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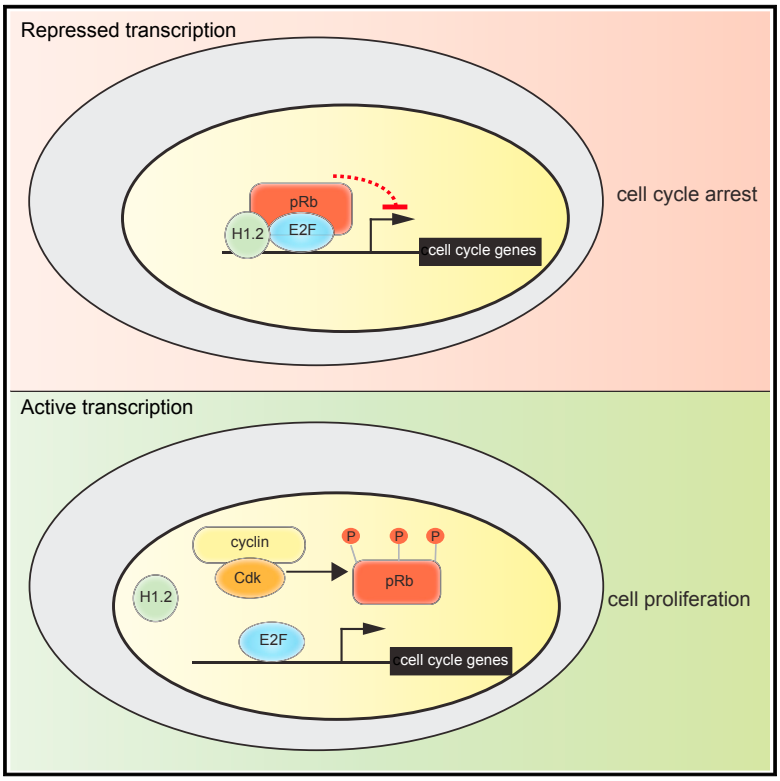
Linker Histone H1.2 Directs Genome-wide Chromatin Association of the Retinoblastoma Tumor Suppressor Protein and Facilitates Its Function

Graphical Abstract



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In Brief

Munro et al. demonstrate that pRb interacts with linker histone H1.2. The pRb-H1.2 complex is enriched on the chromatin of a subset of E2F target genes associated with cell-cycle control. Moreover, H1.2 influences the genome-wide chromatin-binding properties of pRb and impacts transcriptional repression and cell-cycle control.

Highlights

- The H1.2 linker histone is a major and significant pRb interaction partner
- H1.2 and pRb reside in a chromatin-bound complex on diverse E2F target genes
- H1.2 influences global chromatin association and genome-wide distribution of pRb
- H1.2 enhances transcriptional repression by pRb and facilitates cell-cycle arrest

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Linker Histone H1.2 Directs Genome-wide Chromatin Association of the Retinoblastoma Tumor Suppressor Protein and Facilitates Its Function

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SUMMARY

The retinoblastoma tumor suppressor protein pRb is a master regulator of cellular proliferation, principally through interaction with E2F and regulation of E2F target genes. Here, we describe the H1.2 linker histone as a major pRb interaction partner. We establish that H1.2 and pRb are found in a chromatin-bound complex on diverse E2F target genes. Interrogating the global influence of H1.2 on the genome-wide distribution of pRb indicated that the E2F target genes affected by H1.2 are functionally linked to cell-cycle control, consistent with the ability of H1.2 to hinder cell proliferation and the elevated levels of chromatin-bound H1-pRb complex, which occur in growth-arrested cells. Our results define a network of E2F target genes as susceptible to the regulatory influence of H1.2, where H1.2 augments global association of pRb with chromatin, enhances transcriptional repression by pRb, and facilitates pRb-dependent cell-cycle arrest.

INTRODUCTION

The replication-dependent linker H1 histones are generally believed to be involved in repressing gene expression through compacting chromatin into higher order structures (Misteli et al., 2000). There are seven somatic H1 subtypes in human cells (H1.1 to H1.5, H1.0, and H1X) that exhibit considerable sequence divergence in the tail regions (Harshman et al., 2013). Because of the differences in intracellular localization and levels between cell types, it has been speculated that H1 subtypes take on different functional roles in addition to the established general effects on chromatin compaction (Biterge and Schneider, 2014). It has been reported that H1.2 associates with a stable protein complex that influences p53 activity (Kim et al., 2012), and murine H1B (equivalent to human H1.2) interacts with the homeobox protein MSX1 to prevent activation of the MYOD gene, thereby delaying myoblast differentiation (Lee et al., 2004), suggestive of gene-specific regulatory effects.

Despite the generally held view that the principal role of H1 histone is to dampen transcription and maintain transcriptional inactivity, some studies have highlighted a role in transcriptional activation (Clausell et al., 2009; Kim et al., 2013). For example, H1-containing chromatin is remodeled by SWI/SNF (switch/sucrose non-fermentable) complexes (Clausell et al., 2009), and H1.2 stably interacts with CUL4A and PAF1 to generate active chromatin (Kim et al., 2013). Thus, it appears that H1 histones are generally dedicated to repressive roles in gene expression, although transcription-factor-specific roles are likely.

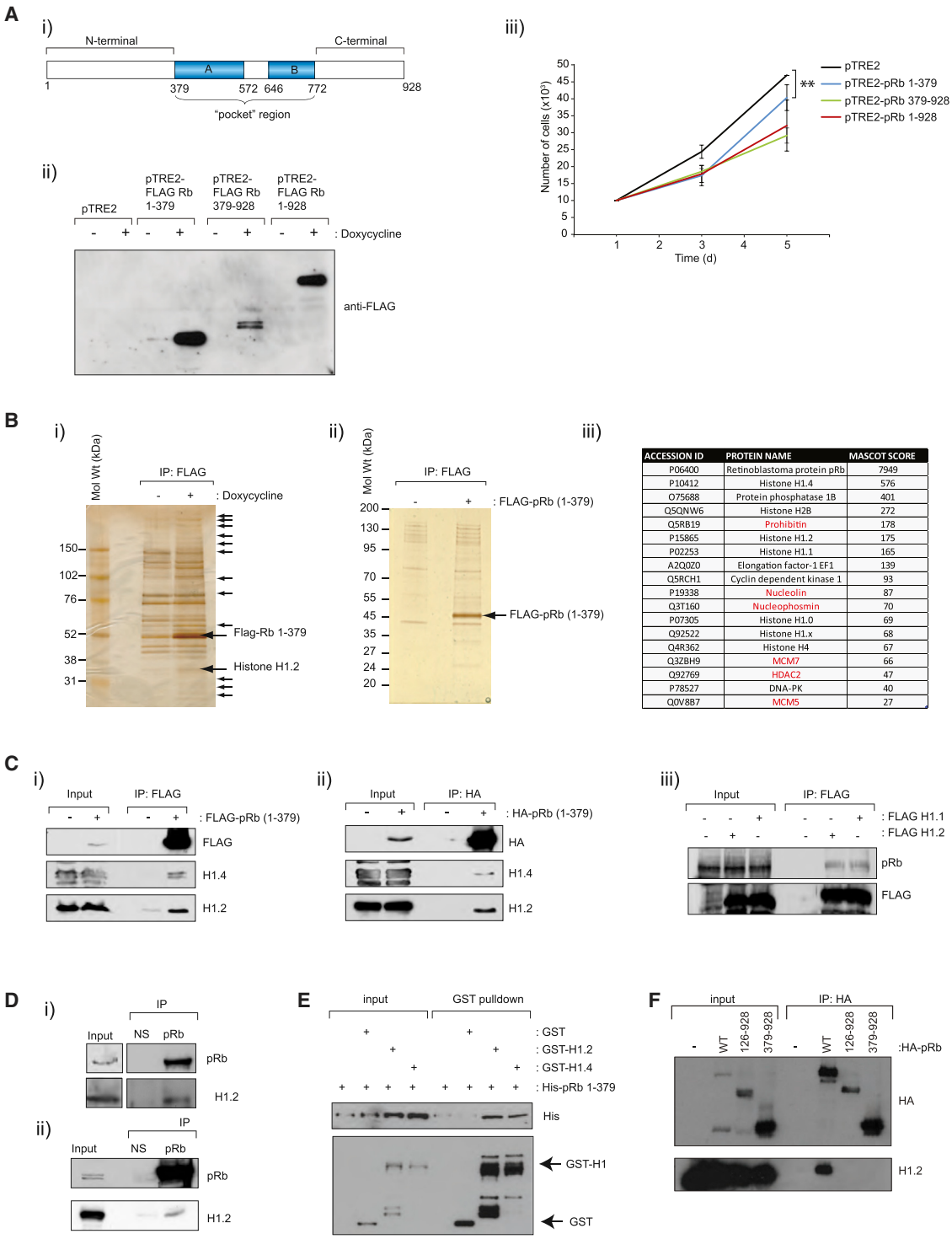
The pRb tumor suppressor protein acts as an important gatekeeper in regulating cell-cycle transition through G1 into S phase, and mutation in the Rb gene represents one of the most frequent events in human cancer, contributing to cancer initiation and progression (Munro et al., 2012). Mechanistically, pRb is a transcriptional regulator with its principal target being the E2F family of transcription factors. The E2F family regulates various target genes involved with cell-cycle progression and diverse cell fates, which thereby allows pRb to influence numerous aspects of cell biology.

In this study, we uncover a role for the H1.2 linker histone in directing the genome-wide association of pRb with chromatin. We have found that H1.2 interacts with pRb and thereby facilitates pRb binding near E2F target genes. Our results suggest a selective role for histone H1.2, mediated through modulating the chromatin-binding properties of pRb, which, in turn, allows H1.2 to exert global effects on the E2F gene network and thereby influence cell-cycle control.

RESULTS

Linker H1 Histones in the pRb Interactome

We generated Tet-On stable cell lines that, upon induction, expressed FLAG-pRb 1-379, 379-928, or wild-type (WT) 1-928 (Figure 1A, i and ii). All three pRb derivatives displayed a nuclear



ACCESSION ID	PROTEIN NAME	MASCOT SCORE
P06400	Retinoblastoma protein pRb	7949
P10412	Histone H1.4	576
O75688	Protein phosphatase 1B	401
Q5QNW6	Histone H2B	272
Q5RB19	Prohibitin	178
P15865	Histone H1.2	175
P02253	Histone H1.1	165
A2Q0Z0	Elongation factor-1 EF1	139
Q5RCH1	Cyclin dependent kinase 1	93
P19338	Nucleolin	87
Q3T160	Nucleophosmin	70
P07305	Histone H1.0	69
Q92522	Histone H1.x	68
Q4R362	Histone H4	67
Q3ZBH9	MCM7	66
Q92769	HDAC2	47
P78527	DNA-PK	40
Q0V8B7	MCM5	27

Figure 1. pRb Interacts with Linker Histone H1.2

(A) (i) Diagram of pRb with the A and B domains of the pocket shown in blue. The N- and C-terminal regions are also indicated. (ii) U2OS Tet-On-inducible cells expressing pTRE2 control vector, FLAG-pRb 1-379, FLAG-pRb 379-928, and FLAG pRb 1-928 were grown with (+) or without (-) doxycycline (1 μg/mL) for 48 hr. Cell lysates were prepared and immunoblotted with FLAG and actin antibodies. (iii) U2OS Tet-On-inducible cells expressing FLAG-pRb 1-379, FLAG-pRb 379-928, and FLAG pRb 1-928 or pTRE2 empty vector were seeded at a density of 1×10^4. Cell counts were performed at 3 and 5 days (d). Graph indicates average ± SD (n = 3; **$p < 0.01$, Student's t test).

