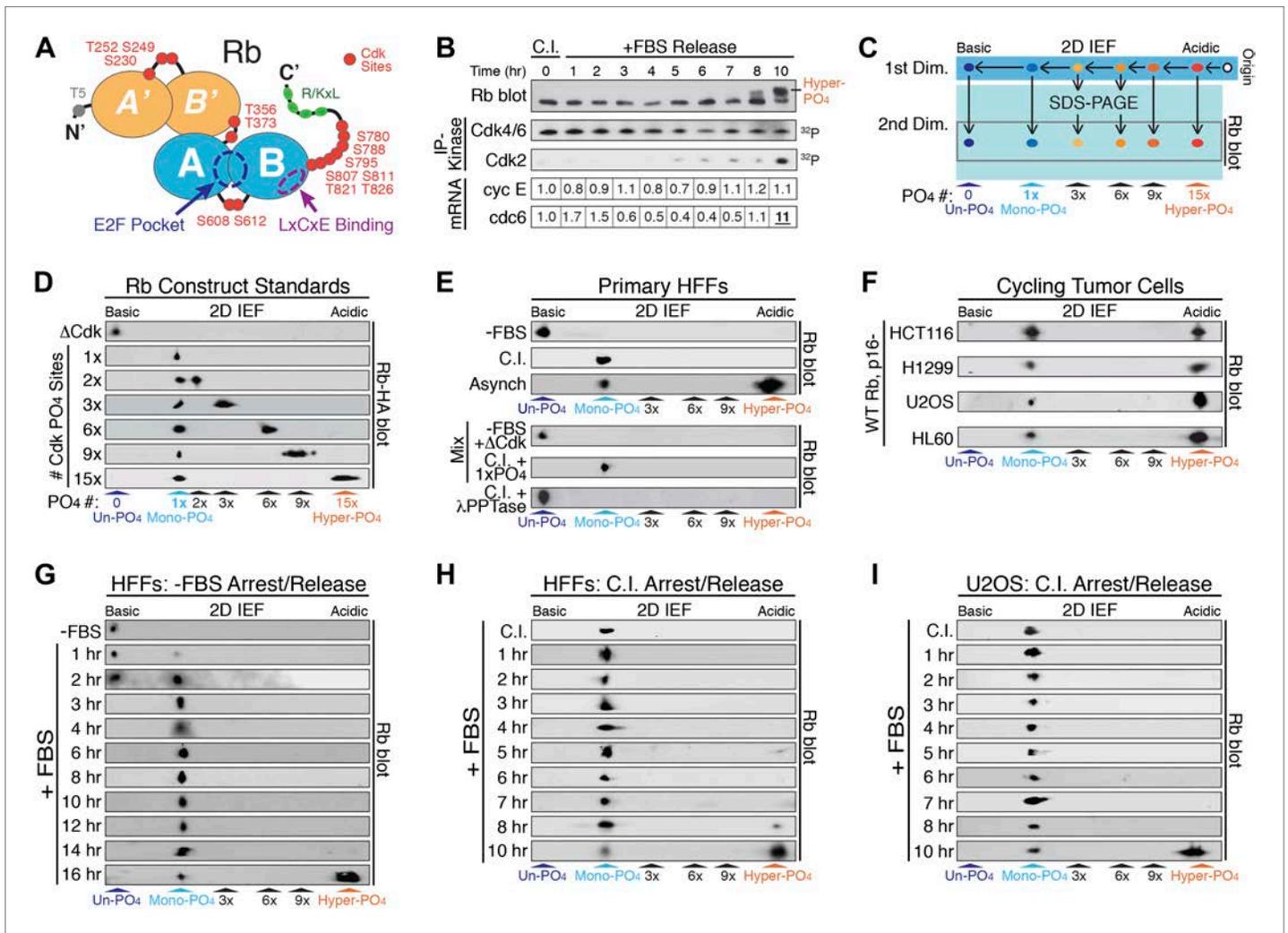


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## Figures and figure supplements

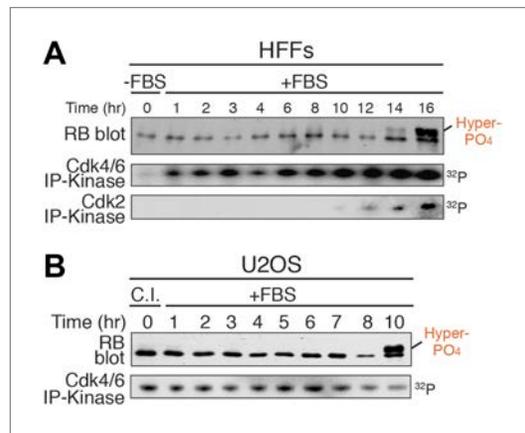
Cyclin D activates the Rb tumor suppressor by mono-phosphorylation

