Degradation of cyclin A is regulated by acetylation

Francesca Mateo[1], Miriam Vidal-Laliena[1], Nuria Canela[1], Luca Busino[2], Marian A. Martinez-Balbas[3], Michele Pagano[2], Neus Agell[1] and Oriol Bachs[1+].

[1]Department of Cell Biology and Pathology, Faculty of Medicine, University of Barcelona, Casanova 143, 08036-Barcelona, Spain, [2]Department of Pathology and NYU Cancer Institute, New York University School of Medicine, 550 First Ave., MSB 599 New York, NY 10016,USA and [3]Instituto de Biología Molecular de Barcelona, Consejo Superior de Investigaciones Científicas (CSIC). Josep Samitier 1,5. Parc Científic de Barcelona. 08028-Barcelona. Spain.

+ Corresponding author. Tel: 34 93 403 52 86, Fax: 34 93 402 19 07;
E-mail: obachs@ub.edu

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Abstract

Cyclin A accumulates at the onset of S phase, remains high during G_2 and early mitosis and is degraded at prometaphase. Here, we report that cyclin A directly interacts with the acetyltransferase P/CAF and is acetylated at lysines 54, 68, 95 and 112. Maximal acetylation occurs simultaneously to ubiquitylation at mitosis, indicating a role of acetylation on cyclin A stability. A non-acetylatable mutant in which these lysines were substituted by arginines (cycA 4R) cannot be ubiquitylated, is more stable than cycA WT and arrests cell cycle at mitosis. Despite of that, it interacts with the proteins needed for its degradation (cdks, Cks, Cdc20, Cdh1 and APC/C). CycA 4R shows higher affinity for cdks than cycA WT and cycA 4R-cdk complexes display higher kinase activity than control complexes. All these results indicate that cyclin A acetylation at specific lysines is crucial for cyclin A stability and also plays a role in the regulation of cyclin A-cdk activity.

Introduction

Cell cycle progression is governed by the family of cyclin dependent kinases (cdks) (Morgan, 1997). Their activities are regulated by binding to regulatory subunits called cyclins, phosphorylation and binding to inhibitory proteins (Sherr and Roberts, 1999). During cell cycle, specific pairs of cyclin-cdks are formed and activated. Cdk1 together with cyclins A and B governs G_2/M transition. G_1 progression is under the control of cyclin D-cdk4/6. Cyclin E-cdk2 triggers DNA synthesis and cyclin A-cdk2 drives S phase progression (Malumbres and Barbacid, 2005). Whereas the levels of most cdks are relatively constant during cell cycle those of cyclins fluctuate, and in that way, they bind to and activate specific cdks.

Cyclin A levels are low during G_1 but they increase at the onset of S phase, when it contributes to the stimulation of DNA synthesis (Rosenberg et al., 1995; Resnitzky et al., 1995). The amount of cyclin A remains high after S phase and in early mitosis when, by associating with cdk1, it drives the initiation of chromosome condensation and possibly nuclear envelope breakdown (Furuno et al., 1999; Pagano and Draetta, 1991; Gong et al., 2007). It is destroyed during prometaphase by the Anaphase Promoting Complex/Cyclosome (APC/C) via proteasome (den Elzen and Pines, 2001). Cyclin B levels rise during G_2 and then it binds to cdk1. This complex promotes the completion of chromosome condensation and spindle assembly, thus driving cell cycle progression until metaphase. Cyclin B is degraded during metaphase, significantly later than cyclin A (Hagting et al., 2002). Because each cyclin is responsible for the phosphorylation of a specific subset of cdk substrates, it is expected that after their degradation their specific substrates would be dephosphorylated. Thus, the ordered destruction of the different cyclins helps to order the sequence of events in

late mitosis (Bloom and Cross, 2007). In fact, on time degradation of cyclins A and B is a key event for mitosis progression and non-degradable mutants of cyclin A cause cell arrest in metaphase, whereas those of cyclin B block cells during anaphase (Parry and O'Farrell, 2001; Sullivan and Morgan, 2007).

The signals that trigger cyclin A degradation at prometaphase are still a matter of controversy. Degradation is induced by APC/C bound to the targeting subunit Cdc20 (APC/C^{Cdc20}) that is activated by phosphorylation by cyclin B-cdk1. Cyclin A degradation is spindle-checkpoint independent and thus, it starts as soon as APC/C^{Cdc20} is activated (den Elzen and Pines, 2001; Geley et al., 2001). In contrast, cyclin B1 degradation by APC/C^{Cdc20} is sensitive to the spindle assembly checkpoint. Therefore, at prometaphase unattached sister chromatids generate signals that allow inhibitory components of the spindle-assembly checkpoint, such as Mad2, to bind to Cdc20 and block its ability to interact with cyclin B1 (Fang et al., 1998; Sudakin et al., 2001). Moreover, a recent report indicates that to maintain the cell cycle arrest induced by the spindle assembly checkpoint, Cdc20 must be ubiquitylated and degraded(Nilsson et al., 2008). Only when all chromatids are attached to the mitotic spindle at metaphase, the spindle-assembly checkpoint becomes inactivated and then cyclin B1 can be degraded. This different behaviour of cyclin A and cyclin B degradation by the same APC/C complex indicates that distinct signals participate in targeting these cyclins for ubiquitylation and the subsequent degradation during mitosis (Geley et al., 2001).

In general, cyclins have a “destruction box” which is a sequence that is recognized by the ubiquitylation machinery in order to degrade these proteins (Glotzer et al., 1991). Cyclin A also has an extended “destruction

box" that includes aa 47-72 (Klotzbucher et al., 1996). However, in order to totally avoid cyclin A ubiquitylation and degradation the first 171 aa of cyclin A must be eliminated, revealing that in addition to the extended "destruction box" more sequences from the amino terminus are needed for cyclin A degradation (Fung et al., 2005). Moreover, the association of cyclin A to its cdk partner is needed for its degradation, suggesting that Cdc20 binds to cyclin A through an extended motif that includes its amino terminus but also its cdk partner (Wolthuis et al., 2008).

Here we report that cyclin A can be acetylated *in vivo* and *in vitro* by the acetyltransferase P/CAF at four specific lysine residues located in its amino terminus. When these residues are substituted by arginines, cyclin A cannot be ubiquitylated, is much more stable and causes cell cycle arrest at G_2/M. Therefore, our results indicate that acetylation is a critical signal in the regulation of cyclin A degradation.

