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Irreversible APC^{Cdh1} Inactivation Underlies the Point of No Return for Cell-Cycle Entry

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SUMMARY

Proliferating cells must cross a point of no return before they replicate their DNA and divide. This commitment decision plays a fundamental role in cancer and degenerative diseases and has been proposed to be mediated by phosphorylation of retinoblastoma (Rb) protein. Here, we show that inactivation of the anaphase-promoting complex/cyclo-some (APC^{Cdh1}) has the necessary characteristics to be the point of no return for cell-cycle entry. Our study shows that APC^{Cdh1} inactivation is a rapid, bistable switch initiated shortly before the start of DNA replication by cyclin E/Cdk2 and made irreversible by Emi1. Exposure to stress between Rb phosphorylation and APC^{Cdh1} inactivation, but not after APC^{Cdh1} inactivation, reverted cells to a mitogen-sensitive quiescent state, from which they can later re-enter the cell cycle. Thus, APC^{Cdh1} inactivation is the commitment point when cells lose the ability to return to quiescence and decide to progress through the cell cycle.

In Brief

Live cell imaging of cell-cycle reporters, reveals that cells commit to cell-cycle entry much later than the restriction point, and that there's a window of time during, which a cell can return to quiescence, rather than moving forward through the, cycle.

Graphical Abstract

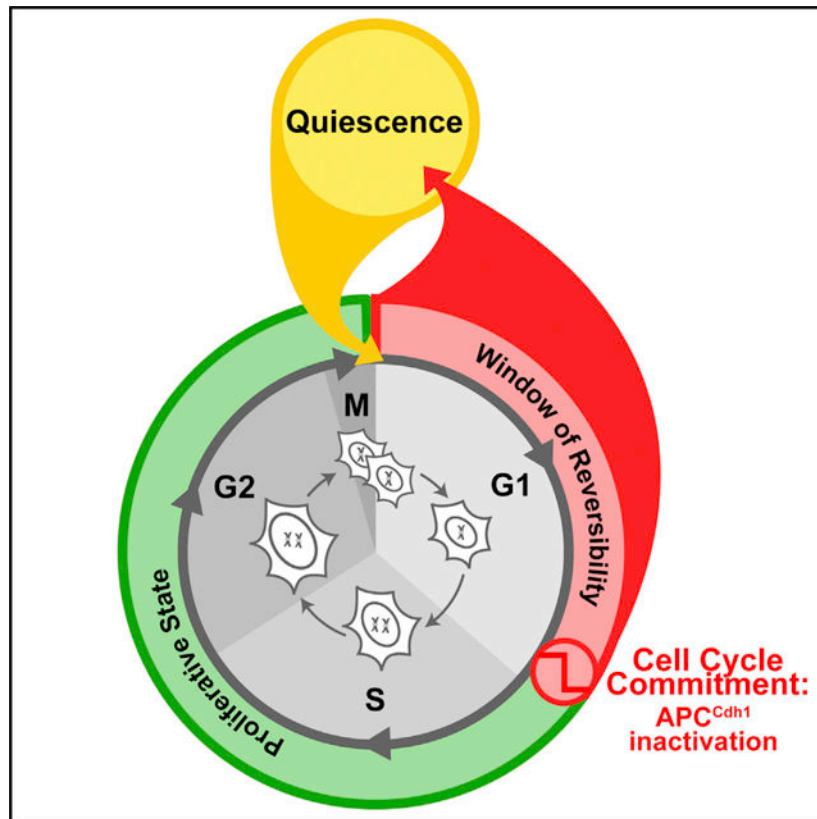
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AUTHOR CONTRIBUTIONS

Conceptualization, S.D.C. and T.M.; Methodology, S.D.C., M.C., S.L.S., and A.J.; Investigation, S.D.C., M.C., and A.J.; Writing - Original Draft, S.D.C. and T.M.; Writing - Review & Editing, S.D.C., M.C., S.L.S., A.J., and T.M.; Visualization, S.D.C.; Supervision, T.M.; Funding Acquisition, S.D.C. and T.M.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2016.05.077>.



INTRODUCTION

Many mammalian cells spend much of their time in a quiescent state in which they retain the potential to proliferate (Hsu et al., 2014). The decision of quiescent cells to enter the cell cycle must be tightly regulated to ensure that tissue homeostasis is maintained.

Dysregulation of this fundamental decision causes cancer and degenerative diseases (Hanahan and Weinberg, 2000). Quiescent cells enter the proliferating state in G1 before DNA replication starts (S) and also exit the proliferating state to go back to quiescence in G1 after completion of mitosis (M) (Pardee, 1974). Cells must already commit in G1 to complete a round of DNA replication and mitosis to prevent damage and ensure a faithful replication.

A long-standing question in cell biology has therefore been how quiescent cells make this decision to enter the cell cycle and commit to complete S and M phase (Planas-Silva and Weinberg, 1997). Pardee proposed over 40 years ago that a specific time point must exist until which cells can reverse their trajectory and return to the quiescent state and after which they cannot return to quiescence and will replicate their DNA and divide (Pardee, 1974) (Figure 1A). By pulsing external proliferation-promoting stimuli (mitogens), the study defined a “restriction point” early in G1 long before DNA replication begins when cells lose their need for mitogens and still complete the cell cycle. This and other studies proposed that the restriction point, characterized by mitogen sensitivity, might also constitute the commitment decision or point of no return for cell-cycle entry (Pardee, 1974; Zetterberg and

Larsson, 1985). The molecular basis for the restriction point has been proposed to be the hyperphosphorylation of the tumor suppressor retinoblastoma protein (Rb) and the consequent liberation of the cell-cycle transcription factor E2F (referred to as pRb-E2F activation) (Narasimha et al., 2014; Yao et al., 2008).

In addition to mitogen signaling, different stress signals including osmotic stress or UV/ionizing irradiation are known to inhibit or delay proliferation before cells start S phase (Deckbar et al., 2011; Erol, 2011). Several studies also observed that cells could be arrested in G1 even after Rb phosphorylation has already been induced (Duli et al., 1994; Vousden and Prives, 2009). This raises the question of whether cells that experience stress after Rb phosphorylation are already committed to the cell cycle but can pause before entering S phase or whether cells can return to quiescence even after Rb has been phosphorylated. The latter would argue that the point of no return must be after Rb phosphorylation.

If cells are able to return to quiescence after pRb-E2F activation, it is conceivable that inactivation of the E3 ubiquitin ligase anaphase-promoting complex/cyclosome-Cdh1 (APC^{Cdh1}), which is believed to occur after pRb-E2F activation and before DNA replication, may represent the true commitment point for cell-cycle entry. If correct, such a hypothesis predicts that stress perturbations would cause a reversible exit to quiescence until the inactivation of APC^{Cdh1} , but not after APC^{Cdh1} inactivation. APC^{Cdh1} is an interesting candidate for a commitment mechanism, as it functions to degrade many proteins necessary for S phase. Cycling cells activate APC^{Cdh1} at the end of mitosis, and quiescent cells maintain active APC^{Cdh1} to prevent re-entry into the cell cycle (Eguren et al., 2011). Cells lacking Cdh1 have a shortened G1 phase, accumulate DNA damage, and undergo apoptosis (Peters, 2006). Based on biochemical bulk-cell analysis, cells exiting mitosis have been proposed to gradually inactivate APC^{Cdh1} prior to S phase entry through the autonomous degradation of the E2 ligase UBCH10 (Rape and Kirschner, 2004). However, this inactivation mechanism has been called into question (Chang et al., 2014; Walker et al., 2008) and alternative inactivation mechanisms have been proposed (Huang et al., 2011; Lukas et al., 1999; Miller et al., 2006; Wang and Kirschner, 2013). This leaves the question open of how APC^{Cdh1} is inactivated and when it occurs in respect to the restriction point, pRb-E2F activation, the start of DNA replication, and cell-cycle commitment.

Our study shows that, in three cell types and in nearly every one out of thousands of individual cells analyzed, APC^{Cdh1} inactivates rapidly within <1 hr to <1% of its previous G1 activity shortly before cells start to replicate their DNA. APC^{Cdh1} inactivation is initiated by cyclin E/Cdk2 before being greatly accelerated and made irreversible by the APC^{Cdh1} -inhibitor Emi1. Strikingly, we found that stress stimuli can induce reversible exit from the cell cycle to a mitogen-sensitive quiescent state long after cells have phosphorylated Rb and crossed the restriction point but only until APC^{Cdh1} inactivation. APC^{Cdh1} inactivation exhibits marked hysteresis only in the presence of Emi1, with stress stimuli preventing APC^{Cdh1} inactivation before, but not restoring the activity after APC^{Cdh1} has been inactivated. Thus, APC^{Cdh1} is inactivated by a bistable irreversible switch long after the restriction point and shortly before the G1/S boundary and exhibits all necessary characteristics to be the commitment point when cells decide to progress through the cell cycle and divide.

RESULTS

Rapid, Near-Complete Inactivation of APC^{Cdh1} Is Triggered Shortly before the Start of DNA Replication

Our study focused on two questions: (1) can stress inputs trigger a return to quiescence after the restriction point and pRb-E2F induction and (2) does APC^{Cdh1} inactivation, instead of pRb-E2F induction, function as the point of no return after which cells commit to replicate their DNA and divide (Figure 1A). To measure APC^{Cdh1} inactivation kinetics in single cells, we used non-transformed human MCF10A breast epithelial cells stably expressing one half of the Fucci reporter system, comprised of mCherry conjugated to a peptide from Geminin containing its APC degron motif (APC-degron; Figure 1B) (Sakaue-Sawano et al., 2008). We analyzed changes in the concentration of the APC-degron reporter during the cell cycle by tracking thousands of single living cells over 24–48 hr using automated 96-well microscopy (Figure S1A).

Control experiments showed that, in mitogen-starved cells and in newly born cells after mitosis, two situations where APC^{Cdh1} is known to polyubiquitinate substrates and cause their degradation, APC-degron reporter levels were very low (Figures 1C, 1D, S1A, and S1B). Treatment with either Cdh1 small interfering RNA (siRNA) or the small molecule APC inhibitor proTAME caused buildup of the APC-degron reporter (Figures S1C–S1H). Furthermore, while mutations of the APC degron motifs showed that the two KEN boxes in the reporter were largely dispensable (Geminin: amino acid [aa]13–15 and aa87–89), an additional mutation of the RxxL motif to alanines (AxxA; Geminin: aa23–26) completely prevented reporter degradation, arguing that the degradation of the reporter is mediated by APC^{Cdh1} activity (Figure 1E). We further found a close match between the reporter levels and the levels of endogenous cyclin A2 (Figure 1F), endogenous Geminin (Figure S1I), fluorescently tagged Dbf4 (Figure S1J) (Ferreira et al., 2000), and kinase-dead Aurora A (Figure 1G) (Floyd et al., 2008), arguing that changes in the reporter concentration are a direct reflection of APC^{Cdh1} activity against different known APC^{Cdh1} substrates. Finally, treatment of cells with the proteasome inhibitor MG132 did not change the accumulation rate of the reporter in S and G2, arguing that the reporter is synthesized during the cell cycle at a near constant rate and also that APC^{Cdh1} is nearly completely inactive during S and G2 (Figures S1K and S1L).

