (*D*) Schematic of His_6-tagged Oct4 mutants. N, N-terminal region; POUs, POU-specific domain; Homeo, Homeo domain; C, C-terminal region. (*E*) Domain mapping by His_6-tagged Oct4 mutants. Recombinant His_6-tagged full-length or truncated Oct4 were bound to Ni-NTA beads and then added to HeLa cell extracts. Shown are immunoblots of the indicated proteins after precipitation.

buffer the potentially problematic extended cyclin expression while maintaining normal mitotic entry and genomic integrity. In this study, we discovered that Oct4 has an unexpected, nontranscriptional function in blocking Cdk1 activation, which helps regulate mitotic entry.

Results

Oct4 Forms a Complex with Cyclin–Cdk1. To explore the reported interaction between Oct4 and Cdk1, we used J1-derived ES cell lines expressing a biotin ligase (BirA) alone or together with a biotinylatable Oct4 (BirA-^{bio}Oct4) (37). As expected, we detected Cdk1, as well as cyclin A and B, in association with ^{bio}Oct4 when precipitated by streptavidin (SA) beads (Fig. 1*A*). Phosphorylated, inactive Cdk1 was preferentially bound to Oct4, but this phosphorylation was not required for binding (Fig. S1 *A*–*F*). We also found Oct4 in Cdk1 immunoprecipitations (Fig. 1*B*). In contrast to cyclin B, only a small fraction of the total Oct4 coprecipitated with Cdk1 (Fig. 1*B*), suggesting that only a fraction of Oct4 interacts with Cdk1, or that the interaction is weak. By gel filtration of ES-cell extracts, we found that unphosphorylated Cdk1 (which also lacked a cyclin partner) did not interact with Oct4, indicating that Cdk1 did not associate with Oct4 unless its cognate cyclin was bound (Fig. S1*D*). Oct4–Cdk1 complexes could also be formed de novo through the addition of exogenous mitotic cyclins (A or B) to ES-cell extracts (Fig. 1*C*). Oct4 binding was specific to Cdk1 because neither ^{bio}Oct4 nor endogenous Oct4 bound to Cdk2 or its associated cyclin E (Fig. S1 *H* and *I*), as previously reported (39). Interestingly, cyclin A bound both Cdk1 and Cdk2 (Fig. S1*J*), but Oct4 associated only with Cdk1, indicating that Oct4 discriminates cyclin A-bound Cdk1 or Cdk2.

To dissect the region of Oct4 mediating the cyclin–Cdk1 interaction, we generated Oct4 mutants with deletions in either one or multiple of the N, POUs, Homeo, and C domains (Fig. 1*D*, Fig. S1*K*, and Table S1), which revealed that the N-terminal domain was completely dispensable for binding to cyclin–Cdk1 whereas other regions, particularly the Homeo and C-terminal domains, were important for binding because their loss strongly reduced binding of Oct4 to the cyclin–Cdk1 complex (Fig. 1*E* and Fig. S1*L*). Oct4, as well as the ΔN mutant, also precipitated Wee1, a factor known to associate with the cyclin–Cdk1 complexes (Fig. 1*E*) (15). These data suggest that multiple domains of Oct4 mediate the cyclin–Cdk1 interaction or that the interaction is disrupted by modest changes in Oct4 folding.

Oct4 Inhibits Cdk1 Activation in Vitro. The interaction between Oct4 and cyclin–Cdk1 suggested that either Oct4 is a substrate of Cdk1 and itself regulated during the cell cycle, or that Cdk1 activity is modulated by Oct4, implicating Oct4 as a direct regulator of the cell cycle. To investigate whether Oct4 modulates Cdk1 activity, we adapted a Cdk1 activation assay that had been developed for somatic-cell extracts for use with ES-cell extracts (15). This system, which recapitulates the physiological activation of Cdk1, revealed the key similarities between somatic and ES cells in regard to the biochemical regulation of Cdk1. After a 20-min lag, cyclin B (2,000 nM) activated Cdk1 (Fig. S2*A*), and that activity was cyclin B concentration-dependent, as assessed by phosphorylation of histone H1, Wee1, and Securin (Fig. S2 *B* and *C*). Similar to the somatic-cell extract system, low levels of Cdk1 activity were sustained by low concentrations of cyclin B whereas a sharp increase of Cdk1 activity was achieved when cyclin B rose above a threshold concentration (Fig. S2*B*), suggesting that ES cells have functional WEE1 and CDC25 feedback loops (15).

