

## SHORT COMMUNICATION

## Cell cycle-dependent acetylation of Rb2/p130 in NIH3T3 cells

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The retinoblastoma protein (pRb) and the pRb-related proteins, p130 and p107, form the ‘pocket protein’ family of cell cycle regulatory factors. A well characterized function of these proteins is the cell cycle-dependent regulation of E2F-responsive genes. The biological activity of pocket proteins is regulated by phosphorylation and for the founding member pRb it has been shown that acetylation also has an important role in modulating its function during the cell cycle. Here, we show that hyperphosphorylated retinoblastoma 2 (Rb2)/p130 also exists in an acetylated form in NIH3T3 cells. Acetylated p130 is present in the nucleus but not in the cytoplasm. Acetylation is cell cycle dependent, starting in S-phase and persisting until late G<sub>2</sub>-period. Using recombinant p130 and truncated forms for *in vitro* acetylation by the acetyltransferase p300, we could identify K1079 in the C-terminal part as the major acetylation site by mass spectrometry. Minor acetylation sites were pinpointed to K1068 and K1111 in the C-terminus, and K128 and K130 in the N-terminus. The human papilloma virus 16 protein-E7 preferentially binds to acetylated p130 and significantly increases *in vitro* p130 acetylation by p300.

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The retinoblastoma gene family consists of three members, retinoblastoma (Rb)/p105, p107 and Rb2/p130. The family is also referred to as the ‘pocket protein’ family, as these proteins are characterized by a conserved pocket region through which they can bind viral and cellular proteins, such as the E2F family of transcription factors (Claudio *et al.*, 2002; Du and Pogoriler, 2006). The Rb family proteins have important roles in proliferation control by transcriptional regulation of cell cycle progression through E2F, especially at the G<sub>1</sub>–S transition (Cobrinik, 2005); moreover, pocket proteins can target chromatin modifiers to E2F promo-

ters and interact with chromatin remodeling enzymes (Giacinti and Giordano, 2006; Macaluso *et al.*, 2006).

Chromatin modifying enzymes not only modify histones, but indeed a huge variety of non-histone regulatory and structural proteins that interact with chromatin, such as protooncogene and tumor suppressor proteins, transcription factors and enzymes (Gu and Roeder, 1997; Vervoorts *et al.*, 2003; Zhang *et al.*, 2003; Choi *et al.*, 2003; Meraner *et al.*, 2006). Retinoblastoma protein (pRb) has been shown to be acetylated by p300/CBP, as well as P/CAF (Nguyen *et al.*, 2004) and the modification changes its properties with respect to phosphorylation by cyclin-dependent kinases (Chan *et al.*, 2001).

The cell cycle-dependent phosphorylation of more than 20 distinct residues has been identified for Rb2/p130 (Canhoto *et al.*, 2000; Hansen *et al.*, 1999, 2001). The majority of sites can be phosphorylated by CDK-2, 4 or 6, predisposing p130 for ubiquitination, and thus proteosomal degradation (Tedesco *et al.*, 2002). However, in contrast to the common picture of pocket protein inactivation through phosphorylation by CDKs, p130 actively associates with E2F-4 in a distinct phosphorylation state as cells enter G<sub>0</sub> (Mayol *et al.*, 1996); this modification state is independent from CDK activity and has been ascribed to glycogen synthase kinase 3 (Litovchick *et al.*, 2004). Moreover, mapping of phosphorylation sites revealed only 3 out of 22 CDK consensus sites being conserved between pRB and p130, whereas 10 phosphorylated serine/threonine residues are conserved between p107 and p130, indicating pronounced differences in the functional consequences of modification among the three pocket proteins.