Since polyubiquitinated substrates are rapidly degraded (Xu and Qu, 2012), changes in the expression of the APC^{Cdh1} reporter can therefore be used to derive a time course of the activity of APC^{Cdh1} (Figure 1H; Movie S1). This conversion to activity is possible since (1) the promoter region of the reporter construct is unregulated (Figures S1M and S1N), (2) the reporter degradation in quiescence, G1, S, and G2 is primarily regulated by APC^{Cdh1}, and (3) the noise in our measurement of reporter intensity changes is very low (Figure S2A; Experimental Procedures).

Because the time of inactivation of APC^{Cdh1} after mitosis or mitogen stimulation was highly variable within the cell population, we aligned APC^{Cdh1} activity time courses from each cell in silico. Markedly, the aligned time courses showed that APC^{Cdh1} was turned off in each cell with stereotypic rapid kinetics with 90% to 10% inactivation within ~50 min (Figure 1I).

By comparing the maximal attained fluorescence intensity after 24 hr of buildup (Figure S2B) and the minimal steady-state intensity in G1 (Figure S2A), APC^{Cdh1} must be inactivated >99% during S and G2 compared to its active state during quiescence and G1. We find an identical rapid and near-complete inactivation of APC^{Cdh1} at the end of G1 in MCF10A, HeLa, and non-trans-formed fibroblast cells (Bj-5Ta; Figure S2C) as well as in cells that were already cycling rather than coming out of quiescence (Figure S2D).

To determine when the inactivation of APC^{Cdh1} occurs relative to DNA replication, we exposed cells to either BrdU or EdU (markers for DNA replication) for 5 min at the end of a time-lapse image series, fixed cells, and then automatically mapped each fixed cell back to its own live-cell time course (Figure S2E). We found that cells incorporated EdU only after APC^{Cdh1} had been inactivated to less than 10% of its maximal activity (Figures 1J and S2F). There was an almost perfect correlation between cells inactivating APC^{Cdh1} and an immediate start of DNA replication. This temporal correlation suggests that APC^{Cdh1} inactivation is rate limiting for DNA replication. In support of this hypothesis, Cdh1 knockdown causes premature APC^{Cdh1} inactivation and premature DNA replication as has been reported in other cell types (Figure 1K) (Sigl et al., 2009; Yuan et al., 2014). To show how APC^{Cdh1} inactivation fits into the cell-cycle timeline, Figure 1L illustrates that APC^{Cdh1} is turned on in every cell at the end of mitosis, stays on during quiescence and in G1, and only turns off just prior to DNA replication.

APC^{Cdh1} Inactivation Is Irreversible Once Completed

Since previous in vitro studies found that the APC^{Cdh1} component Cdh1 can be phosphorylated and inactivated by cyclin A/Cdk2 (Kramer et al., 2000; Lukas et al., 1999), we simultaneously measured Cdk2 activation (Figure 2A) (Spencer et al., 2013) and APC^{Cdh1} inactivation in the same cell. Markedly, each cell starts to gradually increase Cdk2 activity and only 3 to 5 hr later, when Cdk2 activity reaches a critical intermediate level, will APC^{Cdh1} inactivation be triggered (Figures 2B–2D). The tight correlation between the increase in Cdk2 activity and APC^{Cdh1} inactivation in thousands of individual cells together with the earlier finding that Cdh1 can be inactivated by Cdk2, suggests that Cdk2 buildup is a rate-limiting step for APC^{Cdh1} inactivation. However, when testing for a potential role of cyclin A, we found no significant change in the kinetics of APC^{Cdh1} inactivation after cyclin A2 knockdown in either MCF10A or HeLa cells (Figures S2G and S2H). The knockdown of cyclin A2 was effective since it diminished the increase in Cdk2 activity in S phase (Figures 2E–2G). However, when we used siRNA to knockdown cyclin E1 and E2 or a small molecule Cdk1/2 inhibitor to inhibit Cdk2 activity (Figure 2F), APC^{Cdh1} inactivation was in both cases abolished (Figures 2H, 2I, and S2I), arguing that cyclin E/Cdk2 is required for the inactivation of APC^{Cdh1}.

Since rapid and identical APC^{Cdh1} inactivation kinetics in every cell is a hallmark of a bistable switch mechanism (Figure 1I), we next tested whether APC^{Cdh1} inactivation shows hysteresis, the other required characteristic of an irreversible switch. Strikingly, when the same Cdk1/2 inhibitor that prevents APC^{Cdh1} inactivation is added after APC^{Cdh1} inactivation, APC^{Cdh1} activity remained off, demonstrating that APC^{Cdh1} inactivation shows hysteresis and has the characteristic of an irreversible switch mechanism (Figure 2J).

Rapid and Irreversible APC^{Cdh1} Inactivation Is Mediated by the APC^{Cdh1} Inhibitor Emi1

We next determined whether additional factors control the rapid and irreversible characteristic of APC^{Cdh1} inactivation. One candidate is Skp2, which is part of a cullin SCF^{Skp2} E3 ligase important for S phase fidelity and has been suggested to mediate APC^{Cdh1} inactivation (Barr et al., 2016; Fukushima et al., 2013). However, application of a pan-cullin inhibitor that blocks Skp2-mediated degradation of Cdh1 did not change the kinetics of APC^{Cdh1} inactivation. The same experiment also excludes a range of cullin-containing E3 ligases as potential regulators (Figures S3A–S3D) (Soucy et al., 2009). Also, knockdown of the deubiquitinating protein Usp37, another regulator of APC^{Cdh1} (Huang et al., 2011), had only a small effect on the inactivation kinetics of APC^{Cdh1} (Figures 3A and 3B). In contrast, when we used siRNA to knockdown the early mitotic inhibitor 1 (Emi1, Fbxo5), a previously identified inhibitor of APC^{Cdh1} (Miller et al., 2006), we found that the rate of APC^{Cdh1} inactivation was markedly slowed (Figure 3C). Furthermore, the APC^{Cdh1} inactivation rate became bimodal (Figures 3C, S3E, and S3F) despite a continuous distribution of siRNA knockdown efficiency (Figures S3G and S3H), suggesting a threshold level of Emi1 protein is needed to trigger rapid APC^{Cdh1} inactivation. Conversely, overexpression of wild-type Emi1 almost immediately inactivated APC^{Cdh1} after mitosis (Figure S3I) at an even faster rate compared to control cells (Figures 3D and 3E). Together, this shows that Emi1 has a fundamental role in controlling the rate of APC^{Cdh1} inactivation.

We next explored the possibility that Emi1 not only speeds up APC^{Cdh1} inactivation but is also required to render APC^{Cdh1} inactivation irreversible. We knocked down Emi1 using siRNA and then added a Cdk1/2 inhibitor and monitored APC^{Cdh1} activity (Figure 3F). Strikingly, while Cdk1/2 inhibition in control cells does not reactivate APC^{Cdh1} once it has been turned off, Cdk1/2 inhibition in Emi1 knockdown cells caused an immediate reactivation of APC^{Cdh1} (Figures 3G–3J and S3J). We observed this APC^{Cdh1} reactivation when cells were in the process of turning off APC^{Cdh1} during the G1/S transition as well as when cells were in S or G2 phase and the APC^{Cdh1} was completely off. Thus, while Cyclin E/Cdk2 is necessary to initiate the inactivation of APC^{Cdh1}, Emi1 has a dual role to make APC^{Cdh1} inactivation rapid as well as irreversible, ensuring that APC^{Cdh1} activity does not turn back on once cells have started to replicate their DNA.

Single-Cell Time-Course Analysis Shows a Large Time Gap from the Restriction Point and Rb Phosphorylation to APC^{Cdh1} Inactivation

Our finding of a rapid stereotypic inactivation of APC^{Cdh1} shortly before DNA replication was unexpected based on the previous, more gradual inactivation seen in bulk-cell measurements. The existence of an irreversible regulatory step close before DNA replication suggested that APC^{Cdh1} inactivation might be the commitment point for cell-cycle entry or, alternatively, it was conceivable that APC^{Cdh1} inactivation represents merely a temporary cell-cycle checkpoint in which cells pause after the decision to commit to the cell cycle has already been made with the activation of pRb-E2F. In a third plausible model, APC^{Cdh1} inactivation might be triggered simultaneously along with the restriction point and pRb-E2F activation. To validate or exclude this third model, we performed single-cell analysis to measure when mitogen sensitivity is lost and when APC^{Cdh1} is inactivated. We starved cells of mitogens for 48 hr and released them with pulses of mitogens at variable durations.

Rather than observing a sharp transition, the probability of cells in the population entering the cell cycle gradually increased the longer mitogens were present, with a pulse of 8 hr resulting in half of the cells entering the cell cycle while the other half stayed quiescent (Figure 4A). Inhibition of the MAPK pathway, a pathway known to mediate the mitogen signaling response, showed the same time dependence as mitogen withdrawal (Figure 4A). When we monitored the timing of APC^{Cdh1} inactivation in the same cells, we found that individual cells rapidly inactivated APC^{Cdh1} at different times from 8 hr to >24 hr after mitogen stimulation, with half of the cells inactivating APC^{Cdh1} 16 hr after release from mitogen starvation (Figures 4B and 4C). This argues that a long time gap separates the restriction point from APC^{Cdh1} inactivation.

During a similar time window when cells lost their need for mitogens, the fraction of cells that transitioned to a state where Rb was phosphorylated at a dual Serine 807 and 811 site (hereafter Rb phosphorylation) increased from 5–12 hr (Figure 4C), again long before APC^{Cdh1} was inactivated ~8 hr later. The bimodal distributions in histograms of single-cell Rb phosphorylation levels in thousands of cells over time suggested that Rb phosphorylation rapidly increases to a maximal level in every cell but in each cell after a different delay time (Figure 4D), consistent with the previously shown rapid transition to hyperphosphorylated Rb (Havens et al., 2006). Also, since Rb has multiple phosphorylation sites that may in principle be regulated at different times, we directly tested whether the rapid maximal phosphorylation of S807/S811 is reflective of the activation of the transcription factor E2F (Weinberg, 1995; Yao et al., 2008; Zetterberg et al., 1995). Single-molecule RNA fluorescence in situ hybridization (FISH) analysis of the expression of the E2F target genes *E2F1* and *CCNE1* (cyclin E1) shows that both mRNA transcripts are induced only in cells with maximal pRb pS807/S811, but not in cells with low pRb S807/S811, regardless of the time since mitogen release and before the APC^{Cdh1} inactivation switch (Figure 4E). Taken together, our data suggest a timeline in which a typical MCF10A cell entering the cell cycle out of quiescence crosses the restriction point, rapidly hyperphosphorylates Rb, and induces E2F after ~8 hr and then triggers the APC^{Cdh1} inactivation switch after another 8 hr delay (Figure 4F). This demonstrates that, when cells come out of quiescence, pRb-E2F activation and APC^{Cdh1} inactivation are each rapidly executed as sequential events separated by a long time gap.

