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Dynamic site-specific recruitment of RBP2 by pocket protein p130 modulates H3K4 methylation on E2F-responsive promoters

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ABSTRACT

The Histone 3 lysine 4 methylation (H3K4me3) mark closely correlates with active transcription. E2F-responsive promoters display dynamic changes in H3K4 methylation during the course of cell cycle progression. However, how and when these marks are reset, is not known. Here we show that the retinoblastoma binding protein RBP2/KDM5A, capable of removing tri-methylation marks on H3K4, associates with the E2F4 transcription factor via the pocket protein—p130—in a cell-cycle-stage specific manner. The association of RBP2 with p130 is LxCxE motif dependent. RNAi experiments reveal that p130 recruits RBP2 to E2F-responsive promoters in early G1 phase to bring about H3K4 demethylation and gene repression. A point mutation in LxCxE motif of RBP2 renders it incapable of p130-interaction and hence, repression of E2F-regulated gene promoters. We also examine how RBP2 may be recruited to non-E2F responsive promoters. Our studies provide insight into how the chromatin landscape needs to be adjusted rapidly and periodically during cell-cycle progression, concomitantly with temporal transcription, to bring about expression/repression of specific gene sets.

INTRODUCTION

Cell cycle progression involves the temporal transcriptional regulation of large sets of genes. These gene sets, which are activated during one phase, must also be repressed during a later phase of the cell cycle. The cyclic changes in gene expression pattern are accompanied by conforming alterations in chromatin signatures, which must be re-established in each cell cycle. Methylation of Histone 3 lysine 4 (H3K4) correlates closely with transcription activation. Consequently, the levels of H3K4 methylation are dynamically regulated during the cell cycle (1).

H3K4 methylation, basically, is regulated by two set of enzyme families—the histone methyltransferases (HMTs) depositing these marks—mixed lineage leukemia (MLL) and SET family; and the demethylases which remove it—the KDM5 family. Human KDM5 family consists of four members (RBP2/KDM5A, Plu-1/KDM5B, SMCX/KDM5C and SMCY/KDM5D), all of which are capable of demethylating H3K4me 3/2/1 mark (2–6). These multi-domain proteins contain a conserved catalytic N- and C-terminal Jumonji (JmjN/JmjC) domain, a DNA binding AT-rich Interacting domain (ARID), a C5HC2 zinc finger, a Plu-1 domain and two to three Plant homeodomain (PHD) fingers (2). Even though KDM5 members contain several domains capable of binding DNA, it is not clear how they are recruited to specific target genes. Few different mechanisms for chromatin binding have been proposed. For example, ARID domain of KDM5A/RBP2 was shown to bind to sequence-specific DNA motif (7). Other report implicates the PHD3 domain of RBP2, which recognizes H3K4me 3/2 marks to bind chromatin (8). Similarly, PHD1 finger has been shown to bind to unmethylated H3K4 residue (8,9). However, H3K4me3/0 recognition cannot provide target-gene specificity. Therefore, like with other chromatin modifiers, additional factors are likely to contribute to site-specific recruitment.

The KDM5 proteins were discovered earlier but their function as an H3K4me3/2 histone demethylase was discovered later (2–6, 10–13). For instance, RBP2 was initially isolated as a retinoblastoma (pRb) binding protein (13). pRb is a well-characterized tumor suppressor that regulates cell cycle by repressing E2F-family of transcription factors. Though initial reports found that RBP2 and pRb had antagonistic role in differentiation (14), subsequently, it was discovered that RBP2 regulates a large number of E2F-reponsive cell-cycle genes (15–18). Indeed, genome wide analysis revealed that RBP2 co-occupies a large sub-set of E2F4-bound target promoters to induce H3K4 demethy-

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lation and gene repression during differentiation (15,18). Both proteins have been found together in different multi-protein complexes including with pocket protein p130 (15) and Sin 3 (18) but no consensus, on how RBP2 may be recruited to E2F4 target promoters, has emerged. These reports also raise the question of RBP2 involvement, if any, in regulating E2F target genes during cell cycle progression.

The E2Fs regulate cell-cycle genes by periodical and reversible recruitment of the E2F-DP heterodimer to gene promoters. In G_0 or early G1 cells, the E2F-responsive promoters are bound by E2F4/p130 complex and at this time p130 recruits chromatin remodeling enzymes like the Sin3-HDAC, Su(Var) 39 HMT and SWI/SNF to repress transcription (19,20). As cells progress into S phase, E2F4 complex dissociate from genes, giving way to E2F1/pRb complex. E2F1, when freed of pRb by action of cyclin-CDK complexes, recruits histone acetyltransferase and H3K4 HMTs leading to increase in H3 and H4 acetylation, and H3K4 trimethylation; marks associated with active transcription (20,21). These events provide a model in which E2Fs periodically and reversibly recruit histone modifying enzyme complexes to cell-cycle-regulated gene promoters to reset the chromatin landscape during cell-cycle progression. While the mechanism to reverse acetylation marks on E2F-responsive promoters has been worked out in detail, it is still unclear how H3K4me3 marks are removed.

Here, we show that RBP2 associates with E2F4 and pocket protein p130 in a reversible fashion during the cell cycle to bring about the demethylation of H3K4me3 of E2F-responsive promoters. Its interaction with p130 is LxCxE motif dependent. Using p130 shRNA, we show that p130 is required to recruit RBP2 to E2F responsive promoters. We also look at the general mechanism by which RBP2 may be recruited to the chromatin to repress transcription.

MATERIALS AND METHODS

Cloning and site-directed mutagenesis

GST-tagged constructs of full-length E2F4, its deletion mutants, E2F1 and T/E1A domain of p130 protein were cloned by ligating PCR generated fragments into BamH1 linearized pGEX4t1 vector. T/E1A domain of p130 and GFP were cloned in Xho1 linearized pET-14b vector. The PCR products were amplified by high fidelity Phusion Polymerase (NEB) using pCMV E2F4, pCMV E2F1 or cDNA synthesized from U2OS cells (T/E1A domain of p130) as a template. Similarly, fragments of RBP2 were cloned into Sma1 linearized pGEX4t1 vector. SFB–tagged RBP2 was cloned into pcDNA5/FRT vector (Invitrogen). RBP2 was amplified using pcDNA4/TO-RBP2 construct (kind gift from Jun-ichi Nakayama). Single point mutation in GST-RBP2 D5 (E1377K), SFB-RBP2 (E1377K), SFB-RBP2 (W1625A), SFB-RBP2 (H483G, E485Q) and in GST-E2F4^{TAD} M1 (Y392H) and GST-E2F4^{TAD} M2 (D404G, L405 P, F406 L, and D407G) constructs were generated by PCR based mutagenesis. All clones and mutants were verified by sequencing the entire construct.

Cell culture and synchronization

Mouse embryonic fibroblasts, HeLa, and IMR90-tert cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin. Cells stably expressing pcDNA 5/FRT-SFB RBP2 and other point mutants were obtained by transfecting HeLa Flp-In cells (kind gift from S.S. Taylor) as described (22). Cell synchronization was achieved by double thymidine or Nocodazole block. Cells in different stages were collected based on timed release from G1/S phase or G2/M boundary as described before (23). Cells were transfected with shRNAs against p130 transcript (Sigma) using polyethylenimine (Polysciences Inc.), synchronized in early G1 phase, and harvested after 60 h for ChIP (1.5×10^7 for each antibody) or immunoblot analysis (see also Supplementary Figure S3).

Co-immunoprecipitation and immunoblot analysis

Nuclear extract was made as described (24). Co-immunoprecipitation experiments were done using anti-E2F4 (c-20, sc-866, Santa Cruz) or anit-RBP2 antibody (A300-897A, Bethyl Labs, #3876, Cell Signaling Technology) and analyzed by immunoblots scanned on Odyssey infrared imager (LI-COR) as described (23). For pull down experiments with GST tagged proteins, equal amount of purified bead-bound proteins were incubated with HeLa, IMR90-tert cell or MEFs nuclear extract overnight. In case of SFB-tagged proteins, cells were lysed in NETN (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, containing freshly added protease inhibitors, PMSF and Complete EDTA free; Roche) on ice for 20 min, centrifuged, and supernatant was incubated with S-protein beads (Novagen) overnight.

Protein expression and purification

All the GST-tagged and His-tagged proteins were expressed in Rosetta Gami DE3 *Escherichia coli* strain (Novagen). Protein production was induced by adding 0.1 mM IPTG for 6 h at 18°C. The cells were pelleted and lysed in lysis buffer (50 mM Tris pH 7.5,150 mM NaCl, 0.5% NP-40 and 1 mM PMSF), incubated with glutathione agarose beads (Sigma) (or Talon beads; Clontech, in case of His-tagged proteins) at 4°C for 2 h. Subsequently, the beads were washed with ice-cold lysis buffer 3–4 times. Protein concentration was estimated by SDS-PAGE followed by CBB staining.

Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were done as described (25) with the minor modifications. Detailed protocol, antibodies and primers used are provided in supplemental information.

Chromatin affinity purification (ChAP) assay

Approximately 1.5×10^7 stable HeLa Flp-In cells or 3×10^7 transiently-transfected IMR90-tert cells expressing SFB-tagged RBP2 or point mutants were used. Cells were

fixed with 1% formaldehyde for 10 mins at room temperature followed by quenching by 0.125 M Glycine. Two PBS washes were given and the cells were lysed in cell lysis buffer as described in ChIP protocol. This was followed by nuclear lysis and sonication (see ChIP protocol in Supplemental information). The chromatin was diluted by 1× IP dilution buffer and incubated with S-protein beads overnight. The beads were washed sequentially with dialysis buffer two times, IP wash buffer three times followed by TE one time. The DNA was eluted in elution buffer, containing Proteinase K, overnight at 65°C. This was followed by Phenol extraction, RNase treatment and second round of Phenol extraction. The DNA samples were precipitated at –20°C overnight by adding 100% ethanol and analyses by real time PCR.

RNA isolation and real time PCR

RNA isolation and real time PCR experiments were performed as described (26). Primer sequences are provided in Supplementary data.

Statistical analysis

For comparison of two groups Student's *t*-test was used. For comparison of multiple groups Two-way ANOVA followed by Tukeys's or Dunnett's test was employed. The kind of analysis done is indicated in the figure legend.

RESULTS

RBP2 associates with E2F4

In order to study the E2F4 association with RBP2, we expressed E2F4 as a C terminal fusion of Glutathione-S-transferase (GST) protein. Bacterially expressed GST-E2F4 was purified by affinity chromatography on glutathione-agarose beads and incubated with mouse embryonic fibroblasts (MEFs) nuclear extract (NE). E2F4-bound protein complexes recovered from the beads were resolved by SDS-PAGE and analyzed by immunoblotting. As shown in Figure 1A, RBP2 was significantly enriched in the GST-E2F4 sample. We also tested the association of RBP2 with another E2F protein: E2F1, and found that E2F1 was unable to pull down RBP2 under our experimental conditions (see Figure 1A, panel b).

In the same way, a HeLa Flip-In cell line, expressing triple-epitope (SFB short for—S protein-Flag-streptavidin binding peptide,) tagged-RBP2 was used to pull down RBP2 protein using S-protein agarose beads. E2F4 was visibly enriched in SFB-RBP2 over SFB-GFP pull down fractions indicating that RBP2 could associate with E2F4 in mammalian cells (Please see Supplementary Figure S1A in the Supplemental Data available with this article online).

RBP2 associates with E2F4 during early G1 phase

The cellular levels of RBP2 have been reported not to change during cell cycle (15). In contrast, subcellular localization of endogenous E2F4 is known to change during the cell cycle (27,28). Although E2F4 is present in both nucleus and cytoplasm throughout the cell cycle, it is mostly nuclear in G1 phase (27,28). This is also the cell cycle phase when E2F4 is active in repressing E2F-responsive promoters (29,30). Therefore, we questioned if E2F4-RBP2 association is cell cycle stage-specific, and probed for E2F4-RBP2 association in different stages of cell cycle. We synchronized MEFs using nocodazole and isolated cells at various stages by differential timed release of cells in normal media. Cell populations were synchronized for early G1 ($G1_E$), late G1 ($G1_L$), G1/S, and S phases (Supplementary Figure S1C). Consistent with previous report (15), we observed no significant change in the cellular or nuclear levels of RBP2 during the different cell cycle phases (Supplementary Figure S1D and S1E). As shown in Figure 1B, MEFs synchronized in early G1 showed substantial association between RBP2 and E2F4 when compared to MEFs synchronized in G1/S. The cell cycle stage-specific associations observed here are not due to limiting nuclear levels of E2F4, as ample E2F4 protein was present in the NE of both phases analyzed here (Supplementary Figure S1E). Moreover, the amount of endogenous E2F4 immunoprecipitated in both phases was comparable (Figure 1B).

In order to ascertain that RBP2-E2F4 association was not limited to one cell type, we performed similar experiments in HeLa cells. HeLa cells were synchronized in G1/S by using double thymidine block and cells were harvested at different time points as indicated (Supplementary Figure S2A). We observed that RBP2 was present in all cell cycle phases analyzed here (Supplementary Figure S2B and S2C). Similar to our observations in MEFs, RBP2 showed significant association with E2F4 in the $G1_E$ fraction than in the G1/S fractions in HeLa cells as well (Supplementary Figure S2D). This indicates that RBP2 associates with E2F4 in a temporal fashion irrespective of cell type (transformed as well as untransformed cells) or method of synchronization (double thymidine as well as nocodazole block).

RBP2 associates with the transactivation domain of E2F4

In order to map the domain of E2F4, which associates with RBP2, we made GST fusion of E2F4 truncations. Figure 1C illustrates the human E2F4 protein and three deletions used here. We systematically deleted the Transactivation domain (TAD); and the Dimerization domain, used to form heterodimer with DP protein, from the C-terminal of E2F4. Surprisingly, when compared to full-length E2F4 protein, none of the two deletions showed any association with RBP2 (Figure 1D) indicating that either RBP2 only interacted with the full-length E2F4 protein or the TAD was important for this association. We next made the GST fusion of E2F4 TAD (GST-$E2F4^{TAD}$) and probed it for association with RBP2. The 76 amino-acid TAD was able to pull down RBP2 from the HeLa cell NE, indicating that this domain was sufficient for association with RBP2 (Figure 1E panel a, lane 5).

E2Fs including E2F4 are known to interact with the pocket proteins via their Transactivation domain (31,32; see the schematic in Figure 1C). RBP2 was discovered in a screen for cellular proteins that bind to pRb and has been reported to interact with the second pocket protein—p107 (13,33). Therefore, we wanted to ascertain if the E2F4-RBP2 association observed here was direct or via the pocket

