DNA damage during S-phase mediates the proliferation-quiescence decision in the subsequent G1 via p21 expression

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Following DNA damage caused by exogenous sources, such as ionizing radiation, the tumour suppressor p53 mediates cell cycle arrest via expression of the CDK inhibitor, p21. However, the role of p21 in maintaining genomic stability in the absence of exogenous DNA-damaging agents is unclear. Here, using live single-cell measurements of p21 protein in proliferating cultures, we show that naturally occurring DNA damage incurred over S-phase causes p53-dependent accumulation of p21 during mother G2- and daughter G1-phases. High p21 levels mediate G1 arrest via CDK inhibition, yet lower levels have no impact on G1 progression, and the ubiquitin ligases CRL4Cdt2 and SCFSkp2 couple to degrade p21 prior to the G1/S transition. Mathematical modelling reveals that a bistable switch, created by CRL4Cdt2, promotes irreversible S-phase entry by keeping p21 levels low, preventing premature S-phase exit upon DNA damage. Thus, we characterize how p21 regulates the proliferation-quiescence decision to maintain genomic stability.
Cell cycle regulation balances the requirement of proliferation during tissue growth and homeostasis, with the need to ensure that damaged DNA is not propagated to future generations. Checkpoints have evolved to achieve control of cell cycle progression in response to DNA damage. The importance of DNA damage checkpoints is highlighted by the fact that their dysregulation is the fundamental basis of oncogenesis.\(^1\)

The tumour suppressor and transcriptional regulator p53 is stabilized in response to DNA damage and regulates the expression of numerous target genes involved in the DNA damage response.\(^2\) Amongst the transcriptional targets of p53 is the Cyclin-dependent kinase (CDK) inhibitor p21 (refs 4,5). By inhibiting CDK activity, p21 mediates cell cycle arrest.\(^6\)\(^,\)\(^7\) downstream of p53, in response to DNA damage caused by exogenous sources, such as ionizing irradiation or chemical agents.\(^8\)\(^,\)\(^9\). However, the role of p21 in cell cycle control in cells proliferating in the absence of such DNA damage is less clear. Moreover, whether p21 acts as a tumour suppressor in vivo is controversial.\(^10\) For example, in p21 knockout (KO) mice exposed to exogenous DNA damage, both accelerated\(^11\)\(^–\)\(^14\) and inhibited\(^15\) tumourigenesis have been observed. However, the fact that p21KO mice develop spontaneous tumours,\(^15\) albeit later than tumourigenesis have hypo-phosphorylated pRb (Supplementary Fig. 1e). Population growth kinetics and cell cycle timing were unaffected by introduction of the GFP tag into p21 and p21-GFP is able to bind CDK2 (Supplementary Fig. 1d–f). Further confirmation that p21 function is retained by p21-GFP is that cells expressing p21-GFP are able to enter G1 arrest in high-serum growth conditions (Fig. 1f), whereas p21siRNA and p21KO cells are not (Fig. 2b).

Live cell tracking revealed marked cell-to-cell variability in p21-GFP levels amongst G1 and G2 hTert-RPE1 cells, within the same population (Fig. 1c). This variability was also seen in hTert-RPE1 cell fixed and immunostained for unlabelled p21 (Supplementary Fig. 2a). G1 cells have the highest and most variable p21-GFP levels, and there exists a strong correlation between p21-GFP levels and G1 phase length (\(R = 0.62**;\) Fig. 1d). G2 is associated with lower and less variable p21-GFP levels and correlation between G2 length and p21-GFP levels is weaker than in G1 (\(R = 0.51**;\) Supplementary Fig. 2b).

Cells express undetectable levels of p21-GFP during S-phase (Fig. 1c), consistent with previous reports.\(^23\)\(^,\)\(^24\). We do not observe p21-GFP degradation during mitosis, and mitotic p21-GFP expression does not affect mitotic timing (Supplementary Fig. 2c,d).

The lack of p21 degradation during mitosis would allow daughter cells to inherit p21 protein from their mother. Indeed, there is a positive correlation between p21-GFP levels in the mother cell G2 (G2M) and daughter cell G1 (G1D; \(R = 0.75**;\) Supplementary Fig. 2e). Moreover, G1D p21-GFP levels in sister cells are positively correlated (\(R = 0.81**;\) as well as G1D length between sisters (\(R = 0.53**;\) Fig. 1e). Despite the strong correlation in p21-GFP levels in G2M with p21-GFP levels in G1D, the phase lengths of G2M and G1D correlate poorly (\(R = 0.07, P = 0.36\) Pearson’s Correlation). Instead, we find G1D p21-GFP levels correlate better with combined G2M + G1D length (\(R = 0.68**\) than with G1D length alone (\(R = 0.62**\)) suggesting that G2M length is not predictive of G1D because of noise in the timing of mitotic entry (Methods). As such, we find that the maximum p21-GFP level reached in G1D is most reflective of the time between S-phase exit in the mother cell and S-phase entry in daughter cells. Given that much weaker correlations are observed between phase lengths before and after S-phase (Methods), we suggest that stochastic events occurring in S-phase determine p21 levels in, and the length of, the combined G2M + G1D phase.

Although p21-GFP levels begin to rise during G2, p21-GFP levels further increase after mitosis (Fig. 1b,c,f). In 21% (47/219) of G1 cells, p21-GFP accumulates to a high level shortly after mitosis and these cells enter a state we define as G1pm arrest (21% chance of any cell arresting, 0.21^2 = 0.044). As such, conditions leading to arrest are frequently inherited by both daughters.
daughter cells. Finally, where either single or twin arrest occurs in daughter cells, mean G2M p21-GFP levels are higher than those in G2M of cycling daughter cells (twin arrest = 9.6 a.u.; single arrest = 8.0 a.u.; cycling = 5.9 a.u.; Fig. 1g). Thus, we propose that factors contributing to G1pm arrest are detected prior to mitosis, and the single arrest we observe is caused by asymmetric inheritance of these factors; though p21 levels can increase further in G1 to promote G1pm arrest. These observations suggest that the proliferation-quiescence decision in G1, in unperturbed cells, is regulated by p21 levels, which in turn are determined by events occurring in the mother cell S-phase.

p53 drives p21 heterogeneity in unperturbed conditions. We sought to determine factor(s) driving heterogeneity in p21 levels. Multiple transcription factors have been linked to p21 regulation10; the best characterized being p53. Depletion of p53 by siRNA resulted in a large reduction in basal p21-GFP levels, compared to control siRNA (Fig. 2a; Supplementary Fig. 3a–c). Furthermore, G1 length became more homogeneous (Fig. 2a) and no cells analysed entered G1pm arrest (Fig. 2b). Thus, both G1pm arrest and G1 delay are p53-dependent, and p53 signalling is necessary for p21 accumulation in hTert-RPE1 cells.
We further analysed the dependence of p21 expression on p53 by adding the inhibitor Nutlin-3 to stabilize p53 protein. On Nutlin-3 addition, we see a net increase in p21-GFP expression (Supplementary Fig. 3d). However, p21-GFP shows a cell cycle phase-dependent response to Nutlin-3. Nutlin-3-treated cells in G1 or G2 accumulate p21-GFP continuously and...
arrest in G1 (Fig. 2c), while cells in S-phase maintain low p21-GFP during S-phase, before rapidly accumulating p21-GFP after the S/G2 transition (Fig. 2c; Supplementary Movies 2,3). In these cells we observe that G2 arrest correlates with the rate of p21-GFP accumulation, meaning that cells with the highest rate of p21-GFP accumulation arrest in G2, while cells accumulating p21-GFP more slowly undergo mitosis before entering a G1pm arrest (Fig. 2d; Methods).