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localization in induced cells (Figure S1A, i), and caused growth suppression in cell-proliferation assays, with fewer cells evident in the pRb-induced compared to the induced control cell line (Figure 1A, iii). The WT, 379-928, and 1-379 cells each exhibited varying levels of growth inhibition, with WT pRb exhibiting the most significant level (Figure 1A, iii). Notably, pRb 1-379 was also quite active, compared to the control cell line in a colony formation assay (Figure S1B, i and ii).

Given that the ability of pRb to inhibit cell proliferation is principally attributed to its pocket region (379-928), we were intrigued that the N-terminal domain could also suppress cell proliferation, and because the N-terminal domain is poorly characterized, we next used mass spectrometry to identify pRb-interacting proteins, focusing on the pRb 1-379 region. A number of proteins were identified that co-purified with FLAG-pRb 1-379 but not the empty vector control (Figure 1B, iii). We were intrigued by the presence of the linker H1 histone family, including H1.2 and H1.4 (Figure 1B, i and iii), which prompted us to further explore the role of H1. For this, chromatin bound to pRb was purified from FLAG-pRb 1-379-inducible cells, where approximately 5% of the total cellular pRb remained chromatin bound (Figure S1C). Mass spectrometry of the pRb-associated chromatin similarly revealed the presence of H1 histones, including H1.4, H1.2, H1.1, H1.0, and H1x, together with the core histones H2B and H4 (Figure 1B, iii).

We verified that the interaction between pRb and H1 histones occurred in cells, focusing on subtypes H1.2 and H1.4 (we were restricted to investigating these two H1 subtypes due a lack of other suitable subtype-specific antibodies) by immunoprecipitation of pRb from both FLAG-pRb-inducible cells and cells transfected with HA (hemagglutinin)-pRb 1-379, where an interaction was evident (Figure 1C, i and ii). Additionally, endogenous pRb was found to immunoprecipitate with ectopic H1 subtypes (Figure 1C, iii), and an interaction between endogenous pRb and H1.2 was evident in a number of cell types (Figures 1D, i and ii, and S1D). However, because pRb and H1 histones are chromatin associated, it was necessary to rule out that the interaction was mediated indirectly through a separate association of each protein with DNA. We pre-incubated cell lysates with DNase to digest any DNA prior to immunoprecipitation, which actually enhanced the interaction between pRb and H1.2, therefore suggesting that the interaction is not bridged by DNA (Figure S1E). Furthermore, we expressed and purified recombinant GST (glutathione S-transferase)-H1.2, GST-H1.4, and His-pRb 1-379 and performed in vitro GST and Ni-NTA binding assays, where His-pRb bound efficiently to GST-H1.2 or GST-H1.4, but not to GST alone (Figures 1E and S1F), suggesting a specific and direct interaction between pRb and histone H1 subtypes H1.2 and H1.4. We also studied which region of pRb interacts with H1.2. Truncations of HA-tagged pRb were expressed, and the ability to associate with endogenous H1.2 was assessed. While no binding of H1.2 to pRb 1-100 was evident, we observed that H1.2 could associate with other derivatives of pRb (including the low-penetrant point mutant R661W) (Figure S1G). It is consistent with a role for the N-terminal region of pRb for H1.2 binding that deleting the first 126 amino-acid residues of pRb in the context of the full-length protein prevented the interaction with H1.2 (Figure 1F); nuclear accumulation of pRb 126-928 occurred as expected (Figure S1H). Thus, the N-terminal region of pRb is responsible for the interaction with H1.2.

H1 Histones Associate with the Promoters of E2F Target Genes

Because pRb binds to E2F, we next examined whether H1 histones are present in the chromatin environment of E2F target genes. FLAG-tagged H1 subtypes were expressed in U2OS cells, and chromatin immunoprecipitation (ChIP) analysis was performed (Figure S2A, i). All of the H1 subtypes examined were detected in the region of the E2F-binding site on a number of target genes (Figure S2A, ii). We then addressed whether pRb and H1 coexist in a chromatin-bound complex, which we tested by performing sequential ChIP analyses. We detected endogenous pRb and H1.2 as a chromatin-bound complex on E2F target genes in diverse cell types (Figures 2A, i and ii, and

(B) In (i), FLAG-pRb 1-379-inducible cells were grown with (+) or without (-) doxycycline for 48 hr. FLAG immunoprecipitation (IP) was performed followed by elution with FLAG peptide. The eluted protein complexes were subjected to silver staining, and bands of notable difference (indicated by arrows) were excised and subjected to tryptic digestion and nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS). Mol wt, molecular weight. (ii) Chromatin was isolated from FLAG-pRb (1-379) or pTRE2 control cells (both grown in the presence of doxycycline). FLAG immunoprecipitations were performed, followed by elution with FLAG peptide. 20% of each eluted immunocomplexes was analyzed by electrophoresis, and silver staining of the gel was performed. The remaining 80% of the eluates were subjected to in-solution tryptic digestion, and proteins were identified using nano-LC-MS/MS. Comparison between proteins immunoprecipitated from the control vector cell line and pRb 1-379 allowed the identification of proteins that selectively bound to pRb 1-379. (iii) Proteins were identified as pRb 1-379-interacting proteins by mass spectrometry in pRb-associated chromatin, and whole-cell extracts are listed in tabular form, together with Mascot scores. Previously reported interacting proteins are highlighted in red; for example, nucleophosmin, MCM7, and nucleolin (Grinstein et al., 2006; Sterner et al., 1998; Takemura et al., 2002).

(C) In (i), FLAG-pRb 1-379-inducible cells were grown in the presence of doxycycline for 48 hr. Cell lysates were immunoprecipitated with anti-FLAG, followed by immunoblotting with H1.2, H1.4, or FLAG antibodies. (ii) U2OS cells were transfected with HA-pRb 1-379 or control vector. 48 hr post-transfection, cell lysates were prepared, and HA-pRb was then immunoprecipitated, followed by immunoblotting with antibodies against H1.2, H1.4, or HA. (iii) U2OS cells were transfected with FLAG H1.1, FLAG H1.2, or control vector. 48 hr post-transfection, lysates were immunoprecipitated with anti-FLAG, followed by immunoblotting with pRb and FLAG antibodies.

(D) In (i), U2OS cell lysates were immunoprecipitated with non-specific (NS) or pRb antibodies, followed by immunoblotting with H1.2 and pRb antibodies (note that the blot has been spliced between input and IP, as different exposures were required to show input and IP at appropriate levels). (ii) MCF-7 cell lysates were immunoprecipitated with non-specific (NS) or pRb antibodies, followed by immunoblotting with H1.2 and pRb antibodies.

(E) GST-H1.2, GST-H1.4, or GST were incubated in the presence of bacterially expressed His-pRb (1-379) together with Glutathione Sepharose beads. Following extensive washes, immunoblotting was performed with GST and His antibodies.

(F) U2OS cells were transfected with the indicated HA-tagged pRb N-terminal truncations. 48 hr post-transfection, lysates were immunoprecipitated with anti-HA, followed by immunoblotting with H1.2 and HA antibodies.