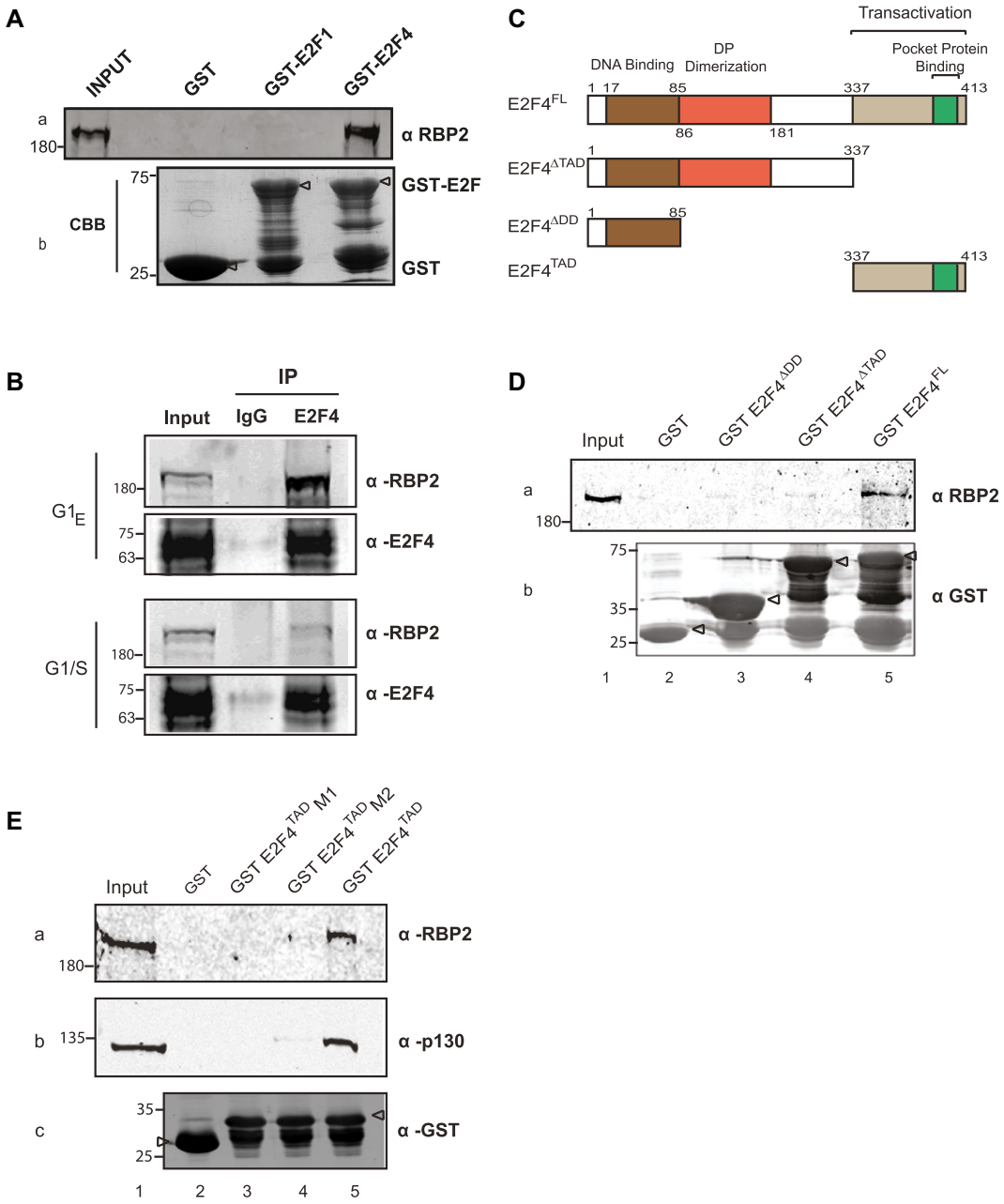

Figure 1. RBP2 associates with the Transactivation Domain of E2F4. (**A**) Association of GST-E2F4 with endogenous RBP2. Bacterially expressed GST, GST-E2F4 and GST-E2F1 were used for pull down experiment using nuclear extract (NE) produced from Mouse embryonic Fibroblasts (MEFs). The blot was probed with anti-RBP2 antibody (panel a). Panel b shows the bead-bound GST, GST-E2F1 or GST-E2F4 proteins stained with Coomassie Brilliant Blue (CBB). (**B**) Cell cycle-stage specific immunoprecipitation of E2F4 from mouse embryonic Fibroblasts. MEFs were synchronized by Nocadazole block and timed release in normal media to obtain cells populations in early G1 ($G1_E$) or G1/S stage (see also Supplementary Figure S1 and S2). NE from these phases was immunoprecipitated by E2F4 or IgG antibody. The blots were probed with anti-RBP2 and anti-E2F4 antibodies as indicated. E2F4 antibody used for immunoblotting is described in Supplementary Figure S1B. (**C**) Schematic structure of E2F4 protein. Full-length and deletions of E2F4 used in this study are shown. All deletions were expressed as C-terminal fusion of GST protein. Functional domains are indicated on top. Numbers indicate amino acids. FL, full length; TAD, transactivation Domain; DD, DP protein dimerization domain. (**D**) Mapping of E2F4 domain which associates with RBP2. GST-tagged $E2F4^{FL}$ (lane 5) and its deletions: GST-$E2F4^{\Delta TAD}$ (lane 4) and GST-$E2F4^{\Delta DD}$ (lane 3), were used for pull down experiment from HeLa NE. The blots were probed with anti-RBP2 (panel a) and GST (panel b) antibody. (**E**) The Association between E2F4 and RBP2 is mediated by p130. GST-$E2F4^{TAD}$ (wild type, lane 5) and its mutant forms GST-$E2F4^{TAD}$ M1 (Y 392 H, lane 3) and GST-$E2F4^{TAD}$ M2 (D 404 G, L405 P, F 406 L, and D 407 G, lane 4) (see also Supplementary Figure S3A) were used for pull down experiment from HeLa cell NE. The blot was probed with anti-RBP2 (panel a), p130 (panel b) and GST (panel c) antibody. (A–B, D–E) The positions of the molecular weight markers are indicated on the *left*.

protein associated with E2F4. As shown above, we observed maximum E2F4-RBP2 association in $G1_E$ phase (Figure 1B) and p130 associates with E2F4 at this time (34). Correspondingly, we probed the immunoblot with p130 antisera. As expected, p130 was present in the GST-E2F4^{TAD} pull down (Figure 1E panel b). In order to establish that this observed RBP2-E2F4 association was being mediated by the pocket protein (p130 here), we took advantage of the conservation of pocket protein-binding domain in E2Fs (31,32) and created two sets of amino acid mutations in the GST-E2F4^{TAD}, M1 (Tyr 392 His) and M2 (Asp 404 Gly, Leu 405 Pro, Phe 406 Leu, and Asp 407 Gly) (Supplementary Figure S3A). Both sets of amino acids mutations have been shown to abolish the E2F—pocket protein interaction previously (31,32). p130 associated with the wild type GST-E2F4^{TAD} but not GST-E2F4^{TAD} M1 and GST-E2F4^{TAD} M2 (Figure 1E panel b, compare lane 5 with lane 3 and 4). These results are consistent with the conservation of E2F—pocket protein-binding and the interaction of pRb with other E2Fs (31,32). However, when probed for RBP2, only wild type GST-E2F4^{TAD} (Figure 1E, panel a) showed significant association with RBP2 but not the mutants. These results indicates that RBP2-E2F4 association might be mediated by the pocket protein p130 and when we disrupt the E2F4-p130 interaction, p130-bound RBP2 is also not detected.

p130 interacts with RBP2

Our results suggested that p130 could be mediating the association between RBP2 and E2F4 proteins. To test this hypothesis, we depleted p130 in HeLa cells using shRNA (Supplementary Figure S3B and S3C). As individual shRNAs were not able to achieve desired levels of p130 knockdown, we used a combination of two shRNAs (#3 + 5, Supplementary Figure S3C, Figure 2A panel a). We used GST-E2F4^{TAD} to pull down RBP2, as before, from nuclear extracts of cells treated with scrambled non-targeting or p130 shRNA. While in the samples treated with scrambled shRNA, GST-E2F4^{TAD} showed robust association with RBP2, the E2F4-RBP2 association was substantially decreased in p130 shRNA treated samples (Figure 2A). These results indicated that for E2F4 to interact with RBP2, p130 is important.

Next we wanted to test if p130 will interact with RBP2. To achieve this, we fused the conserved T antigen /E1A protein-interacting domain (T/E1A domain, residues 417–1024), also called the 'small pocket' of p130 to C terminal of GST and used this fusion protein to pull down RBP2 from MEFs NE as described above. p130 was able to pull down RBP2 robustly and specifically (Figure 2B). We also performed the similar experiments with nuclear extracts from HeLa and IMR90-tert cells (Supplementary Figure S3D and S3E). In both cases, p130 could pull down substantial amounts of RBP2, indicating that RBP2-p130 interaction was seen in multiple cell types. As expected, E2F4 was interacting with p130 in all cases (see panel b in Figure 2B, Supplementary Figure S3D, S3E). The interaction between endogenous RBP2 and p130 proteins was tested by immunoprecipitating RBP2 using specific antibody from HeLa cells. Both endogenous p130 as well as E2F4 associated strongly with RBP2 as shown (Figure 2C).

