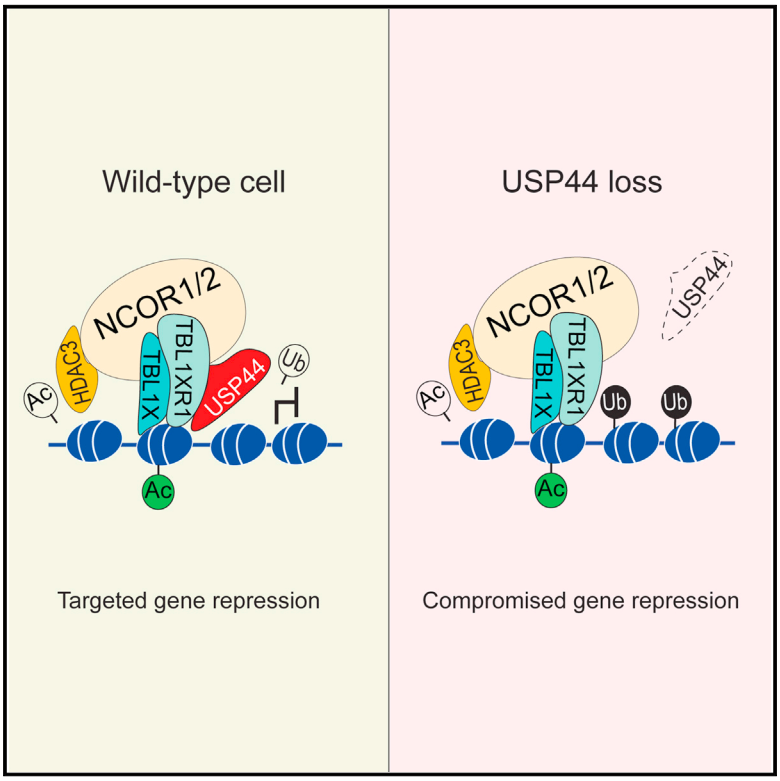
Article

Cell Reports

USP44 Is an Integral Component of N-CoR that Contributes to Gene Repression by Deubiquitinating Histone H2B

Graphical Abstract



Authors

Xianjiang Lan, Boyko S. Atanassov, Wenqian Li, ..., Michael P. Washburn, Jerry L. Workman, Sharon Y.R. Dent

Correspondence

sroth@mdanderson.org

In Brief

Ubiquitin-specific protease 44 (USP44) is involved in the DNA damage response, spindle checkpoint regulation, stem cell differentiation, and cancer development. Lan et al. now find that USP44 is an integral subunit of the N-CoR complex and contributes to N-CoR-mediated gene repression through histone H2B deubiquitination.

Highlights

- USP44 is an integral subunit of the N-CoR complex
- USP44 associates with N-CoR through direct interaction with TBL1X/TBL1XR1
- USP44 contributes to N-CoR-mediated repression of target genes
- USP44 or TBL1XR1 ablation impairs the invasiveness of breast cancer cells

Lan et al., 2016, Cell Reports *17*, 2382–2393
November 22, 2016
http://dx.doi.org/10.1016/j.celrep.2016.10.076

OPEN ACCESS
CellPress

Cell Reports
Article

USP44 Is an Integral Component of N-CoR that Contributes to Gene Repression by Deubiquitinating Histone H2B

Xianjiang Lan,[1,2,3] Boyko S. Atanassov,[1,2] Wenqian Li,[1,2,3] Ying Zhang,[4] Laurence Florens,[4] Ryan D. Mohan,[5] Paul J. Galardy,[6] Michael P. Washburn,[4,7] Jerry L. Workman,[4] and Sharon Y.R. Dent[1,2,8,*]

[1]Department of Epigenetics and Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, TX 78957, USA
[2]Center for Cancer Epigenetics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[3]Program in Epigenetics and Molecular Carcinogenesis, Graduate School of Biomedical Sciences, The University of Texas MD Anderson Cancer Center, Smithville, TX 78957, USA
[4]Stowers Institute for Medical Research, Kansas City, MO 64110, USA
[5]University of Missouri-Kansas City, Kansas City, MO 64110, USA
[6]Department of Pediatric and Adolescent Medicine, Mayo Clinic, Rochester, MN 55905, USA
[7]Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS 66160, USA
[8]Lead Contact
*Correspondence: sroth@mdanderson.org
http://dx.doi.org/10.1016/j.celrep.2016.10.076

SUMMARY

Decreased expression of the USP44 deubiquitinase has been associated with global increases in H2Bub1 levels during mouse embryonic stem cell (mESC) differentiation. However, whether USP44 directly deubiquitinates histone H2B or how its activity is targeted to chromatin is not known. We identified USP44 as an integral subunit of the nuclear receptor co-repressor (N-CoR) complex. USP44 within N-CoR deubiquitinates H2B in vitro and in vivo, and ablation of USP44 impairs the repressive activity of the N-CoR complex. Chromatin immunoprecipitation (ChIP) experiments confirmed that USP44 recruitment reduces H2Bub1 levels at N-CoR target loci. Furthermore, high expression of USP44 correlates with reduced levels of H2Bub1 in the breast cancer cell line MDA-MB-231. Depletion of either USP44 or TBL1XR1 impairs the invasiveness of MDA-MB-231 cells in vitro and causes an increase of global H2Bub1 levels. Our findings indicate that USP44 contributes to N-CoR functions in regulating gene expression and is required for efficient invasiveness of triple-negative breast cancer cells.

INTRODUCTION

Histone H2B is monoubiquitinated on lysine 123 in yeast and the corresponding lysine 120 in mammals (hereafter referred to as H2Bub1). H2Bub1 contributes to the creation of open and accessible chromatin structures (Fierz et al., 2011), and it plays important roles in transcriptional initiation and elongation (Henry et al., 2003; Pavri et al., 2006; Xiao et al., 2005), mRNA processing (Pirngruber et al., 2009), DNA damage response (Moyal et al., 2011), DNA replication (Trujillo and Osley, 2012), and maintenance of stem cell multipotency (Fuchs et al., 2012; Karpiuk et al., 2012). H2Bub1 is required in yeast and human cells for histone H3 methylation on lysine 4 (H3K4) and lysine 79 (H3K79) (Briggs et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Wu et al., 2011), which further contribute to transcriptional activity. The importance of H2Bub1 to human health is highlighted by several studies that indicate H2Bub1 levels are decreased in many aggressive cancers, including colorectal cancer, metastatic breast cancer, testicular seminoma, parathyroid cancer, and lung cancer (Fuchs and Oren, 2014; Prenzel et al., 2011; Urasaki et al., 2012).

H2Bub1 levels are governed by a balance in the activities of specific ubiquitin ligases, such as RNF20/40 (Kim et al., 2005) and deubiquitinases that target this modification. Several deubiquitinases, including USP22 (Zhang et al., 2008; Zhao et al., 2008), USP27X, USP51 (Atanassov et al., 2016), USP3 (Nicassio et al., 2007), USP7 (van der Knaap et al., 2010), USP42 (Hock et al., 2014), and USP49 (Zhang et al., 2013), remove ubiquitin from histone H2B. USP44 also has been implicated in H2Bub1 deubiquitination (Fuchs et al., 2012). In addition, USP44 functions in DNA repair and in regulation of the spindle checkpoint via Cdc20 deubiquitination (Mosbech et al., 2013; Stegmeier et al., 2007). However, USP44 is not an active enzyme when in isolation, indicating that this deubiquitinase (DUB), as previously reported for other USPs (Atanassov et al., 2016; Kee et al., 2010; Lan et al., 2015; Lang et al., 2011; Zhang et al., 2013), requires activating cofactor proteins to productively engage its substrates for deubiquitination.

Here we report the use of tandem affinity purification and multidimensional protein identification technology (MudPIT) to identify USP44-interacting proteins. We found that USP44 interacts with several components of the nuclear receptor co-repressor complex (hereafter N-CoR complex). As part of N-CoR, USP44 deubiquitinates H2B both in vivo and in vitro, and it contributes to N-CoR-mediated repression of target genes, including a

genome-integrated luciferase reporter (Vaquero et al., 2004) and the endogenous *Angptl4* gene (Jin et al., 2011). Chromatin immunoprecipitation (ChIP) experiments revealed that USP44 is recruited to N-CoR target loci, where it deubiquitinates histone H2B.

USP44 has been reported to be a marker for breast cancer stem cells (Liu et al., 2015). Interestingly, depletion of the N-CoR component, TBL1XR1, which is also highly expressed in breast cancer cells (Kadota et al., 2009; Li et al., 2014), also leads to increased global H2Bub1 levels. Overall, our study demonstrates that USP44 associates with the N-CoR complex to regulate H2Bub1 levels at N-CoR target genes and that compromising the function of USP44 or the N-CoR complex impairs the invasiveness of breast cancer cells.

RESULTS

USP44 Is a Subunit of the N-CoR Complex

To further define USP44 functions, we tested the ability of recombinant USP44 to deubiquitinate H2Bub1 in vitro (Figures S1A and S1B, compare lane 1 to lanes 2 and 3). Like most other H2B DUBs identified to date (USP22, USP27x, USP49, and USP51), we found that recombinant USP44 is not active in isolation. The protein domain structure of USP44 is very similar to that of the other H2B DUBs (Figure 1A) that require association with partner proteins for full enzymatic activity (Lang et al., 2011; Zhang et al., 2013), so we reasoned that USP44 also might require partners to acquire enzymatic activity toward H2Bub1. To test this idea, we used a proteomic approach to identify USP44-interacting proteins. We isolated nuclear extracts from 293T cells that stably express USP44 fused with N-terminal FLAG and hemagglutinin (HA) affinity tags (FH-USP44), and then we performed tandem affinity purification (TAP) followed by MudPIT analysis to uncover USP44-associated proteins. As expected, CETN2 (centrin2), the only known USP44-interacting protein (Zhang et al., 2012), was one of the major proteins identified (Figure 1B). Surprisingly, the majority of the other USP44-associated proteins identified are subunits of the N-CoR complex, including TBL1X, TBL1XR1, HDAC3, NCOR1, and NCOR2 (also called SMRT) (Yoon et al., 2003). Moreover, a number of proteasomal subunits, including PSMC1, PSMC2, PSMC4, and PSMC5, which associate with N-CoR for its degradation (Perissi et al., 2004), also were identified as USP44-interacting proteins. None of these USP44 partners were detected in the FH-vector control purified in parallel (Figure 1B).