G1pm arrest is p21-dependent but G1 delay is p21-independent. Since p21-GFP levels correlate with G1 length, and depletion of p53 leads to a more homogenous G1 length, we sought to determine if p21 regulates G1 length. Following p21 depletion by siRNA, only 7% (5/75) of p21-depleted cells entered a G1pm arrest, compared to 17.2% (11/64) of control siRNA-treated cells (Fig. 2b), but we saw no change in G1 length distribution (Fig. 2a). To confirm the effect of depleting p21 by siRNA, we generated CRISPR/Cas9 deletions in the CDKN1A gene using two different gRNAs, p21KO1 and p21KO2 (Supplementary Fig. 3e; Supplementary Note 1). We observed only one p21KO cell entering G1pm arrest (1/172; Fig. 2b), in spite of our observation that p21KO cells have increased levels of DNA damage, as measured by γH2AX levels (Supplementary Fig. 3f). As observed following p21 depletion by siRNA, p21KO in two independently generated clones did not change G1 length (Supplementary Fig. 3g). As such, we find that heterogeneity in G1 length is p53-dependent but p21-independent, while G1pm arrest is both p21- and p53-dependent. Moreover, that cells lacking p21 have increased levels of DNA damage suggests that p21 is important in maintaining long-term genome stability, consistent with recent work.

High p21 levels are required to inhibit CDK2 activity. Since p21 is a CDK inhibitor, we sought to characterize how CDK2 activity changed in p21- versus p53-depleted cells. We generated an hTert-RPE1 mRuby-PCNA cell line expressing a live cell reporter of CDK2 activity28 and depleted p21 or p53 by siRNA. In control siRNA-depleted cells, we see heterogeneity in CDK2 activity following mitosis (Fig. 2e) and there is significant negative correlation between the minimum level of CDK2 activity and G1 length ($R = -0.67^{*}$). In addition, 13.7% (7/51) of the population enter a CDK2low state (red curves; CDK activity <0.75 a.u. at 600 min, Fig. 2e), which, although arbitrarily defined, is similar to the percentage of cells entering a p21high state (red curves, Fig. 1f). This suggests that the CDK2low state corresponds to the p21high state and represents G1pm arrest before the RP. Indeed, if we fix unperturbed hTert-RPE1 cells co-expressing mRuby-PCNA and the CDK2 activity sensor and immunostain for p21, we see that CDK2low cells in G1 represent 16% of the population and that these cells express high levels of p21 (Supplementary Fig. 3h).

Consistent with p53 siRNA treatment in p21-GFP hTert-RPE1 cells, p53 depletion in CDK2 activity sensor expressing cells leads to a more homogenous G1 length and cells do not enter G1pm arrest (0/25; Fig. 2e). However, following p21 siRNA we still see a strong negative correlation between the minimum CDK2 activity and G1 length ($R = -0.63^{**}$). We only observed one p21-depleted cell entering a CDK2low state (1/68), suggesting that the CDK2low G1pm arrest state is due to p21-dependent inhibition of CDK activity, consistent with previous work (Fig. 2e). To determine how different levels of p21 correlate with CDK2 activity, we imaged hTert-RPE1 p21-GFP cells co-expressing an mRuby-tagged CDK2 activity sensor. We found in cycling G1 cells that CDK2 activity is able to increase in the presence of p21 protein (Fig. 2f, grey box). Moreover, CDK2 activity remains high throughout G2 in unperturbed cells, irrelevant of p21-GFP levels (Fig. 2f, brown box). Importantly, at high p21-GFP levels in G1 cells, CDK2 activity is inhibited and cells enter G1 arrest (Fig. 2g).

These data further support the notion that p53 signalling induces p21 accumulation over G2M and G12 and, at sufficiently high p21 levels in G1, CDK2 activity is inhibited leading to a p21-dependent G1pm arrest. However, at low-intermediate levels, p21 is a poor CDK2 inhibitor and a G1 delay is not p21-dependent.

**Basal p21 expression correlates with DNA damage foci.** Given our observation that p21 heterogeneity in unperturbed hTert-RPE1 cells is p53-mediated, reflective of the time between S/G2 in the mother and G1/S in the daughter, and that p21 starts to increase in G2, we postulated that p21 levels increase in response to endogenous DNA damage occurring during S-phase in the mother cell. To assess this, we immunostained unperturbed hTert-RPE1 cells with the DNA damage marker γH2AX and found a striking correlation between p21-GFP expression in G1 and G2 cells and the presence of a single nuclear focus of γH2AX (Fig. 3a–c). We found a similar correlation between foci of 53BP1 and PS1981-ATM with p21-GFP levels (Supplementary Figs 4a,b). These single foci represent endogenous DNA damage (since no exogenous damaging agents were added) and resemble stretches of incompletely replicated DNA29, leaving single-stranded DNA (ssDNA) gaps in G2 cells. Indeed, in unperturbed hTert-RPE1 cells we see colocalization of the ssDNA marker, RPA2, with single γH2AX foci in G2, and an increase in both RPA2 and γH2AX foci is seen after treatment with the DNA polymerase inhibitor, aphidicolin (Supplementary Fig. 4c). Taken together, we hypothesize that endogenous DNA damage generated in unperturbed S-phases could trigger p21 expression and be the molecular basis for the observed heterogeneity in p21 levels.

To explore the link between endogenous DNA damage and p21 expression, we generated hTert-RPE1 cells co-expressing p21-GFP and mRuby-53BP1. 53BP1 localizes to DNA Double Strand Breaks (DSBs) during G1 (ref. 30). Live cell imaging in unperturbed cells revealed that DSBs co-segregate with p21-GFP expression in G1 (Supplementary Movie 4; Supplementary Fig. 4d). To determine how the presence of DNA damage foci in G1 affects proliferation, we generated a cell line co-expressing mRuby-PCNA and GFP-53BP1. Timelapse imaging revealed that in the majority of cell divisions, sister G1 cells behave similarly such that in 47% (67/142) of mitoses neither sister has a 53BP1 focus, in 24% (34/142) both sister cells have a focus, and in 29% (41/142) only one sister has a focus (Fig. 3d). When both sister cells have a 53BP1 focus, the timing of focus appearance shows a small, but significant, positive correlation ($R = 0.39^{*}$; Fig. 3e). Moreover, G1 cells displaying a 53BP1 focus have a longer G1 phase than cells never displaying a focus (Fig. 3f). In 81% (48/59) of G1 cells expressing GFP-53BP1 foci, foci start to decrease in size before S-phase, suggesting that most hTert-RPE1 cells repair DNA damage prior to the G1/S transition. In unperturbed mRuby-PCNA 53BP1-GFP expressing cells, 22% (50/222) of cells enter a G1pm arrest, and of these, 82% (41/50) have at least one 53BP1 focus, indicating a high correlation between G1pm arrest and the presence of DNA damage.

