

The ubiquitin-selective chaperone Cdc48/p97 associates with Ubx3 to modulate monoubiquitylation of histone H2B

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ABSTRACT

Cdc48/p97 is an evolutionary conserved ubiquitin-dependent chaperone involved in a broad array of cellular functions due to its ability to associate with multiple cofactors. Aside from its role in removing RNA polymerase II from chromatin after DNA damage, little is known about how this AAA-ATPase is involved in the transcriptional process. Here, we show that yeast Cdc48 is recruited to chromatin in a transcription-coupled manner and modulates gene expression. Cdc48, together with its cofactor Ubx3 controls monoubiquitylation of histone H2B, a conserved modification regulating nucleosome dynamics and chromatin organization. Mechanistically, Cdc48 facilitates the recruitment of Lge1, a cofactor of the H2B ubiquitin ligase Bre1. The function of Cdc48 in controlling H2B ubiquitylation appears conserved in human cells because disease-related mutations or chemical inhibition of p97 function affected the amount of ubiquitylated H2B in muscle cells. Together, these results suggest a prominent role of Cdc48/p97 in the coordination of chromatin remodeling with gene transcription to define cellular differentiation processes.

INTRODUCTION

Genomic DNA in eukaryotic cells is arranged into nucleosomal repeat units to form a highly organized nucleoprotein structure called chromatin. Each nucleosome corresponds to 147 bp of DNA wrapped around a histone octamer core particle that is composed of two copies of histone H2A, H2B, H3 and H4. Different regulation pathways ensure the correct coordination between chromatin dynamics and DNA-associated processes, since access to DNA is required for DNA replication, DNA repair and mRNA transcription. In particular, post-translational modifications of histones, including ubiquitylation and de-ubiquitylation of histone H2B, define chromatin remodeling important for DNA replication and transcription. Besides nucleosome dynamics, this mark regulates trimethylation of histone H3 on both lysine 4 by the SET1 complex and lysine 79 by the DOT1 complex through a so-called trans-tail pathway, and facilitates recruitment of processing and nuclear export machineries on nascent mRNA transcripts (1–7).

Ubiquitylation of histone H2B is a highly conserved process in eukaryotes. In *Saccharomyces cerevisiae*, H2B is mainly monoubiquitylated but can also be polyubiquitylated on lysine 123 (K123) by the ubiquitin conjugation enzyme Rad6 and the ubiquitin RING ligase Bre1 (8–11). Modification of lysine 120 (K120) of human H2B is catalyzed by hRad6 and RNF20/RNF40. Cofactors of these enzymes have been described including Lge1 in yeast or WAC in mammals, which support the recruitment of the H2B E3 ligase to active transcription sites (12,13). The ubiq-

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ubiquitin moiety on H2B can be cleaved off by two distinct deubiquitylation enzymes (DUBs) in yeast, Ubp10 and Ubp8, a subunit of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) acetylation complex (14,15). Deubiquitylation of H2B facilitates the recruitment of Ctk1, a kinase that phosphorylates Ser-2 of the C-terminal domain of RNA polymerase II large subunit (CTD) and the methyltransferase Set2. Cycles of ubiquitylation/deubiquitylation of H2B are thus required for efficient gene expression (16).

Recent studies indicate that ubiquitylation of histone H2B undergoes major changes during mammalian cell differentiation. In particular, monoubiquitylation of H2B K120 increases during differentiation of human or murine stem cells into diverse lineages, such as osteoblasts, adipocytes, oligodendrocytes and keratinocytes (17,18). Such an up-regulation is required for stem cell differentiation and is associated with transcriptional reprogramming. In contrast, H2B ubiquitylation is down-regulated upon differentiation of human myoblasts based on inefficient recruitment of the E3 ligase RNF20 to chromatin in myotubes (19).

Cdc48, also called p97 or VCP in mammals, is an evolutionary conserved AAA-ATPase consisting of two adenosine triphosphatase (ATPase) domains flanked by N- and C-terminal extensions. Cdc48 primarily interacts with ubiquitylated proteins and uses the energy of adenosine triphosphate (ATP) hydrolysis to segregate ubiquitylated substrates from protein complexes, membranes or chromatin for proteasomal degradation (20–22). Along this line, it has been recently shown that the Rpb1 subunit of the yeast RNA polymerase II is ubiquitylated when stalled at sites of ultraviolet-induced DNA lesions, and extracted from chromatin by Cdc48-dependent segregation (23). Interestingly, mutations in human p97 are known to cause inclusion-body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD), however, the mechanistic defects are still unclear (24,25).

The broad spectrum of cellular functions controlled by Cdc48/p97 is mediated by its multiple substrate-recruiting or substrate-processing cofactors, including E3 ligases and DUBs (26,27). The 'Ubiquitin Regulatory X' (UBX) or UBX-like containing proteins form the largest family of Cdc48/p97 cofactors conserved from yeast to mammals. The UBX domain interacts with the N-terminal region of Cdc48/p97 via conserved structural features. UBX domain proteins display an adaptor function that recruits Cdc48/p97 to specific substrates and cellular locations (28,29).

Here, we identify Cdc48 and its cofactor Ubx3 as novel regulators of H2B ubiquitylation. Cdc48 is recruited co-transcriptionally and supports monoubiquitylation of H2B on actively transcribed genes by controlling the recruitment of Lge1, a cofactor of the E3 ligase Bre1 in yeast. Intriguingly, the dynamics of H2B ubiquitylation during muscle differentiation are also affected in primary muscle cells of IBMPFD patients. These results suggest that the involvement of Cdc48 in H2B monoubiquitylation is an evolutionarily conserved phenomenon that defines chromatin landscape changes important for developmental processes including muscle differentiation.

MATERIALS AND METHODS

Yeast strains

The *S. cerevisiae* strains used in this study are listed in Supplementary Table S1.

Preparation of yeast total extracts

Yeast cells grown in YPD or synthetic medium were collected during the exponential growth phase (OD₆₀₀ of 1.5 or 0.8, respectively). Total protein extracts were prepared by the NaOH-trichloroacetic acid (TCA) lysis method (30). Alternatively, cells were collected and resuspended in ice-cold lysis buffer (50 mM Hepes–KOH at pH 7.4, 150 mM NaCl, 1 mM DTT, 5% glycerol, 0.1% Triton X-100, 5 mM *N*-Ethylmaleimide, protease inhibitor cocktail (Roche)) as 30 OD₆₀₀ per milliliter. To analyze coimmunoprecipitation between Ubx3-HA and Lge1-Myc, cells were lysed in 20 mM Hepes–KOH at pH 7.5, 75 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.5% Triton X-100, protease inhibitor cocktail (Roche). Cell suspensions were lysed at 4°C with glass beads in a magnalyser (Roche), and centrifuged at 13 000 rpm for 20 min at 4°C prior to TCA precipitation and western blotting analysis.