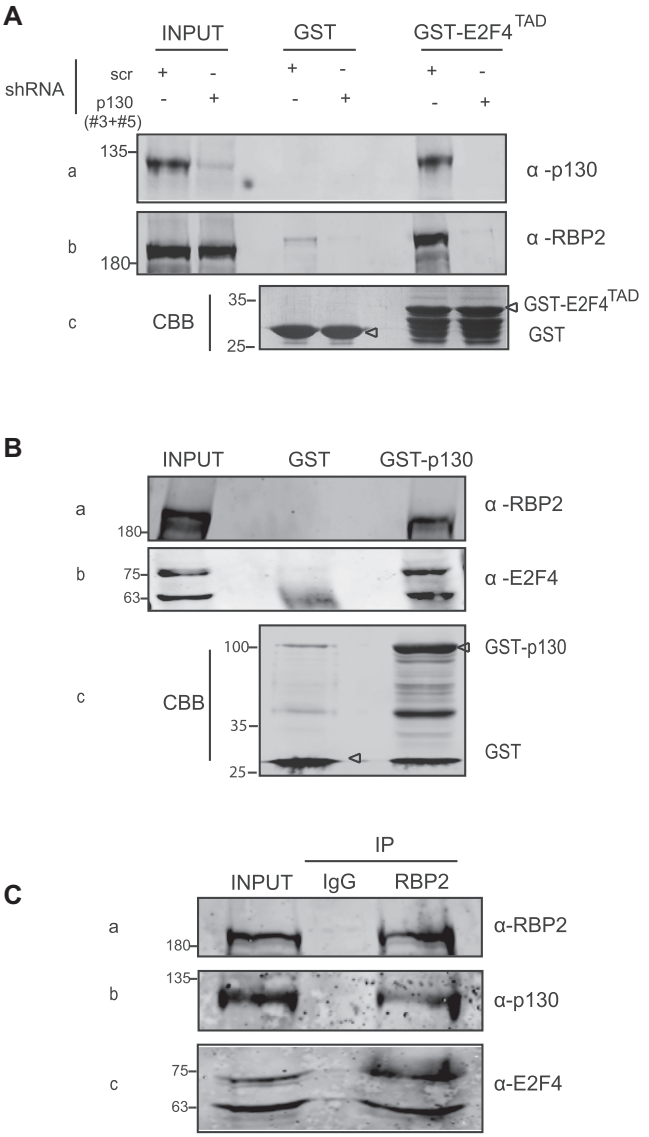

Figure 2. p130 interacts with RBP2. (**A**) The association of E2F4 with RBP2 is mediated by p130. HeLa cells were transfected with shRNA either targeting p130 (two shRNA: #3and #5,were used for efficient knockdown) or Scramble (non-targeting) shRNA (see also Supplementary Figure S3B and S3C). Nuclear extract was prepared from both types of cells and used for pull-down experiment using GST or GST-E2F4 ^{TAD}. The blot was probed with antibody against p130 (panel a) and RBP2 (panel b). Panel c shows bead-bound GST or GST-E2F4^{TAD} proteins stained with CBB. (**B**) The interaction between p130 and RBP2. MEFs were used for making nuclear extract and pull-down experiment was performed using GST or GST-p130 T/E1A (small pocket). The blot was probed with antibodies against RBP2 (panel a) and E2F4 (panel b). Panel c shows the CBB staining of bead-bound protein used in each lane (see also Supplementary Figure S3D and S3E). (**C**) Endogenous RBP2 interacts with endogenous p130. Endogenous RBP2 was immunoprecipitated from Hela cell NE with anti-RBP2 or IgG antibody and immunoblotted with anti- RBP2 (panel a), anti-p130 (panel b) and anti-E2F4 (panel c) antibody. (A–C) The positions of the molecular weight markers are indicated on the *left*.

p130–RBP2 interaction is LxCxE motif dependent

RBP2 is a large protein with multiple domains like PHD, Zinc finger etc. (see Introduction and Figure 3A). In order to map the domain of RBP2, which interacted with p130,

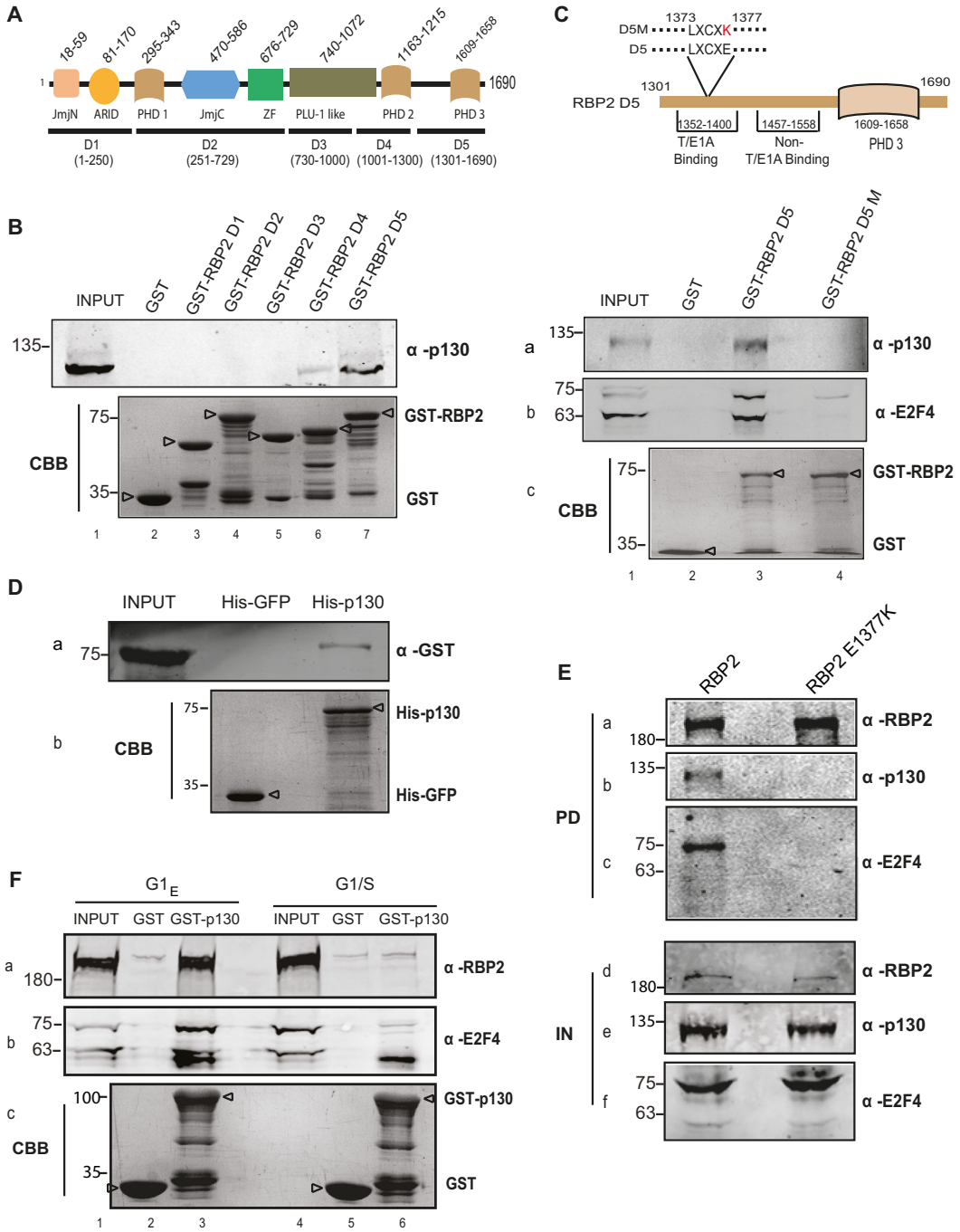

Figure 3. p130 interacts with RBP2 via the LxCxE motif. (**A**) Schematic structure of RBP2 protein. Functional domains are indicated at the bottom. Deletions of RBP2 (D1 to D5) used in this study are indicated below the domains in bold lines. All deletions were fused to C-terminal of GST. Numbers indicate amino acid residues. JmjN/JmjC, N/C-terminal Jumonji domain; ARID, AT-rich interacting domain, PHD, plant homeodomain; ZF, zinc finger. (**B**) Mapping of p130-interacting domain in RBP2. GST and GST-tagged deletions of RBP2 were used for pull down experiment. The blot was probed with anti-p130 antibody (top panel). Bead-bound GST or GST-RBP2 deletion proteins stained with CBB are shown in bottom panel. (**C**) The interaction between RBP2 and p130 is LxCxE motif dependent. On the top, the schematic shows the position of LxCxE motif in deletion RBP2 D5. RBP2 D5 mutant (E1377K) was created by changing Glutamate 1377 to Lysine (see also Supplementary Figure S3F). Numbers indicate amino acid residues. At the bottom, GST-RBP2 D5 (wild type, lane 3) and its mutant GST-RBP2 M (E1377K, lane 4) were used for pull-down experiment. The blot was probed with anti-p130 (panel a) and E2F4 (panel b) antibody. Bead-bound GST or GST-RBP2 deletions stained with CBB are shown in panel c. (**D**) *In vitro* interaction between RBP2 and p130. Bacterially expressed His-p130 T/E1A or His-GFP were used for pull-down of GST-RBP2 D5 from the bacterial lysate. The blot was probed with anti-GST antibody (panel a). Panel b shows the amount of proteins used for pull-down. (**E**) LxCxE motif in full-length RBP2 is major determinant for interaction with p130. Lysate from HeLa cells stably expressing SFB-RBP2 wt or SFB-RBP2 E1377K were used for pull-down experiments using S-protein beads. The blot was probed with RBP2 (panel a, d), p130 (panel b, e) and E2F4 (panel c, f) antibody. Pull-down (PD) and input (IN) are shown as indicated. (**F**) Cell-cycle phase selective association of p130 and RBP2. GST or GST-p130 T/E1A (GST-p130) was used for pull down experiments from early G1 ($G1_E$) and G1/S NE derived from MEFs. The blots were probed with anti-RBP2 (panel a) and E2F4 (panel b) antibody. Panel c shows bead-bound GST or GST-p130 stained with CBB. **(B-F)** The positions of the molecular weight markers are indicated on the *left*.

we created five fragments of RBP2 protein as shown in the schematic in Figure 3A and expressed them as GST fusions. Although fragment 4 of RBP2 (D4, see Figure 3B) showed weak association with p130, only fragment 5 (D5, Figure 3B, compare lane 6 and 7) was able to pull down p130 reproducibly. Fragment 5 contains the PHD3 domain of RBP2 as well as the conserved leucine-X-cysteine-X-glutamic acid motif (LxCxE, where X is any amino acid, Figure 3C and S3F) (33). The proteins having LxCxE motifs are known to associate with pocket proteins; and the T/E1A domain of both pRb and p107 interact with RBP2 via the LxCxE motif (33). Interestingly, the mutation in LxCxE motif in RBP2 is sufficient to abrogate its interaction with p107 but not pRb (33). RBP2 has 15kDa fragment ('non-T/E1A binding' domain), which contributes to pRb interaction in LxCxE mutant background. This 15kDa fragment is independent of LxCxE motif but present in our RBP2 D5 fragment (Figure 3C, top). Therefore, to test if binding of RBP2 to p130 was LxCxE motif-dependent, we mutated glutamic acid to lysine (here E1377K), a mutation that is known to abrogate the LxCxE mediated interactions (33). As shown in Figure 3C (panel a), the RBP2 D5 (E1377K) mutant could not pull down p130 like the wild type. The RBP2 D5 (E1377K) mutant also lost its ability to associate with E2F4 (Figure 3C, panel b) further confirming our results from Figures 1E and 2A, that RBP2 does not associate directly with E2F4. We further tested the association between RBP2 D5 and His-p130 T/E1A domain and found them to interact, albeit weakly (Figure 3D). Even though bacterially expressed RBP2 associates with bacterially expressed p130, this interaction may be stabilized in cells, most likely by a post translational modification.

