Acetylation of Rb by PCAF is required for nuclear localization and keratinocyte differentiation

Adam Pickard, Ping-Pui Wong and Dennis J. McCance*

Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, BT9 7BL, UK

*Author for correspondence (d.mccance@qub.ac.uk)

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Summary

Although the retinoblastoma protein (Rb) functions as a checkpoint in the cell cycle, it also regulates differentiation. It has recently been shown that Rb is acetylated during differentiation; however, the role of this modification has not been identified. Depletion of Rb levels with short hairpin RNA resulted in inhibition of human keratinocyte differentiation, delayed cell cycle exit and allowed cell cycle re-entry. Restoration of Rb levels rescued defects in differentiation and cell cycle exit and re-entry; however, re-expression of Rb with the major acetylation sites mutated did not. During keratinocyte differentiation, acetylation of Rb is mediated by PCAF and it is further shown that PCAF acetyltransferase activity is also required for normal differentiation. The major acetylation sites in Rb are located within the nuclear localization sequence and, although mutation did not alter Rb localization in cycling cells, the mutant is mislocalized to the cytoplasm during differentiation. Studies indicate that acetylation is a mechanism for controlling Rb localization in human keratinocytes, with either reduction of the PCAF or exogenous expression of the deacetylase SIRT1, resulting in mislocalization of Rb. These findings identify PCAF-mediated acetylation of Rb as an event required to retain Rb within the nucleus during keratinocyte differentiation.

Key words: Differentiation, Retinoblastoma, Acetylation, PCAF, SIRT1

Introduction

The retinoblastoma protein (Rb) belongs to a family of proteins called 'pocket proteins', which also includes p107 and p130. These proteins have common functionality including cell cycle control via interactions with E2F transcription factors (Dyson, 1998; Nevins, 1992). However, Rb differs from p107 and p130 in that it has been shown to have a role in regulating cellular differentiation. Ablation of the *Rb1* gene in mice results in embryonic lethality with defects in proliferation, cell death and differentiation in various embryonic tissues (Clarke et al., 1992; Lee et al., 1992). p107- and p130-knockout mice, however, are viable (Cobrinik et al., 1996; Lee et al., 1996). Furthermore, numerous in vivo and in vitro models of differentiation have shown that Rb is crucial in controlling the differentiation process (for a review, see Khidr and Chen, 2006).

During differentiation, Rb function is altered by post-translational modifications. In the switch between actively growing cells and differentiated cells, Rb becomes hypophosphorylated (Martinez et al., 1999) and acetylated (Chan et al., 2001; Nguyen et al., 2004). Hypophosphorylation of Rb is required for cell cycle arrest through sequestration of E2F transcription factors; however, the ability of Rb to induce differentiation is independent of E2F binding (Sellers et al., 1998). Relatively little is known about Rb acetylation but it appears to be essential for differentiation of muscle cells (Nguyen et al., 2004). Acetylation is known to regulate various aspects of protein biology including: stability, function (including DNA binding, transcriptional activity and enzymatic activity), protein-protein interactions and localization (Spange et al., 2009). It is suggested that acetylation regulates interaction with E2F1 in cycling cells (Markham et al., 2006), yet the effects of acetylation on other aspects of Rb functions have not been determined. The major acetylation sites (lysines 873 and 874) reside within a nuclear localization sequence (NLS) close to the C-terminus of the protein (Zacksenhaus et al., 1993). Rb mutants lacking the NLS cannot inhibit cell growth in Rb-null sarcoma ostogenic (Saos)-2 cells and, importantly, are unable to induce differentiation (Zacksenhaus et al., 1993). We report in this study that, during keratinocyte differentiation, Rb is acetylated by the acetyltransferase PCAF and, through mutation of lysines 873 and 874, show that acetylation is required for differentiation and to retain Rb within the nucleus. Knockdown of endogenous PCAF levels or exogenous expression of the deacetylase SIRT1 also resulted in the mislocalization of Rb, suggesting that acetylation is a mechanism for controlling Rb localization during differentiation.

Results

Rb is acetylated during differentiation

Rb acetylation has been observed during differentiation of the monoblastoid cell line U937 (Chan et al., 2001) and the murine muscle cell line C2C12 (Nguyen et al., 2004). Here, the acetylation status of Rb was assessed during calcium-mediated differentiation of primary human keratinocytes. Whole-cell lysates were obtained from early pass keratinocytes that were cycling, grown to confluence (0 hours) or differentiated for various times by serum withdrawal and addition of 1.5 mM $CaCl_2$. Acetylation was assessed by immunoprecipitation of Rb and western blot analysis to detect acetylated lysine residues (Fig. 1A). Rb was acetylated following 72 hours differentiation, confirmed by expression of the differentiation marker keratin 1 (K1). Acetylation was observed during differentiation in three independently isolated batches of keratinocytes, and the reciprocal immunoprecipitations using anti-acetylated lysine also confirmed acetylation of Rb during differentiation (data not shown). Protein extracts obtained from mouse skin were also subjected to immunoprecipitation with Rb

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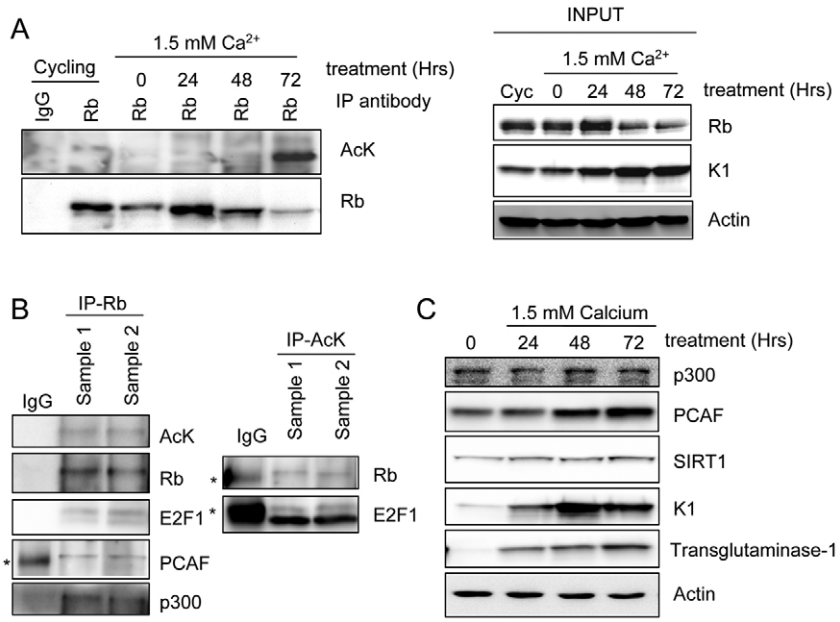