Purification of ubiquitylated proteins

Cells transformed with a plasmid encoding 6His-ubiquitin under the *CUP1* promoter were grown on selective media and stimulated overnight with 0.1 mM of CuSO₄. A total of 100 OD₆₀₀ of cells were lysed in 6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris–HCl pH 8.0, 0.1% Triton X-100 plus 5 mM imidazole, 10 mM beta-mercaptoethanol, protease inhibitors, 20 mM NEM and 100 μM MG132. Purification was performed on Ni²⁺-NTA-agarose beads pre-washed with lysis buffer and incubated for 2 h at room temperature. The beads were washed with 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris–HCl pH 6.3, 10 mM beta-mercaptoethanol, 0.2% Triton X-100 prior elution and western blot analysis using anti-H2B (Active Motif) and anti-His6 antibodies (4,31).

Chromatin immunoprecipitation (ChIP) analysis

ChIPs were performed as previously described (32), with the following modifications. Cells were crosslinked with 1.2% formaldehyde for 10 min. Sonicated extracts were centrifuged for 20 min at 10 000 × *g* prior to overnight immunoprecipitation, using specific antibodies coated Protein G-Sepharose beads. After reversing crosslinking, real-time qPCR was performed using the SYBR Green mix (Roche) and the Light Cycler 480 system (Roche) with gene-specific primers corresponding to 150 bp fragments described in Supplementary Table S2. The antibodies used in this ChIP assay are an anti-Cdc48 antibody kindly provided by T. Sommer, an anti-CTD antibody that recognizes all forms of CTD except the Ser2 phosphorylated form (8WG16 antibody; MMS126R, Covance), anti-HA antibody (HA.11 antibody; MMS-101R-B, Covance), an anti-Rpb3 antibody (W0012, Neoclone), an anti-yeast

H2B (Active Motif; 39237) and an anti-human ubiquityl-histone H2B that cross-reacts with the yeast protein (D11; 5546S, Cell Signaling).

Non-specific signals were systematically assessed by analysing immunoprecipitated DNA using primers for intergenic regions and used to normalize results when indicated.

Chromatin double immunoprecipitation (ChDIP) analysis

ChDIPs were performed as previously described (33,34), with the following modifications. Cells were transformed with plasmids encoding a Flag tagged version of wt or mutated HTB1 (pRS314-*HTB1*-Flag WT or pRS314-*htb1*-Flag K123R). Note that 3 mg of crosslinked chromatin were used for each overnight immunoprecipitation with 100 μ l of anti Flag M2 coated-beads (Sigma-Aldrich). Immunoprecipitates were eluted by an overnight incubation with 100 μ g/ml of 3 \times -Flag peptide (Sigma-Aldrich) prior the second immunoprecipitation with anti-ubiquitin antibodies (FK2 clone, Enzo Life Sciences) and Protein G-Sepharose beads.

RNA isolation and amplification

Total RNA isolation was performed by the hot acid phenol method (Sigma Aldrich). cDNA from total RNAs were obtained by retro-transcription with random oligonucleotides (Roche) using the SuperScriptTM II reverse transcriptase (Invitrogen). Real-time qPCR was then performed using the SYBR Green mix (Roche) and the Light Cycler 480 system (Roche) with gene-specific primers described in Supplementary Table S2.

Human myoblast culture and cell extracts

Human myoblasts are provided from the Muscle Tissue Culture Collection (MTCC), which is part of the German network on muscular dystrophies (MD-NET) and the German network for mitochondrial disorders (mito-NET, project D2, 01GM1113A) funded by the German Ministry of Education and Research (BMBF, Bonn, Germany). The MTCC is a partner of EuroBioBank (www.eurobiobank.org) and TREAT-NMD (www.treat-nmd.eu).

Normal and IBMPFD primary human myoblasts (VCP-Mutation R155H with age- and sex-matched controls) were cultured in skeletal muscle cell growth medium (Provitro; 201 0602 with supplement mix 218 0602 added) at 37°C and 5% CO₂. To induce differentiation, cells were grown to near confluence and skeletal muscle cell growth medium with 15% fetal calf serum was replaced by differentiation medium (Dulbecco's modified Eagle's medium (DMEM) with 2% horse serum). Myogenesis was monitored by western blot analysis of the myogenic differentiation marker MyoD. Differentiated myotubes were separated from undifferentiated cells with diluted trypsin. Cells were lysed for western blotting in Laemmli sample buffer (63 mM Tris pH 6.8, 10% glycerol, 2.5% sodium dodecyl sulphate (SDS), bromophenol blue, 5% β -mercaptoethanol) for 4 min at 95°C. Western blot analyses were performed using standard procedures.

Stably transfected wild-type (wt) p97 and p97^{EQ} U2OS cell lines, provided by C.C. Wehl (35), were grown at 37°C in

humidified air containing 5% CO₂ in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1000 UI/ml penicillin, 1000 UI/ml streptomycin with the addition of selective antibiotics (100 μ g/ml hygromycin B and 100 μ g/ml Zeocin). p97 and/or p97^{EQ} expression was induced with 1 μ g/ml tetracycline for 16 h. Next, total cell lyses were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blot analysis. C2C12 mouse myoblasts were routinely maintained in DMEM supplemented with 10% heat-inactivated FBS, 1000 UI/ml penicillin, 1000 UI/ml streptomycin and 2 mM L-Glutamine at 37°C in humidified air containing 5% CO₂. C2C12 myoblasts were treated with various concentration of DBeQ for 2 h. Next, total cell lyses were separated by SDS-PAGE and subjected to western blot analysis.

Western blot analyses were performed with anti-c-Myc 1/10 000 (Sigma), anti-VCP 1/5000 (ab1433 Abcam), anti-H2B 1/1000 (gift from D. Devys), anti-H2Bub 1/1000 (Medimabs), anti-MyoD1 1/1000 (Dako) and anti- α -tubulin 1/5000 (Sigma). Proteins were detected by immunoblotting using an ECL kit (GE Healthcare).