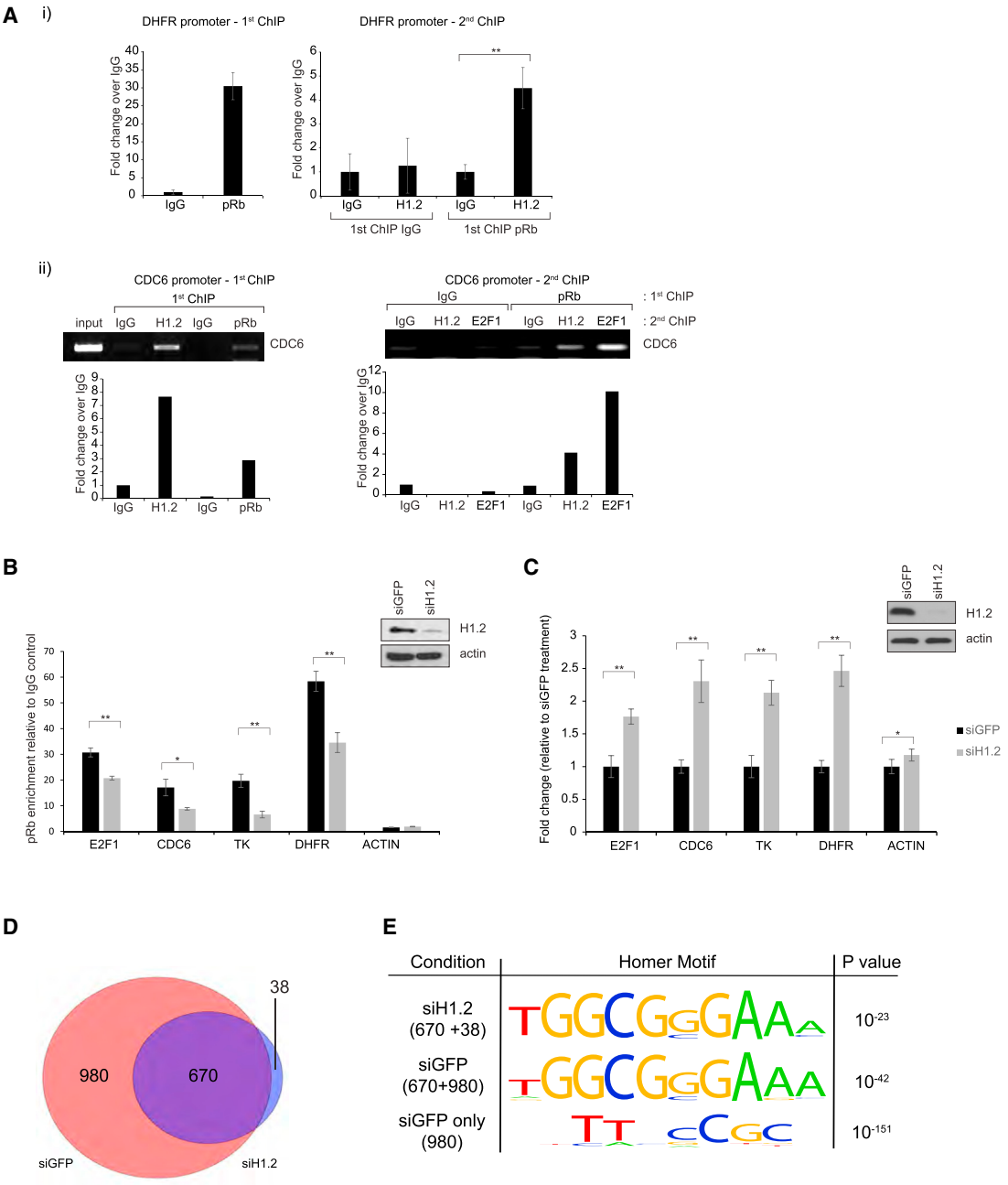

Figure 2. H1.2 Regulates Chromatin-Bound pRb and E2F Target Gene Expression

(A) In (i), chromatin from T98G cells was immunoprecipitated with control immunoglobulin G (IgG) or pRb antibodies. A sequential re-immunoprecipitation (re-IP) was performed with eluted IgG and pRb material with control IgG or H1.2 antibodies. The presence of the H1/pRb complex on the DHFR promoter was analyzed by qPCR. Graphs indicate average ± SD (n = 3; **p < 0.01, Student's t test). (ii) Chromatin from MCF7 cells was immunoprecipitated with control IgG, pRb, or H1.2 antibodies. A sequential re-IP was performed with eluted pRb material with control IgG, H1.2, or E2F-1 antibodies. The binding of the complex to the CDC6 promoter was analyzed by PCR. E2F-1 was included as a positive control for the pRb ChIP-reIP. Quantification of the ChIP signal is shown in graphical format below.

(B) MCF7 cells were transfected with GFP or H1.2 siRNA (20 nM) for 72 hr. ChIP was performed with control IgG and pRb antibodies. ChIP activity on E2F promoters was assessed by qPCR. Corresponding immunoblot is shown on the right. *p < 0.05; **p < 0.01, Student's t test. Graph indicates mean ± SEM (n = 5).

(C) U2OS cells were transfected with GFP or H1.2 siRNA (20 nM) for 72 hr. E2F target gene RNA levels were assessed by qPCR. Transcript levels were normalized to housekeeping gene GAPDH. Corresponding immunoblot is shown on the right. Graph indicates average ± SD (n = 3; *p < 0.05; **p < 0.01, Student's t test).

(D) Venn diagram showing the overlap of pRb peaks between treatment conditions; namely, siGFP control conditions (+ H1.2) and H1.2 knockdown (−H1.2). The siGFP treatment (red) yielded 1,650 pRb peaks, and the siH1.2 treatment (blue) yielded 708 peaks. There were 670 shared peaks (purple) between the two conditions, and 980 and 38 peaks unique to each condition, respectively.

(E) Motif analysis of the most enriched de novo peak identified in the siGFP and siH1.2 condition and among peaks unique to the siGFP condition.

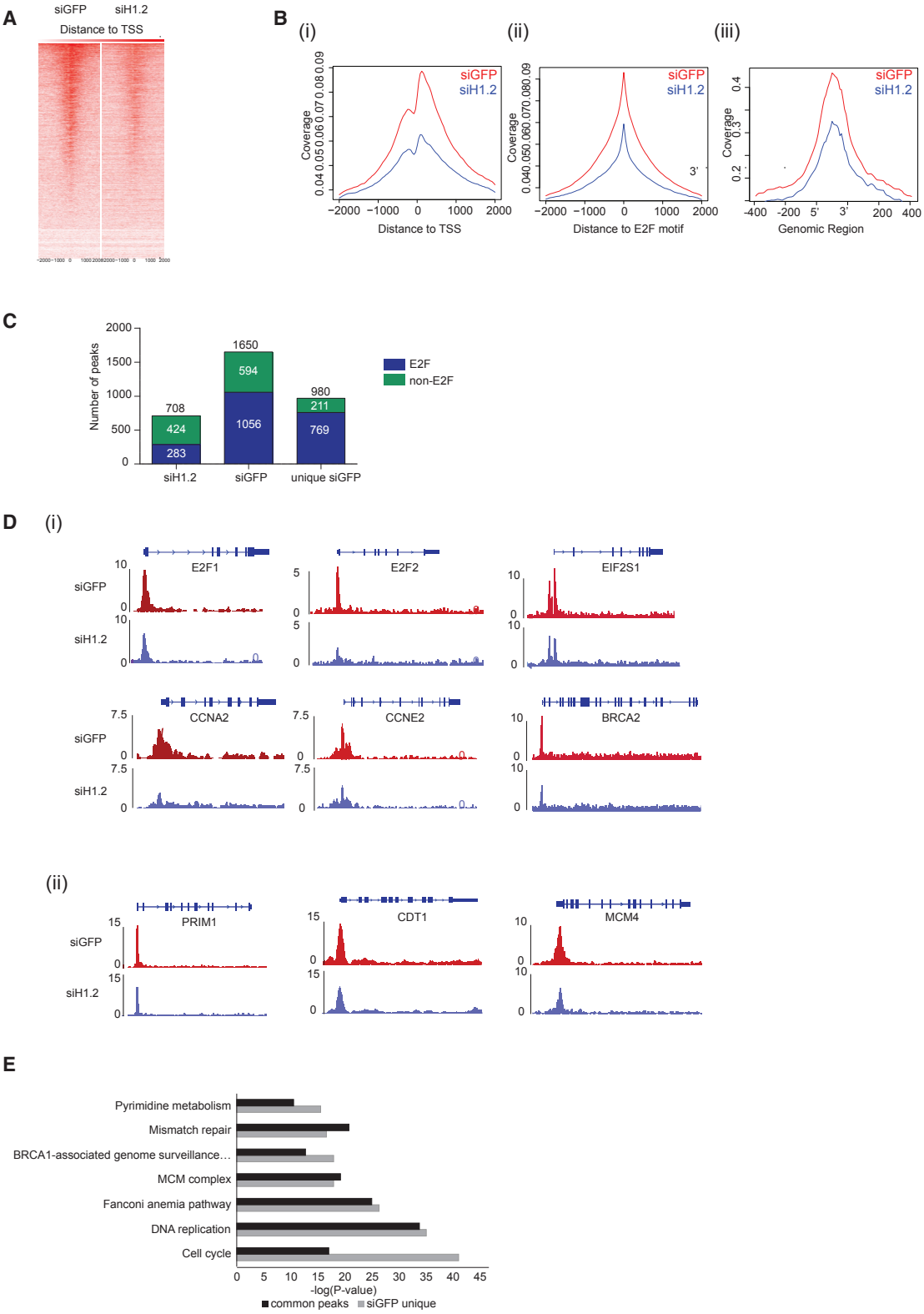

Figure 3. H1.2 Regulates the Genome-wide Association of pRb with Chromatin

(A) Heatmap analysis of pRb binding around transcription start sites (TSSs) under siH1.2 or siGFP treatment. Each panel represents 2,000 bp upstream and 2,000 bp downstream of the transcription start site.

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S2B). Further, depletion of H1.2 resulted in a reduction in the amount of chromatin-bound pRb on a variety of E2F target genes (Figure 2B). Significantly, the expression of the E2F target genes increased upon H1.2 depletion (Figure 2C). Moreover, H1.2 expression augmented the E2F1 transcriptional repression mediated by pRb in reporter-based assays (Figure S2C). Overall, pRb and H1.2 co-exist in the chromatin environment of E2F target genes, where H1.2 contributes to transcriptional inactivation.

In order to clarify whether H1 histones have a global effect on pRb, we performed a genome-wide chromatin-binding analysis by ChIP sequencing (ChIP-seq). We used MCF7 cells, which express WT pRb, and studied the genome-wide distribution of the pRb ChIP complex, as well as the impact that H1.2 has on the genomic distribution of pRb. Initially, we confirmed that the ChIP enrichment was specific to pRb by generating MCF7 CRISPR cells that lack pRb; there was no detectable pRb signal apparent upon the E2F1 promoter in $pRb^{-/-}$ cells compared to their WT counterparts (Figure S2D). Endogenous pRb was immunoprecipitated from MCF7 cells that had been treated with either a control or an H1.2 small interfering (si)RNA (siRNA). Chromatin was isolated, libraries prepared, and the DNA was subjected to deep sequencing. We identified 1,650 and 708 specific peaks in the control siRNA and the H1.2 siRNA, respectively, with an overlap of 670 peaks (Figure 2D). As anticipated, a very strong enrichment of the E2F binding-site motif was identified in all conditions (Figure 2E). Minor binding-site differences were apparent when the "siGFP-only" condition was analyzed, the significance of which has yet to be explored.

Aligning the peaks across the human genome revealed that the majority of the pRb peaks mapped to promoter and intergenic regions (Figures S3A and S3B). The number of pRb peaks observed at promoter regions decreased upon H1.2 depletion, which was accompanied by an increased association of pRb at intergenic regions (Figures S3A and S3B). Moreover, this was consistent with H1.2 depletion, which caused reduced pRb enrichment at transcription start sites (Figures 3A and 3B, i and iii). Importantly, there was a significant reduction in the association of pRb with E2F target genes in the absence of H1.2 (from 1,056 to 283 peaks, which represents a 73% decrease; Figures 3B, ii, and 3C), contrasting with the effect on non-E2F regions, where the absence of H1.2 caused a proportional increase in the binding of pRb (Figure 3C); in the absence of H1.2, pRb binding to non-E2F genomic regions increased from 36% to 60%. Moreover, for certain E2F target genes, the level of pRb binding was reduced in the absence of H1.2—for example, E2F1, E2F2, CCNA2, CCNE2, EIF2S1, and BRCA2 (Figure 3D, i)—whereas for others such as PRIM1, CDT1, and MCM4, binding was minimally affected (Figure 3D, ii). Overall, these results indicate that H1.2 influences the ability of pRb to associate with the promoter regions of E2F target genes.

Cyclin/Cdk Activity Regulates the H1-pRb Interaction

Gene Ontology (GO) analysis revealed that the most significant group of pRb target genes affected by the loss of H1.2 was connected with the cell cycle (Figure 3E). Therefore, we surmised that the chromatin-bound H1-pRb interaction could be influenced by the cell cycle, which we examined in cells that had been growth arrested by either serum starvation or treatment with CDK inhibitors. We performed a sequential ChIP analysis (anti-pRb followed by anti-H1.2 or anti-pan H1 antibody) where, in serum-starved MCF7 cells, an increased level of the chromatin-associated H1-pRb complex on the CDC6 promoter was evident, compared to asynchronous cultures of growing cells (Figures 4A and S4A, i) (as a negative control, ChIP-binding activity to the actin promoter was assessed; Figure S4A, ii and iii). Further, in MCF7 cells growth arrested by treatment with the CDK inhibitor roscovitine (Meijer et al., 1997), the H1-pRb complex was more evident in CDK-inhibitor-treated cells (Figures 4B and S4B). These results indicate that the chromatin-bound H1.2-pRb complex is influenced by cell-cycle progression, with its appearance enhanced in growth-arrested cells. This is compatible with the biological role of pRb, which is principally exerted at the G1-to-S phase transition (Munro et al., 2012).