Fig. 1. Rb is acetylated during keratinocyte differentiation. (**A**) Rb immunoprecipitations from primary human foreskin keratinocytes (HFKs) at different stages through differentiation were analysed by western blotting. Acetylation was detected using an antibody raised against acetylated lysine (anti-AcK). Differentiation was confirmed in these cells by assessing expression of keratin-1 (K1). (**B**) Immunoprecipitations from two samples of mouse skin using either anti-Rb or anti-AcK antibodies. Both precipitations identified that pRb is acetylated *in vivo* and also that pRb is associated with p300, PCAF and E2F1. * indicates non-specific bands in IgG control. (**C**) Western blot analysis of p300 and PCAF during calcium-mediated differentiation shows that during differentiation the steady-state levels of PCAF increase whereas p300 remains relatively constant; differentiation was monitored by K1 and transglutaminase-1 expression.

and acetylated lysine antibodies and identified that Rb is acetylated in vivo (Fig. 1B). As both p300 and PCAF have previously been shown to acetylate Rb and both are associated with Rb in mouse skin (Fig. 1B), their expression was monitored during keratinocyte differentiation. The expression of p300 did not alter significantly during differentiation, whereas PCAF levels increased during the time-course (Fig. 1C). Both p300 and PCAF are known to traffic between the nucleus and cytoplasm (Chen et al., 2007; Santos-Rosa et al., 2003); therefore, their localization during keratinocyte differentiation was investigated. In confluent cells, p300 and PCAF staining was diffuse throughout the cell. Following initiation of differentiation, p300 became almost exclusively nuclear and PCAF exclusively cytoplasmic (Fig. 2A). However, later in differentiation, both p300 and PCAF were localized in the nucleus. Immunohistochemical staining of human foreskin with antibodies against PCAF also demonstrated that the localization of PCAF was cytoplasmic in basal epithelial cells (undifferentiated cells) and nuclear in parabasal cells (differentiated; Fig. 2B). Immunohistochemical staining also showed that Rb and p300 are nuclear during skin differentiation (Fig. 2B). In keratinocytes differentiated for 72 hours, areas that had nuclear PCAF staining were also the regions that were positive for differentiation markers (Fig. 2C). As both p300 and PCAF were localized within the nucleus when Rb was acetylated, levels of the two acetyltransferases were individually reduced using short hairpin RNAs (shRNA) to determine if they are required for keratinocyte differentiation.

p300 and PCAF are required for keratinocyte differentiation

Primary human foreskin keratinocytes (HFKs) were transduced with retroviruses that express shRNA targeted to either p300 or PCAF and western blots were used to determine the knockdown (Fig. 3A). Knockdown of either PCAF or p300 levels resulted in inhibition of differentiation, as determined by reduced keratin 1 and transglutaminase 1 (TG1) expression (Fig. 3B). Knockdown of PCAF and p300 with alterative shRNA molecules gave similar results (data not shown). The effects of p300 and PCAF knockdown on Rb acetylation were investigated by an in vitro acetylation assay. Cell lysates from control or knockdown cells at 0 and 72 hours after differentiation were used to acetylate the large pocket of human Rb (GST-Rb-LP). Knockdown of PCAF levels resulted in a reduction of Rb acetylation following 72 hours differentiation compared with control cells, whereas knockdown of p300 had little effect on Rb acetylation (Fig. 3C). This suggests that although p300 and PCAF are required for keratinocyte differentiation, only PCAF acetylates Rb. To further investigate the role of PCAF during differentiation, PCAF levels were depleted using siRNA targeted to the 3′ UTR and then rescued using adenovirus-encoding full-length PCAF or an acetyltransferase-inactive mutant with a deletion in the active site, PCAFΔHAT. The levels of PCAF expression in keratinocytes were assessed by western blot prior to the onset of differentiation in organotypic rafts (siPCAF-UTR/AdGFP; Fig. 3D). Knockdown of PCAF reduced the level of differentiation with the expression of keratin 1, keratin 10 and involucrin all significantly reduced (Fig. 3E). Re-expression of PCAF in PCAF-depleted keratinocytes restored the differentiation programme, as seen by the re-expression of differentiation markers (siPCAF-UTR/AdPCAF; Fig. 3E). However, expression of PCAFΔHAT was unable to restore differentiation in PCAF-depleted cells (siPCAF-UTR/AdPCAFΔHAT; Fig. 3E), indicating that the acetylation function of PCAF is required during differentiation. In fact, the mutant PCAF appeared to act in a dominant-negative manner and caused a reduction in the expression of differentiation markers in control-treated keratinocytes (si-scram/AdPCAFΔHAT; Fig. 3E).

Rb acetylation is required for keratinocyte differentiation

In order to investigate the significance of Rb acetylation in primary keratinocytes, cells were firstly infected with shRNA targeted to the *Rb1* transcript. HFKs depleted of Rb using two different shRNA molecules did not differentiate as efficiently as control-infected HFKs (supplementary material Fig. S1A,B) demonstrated by the reduced expression of keratin 1 and transglutaminase 1. Despite reduced expression of differentiation-specific proteins, Rb-depleted cells have the morphology of differentiated cells as there is an increase in the formation of cell-cell contacts, as visualized by