**Anil M Narasimha, et al.**



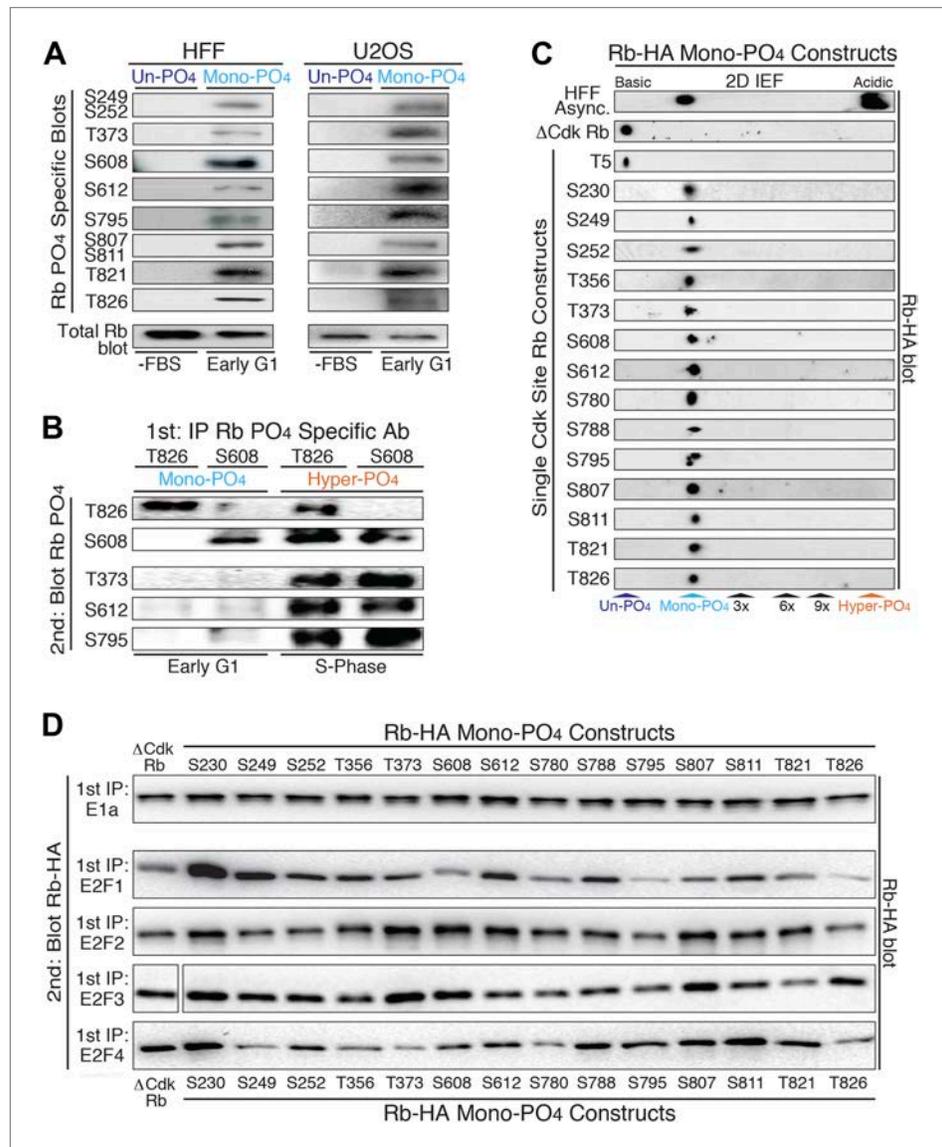
**Figure 1.** Rb is exclusively mono-phosphorylated in early G<sub>1</sub> phase. **(A)** Schematic diagram of human Rb Cdk phosphorylation sites, A'/B' and A/B pocket domains. **(B)** Kinetic analysis of contact inhibited early G<sub>1</sub> phase arrested (+FBS) and released primary Human Fibroblasts (HFFs) by 1D SDS-PAGE Rb immunoblot, anti-Cdk4/6, and anti-Cdk2 immunoprecipitation-kinase assay, and qRT-PCR of cyclin E and cdc6 mRNA normalized to B-2-microglobulin levels. **(C)** Schematic diagram of two-dimensional isoelectric focusing (2D IEF). Immunoprecipitated Rb is loaded at origin on acidic end of IEF strip and separated first by pI. IEF strip is then soaked in SDS, run in second dimension into SDS-PAGE and immunoblotted for Rb. **(D)** 2D IEF Rb-HA immunoblot of Rb construct standards expressed in cycling cells and containing 0 ( $\Delta$ Cdk), 1x, 2x, 3x, 6x, 9x or 15x Cdk phosphorylation sites. **(E)** Top panels: 2D IEF Rb immunoblot of primary HFFs serum deprived G<sub>0</sub> arrested (-FBS), contact inhibited early G<sub>1</sub> phase arrested (+FBS), or asynchronously cycling. Bottom panels: 2D IEF Rb immunoblot of serum deprived G<sub>0</sub> arrested (-FBS) HFFs mixed with  $\Delta$ Cdk Rb standard and contact inhibited treated with  $\lambda$  phosphatase. **(F)** 2D IEF Rb immunoblot from cycling human tumor cell lines expressing wild-type Rb and deregulated cyclin D:Cdk4/6 due to p16 deletion, HCT116 colon carcinoma, H1299 lung adenocarcinoma, U2OS osteosarcoma, HL60 promyelocytic leukemia. **(G)** 2D IEF Rb immunoblot from serum deprived G<sub>0</sub> arrested (-FBS) and released (+FBS) primary HFFs from 0 to 16 hr **(H)** 2D IEF Rb immunoblot from contact inhibited early G<sub>1</sub> phase arrested (+FBS) and released HFFs from 0 to 10 hr **(I)** 2D IEF Rb immunoblot from contact inhibited early G<sub>1</sub> phase arrested (+FBS) and released U2OS from 0 to 10 hr.

