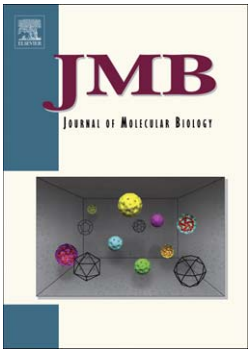

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Inhibition of cell division and DNA replication impair mouse naïve pluripotency exit

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Abstract

The cell cycle has gained attention as a key determinant for cell fate decisions, but the contribution of DNA replication and mitosis in stem cell differentiation has not been extensively studied. To understand if these processes act as 'windows of opportunity' for changes in cell identity, we established synchronized cultures of mouse embryonic stem cells (mESC) as they exit the ground state of pluripotency. We show that initial transcriptional changes in this transition do not require passage through mitosis, and that conversion to primed pluripotency is linked to lineage priming in the G1 phase. Importantly, we demonstrate that impairment of DNA replication severely blocks transcriptional switch to primed pluripotency, even in the absence of p53 activity induced by the DNA damage response. Our data suggest an important role for DNA replication during mESC differentiation, which could shed light on why pluripotent cells are only receptive to differentiation signals during G1, that is, before the S-phase.

Introduction

During embryonic development, cells present distinct transcriptional profiles as they commit to the different lineages of the organism. This conversion is associated with global changes in the structure of chromatin, DNA methylation and the configuration of nuclear architecture [1]. Although there is overwhelming evidence about the epigenetic and transcriptional differences between distinct cellular states, a general mechanism that explains how these transitions are orchestrated remains to be established.

In recent years, a few intriguing hypotheses have linked the changes in the epigenetic states to two major events related to the structure of DNA: cell division and DNA replication. During mitosis gene expression is globally arrested and many transcriptional regulators are ejected from chromatin [2]. M to G1 phase transition is characterized by chromosome decondensation and major changes in the 3D positioning of chromosomes, allowing for the reconfiguration of the nuclear architecture [3]. Additionally, during DNA replication, chromatin is disrupted ahead of the replication fork and must be later reestablished onto newly synthesized DNA [4]. Although the mechanisms of epigenetic inheritance between parental and daughter DNA strands are still poorly understood, it is clear that the replication machinery plays a pivotal role [5,6]. Indeed, many epigenetic modifiers associate with replication forks, and maintenance of DNA methylation patterns and passive demethylation are tightly coupled to DNA synthesis [7,8]. For these reasons, cell division and DNA replication could provide ´windows of opportunity´ to change the epigenetic and transcriptional landscape of cells during differentiation [9,10]. This connection, however, has not been extensively studied in the context of pluripotent stem cell differentiation.

In this scenario, mouse embryonic stem cells (mESCs) provide an excellent *in vitro* model to study transcriptional and epigenetic transitions. They are derived from embryos at the blastocyst stage and are transcriptionally similar to cells of the preimplantation epiblast when cultured in ground state conditions [11]. They can give rise to all the cell lineages of the organism, including the germline, ability that has been termed ‘naïve’ pluripotency. Importantly, in vitro differentiation of mESCs recapitulates early embryonic development [12], and they can be induced to differentiate to cells in an early ‘primed’ state of pluripotency similar to the post-implantation epiblast.

Here, we sought to determine the possible contribution of cell division and DNA replication to stem cell differentiation. By generating synchronized cultures of mESCs as they exit the

ground state of pluripotency, we demonstrate that changes in gene expression begin in the same generation that receives the differentiation signals and that inhibition of cell division affects the transcriptional change of several important markers. Consistent with recent observations for other cell transitions [13,14], we then show that conversion from naïve to early primed pluripotency is associated to lineage priming in the G1 phase. Our results also reveal that inhibition of DNA synthesis severely impairs the exit from the naïve pluripotent ground state, independently of p53 activity induced by DNA damage pathways. This renders DNA replication as an important process that could allow transcriptional change of critical genes during differentiation.

Results

Mouse embryonic stem cells were routinely cultured in the defined medium N2B27 in the presence of 2i and LIF (2i+LIF medium). In these conditions, they form homogeneous populations in the naïve ground state of pluripotency, since they resemble the pre-implantation epiblast more closely than when cultured in FBS containing medium [11]. As mESCs exit naïve pluripotency, they first transit through a primitive ectoderm-like state before adopting somatic cell fates [15,16]. This transition, in which cells acquire a post-implantation epiblast transcriptional signature, has been well documented in the initial steps of neural induction in adherent monoculture [15,17,18], and generates a primitive ectoderm-like cell (PELC) population that is transcriptionally similar to the recently described epiblast-like cells (EpiLCs) [19]. We thus adopted this protocol to analyze the conversion from naïve to primed pluripotency. We induced the differentiation of mESCs to PELCs by plating them in N2B27 without 2i and LIF (Diff medium). Differentiating cells rapidly displayed a morphological transformation, characterized by reduced cell-to-cell interactions, increased cytoplasm size and cell protrusions (Fig. 1A). Gene expression

analysis at 24 and 48 h of differentiation confirmed the downregulation of naïve pluripotency markers and upregulation of post-implantation epiblast markers (Fig. 1B). Nanog protein levels rapidly decreased during PELC induction, while Oct4 remained expressed (Fig. 1C). In addition, most of the cells expressed the post-implantation epiblast marker Otx2 by 48 h, demonstrating the high efficiency of this transition.

It has recently been shown that individual PSCs activate developmental pathways in an asynchronous manner, and there is increasing evidence indicating that this is a consequence of cell fate decisions being made exclusively during the G1 phase of the cell cycle [13,14]. Since we intended to analyze gene expression patterns in whole populations, it was important that all cells received the differentiation signals in the G1 phase. For this reason, we devised a strategy to generate synchronized cultures of mESCs by taking advantage of the Fucci cell cycle reporter system (Fig. 1D), which allows to FACS sort cells at specific phases of the cell cycle due to the differential expression of hCdt1-mCherry and hGeminin-mVenus [20]. Thus, we established a cell line expressing the Fucci fluorescent reporters together with an H2B nuclear marker fused to the mCerulean fluorescent protein (Fig. 1E). This cell line, which presented a normal cell cycle distribution and the expected fluorescence patterns for the Fucci and H2B reporters (Fig. 1F and G), allowed us to generate synchronized cultures by sorting the recently divided cells in early G1, which do not express any of the two fluorescent proteins (Fig. 1H). We sorted early G1 cells directly into 2i+LIF or Diff medium and confirmed that they could progress through the cell cycle (Fig. 1H, Fig. S1A). By taking advantage of the H2B reporter, we determined the degree of synchronization in both conditions by tracking single cell divisions using time-lapse experiments immediately after sorting (Fig. 1I). Analysis of the time distribution of cell divisions enabled us to build the frequency distribution of cells in each generation along time (Fig. 1I). Both undifferentiated and differentiating cells could be efficiently synchronized, with

some expected overlap due to the variance in the total cell cycle length. Importantly, the vast majority of the sorted cells remained in the first cell generation for the subsequent 10 hours after sorting, with only 0.54% of differentiating cells dividing before this time (dashed line in Fig. 1I). Of note, cells that were immediately sorted into 2i+LIF medium displayed a cell cycle length slightly longer than the reported for mESCs. This effect, probably a consequence of the stress of experimental manipulations -cell dissociation, maintenance in ice and the cell sorting itself- was reverted in the second cell generation, which exhibited a mean cell cycle length of 15 h (Fig. S2). As we will show in the next section, the sorting conditions did not alter the maintenance of the naïve ground state of pluripotency when cultured in 2i+LIF medium, thus providing an appropriate synchronization method for analyzing population-wide gene expression changes.

Cell division is not fundamental for initial transcriptional changes as mESCs exit the naïve pluripotent state

Cell division has been proposed as a stage in which cells could drastically alter their transcriptional patterns during cell identity transitions [21–23]. To study this, we evaluated the transcriptional dynamics of mESCs in the Fucci synchronized cultures when cells were maintained in 2i+LIF medium (control) or as they transitioned to the early primed state of pluripotency (Fig. 2A). Considering that sorted early G1 cells did not divide for the first 10 h, we reasoned that we could infer if cell division was fundamental for changes in cell identity by evaluating gene expression patterns with high temporal resolution. In view of the results of Fig. 1H, we sampled cells in both conditions at 0, 5, 10, 18, 23, 30 and 36 h. We first analyzed if cell synchronization by this method affected the maintenance of the naïve pluripotent ground state when cells were cultured in 2i+LIF medium. mESCs sorted and maintained in these conditions preserved the naïve pluripotent status, as evaluated by cell

morphology and stable expression of naïve and primed key pluripotency markers (Fig. 2B). Next, we studied the expression dynamics of the selected genes when early G1 cells were sorted and cultured in Diff medium. As shown in Fig. 2B, most of the evaluated markers significantly changed their expression levels before 10 h, demonstrating that cell division was not necessary for the initial transcriptional changes that occur as cells exit the ground state of pluripotency. An interesting exception to this behavior was the primitive ectoderm marker FGF5, whose expression levels were only significantly upregulated after cells divided. Thus, in the case of this gene, we cannot rule out that mitosis is an important process that modulates its transcriptional dynamics.