We set out to study the posttranslational acetylation of p130 during the cell cycle of NIH3T3 cells, as a screening for acetylated non-histone nuclear proteins revealed p130 to be modified by acetylation. NIH3T3 cells were synchronized in G<sub>0</sub> by serum starvation for 72 h. After stimulation through serum addition cells were harvested at 2 h intervals for a total of 24 h. Nuclear extracts were then prepared and analyzed by immunoblotting with monoclonal antibodies against p130 (BD Transduction Laboratories, San Jose, CA, USA) and acetylated lysine. Serum-starved cells in G<sub>0</sub> showed substantial amounts of hypo-phosphorylated p130 and low amounts of the slower migrating hyperphosphorylated form of p130. During the time course of the experiment the abundance of hypophosphorylated p130 continuously decreased, whereas the amount of the hyperphosphorylated form increased

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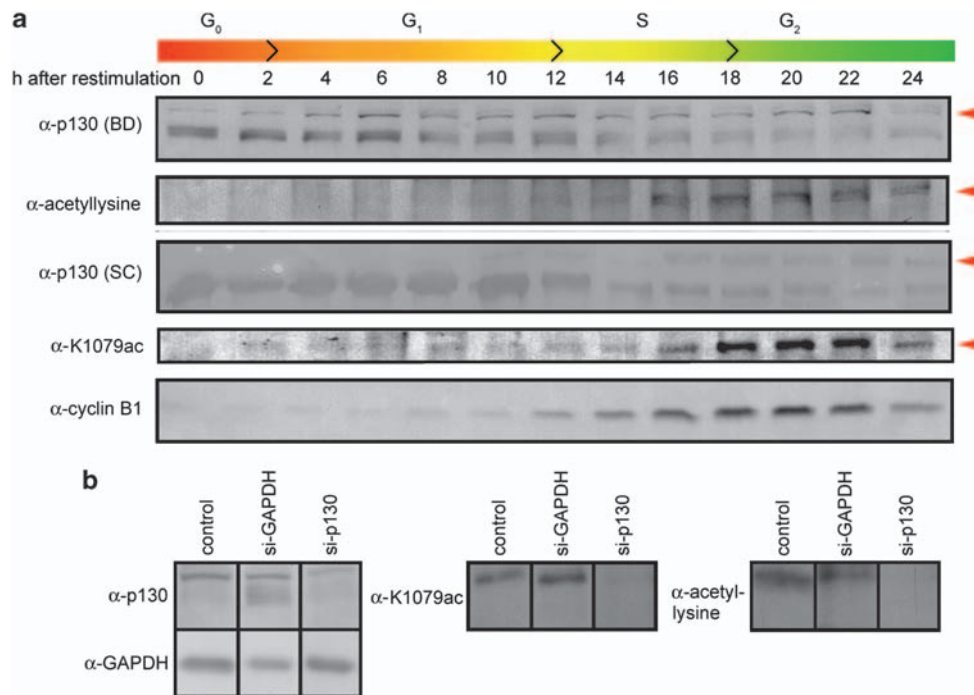
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significantly until 6–8 h after serum addition and stayed on a high level until G<sub>2</sub>-period (Figure 1a). A corresponding cell cycle pattern of hypo- and hyperphosphorylated forms was also obtained when polyclonal p130 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunoblotting. This pattern of hypo- and hyper-phosphorylated p130 has been previously reported for a time period of 12 h after serum addition in T98G cells by Hansen *et al.* (2001). In G<sub>0</sub> and G<sub>1</sub> immunoblotting with anti-acetyl lysine antibodies did not yield an immunosignal with either hypo- or hyper-phosphorylated p130. However, in early S-phase the hyperphosphorylated form of p130 shifted into the acetylated form as anti-acetyl lysine antibodies yielded an immunosignal that co-migrated with hyperphosphorylated p130; this band persisted until late G<sub>2</sub>-period (Figure 1a). This result was confirmed with a specific antibody ( $\alpha$ -K-1079ac) that we raised against a C-terminal p130 peptide from amino acids 1074–1085 containing K-1079 in the acetylated state (SNSPSK<sub>ac</sub>RLREIN; see results below). The cell cycle distribution of cells was monitored by immuno-

detection of cyclin B1 (Figure 1a) and fluorescence-activated cell sorting analysis (not shown). These results suggest that a significant proportion of p130 in a hyperphosphorylated state is acetylated in early S-phase and is present in this state until late G<sub>2</sub>-period.