Results

The putative *in vivo* acetylation of cyclin A was analyzed in HCT116 cells transfected with HA-cyclin A. By immunoprecipitation (IP) with anti-HA antibodies followed by western blotting (WB) with anti-acetylated lysines (acetylK), we observed that cyclin A was acetylated in the cells (Figure 1a). We subsequently aimed to identify the acetyltransferases that could be responsible for this acetylation. Thus, *in vitro* assays, using GST-cyclin A as a substrate and different acetyltransferases as enzymes were performed. As shown in Figure 1b, cyclin A was acetylated by P/CAF but not by CBP or Tip60. To further determine whether P/CAF was also involved in the *in vivo* acetylation of cyclin A, cells were transfected with Flag-P/CAF and HA-cyclin A and treated with control or P/CAF-specific siRNAs. We then performed IP on cell extracts with anti-HA and the levels of cyclin A acetylation were determined by WB. Results revealed that the decrease of P/CAF strongly reduced cyclin A acetylation (Figure 1c), indicating a role of P/CAF in the *in vivo* acetylation of cyclin A.

Among other regulatory functions, acetylation of lysine residues might affect protein stability in different ways. Thus, we decided to investigate whether acetylation could participate in the regulation of cyclin A stability. Cyclin A degradation largely depends on its N-terminal region and the lysine residues involved in cyclin A ubiquitylation and degradation are located in the first 171 aa of its sequence (Figure 2a) (den Elzen and Pines, 2001; Geley et al., 2001). To study whether the acetylation sites were located in this cyclin A region that contains 12 lysines, *in vitro* spot mapping assays were performed. Thus, 13 peptides (each one containing one or two consecutive lysines) belonging to this N-terminal region of cyclin A were synthesized and spotted on a membrane that was subjected to acetylation

assays with P/CAF (supplementary Figure S1). An acetylatable peptide from histone H3 was used as a control. Results indicated that peptides containing K54, K68, K95 and K112 were acetylated (Figure 2b). To analyze whether these lysines were the major acetylation sites in the full length protein, we generated a cyclin A mutant in which lysines 54, 68, 95 and 112 were substituted by arginines (cycA 4R). This mutant was used for *in vitro* acetylation assays with P/CAF. Results indicated that differently from cyclin A WT (cycA WT) that was clearly acetylated by P/CAF, cycA 4R was not (Figure 2c). Finally, to further determine whether these four lysines were the major *in vivo* acetylation sites, cells were tranfected with Flag-cycA WT or Flag-cycA 4R, subsequently subjected to IP with anti-Flag and then cyclin A acetylation was determined by WB. Results revealed that cycA WT was acetylated whereas the mutant cycA 4R was not, indicating that these four lysines are the major *in vivo* acetylation sites (Figure 2d).

The putative *in vivo* interaction between cyclin A and P/CAF was first analyzed by fluorescence microscopy in CFP-cyclin A and YFP-P/CAF transfected cells. Results showed that both proteins co-localized in the nucleus (Figure 3a). The interaction between both proteins was further determined by IP experiments with anti-HA in cells co-transfected with YFP-P/CAF and HA-cyclin A. Subsequent WB analysis demonstrated the co-immunoprecipitation of cyclin A, P/CAF and cdk2 (Figure 3b). Finally, Surface Plasmon Resonance analyses demonstrated the direct association between cyclin A and P/CAF (Figure 3c). We were also interested in determining the interaction between cyclin A and P/CAF during the cell cycle. For that purpose we first analyzed the levels of cyclin A and P/CAF in cells synchronized at different phases of the cell cycle. We observed that the levels of P/CAF were high during S phase and G_2/M, decreased at

metaphase and remained low during G_1. This behaviour was similar to that observed for cyclin A (Figure 3d, left panel). Cdk2 was detected overall the cell cycle although the levels slightly varied depending on the cell cycle phase. To determine the interaction between cyclin A and P/CAF, IP experiments with anti-HA were performed in cells transfected with YFP-P/CAF and HA-cyclin A. WB analysis of the immunoprecipitates indicated that cyclin A and P/CAF mostly interacted during S phase and G_2/M, when both proteins were most abundant. At these specific points of the cell cycle cyclin A also interacted with cdk2. We also aimed to determine the acetylation status of cyclin A. Interestingly, maximal acetylation of cyclin A was observed during G_2/M although the protein was also acetylated during S phase (Figure 3d, right panel).

To study the role of acetylation on cyclin A stability, we determined the half-life of cycA WT and cycA 4R in transfected cells. Figure 4a shows that the half-life of cycA WT is of around 6 h whereas that of cycA 4R is much longer. As it has been reported that non-degradable forms of cyclin A (lacking a part of the N-terminus) cause arrest of cells in metaphase (Geley et al., 2001; Fung et al., 2005), we aimed to study the effect of the cycA 4R mutant on cell cycle progression. FACS analysis revealed a substantial block in G_2/M in cells transfected with the cycA 4R mutant, whereas only a slight effect was observed in cells transfected with cycA WT (Figure 4b).

We further explored the possibility that the increased stability of cycA 4R could be due to defects in its ubiquitylation. Thus, *in vivo* ubiquitylation assays were performed. Cells were transfected with HA-ubiquitin plus Flag-cycA WT or Flag-cycA 4R, then subjected to IP with anti-Flag, and finally the ubiquitylation levels were analyzed by WB with anti-Flag and anti-HA. Results indicated that cycA WT was clearly ubiquitylated whereas

ubiquitylation of cycA 4R was strongly reduced (Figure 5a). The low levels of ubiquitylation and degradation shown by cycA 4R could be due to two different possibilities: 1) these four lysines are actually ubiquitylation sites or 2) these four lysines are acetylation sites needed for the subsequent ubiquitylation of other lysines. To discriminate between these two possibilities we studied the *in vivo* ubiquitylation of Flag-cycA 4Q, a mutant in which lysines 54, 68, 95 and 112 of cyclin A were substituted by glutamines. This form is considered to be a pseudoacetylated mutant because of the structure similarity between glutamine (Q) and the acetylated-lysine residue (Hecht et al., 1995; Li et al., 2002). Results indicated that differently from cycA 4R, the mutant cycA 4Q was ubiquitylated similarly to cycA WT (Figure 5a). Interestingly, the half-life of cycA 4Q is shorter than that of cycA 4R but not as short as that of cycA WT, indicating that cycA 4Q can be degraded although not so efficiently as cycA WT (supplementary Figure S2b). Therefore, these results indicate that K54, K68, K95 and K112 are not ubiquitylation sites but acetylation sites needed for the ubiquitylation of other lysines and for the subsequent degradation of cyclin A.

