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Phosphorylation by cyclin-dependent kinase-9 controls ubiquitin-conjugating enzyme-2A function

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Cyclin-dependent kinase-9 (CDK9) plays a central role in transcriptional elongation and controls multiple cotranscriptional histone modifications, including histone H2B monoubiquitination (H2Bub1). Like other CDK9-dependent histone modifications, the role of CDK9 in maintaining H2Bub1 was shown to be partially dependent upon the phosphorylation status of Ser2 of the RNA polymerase II (RNAPII) C-terminal domain (CTD). Since mutation of Ser2 within the RNAPII CTD resulted in a milder effect on H2Bub1 compared with CDK9 knockdown, we explored whether another CDK9 target may also influence H2Bub1. Based on its homology to yeast Bur1, we hypothesized that CDK9 may directly phosphorylate and activate the ubiquitin-conjugating enzyme utilized for H2B monoubiquitination. Indeed, we demonstrate that UBE2A specifically interacts with CDK9, but not CDK2. Furthermore, UBE2A is phosphorylated by CDK9 in vitro and increases UBE2A activity. Interestingly, CDK9 knockdown not only decreases UBE2A phosphorylation and H2Bub1, but also significantly impairs the induction of UBE2A-dependent monoubiquitination of proliferating cell nuclear antigen (PCNA). Thus, we provide the first evidence that CDK9 is required for the activity of UBE2A in humans, and that its activity is not only required for maintaining H2Bub1, but also for the monoubiquitination of PCNA. The common involvement of these two ubiquitinations in distinct DNA repair pathways may provide a mechanistic rationale for further exploring CDK9 as a combinatorial target for increasing the efficacy of existing cancer therapies based on the induction of DNA damage and are repaired by mechanisms which require H2Bub1 and/or PCNA ubiquitination.

Introduction

The correct packaging of DNA into chromatin plays an essential role in all nuclear DNA-associated processes, including transcription, co-transcriptional processes, DNA replication and DNA repair. In particular, the posttranslational modification of the core histones H2A, H2B, H3 and H4 by phosphorylation, acetylation, methylation or ubiquitination affects these processes in a positive or negative manner depending upon the specific type and location of the modification as well as the surrounding context.[1]

Monoubiquitination of the C-terminal tail of histone H2B (H2Bub1[5]) is conserved from yeast (K123) to human (K120).[2] H2Bub1 is associated with transcriptional elongation on active genes.[3-7] Consistent with this, H2Bub1 is dependent upon the WW domain-containing adaptor with coiled coil protein (WAC) which binds directly to the elongating form of RNA polymerase II (RNAPII).[8] In addition to its role in transcriptional regulation, H2Bub1 also plays a role in DNA repair in both yeast and humans.[9-13] These different functions appear to cooperate to serve a tumor suppressor function in humans.[5,8,14-16]

Like other ubiquitination reactions, H2B monoubiquitination requires the activity of E1, E2 and E3 ubiquitinating enzymes, which subsequently transfer the activated ubiquitin molecule to the target protein.[17] The E2 ubiquitin-conjugating enzyme for H2B in yeast is called Rad6.[18] In humans, this function is assigned primarily to the Rad6 ortholog UBE2A.[19] Bre1 serves as an E3 ubiquitin ligase for H2B in the yeast *Saccharomyces cerevisiae*.[20] In humans, Bre1 has two orthologs, RNF20 and RNF40, which form an obligate heterodimer where each are required for maintaining H2Bub1 levels.[4,7,21-23]

Like most E2 enzymes, Rad6 is involved in the ubiquitination of multiple substrates. Proliferating cell nuclear antigen (PCNA) is ubiquitinated at lysine 164 by a complex containing Rad6 and the E3 ubiquitin ligase Rad18 both in yeast[24] and in humans.[25] Monoubiquitinated PCNA can subsequently be polyubiquitinated by the Rad5/MMS2/Ubc13 complex via a K63-linked chain.[24] This promotes the recruitment of DNA polymerase η to activate the translesion synthesis DNA repair pathway.[26-28] The N-end rule pathway of protein degradation also utilizes Rad6, where it serves as an E2 enzyme for the ubiquitin ligase Ubr1.[29]

