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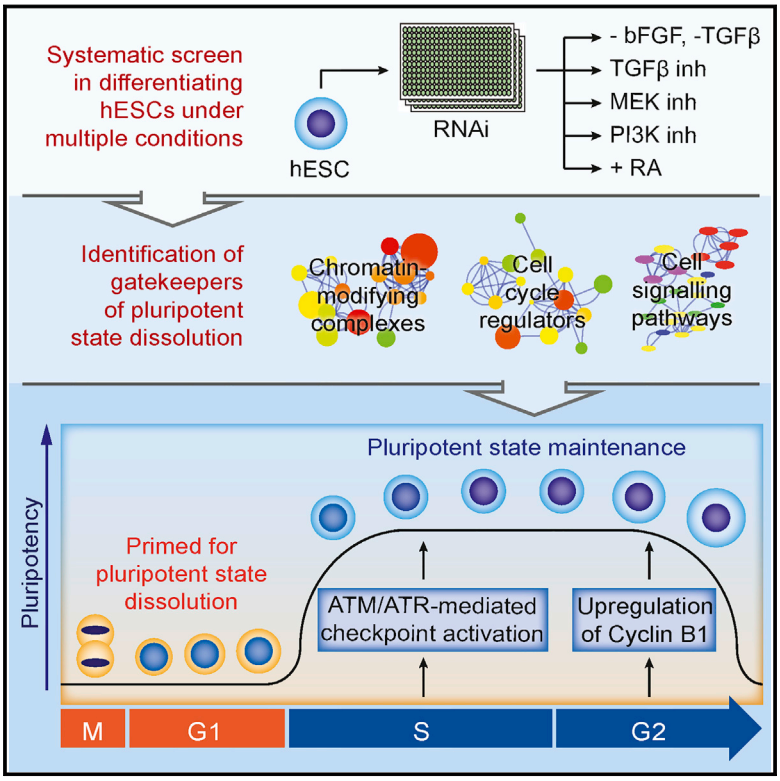
Deterministic Restriction on Pluripotent State Dissolution by Cell-Cycle Pathways

Graphical Abstract



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In Brief

A high-throughput RNAi screen reveals the intrinsic roles of the S and G2 phases in regulating pluripotency independent of G1 phase, functionally establishing that pluripotency control is hardwired to the cell-cycle machinery.

Highlights

- Functional screening reveals gatekeepers of pluripotent state dissolution in hESCs
- The S and G2 phases of the cell cycle actively promote the pluripotent state
- The regulation of pluripotency by S and G2 phases is intrinsic and independent from G1
- The ATM/ATR-CHEK2-p53 axis enhances the TGF-β pathway to prevent PSD

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Deterministic Restriction on Pluripotent State Dissolution by Cell-Cycle Pathways

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SUMMARY

During differentiation, human embryonic stem cells (hESCs) shut down the regulatory network conferring pluripotency in a process we designated pluripotent state dissolution (PSD). In a high-throughput RNAi screen using an inclusive set of differentiation conditions, we identify centrally important and context-dependent processes regulating PSD in hESCs, including histone acetylation, chromatin remodeling, RNA splicing, and signaling pathways. Strikingly, we detected a strong and specific enrichment of cell-cycle genes involved in DNA replication and G2 phase progression. Genetic and chemical perturbation studies demonstrate that the S and G2 phases attenuate PSD because they possess an intrinsic propensity toward the pluripotent state that is independent of G1 phase. Our data therefore functionally establish that pluripotency control is hardwired to the cell-cycle machinery, where S and G2 phase-specific pathways deterministically restrict PSD, whereas the absence of such pathways in G1 phase potentially permits the initiation of differentiation.

INTRODUCTION

The human pluripotent stem cell state is facilitated by an intricate regulatory network controlled chiefly by master transcription factors (Boyer et al., 2005). These master regulators form multiple regulatory connections with other transcription factors, epigenetic modifiers, signal transduction pathways, non-coding RNAs, and other regulators that, together, maintain self-renewal and pluripotency (Ng and Surani, 2011; Young, 2011). Sustenance of this internal regulatory network is dependent on external cues from the cell culture environment. Human embryonic stem cells (hESCs) mainly rely on the basic fibroblast growth factor (bFGF) and Activin/transforming growth factor β (TGF-β) pathways for self-renewal (Beattie et al., 2005; Xu et al., 2005). Withdrawal of these signaling pathways causes the shutdown of the complex hESC regulatory network; we call this process pluripotent state dissolution (PSD). Because many applications of hESCs require their complete and efficient differentiation, it is necessary to obtain detailed knowledge of how the hESC regulatory network is dissolved during differentiation. However, because the maintenance of the pluripotent state requires multiple interactions between regulatory pathways and factors, the dissolution of such a state is likely to be an equally complex process with multiple routes from which it can be enforced. Although several studies have identified regulators of PSD in mouse embryonic stem cells (mESCs) (Betschinger et al., 2013; Guo et al., 2011), there is a lack of knowledge about how PSD is regulated in hESCs.

Systematic studies like high-throughput functional genomics assays have greatly advanced the knowledge about the regulatory networks of ESCs. The unbiased nature of functional genomics makes it a powerful discovery tool for the identification of key protein complexes and pathways by detecting multiple crucial hits from the same pathway or complex. However, for hESCs, most studies rely on expanding the hESC regulatory network using previously known factors, and, to date, only one arrayed high-throughput functional genomics study has been performed in hESCs (Chia et al., 2010). Therefore, there is a lack of knowledge in the molecular understanding of the regulatory network that governs PSD of hESCs.

To address this deficiency, we undertook a large-scale high-throughput RNAi screen in differentiating hESCs for the de novo identification of the molecular pathways regulating PSD. To be comprehensive in dissecting PSD, we probed five differentiation conditions to discover both context-dependent and universal gatekeepers. Strikingly, we found a strong enrichment of cell-cycle hits clustering specifically in S and G2 phases but not in other phases of the cell cycle. Genetic and chemical manipulations of cell-cycle progression established an intrinsic propensity toward pluripotency maintenance in S and G2 phases, enacted by the ataxia telangiectasia mutated (ATM)/ATR-mediated replication checkpoint and Cyclin B1 pathways. Our study reveals a link that hardwires the hESC pluripotency network to the cell-cycle

machinery, where S and G2 phase-specific pathways deterministically restrict PSD and the absence of such pathways at G1 phase could permit PSD.

RESULTS

A High-Throughput RNAi Screen under Multiple Conditions Identifies Genes Important for PSD in hESCs

The process of ESC differentiation can be conceptually subdivided into two major steps: PSD, where the transcriptional network of pluripotency is shut down, and lineage specification, where a new transcriptional program corresponding to a specific somatic lineage is assembled. Although the regulation of lineage specification could differ greatly differ between different lineages, PSD is an early event for the ESC differentiation process. To obtain evidence that PSD is distinct from lineage specification, we looked at the expression kinetics of pluripotency and lineage-specific genes upon withdrawal of the self-renewal factors bFGF and TGF-β. 48 hr after induction of differentiation, the downregulation of many hESC-specific genes (Assou et al., 2007) began (Figure S1A), but the upregulation of lineage-specific factor expression was only evident at 96 hr (Figure S1B). An examination of gene expression of single cells showed similar results (Figure S1C). Therefore, these data indicate that PSD occurs immediately at the onset of differentiation, distinct from lineage specification (Figure S1D). Because *NANOG* is decreased significantly at both early and late time points (Figure S1A), we assigned *NANOG* downregulation as a marker for PSD and created a *NANOG-GFP* hESC line as a reporter of PSD (Figures S1E–S1H).

To achieve robust and unbiased identification of universal and specific factors required for PSD, we carried out a high-throughput RNAi screen under five differentiation conditions (Figure 1A). Under the first condition, we removed the two important cytokines bFGF and TGF-β from hESC medium (Beattie et al., 2005; Xu et al., 2005). Under the second to fourth conditions, we individually perturbed signaling of the TGF-β pathway as well as the mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) branches of the bFGF pathway. Under the fifth condition, we introduced retinoic acid (RA), a potent inducer of differentiation. All conditions led to PSD, as indicated by the efficient loss of hESC identity (Figures S1I–S1K). Cells were transfected with siRNAs 24 hr before induction of differentiation. The degree of preservation of hESC identity was then measured through the average *NANOG*-GFP fluorescence intensity per cell; hESCs depleted of PSD effectors are expected to retain higher GFP signals. We screened a total of 4,558 genes in triplicate for all five conditions, summing up to 68,370 data points (Table S1).

We first ensured the quality of the screen by checking for intra-plate layout effects (Figure 1B; Figure S2A), proper inter-plate alignment (Figure S2B), and good correlation between replicates (Figure 1C; Figure S2C). Genes that reproducibly scored above noise ($z > 1.25$ or $z > 1.5$ in at least two replicates) were regarded as hits (Figure 1D). Hits obtained were observed to maintain a visible GFP signal (Figure 1E). We detected no cell number bias in hit selection (Figure S2D), eliminating the possibility that housekeeping genes could be misidentified as hits. We further conducted counter-screens using a hESC line harboring an *ACTIN-GFP* reporter (Table S2) and found no overlap with the *NANOG-GFP* hits (Figure S2E). Collectively, these results ascertain that the RNAi screen identified genes important for PSD rigorously and reliably.

