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Supplemental information

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

Dilyana Georgieva Kirova, Kristyna Judasova, Julia Vorhauser, Thomas Zerjatke, Jacky Kieran Leung, Ingmar Glauche, and Jörg Mansfeld

Figure S1

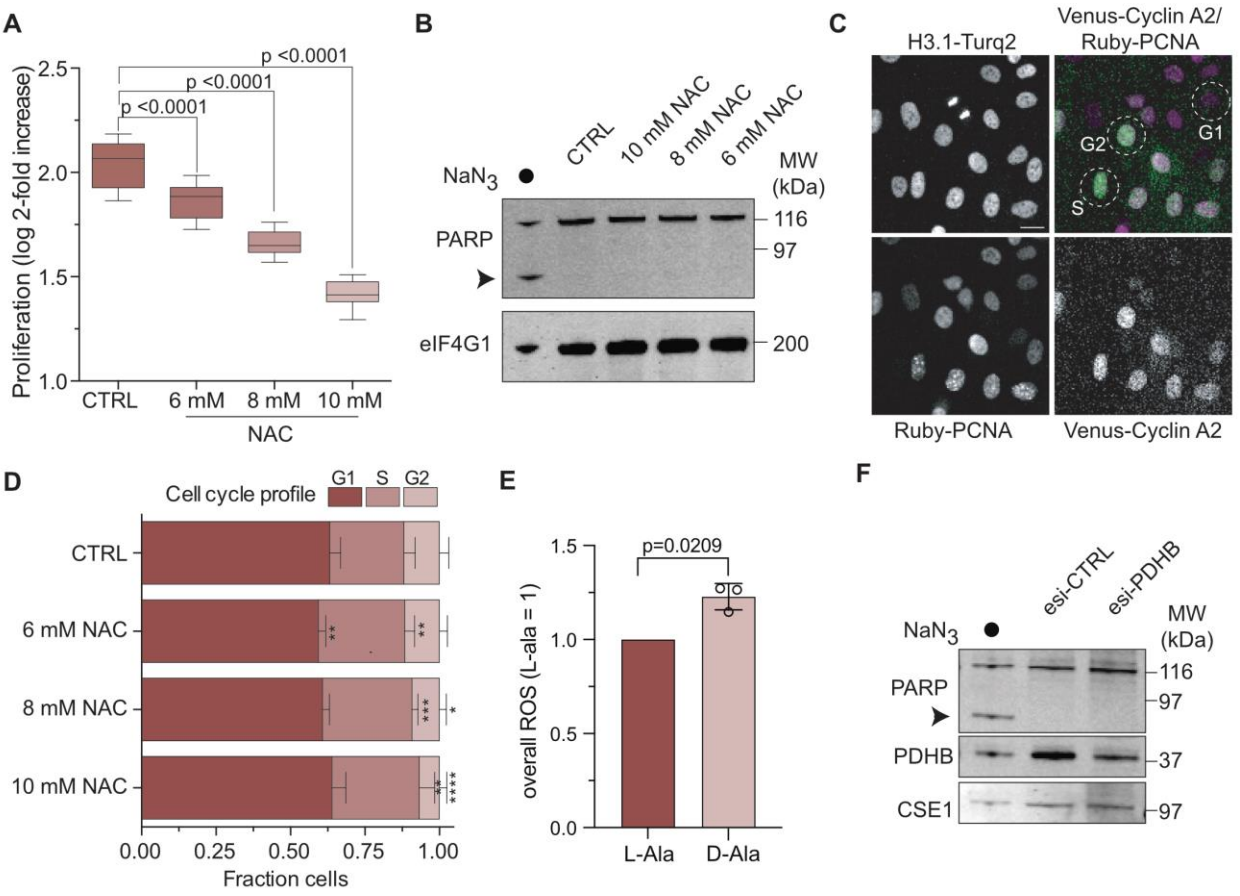

Figure S1, related to Figure 1 and Figure 3. Interference with ROS slows cell proliferation and does not induce apoptosis. (A) Proliferation of RPE-1 cells in the presence of NAC. Boxplots indicate the median log2-fold proliferation of cells for 48 hours. Significance according to one-way ANOVA with 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 (NaN_3) 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

