A ROS-dependent mechanism promotes CDK2 phosphorylation to drive progression through S phase

Graphical abstract

Highlights

- Mitochondrial ROS drive cell cycle progression and proliferation
- Cyclin-dependent kinase 2 (CDK2) is increasingly oxidized during the cell cycle
- The oxidation state of a conserved cysteine on CDK2 regulates KAP binding
- CDK2 oxidation promotes T-loop phosphorylation and DNA replication

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In brief

Reactive oxygen species (ROS) are by-products of mitochondrial respiration. Kirova et al. find that mitochondrial ROS increase during the cell cycle and oxidize cyclin-dependent kinase 2 (CDK2) to regulate its interaction with KAP phosphatase. CDK2 oxidation promotes T-loop phosphorylation and the CDK2 activity needed for DNA replication and proliferation.
A ROS-dependent mechanism promotes CDK2 phosphorylation to drive progression through S phase

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SUMMARY
Reactive oxygen species (ROS) at the right concentration promote cell proliferation in cell culture, stem cells, and model organisms. However, the mystery of how ROS signaling is coordinated with cell cycle progression and integrated into the cell cycle control machinery on the molecular level remains unsolved. Here, we report increasing levels of mitochondrial ROS during the cell cycle in human cell lines that target cyclin-dependent kinase 2 (CDK2). Chemical and metabolic interferences with ROS production decrease T-loop phosphorylation on CDK2 and so impede its full activation and thus its efficient DNA replication. ROS regulate CDK2 activity through the oxidation of a conserved cysteine residue near the T-loop, which prevents the binding of the T-loop phosphatase KAP. Together, our data reveal how mitochondrial metabolism is coupled with DNA replication and cell cycle progression via ROS, thereby demonstrating how KAP activity toward CDKs can be cell cycle regulated.

INTRODUCTION
Reactive oxygen species (ROS) are oxygen-containing molecules with high chemical reactivity to “steal” electrons from molecules (oxidation). Excessive exposure to ROS during oxidative stress or from external sources such as ionizing agents can oxidize proteins, cause DNA mutations, and trigger lipid peroxidation (Schieber and Chandel, 2014). The major sources of intracellular ROS are superoxide anions (O2•−) produced from membrane-associated NADPH oxidases (NOXs) and mitochondria, in response to growth factor (GF) signaling or as by-products of oxidative phosphorylation, respectively (Schieber and Chandel, 2014; Bedard and Krause, 2007; Murphy, 2009). O2•− molecules are subsequently converted to hydrogen peroxide (H2O2), either spontaneously or catalytically by superoxide dismutases (Mölter et al., 2019; Wang et al., 2018). H2O2 is more stable, membrane-permeant, and at physiological concentrations can act as a signaling molecule in a variety of biological processes such as GF signaling, proliferation, differentiation, and adaptation to hypoxia (Holmström and Finkel, 2014; Reczek and Chandel, 2017; Shadel and Horvath, 2015). ROS signaling is in part mediated by reversible oxidation of cysteine thiols to sulfenic acids (R-SOH) and intra- or inter-molecular disulfide bonds, which act as reversible posttranslational modifications (PTMs) that can regulate activity, localization, stability, and interactions of proteins (Reddie and Carroll, 2008).

Low levels of ROS, in particular of H2O2, have been shown to promote proliferation of stem cells, differentiated cells, and cancer cells (Armstrong et al., 2010; Gurusamy et al., 2009; Adusumilli et al., 2021; Alfaro et al., 2017; Moll et al., 2018; Sigaud et al., 2005; Safford et al., 1994; Irani et al., 1997; Ogrunc et al., 2014). Mechanistically, this proliferative effect is best understood for GF signaling, where NOX-derived H2O2 activates membrane-associated receptor tyrosine kinases while inhibiting counteracting phosphatases (Holmström and Finkel, 2014), so that sustained GF signaling can initiate transcriptional programs to promote the G0/G1 and G1/S transitions (Burhans and Heintz, 2009; Chiu and Dawes, 2012). Mitochondrial ROS can also activate transcriptional programs linked to proliferation (Owusu-Ansah et al., 2008; Weinberg et al., 2010; Tsai et al., 2011; Connor et al., 2005); however, whether or not ROS directly regulate the cell cycle independent of transcriptional signaling remains elusive.

A potential target of mitochondrial ROS could be the central regulatory cell cycle network mediated by cyclin-dependent kinases (CDKs). CDKs are activated by a conserved two-step
mechanism: binding of a cell cycle stage-specific cyclin and phosphorylation of a threonine residue (T160 in cyclin-dependent kinase 2 [CDK2]) within the activation segment of the kinase domain (T-loop) (Morgan, 2007). T-loop phosphorylation is carried out by a trimeric CDK-activating kinase (CAK, a complex of CDK7, cyclin H and MAT1) and opposed by CDK-associated phosphatase (KAP) and protein phosphatase 2C-like proteins (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Fisher and Morgan, 1994; Mäkelä et al., 1994; Poon and Hunter, 1995; Hannon et al., 1993; Gyuris et al., 1993; Cheng et al., 1999).

Whether T-loop phosphorylation is regulated to ensure that the correct CDK becomes fully activated at the right time is still debated; since CAK is considered constitutively active (Fisher, 2005) and KAP activity regulation is unclear. Cell cycle stage and CDK specificity of T-loop phosphorylation, i.e., on CDK2 and CDK1, is in part achieved by preferential binding of CAK to monomeric CDK2 and cyclin-complexed CDK1, respectively (Larochelle et al., 2007; Merrick et al., 2008; Fisher and Morgan, 1994; Desai et al., 1995). Although KAP binds to both monomeric and cyclin-complexed CDKs (Poon and Hunter, 1995; Gyuris et al., 1993; Hannon et al., 1994), it can only dephosphorylate monomeric CDKs, i.e., once the bound cyclin is degraded (Poon and Hunter, 1995). This results in a conceptual problem for CDK2 activation during S phase, when cyclin E is degraded and CDK2 switches from cyclin E to cyclin A, meaning that T160 can be targeted by CAK and KAP at the same time.

Here, we employ retina pigment epithelial cells (RPE-1) as a non-transformed cell cycle model to monitor and interrogate intracellular ROS production along with cell cycle progression and CDK2 activation. We uncover that the levels of mitochondrial ROS increase in a cell cycle-dependent manner that recapitulates the activity of S phase regulator, CDK2. We show that oxidation of a cysteine that is conserved in CDK2 but not in other CDKs ensures T-loop phosphorylation required for efficient DNA replication and S phase progression. Our data reveal a regulatory mechanism for CDK2 activity via KAP that provides feedback from mitochondrial metabolism to cell cycle control and proliferation.

**RESULTS**

**Proliferation and S phase progression require oxidative events**

To investigate the interplay between physiological ROS signaling and cell cycle progression, we chose human telomerase reverse transcriptase (hTERT)-immortalized RPE-1 cells as a well-established model cell line with unperturbed cell cycle control (Bodnar et al., 1998). We first assessed whether RPE-1 cells require oxidative events for normal proliferation, as has been reported for other experimental models (Wartenberg et al., 1999; Havens et al., 2006; Murrell et al., 1990; Ohguro et al., 1999; Paul et al., 2014; Alfar et al., 2017). Indeed, treating RPE-1 cells with the antioxidant N-acetyl-L-cysteine (NAC) for 48 h reduced proliferation in a dose-dependent manner (Figure S1A). The decrease in proliferation was not due to induction of apoptosis as monitored by poly ADP-ribose polymerase (PARP) cleavage (Figure S1B). To determine the cell cycle stages sensitive to reducing conditions, we employed RPE-1 cells expressing three endogenous proteins tagged with different fluorescent proteins: (1) proliferating cell nuclear antigen (Ruby-PCNA) to detect replication foci in S phase, (2) cyclin A2-Venus to distinguish cyclin A2-negative G1 cells from cyclin A2-positive S and G2 cells, and (3) histone 3.1-turquoise2 (H3.1-Turq2) to segment nuclei (Zer-jatke et al., 2017). By assessing PCNA foci and cyclin A2 expression, we could classify cells as G1, S, or G2 phase based on snapshots (Figure S1C). This analysis revealed that the addition of NAC at all concentrations significantly increased the proportion of cells in S phase. We also observed that low (6 mM) concentrations of NAC reduced the number of cells in G1, whereas higher concentrations (8 and 10 mM) reduced the cells in G2 (Figure S1D). NAC is a general reductant and does not discriminate between different forms of ROS; therefore, we tested a membrane-permeant polyethylene glycol-linked catalase (PEG-CAT) to specifically target H2O2, which is considered to be the second messenger of ROS signaling (Winterbourn, 2008). Here, we combined Ruby-PCNA and H3.1-Turq2 with Fucci-Gem, a well-established anaphase promoting complex/cyclosome (APC/C) activity reporter that accumulates after the G1/S transition when APC/C is inactivated (Sakae-Sawano et al., 2008). Fucci-Gem marks S and G2 phase cells which can be subsequently separated based on replication foci with Ruby-PCNA (Figure 1A). As with NAC, adding PEG-CAT strongly reduced cell proliferation (Figure 1B) and significantly increased the fraction of S phase at the expense of G1 (Figure 1C). To assess whether ROS directly promote RPE-1 cell proliferation, we increased the intracellular levels of ROS by using a genetically encoded D-amino acid oxidase (DAO) from Rhodosporidium toruloides, which converts medium-supplied D-alanine to a keto-acid, ammonia, and H2O2 (Lee and Chu, 1996; Figure 1D). The increase of H2O2 in response to D- but not L-alanine can be directly monitored by imaging of the ratio-metric H2O2 sensor HyPer2 fused to the N terminus of DAO (Figure 1E; Matlashov et al., 2014). DAO was further linked to a nuclear export sequence (NES, HyPer2-DAO-NES) to recapitulate mitochondrial ROS released through the outer mitochondrial membrane in the form of H2O2 (Shadel and Horvath, 2015). Indeed, the lowest concentration of D-alanine we applied (0.5 mM) significantly increased proliferation, whereas higher concentrations (2.5 or 5 mM) or addition of PEG-CAT had adverse effects (Figure 1F). Of note, the pro-proliferative effect of D-alanine at low concentrations and anti-proliferative effect at higher concentrations highlight the dual role of H2O2, which is dependent on dose and the antioxidant capacity of the cells (Gough and Cotter, 2011). These results suggest that oxidative events, mediated by H2O2, are required for normal proliferation of RPE-1 cells.

**ROS levels increase during the cell cycle**

We further investigated how physiological ROS production is correlated with cell cycle progression. We employed the ROS probe CellRox Deep Red to determine the overall ROS content in living cells that express Ruby-PCNA and cyclin A2-Venus to define the cell cycle phases. Compared with G1, cells in S and G2 phases showed increased labeling of CellRox Deep Red (Figure 2A). In agreement, flow cytometry analysis revealed significant increases in cellular ROS from G1 to S and G2/M phases indicative of ROS fluctuations during the cell cycle (Figure 2B). Notably, the ~25% increase of ROS from G1 to S phase is of the same magnitude as pro-proliferative ROS production by HyPer2-DAO-NES following 0.5 mM D-alanine treatment.
These measurements were performed at atmospheric oxygen concentration (21%), which might affect the redox status of cells that experience lower oxygen concentration in their in vivo tissue context. Thus, we repeated our measurements with RPE-1 cells grown at the oxygen concentration reported for the retinal epithelium (6.3%). These data were consistent and showed a steeper increase of ROS from G1 to S phase (Figure 2C). Measuring ROS in primary human foreskin fibroblasts (BJ) grown at their reported in vivo oxygen concentration (4%) gave a similar result (Figure 2D; Balin et al., 2002).

Notably, CellRox Deep Red labeling of RPE-1 cells appeared to be reminiscent of mitochondrial networks. Labeling of cells with CellRox Deep Red and the mitochondrial marker MitoTracker Green displayed overlapping signals (Figure 2E), suggesting that the cell cycle-correlated ROS dynamics we detected likely recapitulate changes in mitochondrial ROS. Indeed, detection of mitochondrial ROS with MitoSox Red dye mirrored the localization and cell cycle dynamics of overall ROS we observed (Figures 2E and 2F). An increase of mitochondrial ROS in S and G2/M could reflect mitochondrial activity, i.e.,...
Figure 2. Mitochondrial ROS increase in S and G2 phase

(A) ROS detection in RPE-1 cells with CellRox Deep Red. Cell cycle classification based on cyclin A2-Venus and Ruby-PCNA (see also Figure S1B). Scale bars, 25 μm.

(B and C) Flow cytometry analysis of RPE-1 cells stained with CellRox Deep Red grown at the indicated %O2. Bars represent the mean ± SD intensity normalized to G1 levels. Significance according to one-sample t test (G1 versus S) and two-tailed paired t test (S versus G2) (B, n = 3, N = 3; C, n = 3, N = 9).