Informatics Analyses Reveal Pathways that Regulate PSD in a Context-Specific Manner

The screen was designed to enable the identification of both context-dependent and universally important processes that regulate PSD (Figure 2A). We examined the hits from the five distinct conditions using protein interaction network (Figure 2B), Reactome (Table S3), and gene ontology (Table S4) analyses to look for context-dependent effectors of PSD.

First, we observed that the hits included factors that are associated with the primary pathways transducing the distinct initial differentiation cues. These include the repressors SKI and SKIL for TGF-β inhibition (Figure 2B), the ERK2 inactivator DUSP6 for MEK inhibition (Table S1), and the RA receptor RXRA and the transcriptional complexes RNA polymerase II and transcription factor II D (TFIID)/Mediator for +RA (Figure 2B; Table S1). This is reassuring because perturbation of these primary pathways is expected to nullify the differentiation-inducing effect stemming from the same pathway, demonstrating the robustness of the high-throughput screening assay.

Interestingly, the RNA splicing machinery seems to play a role in PSD, especially during RA addition and MEK inhibition (Tables S3 and S4). This is notable because, although multiple RNA splicing factors have been reported to control the pluripotent state of hESCs (Gabut et al., 2011; Lu et al., 2013), it has been unknown whether they also regulate its dissolution. RNA splicing has been shown in other cell types to interact with the MEK and RA pathways (Shilo et al., 2014; Wang et al., 2015). Similar mechanisms might be in place in hESCs, explaining why RNA splicing could affect PSD triggered specifically by MEK inhibition or RA introduction. Our study therefore opens the door for studying the crosstalk between the splicing machinery, PSD, and these developmental pathways.

Besides RNA splicing, studying the functional genetics of PSD further revealed novel nodes of contact between signaling pathways and pluripotency. For example, we observed an enrichment of nucleosome-remodeling deacetylase (NuRD) complex members under conditions where bFGF-MEK signaling is abolished (Figure 2B; Table S4). The NuRD complex has an established role in promoting PSD in mESCs (Kaji et al., 2006; Reynolds et al., 2012), and our results demonstrate the conservation of this function in hESCs. Interestingly, although the leukemia inhibitory factor (LIF)-Stat3 pathway opposes the action of the NuRD complex in mESCs (Hu and Wade, 2012), this role seems to be assumed by the bFGF-MEK pathway in hESCs. On the other hand, a strong enrichment of histone demethylases, specifically those targeting histone 3 lysine 4 (H3K4), was observed under the PI3K pathway inhibition condition (Figure 2B; Tables S3 and S4). This points toward an epigenetic link between the PI3K pathway and the pluripotency network through the maintenance of activating H3K4 methylation marks.

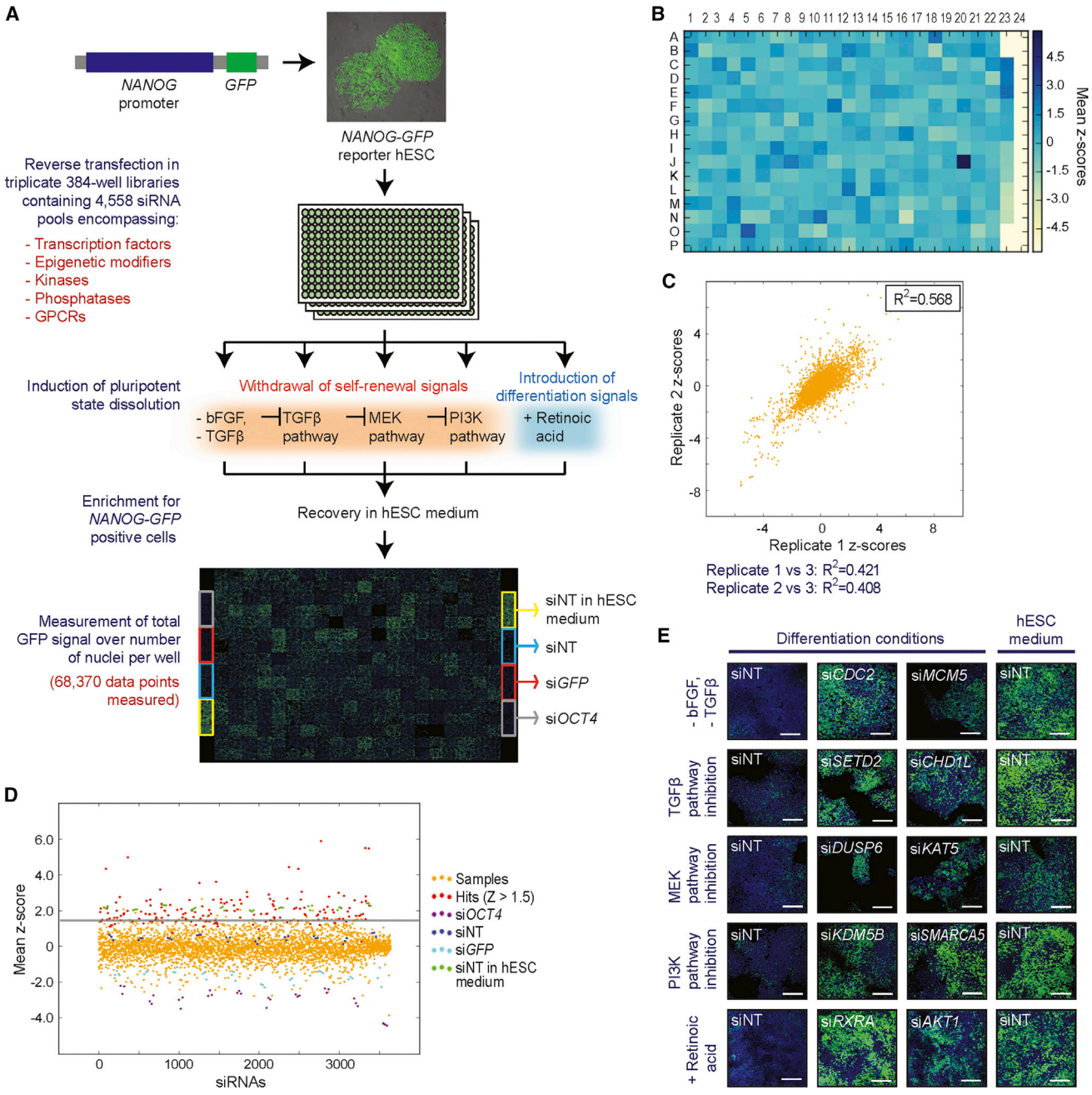


Figure 1. A High-Throughput RNAi Screen for Regulators of PSD

(A) Schematic of the siRNA screen. siNT, non-targeting siRNA; siOCT4, siRNA against *POU5F1* (OCT4); siGFP, siRNA against *GFP*.

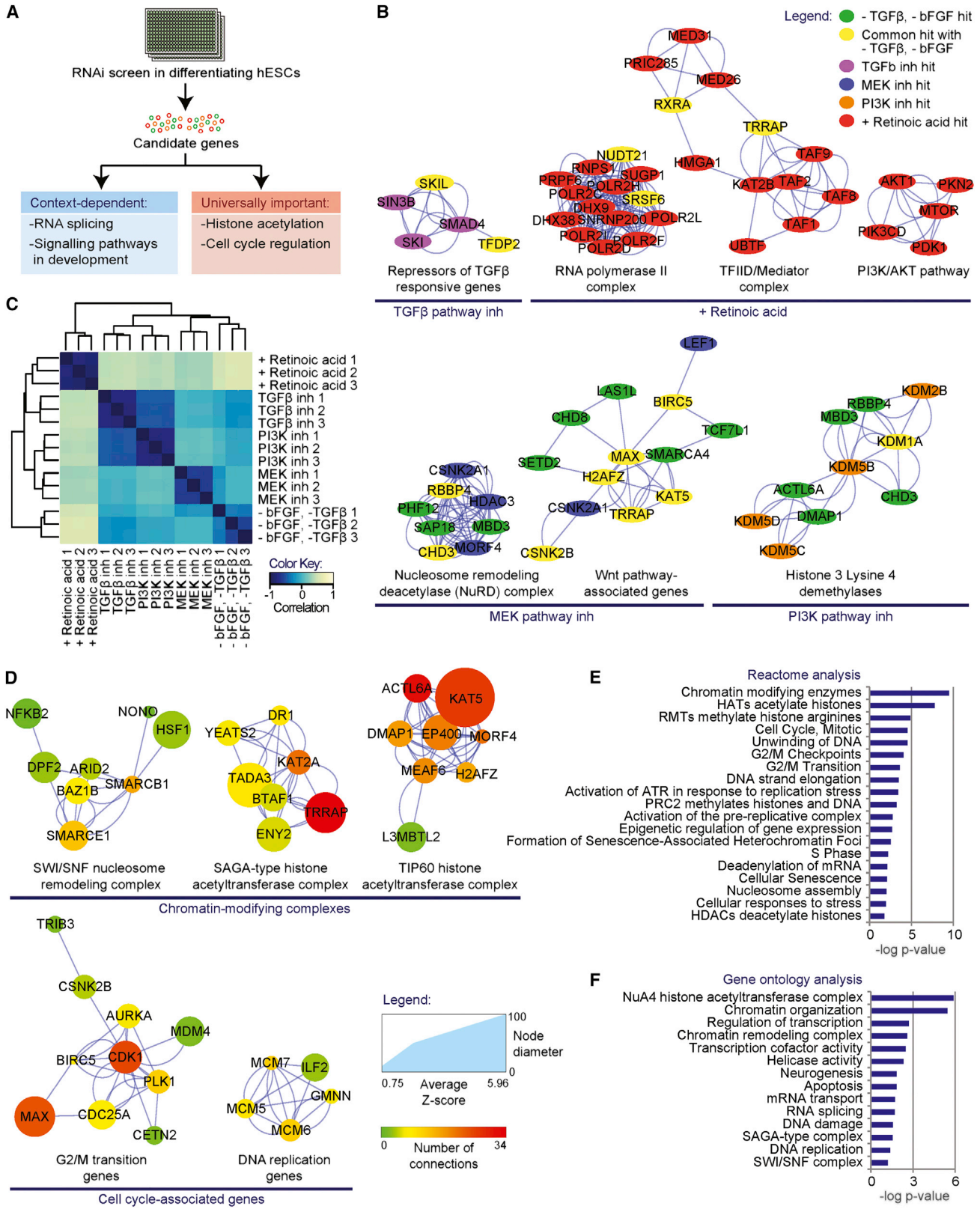
(B) Representative plate heatmap from the −bFGF, −TGF-β condition.