Next we analyzed the acetylation and ubiquitylation of cyclin A during cell cycle. Thus, Flag-cycA WT-transfected cells were synchronized by a double thymidine block and subjected to IP with anti-Flag at different times after the release of the blockade. When WB with anti-acetylK was performed, a peak of cyclin A acetylation was observed at 4-6 h after the release (Figure 5b, bottom panel). Interestingly, during the same period of time a peak of cyclin A ubiquitylation was also observed (Figure 5b, upper panel). The simultaneous acetylation and ubiquitylation of cyclin A supports that acetylation is involved in cyclin A ubiquitylation and degradation. FACS analysis revealed that under our experimental conditions at 4-6 h after

the release of the double-thymidine block cells are in mitosis (supplementary Figure S3).

We further aimed to analyze the mechanisms underlying the decreased ubiquitylation of cycA 4R. It is known that to be degraded cyclin A has to form a cyclin A-Cdk-Cks complex that is recruited to the phosphorylated APC/C by its Cks protein. Cdc20 attached to this complex causes cyclin A to be degraded (Wolthuis et al., 2008). Thus, we analyzed the interactions of Flag-cycA WT and Flag-cycA 4R with Cdc20, Cdh1, APC3, Cks1/2, cdk1 and cdk2 in asynchronously growing cells. Results showed that the interaction with Cdc20 and APC3 was similar in both cases but cycA 4R showed an increased interaction with cdk1, cdk2, Cks and Cdh1 (Figure 6a). The interaction between the mutant cycA 4R and Cdc20 was further confirmed by IP experiments with anti-Flag in cells transfected with Flag-Cdc20 plus CFP-cycA WT or CFP-cycA 4R (Figure 6b, upper panel). Moreover, the increased interaction of Cdh1 with cycA 4R was also confirmed by IP experiments with anti-HA in cells transfected with HA-Cdh1 plus Flag-cycA WT or Flag-cycA 4R (Figure 6b, bottom panel). Experiments carried out in cells synchronized in S phase or G_2/M revealed that the interactions of cycA WT or cycA 4R with these proteins at these specific points are similar to those observed in asynchronously growing cells (supplementary Figure S4).

The increased interaction of cycA 4R with cdk1 and cdk2 is of particular interest because it might affect their kinase activity. To analyze this possibility, cells transfected with Flag-cycA WT or Flag-cycA 4R were subjected to IP with anti-Flag and the associated cdk activity was determined in the immunoprecipitates. We observed that cycA 4R-cdk complexes display higher kinase activity than that of cycA WT-cdk complexes (Figure

6c). When cells were subjected to IP with anti-cdk2 we also observed an increased association of cycA 4R with cdk2 respect to that shown by cycA WT, and also a higher kinase activity in the cycA 4R-cdk2 complexes (Figure 6d). So, in addition to the role in cyclin A stability, lysines 54, 68, 95 and 112 also play a role in the regulation of cyclin A interaction with cdk and its associated kinase activity.

Discussion

The different timing of cyclin A and cyclin B degradation at mitosis and the diverse sensitivity of these cyclins to the spindle assembly checkpoint indicates that specific mechanisms target each one of these cyclins for degradation (van Leuken et al., 2008). We report here that cyclin A acetylation by the acetyltranferase P/CAF participates in the signalling pathway that targets cyclin A for degradation at early mitosis. Acetylation is a post-translational modification occurring at the Nε-amino-group of lysines that might regulate protein functions in many different ways as for instance, protein-protein and protein-DNA interactions and protein stability. Lately, a number of reports have revealed that lysine acetylation might act as a direct signal enhancing protein degradation for proteins such as E2F1 (Galbiati et al., 2005), HIF-1α (Jeong et al., 2002), SV40 T antigen (Shimazu et al., 2006), and pRB (Leduc et al., 2006). Moreover, the interplay between lysine acetylation and ubiquitylation has been reinforced by the evidence that at least four acetyltransferases (p300, CBP, P/CAF and TAF1) possess intrinsic ubiquitin activating/conjugating or ligase activities (Sadoul et al., 2008).

We report here that P/CAF acetylates cyclin A at lysines K54, K68, K95 and K112 and that these lysines are the major acetylation sites both *in vivo* and *in vitro*. These specific residues are located in the amino terminal domain of cyclin A that has been involved in the stability of the protein (Wolthuis et al., 2008). In fact, two of these lysines, K54 and K68 were already described as important residues for the ubiquitylation and degradation of cyclin A. Specifically, the authors reported that substitution of K37, K54 and K68 by arginines generates a more stable cyclin A but this mutant was still ubiquitylated (Fung et al., 2005).

Our results indicate that lysines K54, K68, K95 and K112 are critical residues for ubiquitylation and degradation of cyclin A. A possible role of these lysines in these processes is to act as acetylation sites needed for the subsequent ubiquitylation of other lysine residues. This is supported by the evidence that the ubiquitylation of the non-acetylatable mutant cycA 4R is strongly reduced whereas the pseudoacetylated mutant cycA 4Q was ubiquitylated similarly to cycA WT. As glutamines cannot be ubiquitylated, the ubiquitylation sites in the cycA 4Q mutant must be other lysines probably located in the amino terminus of cyclin A. Thus, these results suggest that likely cyclin A acetylation at these specific lysines signals this protein for the subsequent ubiquitylation and degradation.

According to our observations, P/CAF is the principal histone acetyltransferase involved in cyclin A acetylation. In addition to acetylate histones, P/CAF also participates in the reversible acetylation of various transcriptional regulators as the general transcription factors $TF_{II}E\beta$ and $TF_{II}F$ (Imhof et al., 1997) and the sequence-specific transcription factors E2F1 (Martinez-Balbas et al., 2000), c-myc (Patel et al., 2004), myo D (Patel et al., 2004) and p53 (Gu and Roeder, 1997; Sakaguchi et al., 1998). It has been implicated in many important cellular processes such as transcription, differentiation, proliferation and apoptosis (Schiltz and Nakatani, 2000). In the cell P/CAF is a subunit of multiprotein complexes that posses global histone acetylation activity and locus-specific co-activator functions together with acetyl transferase activity on non-histone susbtrates (Nagy and Tora, 2007). Recently, it has been described that in addition to acetylate p53, the intrinsic ubiquitylation activity of P/CAF controls the stability of the oncoprotein Hdm2, indicating an important role of this acetylase in the DNA damage checkpoint (Linares et al., 2007).

Interestingly, the levels of P/CAF oscillate during cell cycle similarly to those of cyclin A. They are low at G_1, increase during S phase and remain high during G2 and early mitosis to finally decrease before metaphase. As the decrease of cyclin A is produced by degradation by the APC/C^{Cdc20} complex, the similar behaviour of P/CAF suggests that it could also be an APC/C^{Cdc20} substrate. However, this is something that needs to be investigated.