To determine an effect of Oct4 on Cdk1 activation, Oct4 was immunodepleted from ES-cell extracts (Fig. 2*A*). We focused on doses of cyclin B that produced complexes of cyclin B–Cdk1 at concentrations that were equal to or less than the amount of Oct4 in the cell extracts, with the rationale that any Oct4 effect would be more difficult to detect if it were at a much lower concentration than cyclin B–Cdk1. The Oct4-depleted extracts

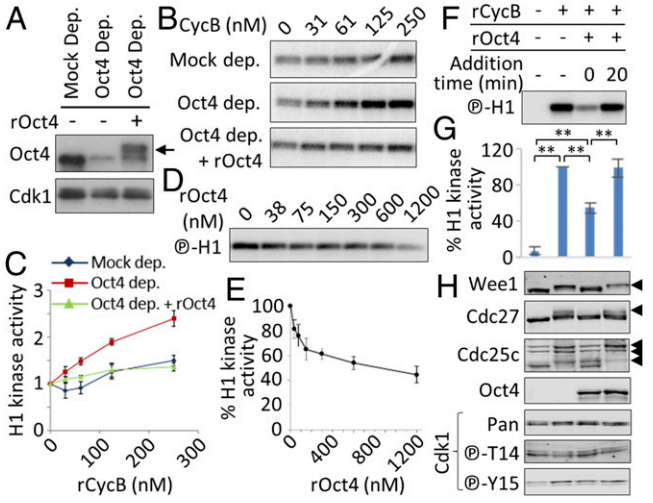

Fig. 2. Oct4 inhibits Cdk1 activation in a cell extract-based kinase assay. (*A*) Representative immunoblots for Oct4 (*Top*) and Cdk1 (*Bottom*) after Oct4 immunodepletion and recombinant His_6-Oct4 add-back. Arrow, His_6-Oct4. (*B*) Representative autoradiogram of Cdk1 kinase assay in ES-cell extracts by adding increasing amounts of cyclin B after mock depletion, Oct4 depletion, or Oct4 depletion with His_6-Oct4 add-back. (*C*) Quantification of three independent Cdk1 kinase assays in ES-cell extracts. Error bar, SEM. (*D*) Representative autoradiogram of Cdk1 kinase assay in HeLa-cell extracts with 2,000 nM cyclin B by adding increasing amounts of His_6-Oct4. (*E*) Quantification of four independent Cdk1 kinase assays in HeLa-cell extracts. Error bar, SEM. (*F*) Representative autoradiogram of Cdk1 kinase assay in HeLa-cell extracts with 2,000 nM cyclin B and 2,000 nM Oct4. Oct4 was added with cyclin B or 20 min later. (*G*) Quantification of *F* (n = 3–6). Error bar, SEM. (*H*) Representative immunoblots for indicated proteins during the experiment shown in *F*. Arrowheads, phosphorylated proteins.

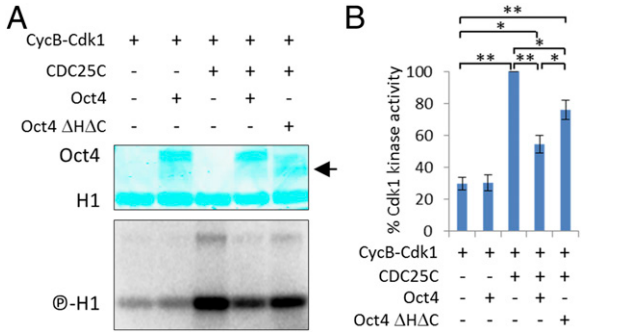

Fig. 3. Oct4 inhibits Cdk1 activation in a recombinant protein-only Cdk1 kinase assay. (*A*) Coomassie-stained gel (*Top*) and autoradiogram (*Bottom*) of a representative cyclin B–Cdk1 kinase assay. The amounts of cyclin B–Cdk1 and CDC25C were too low for detection by Coomassie stain. Arrow represents position of mutant Oct4. (*B*) Quantification of nine independent kinase assays. Error bar, SEM; $*P < 0.05$; $**P < 0.01$.

exhibited significantly higher Cdk1 activities at cyclin B levels above 31 nM compared with mock-depleted controls. Addition of physiological levels of recombinant Oct4 to Oct4-depleted extracts reduced Cdk1 activity, demonstrating that Oct4 is the factor mediating this effect (Fig. 2 *B* and *C* and Fig. S2*D*).