In order to further substantiate that hyperphosphorylated p130 is acetylated in S-phase, we performed small interfering RNA knockdown of p130 and analyzed S-phase nuclear extracts at 16 h after serum addition by immunoblotting using antibodies against p130, acetylated lysine residues and the specific antibody against p130 acetylated at K-1079. The immunoblot with anti-p130 antibodies clearly shows that the hypo- and hyperphosphorylated protein bands significantly decreased in si-p130 transfected cells; in line with this, the immunoblot with the antibody against the acetylated p130 peptide 1074–1085 ( $\alpha$ -K-1079ac) showed a significant decrease of the slower migrating, hyperphosphorylated p130 form (Figure 1b). A corresponding result was obtained with anti-acetyl lysine antibodies; a glyceraldehyde 3-phosphate dehydrogenase knockdown control corroborated the results (Figure 1b).

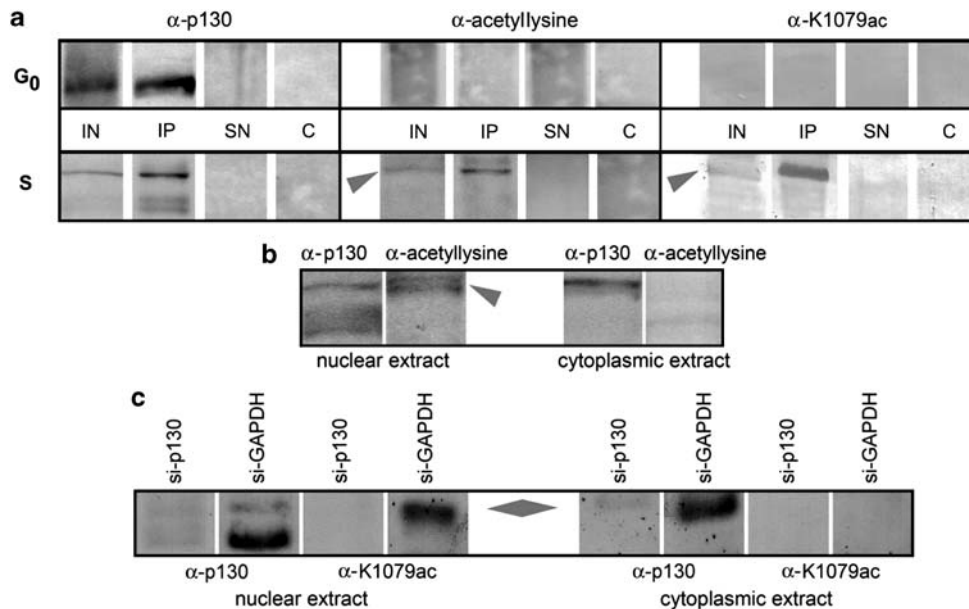


**Figure 1** Cell cycle dependent abundance of p130 forms in NIH3T3 cells. **(a)** NIH3T3 cells were serum starved for 72 h. After serum addition cells were harvested at 2 h intervals. Nuclei were isolated and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein from each sample were subjected to SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) with subsequent blotting to nitrocellulose. p130 was detected either with a monoclonal antibody (BD; BD Transduction Laboratories; No.610262) or with polyclonal antibodies (SC; Santa Cruz, sc-317). Acetyl lysine was detected with a polyclonal antibody (Upstate, Millipore-Merck, Darmstadt, Germany; No.06-933), acetylated p130 with a specific antibody that was raised in rabbits against a C-terminal p130 peptide (aa 1074–1085) where K-1079 is acetylated ( $\alpha$ -K-1079ac), and cyclin B1 with a monoclonal antibody (Santa Cruz; sc-245). Arrows highlight the hyperphosphorylated, acetylated p130 forms. **(b)** Effect of si-p130 knockdown in synchronized S-phase cells at 16 h after serum addition. Small interfering RNA (siRNA) was purchased from Dharmacon (Lafayette, CA, USA; RBL2-p130-mouse siGENOME-ON-TARGET plus set of four, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-mouse control) and resuspended in DEPC treated PBS. NIH-3T3 cells were grown to 90% confluency, and transfected with a mix of 4 RBL2/p130 siRNAs (100 nm each) or GAPDH siRNA and metafectene (ratio 1:4) according to the manufacturer's instructions in serum free medium. Cells were serum starved for 72 h, before stimulation with 10% fetal calf serum to re-enter the cell cycle for 16 h. Cells were then harvested, washed and nuclear extracts were prepared using NE-PER. Proteins were separated by SDS–10% PAGE, blotted onto nitrocellulose and immunodetected using the indicated antibodies. Bands were visualized using electrochemiluminescence.