Cyclin A associates with P/CAF during S phase and this interaction is maintained until early mitosis, then before metaphase this complex is disrupted. Concomitant to its association with P/CAF, cyclin A becomes acetylated. A more detailed time-course analysis indicates that cyclin A acetylation increases at early mitosis simultaneously to cyclin A ubiquitylation. All these data support that acetylation by P/CAF targets cyclin A for its ubiquitylation.

To be degraded cyclin A has to be bound to a cdk subunit which in turn has to be associated with a Cks protein. This cyclin A-cdk-Cks complex is then recruited to the phosphorylated APC/C by its Cks subunit. The Cdc20 attached to cyclin A causes cyclin A to be degraded regardless of the spindle checkpoint being active or not (Wolthuis et al., 2008). Thus, a possibility is that cyclin A acetylation at these specific lysines might be required for the interaction with some of these proteins of the ubiquitylation machinery. However, the analysis of the interactions of the non-acetylatable mutant cycA 4R with the proteins of the APC/C^{Cdc20} ubiquitylation complex ruled out this possibility because this mutant retains the ability to interact with all the proteins of the complex (Cdc20, Cdh1, APC3, cdks and Cks). Thus, the lack of ubiquitylation is not due to a reduced ability to form ubiquitylation complexes. A possible interpretation of these results is that

acetylation of lysines 54, 68, 95 and 112 is needed for the correct incorporation of ubiquitin molecules on specific sites of cyclin A.

An unexpected result was the observation that the levels of cdk1, cdk2 and Cks associated with cycA 4R are much higher than those bound to cycA WT. These results might be interpreted in the sense that cycA 4R displays a much higher affinity for these proteins than cycA WT. As Cks does not directly interact with cyclin A but associates with the cdk subunits, probably the higher affinity of cycA 4R for this protein is a consequence of its increased affinity for the cdks. The increased affinity of cycA 4R for the cdks is accompanied by a higher activity of these complexes compared to that displayed by cycA WT-cdks. Likely, this increased kinase activity is due to the fact that cycA 4R is able to generate more complexes with cdks than cycA WT does. The putative role of the augmented affinity of cycA 4R for cdks and the subsequent elevated kinase activity of these complexes on the ubiquitylation block still remains unknown.

Likely, this increased kinase activity is due to the fact that cycA 4R is able to generate more complexes with cdks than cycA WT does.

As a summary, results presented here reveal that acetylation at specific lysines is a new mechanism that targets cyclin A for degradation at early mitosis. In addition to that, our results also revealed an unexpected new mechanism for the regulation of cdk activity that depends on the integrity of four specific lysines of cyclin A.

Materials and methods

Plasmids

cDNA of wild type cyclin A was cloned into pGEX6P1, pEFHA, pcDNA3-Flag and pECFP-C1 vectors. CycA 4R and 4Q mutants were generated by site-directed mutagenesis. pCX-Flag-P/CAF, pGEX2TKP-$HAT_{P/CAF}$ (352-658), pGEX4T2-P/CAF (full length), pGEX2T-CBP and pGEX2T-Tip60 were a kind gift from MA. Martínez-Balbás. pcDNA3.1-HA-Cdh1 and Flag-Cdc20 were a kind gift from M. Pagano. P/CAF shRNA and control shRNA were purchased from Sigma.

Antibodies and reagents

Antibodies against cyclin A (H-432), cdk2 (M-2), Cdc20 (H175) and Cks1/2 (FL-79) were purchased from Santa Cruz Biotechnology. Anti-Cdh1 (34-2000) was purchased from Zymed. Anti-acetylK (#9441) and anti-phospho-histone H3 (Ser 28) (#9713) were from Cell Signaling. Antibodies against Flag (F7425), HA (H6908) and P/CAF (P7493) were purchased from Sigma, and APC3/Cdc27 (ab10538) from Abcam. For IPs we used monoclonal anti-HA agarose and monoclonal anti-Flag M2 affinity gel from Sigma. [^{14}C]acetylCoA was purchased from Perkin Elmer. Thymidine, Nocodazol and Cycloheximide were from Sigma. ALLN was from Calbiochem.

Protein purification and in vitro acetylation

Protein expression and purification was performed as described (Canela et al., 2006). Acetylase assays were performed as described (Martinez-Balbas et al., 2000). For cyclin A acetylation assays, 1-10 µl of the different acetylases (5,000-10,000 cpm activity on histones) were incubated with 6

µM of purified GST or GST-cyclin A and 0.02 µCi [^{14}C]acetylCoA. For the spot-mapping experiment, the membrane was incubated in 3 ml of HAT buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 0.1 mM EDTA, 5% glycerol, 0.1% NP-40) in the presence of GST-HAT$_{P/CAF}$ and [^{14}C]acetylCoA, for 30 min at 30ºC. Then, the membrane was washed, dried and subjected to autoradiography.

Immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1%SDS, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na_3VO_4, 0.5 µg/µl aprotinin, 10 µg/µl leupeptin) for 30 min on ice. After centrifugation, lysates (0.2-2 mg of protein) were incubated with Flag or HA agarose beads for 2 h at 4ºC. After 3 washes with RIPA buffer, Laemmli buffer was added to the samples and they were electrophoresed.

Surface Plasmon Resonance experiments

The Surface Plasmon Resonance analysis was performed at room temperature using a Biacore T100 (Biacore International AB). P/CAF purified protein was immobilized on a carboxymethylated dextran sensor chip (CM5) using the amine coupling method as described by the manufacturer. A blank immobilization was performed using the same method and was used as the reference surface. Purified full-length cyclin A was diluted in HBS-EP buffer (Biacore International AB) and was injected over the flow cells at a flow rate of 30 µl/min for 60 s. Following a dissociation time of 120 s, final regeneration of the surface was performed

with a short pulse of 0.05% (w/v) SDS. The interaction between P/CAF and cyclin A was detected and presented as a sensorgram by plotting resonance units against time.

Cell culture, synchronization and treatments

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfected synchronized cells were obtained as described (Donzelli et al., 2002). Specific experiments were performed with cells that were cultured in the presence of 10 μg/ml cycloheximide.

Flow citometry analyses

Cells were fixed with 70% cold ethanol for 2 h at 4ºC, washed with PBS, and finally incubated with 50 μg/ml of propidium iodide (Sigma) and 200 μg/ml RNase for 30 min at room temperature. Analysis of DNA content was carried out in a BD Biosciences FACS Canto II. Data was analysed with WinMDI 2.9 software.