RESULTS

Cdc48 is recruited to actively transcribed genes

Recently, Cdc48 has been linked to chromatin-associated degradation mainly involved in DNA damage repair pathways (23). To determine whether Cdc48 also regulates transcription-associated pathways, we followed its recruitment to actively transcribed genes by ChIP and qPCR of the constitutively expressed *PMA1* gene, the galactose-inducible *GAL10* gene and the heat-inducible *HSP104* gene using different primer pairs (Figure 1, Supplementary Figure S1A). Our results reveal that Cdc48 binds to the *PMA1* gene with an up to 6-fold enrichment all along the gene compared to the non-transcribed intergenic region (Figure 1A). In contrast, the recruitment of the thermosensitive mutant protein *cdc48-6* was severely decreased at 30°C, whereas its expression level was not affected (Supplementary Figure S1B). Analysis of the inducible *GAL10* gene indicated that Cdc48 was barely detectable on this gene when cells were grown in glucose and only associates with *GAL10* during its activation with galactose. Upon induction, Cdc48 is recruited on *GAL10* with identical kinetics to RNA polymerase II as analyzed using its Rpb3 subunit (Figure 1B and C). Similarly, Cdc48 accumulated strongly on the heat-inducible *HSP104* gene upon shifting wt yeast cultures from 23°C to 42°C for 30 min, while this temperature stress simultaneously resulted in a major defect of both CTD and Cdc48 recruitment on *PMA1* (Supplementary Figure S1A). Together these results support the idea that Cdc48 associates with active genes in a transcription-coupled manner.

Cdc48 is known to associate with several alternative co-factors that are involved in substrate recruitment and processing. Among those, the Ufd1/Npl4 heterodimer is required for the role of Cdc48 in endoplasmic reticulum (ER)-associated protein degradation and ubiquitin-dependent extraction from chromatin (22,27). Interestingly, Cdc48 recruitment was also affected in a thermosensitive mutant lacking functional Npl4 (Supplementary Figure S1C), suggesting a role for the Ufd1/Npl4 complex in this pro-

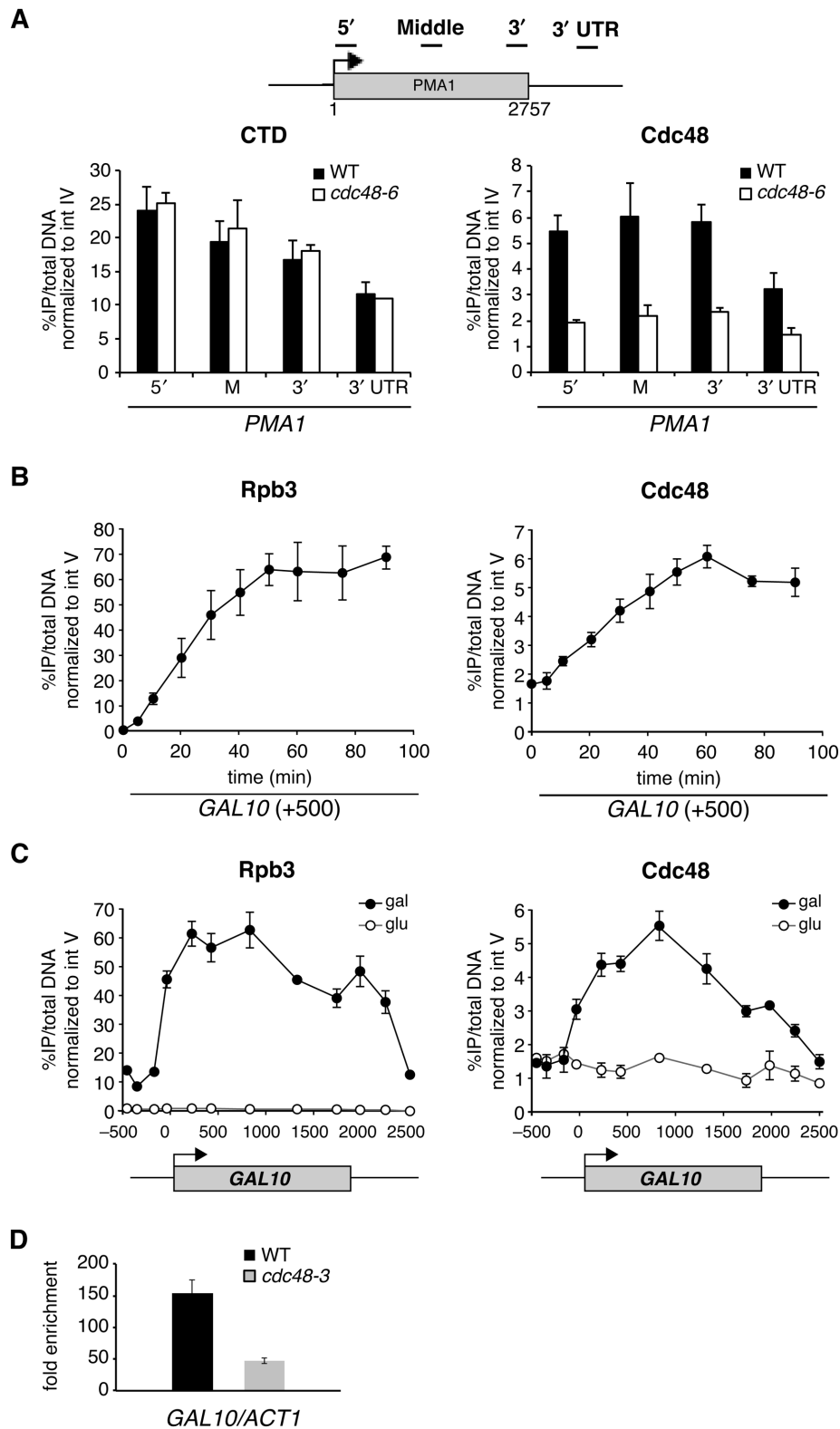


Figure 1. Cdc8 is cotranscriptionally recruited on active genes. (A) Recruitment of Cdc8 on the *PMA1* gene was analyzed by ChIP experiments performed on extracts prepared from WT or *cdc48-6* cells grown overnight at 30°C using anti RNA polymerase II (CTD) or Cdc48 antibodies. Histograms depict the mean and standard deviations of at least three independent experiments. (B) Yeast cells were induced with galactose and ChIP performed to monitor recruitment of Rpb3 (left) or Cdc48 to the +500 position of the *GAL10* ORF at the indicated timepoints. Error bars depict the mean and standard error of at least three independent experiments. (C) Yeast were grown in raffinose and transferred to media containing either galactose or glucose, as indicated, for 60 min. ChIP was then performed with primers as indicated spanning the *GAL10* locus. (D) *GAL10* transcripts were analyzed by RT-qPCR in wt and *cdc48-3* cells and normalized to *ACT1* transcripts.

cess. These observations indicate that interaction with transcriptionally active gene loci requires an intact function of Cdc48.

To analyze whether Cdc48 is not only recruited on active gene loci but directly influences the transcription process, the level of *GAL10* mRNA was analyzed by reverse transcriptase-qPCR (RT-qPCR) in wt and thermosensitive *cdc48-3* mutant cells. As shown in Figure 1D, preventing Cdc48 function severely impairs the expression of these inducible transcripts. Together these results indicate that an intact function of the Cdc48 complex is required for an appropriate transcriptional response.

