Phosphorylation of USP15 and USP4 Regulates Localization and Spliceosomal Deubiquitination

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Abstract

Deubiquitinating enzymes have key roles in diverse cellular processes whose enzymatic activities are regulated by different mechanisms including post-translational modification. Here, we show that USP15 is phosphorylated, and its localization and activity are dependent on the phosphorylation status. Nuclear-cytoplasmic fractionation and mass spectrometric analysis revealed that Thr149 and Thr219 of human USP15, which is conserved among different species, are phosphorylated in the cytoplasm. The phosphorylation status of USP15 at these two positions alters the interaction with its partner protein SART3, consequently leading to its nuclear localization and deubiquitinating activity toward the substrate PRP31. Treatment of cells with purvalanol A, a cyclin-dependent kinase inhibitor, results in nuclear translocation of USP15. USP4, another deubiquitinating enzyme with a high sequence homology and domain structure as USP15, also showed purvalanol A-dependent changes in activity and localization. Collectively, our data suggest that modifications of USP15 and USP4 by phosphorylation are important for the regulation of their localization required for cellular function in the spliceosome.

Introduction

Human ubiquitin-specific protease 15 (USP15) mRNAs are ubiquitously found in various tissues and organs, whereas the protein levels are most abundantly found in the testes, thyroid gland, adrenal gland, and pancreas [1]. It is localized in both the cytoplasm and nucleus, and diverse biological functions have been reported depending on its cellular localization. In the cytoplasm, USP15 is involved in a number of cellular pathways such as neuro-inflammation [2], T-cell activation [3], Nrf2–Keap1 pathway [4], and bone morphogenetic protein signaling [5]. USP15 is also identified as a crucial regulator of the COP9 signalosome [6] and TGF-β activity regulating TGF-β-dependent oncogenesis in glioblastoma [7] and counteracts R-SMAD mono-ubiquitination, which regulates promoter recognition of SMAD complexes [8]. In the nucleus, USP15 regulates spliceosome dynamics and function in chromatin by deubiquitinating PRP31 and H2B, respectively [9,10].

In earlier studies, we have shown that USP15 binding to squamous cell carcinoma antigen recognized by T cells 3 (SART3) has an important role in the translocation of USP15 from the cytoplasm to the nucleus and serves as a targeting factor for co-localization of USP15 with its substrate PRP31 to regulate the spliceosome dynamics [10]. SART3 also has the same role for USP4, another deubiquitinating enzyme (DUB) with high sequence homology and domain structure as USP15, in translocation into the nucleus and to its substrate PRP3 in the spliceosome [11]. We and others have