(C) Scatterplot showing the correlation between screen replicates under the −bFGF, −TGF-β condition.

(D) Representative dot plot of the results of the transcription factor and epigenetic modifier subset from the −bFGF, −TGF-β condition. The gray line indicates the cutoff *Z* score (>1.5).

(E) Representative images for *NANOG*-GFP fluorescence (green) and Hoechst staining (blue) for hits under the five differentiation conditions. Scale bars, 200 μm. See also Figures S1 and S2 and Tables S1 and S2.

Finally, we noticed that developmental pathways play highly context-dependent roles in PSD. For instance, Wnt signaling-associated factors, particularly those regulating β-catenin-mediated transcription, were enriched in hits during inactive bFGF-MEK signaling (Figure 2B; Tables S3 and S4). This is in agreement with the identified cross-regulation between these two pathways (Ding et al., 2005; Singh et al., 2012) and the pro-differentiation role of nuclear β-catenin in hESCs (Davidson



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et al., 2012; Dravid et al., 2005). On the other hand, negative regulators of the PI3K pathway are enriched under the −bFGF, −TGFβ condition (Table S3), whereas positive regulators are enriched under the +RA condition (Figure 2B; Table S3). The PI3K pathway therefore seems to help uphold pluripotency in hESCs (Figures S1I–S1K) in coordination with both the bFGF and TGFβ pathways (Singh et al., 2012) but promotes differentiation instead when acting downstream of RA. These results emphasize the importance of studying the role of developmental pathways in the proper context.

Therefore, separate analyses of the various screening conditions demonstrate the robustness of our screen results in addition to identifying context-dependent processes that are crucial for PSD.

Combined Analysis Uncovers Epigenetic Mechanisms that Universally Regulate PSD upon Withdrawal of Self-Renewal Signals

Hierarchical clustering of the results from the five conditions revealed that the +RA condition is excluded from a tight cluster comprising the other four conditions (Figure 2C). This was conceivable given that the +RA condition introduces a differentiation signal in contrast to the withdrawal of self-renewal signals under the other four conditions. Therefore, we next performed a combined analysis of the four clustering conditions (Figures 2D–2F) to find central pathways that are important for PSD induced by the removal of self-renewal signals.

The combined analysis identified members of multiple chromatin-modifying complexes important for PSD. Histone acetyltransferase (HAT) complex proteins appear to be top hits (Figures 2D–2F), most prominently those that belong to Spt-Ada-Gcn5-acetyltransferase (SAGA)-type (TATA-binding protein-free TAF-containing complex [TFTC]/SPT3-TAF9-GCN5-acetylase complex [STAGA]) and TIP60 (NuA4) HAT complexes. Strikingly, the catalytic subunit of the TIP60 complex *KAT5* is among the top five hits of all four conditions (Table S1), underlining the importance of this complex in PSD. Although histone acetylation levels are known to be higher in undifferentiated ESCs (Legartová et al., 2014), a global but transient increase in histone acetylation occurs during PSD (Golob et al., 2008), potentially necessitating the activity of HAT complexes at the onset of PSD. Nucleosome remodeling is also essential because knockdown of multiple Switch/sucrose non-fermentable (SWI/SNF) family members prevented efficient downregulation of *NANOG* in differentiating hESCs (Figures 2D and 2F). Notably, certain members of these complexes, such as *Trrap* of the NuA4 complex and *Arid1a* of the SWI/SNF complex, have a conserved function in shutting down pluripotency in ground-state mESCs (Betschinger et al., 2013). Therefore, our study systematically highlights the importance of epigenetic regulations in the PSD of hESCs.

The Cell-Cycle Machinery Deterministically Regulates PSD

Besides epigenetic modifiers, our combined analysis also revealed that genes involved in cell-cycle regulation were among the most enriched (Figures 2D–2F), particularly those that are involved in DNA replication during S phase or in G2-to-M transition (Figures 2D and 2E). On the contrary, we found no strong enrichment of processes specific to other phases of the cell cycle, implying that specific cell-cycle regulations influence the cell fate transitions of pluripotent cells. Although the cell cycle has been found to influence cell fate decisions in perspectives other than proliferation (Lee et al., 2014; Rodier et al., 2009), knowledge about the regulation of PSD by the cell cycle is limited, especially for G1-independent cell-cycle states such as S and G2 phases. To explore this topic, we first functionally validated the effect of cell-cycle genes in PSD by impeding DNA replication or prolonging the gap phases using both genetic and chemical approaches (Figure 3A).

Genetic validation of the S- and G2-associated hits confirmed that their depletion (Figures S3A and S3B) impedes pluripotency gene downregulation after removal of self-renewal signals (Figures 3B and 3C). Because their depletion concomitantly perturbs progression through the cell cycle (Figures S3C and S3D), these results suggest that extending the S and G2 phases of the hES cell cycle may dominantly impede PSD. In contrast, although lengthening of G1 phase is associated with differentiation, artificially extending G1 phase by knocking down CDK4/6, depleting Cyclin D, or overexpressing the CDK inhibitor p21 (Figures S3E–S3G) did not significantly affect the downregulation of pluripotency markers upon removal of self-renewal signals (Figure 3D). These findings suggest that the lengthening of G1 phase does not elicit a deterministic effect on PSD of hESCs. *NANOG*-GFP fluorescence was also preserved (Figure S3H), and differentiation marker upregulation was inhibited (Figures S3I–S3K) upon knockdown of S and G2 phase progression genes but not upon G1 phase prolongation, corresponding with the observed changes in pluripotency marker expression.

Because depletion of cell-cycle-related hits impeded S and G2 phase progression, we next validated whether direct manipulation of these cell-cycle phases using chemical inhibitors can similarly affect PSD. With proper dosage, we managed to enrich, but not completely arrest, hESCs in specific cell-cycle phases (Figure S4A) without inducing extensive apoptosis (Figures S4B and S4C). In concordance with our screen results, we found

Figure 2. Identification of Context-Dependent and Universally Important Regulators of PSD

(A) Diagram outlining the major findings from the RNAi screen.

(B) Protein-protein interaction networks of genes that are uniquely enriched for the different screening conditions. The node color indicates the screening conditions under which the gene was identified as a hit. See also Tables S3 and S4.

(C) Heatmap depicting hierarchical clustering by Euclidian distance between different screening conditions.

(D) Enriched gene clusters from the protein-protein interaction network analysis of the combined hits. The node size indicates the average *Z* score of the hits, whereas the node color indicates the degree of integration (number of edges) of the gene with the entire network of hits.

(E) Overrepresented pathways ($p < 0.05$) from the combined hits as determined using the web resource Reactome.

(F) Representative terms ($p < 0.05$) for enriched functional annotation clusters from the combined hits.