Monoubiquitylated H2B levels are reduced in *cdc48* mutants

Cdc48 primarily interacts with ubiquitylated proteins and functions as a ubiquitin-selective segregase in the nuclear chromatin environment both in yeast and higher eukaryotic cells (22). Yeast histone H2B represents one of the most abundant chromatin-associated targets for ubiquitylation. Interestingly, ubiquitylated H2B (Ub-H2B) is mainly associated with transcribed gene regions similar to the distribution of Cdc48 (Figure 1) (36). In addition, preventing H2B ubiquitylation by deletion of Rad6, the Bre1 RING domain or mutation of K123 prevents RNA polymerase II recruitment on galactose inducible genes *GAL1* and *GAL10* but not on *PMAl* and results in a decreased expression of GAL transcripts (4,37). Despite the correlation between the distribution of Ub-H2B and Cdc48 on active genes and their effect on RNA polII recruitment, association of Cdc48 with the *PMAl* gene was not affected by the mutation of H2B K123 (not shown). This observation indicates that ubiquitylation of H2B is not sufficient to recruit Cdc48 to active genes. To determine whether Cdc48 might influence the amount of Ub-H2B, we analyzed H2B modification in total extracts from wt and *cdc48-6* cells and compared the H2B profile with cells deleted for the H2B ubiquitin ligase Bre1. This approach revealed reduced levels of Ub-H2B in both *cdc48-6* and *cdc48-3* cells grown at the semi-restrictive temperature compared to wt (Figure 2A and Supplementary Figure S2). To further support these findings, copper-inducible His-tagged ubiquitin was expressed in wt and *cdc48-6* mutant cells grown at 23°C or the semi-permissive temperature of 30°C followed by purification of ubiquitylated proteins with nickel NTA agarose and western blot analysis using anti-H2B antibody. This assay confirmed reduced levels of His6-Ub-H2B in *cdc48-6* grown at 30°C (Figure 2B, upper panel). In contrast to ubiquitylation, the amount of unmodified H2B was not changed under the same conditions (Figure 2A and B middle panel).

Ubiquitylation of H2B is known to be regulated on transcribed genes for an optimal transcriptional activation and elongation (16). To analyze whether Cdc48 facilitates ubiquitylation or instead protects H2B from deubiquitylation, we performed epistasis analysis combining the *cdc48-6* mutant with deletions of either *UBP8* or *UBP10*, which specifically deubiquitylate Ub-H2B. As previously described, the deletion of *UBP8* strongly enhanced the amount of Ub-H2B. However, in combination with *ubp8Δ*, the *cdc48-6* mutation still led to a decreased detection of Ub-H2B at 30°C. Similar results were observed for *ubp10Δ* although

this deletion strain showed only limited consequences on Ub-H2B (Figure 2C). As Ubp8 and Ubp10 display partially redundant activities, deletion of both DUBs resulted in a synergistic increase in the level of Ub-H2B (Figure 2C; (38)). Despite the major accumulation of Ub-H2B in *ubp8Δ ubp10Δ* cells, disruption of functional Cdc48 was still able to reduce the level of Ub-H2B (Figure 2C).

Together these results suggest that Cdc48 regulates the monoubiquitylation of H2B at least on bulk histones. Although an effect of Cdc48 on de-ubiquitylation cannot be excluded, these data indicate that Cdc48 stimulates the H2B ubiquitylation process.

Cdc48 regulates ubiquitylation of H2B on actively transcribed genes

To determine whether Cdc48 modulates the occupancy of Ub-H2B on active genes, we used a ChDIP assay to detect ubiquitin-modified Flag-H2B combined with qPCR on different active genes (15,33,39). We calculated the level of Ub-H2B relative to the level of H2B present on each region of considered genes. In agreement with previous studies, Ub-H2B occupancy is decreased upon exchanging the ubiquitylation site K123 against arginine (K123R) and increased upon *UBP8* deletion (15,33,39). As shown before, mutation in *CDC48* led to a decrease of Ub-H2B associated to five different genes both in a wt and in *ubp8Δ* cells (compare *cdc48-6* with wt cells, and *cdc48-6 ubp8Δ* with *ubp8Δ* cells), whereas no significant change could be observed on unmodified H2B (Figure 2D and Supplementary Figure S3). These results indicate that Cdc48 improves the monoubiquitylation of H2B on actively transcribed genes. Although Ubp8, and more generally the Ubp8-containing deubiquitylation module, is part of the histone acetylation SAGA complex, we were not able to detect differences between SAGA (*ADH1*, *GCV2*, *PHO84*) and TFIID-dependent (*ACT1*, *PMAl*, *PHO84*) genes (40,41).

Ubx3, a cofactor of Cdc48 for H2B ubiquitylation

To further decipher how Cdc48 controls H2B ubiquitylation, we analyzed the steady-state levels of Ub-H2B in mutants lacking each of the seven *S. cerevisiae* UBX proteins. H2B conjugated by either endogenous or copper-inducible His-tagged ubiquitin was severely decreased in *ubx3Δ* cells, whereas comparable modified H2B was observed in wt and other mutant cells (Figure 3A). Deletion of *UBX3* induced a decrease of Ub-H2B at four different genes indicating that Ubx3, as Cdc48, improves the monoubiquitylation of H2B on actively transcribed genes (Figure 3B). We confirmed that a C-terminal HA-tagged version of Ubx3 (Ubx3-HA) specifically interacts with Cdc48 (Figure 3C; (42)). In addition, both Cdc48 and Ubx3 were found to interact with H2B in coimmunoprecipitation assays (not shown). Finally, in combination with *ubp8Δ*, *UBX3* deletion led to a decreased accumulation of Ub-H2B at 30°C indicating that Ubx3, like Cdc48, is required for efficient ubiquitin conjugation of H2B (Figure 3D). Together these data provide evidence that Ubx3 acts as a major Cdc48 cofactor to facilitate H2B ubiquitylation.