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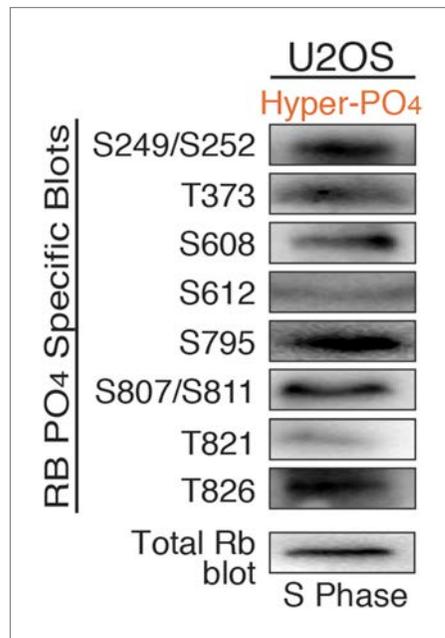


**Figure 1—figure supplement 1.** Rb is exclusively mono-phosphorylated in early G<sub>1</sub> phase.

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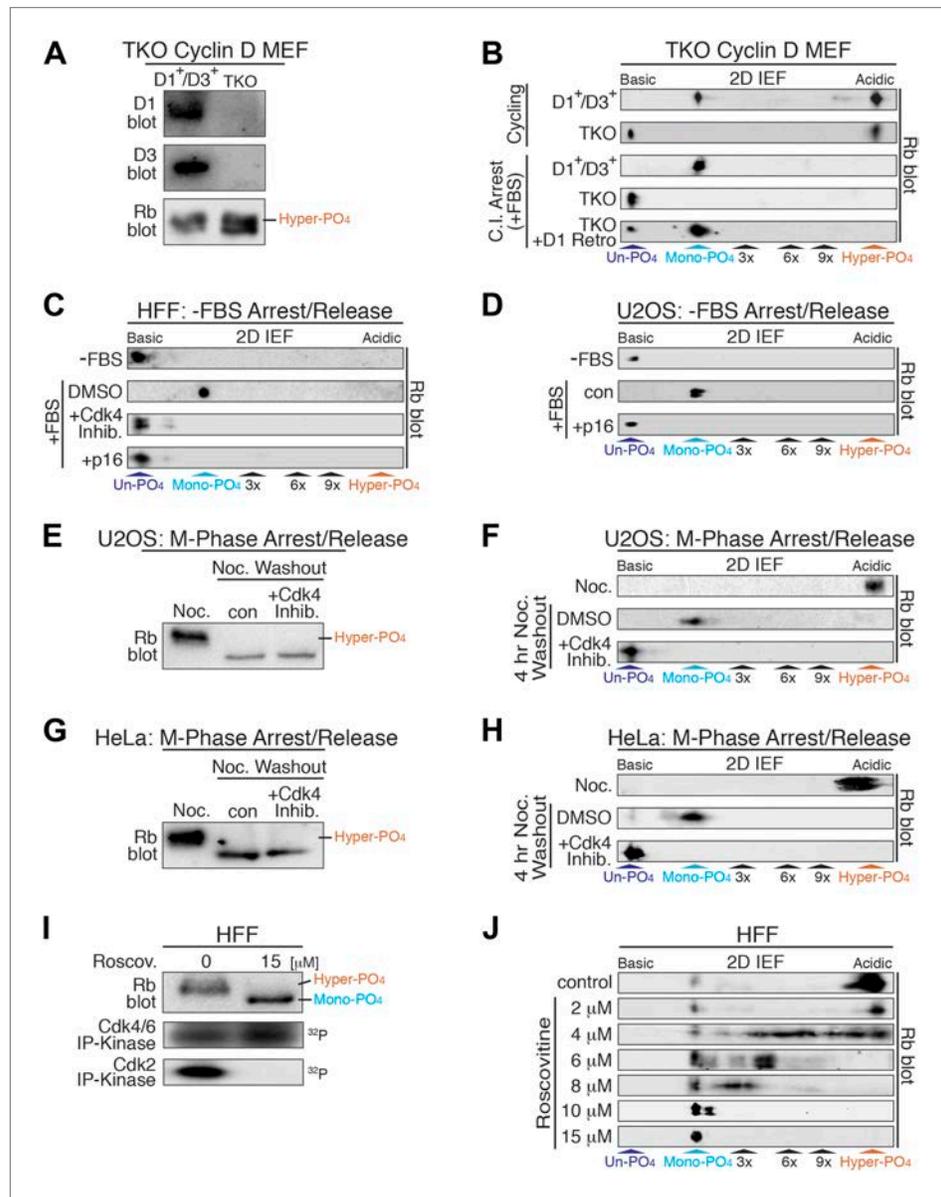


**Figure 2.** Mono-phosphorylated Rb exists as fourteen individual isoforms. **(A)** Phospho-specific Rb immunoblot of un-phosphorylated Rb (G<sub>0</sub>, -FBS) and mono-phosphorylated Rb (contact inhibited early G<sub>1</sub>, +FBS) from HFF and U2OS cells. **(B)** T826 and S608 phospho-specific Rb immunoprecipitation of mono-phosphorylated Rb (contact inhibited early G<sub>1</sub>, +FBS) and hyper-phosphorylated Rb (S phase) from HFF cells, followed by phospho-specific Rb immunoblot analysis, as indicated. Note the absence of other phosphates at these locations on mono-phosphorylated Rb, but present on hyper-phosphorylated Rb. **(C)** 2D IEF Rb-HA immunoblot of single Cdk site Rb-HA constructs expressed in cycling cells. Numbering indicates single Cdk site location on Rb. **(D)** Immunoblot of Rb-HA single Cdk site constructs and control ΔCdk Rb construct from co-immunoprecipitated and co-expressed E1a, E2F1, E2F2, E2F3 or E2F4 (Myc tagged) as indicated. Numbering indicates single Cdk site location on Rb. Note that all 14 avidly bind to at least two E2F family members and there are no completely inactive mono-phosphorylated Rb isoforms. DOI: 10.7554/eLife.02872.005



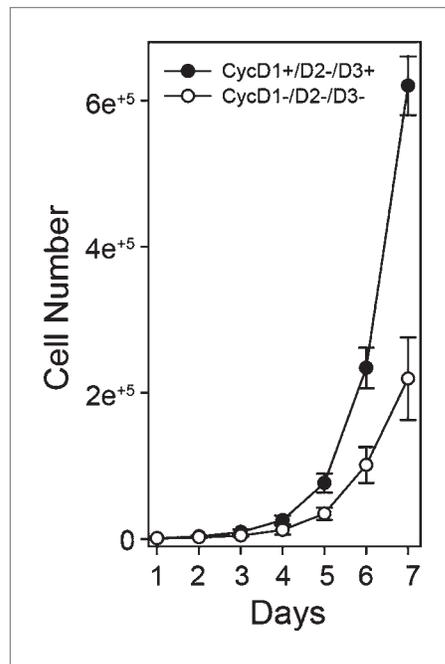
**Figure 2—figure supplement 1.** Mono-Phosphorylated Rb exists as fourteen individual isoforms.