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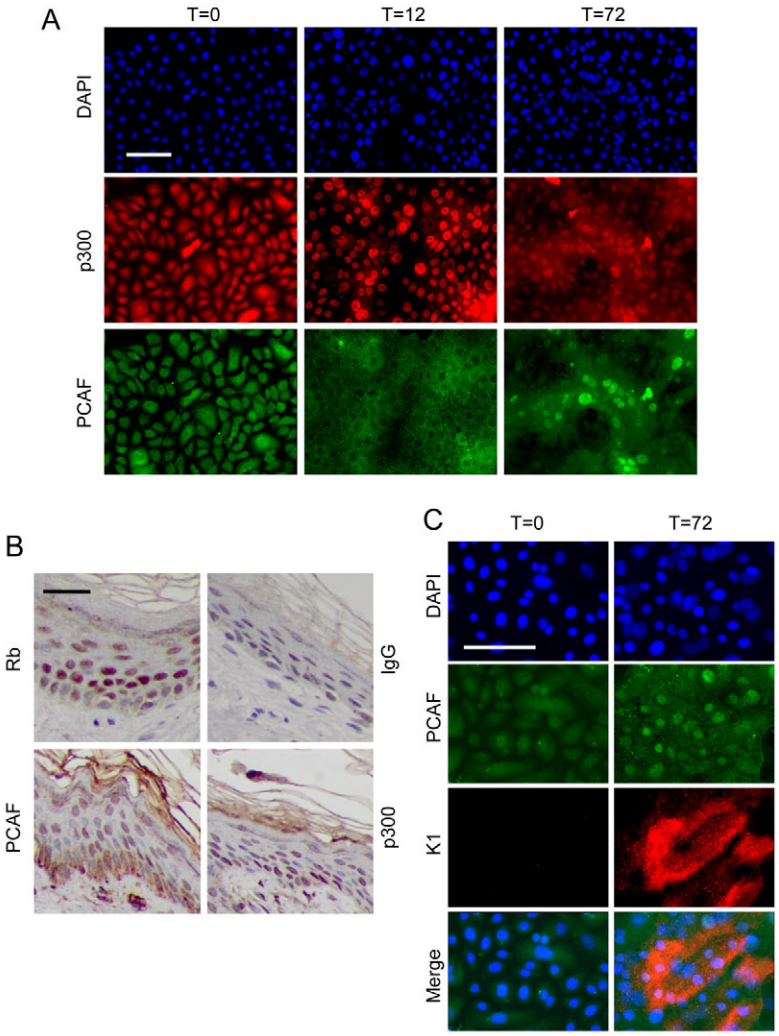

Fig. 2. Localization of PCAF and p300 during differentiation. (**A**) Immunofluorescence analysis of differentiating keratinocytes for expression and localization of p300 and PCAF shows that, shortly after addition of differentiation stimulus, p300 is localized mainly within the nucleus, whereas PCAF is almost exclusively cytoplasmic. Following 72 hours differentiation, both PCAF and p300 were identified in the nucleus. Scale bar: 200 μm. (**B**) Immunohistochemical staining of human foreskin identifies pRb and p300 localized within the nucleus throughout the epithelium, whereas PCAF stains positively in the cytoplasm of the basal cells and then becomes nuclear as cells differentiate. Scale bar: 100 μm. (**C**) Immunofluorescence analysis of differentiated keratinocytes shows that cells with nuclear PCAF are also positive for the differentiation marker K1. Scale bar: 200 μm.

staining for E-cadherin following 72 hours of differentiation. This suggests that not all aspects of calcium-mediated differentiation are affected by loss of Rb. Infection of Rb-knockdown HFKs (generated using shRNA targeted to the 3′ UTR region of the Rb transcript) with a recombinant adenovirus encoding either an HA-tagged wild-type Rb (RbWT) or an HA-tagged mutated Rb that cannot be acetylated (RbKK873/874RR, Ad-RbRR) (Markham et al., 2006; Nguyen et al., 2004; Wong and Weber, 2007; Zhang et al., 1998) was used to assess the role of Rb acetylation during differentiation. RbWT and RbRR expressed by recombinant adenoviruses were functional proteins, as measured by their ability to arrest Saos-2 cells (supplementary material Fig. S2A). Expression of RbWT protein was able to restore expression of differentiation-specific markers K1 and TG1 in response to calcium (Fig. 4A) and K1, K10 and loricrin in organotypic culture (supplementary material Fig. S3); however, expression of the acetylation mutant RbRR could not. The exogenous expression of RbWT and RbRR was equivalent, as observed by western blotting using an anti-HA antibody (Fig. 4A).

Because expression of RbWT was able to restore K1, TG1 and loricrin expression and RbRR was not, suggests that the acetylation sites are important even during the early stages of differentiation. Rb is known to play a key role in controlling proliferation and cell cycle exit (Buttitta and Edgar, 2007); therefore, we examined whether knockdown of Rb in primary keratinocytes would alter cell cycle exit and if this could be reversed by expression of RbWT and RbRR. The proliferative capacity was monitored at various times after addition of a differentiation stimulus, using BrdU incorporation to assess the number of cells in S-phase. shRb-UTR-knockdown cells had an increased proportion of cells incorporating BrdU compared with controls in subconfluent (data not shown) and confluent cultures (Fig. 4B; time 0, shRb-UTR + AdGFP vs sh-scram + AdGFP), suggesting that these cells are more proliferative. Following 24 hours differentiation, control cells rapidly exited the cell cycle, whereas shRb-UTR cells retained their proliferative capacity, suggesting a delay in cell cycle exit (Fig. 4B). Restoration of Rb levels in shRb-UTR cycling cells with infection of Ad-RbWT or Ad-RbRR reduced the number of proliferating cells (Fig. 4B), suggesting that Rb acetylation does not have a role controlling the cell cycle. However, upon differentiation, RbRR-rescued cells were unable to exit the cell cycle as efficiently as cells expressing RbWT and this might explain why these cells do not differentiate. This is also apparent in organotypic rafts where BrdU-positive cells were observed in the suprabasal layers of the cultures (supplementary material Fig. S3). To further assess the differentiation status of these cells, cells were returned to full growth medium 48 hours after addition of a differentiation stimulus, shRb-UTR cells infected with Ad-GFP