H1 Histones Influence Cell Growth

Since the ability of pRb to control cell proliferation requires pRb-dependent regulation of E2F activity (Frolov and Dyson, 2004), and because the transcription properties of pRb are influenced by the interaction with H1.2, we reasoned that H1.2 may impact on cell growth. Therefore, we developed CRISPR cell lines, derived from U2OS (expressing WT pRb), MCF7 (expressing WT pRb), and SAOS2 (expressing MT pRb) cells, in which we disrupted the endogenous H1.2 gene; immunoblotting and immunostaining confirmed that H1.2 protein was undetectable (Figures S4C–S4E). An analysis of chromatin-associated pRb by ChIP analysis confirmed the earlier results (Figure 2C) that H1.2 augments pRb binding, as pRb ChIP activity was diminished in the $H1.2^{-/-}$ cells, compared to their WT counterparts (Figure 4C, i). Concomitant with decreased pRb binding, the transcriptional activity of the E2F target gene, CDC6, was enhanced in the $H1.2^{-/-}$ cells (Figure 4C, ii).

It was necessary to rule out the possibility that H1.2 depletion affected the association of any transcription factor or chromatin-associated protein with DNA and to confirm that the observed effects were specific to pRb binding at localized regions. To this end, ChIP analysis of SP1 and RNA polymerase II (PolII;

(B) Normalized coverage plots of either siGFP (red) or siH1.2 (blue) treatment around the transcription start site (i), around all E2F-binding motifs identified computationally (ii), or around all peaks from the experiment (iii).
(C) Total number of pRb peaks for siH1.2 (708) and siGFP (1,650) treatment, and peaks unique to siGFP (970) condition. Peaks corresponding to the E2F-binding motif (blue) or not (green) are indicated.
(D) Coverage tracks around E2F target gene promoters that show a decrease in peak size with siH1.2 treatment (E2F1, E2F2, EIF2S1, CCNA2, CCNE2, and BRCA1) (i) or no significant change (PRIM1, CDT1, and MCM4) (ii).
(E) GO analysis of pRb association with promoters in the presence and absence of H1.2. Comparison of pRb binding to promoter regions common to both conditions (siGFP and siH1.2) and promoter regions unique to siGFP treatment alone. Analysis was performed using HOMER (v.4.8).

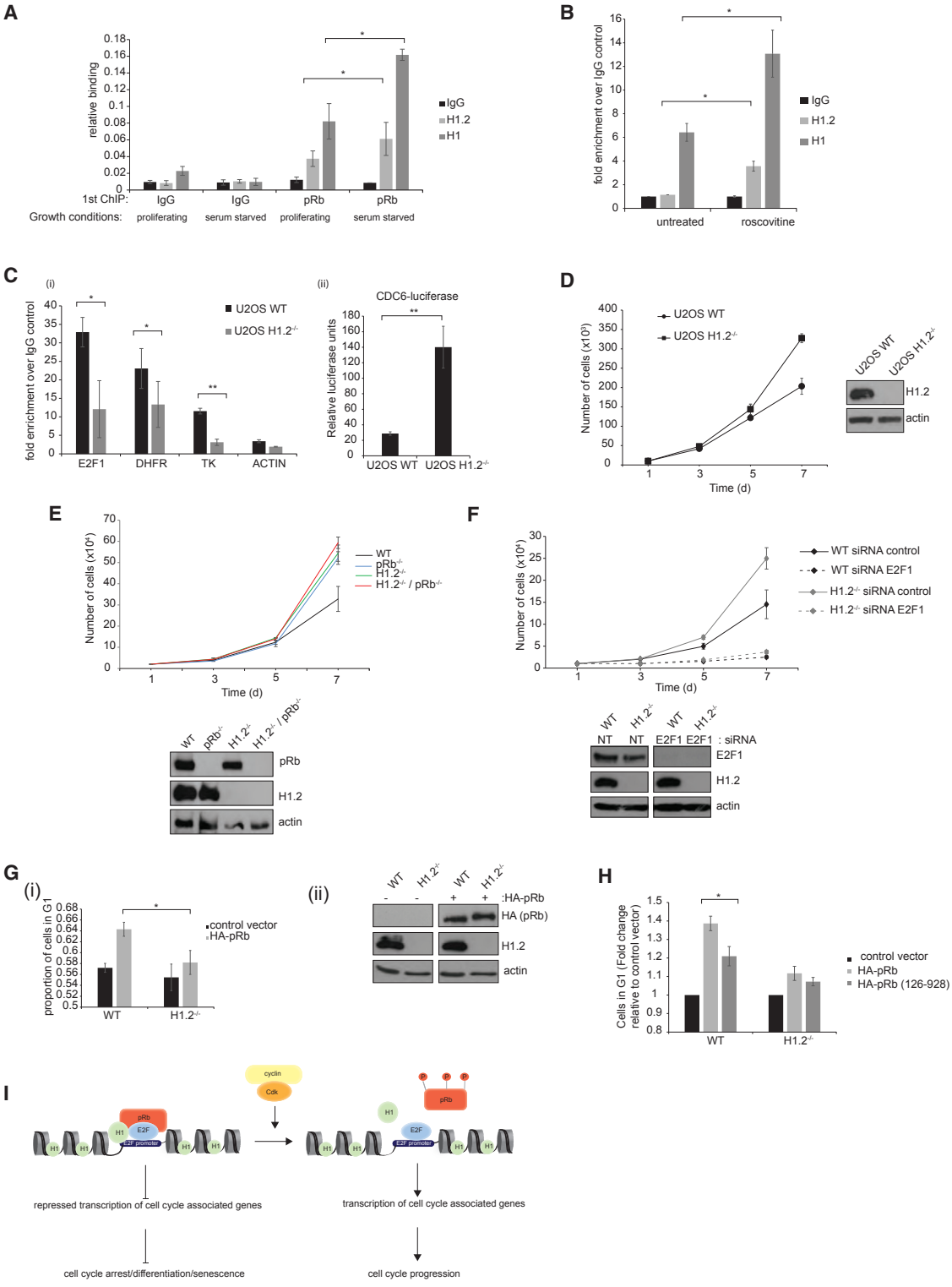

Figure 4. H1.2 Facilitates the Growth Regulatory Properties of pRb

(A) MCF7 cells were grown in normal growth conditions or under conditions of serum starvation for 72 hr. Chromatin extracts were immunoprecipitated with control IgG (IgG) or pRb antibodies. ChIP re-IP was performed with the eluted IgG and pRb material with control IgG, H1.2, or H1 antibodies. Binding of the complex to the CDC6 promoter was analyzed by qPCR.

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POLR2A) binding was performed in H1.2$^{-/-}$ cells; significantly, there was no effect upon H1.2 depletion on the association of either SP1 or PolII with chromatin (Figure S4F, i and ii). We additionally performed ChIP analysis of p53 binding to p53 target genes (where significant levels of H1 were present; Figure S4G, i) and found that H1.2 depletion did not affect p53 association with chromatin (Figure S4G, ii), thus confirming the specificity of the effect of H1.2 on pRb.

An analysis of the growth properties of the H1.2$^{-/-}$ U2OS and H1.2$^{-/-}$ MCF7 cells indicated that the H1.2$^{-/-}$ cells grow faster than their WT counterparts (at 5 and 7 days), reflecting a shorter doubling time of the H1.2$^{-/-}$ cells (U2OS cells: 23.8 hr compared to 27.6 hr; Figures 4D and S4H). As anticipated, Rb$^{-/-}$ cells also grew faster than WT cells (Figure 4E). Moreover, a similar level of increased proliferation was observed in cells that lack H1.2, or both H1.2 and pRb (Figure 4E), which is consistent with pRb and H1.2 acting through a shared mechanism and implying that H1.2 is functionally involved in pRb-dependent growth control. Further, the increased growth rate of H1.2$^{-/-}$ cells was dependent on E2F1 activity, because the increase in growth rate was no longer evident in H1.2$^{-/-}$ cells with depleted E2F1 levels (Figure 4F).

We then used SAOS2 cells, which undergo G1 arrest upon the expression of ectopic WT pRb (Li et al., 1995). The level of G1 cells apparent upon WT pRb expression was compromised in H1.2$^{-/-}$ SAOS2 cells, compared to H1.2-expressing cells (Figures 4G, 4H, and S4I), thus establishing a role for H1.2 in pRb-dependent growth control. Furthermore, expression of pRb (126-928), which is unable to bind to H1.2 (Figure 1F), demonstrated a diminished ability to elicit G1 arrest, compared to WT pRb in H1.2-expressing cells (Figure 4H), thus supporting the hypothesis that the H1.2 interaction is important for pRb-dependent growth control. In sum, these results suggest that the linker histone, H1.2, is functionally important in mediating the growth-regulating effects of pRb.

DISCUSSION

H1 histones have traditionally been regarded as widespread, if not general, repressors of global transcription, mediated through their ability to compact chromatin (Misteli et al., 2000). There are, however, an increasing number of reports that suggest that H1 histones exert gene-specific effects, which can be either positive or negative in how they influence gene expression (Biterge and Schneider, 2014).

We identified linker histones as interaction partners for pRb. The interaction between H1 histone and pRb occurs on chromatin and augments the binding of pRb to E2F target gene promoters. We focused on the role of H1.2, which, by genome-wide analysis of the chromatin-binding properties of pRb by ChIP-seq, highlighted a requirement for H1.2 for the efficient recruitment of pRb to the global network of E2F target genes, with genes involved in cell-cycle progression being particularly sensitive to the influence of H1.2. We propose, therefore, that the H1.2-pRb interaction facilitates the regulation, at a global level, of the E2F target gene network. The enhanced chromatin association, which occurs in arrested cells, is consistent with a model in which H1.2 augments transcriptional repression by pRb and thereby assists cell-cycle arrest (Figure 4I).

In conclusion, our results advance our understanding of the biological role of H1 histones by describing a new interaction with pRb, which enables H1.2 to influence the E2F pathway and the expression of downstream target genes and, consequently, impact cellular proliferation. Our study supports the idea that H1 histones, while able to mediate general repressive effects on transcription by facilitating chromatin compaction, are, in addition, endowed with selective interaction partners, such as pRb, which enables them to preferentially target and regulate key gene networks, like the extensive network controlled by the pRb-E2F pathway.

EXPERIMENTAL PROCEDURES

Expanded details of methods are listed in the Supplemental Experimental Procedures.