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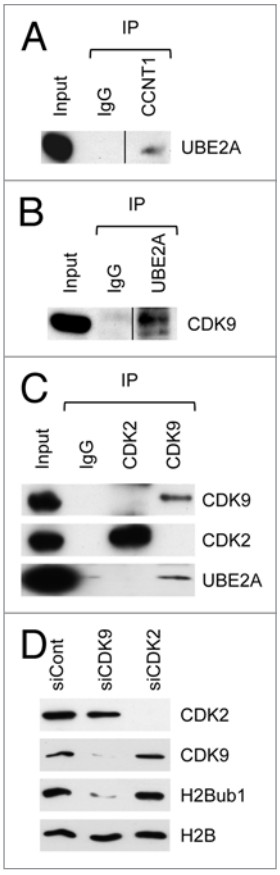

Figure 1. UBE2A interacts with CDK9 but not CDK2. (A–C) Protein lysates from H1299 cells were used for co-immunoprecipitation with antibodies against endogenous Cyclin T1 (A), UBE2A (B), CDK2 or CDK9 (C). The presence of co-immunoprecipitated proteins was detected by western blot using antibodies to UBE2A (A and C), CDK9 (B and C) or CDK2 (C). (D) H1299 cells were transfected with siRNAs against CDK9, CDK2 or with control siRNAs for 48 h. Protein lysates were harvested and analyzed by western blot with antibodies to CDK2, CDK9, H2Bub1 and H2B. H2B serves as a loading control.

The Bur1/Bur2 complex consists of the cdc28-related kinase Bur1 and Cyclin T family member Bur2.[30] Bur1 was shown to control H2B monoubiquitination by direct phosphorylation of the highly conserved serine residue at position 120 of Rad6.[31] In human cells, UBE2A was also shown to be phosphorylated at the conserved serine 120 by cyclin-dependent kinases (CDKs) 1 and 2.[32]

The closest ortholog of Bur1 in humans is the positive transcription elongation factor-b (P-TEFb) complex component CDK9.[30,33] Like Bur1, CDK9 plays an important role in transcriptional elongation and is required for maintaining global H2Bub1 levels in humans.[21] The primary function of CDK9 in transcriptional elongation has been ascribed to its ability to phosphorylate the RNAPII CTD and the negative elongation factors NELF-E (in humans) and SUPT5H.[23] However, to date, no role for CDK9 in the phosphorylation of UBE2A or its function has been reported. Here, we investigated the interaction between CDK9 and UBE2A and demonstrate that CDK9 phosphorylates UBE2A and regulates UBE2A-mediated monoubiquitination of both H2B and PCNA.

Results

CDK9 interacts with UBE2A. Based on the interaction between Bur1 and Rad6 in yeast[20] and the requirement of both UBE2A and CDK9 for H2Bub1 in humans, we hypothesized that UBE2A may also interact with P-TEFb in human cells. Therefore, we performed co-immunoprecipitation studies using antibodies against UBE2A, CDK9 and Cyclin T1. Consistent with our hypothesis, we observed an interaction between endogenous UBE2A and Cyclin T1 (**Fig. 1A**) as well as CDK9 (**Fig. 1B**).

Since a previous study reported interactions between UBE2A and CDK2,[32] we also examined this interaction. However, while UBE2A was coimmunoprecipitated with CDK9, no interaction between UBE2A and CDK2 could be observed in human cells (**Fig. 1C**). Furthermore, only knockdown of CDK9 but not CDK2 resulted in reduced H2Bub1 levels (**Fig. 1D**). Thus UBE2A preferentially interacts with CDK9 but not CDK2 to control H2B monoubiquitination.

CDK9 phosphorylates UBE2A in vitro and in vivo. We next tested whether CDK9 is capable of site-specifically phosphorylating UBE2A. Since serine 120 is highly homologous between yeast Rad6 and human UBE2A we utilized GST-fusion proteins containing wild type or a S120A mutant of UBE2A (**Fig. 2A**) as substrates in an in vitro kinase assay. As reported previously, the RNAPII CTD was significantly phosphorylated by CDK9 in vitro (**Fig. 2B**). Similarly, UBE2A but not a S120A mutant or GST alone was significantly phosphorylated by CDK9 (**Fig. 2C**). Quantification of the intensity of the bands and normalizing to the molecular weights of proteins showed that the S120A mutant was phosphorylated at a level similar to GST alone, while only wild-type UBE2A was specifically phosphorylated above the background level (**Fig. 2D**).

In order to investigate UBE2A phosphorylation in cells, we generated a mouse monoclonal antibody that preferentially recognizes the S120-phosphorylated form of UBE2A, but the unphosphorylated, or S120A mutant form of UBE2A to a much lesser degree (**Fig. 2E**). Importantly, the signal of this antibody in western blot is strongly decreased following the knockdown of either CDK9 or UBE2A, while total UBE2A levels are not significantly decreased following CDK9 knockdown (**Fig. 2F**). Thus these data suggest that the phosphorylation of UBE2A in vivo depends on CDK9 activity.