In order to ascertain that RBP2 associated with p130 only via the LxCxE motif, we mutated the LxCxE motif (E1377K) in full-length RBP2. As described in Supplementary Figure S1A, we made cell lines using HeLa Flip-In system which stably expressed SFB-RBP2 and SFB-RBP2 (E1377K). We used cell lysate to pull down full-length SFB-RBP2 and SFB-RBP2 (E1377K) and probed for associated proteins (Figure 3E). When full-length LxCxE point mutant [SFB-RBP2 (E1377K)] was used for pull down, the RBP2-p130 interaction was abrogated (as also seen in Figure 3C). These results indicated that RBP2 LxCxE is the primary motif for interaction with p130 and a point mutation in this motif is enough to destabilize the p130–RBP2 interaction. We also note that in its interaction with RBP2, p130 behaves like p107 (33; this study).

RBP2 interacts with p130 and E2F4 in early G1 phase

We detected the association of RBP2 with E2F4 in early G1 phase. Is the interaction of p130 with RBP2 also cell-cycle-stage specific or RBP2 and p130 associate with each other throughout the cell cycle? In order to answer this question we used MEFs NE from cell populations in early G1 and G1/S phase to probe for p130–RBP2 association. RBP2 associated with p130 in early G1 phase and not G1/S (Figure 3F). We probed the same blot for E2F4 and, as expected, E2F4 associated with p130 primarily in early G1 phase. The same results could be reproduced in differential synchronized HeLa cells (Supplementary Figure S3G). Our results suggest that RBP2 complexes with p130 and E2F4 in early G1, a phase in which E2F4 and p130 actively repress E2F-responsive promoters.

Binding of RBP2 to E2F responsive promoters is regulated during cell cycle

RBP2 has been shown to bind to E2F-responsive promoters during differentiation (15,18). To extend this observation to dividing cells, we asked whether RBP2 associated with E2F-responsive promoters during the cell cycle. As the interaction of RBP2 with p130 and E2F4 is primarily seen in early G1, we used double-thymidine synchronized HeLa cells from two cell-cycle stages for performing our chromatin immunoprecipitation (ChIP) experiments—early G1, where these promoters are inactive due to repressive E2Fs binding, and G1/S phase, where these promoters are active and repressive E2Fs are displaced by activating E2Fs (30). We selected 6 E2F-regulated promoters that have been studied before (15,17,18,23,30). For negative control, we used U2 snRNA gene ($U2_C$) (23). We also analyzed two mitochondrial promoters to which RBP2 binds, but these promoters are not known to be E2F-responsive or cell cycle regulated—ATP50 and MTRF1 (17,35,36; this study). Consistent with previous reports, we observed that association of E2F4 and p130 proteins on these E2F-responsive promoters was prominent in early G1 while E2F1 protein showed binding predominantly in G1/S fraction (Figure 4A). Consistent with our hypothesis and cell cycle stage-specific association of RBP2 with E2F4 and p130, RBP2 bound these promoters primarily in early G1.

Previously we have shown that H3K4me3 was deposited on E2F-responsive promoters in G1/S and S phase, by recruitment of H3K4 HMTs in these cell-cycle phases, to activate transcription (23,37). In accordance with previous results, we observed high fold enrichment of H3K4me3 mark on E2F-responsive promoters in G1/S over early G1 samples (Figure 4B, Supplementary Figure S4A). However, we could not obtain reproducible variation in H3K4 dimethyl mark in the two fractions (Figure 4B).

Next we used MEFs, synchronized using nocodazole block, to perform similar ChIP experiments in early G1 and G1/S phase. Consistent with our observations in HeLa cell, RBP2 like p130 and E2F4, was enriched in early G1 on E2F-responsive promoters when compared to G1/S (Figure 4C, Supplementary Figure S4B). Correlatively the H3K4me3 mark was low in early G1 and high in G1/S fractions while no significant variations were observed in histone H3 (Figure 4D, Supplementary Figure S4C).

p130 recruits RBP2 to E2F-responsive promoters for demethylation

Our results so far suggest that RBP2 may be recruited to E2F-responsive promoters by p130 to erase the H3K4me3 mark and prepare the promoters for next cycle of activation. If this hypothesis is correct then loss of p130 by RNAi should lead to loss of RBP2 recruitment to E2F-responsive promoters during the early G1 phase. We put our hypothesis to test by depleting p130 in HeLa cells using shRNA as described before (Supplementary Figure S3C), synchronizing them in early G1 and performing ChIP with these cells.