(D) Equivalent analysis as in (C) with primary BJ fibroblasts grown at 4% O2. Significance according to one-sample t test (G1 versus S) and two-tailed paired t test (S versus G2) (n = 4, N = 5).

(E) Co-labeling of RPE-1 cells with CellRox Deep Red, MitoSox Red, and MitoTracker Green. Overlay indicates CellRox (yellow) and MitoTracker (cyan) co-localization. Scale bars, 25 μm.

(F) Flow cytometry analysis of RPE-1 cells stained with MitoSox Red. Bars represent the mean ± SD intensity normalized to G1 levels. Significance according to one-sample t test (G1 versus S) and a two-tailed paired t test (S versus G2) (n = 4, N = 4).

(G) Flow cytometry analysis of RPE-1 cells co-labeled with CellRox Deep Red and MitoTracker. Bars represent the mean ± SD CellRox/MitoTracker ratio. Significance according to paired one-way ANOVA with Holm-Sidak’s multiple comparisons test (n = 3, N = 3).

(H) Flow cytometry quantification of cell volume and mitochondria based on forward scatter and MitoTracker Green. Data indicate the mean log2-fold increase ± SD normalized to G1 phase. Significance according to multiple t tests with Holm-Sidak’s multiple comparisons test (n = 3, N = 3).
Figure 3. Mitochondrial ROS drive progression through S phase

(A) Illustration of mitochondrial metabolite, and ROS flux and the PDH complex as the gatekeeper between glycolysis and TCA cycle.

(B) Western blot analysis of cells treated for 48 h with control (esi-CTRL) or esiRNA targeting the β-subunit of the PDH complex (esi-PDHB).

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due to metabolic switch from glycolysis to oxidative phosphorylation or increased mitochondria mass relative to cell volume. To test these possibilities, we performed flow cytometry analysis of cells stained for mitochondrial ROS with MitoSox Red and the overall mitochondrial content with membrane potential-independent dye MitoTracker Green. We observed an equal ratio of MitoSox Red to MitoTracker Green throughout the cell cycle, which implies that the increase of mitochondrial ROS is correlated to an increase in mitochondria (Figure 2G). Like others before (Havens et al., 2006), we observed that mitochondrial content increased faster than cell volume (Figure 2H), providing a possible explanation for the elevated concentrations of cellular ROS in S and G2 or M phases. Taken together, our data reveal that cellular ROS levels fluctuate during the cell cycle in both normoxic and physiological oxygen conditions, in a manner that correlates with mitochondrial ROS dynamics. Our data also suggest that the increase in cellular ROS largely results from a relative increase in mitochondria in S and G2/M compared with G1 phase.

Mitochondrial ROS drive progression through S phase

Mitochondrial ROS are produced as a by-product of oxidative phosphorylation. Thus, reducing the influx of metabolites into the tricarboxylic acid (TCA) cycle should decrease the amount of O2− released from the electron transport chain (ETC) and limit the concentration of mitochondrial-derived H2O2 in the cytoplasm. We employed endoribonuclease-prepared siRNAs (esi-RNAs) to downregulate PDHB, a subunit of the mitochondrial pyruvate dehydrogenase (PDH) complex that converts pyruvate to acetyl-CoA and acts as a gatekeeper between glycolysis in the cytosol and oxidative phosphorylation in mitochondria (Figure 3A). Depleting PDHB protein levels below 25% (Figures 3B and 3C) was sufficient to significantly reduce cellular H2O2 detected by HyPer7, a ratio-metric H2O2 sensor with improved sensitivity (Pak et al., 2020; Figure 3D). Importantly, reducing ROS production via PDHB depletion did not affect the ADP/ATP ratio indicating that cells were able to maintain ATP levels likely by glycolytic flux (Figure 3E).

PDHB-depleted cells proliferated slower (Figure 3F) but did not induce apoptosis based on PARP cleavage (Figure S1F). In support of our results from treatment with NAC and PEG-CAT (Figures 1 and S1) from single-cell time-lapse microscopy, PDHB-depleted cells took significantly longer to progress through S phase (Figure 3G). We noticed that PDHB depletion in this experimental setup caused a more prolonged and heterogeneous duration of G1 phase. Unchanged ATP levels and decreased levels of p21, however, indicated that longer G1 phases were not caused by activation of a metabolic checkpoint that arrested cells at the G1/S transition (Jones et al., 2005; Mitra et al., 2009). Since we use PCNA foci to identify S phase and PDHB depletion reduced the number of PCNA foci (Figure 4), our analysis likely under-estimates the duration of S phase in favor of G1 phase (Zerjatke et al., 2017). We also assessed the cell cycle distribution of PDHB-depleted cells by western blotting for established cell cycle markers and by single-cell analyses for the S phase markers cyclin A2 and Fucci-Gem. Western blot analysis (Figure 3J) showed that the levels of G1 and G2 phase markers p21 and cyclin B1 were lower in PDHB-depleted cells (Figures 3K and 3L), whereas the S phase protein geminin accumulated (Figure 3M). In agreement with a delay in S phase but not G1 phase, PDHB depletion increased the proportion of Fucci-Gem-positive cells (Figure 3H) as well as cyclin A2-Venus intensity (Figure 3I), which are two markers that only accumulate after the G1/S transition. Our findings indicate that decreasing metabolic flux by interfering with the PDH complex reduces H2O2 to result in slower cell proliferation. The increased time required to progress through S phase and the accumulation of S, but not of G1 or G2 phase markers, supports a major cell cycle-associated role of mitochondrial ROS in S phase.

Mitochondrial ROS promote DNA replication

Since reducing mitochondrial ROS levels via PDHB depletion slowed S phase, we monitored the intensity and number of PCNA foci during time-lapse imaging as a surrogate to assess the fidelity of DNA replication. Replication foci in control cells showed the stereotypical spatiotemporal pattern described for replication factories (Leonhardt et al., 2000): multiple small foci in early S phase, concentration of foci in mid S phase, and formation of large clusters at the nuclear periphery in late S phase (Figure 4A; Video S1). In contrast, PCNA foci in PDHB-depleted cells were barely detectable until the very end of S phase at the same magnification used (Figure 4A; Video S2). To exclude potential off-target effects of esi-PDHB treatment, we targeted another subunit of the PDH complex, PDHA (Figure 4B). Quantifying the number of PCNA foci in S phase cells confirmed that depleting either PDHA or PDHB decreased the number of detectable PCNA foci in S phase (Figure 4C). To strengthen the link between mitochondrial ROS and DNA replication, we also

(C) Quantification of PDHB levels in (B) normalized to esi-CTRL. Bars indicate the mean ± SD. Significance according to two-tailed one-sample t test (n = 3, N = 3).

(D) Analysis of H2O2 levels after PDHB depletion in RPE-1 cells stably expressing HyPer7. Boxplots indicate the median HyPer7 ratio. Significance according to two-tailed unpaired t test (n = 3, esi-CTRL N = 80, esi-PDHB N = 82).

(E) ATP quantification in response to PDHB depletion. Boxplots indicate the median ADP/ATP ratio. Significance according to two-tailed unpaired t test (n = 3, N = 12 esi-CTRL, N = 11, esi-PDHB).

(F) Proliferation of PDHB-depleted cells. Boxplots indicate the median log2-fold change in proliferation. Significance according to two-tailed unpaired t test (n = 3, N = 9).

(G) Single-cell time-lapse analysis. Boxplots indicate the median duration of cell cycle phases. Significance according to Kruskal-Wallis’ test and Dunnett’s correction for multiple comparisons. (n = 3, N is indicated in the graph).

(H) Quantification of cells in S/G2 phase based on Fucci-Gem after treatment with esi-CTRL or esi-PDHB. Boxplots indicate the median fraction of Fucci-Gem-positive cells. Significance according to two-tailed unpaired t test (n = 3, N = 9).

(I) Quantification of cyclin A2-Venus expression after treatment with esi-CTRL or esi-PDHB. Boxplots indicate the median cyclin A2-Venus intensity. Significance according to two-tailed unpaired t test (n = 3, N = 9).

(J-M) Western blot analysis of cell cycle markers after PDHB depletion, and (K-M) graphs showing quantification of the data normalized to esi-CTRL. Bars represent the mean ± SD. Significance according to two-tailed one-sample t test (n = 3, N = 5 [p21], N = 3 [cyclin B1, geminin]).
assessed 5-ethyl-2′-deoxyuridine (EdU) incorporation in PDHB-depleted cells or cells treated for 48 h with the mitochondrial antioxidant MitoTEMPO. Indeed, both treatments significantly reduced the fraction of EdU-positive cells compared with the control (Figures 4D and 4E). Together, these data show that interfering with the TCA cycle and treatment with mitochondrial antioxidants affect DNA replication during S phase. Since interfering with the PDH complex reduced cellular H2O2 and hindered DNA replication, our findings support that mitochondrial ROS promote DNA replication.

**CDK2 activity and T-loop phosphorylation are sensitive to mitochondrial ROS**

CDK2 not only mediates the initiation of DNA replication but also regulates the spatiotemporal pattern of replication in the nucleus (Sansam et al., 2015). Since we observed changes in PCNA foci formation and dynamics in response to PDH complex perturbation (Figure 4), we investigated whether mitochondrial ROS promote CDK2 activity in S phase. To assess CDK2 activity in living cells, we created an RPE-1 cell line with stable expression of a CDK2 activity sensor which is nuclear upon low CKD2 activity and becomes progressively more cytoplasmic as CDK2 activity increases (Spencer et al., 2013; Figure 5A). Depleting PDHB for 48 h strongly increased the number of cells with nuclear expression of the CDK2 sensor indicative of low CDK2 activity (CDK2 low) from 34% to 61% (Figures 5B and 5C). It is important to note that the CDK2low cells are unlikely to be quiescent or arrested before the restriction point (Spencer et al., 2013) because the same treatment resulted in the accumulation of multiple cell...
Figure 5. CDK2 activity and T-loop phosphorylation is sensitive to mitochondrial ROS

(A) Schematic showing CDK2-activity-dependent localization of the CDK2 sensor.

(B) CDK2 sensor localization in RPE-1 cells 48 h after esi-CTRL and esi-PDHB transfection. Note that the contrast of each image was adjusted separately to prevent the saturation of nuclear CDK2 sensor in esi-PDHB treatment. Scale bars, 50 μm.

(C) Scatter plots show scaled intensities of cytoplasmic and nuclear CDK2 sensor in single cells, as treated in (B). The percentage of CDK2\text{low} and CDK2\text{high} is indicated (n = 2, N = 6,000).

(D and E) (D) Western blot analysis of T160 phosphorylation from cells treated with esi-PDHB, and (E) quantification of the data. Bars indicate the mean ± SD. Significance according to two-tailed one-sample t test (n = 4, N = 4).
cycle markers expressed downstream of the G1/S transition, such as cyclin A2 and geminin (Figure 3).

Next, we addressed how mitochondrial metabolism and ROS production affect CDK2 activity on the molecular level. Full activity of CDK2 requires binding of E or A-type cyclins and phosphorylation of T160 within its T-loop. Because PDHB-depleted cells can enter S phase as indicated by an increase of Fucci-Gem-positive cells (Figure 3H) and express cyclin A2 (Figure 3I), we investigated whether T160 phosphorylation was impacted. Indeed, quantitative western blot analysis of lysates from control and PDHB-depleted cells with a phosphorylation-specific T160 antibody (CDK2 pT160) showed a ~50% reduction in T-loop phosphorylation (Figures 5D and 5E). Importantly, this difference was not due to an enrichment of PDHB-depleted cells in G1 phase since repeating the experiment in cells synchronized at the beginning of S phase with thymidine gave a comparable result (Figures S2A and S2B). To confirm these findings, we also starved cells of glutamine (Gln) for 6 h to reduce mitochondrial ROS production (Oh et al., 2020). As with depleting PDHB, 6 h of Gln starvation strongly increased the number of CDK2-low

(F) Scatter plots show scaled intensities of cytoplasmic and nuclear CDK2 sensor in single cells after 6 h of glutamine (Gln) starvation at 21% O2 (n = 3, N = 6,000). (G and H) (G) Western blot analysis detecting T160 phosphorylation in cells treated with glutamine starvation (−Gln) and (H) quantification of the data. Bars represent the mean ± SD. Significance according to two-tailed one-sample t test (n = 3, N = 3).

(E and F) (E) Western blot analysis with cytoplasmic ROS production by HyPer2-DAO-NES, and (F) quantification data. Bars represent the mean ± SD normalized to t = 0. Significance according to two-tailed one-sample t test (n = 4, N = 4).

Figure 6. Genetically enabled ROS production increases T-loop phosphorylation

(A and B) (A) Ratio imaging of RPE-1 cells stably expressing nuclear HyPer2-DAO-NLS during 2 h of treatment with 10 mM D- or L-alanine (D-ala, L-ala), and (B) single-cell analysis of nuclear ROS production normalized to the ratio at t = 0. Scale bars, 10 μm.