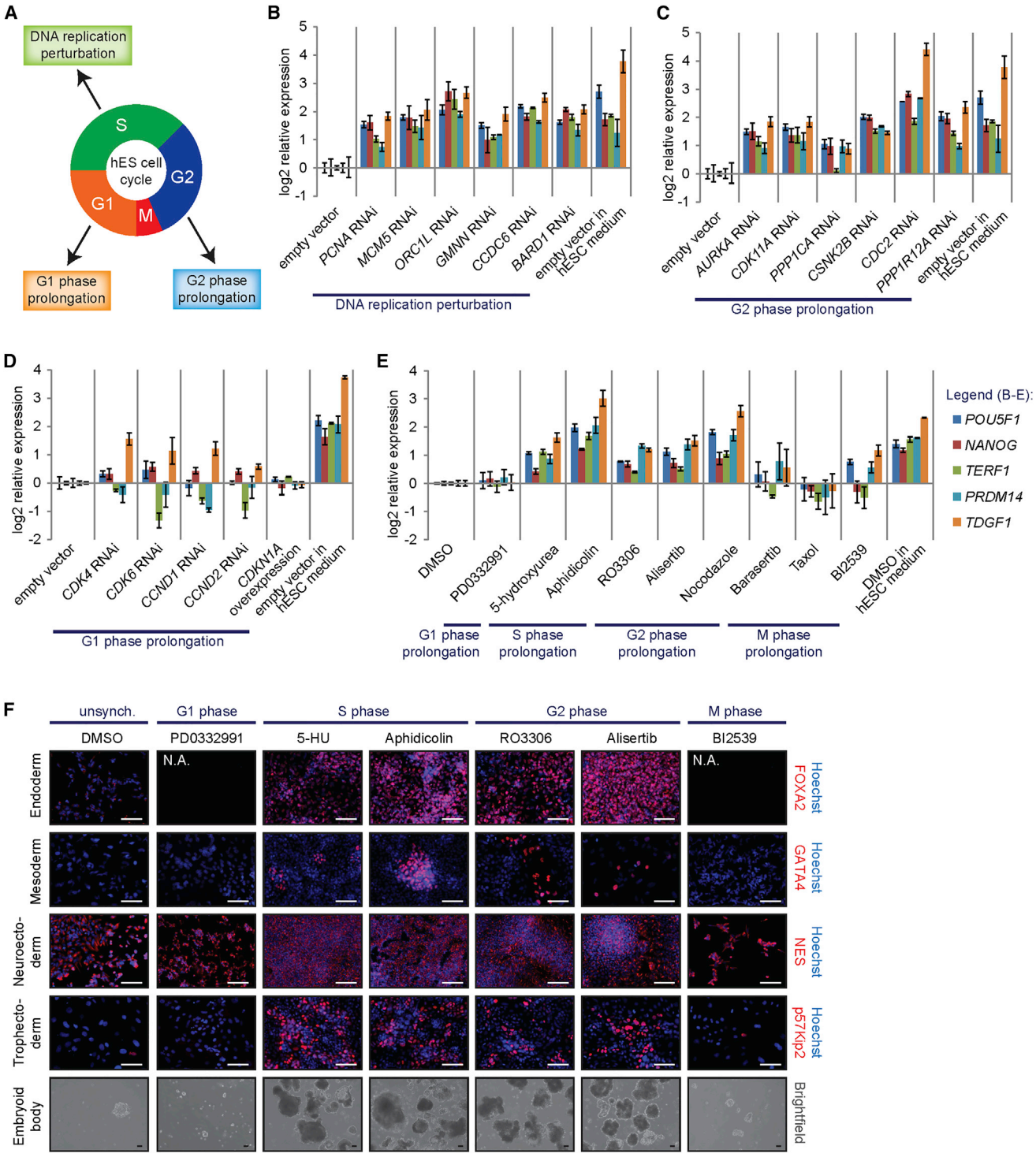

Figure 3. Deterministic Regulation of the Cell Cycle on PSD

(A) Schematic outlining the cell-cycle perturbations at specific phases.

(B–E) qPCR for pluripotency genes upon genetic perturbations of (B) DNA replication, (C) G2 phase progression, and (D) G1 phase progression or upon (E) treatment with small molecules enriching for specific cell-cycle phases under the −bFGF, −TGF-β condition. Triplicate data are represented as mean ± SD.

(F) Images for embryoid bodies and immunofluorescence staining of differentiated cells derived from H1 hESCs treated with various cell-cycle inhibitors under the −bFGF, −TGF-β condition. N.A., no cells survived. Scale bars, 100 μm.

See also Figures S3 and S4.

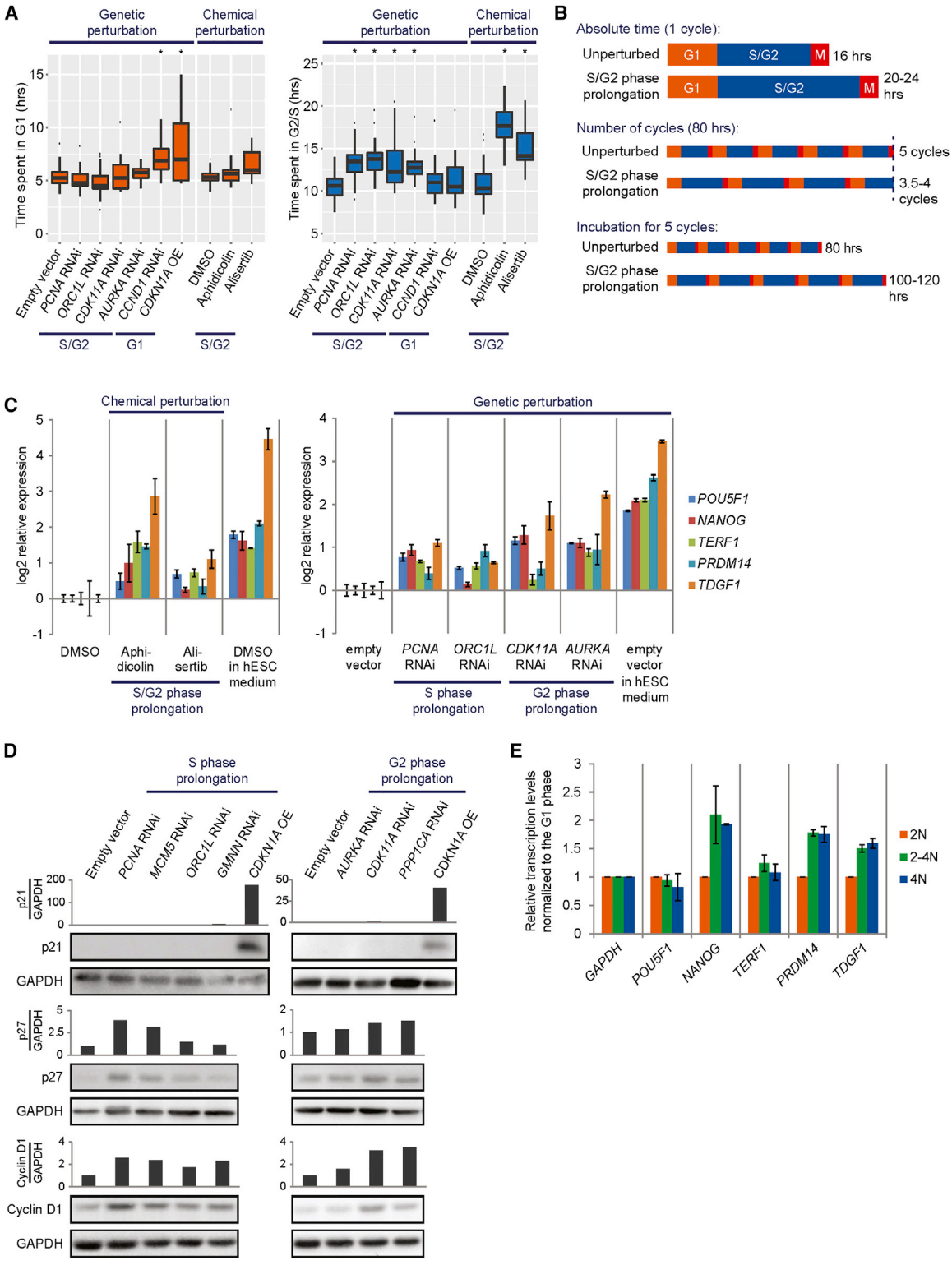

Figure 4. The Propensity for Promoting Pluripotency in S and G2 Phases Is Intrinsic and G1 Phase Independent

(A) Boxplots depicting time spent in G1 and S/G2 phases as calculated using live cell imaging of FUCCI H1 hESCs after various cell-cycle perturbations. *$p < 0.05$ compared with empty vector or DMSO control.

(B) Diagram depicting the difference in lengths of time-controlled and cell-cycle number-controlled experiments.

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that perturbing DNA replication in S phase as well as delaying mitotic transition from G2 phase consistently deterred PSD in different hESC lines (Figure 3E; Figures S4D–S4G). In contrast, inhibitors that led to an elongation of G1 and M phases did not exhibit PSD attenuation (Figure 3E; Figures S4D–S4G), proving that restriction of PSD is not conferred by simply locking cell-cycle progression. Importantly, these observed changes in marker expression ultimately influenced the functional pluripotency of hESCs (Figure 3F).

Because cell-cycle manipulations in ESCs inevitably lead to a certain degree of cell death (Ruiz et al., 2011; Figures S4B and S4C), we wanted to ascertain that the effects of cell-cycle perturbation on PSD are not just a secondary effect of cell death. We observed that the levels of induced cell death did not correlate with the effects of cell-cycle perturbation on PSD (Figure S4H). Additionally, inhibition of apoptosis during perturbation of S or G2 phase did not alter the observed delay in PSD (Figure S4I). These findings collectively indicate that the effects of cell-cycle perturbation on PSD are independent of cell death.

Together, these data demonstrate that the cell cycle can dominantly influence PSD. More importantly, these results also indicate that resistance to PSD is potentially mediated by specific cell-cycle events in S and G2 phases.

The Propensity of S and G2 Phases toward Pluripotent State Maintenance Is Intrinsic and G1 Phase Independent

G1 phase of the cell cycle has been correlated to susceptibility to differentiation, attributed to a higher expression of differentiation markers (Singh et al., 2013) and the enrichment of cell-cycle factors like cyclin-dependent kinase (CDK) inhibitors and G1 cyclins that contribute to lineage specification (Li et al., 2012; Pauklin and Vallier, 2013). Because the reduction in proportion of cells in G1 phase was observed when we delayed progression in the S and G2 phases (Figures S3C and S3D and S4A), it can be argued that the resulting PSD block could be an indirect effect of an inaccessibility to G1 phase or G1-associated factors that initiate differentiation.

