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Noncanonical regulation of alkylation damage resistance by the OTUD4 deubiquitinase

Yu Zhao[1], Mona C Majid[1], Jennifer M Soll[1], Joshua R Brickner[1], Sebastian Dango[2] & Nima Mosammaparast[1,*]

Abstract

Repair of DNA alkylation damage is critical for genomic stability and involves multiple conserved enzymatic pathways. Alkylation damage resistance, which is critical in cancer chemotherapy, depends on the overexpression of alkylation repair proteins. However, the mechanisms responsible for this upregulation are unknown. Here, we show that an OTU domain deubiquitinase, OTUD4, is a positive regulator of ALKBH2 and ALKBH3, two DNA demethylases critical for alkylation repair. Remarkably, we find that OTUD4 catalytic activity is completely dispensable for this function. Rather, OTUD4 is a scaffold for USP7 and USP9X, two deubiquitinases that act directly on the AlkB proteins. Moreover, we show that loss of OTUD4, USP7, or USP9X in tumor cells makes them significantly more sensitive to alkylating agents. Taken together, this work reveals a novel, noncanonical mechanism by which an OTU family deubiquitinase regulates its substrates, and provides multiple new targets for alkylation chemotherapy sensitization of tumors.

Introduction

Cells have evolved multiple pathways for maintaining their genomes, which are continuously subjected to endogenous and exogenous damaging agents. Alkylation is a major mechanism by which nucleic acids are modified, and repair of these adducts is critical since they are mutagenic and may cause DNA breaks (Sedgwick et al, 2007; Fu et al, 2012). Alkylation damage is repaired by at least three different pathways, including base-excision repair (BER), suicidal methyltransferases such as methyl-guanine methyltransferase (MGMT), and direct methylation/alkylation reversal by the AlkB family of demethylases (Drablos et al, 2004; Sedgwick et al,

2007; Fu et al, 2012). Originally discovered in *Escherichia coli*, the AlkB protein catalyzes oxidative demethylation of cytotoxic adducts of DNA bases alkylated at the N1 position of purines and N3 position of pyrimidines (Falnes et al, 2002; Trewick et al, 2002). AlkB proteins may also dealkylate more complex N-linked lesions, such as those produced when oxidized lipids react with DNA (Delaney et al, 2005). By reversion of these modified bases, the bacterial AlkB protein mitigates the toxicity and mutagenicity of these adducts (Delaney & Essigmann, 2004).

At least nine AlkB homologues exist in humans (ALKBH1–ALKBH8 and FTO), but only ALKBH2 and ALKBH3 have been shown to have a similar enzymatic activity as that of the bacterial protein (Duncan et al, 2002; Aas et al, 2003; Lee et al, 2005). While ALKBH2 appears to serve as the primary alkylation repair demethylase in most normal tissues, ALKBH3 appears to supplant this function in prostate and non-small cell lung cancer, where it is overexpressed (Konishi et al, 2005; Ringvoll et al, 2006; Dango et al, 2011). More recent work has demonstrated that in pancreatic adenocarcinoma, increased ALKBH3 correlated with higher pathological stage and poorer prognosis, and knockdown of ALKBH3 reduced tumor proliferation and induced apoptosis in mouse xenograft models (Yamato et al, 2012). Similarly, in urothelial carcinoma, loss of ALKBH3 expression reduced tumor cell survival and induced cell cycle arrest (Shimada et al, 2012). Consistently, ALKBH3 expression levels correlated with tumor grade and tumor size at the time of diagnosis in this cancer. On the other hand, ALKBH2 has been shown to be an important factor in mediating alkylation damage repair in glioblastoma (Fujii et al, 2013; Johannessen et al, 2013). Both of these two enzymes cooperate with other alkylation repair pathways to protect against inflammation-induced carcinogenesis, strongly suggesting that the AlkB family is critical for repair of environmentally produced etheno lesions induced by inflammation, as well as reversal of alkylation adducts (Calvo et al, 2012). Thus, the regulation of these enzymes is likely to be critical in a host of different contexts.

The regulation of alkylation repair is understood best in *E. coli*, in which relevant repair enzymes, including AlkB, the AlkA DNA glycosylase, and others, are induced upon the treatment of cells with low levels of an alkylating agent, rendering the cells resistant to subsequent doses of alkylating agents that are lethal without prior

1   Department of Pathology and Immunology, Division of Laboratory and Genomic Medicine, Washington University in St. Louis, St. Louis, MO, USA
2   Division of General, Visceral and Pediatric Surgery, University Medical Center Göttingen, Göttingen, Germany
    *Corresponding author. Tel: +1 314 747 5472; E-mail: mosammaparast@wustl.edu

adaptation (Kleibl, 2002). Mechanistically, this involves the Ada protein, a suicide alkyltransferase which forms a potent transcriptional activator upon methylation, inducing its own transcription as well as AlkA and AlkB (Kleibl, 2002). However, the adaptive response does not exist in yeast or in mammals, and thus little is known about how this enzyme class is regulated. Recent work has suggested that Mgt1, the yeast homologue of Ada and mammalian MGMT, is targeted for degradation by the ubiquitination machinery (Hwang et al, 2009), but whether this ubiquitination is a constitutive housekeeping pathway or differentially regulated is unknown.

In this regard, ubiquitination plays a key role in regulated proteolysis through the ubiquitin–proteasome system, but also promotes a diverse set of cellular processes, including protein trafficking and the DNA damage response (Komander & Rape, 2012; Kulathu & Komander, 2012). Ubiquitination is reversible, and specific deubiquitinases (DUBs) remove ubiquitin chains from target proteins to regulate their functions. Over 75 DUBs are encoded by the human genome, each of which falls into six distinct DUB families, including the ovarian tumor (OTU) family of DUBs, which has emerged as an important player in the modulation of many distinct pathways (Reyes-Turcu et al, 2009). Notably, one of the OTU family members, OTUB1, modulates the response to DNA breaks by countering ubiquitination in a noncanonical fashion (Nakada et al, 2010). Instead of utilizing its own DUB activity, OTUB1 binds to and inhibits specific E2 ligases involved in the DNA damage response (Nakada et al, 2010). To date, this mechanism of inhibition appears to be specific for OTUB1, as other members of the OTU family function via their enzymatic deubiquitination activities (Hu et al, 2013; Keusekotten et al, 2013; Luo et al, 2013; Kato et al, 2014). Furthermore, while certain ubiquitin ligases, including cullins, coalesce into multienzyme complexes (Lydeard et al, 2013), functional interactions between multiple DUBs have not been observed.

In this study, we demonstrate a critical role for the uncharacterized OTU family DUB, OTUD4, in the regulation of human ALKBH2 and ALKBH3 demethylases. OTUD4 promotes resistance to alkylation damage by countering ubiquitination and proteasomal targeting of these enzymes. Surprisingly, we find that OTUD4 catalytic activity is completely dispensable for its regulatory function. Rather, it serves to recruit an alternative set of DUBs, USP7, and USP9X, which directly deubiquitinate the AlkB proteins. Thus, OTUD4 serves primarily as a scaffold, bridging AlkB proteins to a multi-DUB complex. In turn, loss of OTUD4, USP7, or USP9X increases sensitivity to alkylation damage, raising the possibility that these DUBs could be targeted for inhibition to sensitize tumors to alkylating agents.

Results

OTUD4 associates with human AlkB homologues ALKBH2 and ALKBH3

To gain greater insight into how the ALKBH3 pathway is regulated, we purified ALKBH3 from the PC-3 prostate cancer cell line using tandem affinity purification as previously described (Nakatani & Ogryzko, 2003). The resulting proteins were subjected to mass spectrometry analysis (Fig 1A; Supplementary Table S1). ALKBH3 interacted with members of the ASCC3 helicase complex, as was shown previously (Dango et al, 2011). Interestingly, we also found peptides corresponding to OTUD4 that were enriched in association with ALKBH3 (Supplementary Table S1). OTUD4 encodes a deubiquitinase of the OTU family (Reyes-Turcu et al, 2009). We confirmed that OTUD4 associates with ALKBH3 by immunoprecipitation of Flag-tagged ALKBH3 expressed in PC-3 cells (Fig 1B). One of the other human AlkB homologues, ALKBH2, also co-immunoprecipitated OTUD4 (Fig 1B). To determine whether ALKBH3 interacted specifically with OTUD4 or with other members of the OTU DUB family, we expressed and immunoprecipitated Flag-OTUD4 as well as Flag-OTUB1 and Flag-OTUB2 (Fig 1C). Consistent with our mass spectrometry data, which did not identify any peptides corresponding to OTUB1 or OTUB2 in association with ALKBH3, OTUD4 preferentially immunoprecipitated ALKBH3. As expected, however, all three DUBs interacted with polyubiquitin chains (Supplementary Fig S1A). Importantly, the interaction between ALKBH3 and OTUD4 could be observed at the level of endogenous proteins (Fig 1D), providing further evidence that these proteins are associated in vivo. Finally, we expressed MBP-tagged OTUD4 and Flag-tagged ALKBH3 in bacteria and found that Flag-ALKBH3 was pulled down with MBP-OTUD4 (Fig 1E). This suggested that OTUD4 and ALKBH3 likely bind to one another in a direct fashion.

Characterization of the deubiquitinase activity of OTUD4

A recent study that characterized many human OTU domain-containing proteins suggested that the OTU domain of OTUD4 has DUB activity with preferential activity against K48-linked chains (Mevissen et al, 2013). We purified the OTU domain of OTUD4 (OTUD4$_{CD}$; Supplementary Fig S1B) as a GST fusion and confirmed its preference for K48-linked diubiquitin as its substrate, although detectable activity against K11- and K63-linked diubiquitin was also observed (Supplementary Fig S1C). Mutation of the catalytic cysteine residue to alanine (C45A) completely abrogated DUB activity against K48-linked Ub$_{2-7}$ (Fig 1F). We did not observe any detectable activity against K63-linked Ub$_{2-7}$ for OTUD4$_{CD}$ (Supplementary Fig S1D). Furthermore, we did not detect deSUMOylase activity using the OTUD4$_{CD}$ domain, in contrast to the deSUMOylase SENP2 (Supplementary Fig S1E). We should note, however, that certain other OTU proteins have accessory domains that may alter the substrate specificity of the full-length DUB (Mevissen et al, 2013).