These data suggest that DNA damage is inherited from the mother cell leading to 53BP1 localization to DSBs in daughter cells, that correlates with both a G1 delay and G1pm arrest. Despite p21 levels correlating with the presence of 53BP1 foci and a G1 delay, only G1pm arrest, but not G1 delay, is p21-dependent.
Impaired DNA replication can induce p21 expression. Our data suggest that stochastic DNA damage occurring during normal DNA replication could generate the observed heterogeneity in p21 expression. To determine if p21 expression can be induced by impaired DNA replication during S-phase, we treated cells with low dose aphidicolin. Aphidicolin treatment caused an increase in the proportion of cells with a single γH2AX focus, in the number of γH2AX foci per nucleus, and in p53 expression (Fig. 4a; Supplementary Fig. 5a). In timelapse imaging of aphidicolin-treated cells, we observed a longer S-phase, indicative of replication fork slowing, and a marked increase in p21-GFP levels following S/G2, compared to DMSO-treated cells (Fig. 4b). Following mitosis, 62.5% (20/32) of aphidicolin-treated cells entered G1pm arrest, compared to 13.2% (5/38) of DMSO-treated cells (Fig. 4b). In addition, 25% (8/32) of aphidicolin-treated cells arrested in G2 with high p21-GFP expression, a phenotype rarely observed in unperturbed cells. Of note, we observed that after aphidicolin treatment of hTert-RPE1 cells expressing mRuby-PCNA and a CDK2 activity sensor, that cells arresting in G2 eventually downregulate CDK2 activity (Fig. 4d; top panel, bottom panel).
yellow curves). This suggests that when levels of damaged DNA, and corresponding p21 levels, exceed a threshold, CDK2 activity is inhibited and cells undergo G2 arrest. Overall, these data show that by inducing aberrant DNA replication with aphidicolin, cells activate p21 expression, downstream of p53, which promotes either a G1 or G2 arrest.

To further examine if DNA damage during S-phase can induce p21 accumulation in G2, we treated cells with an ATR kinase inhibitor (ATRi; AZD6738) to induce destablization of stalled DNA replication forks. Treatment with ATRi leads to an increase in the proportion of cells with a single γH2AX focus, in the number of γH2AX foci per nucleus, and in p53 expression.
(Fig. 4c; Supplementary Fig. 5b). Similar to aphidicolin treatment, cells treated with ATRi display a marked increase in p21-GFP levels in cells in G1, compared to DMSO-treated cells (Fig. 4b). The number of cells arresting in G1 following mitosis also increased from 13.2% (5/38) in DMSO-treated cells to 52.5% (21/40) in ATRi-treated cells (Fig. 4b). However, unlike aphidicolin treatment, we saw a small, but significant, decrease in S-phase length when compared to DMSO-treated cells (Supplementary Fig. 5c), and no cells were observed to arrest in G2, consistent with G2 arrest being ATR-dependent33. Taken together, these data demonstrate that in the absence of ATR activity, DNA damage in S-phase leads to accumulation of p21 protein that frequently exceeds the threshold required for cell cycle arrest.

To determine if p21 and/or p53 is required for the cellular response to impaired DNA replication, we depleted p21 or p53 by siRNA and treated hTert-RPE1 cells expressing mRuby-PCNA and the CDK2 activity sensor with aphidicolin. p53-depleted cells treated with aphidicolin were unable to arrest in G1 (0/32) (Fig. 4d). G1 length was more homogeneous and G2 arrest was absent. However, in a fraction of cells, an extended G2 phase was observed. In p21-depleted cells treated with aphidicolin, there was no G2 arrest (0/42; although similar to p53-depleted cells extended G2 lengths were observed), and G1p3 arrest was severely compromised compared to control siRNA-treated cells (7/42 p21-depleted cells arrest versus 17/24 in control siRNA-depleted cells; Fig. 4d). These data support our conclusion that G1p3 arrest is p21- and p53-dependent, and also reveal a p53- and p21-dependent G2 arrest.

Skp2 and Cdt2 degrade p21 with different rates and timings.

Since p21-GFP is absent in all cells during S-phase, we hypothesized that, when high levels of p21 promote cell cycle arrest, its removal is required for efficient DNA replication. Both SCFSkp2 and CRL4Cdt2 have been implicated in p21 degradation at the G1/S transition34-38 and we wanted to determine the relative contribution of these two pathways to p21 removal.

Following Skp2 depletion by siRNA, we find that p21-GFP is still degraded prior to and over S-phase (Fig. 5a; Supplementary Fig. 6a,b). However, p21-GFP degradation becomes switch-like at the G1/S transition in Skp2-depleted cells (Fig. 5a). While in control siRNA-depleted cells p21-GFP levels correlate with G1 length ($R = 0.76^*$), in Skp2-depleted cells G1 length was longer and showed weaker correlation with p21-GFP levels ($R = 0.40^{**}$; Fig. 5b). These data suggest that SCF$^{Skp2}$ plays an important role in degrading p21 prior to the G1/S transition and is responsible for the slower rate of p21 degradation during G1. Additionally, in control siRNA-treated cells we observed a ‘burst’ in p21-GFP expression after mitosis. However, following SCF$^{Skp2}$ depletion, p21-GFP levels in G2 increased much more rapidly and no post-mitotic burst in p21-GFP was observed (Fig. 5a).

After Cdt2 depletion by siRNA, notably fewer cells entered S-phase than control-depleted cells, and only cells that removed p21-GFP to undetectable levels entered S-phase (Fig. 5a,b; Supplementary Fig. 6c; Methods). In Cdt2-depleted cells, p21-GFP levels increased immediately after S-phase entry (Fig. 5a) and this increase was associated with a longer S-phase. We observed anti-correlated cycles of p21-GFP expression with mRuby-PCNA foci over short time frames—suggestive of interrupted cycles of incomplete DNA replication (Supplementary Fig. 6d; Supplementary Movie 5). Cell death was also observed following Cdt2 depletion. Finally, switch-like degradation of p21-GFP at the G1/S transition was abolished when Cdt2 was depleted (Fig. 5a). Thus, our data show that CRL4$^{Cdt2}$ is essential for degrading p21 during S-phase and is responsible for the switch-like, rapid degradation of p21 at the G1/S transition. Cdt2 depletion phenotypes were rescued by p21 and Cdt2 co-depletion—where cells had normal S-phase lengths (Supplementary Fig. 6e), and no interrupted DNA replication or cell death was observed. These data show that even the presence of low levels of p21 during S-phase can perturb DNA replication, cause premature S-phase exit, and cell death.

Depleting both Skp2 and Cdt2 prevented the vast majority of cells from entering S-phase (Fig. 5a). Cells that did enter S-phase had p21-GFP levels below our detection limit (Fig. 5b). Of note, we observed that after depletion of only Cdt2, or after Cdt2 and Skp2 co-depletion, overall p21-GFP levels increased over time, indicative of accumulating DNA damage (Fig. 5c). Thus we find that p21 removal at the G1/S transition promotes efficient DNA replication, limiting DNA damage, and that SCF$^{Skp2}$ and CRL4$^{Cdt2}$ cooperate to degrade p21 with different rates and timings.

Two double-negative feedback loops control p21 degradation.

To understand the mechanism behind p21 dynamics and its removal, we sought to mathematically model p21 regulation. We constructed a wiring diagram based on well-established reactions that make up the p21 control network (Fig. 6a; Methods). In our model, two double-negative feedback loops control p21 expression: (i) phosphorylation by CDK2 targets p21 for Skp2-dependent degradation24,39, while high levels of p21 inhibit CDK2 activity5; and (ii) PCNA in active replication complexes (aRCs) recruits CRL4$^{Cdt2}$ to degrade p21 (refs 24,40), while p21 can bind to PCNA interfering with its function as a processivity factor during DNA replication41,42.