To further analyze the relationship between cell division and the transcriptional changes that occur during naïve pluripotency exit, we decided to block the progression to the second cell generation as early G1 cells were set to differentiate. There are several approaches to inhibit cell division, most of which make use of drugs that arrest cells at the metaphase of mitosis. However, as we previously mentioned, during this phase transcription is globally inhibited, so it would not be a proper condition to analyze changes in gene expression. To circumvent this limitation, we evaluated the effect of blocking cell division by RO3306, a potent inhibitor of cyclin-dependent kinase 1 (Cdk1) that arrests cells at the G2-phase of the cell cycle and displays minor pleiotropic effects to other Cdks [24]. Cells cultured for 15 h in the presence of this drug displayed normal interphase nuclei, could enter mitosis within 30 min after change to fresh medium and effectively divided 2 h later (Fig. 3 A-C). We next analyzed the expression patterns of early G1 cells sorted in (i) Diff medium alone or (ii) Diff medium plus RO3306 at 10 h, that is, before cells began to divide (Figure 3D). Addition of this drug effectively blocked cell division, as analyzed by time-lapse microscopy experiments that were performed together with each biological replicate (data not shown). Gene expression analysis after this treatment revealed different effects

on the analyzed genes (Fig. 3E). While Esrrb, Otx2 and Klf4 expression levels were not affected by addition of RO3306, others such as FGF5, Oct6, Dnmt3A and Nanog displayed reduced levels of transcriptional change compared to the control condition. Among these, the largest and most significant effect was observed for the primitive ectoderm markers FGF5 and Dnmt3A and for the naïve marker Nanog. Overall, these results suggest that although cell division was not fundamental to trigger the transcriptional changes associated with naïve pluripotency exit, it could be an important process to regulate the expression levels of key developmental factors, especially for the primitive ectoderm marker FGF5.

Exit from naïve to primed pluripotency is coupled to transition through the G1 phase

As we previously mentioned, it has been shown that embryonic stem cells are 'primed' to initiate cell fate decisions during the G1 phase of the cell cycle. Most of the evidence, however, comes from studies performed in human ES cells (hESCs) [14,25–28], with only one publication in mouse ES cells [13]. mESCs and human ESCs are believed to represent different stages of embryonic development and the maintenance of the pluripotent state is supported by different signaling pathways [29]. Interestingly, although both cell types have a short length of the G1 phase, they also show remarkable differences in the activity of cell cycle regulators, with mESCs not expressing members of the cyclin D family and having a hyper-phosphorylated retinoblastoma protein [30]. For these reasons, more research is needed to describe a general mechanism that accounts for the lineage priming in G1 in both cell types. Importantly, the requirement of cells to start differentiation from the G1 phase has not yet been studied in the transition from the naïve ground state towards

primed pluripotency [31]. Thus, we decided to use our experimental set-up to study this in more detail in a mouse model.

We compared the patterns of gene expression between cells that received the differentiation signals in G1 to those of cells that received it after this phase was completed. To this end, we first calculated the time necessary for early G1 cells sorted and maintained in 2i+LIF medium to transit to the S phase, by analyzing time-lapse movies performed right after the sorting of cells. By analyzing the Fucci fluorescence dynamics in individual cells (Fig. 4A), we observed that 5 h after sorting more than 80% of the cells had completed G1 (Fig. 4B). Although in our experimental conditions the length of G1 was slightly longer than the reported for mESCs, we speculate that this might have been a consequence of the sorting conditions. As we observed for the cell cycle length (Fig. S2), this effect was reverted in the second cell generation (data not shown).

With this information, we studied the relationship between differentiation to the primed state and the passage through G1 by comparing gene expression patterns under different culture conditions: (i) early G1 cells sorted and cultured directly in Diff medium (‘Diff in G1’), (ii) sorted and maintained in 2i+LIF medium (‘Undiff’) and (iii) sorted and cultured in 2i+LIF for 5 h and the switched to Diff medium (‘Diff post-G1’) for the rest of the experiment (Fig. 4C). We reasoned that if differentiation signals could effect a response in any phase of the cell cycle, then it would be expected a shift in the patterns of gene expression of 5 h when comparing ‘Diff post-G1’ and ‘Diff in G1’ conditions. On the contrary, if cells were sensitive to differentiation cues exclusively during G1, then it would be expected a shift of at least the duration of the cell cycle, since the differentiating signals would only be received in the following G1 phase.

Gene expression analysis comparing 'Diff post-G1' and 'Diff in G1' showed that Nanog, Otx2, Esrrb and Dnmt3A displayed a shift significantly longer than 5 h and compatible with the length of the cell cycle for our experimental setup (Fig. 4D). Oct6 also showed a similar pattern, although the expression levels at early time points were higher than the undifferentiated control, probably due to a low percentage of cells that had not finished G1 after media change and were thus still responsive. Strikingly, FGF5 and Klf4 did not display expression changes for up to 48 h after sorting. A possible explanation to this puzzling observation could be that the initial 5 h in which 'Diff post-G1' were cultured in 2i+LIF medium were sufficient for cells to secrete local factors (e.g., specific components of the extracellular matrix) that specifically affected the behavior of these genes during differentiation. However, the overall analysis indicates that the evaluated genes presented a gene expression shift longer than 5 h and, in most cases, compatible with cells receiving the differentiation signal in the following G1 phase. Thus, our results suggest that differentiation from the ground state to primed pluripotency is also subjected to lineage priming in G1, thus broadening the spectrum of cell transitions that respond to differentiation cues in a cell cycle dependent manner.

Inhibition of DNA replication impairs transcriptional change during as mESCs exit naïve pluripotency

Recent reports began to shed light on the molecular pathways involved in lineage priming in G1 [14,25]. However, current mechanistic explanations have been proposed in the context of hESCs that, as we mentioned, bare significant differences to mESCs regarding developmental stage and cyclin Ds regulation, thus limiting the generalization of the results. We reasoned that if DNA replication acts as a 'window of opportunity' that allows a global

epigenetic reconfiguration as cells differentiate, this could explain why PSCs are only receptive to differentiation cues within G1, that is, before the S phase. If this is correct, interrupting DNA synthesis during differentiation should impair transcriptional changes. To evaluate this possibility we inhibited DNA replication with excess thymidine as mESCs exited the ground-state of pluripotency [32]. This treatment effectively blocked DNA synthesis in the Fucci line and was reversible shortly after drug release, as judged by incorporation of the nucleotide analog EdU and propidium iodide staining (Fig. 5 A,B).

To evaluate the effect of inhibiting DNA replication on the transcriptional change we sorted early G1 cells and cultured them either in Diff medium alone (control) or in Diff medium with excess thymidine (Fig. 5C). Surprisingly, gene expression analysis revealed that inhibition of DNA synthesis severely affected the transcriptional change towards primed pluripotency, with the strongest effect being for the naïve pluripotency markers Nanog, Esrrb and Klf4 (Fig. 5D). Indeed, transcriptional change for these genes was almost completely impaired, with expression levels similar to the undifferentiated state (time 0 h). On the other hand, we observed that upregulation of primed markers displayed a milder but significant restriction compared to Diff medium alone. To further validate these results, we blocked DNA synthesis with aphidicolin, an inhibitor of DNA polymerases α, δ and ε, and obtained a similar effect although with minor differences, likely due to pleiotropic effects caused by pharmacological inhibition (Fig. S3). Importantly, when cultures were released from thymidine block at 18 h post-sorting and cell cycle progression was allowed (Fig. 5C), both naïve and primed markers resumed the transcriptional changes to primitive ectoderm-like cells, suggesting a causal connection between DNA replication and transcriptional changes (Fig. 5D). An important consideration is that inhibiting DNA synthesis inevitably blocks cell division. To analyze if the effect we observed under thymidine treatment was in fact a consequence of the repression of cell division and not of DNA synthesis, we com-

pared the transcriptional dynamics of synchronized differentiating cells under thymidine or RO3306 treatments. If this was the case, gene expression dynamics between both treatments should be highly similar, since they would reflect the same underlying process. Comparison of both experimental conditions showed that, for most of the analyzed genes, the effect of blocking DNA synthesis was stronger than that of inhibiting cell division, indicating that the main effect was indeed a consequence of DNA replication (Fig. S4). However, the expression patterns of FGF5 and Dnmt3A under both treatments was strikingly similar, suggesting that these genes may have been actually affected by the impairment of mitosis, in agreement with our previous results (Fig 3E). Overall, our data show that blocking DNA replication as cells exit the naïve ground state towards primed pluripotency prevents them from efficiently differentiating, with the strongest effect observed in the naïve pluripotency markers.