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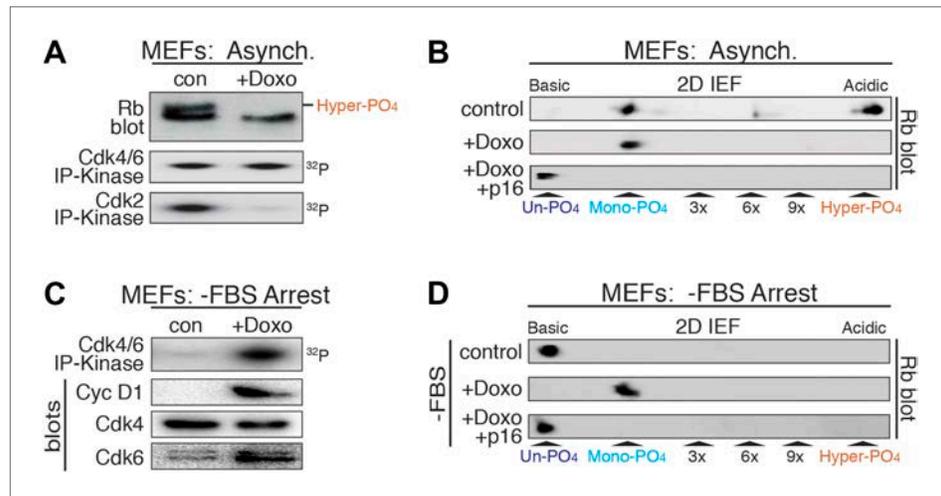


**Figure 3.** Cyclin D:Cdk/6 is the Rb mono-phosphorylation kinase. **(A)** Immunoblot of cyclin D1, D3, Rb in cycling parental D1<sup>+</sup>/D3<sup>+</sup> MEFs and triple knockout (TKO) cyclin D<sup>-</sup> MEFs. **(B)** 2D IEF Rb immunoblot from cycling parental D1<sup>+</sup>/D3<sup>+</sup> MEFs and TKO cyclin D<sup>-</sup> MEFs, and contact inhibited early G<sub>1</sub> phase arrested (+FBS) parental D1<sup>+</sup>/D3<sup>+</sup> MEFs and TKO cyclin D<sup>-</sup> MEFs plus/minus retroviral cyclin D1 expression. **(C)** 2D IEF Rb immunoblot from serum deprived G<sub>0</sub> arrested (-FBS) and released (+FBS) HFFs plus control DMSO, Cdk4 inhibitor (PD0332991) or retroviral p16 expression. **(D)** 2D IEF Rb immunoblot from serum deprived G<sub>0</sub> arrested (-FBS) and released (+FBS) p16-deleted U2OS tumor cells or released (+FBS) plus TET-induced p16 expression. **(E, F)** 1D SDS-PAGE **(E)** and 2D IEF **(F)** Rb immunoblot of G<sub>2</sub>/M phase nocodazole arrested (Noc.) and released U2OS cells plus DMSO (con) or Cdk4 inhibitor (PD0332991). **(G, H)** 1D SDS-PAGE **(G)** and 2D IEF **(H)** Rb immunoblot of G<sub>2</sub>/M phase nocodazole arrested (Noc.) and released HeLa cells plus DMSO (con) or Cdk4 inhibitor (PD0332991). **(I)** Late G<sub>1</sub> phase primary HFFs were treated with Cdk2 inhibitor Roscovitine [15 μM] or control (DMSO) and analyzed by 1D SDS-PAGE Rb immunoblot, anti-Cdk4/6 and anti-Cdk2 immunoprecipitation-kinase assays. **(J)** 2D IEF Rb immunoblot from late G<sub>1</sub> phase HFFs treated with dose curve of Cdk2 inhibitor Roscovitine or control (DMSO).