To test this hypothesis, we first measured the actual time cells spend in G1 and S/G2 phases using the fluorescent ubiquitination-based cell-cycle indicator (FUCCI) reporter (Sakaue-Sawano et al., 2008). Although hESCs spent more time in S/G2 phase when we interfered with DNA replication and G2 phase progression, the absolute time in G1 phase remained largely unchanged (Figure 4A). However, the collective time spent in G1 phase was still decreased when we allowed the cells to differentiate for the same period of time (Figure 4B). Therefore, we measured pluripotency marker expression after an equal number of cell divisions in untreated and cell-cycle-perturbed hESCs (Figure 4C). In these experiments, we observed that PSD was still attenuated in hESCs with delayed S or G2 phase progression (Figure 4C), ruling out the possibility that the restriction of PSD could be a result of G1 phase inaccessibility.

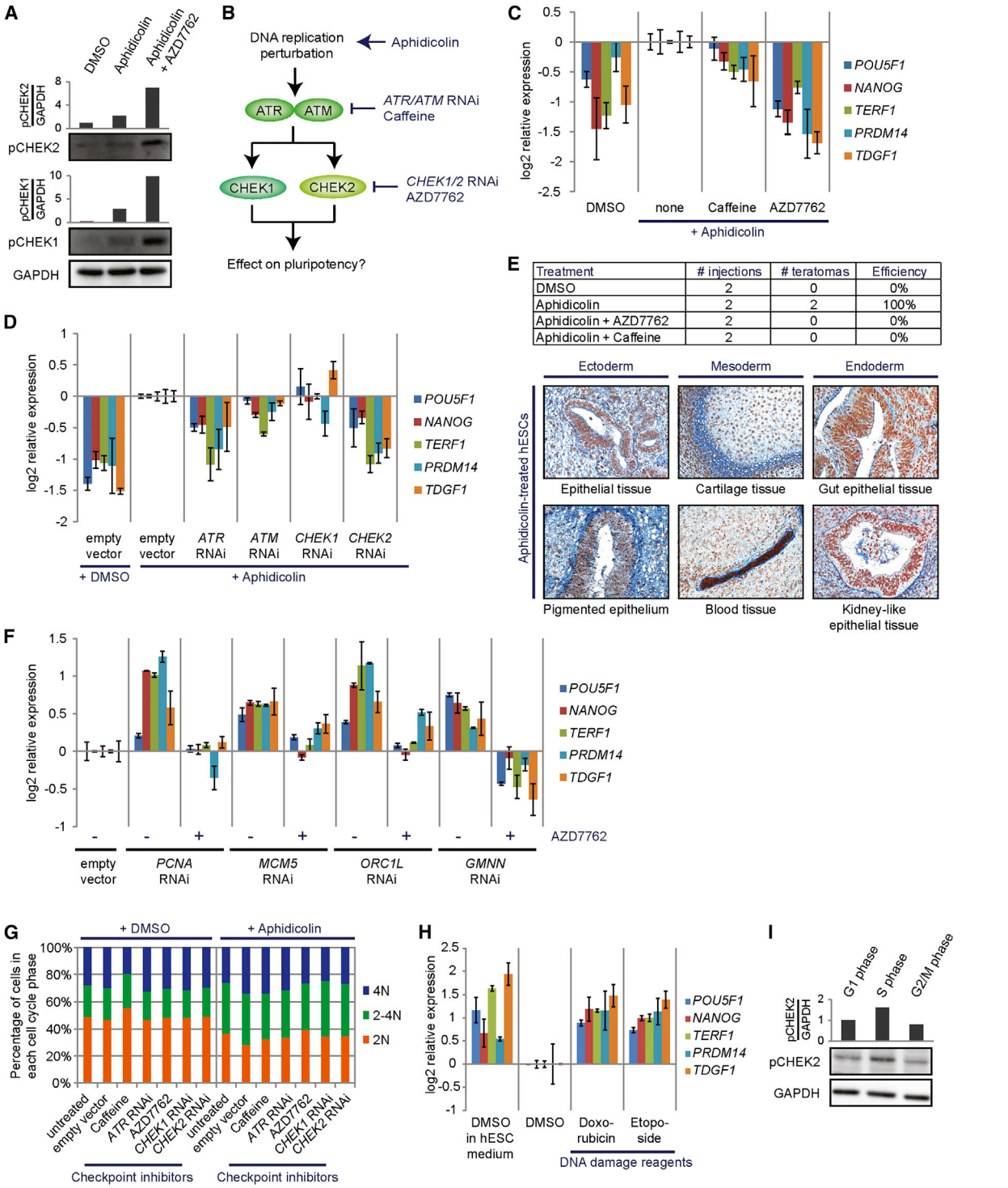
We next checked whether the expression levels of G1 phase-specific factors that are implicated in differentiation were decreased. Neither CDK inhibitors, such as p21 and p27, nor Cyclin D were downregulated upon knockdown of S or G2 phase-related hits (Figure 4D). In fact, the protein levels of these G1-associated factors were increased slightly, indicating that the consequent prevention of PSD is definitely not due to an unavailability of G1-associated lineage specification factors. Because delayed PSD could not be explained via the length of G1 phase or by its associated factors, this phenomenon might stem from a direct effect of S- and G2-specific pathways, which render these phases of the cell cycle intrinsically inclined toward pluripotent state maintenance.

We also examined the transcription of pluripotency factors to look for potential cell-cycle phase-specific regulation. The steady-state levels of pluripotency genes have been found previously to remain similar across the hESC cycle (Singh et al., 2013). However, when we examined de novo transcription levels of pluripotency regulators in different cell-cycle phases, genes such as *NANOG* and *PRDM14* were preferentially transcribed during S and G2/M phases compared with G1 phase (Figure 4E). This suggests that the S and G2 phases may be intrinsically wired into maintaining pluripotency, therefore delaying PSD when progression through these cell-cycle phases is altered.

Activation of the ATM/ATR-Mediated Checkpoint at S Phase Attenuates PSD

To look for the specific cell-cycle machineries that uphold the pluripotency network, we first checked pathways that are activated when S phase is perturbed. Perturbation of DNA replication generates replication stress, which can lead to DNA damage accumulation and subsequent activation of the ATM/ATR-mediated checkpoint (Bakkenist and Kastan, 2003; Zou and Elledge, 2003). Indeed, we observed elevated γH2AX foci (Figure S5A) and CHEK1/2 phosphorylation (Figure 5A; Figure S5B) after perturbation of DNA replication. To test whether this activation of the checkpoint is causative for blocking PSD, we subdued ATM/ATR-mediated checkpoint signaling in hESCs using various checkpoint inhibitors (Figure 5B). Genetic and chemical inhibition of the sensor kinases ATM and ATR or of the effector kinase CHEK2 reversed the block on PSD by aphidicolin, as evidenced by the downregulation of pluripotency markers (Figures 5C and 5D; Figures S5C–S5F). These changes in gene expression translate to functional pluripotency, as observed in teratoma formation assays (Figure 5E). In addition, inhibition of the checkpoint similarly abolished the PSD delay resulting from knockdown of replication-associated hits (Figure 5F). These results from both genetic and chemical approaches demonstrate that signaling through the ATM/ATR-CHEK2 axis directly contributes to pluripotency. Interestingly, in all cases where the checkpoint activated by aphidicolin treatment was abolished, DNA replication remained largely delayed (Figure 5G). This verifies that the PSD block is not a result of a simple S phase lock or the physical state

(C) qPCR for pluripotency genes under the −bFGF, −TGFβ condition upon cell-cycle perturbations, with data collection time points normalized to the number of cell cycles. Triplicate data are represented as mean ± SD.
(D) Western blot for p21, p27, and Cyclin D1 levels upon knockdown of S and G2 phase-associated hits.
(E) qPCR for nascent transcripts of pluripotency genes collected from H1 hESCs sorted into different cell-cycle phases according to DNA content.



Treatment	# injections	# teratomas	Efficiency
DMSO	2	0	0%
Aphidicolin	2	2	100%
Aphidicolin + AZD7762	2	0	0%
Aphidicolin + Caffeine	2	0	0%

Figure 5. ATM/ATR-CHEK2 Prevents PSD upon Replication Arrest

(A) Western blot of phosphorylated CHEK2 and CHEK1 proteins upon treatment with cell-cycle inhibitors.

(B) Outline of manipulations performed on the ATM/ATR pathway.

(C and D) qPCR for pluripotency genes under the −bFGF, −TGFβ condition upon treatment with aphidicolin concomitant with (C) treatment with short hairpin RNAs (shRNAs) against ATM/ATR pathway members or (D) inhibition of the ATM/ATR pathway with small molecules. Triplicate data are represented as mean ± SD.

(E) Table describing teratoma formation efficiencies of H1 hESCs treated with aphidicolin, AZD7762, and caffeine under the −bFGF, −TGFβ condition, and teratoma formation assay for aphidicolin-treated cells.

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of DNA during replication but a direct result of ATM/ATR-mediated checkpoint activation. Finally, activation of the ATM/ATR pathway by DNA damage-inducing reagents similarly attenuated PSD (Figure 5H; Figure S5G), confirming that checkpoint activation can directly block PSD.