Phosphorylation increases UBE2A activity toward H2B. A previous report demonstrated that UBE2A is capable of ubiquitinating histone proteins in an E3-independent manner in vitro in the presence of substrate nucleosomes, ATP, ubiquitin and E1.[19] In order to test whether phosphorylation affects UBE2A function, we performed various in vitro ubiquitination reactions using assembled nucleosomes as a substrate. As noted previously in reference 32, the position analogous to S120 of UBE2A is often replaced by two aspartate (D) or glutamate (E) residues in many other human E2 enzymes (**Fig. 3A**). Thus we produced recombinant GST-fusion proteins not only for wild type, but also catalytically inactive (C88S), phosphorylation-deficient (S120A) and phosphomimicking (N119D/S120D; DD) mutants of UBE2A (**Fig. 3B**). Consistent with a positive role for a negative charge

at this location within E2 enzymes, the DD mutant showed a significantly higher in vitro ubiquitinating activity toward H2B compared with wild-type UBE2A (**Fig. 3C**). In contrast, the catalytically inactive form of UBE2A (C88S) was unable to ubiquitinate H2B. A further indication that phosphorylation increases UBE2A ubiquitin-conjugating activity was shown by combined in vitro kinase and ubiquitination assays, in which UBE2A was incubated together with P-TEFb prior to in vitro ubiquitination assays. Here, the ubiquitin-conjugating activity of UBE2A was also significantly increased following incubation with P-TEFb (**Fig. 3D**).

CDK9 regulates UBE2A activity in vivo. Like most E2 ubiquitin-conjugating enzymes, UBE2A cooperates with multiple E3 ubiquitin ligase complexes. In this case, RAD18 and RNF20/40, which ubiquitinate PCNA and H2B, respectively, both utilize UBE2A for substrate ubiquitination.[19,25] Based on our data indicating that CDK9 regulates UBE2A ubiquitin-conjugating activity, we hypothesized that the monoubiquitination of PCNA may also depend upon CDK9. Indeed, the reduction of PCNA monoubiquitination in UVC-treated CDK9-depleted cells was comparable to the effect of knocking down UBE2A alone (**Fig. 4A**). Similarly, knockdown of Cyclin T1, the major cyclin required for CDK9 activity,[34] also led to a reduction of PCNA monoubiquitination following UVC treatment (**Fig. 4B**).

Discussion

CDK9 plays a significant role in the regulation of transcriptional elongation. It activates RNAPII by direct phosphorylation and also inactivates negative elongation factors.[35-37] The role of CDK9 in directing various chromatin modifications has also been established.[23,38] In particular, we showed that CDK9 activity is necessary for the maintaining of global and gene-specific levels of histone H2B monoubiquitination.[21] In our initial model, which was further supported by another recent report in reference 8, we proposed that CDK9 regulates H2Bub1 at least in part by phosphorylation of Ser2 within the RNA polymerase II C-terminal domain. However, the substitution of Ser2 with an alanine resulted in an incomplete loss of H2Bub1,[21] leading us to hypothesize that additional CDK9 targets, such as UBE2A, might regulate H2Bub1.[23] In this study, we show for the first time that UBE2A, a ubiquitin-conjugating enzyme for H2B, is phosphorylated and activated by CDK9.

The interaction between UBE2A and P-TEFb components was confirmed by co-immunoprecipitation. The in vitro phosphorylation assay confirmed site-specific phosphorylation of UBE2A by P-TEFb. Consistently, the level of phosphorylated UBE2A in vivo was decreased by CDK9 knockdown. In support of a general regulatory function of UBE2A by CDK9, the ubiquitination of PCNA, which depends upon UBE2A activity, was reduced after the knockdown of P-TEFb components.