In vivo ubiquitylation assays

Cells were transfected with indicated plasmids. 24 h after transfection, cells were replated and treated with 100 μM ALLN for 16 h. Then, cells were harvested and subjected to IP.

Immunoprecipitation and kinase assays

After three washes in RIPA buffer and two in kinase buffer (50 mM Hepes pH 7.4, 2.5 mM EGTA, 10 mM $MgCl_2$) immunoprecipitates were resuspended in a final volume of 30 μl of kinase buffer containing 12.5 μM

ATP, 1 μCi of [^{32}P]ATP, 2 mM dithiothreitol, and 2 μg of histone H1 for 30 min at 30°C. Reactions were stopped by the addition of Laemmli buffer. The samples were then electrophoresed on 12% SDS-polyacrylamide gels and then stained with Coomassie Blue and dried. The radioactivity associated to the gels was detected with a PhosphorImager.

Supplementary information is available at the oncogene's web site

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Legends to the Figures

Figure 1 Cyclin A is acetylated by P/CAF *in vivo* and *in vitro*. **(a)** HCT116 cells transfected with HA-cyclin A were subjected to IP with anti-HA or IgG as a control, followed by WB with anti-HA or anti-acetylK. **(b)** Purified GST-cyclin A was subjected to *in vitro* acetylation assays using the catalytic domain of P/CAF (GST-$HAT_{P/CAF}$), GST-CBP or GST-Tip60 in the presence of [^{14}C]acetylCoA. Purified GST was used as a negative control substrate. In the assays with P/CAF or Tip60 their autoacetylation was used as a positive control, whereas in the case of CBP, histones were used as a positive control substrate. Acetylated proteins were visualized by autoradiography (upper panel). A loading control gel was stained with coomassie blue (bottom panel). **(c)** HCT-116 cells were transfected with Flag-P/CAF, HA-cyclin A and control or P/CAF siRNA. Extracts were prepared and expression of P/CAF was analysed by WB (left panel). IPs against HA or IgG as a control were performed with part of the extracts followed by western blot with anti-HA or anti-acetylK (right panel).

Figure 2 Cyclin A is acetylated at lysines 54, 68, 95 and 112. **(a)** Schematic representation of cyclin A lysine residues and domains. (**b**) Thirteen peptides including one or two consecutive lysines from the cyclin A fragment including aa 1-171, were spotted on a membrane (see also Fig S1). As a positive control a peptide from histone H3 was added. The membrane was subjected to *in vitro* acetylation assays with P/CAF and [^{14}C]acetylCoA. Acetylation was visualized by autoradiography. **(c)** Purified GST-cycA WT and GST-cycA 4R were used in *in vitro* acetylation assays with P/CAF. Acetylation was visualized by autoradiography (left panel). A loading

control gel was stained with coomassie blue (right panel). **(d)** HeLa cells were transfected with Flag-cycA WT or Flag-cycA 4R. Cell extracts were subjected to IP with anti-Flag followed by WB with anti-Flag and anti-acetylK.

Figure 3 Cyclin A is acetylated and interacts with P/CAF at S and G_2/M phases of the cell cycle. **(a)** COS cells were transfected with CFP-cyclin A and YFP-P/CAF and co-localization of both proteins was studied by fluorescence microscopy. **(b)** HeLa cells were transfected with HA-cyclin A and YFP-P/CAF. Cell extracts were subjected to IP using HA or IgG as a control followed by WB with antibodies against HA, P/CAF and cdk2. A sample of cell lysate (input) was used as a control. **(c)** The putative direct interaction between P/CAF and cyclin A was studied by Surface Plasmon Resonance as described in methods section. P/CAF was fixed on the matrix and cyclin A was left to circulate on the chip. The interaction was represented in the sensorgram. **(d)** HeLa cells were transfected with HA-cyclin A and YFP-P/CAF and synchronized by a double-thymidine block or nocodazol as described in methods section. Then, the levels of P/CAF, cyclin A and cdk2 were determined by WB (left panel). To confirm the time of mitosis a western blot with antibodies against phosphorylated histone H3 was performed (bottom, left panel). Cell extracts were subjected to IP with anti-HA and the amount of P/CAF, cyclin A, acetylated cyclin A and cdk2 was analyzed by WB (right panel).

Figure 4 CycA 4R is much more stable than cycA WT and induces cell cycle arrest at G_2/M. **(a)** HeLa cells were transfected with Flag-cycA WT or Flag-cycA 4R, treated with cyclohexymide (CHX) and collected at different

times of treatment. Cyclin A levels were analyzed by western blot. Cdk2 levels were used as a loading control. The amount of Flag-cyclin A was quantitated and represented in the graph. Results are the mean ± SE of 8 independent experiments. **(b)** HeLa cells were mock-transfected as a control or transfected with CFP-cycA WT or CFP-cycA 4R. FACS analysis of the population of transfected cells was performed (bottom panel) and represented in a graph (upper panel). Control of expression of CFP-cycA WT and 4R is shown in Figure S2a.

Figure 5 Cyclin A acetylation is simultaneous to its ubiquitylation. **(a)** HeLa cells were transfected with HA-ubiquitin plus Flag-cycA WT, Flag-cycA 4R or Flag-cycA 4Q and treated with the proteasome inhibitor ALLN. Then, they were lysed and subjected to IP with anti-Flag or IgG as a control. The levels of ubiquitylated cyclin A were determined by WB with anti-Flag and anti-HA. **(b)** Flag-cycA WT-transfected cells were treated with the proteasome inhibitor ALLN, collected at different times after the release of a double-thymidine block and subjected to IP with anti-Flag. Cyclin A ubiquitylation was determined by western blot with anti-Flag and cyclin A acetylation with anti-acetylK. A shorter exposure of WB anti-Flag is shown in the middle panel.

Figure 6 Differential interaction of cycA WT and 4R with components of the ubiquitylation machinery and cdk1 and cdk2. **(a)** 293T cells were transfected with Flag-cycA WT or Flag-cycA 4R, then lysed, and IPs with anti-Flag were performed. The presence of APC3, Cdh1, Cdc20, Cks1/2, cdk1 and cdk2 in the immunoprecipitates was determined by WB. **(b)** As described in **(a)**, but co-transfecting Flag-Cdc20 with CFP-cycA WT or 4R,

or co-transfecting HA-Cdh1 with Flag-cycA WT or 4R. **(c, d)** 293T cells were transfected with Flag-cycA WT or 4R, lysed and immunoprecipitated with anti-Flag **(c)** or anti-CDK2 **(d).** Kinase assays of the immunoprecipitates were performed and phosphorylation of histone H1 was detected by PhosphorImager. Kinase activity was normalized to the amount of immunoprecipitated cdk2 and represented in the graphs. Results shown are the mean ± SE of 3 independent experiments. Asterisk indicates an unspecific band detected by anti-Flag antibody.