To further explore possible connections among CETN2, USP44, and N-CoR, we purified FH-tagged CETN2 from 293T cells and analyzed the purified fractions by MudPIT. Consistent with our results above, USP44 was detected as a CETN2-interacting protein. Moreover, RAD23B and XPC, both of which are known to form a complex with CETN2 in the nucleus (Nishi et al., 2005), also were detected in the CENT2-precipitated fractions. However, N-CoR complex subunits were not detected in the CETN2 purification, and XPC and RAD23B were not found in USP44-precipitated fractions (Figure 1B). Thus, these data indicate that USP44 is likely part of two separate complexes, associated with either CETN2 or N-CoR.

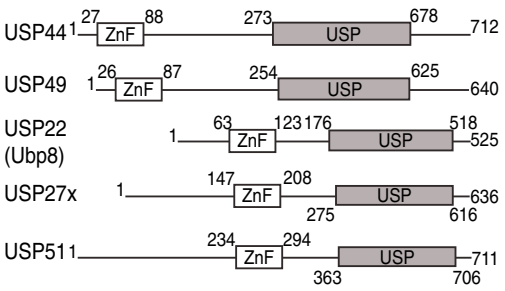
To validate our MudPIT results, we immunoprecipitated ectopically expressed USP44 from 293T nuclear extracts, and we blotted the precipitated fractions for TBL1X, TBL1XR, NCOR1, or HDAC3. These experiments confirmed interactions between endogenous N-CoR complex subunits and FH-USP44, but not another H2Bub1 DUB, USP22 (Figure 1C). Reciprocal immunoprecipitation (IP) using a TBL1X-specific antibody pulled down FH-tagged USP44 (anti-HA blot) and endogenous USP44, as well as other N-CoR complex subunits (Figures 1D and 1E). To further determine whether USP44 is an integral component of the N-CoR complex, we performed gel filtration using FLAG- and HA-purified USP44 eluates. Immunoblots of column fractions showed that USP44 co-purifies with N-CoR subunits (fractions 16 and 17), corresponding to the expected molecular weight of the N-CoR complex (1.5–2 MDa) (Yoon et al., 2003). Importantly, CETN2 was found mostly in fractions that contained little or no N-CoR subunits (fractions 19–23) (Figure 1F), again indicating that USP44 forms separate complexes with these proteins. Further cell fractionation experiments confirmed associations of USP44 and N-CoR complex components with chromatin (Figure S1D). Collectively, these results demonstrate that USP44 associates with the N-CoR complex independently of CETN2 in the nucleus, indicating that this DUB likely functions in multiple processes.

USP44 Interacts Directly with WD40 Repeats in TBL1X and TBLXR1

Several USPs interact with WD40 motif-containing proteins (Villamil et al., 2013). For example, WDR48 interacts with USP1, USP12, and USP46 to activate their DUB activity (Cohn et al., 2009; Yin et al., 2015). Interestingly, the N-CoR components TBLX and TBL1XR1 contain WD40 repeats (Uniprot). Moreover, TBL1X and TBL1XR1 were the top non-CETN2 USP44-interacting proteins detected in our MudPIT analyses (Figure 1B). To determine whether USP44 interacts directly with TBLX1 or TBLXR1, we performed IP experiments using recombinant HA-USP44 and FLAG-TBL1X/TBL1XR1 from baculovirus-infected insect cells. Both TBL1X and TBL1XR1 interacted with USP44, alone and together (Figure 2A). TBL1X and TBL1XR1 contain multiple domains, including LisH domains, F-box domains, and WD40 repeats (Figure 2B). Previous studies demonstrated that the N termini of LisH and F-box domains bind to histones H2B and H4 and also are required for assembly of the N-CoR complex (Oberoi et al., 2011; Yoon et al., 2003).

To determine whether these domains or the C-terminal WD40 repeats of TBL1X/TBL1XR1 are required for interaction with USP44, we created expression constructs for truncated forms of TBLXR1 that contained either the N-terminal LisH and F-box motifs (F1) or the C-terminal WD40 repeats (F2) fused to Gal4 (Figure 2B). Introduction of these constructs into 293T cells expressing FH-USP44 and subsequent FLAG -immunoprecipitations revealed that full-length TBLXR1 and the C-terminal fragment interacted with USP44, but the N-terminal TBL1XR1 construct did not (Figure 2C). These results indicate that the WD repeats in TBXLR1 are both necessary and sufficient for USP44 interaction. Nevertheless, an in vitro reconstituted complex containing USP44, TBL1X, and/or TBLXR1 did not show obviously different DUB activity toward H2Bub1 relative to

A

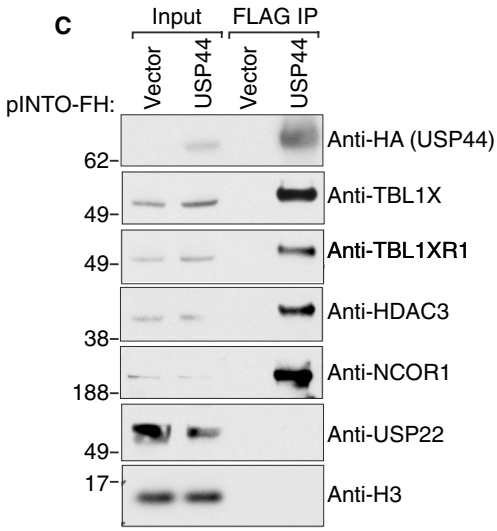


B

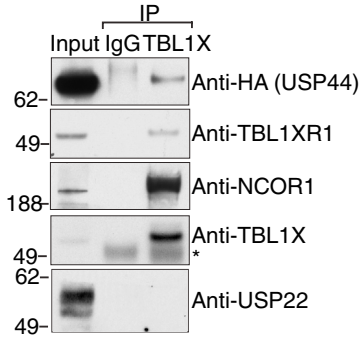
dNSAF: Normalized Distributed Spectral Abundance Factor

	CETN2_dNSAF	USP44_dNSAF	Control_ dNSAF
CETN2	0.7833421	0.06587761	X
USP44	0.00013778	0.03727431	X
RAD23B	0.00575189	X	X
XPC	0.00279162	X	X
TBL1XR1	X	0.00949706	X
TBL1X	X	0.00411709	X
PSMC4	X	0.00294579	X
HDAC3	X	0.00174454	X
PSMC1	X	0.00116108	X
PSMC2	X	0.00090758	X
PSMC5	X	0.00080303	X
NCOR1	X	0.00058988	X
NCOR2	X	0.00043233	X

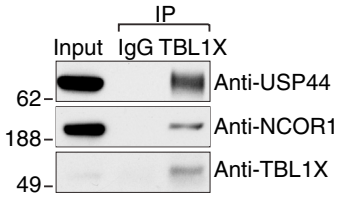
C



D



E



F

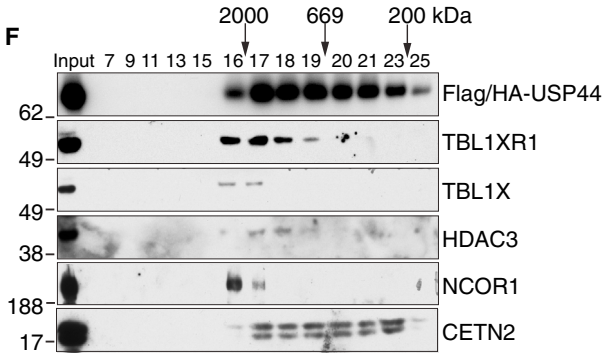


Figure 1. USP44 Associates with the N-CoR Complex

(A) Schematic representation shows the protein domain structures of USP44, USP49, USP22, USP27x, and USP51.

(B) Distributed normalized spectral abundance factor (dNSAF) of partial CETN2-associated polypeptides and USP44-associated polypeptides identified by MudPIT analysis. Bait proteins are highlighted in red, components of the CETN2-XPC complex are highlighted in blue, and components of the N-CoR complex are in green.

(C) Immunoprecipitation (IP) from nuclear extract (NE) of NFH-USP44 293T cells using FLAG beads. pINTO-NFH empty vector-transfected cells were used as a negative control. Precipitated proteins were resolved on SDS-PAGE and analyzed by western blot with the indicated antibodies. H3 serves as loading control. NFH, N-terminal FLAG and HA tags.

(D) IP from NE of NFH-USP44 293T cells using TBL1X antibody, followed by western blot with the indicated antibodies, is shown.

(E) IP from NE of MDA-MB-231 cells using TBL1X antibody, followed by western blot with the indicated antibodies, is shown.

(F) Gel filtration analysis of tandem FLAG- and HA-purified NFH-USP44 complexes. Fractions were resolved on SDS-PAGE, followed by immunoblotting with the indicated antibodies.