Since the situation in cycling cells might be different, we also investigated whether a similar time gap exists in asynchronous cycling cells. Using live-cell tracking software, we automatically called the point of mitosis when the condensed chromosomes separate into two daughter nuclei at anaphase. Approximately 80% of cells kept Rb phosphorylated while in mitosis and in early G1 (Figures 4G, 4H, S4A, and S4B). This population of cycling cells that never loses Rb phosphorylation inactivates APC^{Cdh1} and starts S phase on average 4 hr after anaphase (Figure 4I). The other 20% of cells go through a variable length, quiescent-like state without phosphorylated Rb before later crossing the restriction point, phosphorylating Rb, and re-entering the cell cycle (Figure 4J). Consistent with previous studies (Spencer et al., 2013; Stacey, 2003), we conclude that cells have two distinct time windows when they are sensitive to mitogens, a window early in G0 and G1 when they come out of quiescence and a window in the previous G2, M, and early G1 phase when they are proliferating (Figure 4K). Thus, while the timing greatly varied between individual cells and

stimulation conditions, in all cases, the restriction point and pRb-E2F activation was separated by a long time gap after APC^{Cdh1} inactivation, excluding the model that pRb-E2F induction and APC^{Cdh1} inactivation are part of a single commitment mechanism.

Irreversible Inactivation of APC^{Cdh1} Precedes Checkpoint Arrest at the G1/S Boundary

Our finding that APC^{Cdh1} inactivation is an irreversible step is consistent with APC^{Cdh1} inactivation being either a commitment point or a checkpoint mechanism that allows cells to wait for completion of the buildup in Cdk2 activity before progressing with the cell cycle. Particularly, we considered that APC^{Cdh1} inactivation might be related to a previously described DNA replication stress-mediated checkpoint arrest at the G1/S boundary that can be triggered by aphidicolin, thymidine, or hydroxyurea (Davis et al., 2001). Markedly, we found that cells inactivated APC^{Cdh1} with the same kinetics in the presence of these compounds, arguing that cells undergo a checkpoint arrest at the G1/S boundary only after inactivating APC^{Cdh1} and without altering the APC^{Cdh1} inactivation kinetics (Figure 4L). When cells became arrested at the G1/S boundary and DNA replication stopped, APC^{Cdh1} activity remained off and Cdk2 activity stopped increasing but maintained an intermediate amount of activity, arguing that cells cannot exit to quiescence any more once they reach the G1/S boundary (Figure S4C). Thus, APC^{Cdh1} inactivation is an irreversible bistable switch that is triggered independently and before the previously described cell-cycle checkpoint arrest at the G1/S boundary.

Stress Inputs Can Trigger Reversible Exit to Quiescence until APC^{Cdh1} Is Inactivated, but Not after

With the temporal framework for mitogen sensitivity and pRb-E2F activation, APC^{Cdh1} inactivation, and the G1/S boundary in place, we focused on our main question of whether pRb-E2F activation or APC^{Cdh1} inactivation represents the point of no return for cell-cycle entry. Particularly, we tested how different stresses applied after pRb-E2F activation alter the cell-cycle trajectories of cells. Several studies reported that S phase entry can be prevented by stresses that do not require DNA replication (Deckbar et al., 2011; Havens et al., 2006). While these studies were consistent with a temporary checkpoint arrest mechanism that allows for repair before cells will proceed with the cell cycle, we considered that these arrested cells might instead be able to exit back to a mitogen-sensitive quiescent state. Such a return to quiescence after pRb-E2F activation would argue that the restriction point is not the point of no return for cell-cycle entry and therefore not the commitment point (Figure 5A).

We made use of a previous finding that Cdk2 activity bifurcates after mitosis and stays elevated in those cells that will enter the cell cycle (Figure 5B). This allows for a Cdk2-activity threshold to be selected after mitosis, with cells above the threshold maintaining high Rb phosphorylation and Cdk2 activity with >96% of them inactivating APC^{Cdh1} 4 hr later (Figures 5C, S5A, and S5B; Spencer et al., 2013). We first tested for stress-mediated cell-cycle exit using Neocarzinostatin (NCS), a commonly used drug to generate DNA double-stranded breaks. When we added a 20 min pulse of NCS to cells in G1 that we selected by live-cell analysis to have a Cdk2 activity above the threshold (Figures 5A and 5B), 20% of the NCS-treated cells now inactivated Cdk2 and failed to inactivate APC^{Cdh1}

(Figures 5D, S5D, and S5E) compared to 4% in control cells (Figures 5C, S5C, and S5E). To confirm that these cells lost Rb phosphorylation, we fixed cells 8 hr after NCS addition and measured Rb phosphorylation at S807/S811 by mapping fixed to live cells. We found that cells with low Cdk2 activity also had low levels of phosphorylated Rb and kept APC^{Cdh1} activity on, suggesting that they have entered quiescence (Figure 5E).

Markedly, cells that were exposed to the same pulse of NCS after APC^{Cdh1} inactivation (Figure 5F) showed hysteresis with each cell either continuing to build up Cdk2 activity or maintaining an elevated level of Cdk2 activity. In no case in hundreds of cells did we observe cells that responded by re-activating APC^{Cdh1} (Figures 5F–5H, S5F, and S5G). Cells exposed to NCS after APC^{Cdh1} inactivation went on to complete mitosis several hours later (Figure S5H), indicating these cells were committed to divide. Of note, cells exposed to NCS in late G2 delayed mitosis for several hours but were still able to enter mitosis and divide, suggesting that these committed cells paused to fix damage before completing the cell cycle (Figure S5I).

In the presence of mitogens, NCS-treated cells in G1 that exited to quiescence often again increased Cdk2 activity and re-entered the cell cycle approximately 10 hr later (Figure 5I). However, when we removed mitogens at the same time that we added NCS, cells kept Cdk2 activity low and stayed quiescent (Figure 5J). When we tested whether these cells still responded to mitogens at a later time, they still could be activated by mitogens and re-enter the cell cycle (Figure 5K). Together, these data show that even after passing the restriction point and activating pRb-E2F, but only up until the point when APC^{Cdh1} inactivates, can cells return to a mitogen-sensitive quiescent state (Figure 5L).

We initially considered that stress-mediated exit to quiescence might be a rare event as a majority of cells still entered the cell cycle after NCS addition in late G1. However, when we analyzed cells with increasing Cdk2 activity in G1 phase that were exposed to hyper-osmotic stress, 96% of cells reversibly exited to quiescence and 63% of cells exited to quiescence in response to reactive oxygen stress (H₂O₂ application; Figures 6A–D and S6A–S6C). We again found that cells exposed to these stresses after APC^{Cdh1} inactivation showed hysteresis, maintained Cdk2 activity, and kept APC^{Cdh1} inactivated in response to the same stresses (Figures 6E–6G, S6D, and S6E). Furthermore, stress-mediated exit from the cell cycle in G1 was not restricted to cycling cells as we observed the same stress sensitivity after cells build up Cdk2 activity in cells coming out of quiescence (Figure S6F). Thus, diverse stresses that induce distinct stress pathways mediate a reversible return to a mitogen-sensitive quiescent state even after cells have crossed the restriction point as long as APC^{Cdh1} is not inactivated.

Emi1-Mediated APC^{Cdh1} Inactivation Suppresses Stress Sensitivity and Irreversibly Commits Cells to Progress through the Cell Cycle

To further test the hypothesis that APC^{Cdh1} inactivation mediates cell-cycle commitment, we determined whether Emil renders APC^{Cdh1} inactivation irreversible not only in respect to Cdk2 activity, but also in respect to stress signaling. We knocked down Emil using siRNA and then exposed cells to NCS in G1 phase. Cells treated with Emil siRNA were slightly more susceptible to NCS, as a higher percentage of cells exited the cell cycle prior to

APC^{Cdh1} inactivation (Figures 7A and S7A–S7C). However, cells that continued on to S phase failed to keep APC^{Cdh1} inactivated and lost Cdk2 activity when the same stress was applied (Figure 7A). In contrast, cells treated with control siRNA and were exposed to the same stress were able to maintain APC^{Cdh1} inactivation and enter the next mitosis several hours later. Thus, APC^{Cdh1} inactivation becomes reversible in respect to stress application only in the absence of Emil. This argues that APC^{Cdh1} inactivation is an Emil-regulated bistable switch that results in a loss of a cell's ability to exit to the quiescent state in response to stress. Together, our data argue that the Emil-mediated irreversible inactivation of APC^{Cdh1} is triggered shortly before the G1/S boundary and long after pRb-E2F activation and that APC^{Cdh1} inactivation has all the needed characteristics to be the commitment point after which cells must complete the cell cycle and can no longer return to quiescence before they divide by passing through mitosis.

DISCUSSION

APC^{Cdh1} Inactivation Is a Rapid and Irreversible Switch that Commits Cells to Start DNA Replication

Our study introduces a live-cell analysis approach using geminin-and helicase-B-derived peptide reporters (Sakaue-Sawano et al., 2008; Spencer et al., 2013) to quantitatively compare changes in APC^{Cdh1} and Cdk2 activities in hundreds of individual cells throughout the human cell cycle. Our study provides three main lines of arguments that APC^{Cdh1} inactivation underlies the cell-cycle commitment point. First, inactivation is rapid, bistable, and shows complete hysteresis with respect to Cdk2 inhibition, all critical characteristics of an irreversible decision process. The APC^{Cdh1} inactivation kinetics is the same in every cycling cell and in cells coming out of quiescence and also the same in the three cell models we tested, arguing for a general bistable switch mechanism. Second, in a time window of reversibility that lasts from pRb-E2F until APC^{Cdh1} inactivation, stress perturbations can cause cells to exit to quiescence. Only after APC^{Cdh1} inactivation do cells lose their stress sensitivity, demonstrating a key hysteresis characteristic required for a point of no return or commitment point (Figure 7B). This marked rapid irreversibility is lost upon knockdown of the APC^{Cdh1} inhibitor Emil, arguing that Emil is responsible for the speed, bistability, and hysteresis of the switch mechanism. Third, previous studies already showed that the inactivation of APC^{Cdh1} is a rate-limiting step for entering S phase as knockdown of Cdh1 causes premature DNA replication (Sigl et al., 2009; Yuan et al., 2014). Our single-cell analysis confirmed this data and further shows that APC^{Cdh1} inactivation is followed in nearly every cell by DNA replication within an hour. Thus, APC^{Cdh1} inactivation has the necessary characteristics to be a general commitment point for cell-cycle entry distinct from the much earlier restriction point.