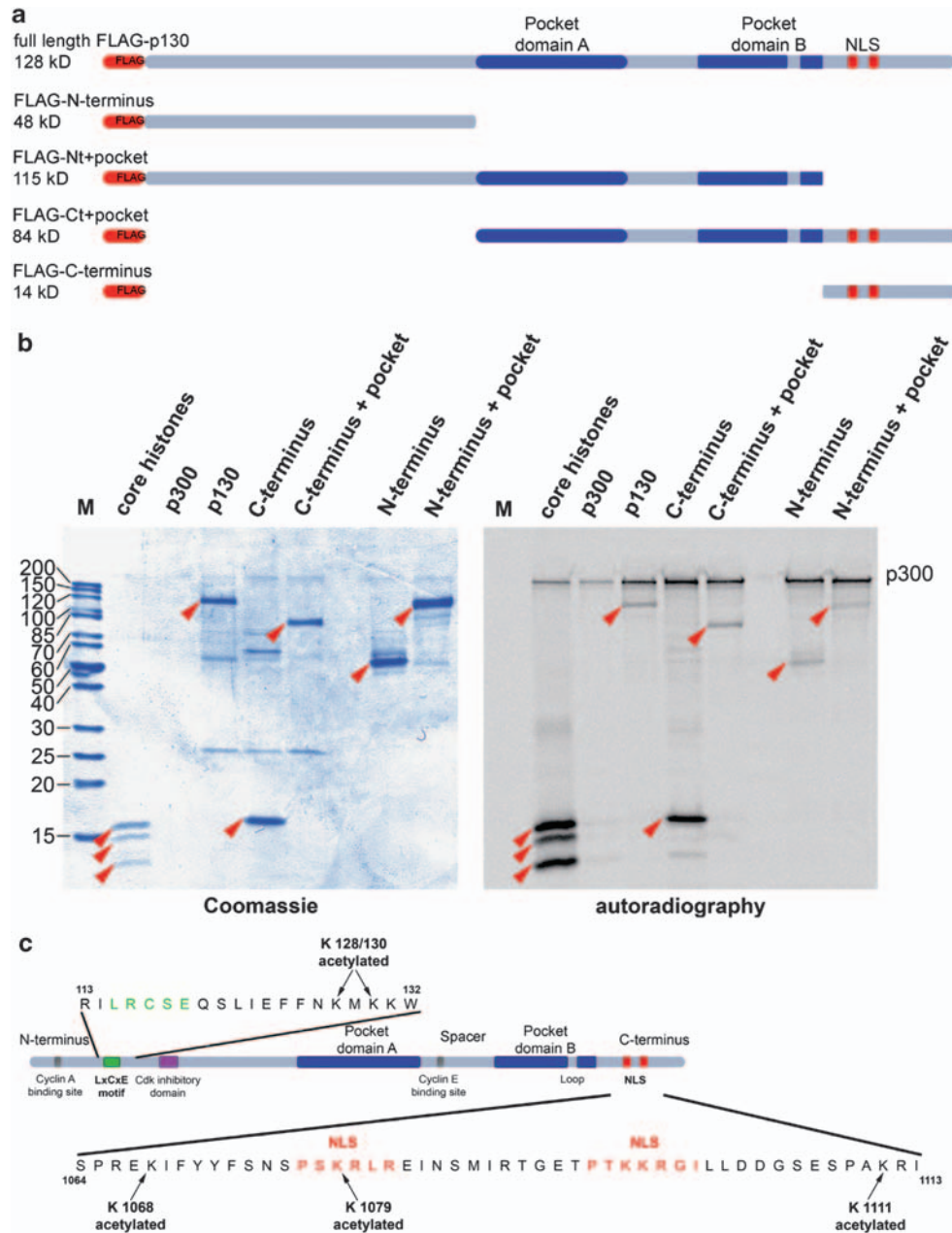
In order to further prove acetylation of hyperphosphorylated p130, we performed immunoprecipitation using anti-p130 antibodies for precipitation of nuclear extracts from NIH3T3 cells in G<sub>0</sub> and S-phase. After electrophoresis, immunoblotting was done with antibodies against p130, acetyl lysine, and the acetylated C-terminal p130 peptide ( $\alpha$ -K-1079ac). Figure 2a shows that anti-p130 antibodies precipitate p130 with the hyperphosphorylated form being acetylated, as detected by anti-acetyl lysine, as well as specific anti-p130-K-1079ac antibodies. To get an idea of a possible intracellular localization of acetylated p130, we analyzed nuclear and cytoplasmic extracts from exponentially growing NIH3T3 cells. Figure 2b shows that anti-acetyl lysine antibodies detected acetylated p130 exclusively in nuclear extracts, although the amount of hyperphosphorylated p130 is higher in the cytoplasmic extract. To corroborate this result, we again used siRNA knock-down of p130 in exponentially growing NIH3T3 cells (Figure 2c). Immunoblotting of nuclear and cytoplasmic extracts of si-p130 transfected cells revealed efficient downregulation of p130 in both forms (hypo- and hyper-phosphorylated), whereas a small interfering-glyceraldehyde 3-phosphate dehydrogenase control yielded the localization pattern as seen in the untransfected wild type (Figures 2b and c). As expected, immunoblotting with the specific antibody against acetylated p130 ( $\alpha$ -K-1079ac) showed the acetylated