(C and D) (C) Western blot analysis showing increased T160 phosphorylation in PDHB-depleted cells after D-ala-driven ROS production by HyPer2-DAO-NLS, and (D) quantification of T160 phosphorylation levels. Bars represent the mean ± SD normalized to t = 0. Significance according to two-tailed one-sample t test (n = 3, N = 3).

(Al) Equations analysis as (F) but with RPE-1 cells grown at 6.3% O2 (n = 3, N = 6,000).
(F) Western blot analysis and (K) quantification of T160 phosphorylation from cells grown at 6.3% O2 as in (G) and (H). Bars represent the mean ± SD. Significance according to two-tailed one-sample t test (n = 3, N = 3).
Figure 7. Preventing C177 oxidation increases KAP binding to CDK2
(A) Schematic of sulfinic acid (SOH) labeling in living cells by BTD and subsequent purification.
(B) Western blot analysis ($n = 3$) of StreptII pull-downs from control (DMSO) and BTD-treated RPE-1 cells. Detection of biotin is shown in Figure S3A.

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cells in both normoxic (21% O2) and physiological (6.3% O2) oxygen conditions (Figures 5F and 5I) and reduced T160 phosphorylation (Figures 5G, 5H, 5J, and 5K). Importantly, 6 h of G1 starvation did not synchronize cells in G1 phase, where pT160 is intrinsically lower (Figure S2C). Finally, addition of 8 and 10 mM NAC for 5 h to S phase cells also reduced T160 phosphorylation, although to a lesser extent (Figures S2D and S2E). Exposure of cells to different concentrations of NAC for 48 h, similar to proliferation experiments in Figure S1, decreased pT160 by up to 83% (Figure S2G) and resulted in accumulation of the CDK2 sensor in nuclei indicative of reduced CDK2 activity (Figure S2F). Importantly, the fraction of cyclin A2-positive cells increased upon NAC treatment in agreement with a delay in S phase but not in G1 (Figure S2H). Collectively, our data show that reducing conditions achieved by metabolic perturbation of oxidative phosphorylation or a chemical reductant decreases T-loop phosphorylation of CDK2, and thus, the degree of CDK2 activity is coupled to mitochondrial metabolism by ROS.

Genetically enabled ROS production increases T-loop phosphorylation

Thus far, our experiments in reducing conditions demonstrate indirectly that ROS are involved in regulating CDK2 activity via T160 phosphorylation. For more direct evidence, we tested if the loss of T160 phosphorylation after PDHB depletion can be rescued by DAO-mediated H2O2 production (Figure 1). As CDK2-cyclin E/A complexes are predominately nuclear, we created an RPE-1 cell line that stably expresses DAO fused to a nuclear localization sequence (NLS, HyPer2-DAO-NLS) that can rapidly generate ROS in response to D- but not to L-alanine (Figures 6A and 6B). Based on the gradual increase in the HyPer2 ratio (Figure 6B), we induced H2O2 for 1 and 2 h and evaluated the consequences on T160 phosphorylation in PDHB-depleted cells. 1 h of stimulating H2O2 production from nuclear DAO was sufficient to increase T160 phosphorylation significantly (Figures 6C and 6D). Because H2O2 produced from mitochondria needs first to pass the nuclear membrane to reach CDK2-cyclin E/A complexes, we repeated the experiment with cytoplasmic HyPer2-DAO-NES (Figure 1E). Cytoplasmic H2O2 production also rescued T160 phosphorylation (Figures 6E and 6F), indicating that mitochondrial ROS can target CDK2 which is predominantly nuclear. These data show that genetically enabled H2O2 production promotes T-loop phosphorylation on CDK2. The observation that cytoplasmic production of H2O2 can target nucleoplasmic CDK2 further supports that mitochondrial ROS can regulate CDK2 activity by acting as a signaling molecule.

Preventing C177 oxidation increases KAP binding to CDK2

Our finding that ROS stimulate CDK2 T-loop phosphorylation led us to hypothesize that either CDK2 itself or the enzymes that regulate T160 phosphorylation (CAK and KAP) are targeted by ROS. To evaluate these possibilities, we took advantage of the membrane-permeant, chemoselective probe BTD to label sulfenic acids in living cells (Gupta and Carroll, 2016). After labeling RPE-1 cells with BTD for 30 min, we performed click chemistry and a streptavidin pull-down to purify sulfenic acid-modified proteins (Figures 7A and S3A). In support of CDK2 oxidation, we detected CDK2 and known sulfenic acid-modified protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in our pull-downs, but not highly abundant ribosomal protein RPL26 which does not contain any cysteine residues (Figure 7B). This is in agreement with several mass spectrometry studies using BTD or alternative probes in H2O2-treated cell extracts or unperturbed cells identifying CDK2 oxidation to sulfenic acid on C177 (Yang et al., 2015; Gupta et al., 2017; Xiao et al., 2020; Shi et al., 2021). C177 is positioned in an unstructured loop near T160 (Figure S3B), conserved in vertebrates (Figure S3C) and only found in CDK2 and not in other related human CDKs (Figure 7C). Next, we assessed whether CDK2 oxidation increases from G1 to S and G2 phase as predicted with an increase in ROS levels during the cell cycle. We expressed CDK2 with a C-terminal hemagglutinin (HA) tag in RPE-1 cells and released them from serum starvation for 8, 18, and 22 h to be synchronized to G1, S, and G2, respectively (Figures S3D and S3E). After labeling with BTD for 30 min, we lysed the cells under reducing conditions and conjugated the fluorescent dye Aza800 to BTD by click chemistry (Figure S3F). Detection of Aza800 on CDK2-HA immunoprecipitates revealed that cysteine oxidation on CDK2 to sulfenic acid increased from G1 phase to S and G2 phase in a manner that decreased T160 phosphorylation (Figures 7D and S3F). To confirm the specificity of this reaction, we performed quantitative mass spectrometry on CDK2-HA purified from S phase cells and determined that CDK2 oxidation indeed decreased T160 phosphorylation (Figure S3F).

Our finding that ROS stimulate CDK2 T-loop phosphorylation led us to hypothesize that either CDK2 itself or the enzymes that regulate T160 phosphorylation (CAK and KAP) are targeted by ROS. To evaluate these possibilities, we took advantage of the membrane-permeant, chemoselective probe BTD to label sulfenic acids in living cells (Gupta and Carroll, 2016). After labeling RPE-1 cells with BTD for 30 min, we performed click chemistry and a streptavidin pull-down to purify sulfenic acid-modified proteins (Figures 7A and S3A). In support of CDK2 oxidation, we detected CDK2 and known sulfenic acid-modified protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in our pull-downs, but not highly abundant ribosomal protein RPL26 which does not contain any cysteine residues (Figure 7B). This is in agreement with several mass spectrometry studies using BTD or alternative probes in H2O2-treated cell extracts or unperturbed cells identifying CDK2 oxidation to sulfenic acid on C177 (Yang et al., 2015; Gupta et al., 2017; Xiao et al., 2020; Shi et al., 2021). C177 is positioned in an unstructured loop near T160 (Figure S3B), conserved in vertebrates (Figure S3C) and only found in CDK2 and not in other related human CDKs (Figure 7C). Next, we assessed whether CDK2 oxidation increases from G1 to S and G2 phase as predicted with an increase in ROS levels during the cell cycle. We expressed CDK2 with a C-terminal hemagglutinin (HA) tag in RPE-1 cells and released them from serum starvation for 8, 18, and 22 h to be synchronized to G1, S, and G2, respectively (Figures S3D and S3E). After labeling with BTD for 30 min, we lysed the cells under reducing conditions and conjugated the fluorescent dye Aza800 to BTD by click chemistry (Figure S3F). Detection of Aza800 on CDK2-HA immunoprecipitates revealed that cysteine oxidation on CDK2 to sulfenic acid increased from G1 phase to S and G2 phase in a manner that decreased T160 phosphorylation (Figures 7D and S3F). To confirm the specificity of this reaction, we performed quantitative mass spectrometry on CDK2-HA purified from S phase cells and determined that CDK2 oxidation indeed decreased T160 phosphorylation (Figure S3F).
that reflected the increase of ROS during the cell cycle we observed (compare Figure 2 and Figure 7D). To investigate the relationship between C177 oxidation and T160 phosphorylation, we mutated C177 to cysteine-mimetic serine (C177S) or alanine (C177A) and expressed these mutant constructs tagged with StreplII into RPE-1 cells synchronized to the beginning of S phase. StreplII pull-downs showed that T160 phosphorylation on the C177S (Figures 7E and 7F) and C177A (Figures S3G and S3H) mutants were markedly reduced, whereas binding of cyclin A2 (Figures 7G and S3I) and cyclin E1 (Figures S3J and S3K) was not significantly impacted.

One possibility is that C177 oxidation regulates the binding of CAK or KAP to CDK2 and, therefore, impacts the phosphorylation status of T160. To test this, we compared the binding of recombinant CAK and KAP with CDK2-StrepII WT and C177S purified from cells synchronized to S phase when CDK2 should be oxidized (Figure S4A). For CAK binding assays, we first dephosphorylated CDK2-StrepII WT and C177S immobilized on beads with λ-phosphatase (Figure S4B) before adding recombinant CAK in the presence or absence of the reducing agent dithiothreitol (DTT) (Figure S4C). Here, we did not observe significant changes in CAK binding to CDK2 by western blotting for CDK7 (Figure S4D). In contrast, when we assessed the binding of recombinant KAP to phosphorylated CDK2, we found that KAP binding was increased 2-fold for the C177S mutant compared with the WT in the absence of DTT (Figures 7H and 7I). In the presence of DTT, KAP binding selectively increased for CDK2 WT but not for the C177S mutant (Figure 7I). Finally, we tested whether endogenous KAP binds to CDK2 in a redox-dependent manner. We performed CDK2 pull-downs from lysates of S phase-synchronized cells treated with or without DTT for 10 min. Indeed, endogenous KAP was only detected in CDK2 pull-downs under reducing conditions and not without DTT in the extract preparation (Figure 7J). In contrast, HA-tagged CDK2 C177S but not WT was able to pull-down endogenous KAP in the absence of DTT, suggesting that mutation of C177S is sufficient to render the CDK2-KAP interaction insensitive to oxidation (Figure 7K).

If CDK2 oxidation on C177 is important for full CDK2 activation and S phase progression then cells expressing C177S in the absence of endogenous CDK2 activity should be impaired in proliferation and DNA replication. Since C177 is vital to proliferation, we were hesitant to introduce a C177S mutation directly to proliferating CDK2 since C177S was expressed more than WT (Figures S4F and S4G). Finally, we assessed the ability of C177S to promote DNA replication. To account for potential differences in cell cycle distribution between WT and C177S cells, we synchronized the cells to G1 phase by treating them with CDK4/6 inhibitor palbociclib for 24 h (Trotter and Hagan, 2020). We released cells in the presence of EdU and evaluated EdU incorporation 4 and 6 h later for early and mid S phase, respectively (Figure S4E; Trotter and Hagan, 2020). In agreement with our conclusion that C177 oxidation promotes CDK2 activity and DNA replication, there were fewer EdU-positive cells for the C177S mutant compared with WT (Figure 7N). Collectively, these data demonstrate that oxidation of C177 located near the T-loop is important to maintain complete T160 phosphorylation and optimal CDK2 activity. Mutation of C177 or reducing CDK2 oxidation increased KAP binding, which supports that ROS promote S phase progression and DNA replication by preventing T160 phosphatase recruitment.

**DISCUSSION**

To proliferate, cells must coordinate cell growth driven by metabolism and cell cycle progression to ensure that DNA and other essential cellular components are duplicated before cell division. Bidirectional cross-talk between cell cycle and metabolic regulation are emerging topics of active research, especially because aberrations in either system are hallmarks of numerous diseases including cancer. In the present study, we uncover that mitochondrial ROS levels continuously increase from G1 to S and G2 phases and oxidize CDK2. Oxidation occurs at a conserved cysteine residue close to the T-loop of CDK2 and prevents the binding of KAP. As a consequence, T-loop phosphorylation can be sustained despite high KAP expression during early S phase (Gyuris et al., 1993), guaranteeing complete CDK2 activation and S phase progression. Thus, we identify a simple and elegant mechanism by which cell cycle-dependent production of mitochondrial ROS promote DNA replication to coordinate cell cycle progression with the metabolic state of the cell.

The concept that the redox environment of cells is coordinated with cell cycle progression dates back to 1931 when Rapkine (Rapkine, 1931) postulated a thiol cycle in dividing urchin eggs. Since then, redox fluctuations similar to the ROS dynamics we report have been inferred by several studies indirectly by reducing agents such as NAC (Menon et al., 2007; Kim et al., 2020) and H2O2 in order of sensitivity. We find that CellRox Deep Red predominantly labels mitochondrial...
structures in living cells, which is consistent with O$_2^-$ production in mitochondria. Our data suggest that the cell-cycle-dependent increase of cellular ROS likely recapitulates the relative increase in mitochondrial content rather than increased respiration (Figure 2). We cannot, however, exclude that bursts of mitochondrial activity undetectable by our analyses could also contribute to ROS production, such as during the G1/S transition (Mitra et al., 2009).