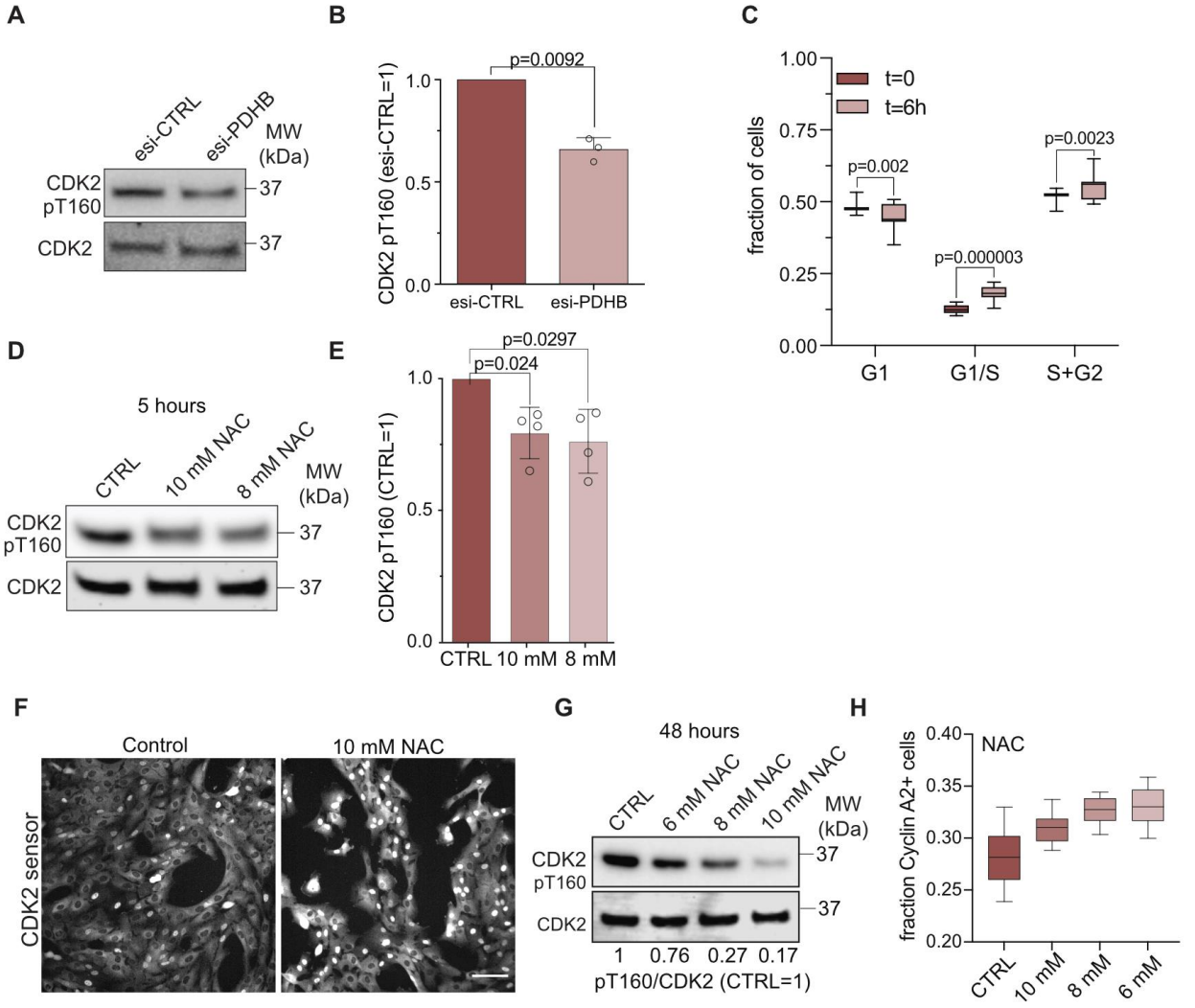

Figure S2, related to Figure 5. Changes in CDK2 T-loop phosphorylation in response to reductive treatments. (A) Western blot analysis showing T160 phosphorylation (pT160) in S phase synchronized RPE-1 cells depleted of PDHB for 48 hours. (B) Quantification of the data shown in (A). Bars indicate the 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