Cyclin E/Cdk2 Initiates APC^{Cdh1} Inactivation and Emil1 Makes APC^{Cdh1} Inactivation Irreversible

Of the proteins previously identified to regulate APC^{Cdh1}, we found that cyclin E/Cdk2 and Emil were the most relevant to trigger APC^{Cdh1} inactivation before the G1/S boundary. In support of this model, Cdh1 has nine potential Cdk consensus phosphorylation sites that, when mutated, prevent Cdh1 from binding the APC (Kramer et al., 2000). Our proposed

model of APC^{Cdh1} inactivation implies that cyclin E and Cdk2 are both necessary for cell-cycle progression, and our data from siRNA knockdown experiments of cyclin E1 and E2 confirm this essential role. However, complete ablation of cyclin E or Cdk2 in mice did not prevent cell proliferation, in apparent conflict with our model. In the case of cyclin E knockout mice (Geng et al., 2003), our experiments in which we overexpress Emil give a plausible explanation of how cells can compensate for the loss of cyclin E (Figures 3D and 3E). We and others observed that high levels of Emil can inhibit APC^{Cdh1} without an increase in cyclin E/Cdk2 activity. In the case of Cdk2 knockout mice, the discrepancy is likely due to compensation for the loss of Cdk2 by upregulation of Cdk1 activity. Indeed, it has been shown that cyclin E can bind and activate Cdk1 in cells derived from a Cdk2 knockout mouse (Aleem et al., 2005), indicating that in the absence of Cdk2, cyclin E/Cdk1 is capable of phosphorylating Cdk2 substrates. Consistent with cyclin E activating Cdk1 as a compensation mechanism, we found that addition of the Cdk1 inhibitor R0-3306 to wild-type mouse embryonic fibroblasts (MEFs) only prevented a small fraction of cells from accumulating the APC^{Cdh1} substrates geminin and cyclin A (Figure S7D), while the Cdk1 inhibitor had a larger effect in Cdk2^{-/-} MEFs (Figure S7E). Thus, while our model suggests that cyclin E/Cdk2 and Emil are the primary mediators of APC^{Cdh1} inactivation, in the absence of cyclin E or Cdk2, there is likely compensation with cyclin E/Cdk1, cyclin A/Cdk2, and upregulated Emil also contributing to the inactivation of APC^{Cdh1}.

While we did not find a significant role for cyclin A/Cdk2 (Lukas et al., 1999) or SCF^{Skp2} (Barr et al., 2016; Fukushima et al., 2013) for the rapid inactivation of APC^{Cdh1} in normal cells, it is likely that these APC^{Cdh1} regulators are still functionally important at later points in the cell cycle when Cdh1 also becomes at least partially degraded (Fukushima et al., 2013). We also observed that knockdown of the APC^{Cdh1}-regulator Usp37 (Huang et al., 2011) had a small but significant effect on the kinetics of APC^{Cdh1} inactivation and may therefore function as an additional regulator of APC^{Cdh1} inactivation.

Different Stresses Can Cause Exit to Quiescence until APC^{Cdh1} Inactivation or, Alternatively, Checkpoint Arrest Immediately after APC^{Cdh1} Inactivation at the G1/S Boundary

Our study tested osmotic, hydrogen peroxide, and double strand DNA break stresses and showed that all three can cause cells to exit to quiescence after pRB-E2F activation until APC^{Cdh1} inactivation. It is likely that many other stresses and signaling pathways known to inhibit proliferation can cause a similar reversible exit to quiescence during this time window. We also show that addition of thymidine, aphidicolin, or hydroxyurea in G1 has no effect on the inactivation kinetics of APC^{Cdh1} but all result in a DNA-damage-mediated arrest at the G1/S boundary with Cdk2 activity remaining elevated and APC^{Cdh1} remaining off. This argues that these G1/S-arrested cells merely pause at the onset of S phase and do not return to quiescence start of DNA replication (Borel et al., 2002).

Thus, two mechanisms have to be distinguished that prevent S-phase entry after the restriction point: (1) immediately acting stress and other signals in G1 that can prevent APC^{Cdh1} inactivation and induce an exit to quiescence versus (2) stress perturbations that

require DNA replication that lead to a checkpoint arrest only after APC^{Cdh1} inactivation at the G1/S boundary without causing exit to quiescence.

Choreography of Cell-Cycle Entry and Exit Is Regulated by Mitogens and Stress

Our study reveals an overall timeline of when cells are sensitive to mitogens and stress during the cell cycle of MCF10A cells (Figure 7B). In cells coming out of quiescence, the time window for mitogen sensitivity is primarily before Rb phosphorylation. In cycling cells, the time window for mitogen sensitivity is primarily during G2 and M of the preceding cell cycle (Spencer et al., 2013; Stacey, 2003). In the latter case, as a corresponding molecular mechanism, we found that the same fraction of cells that lose Rb phosphorylation during mitosis exit the cell cycle after mitosis (Figure 7B, bold dashed gray line), while the remaining cells keep Rb phosphorylated, with most of the cells increasing Cdk2 activity and re-entering the cell cycle (Spencer et al., 2013).

Even though cells with Rb phosphorylated and E2F activated are on a trajectory to enter the cell cycle, they still respond to stress and can reverse Rb phosphorylation, E2F induction, and Cdk2 activation to return back to a mitogen-sensitive quiescent state (Figure 7B, dashed gray lines). Our results argue that mitogen signals are counteracted by diverse stress signals during a window of reversibility that lasts from pRB-E2F activation until APC^{Cdh1} inactivation, after which stress signals can only mediate checkpoint arrest and not a return to quiescence.

From a regulatory perspective, this breaks the cell cycle into three states marked by their E2F and APC^{Cdh1} activities (Figure 7C): (1) quiescence, defined as a state when E2F activity is off and APC^{Cdh1} is on, (2) a window of reversibility, defined as a state when E2F and APC^{Cdh1} are both on, during which cells prepare for proliferation but can still exit to quiescence, and (3) A proliferative state, defined as a state when APC^{Cdh1} is off. We propose that the window of reversibility reflects a competition between slower-acting mitogen signaling and faster-acting stress signaling pathways that can trigger throughout G1 an exit to quiescence until APC^{Cdh1} is inactivated. Such a competition is well suited as a general safety control circuit by giving cells sufficient time to express the E2F target genes needed for proliferation while also providing a mechanism to remain sensitive to inhibitory stimuli and prevent damaged or challenged cells from entering the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Lines

All experiments were done using MCF-10A cells, a non-transformed human mammary epithelial cell line, unless otherwise noted, and were obtained from ATCC (CRL-10317). In addition to MCF10A cells, APC activity was also measured in both HeLa (ATCC, CCL-2) and BJ-5ta cells (ATCC, CRL-4001).

Time-Lapse Microscopy

Cells were plated ~24 hr prior to imaging in full-growth media in a 96-well dish (Costar #3904) such that the density would remain sub-confluent until the end of the imaging period.

Time-lapse imaging was performed in 290 μ L full-growth media. Images were taken in CFP, YFP, and RFP channels every 12 min on an IXMicro microscope (Molecular Devices) with a 10 \times 0.3NA objective. Total light exposure time was kept under 600 ms for each time point. Cells were imaged in a humidified, 37°C chamber in 5% CO₂. Cell tracking and data analysis was done using custom MATLAB scripts.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde, washed three times in PBS, permeabilized with 0.2% triton, and stained overnight at 4°C with anti-phospho-Rb (807/811; Cell Signaling Technology, #8516), anti-cyclin A (Santa Cruz Biotechnology, sc-751), anti-p21 (BD PharMingen, 556430), anti-Geminin (Sigma-Aldrich, HPA049977), or anti-BrdU (Abcam, #ab6326). Primary antibodies were visualized using a secondary antibody conjugated to Alexa Fluor-647 and imaged with a Far Red filter. Cells were treated with 10 mM BrdU for 15 min prior to fixation. For figures showing EdU staining, cells were treated with 10 mM EdU for 15 min and fixed and processed according to manufacturer's instructions (Invitrogen, #C10356).

Inhibitors

The inhibitors used in this study were: proTAME (10 mM, APC inhibitor, Boston Biochem, I-440), MLN-4924 (3 mM, neddylation inhibitor, Active Biochem, #A-1139), Cdk1/2i III (3 mM, EMD Biosciences #217714), neocarzinostatin (40 ng/mL, Sigma-aldrich, N9162), aphidicolin (2 mM, Sigma-aldrich, A0781), thymidine (2mM, Sigma-aldrich, T9250), and hydroxyurea (500 mM, Sigma- aldrich, H8627).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Aleem E, Kiyokawa H, and Kaldis P (2005). Cdc2-cyclin E complexes regulate the G1/S phase transition. *Nat. Cell Biol.* 7, 831–836. [PubMed: 16007079]
- Barr ARH, Heldt FS, Zhang T, Bakal C, and Novák B (2016). A dynamical framework for the all-or-none G1/S transition. *Cell Syst.* 2, 27–37. [PubMed: 27136687]
- Borel F, Lacroix FB, and Margolis RL (2002). Prolonged arrest of mammalian cells at the G1/S boundary results in permanent S phase stasis. *J. Cell Sci.* 115, 2829–2838. [PubMed: 12082144]
- Chang L, Zhang Z, Yang J, McLaughlin SH, and Barford D (2014). Molecular architecture and mechanism of the anaphase-promoting complex. *Nature* 513, 388–393. [PubMed: 25043029]
- Davis PK, Ho A, and Dowdy SF (2001). Biological methods for cell-cycle synchronization of mammalian cells. *BioTechniques* 30, 1322–1326, 1328, 1330–1331. [PubMed: 11414226]