To determine whether other ES cell-specific factors are necessary for the Cdk1 inhibition, we assayed Cdk1 activation by adding increasing amount of recombinant Oct4 (0–1,200 nM) to HeLa cell extracts, which lack other ES cell-specific factors. A mitotic amount of cyclin B (2,000 nM) was added to activate Cdk1. Without any additional ES cell-specific factors, Oct4 was able to inhibit Cdk1 activation. The inhibitory Oct4 effect showed saturation ≥300 nM by inhibiting Cdk1 activation by ∼55% (Fig. 2 *D* and *E*). Interestingly, 300 nM is similar to the endogenous Oct4 level in ES cells, which is 11.2 ± 3.5 ng/μL (SEM) or 280 ± 88 nM (at 2.1 pL by Coulter Counter) (Fig. S2*E*). Indeed, because Oct4 is nuclear localized and because of excluded volume effects inside the cell, its concentration could be effectively higher. Although the cell-extract system may not be quantitatively identical to cells, the cellular concentration of Oct4 makes plausible a mechanism whereby it binds to and inhibits activation of Cdk1.

These results indicated that Oct4 could either directly, but partially, inhibit Cdk1, or that it could block CDC25-mediated Cdk1 activation, which, based on previous experiments (15), would also give a quantitatively similar result to the plateau we observed in Fig. 2 *D* and *E*. At mitotic entry, CDC25 activity converts phosphorylated cyclin–Cdk1 complexes into active Cdk1 kinase. If Oct4 were inhibiting this step, it would indicate that it was blocking the activation of Cdk1. To test whether Oct4 was specifically inhibiting Cdk1 activation, we performed an order-of-addition experiment in which Oct4 was either added simultaneously with cyclin B (at an equimolar concentration) or was added 20 min after cyclin B, allowing for the activation of Cdk1 before Oct4 addition. As expected, Oct4 significantly reduced Cdk1 activity when it was added simultaneously with the cyclin B, but, when Oct4 was added after Cdk1 activation, no inhibition of Cdk1 activity was observed as measured by H1 kinase assay or phosphorylation of endogenous Cdk1 substrates in the cell extracts (Fig. 2 *F*–*H*). The addition of Oct4 to the extracts led to a small change in the mobility of Cdk1 and possibly a small increase in phosphorylation at the inhibitory Thr14 site. Unexpectedly however, the adjacent Tyr15 site seemed to show slightly reduced phosphorylation in the presence of Oct4. Although the Tyr15 result was surprising, these findings do indicate a specific Oct4 effect on Cdk1 activation.

To develop this model further, we developed a kinase assay using only purified proteins. We generated the phosphorylated, but activatable, cyclin B–Cdk1 complex, which exhibited a basal activity toward purified histone H1 but not Oct4, indicating that Oct4 is not a Cdk1 substrate (Fig. 3*A* and Fig. S3*A*). Addition of Oct4 alone did not inhibit basal Cdk1 activity, which is consistent with a model whereby Oct4 does not directly inhibit Cdk1 activity and indicates that Oct4's action is mechanistically different from CKIs such as p27. Addition of recombinant CDC25 activated cyclin B–Cdk1 ∼3.5-fold, whereas addition of both CDC25C and Oct4 together reduced activation by 50% compared to when CDC25C was added alone or together with a nonspecific protein such as BSA (Fig. 3 *A* and *B* and Fig. S3 *B* and *C*). These data demonstrated that Oct4 inhibits activation of Cdk1 by counteracting CDC25 function rather than directly inhibiting Cdk1. An Oct4 mutant lacking the Homeo and C-terminal domains (ΔHΔC) that only weakly binds to cyclin–Cdk1 (Fig. S1 *K* and *L*) exhibited attenuated inhibition on the CDC25-dependent activation (Fig. 3*A* and Fig. S3*C*), indicating that binding of Oct4 to the kinase could be important in inhibiting its activation.