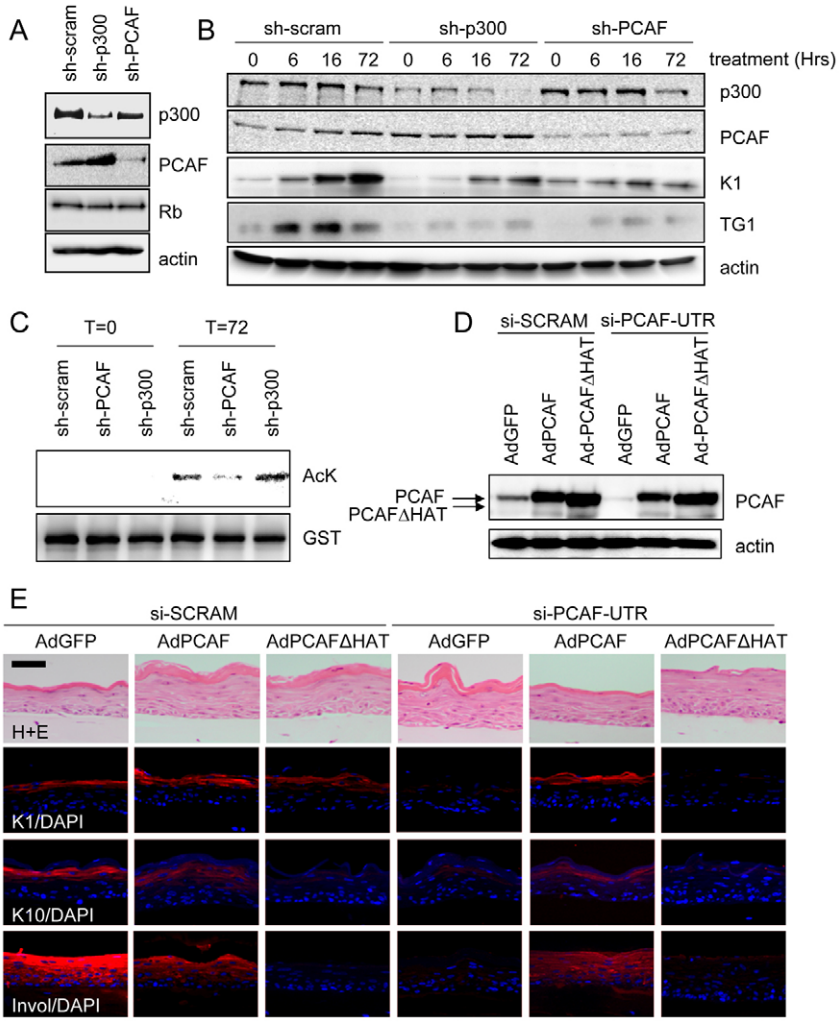

Fig. 3. Knockdown of PCAF inhibits Rb acetylation and keratinocyte differentiation. (**A**) Stable knockdown keratinocytes were generated by retroviral transduction of shRNA targeted to PCAF and p300. Western blot analysis of cell lysates demonstrates knockdown of the targeted proteins; levels of Rb and β-actin show equal loading. (**B**) Stable knockdown cell lines, generated as described in A, were differentiated using calcium treatment. Neither sh-PCAF- nor sh-p300-knockdown cells differentiated as efficiently as control cells, as determined by keratin 1 (K1) and transglutaminase-1 (TG1) expression. (**C**) Lysates from confluent (*t*=0) and cells differentiated for 72 hours were used to acetylate GST-Rb-LP. Induction of acetylation was observed in differentiated control cell lysates, as detected by an anti-acetylated lysine (AcK) antibody. However, Rb acetylation was reduced in PCAF-knockdown cell lysates, whereas it was unaffected by reduction of p300 levels. (**D**) Expression of PCAF in PCAF-depleted cells (siPCAF) and when wild-type (AdPCAF) or an acetylase-deficient mutant (Ad-PCAFΔHAT) PCAF is re-expressed using adenoviruses. PCAFΔHAT lacks 28 amino acids and results in a band running 3 kDa below full-length PCAF. (**E**) Expression of K1, K10 and involucrin in 5-day organotypic cultures of the keratinocytes generated in D, showing that re-expression of wild-type PCAF, but not the acetylase-deficient mutant, is able to rescue differentiation. Scale bar: 100 μm.

showed a significant increase in the percentage of cells incorporating BrdU ($P<0.05$), suggesting that they can re-enter the cell cycle and are therefore not terminally differentiated (Fig. 4B; 48 + 24 hours recovery). Re-expression of RbWT allowed cells to terminally differentiate whereas RbRR could not prevent cell cycle re-entry suggesting that these cells are not irreversibly arrested (Fig. 3B; 48 + 24 hours recovery). Together, this suggests that acetylation at lysine residues 873 and 874 is required for the permanent cell cycle exit of primary human keratinocytes.

Acetylation controls Rb localization during differentiation

In order to understand why RbRR could not rescue differentiation in Rb-knockdown cells, several aspects of Rb function were investigated. RbRR functions identically to RbWT in its ability to reduce cellular proliferation (Fig. 3B; supplementary material Fig. S2A) and this has been attributed to its equivalent ability to bind and inhibit E2F1 transcriptional activity (Markham et al., 2006; Nguyen et al., 2004). It was confirmed that RbRR was able to bind and inhibit E2F1 in primary keratinocytes (data not shown) and, therefore, altered E2F1 activity could not explain the difference in function during differentiation. Likewise, the mutation does not alter protein stability (data not shown). The acetylation sites in Rb are located within an NLS at the C-terminus of the protein and, as acetylation has been shown to modulate the cellular localization of non-histone proteins (Spange et al., 2009), it was postulated that acetylation might regulate the cellular localization of Rb. To investigate the effects of mutating the acetylation sites on Rb localization during keratinocyte differentiation, recombinant adenoviruses were generated encoding wild-type Rb and RbRR with a C-terminal GFP tag. The GFP-tagged Rb proteins were both functional as Ad-GFP-RbWT and Ad-GFP-RbRR were able to arrest Saos-2 cells, whereas Ad-GFP could not (supplementary material Fig. S2B). Like endogenous Rb, both GFP-RbWT and GFP-RbRR were localized to the nucleus in live cycling cells (Fig. 5A), whereas GFP alone was diffuse throughout the cells. Following the onset of differentiation, both RbWT and RbRR continued to be localized in the nucleus; however, following 72 hours differentiation, when the majority of GFP-RbWT is in the nucleus, GFP-RbRR was relocated to the cytoplasm (Fig. 5A). This was confirmed by fractionation of confluent (*t*=0) and differentiated HFKs (*t*=72 hours) expressing GFP-RbWT and GFP-RbRR (Fig. 5B). It is at this time that Rb acetylation was observed and when PCAF translocated to the nucleus (Fig. 2), which suggests that acetylation acts to retain Rb within the nucleus. The separation and purity of the different fractions was assessed using beta-tubulin and p63 as cytoplasmic and nuclear markers, respectively. Immunofluorescence analysis also confirmed that although the RbRR-expressing cells appear to have a differentiated morphology with flattened cells and an increased cytoplasm:nuclear ratio, they do not express the differentiation marker K1 (Fig. 5C). This is