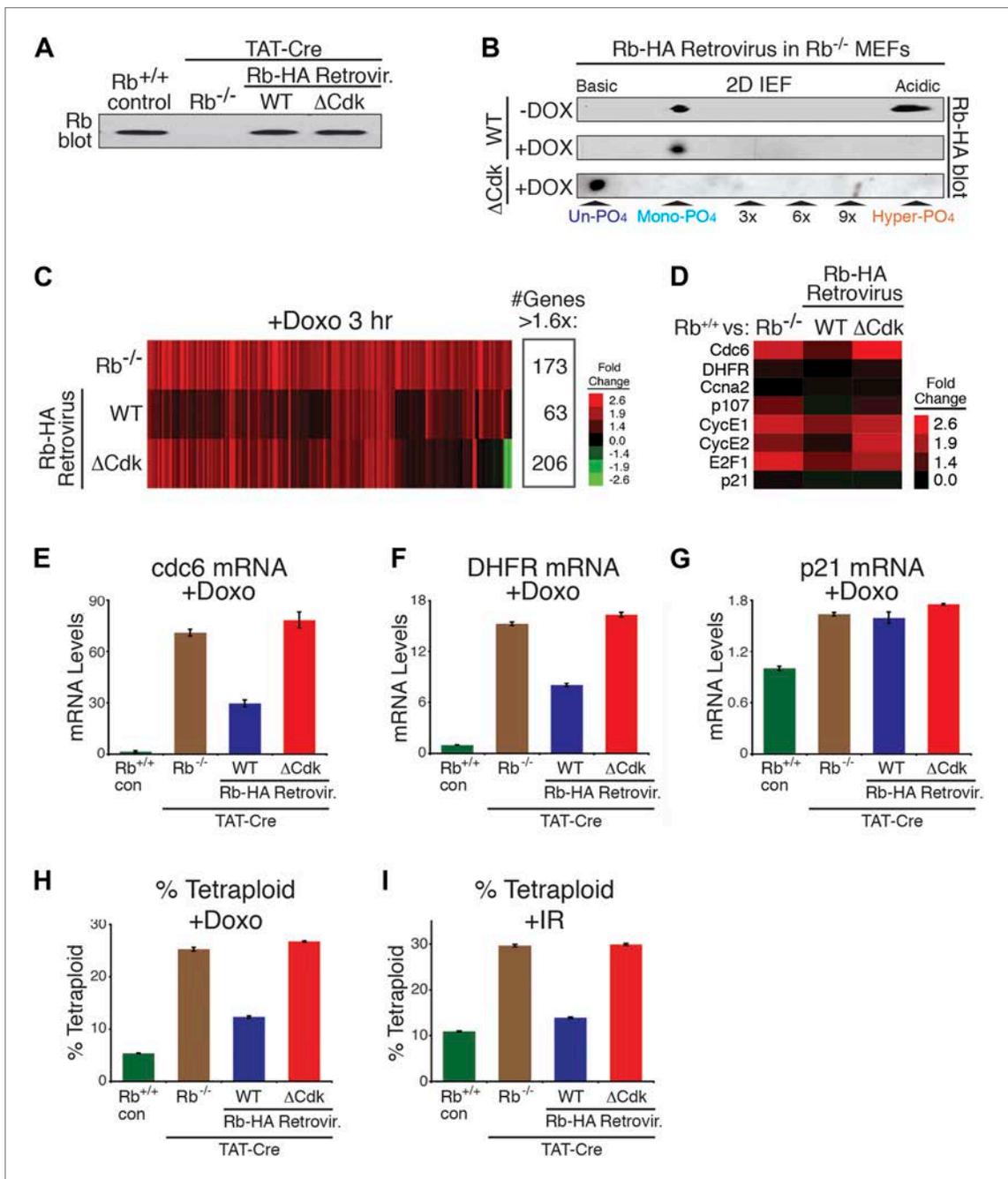
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**Figure 3—figure supplement 1.** Cyclin D:Cdk4/6 is the Rb mono-phosphorylation kinase.  
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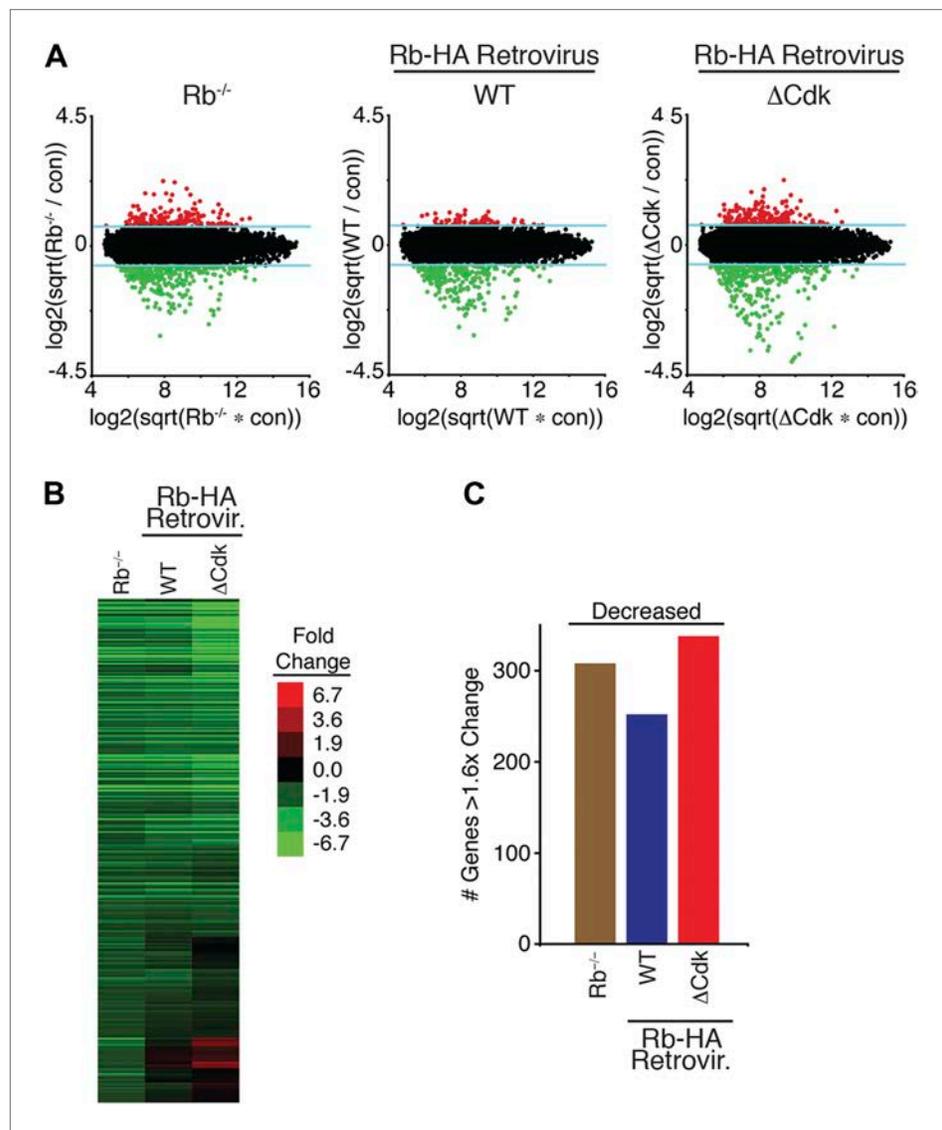