Ectopic Expression of Oct4 Delays Mitotic Entry. The kinase assay data indicated an inhibitory role for Oct4 in Cdk1 activation that, consequently, could delay mitotic entry in cells expressing Oct4. To test this hypothesis, we developed a system to study mitotic entry in intact cells (Fig. 4*A*) using GFP-PCNA (proliferating cell nuclear antigen) (40) and histone H2B-RFP fusion proteins (41). HeLa cells were used initially because their mitotic regulation is well understood, and importantly, they lack endogenous Oct4 expression. The duration of G2 phase was measured by the dispersal of GFP-PCNA foci at the end of S phase and the condensation of H2B-RFP marked chromatin at the start of M phase (Fig. 4 *B*–*D*). To mimic ES cells, human Oct4 was expressed at levels comparable with human ES cells by retroviral transduction of HeLa cells at levels. Nondegradable cyclin A was

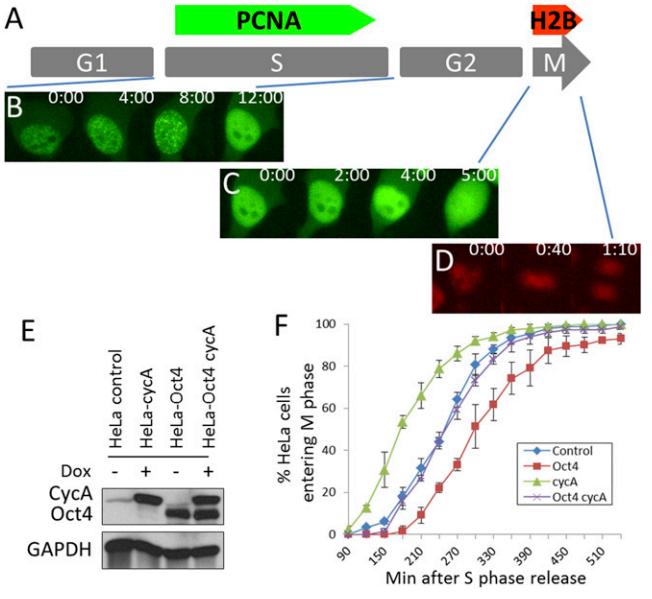

Fig. 4. Ectopic Oct4 expression in HeLa cells delays mitotic entry. (*A*) Schematic of the GFP-PCNA and H2B-RFP dual color system. (*B*) S-phase progression monitored by the GFP-PCNA reporter. (*C*) G2 phase is indirectly monitored by dispersal of the GFP-PCNA foci and condensation of H2B-RFP marked chromosomes. (*D*) M-phase progression monitored by the H2B-RFP reporter. (*E*) Immunoblots on HeLa control, HeLa cells expressing cyclin A (HeLa-cycA), Oct4 (HeLa-Oct4), and both (HeLa-Oct4 cycA) with indicated antibodies. (*F*) Timing of mitotic entry of HeLa control, HeLa-Oct4, HeLa-cycA, and HeLa-Oct4 cycA cells. These data represent three independent experiments (50 cells for each cell line counted per experiment). Error bar, SEM.

expressed under the control of a Tet-On promoter after retroviral transduction (Fig. 4*E* and Fig. S4*A*) to reproduce the high levels of mitotic cyclins in ES cells. We found that G2 phase was completed by 50% of control HeLa cells in 250 min but was elongated to 310 min in HeLa cells expressing Oct4 (Fig. 4*F*). As expected, ectopic cyclin A shortened G2 phase in HeLa cells (17); here, G2 phase was completed in 155 min by 50% of HeLa cells expressing cyclin A whereas it was effectively restored to a normal length (245 min) when Oct4 was coexpressed (Fig. 4*F*). These data suggest that Oct4-mediated Cdk1 inhibition offsets the shortening of G2 phase and premature mitotic entry caused by elevated cyclin A expression. Oct4 expression did not alter the durations of other phases of the cell cycle (Fig. S4 *B–E*). Importantly, although Oct4 expression could rescue G2-phase shortening caused by cyclin A overexpression, the S-phase shortening caused by cyclin A was not rescued (Fig. S4*E*), which is likely explained by cyclin A promoting S-phase progression through Cdk2, which is not a binding partner of Oct4.