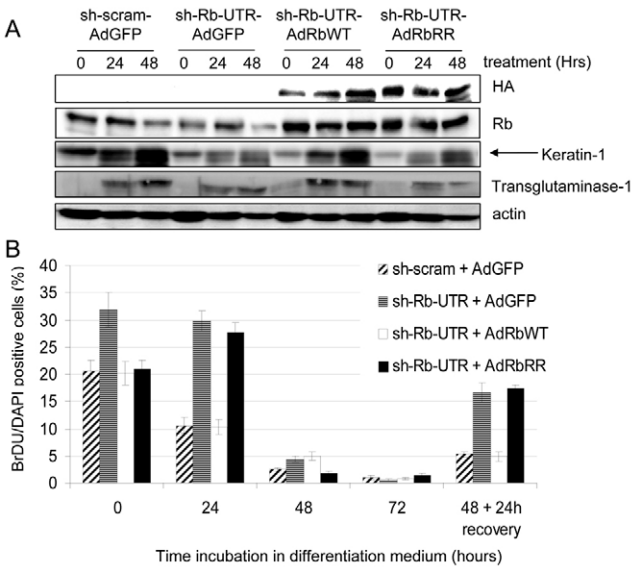

Fig. 4. An acetylation mutant of Rb cannot restore the differentiation capacity of Rb-depleted cells. (**A**) Keratinocytes expressing shRNA targeted to the UTR of the *Rb1* transcript were infected with control recombinant adenovirus (AdGFP), wild-type Rb (AdRbWT) or the acetylation mutant of Rb (AdRbRR). Western blot analysis of differentiating cells shows that re-expression of Rb restored differentiation to knockdown cells, whereas expression of RbRR could not. Exogenous expression of Rb was detected using an HA-antibody and β-actin was used as loading control. (**B**) Adenoviral rescue of Rb levels in Rb-depleted cells restores defects in cell cycle exit and re-entry. The percentage of HFKs within the cell cycle was determined by immunofluorescence detection of BrdU incorporation over a 72-hour time-course of differentiation. Cell cycle re-entry was assessed following 24 hours incubation in normal growth media (recovery). The results demonstrated that lysine residues 873 and 874 are required for cell cycle exit during differentiation and to prevent cell cycle re-entry. This is the average of three experiments.

similar to the appearance of Rb-depleted cells that are differentiated for 72 hours (supplementary material Fig. S1C).

PCAF is required to maintain Rb localization during differentiation

Because acetylation is required for differentiation and appears to regulate Rb localization, we next monitored the localization of endogenous Rb during differentiation of PCAF knockdown using sh-scram and sh-p300 cells as controls. Control and knockdown cells were differentiated for 72 hours and fixed at the indicated times. Indirect immunofluorescence analysis of Rb localization in sh-scram control and p300-knockdown cells showed that Rb localized exclusively in the nucleus throughout 72 hours of differentiation (Fig. 6A). However, in PCAF-knockdown cells following 72 hours differentiation, the majority of Rb was translocated to the cytoplasm (Fig. 6A). Again, it was at this time that PCAF translocated into the nucleus (Fig. 2A) and further implicated PCAF as the acetyltransferase targeting Rb. Cytoplasmic fractions of keratinocytes differentiated for 72 hours also confirm the translocation of endogenous Rb into the cytoplasm of PCAF-knockdown cells (Fig. 6B).

To further investigate the role for acetylation in regulating Rb localization, we exogenously expressed SIRT1, a deacetylase recently shown to deacetylate Rb (Wong and Weber, 2007). HFKs

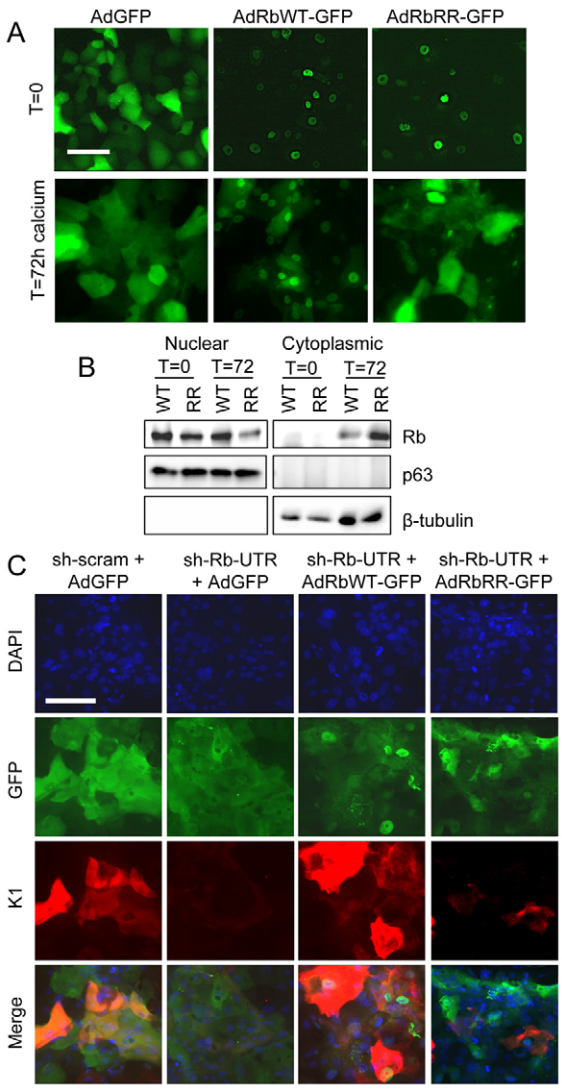

Fig. 5. Mutation of lysine residues 873 and 874 results in mislocalization of Rb during differentiation. (**A**) Primary HFKs were infected with recombinant adenovirus encoding GFP-tagged Rb in wild-type (RbWT-GFP) or an acetylation mutant (RbRR-GFP). Both localize to the nucleus in cycling cells but, upon differentiation, RbRR-GFP is mislocalized to the cytoplasm, whereas the majority of RbWT-GFP is retained in the nucleus. Scale bar: 200 μm. (**B**) Fractionation of infected and differentiated keratinocytes showed that RbRR-GFP was mislocalized to the cytoplasm following differentiation, whereas RbWT-GFP was retained in the nucleus. β-tubulin and p63 were used as markers of cytoplasmic and nuclear fractions, respectively. (**C**) Immunofluorescence analysis of 72-hour differentiated keratinocytes infected with AdGFP, AdRbWT and AdRbRR shows the localization of GFP and expression of the differentiation marker K1. Cells with wild-type Rb also express K1, whereas cells expressing the mutant RbRR do not. Scale bar: 100 μM.

were transfected with SIRT1 or an inactive mutant SIRT1, H363Y, along with HA-tagged Rb. In HFKs cotransfected with empty vector control and pcDNA3-HA-Rb, the exogenous Rb was localized to the nucleus (Fig. 6C). In HFKs cotransfected with SIRT1, Rb was present both in the nucleus and cytoplasm, which suggests that SIRT1 promotes the translocation of Rb. This