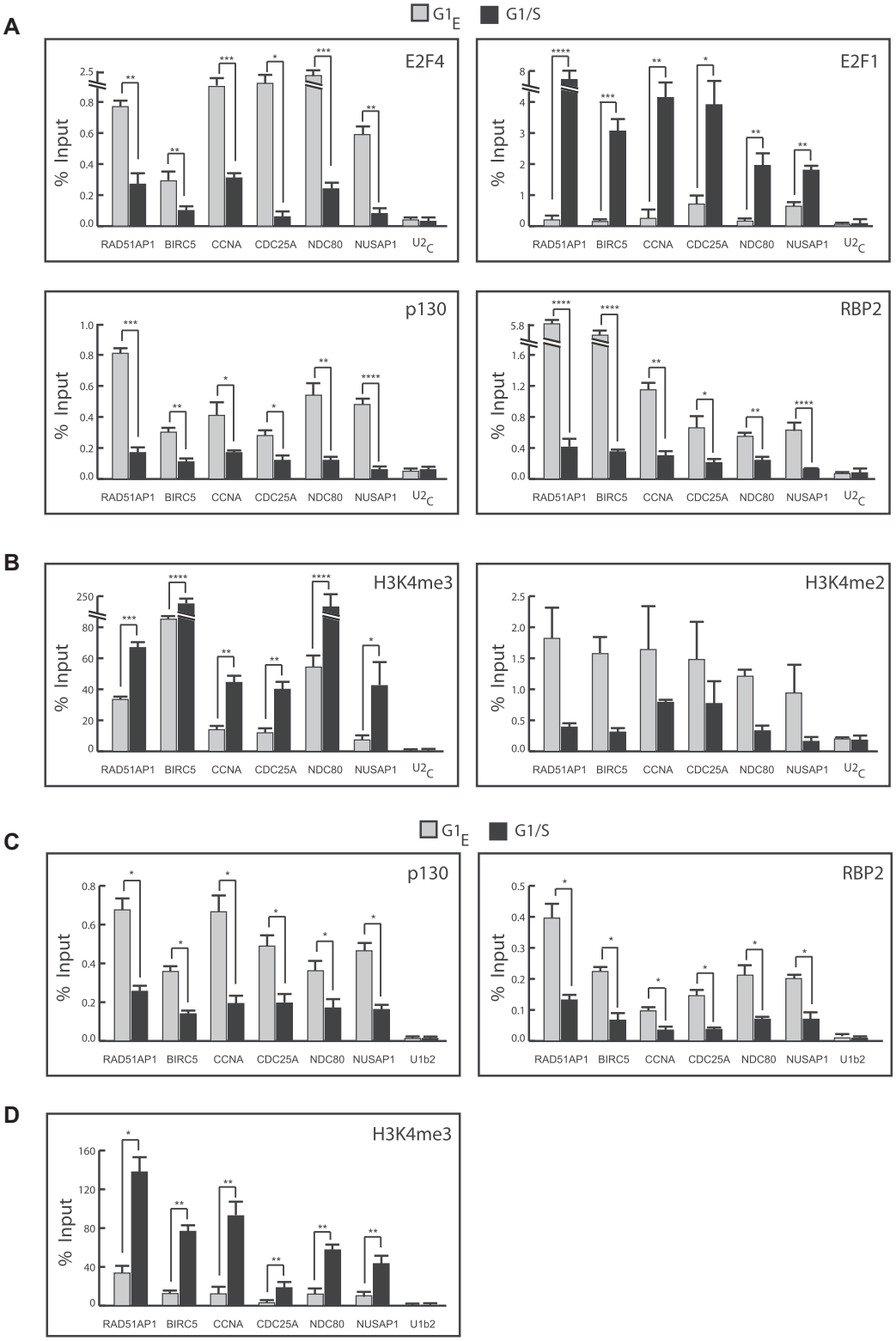

Figure 4. p130 and RBP2 bind to the E2F responsive promoters in a cell-cycle stage specific manner for demethylation of H3K4me3. (A, B) p130 and RBP2 bind to E2F-responsive promoters in early G1 phase. Chromatin immunoprecipitation (ChIP) assay with E2F4, E2F1, p130 and RBP2 (**A**) and, H3K4me3 and H3K4me2 (**B**) antibodies, performed on the HeLa cells synchronised in early G1 ($G1_E$; light grey) and G1/S (black) phase using double thymidine block are shown. See Supplementary Figure S4A for H3 and IgG ChIP. All experiments shown were done at least three, usually more times with similar results. (C, D) ChIP assay with p130 and RBP2 (A) and, H3K4me3 (B) antibodies, performed on mouse embryonic fibroblasts synchronized in early G1 ($G1_E$; grey) and G1/S (black) phase, using Nocodazole block, are shown. See Supplementary Figure S4B for E2F4 and S4C for H3 and IgG ChIP. The data shows average of two independent experiments. (**A–D**) The immunoprecipitated DNA was quantified with real-time PCR and the results are plotted as percent input enrichment. The antibody used is indicated in the top right corner of each box. The error bars represent S.D. Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, ns: not significant, $P > 0.05$.

Consistent with previous reports, we did not observe any significant effect on cell cycle progression in p130-shRNA treated cells (38; Supplementary Figure S5A).

As shown in Figure 5A, p130 shRNA transfection depleted majority of p130 protein. As a consequence, the p130 binding on E2F-responsive promoters was also reduced (Figure 5B). Consistent with our hypothesis, there was analogous decrease in the RBP2 binding to these promoters. However, we also observed a decreased binding of E2F4 on these promoters. It has been shown that the nuclear localization of E2F4 is impaired in absence of p130 (28,39; see also Supplementary Figure S5B) and this can be a reason for low E2F4 binding in our experiments as the cellular levels of E2F4 (and RBP2) were largely unaffected upon p130 knockdown (Figure 5A). In any case, this experiment proves our hypothesis where E2F4 and p130 recruit RBP2 to E2F-responsive promoters, and RBP2 removes the H3K4me3 mark to reset the E2F-responsive promoters and repress transcription. Consistent with the latter, and decreased RBP2 binding, H3K4me3 mark was significantly increased on E2F-responsive promoters, but not globally (Figure 5C and A panel d). However, p130 knockdown did not change the levels of histone H3 in the same experiments (Supplementary Figure S5C). Our results indicate that just like acetylation marks, H3K4me3 also needs to be actively removed during the cell cycle progression.

We also analyzed the non-E2F-responsive promoters ATP50 and MTRF1. First we ensured that these promoters are negative for E2F4 and p130 binding in early G1 phase (Supplementary Figure S5D). Then we checked if the levels of RBP2 varied during early G1 and G1/S phase on these promoters (Supplementary Figure S5E). As expected, RBP2 bound to both promoters in similar fashion during early G1 and G1/S phases. Finally, we tested the effect of p130 knockdown on RBP2-binding on these promoters. ATP50 and MTRF1 did not show any significant variation in RBP2 binding in control vs. knockdown samples (Figure 5B). Similarly, the H3K4me3 levels were largely unaffected on these promoters upon p130 knockdown (Figure 5C, Supplementary Figure S5F). These results indicate that p130 is engaged in recruitment of RBP2 to E2F-responsive promoters specifically and recruitment of RBP2 to non-E2F-responsive promoters may be carried out in different manner.

Different mechanisms for recruitment of RBP2 to chromatin

The dramatic decrease in RBP2 binding upon p130 knockdown was a little surprising, given that RBP2 has three domains capable of binding to the chromatin, namely ARID (sequence specific binding), PHD1 (binds to H3K4me0) and PHD3 (binds to H3K4me3) (7–9). All these bindings have been mapped to single amino acid residue (7–9). In order to understand the role of RBP2 binding at E2F-responsive promoters better, in addition to SFB-RBP2 wild type (wt.; described above) and RBP2 LxCxE mutant (E1377K), we made HeLa Flip-In cell lines stably expressing RBP2 PHD3 mutant (W1625A), incapable of binding H3K4me3 (8). We also created a catalytic dead mutant of RBP2 (H483G, E485Q) based on previously published reports (2,4). Clones expressing similar amount of RBP2 protein were selected for further experiments. [We tried to make cell lines expressing point mutants of ARID and PHD1 domain that have been proposed to abrogate chromatin binding (7,9) however we were unsuccessful despite several attempts.]

We used S-protein-agarose beads to pull down ectopically expressed RBP2 and performed Chromatin Affinity Purification (ChAP) experiments (40,41). A fraction of the beads were analyzed by immunoblot to determine the amount of RBP2 protein being pull down in each cell line and its interaction status with p130 (Supplementary Figure S6A). Consistent with our previous results, RBP2 wt. was able to interact with p130 but not the RBP2 (E1377K) mutant. Other two RBP2 point mutants were capable of interacting with p130, indicating that only LxCxE motif affects the RBP2-p130 protein interaction.

In our ChAP experiments, the recombinant SFB-RBP2 wt. showed specific binding to RBP2 positive promoters (Figure 6A). Pleasantly, LxCxE mutant RBP2 E1377K was impaired in binding to E2F-responsive promoters (Figure 6A) but not the non-E2F responsive promoters, while the catalytic dead mutant RBP2 (H483G, E485Q) showed binding comparable to wild type protein in all cases. We observed the same binding pattern for RBP2 and mutants when we transfected non-transformed cells—IMR90-tert—with above constructs and performed ChAP (Supplementary Figure S6B). Mutation in PHD3 (W1625A) domain also compromised the binding of RBP2 to E2F-responsive promoters, but in contrast to LxCxE mutation, its effects were seen on all promoters including ATP50 and MTRF1 (Figure 6A). These results indicate that while RBP2 may use the PHD3 domain as a general mechanism to bind its target promoters, site-specific recruitment by extrinsic factors (here p130) is also employed.

We also utilized the above cell lines to validate our findings in Figure 5C. We performed H3K4me3 ChIP in cell lines expressing either SFB-RBP2 wt or RBP2 mutant deficient in binding p130 (i.e. E1377K). Expression of wild type RBP2 decreased the levels of H3K4me3 on E2F-responsive promoters, corroborating our observations from Figure 5C. In direct contrast, the LxCxE mutant of RBP2 behaved like the empty vector in its ability to remove H3K4me3 marks (Figure 6B), although histone H3 levels were unchanged in all samples (Supplementary Figure S6C). This observation was not reproduced in non-E2F-responsive promoters, where both the wt. and the LxCxE mutant RBP2 displayed similar effect on H3K4me3 levels (Figure 6B). Similar results were obtained with IMR90-tert cells indicating that RBP2 regulates E2F-specific promoters in different cell types (Supplementary Figure S6D).