To determine whether OTUD4 is a bona fide K48-linked DUB, we purified full-length recombinant wild-type OTUD4 from bacteria. To ensure that the purified protein is full length, we expressed OTUD4 with an N-terminal 6X-His tag as well as a C-terminal Flag tag and isolated the recombinant protein by sequential Ni-NTA and Flag-immunoaffinity purification (Supplementary Fig S1F). Indeed, the full-length protein has activity against K48-linked diubiquitin, and significantly less activity against K11-linked and K63-linked diubiquitin, similar to the catalytic domain alone (Fig 1G). Again, mutation of the catalytic cysteine in the full-length context completely abrogates this activity of OTUD4 (Fig 1H). Taken together, these results demonstrate that OTUD4 is a DUB with preference for K48-linked chains.
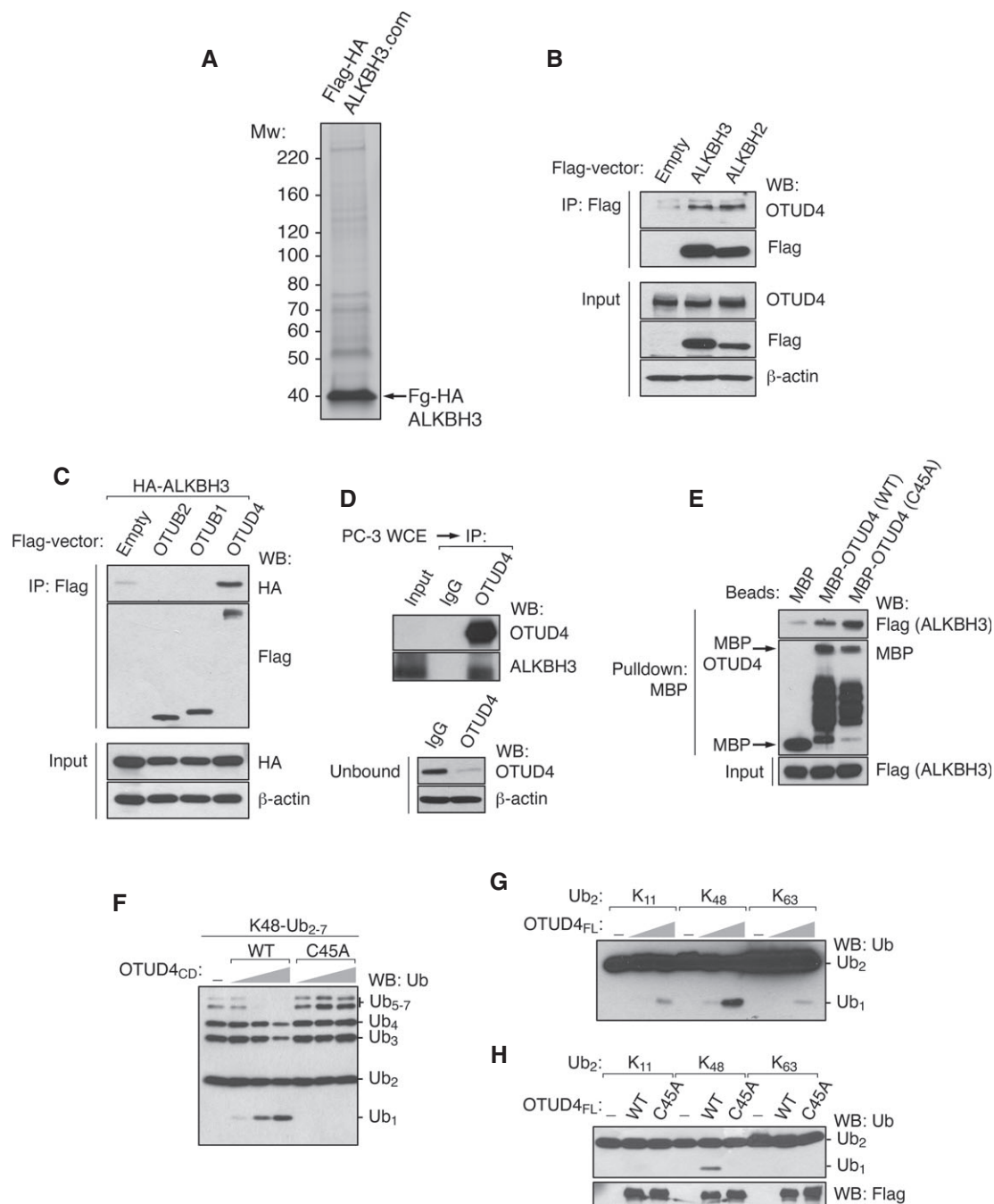
Figure 1. ALKBH3 and ALKBH2 are associated with the OTUD4 deubiquitinase.

A   Silver staining of the Flag-HA-ALKBH3 complex purified by tandem affinity purification. Positions of molecular weight markers (in kDa) are indicated on the left. Mass spectrometry analysis of the complex is shown in Supplementary Table S1.

B   Flag immunoprecipitation was performed from PC-3 cells expressing Flag-ALKBH3, ALKBH2, or empty vector and Western blotted (WB) with the indicated antibodies.

C   Flag immunoprecipitation was performed from 293T cells expressing the indicated vectors and then blotted as shown.

D   PC-3 whole-cell extract (PC-3 WCE) was immunoprecipitated using control IgG or OTUD4 antibody and blotted as shown. Unbound material was also analyzed (bottom).

E   MBP, MBP-OTUD4 (wild-type), or MBP-OTUD4 (C45A) was co-expressed in *E. coli* with His-Flag-ALKBH3. The bound material was analyzed by Western blot after MBP pulldown using the indicated antibodies.

F   The wild-type (WT) or mutant (C45A) versions of the OTUD4 catalytic domain (OTUD4$_{CD}$; 0.13, 0.64, or 3.2 μM) were incubated with K48-linked Ub$_{2-7}$ chains (0.5 μg) for 3 h and analyzed by Western blot.

G   Full-length recombinant His-OTUD4-Flag protein (0.2–1 μM) was incubated with K11-, K48-, or K63-linked diubiquitin (0.3 μM) for 24 h and analyzed by Western blot.

H   His-OTUD4-Flag protein (WT or C45A; 1 μM) was incubated with diubiquitin chains as in (G) and analyzed by Western blot.

OTUD4 regulates ALKBH3 ubiquitination status and stability *in vivo*

Association between the mammalian AlkB homologues and OTUD4 suggested that these demethylases may be regulated by ubiquitination. We first set out to determine whether ALKBH3 is ubiquitinated *in vivo*. Flag-ALKBH3 and HA-ubiquitin were co-transfected, followed by Flag immunoprecipitation. Indeed, we found that ALKBH3 is ubiquitinated *in vivo*, and proteasomal inhibition with MG132 significantly increased ALKBH3-ubiquitin conjugation (Fig 2A). This suggested that ALKBH3-ubiquitin conjugates were K48-linked, which we confirmed using an antibody specific to K48-linked ubiquitin chains (Fig 2B; Supplementary Fig S2A). Immunoprecipitation of tagged ALKBH3 under denaturing conditions confirmed that it is covalently modified by ubiquitin (Fig 2C). Using K48R and K48-only ubiquitin mutants, we determined that K48-linked ubiquitination is the major form of ubiquitin conjugated to ALKBH3 (Fig 2D). To determine whether OTUD4 contributes to the ubiquitination status of ALKBH3, we immunoprecipitated Flag-ALKBH3 upon knockdown of OTUD4. Indeed, loss of OTUD4 significantly increased K48-linked ubiquitination of ALKBH3 relative to control knockdown (Fig 2E).

These results suggested that ALKBH3 may be an unstable protein because it is targeted to the proteasome. Upon translational inhibition with cycloheximide, a significant reduction of ALKBH3 protein levels was observed within hours, but this instability of ALKBH3 was inhibited by MG132 (Supplementary Fig S2B). We then tested whether knockdown of OTUD4 affects ALKBH3 protein levels in PC-3 cells. Strikingly, we found that loss of OTUD4 using two distinct shRNAs resulted in significantly destabilized ALKBH3 in PC-3 cells (Fig 2F). However, ASCC3, the helicase partner of ALKBH3, was not significantly affected by OTUD4 knockdown. This phenotype was not due to an affect on ALKBH3 mRNA levels, as knockdown of OTUD4 did not significantly change the relative amount of ALKBH3 transcript in PC-3 cells (Supplementary Fig S2C). Consistent with this, exogenously expressed ALKBH3 was also affected by OTUD4 knockdown (Y. Zhao & N. Mosammaparast, unpublished observations).

If OTUD4 functions to prevent degradation of ALKBH3 via the proteasome, reduction of OTUD4 should reduce the half-life of ALKBH3. Consistently, knockdown of OTUD4 (to levels which did not fully destabilize ALKBH3) caused a significant reduction in the half-life of ALKBH3 in PC-3 cells (Fig 2G). Furthermore, inhibition of the proteasome with MG132 reversed the destabilizing effect of OTUD4 loss (Fig 2G; Supplementary Fig S2D). We had

previously shown that certain tumor cell lines significantly overexpress ALKBH3 (Dango *et al*, 2011), and based on our current data, it was possible that OTUD4 may be at least partially responsible for this observation. Analysis of a panel of established tumor cell lines revealed a direct correlation between OTUD4 and ALKBH3 protein levels (Supplementary Fig S2E), while ALKBH3 mRNA levels did not correlate well with ALKBH3 protein expression in these cell lines (Supplementary Fig S2F). Furthermore, overexpression of OTUD4 increased protein expression of ALKBH3 at steady state and increased ALKBH3 half-life (Supplementary Fig S2G and H).

Since OTUD4 also interacts with ALKBH2, we wished to determine whether OTUD4 affected the stability of this demethylase. Indeed, knockdown of OTUD4 also destabilized endogenous ALKBH2 (Fig 2H). We then wished to determine whether the destabilization of the ALKBH3 protein is specific to OTUD4 or whether another K48-specific OTU DUB could affect ALKBH3. In contrast to OTUD4, knockdown of OTUB1 did not significantly affect ALKBH3 levels at steady state (Fig 2I), consistent with the specificity of the interaction between these demethylases and OTUD4. We conclude that OTUD4 regulates ALKBH3 by modulating its ubiquitination.