Related to our findings, it has recently been reported that hESCs were impaired to exit the pluripotent state when DNA replication was delayed, but this 'ES maintaining' effect was attributed to the activation of the transcription factor p53 induced by replication fork stalling [33]. In our system, culture with thymidine or aphidicolin for prolonged periods increased the rate of cell mortality (Fig. S5A). These treatments induced the phosphorylation of Chk1 at serine 345 (Fig. S5B), a key effector of the DNA damage response (DDR) [34]. Furthermore, p53 protein levels and its nuclear localization increased upon treatment with both drugs, together with the expression of its transcriptional target Mdm2, indicating DDR pathway activation (Fig. S5C,D).

To analyze if the inhibition of transcriptional change when blocking DNA replication was exclusively explained by activation of p53 and not connected to replication itself, we utilized CRISPR/Cas9 technology to generate a p53-null mESC line (Fig. 6A,B and Fig. S6A). The p53 -/- line presented normal morphology, no changes in cell cycle distribution

and showed no difference in naïve or primed pluripotency markers expression compared to WT cells when cultured in 2i+LIF medium (Fig. S6B, D-E). Importantly, DNA replication was still inhibited in p53 -/- cells after thymidine or aphidicolin treatment (Fig. S6C) and, as expected, these cells displayed reduced levels of cell death compared to WT cells (Fig. S6H,I). Upon induction of differentiation to the primed state, p53 -/- and WT cells displayed similar morphology and expression of naïve pluripotency markers (Fig. S6F-G). However, absence of p53 significantly restricted the upregulation of the primed markers FGF5, Dnmt3A, Oct6 and Otx2 (Fig. S6G), supporting a role for p53 during cell differentiation, and in agreement with a previous report [35].

Direct knockout of p53 using CRISPR in our Fucci cells was inconvenient due to their expression of fluorescent proteins and antibiotic resistance genes, which impaired selection of transfected cells. Thus, for these experiments, we generated synchronized cultures by incubating cells with RO3306, which proved to be a very efficient protocol that did not affect the cell cycle phase lengths nor the maintenance of the pluripotent ground state when cultured in 2i+LIF medium (Fig. S7). Furthermore, inhibition of DNA replication by either excess thymidine or aphidicolin using this alternative synchronization method continued to repress transcriptional change in WT mESCs cultured in Diff medium, supporting our previous findings (see below).

We designed a strategy where synchronized WT or p53 -/- cells were set to differentiate for 28 h in the presence of (i) DMSO (control), (ii) thymidine or (iii) aphidicolin (Fig. 6C). We reasoned that if activation of p53 as a consequence of inhibiting DNA replication could account for the entire effect on the repression of transcriptional change, p53 -/- cells set to differentiate in the presence of inhibitors of DNA replication should differentiate normally, with no differences in gene expression compared to p53 -/- cells grown in DMSO. As shown in Fig. 6D, gene expression analysis of the naïve pluripotency markers revealed a

significant although partial recovery of the transcriptional change in the p53 -/- cells compared to WT cells upon treatment with thymidine or APH. Although these results support and validate a role for p53 in the inhibition of transcriptional change in mESCs, they also indicate that this protein is only responsible for a fraction of the effect, since APH and thymidine treated cells did not reach the same levels of expression that the control cells. Thus, our results indicate that inhibiting DNA replication by two complementary approaches impairs the efficient exit of the ground state of pluripotency even in the absence of p53, suggesting that processes coupled to DNA replication might be necessary to allow the epigenetic and transcriptional reconfiguration that takes place during naïve pluripotency exit.

Discussion

In this work, we have investigated the relationship between the changes in cell identity during naïve pluripotency exit and two processes that are coupled to major epigenetic transformations: cell division and DNA replication. By generating synchronized cultures of mESCs we show that when differentiation signals are provided in the G1 phase, the initial transcriptional changes associated with the exit of the pluripotent ground state begin in the same cell generation, that is, before mitosis. Interestingly, we show that blocking cell division had a significant effect on the expression dynamics of multiple genes, with the highest effect being on FGF5, Dnmt3A and Nanog, while others like Klf4 and Esrrb remained unchanged. From these evidences, we speculate that the epigenetic changes that take place during mitosis could be important for the regulation of specific genes. Our results are in agreement with a recent work by John Gurdon's lab, where they analyzed the reprogramming of mammalian nuclei after transplantation to amphibian oocytes [36]. The authors concluded that mitosis is not fundamental, but that it might be a process that enhances

transcriptional change during identity transitions, which they named ‘mitotic advantage’ [22]. In this context, our results provide initial experimental evidences in the field of pluripotent stem cells that are relevant to deciphering the relationship between cell division and transcriptional transitions. Future research in the ‘mitotic bookmarking’ of cells [2], an area of increasing interest, will also be important to determine this relation.

Another aspect of the cell cycle related to cell identity changes is the recent observation that ES cells are only responsive to differentiation signals during the G1 phase, a process termed ‘lineage priming in G1’ [13,14]. Most of the evidence in this regard comes from experiments performed in human ES cells, which bare significant differences with mouse ES cells at multiple levels such as developmental stage, signaling pathways and activity of cell cycle regulators [29]. Importantly, whether the transition from the pluripotent ground state to the primed state is also subjected to lineage priming in G1 had not yet been addressed [31]. By studying gene expression patterns when cells received the differentiation signals in G1 or after this phase was completed, we show that most of the genes analyzed responded in a cell-cycle dependent manner compatible with lineage priming in G1. Although the mechanisms that could account for this behavior in ES cells are not fully resolved, in recent years this has been addressed by several groups in human ES cells. It was shown that cyclin D1, which is expressed exclusively during the G1 phase, regulates the sub-cellular localization of the transcription factors Smad2/3, members of the TGF-β pathway that are critical for the properties of hESCs [14]. Recently, the same authors observed that cyclin D1 also acts as a transcriptional regulator for developmental genes in a cell cycle dependent manner [37]. However, mESCs do not express the D family of cyclins, suggesting that there may be other mechanisms to explain this behavior.

A process within the cell cycle that could also be related to the epigenetic and transcriptional transitions is DNA replication [4,9]. In this work, we hypothesized that if this event

was important to allow the new epigenetic configurations that precede transcriptional transitions, this could contribute to a mechanistic explanation of why pluripotent cells are only responsive to differentiation cues during G1, that is, before the S phase. In that sense, the relationship between the process of DNA replication and changes in cell identity has recently began to be addressed. Tsubouchi and collaborators demonstrated that DNA replication is fundamental for the nuclear reprogramming of somatic cells after cell fusion experiments with mESCs [38]. On the other hand, Wang and collaborators recently showed the importance of DNA synthesis to allow transcriptional transitions, both during nuclear transfer experiments and during the first segmentations of mouse embryos [39]. Despite these recent observations, the relationship between the process of DNA replication and cell differentiation has not yet been thoroughly evaluated in pluripotent stem cells, an experimental system that could contribute to major advances in this field. Here, we addressed this subject by analyzing the effect of blocking DNA replication on the transcriptional dynamics of synchronized cells transiting to the early primed state of pluripotency. We show that blocking DNA replication by different treatments severely impaired expression changes of several key developmental genes, of which the naïve markers displayed the strongest effect. Our results are also in agreement with recent observations in hESCs, which indicate that the transcription factor p53 is implicated in this process. However, by generating a p53 knock-out mESCs line we show that this protein is only responsible of a fraction of the effect, suggesting a possible role for processes coupled to DNA replication. In this context, a recent report has also shown that the bivalent marks on chromatin deposited at the promoters of multiple developmental genes are only present during the G1 phase, and that they are resolved upon transit to the S phase [40]. Thus, it would be interesting to analyze if DNA replication is connected to this fast epigenetic transformation.