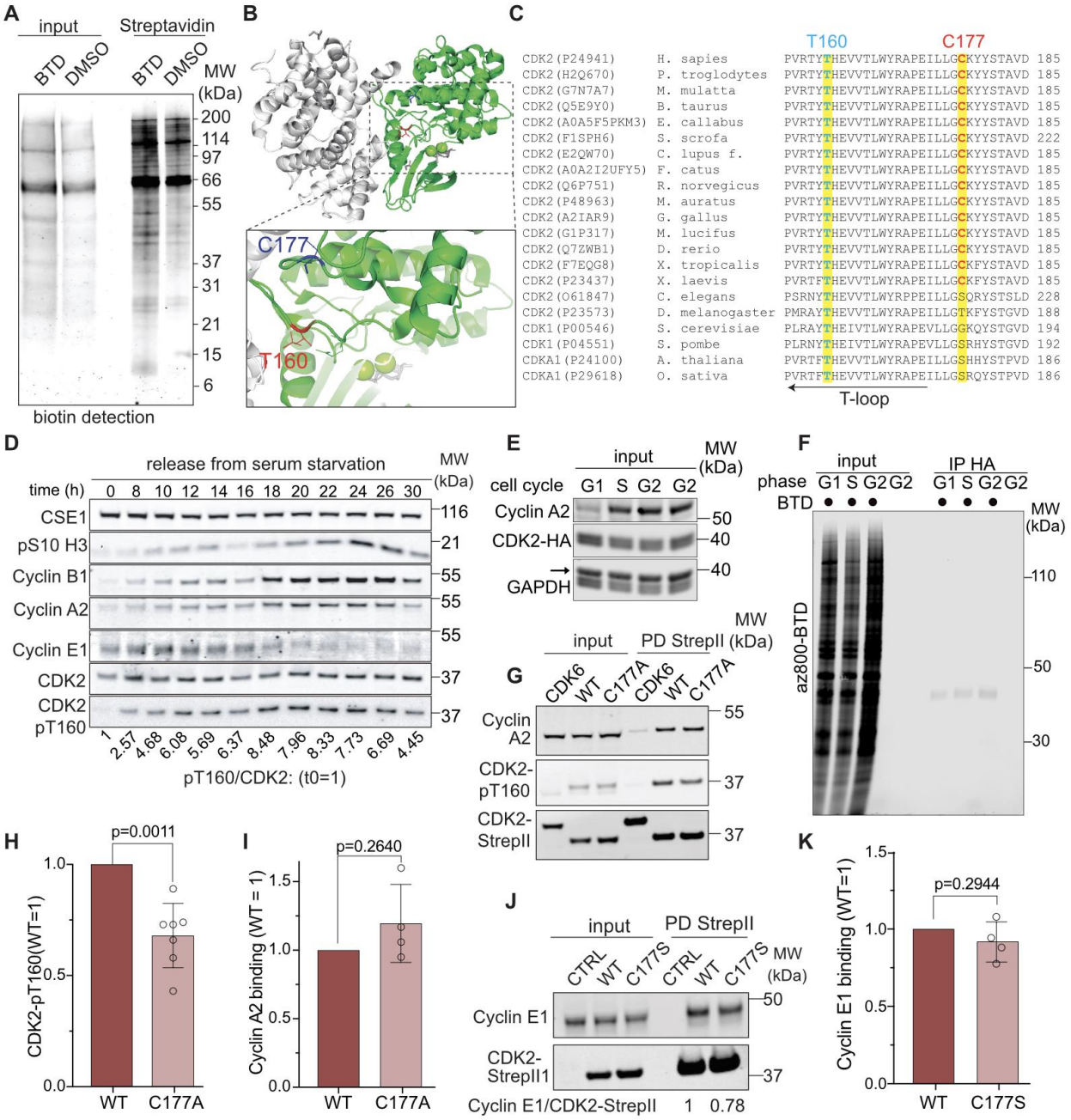

Figure S3, related to Figure 7. CDK2 oxidation during the cell cycle and C177 mutagenesis decrease T160 phosphorylation. (A) Biotin detection of Western Blot analysis in Figure 7B showing all BTD-biotin labeled proteins. (B) Structure of CDK2-cyclin A (PDB: 4I3Z) highlighting T160 (red) and C177 (blue). (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 StrepII pull-downs using S-phase synchronized RPE-1 cells transiently expressing CDK2-WT-StrepII (WT) or CDK2-C177A-StrepII (C177A) or CDK6-StrepII 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 StrepII pull-downs using S-phase synchronized RPE-1 cells transiently expressing CDK2-WT-StrepII (WT) or CDK2-C177S-StrepII (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