- Deckbar D, Jeggo PA, and Löbrich M (2011). Understanding the limitations of radiation-induced cell cycle checkpoints. *Crit. Rev. Biochem. Mol. Biol.* 46, 271–283. [PubMed: 21524151]
- Duli V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, Elledge SJ, and Reed SI (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* 76, 1013–1023. [PubMed: 8137420]
- Eguren M, Manchado E, and Malumbres M (2011). Non-mitotic functions of the Anaphase-Promoting Complex. *Semin. Cell Dev. Biol.* 22, 572–578. [PubMed: 21439391]
- Erol A (2011). Genotoxic stress-mediated cell cycle activities for the decision of cellular fate. *Cell Cycle* 10, 3239–3248. [PubMed: 21921675]
- Ferreira MF, Santocanale C, Drury LS, and Diffley JF (2000). Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. *Mol. Cell. Biol.* 20, 242–248. [PubMed: 10594027]
- Floyd S, Pines J, and Lindon C (2008). APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. *Curr. Biol.* 18, 1649–1658. [PubMed: 18976910]
- Fukushima H, Ogura K, Wan L, Lu Y, Li V, Gao D, Liu P, Lau AW, Wu T, Kirschner MW, et al. (2013). SCF-mediated Cdh1 degradation defines a negative feedback system that coordinates cell-cycle progression. *Cell Rep.* 4, 803–816. [PubMed: 23972993]
- Geng Y, Yu Q, Sicinska E, Das M, Schneider JE, Bhattacharya S, Rideout WM, Bronson RT, Gardner H, and Sicinski P (2003). Cyclin E ablation in the mouse. *Cell* 114, 431–443. [PubMed: 12941272]
- Hanahan D, and Weinberg RA (2000). The hallmarks of cancer. *Cell* 100, 57–70. [PubMed: 10647931]
- Havens CG, Ho A, Yoshioka N, and Dowdy SF (2006). Regulation of late G1/S phase transition and APC Cdh1 by reactive oxygen species. *Mol. Cell. Biol.* 26, 4701–4711. [PubMed: 16738333]
- Hsu YC, Li L, and Fuchs E (2014). Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. *Cell* 157, 935–949. [PubMed: 24813615]
- Huang X, Summers MK, Pham V, Lill JR, Liu J, Lee G, Kirkpatrick DS, Jackson PK, Fang G, and Dixit VM (2011). Deubiquitinase USP37 is activated by CDK2 to antagonize APC(CDH1) and promote S phase entry. *Mol. Cell* 42, 511–523. [PubMed: 21596315]
- Kramer ER, Scheuringer N, Podtelejnikov AV, Mann M, and Peters JM (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Mol. Biol. Cell* 11, 1555–1569. [PubMed: 10793135]
- Lukas C, Sørensen CS, Kramer E, Santoni-Rugiu E, Lindene C, Peters JM, Bartek J, and Lukas J (1999). Accumulation of cyclin B1 requires E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex. *Nature* 401, 815–818. [PubMed: 10548110]
- Miller JJ, Summers MK, Hansen DV, Nachury MV, Lehman NL, Loktev A, and Jackson PK (2006). Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. *Genes Dev.* 20, 2410–2420. [PubMed: 16921029]
- Narasimha AM, Kaulich M, Shapiro GS, Choi YJ, Sicinski P, and Dowdy SF (2014). Cyclin D activates the Rb tumor suppressor by monophosphorylation. *eLife* 3, e02872.
- Pardee AB (1974). A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. USA* 71, 1286–1290. [PubMed: 4524638]
- Peters JM (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell Biol.* 7, 644–656. [PubMed: 16896351]
- Planas-Silva MD, and Weinberg RA (1997). The restriction point and control of cell proliferation. *Curr. Opin. Cell Biol.* 9, 768–772. [PubMed: 9425340]
- Rape M, and Kirschner MW (2004). Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. *Nature* 432, 588–595. [PubMed: 15558010]
- Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, et al. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487–498. [PubMed: 18267078]
- Sigl R, Wandke C, Rauch V, Kirk J, Hunt T, and Geley S (2009). Loss of the mammalian APC/C activator FZR1 shortens G1 and lengthens S phase but has little effect on exit from mitosis. *J. Cell Sci.* 122, 4208–4217. [PubMed: 19861496]

- Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, et al. (2009). An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 458, 732–736. [PubMed: 19360080]
- Spencer SL, Cappell SD, Tsai FC, Overton KW, Wang CL, and Meyer T (2013). The proliferation-quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit. *Cell* 155, 369–383. [PubMed: 24075009]
- Stacey DW (2003). Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. *Curr. Opin. Cell Biol.* 15, 158–163. [PubMed: 12648671]
- Vousden KH, and Prives C (2009). Blinded by the Light: The Growing Complexity of p53. *Cell* 137, 413–431. [PubMed: 19410540]
- Walker A, Acquaviva C, Matsusaka T, Koop L, and Pines J (2008). UbcH10 has a rate-limiting role in G1 phase but might not act in the spindle checkpoint or as part of an autonomous oscillator. *J. Cell Sci.* 121, 2319–2326. [PubMed: 18559889]
- Wang W, and Kirschner MW (2013). Emi1 preferentially inhibits ubiquitin chain elongation by the anaphase-promoting complex. *Nat. Cell Biol.* 15, 797–806. [PubMed: 23708001]
- Weinberg RA (1995). The retinoblastoma protein and cell cycle control. *Cell* 81, 323–330. [PubMed: 7736585]
- Xu L, and Qu Z (2012). Roles of protein ubiquitination and degradation kinetics in biological oscillations. *PLoS ONE* 7, e34616.
- Yao G, Lee TJ, Mori S, Nevins JR, and You L (2008). A bistable Rb-E2F switch underlies the restriction point. *Nat. Cell Biol.* 10, 476–482. [PubMed: 18364697]
- Yuan X, Srividhya J, De Luca T, Lee JH, and Pomeroy JR (2014). Uncovering the role of APC-Cdh1 in generating the dynamics of S-phase onset. *Mol. Biol. Cell* 25, 441–456. [PubMed: 24356446]
- Zetterberg A, and Larsson O (1985). Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* 82, 5365–5369. [PubMed: 3860868]
- Zetterberg A, Larsson O, and Wiman KG (1995). What is the restriction point? *Curr. Opin. Cell Biol.* 7, 835–842. [PubMed: 8608014]

Highlights

- Rapid inactivation of APC^{Cdh1} is triggered minutes before, DNA replication starts
- Cyclin E/Cdk2 starts and Emi1 executes rapid and., irreversible APC^{Cdh1} inactivation
- A large time gap separates pRb-E2F activation from APC^{Cdh1}, inactivation
- Cells can reverse back to quiescence until APC^{Cdh1}, inactivation, but not after it

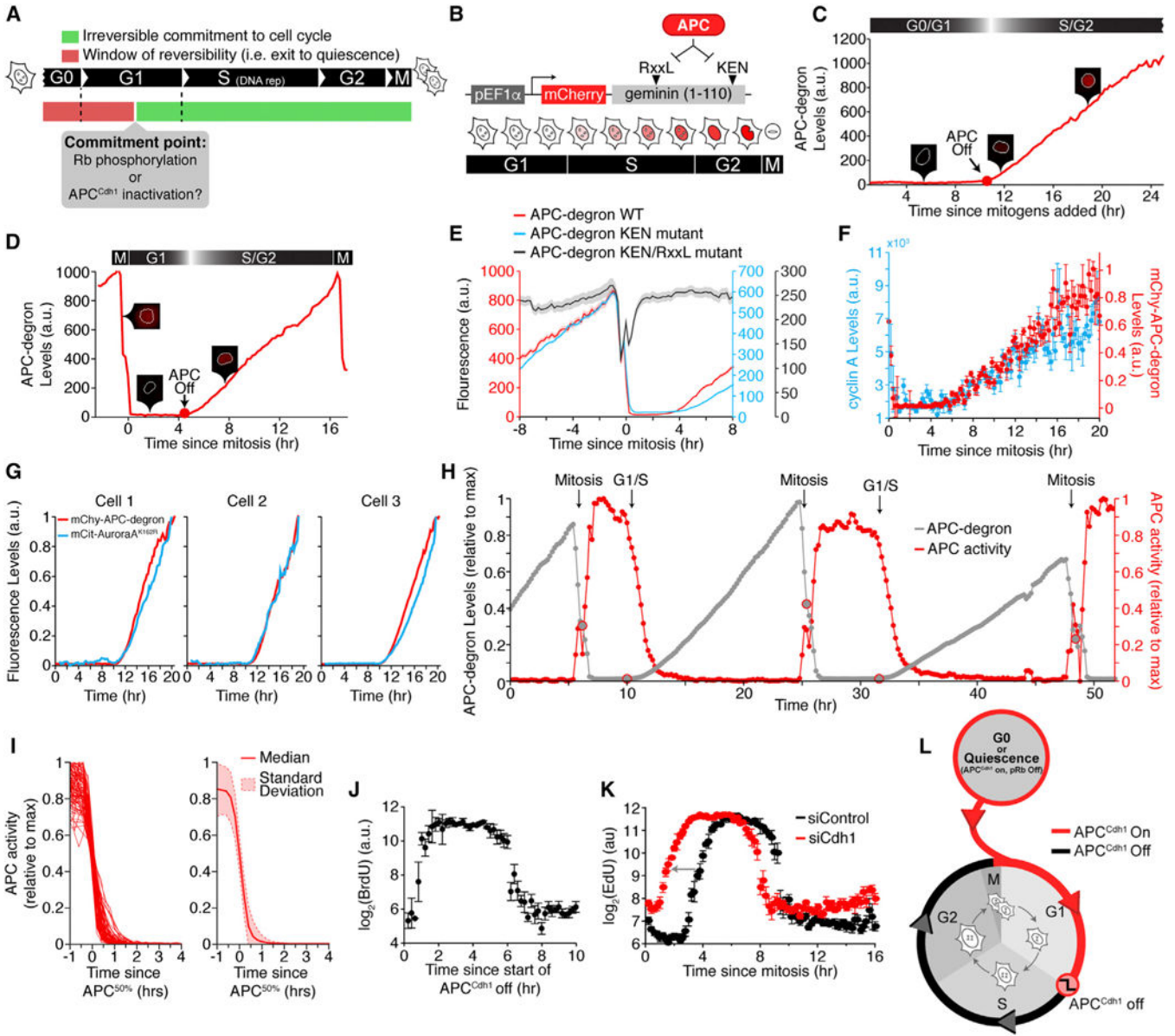


Figure 1. Rapid and Near-Complete APC^{Cdh1} Inactivation Shortly before S Phase Entry

(A) Schematic diagram of the cell-cycle commitment model.

(B) Schematic diagram of the APC-degron reporter (Geminin: aa1–110).

(C) Single-cell trace of APC-degron reporter levels in a representative cell released from mitogen starvation. Inset: snapshots of the APC-degron reporter.

(D) Single-cell trace of the APC-degron reporter in a representative cycling cell as in (C).

(E) MCF10A cells expressing mVenus-APC-degron wild-type and either mChy-APC-degron KEN mutant or mChy-APC-degron KEN/RxxL mutant. Lines are median traces ± SEM. (n = 205 cells, wild-type; n = 800, KEN; n = 600, RxxL).

(F) Cells were imaged for ~4 hr then fixed and stained with α-cyclin A2. Cells were binned by the time since mitosis. Data represent median intensity ± SEM of either cyclin A2 or APC-degron reporter.

(G) HeLa cells transfected with the APC-degron reporter and mCitrine-Aurora-A K162R (three representative cells shown).

(H) APC-degron reporter levels and the derived APC activity for a single cell. Time of mitosis and the G1/S transition are computationally identified.

(I) Left: Single-cell traces of APC^{Cdh1} activity computationally aligned to 50% APC^{Cdh1} activity (random selection of 91 out of 431 cells analyzed). Right: Median APC^{Cdh1} activity trace \pm SD (n = 861).

(J) Scatterplot of BrdU levels versus the time since APC^{Cdh1} started to inactivate. Fixed cells were mapped back to live-cell data. Single-cell data were binned and data points are median \pm SEM (n = 1100).

(K) MCF10A cells were treated with either control siRNA or Cdh1 siRNA. Fixed cells were mapped back to live-cell data. Single-cell data were binned and data points are median \pm SEM. n > 30,000 cells per condition. Arrow highlights the shift in timing of EdU incorporation after Cdh1 siRNA.

(L) Schematic when APC^{Cdh1} is on and off during the cell cycle. See also Figure S1.