**Figure 4.** DNA damage induces cyclin D:Cdk4/6 activity and mono-phosphorylated Rb. **(A)** Asynchronously cycling MEFs (+FBS) treated with Doxorubicin (+Doxo, 100 ng/ml) were analyzed by 1D SDS-PAGE Rb immunoblot, anti-Cdk4/6 and anti-Cdk2 immunoprecipitation-kinase assays. **(B)** 2D IEF Rb immunoblot from asynchronously cycling MEFs (+FBS) treated with control or Doxorubicin (+Doxo) or Doxorubicin plus retroviral p16 (+Doxo/+p16) expression. **(C)** Serum-deprived G<sub>0</sub> arrested MEFs (–FBS) treated with Doxorubicin (+Doxo/–FBS) were immunoblot analyzed for cyclin D1, Cdk4, Cdk6, and Cdk4/6 immunoprecipitation-kinase activity. Note activation of cyclin D:Cdk4/6 in absence of serum growth factors. **(D)** 2D IEF Rb immunoblot from serum-deprived G<sub>0</sub> arrested MEFs (–FBS) treated with control or Doxorubicin (+Doxo) or Doxorubicin plus retroviral p16 (+Doxo/+p16) expression.  
DOI: [10.7554/eLife.02872.009](https://doi.org/10.7554/eLife.02872.009)



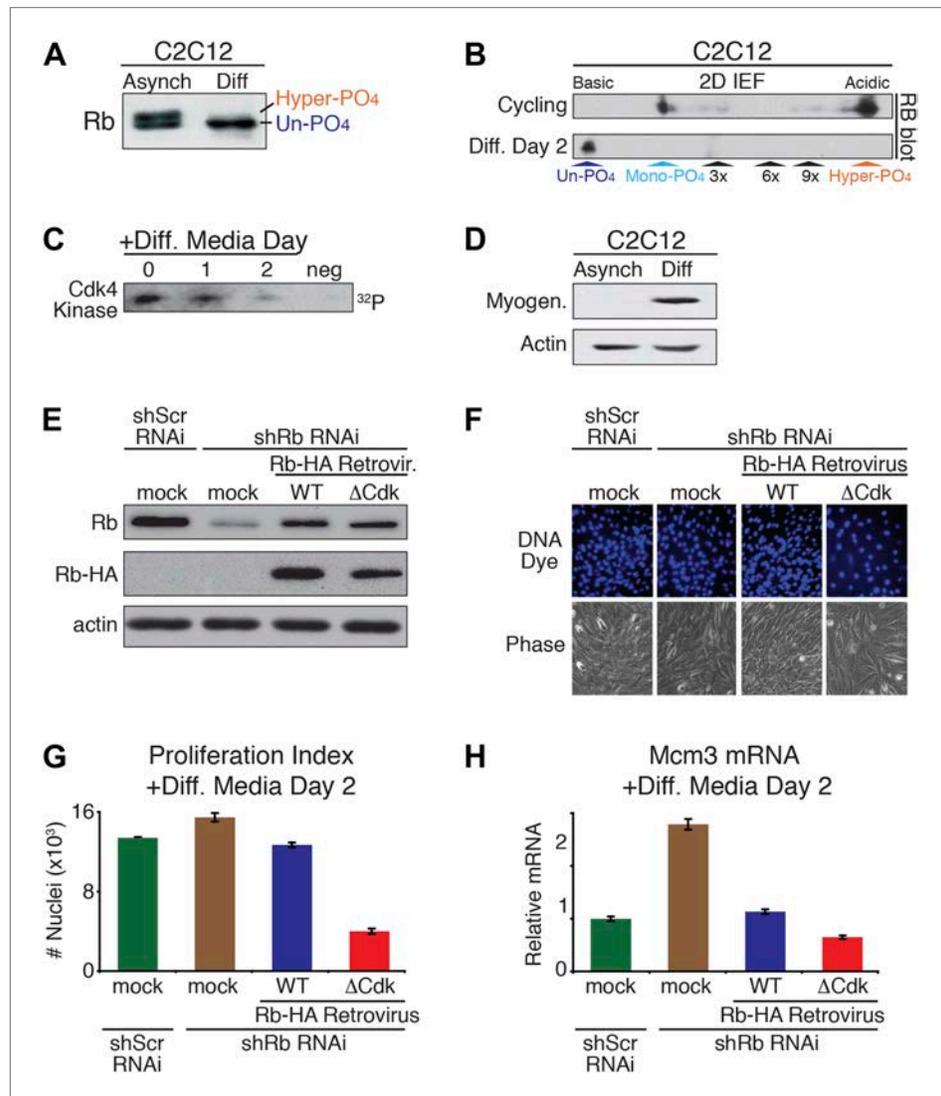
**Figure 5.** Mono-phosphorylated Rb is active during a DNA damage response. **(A)** Rb immunoblot of contact inhibited parental MEFs, conditionally deleted Rb<sup>-/-</sup> MEFs, and retrovirally-expressed wild type (WT) Rb-HA or non-phosphorylatable ΔCdk Rb-HA in deleted Rb<sup>-/-</sup> MEFs. **(B)** 2D IEF Rb immunoblot of cycling and doxorubicin (+Dox) (100 ng/ml) treated WT Rb-HA MEFs, and ΔCdk Rb-HA MEFs in deleted Rb<sup>-/-</sup> background. **(C)** Microarray heat map of mRNA levels from early G<sub>1</sub> phase Rb<sup>-/-</sup> MEFs, WT Rb-HA MEFs, and ΔCdk Rb-HA MEFs compared to parental MEFs treated with Doxorubicin (+Doxo) for 3 hr. Only genes increased >1.6-fold are shown. **(D)** Focused mRNA expression analysis of E2F target genes and p21 control gene from **(C)**. **(E–G)** qRT-PCR mRNA analysis of endogenous E2F-dependent target genes, *cdc6* **(E)** and *DHFR* **(F)**, and a non-E2F control gene, *p21* **(G)**, from parental MEFs (con), Rb<sup>-/-</sup> MEFs, WT Rb-HA MEFs, and ΔCdk Rb-HA MEFs in deleted Rb<sup>-/-</sup> background treated with doxorubicin (+Doxo). Mean values were normalized to β<sub>2</sub>-microglobulin levels, reported as fold change from parental MEFs (con). Error bars indicate SEM from three independent experiments. **(H and I)** Quantification of percent tetraploid (>4n DNA) nuclei in parental MEFs (con), Rb<sup>-/-</sup> MEFs, WT Rb-HA MEFs, and ΔCdk Rb-HA MEFs in deleted Rb<sup>-/-</sup> background four days after treatment with Doxorubicin (+Doxo) **(H)** or 2 days post-treatment with ionizing radiation (20 grays) **(I)**. Error bars indicate SEM from three independent experiments.