Consistent with our data, stochastic simulations show p21 degradation prior to the G1/S transition, the absence of p21 during S-phase, and its re-accumulation in G2 (Fig. 6b; Supplementary Fig. 7a). p21 loss in G1 is mainly caused by increasing CDK2 activity, which promotes Skp2-dependent degradation at a comparatively low rate. Once CDK2-Cyclins reach a threshold, PCNA loading onto DNA is triggered, aRCs accumulate, and strong Cdt2-dependent degradation removes residual p21 while the cell enters S-phase. At the end of S-phase, aRCs disappear and p21 re-accumulates. We also see significant intra- and inter-cellular variability in p21 levels due to the stochasticity of DNA damage and p53, supporting our experimental observations (Fig. 6c). However, p21 remains low over S-phase even when damage occurs, consistent with data from our experiments (Figs 2c,d and 4b). This is due to the presence of a bistable switch, which creates two distinct cellular states with either high or low p21 level depending on the cell cycle stage (Fig. 6d). In G1, CDK2 activity is low and RCs are inactive such that p21 is attracted to the upper steady state. As CDK2-Cyclin accumulates and origins fire, p21 is lost and cells enter S-phase. The existence of a bistable region between both states allows the S-phase level of p21 to be insensitive to DNA damage, while damage in G1 (and G2, Supplementary Fig. 7b) leads to an increase in p21 and in the CDK2-Cyclin threshold required to enter S-phase. Hence, large amounts of damage can cause G1p3 arrest (Fig. 6d, topmost curve).

We sought to test the relative importance of the Skp2- and Cdt2-degradation pathways for bistability. Skp2 depletion leaves the bistable switch intact such that S-phase levels of p21 remain low (Supplementary Fig. 7c). However, Skp2-depleted cells show a more rapid p21 degradation prior to S-phase and a more rapid re-accumulation in G2 (Fig. 6e, left panels). Cdt2 depletion almost completely abolishes bistability, which makes the G1/S transition easier to reverse (Supplementary Fig. 7c), and leads to p21 expression during S-phase (Fig. 6e, right panels). This latter phenotype is more pronounced in cells depleted of...
both Skp2 and Cdt2 (Supplementary Fig. 7d). As found experimentally (Supplementary Fig. 6d,e), p21 expression during S-phase can cause premature S-phase exit, which modelling suggests is due to PCNA inhibition (Supplementary Fig. 7e). Hence, strong Cdt2-dependent degradation of p21 ensures an irreversible G1/S transition and undisturbed DNA replication.

In summary, we have developed a model that not only explains p21 dynamics in unperturbed and DNA damaged cells but also correctly predicts the effects of Skp2 and Cdt2 depletion experiments. Moreover, modelling confirms that stochastic DNA damage events are a major source of inter- and intra-cellular heterogeneity in p21 levels, and reveals a
bistable switch that makes S-phase cells unable to accumulate p21 protein.

**Arrest in response to serum starvation is not p21-dependent.**

Given that passage through the RP depends on mitogen-signaling and thus serum concentration, we wanted to determine how p21-GFP levels change in hTert-RPE1 cells during G1pm arrest in response to serum starvation. As cells exit mitosis in the absence of serum, the majority (90%; 26/29) enter G1pm arrest (Fig. 7a). Importantly, these arresting cells have variable p21-GFP levels, and many serum-starved G1pm-arrested cells have low p21-GFP levels, comparable to those measured during G1 in cycling cells (Fig. 1f). Moreover, unlike cells in serum, G2Md p21-GFP levels do not correlate with G1pm arrest ($R = 0.0989$, $P = 0.6098$). Therefore, while G1pm arrest in the presence of high serum is characterized by high p21 expression, G1pm arrest after serum withdrawal is not.

Since we did not observe a correlation between p21-GFP levels and G1pm arrest after serum starvation, we wanted to know if cells could arrest in G1 in response to serum withdrawal in the absence of p21 protein. We serum-starved WT, p21KO1 and p21KO2 hTert-RPE1 cells and measured their cell cycle profiles by FACS. We found that p21KO cells arrested in G1 as efficiently as WT cells in response to serum withdrawal (Fig. 7b).
This indicates that p21 is not required for G1pm arrest after serum removal.

Thus there exist two molecularly distinct G1pm arrest states—one that occurs in the presence of serum and is p21-dependent, and another that occurs following serum withdrawal that is p21 independent. Furthermore, the p53- and p21-dependent G1pm arrest we observe in cells grown in high serum is principally caused by endogenous DNA damage.

**Discussion**

In this study, we sought to characterize and understand the role of p21 in regulating the proliferation-quiescence decision and genome stability in unperturbed cells. In response to DNA damage generated during S-phase, p21 accumulation begins in G2M and continues into G1D, where, at sufficiently high levels, p21 inhibits CDK activity and promotes G1 arrest following mitosis.

Our experiments demonstrate that in unperturbed cycling cells, heterogeneity in p21 levels is fully accounted for by p53. This is consistent with a recent report demonstrating that, in cells expressing both p53-YFP and p21-CFP, p21-CFP levels peak following peaks in p53-YFP (ref. 43). The authors also observed a spike in p53 and p21 levels following mitosis, agreeing with our observation that p21-GFP levels increase rapidly in G1 post-mitosis. This may be explained in part by ssDNA breaks generated by incomplete DNA replication in the mother cell being converted to DSBs during mitosis44,45. A post-mitotic increase in p21 levels can also be explained by SCF$^\text{Skp2}$-mediated p21 degradation being turned off in G1 by increasing APC/C$^\text{Cdh1}$ activity46,47. Since we observe significantly higher levels of p21 in G2, as well as a reduced p21 increase in G1, following Skp2 depletion, this suggests that the post-mitotic burst in p21 is likely to be a combination of both SCF$^\text{Skp2}$-inactivation and additional DNA damage occurring over mitosis.

Spencer et al.22 demonstrated that p21 is responsible for a bifurcation in CDK2 activity following mitosis, and that this underpins the proliferation-quiescence decision in cycling cells. We find that the source of bifurcation in the p21-dependent proliferation-quiescence decision is endogenous DNA damage occurring in the mother cell (Fig. 7c). Strikingly, we find that while p21 is responsible for G1pm arrest, heterogeneity in G1 length is not p21-dependent. Because heterogeneity in G1 length is p53-dependent, this explains the correlation between p21 levels and G1 phase length; p53 activity is leading to transcriptional activation of p21, as well as causing a G1 delay. Thus, p21 is acting as a readout of p53 activity, while having an insignificant effect on G1 phase length. Determining the mechanisms responsible for this p53-dependent G1 delay remains an open challenge.

Our data and modelling suggest that p21 degradation has the hallmarks of bistability due to two double-negative feedback loops involving SCF$^\text{Skp2}$ and CRL4$^\text{Cdt2}$. Thus we provide experimental
evidence for, and extend on, previous work which concluded that the decision to remain quiescent or proliferate is bistable because of feedback regulation between p21 and CDK2 via SCF<sup>Kip2</sup> (ref. 21). Moreover, our finding that CRAP<sup>Δδδ</sup> is required for p21 degradation at the G1/S transition and over S-phase is consistent with previous work by Kim et al. who find, in line with our experiments, that DNA re-replication occurs in the absence of CRAP<sup>Δδδ</sup> and that this is p21-dependent<sup>38</sup>. We speculate that p21 acts downstream of pulsatile p53 signalling to integrate information on DNA damage during mother G2/M- and the daughter G1- phases that then determines the outcome of the proliferation-quiescence decision in G1. Complete degradation of p21 before S-phase entry would ensure that information on DNA damage generated during the previous cycle is fully erased before cells commit to a new cell cycle. This would allow the process of gathering information on DNA damage to begin anew following S/G2 passage. The presence of an information-gathering phase and a reset phase would allow cells to make a decision on whether to proliferate or quiesce in response to DNA damage that remains consistent across populations and generations of cells.