Previous studies have suggested that mitochondria in Drosophila and human cells are needed to provide the energy and buildup of cyclin E required to enter S phase (Mandal et al., 2005; Mitra et al., 2009; Robbins and Morrill, 1969). Our work identifies that mitochondrial ROS are a key driver of S phase by ensuring full CDK2 activity necessary for DNA replication (Figures 1, 3, and 4). We find no evidence that restricting the metabolic influx of acetyl-CoA into the TCA cycle decreases ATP levels and activates a metabolic checkpoint that restricts entry into S phase in a p53- and p21-dependent manner (Figure 3; Jones et al., 2005; Mitra et al., 2009). Furthermore, mitochondrial ATP production appears to be dispensable for human cells with access to sufficient glucose (Sullivan et al., 2015). Towards the end of G1 phase, mitochondria form a transient hyperfused network that drives cells into S phase, by an undefined mechanism that possibly involves p53 and boosting the levels of cyclin E (Mitra et al., 2009). Our data indicate that a limiting function of mitochondria seems to employ two sequential and distinct mechanisms to regulate S phase via CDK2 activity: energy-driven expression of cyclin E to initiate S phase and ROS-sensitive cysteine-reactive probe that prevents C177 oxidation but still allows for KAP binding.

In contrast to CDK2, CAK and KAP sequentially bind to different cyclins during the cell cycle and phosphorylation and de-phosphorylation is lacking. In higher organisms, coordination is needed to avoid unnecessary cycles of phosphorylation to drive DNA replication. Thus, mitochondria seem to employ two sequential and distinct mechanisms to regulate S phase via CDK2 activity: energy-driven expression of cyclin E to initiate S phase and ROS-sensitive cysteine-reactive probe that prevents C177 oxidation but still allows for KAP binding.

We propose a model by which the increase in mitochondrial ROS in S phase prevents KAP binding to CDK2 to ensure that T-loop phosphorylation of CDK2 can be sustained at times of high KAP expression (Gupta et al., 2017; Xiao et al., 2019). An exception to this rule would include CDK2 in Drosophila which has no C177, but here, CDK2 does not switch between cyclins because cyclin A only interacts with CDK1 in this organism (Harper and Elledge, 1998). A cysteine in the position of C177 is also absent in unicellular organisms and plants, where the function of CDK2 is carried out by CDK1.

The structure of KAP in association with CDK2 pT160 presents no clear evidence of how C177 oxidation could hinder KAP binding (Song et al., 2001). However, only residues 25–198 of KAP are resolved and truncation of residues 1–34 completely abolishes its interaction with CDK2 (Yeh et al., 2003). Hence, it is conceivable that the N terminus of KAP directly binds to CDK2 in a C177 oxidation-sensitive manner. Alternatively, we are currently investigating whether another interactor binds to CDK2 in more oxidizing conditions and thereby prevents the recruitment of KAP.

**Limitations of the study**

Although proteomics identified C177 as the only oxidized cysteine of CDK2 (Yang et al., 2015; Gupta et al., 2017; Xiao et al., 2020; Shi et al., 2021), we cannot exclude that the other two cysteines of CDK2 can be oxidized as well. The crystal structure of CDK2, however, indicates that only C177 is solvent exposed (Brown et al., 1999). KAP itself contains several redox-sensitive cysteines (Xiao et al., 2020), and cysteine 140 which is crucial for KAP activity is readily oxidized in vitro (Song et al., 2001). Thus, the increase of ROS during S and G2 phase might also negatively regulate KAP activity or its interaction with CDK2 to feedback to CDK2 activity. Our data imply that the levels of ROS and CDK2 oxidation are reduced once cells enter the next cycle. Whether this is a consequence of diminished ETC activity in mitosis/early G1 phase or an active process that might involve the antioxidant system or CDK2 turnover are intriguing questions to be addressed in the future.
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CDK2                | BD Bioscience | Cat# 610146; RRID: AB_397547 |
| CDK2 pT160          | Cell Signaling Technology | Cat# 2561; RRID: AB_2078685 |
| CDK4                | Cell Signaling Technology | Cat# 12790; RRID: AB_2631166 |
| CDK7                | Cell Signaling Technology | Cat# 2916; RRID: AB_2077142 |
| CSE1                | Abcam | Cat# ab54674; RRID: AB_940806 |
| CSE1                | Abcam | Cat# ab96755; RRID: AB_10865417 |
| Cyclin A2           | Santa Cruz | Cat# sc-596; RRID: AB_631330 |
| Cyclin B1           | BD Pharmingen | Cat# 554177; RRID: AB_395288 |
| Cyclin E1           | Bethyl | Cat# A301-566A; RRID: AB_1039994 |
| eIF4G               | Cell Signaling Technology | Cat# 2469; RRID: AB_2096028 |
| GAPDH               | Cell Signaling Technology | Cat# 2118; RRID: AB_561053 |
| Geminin             | Cell Signaling Technology | Cat# 5165; RRID: AB_10623289 |
| KAP (CDKN3)         | Abcam | Cat# ab175393 |
| Mouse anti-HA Tag (YPYDVPDYA) | Custom-made | N/A |
| p21                 | Cell Signaling Technology | Cat# 2947; RRID: AB_823586 |
| PARP                | Cell Signaling Technology | Cat# 9542; RRID: AB_2160739 |
| PDHA                | GeneTex | Cat# GTX104015; RRID: AB_1951155 |
| PDHB                | GeneTex | Cat# GTX119625; RRID: AB_11163863 |
| RPL26               | Bethyl | Cat# A300-686A; RRID: AB_530289 |
| Streptavidin        | Abcam | Cat# ab76949; RRID: AB_1524455 |
| α-tubulin           | Sigma Aldrich | Cat# T5168; RRID: AB_477579 |
| β-actin             | Sigma Aldrich | Cat# A5441; RRID: AB_476744 |
| IRDye 680RD Donkey anti-Rabbit IgG (H+L) | LI-COR Biosciences | Cat# 926-68073; RRID: AB_10954442 |
| IRDye 800CW Donkey anti-Rabbit IgG (H + L) | LI-COR Biosciences | Cat# 926-32213; RRID: AB_621848 |
| IRDye 800CW Donkey anti-Mouse IgG (H + L) | LI-COR Biosciences | Cat# 926-32212; RRID: AB_621847 |
| IRDye 800RD Donkey anti-Rabbit IgG (H + L) | LI-COR Biosciences | Cat# 926-68073; RRID: AB_10954442 |
| Anti-rabbit IgG, HRP-linked Antibody | Cell Signaling Technology | Cat# 7074; RRID: AB_2099233 |
| Anti-mouse IgG, HRP-linked Antibody | Cell Signaling Technology | Cat# 7076; RRID: AB_330924 |
| **Virus strains**   |        |            |
| AAV Helper-free System | Stratagene | Cat# 240071 |
| **Chemicals, peptides, and recombinant proteins** | | |
| 6-Benzylaminopurine (6-BAP) | Sigma Aldrich | Cat# B3408 |
| AF647-Picolyl-azide | Jena Biosciences | Cat# CLK-1300 |
| Amphoter cin B       | Sigma Aldrich | Cat# A2942 |
| AZDye 800 Picoly Azide (Az800) | Click Chemistry Tools | Cat# 1563-5 |
| Benzonase           | Sigma Aldrich | Cat# E1014 |
| Biotin              | Sigma Aldrich | Cat# B4639 |
| BSA (Fraction V)    | Roth | Cat# 8076.4 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **BTD** | Kindly provided by Kate Carroll, Scripps, UF, USA and André Nadler, MPI-CBG Dresden, Germany | https://doi.org/10.1021/jacs.7b01791 |
| CellRox Deep Red | Invitrogen | Cat# C10422 |
| cOmplete™, EDTA-free Protease Inhibitor Cocktail | Merck | Cat# 4693132001 |
| Copper(II) sulfate | Sigma Aldrich | Cat# C1297 |
| D-alanine | Sigma Aldrich | Cat# A7377 |
| DMSO | Sigma Aldrich | Cat# D2650 |
| Dry milk powder | Roth | Cat# T145.3 |
| DTT | Sigma Aldrich | Cat# D0632 |
| Formaldehyde | Thermo Scientific | Cat# 28908 |
| Gelantine | VWR | Cat# 1040700500 |
| Glutamax | Gibco | Cat# 35050038 |
| Glycerol | VWR | Cat# 24386.298 |
| Hoechst 33342 | Sigma Aldrich | Cat# 14533 |
| Iodoacetamide (IAA) | Sigma Aldrich | Cat# A3221 |
| IRDye 800CW Streptavidin | LI-COR Biosciences | Cat# 926-32230 |
| L-alanine | Sigma Aldrich | Cat# A7469 |
| Lambda Protein Phosphatase | New England Biolabs | Cat# P0753 |
| MES running buffer (Bolt) | Invitrogen | Cat# B0002 |
| MitoSOX | Invitrogen | Cat# M36008 |
| MitoTEMPO | Sigma Aldrich | Cat# SML0737 |
| MitoTracker Green | Invitrogen | Cat# M7514 |
| MOPS SDS running buffer (Bolt) | Invitrogen | Cat# B0001 |
| N-acetyl-L-cysteine | Sigma Aldrich | Cat# A7250 |
| Neomycin (G418) | Sigma Aldrich | Cat# G8168 |
| NuPage LDS sample buffer | Invitrogen | Cat# NP0007 |
| OPTIMEM | Gibco | Cat# 31985047 |
| Palbociclib | Sigma Aldrich | Cat# PZ0383 |
| Paraformaldehyde (PFA) | Merck | Cat# 1.040005 |
| PEG-Catalase | Sigma Aldrich | Cat# C4963 |
| Penicillin-streptomycin | Sigma Aldrich | Cat# P0781 |
| PhosSTOP | Merck | Cat# 4906837001 |
| Picoly-azide PEG4-biotin | Jena Biosciences | Cat# CLK-1167 |
| PP1 Analog III (3MB-PP1) | Merck | Cat# 529582 |
| Puromycin | Sigma Aldrich | Cat# P8833 |
| Recombinant CAK | ThermoFisher Scientific | Cat# PV3968 |
| Recombinant KAP | Sigma Aldrich | Cat# SRP5175 |
| RNAiMAX | ThermoFisher Scientific | Cat# 13778150 |
| SIR-DNA | Spirochrome | Cat# SC007 |
| Sodium Azide | Sigma Aldrich | Cat# S2002 |
| Sodium bicarbonate | Gibco | Cat# 25080094 |
| Sodium L-ascorbate | Sigma Aldrich | Cat# A7631 |
| Soy protein isolate | Amazon/Vitasgy | ASIN# B01FK8PROK |
| TCEP | Sigma Aldrich | Cat# C4706 |
| Thymidine | Sigma Aldrich | Cat# T9250 |
| Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)-methyl]-amin (TBTA) | Sigma Aldrich | Cat# 678937 |

(Continued on next page)
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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Triton X-100        | Sigma Aldrich | Cat# T8787 |
| Trypsin-EDTA solution 10x | Sigma Aldrich | Cat# 59418C |
| Tween 20            | VWR    | Cat# M147  |
| ADP/ATP ratio assay kit | Sigma Aldrich | Cat# MAK135-1KT |
| Click-IT Plus EdU Alexa Fluor 488 Imaging Kit | ThermoFisher Scientific | Cat# C10637 |
| Luminata Forte Western HRP Substrate | Millipore | Cat# WBLUF0500 |
| ProteoSilver stain kit | Sigma Aldrich | Cat# PROTSIL1 |
| Super Signal West Femto Maximum Sensitivity Substrate | ThermoFisher Scientific | Cat# 34095 |

**Critical commercial assays**

**Experimental models: Cell lines**

| RPE-1 FRT/TR mRuby-PCNA, Histone3.1-mTurquoise2, Cdk2 sensor (DHB-Venus) | Kindly provided by Robert Fisher, Icahn School of Medicine, New York, USA |
| RPE-1 FRT/TR mRuby-PCNA, Histone3.1-mTurquoise2, Cdk2 sensor (DHB-mCherry) | this study |
| RPE-1 FRT/TR mRuby-PCNA + Histone 3.1-iRFP + Hyper2-DAO-NES | this study |
| RPE-1 FRT/TR Hyper2-DAO-NLS | this study |
| RPE-1 FRT/TR CDK2 sensor (DHB-mCherry), NLS-DAO-HyPer2 | this study |
| RPE-1 FRT/TR Hyper2-DAO-NES | this study |
| RPE-1 FRT/TR CDK2 sensor (DHB-mCherry), NES-DAO-HyPer2 | this study |
| RPE-1 FRT/TR mKO2-hCdt1(30-120), Clover-hGeminin(1-110) | this study |
| RPE-1 FRT/TR Hyper7 + mRuby-PCNA + Histone 3.1-iRFP | this study |
| RPE-1 CDK2-as + CDK2 WT + eGFP (pool of 5 independent clones) | this study |