OTUD4 catalytic activity is dispensable for ALKBH3 stability

Given its primary enzymatic function, we predicted that the catalytic activity of OTUD4 is critical for its role in stabilization of ALKBH3. To test this hypothesis, we stably expressed tagged shRNA-resistant wild-type and catalytically inactive (C45A) forms of OTUD4 in PC-3 cells, with concurrent knockdown of the endogenous OTUD4. As expected, the wild-type OTUD4 was capable of rescuing ALKBH3 levels (Fig 3A). Surprisingly, however, the catalytically inactive version of OTUD4 was also capable of rescuing the stability of ALKBH3 in these cells (Fig 3A). To further confirm that the catalytic activity of OTUD4 is not important for its function in stabilizing ALKBH3, we also performed the rescue experiment using a different catalytic mutant (C45S), as well as a N-terminal deletion that removes 65 amino acids (NΔ65), which deletes the N-terminal half of the OTU domain (Fig 3B). Consistent with the C45A mutant, these two mutants of OTUD4 also rescued ALKBH3 stability. We then tested whether either wild-type or mutant versions of the protein affected ALKBH3 ubiquitination *in vivo*. Indeed, when we co-express HA-Ub with either wild-type or the C45A mutant OTUD4 and immunoprecipitate Flag-ALKBH3,

Figure 2. OTUD4 promotes ALKBH3 deubiquitination and stability.

A   Flag immunoprecipitation was performed from 293T cells expressing Flag-ALKBH3 and HA-Ub in the presence of MG132 where indicated, followed by Western blotting. The positions of immunoglobulin heavy chain (Ig-HC) and monoubiquitinated ALKBH3 (ALKBH3-Ub$_1$) are shown.

B   Samples from (A) were analyzed using an antibody specific for K48-linked polyubiquitin.

C   Flag-ALKBH3 and HA-Ub were expressed as in (A), and Flag immunoprecipitation was performed after SDS denaturation.

D   Flag-ALKBH3 and the indicated HA-ubiquitin vectors were transfected as in (A), and Flag immunoprecipitation was performed after SDS denaturation.

E   293T cells expressing Flag-ALKBH3 were transduced with lentivirus expressing control or two distinct shRNAs specific for OTUD4. After SDS denaturation and Flag immunoprecipitation, the bound material was analyzed by Western blot as shown.

F   PC-3 cells were transduced with lentivirus as in (E), selected, and analyzed by Western blot using the indicated antibodies.

G   PC-3 cells transduced with lentiviral shRNAs were treated with cycloheximide (120 μg/ml) and MG132 (30 μM) as indicated, and whole-cell extracts were analyzed by Western blot.

H   PC-3 cells were transduced as in (F) and analyzed by Western blot using the antibodies as shown.

I   PC-3 cells were transduced with control or two OTUB1-specific shRNA vectors, selected, and analyzed by Western blot using the indicated antibodies.
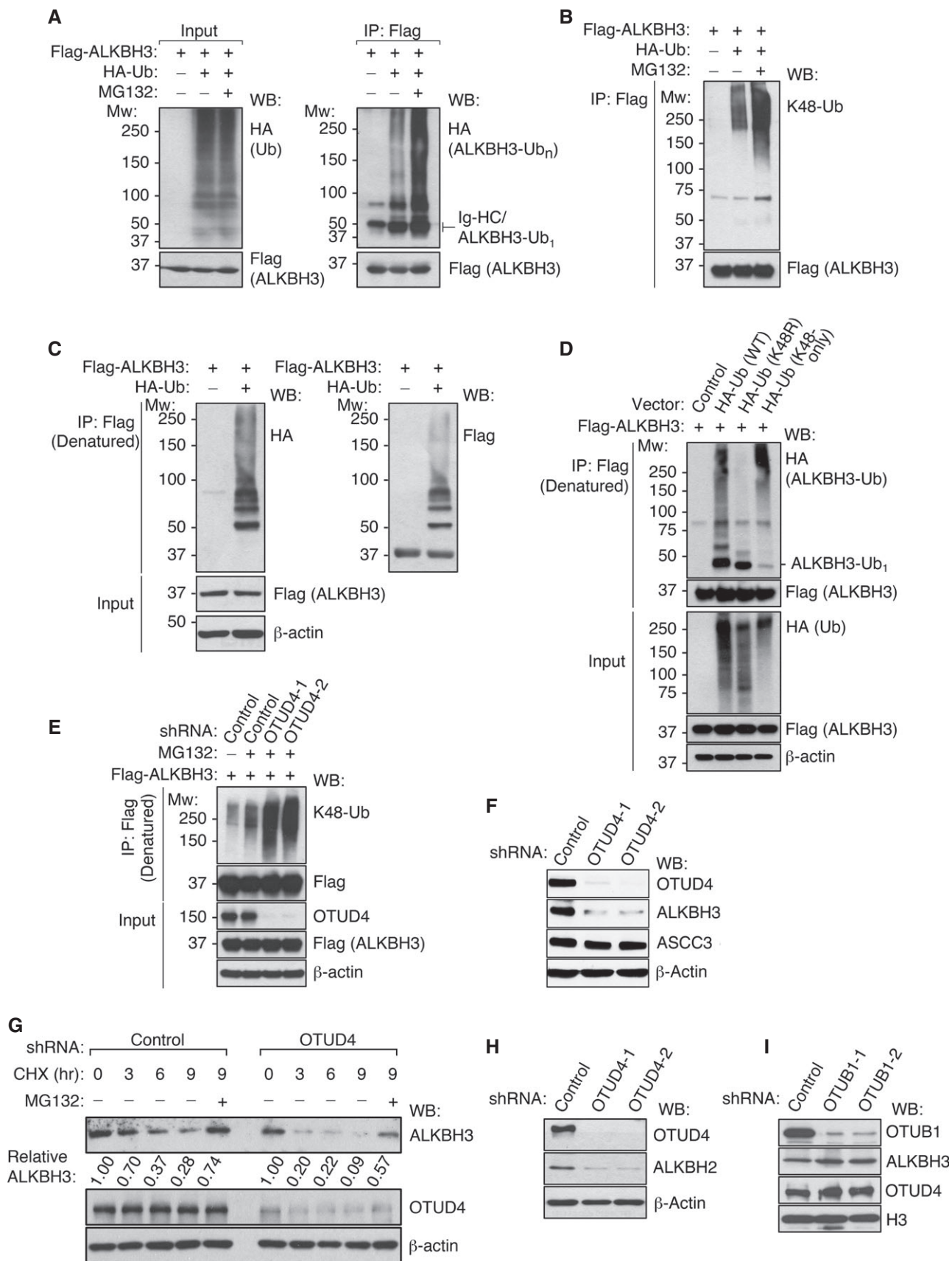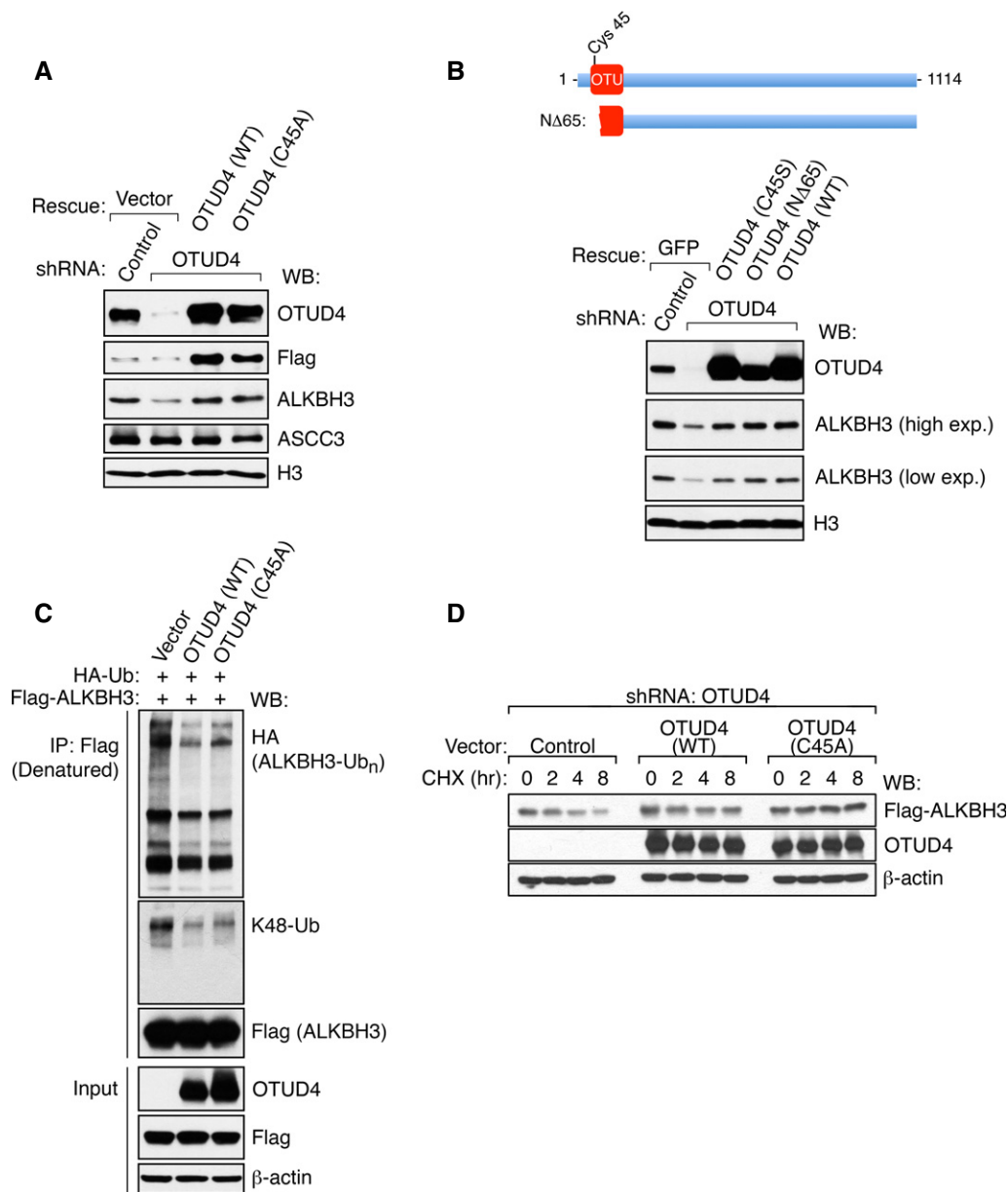
Figure 2.