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Figure 1

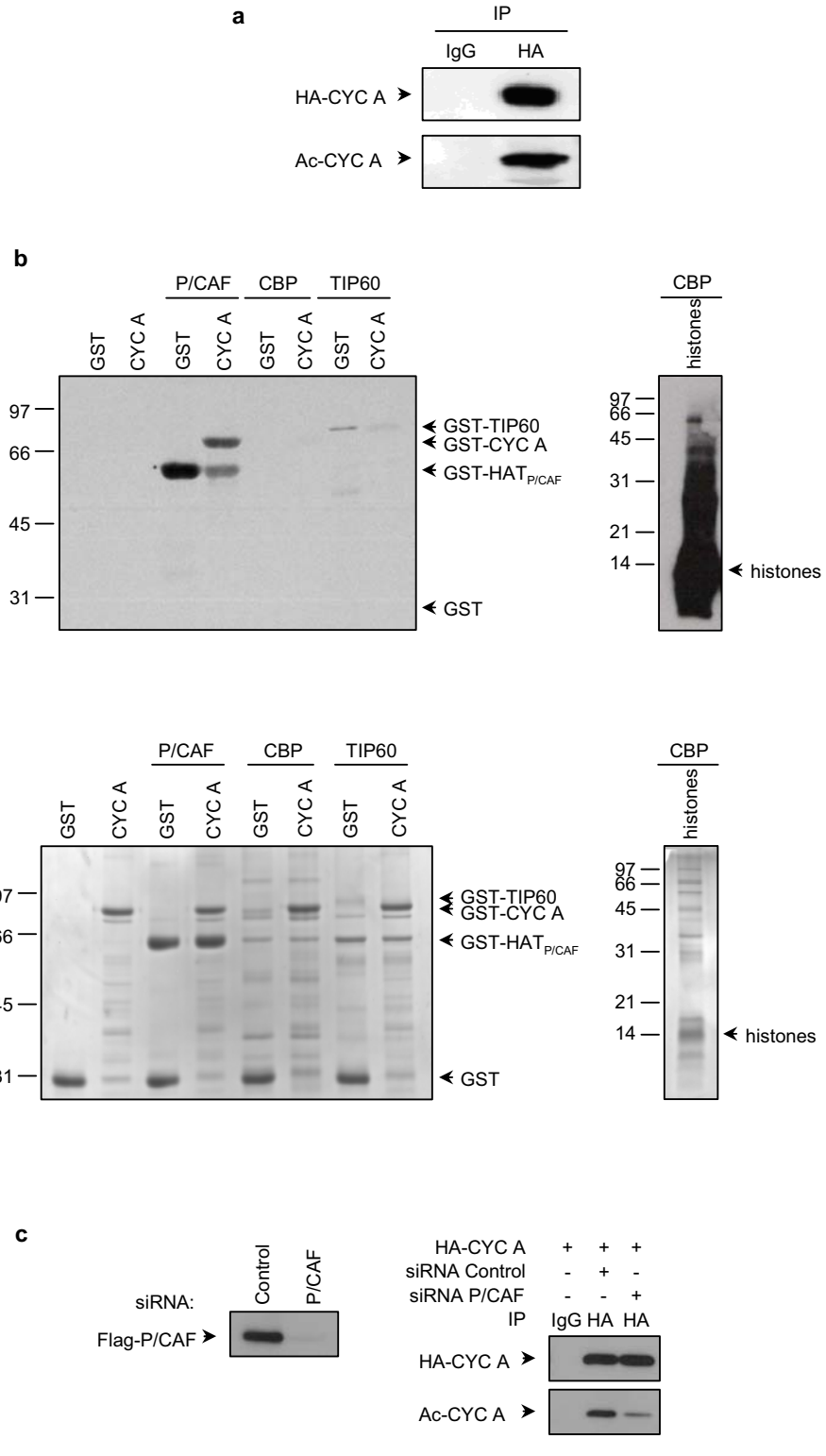
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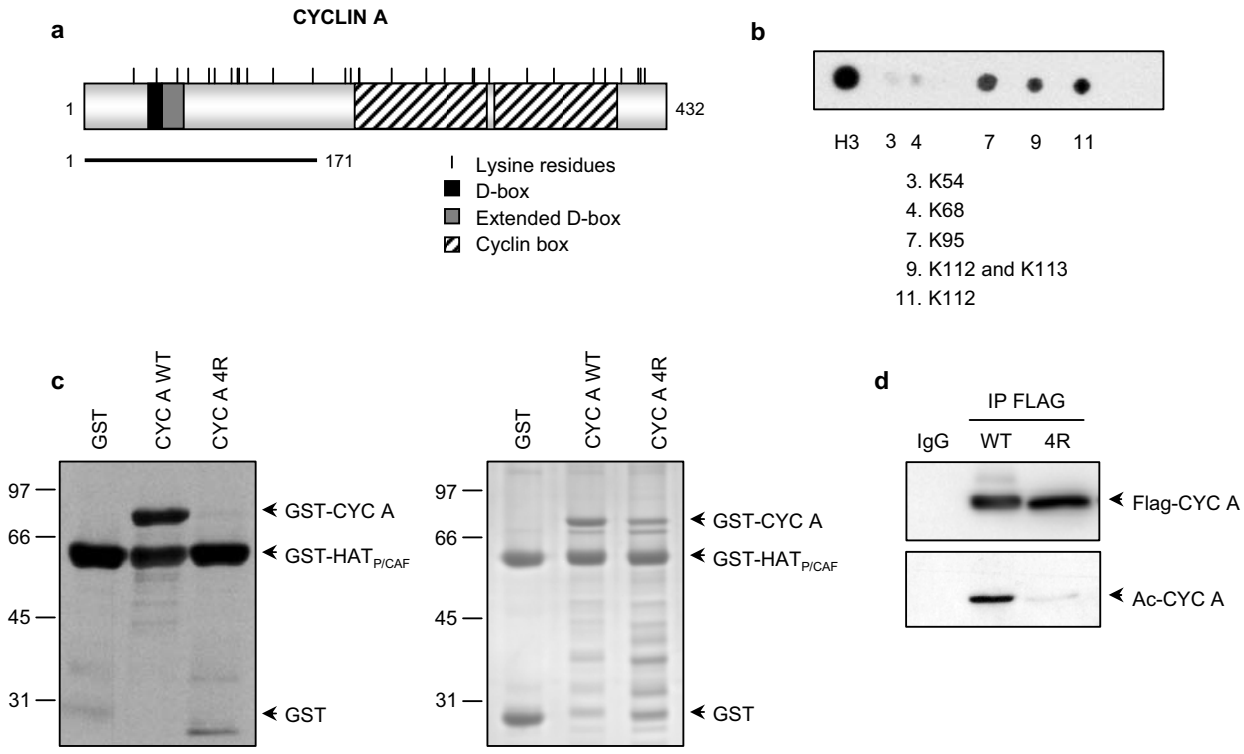
Figure 2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7. K95