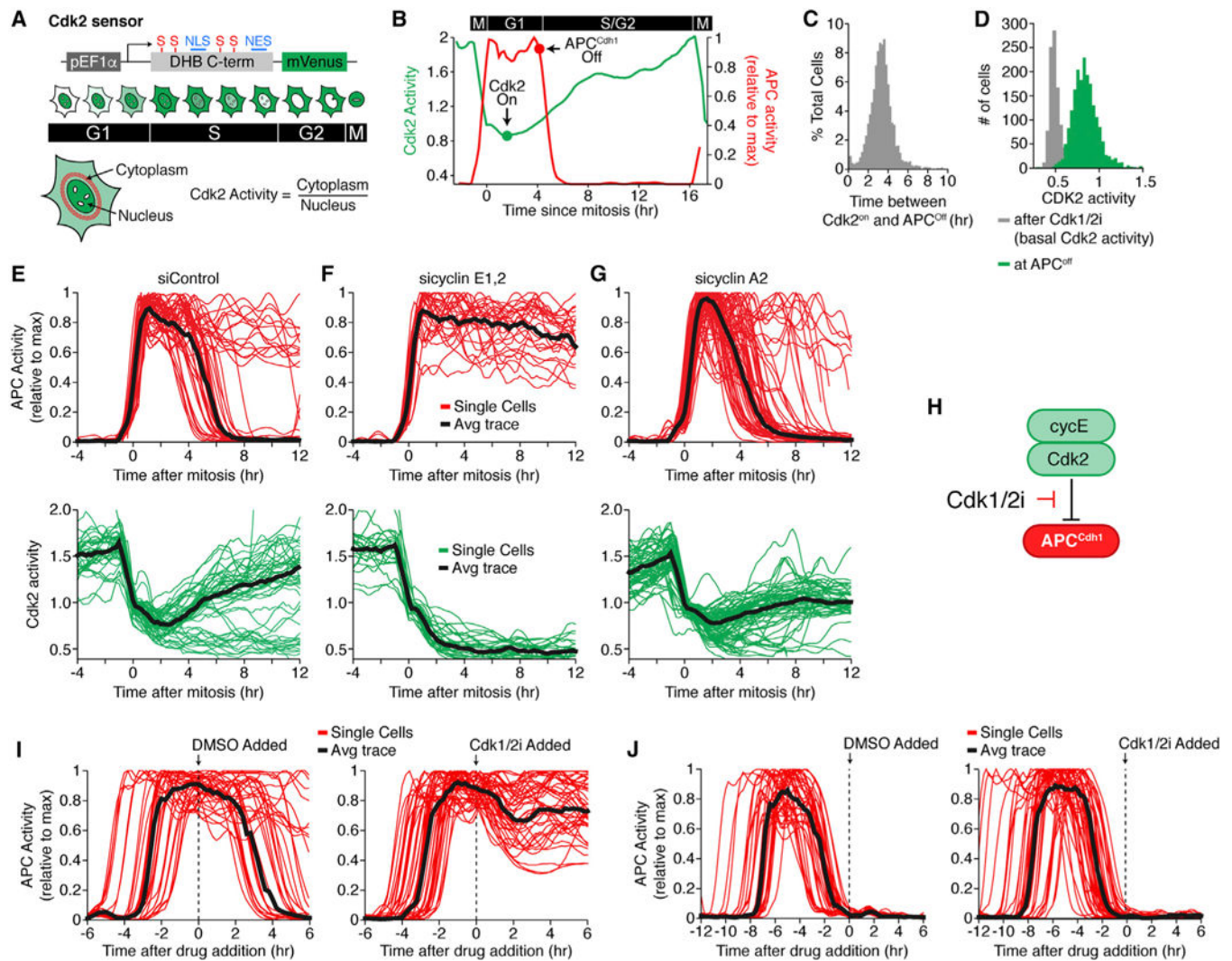


Figure 2. APC^{Cdh1} Inactivation Is Irreversible and Requires Cyclin E/Cdk2

(A) Schematic of the Cdk2 sensor. Cdk2-mediated phosphorylation controls export of the reporter from the nucleus. NLS, nuclear localization signal; NES, nuclear export signal; S, CDK consensus site on serine.

(B) APC^{Cdh1} and Cdk2 activities in a representative single cycling cell.

(C) Histogram of the time between the initial rise of Cdk2 activity and APC^{Cdh1} inactivation. $n > 1000$ cells.

(D) Histogram of Cdk2 activity when APC^{Cdh1} inactivates (green). Basal Cdk2 activity after treatment with a Cdk1/2 inhibitor (gray) is added for reference.

(E-G) APC and Cdk2 activity aligned in silico to mitosis treated with either control (E), cyclin E1 and E2 (F), or cyclin A2 siRNA (G). Grey line is median trace. $n = 54, 26,$ and $48,$ respectively.

(H) Schematic diagram showing that cyclin E/Cdk2 mediates inactivation of APC^{Cdh1} and that Cdk2 inhibition is expected to prevent APC^{Cdh1} inactivation.

(I and J) APC activity traces treated with either DMSO or Cdk1/2 inhibitor. Time of treatment is indicated by the dashed line. (I) Cells treated while in G1 phase ($n = 41$, DMSO;

n = 45, Cdk1/2i).(J) Cells treated while in S phase (n = 47, DMSO; n = 45, Cdk1/2i). Black line is median trace. See also Figure S2.

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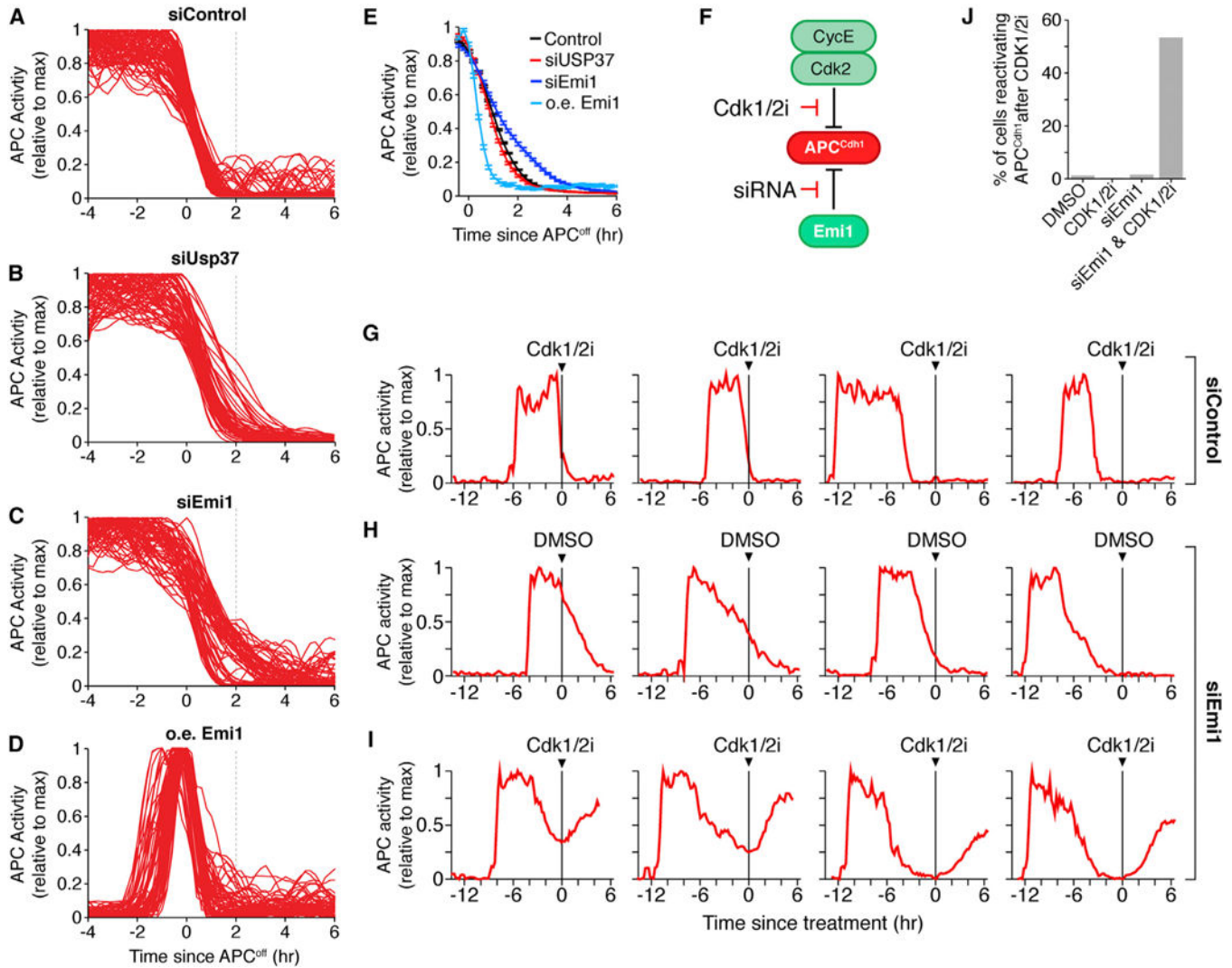


Figure 3. Emi1 Is Required for Rapid and Irreversible APC^{Cdh1} Inactivation

(A-D) Single-cell APC activity traces aligned to the time that APC^{Cdh1} starts to turn off in cells treated with either control (A), Usp37 (B), or Emi1 siRNA (C) or cells overexpressing (oe) wild-type Emi1 (D). Dashed line added for reference between panels.

(E) Median APC activity trace ± SEM from cells in (A-D). n = 276, 201, 214, 152, respectively.

(F) Schematic of experimental design for (G-I).

(G-I) Single-cell APC activity traces treated with either control (G) or Emi1 siRNA (H and I). Cells were exposed to either DMSO (H) or Cdk1/2 inhibitor (G and I) at the indicated time. Data from all cells is in Figure S3J.

(J) Bar graph of the percent of cells that reactivate APC^{Cdh1} upon Cdk1/2 inhibitor addition. n ≈ 250 cells per condition. See also Figure S3.