(Continued on next page)
| Reagent or Resource | Source | Identifier  |
|--------------------|--------|------------|
| RPE-1 CDK2-as + CDK2 C177S + mRuby (pool of 5 independent clones) | this study | Table S1: #20 |
| RPE-1 FRT/TR CDK2-HA (WT) | this study | Table S1: #21 |
| RPE-1 FRT/TR CDK2-HA (C177S) | this study | Table S1: #22 |
| Oligonucleotides | | |
| esi-PDHB | Eupheria | HU-04685-1 |
| esi-CTRL (5’-TGTCCTTTAACA CACTGCTACGAGCTACGAGTACATT GCGATACACAGCTACGAGTACATT TAAACGGCCTTTCCAGTATCGT GCATCTATATTTCCGGGGACG ATAAATAATGGGCTTCTTACA AGTCTATGATACATGCTTACA GATGGGACGTTCGTTACATGAAA TAAGACTTACACACACTCCAA TTACATTTCGCGCACTGGTCGTC (GCCAG-3’) | Eupheria | custom design |
| si-PDHA (5’-AGGUUGUGUGCUAA AGGGAAA-3’) | Eurofins | custom design |
| si-CTRL (5’-UGGUUUACAUGU CGACUAA-3’) | Eurofins | custom design |
| Oligonucleotides used for PCR and cloning | IDT, Sigma Aldrich | See Table S2 |
| Recombinant DNA | | |
| pAAV-Histon3.1-iRFP | this study | Table S2: #1 |
| pcDNA3-mAG-Geminin (1/110) | N/A | https://doi.org/10.1016/j.cell.2007.12.033 |
| pAAV-H3.1-mTurquoise2 | Our lab has developed | https://doi.org/10.1016/j.celrep.2017.05.022 |
| CSII-CDK2 sensor (Venus) | Kindly provided by Sabrina Spencer, University of Colorado, USA | https://doi.org/10.1016/j.cell.2013.08.062 |
| pIRESNeomycin3-Cdk2-sensor (Venus) | this study | Table S2: #5 |
| CAGGS-NLS-Flag-Cas9-ires-Puromycin | Kindly provided by Francis Stewart, TU Dresden, Germany | https://doi.org/10.1038/srep25529 |
| pIRES-EGFP | Clontech | 6029-1 |
| pIRESNeo3 | Clontech | 6988-1 |
| pIRESNeomycin3-CAGGS promoter | this study | Table S2: #8 |
| CSII-CDK2 sensor (DHB-mCherry) | Kindly provided by Sabrina Spencer, University of Colorado, USA | https://doi.org/10.1016/j.cell.2013.08.062 |
| pIRESNeomycin3-CDK2 sensor (DHB-mCherry) | this study | Table S2: #10 |
| pIRES-Puro3 | Clontech | 6986-1 |
| pIRESPuromycin3-CDK2 sensor (DHB-mCherry) | this study | Table S2: #12 |
| pLL3.7m-Clover-Geminin(1-110)-ires-mKO2-Cdt(30-120) | Addgene | RRID:Addgene_83841 |
| pC1-HyPer-3 | Addgene | RRID:Addgene_42131 |
| pC1-Clover-Geminin(1-110)-ires-mKO2-Cdt(30-120) | this study | Table S2: #15 |
| pC1-Clover-Geminin(1-110)-ires-Histone3.1-Turquoise2 | this study | Table S2: #16 |
| pAAV-Hyper2-DAO-NLS | Kindly provided Vsevolod Belousov, IBCh RAS, Russia | https://doi.org/10.1089/ars.2013.5618 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pcS2-HyPer7         | Kindly provided Vsevolod Belousov, IBCh RAS, Russia | https://doi.org/10.1016/j.cmet.2020.02.003 |
| piRESNeomycin3-CDK2-StrepII-WT | this study | Table S2: #19 |
| piRESNeomycin3-CDK2-StrepII-C177S | this study | Table S2: #20 |
| piRESNeomycin3-CDK2-StrepII-C177A | this study | Table S2: #21 |
| piRESNeomycin3-Cdk6-StrepII-WT | this study | Table S2: #22 |
| pAAV-Hyper2-DAO-NES | Kindly provided Vsevolod Belousov, IBCh RAS, Russia | https://doi.org/10.1089/ars.2013.5618 |
| pMito-iRFP713       | Addgene RRID:Addgene_45465 | |
| piRES2-CDK2(WT)-IRE2-eGFP | this study | Table S2: #25 |
| piRES2-CDK2(C177S)-IRE2-mRuby | this study | Table S2: #26 |
| pDNA5 FRT/TO CDK2(WT)-HA | this study | Table S2: #27 |
| piRESNeomycin3_CDK2(C177S)-HA | this study | Table S2: #28 |
| piRESNeomycin3_CDK2(WT)-HA | this study | Table S2: #29 |
| piRESNeomycin3-CDK2(C177S)-HA | this study | Table S2: #30 |

Software and algorithms

| Affinity Designer | Serif (Europe) Ltd | N/A |
| Fiji | N/A | https://doi.org/10.1038/nmeth.2019 |
| FlowJo | Becton, Dickinson and Company | N/A |
| Mathematica 12.1 | Wolfram Research Inc. | N/A |
| MetaXpress 5 and 6 | Molecular Devices | N/A |
| Papers2 | Mekentosj B.V. | N/A |
| Prism 6-9 | Graph Pad | N/A |
| TraCurate | N/A | https://doi.org/10.1016/j.softx.2021.100656 |

Other

| Auto-fluorescence-reduced imaging DMEM | Gibco | https://doi.org/10.1007/978-1-60327-993-2_7 |
| DMEM | Gibco | 41966052 |
| DMEM/F12 | Sigma Aldrich | D6421 |
| Dynabeads Protein G | Invitrogen | 10004D |
| Fetal Bovine Serum | Gibco | 10500064 |
| Fetal Bovine Serum, dialyzed | Gibco | 26400044 |
| Immobilon-FL PVDF | Millipore | IPFL00010 |
| Leibovitz’s L-15 Medium, no phenol red | Life Technologies | 21083027 |
| Minimum Essential Medium Eagle (MEM) | Sigma Aldrich | M2279 |
| Magnetic streptavidin beads | Pierce | 88816 |
| MagStrep "type3" XT beads | IBA | 2-4090-002 |

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests should be directed to and will be fulfilled by the corresponding author: Dr. Jörg Mansfeld (jorg.mansfeld@icr.ac.uk)

**Materials availability**
Generated plasmids and cells are available from the lead contact upon request or have been deposited on Addgene.

**Data and code availability**
- This paper does not contain standardized datatypes. All other datatypes will be shared by the lead contact upon request.
- This paper does not report original code.
EXPETIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and cell culture
All cell lines were cultured according to standard mammalian tissue culture protocol at 37°C in 5% CO2, assessed for authenticity by imaging, and tested for mycoplasma contamination. For hypoxia experiments, cells were adapted to 6.3% (RPE-1) and 4% O2 (BJ) in Whitley H35 hypoxostation (Don Whitley Scientific) for 3-4 days before the experiment. Prior to imaging or flow cytometry analyses, cells were fixed at the indicated concentrations of O2 in 3.7% PFA/PBS for 15 minutes. hTERT RPE-1 (Gender: female, RRID:CVCL_4388), hTERT RPE-1 FRT/TR (Gender: female, RRID:CVCL_VP32), hTERT-RPE-1 CDK2 as (Gender: female; Merrick et al., 2011) and the thereof derived cell lines listed in the key resources table and Table S2 were grown in DMEM/F12 supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, 1% (v/v) Glutamax, 0.5 μg/mL Amphotericin B and 0.26% sodium bicarbonate. AAV-293 (Gender: female, RRID:CVCL_6871) cells were grown in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, 1% (v/v) Glutamax, 0.5 μg/mL Amphotericin B. BJ fibroblasts (Gender: male, RRID:CVCL_3653) were grown in MEM supplemented with 10% FBS, 1% (v/v) penicillin-streptomycin and 1% (v/v) Glutamax. For BJ fibroblasts, the surface of cell culture plates was coated with 1% gelatin prior to seeding. Related to Figures 1, 2, 3, 4, 5, 6, 7, and S1–S3.

Plasmids and cell line generation
All plasmids and cell lines used in this study including vector backbones, PCR primers, templates, restriction sites, parent cell lines, inserts, methodology, and sources are listed in Tables S1 and S2. Gene knock-in of mRuby-PCNA and Histone 3.1-iRFP were created by rAAV-mediated gene targeting according to Zerjatke et al. (2017) and Collin et al. (2013). Stable cell lines were created by electroporation of plasmids using a Neon Transfection System (Thermo Fisher) according to the manufacturer’s instructions. After 14-21 days, single positive clones were picked after selection with 400 μg/mL neomycin or 0.5 μg/mL puromycin. RPE-1 cell lines ectopically expressing HyPer2-DAO-NES or HyPer2-DAO-NLS were generated by viral transduction using an AAV gene targeting system (Stratagene) according to the manufacturer’s instructions, and single HyPer2-fluorescent clones were selected by cell sorting on a BD FACSaria III (BD Biosciences). All cell lines used in this study including parental cell lines, plasmids, methods of creation, and references are described in Table S1. For transient transfection, 8 μg of CDK2-StrepII WT or C177S plasmid were electroporated per 10^6 cells, followed by extract preparation 48 hours later. Related to Figures 1, 3, 5, 6, 7, and S3.

METHOD DETAILS

Cell treatments
To synchronize RPE-1 cells at the beginning of S phase, 2.5 or 10 mM thymidine was added to the growth medium for 24 hours. To synchronize cells in G1 phase, 150 nM Palbociclib was supplied to media for 24 hours. For glutamine starvation experiments, cells were washed once with PBS, incubated for 6 hours in DMEM/F12 without glutamine and then supplemented with 10% (v/v) dialyzed FBS, 1% (v/v) penicillin-streptomycin, 0.5 μg/mL Amphotericin B, and 0.26% sodium bicarbonate. Stock solutions (500 mM) of N-acetyl-L-cysteine (NAC) in 7.5% sodium bicarbonate were always prepared freshly, adjusted with NaOH to pH 7.4-7.5, and added to cell culture media at the indicated concentrations and time. PEG-Catalase was dissolved in 50% glycerol/H2O and added to the cells at the indicated amounts. Stock solutions (1 M) of D- and L-alanine in H2O were stored at -20°C and diluted to the indicated final concentration in growth media 24 hours after cell seeding for proliferation experiments (Figure 1) or 48 hours after esi-RNA transfection (Figure 5). MitoTEMPO was dissolved in DMSO and added at the indicated concentrations. Endogenous CDK2 in RPE-1 CDK2 as cells was inhibited by adding 10 μM 3MB-PP1 for the duration of the experiment. To ensure normal CDK-cyclin pairing (Merrick et al., 2011) 0.5 μM 6-benzylaminopurine (6-BAP) was added during cultivation and experiments as indicated. As a positive control for apoptosis (Figure S1) 50 μM sodium azide was added to RPE-1 cells for 24 hours. Related to Figures 1, 4, 5, 6, 7, and S1–S3.

RNA interference
esi-RNA or si-RNA oligonucleotides were delivered using RNAiMAX by reverse transfection according to the manufacturer’s instructions. Briefly, transfections contained 6.5 ng esi-RNA per 96 well, 49.5 ng esi-RNA per μ-Slide 8 well, 1 μg esi-RNA per 6 well, or 50 nM of si-RNA mixed with 0.2 μL, 0.6 μL, and 2 μL of RNAiMAX in OPTIMEM, respectively. RNAi-treated cells were analyzed 48 hours after transfection with the exception of long-term time-lapse imaging (Figures 3G and 4A), which was started already after 24 hours. Sequences for esi-and siRNAs are listed in the key resources table. Related to Figures 3, 4, 5, 6, S1, and S2.