Oct4 Delay of Mitotic Entry Is Transcription-Independent. The kinase assay data demonstrated that Oct4 inhibits the activation of Cdk1 biochemically. To test whether the transcriptional function of Oct4 played a role in delaying mitotic entry in intact cells, we introduced a previously described transcriptionally inactive mouse Oct4 mutant into HeLa cells using retroviral transduction (42). We confirmed that this mutant was expressed at similar levels to that in mouse ES cells, nuclear localized and associated with Cdk1 (Fig. 5*A* and Fig. S5 *A–C*).

This Oct4 mutant failed to activate transcriptional targets and was unable to reprogram somatic cells into iPSCs (Fig. 5*B*, Fig. S5*D*, and Table S2). Additionally, microarray analysis on HeLa control cells and HeLa cells expressing the mutant Oct4 revealed that the mutant did not alter the global expression profile, as evaluated by the Pearson's correlation coefficient (r^2) and hierarchical clustering analysis (Fig. 5*C* and Fig. S5 *E* and *F*). When we performed time-lapse live-cell imaging, we found that, similar to cells expressing WT Oct4, HeLa cells expressing the transcriptionally inactive Oct4 had a significantly longer G2 relative to control cells (Fig. 5*D*), demonstrating that Oct4 delays mitotic entry in a nontranscriptional manner.

Interestingly, we found that WT Oct4, like the transcriptionally inactive version, also did not effect global gene expression in HeLa cells (Fig. 5*C* and Fig. S5 *E* and *F*), possibly because HeLa cells lack essential transcriptional coactivators of Oct4 (43). These results make it unlikely that any cell cycle effect in HeLa cells expressing Oct4 is caused by transcriptional alteration of downstream targets and further strengthen the conclusion that Oct4 can affect mitotic entry through a mechanism independent of transcription.

Oct4 Promotes G2 Duration in ES Cells. These results indicated that Oct4 inhibits Cdk1 activation and ensures normal G2 progression, which possibly serves to counteract the high cyclin environment of ES cells. Consequently, we expected to observe a shortened G2 phase after Oct4 down-regulation in ES cells. However, testing this idea is difficult because Oct4 down-regulation causes large changes in gene expression, leading to ES cell differentiation and a consequent slowing of cell cycle progression and G2-phase elongation (23, 24). To distinguish a direct molecular effect for Oct4 on mitotic entry through blocking Cdk1 activation from an indirect effect mediated through changes in gene expression that lead to differentiation, we analyzed cells during the first cell cycle immediately after Oct4 down-regulation but before significant effects on differentiation. To achieve rapid Oct4 down-regulation, we used ZHBTc4 ES cells, in which endogenous Oct4 has been deleted and Oct4 is expressed from a Tet-Off promoter (44). An 80% decrease of Oct4 protein in ZHBTc4 cells could be achieved after 12 h of doxycycline treatment (Fig. 6 *A–C*). To eliminate differentiated cells from