In summary, in this work we contribute with experimental evidence that links the changes in cell identity with the processes of cell division and DNA replication during the exit of the naïve ground state of pluripotency. Although further validation of our results in ESCs derived from other mouse strains as well as from different species will be important, we believe our work constitute a first and informative approach to this issue, in a context where more experimental evidence is needed in the field. In that sense, it would also be important to determine the role of the factors that remain associated to chromatin during mitosis and, on the other hand, to analyze the epigenetic marks present on chromatin before and after DNA replication during cell differentiation. Although this type of studies are greatly limited by technical aspects, we believe that the application and development of new technologies, such as CRISPR and the ones derived from high throughput sequencing will be critical to understand the role of the cell cycle in cell differentiation.

Materials and methods

Full methods are available on the Supplemental Data

Cell lines, culture of mESCs and differentiation to PELCs

W4 mESCs were provided by the Rockefeller University Core Facility. Cells were cultured using the chemically defined medium N2B27 with 1000 U/ml LIF (Millipore), 1 μM PD0325901 (Tocris) and 3 μM CHIR99021 (Tocris), hereafter called '2i+LIF medium'. N2B27 formulation is described elsewhere [41]. Cells were maintained on 0,1% gelatin coated dishes, passaged every three days using TrypLE (Gibco) and grown at 37°C in a 5% CO2 (v/v) incubator. To induce PELC differentiation, mESCs were first cultured in 2i+LIF medium for 24 h at high density (1 x 10^5 cells/cm^2), then plated at 1 x 10^4 cells/cm^2 onto gelatin coated dishes in N2B27 without LIF and 2i (Diff medium) and grown for 24 -

48 h. Construction of W4 Fucci-H2B and p53 -/- ES cells is described in the Supplemental Methods.

Cell Synchronization and Live Imaging

Synchronization procedures are explained in detail in the Supplemental Methods. Briefly, Fucci early G1 mESCs (hCdt1-mCherry/mVenus-hGeminin double negative population) were sorted in a BD FACSAria II flow cytometer (BD Biosciences) and cultured in 2i+LIF or Diff media. Alternatively, we synchronized cells using 6 μM RO3306 (Sigma) for 15 h and then washing the cells with fresh medium. For time-lapse experiments, cells were plated in μ-Dish 35 mm dishes (IBIDI) and imaged in a LCV110 VivaView Incubator Microscope (Olympus).

Inhibition of DNA replication

DNA synthesis was inhibited with 1.25 mM thymidine (Sigma) or with 0.5 μg/ml aphidicolin (Sigma). Replication analysis was performed by Click-it EdU incorporation kit (Thermo Fisher Scientific) according to manufacturer's instructions.

Gene expression analysis

Total RNA was extracted with Trizol (Thermo Fisher Scientific) following manufacturer's instructions and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR was performed in a LightCycler 480 Real Time PCR system. Gene expression was normalized to the geometrical mean of GAPDH and PGK1 house-keeping values. Primers are listed in the Supplementary Methods.

Statistical Analysis

Statistical significance was analyzed using either paired Student's t-test or randomized block design (RBD) ANOVA. Comparisons between means were assessed using the Tukey test.

Author Contributions

A.W. and A.G. designed and A.W. performed most of the experiments. C.V.E., C.S., M.S.C. and I.M. performed some of the experiments. A.D. constructed the Fucci-H2B line and M.Z.O. and A.R.M. constructed the Fucci and CRISPR plasmids, respectively. S.M. and A.B. provided equipment and reagents and, together with L.B., participated with helpful discussions and ideas. A.W. and A.G. wrote the manuscript.

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References

[1] M. Li, G.-H. Liu, J.C. Izpisua Belmonte, Navigating the epigenetic landscape of pluripotent stem cells., Nat. Rev. Mol. Cell Biol. 13 (2012) 524–35.

doi:10.1038/nrm3393.

[2] S. Kadauke, G. a Blobel, Mitotic bookmarking by transcription factors., Epigenetics Chromatin. 6 (2013) 6. doi:10.1186/1756-8935-6-6.

[3] T. Cremer, V. Zakhartchenko, Nuclear architecture in developmental biology and cell specialisation, Reprod. Fertil. Dev. 23 (2010) 94–106. doi:10.1071/RD10249.

[4] A. Corpet, G. Almouzni, Making copies of chromatin: the challenge of nucleosomal organization and epigenetic information, Trends Cell Biol. 19 (2009) 29–41. doi:10.1016/j.tcb.2008.10.002.

[5] R. Margueron, D. Reinberg, Chromatin structure and the inheritance of epigenetic information., Nat. Rev. Genet. 11 (2010) 285–96. doi:10.1038/nrg2752.

[6] C. Alabert, A. Groth, Chromatin replication and epigenome maintenance, Nat. Rev. Mol. Cell Biol. 13 (2012) 153–167. doi:10.1038/nrm3288.

[7] E. Meshorer, T. Misteli, Chromatin in pluripotent embryonic stem cells and differentiation., Nat. Rev. Mol. Cell Biol. 7 (2006) 540–546. doi:10.1038/nrm1938.

[8] B.M. Sirbu, F.B. Couch, J.T. Feigerle, S. Bhaskara, S.W. Hiebert, Analysis of protein dynamics at active , stalled , and collapsed replication forks, (2011) 1320–1327. doi:10.1101/gad.2053211.Venkitaraman.

[9] G. Almouzni, H. Cedar, Maintenance of epigenetic information, Cold Spring Harb. Perspect. Biol. 8 (2016).

[10] Y. Ma, K. Kanakousaki, L. Buttitta, How the cell cycle impacts chromatin architecture and influences cell fate, Front. Genet. 6 (2015) 1–18.

doi:10.3389/fgene.2015.00019.

[11] T. Boroviak, R. Loos, P. Bertone, A. Smith, J. Nichols, The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification., Nat. Cell Biol. 16 (2014) 516–28. doi:10.1038/ncb2965.

[12] G. Keller, Embryonic stem cell differentiation : emergence of a new era in biology and medicine, (2005) 1129–1155. doi:10.1101/gad.1303605.).

[13] D. Coronado, M. Godet, P.-Y.Y. Bourillot, Y. Tapponnier, A. Bernat, M. Petit, M. Afanassieff, S. Markossian, A. Malashicheva, R. Iacone, K. Anastassiadis, P. Savatier, A short G1 phase is an intrinsic determinant of naïve embryonic stem cell pluripotency., Stem Cell Res. 10 (2013) 118–31. doi:10.1016/j.scr.2012.10.004.

[14] S. Pauklin, L. Vallier, The cell-cycle state of stem cells determines cell fate propensity., Cell. 155 (2013) 135–47. doi:10.1016/j.cell.2013.08.031.

[15] M.P. Stavridis, B.J. Collins, K.G. Storey, Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation., Development. 137 (2010) 881–890. doi:10.1242/dev.043117.

[16] T. Kalkan, N. Olova, M. Roode, C. Mulas, H.J. Lee, I. Nett, W. Reik, P. Bertone, A. Smith, Tracking the embryonic stem cell transition from ground state pluripotency, (2016).

[17] C. Cai, J. Thorne, L. Grabel, Hedgehog serves as a mitogen and survival factor during embryonic stem cell neurogenesis., Stem Cells. 26 (2008) 1097–108. doi:10.1634/stemcells.2007-0684.

[18] E. Abranches, M. Silva, L. Pradier, H. Schulz, O. Hummel, D. Henrique, E. Bekman,

Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo., PLoS One. 4 (2009) e6286. doi:10.1371/journal.pone.0006286.

[19] G. Nair, E. Abranches, A.M. V Guedes, D. Henrique, A. Raj, Heterogeneous lineage marker expression in naive embryonic stem cells is mostly due to spontaneous differentiation., Sci. Rep. 5 (2015) 13339. doi:10.1038/srep13339.

[20] A. Sakaue-sawano, H. Kurokawa, T. Morimura, A. Hanyu, H. Hama, H. Osawa, S. Kashiwagi, K. Fukami, T. Miyata, H. Miyoshi, T. Imamura, M. Ogawa, Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression, Cell. (2008) 487–498. doi:10.1016/j.cell.2007.12.033.

[21] D. Egli, G. Birkhoff, K. Eggan, Mediators of reprogramming: transcription factors and transitions through mitosis., Nat. Rev. Mol. Cell Biol. 9 (2008) 505–516. doi:10.1038/nrm2439.

[22] R.P. Halley-stott, Nuclear reprogramming and mitosis – how does mitosis enhance changes in gene expression?, Transcription. 1264 (2015) 1–4. doi:10.1080/21541264.2015.1014262.

[23] C. Deluz, E.T. Friman, D. Strebinger, A. Benke, M. Raccaud, A. Callegari, M. Leleu, S. Manley, D.M. Suter, A role for mitotic bookmarking of SOX2 in pluripotency and differentiation., Genes Dev. (2016) 1–13. doi:10.1101/gad.289256.116.