9. K112 and K113
11. K112
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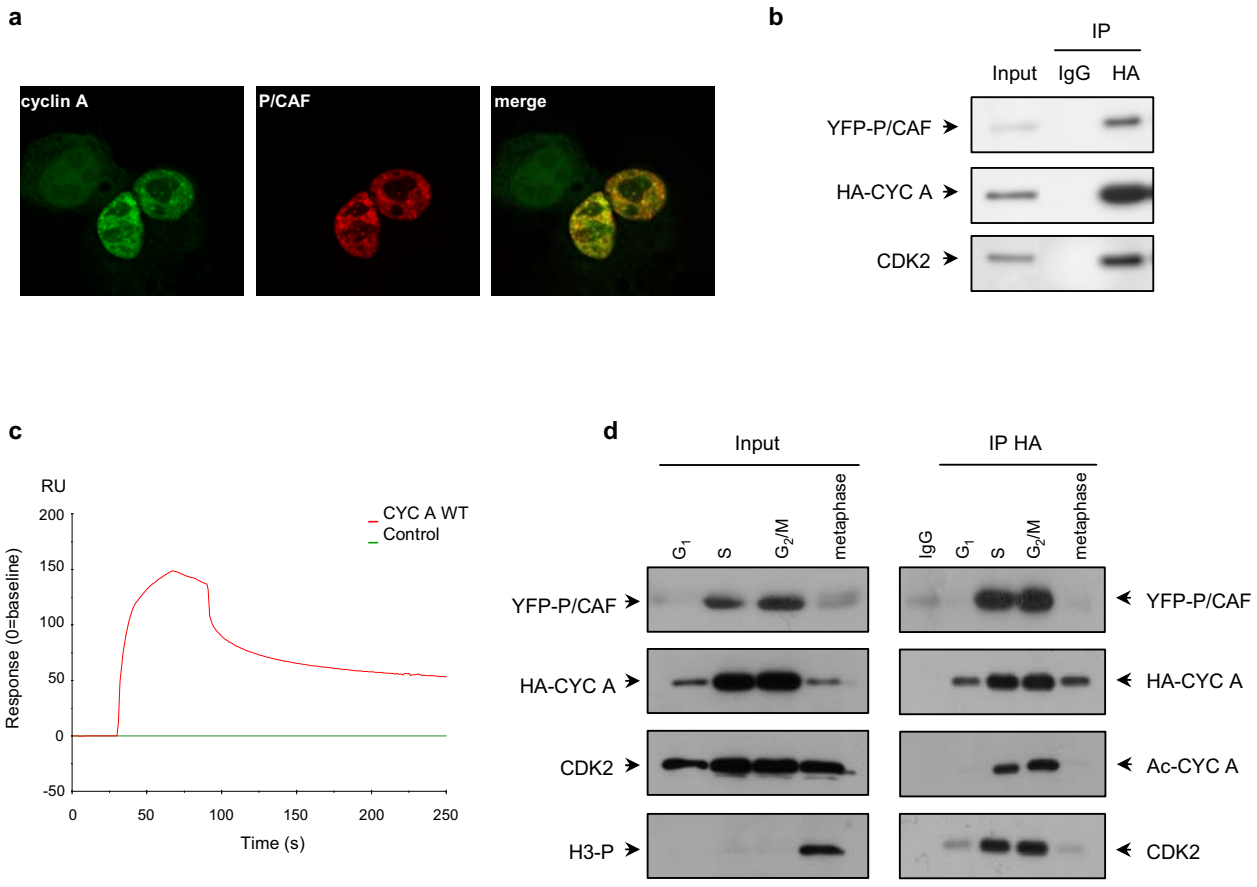
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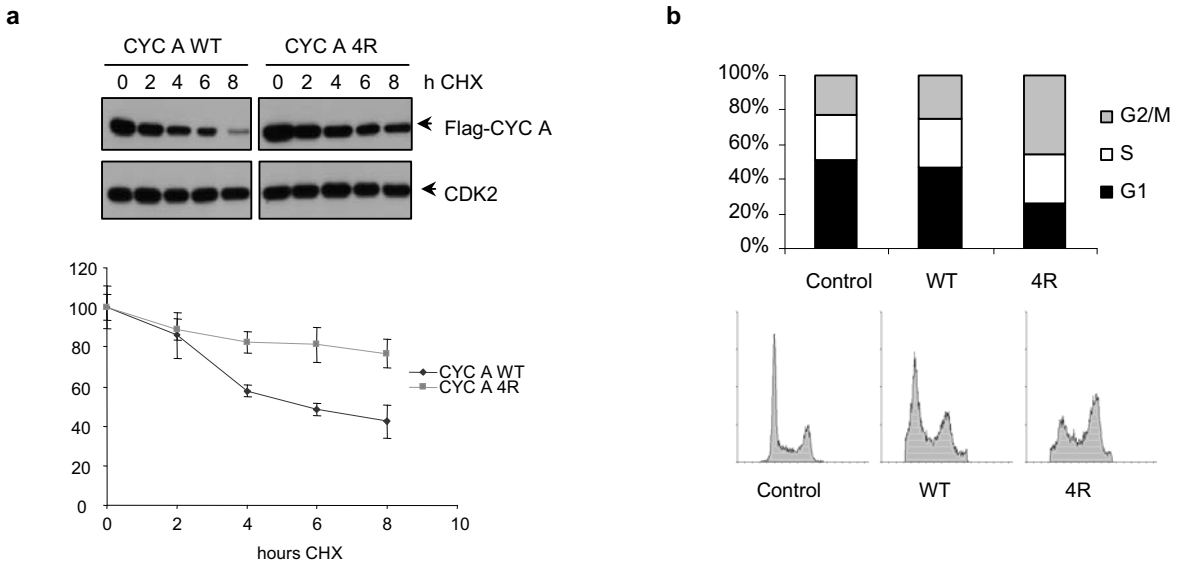