Cell Culture and Transfection

U2OS, HeLa, MCF7, T98G, and SAOS2 cells were cultured in DMEM (GIBCO) supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin

(B) MCF7 cells were treated with roscovitine (20 μM) for 16 hr or were untreated. Chromatin extracts were immunoprecipitated with control IgG or pRb antibodies. ChIP re-IP was performed with the eluted pRb material with control IgG, H1.2, or H1 antibodies. Binding of the complex to the CDC6 promoter was analyzed by qPCR.
(C) In (i), pRb ChIPs in U2OS WT (WT) and U2OS H1.2$^{-/-}$ CRISPR cell lines. ChIP analysis was performed with either control IgG or pRb antibodies. Binding to the indicated E2F promoters was assessed by qPCR. (ii) U2OS WT and U2OS H1.2$^{-/-}$ CRISPR cell lines were transfected with expression vectors encoding E2F-1 and CDC6-luciferase for 48 hr, and pCMV-βGal was included to monitor transfection efficiency. Relative luciferase activity (luciferase units per unit of βGal) is shown.
(D) U2OS WT and U2OS H1.2$^{-/-}$ cells were seeded in triplicate. Cell counts were performed at 3, 5, and 7 days post-seeding. Corresponding immunoblots are shown on the right.
(E) U2OS WT, U2OS pRb$^{-/-}$, U2OS H1.2$^{-/-}$, and double-knockout U2OS pRb$^{-/-}$/H1.2$^{-/-}$ cells were seeded at a density of 2 × 10^4 cells in triplicate. Cell counts were performed 3, 5, and 7 days post-seeding. Corresponding immunoblots for each cell line are shown below.
(F) U2OS WT and U2OS H1.2$^{-/-}$ cells were transfected with E2F1 siRNA (20 nM) or control siRNA (20 nM). 24 hr later, cells were trypsinized, counted, and seeded at a density of 1 × 10^4 cells in triplicate. Cell counts were performed 3, 5, and 7 days post-seeding. Corresponding immunoblots are shown below.
(G) In (i), SAOS2 WT and SAOS2 H1.2$^{-/-}$ CRISPR cell lines were transfected with either control plasmid or HA-pRb. 48 hr later, cells were harvested for flow-cytometry analysis. Graph shows the proportion of cells in G1 phase of the cell cycle. (ii) Corresponding immunoblots for (i).
(H) SAOS2 WT and SAOS2 H1.2$^{-/-}$ CRISPR cell lines were transfected with either control plasmid, HA-pRb, or HA-pRb (126-928). 48 hr later, cells were harvested for flow cytometry analysis. Graph shows the fold change in cells in G1 phase of the cell cycle.
(I) Model depicting the relationship between pRb and histone H1.2. H1.2 associates with pRb at E2F-regulated promoters and augments the ability of pRb to silence transcription, potentially resulting in scenarios such as cell-cycle arrest, differentiation, or senescence. Under conditions favorable to cell growth, pRb is phosphorylated by cyclin-CDK, resulting in the dissociation of the pRb-H1.2 complex from chromatin and active transcription of cell-cycle-associated genes by E2F and cell-cycle progression.
Data in (A)–(H) indicate average ± SD (n = 3). *p < 0.05; **p < 0.01, Student's t test.

(GIBCO) at 37°C in 5% CO_2. Cell lines were transfected with GeneJuice (Novagen). Transfections included pCMV-βGal (β-galactosidase) as an internal control to normalize transfection efficiency. For siRNA-knockdown experiments, cells were transfected with 20 nM siRNA using Oligofectamine Transfection Reagent (Invitrogen). siRNA sequences are available upon request.

Antibodies

The following antibodies were used: anti-FLAG peptide monoclonal antibody M2 (Sigma), anti-FLAG peptide monoclonal antibody M2-coupled agarose beads (Sigma), anti-HA11 monoclonal antibody (Covance), E2F-1 (C20 and KH95, Santa Cruz Biotechnology), pRb (4H1, Cell Signaling Technology), G3-245 (Becton Dickinson) and IF8 (Santa Cruz), GAPDH (V18, Santa Cruz) and β-Actin (Sigma), anti-H1.2 (ab17677 and ab4086, Abcam), anti H1.4 (from Millipore), anti-H1 (sc-8030, Santa Cruz), SP1 (sc59, Santa Cruz), and RNA PolII (sc55492, Santa Cruz).

ACCESSION NUMBERS

The accession number for the sequencing data reported in this paper is GEO: GSE98728.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.053.

AUTHOR CONTRIBUTIONS

S.M. designed and performed the experiments, analyzed the data, and wrote the paper. M.F. and S.M.C. performed additional experiments. E.S.H. and U.O. performed and analyzed the ChIP-seq experiment. R.K. and B.M.K. performed the mass spectrometry sample analysis. N.B.L.T. directed the research and wrote the paper.

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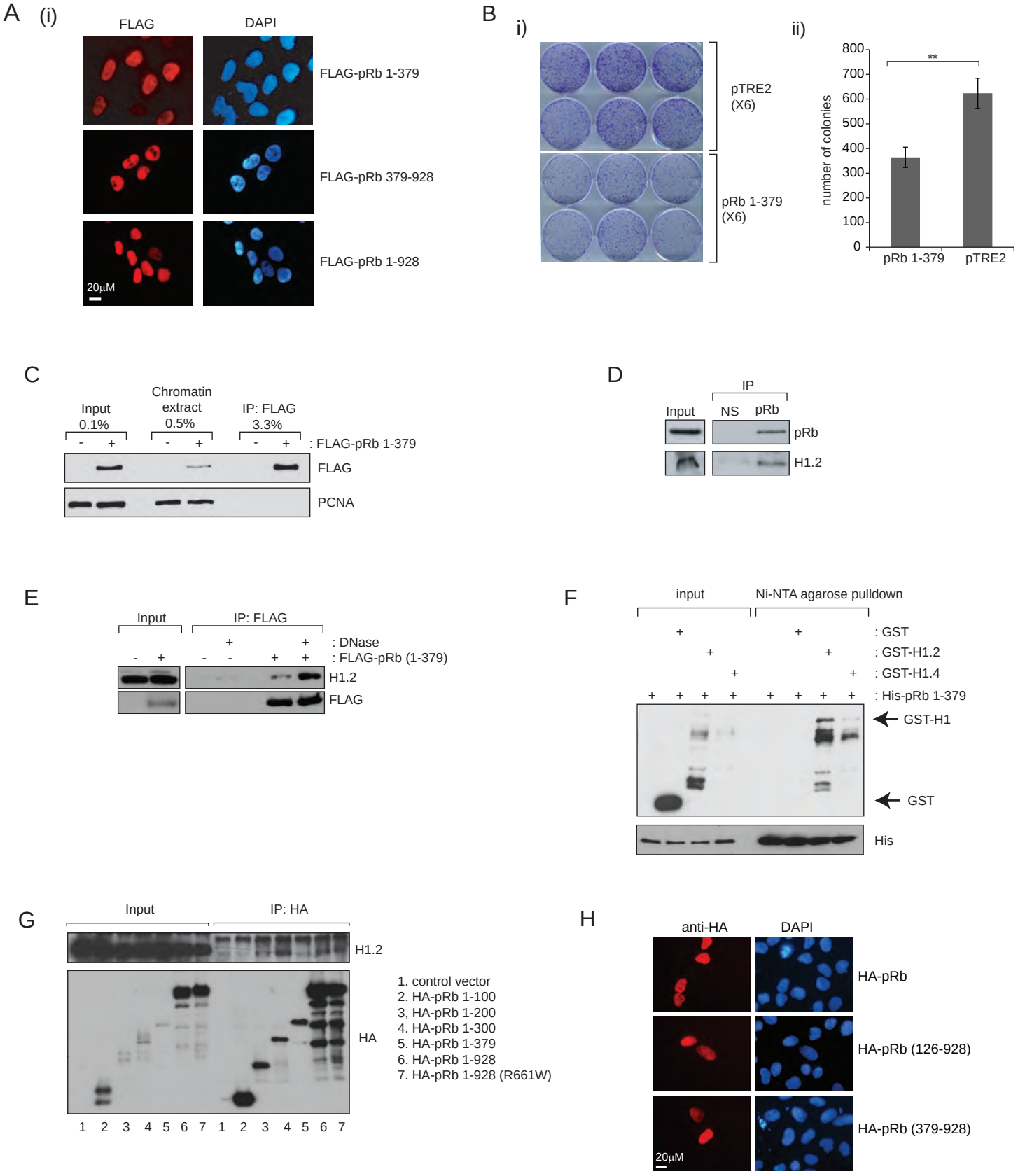
Cell Reports, Volume *19*

Supplemental Information

Linker Histone H1.2 Directs Genome-wide Chromatin Association of the Retinoblastoma Tumor Suppressor Protein and Facilitates Its Function

Shonagh Munro, Edward S. Hookway, Melanie Floderer, Simon M. Carr, Rebecca Konietzny, Benedikt M. Kessler, Udo Oppermann, and Nicholas B. La Thangue

SI Figure 1


A (i)
FLAG
DAPI
FLAG-pRb 1-379
FLAG-pRb 379-928
FLAG-pRb 1-928
20μM
B
i)
pTRE2 (X6)
pRb 1-379 (X6)
ii)
number of colonies
800
700
600
500
400
300
200
100
0
**
pRb 1-379
pTRE2
C
Input 0.1%
Chromatin extract 0.5%
IP: FLAG 3.3%
: FLAG-pRb 1-379
FLAG
PCNA
D
IP
Input
NS
pRb
pRb
H1.2
E
Input
IP: FLAG
: DNase
: FLAG-pRb (1-379)
H1.2
FLAG
F
input
Ni-NTA agarose pulldown
: GST
: GST-H1.2
: GST-H1.4
: His-pRb 1-379
GST-H1
GST
His
G
Input
IP: HA
H1.2
HA
1. control vector
2. HA-pRb 1-100
3, HA-pRb 1-200
4. HA-pRb 1-300
5. HA-pRb 1-379
6. HA-pRb 1-928
7. HA-pRb 1-928 (R661W)
1 2 3 4 5 6 7 1 2 3 4 5 6 7
H
anti-HA
DAPI
HA-pRb
HA-pRb (126-928)
HA-pRb (379-928)
20μM

Supplemental Figure Legends

SI Figure 1, related to Figure 1

A U2OS Tet-On inducible cells were grown in the presence of doxycycline (1µg/ml) for 48h. Protein localisation was assessed by immunofluorescence with FLAG antibody (red). DAPI was used to visualise nuclei.

B FLAG-pRb (1-379) inducible cells and pTRE2 empty vector control cells were seeded at a density of 200 cells per well (6 replicates). Colony formation was measured after two weeks of culture in the presence of doxycycline (1µg/ml). Subsequently, cells were stained with crystal violet (i) and the number of colonies counted (ii). ***P<0.01* (Student's *t*-test) Graphs represent mean ± SD, n=5.