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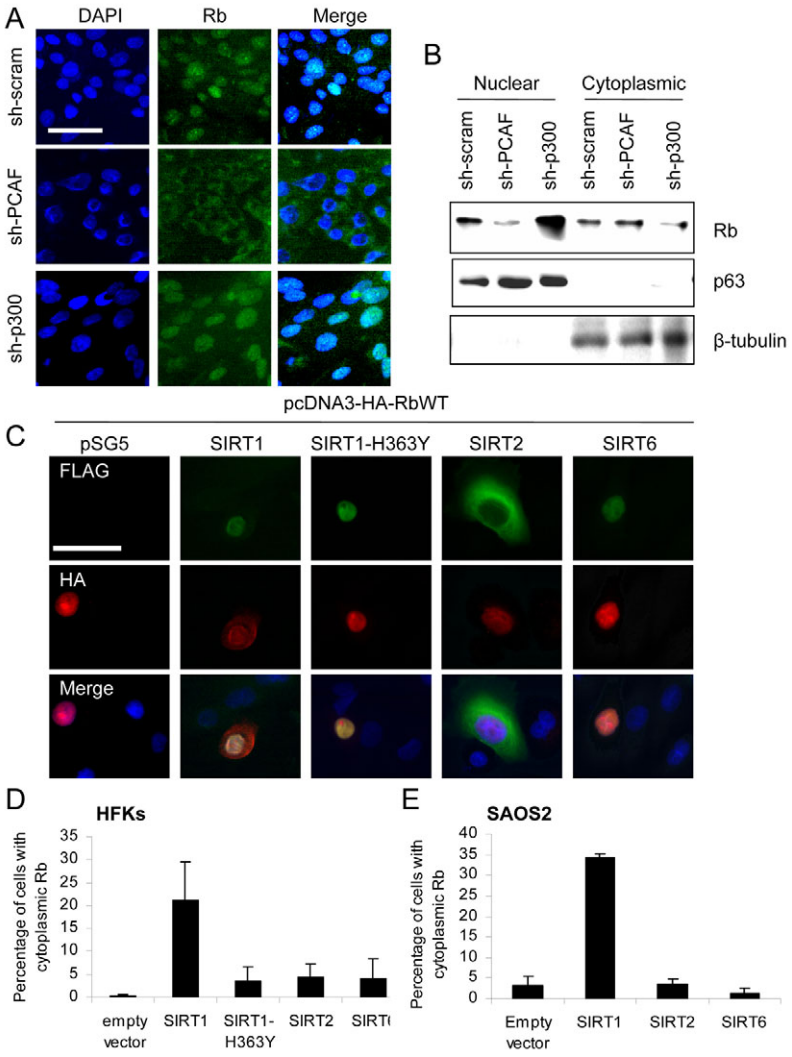

Fig. 6. Acetylation controls Rb localization. (**A**) Control, sh-PCAF and sh-p300 retroviral stable keratinocyte lines were differentiated with calcium for the indicated times and cells were fixed and stained with an anti-Rb antibody. In control and p300-knockdown cells, Rb localized to the nucleus throughout differentiation, whereas, in sh-PCAF cells, Rb was mainly cytoplasmic following 72 hours differentiation. Scale bar: 100 µm. (**B**) Following 72 hours differentiation, nuclear and cytoplasmic fractions were separated from the stable lines used in A and western blotting showed that endogenous Rb levels were significantly reduced in the nucleus of sh-PCAF cells but not in the scrambled control (sh-scram). β-tubulin and p63 were used as cytoplasmic and nuclear markers. (**C**) Immunofluorescence analysis of primary human keratinocytes cotransfected with pcDNA3-HA-Rb and Flag-tagged members of the family of SIRT deacetylases showed that SIRT1 mislocalizes Rb to the cytoplasm. A mutant form of SIRT1 (SIRT1-H363Y) that lacks deacetylase activity did not mislocalize Rb. Mislocalization of Rb is a targeted function of SIRT1, as expression of SIRT2 or SIRT6 did not alter Rb localization. Scale bar: 100 µm. (**D**,**E**) Keratinocytes and Saos-2 cells transfected as in C were counted based on the localization of Rb. The percentage of cells with cytoplasmic Rb staining is shown from three independent experiments.

translocation was dependent on SIRT1 deacetylase activity as, in cells transfected with the mutant SIRT1-H363Y, Rb remained in the nucleus. In addition, expression of SIRT2 (localized in the cytoplasm) and SIRT6 (localized in the nucleus) had no effect on Rb localization (Fig. 6C). Quantification of the percentage of cells where Rb staining was evident in the cytoplasm of cotransfected HFKs is shown in Fig. 6D. Similar effects of SIRT1 mislocalization of Rb were also observed in Saos-2 cells cotransfected with pcDNA3-HA-Rb (Fig. 6E). Taken together, these results suggest that acetylation is a mechanism for retaining Rb within the nucleus during differentiation.

Rb maintains keratinocyte differentiation

Our data suggests that acetylation is required to retain Rb within the nucleus during the later stages of differentiation. In order to test why cells might require Rb at this time, an Rb-specfic RNAi molecule was transfected into HFKs that had been differentiated for 72 hours. At this time, almost all cells had exited the cell cycle and control cells could not be induced to re-enter the cycle (Fig. 4B). Following transfection, cells were grown for 24 hours in full growth medium and pulsed with BrdU. The number of cells in S-phase was determined by immunofluorescence. The reduction of Rb levels resulted in a significant increase in the number of cells able to re-enter the cell cycle, suggesting that the continued expression of Rb during differentiation is important for terminal differentiation (Fig. 7A). Western blot analysis was used to confirm the knockdown of Rb levels (Fig. 7B). Blotting also showed that Rb depletion resulted in a reduced expression of K1, TG1 and loricrin, suggesting that these cells have either dedifferentiated or, because they have not permanently exited the cell cycle, have not terminally differentiated and so exhibit low levels of differentiation markers (Fig. 7B). This can be more clearly observed where cells that are positive for BrdU have reduced K1 staining in si-Rb-treated cultures (Fig. 7C). Co-staining Rb-depleted cells for BrdU and E-cadherin shows that BrdU-positive cells have a differentiated flattened morphology (supplementary material Fig. S1C). Three-dimensional visualisation of confocal images shows that BrdU-positive nuclei are in the same plane as the E-cadherin staining (supplementary material Fig. S4). Although the Rb depleted cells can re-enter the cell cycle on growth stimulation, they do not continue to proliferate as cells replated after 72 hours of differentiation do not grow out into colonies (data not shown). In summary, our results indicate that Rb acetylation is an important modification that acts to retain Rb within the nucleus during differentiation, an event required to maintain the differentiated state.