**Methods**

**Cell culture.** hTert-RPE1 cells were from ATCC and were maintained in DMEM (Gibco) 10% FBS (Sigma) and 1% Penicillin-Streptomycin (PS; Gibco) at 37 °C and 5% CO₂. Cell lines were checked by PCR on a monthly basis for absence of mycoplasma.

**Generating mRuby-PCNA tagged hTert-RPE1 cell line.** One allele of the endogenous PCNA gene was tagged at the N-terminus with a cDNA encoding the mRuby fluorophore using AAV-directed homologous recombination. To generate the targeting construct, Left and Right Homology Arms (LHA, RHA) flanking mRuby cDNA were amplified by PCR from hTert-RPE1 genomic DNA using the following primers: P1 NotI: 5'-cggggtctggatcagctgagc-3' and P2: 5'-ctgtagattgtgatcagctgagc-3' to amplify the LHA including the first 20 bp of mRuby; P3: 5'-ttgttgctgcagccacggagacctgcagtcaag-3' including the first 20 bp of PCNA LHA; and P4 XbaI: 5'-ctcgattgcagctcctcctgcccagggcggc-3' to amplify mRuby and create a Gly-Gly-Gly-Ser-Arg linker in front of the first PCNA exon. P5 XbaI: 5'-tcattagctgagcgcgctgtgc-3' and P6 NotI: 5'-gcgggctcgacggagagatcagc-3' to generate the RHA. LHA and mRuby fragments were fused by PCR using P1 and P4 and cloned by three-way ligation, together with the RHA into the NotI sites of pAAV-MCS (Stratagene). The complete targeting construct was packed into pAAV viruses using an AAV helper-free system (Stratagene), according to the manufacturer’s instructions, followed by transduction of hTert-RPE1 cells. Four days after transduction, single mRuby positive integrands were sorted by flow cytometry into 96-well plates and analysed for correct targeting of PCNA by Western blotting.

**Generating p21-GFP-tagged hTert-RPE1 cell lines.** The endogenous CDKN1A gene was tagged at the C-terminus with CRISPR-mediated gene tagging. The gRNA 5'-TTTTGGGCTCCTGCGCTTCAGG-3' (PAM site in bold) downstream of the CTKN1A stop codon was selected using crispr.mit.edu. Forward and reverse oligos for the gRNA were annealed and ligated into the Bsal cut pX330 bicstronic Cas9 and sgRNA expression plasmid<sup>22</sup>. P3X30-06-Chimeric_BB-CBh-hspCas9 was a gift from Feng Zhang (Addgene plasmid #42230). For the homology donor plasmid, we PCR amplified CDKN1A homology arms from hTert-RPE1 genomic DNA using the high-fidelity Q5 DNA polymerase (NEB). To PCR amplify the LHA we used the forward primer with a NotI site: 5'-cgccgctggagcctggc-3' and the reverse primer with a Sall site: 5'-ctcgattgcagctcctcctgcccagggcggc-3'. To PCR amplify the RHA we used the forward primer with a Sall site: 5'-gttggctgcagccacggagacctgcagtcaag-3' to amplify the LHA including the first 20 bp of mRuby; 5'-ttgttgctgcagccacggagacctgcagtcaag-3' including the first 20 bp of PCNA LHA; and 5'-ctcgattgcagctcctcctgcccagggcggc-3' to amplify mRuby and create a Gly-Gly-Gly-Ser-Arg linker in front of the first PCNA exon. P5 XbaI: 5'-tcattagctgagcgcgctgtgc-3' and P6 NotI: 5'-gcgggctcgacggagagatcagc-3' to generate the RHA. LHA and mRuby fragments were fused by PCR using P1 and P4 and cloned by three-way ligation, together with the RHA into the NotI sites of pAAV-MCS (Stratagene). The complete targeting construct was packed into pAAV viruses using an AAV helper-free system (Stratagene), according to the manufacturer’s instructions, followed by transduction of hTert-RPE1 cells. Four days after transduction, single mRuby positive integrands were sorted by flow cytometry into 96-well plates and analysed for correct targeting of PCNA by Western blotting.

**Generating p21KO cell lines.** We generated two CDKN1A nickase targeting vectors, based on the methods described in ref. 49. For p21KO1, we used the two gRNA sequences: 5'-GTATGGCTTGACCCATGCTC-3' and 5'-TCTAAGACCCAGGGTAC-3'. These were annealed and ligated into the dual sgRNA All-in-One Cas9<sup>0106</sup> GFP nickase vector<sup>23</sup>. After sequencing, plasmids were transfected into hTert-RPE1 cells expressing mRuby-PCNA. Three days post transfection, GFP positive cells were sorted to single-cell density into three 96-well plates containing 1:1 conditioned fresh growth media. Clones were left to expand for 7–10 days and then screened for p21 expression by immunostaining. Clones deemed to be negative based on p21 immunostaining were characterized further by genotyping.

For genotyping of p21KO lines, genomic DNA was extracted using the Flexigene DNA kit, according to the manufacturer’s instructions. The p21 gene was amplified using nested PCR with primers: CDKN1A_KO_FWD1 5'-ATGTCAGACCAGCCTG-3' and CDKN1A_KO_REV1 5'-TTAGGCTTTCCTCCTT-3', followed by CDKN1A_KO_FWD2 5'-GGATGTCCGTCAGAACCCAT and CDKN1A_KO_REV2 5'-GTGGAGAAGGTAGACGTGG-3'. The resultant PCR product was ligated into the pEt2.1b Cloning Vector (Fermentas). Plasmid DNA was purified from individual colonies using the Qiagen Miniprep kit and sent for sequencing using the pEt_forw primer. The results of the genotyping are shown in Supplementary Note 1. Note that for p21KO1A, 10/10 colonies showed the same genotype suggesting that both alleles have the same deletion.

**Live cell imaging.** Live cell imaging was performed on the Opera HC spinning disk confocal microscope (PerkinElmer), with atmospheric control to maintain cells at 37 °C, 5% CO₂ and 80% humidity. Cells were plated on 384 well CellCarrier (PerkinElmer) plates at a density of 1,000 cells/well 24 h prior to imaging. Cells were imaged using a 20X (N.A. 0.45) objective at 10 min intervals for 24-72 h in Phenol-Red Free DMEM + 10% FBS and 1% P/S. Image processing was performed in Matlab using custom-made scripts.

**Testing 488 nm exposures for live imaging.** Since laser irradiation can induce DNA damage, and thus p21 expression, we wanted to be sure that our imaging conditions were not inducing p21 expression. Therefore, we imaged hTert-RPE1 cells at 36 h post 488 nm laser exposures where the laser power was greater than the 20% used in our other experiments; exposure time remained constant (120 ms). The results are shown in Fig. 1d. Exposure...
1 = 20%, Exposure 2 = 30%, Exposure 3 = 40% of 488 nm laser power. Across these three different exposures, we saw no difference in cell cycle timing or amount of p21-GFP expressed. Moreover, a two-sample Kolmogorov-Smirnov test produced the null result indicating both samples came from the same continuous distribution for both maximum G1 p21 after correction for 1.5X and 2X exposure difference: 20% versus 30% $P = 0.938$ and 20% versus 40% $P = 0.243$; and G1 length with no correction: 20% versus 30% $P = 0.916$ and 20% versus 40% $P = 0.607$.