Figure 3. OTUD4 catalytic activity is dispensable for ALKBH3 stabilization.

A   PC-3 cells stably expressing shRNA-resistant Flag-OTUD4 (WT or catalytically inactive C45A) or empty vector control were transduced with control or OTUD4 shRNA vectors and selected, and whole-cell extracts were Western blotted with the indicated antibodies.

B   Schematic of the OTUD4 protein is shown on top, along with the NΔ65 deletion and the position of the catalytic cysteine residue. PC-3 cells stably expressing the indicated versions of shRNA-resistant Flag-OTUD4 were transduced as in (A) and Western blotted as shown. Higher and lower exposures of the ALKBH3 Western blot are indicated as "high exp" and "low exp", respectively.

C   293T cells expressing Flag-ALKBH3, HA-Ub, and OTUD4 shRNA were reconstituted with shRNA-resistant OTUD4 (WT or catalytically inactive C45A) or empty vector control. Flag-ALKBH3 was immunoprecipitated under denaturing conditions and blotted as shown to determine its ubiquitination status.

D   Cells expressing the same vectors as in (C) were treated with cycloheximide (120 μg/ml) for the indicated period of time, and whole-cell extracts were analyzed by Western blot as shown.

we see reduction of ALKBH3 ubiquitination with both forms of OTUD4 (Fig 3C). Furthermore, both WT and the C45A mutant OTUD4 stabilized ALKBH3 in cycloheximide chase experiments (Fig 3D). Taken together, these results suggest that OTUD4 modulates ALKBH3 ubiquitination independent of its own catalytic activity.

OTUD4 interacts with USP7 and USP9X

To our knowledge, only one other DUB, OTUB1, is capable of countering ubiquitination independent of its own catalytic activity, which functions by binding to and inhibiting E2 ubiquitin ligases (Nakada *et al*, 2010; Herhaus *et al*, 2013). The domain required for E2

Figure 4. OTUD4 forms a complex with USP9X and USP7.

A, B Silver staining of the OTUD4 complex separated on a 4–15% SDS–PAGE gel. The OTUD4 complex was immunopurified using anti-Flag resin from cytosol (A) or nuclear extract (B) prepared from PC-3 cells stably expressing Flag-HA-OTUD4. The purification from cells expressing empty vector is also shown. Positions of molecular weight markers (in kDa) are indicated.

C Flag-HA-OTUD4 was expressed in PC-3 cells, and immunofluorescence was performed using an anti-HA antibody, with Hoechst as nuclear counter stain.

D Flag immunoprecipitation was performed from 293T cells expressing Flag-OTUD4, OTUB1, OTUB2, or empty vector and then blotted with the indicated antibodies.

E, F PC-3 whole-cell extract (PC-3 WCE) was immunoprecipitated using control IgG or the indicated DUB antibody and then blotted as shown.

G HA immunoprecipitation was performed from 293T cells expressing Flag-OTUD4 and HA-USP7 or HA vector and then blotted with the indicated antibodies.

H Flag immunoprecipitation was performed from 293T cells expressing Flag-tagged GFP, OTUD4, or OTUD4 (C45A) and then blotted as shown.

I MBP, MBP-OTUD4, or MBP-OTUD4 (C45A) were co-expressed in *E. coli* with His-Flag-USP7. The bound material was analyzed by Western blot after MBP pulldown using the indicated antibodies.

J Flag immunoprecipitation was performed from 293T cells expressing Flag-ALKBH3 and HA-USP7, along with OTUD4 (WT), OTUD4 (C45A), or empty vector as indicated, and then blotted as shown. Higher and lower exposures are indicated as "high exp" and "low exp", respectively.

K Flag immunoprecipitation was performed as in (J) after expression of the indicated vectors and shRNAs in 293T cells.

binding in OTUB1 is missing in OTUD4, suggesting that OTUD4 counteracts ubiquitination through another mechanism. To determine this, we purified tagged OTUD4 stably expressed from PC-3 cytosol and nuclear extracts and identified its associated partners by tandem mass spectrometry (Fig 4A and B; Supplementary Fig S3A). We purified OTUD4 from both compartments because, while OTUD4 is primarily cytoplasmic, immunofluorescence suggested that OTUD4 is present in the nucleus as well (Fig 4C; Supplementary Fig S3B). Our mass spectrometry results suggested that OTUD4 is associated with two additional DUBs, USP7 and USP9X (Supplementary Table S2). Therefore, it was possible that OTUD4 promoted the stabilization of ALKBH3 by serving to recruit these additional DUBs.

Using co-immunoprecipitation, we demonstrated that OTUD4, but not OTUB1 or OTUB2, interacted specifically with USP7 and USP9X (Fig 4D). The interaction between OTUD4 and USP9X occurred predominantly in the cytoplasm, although a small amount of USP9X immunoprecipitated with OTUD4 from nuclear extract (Supplementary Fig S3C and D). Immunoprecipitation of OTUD4 from PC-3 cell extracts demonstrated the interaction between OTUD4 and USP7 as well as USP9X at the endogenous level (Fig 4E). We were also able to observe co-immunoprecipitation of OTUD4 upon immunoprecipitation of endogenous USP9X and HA-USP7 (Fig 4F and G). The observed interactions between OTUD4 and USP7/USP9X should be independent of the catalytic activity of OTUD4, and as expected, the C45A mutant form of OTUD4 was also able to immunoprecipitate both DUBs similar to wild-type OTUD4 (Fig 4H). We co-expressed Flag-tagged USP7 and MBP-tagged OTUD4 in bacteria and performed MBP pulldowns (Fig 4I); we also tested whether MBP-OTUD4 could interact with *in vitro* transcribed and translated USP9X (Supplementary Fig S3E). These results demonstrated that recombinant forms of these DUBs could interact, suggesting OTUD4 associates directly with USP7 and USP9X.

OTUD4 promotes the association of ALKBH3 and USP7/USP9X

If OTUD4 functions in association with these additional DUBs, it may serve to help recruit these DUBs to substrates such as ALKBH3. This could explain why OTUD4 promotes ALKBH3 stability independent of its own DUB activity. To test this, we performed immunoprecipitation of Flag-ALKBH3 in 293T cells with or without the expression of untagged OTUD4. Without exogenous OTUD4, we found a small but reproducible amount of HA-USP7 and endogenous USP9X in association with ALKBH3 (Fig 4J). Expression of OTUD4 significantly increased the amount of HA-USP7 and USP9X

associated with ALKBH3 (Fig 4J, compare IP lanes 2 and 3). The OTUD4-mediated association between ALKBH3 and USP7/USP9X was independent of OTUD4 activity (Fig 4J, compare IP lanes 3 and 4). However, the association between ALKBH3 and ASCC3 was not increased by exogenous expression of OTUD4, suggesting that OTUD4 specifically promotes the interaction between ALKBH3 and USP7/USP9X. To confirm these results, we knocked down OTUD4 in 293T cells and immunoprecipitated Flag-ALKBH3. Loss of OTUD4 using two distinct shRNAs significantly reduced the amount of USP7 and USP9X that was associated with Flag-ALKBH3 (Fig 4K). However, a minimal amount of binding between ALKBH3 and USP7/USP9X could be observed without OTUD4 (Fig 4K; Supplementary Fig S3F). We then tested the converse notion; that is, we wished to modulate the expression of USP7 and USP9X to determine whether the association between ALKBH3 and OTUD4 would also be altered. However, upon knockdown of either USP7 or USP9X, we did not observe a change in the amount of HA-OTUD4 that was immunoprecipitated with Flag-ALKBH3 (Supplementary Fig S3G). Furthermore, overexpression of wild-type or catalytically inactive USP7 did not result in any apparent change in the interaction between OTUD4 and ALKBH3 (Supplementary Fig S3H). These results are consistent with the model that OTUD4 serves to promote the association of ALKBH3 and USP7/USP9X to assemble a DUB complex.