and hyperphosphorylated p130 in nuclear extracts of the small interfering-glyceraldehyde 3-phosphate dehydrogenase control, but neither in the cytoplasmic extract of the small interfering-glyceraldehyde 3-phosphate dehydrogenase control nor in the nuclear extracts of si-p130 knockdown cells (Figure 2c). From these results, we conclude that acetylated, hyperphosphorylated p130 is exclusively located in the nucleus, although the main proportion of hyperphosphorylated p130 resides in the cytoplasm (in a non-acetylated state).

As the total amount of p130 is very low in NIH3T3 cells, we expressed N-terminally FLAG-tagged p130 (Figure 3a) in insect cells and used the recombinant protein for *in vitro* acetylation by the acetyltransferase p300. Using radioactive acetyl-CoA as a co-substrate, electrophoretic analysis and subsequent autoradiography of the assay product revealed that p130 is acetylated by p300 *in vitro* (Figure 3b; lane 4, autoradiography panel). Corresponding assays were performed with non-radioactive acetyl-CoA and acetylated p130, as well as non-acetylated p130 were subsequently analyzed by mass spectrometry. This revealed a dominant acetylation site at K-1079 (Supplementary Information; Figure 1) and minor acetylation at K-1068 and K-1111 in the C-terminus and at K-128 and K-130 in the N-terminus. To substantiate these findings, we expressed additional truncated p130 forms in insect cells (Figure 3a): FLAG-tagged versions



**Figure 2** p130 from nuclear extracts exists in an acetylated form. (a) Nuclear extracts from synchronized NIH3T3 cells in G<sub>0</sub>-phase (0h after serum addition) or S-phase (16h after serum addition) were immunoprecipitated with antibodies against p130 (BD Transduction Laboratories) coupled to Protein-G-Sepharose beads. After washing, beads (IP), supernatant (SN), as well as nuclear extract (input, IN) were subjected to SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) with subsequent immunoblotting using antibodies against p130 (BD), acetyl lysine or K-1079ac, respectively. As an immunoglobulinG control, nuclear extract lanes were also incubated with secondary antibodies alone (c, control). Proteins were visualized with alkaline phosphatase conjugated secondary antibodies. (b) Exponentially growing NIH3T3 cells were fractionated into nuclear and cytoplasmic extracts. After SDS–10% PAGE and blotting onto nitrocellulose, p130 and acetylated p130 were detected by the corresponding antibodies. (c) Exponentially growing NIH3T3 cells were transfected with si-p130 or small interfering-glyceraldehyde 3-phosphate dehydrogenase (si-GAPDH) for 72 h as described in the legend of Figure 1. After harvesting, cells were fractionated and samples were processed and analysed as described above. Arrows highlight the position of acetylated p130.