RBP2 regulates E2F-responsive promoters

Finally, we checked the mRNA levels of E2F-responsive genes in cells stably expressing the various recombinant RBP2 proteins. Recombinant RBP2 expression causes decrease in gene expression (2,4–6). Consistent with these observations, we also detected a decrease in transcript levels in samples expressing wild type RBP2 when compared to untransfected HeLa and IMR90-tert cells (Supplementary Figure S7A and S7B respectively). However, the cells ex-

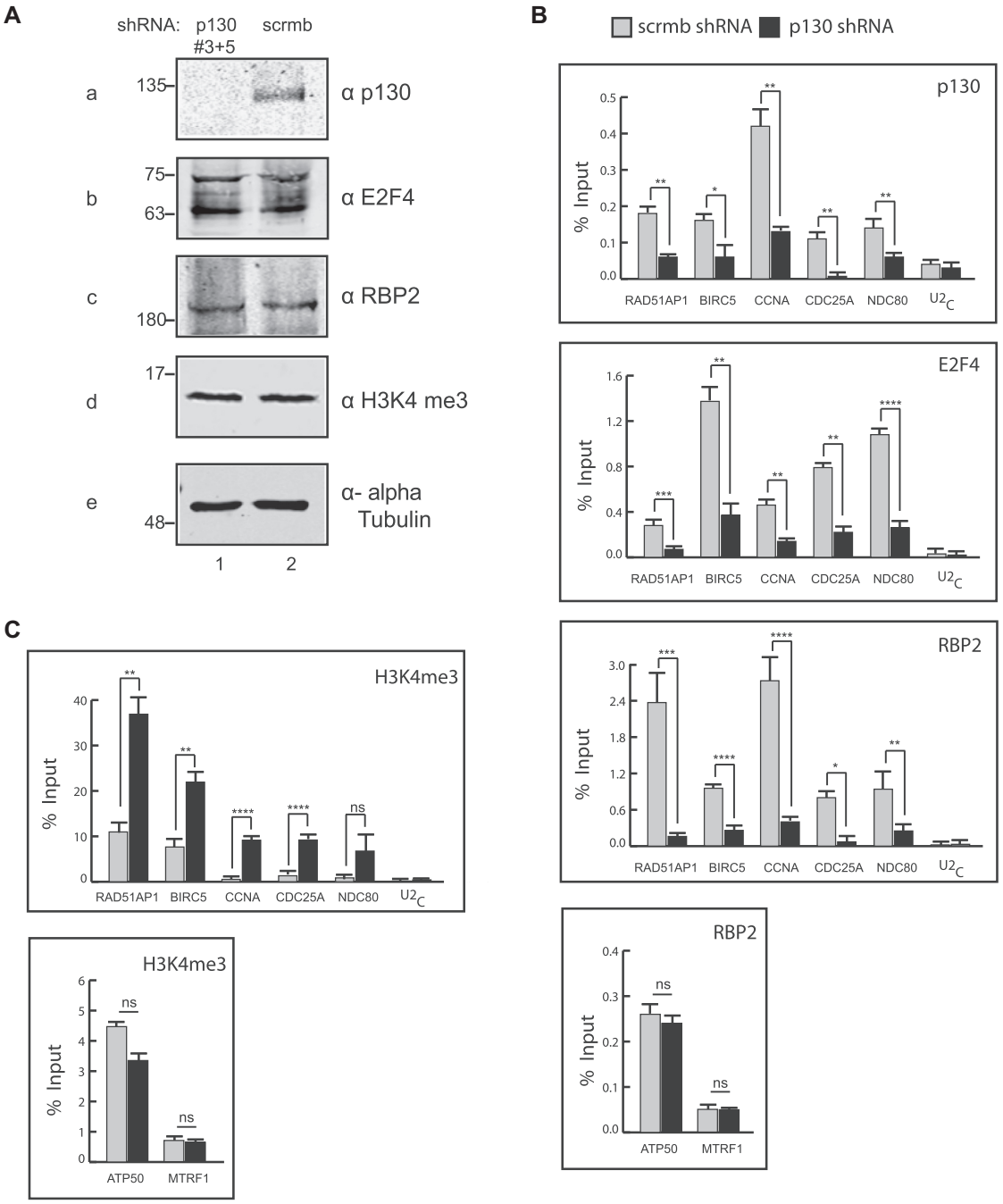

Figure 5. Removal of p130 leads to decreased recruitment of RBP2 and increase H3K4me3 on E2F promoters. (**A**) Western blot showing the levels of different proteins upon p130 knockdown. HeLa cells treated with two p130 shRNAs (shRNA#3 and shRNA #5), or scrambled (scrmb) shRNA were subject to immunoblot analyses. The blot was probed p130 (panel a), E2F4 (panel b), RBP2 (panel c), H3K4me3 (panel d) and alpha-tubulin (panel e) antibodies. See also Supplementary Figure S5. The positions of the molecular weight markers are indicated on the *left*. (**B, C**) Knockdown of p130 leads to decrease in recruitment of RBP2. HeLa cells transfected with shRNA, which either targets p130 transcripts (shRNA #3 and #5; black) or non-specific scramble (grey), were used for performing ChIP experiment with indicated antibodies in early G1 phase. Scrmb; scramble shRNA. See Supplementary Figure S5C for H3 and IgG ChIP. The immunoprecipitated DNA was quantified with real-time PCR and the results are plotted as percent input enrichment. The antibody used is indicated in the top right corner of each box. All experiments shown are done at least three times with similar results; the results from single representative experiment is shown here. The error bars represent S.D. Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, ns: not significant, $P > 0.05$.

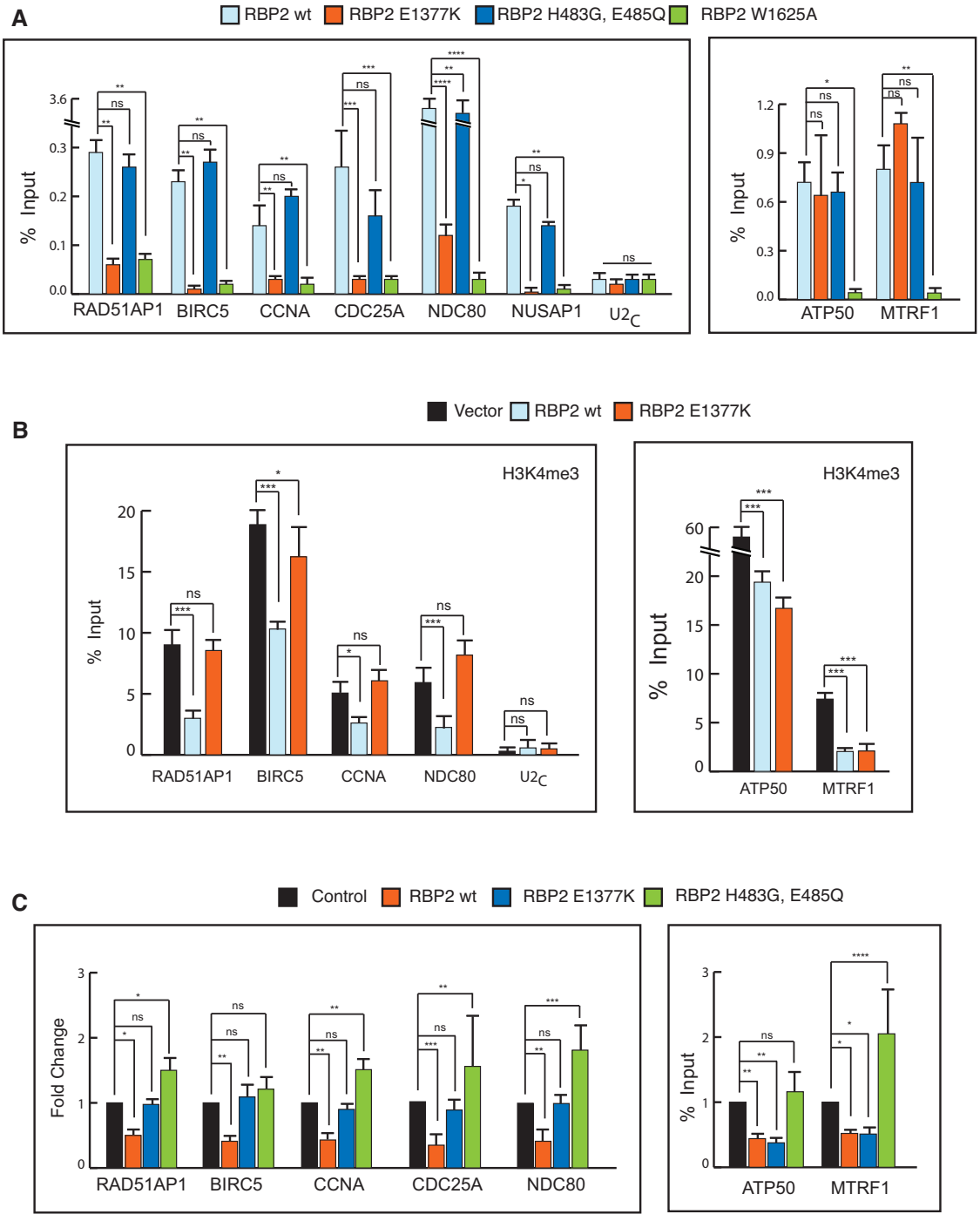

Figure 6. Interaction with p130 is essential for the recruitment of RBP2 onto the E2F-responsive promoters. (**A**) Mutation of LxCxE motif in RBP2 leads to loss of binding of RBP2 to the E2F-responsive promoters. Chromatin affinity purification (ChAP) assay was performed in HeLa cells stably expressing either wild type (wt.) SFB-RBP2, SFB-RBP2 (E1377K), SFB-RBP2 (H483G, E485Q) or SFB-RBP2 (W1625A) are shown. The amount of RBP2 pulled down in each sample is shown in Supplementary Figure S5A. The error bars represent S.D. Tukey's multiple comparison test $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, ns: not significant, $P > 0.05$. (**B**) ChIP assay was performed in cells expressing either RBP2 wt., RBP2 E1377K or empty SFB-pcDNA5-FRT vector. The H3K4me3 levels on different promoters are shown. H3 levels are shown in Supplementary Figure S6B. The error bars represent S.D. Tukey's multiple comparison test was employed to calculate the significance $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, ns: not significant, $P > 0.05$. (**C**) RBP2 affects transcription of E2F-responsive gene. G1/S synchronized cells were treated with either siRNA against 3′UTR region of RBP2 or control (luciferase) siRNA in cells stably expressing either empty vector, RBP2 wt., RBP2 E1377K or RBP2 H483G, E485Q. cDNA synthesized from total RNA was amplified using real-time PCR for indicated mRNAs. All signals were normalized to actin mRNA levels from respective samples. The expression level is relative to control siRNA-treated cells (which is arbitrarily set to 1) from respective cell line but for ease of understanding only one control bar is shown. The error bars represent S.D. Dunnett's multiple comparison test was employed to calculate the significance $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, ns: not significant, $P > 0.05$.