Proliferation experiments and EdU detection
To determine initial and final number of cells for proliferation experiments, complete 96 wells were imaged 5 hours after seeding (for esi/si-RNA experiments) or just prior to compound application and at the end of the experiment. Cell numbers were determined by detection and segmentation of nuclei using fluorescently tagged histone 3.1 or staining of DNA with 200 nM SIR-DNA (Spirochrome). For cell growth competition, RPE-1 CDK2 as cells stably expressing CDK2 WT and GFP from the same RNA (CDK2 WT_iRES2_eGFP) or CDK2 C177S and mRuby (CDK2 C177S_iRES2_mRuby) were seeded into the same 6 well in the presence of 10 μM 3MB-PP1. Cell
numbers of WT and C177S cells were determined by splitting ~1,000 – 15,000 cells from a 6 well into a 96 well plate, followed by incubation with 200 mM SIR-DNA and imaging 1 hour after seeding, when attachment of cells was complete. WT (GFP positive) and C177S (mRuby positive) cells were identified by single-cell analysis based on nuclear segmentation and GFP/mRuby detection using the segmentation and filter modules of MetaXpress Custom Module Editor (Molecular Devices). The ratio of WT/C177S cells on day 0 was set to 1 and the procedure was repeated on days 3 and 6. DNA replication was assessed by adding 10 μM EdU to cells for 45 minutes (Figures 4D and 4E) or the duration of the experiment (Figure 7N). Afterwards, cells were fixed in 4% PFA/PBS for 15 minutes at RT, washed twice in 3% BSA/PBS and extracted for 20 minutes in 0.5% Triton X-100/PBS, followed by two 5 minutes washes in 3% BSA/PBS. Subsequently, EdU was labeled with Alexa 488 using a Click-IT Plus Edu Alexa Fluor 488 Imaging Kit according to the manufacturer’s instructions (Figures 4D and 4E) or by the following protocol: a click mixture of PBS containing 4 mM CuSO4, 5 mM AF647-Picolyl-azide and 10 mM sodium ascorbate was added to cells for 1 hour at RT in the dark (Figure 7N); after removal of the click mixture, cells were washed once in 3% BSA/PBS and PBS followed by staining of DNA with 2 μM Hoechst 33342 for 30 minutes, two washes with PBS, and image analysis. Related to Figures 4 and 7.

Lysate, extracts and interaction studies
To obtain total cell lysates, RPE-1 cells were washed once in PBS followed by the addition of 1x NuPAGE LDS Sample buffer containing 100 mM DTT. For extracts, cells were trypsinized with 2x Trypsin-EDTA, washed once in PBS, resuspended in extraction buffer P (30 mM Tris pH 7.5, 0.25% NP-40, 2.5 mM MgCl2, 175 mM NaCl, 10% glycerol, 1 mM DTT, cOmplete protease inhibitors (Merck), phosSTOP phosphatase inhibitors (Merck), incubated for 20 minutes on ice and centrifuged at 13,000g for 15 minutes at 4°C. Cleared extracts were mixed 3:1 (v/v) with 3x NuPAGE LDS/DDT Sample buffer and boiled at 95°C for 5 minutes. For Strepl pull-downs RPE-1 cells were lysed with extraction buffer S (50 mM Tris pH 8, 150 mM NaCl, 2.5 mM MgCl2, 5% glycerol, 1% Triton X-100), cOmplete protease inhibitors and incubated for 20 minutes on ice. Extracts were cleared (13,000g, 15 minutes, 4°C) and added to MagStrep type3 XT beads equilibrated with extraction buffer and incubated for 30 minutes on a rotating wheel at 4°C. To assess binding of endogenous KAP to CDK2-Strepl-WT, living cells were incubated with 5 mM DTT at 37°C for 10 minutes prior to extraction in buffer S (+/− 20 mM DTT). For analyses of CDK2 T160 phosphorylation, cyclin A2 binding, cyclin E1 binding and endogenous KAP, binding beads were washed 3x in extraction buffer S and precipitates were eluted in NuPAGE LDS/DDT sample buffer at 95°C for 5 minutes. For binding assays with recombinant GST-KAP or CAK, CDK2-WT-Strepl and CDK2-C177S-Strepl immobilized on MagStrep type3 XT beads were washed 1x in extraction buffer S +/− 10 mM DTT. For CAK binding assays CDK2 was dephosphorylated by washing beads once in λ-phosphatase buffer with 1 mM MnCl2 and incubated with 400 units of λ-phosphatase for 30 minutes at 4°C. Subsequently, CDK2 WT or C177S beads were incubated with or without 10 mM DTT in extraction buffer S including 0.1 μg/μL bovine serum albumin (BSA) for 30 minutes at 4°C. KAP binding assays were performed for 30 minutes on a rotating wheel at 4°C with 80 ng KAP supplied to binding buffer K (50 mM Tris pH 6.8, 150 mM NaCl, 0.1 μg/μL BSA) +/− 1 mM DTT. CAK binding assays were performed for 1 hour on a rotating wheel at 4°C with 125 ng of CAK diluted in binding buffer C (8 mM MOPS/NaOH pH 7, 0.2 mM EDTA, 150 mM NaCl) +/− 1 mM DTT. After binding, beads were washed twice with extraction buffer S and eluted with NuPAGE LDS/DDT sample buffer for 5 min at 95°C. For binding of endogenous KAP to CDK2-WT-Strepl, living cells were incubated with 5 mM DTT at 37°C for 10 minutes before extract preparation in buffer S +/− 20 mM DTT. Staining of CDK2-Strepl pull-downs was performed using a ProteoSilver staining kit according to the manufacturer’s instructions. Related to Figures 3, 4, 5, 6, 7, and S1–S3.

BTD labeling
RPE-1 cells were grown to 80-90% confluency in 15 cm dishes (Greiner Bio-One), washed once in PBS and labeled for sulfenic acids with 1 mM of the sulfenic acid-reactive probe BTD (Gupta et al., 2017). BTD stocks were dissolved in DMSO and added to the growth medium for 30 minutes at 37°C (0.5% final concentration DMSO). Subsequently, cells were washed twice with PBS and fixed in PBS/1% formaldehyde (FA) supplemented with 10 mM iodoacetamide (IAA) for 15 minutes. After further alkylation in PBS/10 mM IAA for 15 minutes, cells were reduced with 40 mM DTT/PBS for 30 minutes, washed once with PBS, permeabilized with 90% methanol at -20°C for 15 minutes, washed once with PBS and treated with 15 μg/ml benzonase per 15 cm dish for 15 minutes. After an additional wash in PBS, cells were blocked with PBS/5% BSA for 1 hour, washed once with PBS, and incubated with 0.5 nmol picolyl-azide PEG4-biotin in 7.5 mM PBS per dish. Clicking of biotin-azole to the alkeno group of BTD was catalyzed by adding a 2x concentrated click mix to reach a final concentration of 1 mM CuSO4, 0.1 mM Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)-methyl]-amin (TBTA) and 1 mM sodium ascorbate. After 1 hour clicking at RT, cells were washed once with PBS, thrice in PBS/0.1% Tween 20/10 mM EDTA for 10 minutes, once with PBS and scraped in 1 mL of PBS containing 2% SDS/5 mM DTT. Samples were then boiled at 95°C for 45 minutes to reverse the FA crosslink, cleared for 15 minutes at 13,000g, the supernatants diluted 6 times in PBS and incubated with PBS-equilibrated magnetic streptavidin beads on a wheel overnight at 4°C. Unbound material was removed, and beads were washed 4x in 4 M urea/0.5% SDS/25 mM HEPES, 4x in PBS/0.5% SDS and then transferred to a clean microfuge tube. After two more PBS washes, bound proteins were eluted by NuPAGE LDS sample buffer supplemented with 2.5 mM biotin/100 mM DTT at 95°C for 15 minutes (Figures 7B and S2J). To investigate the cell cycle-dependent oxidation of CDK2, RPE-1 cells expressing CDK2-HA WT were grown to 50% confluency and serum-starved for 24 hours. According to Figure S2J, cells were labeled at 8, 18 and 22 hours representing G1, S and G2 phases for 30 minutes at 37°C in media containing 1% FBS to minimize quenching of the BTD probe by PBS. Subsequently, cells were washed twice with PBS and lysed in 50 mM Tris pH 8, 400 mM NaCl, 5% glycerol, 1% SDS, supplemented with fresh 10 mM TCEP and cOmplete protease inhibitors for 15 minutes at RT. Lysates were alkylated with IAA (40 mM) for
15 minutes at RT in the dark and stored at -80 °C until all time points were collected. Lysates were sonicated to shear DNA, cleared at 13,000 x g, and then a click reaction to conjugate BTD to Az800 (AZDye 800 Picolyl Azide) was performed as described above. Afterwards, proteins were precipitated by chloroform/methanol to remove click reagents and protein pellets were resuspended in 20 mM Tris pH 8, 0.5 M Urea and 0.5% SDS. Lysates were sonicated and diluted 1:7 with Buffer A containing 20 mM Tris pH 8, 120 mM NaCl and 1% Triton and incubated with protein G Dynabeads coupled to HA antibodies for 1 hour at 4 °C. Beads were then washed once with Buffer A supplemented with 120 mM NaCl and thrice with Buffer A supplemented with 400 mM NaCl. CDK2-HA was eluted by boiling beads for 10 minutes at 65 °C in NuPage LDS sample buffer, followed by transfer to a new tube, addition of 100 mM DTT and analysis by SDS-PAGE (Figures 7D and S3B). Related to Figures 7, S2, and S3.

**SDS-PAGE and Western blot analyses**

Proteins were separated by SDS-PAGE using Bis-Tris 4–12% Bolt gradient gels in MES or MOPS buffers in a Mini Gel Tank (Thermo Fisher). Western blot analyses were performed using a wet transfer Criterion Blotter (BioRad) in MOPS/20% ethanol transfer buffer using Immobilon-blot PVDF membranes. Membranes were blocked for 1 hour at RT in 5% dry milk or 5% soy protein isolate (for CDK2 pT160 detection) prepared in PBS/0.2% Tween 20. Primary antibodies were added overnight at 4 °C, followed by 3x washing in PBS/0.02% Tween 20 and incubation with secondary antibodies for 1 hour at RT. For quantitative detection, fluorescently-labeled secondary antibodies were used with a near-infrared scanning system (Odyssey, Li-COR). Alternatively, detection was performed with horseradish peroxidase (HRP)-conjugated antibodies and Luminata Forte Western HRP Substrate or Super Signal West Femto Maximum Sensitivity Substrate on an ImageQuant LAS4000 system (Amersham Biosciences). Related to Figures 3, 4, 5, 6, 7, and S1–S3.

**ROS labeling**

For flow cytometry analyses by the LSR Fortessa FACS (BD Bioscience) analyzer, 10⁶ asynchronous cells were labeled for 30 minutes with 5 μg/mL Hoechst 33342, trypsinized, washed in PBS and transferred to CO₂-independent L15 medium supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin, 1% (v/v) Glutamax, 0.5 μg/mL Amphotericin B and 0.26% sodium bicarbonate. Then, 5 μg/mL Hoechst 33342 and 5 μM CellRox Deep Red and/or MitoSox Red were added for 30 minutes or 100 nM MitoTracker Green for 10 minutes. ROS labeling of adherent cells was performed in living RPE-1 cells expressing endogenously tagged histone 3.1-Turquoise2, Ruby-PCNA, cyclinA2-Venus using the reagents, concentrations and labeling times indicated above, followed by a brief wash in PBS to remove the excess of the free dye and incubating cells in imaging DMEM (described below). The relative increase of cell volume versus mitochondria during the cell cycle was determined by forward scatter and MitoTracker Green, respectively, and normalized to the G1 population. Related to Figures 2 and S1.

**ADP/ATP ratio**

The ADP/ATP ratio was determined using an ADP/ATP ratio assay kit 48 hours after PDHB depletion in a 96 well plate. Measurements were performed according to the manufacturer’s instructions on a Glomax Luminometer (Promega). Related to Figure 3.