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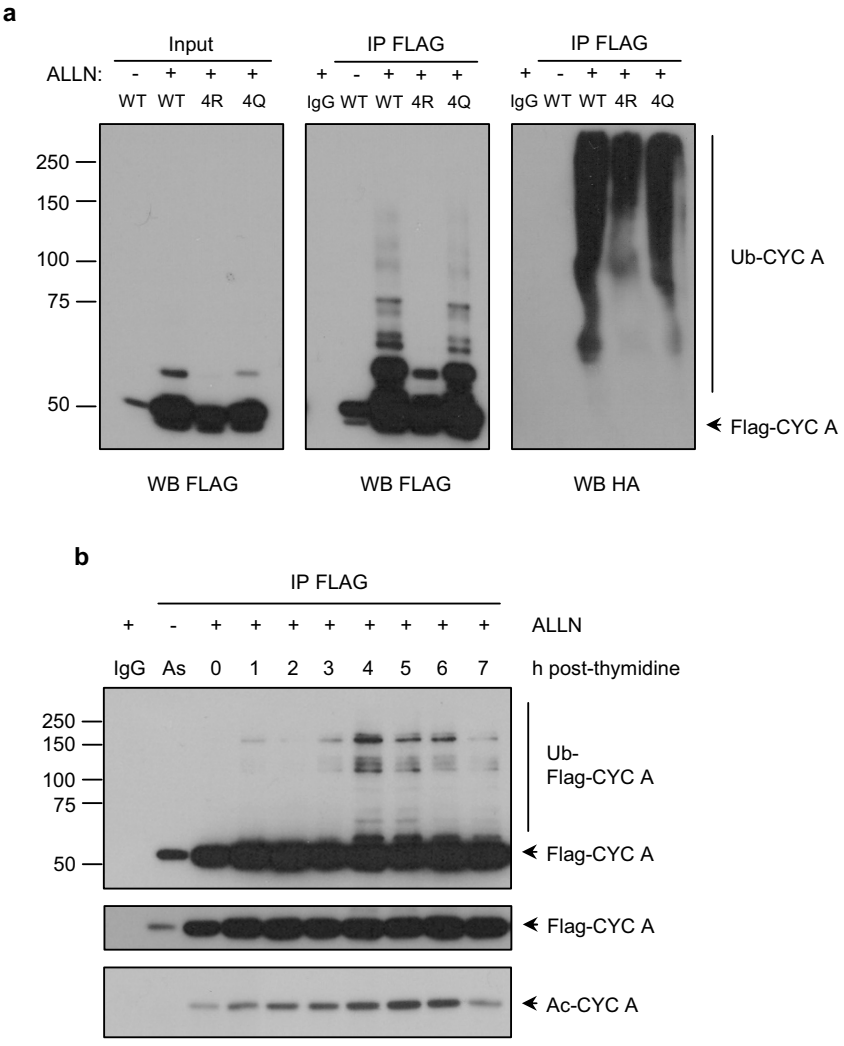
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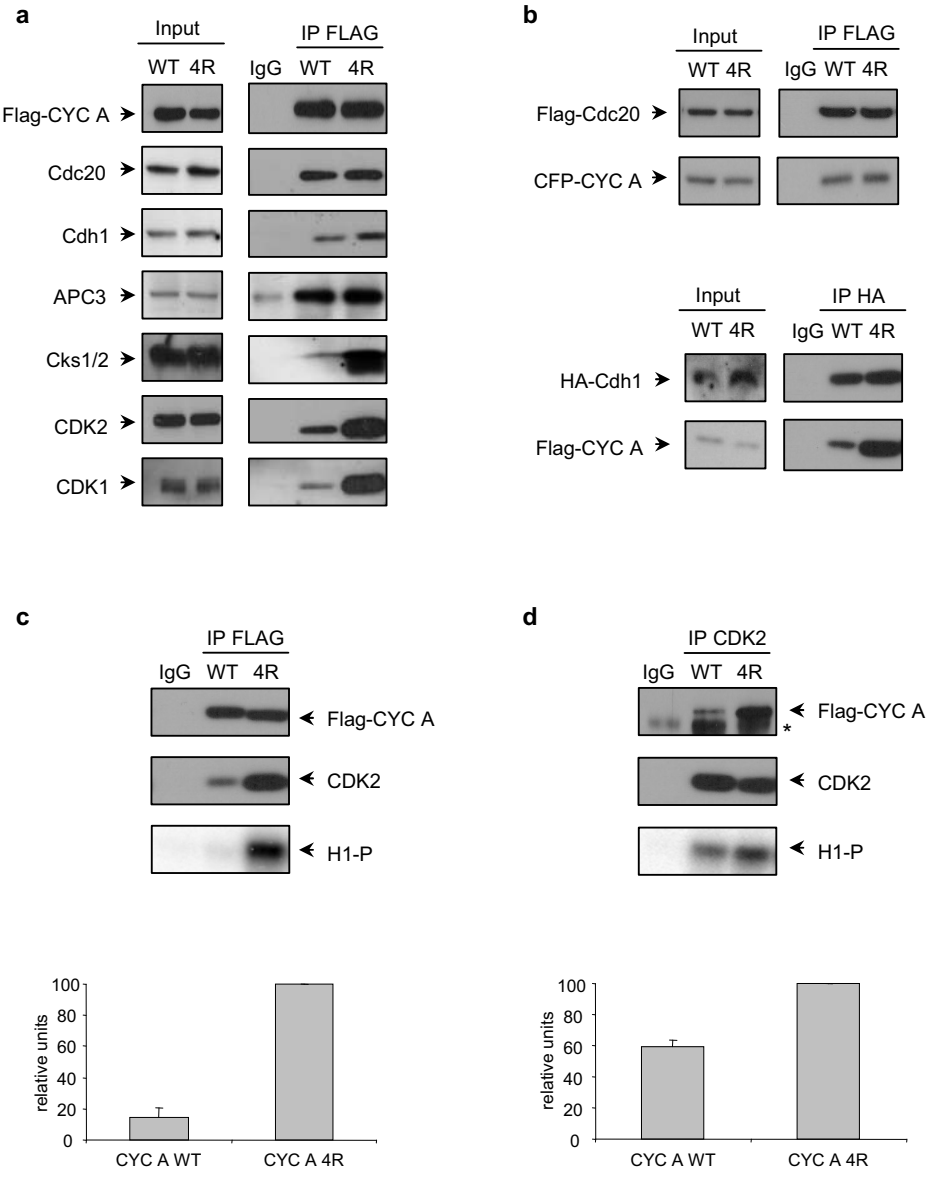
Figure 6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