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Supplemental Information

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

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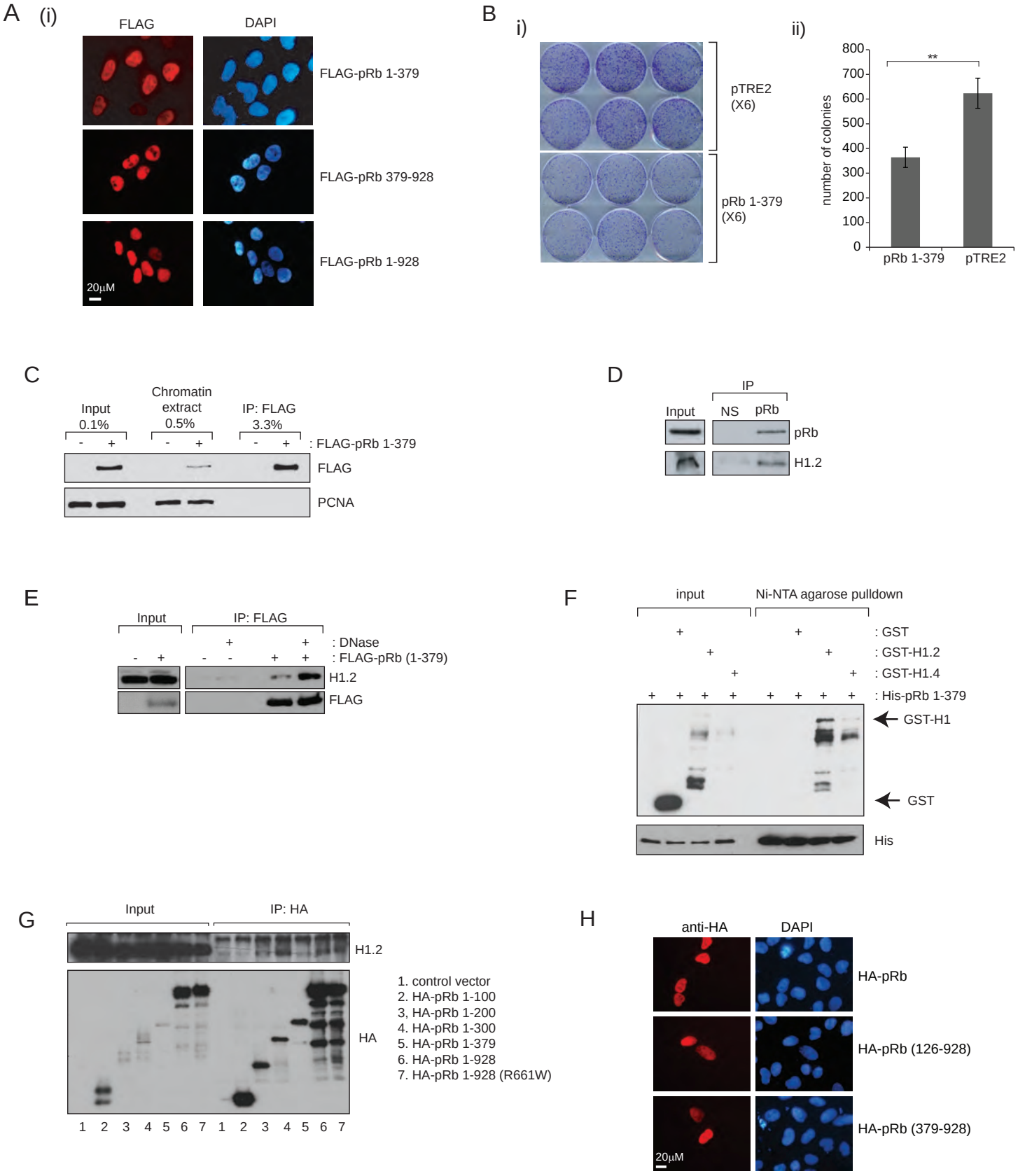
SI Figure 1


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.