Inhibitors. Inhibitors used were ATRi (AZD6738; Selleckchem), Aphidicolin (Sigma), Camptothecin (Sigma), Exisporide (Sigma) and Nutlin-3 (Sigma). All drugs were reconstituted in DMSO and stored in aliquots at -20 °C.

siRNA transfection. For live cell imaging, cells were either transfected 24 h prior to imaging, or, in cases where we wanted to capture the first cell cycle after protein loss, 6 h prior to imaging. Cells were transfected with 20 nM final concentration of siRNA using Lipofectamine RNAiMAX. According to the manufacturer’s protocol (Invitrogen). Briefly, per well of a 384 well plate, 40 nL of Lipofectamine RNAiMAX was mixed with siRNA in 10 μL OPTI-MEM. To this, 20 μL cells in medium, 20% versus 30% $P = 0.938$ and 20% versus 40% $P = 0.243$; and G1 length with no correction: 20% versus 30% $P = 0.916$ and 20% versus 40% $P = 0.607$.

Cloning 53BP1-mRuby. GFP-53BP1 plasmid was a generous gift from Jiri Lukas40. This plasmid contains full-length mouse 53BP1 cDNA tagged with GFP. To visualize endogenously tagged p21-GFP and 53BP1 in the same cell line, we changed the GFP tag for an mRuby tag in the 53BP1 plasmid. We removed the GFP tag with BstXI and BseE1 double digestion. We PCR amplified mRuby with following primers: forward 5'-gtgatgagacgattgattc-3' and reverse 5'-tggcagccctgccggcggg-3'. Digested mRuby PCR product was ligated into the cut 53BP1 vector and sequenced to check for in-frame ligation.

Generating GFP- and mRuby-53BP1 stable cell lines. HtRetRPE1 cells were plated in six well plates to ensure a density of 80% confluency on the day of transfection. Cells were transfected with 4 μg of plasmid using Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen). After 24 h incubation, cells were transferred to a T-25, and after a further 24 h, cells were sorted to single-cell density in 96-well plates containing 50% fresh media:50% conditioned media with 0.25 mg ml$^{-1}$ G418 (Sigma), using the BD FACS Aria. After 10–14 days selection, single-cell clones were visualized for appropriate expression of 53BP1 and those clones were expanded.

Immunostaining. Cells were fixed in an equal volume of media to warm 8% formaldehyde in PBS for 5 min at 37 °C. Cells were permeabilized in PBS:0.2% TritonX-100 for 5 min at RT and then blocked in 2% BSA in PBS (Blocking buffer) for 1 h at RT. Cells were incubated in primary antibody diluted in Blocking buffer for 1 h at RT or overnight at 4 °C. Cells were washed three times in PBS and then incubated with Alexa-conjugated secondary antibodies (ThermoFisher) diluted in Blocking buffer for 1 h at RT. Cells were washed three times in PBS and incubated in 1 μg ml$^{-1}$ Hoechst 33528 (Sigma) diluted in PBS for 15 min at RT. Cells were imaged on the Opera HC spinning disc confocal microscope (PerkinElmer). All imaging data was uploaded to the Columbus image analysis database (PerkinElmer) for visualization and analysis.

For RPA2 immunostaining, cells were plated onto glass coverslips. Cells were briefly washed in PBS and then pre-extracted using ice-cold PBS:0.2% TritonX-100 for 1 min. Cells were then fixed in warm 4% formaldehyde and processed as usual. Cells on coverslips were imaged on a Zeiss LSM710 confocal microscope using a 60x oil, N.A. 1.4 objective.

Primary antibodies used for immunostaining in this study are: p21 (BD 556430, 1:1,000), p53 (CST 2527, 1:1,000), 53BP1 (CST 4927, 1:1,000), γH2AX (CST 9718, 1:2,000), P-S1981- ATM (CST 4526, 1:500), RPA2 (Abcam ab2175, 1:250).

Automated cell tracking. Live imaging data was analysed using custom image analysis scripts (see below and Supplementary Software 1). The exception to this was analysis of HtRet-RPE1 mRuby-PCNA cells expressing GFP-53BP1, which were manually analysed using Velocity software (PerkinElmer) as we were unable to achieve reliable tracking of 53BP1 foci, and HtRet-RPE1 p21-GFP cells expressing CDK2L-mRuby (CDK2 sensor28) since there was no constant nuclear signal to track reliably.

For automated tracking of HtRet-RPE1 cells expressing fluorescent markers in higher throughput we developed a cell-tracking pipeline in the commercial software package Matlab. The pipeline tracks cells and extracts data from object sequences of uncompressed TIF images, captured from live cell imaging. Segmentation is based on well-established methods, while tracking is an implementation of a probabilistic tracking algorithm defined in refs 51,52. The code for cell tracking can be found online and contains an example (cellcycle.org.uk; Resources). The key steps in this pipeline are outlined below.

1. Nuclear segmentation:
   1. Initial background correction is performed on individual images; here a highly blurred image (very wide Gaussian kernel) is negated from the original image.
   2. Nuclei from channels with a nuclear probe are combined to maximize the quality of nuclear segmentation, this principally involves channels containing the p21-GFP and mRuby-PCNA signal.
   3. Nuclei are subsequently segmented from the filtered, combined image using Marker Controlled Watershed Segmentation.
   4. Segments with intensity or size below a threshold are filtered out, as these correspond to mis-segmented background noise.
   5. Segmented images are saved as equally sized zero matrices with each nucleus region uniquely labelled.

2. Feature extraction: features describing morphology and fluorescent marker levels and localization for each channel are calculated in each frame. Importantly, here we extract intensity and texture features from unfiltered images. The reasoning behind this is being that across the experiments we perform, average intensity levels can vary over several orders of magnitude for example, under Nutlin-3 treatment; plate-wise or well-wise filtering here can mask true levels of p21 relative to controls. Specifically the features we extract are:
   1. Cell area: area in pixels of the segmented nuclei
   2. Major axis length: length of the ellipse that is equivalent based on second order moments to the segment
   3. Minor axis length: width of the ellipse that is equivalent based on second order moments to the segment
   4. Eccentricity: eccentricity of the ellipse that is equivalent based on second order moments to the segment
   5. Equivalent diameter: diameter of the circle with the same area as the segment
   6. Solidity: percentage of pixels in the convex hull surrounding the segment which are also in the segment
   7. Perimeter: length of the segment
   8. Roundness: defined as $4π \times \text{area}/\text{perimeter}^2$ (ref. 53)
   9. PCNA mean intensity
   10. s.d. of PCNA intensity
   11. Kurtosis of PCNA intensity
   12. Foci strength: PCNA intensities below the mean are removed, the s.d. of pixels above the mean is then calculated. This identifies bright spots, and ignores dark spots in the nucleus, for example, nucleoli.
   13. p21 mean intensity
   14. γH2AX s.d.
   15. Mean silhouette score: K-mean clustering ($K = 2$) is performed on nuclei segments, and the mean silhouette score of the two clusters calculated. This feature identifies dumbbell shapes and is indicative of double nuclei segments. These features are defined in the single-cell track matrices presented online. In the matrices the first column gives cell index, the second time point and third migration distance. Subsequent columns are features as defined above. For CDK2 activity data, p21 intensities are...
replaced with CDK2L-GFP nuclear intensity, CDK2L-GFP ring region intensity, and the ratio of these two values. The ring region, here defined as a seven pixel wide ring surrounding the nucleus, has been shown to perform effectively in measuring nuclear translational movement.