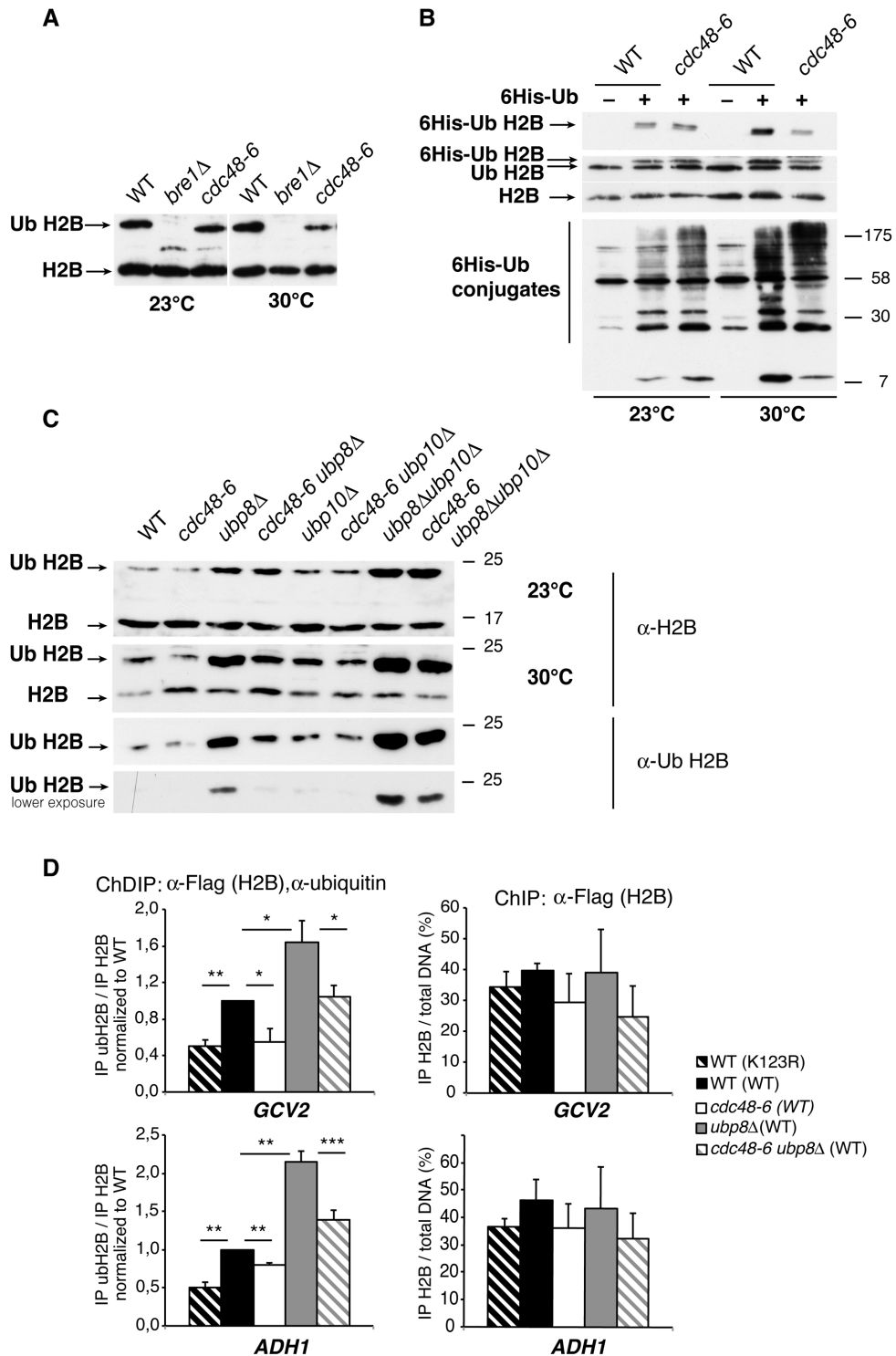


Figure 2. Cdc48 facilitates ubiquitylation of the histone H2B on active genes. (A) Wt, *cdc48-6* or *bre1* Δ cells were grown at 23°C or 30°C. Expression levels of H2B and Ub-H2B in whole cell lysates were analyzed by western blotting using anti H2B antibody. (B) Wt or *cdc48-6* cells grown at 23°C or 30°C were transformed (+) or not (-) with a plasmid encoding 6His-ubiquitin. 6His-ubiquitin conjugates were Ni-purified from cell extracts and examined by western blotting with an anti-H2B (upper panel). Expression levels of H2B and Ub-H2B in whole cell lysates were analyzed with anti H2B blot (middle panel). 6His-ubiquitin expression and efficiency of purification was controlled using an anti-6His antibody (lower panel). Note the accumulation of high-molecular mass ubiquitylated proteins in *cdc48-6* cells at 30°C. (C) Total extracts from indicated cells grown at 23°C or 30°C were analyzed by western blotting using anti-H2B or anti-Ub-H2B antibodies. (D) Indicated cells were transformed with plasmids encoding for a Flag-tagged version of H2B (WT) or H2B-K123R (K123R) and grown at 30°C. Double ChIP on *GCV2* and *ADH1* genes was performed first with anti-Flag antibodies (right panels) and then anti-ubiquitin antibodies. The ratio between both was then normalized to the wt strain (left panels). Histograms depict the mean and standard deviations of at least three independent experiments. Significance of the differences observed between both strains was evaluated using Student's *t*-test (**P* = 0.01–0.05; ***P* = 0.001–0.01; ****P* < 0.001).