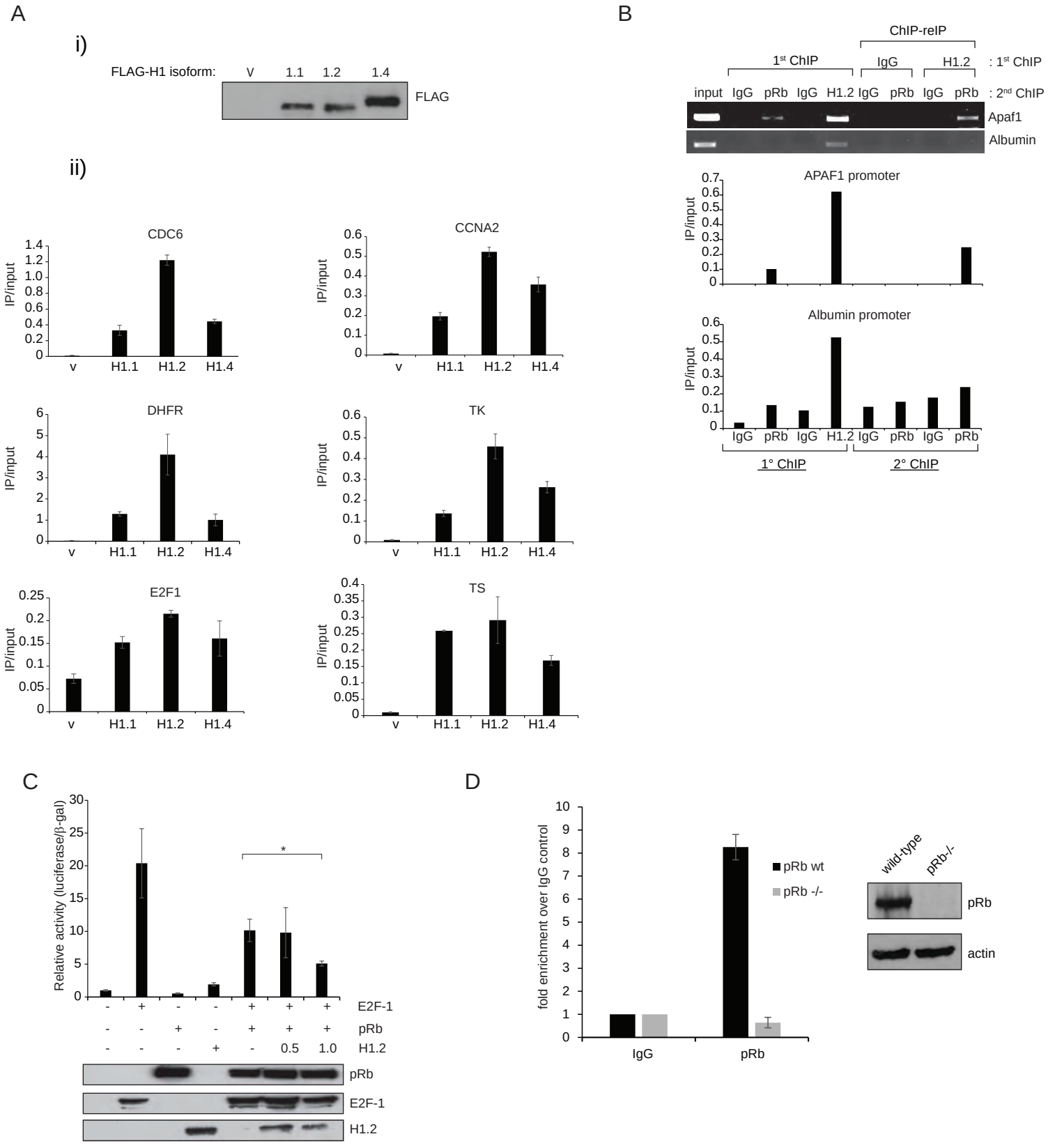
E FLAG-pRb (1-379) inducible cells and pTRE2 control cells were grown in the presence doxycycline for 48h. FLAG immunoprecipitation was performed with (+) or without (-) DNase (50 U) followed by immunoblotting with H1.2 and FLAG antibodies as indicated.

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).