**Figure 3** Acetylation of p130 by p300 acetyltransferase *in vitro* takes place predominantly in the C-terminus and the major acetylation site can be pinpointed to K-1079. (a) Schematic representation of N-terminally FLAG-tagged full-length p130, as well as truncated versions expressed in Sf9 cells (baculovirus system). (b) Full-length and truncated versions of p130 were used for *in vitro* acetylation assays. Proteins were incubated with p300 and  $20 \mu\text{M}$   $^{14}\text{C}$ -acetylCoA for 30 min at  $37^\circ\text{C}$ , visualized by Coomassie blue stain (left panel) and autoradiography (right panel) after SDS-10% polyacrylamide gel electrophoresis (SDS-10% PAGE). Histones and p300 were used as controls. (c) Lysine residues found to be acetylated by MALDI-TOF/TOF mass spectrometry are shown in their respective sequence environment. Ct, C-terminus; NLS, nuclear localization sequence; Nt, N-terminus.

of the N-terminus (48 kDa), N-terminus + pocket domain (115 kDa), C-terminus + pocket domain (84 kDa) and C-terminus (14 kDa). We used these proteins for radioactive *in vitro* assays with p300 and subsequently analyzed them by SDS-polyacrylamide gel electrophoresis and autoradiography. Figure 3b shows that the five recombinant proteins were produced in sufficient quantity and quality (Coomassie panel) and approximately equal amounts were used for the p300-histone acetyltransferase assay. As control we used chicken

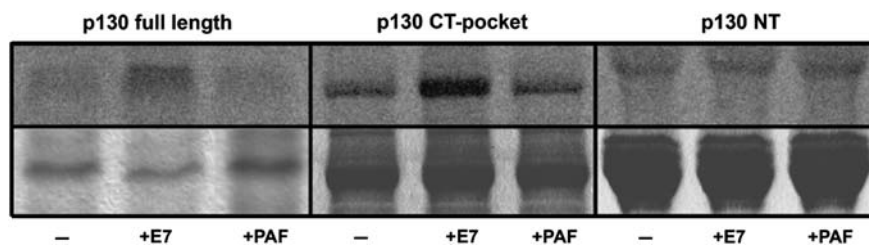
erythrocyte histones. Autoradiography revealed that full-length p130 and C-terminus + pocket domain construct were labeled roughly equally, whereas the isolated C-terminus exhibited particularly high acetylation by p300 (Figure 3b, autoradiography panel). The truncated p130 versions comprising the N-terminus and the N-terminus + pocket were acetylated to a lesser extent. As expected, autoacetylation of p300 and prominent acetylation of core histones are visible. Note, that no unspecific acetylation of contaminating minor proteins

could be detected, emphasizing the specificity of p130 acetylation.

The fact that the C-terminus and the constructs that contain the C-terminus are acetylated stronger than the N-terminal constructs is in line with mass spectrometry results that clearly identified a major acetylation site at K-1079, whereas the other acetylated residues represented minor sites. Acetylated sites are depicted in Figure 3c. The major acetylatable K-1079 is located within one of two C-terminal nuclear localization sequences, implying a possible effect on subcellular localization of p130. Therefore, we performed transient transfection experiments with C-terminal p130/GFP constructs where K-1079 was mutated to arginine or glutamine, but could not observe a significant change of nuclear localization of the reporter protein by microscopy of transfected cells (results not shown), indicating that a more subtle mechanism is modulating nuclear localization of p130 rather than a simple K-1079 acetylation. This is in line with previous results (Chestukhin *et al.*, 2002), showing that functional deletion of one of the two nuclear localization sequences alone was not sufficient to abolish nuclear localization of p130, whereas only deletion of both resulted in reduced nuclear localization (Chestukhin *et al.*, 2002). In this respect, it is interesting that all three acetylated lysines in the C-terminus (K-1068, K-1079 and K-1111) are in close neighborhood (3 or 4 amino acids) to documented phosphorylation sites (S-1064, S-1076 and S-1108; Hansen *et al.*, 2001); in all three cases the phosphorylated serine is followed by a proline. It is tempting to speculate that the prolines might be substrates of peptidyl-proline isomerases, which would induce secondary structure changes. It has been shown that a proline isomerase acts on prolines 30 and 38 of histone H3, thereby modulating methylation of a neighboring lysine (Nelson *et al.*, 2006). An interrelation between proline isomerization and p300 recruitment has been demonstrated for p73 (Mantovani *et al.*, 2004). The fact of phosphorylation and acetylation sites together with proline residues being clustered within few amino acids, and all clusters being located within a region of only 50 amino acids, suggests that the C-terminus of p130 may be a region of particular regulatory potential in the sense of a protein modification code (Brosch *et al.*, 2008; Sims and Reinberg, 2008).