3. Segment probability: segments can be one of five defined objects, either correctly segmented (one nucleus, mitotic nucleus, nucleus exiting mitosis) or incorrectly segmented (no nuclei, more than one nuclei). Probabilistic tracking maximizes the likelihood that a track corresponds to the movements and divisions of a single nucleus. Thus for tracking, probabilities are assigned to each of the five possibilities mentioned, for each segment, in each frame such that tracking can maximize the likelihood a single nucleus is followed. This is performed by manually examining the examples of each of the defined objects, for example, mitotic nuclei, or multiple nuclei, from all segmented objects within a graphical user interface (GUI). Subsequently, a support vector machine classifier is trained on these manually selected examples. The classifier is used to assign a probability to every segment, in every frame, of it being one of these objects. As such, every segmented object, in every frame, is assigned five posterior probabilities corresponding to the likelihood of it being one nucleus; mitotic nucleus; nucleus exiting mitosis; no nuclei; more than one nuclei.

4. Probabilistic tracking: The goal of automated cell tracking is to generate a set of tracks that correspond to individual nuclei as they move across the field of view (FOV), and to capture cell division events, such that the lineage of nuclei is recorded. Briefly, in the automated probabilistic tracking algorithm used, hill-climbing optimization is performed to maximize a cost function designed to satisfy constraints described above. Specifically, the cost function penalizes large movements, and rewards tracks which follow segments that have a high probability of being a single cell; or an object with a high probability of being mitotic where in subsequent frames two objects exist that have a high probability of being mitotic exit cells. Optimization is performed by iteratively adapting a best scoring track, from the set of all possible new tracks, given the current set of tracks. To find the highest scoring track, given the current set of tracks, a dynamic programming technique is used. Notably, the algorithm can handle gaps and nuclei entering and exiting the FOV.

5. Manual correction: automated tracking performs the majority of work in tracking the hTERT-RPE1 cells, however these cells are highly motile, and some of the conditions we use increase motility even further. As a result, the error rate in tracking ranges from 1 mistake, per cell, per 300 frames; to 1 mistake, per cell, per 20 frames. In the cell cycle analysis we are performing it is essential we obtain near 100% accuracy. As such, we developed a GUI to efficiently amend errors in automated tracking, as well as compare the feature values of tracked cells against the original confocal microscopy images. This allows us to achieve nearly 100% accuracy in tracking, and more importantly if we identify outliers, or unexpected results in later analysis we can return to the videos and visually inspect whether the result is genuine or the product of a tracking error.

Defining G1/S and S/G2 transitions. We define G1/S transition as the point at which mRuby-PCNA intensity begins to increase and we see the appearance of PCNA foci. This definition is consistent at their maximum strength (Fig. 1a); after this we see rapid disassembly of foci (Fig. 1b). All transitions were manually marked on cell tracks, for further analysis of the combined length of G2M and G1D would correlate better with the maximum p21 accumulation (Fig. 1c traces) was generally similar to those seen in imaging (p21-GFP background expressing cells). No adjustment for this decrease was made, such that data remains dynamically consistent, prior to pooling of the data.

Result p21 levels decreased over imaging, this is due to intensity adjustments made by the microscope, due to increasingly bright images, (more p21-GFP expressing cells). No adjustment for this decrease was made, such that data remains faithful to the original imaging data sets, though baseline levels for arrest thresholds are defined at 15 a.u. For all p21-GFP traces except Fig. 1b, p21-GFP levels were normalized using the lowest of p21-GFP across all tracks. This value (4 a.u.) was generally similar to those seen in imaging (p21-GFP background levels were 12 a.u. in the first frame at the 10th percentile, while p21-GFP background levels were 2 a.u. in the last frame at the 100th percentile). The lowest track level was used rather than imaging baseline values, to avoid negative values.

Maximum and mean p21 expression levels and P-values. p21-GFP values, used for population mean, s.d. and correlation measures in the main text are derived from the maximum p21-GFP value reached within a single cell in the cell cycle phase defined. The maximum p21-GFP value was used rather than the mean p21 value, as this generally gave stronger correlations to cell cycle phase length, though in all cases analysed for both mean and maximum the mean value also provided a statistically significant correlation. However both maximum G1 D and G1 length were not normally distributed as determined by a one sample Kolmogorov-Smirnov test (G1 p21 levels, $P = 1.8677e-04$; G1 length, $P = 0.0012$). Following log transformation, the sample fell under the normal distribution (G1 p21 levels, $P = 0.1467$; G1 length, $P = 0.2408$). Therefore, we calculated the Pearson correlation coefficient on the log-transformed data as well, since Pearson's correlation does not fully describe the association of data which does not fall under a bivariate normal distribution. While correlation values did change, at no point did a previously significant result become insignificant. As such, for simplicity and to match that data presented in figures, correlations determined on the untransformed data are given in the main text.

Supplementary Table 1 gives the values quoted in the main text (derived from the maximum p21 value per cell, per phase) alongside the respected value derived from the mean p21 level per cell. Full P-values are also quoted in this table; otherwise $P < 0.01$ is marked by **, and $P < 0.05$ is denoted by *. Correlations between p21-GFP levels (mean and maximum) with phase lengths following S-phase are also quoted. Results following log transformation of the max p21 levels versus length data are also given.

Mitotic timing masks correlation between G2M and G1D lengths. Given that we observe strong correlation between i) p21-GFP levels in the mother G2 (G2M) and daughter G1 (G1D), ii) p21-GFP levels in G2M and G2M length, and iii) p21-GFP levels in G1D and G1D length, we were initially surprised to find no correlation between G2M length and G1D length; we were initially surprised to find no correlation between S-phase entry is compromised following Cdt2 depletion.
which cells are captured for an entire S-phase, and periods of 100 min before and 200 min after (as plotted in Fig. 5a). We found the average number of traces per FSV to be 32.5 for control siRNA, 27 for Skp2 siRNA, 12.3 for Cdt2 siRNA, and 6.3 for Cdt2 and Skp2 co-depletion.

Mathematical modelling. Based on the wiring diagram in Fig. 6a, we derived a set of nonlinear ordinary differential equations to describe the rate of change of components in the p21 control network over time (see Supplementary Software 2). Reaction kinetics were chosen according to the law of mass action with a few exceptions listed below.

Protein transcription and translation: Part of the noise in cell cycle progression stems from the low copy numbers of mRNAs that encode for regulatory proteins. To account for this, we modelled the level of mRNAs of p21 (mRNAp21), cyclins (mRNACy), and p53 (mRNAp53).

\[
\frac{d[mRNA_{p21}]}{dt} = k_{syn}^{Cy} \cdot [mRNA_{p21}] - k_{deg}^{Cy} \cdot [mRNA_{p21}] - [mRNA_{p21}],
\]

\[
\frac{d[mRNA_{Cy}]}{dt} = k_{syn}^{Cy} \cdot [mRNA_{Cy}] - k_{deg}^{Cy} \cdot [mRNA_{Cy}],
\]

\[
\frac{d[mRNA_{p53}]}{dt} = k_{syn}^{p53} \cdot [mRNA_{p53}],
\]

where mRNAs are synthesized and degraded with the constitutive rates \(k_{syn}^{Cy}\) and \(k_{syn}^{p53}\), respectively, and \(k_{deg}^{Cy}\) denotes the rate of p53-dependent synthesis of p21 mRNAs. Here and in the rest of the model we assumed that cyclin corresponds to the sum of CyclinE and CyclinA, which both activate CDK2. p21 (p21), cyclins (Cy) and p53 (p53) are produced from their mRNAs according to the following equations.