A deubiquitinase recruiting domain in OTUD4 promotes ALKBH3 stability

If the scaffolding model for OTUD4 is correct, then it must contain a region outside the OTU domain that recruits these additional DUBs to promote ALKBH3 stability. We created a panel of OTUD4 deletions (Fig 5A) and determined by co-immunoprecipitation that a region comprised of residues 181–550 was necessary and sufficient to bind to both USP7 and USP9X (Fig 5B). Co-immunoprecipitation of USP7 or USP9X demonstrated an increased association between USP7 and USP9X upon OTUD4 overexpression, suggesting that the USP7 and USP9X binding regions on OTUD4 are not completely overlapping (Supplementary Fig S4A and B). We designate the 181–550 region as the deubiquitinase recruiting domain (DRD) of OTUD4. Indeed, when purified from 293T cells, this domain has significant DUB activity, in contrast to the recombinant DRD purified from *E. coli* (Supplementary Fig S4C and D; Fig 5C). The DUB activity co-purified with the DRD could be at least partially inhibited upon addition of a USP9X inhibitor, WP1130 (Kapuria *et al*, 2010),
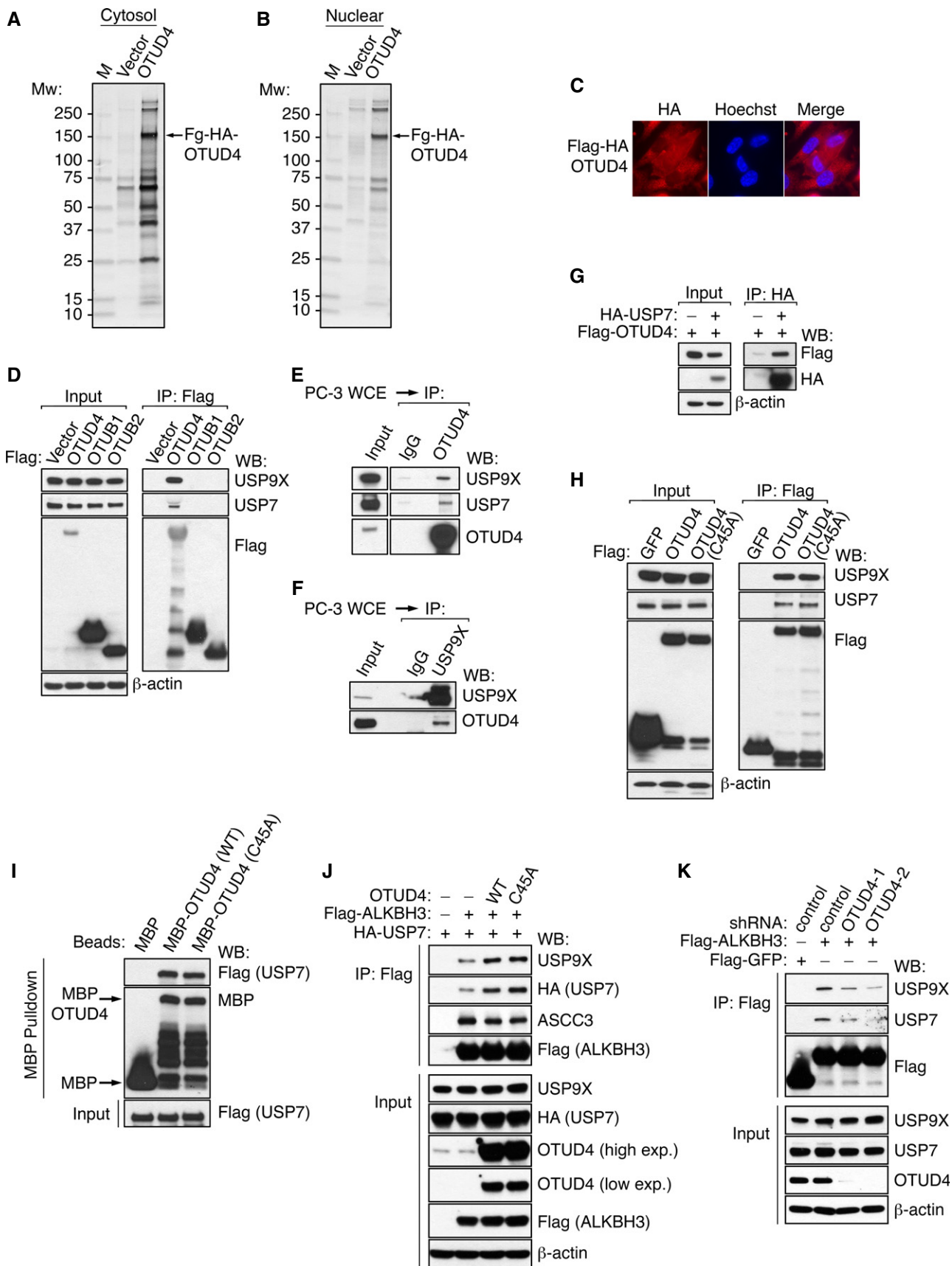
Figure 4.

Figure 5.   A deubiquitinase scaffolding domain in OTUD4 is sufficient for ALKBH3 deubiquitination.

A   Schematic of OTUD4 deletions used in this study.
B   Flag-tagged OTUD4 deletions were expressed in 293T cells, immunoprecipitated, and blotted with the antibodies as shown.
C   *E. coli*-purified MBP-tagged OTUD4 fragments (0.8, 2.4, or 7.2 μg), or 293T-purified Flag-GFP or Flag-OTUD4$_{181-550}$ fragments (1–3 μg) were used to test for DUB activity using K48-linked Ub$_{2-7}$ (0.1 μg).
D   Flag-OTUD4$_{181-550}$ purified from 293T cells (0.2 μg) was pre-incubated with DMSO, NEM (500 μM), USP9X inhibitor (WP1130; 40–100 μM), or USP7 inhibitor (P22077; 40 μM) as indicated, and tested for DUB activity as in (C).
E   Binding was tested between immobilized MBP or MBP-OTUD4 deletions (6 μg each) and His-USP7 (1 μg) or His-Flag-ALKBH3 (1 μg). Bound material was analyzed by Western blot using the indicated antibodies.
F   Immobilized GST or GST-ALKBH3 (0.5 μg) was incubated with MBP-OTUD4-DRD (181–550) or MBP-OTUD4-1-180. After initial complex formation, binding to His-USP7 (1 μg) was tested as in (E).
G   293T cells expressing the indicated vectors were treated with MG132 (30 μM). After Ni-NTA purification, the bound material was analyzed by Western blot as shown. Quantification of ubiquitinated ALKBH3 was determined by densitometry.

or a USP7 inhibitor, P22077 (Altun *et al*, 2011), but completely inhibited when the two inhibitors are used simultaneously (Fig 5D). We should note that ALG13, another OTU domain-containing protein, also contains a region with significant homology to the DRD of OTUD4, suggesting that at least one other OTU domain DUB may function via this mechanism (Supplementary Fig S4E).

Next, we demonstrated that recombinant, MBP-tagged OTUD4-DRD interacted with purified, recombinant His-tagged USP7; however, residues 1–180 and 1–300 of OTUD4 were insufficient for such interaction (Fig 5E, left panel). We used the same panel of MBP-tagged OTUD4 deletions to determine the ALKBH3 interaction domain within OTUD4. At least two distinct domains within OTUD4 (residues 1–180, as well as the DRD itself) were able to interact directly with recombinant ALKBH3 (Fig 5E, right panel; Supplementary Fig S4F). Since the DRD is sufficient to interact directly with both ALKBH3 and USP7, we reasoned that we could reconstitute this scaffolding function of the DRD *in vitro*. In fact, while GST-tagged ALKBH3 interacted poorly with His-USP7, the presence of MBP-DRD significantly increased the apparent affinity of these two proteins for each other (Fig 5F). These data suggested that the DRD should be sufficient to rescue the function of OTUD4 with regard to ALKBH3 deubiquitination. Indeed, expression of the DRD in OTUD4-deficient cells appeared to reduce ALKBH3 ubiquitination similar to full-length OTUD4 (Fig 5G). In contrast, residues 1–180 or 1–300 of OTUD4 were not capable of promoting ALKBH3 deubiquitination in these cells, despite the fact that both of these domains interact with ALKBH3 directly and have intrinsic DUB activity. Although we have not confirmed this via reciprocal immunoprecipitation of ubiquitinated proteins to analyze the status of ALKBH3 ubiquitination, our data suggest that the OTUD4 DRD serves as a DUB scaffold to promote ALKBH3 stability.

Loss of USP7 or USP9X increases ALKBH3 ubiquitination and affects ALKBH3 stability

If recruitment of USP7 and USP9X is critical for OTUD4 function, then these additional DUBs should also be important in promoting ALKBH3 stability through their DUB activities. Therefore, we tested to see whether loss of either USP7 or USP9X would also destabilize ALKBH3. Indeed, shRNA-mediated knockdown of either DUB in PC-3 cells significantly reduced ALKBH3 protein levels at steady state (Fig 6A). This was observed with two distinct shRNAs targeting USP9X and USP7. Consistent with a role for these additional DUBs in stabilizing ALKBH3, knockdown of USP9X or USP7 significantly reduced the half-life of ALKBH3 relative to control

knockdown (Fig 6B; Supplementary Fig S5A). As with OTUD4, loss of USP9X or USP7 did not significantly alter ALKBH3 mRNA levels (Supplementary Fig S5B). Conversely, overexpression of USP9X or USP7 stabilized ALKBH3 (Fig 6C). We then tested whether the catalytic activity of these additional DUBs is critical in this pathway. Expression of wild-type but not a catalytically inactive form of USP7 (C223S) reduced the ubiquitination of ALKBH3 in 293T cells (Fig 6D). Similarly, expression of USP9X reduced the ubiquitination of ALKBH3, but this apparent deubiquitination was reduced with the USP9X inhibitor WP1130 (Supplementary Fig S5C). Furthermore, knockdown of either USP7 or USP9X increased the level of ALKBH3 K48-linked ubiquitination (Fig 6E), suggesting these DUBs, as well as OTUD4, function to counter ALKBH3 ubiquitination. This is in contrast with OTUD4, which can reduce ALKBH3 ubiquitination independent of its catalytic activity (see Fig 3C).

We sought additional evidence that OTUD4 functions as a DUB scaffold. To this end, we tested the ability of USP9X to deubiquitinate ALKBH3 in 293T cells with or without OTUD4. In the absence of OTUD4, expression of USP9X reduced ALKBH3 ubiquitination very modestly (Fig 6F). In contrast, when OTUD4 is expressed, USP9X robustly deubiquitinated ALKBH3 (Fig 6F; compare lanes 2–4 and 6–8). Thus, while USP9X promotes ALKBH3 deubiquitination, it optimally requires OTUD4 for this function. We further tested the epistatic relationship of these DUBs. While loss of OTUD4 increased ALKBH3 K48 ubiquitination, concurrent knockdown of OTUD4 with USP9X or USP7 did not further increase ALKBH3 ubiquitination (Fig 6G). These results suggest that OTUD4 functions in the same pathway as USP9X/USP7 in promoting ALKBH3 deubiquitination.