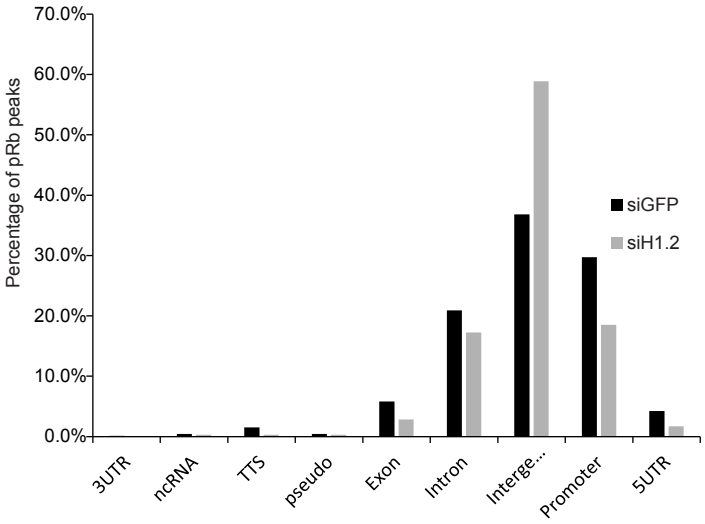
B U2OS chromatin extracts were immunoprecipitated with control IgG (IgG), pRb or H1.2 antibodies as indicated. A ChIP re-IP was performed with the eluted H1.2 material and corresponding non-specific control material with control IgG or pRb antibodies. The binding of the complex to the APAF1 promoter 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).

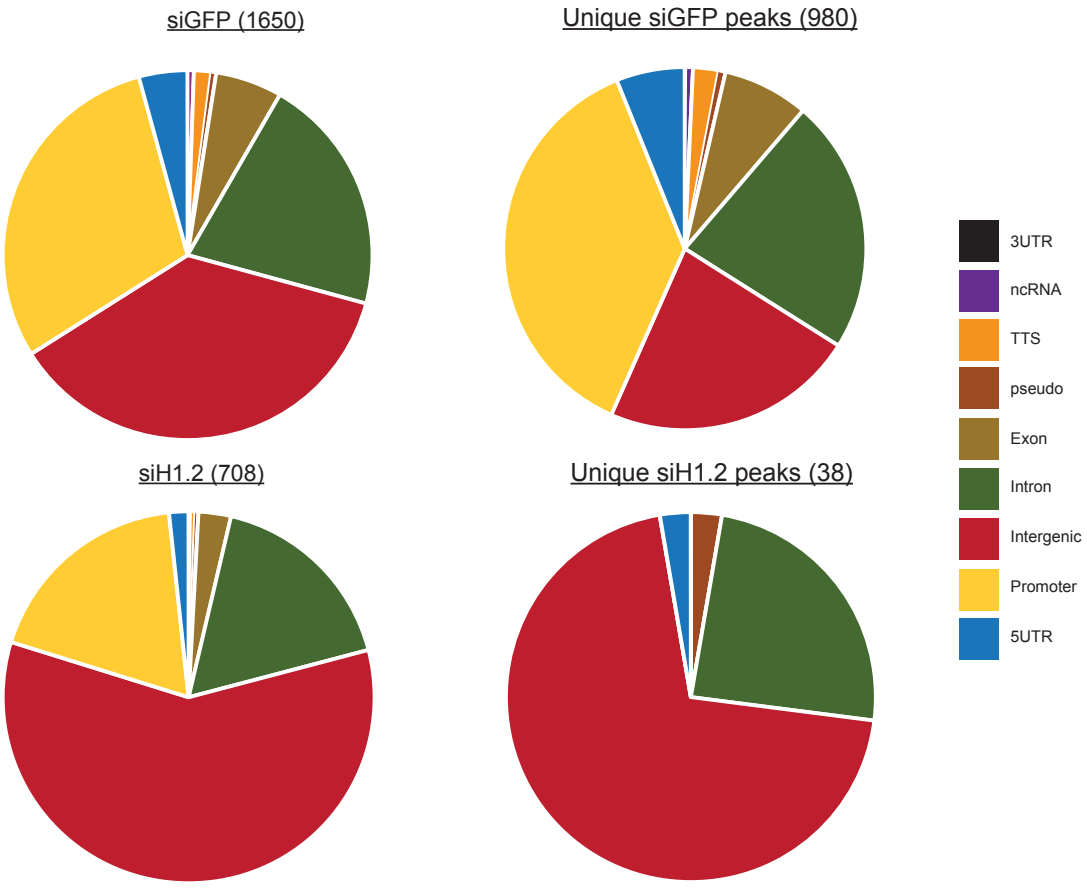
D (i) pRb chromatin immunoprecipitations in MCF7 wild-type (WT) and MCF7 $pRb^{-/-}$ CRISPR cell lines. ChIP analysis was performed with either control IgG or pRb antibodies. Binding to the indicated E2F1 promoter was assessed by qPCR. Graph represents mean ± SD (n=3).