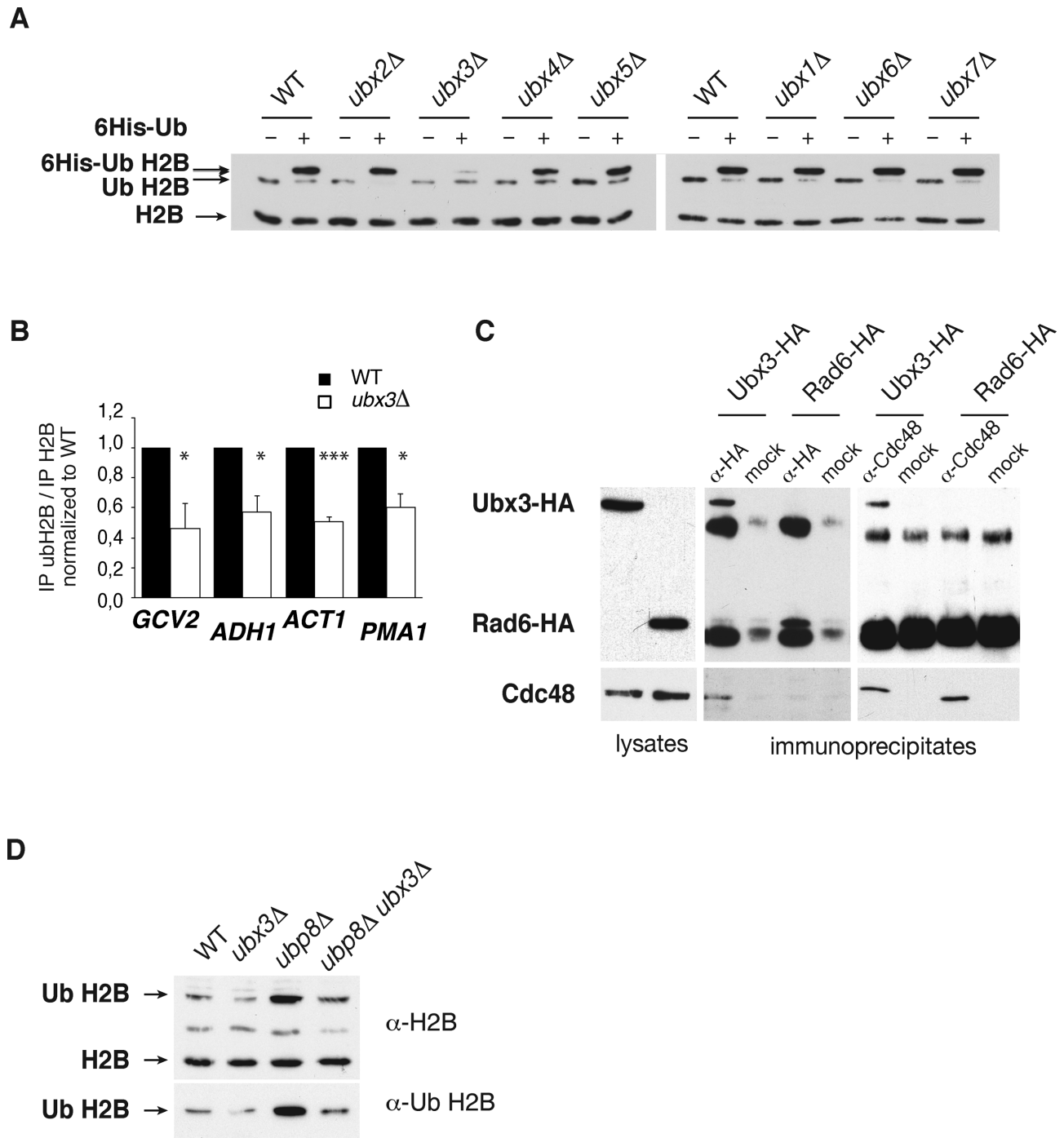


Figure 3. Ubx3 is a cofactor of Cdc48 for ubiquitylation of H2B. (A) Wt or *ubxΔ* cells grown at 30°C were transformed (+) or not (-) with a plasmid encoding 6His-ubiquitin. Expression levels of H2B and Ub-H2B in whole cell lysates were analyzed with anti H2B blot. (B) Ubiquitylation of H2B an actively transcribed genes was analyzed by ChIP using anti-UbH2B and anti H2B antibodies in wt and *ubx3Δ* cells. The Ub-H2B/H2B ratio in wt and mutant cells was normalized to the wt cells. Histograms depict the mean and standard deviations of at least three independent experiments. Significance of the differences observed between both strains was evaluated using Student's *t*-test (**P* = 0.01–0.05; ***P* = 0.001–0.01; ****P* < 0.001). (C) Lysates from wt cells expressing Ubx3-HA or Rad6-HA as a control were immunoprecipitated using anti-HA, anti-Cdc48 or mock antibodies and analyzed by western blotting with the indicated antibodies. (D) Total extracts from indicated cells were analyzed by western blotting using anti-H2B or anti-Ub-H2B antibodies.

The Cdc48/Ubx3 complex controls the recruitment of Lge1, a cofactor of the Bre1 ligase, on active genes

As previously mentioned, ubiquitylation of yeast H2B is achieved by Rad6 as an E2, Bre1 as the E3 ubiquitin ligase and its Lge1 cofactor (8–12). To understand how Cdc48 and Ubx3 facilitate ubiquitylation of H2B, we first performed genetic analyses and found that combining *LGE1* deletion with the *cdc48-6* mutation or *ubx3* deletion led to synthetic sickness of double mutant cells (Figure 4A) thus indicating a clear genetic interaction between *LGE1* and both *CDC48* and *UBX3*. In agreement with this result, the cotranscriptional recruitment of Lge1-HA on transcribing genes was severely reduced in *cdc48-6* compared to wt cells without significant effects on CTD association with indicated genes (Figure 4B). Importantly, genomic C-terminal HA-tagging of Lge1, as well as Rad6 and Bre1, did not affect their enzymatic activity (Supplementary Figure S4A). The lowered cotranscriptional recruitment of Lge1 was correlated with decrease of Lge1-HA expression level in *cdc48-6* cells at 30°C, whereas *cdc48-6* did not specifically affect tagged Rad6-HA and Bre1-HA protein level (Figure 4C). To further determine whether the Cdc48-regulated recruitment of Lge1 to active genes was only due to this expression regulation and/or to a direct effect, we analyzed the expression of Lge1-HA and Bre1-HA upon *UBX3* deletion. As shown in Figure 4D, expression of Bre1 and Lge1 were comparable in wt and mutant strain. ChIP analysis revealed that cotranscriptional recruitment of Lge1-HA was impaired upon *UBX3* deletion whereas recruitment of Bre1-HA was unaffected (Figure 4E and not shown).

Coimmunoprecipitation experiments between Ubx3 and Lge1 (Figure 4F) further confirm that Cdc48 cooperates with its cofactor Ubx3 in cotranscriptional recruitment of the H2B ubiquitylation machinery to active genes by interaction between Ubx3 and Lge1.

p97 controls H2B ubiquitylation in human cells

The identification of Cdc48 as a regulator of H2B ubiquitylation in yeast prompted us to analyze whether this function is conserved across evolution. To determine whether p97 might influence ubiquitylation of H2B, C2C12 myoblast cells were treated with increasing doses of N2,N4-dibenzylquinazoline-2,4-diamine (DBEQ), a selective and reversible ATP-competitive p97 inhibitor (43). The ubiquitylation status of H2B was analyzed by western blotting using specific antibodies (Figure 5A). Densitometric analysis revealed that the Ub-H2B/H2B ratio increased by a factor of 2.4 and 2.5 upon treatment with 5 and 10 μM DBEQ, respectively. To confirm that inhibiting the ATPase activity of p97 led to an increased Ub-H2B/H2B ratio, H2B ubiquitylation was analyzed using U2OS cells stably expressing tetracycline-inducible and myc-tagged WT or ATPase inactive E578Q mutant p97 (35). While expression of tetracycline-induced wt p97 did not change expression of Ub-H2B, induction of p97 E578Q mutant led to a 4-fold increase of the Ub-H2B/H2B ratio (Figure 5B). These experiments show that inhibiting the ATPase activity of p97 promotes an increase in monoubiquitylated H2B.