C FLAG-pRb (1-379) inducible cells were cultured for 48h in the presence of doxycycline. Chromatin was extracted and digested with benzonase for 30 minutes on ice. Subsequently, immunoprecipitation of the chromatin fraction was performed with FLAG-antibody. Fractions of whole cell extract (WCE, 0.1%), chromatin extract (0.5%) and eluate were analysed by immunoblotting for FLAG and PCNA. pTRE2 empty vector cell line was used as a control (-). Approximately 5% of total pRb chromatin bound (quantitation of chromatin pRb signal compared to WCE signal is 25%, but 5X more chromatin loaded proportionally (25/5=5%).

D HeLa cell lysates were immunoprecipitated with control IgG non-specific (NS) or pRb antibodies, followed by immunoblotting with pRb and H1.2 antibodies.

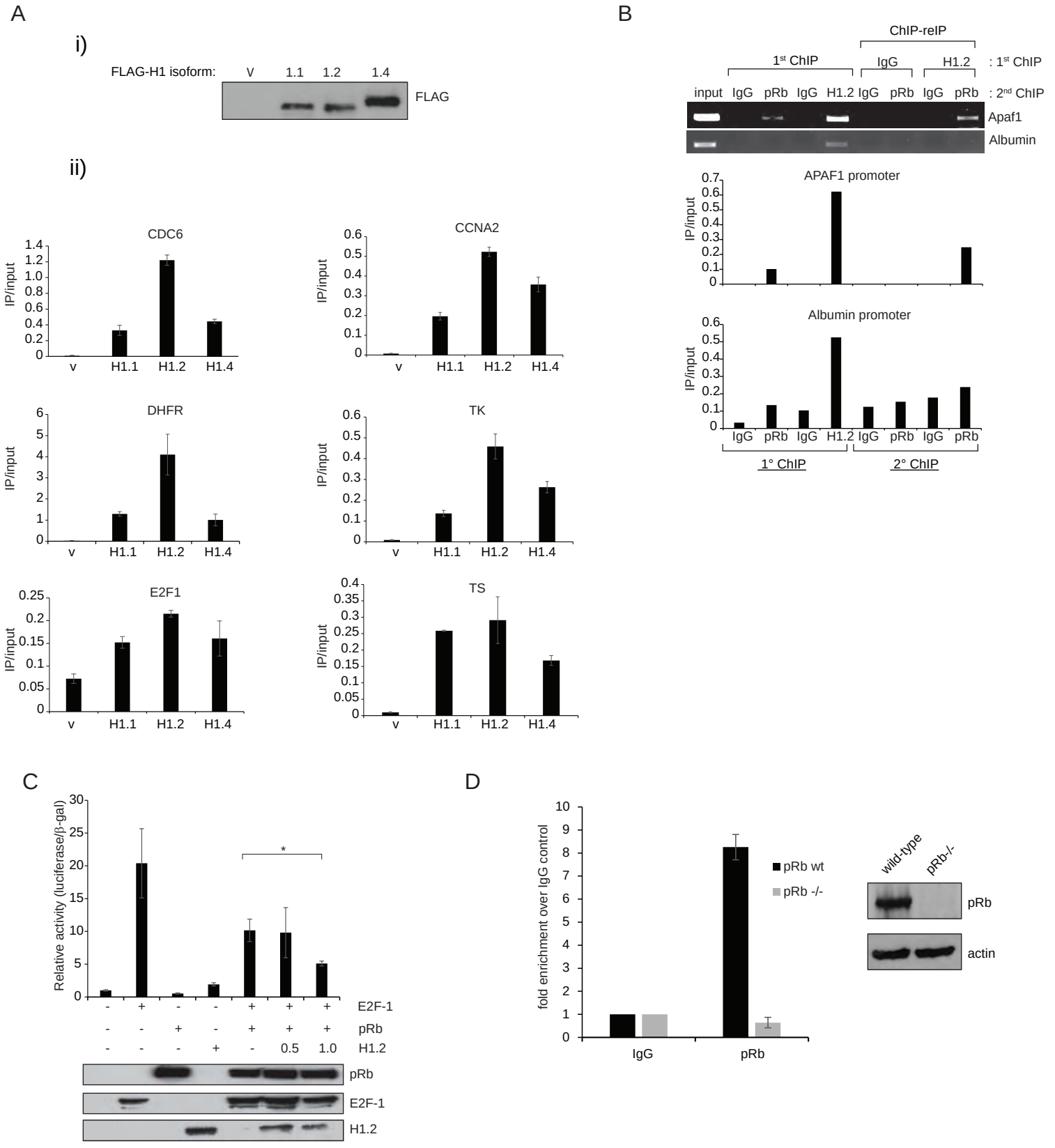
E FLAG-pRb (1-379) inducible cells and pTRE2 control cells were grown in the presence doxycycline for 48h. FLAG immunoprecipitation was performed with (+) or without (-) DNase (50 U) followed by immunoblotting with H1.2 and FLAG antibodies as indicated.

F Bacterially expressed GST-H1.2, GST-H1.4 and GST alone were incubated in the presence of bacterially expressed His-pRb (1-379) together with Ni-NTA agarose. Following extensive washes, immunoblotting was performed with GST and His antibodies as indicated.

G U2OS cells were transfected with expression vectors expressing different HA-tagged pRb C-terminal truncations and mutations. 48h post-transfection, lysates were immunoprecipitated with anti-HA followed by immunoblotting with H1.2 and HA antibodies as indicated.

H U2OS cells were transfected with expression vectors expressing different HA-tagged pRb truncations. 48h post-transfection, protein localisation was assessed by immunofluorescence with HA antibody (red). DAPI was used to visualise nuclei.

SI Figure 2


A
i)
FLAG-H1 isoform: V 1.1 1.2 1.4
FLAG
ii)
CDC6
CCNA2
DHFR
TK
E2F1
TS
IP/input
v H1.1 H1.2 H1.4
B
ChIP-reIP
1st ChIP
IgG
H1.2
: 1st ChIP
input IgG pRb IgG H1.2 IgG pRb IgG pRb
: 2nd ChIP
Apaf1
Albumin
APAF1 promoter
Albumin promoter
IP/input
IgG pRb IgG H1.2 IgG pRb IgG pRb
1° ChIP
2° ChIP
C
Relative activity (luciferase/β-gal)
*
E2F-1
pRb
H1.2
D
fold enrichment over IgG control
pRb wt
pRb -/-
IgG
pRb
wild-type
pRb-/-
pRb
actin

SI Figure 2, related to Figure 2

A (i) U2OS cells were transfected with expression vectors for FLAG H1.1, H1.2 and H1.4, or control vector (v) and protein expression was confirmed using FLAG antibody.

(ii) ChIP was performed on chromatin isolated from the transfected cells as indicated. The ability of the H1 subtypes to associate with various E2F target gene promoters was analysed by qPCR. Graphs represents mean ± SD (n=3).

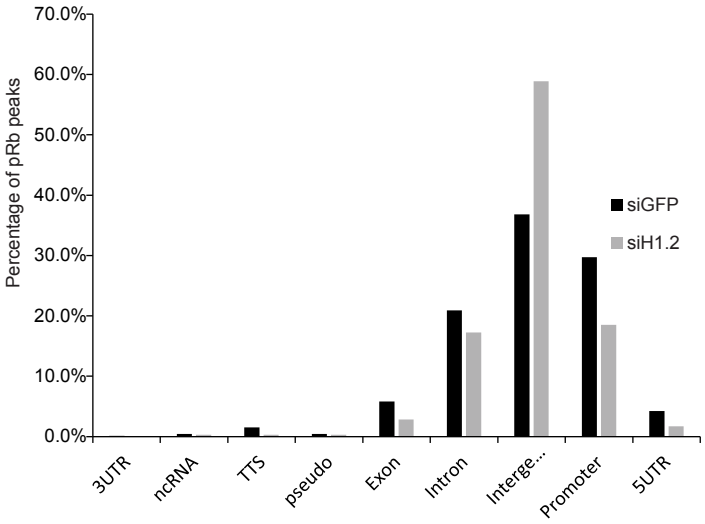
B U2OS chromatin extracts were immunoprecipitated with control IgG (IgG), pRb or H1.2 antibodies as indicated. A ChIP re-IP was performed with the eluted H1.2 material and corresponding non-specific control material with control IgG or pRb antibodies. The binding of the complex to the APAF1 promoter was analysed by PCR. The albumin promoter was included as a negative control for the pRb ChIP. Quantification of the ChIP signal is shown in graphical format below.

C U2OS cells were transfected with expression vectors encoding E2F-1, pRb and H1.2, together with DHFR-luciferase and pCMV-bgal to monitor the transfection efficiency. Cells were harvested 48h post-transfection. Relative luciferase activity (luciferase/β-gal) is shown together with the expression level of the ectopic proteins underneath. *$*P<0.05$* (Student's *t*-test), graph represents mean ± SD (n=3).

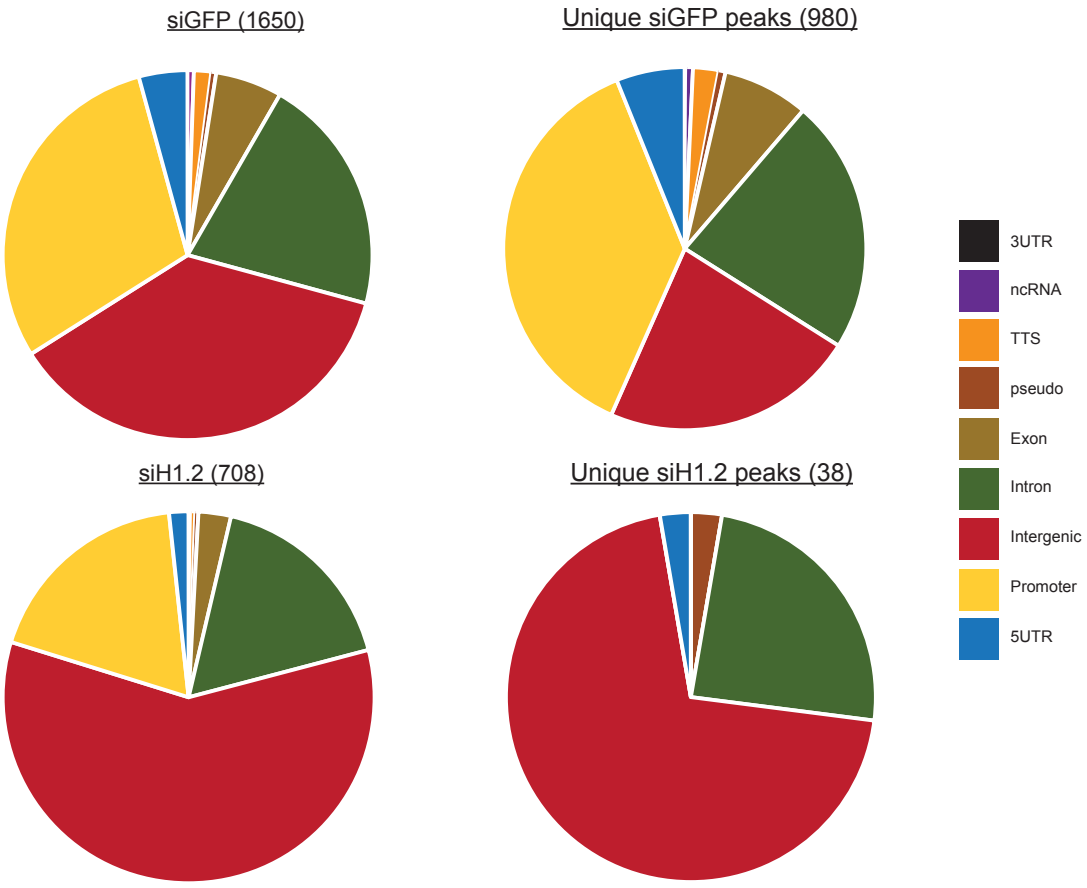
D (i) pRb chromatin immunoprecipitations in MCF7 wild-type (WT) and MCF7 $pRb^{-/-}$ CRISPR cell lines. ChIP analysis was performed with either control IgG or pRb antibodies. Binding to the indicated E2F1 promoter was assessed by qPCR. Graph represents mean ± SD (n=3).