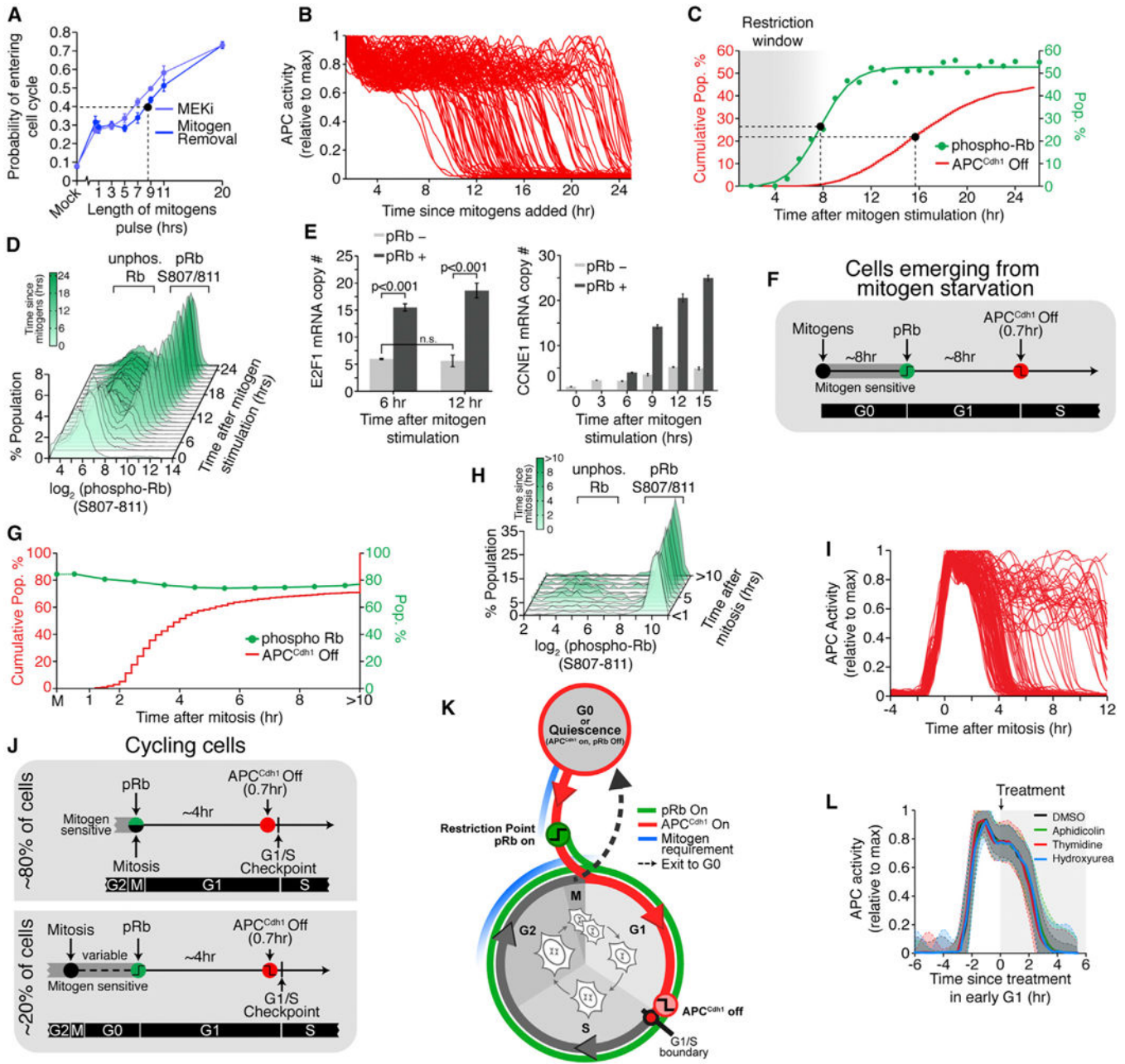


Figure 4. A Large Time Gap Separates the Restriction Point and pRb-E2F Induction from APC^{Cdh1} Inactivation

(A) Probability of entering the cell cycle (i.e., EdU incorporation after 24 hr) as a function of the length of mitogen or MEKi pulse after release from starvation. Dashed line indicates when 50% of cells enter the cell cycle. Error bars are SD from four experiments, n > 3000 cells per data point.

(B) APC activity in cells released from mitogen-starvation (n = 114).

(C) Cumulative distribution function (CDF) of the time cells inactivated APC^{Cdh1} after release from mitogen-starvation (red; n = 9,068). Scatterplot of the percent of cells with phospho-Rb (S807/S811) as a function of time since mitogen stimulation (n > 15,000 cells

per data point). Green line is sigmoidal best-fit line. Dashed lines indicate when half max is reached.

(D) Histograms of pRb807/811 levels taken at 1 hr intervals after release from mitogen starvation ($n > 15,000$ cells per histogram).

(E) Single-cell mRNA FISH of E2F1 and cycE1 after release from mitogen starvation. Cells were separated into pRb negative and positive based on bimodal histogram as in (D). Error bars are SEM from four experiments, $n > 1000$ cells per condition.

(F) Schematic diagram of the timing of cell-cycle events in cells released from mitogen starvation.

(G) Scatterplot of the percent of cells with pRb807/811 at each bin relative to mitosis (green; $n \ll 400$ cells per data point). CDF of the time cells inactivated APC^{Cdh1} relative to mitosis (red; $n = 1042$).

(H) Histograms of pRb807/811 levels divided into bins since mitosis. ($n \approx 400$ cells per histogram).

(I) Single-cell traces of APC activity in asynchronous cells, aligned to mitosis ($n = 140$).

(J) Schematic of the timing of cell-cycle events in cycling cells. Following mitosis, cells either maintain Rb hyper-phosphorylation and stay in the cell cycle or they lose Rb phosphorylation and enter a transient G0-like state.

(K) Schematic showing when cells are sensitive to mitogens relative to Rb phosphorylation and APC^{Cdh1} inactivation.

(L) Median traces \pm SD of APC activity in cells treated with the indicated drug in G1 phase. $n = 113$, control; $n = 107$, aphidicolin; $n = 116$, thymidine; $n = 84$, hydroxyurea. See also Figure S4.

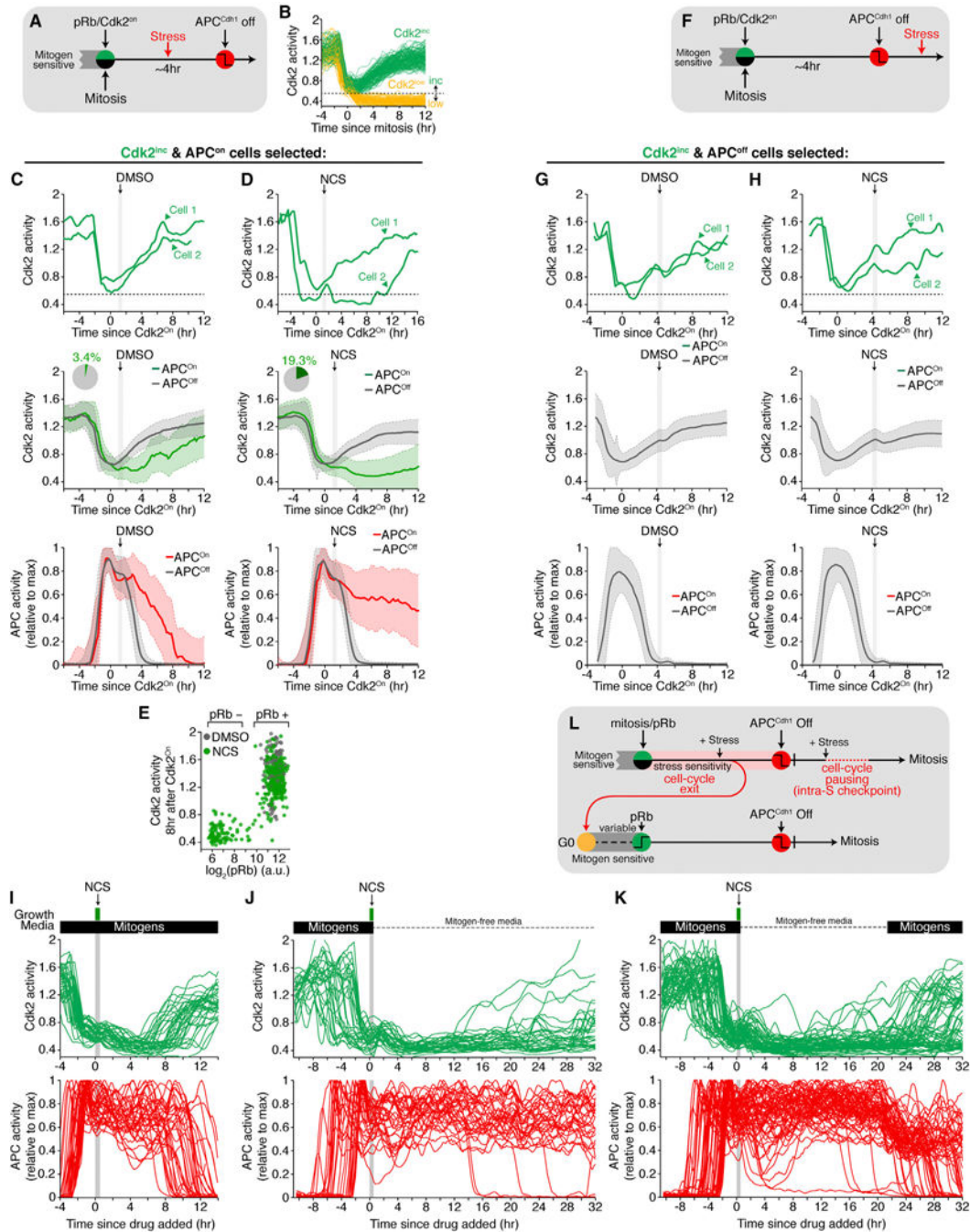


Figure 5. Stress Signaling Can Induce Cell-Cycle Exit to Mitogen-Regulated Quiescence after pRb-E2F Induction but Only until APC^{Cdh1} Inactivation

(A) Experimental setup for stress administration after the restriction point but before APC^{Cdh1} inactivation.

(B) Schematic showing cells that have increasing Cdk2 activity (Cdk2^{inc}) or basal Cdk2 activity (Cdk2^{low}) 2 hr after mitosis. Horizontal dashed line indicates the threshold level of Cdk2 activity used to categorize a cell as either Cdk2^{inc} or Cdk2^{low}. Only Cdk2^{inc} cells were considered in Figures 5C–5H.

(C and D) Cdk2 and APC^{Cdh1} activities in cells exposed to NCS after Cdk2 activity started to rise. Light gray band represents time when cells were exposed to stress. Top: two individual cells are shown. Middle: median Cdk2 activity \pm SD. Bottom: median APC^{Cdh1} activity. Traces are colored if Cdk2 inactivated after initially turning on or are gray if Cdk2 stayed active and APC^{Cdh1} inactivated. Inset: percentage of cells that inactivated Cdk2 activity despite initially turning on. (C) DMSO or (D) 40ng/mL Neocarzinostatin (NCS) (n = 270, DMSO; n = 554, NCS; see Figure S5E).

(E) Scatterplot of pRb807/811 levels versus Cdk2 activity 8 hr after the initial rise in Cdk2 activity.

(F) Experimental setup for stress administered after APC^{Cdh1} inactivation. (G and H) Cdk2 and APC^{Cdh1} activities exposed to stress after APC^{Cdh1} inactivation. Top: two cells are highlighted. Middle: median Cdk2 activity \pm SD. Bottom: median APC^{Cdh1} activity.

(G) DMSO or (H) 40ng/mL Neocarzinostatin (NCS). Traces are colored if Cdk2 inactivated despite initially turning on or are gray if Cdk2 stayed active and APC^{Cdh1} inactivated. Note no traces were colored (n = 587, DMSO; n = 1284).

(I) Cdk2 and APC^{Cdh1} activity traces of cells treated with NCS after crossing the restriction point. These cells inactivated Cdk2, only to re-activate Cdk2 and reenter the cell cycle several hours later (n = 35).