Given that H2B ubiquitylation is down-regulated upon differentiation of human myoblasts into myotubes, it is in-

triguing to note that missense mutations of p97 are linked to a dominantly inherited myopathy (IBMPFD) (19). We therefore analyzed ubiquitylation of H2B along differentiation of primary myoblasts of IBMPFD myopathy patients compared to myocytes from unaffected age- and sex-matched individuals. The myopathy patient cells carry a point mutation on a highly conserved residue R155H that does not affect the ATPase activity (44–46). As shown in Figure 5C, the p97^{R155H} mutation did not significantly alter the *in vitro* differentiation into myotubes as indicated by the expression of MyoD. As previously described, H2B ubiquitylation was severely decreased early in differentiation (48 h after induction of differentiation), whereas this reduction was significantly delayed in IBMPFD patient cells. Together these data show that p97 (functional ATPase activity and intact N-terminal domain) is required for controlling the appropriate Ub-H2B/H2B ratio in human muscle cells.

DISCUSSION

Our results indicate a multifaceted role for Cdc48/p97 in controlling transcriptional processes. In yeast, we demonstrate that Cdc48 together with its core cofactor Npl4, is recruited to active chromatin in a transcription-coupled manner, associates with the transcribed portion of the genes and is required for the proper expression of inducible genes. Moreover, Cdc48 complexes are critical for maintaining correct levels of ubiquitylated H2B. By combining Cdc48 mutations with deletion of H2B-specific deubiquitylating enzymes, Ubp8 and Ubp10, we found that Cdc48 acts primarily on the ubiquitylation process, suggesting that it stimulates the recruitment or activity of the ubiquitylation machinery. Indeed, Cdc48 is required for the cotranscriptional recruitment of the Lge1 cofactor to chromatin. This newly described function of Cdc48 is mediated by a specific cofactor from the UBX family, Ubx3. Our results favor the model that Cdc48/Ubx3 facilitate or stabilize the binding of the H2B ubiquitylation machinery to active genes via interaction between Ubx3 and Lge1. These results are consistent with a role of Cdc48 as a platform coordinating the recruitment of processing factors (26), here Lge1, with specific targets on chromatin and suggest a possible function for this chaperone in chromatin remodeling. Beyond Lge1, the association of Cdc48 with transcribing genes raises the intriguing possibility that Cdc48 is a common integrator that controls multiple facets of the transcriptional process.

In human cells, different approaches affecting the catalytic activity of p97 or its cofactor-binding N-terminal domain led to a significant increase of Ub-H2B level. In particular, we observed that loss of H2B ubiquitylation during myogenic differentiation was significantly reduced in IBMPFD patient cells. Pathogenic mutations in p97 are primarily in the N-terminal domain implicated in ubiquitin binding and protein–protein interactions (45,47). The R155H mutation affects a highly conserved residue and is by far the most common, accounting for ~50% of all affected individuals (44,47,48). The p97^{R155H} mutation is neither associated with altered binding of known cofactors nor with decrease in ATPase activity (44,45). Since this mutation did not significantly influence the myoblast differentiation, changes in Ub-H2B level were likely not caused by