The OTUD4 complex is required for alkylation damage resistance and repair

Since OTUD4 is critical for ALKBH3 stability and ALKBH3 plays a critical role in proliferation and alkylation damage resistance in prostate cancer and certain other tumor cell lines (Konishi *et al*, 2005; Dango *et al*, 2011; Tasaki *et al*, 2011), we tested whether OTUD4 was critical for PC-3 cell proliferation. Knockdown of OTUD4 significantly reduced proliferation of PC-3 cells in culture (Fig 7A), as did loss of USP7 and USP9X (Fig 7B). Furthermore, knockdown of OTUD4 caused a significant increase in the sensitivity of these cells to the alkylating agent methyl methanesulfonate (MMS) (Fig 7C). This was observed using two distinct OTUD4-specific shRNAs. We then wished to determine whether this role of OTUD4 in alkylation damage resistance is applicable to other cells that depend on the ALKBH3 pathway. Knockdown of OTUD4 in H23
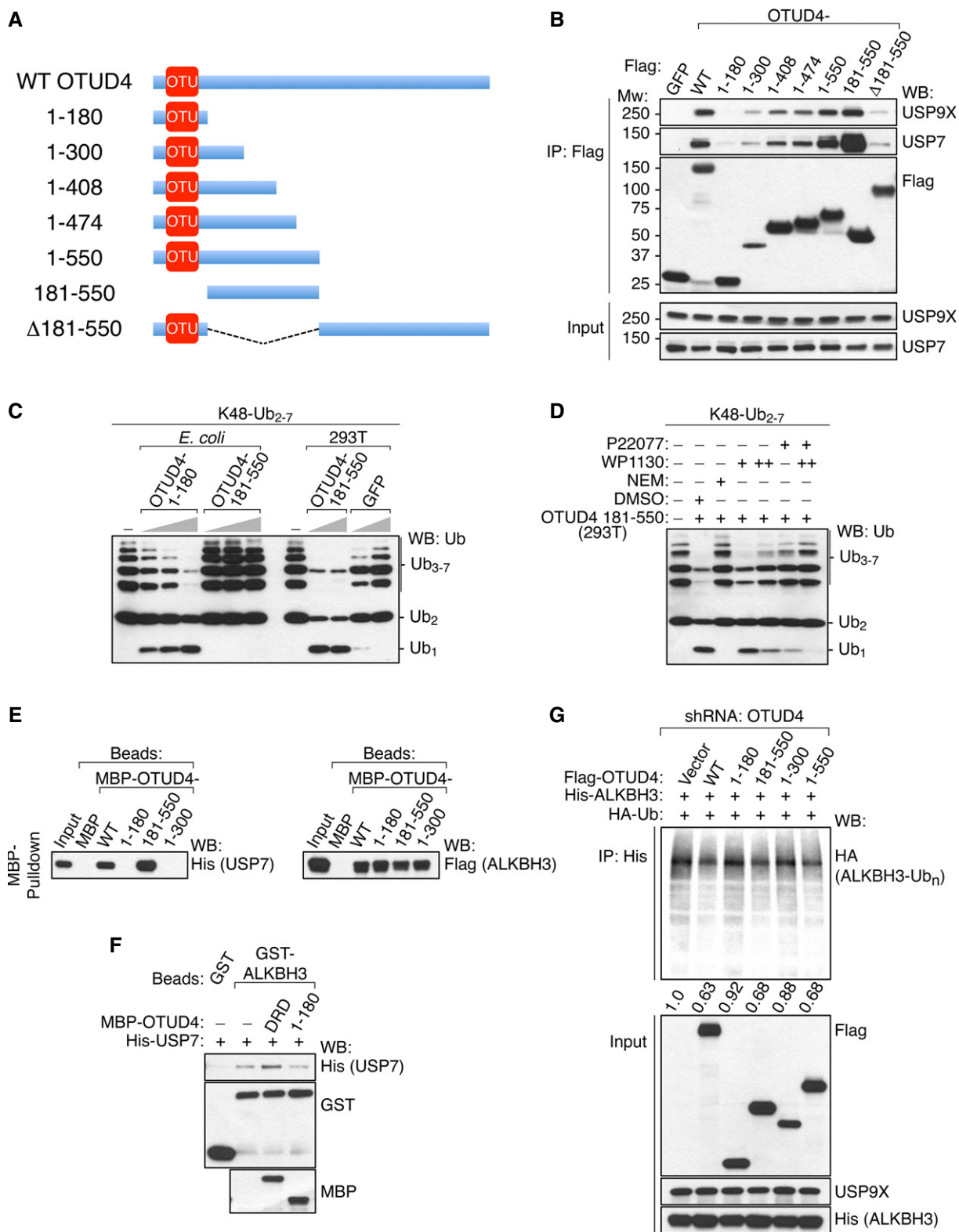
Figure 5.

or U2OS cells also destabilized ALKBH3 at steady state (Supplementary Fig S6A). Consistent with this, knockdown of OTUD4 also caused MMS hypersensitivity in H23 cells (Fig 7D). Certain DUBs

are thought to play a role in the localization of their targets; for example, the localization of the phosphatase PTEN is regulated by the USP7 DUB (Song *et al*, 2008). However, knockdown of OTUD4
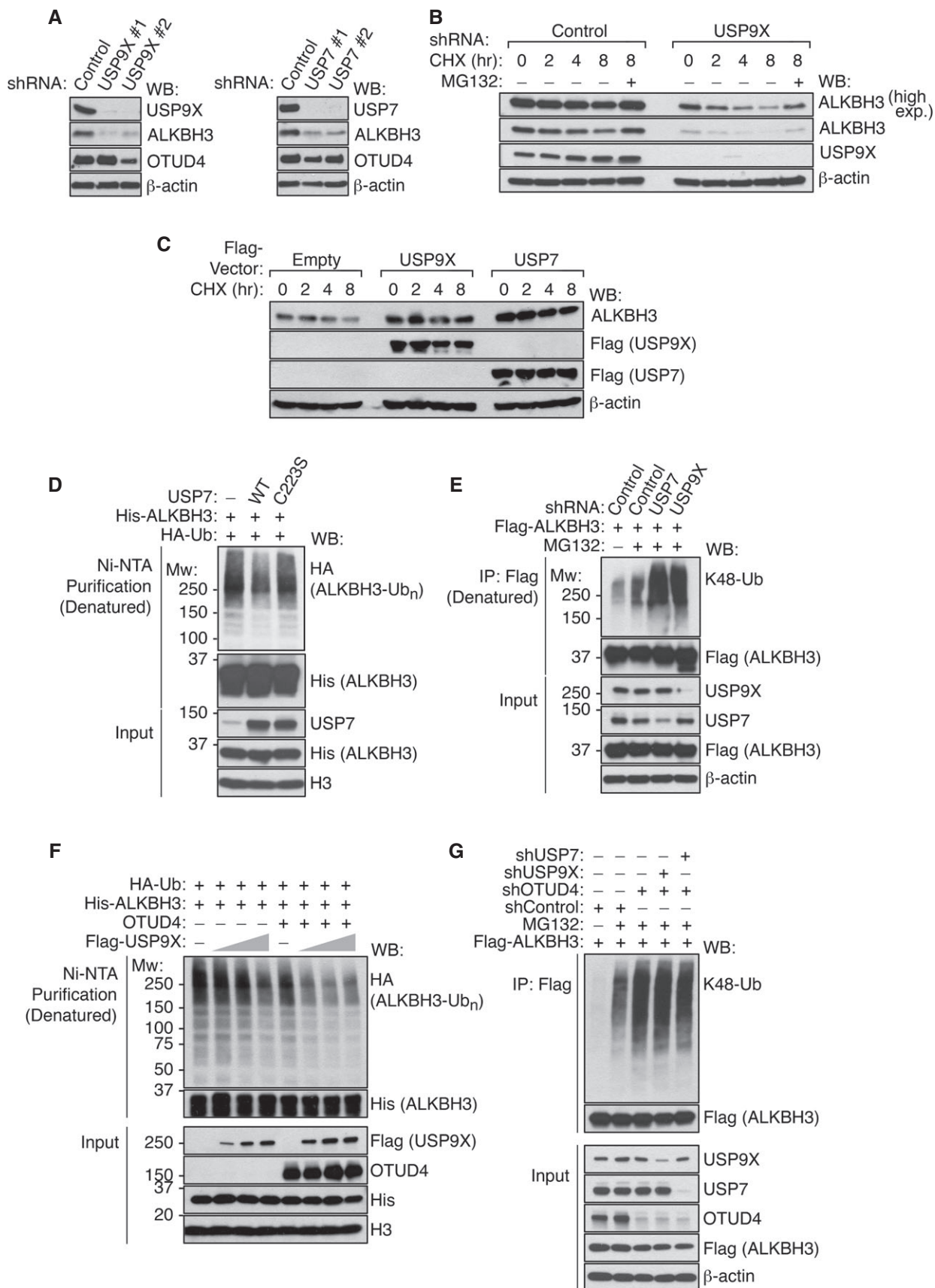
Figure 6.

Figure 6.   USP7 and USP9X promote ALKBH3 deubiquitination and stabilization.

A   PC-3 cells were transduced with lentivirus expressing control or two distinct shRNAs targeting USP9X (left) or USP7 (right), selected, and analyzed by Western blot using the indicated antibodies.

B   PC-3 cells transduced with control or USP9X-specific lentiviral shRNAs were treated with cycloheximide (120 μg/ml) and MG132 (30 μM) as shown. Whole-cell extracts were collected at the indicated times and analyzed by Western blot. A higher exposure of the ALKBH3 Western blot is shown and is indicated as "high exp".

C   293T cells transfected with Flag vector, Flag-USP9X, or Flag-USP7 were treated with cycloheximide (120 μg/ml). Whole-cell extracts were collected and analyzed by Western blot as in (B).

D   293T cells expressing the indicated vectors were transfected with wild-type or catalytically inactive USP7 and treated with MG132 (30 μM). After Ni-NTA purification, the bound material was analyzed by Western blot as shown.

E   293T cells expressing Flag-ALKBH3 were transduced with control, USP7, or USP9X shRNAs and treated with MG132 (30 μM) as indicated. After SDS denaturation and Flag immunoprecipitation, the bound material was analyzed by Western blot as shown.

F   293T cells expressing an shRNA against OTUD4 were reconstituted with vector or shRNA-resistant OTUD4 as well as the other indicated vectors. After treatment with MG132 (30 μM) and Ni-NTA purification, the bound material was analyzed by Western blot as shown.

G   293T cells expressing Flag-ALKBH3 were transduced with the indicated combination of shRNAs and treated with MG132 (30 μM) as shown. The samples were Flag-immunoprecipitated after SDS denaturation and analyzed as in (E).

did not affect the localization of either ALKBH3 or ALKBH2, with or without MMS treatment (Fig 7E and F; Supplementary Fig S6B). We conclude from these data that OTUD4 promotes alkylation damage resistance likely by affecting the levels of these demethylases, but without affecting their subcellular localization.