[24] L.T. Vassilev, C. Tovar, S. Chen, D. Knezevic, X. Zhao, H. Sun, D.C. Heimbrook, L. Chen, Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1., Proc. Natl. Acad. Sci. 103 (2006) 10660–10665. doi:10.1073/pnas.0600447103.

[25] A.M. Singh, J. Chappell, R. Trost, L. Lin, T. Wang, J. Tang, H. Wu, S. Zhao, P. Jin, S. Dalton, Cell-Cycle Control of Developmentally Regulated Transcription Factors Accounts for Heterogeneity in Human Pluripotent Cells, Stem Cell Reports. 1 (2013) 1–13. doi:10.1016/j.stemcr.2013.10.009.

[26] Y. Sela, N. Molotski, S. Golan, J. Itskovitz-Eldor, Y. Soen, Human embryonic stem cells exhibit increased propensity to differentiate during the G1 phase prior to phosphorylation of retinoblastoma protein, Stem Cells. 30 (2012) 1097–1108. doi:10.1002/stem.1078.

[27] A.A. Filipczyk, A.L. Laslett, C. Mummery, M.F. Pera, Differentiation is coupled to changes in the cell cycle regulatory apparatus of human embryonic stem cells, Stem Cell Res. 1 (2007) 45–60. doi:10.1016/j.scr.2007.09.002.

[28] S. Chetty, E.N. Engquist, E. Mehanna, K.O. Lui, A.M. Tsankov, D.A. Melton, A Src inhibitor regulates the cell cycle of human pluripotent stem cells and improves directed differentiation, J. Cell Biol. 210 (2015) 1257–1268. doi:10.1083/jcb.201502035.

[29] L. Weinberger, M. Ayyash, N. Novershtern, J.H. Hanna, Dynamic stem cell states : naive to primed pluripotency in rodents and humans, Nat. Publ. Gr. (2016). doi:10.1038/nrm.2015.28.

[30] A.M. Singh, S. Dalton, The Cell Cycle and Myc Intersect with Mechanisms that Regulate Pluripotency and Reprogramming, Cell Stem Cell. 5 (2009) 141–149. doi:10.1016/j.stem.2009.07.003.

[31] S. Dalton, Linking the Cell Cycle to Cell Fate Decisions, Trends Cell Biol. 25 (2015) 592–600. doi:10.1016/j.tcb.2015.07.007.

[32] R. Rodriguez, M. Meuth, Chk1 and p21 Cooperate to Prevent Apoptosis during DNA Replication Fork Stress, Mol. Biol. Cell. 17 (2006) 402–412. doi:10.1091/mbc.E05.

[33] K.A.U. Gonzales, H. Liang, Y.-S. Lim, Y.-S. Chan, J.-C. Yeo, C.-P. Tan, B. Gao, B. Le, Z.-Y. Tan, K.-Y. Low, Y.-C. Liou, F. Bard, H.-H. Ng, Deterministic Restriction on Pluripotent State Dissolution by Cell-Cycle Pathways, Cell. 162 (2015) 564–579. doi:10.1016/j.cell.2015.07.001.

[34] K.A. Cimprich, D. Cortez, ATR: an essential regulator of genome integrity., Nat. Rev. Mol. Cell Biol. 9 (2008) 616–27. doi:10.1038/nrm2450.

[35] M. Shigeta, S. Ohtsuka, S. Nishikawa-Torikai, M. Yamane, S. Fujii, K. Murakami, H. Niwa, Maintenance of pluripotency in mouse ES cells without Trp53., Sci. Rep. 3 (2013) 2944. doi:10.1038/srep02944.

[36] R.P. Halley-Stott, J. Jullien, V. Pasque, J. Gurdon, Mitosis Gives a Brief Window of Opportunity for a Change in Gene Transcription, PLoS Biol. 12 (2014) 1–13. doi:10.1371/journal.pbio.1001914.

[37] S. Pauklin, P. Madrigal, A. Bertero, L. Vallier, Initiation of stem cell differentiation involves cell cycle-dependent regulation of developmental genes by Cyclin D, Genes Dev. (2016) 421–433. doi:10.1101/gad.271452.115.

[38] T. Tsubouchi, J. Soza-Ried, K. Brown, F.M. Piccolo, I. Cantone, D. Landeira, H. Bagci, H. Hochegger, M. Merkenschlager, A.G. Fisher, DNA synthesis is required for reprogramming mediated by stem cell fusion, Cell. 152 (2013) 873–883. doi:10.1016/j.cell.2013.01.012.

[39] B. Wang, M.J. Pfeiffer, C. Schwarzer, M.J. Araúzo-Bravo, M. Boiani, DNA

replication is an integral part of the mouse oocyte's reprogramming machinery, PLoS One. 9 (2014). doi:10.1371/journal.pone.0097199.

[40] A.M. Singh, Y. Sun, L. Li, W. Zhang, T. Wu, S. Zhao, Z. Qin, S. Dalton, Cell-Cycle Control of Bivalent Epigenetic Domains Regulates the Exit from Pluripotency, Stem Cell Reports. 5 (2015) 323–336. doi:10.1016/j.stemcr.2015.07.005.

[41] I. Ismailoglu, Q. Chen, M. Popowski, L. Yang, S.S. Gross, A.H. Brivanlou, Huntingtin protein is essential for mitochondrial metabolism, bioenergetics and structure in murine embryonic stem cells, Dev. Biol. 391 (2014) 230–240. doi:10.1016/j.ydbio.2014.04.005.

Figure Legends

Fig. 1. Transition to early primed pluripotency and cell synchronization

(A) Morphology of cells cultured in 2i+LIF medium or in Diff medium for 36h. (B) RT-qPCR analysis of W4 mESCs upon PELC induction. Results are presented as means ± SEM (n = 3). Data was relativized to undifferentiated cells. Different letters indicate significant differences ($p<0.05$). (C) Representative immunostainings of Nanog, Oct4 and Otx2 for W4 mESCs grown in 2i+LIF medium (Undiff) or cultured in Diff medium for the indicated times (Diff). Nuclei were counterstained with DAPI. Scale bar, 50 µm. (D) Diagram of Fucci system. (E) Representative Fucci-H2B colonies cultured in 2i+LIF medium. Scale bar, 100 µm. (F) Cell cycle distribution of parental W4 and Fucci-H2B mESCs after propidium iodide staining and flow cytometric analysis. (G) Fucci-H2B cells fluorescence patterns during cell cycle progression visualized by time-lapse imaging. Arrows indicate parental and daughter cells. (H) *Left*, flow cytometry dotplot showing Fucci fluorescence distribution. *Right*, experimental approach for generating synchronized cultures of Fucci cells. (I) *Top,* strategy followed for quantifying cell synchrony. *Bottom,* frequency distribution of cell generations after the sorting and tracking of cells in 2i+LIF medium (n = 510 cells) or Diff medium (n = 660 cells). Results are from three independent experiments.

Fig. 2. mESCs initiate the transcriptional transition to primed pluripotency before cell division

(A) Diagram of the experimental design. (B) RT-qPCR of the indicated markers in Fucci synchronized cultures maintained in 2i+LIF medium or Diff medium. Samples were collected at 0, 5, 10, 18, 23, 30 and 36 h post sorting. Results are presented as means ±

SEM for 4 independent experiments and plotted in log2 scale. Data was relativized to expression levels of early G1 cells immediately after sorting (T0). * indicate significant differences ($p<0.05$) between 2i+LIF and Diff conditions for each indicated time. Vertical dashed lines denote the time at which cells begin to divide.

Fig. 3. Inhibition of cell division affects the transcriptional change of specific genes.

(A) Representative colonies of W4 mESCs after 15 h incubation with RO3306 (left panels) or 30 min after drug release (right panels). Arrows indicate mitotic cells as judged by morphology. (B) W4 Fucci-H2B cells released for 30 min after RO3306 treatment for 15 h, showing cells mostly in metaphase. H2B-mCerulean fluorescence is shown in red. Bar, 50 µm. (C) Cell cycle distribution of (i) control mESCs (untreated), (ii) cells incubated with RO3306 for 15 h and (iii) cells released for 2 h from G2 block. Cell cycle distribution was analyzed by flow cytometry based on propidium iodide staining. (D) Diagram of the experimental design used to analyze the effect of impairing cell division. (E) RT-qPCR of the indicated markers after early G1 cells were cultured for the indicated times in Diff medium alone or in Diff medium with the addition of RO3306 at 10 h post sorting. Results are presented as means ± SEM for three independent experiments and plotted in log2 scale. Data was relativized to expression levels of early G1 cells immediately after sorting (T0). * indicate significant differences ($p<0.05$) between both conditions for each indicated time. Dotted lines indicate the time of addition of RO3306 (10 h), previous to the onset of cell division.