SI Figure 3

A



B

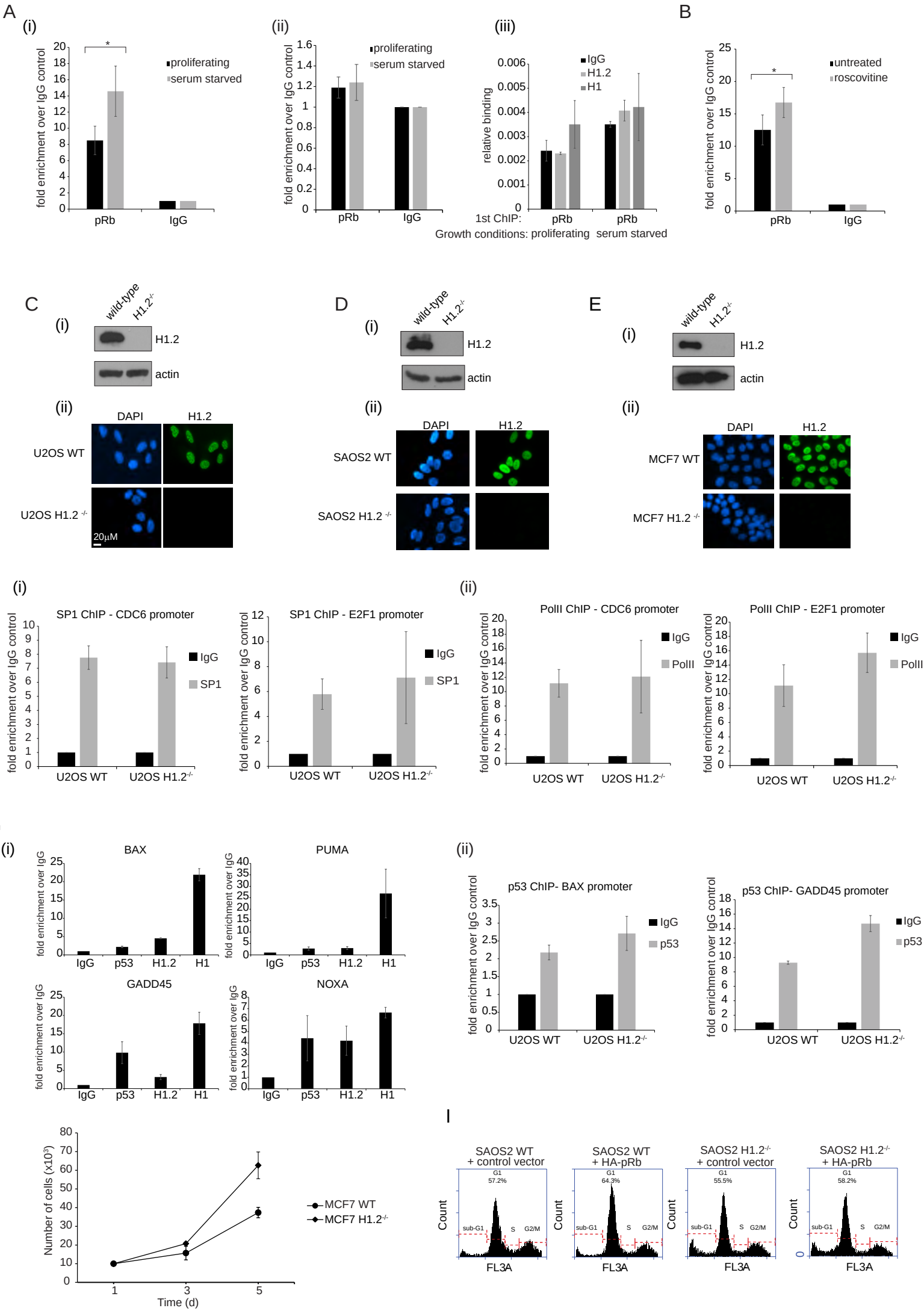


SI Figure 3, related to Figure 3

A Graphical representation different genomic regions for pRb peaks recovered under siGFP (control) or siH1.2.

B Pie chart representation of locations of pRb ChIP-seq peaks in the genome. Charts show siGFP alone, siH1.2 alone, peaks unique to siGFP and peaks unique to siH1.2

SI Figure 4


A
(i)
fold enrichment over IgG control
proliferating
serum starved
pRb
IgG
(ii)
fold enrichment over IgG control
proliferating
serum starved
pRb
IgG
(iii)
relative binding
IgG
H1.2
H1
1st ChIP: pRb pRb
Growth conditions: proliferating serum starved
B
fold enrichment over IgG control
untreated
roscovitine
pRb
IgG
C
(i)
wild-type
H1.2 -/-
H1.2
actin
(ii)
DAPI
H1.2
U2OS WT
U2OS H1.2 -/-
20μM
D
(i)
wild-type
H1.2 -/-
H1.2
actin
(ii)
DAPI
H1.2
SAOS2 WT
SAOS2 H1.2 -/-
E
(i)
wild-type
H1.2 -/-
H1.2
actin
(ii)
DAPI
H1.2
MCF7 WT
MCF7 H1.2 -/-
F
(i)
SP1 ChIP - CDC6 promoter
fold enrichment over IgG control
IgG
SP1
U2OS WT
U2OS H1.2 -/-
SP1 ChIP - E2F1 promoter
fold enrichment over IgG control
IgG
SP1
U2OS WT
U2OS H1.2 -/-
(ii)
PolII ChIP - CDC6 promoter
fold enrichment over IgG control
IgG
PolII
U2OS WT
U2OS H1.2 -/-
PolII ChIP - E2F1 promoter
fold enrichment over IgG control
IgG
PolII
U2OS WT
U2OS H1.2 -/-
G
(i)
BAX
PUMA
GADD45
NOXA
fold enrichment over IgG
IgG
p53
H1.2
H1
(ii)
p53 ChIP- BAX promoter
fold enrichment over IgG control
IgG
p53
U2OS WT
U2OS H1.2 -/-
p53 ChIP- GADD45 promoter
fold enrichment over IgG control
IgG
p53
U2OS WT
U2OS H1.2 -/-
H
Number of cells (x10³)
Time (d)
MCF7 WT
MCF7 H1.2 -/-
I
SAOS2 WT + control vector
SAOS2 WT + HA-pRb
SAOS2 H1.2 -/- + control vector
SAOS2 H1.2 -/- + HA-pRb
Count
FL3A
sub-G1
G1
S
G2/M
G1 57.2%
G1 64.3%
G1 55.5%
G1 58.2%

SI Figure 4, related to Figure 4

A (i) Results of the first ChIP for Figure 4A. MCF7 cells were grown in normal growth conditions (proliferating) or under conditions of serum starvation for 72h. Chromatin from MCF7 cells was immunoprecipitated with control IgG or pRb antibodies as indicated. The presence of pRb on the CDC6 promoter was analysed by qPCR. **P<0.05* (Student's *t*-test), graph represents mean ± SD (n=3). (ii) Chromatin from A (i) was analysed for the presence of pRb on the actin promoter by qPCR, graph represents mean ± SD (n=3). (iii) Chromatin from Figure 4A was analysed for actin chromatin enrichment under the indicated conditions, graph represents mean ± SD (n=3).

B Results of the first ChIP for Figure 4B. MCF7 cells were treated with or without roscovitine (20μM) for 16h. Chromatin from MCF7 cells was immunoprecipitated with control IgG or pRb antibodies as indicated. The presence of pRb on the CDC6 promoter was analysed by qPCR. **P<0.05* (Student's *t*-test), graph represents mean ± SD (n=3).

C (i) Wild-type U2OS and H1.2$^{-/-}$ cell lysates were immunoblotted for H1.2 and actin. (ii) Wild-type U2OS cells and H1.2$^{-/-}$ cells H1.2 protein expression was assessed by immunofluorescence with H1.2 antibody (green). DAPI was used to visualise nuclei.

D (i) Wild-type SAOS2 and H1.2$^{-/-}$ cell lysates were immunoblotted for H1.2 and actin. (ii) Wild-type SAOS2 cells and H1.2$^{-/-}$ cells protein expression was assessed by immunofluorescence with H1.2 antibody (green). DAPI was used to visualise nuclei.

E (i) Wild-type MCF7 and H1.2$^{-/-}$ cell lysates were immunoblotted for H1.2 and actin. (ii) Wild-type MCF7 cells and H1.2$^{-/-}$ cells protein expression was assessed by immunofluorescence with H1.2 antibody (green). DAPI was used to visualise nuclei.

F (i) SP1 chromatin immunoprecipitations in U2OS wild-type (WT) and U2OS H1.2$^{-/-}$ CRISPR cell lines. ChIP analysis was performed with either the appropriate control IgG or SP1

antibodies. Binding to the E2F1 and CDC6 promoters was assessed by qPCR. Graph represents mean ± SD (n=3).

(ii) RNA PolII chromatin immunoprecipitations in U2OS wild-type (WT) and U2OS H1.2$^{-/-}$ CRISPR cell lines. ChIP analysis was performed with either the appropriate control IgG or PolII antibodies. Binding to the E2F1 and CDC6 promoters was assessed by qPCR. Graph represents mean ± SD (n=3).