We sought additional evidence that OTUD4 promotes alkylation damage resistance through the stabilization of mammalian AlkB homologues. Previously, we had demonstrated that PC-3 cells do not depend on ALKBH2 for resistance to MMS, but instead rely primarily on ALKBH3 for this function (Dango *et al*, 2011). Therefore, if ALKBH3 is expressed sufficiently in these cells, MMS resistance should be normalized, even in the absence of OTUD4. We created an ALKBH3 overexpressing PC-3 cell line that expresses a greater amount of exogenous ALKBH3 even upon OTUD4 knockdown (Supplementary Fig S6C). As expected, knockdown of OTUD4 without ALKBH3 overexpression increased the sensitivity of these cells to MMS (Fig 7G). However, with OTUD4 knockdown and ALKBH3 overexpression, the cells recovered their alkylation damage resistance significantly, to levels near that of control knockdown (Fig 7G). These data strongly suggest that OTUD4 promotes alkylation damage resistance through regulation of ALKBH3 in PC-3 cells. We then tested whether the catalytic activity of OTUD4 was critical for MMS sensitivity. Consistent with the rescue of ALKBH3 stability, both the wild-type and the C45A mutant forms of OTUD4 rescued MMS sensitivity in PC-3 cells (Fig 7H). Finally, since USP9X and USP7 also contribute to ALKBH3 stability, we tested whether knockdown of these proteins caused MMS sensitivity. Loss of either

of these DUBs also increased MMS sensitivity, although we consistently observed a greater degree of MMS sensitivity with loss of USP9X compared to USP7 (Fig 7I and J). This is consistent with a more prominent destabilization of ALKBH3 with USP9X knockdown compared to USP7 knockdown (see Fig 6). Culturing PC-3 cells with the USP9X inhibitor WP1130 destabilized ALKBH3 and caused a moderate but significant increase in MMS sensitivity (Fig 7K and L). Global levels of genomic 3-methylcytosine, a major substrate for ALKBH3, were significantly increased in PC-3 cells upon knockdown of OTUD4 or USP9X (Fig 7M), further underscoring the role of these deubiquitinases in the regulation of ALKBH3. We also purified Flag-ALKBH3 from 293T cells where each of these three DUBs were knocked down, and determined demethylase activity *in vitro* (Supplementary Fig S6D). However, there was no intrinsic difference in the demethylation activity of ALKBH3, suggesting that the increase in MMS sensitivity is due primarily to ALKBH3 loss and not a change in the repair activity of ALKBH3 itself. In all, our data demonstrate a novel molecular mechanism by which different DUBs cooperate to promote alkylation damage resistance.

Discussion

Here, we demonstrate that OTUD4 may serve as a master regulator of alkylation damage resistance through stabilization of the human AlkB homologues. A number of distinct lines of evidence support this role for OTUD4. First, OTUD4 interacts specifically with

Figure 7.   Loss of OTUD4, USP9X, or USP7 causes alkylation damage sensitivity.

A, B   PC-3 cells were transduced with the indicated shRNAs. After selection, growth was determined by MTS assay.

C, D   PC-3 (C) or H23 (D) cells were transduced with the indicated shRNAs, selected, and exposed to the indicated concentration of MMS for 1 h. Survival was assessed 48 h later by MTS assay.

E, F   PC-3 cells stably expressing Flag-HA-ALKBH3 (E) or Flag-HA-ALKBH2 (F) were transduced with control or OTUD4-specific shRNA vectors. After selection, immunofluorescence was performed using an anti-HA antibody, with Hoechst as nuclear counter stain. Scale bar, 10 μm.

G   The four PC-3 cell lines expressing the indicated combination of vector and shRNA were treated with 1 mM MMS for 1 h and assessed for survival by MTS assay. Growth was normalized to cells expressing empty vector with control shRNA.

H   PC-3 cells were transduced as shown. After selection, cells were exposed to 1 or 2 mM MMS for 1 h and assessed for survival as in (G).

I, J   PC-3 cells expressing shRNAs against USP9X (I) or USP7 (J) were treated with 2 mM MMS for 1 h and assessed for survival as in (G).

K   PC-3 cells were treated with DMSO or the USP9X inhibitor WP1130 (1 μM) for 18 h, followed by treatment with cycloheximide (120 μg/ml) as shown. Whole-cell extracts were collected at the indicated times and analyzed by Western blot.

L   PC-3 cells incubated with DMSO or WP1130 (1.5 μM) were treated with 1 mM MMS for 1 h, and survival was assessed 48 h later by MTS assay.

M   Genomic DNA was purified from PC-3 cells expressing the indicated shRNAs. Equal amounts of total genomic DNA from each cell line (600, 300, or 100 ng) were dot-blotted and probed with an anti-3-methylcytosine antibody.

N   Proposed model for OTUD4 function in mediating ALKBH3 deubiquitination.

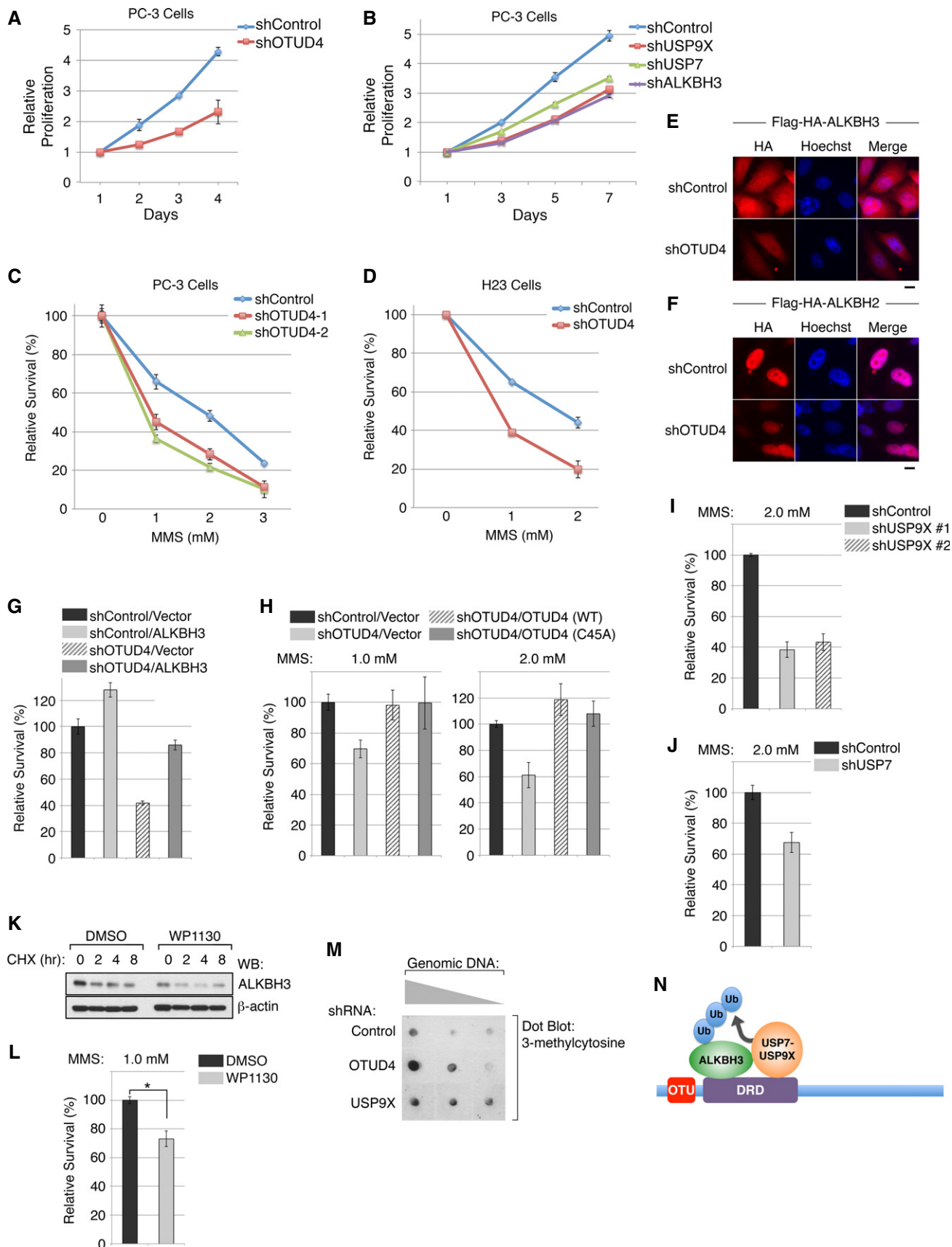Data information: Error bars indicate ± SD of triplicate experiments. *P < 0.05.

Figure 7.

ALKBH2 and ALKBH3 and encodes a K48-specific DUB (Fig 1). Consistently, ALKBH3 is subjected to K48-linked ubiquitination and proteasomal degradation (Fig 2A–D). OTUD4 antagonizes ALKBH3 ubiquitination and stabilizes both ALKBH2 and ALKBH3 in vivo (Fig 2E–H). ALKBH3 protein levels do not correlate well with ALKBH3 mRNA levels in various tumor cell lines but do correlate with OTUD4 levels (Supplementary Fig S2). Finally, overexpression of ALKBH3 in PC-3 cells, which depend primarily on ALKBH3 instead of ALKBH2 for alkylation damage resistance, is sufficient to rescue alkylation damage sensitivity upon loss of OTUD4 (Fig 7G).