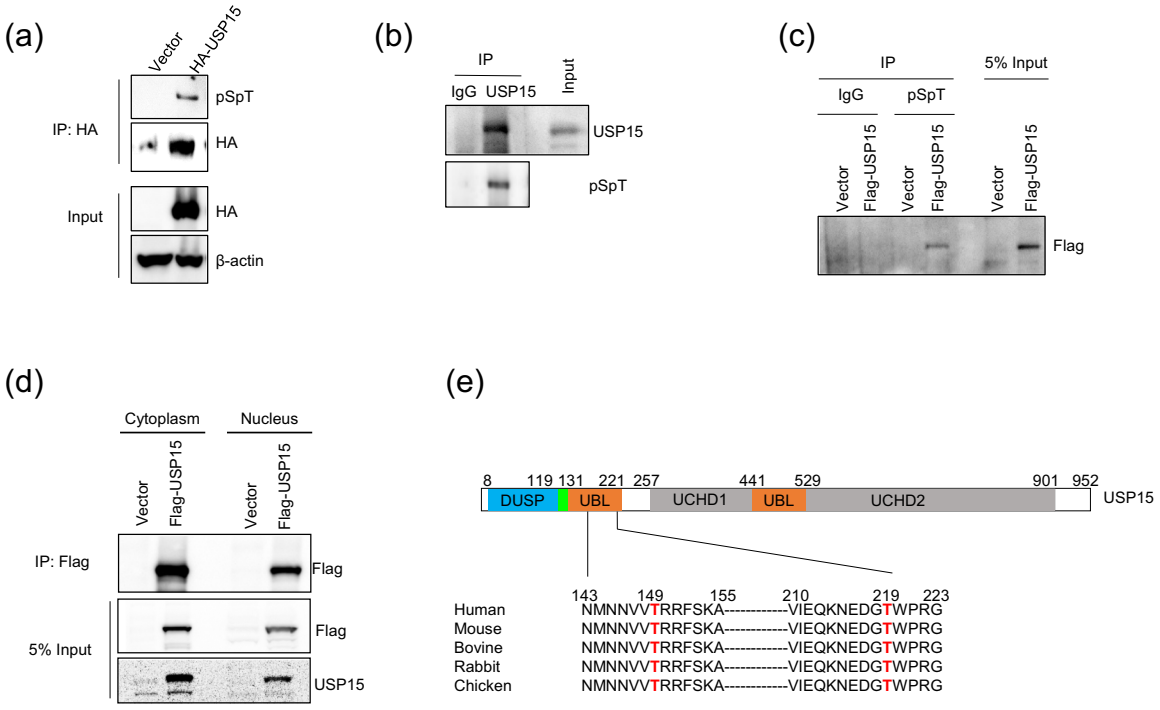


Fig. 1. USP15 is phosphorylated in cells. (a) HA-USP15-transfected HeLa cell lysates were purified using anti-HA-agarose bead, the beads were washed, bound proteins were eluted with 2× SDS sample buffer, and co-precipitated proteins were detected by Western blot with anti-pSer/Thr antibody. (b) Lysates of HeLa cells were immunoprecipitated with either control IgG or anti-USP15 antibodies, and co-precipitated phospho-USP15 proteins were detected by the anti-pSer/Thr antibody. To confirm the accurate size of phosphorylated USP15, the input lysate was loaded in the same gel and the membrane was aligned with the IP membrane by merging the molecular weight marker after blotting with USP15 antibody. (c) Control pCS2 or Flag-USP15-transfected HeLa cells were immunoprecipitated with either IgG or anti-pSer/Thr antibody. Co-precipitated phospho-USP15 proteins were detected by anti-Flag antibody. (d) Flag-USP15-transfected HeLa cells were centrifuged at 7000 rpm for 5 min and washed by suspending the pellet in 1× DPBS, and the nuclear-cytoplasmic fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Extracted fractions were immunoprecipitated with anti-Flag agarose beads, and elutes were separated by SDS-PAGE and detected by Western blot. (e) Schematic overview showing that USP15^{Thr149} and USP15^{Thr219} phosphorylation sites are conserved among different species during evolution.

provided the structural basis for the interaction between SART3 and the two DUBs; that is, the linker between the DUSP and UBL domains of USP4 (and USP15) binds to the half-a-tetratricopeptide repeat domain of SART3 [12,13].

We further demonstrated that the nuclear localization sequence (NLS) of SART3 binds importin-α in a bipartite manner and is essential for the spliceosomal activities of the two DUBs [12]. However, it is not yet clear when and how the nuclear translocation of USP15 and USP4 by SART3 is regulated.

SART3 interacts directly with the spliceosomal components PRP31 and PRP3, substrates of USP15 and USP4, respectively, guiding the rearrangement of the splicing complex resulting in an active spliceosome. We noted that the loss of USP15 impairs mitotic progression by interfering with mRNA splicing, for example, splicing of α-tubulin and Bub1 [10]**.** This indicates that the function and localization of USP15 may be regulated further by the modification or interaction with partner proteins in the cell cycle because reversible phosphorylation by several cyclin-dependent kinases (CDKs) and protein phosphatase are the most important post-translational modification during cell cycle progression. In addition, USP4 was reported to undergo AKT-dependent phosphorylation in controlling TGF-β signaling [14]. Therefore, it is quite possible that the phosphorylation status of USP15 could be an important regulator of its function and localization.

In this study, we analyzed the differential phosphorylation status of USP15 in the cell. We found that Thr149 and Thr219 of USP15 in the N-terminal UBL domain are differentially phosphorylated in the cytoplasm and nucleus and examined the effect of

phosphorylation at Thr149 and Thr219 of USP15 on its interaction, localization, and substrate targeting in spliceosomal dynamics. This study provides an understanding of how the nuclear translocation of USP15 and USP4 is regulated by posttranslational modifications.