SI Figure 3

A



B

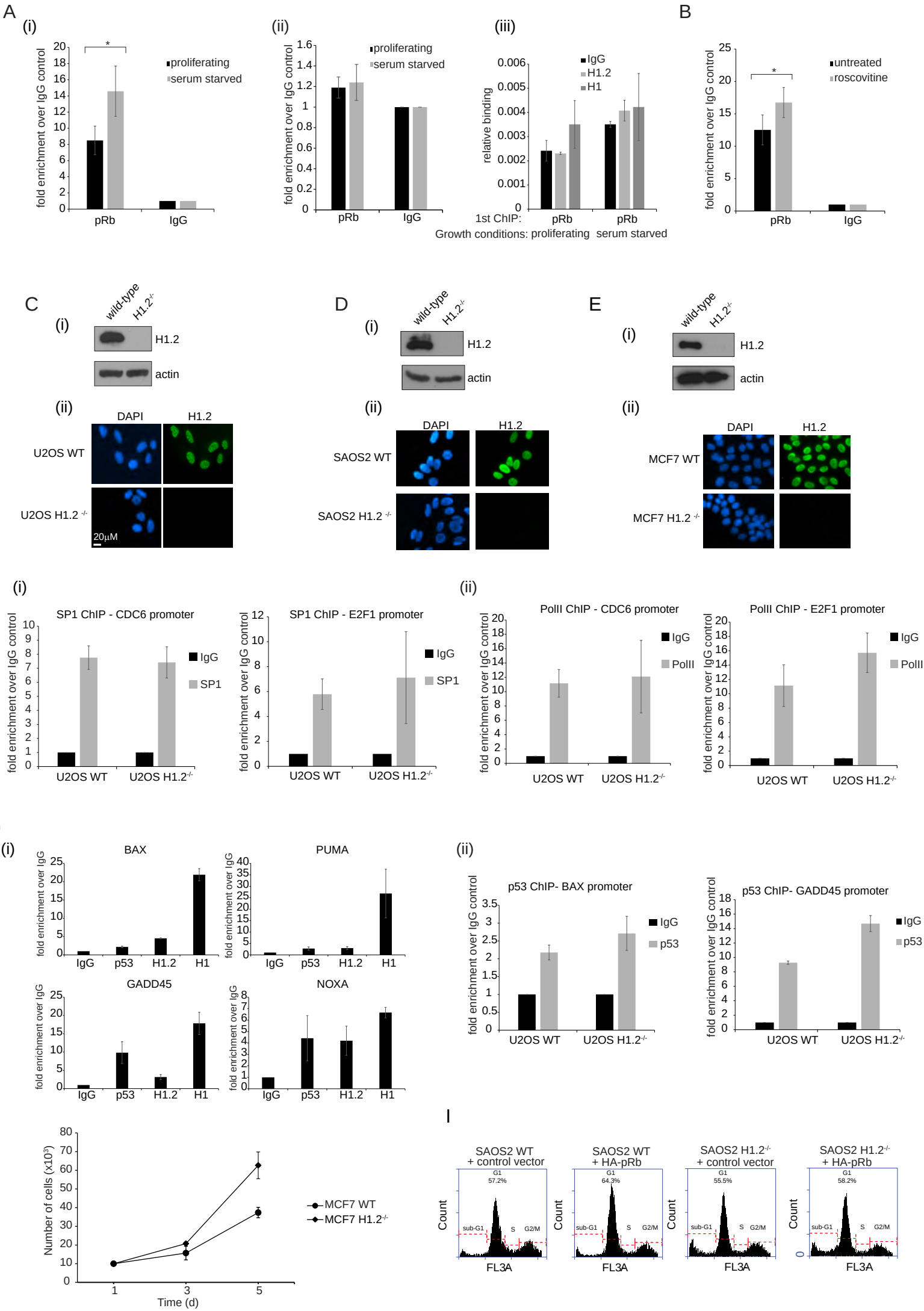


SI Figure 3, related to Figure 3

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.