What is most striking is that we find OTUD4 catalytic activity to be apparently dispensable for its stabilization function and alkylation damage resistance (Figs 3 and 7H). To our knowledge, virtually all other characterized OTU family DUBs, including OTUB2, OTUD5, OTUD7B/Cezanne, and OTULIN, regulate their targets through their catalytic activities (Hu et al, 2013; Keusekotten et al, 2013; Luo et al, 2013; Kato et al, 2014). One notable exception is OTUB1, which can regulate its targets by binding E2 ubiquitin ligases, directly inhibiting these enzymes (Nakada et al, 2010; Herhaus et al, 2013). Our studies support the notion that OTUD4 functions in a manner distinct from OTUB1 by forming a complex with additional DUB activities, which were revealed to be USP7 and USP9X by proteomic analysis of the OTUD4 complex (Fig 4). We propose that while ALKBH3 can associate (albeit weakly) with these additional deubiquitinases, the primary role of OTUD4 in the regulation of ALKBH3 appears to be promoting its association with USP7 and USP9X (Fig 7N). Consistent with a role in promoting OTUD4 function, knockdown of either USP7 or USP9X caused destabilization of ALKBH3 and alkylation damage sensitivity (Figs 6 and 7). Both of these DUBs have been previously shown to play a role in regulating other substrates important in various aspects of the response to DNA damage, including regulation of p53 and the stabilization of the nucleotide excision repair factor UVSSA for USP7, and the Bcl2-like anti-apoptotic factor MCL1 for USP9X (Lee & Gu, 2010; Schwickart et al, 2010; Schwertman et al, 2012; Zhang et al, 2012). In the case of ALKBH3, it appears that loss of any one of these three DUBs results in its destabilization. This may be due to the distinct subcellular localization pattern for USP7 and USP9X, which are predominantly nuclear and cytoplasmic, respectively (Nathan et al, 2008). This is consistent with our proteomics data, which demonstrated peptides corresponding to USP7 only in the nuclear OTUD4 complex. Thus, ALKBH3, which is present in both cellular compartments, may be stabilized in the cytosol primarily by OTUD4-USP9X, while OTUD4-USP7 would take on this role in the nucleus. However, we believe that OTUD4-USP9X is the predominant complex that deubiquitinates ALKBH3, since OTUD4 is primarily cytoplasmic, and USP9X plays a greater role in ALKBH3 function than USP7 (Figs 6 and 7). Interestingly, another group previously demonstrated that USP7 and USP9X can stabilize the same target molecule, MARCH7 (Nathan et al, 2008). It is intriguing to consider whether OTUD4 may also serve to promote the association of MARCH7 with USP7 and USP9X or whether OTUD4 may function in this noncanonical manner with other targets.

What could be the function of the OTUD4 catalytic activity? It remains to be determined whether such a mechanism could be applied more generally to other OTUD4 substrates. As mentioned earlier, the noncanonical mechanism of E2 inhibition by OTUB1 is important for certain targets (Nakada et al, 2010; Herhaus et al,

2013), while its DUB activity is important for others (Goncharov et al, 2013). We hypothesize that the same may be true for OTUD4, such that for certain substrates, its own catalytic activity may be critical for deubiquitination, while it is dispensable for ALKBH3. While additional substrates for OTUD4 have yet to be identified, a recent whole-exome sequencing study demonstrated that OTUD4 is mutated in an inherited disorder that causes ataxia, dementia, and hypogonadotropism (Margolin et al, 2013). OTUD4 knockdown experiments in zebrafish recapitulated many of these phenotypes, which were rescued by the expression of human OTUD4. However, we should note that the human OTUD4 clone that was used for these studies was missing the first 65 amino acids of OTUD4 (Margolin et al, 2013), which would encode a protein that lacks its own DUB activity. Our studies suggest that this OTUD4-NΔ65 rescues ALKBH3 stability (Fig 3B), consistent with the notion that its scaffolding function is maintained. Taken together, these results suggest that many of the physiological functions of OTUD4 may be independent of its own catalytic activity.

This novel mechanism of cooperativity between different DUBs has a number of important consequences, not only for DUB biology but also for targeting DUBs for therapeutic intervention. One function may be to expand the substrate repertoire of the associated DUBs. This is consistent with the ability of OTUD4 to promote the association of ALKBH3 with USP7 and USP9X (Figs 4 and 5). In fact, a large proteomic study that systematically identified DUB interacting partners suggested that DUBs in the OTU family may interact with DUBs of different classes, suggesting that the noncanonical mechanism of DUB function revealed here may be more generalizable (Sowa et al, 2009). However, it has yet to be determined whether these DUB–DUB interactions are direct or whether they are mediated by binding a common interacting partner, such as polyubiquitin chains. The OTU domain-containing protein ALG13 contains a domain with homology to the OTUD4 DRD, and it is possible that ALG13 may also act as a DUB scaffolding protein (Supplementary Fig S4E). A DUB cooperativity model may also explain why certain proteins have been shown to be substrates for multiple DUBs. It is intriguing to consider whether some of the numerous DUBs that have been shown to act on p53 (Brooks & Gu, 2011) may actually function within a DUB complex. Functional overlap between DUBs within a complex also implies that small molecule inhibition of one specific DUB may have consequences for other substrates interacting with the complex. Indeed, we demonstrate that use of the USP9X inhibitor WP1130 decreases ALKBH3 stability and increases alkylation damage sensitivity in prostate cancer cells (Fig 7K and L). Thus, it may be possible to modulate alkylation repair sensitivity in tumors by the use of these inhibitors. This could have important implications for tumor therapy, as alkylating agents are one of the most commonly used chemotherapeutic drugs used for cancer and resistance to such agents is common (Fu et al, 2012).

Materials and Methods

Plasmids

Human ALKBH2 and ALKBH3 cDNAs were isolated as previously described (Dango et al, 2011) and subcloned into pHAGE-CMV-Flag,

pHAGE-CMV-3xHA, or pMSCV-Flag-HA by gateway recombination (Sowa *et al*, 2009). An OTUD4 cDNA that contained codons 66–1114 was obtained from Open Biosystems (MGC clone 126483). The remaining N-terminal codons were inserted using long oligonucleotides and confirmed by sequencing. For recombinant protein expression, cDNAs were cloned into pGEX-4T1, pMAL-C5e, or pET28a-Flag. OTUD4 mutants were produced by PCR-mediated mutagenesis. For the shRNA-resistant OTUD4 clone, seven silent mutations were introduced in the ORF region corresponding to the sequence targeted by shOTUD4-1. The pFlag-OTUB1 and OTUB2 vectors were kind gifts of Deborah Lenschow (Washington University in St. Louis); the USP7 cDNA and the USP9X-Flag vector were kind gifts of Anindya Dutta (University of Virginia) and Vishva Dixit (Genentech), respectively. The pcDNA-HA-Ub vector was a kind gift Yang Shi (Harvard Medical School).

Purification of ALKBH3 and OTUD4 protein complexes and MS/MS identification

The tandem affinity purification of ALKBH3 was been previously described (Dango *et al*, 2011). Briefly, Flag-HA-ALKBH3 was stably expressed after transduction of pMSCV-Flag-HA-ALKBH3 retrovirus into PC-3 cells. Nuclear extract was prepared from the stable cell line, and the ALKBH3 complex was purified using anti-Flag (M2) resin (Sigma), followed by purification using anti-HA (F-7) resin (Santa Cruz) in TAP buffer (50 mM Tris–HCl pH 7.9, 100 mM KCl, 5 mM MgCl$_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT, and protease inhibitors). The OTUD4 complexes were similarly purified, except that both nuclear and cytosolic extracts were used separately for the purification, and a single anti-Flag purification was performed. After peptide elution, the complexes were TCA-precipitated and associated proteins were identified by LC-MS/MS at the Taplin Mass Spectrometry Facility (Harvard Medical School) using an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (ThermoFisher) and Sequest software (Eng *et al*, 1994). The details of the MS analysis are described in the Supplementary Materials and Methods.

Immunoprecipitation and Western blotting

Immunoprecipitation was performed as previously described (Zhao *et al*, 2013) with minor modifications. Briefly, 293T cells were transfected with plasmids as indicated using TransIT-293 (Mirus Bio) for 2–3 days. Cells were harvested, resuspended in lysis buffer (50 mM Tris–HCl pH 7.9, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM DTT, and protease inhibitors), cleared by centrifugation, and incubated at 4°C with M2-agarose beads (Sigma) or HA-agarose beads (Santa Cruz). The beads were then washed in the same buffer five times, and bound material was eluted using Laemmli buffer and analyzed by Western blotting. Endogenous immunoprecipitation was performed from PC-3 cell extracts as previously described (Dango *et al*, 2011) with minor modifications. Cells were harvested as above, resuspended in lysis buffer, and incubated overnight at 4°C with 3 μg of antibody and 2.5 mg of BSA. The extract was then incubated with PrA-Sepharose for 1 h at 4°C, centrifuged, and washed extensively in lysis buffer. For immunoprecipitation after denaturation, the extracts were resuspended in TBS with 1% SDS, boiled, and then diluted with lysis buffer to 0.1% SDS before immunoprecipitation. Bound material was eluted using Laemmli sample

buffer and analyzed by Western blotting. All Western blotting was carried out using PVDF membrane and the indicated antibodies. Protein bands were visualized by enhanced chemiluminescence (Bio-Rad).

Protein purification

GST-, 6X-His-, and MBP-tagged recombinant proteins were purified from *E. coli* using glutathione-Sepharose, Ni-NTA agarose, or amylose resin as described (Dango *et al*, 2011). The 6x-His-OTUD4-Flag protein was purified on Ni-NTA agarose, dialyzed in TAP buffer, followed by purification using anti-Flag (M2) agarose, and eluted with Flag peptide (Sigma). For purification of OTUD4 from 293T cells, Flag-OTUD4 was expressed by transfection or by stable lentiviral expression. Cells were harvested in 1× PBS, resuspended in lysis buffer, and incubated with anti-Flag (M2) agarose after by centrifugation. The bound material was eluted using Flag peptide. Flag-tagged USP9X was produced using the pRK-USP9X-Flag vector and the SP6 transcription/translation kit (Pierce) according to the manufacturer's instructions.

In vitro deubiquitinase assays

All ubiquitin substrates, including poly-SUMO, and His-SENP2 were from Boston Biochem. The USP9X inhibitor (WP1130) and USP7 inhibitor (P22077) were from Calbiochem. Deubiquitination (DUB) assays were performed as described (Licchesi *et al*, 2012) with minor modifications. Briefly, all DUB assays were performed in TAP buffer at 37°C containing the indicated amount of enzyme and substrate in a volume of 25 μl. The reactions were typically incubated for 3 h, unless indicated otherwise. Reactions were stopped by the addition of Laemmli buffer and analyzed by Western blot.

MMS cytotoxicity assay, immunofluorescent microscopy, and antibodies

MMS cytotoxicity and immunofluorescent microscopy were performed as described in the Supplementary Materials and Methods. All antibodies used in this study are shown in Supplementary Table S3.

Author contributions

YZ, MCM, JMS, JRB, SD, and NM designed and carried out experiments. NM supervised the project and wrote the manuscript with input from YZ.

Conflict of interest

The authors declare that they have no conflict of interest.

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