Fig. 4. Exit from naïve to primed pluripotency is coupled to transition through the G1 phase.

(A) Representative single cell fluorescence dynamics for the Fucci markers after early G1 cells were sorted into Diff medium and subjected to time-lapse imaging. (B) Proportion of cells of the first generation that completed G1 along time after sorting, evaluated by time-lapse imaging as in A; n = 75, three independent experiments. (C) Diagram of the experimental design. (D) RT-qPCR analysis of the indicated markers based on the experimental design depicted in C. Results are presented as means ± SEM for three independent experiments and plotted in log2 scale. Data was relativized to expression levels of early G1 cells immediately after cell sorting (T0). Different letters indicate significant differences between conditions for each indicated time ($p<0.05$). Dotted lines indicate the time of media change for the 'Diff post-G1' condition (5 h).

Fig. 5. Inhibition of DNA replication impairs transcriptional change during naïve pluripotency exit

(A) Flow cytometric analysis of the replicative state was performed by EdU incorporation and propidium iodide staining. mESCs were cultured in 2i+LIF medium in the following conditions: (i) mock treated cells (Control), (ii) cells incubated for 5 h with excess thymidine (Thymidine) and (iii) cells incubated for 5 h with excess thymidine and then released from block for 1 h by medium change (Thymidine + 1 h release). The gates are indicated with dotted lines, and depict the different subpopulations of cells according to the phase of the cell cycle. (B) Quantification of EdU positive cells (EdU+) in each condition from three independent experiments as in A. Results are presented as means ± SEM. Different letters indicate significant differences ($p<0.05$). (C) Diagram of the experimental design showing

the three conditions: Control, Thymidine and Thymidine Release. (D) RT-qPCR analysis of the indicated markers based on the experimental design depicted in C. Results are presented as means ± SEM for three independent experiments and plotted in log2 scale. Data was relativized to expression levels of early G1 cells immediately after cell sorting (T0). Different letters indicate significant differences between conditions for each indicated time ($p<0.05$). Dotted lines indicate the time of medium change to release thymidine block (18 h).

Fig. 6. Transcriptional change upon inhibiting DNA replication remains partially inhibited in the absence of p53

(A) Diagram of CRISPR mediated p53 knockout generation. A sequence next to the p53 first codon in exon 2 was targeted using a specific sgRNA. (B) CRISPR mediated p53 knockout. Representative immunoblot showing p53 depletion in p53 -/- mESCs cells compared to W4 parental cells, performed with a p53 antibody that recognizes the C-terminal domain. GAPDH was used as loading control. (C) Diagram of the experimental design. (D) RT-qPCR analysis of the indicated markers based on the experimental design depicted in C. WT or p53 -/- mESCs were synchronized by RO3306 treatment and then set to differentiate for 28 h in Diff medium in the presence of DMSO, thymidine (Thym) or aphidicolin (APH). Results of three independent biological replicates are presented for each condition. Values were relativized to WT cells cultured in 2i+LIF medium for each biological replicate. The black line in each condition represents the mean of the three replicates. Different letters at the top of the chart indicate significant differences between the groups ($p<0.05$) and the p-value for each gene is indicated in the bottom-right corner of each chart. Dotted lines show the reference expression levels of WT cells cultured in 2i+LIF.