The activity of the ATM/ATR pathway on PSD is specifically downstream of the S phase replication perturbation because increased CHEK2 phosphorylation was not observed when perturbing other cell-cycle phases (Figure S5H). Besides, inhibiting checkpoint signaling did not change PSD kinetics when perturbing the other cell-cycle phases (Figure S5I). Interestingly, we observed higher activity of the ATM/ATR pathway in unperturbed hESCs in S phase compared with other cell-cycle phases (Figure 5I), suggesting that this pathway may also contribute to the intrinsic propensity of S phase toward pluripotent state maintenance. In summary, these results demonstrate the ATM/ATR-mediated checkpoint is the dominant PSD inhibitor downstream of DNA replication perturbation and, likely, also during normal S phase progression.

The ATM/ATR-Mediated Checkpoint Activates p53 to Enhance TGF-β Signaling and Uphold Pluripotency

To obtain a mechanistic connection between ATM/ATR-CHEK2 activation and pluripotent state retention in hESCs, we performed a time course microarray analysis in hESCs treated with the DNA replication inhibitor aphidicolin and the checkpoint inhibitor AZD7762 (Figure 6A). Upon aphidicolin treatment, we observed an upregulation of genes involved in active TGF-β signaling and a simultaneous downregulation of BMP4 pathway genes and TGF-β pathway antagonists (Figures 6B and 6C; Figures S6A and S6B). The changes in TGF-β pathway gene expression led to enhanced TGF-β signaling, as indicated by increased SMAD2 phosphorylation (Figure 6D; Figure S6C), and can be mimicked by knockdown of replication-related hits (Figure 6E; Figure S6D). Importantly, the augmentation of TGF-β signaling can be reversed by checkpoint inhibitor treatment (Figures 6B–6D; Figure S6E), confirming that the effect on TGF-β signaling is directly from checkpoint activation. These results therefore establish that the ATM/ATR-CHEK2 axis augments TGF-β signaling.

The TGF-β pathway has a well-known role in promoting the human pluripotent state (James et al., 2005), which we observed to occur in a dose-dependent manner (Figure S6F). Therefore, it can be inferred that heightened TGF-β signaling is responsible for PSD inhibition during replication perturbation. However, it is also possible that the block in PSD occurs upstream of the changes in TGF-β-related gene expression. We therefore compared time course expression profiles upon the withdrawal of self-renewal signals and observed that TGF-β-related gene expression changes occur earlier and to a greater degree compared with pluripotency marker expression changes (Figure 6C). This implies that the changes in TGF-β-related gene expression are under closer control of the ATM/ATR-mediated checkpoint compared with pluripotency genes. In addition, these changes were detected even when cells were in hESC medium (Figure S6E), and altered TGF-β-related expression does not simply correlate with pluripotency status (Figure S6G). These data rule out the possibility that changes in TGF-β-related gene expression could be a secondary effect from delayed PSD and support the notion that pluripotent state preservation occurs as a result of the initial augmentation of TGF-β signaling.

Changes in TGF-β-related gene expression upon checkpoint activation indicate an underlying transcriptional regulatory mechanism. To find out the responsible transcription factor, we first looked at the canonical transcription factor p53 because it is activated by the ATM/ATR-CHEK2 axis during DNA damage response in S phase (Banin et al., 1998; Hirao et al., 2000; Tibbetts et al., 1999; Figure 6D; Figures S6C, S6D, and S6H) and was observed to have binding sites near TGF-β-related genes in hESCs (Akdemir et al., 2014). We therefore manipulated p53 levels in hESCs to observe its effect on the prevention of PSD by checkpoint activation (Figure 6F). *TP53* knockdown (Figure S6I) in checkpoint-activated hESCs not only abolished the delay in PSD (Figure 6G) but also reversed the altered expression of TGF-β-related genes (Figure 6H), proving its necessity in impeding PSD. Moreover, stabilization of p53 without DNA damage using Nutlin-3 (Figures S6J and S6K) is sufficient to delay pluripotency marker downregulation (Figure 6I) and alter TGF-β-related gene expression (Figure 6J) in the absence of self-renewal factors. Importantly, Nutlin-3 treatment did not induce significant changes in cell-cycle profile of hESCs (Figure S6L), confirming that p53 can directly delay PSD independent of cell-cycle changes. These data demonstrate both the necessity and sufficiency of p53 in upholding the human pluripotent state. Finally, in line with the elevated ATM/ATR activity during S phase (Figure 5I), we observed higher p53 protein and SMAD2 phosphorylation levels in S phase-sorted hESCs (Figure S6M), implying that the mechanisms discussed work similarly during normal progression through the cell cycle.

Collectively, our data strongly argue for a detailed mechanism behind how S phase perturbation upholds pluripotency (Figure 6K). In the presence of replication stress or DNA damage, p53 stabilization by ATM/ATR-CHEK2 signaling enhances TGF-β pathway signaling, which consequently sustains *NANOG* expression (Vallier et al., 2009; Xu et al., 2008) and preserves the pluripotent state.

High Levels of Cyclin B1 in G2 Phase Attenuate PSD

We finally looked at how the enrichment of hESCs at G2 phase could delay PSD (Figure 7A). Genetic and chemical perturbations

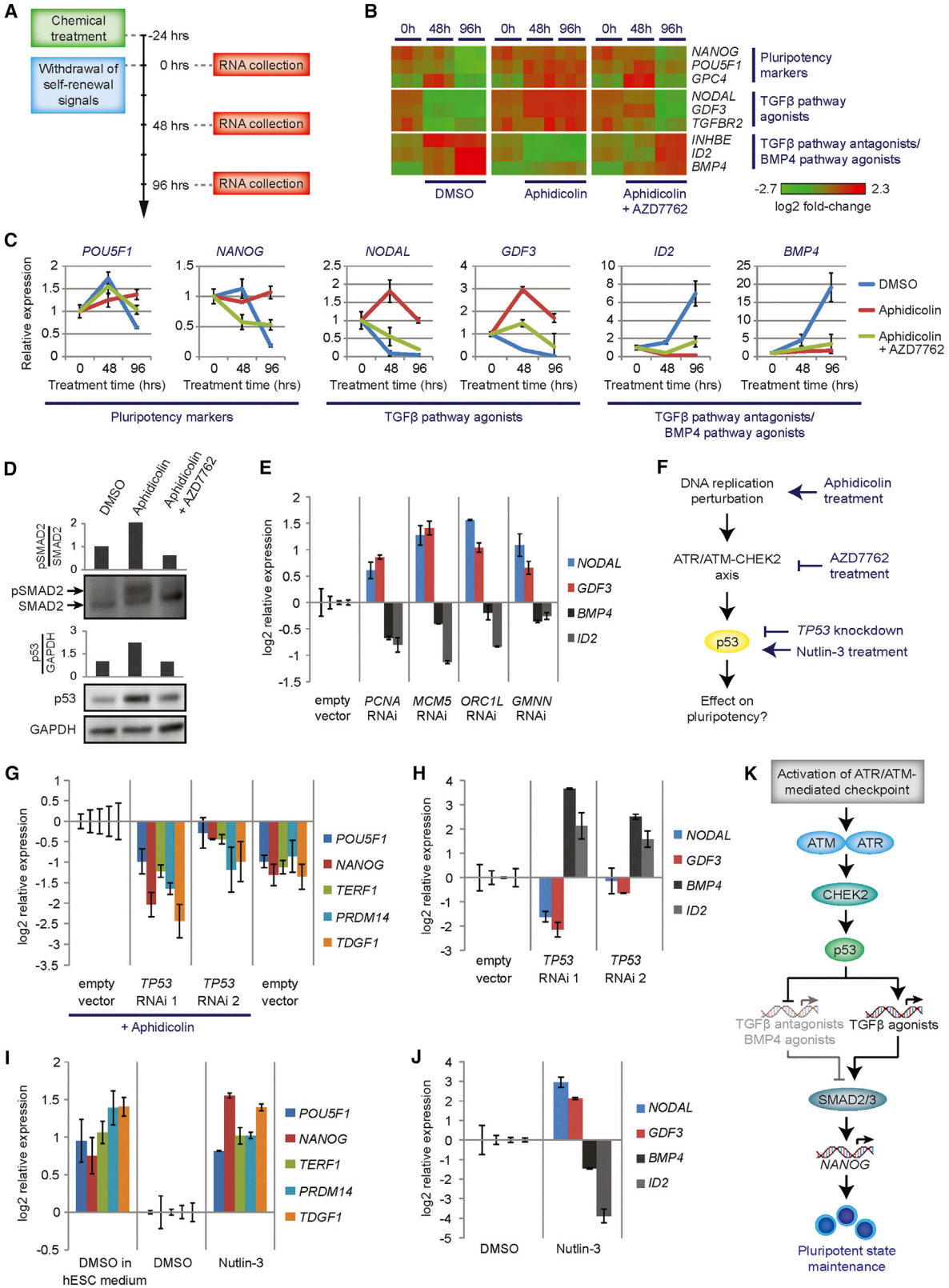
(F) qPCR for pluripotency genes upon knockdown of S phase-associated hits together with DMSO or AZD7762 treatment under the −bFGF, −TGFβ condition. Triplicate data are represented as mean ± SD.

(G) Fluorescence-activated cell sorting (FACS) quantification of Hoechst staining indicating the cell-cycle status of H1 hESCs after ATM/ATR pathway inhibition in hESC medium.

(H) qPCR for pluripotency genes under the −bFGF, −TGFβ condition upon treatment with DNA damage-inducing reagents. Triplicate data are represented as mean ± SD.