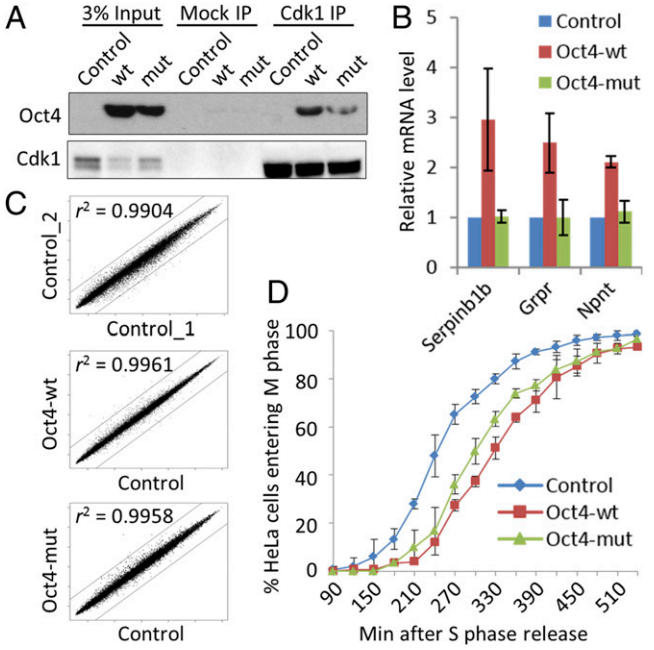

Fig. 5. Oct4-mediated mitotic entry delay is transcription-independent. (*A*) Coprecipitation of Oct4 from HeLa cells expressing native Oct4 (wt) or a transcriptionally inactive mutant (mut) with Cdk1 antibody. Shown are immunoblots of indicated proteins. Mock IP was performed using total IgG. (*B*) Q-PCR analyses on Oct4 target genes in mouse embryonic fibroblasts (MEFs), MEFs expressing the native Oct4 (Oct4-wt) and the mutant Oct4 (Oct4-mut). Genes presented were induced reproducibly by Oct4-wt in three experiments. (*C*) Genome-wide expression profiling on HeLa control, HeLa Oct4-wt, and HeLa Oct4-mut cells. Presented are the Pearson's correlation coefficient (r^2) between two biological replicates of HeLa control cells (*Top*), HeLa control and HeLa Oct4-wt cells (*Middle*), and HeLa control and HeLa Oct4-mut cells (*Bottom*). (*D*) Timing of mitotic entry of HeLa control, HeLa Oct4-wt, and HeLa Oct4-mut cells. These data represent three independent experiments (50 cells for each cell line counted per experiment). Error bar, SEM.

the analyses, we excluded cells negative for Nanog (Fig. 6*C*) and expressing the lowest level of Oct4 (10–20% of total Nanog-positive cells), which were differentiated and contained a significantly higher percentage of G1-phase cells (Fig. S6*A*). We also excluded cells with the highest Oct4 expression (20–30% of total Nanog-positive cells), which seemed to be unaffected by the doxycycline, although including these cells would not have altered the analysis. When we analyzed the remaining cells, which were divided equally into three populations based on the intracellular Oct4 levels ($Oct4^{high}$, $Oct4^{medium}$, and $Oct4^{low}$), we observed an increase in the percentage of cells in G2 in an Oct4 dose-dependent manner in all four experiments (Fig. 6*C*). The G1 phase is known to elongate more significantly than other phases when ES cells differentiate (23, 24), as shown by Fig. S6*A*. However, the proportion of G1 phase cells remains similar among these three populations (Fig. 6*C*); therefore, the trend of G2 cells cannot be explained by differentiation-induced cell cycle redistribution. We confirmed that Oct4 was expressed at constant levels throughout the cell cycle (Fig. 6*D*); thus, our observation was not because Oct4 was more abundant in G2 phase. Furthermore, we found a similar shortening of the G2 phase when Oct4 is down-regulated in human ES cells (Fig. S6 *B–D*), suggesting that the mitotic regulatory function of Oct4 is conserved. These results indicate that Oct4 extends G2 in ES cells. Whether the transcriptionally inactive Oct4 mutant has a similar effect on G2 phase in ES cells is difficult to test because the mutant acts as a dominant negative transcription factor, and ES cells expressing the mutant cannot be made (42).

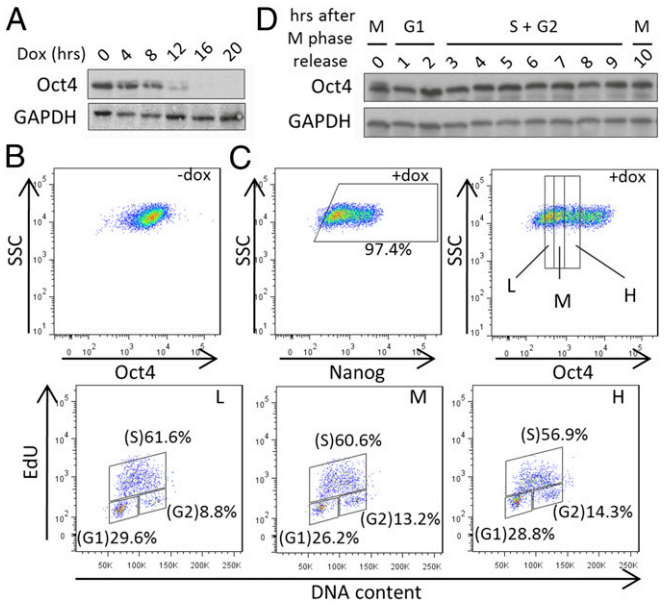

Fig. 6. Oct4 elongates G2 phase in ES cells. (*A*) Oct4 down-regulation in ZHBTc4 cells. Samples were collected at indicated time points with 10 μg/mL doxycycline treatment. Shown are immunoblots for indicated proteins. (*B*) FACS analysis of Oct4 expression level in ES cells. (*C*) FACS analysis of Oct4 down-regulated ES cells. ZHBTc4 cells treated with doxycycline for 12 h were stained for EdU incorporation and Oct4, Nanog, and DNA content. The cells were gated by Oct4 level into $Oct4^{high}$, $Oct4^{medium}$, and $Oct4^{low}$ populations (~20% each of total Nanog-expressing cells). Cells negative for Nanog and expressing very low levels of Oct4 were excluded from the analysis. (*D*) Oct4 levels after release of ES cells from M-phase arrest. Shown are immunoblots for indicated proteins.