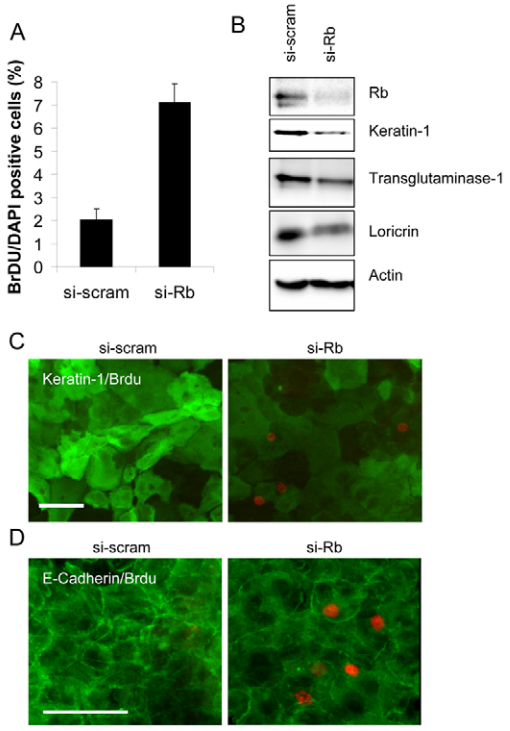

Fig. 7. Rb is required throughout differentiation of primary keratinocytes. (**A**) Following 72 hours of differentiation, Rb levels were depleted using siRNA and BrdU incorporation was measured 24 hours later. Data is from three independent experiments and represents counts from at least 2000 cells in each experiment. (**B**) Western blots of cells treated as in A showed significant depletion of Rb and a reduction in differentiation marker expression. (**C**) Immunofluorescence demonstrated that knockdown of Rb allowed cells to re-enter the cell cycle, with reduced K1 expression. Scale bar: 100 μM. (**D**) E-cadherin and BrdU staining of cells treated as in A show that cells positive for BrdU have a differentiated-like morphology. Scale bar: 200 μM.

Discussion

This study shows that Rb is a key regulator of differentiation and that acetylation is an important modification during this process. We have investigated the role of Rb acetylation in keratinocyte differentiation by mutating the major acetylation sites, lysines 873 and 874, to arginine and then determining the ability of the mutant to restore differentiation in Rb-knockdown keratinocytes. Mutation of the acetylation sites did not affect the ability of Rb to inhibit the proliferation, probably because of the fact that RbRR can interact with and inhibit E2F1 (Markham et al., 2006). This also suggests that inhibition of E2F family members is not sufficient to induce terminal differentiation, as has previously reported in the Saos-2 cell line (Sellers et al., 1998). However, unlike wild-type Rb, the acetylation mutant is unable to induce either early or late differentiation markers and, because Rb is acetylated relatively late during differentiation, this suggests that either the early events are acetylation-independent or the mutated lysine residues can impact functions of Rb. The delay in cell cycle exit observed with the acetylation mutant might also be independent of acetylation, although again we show that lysine residues 873 and 874 appear to be important in controlling the exit. Overall, it appears that Rb is required for permanent cell cycle exit and differentiation but the early functions might be acetylation-independent. Recently, it has been identified that lysine 873 is methylated and mutation of this residue alone was shown to delay the onset of differentiation in Saos-2 and C2C12 cells (Munro et al., 2010), suggesting important roles for these residues.

Although acetylation of Rb at lysines 873 and 874 has been shown to be mediated by both p300 (Chan et al., 2001) and PCAF (Chan et al., 2001; Nguyen et al., 2004), we show here that only knockdown of PCAF reduces Rb acetylation, alters its localization during keratinocyte differentiation and inhibits expression of differentiation markers, implying that PCAF might be the major acetyltransferase targeting Rb in vivo. In addition, acetylation of Rb, nuclear localization and expression of differentiation markers can be reinstated by exogenous expression of active PCAF but not an acetylase-dead mutant. These results also suggest that p300 cannot compensate for the loss of PCAF and confirm previous reports that knockdown of p300 does not affect Rb acetylation during cell cycle (Iyer et al., 2007). We have recently demonstrated that p300 knockdown inhibits keratinocyte differentiation via regulation of $p21^{WAF1/CIP1}$ expression (Wong et al., 2010) and our observation that Rb is acetylated in p300-depleted cells suggests that although acetylation of Rb is required during the differentiation process, alone it is not sufficient to induce differentiation.

The identification of a low-penetrance mutation of Rb1, where exons 24 and 25 are deleted, led us to investigate whether acetylation would affect Rb localization. This mutation generates a protein lacking amino acids 830-888, which removes the NLS and acetylation site of Rb, resulting in its mislocalization to the cytoplasm (Bremner et al., 1997). We have shown that a mutant lacking the acetylation sites is translocated to the cytoplasm upon differentiation, suggesting that acetylation acts to retain Rb in the nucleus. Additionally, either knockdown of PCAF or overexpression of SIRT1, a deacetylase, also led to mislocalization of Rb. Altered Rb localization has been described previously in an array of cancer cell lines and primary cancer tissues, with cytoplasmic or nuclear-cytoplasmic staining of Rb observed in approximately 90% and 60% of tested samples, respectively (Jiao et al., 2008). This was attributed to aberrant phosphorylation of Rb in at least some of the cell lines (Jiao et al., 2006), although our data would suggest that acetylation is another important modification that could control Rb localization. Intriguingly, mislocalization of Rb also correlated with moderate and poorly differentiated primary cancer specimens (Jiao et al., 2008). It has also been proposed that acetylation can inhibit cyclin-E–CDK2-mediated phosphorylation of Rb in in vitro phosphorylation assays (Chan et al., 2001); however, during differentiation, Rb becomes hypophosphorylated and, therefore, acetylation might be an independent method to control Rb localization.

In addition, our results imply that Rb has an important role in the control of differentiation even when the majority of cells have exited the cell cycle, suggesting that Rb is required to maintain differentiation. It is not clear at present if the role of Rb is to maintain permanent cell cycle exit or if this plus other functions of Rb are required for successful differentiation. Rb-depleted cells do change to a differentiation-like morphology, with a high cytoplasm to nuclear ratio, but do not express early or late differentiation-specific markers and a portion of these cells can re-enter the cell cycle. Our results are consistent with a number of recent reports where inhibition of Rb function allows differentiated retinal or muscle cells to re-enter the cell cycle (Ajioka and Dyer, 2008; Blais et al., 2007). Our findings are also supported by recent work that has identified that inactivation of Rb and Hippo in *Drosophila* induces dedifferentiation in the retina (Nicolay et al., 2010).