(I) Western blot for phosphorylated CHEK2 levels in H1 hESCs sorted according to cell-cycle phases based on DNA content.

See also Figure S5.



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of G2 phase progression resulted in a consistent elevation of Cyclin B1 expression (Figure 7B; Figure S7A), but not other cyclins (Figure 7B). Furthermore, Cyclin B1 was higher in hESCs sorted in G2 phase (Figure S7B). Therefore, we hypothesized that Cyclin B1 might be the underlying cause for promoting the pluripotent state during G2 phase. Interestingly, when we knocked down Cyclin B1 in hESCs (Figure S7C), a dramatic downregulation of pluripotency marker expression was observed prior to cell death (Figure 7C; Figure S7D), indicating a tight linkage between Cyclin B1 and pluripotency. Cyclin B1 knockdown enriched cells in G2 phase instead of G1 phase (Figure 7D), strikingly demonstrating a decoupling of the expected cell-cycle profile associated with differentiation. We furthermore overexpressed Cyclin B1 in hESCs (Figure S7E) and demonstrated that overexpression of a single cell-cycle factor can delay PSD (Figure 7E; Figure S7F). Moreover, Cyclin B1 overexpression did not cause a significant enrichment of cells in G2 phase (Figure 7F), indicating that Cyclin B1 prevents PSD downstream of G2 phase prolongation. Importantly, these observations extend to protein levels of pluripotency markers (Figure S7G), are replicable in other hESC lines (Figures S7H and S7I), and, ultimately, affect functional pluripotency (Figure 7G). Together, these results indicate that Cyclin B1 is a connecting node between G2 phase and its ability to prevent PSD. To investigate how Cyclin B1 promotes the pluripotent state, we performed a time course microarray analysis of hESCs overexpressing Cyclin B1. Interestingly, we also found an initial upregulation of TGF-β agonists (Figures S7J and S7K), suggesting that Cyclin B1 might also work through TGF-β to prevent PSD.

DISCUSSION

The Context-Dependent Regulation of PSD

Differentiation necessitates the disintegration of the pluripotency network. However, how differentiation cues lead to the breakdown of this network is ill-defined, especially in hESCs. To identify genes required for PSD, we performed a systematic large-scale RNAi screen under multiple conditions to examine the regulation of PSD given various cues initiating differentiation. First, we observed that the regulation of PSD upon RA introduction is starkly divergent from the conditions under which hESCs were deprived of self-renewal signals (Figure 2C). This implies that the effectors of PSD vary when self-renewal signals are withdrawn versus when a differentiation signal is introduced and that the mode of differentiation induction influences PSD regulation. Second, our multi-conditional screening approach identified both general and context-specific regulators of PSD. We found that the withdrawal of self-renewal signals is associated with several common PSD effectors. Members of chromatin-modifying complexes such as HAT complexes and the SWI/SNF complex were highly enriched, emphasizing a universal need to restructure the chromatin to enable complete cell fate transition during PSD (Figures 2D–2F). Conversely, other PSD regulators, like RNA splicing and Wnt signaling, tend to function in a more context-dependent manner (Figure 2B). Finally, we found that multiple members of certain complexes play a role in PSD (Figures 2B and 2D), providing a starting point to sift out the function of different members of protein complexes by revealing the ones which are specifically important for PSD in hESCs. Together, our large-scale RNAi study provides a unique resource for dissecting the mechanisms behind PSD by providing new insights into the universal roles of various factors and pathways on PSD while raising important notions about the inherent context specificity of the regulatory network governing PSD in hESCs.

The Deterministic Effect of Cell-Cycle States on PSD

During proliferation, cells experience dramatic biochemical and physical changes by going through the cell cycle. This results in distinct cell-cycle states, which cells have evolved to utilize to prime and regulate other events that are not immediately related to proliferation, such as immune response, metabolism, and lineage specification (Lee et al., 2014; Pauklin and Vallier, 2013; Rodier et al., 2009). Despite increasing evidence showing that the cell cycle regulates other cellular processes, there is no direct or functional evidence that cell-cycle states can control the pluripotency network and its dissolution. Here we demonstrate that specific cell-cycle states dominantly block PSD in hESCs. Specifically, when progression through the S or G2 phases of the hES cell cycle is perturbed, cells are enriched at specific cell-cycle states with machineries that trigger a selective preference toward pluripotency maintenance and delay PSD (Figure 3). Particularly, the ATM/ATR-CHEK2-mediated activation of p53 and Cyclin B1 upregulation during arrest at the S and G2 phases, respectively, defines their respective cell-cycle states and exerts control over the pluripotency network. Moreover, these pathways are not only activated at the perturbed states but are also involved in natural cell-cycle progression (Figure 5I; Figures S6M and S7B). Therefore, these pathways and their effects in upholding pluripotency can be applied to normal

Figure 6. Augmentation of the TGF-β and p53 Pathways by the ATM/ATR-Mediated Checkpoint

(A) Schematic of the time course microarray experiment.

(B) Microarray heatmap for differentially expressed genes upon treatment with aphidicolin and AZD7762 under the −bFGF, −TGFβ condition.

(C) Time course qPCR for pluripotency and TGF-β-related genes upon treatment with aphidicolin and AZD7762 under the −bFGF, −TGFβ condition. Triplicate data are represented as mean ± SD.

(D) Western blot of phospho-SMAD2 and p53 proteins upon treatment of H1 hESCs with aphidicolin and AZD7762.

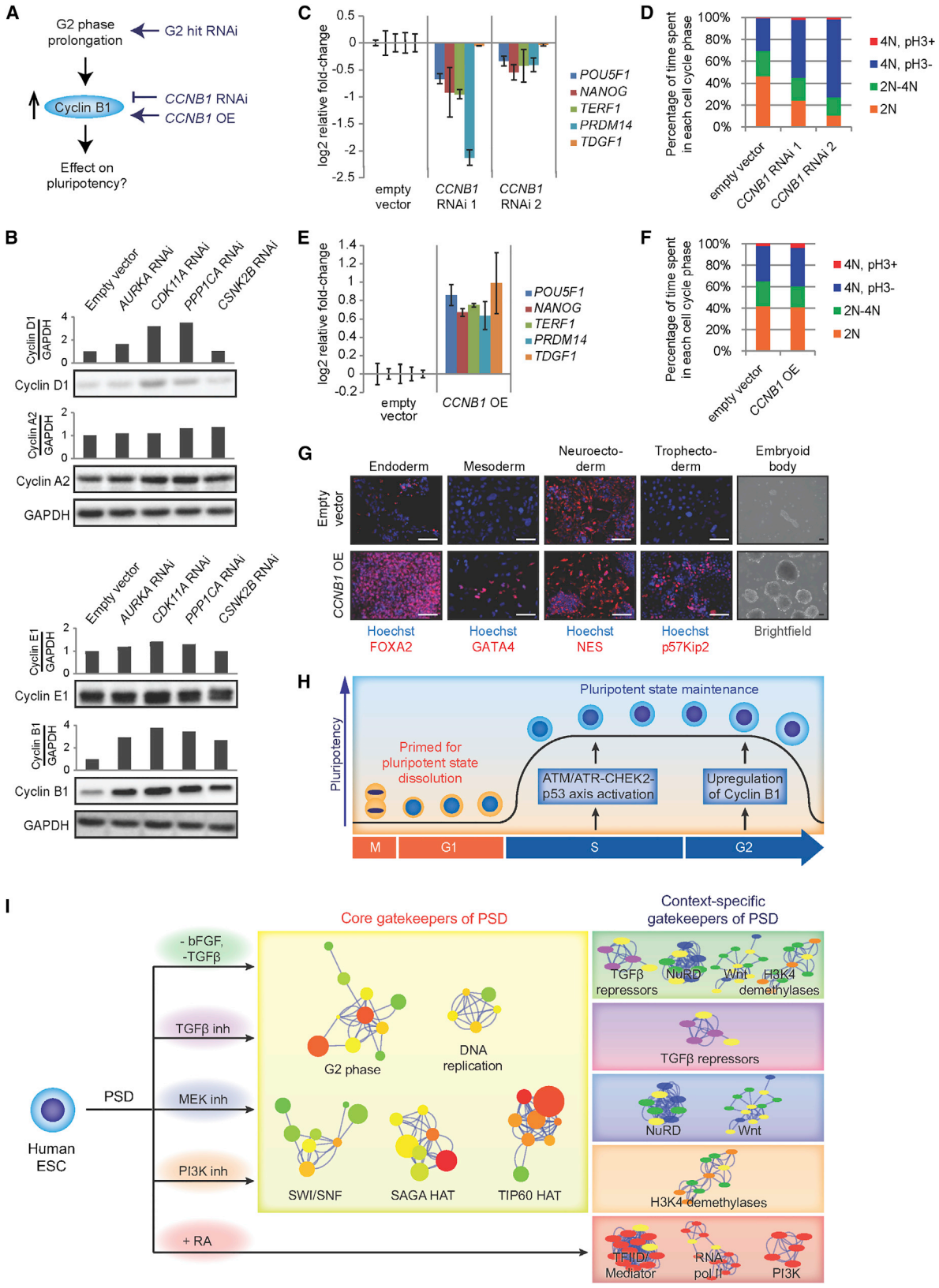
(E) qPCR for TGF-β agonists (color) and antagonists (grayscale) under the −bFGF, −TGF-β condition upon knockdown of replication-related genes.