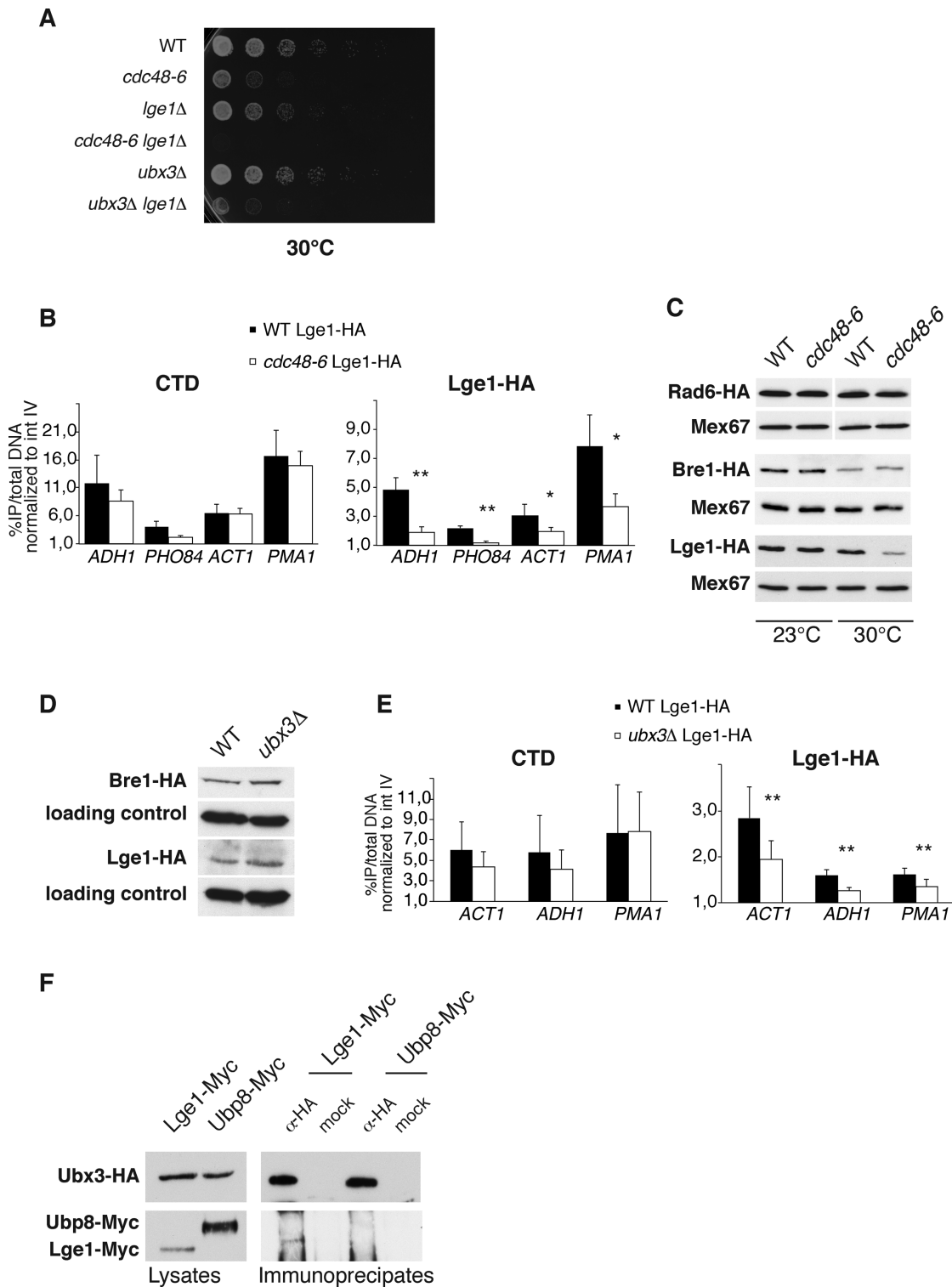


Figure 4. Cdc48 controls the expression and cotranscriptional recruitment of Lge1. **(A)** Note that 5-fold serial dilutions of wt and indicated mutant cells were cultured in rich medium at 30°C. **(B)** ChIP experiments were performed on extracts prepared from WT or *cdc48-6* cells grown overnight at 30°C using anti RNA polymerase II (CTD) or anti-HA (Lge1-HA) antibodies. Histograms depict the mean and standard deviations of at least three independent experiments. Significance of the differences observed between both strains was evaluated using Student's *t*-test (**P* = 0.01–0.05; ***P* = 0.001–0.01; ****P* < 0.001). **(C)** Expression of HA-tagged versions of Rad6, Bre1 and Lge1 were analyzed in total extracts from WT or *cdc48-6* cells by western blotting using anti-HA antibodies or anti-Mex67 antibodies as loading control. **(D)** Expression of Lge1-HA and Bre1-HA were analyzed in total extracts from WT or *ubx3Δ* cells by western blotting using anti-HA antibodies or a non-specific protein as loading control. **(E)** ChIP experiments were performed on extracts prepared from WT or *ubx3Δ* cells grown overnight at 30°C using anti RNA polymerase II (CTD) or anti-HA (Lge1-HA) antibodies and analyzed as in B. **(F)** Lysates from wt cells expressing Ub3-HA and Lge1-Myc or Ubp8-Myc as a control were immunoprecipitated using anti-HA or mock antibodies, and analyzed by western blotting with the anti-HA and anti-Myc antibodies.

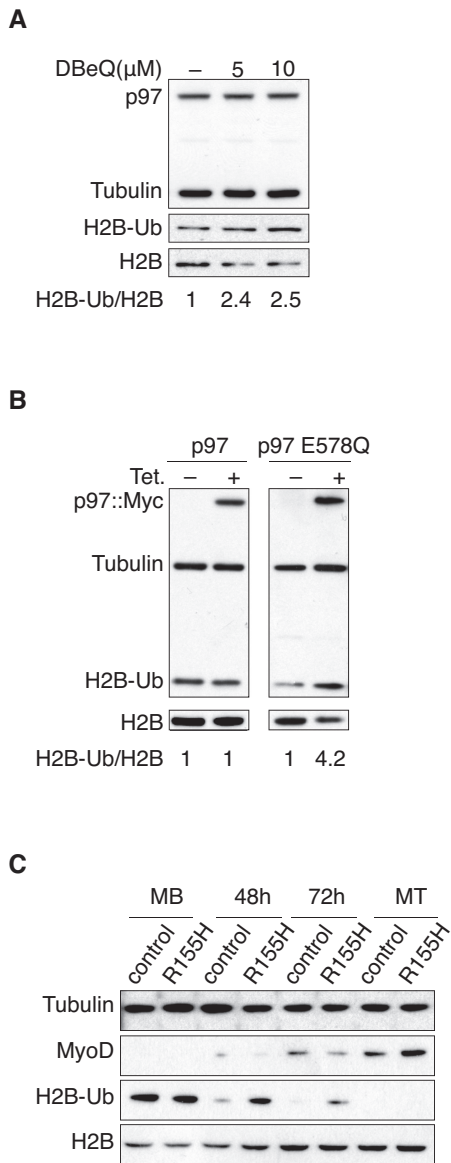


Figure 5. Human p97 is required for a proper level of H2B ubiquitylation in human muscle cells. **(A)** Inhibition of the p97 function by DBE-Q treatment affects in turn histone H2B ubiquitylation. C2C12 myoblasts were treated with indicated concentration of DBE-Q (5 and 10 μM) for 2 h. Next, total cell lysates were separated by SDS-PAGE and subjected to western blot analysis with anti-p97, anti-H2B and anti-H2B-Ub antibodies. α-tubulin was used as a loading control. Densitometric analysis done by Image J software, results were normalized to the control condition and expressed as the Ub-H2B/H2B ratio. **(B)** Overexpression of the ATPase inactive p97 form increases the ubiquitylation level of histone H2B. Overexpression of Myc-tagged p97 or ATPase inactive p97-E578Q mutant in U2OS cells was induced by incubation with tetracycline (1 μg/ml) for 16 h. Next, total lysates from non-induced (-) and tetracycline-induced (+) cells were separated by SDS-PAGE and subjected to western blot analysis with anti-Myc, anti-H2B-Ub and anti-H2B antibodies. α-tubulin was used as a loading control. Densitometric analysis done by Image J software, results were normalized to the control condition and expressed as the Ub-H2B/H2B ratio. **(C)** Delayed depletion of H2B during myogenic differentiation of IBMPFD primary human myoblasts. Lysates of control and IBMPFD primary human myoblasts (MB) and those corresponding to the indicated number of hours after induction of their differentiation (48 and 72 h) were analyzed by western blot using antibodies that recognize the indicated proteins. α-Tubulin was used as a loading control. MT, differentiated myotubes.

reduced cell proliferation. The most likely hypothesis is that the R155 residue affects binding of p97 with specific cofactor(s) and thus reduces Ub-H2B levels. It is worth noting that the yeast Ubx3 has no known human ortholog. In addition, by introducing the R165H (equivalent of R155) mutation in the yeast Cdc48, we could not detect any effect on Ub-H2B (not shown). Similarly, expression of human wt or p97^{R155H} mutant in *cdc48-6* cells did not induce any changes in Ub-H2B levels (not shown). These data suggest that the human cofactor mediating the role of p97 in controlling Ub-H2B in muscle cells is not conserved in yeast. Although there is no ortholog of Lge1 in human cells, there may be additional cofactors that are recruited to chromatin in a p97-dependent manner. For example, the WAC cofactor mediates the cotranscriptional recruitment of the H2B ubiquitin ligase RNF20/40 (13). Further studies will be necessary to analyze whether WAC could be an indirect target of p97 and better understand the regulatory role of p97 that underlies transcriptional elongation and epigenetic changes during myogenic differentiation.

Together, our results suggest that both in yeast and mammals, Cdc48/p97 serves as an integrating platform for modulation of histone ubiquitylation. Interestingly, however, the molecular mechanisms diverge to include different cofactors and different functionalities. Given the fact that Cdc48/p97 is located throughout the cell and exerts regulation of many cellular functions, it might contribute to the integration of cellular processes, adjusting inducible signaling pathways with the regulation of chromatin-associated ubiquitin-based modifications.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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