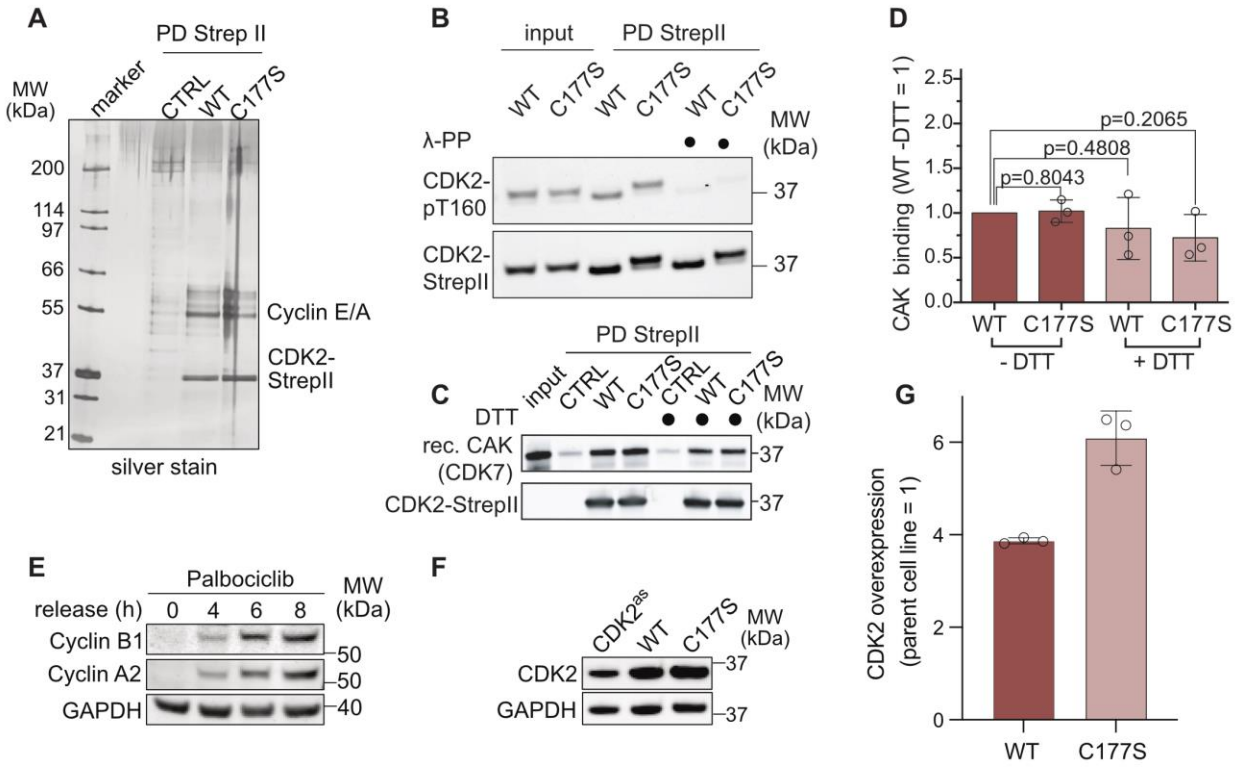

Figure S4, related to Figure 7. CDK2-CAK binding assays and rescue of RPE-1 CDK2^{as} cells with CDK2 WT and C177S. (A-C) Silver staining and Western blot analysis of Strep II pull-downs of CDK2 WT and C177S from lysates of S phase synchronized cells in the absence of DTT. Note, beads-bound CDK2-StrepII 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-StrepII 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 CDK2^{as} cells and CDK2^{as} 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 CDK2^{as} 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	RRID:CVCL_VP32
3	RPE-1 mRuby-PCNA, Histone3.1-mTurquoise2, CylinA2-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	CDK 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'-acgcGTCGACggtgcaggcggagccggaggtgcgggtggggctggaggagcagctgaaggatccgtcgccaggcagcctgacc-3') and (5'-CCCaagcttTCActcttccatcacgccgatctgcc-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	SCII-CDK2 sensor (Venus)	CSII	CDK2 sensor (DHB-Venus)	NA	Ampicillin	NA	PMID 24075009
5	pIRESNeomycin 3-Cdk2-sensor (Venus)	pIRESNeo3	CDK2 sensor (DHB-Venus)	Subcloning of DHB-Venus from plasmid 4 into pIRESNeomycin3 using AgeI, HPAI 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	pIRESNeomycin 3	pIRESNeo3	empty	NA	Ampicillin	Neomycin	Clontech
9	pIRESNeomycin 3-CAGGS promoter	pIRESNeo3	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	pIRESNeomycin 3-CDK2 sensor (DHB-mCherry)	pIRESNeo3	CDK2 sensor (DHB-mCherry)	PCR of DHB-mCherry with primers (5'-Ggaattcaccatgacaaatgatgtcacctgg-3') and (5'-ATAAGAATgcggccgcttacttgtacagctcgtccatgcc-3') from plasmid 10 and cloning into 9 using EcoRI and NotI restriction sites	Ampicillin	Neomycin	this study