(F) Outline of experimental manipulations performed on the ATM/ATR-CHEK2-p53 axis.

(G–J) qPCR of H1 hESCs under the −bFGF, −TGF-β condition for (G) pluripotency genes and (H) TGF-β agonists (color) and antagonists (grayscale) upon knockdown of *TP53* concomitant with treatment with aphidicolin or (I) pluripotency genes and (J) TGF-β agonists (color) and antagonists (grayscale) upon stabilization of p53 with Nutlin-3. Triplicate data are represented as mean ± SD.

(K) Schematic outlining the mechanism behind the regulation of pluripotency by the ATM/ATR-mediated checkpoint.

See also Figure S6.



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progression through S and G2 phases. Overall, our investigation provides the first evidence that cell-cycle machineries corresponding to specific states can directly and dominantly regulate the human pluripotent state.

Furthermore, the employment of the said pathways by the S and G2 phases establish their active role in boosting the pluripotent state. This differs from the current perspective that the S and G2 phases passively retain the pluripotent state given that only G1 phase harbors a propensity to receive extracellular differentiation signals and express lineage-specific factors (Scott et al., 1982; Singh et al., 2013). In fact, we hypothesize that the absence of such pathways in G1 phase may underlie its responsiveness to differentiation cues. Therefore, we propose that the S/G2 and G1 phases shift the weights between pluripotency maintenance and PSD priming across the hES cell cycle (Figure 7H). This model advocates that a balance between cell-cycle phases is critical for ESC fate determination, unlike the previously recognized G1-centric model.

Consequences of Fate Choices upon DNA Damage in Pluripotent Stem Cells

During proliferation, stem cells can encounter various situations, like replication stress, DNA damage, and checkpoint activation. How stem cells make their fate choices in response to these special but prevalent cell-cycle events has direct consequences on genome stability, tissue development, and stem cell maintenance. Here we demonstrate that pluripotent stem cells resist cell fate changes in the presence of replication-induced DNA damage (Figure 5). We further show that the DNA damage-induced checkpoint stabilizes p53 to regulate TGF-β pathway gene expression and retain pluripotency marker expression (Figure 6). As a hub transcription factor, p53 is known to enact diverse functions under different cellular contexts and activation dynamics (Aylon and Oren, 2007; Chang et al., 2011; Ubil et al., 2014). Although p53 activation has been thought to trigger apoptosis or differentiation in hESCs (Qin et al., 2007), chromatin binding of p53 differs depending on whether it is activated by a differentiation signal or DNA damage (Akdemir et al., 2014). Here we demonstrate a new perspective where p53 acts to maintain the human pluripotent state upon the withdrawal of self-renewal factors (Figures 6F–6J). By retaining the pluripotent cell fate in the face of DNA damage, cells benefit from an increased efficiency of DNA damage repair because ESCs are known to express higher levels of homologous recombination and damage repair proteins (Momcilovic et al., 2010; Tichy et al., 2010). Furthermore, if the damage incurred is beyond the capacity to repair, then hESCs have the choice to undergo apoptosis more effectively because of a lower apoptotic threshold in hESCs compared with differentiated cells (Dumitru et al., 2012; Liu et al., 2013). As a result, pluripotent stem cells can more efficiently resolve damage before committing to differentiation or, if the damage is beyond repair, terminate themselves to avoid the potential detriment of giving rise to lineage progenitors with damaged genomes.

Conclusions

In conclusion, our systematic functional screening of hESCs under multiple differentiation conditions enabled the identification of both universal and specific gatekeepers for PSD (Figure 7I). We unravel a universal regulatory role for HAT complexes and the SWI/SNF complex in PSD, whereas the NuRD complex, RNA splicing, and signaling pathways regulate PSD in a context-specific manner. Our screen unexpectedly discovered a strong enrichment of cell-cycle hits clustering specifically in S and G2 phases but not in other phases of the cell cycle. Our mechanistic studies demonstrate that the S and G2 phases possess an intrinsic propensity toward the pluripotent state, mediated by the ATM/ATR-CHEK2-p53 and Cyclin B1 pathways, respectively. Therefore, we introduce a new paradigm for the coupling of cell cycle and pluripotency in which the S and G2 phases employ pathways that inhibit PSD and maintain ESC identity, whereas the absence of such pathways in the G1 and M phases potentially contributes to their amenability to PSD (Figure 7H).

EXPERIMENTAL PROCEDURES

Cell Culture

The hESC lines H1 (WA-01), HES2, HES3, *NANOG-GFP* H1 reporter, and *ACTIN-GFP* H1 reporter were used for this study. They were cultured feeder-free on Matrigel (BD Biosciences) with mTeSR1 medium (STEMCELL Technologies). The medium was changed daily. The hESCs were routinely subcultured with 1 U/ml Dispase in DMEM/F12 (STEMCELL Technologies) every 4–5 days. For experiments, hESCs were passaged using TrypLE Express (Life Technologies) before treatment. See Supplemental Experimental Procedures for experimental treatment protocols.

High-Throughput RNAi Screening

Pooled small interfering RNAs (siRNAs) (2.5 μl of 1 μM; Dharmacon, Ambion) were printed on 384-well plates (Greiner) coated with 30×-diluted Matrigel (BD Biosciences) and frozen at −80°C before use. Lipofectamine RNAi Max (Invitrogen) diluted 200× in 5 μl OptiMEM (Invitrogen) was added per well

Figure 7. Cyclin B1 Attenuates PSD upon G2 Phase Prolongation

(A) Outline of manipulations performed on Cyclin B1.

(B) Western blot of cyclin levels upon knockdown of G2-associated hits.

(C) qPCR for pluripotency genes upon knockdown of Cyclin B1 in hESC medium. Triplicate data are represented as mean ± SD.

(D) FACS quantification indicating the cell-cycle status of H1 hESCs based on DNA content after Cyclin B1 knockdown in hESC medium.

(E) qPCR for pluripotency genes under the −bFGF, −TGF-β condition upon overexpression of Cyclin B1. Triplicate data are represented as mean ± SD.

(F) FACS quantification indicating the cell-cycle status of H1 hESCs based on DNA content after Cyclin B1 overexpression in hESC medium.

(G) Images for embryoid bodies and immunofluorescence staining of differentiated cells derived from H1 hESCs overexpressing Cyclin B1 under the −bFGF, −TGF-β condition. Scale bars, 100 μm. See also Figure S7.

(H) Model for the deterministic regulation of the cell cycle on PSD. The G2 and S phases are inclined toward pluripotent state maintenance because of active pluripotent state-promoting pathways.

(I) Summary of the findings from the high-throughput RNAi screen for PSD. Both core and context-specific gatekeepers were uncovered, providing a mechanistic model of PSD regulation in hESCs. The color scheme for core gatekeepers follows Figure 2D, whereas the color scheme for context-specific gatekeepers follows Figure 2B.

and incubated at room temperature for 20 min. 3,000 *NANOG-GFP* H1 hESCs in 45 μl mTeSR1 (STEMCELL Technologies) were seeded into each well. 24 hr after seeding, mTeSR1 was replaced with the following differentiation media: −bFGF, −TGF-β condition (mTeSR1 without select growth factors [STEMCELL Technologies]), TGF-β pathway inhibition (mTeSR + 1 μM A8301 [Stemolecule]), MEK pathway inhibition (mTeSR1 + 2.5 μM PD0325901 [Sigma]), PI3K pathway inhibition (mTeSR1 + 20 μM LY294002 [STEMCELL Technologies]), and +RA (mTeSR1 + 20 μM RA [Sigma]). Cells were incubated in differentiation media for 120 hr for condition 1 and 48 hr for conditions 2–5. Media were then replaced with mTeSR1 and incubated for another 24 hr. Cells were then fixed with 4% paraformaldehyde (Sigma) and stained with Hoechst 3342 (1:4,000, Invitrogen). Microscope images were acquired using ImageXpress Ultra (Research Instruments) at 20× magnification and quantified using MetaXpress image acquisition and analysis software. See Supplemental Experimental Procedures for informatics analysis.

qRT-PCR

RNA extraction using TRIzol (Invitrogen), reverse transcription using Superscript II (Invitrogen), and qPCR using SYBR Green (KAPA) were all performed via standard procedures. Measured transcripts were normalized to GAPDH levels.

Immunoblotting and Immunostaining

Immunoblotting and immunostaining were performed as conventional procedures. See Supplemental Experimental Procedures for details and antibodies used.

Microarray

mRNAs derived from hESCs were reverse-transcribed, labeled, and analyzed on an Illumina microarray platform (HumanHT-12 v4 Expression BeadChips) according to the manufacturer's instructions. Rank invariant normalization was applied.

ACCESSION NUMBERS

The accession numbers for the microarray data reported in this paper are GEO: GSE62062, GSE63215.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.07.001.

AUTHOR CONTRIBUTIONS

K.A.G. designed research, performed experiments, analyzed data, wrote the paper, and supervised the overall project. H.L. designed research, performed experiments, analyzed data, and wrote the paper. Y.S.L., Y.S.C., J.C.Y., C.P.T., B.G., B.L., Z.Y.T., and K.Y.L. performed experiments. F.B. and Y.C.L. supervised the overall project. H.H.N. designed research, wrote the paper, and supervised the overall project.

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