**Microscopy**

Automated microscopy was performed on an ImageXpress Micro XLS wide-field screening microscope (Molecular Devices) equipped with 10x, 0.5 NA and 20x, 0.7 NA Plan Apo air objectives (Nikon) and laser-based autofocus. Excitation and detection were done by a Spectra X light engine (Lumencor) and sCMOS (Andor) camera with the indicated filters: DAPI (Ex: 377/50; Dic: 442-450 / 415-570; Em:447/60); CFP (Ex: 438/24; Dic: 426-450/467-600; Em: 483/32); GFP (Ex: 472/30; Dic: 442-488 / 502-730; Em: 520/35), YFP (Ex: 513/17; Dic: 488-512 / 528-625; Em: 542/27), TexasRed (Ex: 575/25 Dic: 530-585 / 601-800; Em: 624/40) and Cy5 (Ex: 628/40; Dic: 594-651 / 669-726; Em: 692/40). During experiments, cells were maintained in a stage incubator at 37 °C in a humidified atmosphere of 5% CO₂. All cells were grown in black 96 well clear-bottom plates (μclear, Greiner Bio-One). For long-term time-lapse microscopy and ROS imaging, the growth media was exchanged to an auto-fluorescence-reduced imaging DMEM (Schmitz and Gerlich, 2009) supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, 1% Glutamax, 0.5 μg/mL Amphotericin B and 0.26% sodium bicarbonate. Long-term time-lapse microscopy was performed by taking images using a 10x objective at 7 minutes intervals for 48 hours. To determine the initial and final number of cells for proliferation experiments, the complete 96 well was imaged 5 hours after seeding (for esi/si-RNA experiments) or just prior to compound application and at the end of the experiment. Microscopy of cells stained with ROS-sensitive dyes was performed by acquiring maximum image projection using a 20x objective. For ratio imaging of HyPer2-DAO-NLS and HyPer2-DAO-NES cells with ImageXpress Micro XLS, the following filter sets were used: Ex: (438/24) with Em: (542/27), and Ex: (513/17) with Em: (542/27). For CDK2 sensor imaging of cells grown under physiological %O₂, cells were fixed in HyPer2-DAO-NLS and HyPer2-DAO-NES cells with ImageXpress Micro XLS, the following filter sets were used: Ex: (438/24) with Em: (542/27). Related to Figures 1, 2, 3, 4, 5, 6, 7, S1, and S2.
QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis

Image analyses of single time points were performed in MetaXpress 5 and 6 (Molecular Devices) using customized image analysis pipelines. Briefly, images were flat-field and background corrected using a tophat filter and nuclei were segmented based on DNA or Ruby-PCNA labeling to create masks for fluorescence extraction. To determine the initial and final number of cells in proliferation experiments, the complete 96 well was imaged 5 hours after seeding (for esi/si-RNA experiments) or prior to compound application and again at the end of the experiment. Detection of PCNA replication foci was performed using the nuclear speckle plugin from the MetaXpress Custom Module Editor. Fucci analysis was performed by segmenting all nuclei based on SIR-DNA staining and by merging the nuclear intensities of Gem(1-110) and Cdt(30-120) to a common segmentation mask. Gem(1-110)- and Cdt(30-120)-positive cells were assigned by minimum threshold filtering and the cell cycle distribution was indicated as a fraction of the sum of Gem(1-110)- or Cdt(30-120)-positive cells (Figure S2C). Time-lapse analysis of single cells was performed as previously described (Zerjatke et al., 2017). Briefly, all images were background corrected using flat-field correction, and nuclei were segmented based on histone 3.1 labeling using intensity thresholding and subsequent watershed filtering. Time-lapse single-cell tracking was performed with a nearest-neighbor approach and subsequent manual track curation using TraCurate (Wagner et al., 2021). Cell cycle phases were classified according to PCNA mean intensity and distribution as described in Zerjatke et al. (2017). Nuclear CDK2 levels were quantified as mean intensities based on the histone segmentation masks. Cytoplasmic CDK2 levels were quantified as the mean intensities in two cap regions adjacent to the poles of each cell nuclei. Image analysis and quantification were performed with Mathematica 12.1 (Wolfram Research Inc.). Thresholds for CDK2^{Low}/CDK2^{High} cells were determined by histogram analysis of ratios of nuclear and cytoplasmic CDK2 to identify the CDK2^{Low} peak (Spencer et al., 2013). Ratio image analyses were performed in Fiji (Schindelin et al., 2012) using the Ratio Plus plugin as previously described (Kardash et al., 2011). Ratio image analyses of HyPer7 images were performed using a semi-automated Fiji macro. Briefly, the images were background corrected (rolling ball = 50), smoothened and a mask was defined based on the HyPer7 Ex: (464-492)/Em: (500-523) image and binarized. Then, images from both excitations were multiplied with the mask to remove background, converted to 32 bit, and the ratio image was calculated by dividing the HyPer 7 signal from (Ex: 464-492)/(Em: 500-523) by (Ex: 381-401)/(Em: 500-523). Flow cytometry analyses were performed in FlowJo (Becton, Dickinson and Company). Forward scatter values were used as a relative proxy for cell size. Related to Figures 1, 3, 4, 5, 6, 7, S1, and S2.

Statistical methods

Data normalization was performed in Microsoft Excel (Microsoft), and statistical analysis and graph presentation were performed in Prism 6-9 (GraphPad) using the statistical tests indicated in the figure legends. All data are representative of at least three independent repeats unless otherwise stated. The notation n refers to the number of independently performed experiments, and the notation N refers to the number of data points used for statistical analyses and data presentation. A p-value lower than 0.05 was considered statistically significant and individual p-values are indicated in each figure or figure legend. Bar charts indicate the mean ± SD and show all data points for N<10. Box plots show the median and 25th-75th percentiles, and whiskers represent the 5th-95th percentiles. For quantitative Western blot analyses by near-infrared fluorescence detection (Li-COR), band intensities were divided by the intensity of the loading control or total CDK2 levels (for pT160) to correct for sample loading. Time-lapse data of single-cell ratio imaging were smoothened with the 4 neighbors on each side and a 6th order polynomial using Prism 6. For flow cytometry analyses, the intensities of individual cell cycle phases were normalized by the sum of intensities of all cell cycle phases to correct for differences in overall staining intensities between experiments. No randomization or blinding was performed in this study. Related to Figures 1, 2, 3, 4, 5, 6, 7, and S1–S3.
Supplemental information

A ROS-dependent mechanism promotes CDK2 phosphorylation to drive progression through S phase

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Figure S1, related to Figure 1 and Figure 3. Interference with ROS slows cell proliferation and does not induce apoptosis. (A) Proliferation of RPE-1 cells in the presence of NAC. Boxplots indicate the median log2-fold proliferation of cells for 48 hours. Significance according to one-way ANOVA with Dunnett’s multi comparison test (n=3, N=18). (B) Western blot analysis detecting PARP cleavage in cells treated with NAC or 50 µM sodium azide (NaN₃) as a positive control. (C) Cell cycle analysis based on fluorescent markers. Cyclin A2-Venus negative cells are in G1 phase, cyclin A2-Venus positive cells are in S or G2 phase, and PCNA foci identify S phase. Circles in the overlay indicate examples of cells in G1, S and G2 phase, classified according to the expression of cyclin A2-Venus and PCNA foci. Scale bar = 10 µm. (D) Stacked bars indicate the mean ± SD fraction of the cells from (A) in cell cycle phases based on classification from (C). Significance according to one-way ANOVA with Holm-Sidak’s multiple comparisons test: **(6 mM NAC (G1) = 0.0077), ***(6 mM NAC (S) = 0.0085), ***(8 mM NAC (S) = 0.0007), *(8 mM NAC (G2) = 0.0133), ***(10 mM NAC (S) = 0.0046), ****(10 mM NAC (G2) = p<0.0001), (n=3, N=18). (E) Quantification of ROS detected by CellRox Deep Red in HyPer2-DAO-NES expressing RPE-1 cells in Figure 1F in response to 0.5 mM D-alanine (D-ala). Control cells were treated either with 0.5 mM or 5 mM L-alanine (L-ala). Bars represent the mean ± SD. Significance according to two-tailed one-sample t-test (n=3, N=3). (F) Western blot analysis assessing PARP cleavage in cells depleted of PDHB.
Figure S2

(A) Western blot analysis showing T160 phosphorylation (pT160) in S phase synchronized RPE-1 cells depleted of PDHB for 48 hours. (B) Quantification of the data shown in (A). Bars indicate the mean ± SD. Significance according to two-tailed one-sample t-test (n=3, N=3). (C) Cell cycle distribution analysis based on live cell imaging of Fucci-Gem and Fucci-Cdt expressing cells. Boxplots indicate the median fraction of cells in the indicated cell cycle phase at the beginning and after 6 hours of glutamine starvation. Significance according to two-way ANOVA with Sidak’s multiple comparisons test (n=3, N=18). (D) Western blot analysis showing pT160 in S phase-synchronized RPE-1 cells 5 hours after treatment with NAC. (E) Quantification of the data shown in (D). Bars indicate the mean ± SD. Significance according to two-tailed one-sample t-test (n=4, N=4). (F) Localization of CDK2 sensor 48 hours after treatment with NAC in RPE-1 cells. Scale bar = 100 µM. (G) Western blot analysis (n=2) detecting pT160 in RPE-1 cells treated with NAC for 48 hours. (H) Single-cell analysis of Cyclin A2-Venus expressing cells treated as in (G). Boxplots indicate the median fraction of Cyclin A2 positive cells in response to NAC (n=3, N=18).

Figure S2, related to Figure 5. Changes in CDK2 T-loop phosphorylation in response to reductive treatments. (A) Western blot analysis showing T160 phosphorylation (pT160) in S phase synchronized RPE-1 cells depleted of PDHB for 48 hours. (B) Quantification of the data shown in (A). Bars indicate the mean ± SD. Significance according to two-tailed one-sample t-test (n=3, N=3). (C) Cell cycle distribution analysis based on live cell imaging of Fucci-Gem and Fucci-Cdt expressing cells. Boxplots indicate the median fraction of cells in the indicated cell cycle phase at the beginning and after 6 hours of glutamine starvation. Significance according to two-way ANOVA with Sidak’s multiple comparisons test (n=3, N=18). (D) Western blot analysis showing pT160 in S phase-synchronized RPE-1 cells 5 hours after treatment with NAC. (E) Quantification of the data shown in (D). Bars indicate the mean ± SD. Significance according to two-tailed one-sample t-test (n=4, N=4). (F) Localization of CDK2 sensor 48 hours after treatment with NAC in RPE-1 cells. Scale bar = 100 µM. (G) Western blot analysis (n=2) detecting pT160 in RPE-1 cells treated with NAC for 48 hours. (H) Single-cell analysis of Cyclin A2-Venus expressing cells treated as in (G). Boxplots indicate the median fraction of Cyclin A2 positive cells in response to NAC (n=3, N=18).
Figure S3, related to Figure 7. CDK2 oxidation during the cell cycle and C177 mutagenesis decrease T160 phosphorylation. (A) Biotin detection of Western Blot analysis in Figure 7B showing all BTD-biotin labeled proteins. (B) Structure of CDK2-cyclin A (PDB: 4I3Z) highlighting T160 (red) and C177 (blue). (C) Clustal Omega alignment of CDK2 kinases showing that C177 is highly conserved in vertebrates. Organisms in which CDK2 functions are exerted by a single CDK (i.e., in yeast or plants) do not contain a corresponding cysteine residue. (D) Western blot analysis (n=2) showing T-loop phosphorylation and expression of cell cycle markers in RPE-1 cells after releasing from 24h of serum starvation. The ratio of
phosphorylated T160 (pT160) to CDK2 normalized to t=0 is indicated beneath the Western blot. (E) Western blot analysis of the input samples of (F) and data shown in Figure 7D detecting the differential expression of cyclin A2 in G1 versus S and G2 phase samples. (F) Complete scan of the data presented in Figure 7D at a lower intensity to highlight differential BTD labeling of input samples at different stages of the cell cycle. (G-I) Western blot analysis and quantification of StreptII pull-downs using S-phase synchronized RPE-1 cells transiently expressing CDK2-WT-StreptII (WT) or CDK2-C177A-StreptII (C177A) or CDK6-StreptII as a control. Bars represent the mean ± SD of pT160 and cyclin A2. Significance according to two-tailed one-sample t-test. (pT160: n=7, N=7; cyclin A2: n=4, N=4). (J-K) Western blot analysis and quantification of StreptII pull-downs using S-phase synchronized RPE-1 cells transiently expressing CDK2-WT-StreptII (WT) or CDK2-C177S-StreptII (C177S). Bars represent the mean ± SD of Cyclin E1 binding. Significance according to two-tailed one-sample t-test. (n=4, N=4).
Figure S4, related to Figure 7. CDK2-CAK binding assays and rescue of RPE-1 CDK2wt cells with CDK2 WT and C177S. (A-C) Silver staining and Western blot analysis of Strept II pull-downs of CDK2 WT and C177S from lysates of S phase synchronized cells in the absence of DTT. Note, beads-bound CDK2-StrepII was used as shown in Figure 7H and for λ-phosphatase (λ-PP) treatment (B) and subsequent binding assays with recombinant CAK in the presence or absence of DTT (C). (D) Quantification of CAK binding to CDK2-StreplII from (C). Bars represent the mean ± SD. Significance according to two-tailed unpaired one-sample t-test. (CAK: n=3, N=3). (E) Western blot analysis (n=2) of total lysates from RPE-1 cells released from a 24-hour Palbociclib block for 4 and 6 hours (as in Figure 7N), corresponding to early and mid S phase. (F) Western blot analysis of total lysates prepared from parent CDK2wt cells and CDK2wt cells stably expressing untagged CDK2 WT or C177S. (G) Quantification of CDK2 WT and C177S expression shown in (F) normalized to endogenous CDK2 levels in parental RPE-1 CDK2wt cells. Bars represent the mean and ± SD. (n=3, N=3).
## Supplemental Table 1. Generation of cell lines, related to STAR Methods.