Oct4 Helps Maintain Survival and Genomic Integrity of ES Cells. Because premature mitotic entry has been associated with chromosomal abnormalities and apoptosis (17, 45–47), we hypothesized that Oct4 plays a role in maintaining genomic integrity of ES cells. We performed a cytokinesis-block micronucleus assay, in which micronuclei represent broken chromosomes or chromosomes that fail to travel to either daughter during mitosis (48), to evaluate chromosomal missegregation and genomic stability (Fig. 7*A*). We observed a twofold increase of micronuclei during the first cell division after Oct4 down-regulation compared with the control ZHBTc4 cells (Fig. 7*B*). We also observed a twofold increase of Annexin V-positive cells upon Oct4 down-regulation (Fig. 7*C* and Fig. S7), as previously reported (49). Interestingly, treating the cells with the Cdk1 inhibitor RO-3306 significantly reduced apoptosis, indicating that Cdk1 activity contributes to the increased apoptosis (Fig. 7*C* and Fig. S7). Together, these data demonstrated that Oct4 plays a role in maintaining genomic integrity, which would otherwise be at risk due to accelerated progression through M phase.

Discussion

ES cells and non-ICM–derived pluripotent cells, such as iPS cells, undergo rapid cell cycle progression whereas differentiation of pluripotent cells is accompanied by a slower cell cycle, suggesting a connection between pluripotency and an abbreviated cell cycle (23, 24, 50). Here, we present the unexpected finding that Oct4, a transcription factor that is required to maintain pluripotency, directly inhibits the activation of Cdk1 and thereby regulates mitotic entry.

The rapid cell cycle progression of ES cells, which is characterized by reduced oscillation of cyclins and Cdk activities, inactivation of Rb, and sustained E2F transcriptional activity (3, 4, 21, 22, 24), comes with potential risks. Loss of *Rb* is known to accelerate G1-to-S phase transition and lead to mitotic defects (25–28). Additionally, premature or constitutive activation of Cdk1, through increased cyclin A or B levels or the loss of *Wee1*, can lead to genomic instability, including cell death and chromosomal abnormality (17, 45, 46, 51). Because ES cells, like the epiblast cells in embryos, can give rise to all mature tissues and cell types, an increase in genomic instability could have deleterious repercussions throughout the whole organism or lead to death of the embryo. We reasoned that, in the setting of their unique cell cycle regulation, ES cells must have a mechanism to prevent premature Cdk1 activation and the associated risks of early mitotic entry. We believe that the nontranscriptional Oct4-mediated Cdk1 inhibition serves as a mechanism to mitigate the risk of premature mitotic entry in the setting of increased activity and dampened oscillation of Cdks (Fig. 7*D*) that occur as a result of the transcriptional function of Oct4. As Oct4 expression decreases upon differentiation, so does the need for additional Cdk1 inhibition, because the typical somatic pattern of cyclin oscillations and associated kinase activity are restored. Therefore, the Oct4-mediated Cdk1 inhibition is an ES cell-specific mechanism to solve an ES cell-specific problem.

The cyclin B–Cdk1 complex shuttles between the nucleus and cytoplasm. As a nuclear protein, Oct4 might inhibit Cdk1 in the nucleus, similar to Wee1, another important, yet nuclear-localized Cdk1 inhibitor. The nuclear-localized Wee1 kinase phosphorylates and inactivates any nuclear Cdk1, which is then returned to cytoplasm (11). As ES cells express high levels of interphase cyclin A, which is nuclear-localized and activates cyclin B–Cdk1, an additional inhibitory mechanism of nuclear cyclin B–Cdk1 by Oct4 might be a specific requirement of ES cells.