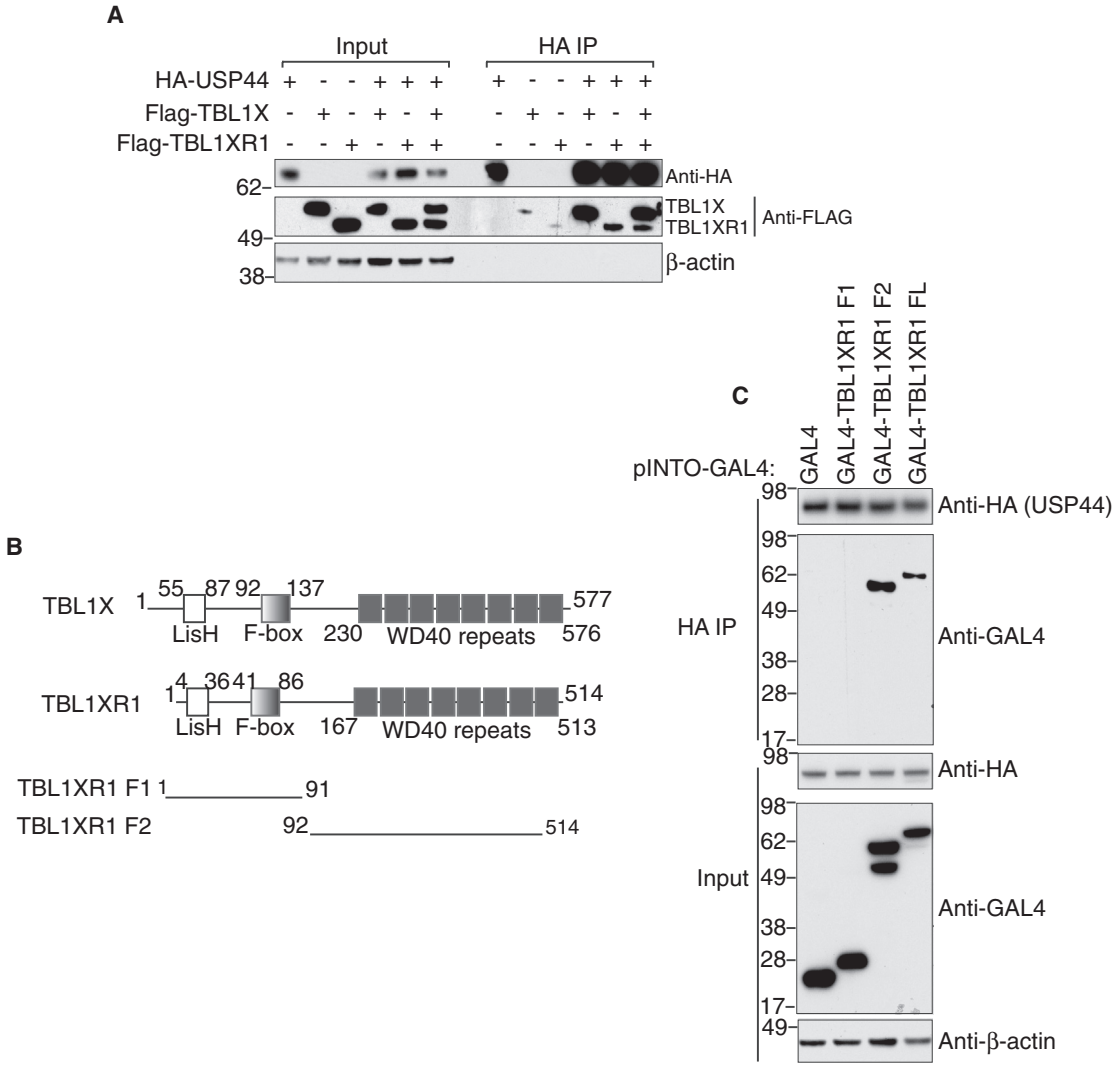


Figure 2. WD40 Repeats of TBL1X/R1 Are Required for the Association of USP44 with the N-CoR Complex

(A) Immunoprecipitation of HA-USP44 from Sf21 cells expressing the indicated proteins. The HA-precipitated fractions were resolved on SDS-PAGE and probed with HA (USP44) or with FLAG (TBL1X and TBL1XR1).

(B) A domain chart of TBL1X and TBL1XR1 proteins. Schematic representation of the TBL1XR1 deletion constructs used in (C) is presented at the bottom.

(C) NFH-USP44-expressing cells were transiently transfected with the indicated GAL4-TBL1XR1-expressing vectors. The USP44-interacting proteins were precipitated using HA beads and purified fractions blotted anti-GAL4 (TBL1XR1, full length or indicated truncations). β-actin serves as loading control.

USP44 alone (Figure S1C, compare lane 1 to lanes 2, 3, and 5), indicating that interactions with TBL1X and TBLXR1 are required for USP44 association with N-CoR but are not sufficient for USP44 activation. Furthermore, ablation of USP44 did not alter the steady-state levels of TBL1X and TBL1XR1 in mouse embryonic fibroblasts (MEFs) (Figure S2), indicating that the N-CoR complex is not likely a target for USP44 DUB activity.

USP44 Deubiquitinates H2Bub1 as Part of N-CoR

To determine whether USP44 is active toward H2Bub1 in vivo, we depleted USP44 in 293T cells using small hairpin RNAs (shRNAs) (Figure 3A). Consistent with previous reports using embryonic stem cells (ESCs) (Fuchs et al., 2012), depletion of USP44 led to a noticeable (~2-fold) increase in H2Bub1 levels in 293T cells (Figures 3A and 3B). To further address USP44 effects on H2Bub1, we overexpressed either wild-type (WT) or a catalytic mutant (C282A) of USP44 (Figure 3C). Overexpression of WT USP44 led to an obvious decrease of H2Bub1 levels, whereas overexpression of the C282A mutant did not (Figures 3C and 3D).

To more directly test the ability of USP44 to deubiquitinate H2Bub1, we utilized our purified FH-USP44-N-CoR complex for in vitro DUB assays using purified core histones as substrate (Figure 3E). H2Bub1 immunoblots indicated that, relative to an FH-vector control purified in parallel, increased amounts of FH-USP44-N-CoR in the reaction led to decreased H2Bub1 levels.

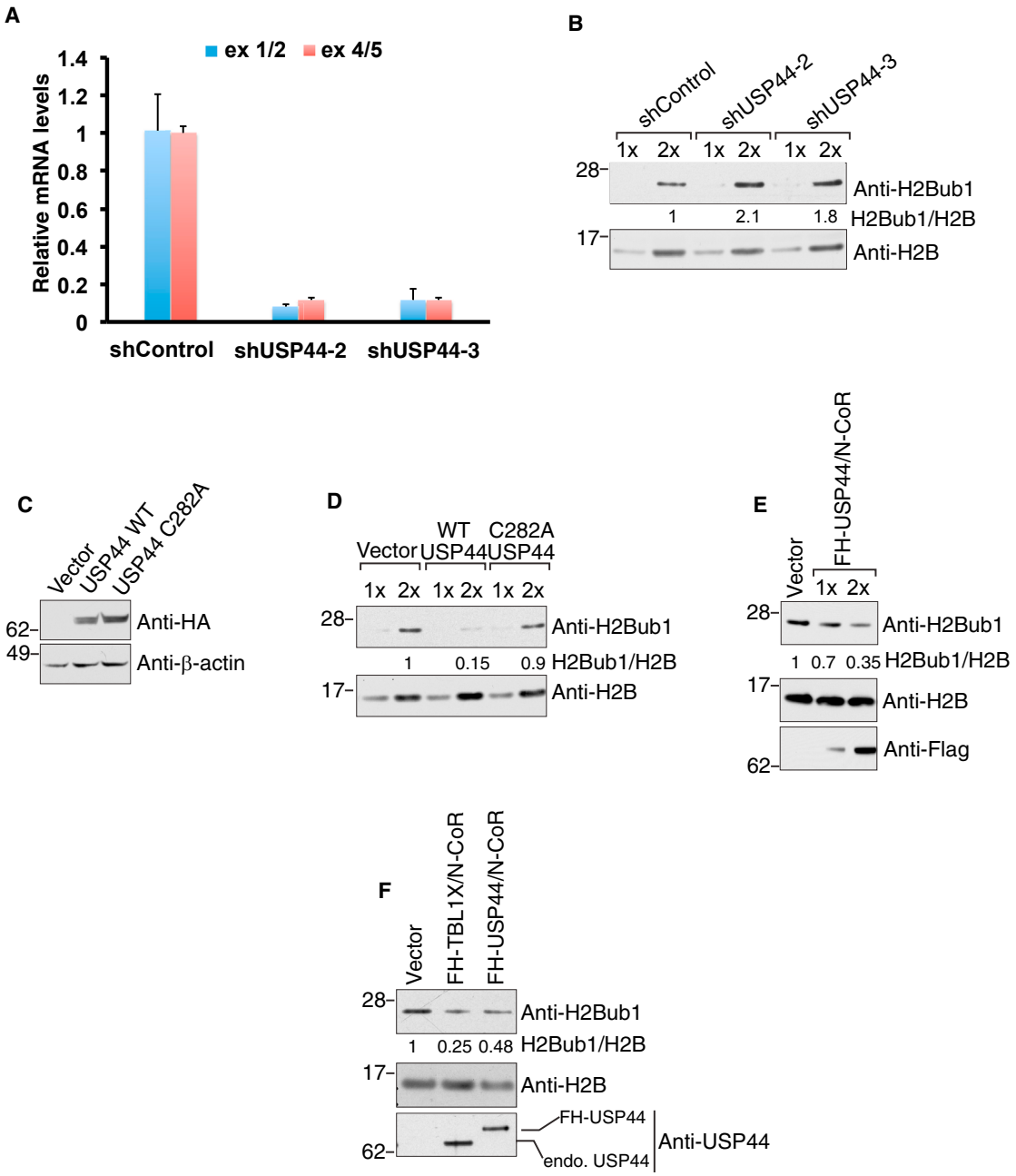


Figure 3. The USP44-N-CoR Complex Deubiquitinates H2B In Vivo and In Vitro

(A) 293T cells were infected with non-targeting shRNA (shControl) or USP44-targeting shRNA (shUSP44) expressing lentiviruses. Then 48 hr after the infection, the KD efficiency of USP44 was detected by real-time qPCR using two different pairs of USP44 primers. GAPDH was used as the internal control for normalization. Each value is the mean of three technical replicates with error bars representing the SD. Ex 1/2, primers on exon 1 and 2 junction; ex 4/5, primers on exon 4 and 5 junction.