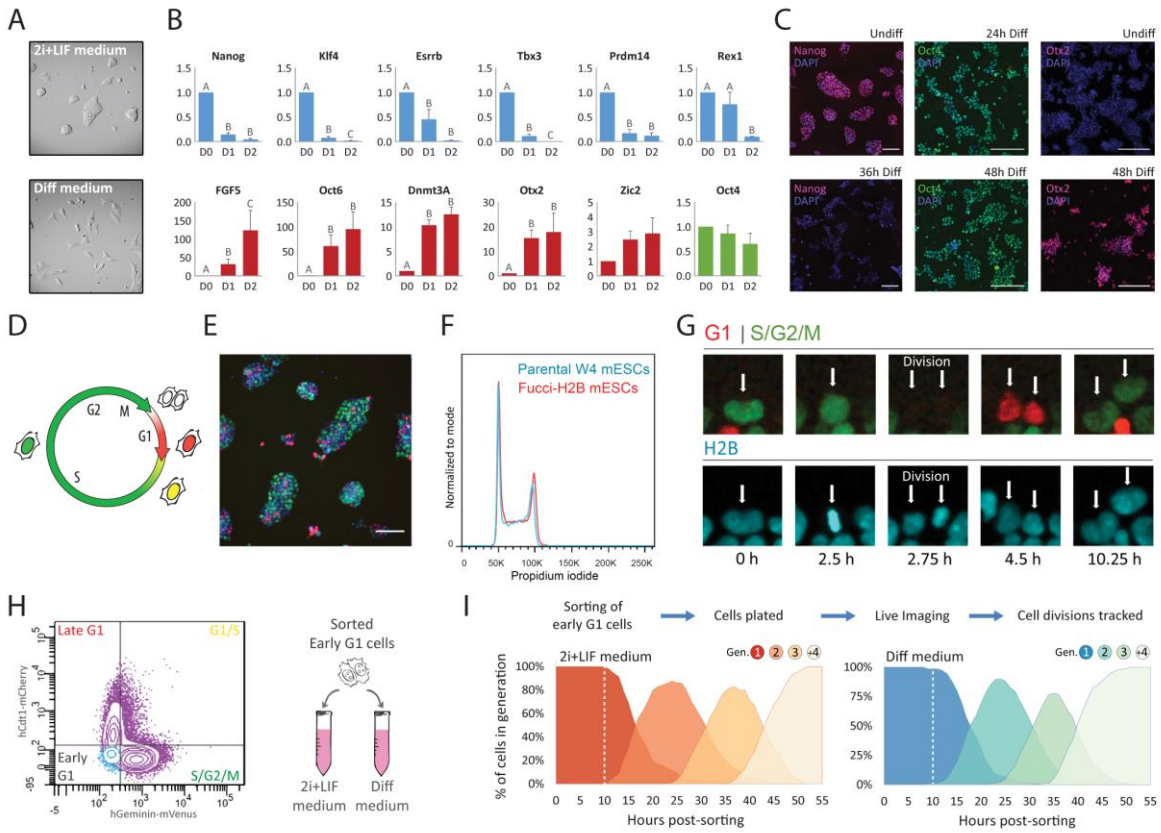

Fig. 1

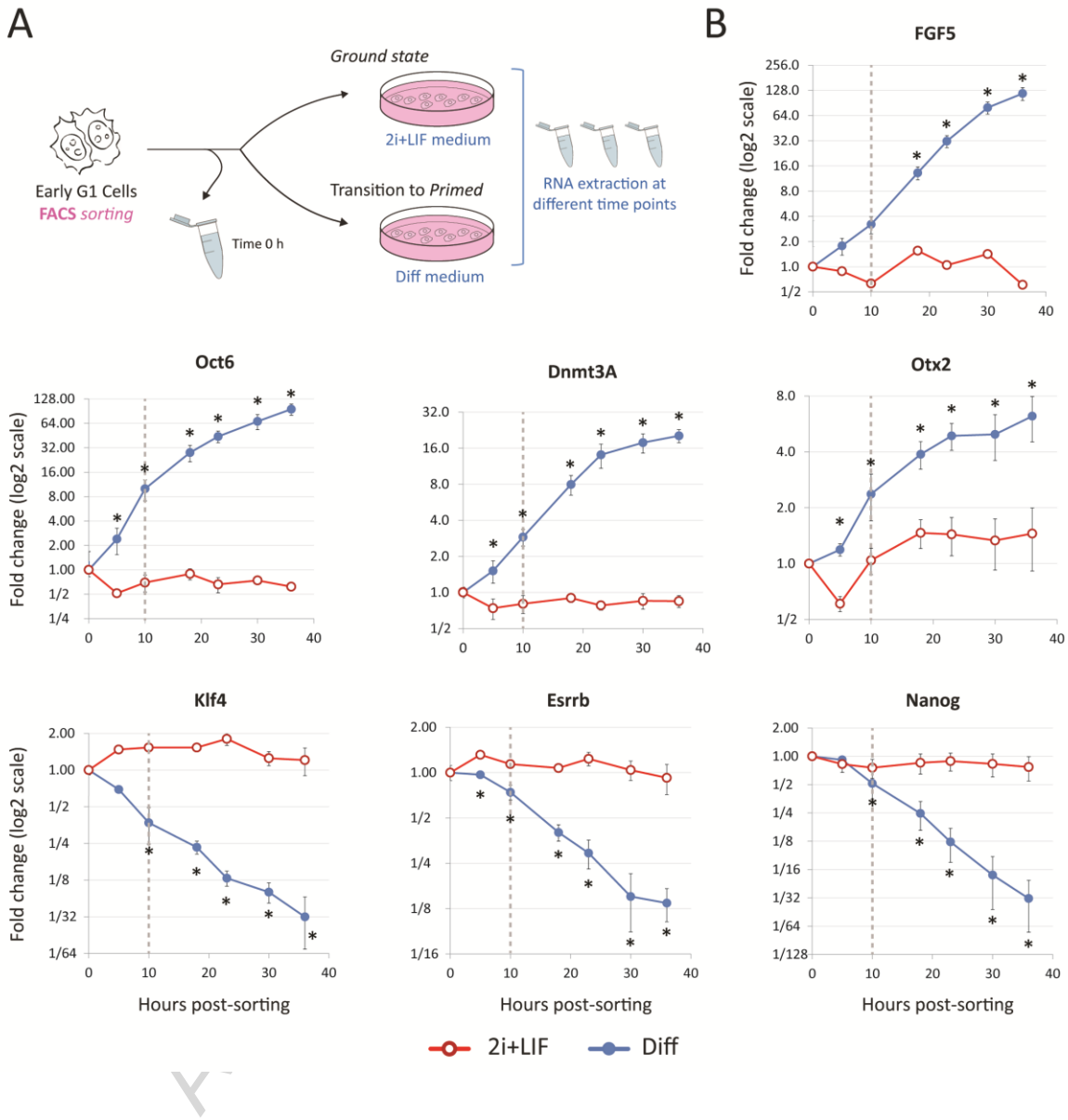

Fig. 2

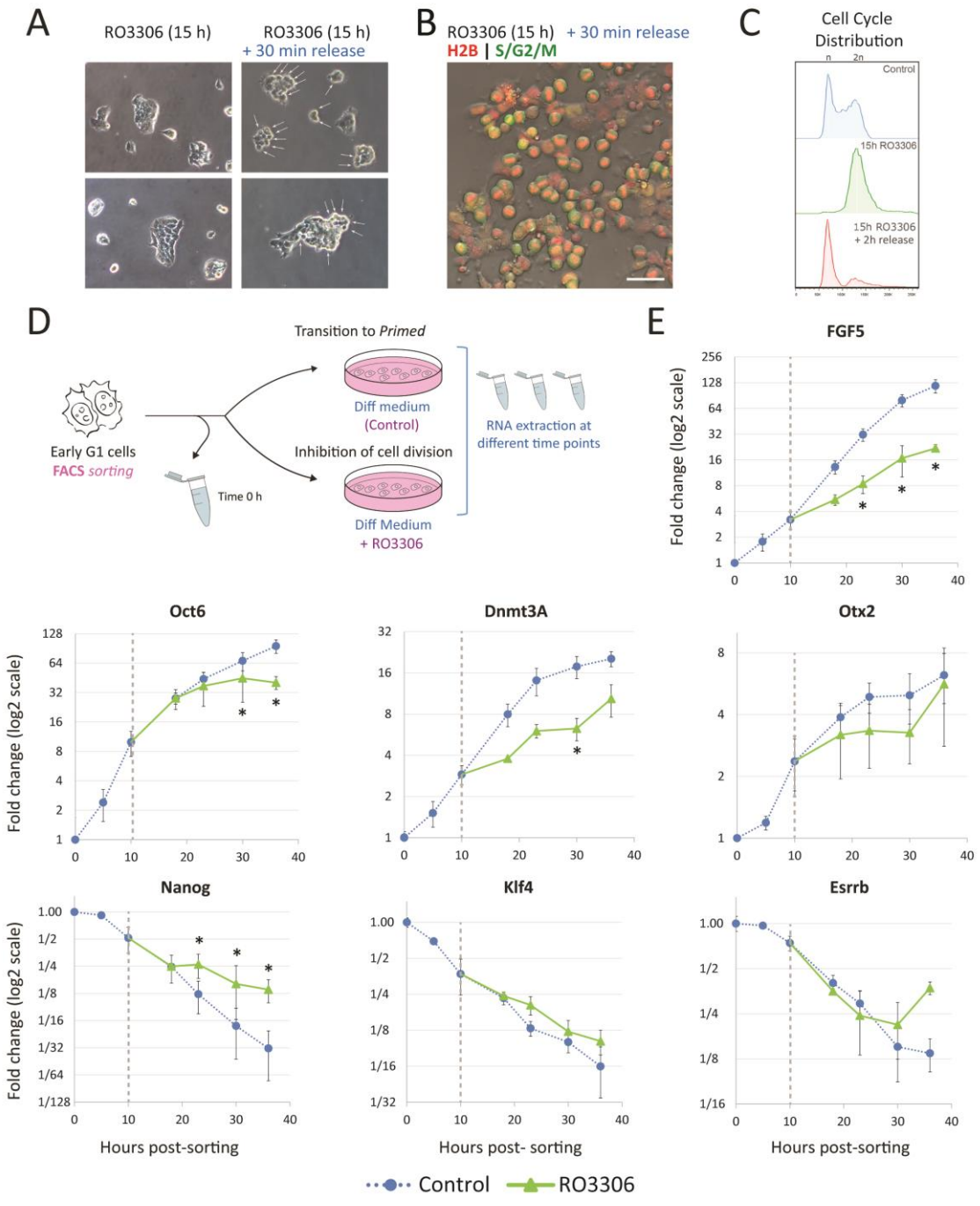

Fig.3

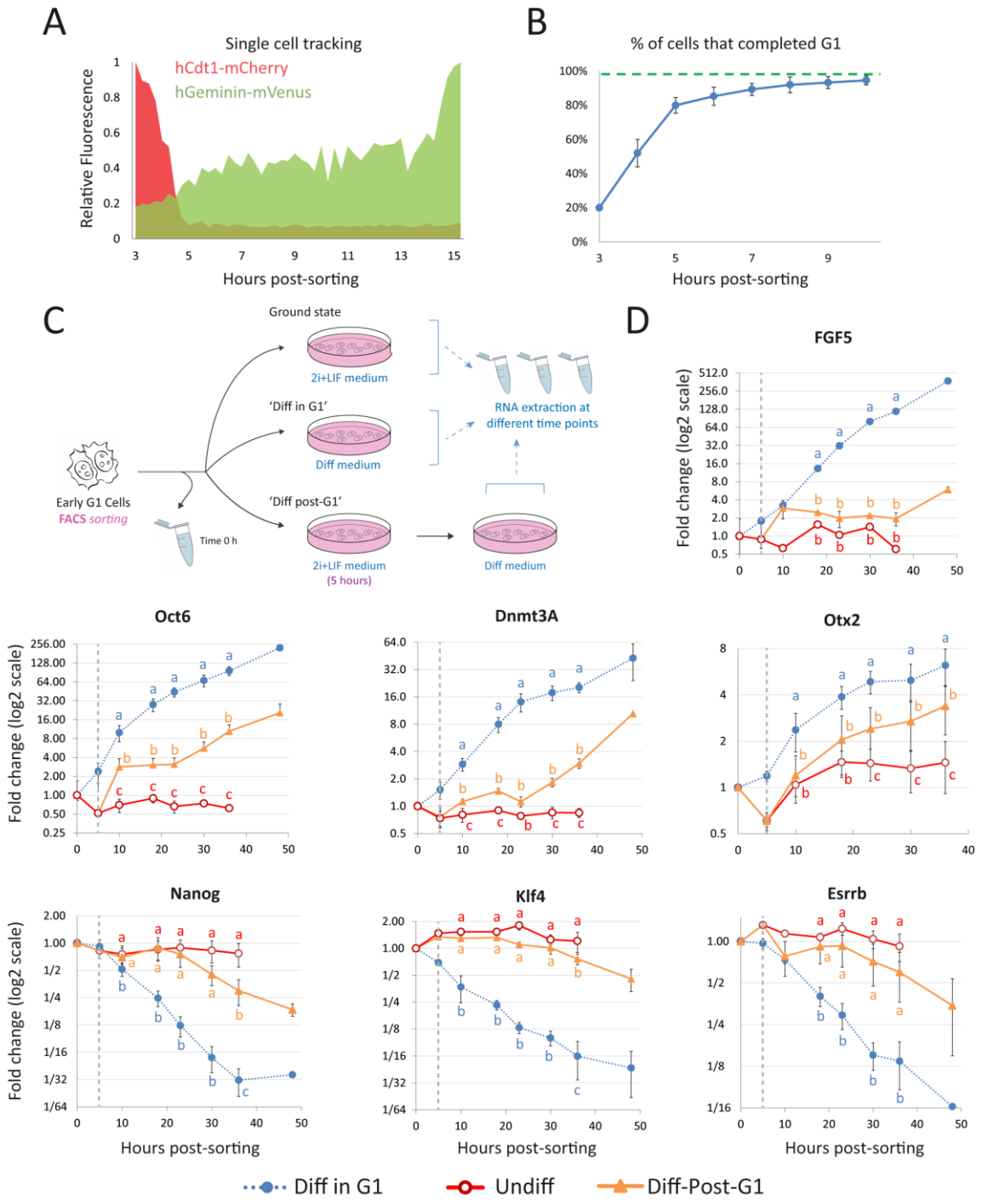

Fig. 4

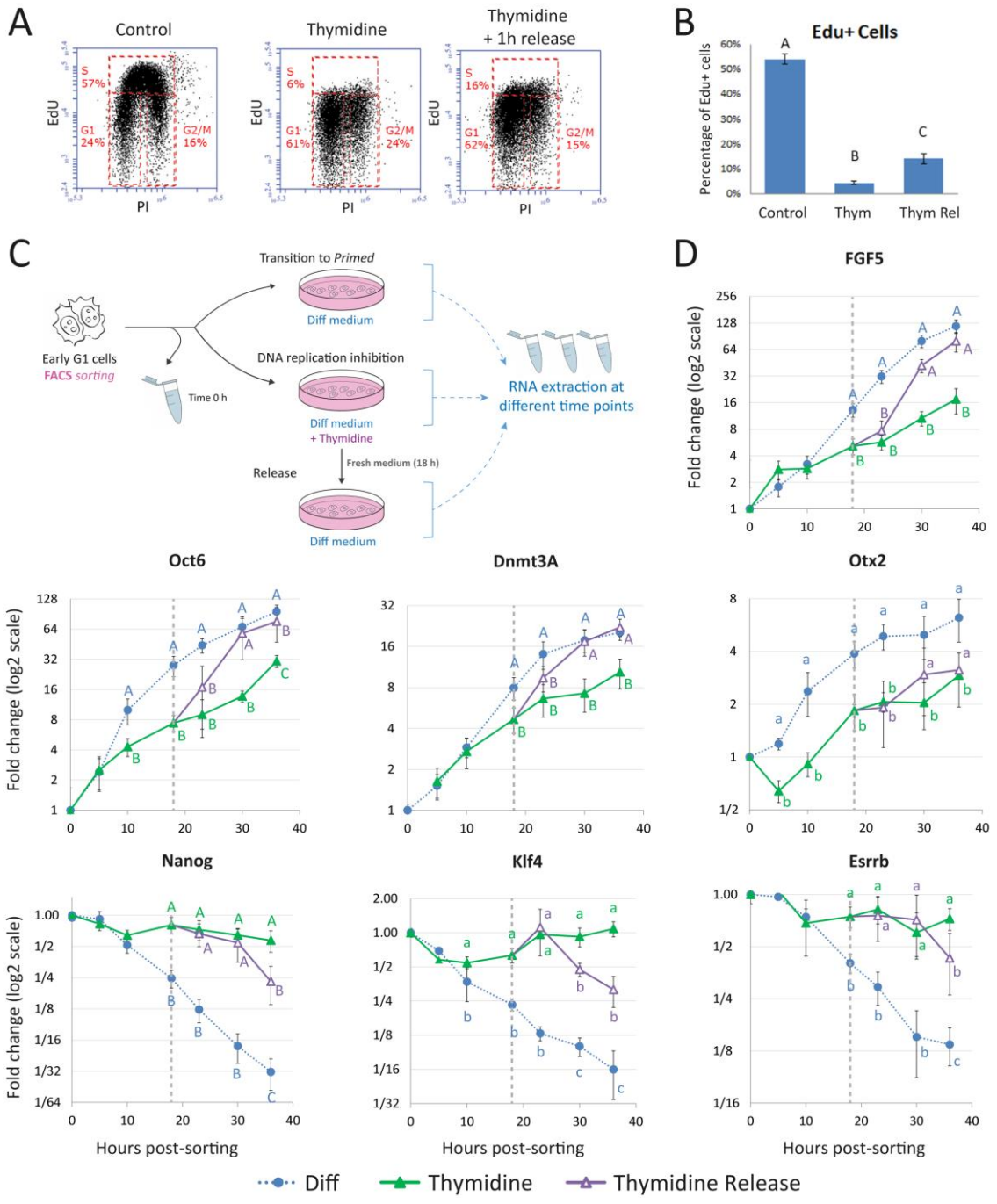

Fig. 5

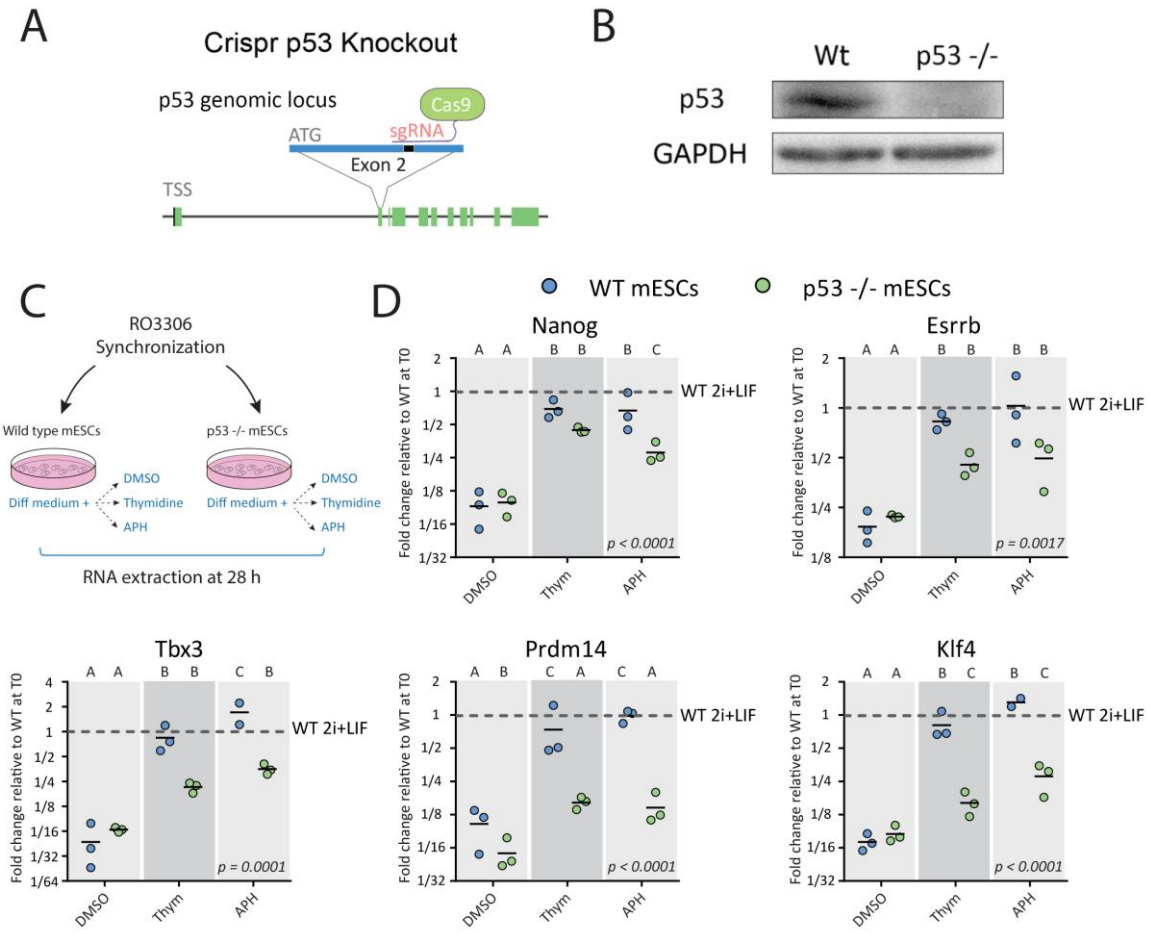

Fig. 6

Highlights

- Transcriptional programs change drastically during stem cell differentiation
- Cell cycle events were proposed to act as instances of global epigenetic remodeling
- Mitosis may enhance but it is not fundamental for transcriptional change
- Transition from naïve to primed pluripotency is coupled to lineage priming in G1
- Inhibition of DNA replication impairs naïve pluripotency exit