| No. | Name                              | Plasmid used | Parental cell line | Insert                          | Method                                      | Reference       |
|-----|-----------------------------------|--------------|--------------------|---------------------------------|---------------------------------------------|-----------------|
| 1   | RPE-1                             | NA           | NA                 | NA                             | NA                                          | RRID:CVCL_4388  |
| 2   | RPE-1 FRT/TR                     | NA           | NA                 | NA                             | NA                                          | PMID: 28564611  |
| 3   | RPE-1 mRuby-PCNA, Histone3.1-mTurquoise2, Cylin2-mVenus | NA           | NA                 | NA                             | NA                                          | PMID: 28564611  |
| 4   | RPE-1 FRT/TR mRuby-PCNA           | NA           | NA                 | NA                             | NA                                          | PMID: 28564611  |
| 5   | RPE-1 FRT/TR mRuby-PCNA, Histone3.1-iRFP | 1            | 4                  | Histone3.1-iRFP                 | endogenous knock-in by rAAV gene-targeting  | this study      |
| 6   | RPE-1 FRT/TR mRuby-PCNA, Histone3.1-iRFP, mAG-hGeminin (1-110) | 2            | 5                  | mAG-Geminin (1-110)             | ectopic expression, electroporation         | this study      |
| 7   | RPE-1 FRT/TR mRuby-PCNA, Histone3.1-mTurquoise2 | 3            | 4                  | Histone 3.1-mTurquoise2         | endogenous knock-in by rAAV gene-targeting  | this study      |
| 8   | RPE-1 FRT/TR mRuby-PCNA, Histone3.1-mTurquoise2, Cdk2 sensor (DHB-Venus) | 5            | 7                  | Cdk2 sensor (DHB-Venus)         | ectopic expression, electroporation         | this study      |
| 9   | RPE-1 FRT/TR CDK2 sensor (DHB-mCherry) | 13           | 1                  | CDK2 sensor (DHB-mCherry)       | ectopic expression, electroporation         | this study      |
| 10  | RPE-1 FRT/TR Clover-hGeminin(1-110), Histone3.1-mTurquoise2, Cdk2 sensor (DHB-mCherry) | 17           | 9                  | Clover-hGeminin(1-110), Histone3.1-mTurquoise2 | ectopic expression, electroporation         | this study      |
| 11  | RPE-1 FRT/TR mRuby-PCNA + Histone 3.1-iRFP + HyPer2-DAO-NES | 18           | 5                  | HyPer2-DAO-NES                  | ectopic expression, viral transduction      | this study      |
| 12  | RPE-1 FRT/TR Hyper2-DAO-NLS       | 18           | 2                  | HyPer2-DAO-NLS                  | ectopic expression, viral transduction      | this study      |
| 13  | RPE-1 FRT/TR CDK2 sensor (DHB-mCherry), NLS-DAO-HyPer2 | 11           | 12                 | CDK2 sensor (DHB-mCherry)       | ectopic expression, electroporation         | this study      |
| 14  | RPE-1 FRT/TR Hyper2-DAO-NES      | 24           | 2                  | NES-DAO-HyPer2                  | ectopic expression, viral transduction      | this study      |
| 15  | RPE-1 FRT/TR CDK2 sensor (DHB-mCherry), NES-DAO-HyPer2 | 11           | 14                 | CDK2 sensor (DHB-mCherry)       | ectopic expression, electroporation         | this study      |
| 16  | RPE-1 FRT/TR mKO2-hCdt1(30-120), Clover-hGeminin(1-110) | 16           | 2                  | mKO2-hCdt1(30-120), Clover-hGeminin(1-110) | ectopic expression, electroporation         | this study      |
| 17  | RPE-1 FRT/TR Hyper7 + mRuby-PCNA + Histone 3.1-iRFP | 19           | 5                  | HyPer7                         | ectopic expression, electroporation         | this study      |
| 18  | RPE-1 CDK2-as                     | NA           | NA                 | NA                             | NA                                          | PMID: 21658603  |
| 19  | RPE-1 CDK2-as + CDK2 WT + eGFP (pool of 5 independent clones) | 26           | 18                 | CDK2-WT_IRES2_eGFP              | ectopic expression, electroporation         | this study      |
| 20  | RPE-1 CDK2-as + CDK2 C177S + mRuby (pool of 5 independent clones) | 27           | 18                 | CDK2-C177S_IRES2_mRuby          | ectopic expression, electroporation         | this study      |
| 21  | RPE-1 FRT/TR CDK2-HA (WT)        | 28           | 2                  | CDK2-WT-HA                      | integration into single FRT site, electroporation | this study      |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 22 | RPE-1 FRT/TR CDK2-HA (C177S) | 29 | 2 | CDK2-C177S-HA | integration into single FRT site, electroporation this study |

NA = not applicable
Supplemental Table 2. Plasmids, related to STAR Methods.

| No. | Name                                      | Backbone | Insert                      | Method                                                                 | Resistance bacteria | Resistance cell line | Source                  |
|-----|-------------------------------------------|----------|-----------------------------|------------------------------------------------------------------------|---------------------|----------------------|-------------------------|
| 1   | pAAV-Histone3.1-iRFP                      | pAAV     | Histone3.1-iRFP             | PCR of iRFP from RRID:Addgene 45465 with primers (5’-acgcGTCGACggtcagcggagccgagagttgcgagggtggcttgaggacagctgaaggatcgcgcagccagcgtgccgacc-3’) and (5’-CCCaagctTACctctcattacgccgatctgcc-3’) from 25 and cloning into 3 using SalI and HindIII restriction sites | Ampicillin          | NA                   | this study              |
| 2   | pcDNA3 mAG-Geminin (1/110)                 | pcDNA3   | mAG-Geminin (1-110)         | NA                                                                     | Ampicillin          | Neomycin             | PMID: 18267078          |
| 3   | pAAV-H3.1-mTurquoise2                     | pAAV     | H3.1-mTurquoise2            | NA                                                                     | Ampicillin          | NA                   | PMID: 28564611          |
| 4   | SCIICDK2 sensor (Venus)                   | CSII     | CDK2 sensor (DHB-Venus)     | NA                                                                     | Ampicillin          | NA                   | PMID 24075009           |
| 5   | pIRESNeo 3-Cdk2-sensor (Venus)            | pIRESNeo 3 | CDK2 sensor (DHB-Venus)     | Subcloning of DHB-Venus from plasmid 4 into pIRESNeo3 using AgeI, HPAl and BamHi restriction sites | Ampicillin          | Neomycin             | this study              |
| 6   | CAGGS-NLS-Flag-Cas9-ires-Puromycin        | NA       | NLS-Flag-Cas9 (WT nuclease) | NA                                                                     | Ampicillin          | Puromycin            | PMID 27216209           |
| 7   | pIRES2-EGFP                               | pIRES    | EGFP                        | NA                                                                     | Kanamycin           | Neomycin             | Clontech                |
| 8   | pIRESNeo 3                                | pIRESNeo 3 | empty                      | NA                                                                     | Ampicillin          | Neomycin             | Clontech                |
| 9   | pIRESNeo 3-CAGGS promoter                 | pIRESNeo 3 | CAGGS promoter              | Subcloning of CAGGS promoter from plasmid 6 into 8 using SnaBI and NheI restriction sites | Ampicillin          | Neomycin             | this study              |
| 10  | CSII-CDK2 sensor (DHB-mCherry)            | CSII     | CDK2 sensor (DHB-mCherry)   | NA                                                                     | Ampicillin          | NA                   | PMID 24075009           |
| 11  | pIRESNeo 3-CDK2 sensor (DHB-mCherry)      | pIRESNeo 3 | CDK2 sensor (DHB-mCherry)   | PCR of DHB-mCherry with primers (5’-Ggaattcaccatgcaaaatgtgcttcagctgg-3’) and (5’-ATAAGAATgccccctgctctgctctgctctgctccgacc-3’) from plasmid 10 and cloning into 9 using EcoRI and NolI restriction sites | Ampicillin          | Neomycin             | this study              |
| 12  | pIRES-Puromycin in3                       | pIRESNeo 3 | NA                         | NA                                                                     | Ampicillin          | Puromycin            | Clontech                |
| 13  | pIRES-Puromycin in3                       | pIRESNeo 3 | CDK2 sensor                 | Subcloning of DHB-mCherry from plasmid 11 into 12 using Ndel and NotI restriction sites | Ampicillin          | Puromycin            | this study              |
| Step | Plasmid | Details | Antibiotics | Comment |
|------|---------|---------|-------------|---------|
| 14   | pLL3.7m | Clover-Geminin(1-110)-IRES-mKO2-Cdt(30-120) | NA | Ampicillin NA RRID:Add gene_83841 |
| 15   | pC1-HyPer-3 | pC1 | HyPer-3 | NA | Kanamycin Neomycin RRID:Add gene_42131 |
| 16   | pC1-Clover-Geminin(1-110)-IRES-mKO2-Cdt(30-120) | pC1 | Clover-Geminin(1-110)-IRES-mKO2-Cdt(30-120) | Subcloning of Clover-Geminin(1-110)-IRES-mKO2-Cdt(30-120) from plasmid 14 into 15 using NheI and SmaI restriction sites | Kanamycin Neomycin this study |
| 17   | pC1-Clover-Geminin(1-110)-IRES-Histone3.1-Turquoise2 | pC1 | Clover-Geminin(1-110)-IRES-Histone3.1-Turquoise2 | PCR of Clover-Geminin(1-110)-IRES-Histone3.1-Turquoise2 with primers (5'-CGgaattcATGGCGCGTACTAAGCAGAC-3') and (5'-CGGGATCCtcacttgtacagctcgtccatgc-3') from 3 and cloned into 16 using EcoRI and BamHI restriction sites | Kanamycin Neomycin this study |
| 18   | pAAV-Hyper2-DAO-NLS | pAAV | Hyper2-DAO-NLS | NA | Ampicillin NA PMID 24020354 |
| 19   | pCS2-HyPer7 | pCS2 | HyPer7 | NA | Ampicillin NA PMID 32130885 |
| 20   | pIRESNeo-3-CDK2-StrepII-WT | pIRESNeo-3 | CDK2-StrepII-WT | PCR of CDK2-StrepII-WT with primers (5'-tgtggtaccgagctcctgaaatcctcctgggctgcaaatattattcacaagcttgaggacatcggagctgggcatctttgctgagatggtgacttgccgggcc-3') and (5'-cGGGATCCtcacttgtacagctcgtccatgc-3') from 20 and cloning into 20 using SacI and BamHI restriction sites | Ampicillin Neomycin this study |
| 21   | pIRESNeo-3-CDK2-StrepII-C177S | pIRESNeo-3 | CDK2-StrepII-C177S | PCR CDK2-StrepII-C177S with primers (5'-tgtggtaccgagctcctgaaatcctcctgggctgcaaatattattcacaagcttgaggacatcggagctgggcatctttgctgagatggtgacttgccgggcc-3') and (5'-cGGGATCCtcacttgtacagctcgtccatgc-3') from 20 and cloning into 20 using SacI and BamHI restriction sites | Ampicillin Neomycin this study |
| 22   | pIRESNeo-3-CDK2-StrepII-C177A | pIRESNeo-3 | CDK2-StrepII-C177A | PCR of CDK2-StrepII-C177A with primers (5'-tgtggtaccgagctcctgaaatcctcctgggctgcaaatattattcacaagcttgaggacatcggagctgggcatctttgctgagatggtgacttgccgggcc-3') and (5'-cGGGATCCtcacttgtacagctcgtccatgc-3') from 20 and cloning into 20 using SacI and BamHI restriction sites | Ampicillin Neomycin this study |
| Step | Description | Original Source | Primers Used | Notes |
|------|-------------|-----------------|--------------|-------|
| 23   | PCR of CDK6 with primers (5'-ccgCTCGAGATGGAGAAGGACGGCCTGTGCGCCGCGCTGACC-3') and (5'-ggGATCCCTCATTTCCTGGAATCTCGGGTGGGCTCCAGCGCCAGGCAGACGGCAGG-3') from cDNA and cloning into 20 using SacI and BamHI restriction sites | | | Ampicillin Neomycin this study |
| 24   | PCR of CDK2 from plasmid 28 with 5'-ccgCTCGAGATGGAGAACTTCCAAAAGGTGGAAAG-3' and 5'-ggGATCCCTCAGGATGGAGAAGGACGGCCTGTGCGCCGCGCTGACC-3' and cloning into the XhoI and BamHI sites of 7 | | | Ampicillin NA PMID 24020354 |
| 25   | PCR of CDK2 from plasmid 29 with 5'-ccgCTCGAGATGGAGAACTTCCAAAAGGTGGAAAG-3' and 5'-ggGATCCCTCAGGATGGAGAAGGACGGCCTGTGCGCCGCGCTGACC-3' and cloning into the XhoI and BamHI sites of 7, where eGFP was replaced by mRuby | | | Kanamycin Neomycin RRID:Add gene_45465 |
| 27   | PCR of CDK2 WT from plasmid 30 (NotI and AfeI) into plasmid 32 (NotI and EcoRV) | | | Ampicillin Neomycin this study |
| 30   | PCR of CDK2 WT from plasmid 20 with 5'-ccgCTCGAGATGGAGAAGGACGGCCTGTGCGCCGCGCTGACC-3' and 5'-ggGATCCCTCAGGATGGAGAAGGACGGCCTGTGCGCCGCGCTGACC-3' and cloning into the XhoI and BamHI sites of plasmid 8 | | | Kanamycin Neomycin this study |
| 31   | PCR of CDK2 C177S from plasmid 31 into plasmid 28 using BsrG1 and NotI | | | Kanamycin Neomycin this study |