(B) The depletion of USP44 leads to a marked increase in global H2Bub1. H2B blots were used as a loading control.

(C) Expression levels of WT or catalytically inactive USP44 proteins in the cells used in (D) are shown.

(D) Expression of WT USP44, but not catalytic inactive USP44, reduces global levels of H2Bub1 in 293T cells. 1× and 2× indicate the amount of purified total histones loaded.

(E and F) (E) Sequential FLAG- and HA-purified USP44 complex or (F) TBL1X complex from 293T cells was used for in vitro DUB assay, using histones as substrate. Both complexes show activity toward H2Bub in vitro. 1× and 2× indicate the amount of purified complexes used in the assay. Blots were quantified using ImageJ software in this and all other figures. Endo., endogenous.

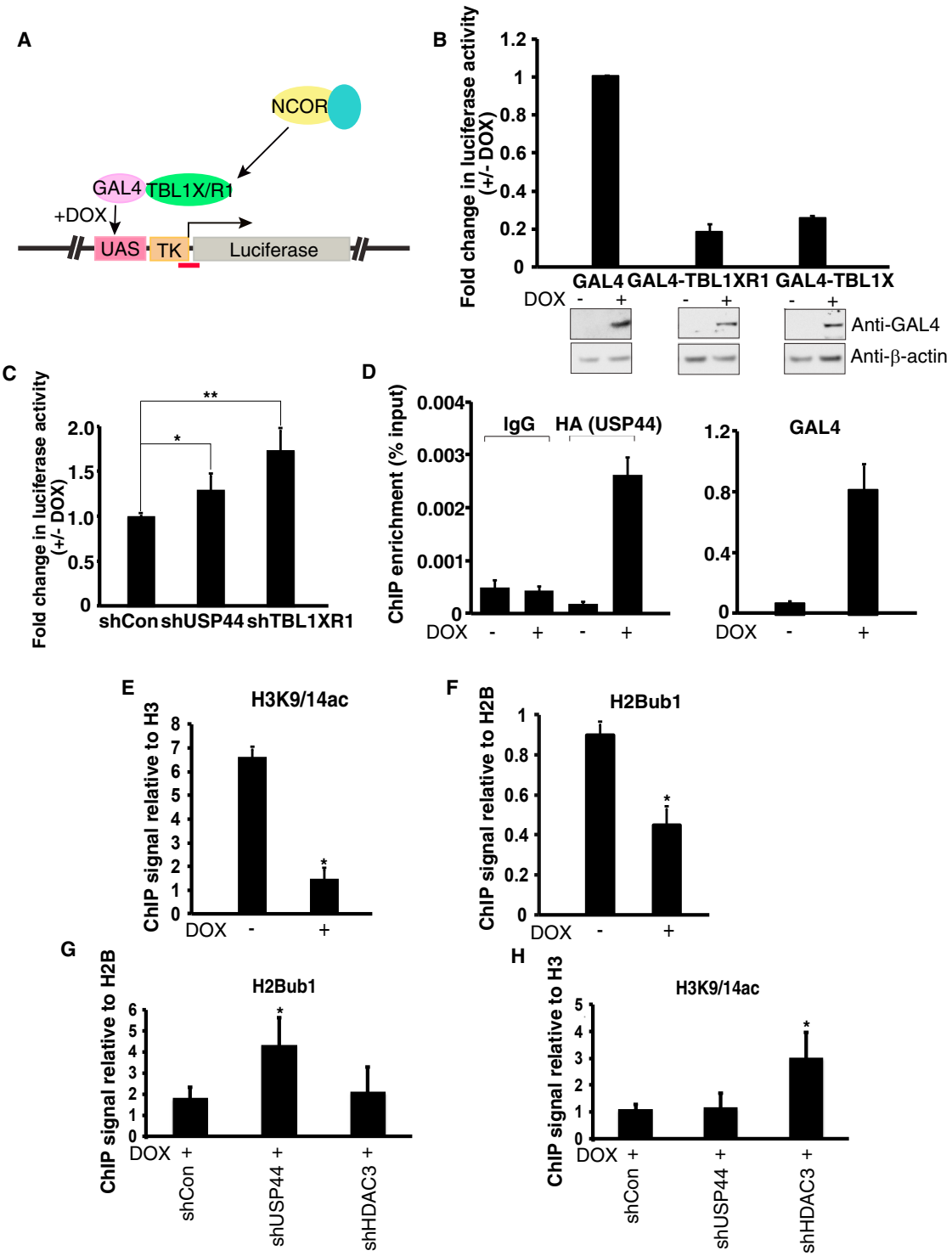


Figure 4. USP44 Facilitates the Repressive Activity of the N-CoR Complex through Deubiquitinating H2B

(A) Schematic representation of the luciferase reporter system. The red line under the scheme indicates the position of the ChIP primers used in the figure.

(B) Fold change in luciferase activity in cells expressing GAL4, GAL4-TBL1XR1, and GAL4-TBL1X, 24 hr after doxycycline (100 ng/mL) induction. The repression extent compared with GAL4 control is indicated on the y axis (the lower the bar, the more repression of the reporter). Each value is the mean of three independent measurements with error bars representing the SD. Expression levels of all GAL4-tagged proteins are shown at the bottom without (–) and with (+) induction.

(legend continued on next page)

To further test whether DUB activity is associated with the N-CoR complex, we repeated these experiments using purified N-CoR from 293T cells stably expressing FH-TBL1X (Figure S3). Incubation of the FH-TBL1X-N-CoR complex with purified histones again led to decreased levels of H2Bub1 (Figure 3F). Moreover, association with CETN2 did not affect the DUB activity of USP44 in vitro (Figures S1A and S1B, compare lane 1 to lanes 4 and 5). These experiments indicate that USP44 is an active deubiquitinase when associated with the N-CoR complex.

USP44 Enhances the Repressive Activity of N-CoR

Given that N-CoR functions as a transcriptional repressor through recruitment of the histone deacetylase HDAC3 (Wong et al., 2014), we next determined whether USP44 also contributes to N-CoR functions using a genome-integrated luciferase reporter system as previously described (Vaquero et al., 2004) (Figure 4A). We generated stable 293 T-Rex cells (Vaquero et al., 2004) containing doxycycline-inducible expression constructs for GAL4-TBL1X, GAL4-TBL1XR1, or GAL4 alone. As expected, luciferase expression and activity was repressed upon the induction of GAL4-TBL1X or GAL4-TBL1XR1 (Figure 4B). We next examined the contribution of USP44 and TBL1XR1 to the repressive activity of GAL4-TBL1XR1. Real-time qPCR and immunoblot analyses confirmed the knockdown (KD) efficiency of USP44 and GAL4-TBL1XR1 (Figure S4). As expected, the depletion of TBL1XR1 led to a marked increase in luciferase activity (Figure 4C). Interestingly, the depletion of USP44 also led to a moderate increase in luciferase activity (Figure 4C). As GAL4-TBL1XR1 is required for the recruitment of both USP44 and HDAC3, the enhanced effect of TBLXR1 KD likely reflects the loss of both HDAC3 and USP44.

We next confirmed promoter recruitment of USP44 together with the N-CoR complex by ChIP-qPCR. As expected, upon doxycycline induction, GAL4-TBL1XR1 and FLAG-HA-USP44 were both recruited to the thymidine kinase (TK) promoter that drives the luciferase reporter (Figure 4D). In addition, histone H3 acetylation at lysines 9 and 14 (H3K9/14ac), known substrates of HDAC3 (Bhaskara et al., 2010), was reduced, consistent with HDAC3 of N-CoR recruitment (Figure 4E). Moreover, H2Bub1 also was reduced (Figure 4F), consistent with N-CoR recruitment of USP44 at this locus. As expected, the depletion of USP44 led to an increase in H2Bub1 at this promoter, but it had no effect on the H3K9/14ac. Conversely, the depletion of HDAC3 led to increased H3K9/14ac, but it had no effect on H2Bub1 at the luciferase promoter (Figures 4G and 4H).

The N-CoR complex is required for transcriptional repression of unliganded peroxisome proliferator-activated receptor (PPAR) target genes (Krogsdam et al., 2002). PPARδ is broadly expressed in most tissues and the activation of PPARδ enhances lipid metabolism. PPARδ is highly expressed in MEFs, and it functions as a transcription repressor through association with HDAC3 and NCOR in the absence of ligand (Shi et al., 2002). GW501516 (GW) is a highly specific synthetic PPARδ ligand and a promising drug candidate for obesity (Evans et al., 2004). Upon ligand treatment, PPARδ becomes an activator and leads to abundant activation of target genes (Mandard et al., 2004). *Angiopoietin-like 4* (*Angptl4*, also named *PGAR* or *FLAF*) is a well-characterized target gene of PPARδ, and it is one of the most significantly induced target genes upon GW treatment in MEFs (Jin et al., 2011; Oliver et al., 2001).

To further validate the repressive activity of USP44 in the context of the N-CoR complex in vivo, we asked if depletion of Usp44 affects the repression of *Angptl4*. As expected, *Angptl4* expression was significantly induced upon treatment with GW ligand, and depletion of Tbl1xr1 led to a marked increase in *Angptl4* expression without or with GW treatment (Figures 5A and 5B). Importantly, *Angptl4* expression also was increased in *Usp44*-null MEFs without or with ligand treatment compared with WT MEFs (Figure 5C; Figure S3B), indicating that USP44 is required for full repression of this N-CoR target gene.