(J) Cdk2 and APC^{Cdh1} activity traces treated with 200 ng/mL NCS for 12 min, then washed with mitogen-free media, and monitored for an additional 32 hr. (n = 33).

(K) Cdk2 and APC traces treated with 200 ng/mL NCS for 12 min, then washed with mitogen-free media, and monitored for an additional 21 hr. After 21 hr, full-growth media was added and cells were monitored for an additional 10 hr. Cells re-entered the cell cycle after stimulation with mitogens (n = 61).

(L) Cells stressed in G1 enter a quiescent state while cells stressed in S or G2 pause before continuing through the cell cycle. See also Figure S5.

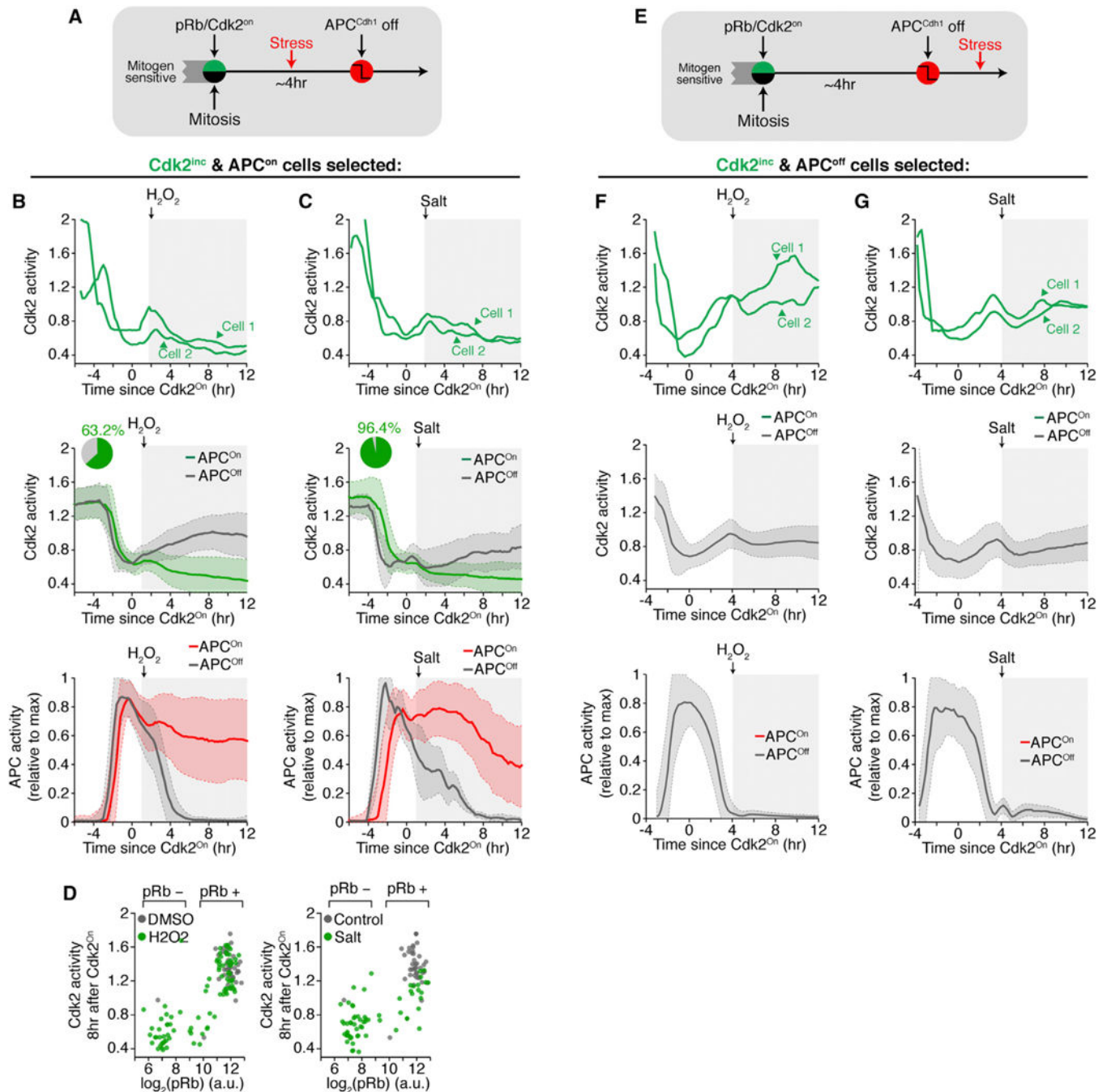


Figure 6. Multiple Types of Stresses Can Cause Cell-Cycle Exit until, but Not after APC^{Cdh1} Inactivation

(A) Experimental setup for stress administration after the restriction point but before APC^{Cdh1} inactivation.

(B and C) Representative examples and median traces \pm SD of Cdk2 and APC activities in cells exposed to stress after Cdk2 activity started to rise. Light gray band represents time when cells were exposed to stress. Top: two cells are highlighted. Middle: median Cdk2 activity. Bottom: median APC activity. Traces are colored if Cdk2 inactivated despite initially turning on or are gray if Cdk2 stayed active and APC^{Cdh1} inactivated. Inset: percentage of

cells that inactivated Cdk2 activity despite initially turning on. (B) 200 μ M Hydrogen peroxide (H₂O₂) or (C) 100 mM NaCl (salt) (n = 821, H₂O₂; n = 223, salt). See Figure S6C.

(D) Scatterplot of pRb807/811 levels versus Cdk2 activity 8 hr after the initial rise in Cdk2 activity (n = 1152, H₂O₂; n = 194, salt).

(E) Experimental setup for stress administered after APC^{Cdh1} inactivation.

(F and G) Median traces \pm SD of Cdk2 and APC activity exposed to stress after APC^{Cdh1} inactivation. Top: two cells are highlighted. Middle: median Cdk2 activity. Bottom: median APC activity. (F) 200 μ M Hydrogen peroxide (H₂O₂) or (G) 100mM NaCl (salt). Traces are colored if Cdk2 inactivated despite initially turning on or are gray if Cdk2 stayed active and APC^{Cdh1} inactivated. Note no traces were colored. See also Figure S6.

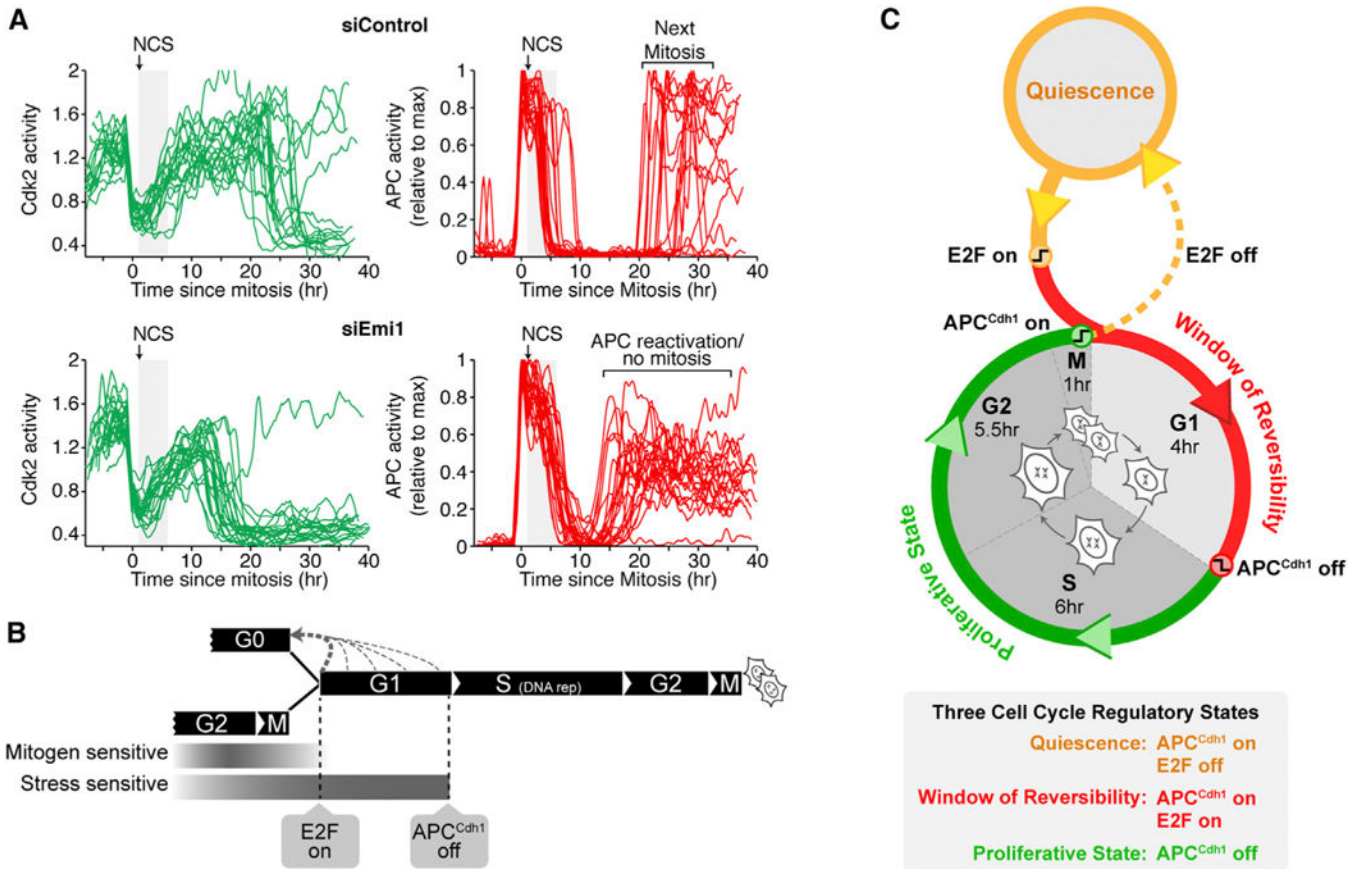


Figure 7. APC^{Cdh1} Inactivation Is a Rapid, Irreversible, and Bistable Switch that Commits Cells to Progress through the Cell Cycle

(A) Cdk2 and APC^{Cdh1} activities in cells exposed to NCS and treated with either control (top) or Emil siRNA (bottom). Cells treated with Emil siRNA re-activate APC^{Cdh1} and inactivate Cdk2. Control siRNA treated cells re-activate APC^{Cdh1} much later during mitosis (n = 28, si Control; n = 23, siEmil).

(B) Windows of mitogen and stress sensitivity during cell-cycle entry and exit. Timing of cell-cycle entry and exit shows that mitogens and stress regulate entry during different time windows. Approximate cell-cycle phase durations for MCF10A are highlighted to place APC^{Cdh1} inactivation and cell-cycle commitment into the overall cell-cycle context.

(C) Cell-cycle entry and exit is characterized by three regulatory states: quiescence, a window of reversibility, and a proliferative state, defined by changes in APC^{Cdh1} and E2F activities. The scheme shows the relationship of these states with the cell-cycle phases. See also Figure S7.