The mode of UBE2A regulation by CDK9-dependent phosphorylation is not yet clear. Interestingly, many members of the UBE2 family in humans have at least one or two negatively charged amino acids at the analogous site phosphorylated in UBE2A, implying that a strong negative charge is necessary at this site. However, in the case of UBE2A, this activity appears to be regulated by CDK9-mediated phosphorylation. Consistent

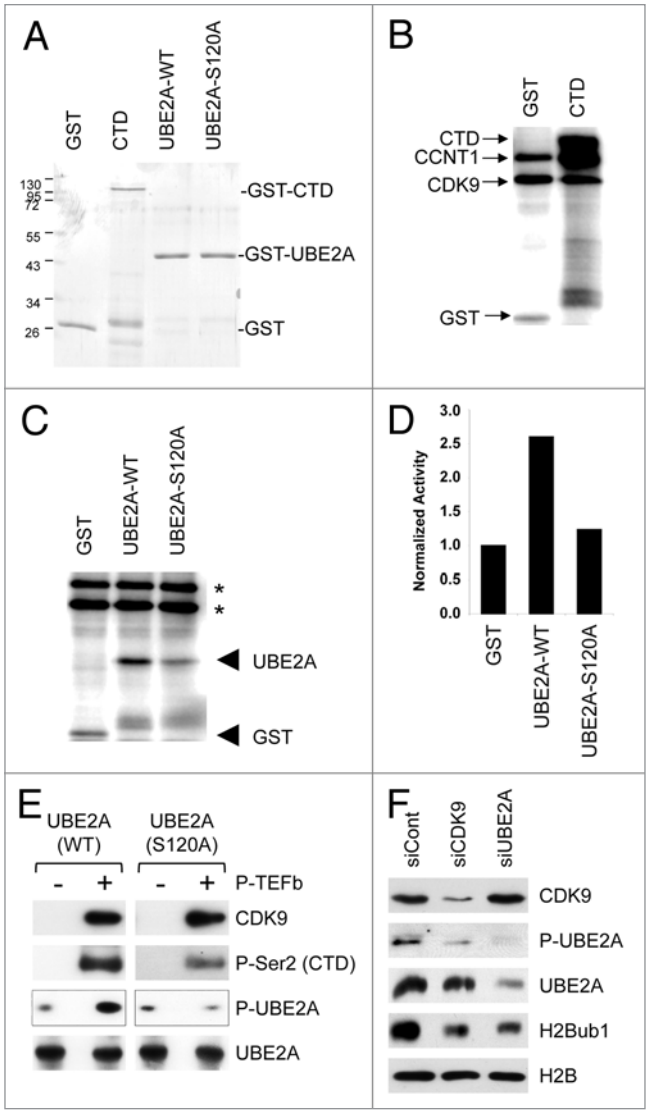

Figure 2. CDK9 phosphorylates UBE2A in vitro and in vivo. (A) Recombinant bacterially expressed GST, GST-CTD (RNAPII), GST-UBE2A-WT or GST-UBE2A-S120A were separated by SDS-PAGE and stained with Coomassie. (B and C) In vitro kinase assays were performed using recombinant GST (B and C), GST-CTD (B), GST-UBE2A-WT (C) or GST-UBE2A-S120A (C) as substrates for recombinant P-TEFb in the presence of [^{32}P]-γ-ATP. The products of reaction were separated by SDS-PAGE and visualized by autoradiography. *Indicates auto-phosphorylated CDK9 and CCNT1. (D) Quantitation of the intensity of the phosphorylated products in (C) using the TYPHOON Scanner Control 3.0 software. Results were normalized to the protein size and presented as fold relative to GST background. (E) Phosphospecificity of a monoclonal anti-phospho-UBE2A antibody. In vitro phosphorylation of GST-UBE2A-WT or GST-UBE2A-S120A was performed as in (C) in presence of GST-CTD and recombinant P-TEFb. Products of the reaction were visualized with western blot using antibodies to CDK9, P-Ser2, P-UBE2A and UBE2A. UBE2A is shown as a loading control. (F) HCT116 cells were transfected with control, CDK9 or UBE2A siRNAs, and cell lysates were analyzed 48 h after transfection by western blot with antibodies to CDK9, P-UBE2A, UBE2A, H2Bub1 and H2B. H2B serves as a loading control.