G (i) Chromatin immunoprecipitations in U2OS cells. ChIP analysis was performed with either the appropriate control IgG, p53, H1.2 or H1 antibodies. Binding to the BAX, GADD45, PUMA and NOXA promoters was assessed by qPCR. Graph represents mean + SD (n=3).

(ii) p53 chromatin immunoprecipitations in U2OS wild-type (WT) and U2OS H1.2$^{-/-}$ CRISPR cell lines. ChIP analysis was performed with either the appropriate control IgG or p53 antibodies. Binding to the BAX and GADD45 promoters was assessed by qPCR. Graph represents mean ± SD (n=3).

H MCF7 wild-type and MCF7 H1.2$^{-/-}$ cells were seeded at a density of 1x10^4 cells in triplicate. Cells were counted at 3 and 5 days post-seeding. Cell doubling times: WT 63.1h and H1.2$^{-/-}$ 45.33h. Graph represents mean ± SD (n=3).

I Accompanying FACs profiles for Figure 4G.

Supplemental Experimental Procedures

Plasmids and expression vectors

pSG5L-HA-pRb, pcDNA3.1-HA-pRb (1-379) and pcDNA3.1-HA-E2F1 have been described previously (Markham et al., 2006; Munro et al., 2010). H1.1, H1.2, H1.4 and H1.5 were amplified from human cDNA and subcloned into p3XFLAG-CMV-7.1 and pGEX-4T-1 expression vectors (Sigma and GE Healthcare Life sciences).

Cell lines

MCF7, U2OS, HeLa, T98G and SAOS2 cells were from Sigma (ECACC).

Generation of Tet-On inducible cell lines

The FLAG-pRb (1-379) and FLAG-pRb (1-928) inducible cell lines were generated in U2OS Tet-On cells, using the Tet-On® gene expression system (Clontech) and cultured in DMEM supplemented with 75μg/ml Hygromycin B (Invitrogen), 100μg/ml G418 (Santa Cruz), 1% Penicillin / Streptomycin (Gibco, Life technologies) and 5% tetracycline-negative FCS (PAA Laboratories), at 37°C and 5% CO_2. Protein expression was induced by adding 1μg/ml doxycycline (Sigma) for 48h unless otherwise stated.

Immunoprecipitation and immunoblotting

Cells were harvested, washed in PBS, and resuspended in TNN buffer [50 mM Tris pH 7.4, 5 mM EDTA, 0.5% Igepal CA-630 (Sigma), 50 mM NaF, 1 mM DTT, 0.2 mM Na_3VO_4, 120 mM NaCl, protease inhibitor cocktail]. Total protein concentration was determined by Bradford Assay (Bio-Rad). Typically 1-1.5 mg of cell extract was added to pre-washed Protein-G agarose beads (Sigma) with 1μg of antibody. After overnight incubation the beads were washed 4 times in TNN prior to protein elution with 3xSDS loading buffer and analysis by immunoblotting with the appropriate antibodies.

FLAG immunoprecipitation for mass spectrometry

Prior to the FLAG immunoprecipitation, the lysates were precleared with mouse non-specific antibody and Protein-G agarose beads for 1h at 4°C. For the isolation of FLAG-tagged proteins, anti-FLAG monoclonal antibody M2-coupled agarose beads (Sigma) were used. Three volumes of IP wash buffer (50 mM Tris-HCl pH 7.5, 150mM NaCl, 5.0% Glycerol, 0.5% Triton X-100, 0. mM EGTA, protease inhibitor cocktail) and beads were added to the whole-cell extract or chromatin fraction and incubated for 2 hours at 4°C on a rotating wheel. Afterwards, the beads were washed 5 times with 1 ml IP wash buffer and centrifuged for 30 sec at 3,300 x g at 4°C. The elution of FLAG-tagged pRb (1-379) was performed by resuspending the beads in 0.5mg/ml FLAG

peptide (Sigma) in 20mMTris-HCl pH 7.5 and incubating for 1h at 100 rpm at 4°C. After centrifugation for 1 min at 3,300 x g at 4°C, the supernatant was collected for further analysis.

Immunostaining

U2OS, MCF7 and SAOS2 cells were stained according to previously published procedures (Markham et al., 2006).

Flow cytometry

Cells were seeded in 60mm dishes and transfected with the indicated expression vectors together with 200ng pBB14–GFP to monitor transfection efficiency, cells were washed in PBS and fixed overnight in 50% ethanol/PBS at 4ºC. Fixed cells were washed in PBS and incubated for 30min with 25U/ml RNase A (Sigma) and 50μg/ml propidium iodide (Sigma) in PBS. The analysis of cell cycle profiles was performed on the BD Accuri C6 flow cytometer.

Mass spectrometry

DTT reducing reagent was added to the eluate after chromatin extraction and FLAG-IP to the final concentration of 5mM and incubated for 60s at room temperature. Then, iodoacetamide alkylating reagent (Sigma) was added to the samples, to a final concentration of 20mM and incubated for 60s at room temperature. Afterwards, a methanol/chloroform extraction for proteins was performed, 600μl methanol and 150μl chloroform were added to 200 μl sample. Samples were then vortexed, and 450μl ultrapure water was added. The samples were centrifuged for 1min at 16,000 x *g* at 4°C and the aqueous phase was removed. 450μl methanol was added to the sample containing organic phase and interphase and samples were vortexed. Subsequently, the samples were centrifuged for 2min at 16,000x*g* at room temperature and the supernatant was removed. The protein pellet was resuspended in 50μl 6 M urea buffer (VWR) by vortexing and sonication for 2 min. The samples were diluted with 250 μl ultrapure water. A trypsin-digest (1:50 ratio trypsin regarding the total protein content) was carried out overnight at 37°C. The samples were purified using Sep-Pak C18 Plus Light Cartridge (Waters). Afterwards, purified peptides were dried down completely by speed-vacuum centrifugation and resuspended in 2% acetonitrile and 0.1% fluoroacetic acid.

Nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) was performed to analyse HPLC-purified peptides, using an Acquity LC instrument (C18 column with a 75μm x 250mm, 1.7μm particle size; Waters) coupled to a Thermo LTQ Orbitrap Elite mass spectrometer (resolution of 120,000 at 400m/z, Top 20, collision-induced dissociation). A gradient of 1–35% acetonitrile was used for 60min at a flow rate of 250 nl/minute. Peak lists containing MS/MS spectra were generated, using MSConvert. Mascot version 2.3

(http://www.matrixscience.com) was used to identify post-translational modifications as well as unknown interaction partners of pRb (1-379). The following settings were used for the search against the Swiss-Prot protein database: the taxonomy restriction "human" (20,306 entries as of June 2014), tryptic restriction and mass deviations of 10 parts per million / 0.5 daltons in the respective MS modes. For the identification of unknown pRb (1-379) interaction partners, the obtained data for pRb (1-379) were additionally compared to the pTRE2 empty vector cell line. Hits were selected based on the emPAI value (Exponentially Modified Protein Abundance Index) that gives an estimation of absolute protein amount by the number of sequenced peptides per protein (Ishihama *et al*., 2005).

Chromatin immunoprecipitation (ChIP)

U2OS cells were maintained in DMEM containing 5% foetal calf serum. Cells were transfected with the indicated plasmids for 48h. Cells were cross-linked with formaldehyde to a final concentration of 1%. ChIP samples were prepared as described previously (Munro et al., 2010). Immunoprecipitations were performed using 1µg anti-E2F-1 (C-20, Santa Cruz), anti-pRb (4H1, Cell Signaling), anti-H1.2 (ab4086, Abcam), anti-H1 (sc-8030, Santa Cruz), anti-HA11 (Covance), anti-FLAG M2 (Sigma) or the relevant non-specific IgG (Santa Cruz). For ChIP-reIP, the first immunoprecipitation was performed with 2µg antibody. Following elution of the first ChIP, chromatin was diluted 10-fold in ChIP-reIP buffer (10mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 1% Igepal) and the second immunoprecipitation performed with 1µg antibody overnight at 4ºC. The secondary ChIP was washed as for the primary ChIP. Following elution, the recovered DNA was analyzed by semi-quantitative or real-time quantitative PCR. Primer sequences are available upon request.

Quantitative PCR

Real-time PCR for the ChIP was carried out on the Stratagene MX3005P. Brilliant III SYBR Green QPCR Master Mix (Agilent) was used according to the manufacturer's instructions.

ChIP-seq

Libraries were prepared from either DNA from specific pRb ChIP material or sonicated input controls using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) and sequenced on a NextSeq 500 (Illumina). Reads were trimmed for adaptors and quality using Trim Galore (version 0.3.7) and aligned to GRCh37 using Bowtie2 (version 2.2.5). Duplicate reads were marked using Picard Tools. Peaks were called using Macs 2 (version 2.1.0), using the sequenced input as control with a false discovery rate of 0.01. Peaks within 500kb of centromeres and peaks overlapping alpha-satellite DNA repeats were filtered. Bedtools (version 2.24.0) was used to identify over-lapping peaks between conditions. Motif analysis

was performed using HOMER (version 4.8) using the region 350bp before the transcription start site and 50bp. Identification of E2F binding site motif was performed *in silico* using the "findMotifsGenome.pl" function from within HOMER using the binding motifs for E2F, E2F1, E2F4, E2F6 and E2F7. A gene was identified as being an E2F target gene if there was at least one identified binding site within 500bp of the transcription start site of a gene. Peak information from the ENCODE project (GEO accession GSM935477) was obtained and genes called as being either E2F or non-E2F using the same criteria Coverage plots were generated using Homer to calculate coverage around the transcription start site. All computationally identified E2F binding sites and all peaks as identified by MACS. Per-gene coverage around the transcription start site was performed using ngs.plot.r (version 2.47.1). Data tracks were visualised in IGV using bedgraphs normalised to 10^7 mapped reads.

CRISPR-Cas9 gene disruption

pSpCas9(BB)-2A-Puro (Addgene plasmid ID: 48139) was used for the generation of H1.2 and pRb knock out cell lines using CRISPR-Cas9 technology. Cell lines were generated as detailed in (Ran et al., 2013).

Luciferase reporter assay

For reporter assays, U2OS cells were transfected with 100ng of luciferase reporter plasmid, 100ng of pCMV-β-galactosidase (β-gal) plasmid as an internal control and the indicated expression plasmids, as previously described (Munro et al., 2010).

Colony formation assay

Cells were seeded in 6 well plates at a density of $1x10^3$ and left for 10-14 days. Surviving colonies were stained with crystal violet, and counted manually.

Cell proliferation assays

Cells were seeded at a density of $2x10^4$ cells in triplicate (day 1). Cell counts were performed 3, 5 and 7 days post-seeding. For proliferation assays with siRNA treatments, cells were transfected with 20nM siRNA, 24h later cells were trypsinized, counted and reseeded at a density of $2x10^4$ cells in triplicate (day 1). Cell counts were performed 3, 5 and 7 days post-seeding.