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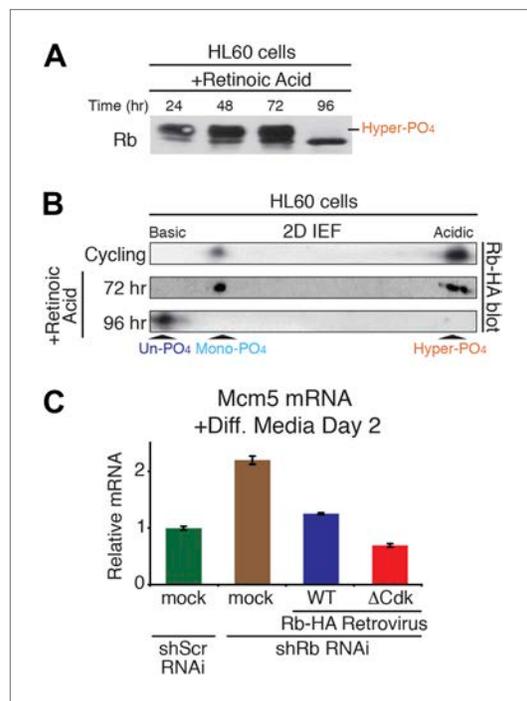
**Figure 5—figure supplement 1.** Mono-phosphorylated Rb is active during a DNA damage response.

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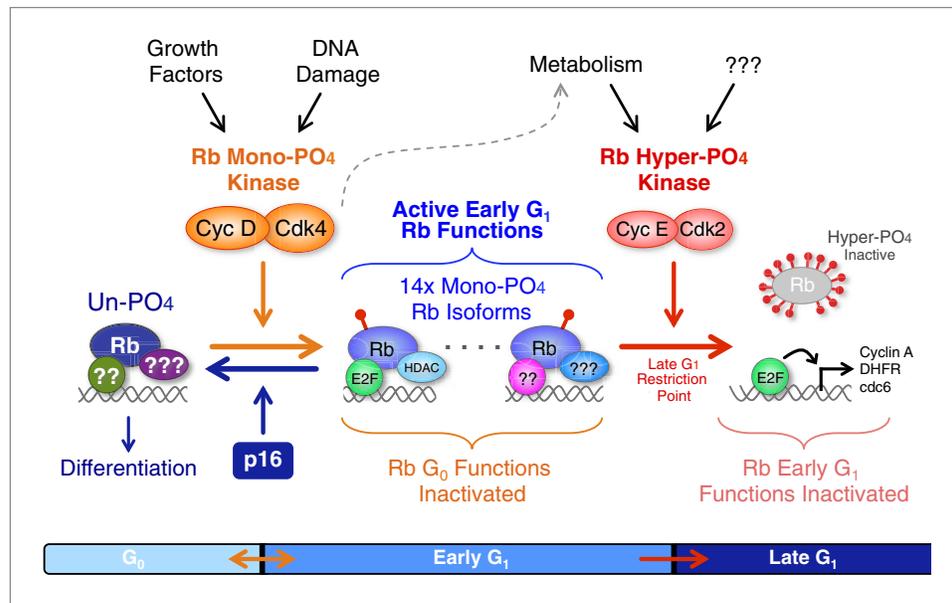
**Figure 6.** Un-phosphorylated Rb promotes cell cycle exit and differentiation. **(A)** Rb immunoblot of cycling C2C12 myoblasts (Asynch), and after 2 days in differentiation medium (Diff). **(B)** 2D IEF Rb immunoblot from cycling C2C12 myoblasts (Asynch) and after 2 days in differentiation medium (Diff). **(C)** Cdk4/6 immunoprecipitation-kinase assay of cycling C2C12 myoblasts (0) and at 1 and 2 days post-addition of differentiation medium (Diff). Negative (neg) control, irrelevant antibody. **(D)** Myogenin immunoblot of cycling C2C12 myoblasts (Asynch), and after 2 days in differentiation medium (Diff). **(E)** Immunoblot of endogenous Rb, and retroviral expressed wild type (WT) Rb-HA or  $\Delta$ Cdk Rb-HA in C2C12 myoblasts co-infected with short hairpin (sh) RNAi retroviruses targeting endogenous Rb (3' UTR) or scrambled (Scr) control. **(F and G)** Proliferation analysis (number of nuclei) of C2C12 myoblasts co-infected with short hairpin (sh) RNAi retroviruses targeting endogenous Rb (3' UTR) or scrambled (Scr) control, and wild type (WT) Rb-HA or  $\Delta$ Cdk Rb-HA retroviruses. Cells were stained with Hoechst 33342 DNA dye, visualized by microscopy **(F)** and quantified by flow cytometry **(G)** (number of nuclei  $\times 10^3$ ) at 2 days post-addition of differentiation medium. Error bars indicate SEM. **(H)** qRT-PCR mRNA analysis of Mcm3 DNA replication factor in C2C12 myoblasts treated as above **(G)** for 2 days in differentiation media. Mean values were normalized to  $\beta$ 2-microglobulin levels and reported as fold change from C2C12 myotubes expressing endogenous Rb (Scr<sup>shRNA</sup>). Error bars indicate SEM from three independent experiments.