12	pIRES-Puromycin3	pIRESPuro3	NA	NA	Ampicillin	Puromycin	Clontech
13	pRIRES Puromycin3-	pIRESNeo3	CDK2 sensor	Subcloning of DHB-mCherry from plasmid 11 into 12 using NdeI and NotI restriction sites	Ampicillin	Puromycin	this study

	CDK2 sensor (DHB-mCherry)		(DHB-mCherry)				
14	pLL3.7m-Clover-Geminin(1-110)-IRES-mKO2-Cdt(30-120)	pLL3.7m	Clover-Geminin(1-110)-IRES-mKO2-Cdt(30-120)	NA	Ampicillin	NA	RRID:Addgene_83841
15	pC1-HyPer-3	pC1	HyPer-3	NA	Kanamycin	Neomycin	RRID:Addgene_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	pIRESNeomycin3-CDK2-StrepII-WT	pIRESNeo3	CDK2-StrepII-WT	PCR of CDK2-StrepII-WT with primers (5'-ccgCTCGAGATGGAGAACTTCCAAAAGGTGGAAAAG-3') and (5'-cgGGATCCTCATTTTTCGAACTGCGGGTGGCTCCAGCCAGCGCCGAGTCGAAGATGGGGTACTGGCTTGGTCACATCC-3') from cDNA and cloning into 8 using XhoI and BamHI restriction sites	Ampicillin	Neomycin	this study
21	pIRESNeomycin3-CDK2-StrepII-C177S	pIRESNeo3	CDK2-StrepII-C177S	PCR CDK2-StrepII-C177S with primers (5'-tgtggtaccgagctcctgaaatcctcctgggctgcaaatattattccacagctgtggacatctggagcctgggcAGcatctttgctgagatggtgactcgccgggcc-3') and (5'-cgGGATCCTCATTTTTCGAACTGCGGGTGGCTCCAGCCAGCGCCGAGTCGAAGATGGGGTACTGGCTTGGTCACATCC-3') from 20 and cloning into 20 using SacI and BamHI restriction sites	Ampicillin	Neomycin	this study
22	pIRESNeomycin3-CDK2-StrepII-C177A	pIRESNeo3	CDK2-StrepIIC177A	PCR of CDK2-StrepII-C177A with primers (5'-tgtggtaccgagctcctgaaatcctcctgggctgcaaatattattccacagctgtggacatctggagcctgggcGCcatctttgctgagatggtgactcgccgggcc-3') and (5'-cgGGATCCTCATTTTTCGAACTGCGGGTGGCTCCAGCCAGCGCC	Ampicillin	Neomycin	this study