Our data suggest that Oct4 regulates mitotic entry by inhibiting Cdk1 activation, possibly by interfering with CDC25. That Oct4 targets this step is perhaps not surprising because it resembles other means through which the cell cycle is regulated: e.g., the DNA damage checkpoint inhibits the activation of Cdks by regulating the CDC25 proteins. How ES cells enter mitosis in the presence of Oct4 remains unclear. Unlike p27 and many

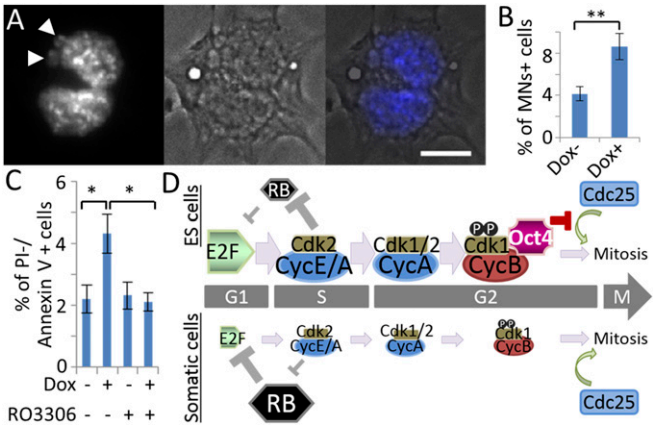

Fig. 7. Oct4 helps maintain genomic integrity. (*A*) A representative image of micronuclei observed in the cytokinesis-block micronucleus assay in ZHBTc4 ES cells. Arrowheads, micronuclei enclosed in a telophase-arrested binuclei cell. (*Left*) DAPI staining; (*Middle*) bright field; (*Right*) merged. (Scale bar: 10 μm.) (*B*) Frequencies of micronuclei in control (dox−) and ZHBTc4 cells treated with doxycycline for 16 h (dox+) from four experiments. Error bar, SEM; two-tailed *t* test, $^{**}P < 0.01$. (*C*) Percentage of Annexin-positive ZHBTc4 cells treated with doxycycline and DMSO or 9 μM RO-3306 for 16 h from five experiments. Error bar, SEM; $^{*}P < 0.05$. (*D*) A model of Oct4 in the regulation of ES cell mitotic entry. (*Top*) In ES cells, a feedback loop due to inactivation of Rb and high levels of cyclins and Cdk activities creates the potential risk for premature mitotic entry. Oct4 serves as an additional, ES cell-specific mechanism to inhibit Cdk1 activation by counteracting CDC25 function and ensure timely mitotic entry. (*Bottom*) In somatic cells, expression of cyclins and activities of Cdk and Rb are regulated by the cell cycle.

other cell cycle inhibitors that are degraded to allow for cell cycle stage transitions, Oct4 remains constant to maintain ES cells undifferentiated. Therefore, Cdk1 must become activated in the presence of Oct4. Most likely, Oct4 is present at a lower level than are cyclin–Cdk1 complexes needed for mitosis; thus, when mitotic cyclins accumulate at mitotic entry, the threshold of Oct4 inhibition is passed. In this way, Oct4 would prevent interphase concentrations of mitotic cyclins from inducing mitosis, but not the mitotic concentrations. The apparent weak affinity of Oct4 for cyclin–Cdk1, which might become even weaker in mitosis (Fig. S1*E*), may also favor the appearance of the uninhibited cyclin–Cdk1 complexes to permit mitosis.

Oct4 is arguably at the very top of the hierarchy of pluripotency regulators, yet how Oct4 maintains pluripotency is only partially understood. Our data reveal that, in addition to its role as a transcriptional regulator, Oct4 helps control the risk of premature mitotic entry caused by high levels of cyclins and high activities of interphase Cdks found in ES cells. As such, our findings reveal a critical role for Oct4 in coupling pluripotency to the unique cell cycle regulation and genome protection of ES cells.

Materials and Methods

The cell-extract kinase assay was performed as described with minor modification (15). For the purified kinase assays, purified His_6-cyclin B was bound to Ni-NTA beads and incubated in cell extracts to generate bound, phosphorylated Cdk1. The cyclin B–Cdk1 was reisolated and incubated with histone H1, Oct4, or His_6-CDC25C with $ATP\gamma P^{32}$. Detailed information is provided in *SI Materials and Methods*.

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