\[
\frac{dp21}{dt} = k_{syn}^{p21} \cdot [mRNA_{p21}] - k_{deg}^{p21} \cdot [p21],
\]

\[
\frac{dp21_c}{dt} = k_{syn}^{p21_c} \cdot [mRNA_{p21_c}] - k_{deg}^{p21_c} \cdot [p21_c],
\]

\[
\frac{dp53}{dt} = k_{syn}^{p53} \cdot [mRNA_{p53}] - k_{deg}^{p53} \cdot [p53],
\]

with \(k_{syn}^{p21}, k_{syn}^{p21_c}\), and \(k_{syn}^{p53}\) denoting the protein synthesis rates, and \(k_{deg}^{p21}, k_{deg}^{p21_c}\) and \(k_{deg}^{p53}\) the corresponding degradation rates. The subscript \(t\) indicates total protein levels, which include the protein in its free from and as part of larger complexes. Also note that since CDK2 is in excess over its cyclins\(^7\), Cy corresponds directly to the total level of CDK2-Cyclin complexes with Cy denoting the level of active CDK2-Cyclin. Protein degradation rates are as follows.

\[
r_{Cy} = k_{cyc}^{Cy} + k_{cyc}^{p21} \cdot [p21] + k_{cyc}^{p53} \cdot [p53],
\]

\[
r_{p21_c} = k_{cyc}^{p21_c} \cdot [p21_c] + k_{cyc}^{p21_c} \cdot [skp2] \cdot Cy + k_{p21_c}^{RC} \cdot [Cdt2] \cdot [RC],
\]

\[
r_{p53} = k_{cyc}^{p53} \cdot [p53] + k_{p53}^{t} \cdot [Dam] + \frac{k_{p53}^{d}}{[Dam] + k_{p53}^{d}}.
\]

The constitutive rates of cyclin and p21 degradation are \(k_{cyc}^{p21}\) and \(k_{cyc}^{p21_c}\), respectively. In addition, CDK2-mediated phosphorylation can promote cyclin degradation with rate \(k_{p21_c}^{RC}\) in an Skp2-dependent manner\(^8\). Here, Skp2 denotes the relative level of the SCFSkp2 ubiquitin ligase. Similarly, p21 degradation occurs with rate \(k_{p53}^{t}\), through a pathway involving CDK2 phosphorylation and Skp2 (refs 43,44), via CRL4\(^{-}\)D2 (Cdt2), which is recruited to chromatin-bound PCNA\(^{-}\)within arCCs (RC\(_C\)). For p53, stabilization has been shown to occur in response to DNA damage\(^1\), which we accounted for by assuming that p53 degradation with rate \(k_{p53}^{t}\) is inversely proportional to the sum of DNA damage (Dam) and an inhibition constant \(k_{p53}^{i}\).

CDK2 inhibition. At high levels, p21 can act as an efficient inhibitor of CDK2 (ref. 6). Hence, we assumed that p21 binds to CDK2-Cyclin complexes converting them from an active form (Cy) into an inactive one (CyP21).

\[
\frac{dp21_c}{dt} = k_{syn}^{p21_c} \cdot [p21_c] \cdot Cy - (k_{cyc}^{p21_c} + k_{p21_c}^{RC} + k_{t}^{p21_c}) \cdot Cy \cdot p21_c,
\]

with \(Cy = Cy - CyP21\).

\[
\frac{dp21_{t}}{dt} = CyP21 \cdot p21 - Cy \cdot (k_{cyc}^{p21_c} + k_{p21_c}^{RC} + k_{cyc}^{p21_c}) \cdot Cy \cdot p21_c,
\]

\[
\frac{dp53}{dt} = k_{syn}^{p53} \cdot [p53] \cdot [Dam]
\]

and an inhibition constant \(k_{p53}^{i}\). Here, \(k_{p53}^{i}\) and \(k_{p53}^{d}\) denote the nuclear import and export rates of PCNA, respectively. The association and dissociation of p21 and free PCNA occur with rate \(k_{p21}^{RC}\) and \(k_{p21}^{RC}\), respectively, while the binding and dissociation rates of PCNA and replication complexes are \(k_{p53}^{RC}\) and \(k_{p53}^{RC}\), respectively. Considering all the complexes that contain p21 the following conservation equation holds.

\[
p21 = p21_c + CyP21 + [p53] - RC\(_C\),
\]

DNA damage. In our model, DNA damage (Dam) can either occur randomly, independent of the cell cycle stage, or specifically during DNA replication.

\[
\frac{ddam}{dt} = k_{d}^{p53} \cdot [p53] + k_{d}^{p53} \cdot [Dam] \cdot [Dam] + [Dam],
\]

with \(k_{d}^{p53} = k_{d}^{p53} + k_{d}^{p53} \cdot [p53] + [Dam] + [Dam] + [Dam],
\]

Here, \(k_{d}^{p53} + k_{d}^{p53}\) correspond to the constitutive and replication-dependent rate of DNA damage induction, respectively. Damage repair occurs with a p53-independent rate \(k_{d}^{p53}\) and a p53-dependent rate \(k_{d}^{p53}\), assuming that p53 expression induces DNA repair processes.

Computation. A deterministic version of the model was prepared using the Systems Biology Toolbox 2 (ref. 67) for MatLab (version 8.6.0 R2015b) and simulated with the CVODE routine from SUNDIALS\(^8\). Bifurcation diagrams were calculated using the freely available software XPP-Aut\(^8\). Different versions of the model are available at http://cellcycle.org.uk/publication. The model was also deposited in the BioModels database\(^9\) and assigned the identifier MODEL1607210001. Kinetic parameters were estimated by comparing deterministic simulations to experimental time-course data of cell cycle regulators and to qualitative experimental observations. Parameter values and non-zero initial
conditions are listed in Supplementary Tables 3 and 4. We simulated a stochastic version of the model using custom-made MatLab code of the stochastic simulation algorithm, also known as Gillespie’s algorithm (reviewed in ref. 71) according to the sorting direct method. To this end, the rate expressions of the deterministic model were converted into propensity functions, that requires the transformation of the relative levels of cell cycle regulators into numbers of molecules. Here, we followed the system in a control volume containing on average 1,000 molecules per AU of proteins, 100 molecules per AU of mRNAs and 1 event per AU of DNA damage. Hence, most of the simulated cell-to-cell variability originates in the stochastic infliction of DNA damage followed by transcriptional noise, while translational noise plays only a minor role. To account for the extrinsic noise observed in our experiments, that is, the variability in p21 levels after cell division, we multiplied the initial p21 level, the initial p21 mRNA level and the p53-independent p21 mRNA synthesis rate ($C_{DNA}$ in equation (1)) for each cell (simulation run) by a single random number drawn from a uniform distribution in the interval $(0,1)$. For the simulation of deletion experiments $Skp2$ and/or $CdK2$ were reduced to 1%.

**Code.** Newly generated code used for cell tracking and feature extraction, and for generating our mathematical model is freely available at http://cellcycle.org.uk/ publication

**Data availability.** The data sets generated during the current study are available from the corresponding authors on reasonable request.

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**Code.** Newly generated code used for cell tracking and feature extraction, and for generating our mathematical model is freely available at http://cellcycle.org.uk/ publication

**Data availability.** The data sets generated during the current study are available from the corresponding authors on reasonable request.

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