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In summary, our results show that acetylation functions to retain Rb in the nucleus in the later stages of differentiation in order to maintain Rb function and, in turn, maintain permanent cell cycle arrest.

Materials and Methods

Keratinocyte culture and generation of stable knockdown cell lines

Primary human foreskin keratinocytes (HFK) were isolated from neonatal foreskins and grown in Epilife medium (Cascade) containing human keratinocyte growth supplement (HKGS, Cascade). Keratinocytes were transduced with retrovirus produced in ϕNYX-GP packaging cell line as described previously (Guess and McCance, 2005; Rheinwald and Green, 1975), using the following plasmids: pSuper-retro constructs expressing short hairpin RNAs (shRNA) against no known annotated gene (sh-scram) or targeting the Rb coding region (sh-Rb-CR) or 3′ UTR (sh-Rb-UTR) were cloned as described previously (Incassati et al., 2006); p300- and PCAF-knockdown lines were generated using plasmids purchased from Origene. To differentiate HFKs, the cells were grown to confluence in the medium described above and then differentiation was induced by addition of 1.5 mM calcium chloride and withdrawal of HKGS (Fang et al., 1998). Organotypic raft culture was as described previously (Menges et al., 2006).

Generation of adenovirus

Recombinant adenoviruses were cloned using ViraPower Adenoviral Gateway Expression Kit (Invitrogen). To generate AdGFP, pMAX-GFP (Amaxa) was digested with *Kpn*I and *Xho*I and inserted into the same sites of pENTR11. AdRbWT and AdRbRR were generated by first subcloning the *Nco*I-*Xho*I fragment from pSG5-RbWT or pSG5-RbRR (Miyake et al., 2000; Nguyen et al., 2004) into the same sites of pENTR11 (pENTR11-*Nco*I-RbWT-*Xho*I and pENTR11-*Nco*I-RbRR-*Xho*I). Then the *Nco*I fragment from the pSG5-RbWT was introduced into pENTR11-*Nco*I-RbWT-*Xho*I and pENTR11-*Nco*I-RbRR-*Xho*I and the directionality confirmed by sequencing.

To generate GFP-tagged Rb, EGFP was first excised from pEGFP-N1 by *Xho*I and *Not*I digestion and ligated into *Sal*I and *Not*I in pENTR11. The *Hin*dIII-*Eco*RI and *Eco*RI-*Eco*RI fragments of pSG5-RbWT were ligated into the vector to generate pENTR-RbWT-stop-N1-GFP. Finally, PCR was conducted to remove the stop codon using the following primers: forward, TcTTgAATTcgcTAgccTATcTccg; and reverse, AgAAggTAccTTcTcTTccTTgTT. pSG5-RbWT or pSG5-RbRR was used as template. The PCR product was digested with *Dra*III and *Kpn*I and ligated into the same sites of pENTR-RbWT-stop-N1-GFP. AdPCAF and AdPCAFΔHAT were generated by ligating the *Eco*RI and *Kpn*I fragment of pCX-PCAF and pCX-PCAFΔHAT, as described previously (Santos-Rosa et al., 2003), into the same sites of pENTR11 and the orientation was corrected by digesting the resulting vector with *Sal*I and *Eco*RI and ligating into *Eco*RI and *Xho*I of pENTR11. All constructs were then recombined into pAd/CMV/V5-DEST. All recombinant adenoviruses were generated using HEK293 cells and were purified and titrated as previously described (Enquist and Lowenstein, 1996).

Transient transfection

Fugene HD was used for transfection of siRNA; control and *Rb1* siRNA were purchased from Ambion. siRNA targeting the UTR of PCAF was as previously described (Linares et al., 2007): 24 hours after transfection, cells were infected with adenovirus encoding PCAF. Fugene 6 was used for transfection of keratinocytes with plasmid DNA, whereas Fugene HD (Roche) was used for siRNA transfections. pECE-Flag-SIRT1 and pECE-Flag-SIRT1-H363Y, described in Brunet et al. (Brunet et al., 2004), were obtained from Addgene (plasmids 1791 and 1792). pcDNA3-Flag-SIRT2 and pcDNA3-Flag-SIRT6, described in North et al. (North et al., 2003), were also obtained from Addgene (plasmids 13813 and 13817).

Immunofluorescence

To determine proliferation in cell cultures, cells were pulsed for 15 minutes with 10 μM BrdU prior to fixing with 4% paraformaldehyde. Following antigen retrieval with citrate buffer (DAKO), cells were stained with 1:200 dilution of anti-BrdU (BD Biosciences). The number of BrdU-positive cells and DAPI-counterstained cells were counted to determine the percentage of proliferating cells. For organotypic rafts, sections were deparaffinised with xylene and then rehydrated with step-down concentrations of ethanol. Sections were blocked using 10% bovine calf serum and 0.2% Triton X-100. Sections were then stained with anti-keratin-1 (1:2000, Covance), anti-keratin-10 (1:100, Biodesign) or anti-loricrin (1:100; Neomarkers). Secondary antibodies were goat anti-mouse and goat anti-rabbit conjugated to fluorophores 488 or 594 (1:400, Invitrogen). Sections were mounted with ProLong Gold Antifade Reagent plus DAPI (Invitrogen).

Protein extracts, immunoprecipitation and in vitro acetylation assays

Whole-cell lysates for western blot were generated using urea buffer (8 M urea, 50 mM Tris pH 7.5, 0.1% v/v beta-mercaptoethanol and protease inhibitor cocktail; Roche). Cell fractions were prepared as previously described (Sen et al., 2006). For immunoprecipitation and in vitro acetylation assays, cells were lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40, 10 mM EDTA, 5 μM trichostatin A and protein inhibitor cocktail. For Rb immunoprecipitation, 1 mg protein lysate was used with 1 μg anti-Rb (BD Biosciences) and 10 μL Protein-G agarose (Santa Cruz). For in vitro acetylation, 500 μg protein lysate was incubated with 0.3 μg purified GST-Rb-LP, as previously described (Nguyen et al., 2004), for 4 hours at 30°C. Additional antibodies used in this study were: monoclonal IgG2A anti-acetylated lysine (Cell Signaling), goat anti-mouse IgG2A (Santa Cruz), goat anti-mouse IgG1 (Santa Cruz), mouse anti-p300 (Abnova), rabbit anti-p300 (Santa Cruz), anti-PCAF (Santa Cruz), anti-HA (Santa Cruz), anti-beta actin (Sigma), anti-TBP (Abcam), anti-β-tubulin (Sigma) and anti-Flag 488 (Sigma).

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