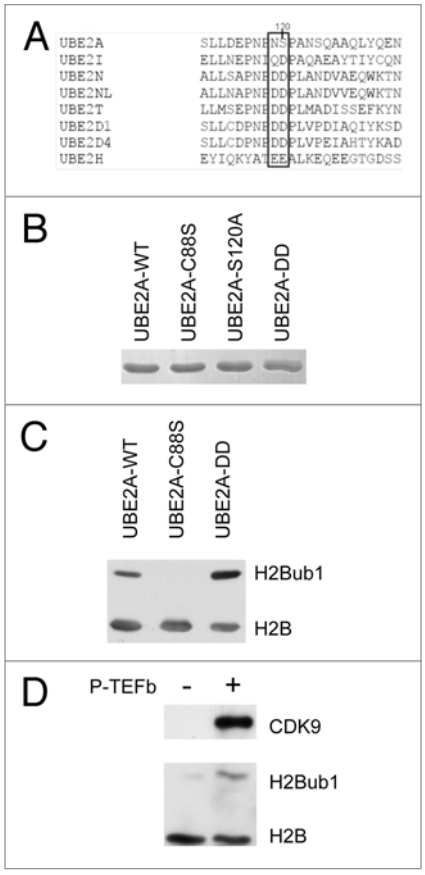

Figure 3. Phosphorylation controls UBE2A ubiquitin-conjugating activity. (A) Amino acid sequence alignment of various human ubiquitin conjugating enzymes (done by ClustalW software). The frame indicates the conservation of the negatively charged amino acids, which are substituted by a serine residue in UBE2A. (B) Recombinant bacterially expressed GST-fusion proteins of wild-type UBE2A (WT), or catalytically inactive (C88S), phosphorylation-deficient (S120A) or phosphomimetic (N119D/S120D; DD) mutants of UBE2A. Proteins were resolved by SDS-PAGE and visualized by Coomassie staining. (C) An in vitro ubiquitination assay was performed in presence of wild-type UBE2A, C88S or DD mutants using assembled recombinant nucleosomes as substrates. Products were analyzed by western blot using antibodies to H2B. The higher migrating band represents H2Bub1. (D) An in vitro ubiquitination assay was performed as in (C) following in vitro phosphorylation of UBE2A by P-TEFb. Protein lysates were resolved by western blot with antibodies to CDK9 and H2B (the higher migrating band represents H2Bub1).

with a direct structural role for S120 phosphorylation in controlling UBE2A ubiquitin-conjugating activity, we observed no change in the interaction of UBE2A with either RNF40 or RAD18 following CDK9 inhibition (data not shown) but do provide evidence that its in vitro activity is dependent upon a negative charge at S120.

In addition to its role in transcription, H2Bub1 was recently shown to play an important role in DNA double-strand break (DSB) repair.[9,11-13] Interestingly, the potent CDK9 inhibitor flavopiridol has been shown to potentiate the effects of various DSB inducers in inducing apoptosis in cancer cells.[39-43] Furthermore, like RNF20 or RNF40 knockdown,[44] CDK9 knockdown has been linked to the induction of replication stress,[45,46] further supporting this link. Different mechanisms of flavopiridol action, including cell cycle regulation and the modulation of PARP activity, have been proposed to explain these effects. Although some authors have mentioned CDK9 as a possible relevant flavopiridol target, no clear connection to CDK9-dependent chromatin modifications or their role in these effects has been made so far.

In addition to the role of H2Bub1 in DNA repair, the ubiquitination of PCNA is an important component in the Fanconi anemia (FA) network, which is essential for the repair of interstrand DNA cross-links.[47] Consistent with a regulatory role for CDK9 in controlling PCNA monoubiquitination and its role in DNA repair by the FA pathway, the combinatorial treatment of flavopiridol and Mitomycin C significantly increased the efficiency of apoptosis induction in breast cancer cells.[48] Thus, based on our results, we now provide a potential mechanistic explanation for the observed effects, which may offer a rational basis for testing more specific CDK9 inhibitors in combination with interstrand cross-linkers.

Based on these results we propose a model in which P-TEFb-mediated UBE2A phosphorylation regulates its activity and subsequent H2B and PCNA monoubiquitination, both of which are necessary for distinct pathways required for the proper repair of different types of DNA damage. Additional mechanistic studies combining more specific CDK9 inhibitors with conventional radiation or chemotherapeutic treatments may uncover new and more effective approaches to cancer treatment.

Materials and Methods

Cell culture. H1299 and HCT116 cells were cultured in phenol red-free high-glucose Dulbecco's modified Eagles medium (DMEM) and McCoy's 5A medium, respectively, supplemented with 10% bovine growth serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate at 37°C under 5% CO_2 atmosphere. Plasmids and siRNAs were transfected using Lipofectamine 2000 or RNAiMAX (Invitrogen GmbH), respectively, according to the manufacturer's instructions. When indicated cells were irradiated with 80 J/m^2 UVC light.

GST protein purification. UBE2A was amplified from cDNA from H1299 cells and cloned into the pGEX-6P1 vector (GE Healthcare) for expression in *E. coli*. Site-directed mutagenesis was performed by amplifying the plasmid with the gene using primers containing the mutation as described in reference 49. GST-UBE2A fusion proteins were expressed and purified from the RilDE BL21 *E. coli* strain (Invitrogen) on glutathione-sepharose beads as described previously in reference 50. Eluted proteins were analyzed by SDS-PAGE and quantitated by Coomassie staining using a serial dilution curve of BSA.