It has been shown that p130 is predominantly abundant in G<sub>0</sub>- and G<sub>1</sub>-phases (Claudio *et al.*, 2002) and rapidly decreases after S-phase (Classon and Dyson, 2001); in NIH3T3 cells we did not observe such a rapid decrease of p130. The modified forms of nuclear p130 rather persisted through G<sub>2</sub>-period (Figure 1), a finding consistent with a role for p130 in active G<sub>2</sub>-complexes (Popov *et al.*, 2005) and in cell cycle exit in G<sub>2</sub> (Jackson *et al.*, 2005).

To demonstrate a functional significance of acetylation for the properties of p130, we analyzed p300-mediated acetylation of p130 in the presence of the human papilloma virus16 protein-E7 (HPV16-E7). Pocket proteins have been shown to be targets of small DNA virus oncoproteins, such as proteins from certain types of papilloma-, polyoma- and adeno-viruses (Felsani *et al.*, 2006; White and Khalili, 2006). HPV16-E7 binds to p130 and targets it for degradation; moreover, histone deacetylases bind to p130 and the E7 protein, thereby inhibiting histone deacetylase binding to the E2F2 promoter and elevating the acetylation of histones associated with E2F-responsive promoters (Roman, 2006). We used full-length p130 protein, the C-terminus+pocket construct and the N-terminus for radioactive *in vitro* acetylation by p300 in the presence or absence of HPV16-E7. Figure 4 shows that E7 protein enhances p300 mediated acetylation of full-length p130; this enhancement was even more pronounced when the C-terminus+pocket was used as a substrate, whereas no stimulating effect was detected with the N-terminus. As control we used a polypeptide of comparable molecular weight, *Penicillium*-antifungal peptide, to rule out an unspecific protein effect. Densitometric evaluation of autoradiograms of Figure 4 revealed that HPV16-E7 enhanced acetylation of p130 full-length protein 1.28-fold, acetylation of p130-C-terminus+pocket 1.65-fold, whereas acetylation of p130-N-terminus was unaffected by HPV16-E7 (Supplementary Table 1). Obviously, the E7 protein has a substrate- and site-specific effect on p300 affinity for p130. The well-known binding of histone deacetylases to p130 might not only be due to a targeting role of p130 for histone deacetylases to distinct promoters, but may also reflect binding of the enzyme to exert its catalytic activity on p130 for modulating its functional properties. The functional changes of p130 induced by de/acetylation most likely change its interaction



**Figure 4** The human papillomavirus-16-E7 protein causes enhanced *in vitro* acetylation of p130 in the C-terminus (Ct). Full-length p130 and truncated versions (C-terminus + pocket, N-terminus (Nt)) were incubated with <sup>14</sup>C-acetylCoA and p300 in the presence or absence of HPV16-E7 protein (2 μg/100 μl). As a control peptide the same amounts of *Penicillium*-antifungal peptide (PAF) were used. Each reaction mix was subjected to SDS-10% polyacrylamide gel electrophoresis (SDS-10% PAGE; lower panel, Coomassie) with subsequent autoradiography (upper panel).

properties with other proteins, as known from a number of acetyltable non-histone proteins.

### Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)



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