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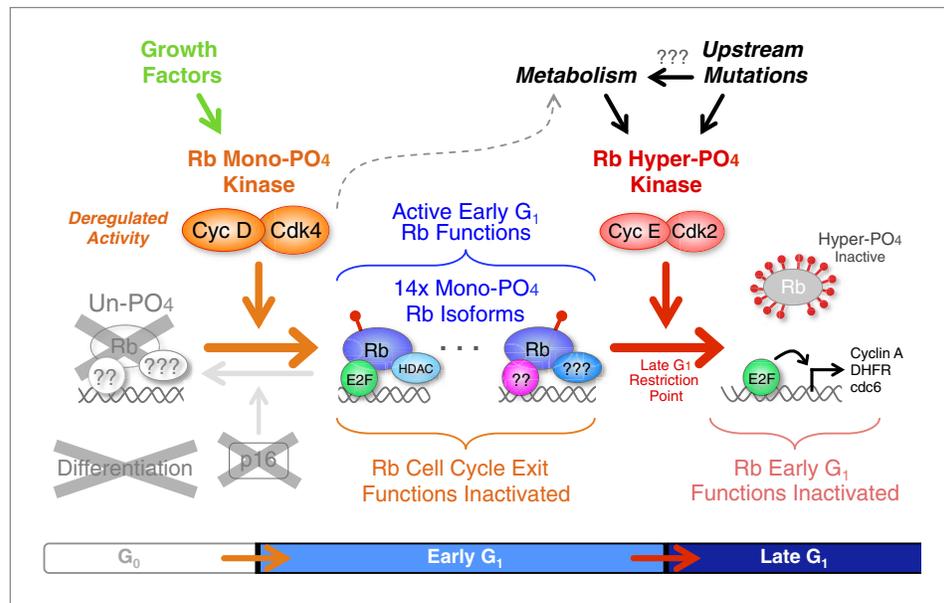
**Figure 6—figure supplement 1.** Un-phosphorylated Rb promotes cell cycle exit and differentiation.

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**Figure 7.** Revised working model of  $G_1$  cell cycle progression. Un-phosphorylated Rb regulates  $G_0$  cell cycle exit and differentiation. Growth factor signaling and DNA damage stimulate activation of cyclin D:Cdk4/6 complexes that diversify Rb into 14 mono-phosphorylated isoforms that independently bind specific cellular factors to regulate early  $G_1$  phase functions and the DNA damage response. Cyclin D:Cdk4/6 mono-phosphorylation of Rb inactivates un-phosphorylated Rb  $G_0$  functions and thereby prevents cells from exiting the cell cycle. Activation of cyclin E:Cdk2 complexes inactivates all 14 mono-phosphorylated Rb isoforms by hyper-phosphorylation (>12x phosphates) at the late  $G_1$  Restriction Point. Cyclin A:Cdk2 and cyclin B:Cdk1 maintain Rb in an inactive hyper-phosphorylated state during S,  $G_2$  and M phases. As cells complete cytokinesis, hyper-phosphorylated Rb is de-phosphorylated by phosphatases and rapidly mono-phosphorylated by cyclin D:Cdk4/6 complexes. We speculate that an unknown metabolic sensor is upstream of cyclin E:Cdk2 activation.

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**Figure 8.** Deregulated cyclin D:Cdk4/6 in cancer mono-phosphorylates Rb to prevent cell cycle exit. Deregulation of cyclin D:Cdk4/6 activity in cells occurs by a variety of mechanisms, including: p16 deletion, cyclin D1, D2 and D3 amplification or overexpression, and mutation or overexpression of Cdk4 or Cdk6. Cyclin D:Cdk4/6 mono-phosphorylation of Rb simultaneously inactivates Rb's G<sub>0</sub> functions and activates Rb's early G<sub>1</sub> phase functions thereby driving cells from a low metabolism G<sub>0</sub> quiescence into a high metabolism early G<sub>1</sub> arrested state that also prevents subsequent cell cycle exit or differentiation. Similar to p53 mutations and Bcl2 overexpression, deregulated cyclin D:Cdk4/6 activity is a well tolerated priming oncogenic mutation that avoids activation of oncogene-induced apoptosis. The net effect is a subtle, but irreversible, oncogenic step forward. We predict that additional oncogenic and metabolic pathways ultimately converge on and activate cyclin E:Cdk2 complexes to inactivate Rb by hyper-phosphorylation at the Restriction Point and drive cells into late G<sub>1</sub> phase.

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