pressing E1377K and W1625A mutant proteins displayed either no change or an unexpected increase (in case of HeLa cells which we are not able to explain) in transcript levels when compared to empty-vector transfected samples, indicating that these promoters were not repressed (Supplementary Figure S7A and S7B respectively). Consistent with these observations, the LxCxE mutant RBP2 did not show any significant change in the cell cycle profile when compared to wild type RBP2 protein (Supplementary Figure S7C).

In order to ensure that endogenous RBP2 is not a factor in our interpretation, we made use of RBP2 RNAi. We used siRNA targeting the 3′UTR of RBP2 mRNA to knock down endogenous RBP2 but not the recombinant RBP2 (expressed from SFB constructs; Supplementary Figure S7D and S7E). After siRNA transfection, we synchronized the cells in G1/S— the phase where expression of these genes has been reported (42). Our results show that similar to observation made in Supplementary Figure S7A and S7B, overexpression of RBP2 causes decrease in gene expression when compared to control (Figure 6C). The LxCxE mutant as well as catalytic dead mutant showed either no change or an increase in gene expression on E2F-responsive genes as observed before (Supplementary Figure S7A and S7B). However, on non-E2F responsive promoters, the LxCxE mutant behaved like the wild type protein, while the catalytic dead mutant was still unable to show repression, indicating that RBP2 catalytic activity was important for the repression of all RBP2 target genes. Altogether, our results indicate that the LxCxE mediated recruitment of RBP2 (by p130) to E2F-responsive promoters is essential for its function as a demethylase and as a transcriptional repressor.

Taken together our results reveal that RBP2 has a role in repression of E2F-responsive promoters and chromatin binding of this protein is requisite for its activity. Our results indicate that while RBP2 may have a general mechanism to reach its target promoters and repress them, site-specific recruitment is also employed during the cell cycle to ensure cyclic, reversible and precise recruitment.

DISCUSSION

The H3K4me3 mark is modulated in a cell cycle stage-specific manner to reset the E2F responsive promoters from active state to repressed state (23). However, experimental evidence on how this was achieved, was lacking. Here, we show that RBP2 interacts with the pocket-protein—p130—via the LxCxE motif and p130 recruits RBP2 to E2F-responsive promoters in a cell cycle-specific manner. During early G1, RBP2 demethylates the E2F-regulated cell-cycle gene promoters to bring about repression. Our findings highlight how, in contrast to permanent silencing, multiple layers of regulation are enforced on epigenetic modifiers to ensure their dynamic, periodical and specific recruitment to cell cycle gene promoters.

RBP2 associates with E2F4 and p130 in early G1 phase

We show that RBP2 associates with E2F4 via p130 in cycling cells. Previously, although a functional relationship has been recognized between RBP2 and E2F4 using genome–wide location analyses during differentiation (15,18), physical association between the two proteins had not been established. Previous efforts to show associations might have been unsuccessful because of the fact that RBP2 is chromatin bound protein and not easily released from the chromatin fraction. We used high salt extraction during our nuclear extract preparation (24), which releases RBP2 from the chromatin and makes it available for interaction. Similar results were reported previously where only high salt extraction was able to produce interaction between pRb and RBP2 (14). Exogenous expression of RBP2 is another way to make this protein available for interaction. Even though we tested the association of E2F4–p130–RBP2 here, E2F5, another 'repressor' E2F has been known to bind p130 (19). It is likely that E2F5 may also play a role in recruitment of RBP2 in regulation of E2F-responsive promoters.

Our results also show that RBP2 and p130 interaction is cell cycle-stage-specific despite the fact that both proteins are present throughout the cell cycle. What limits this association to early G1? Most likely a post-translational modification may be requisite. In support of this hypothesis, out of the three pocket proteins, only p130 is known to undergo phosphorylation during early G1 phase (43,44). RBP2 itself may be post- translationally modified in early G1 leading to this interaction (45–47). In context of the LxCxE binding between pocket proteins and its interacting partner, even though these proteins are capable of binding directly, post-translational/ phosphorylation events are known to regulate this binding (48,49) and may provide a perfect mechanism to turn on and turn off the p130–RBP2 interaction in accordance with growth signals.

The relationship of RBP2 with the three pocket proteins

RBP2 has been shown to interact with pocket proteins—pRb and p107 previously (13,14,33) and p130 (this study). The pocket protein family members contain conserved domain A and B linked by a spacer region, together called the 'pocket' or 'T/E1A binding' domain. This region is important for binding to LxCxE motif present in numerous viral and cellular proteins (50). The LxCxE motif is present in RBP2 and responsible for its interaction with all three pocket-proteins. However, RBP2 can interact with pRb in an LxCxE-motif-independent manner through a 15kDa region called 'non-T/E1A binding' domain (see Figure 3C; 33). This is also reflected in its interaction with pRb Δ663, which shows decreased binding to LxCxE motif (14). The LxCxE-dependent and -independent interactions provide RBP2 with a range of binding-surface affinities for complex formation with its partner pocket proteins. RBP2 is a multi-facet demethylase, which functions in activation as well as repression (14–16,18,51–53). The diverse interactions with pocket proteins provides RBP2 with an opportunity to occur in different complexes, and

function in context-dependent manner (14–16; this study). It also increases the site-specific recruitment possibilities for RBP2—with pRb on some promoters and with p130 on other promoters (14–16).

The different mechanisms of RBP2 to bind chromatin

Different mechanisms for chromatin binding have been proposed for RBP2. The ARID, PHD1 and PHD3 domain have been implicated in recruiting RBP2 to the chromatin (7–9). *In vitro* experiments propose that PHD1 binds unmethylated H3K4 to stimulate catalytic-domain mediated demethylase activity of RBP2, and concurrent binding of RBP2 to its substrate (H3K4 me0 via PHD1) and product (H3K4 me3 via PHD3) provides a positive-feedback mechanism to facilitate the spread of demethylation on chromatin (9). However, others have shown that PHD1 domain has an insignificant effect on *in vitro* enzymatic kinetics of the KDM5 family (54). Recently it was reported that abrogating the recognition of H3K4me2/3 in KDM5 Drosophila homolog—Lid— by a point mutation in PHD3, marginally attenuated promoter binding of only a subset of Lid- target genes (55). Therefore, H3K4me2/3 recognition cannot be the only mechanism for RBP2 to reach its target promoters, more so in a particular cell cycle phase. Previous reports of other KDM5 members reveal associations with gene-specific transcription factors, such as REST and Myc that could mediate their promoter specific recruitment (6,56). Notably, RBP2 itself has been shown to be recruited to hTERT promoter by Mad1 (57). Here, we show that RBP2 is recruited to E2F-responsive promoters by p130. However, we observe that PHD3 domain is also involved in recruiting RBP2 to promoters. Considering these results, it seems likely that a more complex chromatin binding mechanism is in play, which involves both recruitment by co-factor(s) and/or histone modifications, and RBP2 has the opportunity to use more than one domain/motif to stabilize its interaction with chromatin.

There is growing evidence for the oncogenic function of RBP2 in cancer, and its potential role as a drug target in cancer therapy. However, our work here suggests a tumor-suppressive role for RBP2 during cell proliferation. Are the pro-oncogenic functions of RBP2 limited to certain cell types/cancers, or do these predominate in an environment where E2F/pRb pathway has been inactivated? In any case, our work here, highlights the role of RBP2 in cell cycle regulation and puts the focus back on proliferation, rather than cell differentiation, in context of cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author Contribution: Z.U.Z. performed all experiments except the ones mentioned below. M.R.K. performed experiments presented in S1A-B, S2B, S3B-C,G, S5A and made RBP2 (H483G, E485Q) and (W1625A) mutants used in Figure 6 and Supplementary Figure S7. S.T. and Z.U.Z. designed and analyzed the experiments and wrote the manuscript.

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