In vitro kinase assay. The radiophospholabeling was performed as described previously in reference 51, using 150 ng of recombinant CDK9 (Cell Signaling), 500 ng of target protein and 50 μCi of γ-[^{32}P]-ATP. The reaction was performed for 20 min at 30°C and stopped by adding 6x Laemmli buffer and heating at 95°C for 5 min. Samples were subsequently separated by

SDS-PAGE; the gel was dried and analyzed by phosphoimager to detect the phosphorylated proteins. Non-radioactive kinase assays were performed by mixing GST-UBE2A and GST-CTD with 400 ng of recombinant P-TEFb (ProQinase, GmbH) in the reaction buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 6 mM EDTA, 100 μM ATP, 1 mM DTT, 0.1 mM Na_3VO_4 and protease inhibitors. Reaction was performed for 1 h at 30°C.

In vitro ubiquitination assay. GST-UBE2A or its mutant forms were mixed with ubiquitin, E1, and assembled nucleosomes in the reaction buffer containing 2 mM ATP, 2 mM $MgCl_2$, 25 mM Tris (pH 7.6), 50 mM NaCl and 1 mM DDT. The reaction was incubated for 1 h at 37°C. For the coupled phosphorylation-ubiquitination reaction GST-UBE2A was first exposed to an in vitro kinase reaction with P-TEFb as described above and the resulting mix was added to ubiquitination assay.

Protein co-immunoprecipitation. Co-immunoprecipitation was essentially performed as described in reference 52. Briefly, cells were washed with PBS and then scraped in 1 ml Frackelton buffer. Cells were vortexed for 15 sec and tumbled for 45 min at 4°C. Cell lysate was cleared by centrifugation and the supernatant was pre-cleared by adding 50 μl of sepharose beads for 1 h. The pre-cleared supernatant was incubated with 1–3 μg of antibodies for 2 h and 30 μl of protein A or G coupled sepharose beads for 1 h at 4°C. Beads were washed four times with Frackelton buffer and heated at 95°C with 70 μl of 2x Laemmli buffer. The supernatant was analyzed by SDS-PAGE and western blot.

Monoclonal antibody generation. The phospho-UBE2A antibody was generated essentially as previously described in reference 53, using the peptide N-QSL LDE PNP N**S**P ANS QAA QLY QEC-C (Peptide Specialty Laboratories) where the underlined serine residue was phosphorylated. Individual hybridoma clones were initially positively selected for reactivity against the phosphopeptide and then negatively selected against the non-phosphorylated peptide. Positive clones were further screened by western blot analyses before performing subsequent analyses.

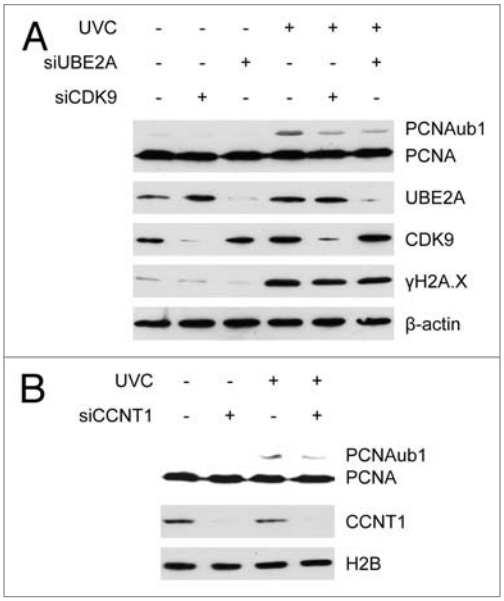

Figure 4. P-TEFb is required for ubiquitination of other UBE2A targets. (A and B) HCT116 cells were transfected with control (A and B), UBE2A (A), CDK9 (A) or CCNT1 (B) siRNAs. Cells were exposed to 80 J/m² UVC 48 h after transfection and grown for an additional 3 h. Proteins lysates were harvested and analyzed by western blot with antibodies to PCNA (higher migrating band represents the monoubiquitinated form of PCNA; PCNAub1), UBE2A, H2B, CDK9, CCNT1 phosphorylated H2AX (γH2A.X) and β-actin. β-actin and H2B were used as loading controls. γH2A.X confirms the induction of DNA damage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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