To test if USP44 is targeted to the promoter of *Angptl4*, we generated a stable cell line expressing HA-tagged USP44 in *Usp44* knockout (KO) MEFs, and we utilized these cells for ChIP-qPCR. These experiments confirmed that USP44 is recruited to the promoter of *Angptl4* gene (Figure 5D). Consistently, upon GW treatment, enrichment of USP44 was decreased at this locus (Figure 5D). In addition, both H3K9/14ac and H2Bub1 increased upon GW treatment (Figures 5E and 5F), consistent with dissociation of the N-CoR complex, including both HDAC3 and USP44, from this locus. Together with our data obtained from the luciferase reporter system, these results strongly indicate that both histone deacetylation and USP44-mediated removal of H2Bub1 contribute to the repression of N-CoR target genes.

USP44-N-CoR Complex Links H2Bub1 to the Invasiveness of Triple-Negative Breast Cancer Cells

USP44 is highly expressed in breast cancer stem cells, and it may contribute to breast cancer aggressiveness (Liu et al., 2015). TBL1XR1 is overexpressed and involved in the aggressiveness of malignant breast cancer cells, including MDA-MB-231 triple-negative cells (Li et al., 2014). Interestingly, we found that USP44 mRNA levels are significantly higher and H2Bub1 levels are lower (but H2Aub1 levels are similar) in

(C) Fold change in luciferase activity in GAL4-TBL1XR1 cells upon KD of USP44 or TBL1XR1. Cells were infected with shControl-, shUSP44-, or shTBL1XR1-expressing lentiviruses for 2 days, and then the GAL4-TBL1XR1 expression was induced by adding 100 ng/mL doxycycline to the medium. After 24-hr induction, luciferase activity was measured. Each value is the mean of three independent measurements with error bars representing the SD.

(D) ChIP experiments monitoring the occupancy HA-USP44 at the promoter of the reporter in GAL4-TBL1XR1 cells transiently expressing HA-USP44, without or with doxycycline (GAL4-TBL1XR1) induction. Normal mouse IgG was used as a negative control in this experiment.

(E and F) (E) ChIP monitoring the enrichment of H3K9/14ac or (F) H2Bub1 at the reporter promoter in GAL4-TBL1XR1 cells, without or with doxycycline induction, is shown.

(G and H) (G) Probing the levels of the H2Bub1 and (H) H3K9/14ac at the reporter promoter in USP44- or HDAC3-depleted cells, with or without GAL4-TBL1XR1 induction. Enrichment is shown as a percentage of either input, histone H3 in the case of H3K9/14ac or histone H2B in the case of H2Bub1. All values represent the average of three technical replicates with error bars indicating SD (*p value < 0.05 and **p value < 0.01 by two-sided t test).

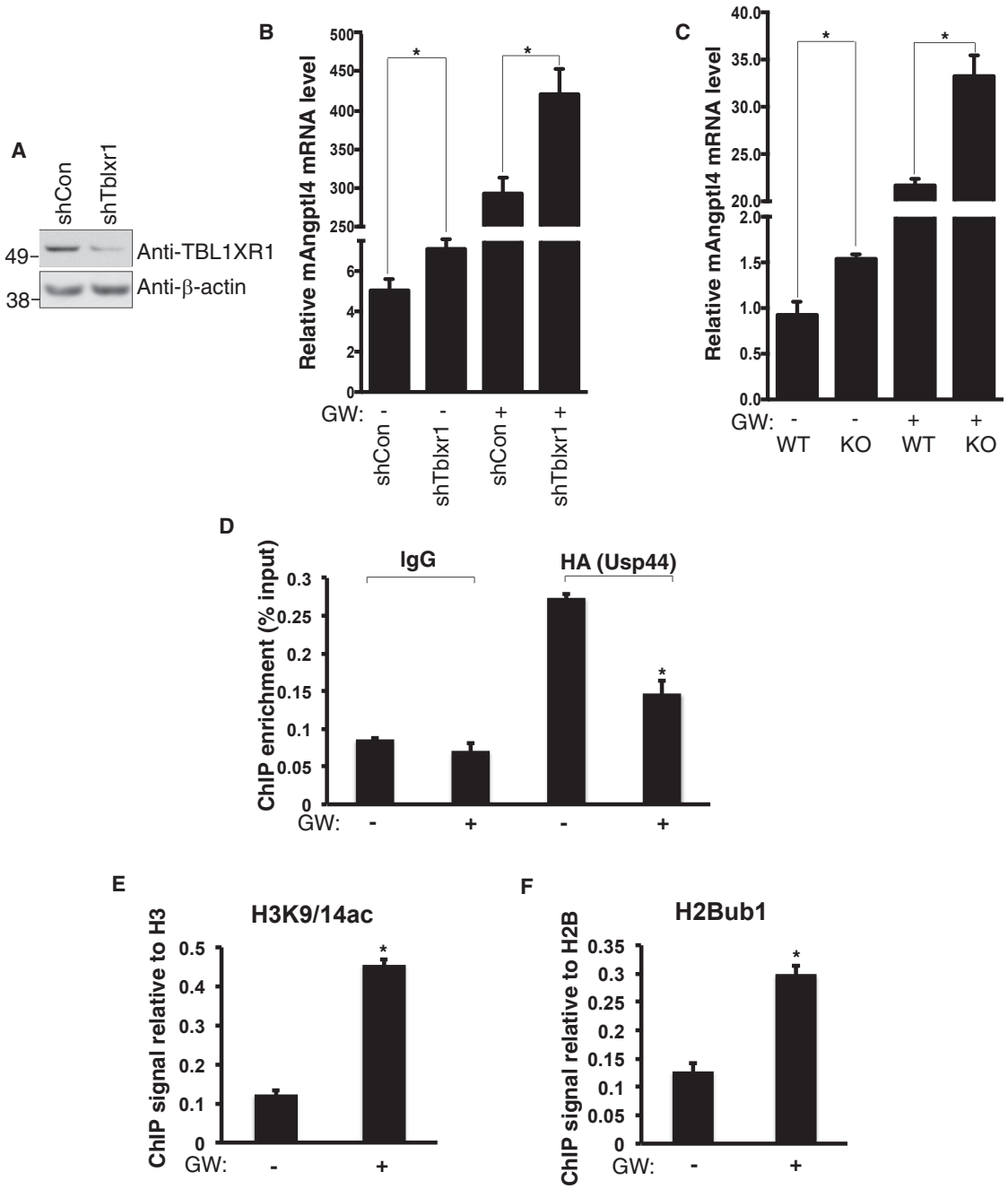


Figure 5. USP44 Contributes to the Repressive Activity of the N-CoR Complex through Deubiquitinating H2B at the Promoter of PPARδ Target Gene *Angptl4*

(A) Immunoblots to demonstrate the efficiency of Tbl1xr1 silencing in MEFs are shown.

(B) MEFs were infected with shControl or shTbl1xr1 for 2 days, followed by DMSO or 100 nM GW501516 (GW) treatment for 24 hr. Total RNA was isolated and the mRNA levels of Angptl4 were determined by real-time qPCR.

(C) Real-time qPCR analysis of Angptl4 mRNA in Usp44 WT and Usp44 KO MEFs, without or with PPARδ-specific ligand GW treatment at 100 nM for 24 hr. Two independent primary Usp44 MEF lines and two independent immortalized MEF lines were analyzed.

(D) Anti-HA ChIP monitoring the occupancy of USP44 at the promoter of *Angptl4* in MEFs stably expressing USP44-HA, without or with GW treatment, is shown.

(E and F) (E) ChIP monitoring the enrichment of H3K9/14ac or (F) H2Bub1 at the promoter of *Angptl4* in MEFs, without or with GW treatment. ChIP enrichment is shown as a percentage of either input, histone H3 in the case of H3K9/14ac or histone H2B in the case of H2Bub1. All values represent the average of three technical replicates with error bars indicating SD (*p value < 0.05 by two-sided t test). The ChIP primers used in this experiment locate at the promoter region of the *Angptl4* gene.