				GAGTCGAAGATGGGGTACTGG CTTGGTCACATCC-3') from 20 and re-cloned into 20 using SacI and BamHI restriction sites			
23	pIRESN eomycin 3-Cdk6-StrepII-WT	pIRESNeo 3	CDK6-StrepII-WT	PCR of CDK6 with primers (5'-ccgCTCGAGATGGAGAAGGACG GCCTGTGCCGCGCTGACC-3') and (5'-cgGGATCCTCATTTTTCGAACTG CGGGTGGCTCCAGCCAGCGCC GGCTGTATTCAGCTCCGAGGT GTTCTGGCTGGGCGGCAGG-3') from cDNA and cloning into 8 using XhoI and BamHI restriction sites	Ampicillin	Neomycin	this study
24	pAAV-Hyper2-DAO-NES	pAAV	Hyper2-DAO-NES	NA	Ampicillin	NA	PMID 24020354
25	pMito-iRFP713	pN1	iRFP713	NA	Kanamycin	Neomycin	RRID:Addgene_45465
26	pIRES2-CDK2(WT)-IRES2-eGFP	pAAV	CDK2 WT + eGFP	PCR of CDK2 from plasmid 28 with 5'-ccgCTCGAGATGGAGAACTTCC AAAAGGTGGAAAAG-3' and 5'-cgGGATCCtcagagtcgaagatggggta ctgg-3' and cloning into the XhoI and BamHI sites of 7	Ampicillin	Neomycin	this study
27	pIRES2-CDK2(C177S)-IRES2-mRuby	pN1	CDK2 C177S + mRuby	PCR of CDK2 from plasmid 29 with 5'-ccgCTCGAGATGGAGAACTTCC AAAAGGTGGAAAAG-3' and 5'-cgGGATCCtcagagtcgaagatggggta ctgg-3' cloning into the XhoI and BamHI sites of 7, where eGFP was replaced by mRuby	Kanamycin	Neomycin	this study
28	pDNA5 FRT/TO CDK2(WT)-HA	pCDNA5 FRT/TO neo	CDK2-WT-HA	Subcloning of CDK2 WT from plasmid 30 (NotI and AfeI) into plasmid 32 (NotI and EcoRV)	Ampicillin	Neomycin	this study
29	pIRESN eomycin 3_CDK2 (C177S) -HA	pCDNA5 FRT/TO neomycin resistance	CDK2-C177S-HA	Subcloning of CDK2 C177S from plasmid 31 into plasmid 28 using BsrG1 and NotI	Ampicillin	Neomycin	this study
30	pIRESN eomycin 3_CDK2 (WT)-HA	pIRESNeo 3	CDK2-WT-HA	PCR of CDK2 WT from plasmid 20 with 5'-ccgCTCGAGATGGAGAACTTCC AAAAGGTGGAAAAG-3' and 5'-gcGGATCCtcacgcatagtcaggaacat cgtatgggtaGCCAGCGCCgagtcgaa gatggggtactggcttggtcacatcc-3' and cloning into the XhoI and BamHI sites of plasmid 8	Kanamycin	Neomycin	this study
31	pIRESN eomycin 3-CDK2(C177S)-HA	pIRESNeo 3	CDK2-C177S-HA	PCR of CDK2 C177S from plasmid 21 with 5'-ccgCTCGAGATGGAGAACTTCC AAAAGGTGGAAAAG-3' and 5'-gcGGATCCtcacgcatagtcaggaacat cgtatgggtaGCCAGCGCCgagtcgaa gatggggtactggcttggtcacatcc-3' and cloning into the XhoI and BamHI sites of plasmid 8	Kanamycin	Neomycin	this study

NA=not applicable