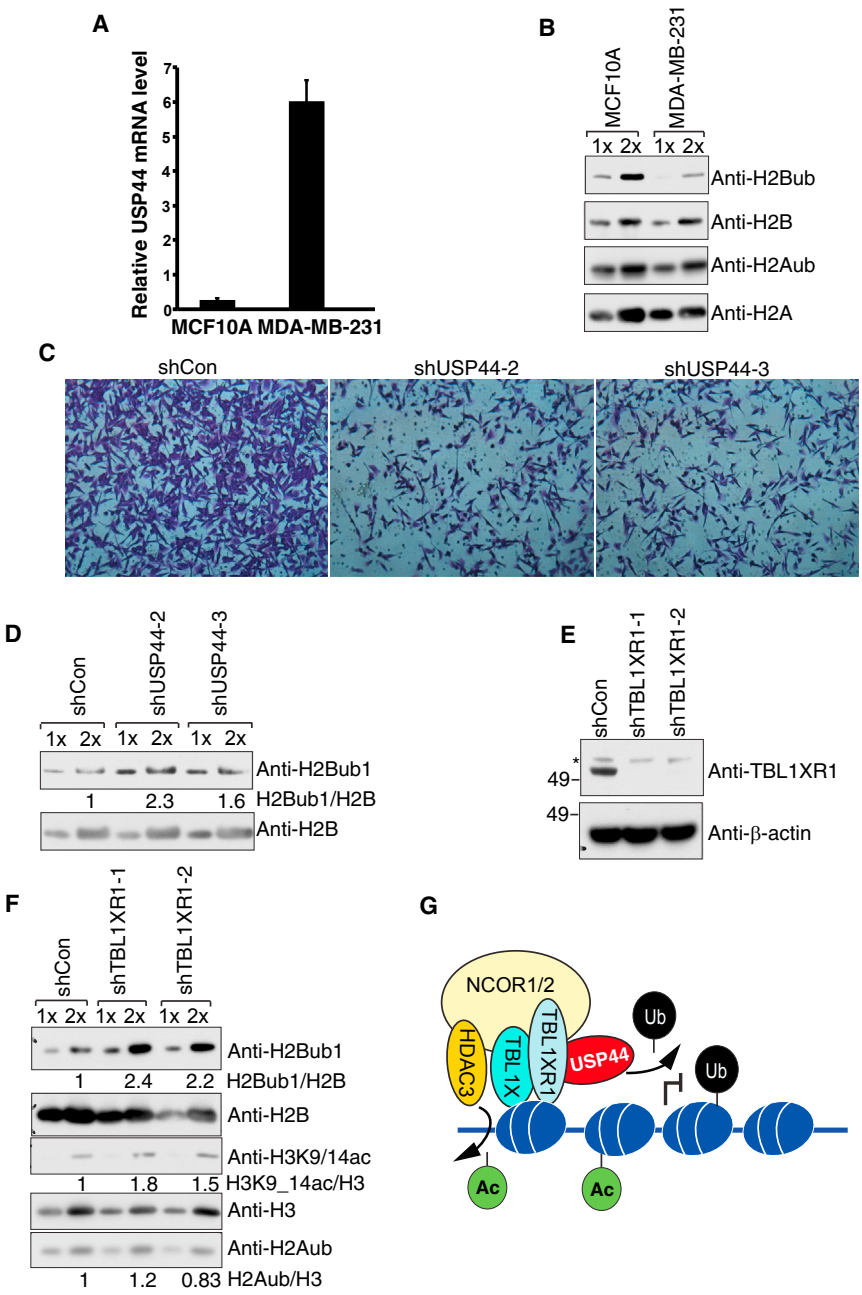


Figure 6. USP44 Regulates the Invasiveness of Breast Cancer Cells

(A) Real-time qPCR analysis of USP44 mRNA in MCF10A cells and MDA-MB-231 cells. GAPDH was used as an internal control.

(B) Histones purified from MCF10A and MDA-MB-231 cells were resolved on SDS-PAGE and blotted with anti-H2Bub or anti-H2Aub antibody. Total H2B or H2A blots were used as the loading control.

(C) MDA-MB-231 cells stably expressing shControl or two different shUSP44s were used to perform the transwell invasion assay. After 48-hr incubation, cells invading to the bottom of Matrigel chambers were stained with crystal violet.

(D) Histones purified from MDA-MB-231 cells stably infected with shControl or shUSP44 were resolved on SDS-PAGE and blotted with the indicated antibodies.

(E) Immunoblots to demonstrate silencing efficiency of TBL1XR1 in MDA-MB-231 cells, using two different shRNAs, are shown.

(F) Histones purified from MDA-MB-231 cells stably expressing shControl or shTBL1XR1 shRNA were resolved on SDS-PAGE and blotted with the indicated antibodies. Total H2B, H3, or H2A serves as the loading control. 1× and 2× indicate the amount of purified total histones.

(G) Model of the function of the USP44-N-CoR complex in cells. The N-CoR complex binds chromatin through TBL1X/TBL1XR1, and it directly recruits USP44 to deubiquitinate histone H2B and HDAC3 to deacetylate histone H3K9/14 to repress target gene transcription.

MDA-MB-231 cells compared to MCF10A normal breast epithelial cells (Figures 6A and 6B). The depletion of USP44 significantly impaired the invasiveness of MDA-MB-231 cells in vitro, and it led to an increase in global H2Bub1 levels (Figures 6C and 6D; Figure S5). The depletion of TBL1XR1 also impaired the invasiveness of these breast cancer cells (Figure S6), consistent with a previous study (Kadota et al., 2009). Accordingly, we found that depletion of TBL1XR1 also led to increased levels of H2Bub1 and moderately increased levels of H3K9/14ac, but no obvious change in H2Aub1 levels (Figures 6E and 6F). Together our results indicate that USP44 contributes to N-CoR functions in regulating gene expression and in modulating invasiveness of triple-negative breast cancer cells.

DISCUSSION

The N-CoR complex functions as a corepressor for various transcription factors, including nuclear receptors, through the recruitment of HDACs (Wong et al., 2014). Here we report that USP44 functions as a second enzymatic subunit of the N-CoR complex and that this complex deubiquitinates histone H2B in vivo and in vitro. Our results indicate that both the deubiquitination of histone H2B by USP44 and deacetylation of histone H3 by HDAC3 contribute to N-CoR-mediated transcriptional repression (Figure 6G).

Several chromatin-modifying complexes contain multiple activities, as we describe here for the USP44-N-CoR complex. The SAGA complex, a well-characterized histone-modifying complex, contains two enzymes, the histone acetyltransferase Gcn5 and the histone deubiquitinase USP22 (Koutelou et al., 2010). Moreover, the MOF-MSL complex is composed of the MOF histone acetyltransferase and the MSL E3 ubiquitin ligase (Wu et al., 2011). These multiple enzyme-containing complexes

reflect the functional coordination of complicated networks of chromatin modifications that are required for the proper regulation of gene transcription (Workman, 2016).

The N-terminal domains of TBL1X and TBL1XR previously were reported to bind histone H2B and H4 (Yoon et al., 2003). Here we found that the C-terminal WD40 repeat domains of these proteins are required to recruit USP44. Given that USP44 functions as a histone DUB, interaction with TBL1X and TBL1XR1 could help USP44 target its substrates for deubiquitination. However, association with TBL1X and TBL1XR1 is not enough to activate the DUB activity of USP44 in vitro (Figure S1C), indicating that additional partners are required. Future work will determine which other subunits of N-CoR are needed for USP44 activity and the structural basis for its activation.

A previous report of global proteomic analyses identified interacting proteins for 75 human DUBs, including USP44, in whole-cell lysates (Sowa et al., 2009). The major USP44-interacting proteins identified were CETN2 and a number of mitochondrial proteins, such as MRPL40, MRPL23, and MRPL53. The USP44-CETN2 complex is known to play an important role in preventing chromosome segregation errors during mitosis (Zhang et al., 2012). The function of USP44 in association with mitochondrial proteins is not yet clear. However, no subunits of the N-CoR complex were identified, which might reflect the use of whole-cell extracts rather than nuclear extracts as used in our study. Indeed, none of the mitochondrial proteins identified in the previous study were found in our MudPIT analyses. Together, these data suggest that USP44 plays multiple roles in different cellular compartments by forming complexes with distinct partners.

Genome-wide ChIP sequencing (ChIP-seq) analyses in human cells show that H2Bub1 is generally associated with highly expressed genes, being enriched at the 5′ beginnings of transcribed regions and also broadly distributed throughout gene bodies (Jung et al., 2012; Minsky et al., 2008). Several studies have highlighted the important roles of H2Bub1 in transcriptional elongation (Fleming et al., 2008; Pavri et al., 2006; Zhang and Yu, 2011). However, the role of H2Bub1 at gene promoters is less clear. It is still an open debate whether relatively low H2Bub1 signals at gene promoters reflect enhanced deubiquitination activity at this region or low ubiquitination of promoter nucleosomes. In yeast, cycles of H2B ubiquitination and deubiquitination are needed for a productive switch from transcription initiation to elongation (Henry et al., 2003). Recent studies indicate a direct link between H2B deubiquitination at promoter regions and decreased transcription in *Drosophila* and mammalian cells (Kessler et al., 2015; Sussman et al., 2013). In addition, in vitro analyses using chemically defined nucleosome arrays containing recombinant H2Bub1 (Fierz et al., 2011) demonstrated that H2Bub1 can disrupt higher-order chromatin architectures, facilitating more open, accessible conformations needed for transcription. Our data indicate that the USP44-N-CoR complex likely represses transcription by removing H2Bub1 via USP44 and histone acetylation via HDAC3 at the promoter.

Reduced global H2Bub1 levels have been observed in multiple advanced cancers, including breast cancer, lung cancer, colorectal cancer, and seminoma (Fuchs and Oren, 2014), raising the possibility that high levels of H2Bub1-specific DUBs could play a causal role in tumor formation or progression. In support of this notion, USP22, a well-known H2Bub1 DUB (Zhang et al., 2008), is highly expressed in various aggressive cancers and is part of an 11-gene death-from-cancer signature that defines tumors with cancer stem cell phenotypes of aggressive growth, metastasis, and resistance to therapy (Glinsky, 2006). Interestingly, USP44 is overexpressed in human T cell leukemias (Zhang et al., 2011). We report here that USP44 is also highly expressed in aggressive breast cancer MDA-MB-231 cells, leading to low global H2Bub1 levels and contributing to the invasiveness of these cells. Consistent with these findings, another essential subunit of the N-CoR complex, TBL1XR1, is also highly expressed in advanced breast cancer cells and contributes to their aggressiveness (Li et al., 2014).

The N-CoR complex is a well-documented co-repressor for unliganded nuclear receptors, and our data demonstrate that USP44 contributes to NCoR-mediated repression of the PPARδ target gene *Angptl4*. However, we were unable to detect an effect of USP44 depletion on the transcription of estrogen receptor (ER) target genes in MCF7 cells (data not shown), which also involves N-CoR. The expression of USP44 in MCF7 and MCF10-A cells is very low (data not shown and Figure 6A). Interestingly, Liu et al. (2015) reported that USP44 expression is significantly upregulated in breast cancer stem cells derived from MCF7 cells and contributes to the aggressiveness of breast cancer cells. Given that MDA-MB-231 cells are triple negative and lack ER expression, USP44 and the N-CoR complex must function to repress other types of genes in these cells. Also, USP44 can deubiquitinate non-histone substrates (Stegmeier et al., 2007), so this DUB may contribute to cancer aggressiveness independently of the N-CoR complex and H2B deubiquitination. Clearly, additional studies are needed to fully understand the multiple contributions of USP44 and other USPs to oncogenesis.

EXPERIMENTAL PROCEDURES

Nuclear Extract Preparation, Affinity Purification for Gel Filtration, Deubiquitination Assay, and MudPIT Analysis

The 293T cells stably expressing FLAG and HA-USP44, CETN2, TBL1X, or TBL1XR1 were established after transfection with pINTO-N-FH-USP44, pINTO-N-FH-CETN2, pINTO-N-FH-TBL1X, pINTO-N-FH-TBL1XR1, or pINTO-N-FH empty vector and following selection in DMEM-H complete medium containing 300 μg/mL Zeocin (Life Technologies, R25001) for ~2–3 weeks. 20–40 × 15-cm plates of stable cells were used for one tandem affinity purification experiment. Nuclear extracts were prepared as described previously (Gao et al., 2014) with modifications.

Gel Filtration and MudPIT Analysis

FLAG-HA-USP44 complex was purified by FLAG-HA affinity purification from nuclear extracts, and the eluted complex was loaded into a superdex 200 column and fractionated by size, as described previously (Lan et al., 2015).

Nuclear extracts were isolated from 293T cells stably expressing USP44 fused with N-terminal FLAG and HA affinity tags (FH-USP44), and then they were analyzed by TAP followed by MudPIT to uncover USP44-associated proteins. TAP of FH-tagged CETN2 (centrin2), the only known USP44-interacting protein, also was performed, followed by MudPIT analysis. An FH-vector control was purified and analyzed in parallel (see the Supplemental Experimental Procedures for details).

The complete mass spectrometry dataset (raw files, peak files, SEQUEST search files, as well as DTASelect result files, and protein sequences fasta file) are available at: ftp://MSV000080144@massive.ucsd.edu. Password XL75987. Proteome Xchange: PXD004957.

Luciferase Activity Assay

The 293T-Rex-luciferase cells (Vaquero et al., 2004) stably transfected with pINTO-GAL4 vector control or inserts of interest were treated with 100 ng/mL doxycycline for 24 hr. Induced cells were lysed by ice-cold lysis buffer (Promega), and then 10–25 μg of the cell lysate was assayed for luciferase activity using luciferase assay substrate (Promega), as described previously (Gao et al., 2014).

More detailed experimental procedures used in this work are presented in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.076.

AUTHOR CONTRIBUTIONS

X.L. performed the experiments and analyzed the data. S.Y.R.D. and B.S.A. aided in study design, data interpretation, and manuscript writing. L.F., R.D.M., and J.L.W. assisted with the analyses of proteomic data. W.L. helped perform ChIP experiments. P.J.G. provided *Usp44* KO MEF cells. Y.Z., L.F., and M.P.W. performed MudPIT and proteomic data analyses.

ACKNOWLEDGMENTS

We thank Dr. Danny Reinberg for the luciferase reporter system and pINTO-N-FH expression vector and Dr. Taiping Chen for the pCAG-C-FH vector. This work was largely supported by NIH grant R01 GM096472 to S.Y.R.D. Y.Z., R.D.M., L.F., M.P.W., and J.L.W. acknowledge support from NIH grant RO1GM 099945 and from the Stowers Institute for Medical Research. Sequencing was done by the Science Park NGS Core, supported by CPRIT Core Facility Support grant RP120348.

Received: April 29, 2016
Revised: September 20, 2016
Accepted: October 19, 2016
Published: November 22, 2016

REFERENCES

Atanassov, B.S., Mohan, R.D., Lan, X., Kuang, X., Lu, Y., Lin, K., McIvor, E., Li, W., Zhang, Y., Florens, L., et al. (2016). ATXN7L3 and ENY2 coordinate activity of multiple H2B deubiquitinases important for cellular proliferation and tumor growth. Mol. Cell *62*, 558–571.

Bhaskara, S., Knutson, S.K., Jiang, G., Chandrasekharan, M.B., Wilson, A.J., Zheng, S., Yenamandra, A., Locke, K., Yuan, J.L., Bonine-Summers, A.R., et al. (2010). Hdac3 is essential for the maintenance of chromatin structure and genome stability. Cancer Cell *18*, 436–447.

Briggs, S.D., Xiao, T., Sun, Z.W., Caldwell, J.A., Shabanowitz, J., Hunt, D.F., Allis, C.D., and Strahl, B.D. (2002). Gene silencing: trans-histone regulatory pathway in chromatin. Nature *418*, 498.

Cohn, M.A., Kee, Y., Haas, W., Gygi, S.P., and D'Andrea, A.D. (2009). UAF1 is a subunit of multiple deubiquitinating enzyme complexes. J. Biol. Chem. *284*, 5343–5351.

Evans, R.M., Barish, G.D., and Wang, Y.X. (2004). PPARs and the complex journey to obesity. Nat. Med. *10*, 355–361.

Fierz, B., Chatterjee, C., McGinty, R.K., Bar-Dagan, M., Raleigh, D.P., and Muir, T.W. (2011). Histone H2B ubiquitylation disrupts local and higher-order chromatin compaction. Nat. Chem. Biol. *7*, 113–119.

Fleming, A.B., Kao, C.F., Hillyer, C., Pikaart, M., and Osley, M.A. (2008). H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. Mol. Cell *31*, 57–66.

Fuchs, G., and Oren, M. (2014). Writing and reading H2B monoubiquitylation. Biochim. Biophys. Acta *1839*, 694–701.

Fuchs, G., Shema, E., Vesterman, R., Kotler, E., Wolchinsky, Z., Wilder, S., Golomb, L., Pribluda, A., Zhang, F., Haj-Yahya, M., et al. (2012). RNF20 and USP44 regulate stem cell differentiation by modulating H2B monoubiquitylation. Mol. Cell *46*, 662–673.

Gao, Z., Lee, P., Stafford, J.M., von Schimmelmann, M., Schaefer, A., and Reinberg, D. (2014). An AUTS2-Polycomb complex activates gene expression in the CNS. Nature *516*, 349–354.

Glinsky, G.V. (2006). Genomic models of metastatic cancer: functional analysis of death-from-cancer signature genes reveals aneuploid, anoikis-resistant, metastasis-enabling phenotype with altered cell cycle control and activated Polycomb Group (PcG) protein chromatin silencing pathway. Cell Cycle *5*, 1208–1216.

Henry, K.W., Wyce, A., Lo, W.S., Duggan, L.J., Emre, N.C., Kao, C.F., Pillus, L., Shilatifard, A., Osley, M.A., and Berger, S.L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev. *17*, 2648–2663.

Hock, A.K., Vigneron, A.M., and Vousden, K.H. (2014). Ubiquitin-specific peptidase 42 (USP42) functions to deubiquitylate histones and regulate transcriptional activity. J. Biol. Chem. *289*, 34862–34870.

Jin, Q., Yu, L.R., Wang, L., Zhang, Z., Kasper, L.H., Lee, J.E., Wang, C., Brindle, P.K., Dent, S.Y., and Ge, K. (2011). Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation. EMBO J. *30*, 249–262.

Jung, I., Kim, S.K., Kim, M., Han, Y.M., Kim, Y.S., Kim, D., and Lee, D. (2012). H2B monoubiquitylation is a 5′-enriched active transcription mark and correlates with exon-intron structure in human cells. Genome Res. *22*, 1026–1035.

Kadota, M., Sato, M., Duncan, B., Ooshima, A., Yang, H.H., Diaz-Meyer, N., Gere, S., Kageyama, S., Fukuoka, J., Nagata, T., et al. (2009). Identification of novel gene amplifications in breast cancer and coexistence of gene amplification with an activating mutation of PIK3CA. Cancer Res. *69*, 7357–7365.

Karpiuk, O., Najafova, Z., Kramer, F., Hennion, M., Galonska, C., König, A., Snaidero, N., Vogel, T., Shchebet, A., Begus-Nahrmann, Y., et al. (2012). The histone H2B monoubiquitination regulatory pathway is required for differentiation of multipotent stem cells. Mol. Cell *46*, 705–713.

Kee, Y., Yang, K., Cohn, M.A., Haas, W., Gygi, S.P., and D'Andrea, A.D. (2010). WDR20 regulates activity of the USP12 x UAF1 deubiquitinating enzyme complex. J. Biol. Chem. *285*, 11252–11257.

Kessler, R., Tisserand, J., Font-Burgada, J., Reina, O., Coch, L., Attolini, C.S., Garcia-Bassets, I., and Azorín, F. (2015). dDsk2 regulates H2Bub1 and RNA polymerase II pausing at dHP1c complex target genes. Nat. Commun. *6*, 7049.

Kim, J., Hake, S.B., and Roeder, R.G. (2005). The human homolog of yeast BRE1 functions as a transcriptional coactivator through direct activator interactions. Mol. Cell *20*, 759–770.

Koutelou, E., Hirsch, C.L., and Dent, S.Y. (2010). Multiple faces of the SAGA complex. Curr. Opin. Cell Biol. *22*, 374–382.

Krogsdam, A.M., Nielsen, C.A., Neve, S., Holst, D., Helledie, T., Thomsen, B., Bendixen, C., Mandrup, S., and Kristiansen, K. (2002). Nuclear receptor corepressor-dependent repression of peroxisome-proliferator-activated receptor delta-mediated transactivation. Biochem. J. *363*, 157–165.

Lan, X., Koutelou, E., Schibler, A.C., Chen, Y.C., Grant, P.A., and Dent, S.Y. (2015). Poly(Q) expansions in ATXN7 affect solubility but not activity of the SAGA deubiquitinating module. Mol. Cell. Biol. *35*, 1777–1787.

Lang, G., Bonnet, J., Umlauf, D., Karmodiya, K., Koffler, J., Stierle, M., Devys, D., and Tora, L. (2011). The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. Mol. Cell. Biol. *31*, 3734–3744.

Li, X., Liang, W., Liu, J., Lin, C., Wu, S., Song, L., and Yuan, Z. (2014). Transducin (β)-like 1 X-linked receptor 1 promotes proliferation and tumorigenicity in human breast cancer via activation of beta-catenin signaling. Breast Cancer Res. *16*, 465.

Liu, T., Sun, B., Zhao, X., Li, Y., Zhao, X., Liu, Y., Yao, Z., Gu, Q., Dong, X., Shao, B., et al. (2015). USP44+ cancer stem cell subclones contribute to breast

cancer aggressiveness by promoting vasculogenic mimicry. Mol. Cancer Ther. *14*, 2121–2131.

Mandard, S., Zandbergen, F., Tan, N.S., Escher, P., Patsouris, D., Koenig, W., Kleemann, R., Bakker, A., Veenman, F., Wahli, W., et al. (2004). The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. J. Biol. Chem. *279*, 34411–34420.

Minsky, N., Shema, E., Field, Y., Schuster, M., Segal, E., and Oren, M. (2008). Monoubiquitinated H2B is associated with the transcribed region of highly expressed genes in human cells. Nat. Cell Biol. *10*, 483–488.

Mosbech, A., Lukas, C., Bekker-Jensen, S., and Mailand, N. (2013). The deubiquitylating enzyme USP44 counteracts the DNA double-strand break response mediated by the RNF8 and RNF168 ubiquitin ligases. J. Biol. Chem. *288*, 16579–16587.

Moyal, L., Lerenthal, Y., Gana-Weisz, M., Mass, G., So, S., Wang, S.Y., Eppink, B., Chung, Y.M., Shalev, G., Shema, E., et al. (2011). Requirement of ATM-dependent monoubiquitylation of histone H2B for timely repair of DNA double-strand breaks. Mol. Cell *41*, 529–542.

Ng, H.H., Xu, R.M., Zhang, Y., and Struhl, K. (2002). Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. J. Biol. Chem. *277*, 34655–34657.

Nicassio, F., Corrado, N., Vissers, J.H., Areces, L.B., Bergink, S., Marteijn, J.A., Geverts, B., Houtsmuller, A.B., Vermeulen, W., Di Fiore, P.P., and Citterio, E. (2007). Human USP3 is a chromatin modifier required for S phase progression and genome stability. Curr. Biol. *17*, 1972–1977.

Nishi, R., Okuda, Y., Watanabe, E., Mori, T., Iwai, S., Masutani, C., Sugasawa, K., and Hanaoka, F. (2005). Centrin 2 stimulates nucleotide excision repair by interacting with xeroderma pigmentosum group C protein. Mol. Cell. Biol. *25*, 5664–5674.

Oberoi, J., Fairall, L., Watson, P.J., Yang, J.C., Czimmerer, Z., Kampmann, T., Goult, B.T., Greenwood, J.A., Gooch, J.T., Kallenberger, B.C., et al. (2011). Structural basis for the assembly of the SMRT/NCoR core transcriptional repression machinery. Nat. Struct. Mol. Biol. *18*, 177–184.

Oliver, W.R., Jr., Shenk, J.L., Snaith, M.R., Russell, C.S., Plunket, K.D., Bodkin, N.L., Lewis, M.C., Winegar, D.A., Sznaidman, M.L., Lambert, M.H., et al. (2001). A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc. Natl. Acad. Sci. USA *98*, 5306–5311.

Pavri, R., Zhu, B., Li, G., Trojer, P., Mandal, S., Shilatifard, A., and Reinberg, D. (2006). Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. Cell *125*, 703–717.

Perissi, V., Aggarwal, A., Glass, C.K., Rose, D.W., and Rosenfeld, M.G. (2004). A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell *116*, 511–526.

Pirngruber, J., Shchebet, A., Schreiber, L., Shema, E., Minsky, N., Chapman, R.D., Eick, D., Aylon, Y., Oren, M., and Johnsen, S.A. (2009). CDK9 directs H2B monoubiquitination and controls replication-dependent histone mRNA 3′-end processing. EMBO Rep. *10*, 894–900.

Prenzel, T., Begus-Nahrmann, Y., Kramer, F., Hennion, M., Hsu, C., Gorsler, T., Hintermair, C., Eick, D., Kremmer, E., Simons, M., et al. (2011). Estrogen-dependent gene transcription in human breast cancer cells relies upon proteasome-dependent monoubiquitination of histone H2B. Cancer Res. *71*, 5739–5753.

Shi, Y., Hon, M., and Evans, R.M. (2002). The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. Proc. Natl. Acad. Sci. USA *99*, 2613–2618.

Sowa, M.E., Bennett, E.J., Gygi, S.P., and Harper, J.W. (2009). Defining the human deubiquitinating enzyme interaction landscape. Cell *138*, 389–403.

Stegmeier, F., Rape, M., Draviam, V.M., Nalepa, G., Sowa, M.E., Ang, X.L., McDonald, E.R., 3rd, Li, M.Z., Hannon, G.J., Sorger, P.K., et al. (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. Nature *446*, 876–881.

Sun, Z.W., and Allis, C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104–108.

Sussman, R.T., Stanek, T.J., Esteso, P., Gearhart, J.D., Knudsen, K.E., and McMahon, S.B. (2013). The epigenetic modifier ubiquitin-specific protease 22 (USP22) regulates embryonic stem cell differentiation via transcriptional repression of sex-determining region Y-box 2 (SOX2). J. Biol. Chem. *288*, 24234–24246.

Trujillo, K.M., and Osley, M.A. (2012). A role for H2B ubiquitylation in DNA replication. Mol. Cell *48*, 734–746.

Urasaki, Y., Heath, L., and Xu, C.W. (2012). Coupling of glucose deprivation with impaired histone H2B monoubiquitination in tumors. PLoS ONE *7*, e36775.

van der Knaap, J.A., Kozhevnikova, E., Langenberg, K., Moshkin, Y.M., and Verrijzer, C.P. (2010). Biosynthetic enzyme GMP synthetase cooperates with ubiquitin-specific protease 7 in transcriptional regulation of ecdysteroid target genes. Mol. Cell. Biol. *30*, 736–744.

Vaquero, A., Scher, M., Lee, D., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004). Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. Mol. Cell *16*, 93–105.

Villamil, M.A., Liang, Q., and Zhuang, Z. (2013). The WD40-repeat protein-containing deubiquitinase complex: catalysis, regulation, and potential for therapeutic intervention. Cell Biochem. Biophys. *67*, 111–126.

Wong, M.M., Guo, C., and Zhang, J. (2014). Nuclear receptor corepressor complexes in cancer: mechanism, function and regulation. Am. J. Clin. Exp. Urol. *2*, 169–187.

Workman, J.L. (2016). CHROMATIN. It takes teamwork to modify chromatin. Science *351*, 667.

Wu, L., Zee, B.M., Wang, Y., Garcia, B.A., and Dou, Y. (2011). The RING finger protein MSL2 in the MOF complex is an E3 ubiquitin ligase for H2B K34 and is involved in crosstalk with H3 K4 and K79 methylation. Mol. Cell *43*, 132–144.

Xiao, T., Kao, C.F., Krogan, N.J., Sun, Z.W., Greenblatt, J.F., Osley, M.A., and Strahl, B.D. (2005). Histone H2B ubiquitylation is associated with elongating RNA polymerase II. Mol. Cell. Biol. *25*, 637–651.

Yin, J., Schoeffler, A.J., Wickliffe, K., Newton, K., Starovasnik, M.A., Dueber, E.C., and Harris, S.F. (2015). Structural insights into WD-repeat 48 activation of ubiquitin-specific protease 46. Structure *23*, 2043–2054.

Yoon, H.G., Chan, D.W., Huang, Z.Q., Li, J., Fondell, J.D., Qin, J., and Wong, J. (2003). Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. EMBO J. *22*, 1336–1346.

Zhang, F., and Yu, X. (2011). WAC, a functional partner of RNF20/40, regulates histone H2B ubiquitination and gene transcription. Mol. Cell *41*, 384–397.

Zhang, X.Y., Varthi, M., Sykes, S.M., Phillips, C., Warzecha, C., Zhu, W., Wyce, A., Thorne, A.W., Berger, S.L., and McMahon, S.B. (2008). The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. Mol. Cell *29*, 102–111.

Zhang, Y., van Deursen, J., and Galardy, P.J. (2011). Overexpression of ubiquitin specific protease 44 (USP44) induces chromosomal instability and is frequently observed in human T-cell leukemia. PLoS ONE *6*, e23389.

Zhang, Y., Foreman, O., Wigle, D.A., Kosari, F., Vasmatzis, G., Salisbury, J.L., van Deursen, J., and Galardy, P.J. (2012). USP44 regulates centrosome positioning to prevent aneuploidy and suppress tumorigenesis. J. Clin. Invest. *122*, 4362–4374.

Zhang, Z., Jones, A., Joo, H.Y., Zhou, D., Cao, Y., Chen, S., Erdjument-Bromage, H., Renfrow, M., He, H., Tempst, P., et al. (2013). USP49 deubiquitinates histone H2B and regulates cotranscriptional pre-mRNA splicing. Genes Dev. *27*, 1581–1595.

Zhao, Y., Lang, G., Ito, S., Bonnet, J., Metzger, E., Sawatsubashi, S., Suzuki, E., Le Guezennec, X., Stunnenberg, H.G., Krasnov, A., et al. (2008). A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. Mol. Cell *29*, 92–101.