Results

Identification of phosphorylation sites on USP15

To investigate the phosphorylation-dependent regulation of USP15, we first examined whether USP15 was phosphorylated in cells. Ectopically overexpressed or endogenous USP15 was immunoprecipitated and immunoblotted with the phosphoserine/threonine (pSpT) antibody. As shown in Fig. 1a and b, both overexpressed and endogenous USP15 were well precipitated and detected by phospho-specific antibodies. In addition, Flag-USP15 was co-precipitated efficiently by pS/pT antibody affinity purification (Fig. 1c), which further confirms the phosphorylation of USP15 in cells. To further assess the consequence of USP15 phosphorylation in cells, we attempted to identify the specific phosphorylation sites based on its localization. For this, we performed nuclear and cytoplasmic fractionation following overexpression of USP15 to find its differential phosphorylation sites in HeLa cells (Fig. 1d). The cytoplasmic and nuclear fractions were immunoprecipitated, and eluted fractions were detected by Coomassie staining or silver staining (Supplementary Fig. S1). Several silver staining gels were collected for the use of a similar amount of coomassie staining gel in mass spectrometry analysis. The phosphorylation sites from isolated USP15 bands were identified by mass spectrometry after in-gel digestion, and the identified phosphopeptides are summarized in Table 1. Among the 14 phosphopeptides identified, only 3 were present in both the cytoplasmic and nuclear fractions, while the rest were found either only in the cytoplasmic fraction (nine) or nuclear fraction (two). After examining these phosphosites, we were particularly interested in T149 and T219 because these two sites are highly conserved among various species, and they are located at the UBL domain (Fig. 1e). UBL domain is reported to be important for the regulation of conformational change and catalytic activity of DUBs [15], and UBL domain of USP15 is involved in the interaction with the partner protein SART3 for nuclear translocation [12].

Phosphorylation at USP15^{Thr149} and USP15^{Thr219} regulates its localization

To find the phosphorylation effect of these two sites of USP15 on its localization, we constructed phosphomutants and phosphomimetics of USP15^{T149} and USP15^{T219} by replacing threonine with alanine and aspartate, respectively. The alanine mutations should abolish phosphorylation of the targeted residues, while the corresponding aspartate mutants could act as phosphomimetics that substitute for the desired phosphorylation. As shown in Fig. 2a, loss of phosphorylation in the phosphomutants, USP15^{T149A} and USP15^{T219A}, redirected USP15 into the nucleus comparatively more than the wild type (WT). In contrast, overexpression of the corresponding phosphomimetics, USP15^{T149D} and USP15^{T219D}, mostly localized in the cytoplasm (Fig. 2a, b and Supplementary Fig. S2a). These results suggest that USP15 phosphorylation at T149 and T219 is important in determining its localization. Next, we investigated whether the USP15 phosphorylation mediated changes in localization are dependent on SART3 by co-expressing SART3 with USP15. The co-expression of USP15 with SART3 led to the nuclear translocation of USP15, which is consistent with previous results [10]. However,

Table 1. Mass spectrometry analysis of phosphorylated peptides of USP15 in cytoplasmic and nuclear fractions[a]

Phosphopeptide		Phosphorylation site
Cytoplasm	Nucleus	
AEGGAADLD**T**QR	AEGGAADLD**T**QR	11
KGDTW**Y**LVD**S**R	KGDTW**Y**LVD**S**R	31, 35
	LVSWY**T**LMEGQEPIARK	105
LCENGNMNNVV**T**RR		149
NEDG**T**WPR		219
	YQEELNFDNPLGMRGEIAK	293
MDPLTKPMQ**Y**KVVVPK		463
IGNILDLC**T**ALSALSGIPADK		478
VVVPKIGNILDLCTAL**S**AL**S**GIPADK		481, 484
TED**T**EHVIIPVCLR		670
RLF**T**FQFNNLGNTDIN**Y**IK		684, 697

[a] The corresponding phosphorylation sites are indicated in bold.

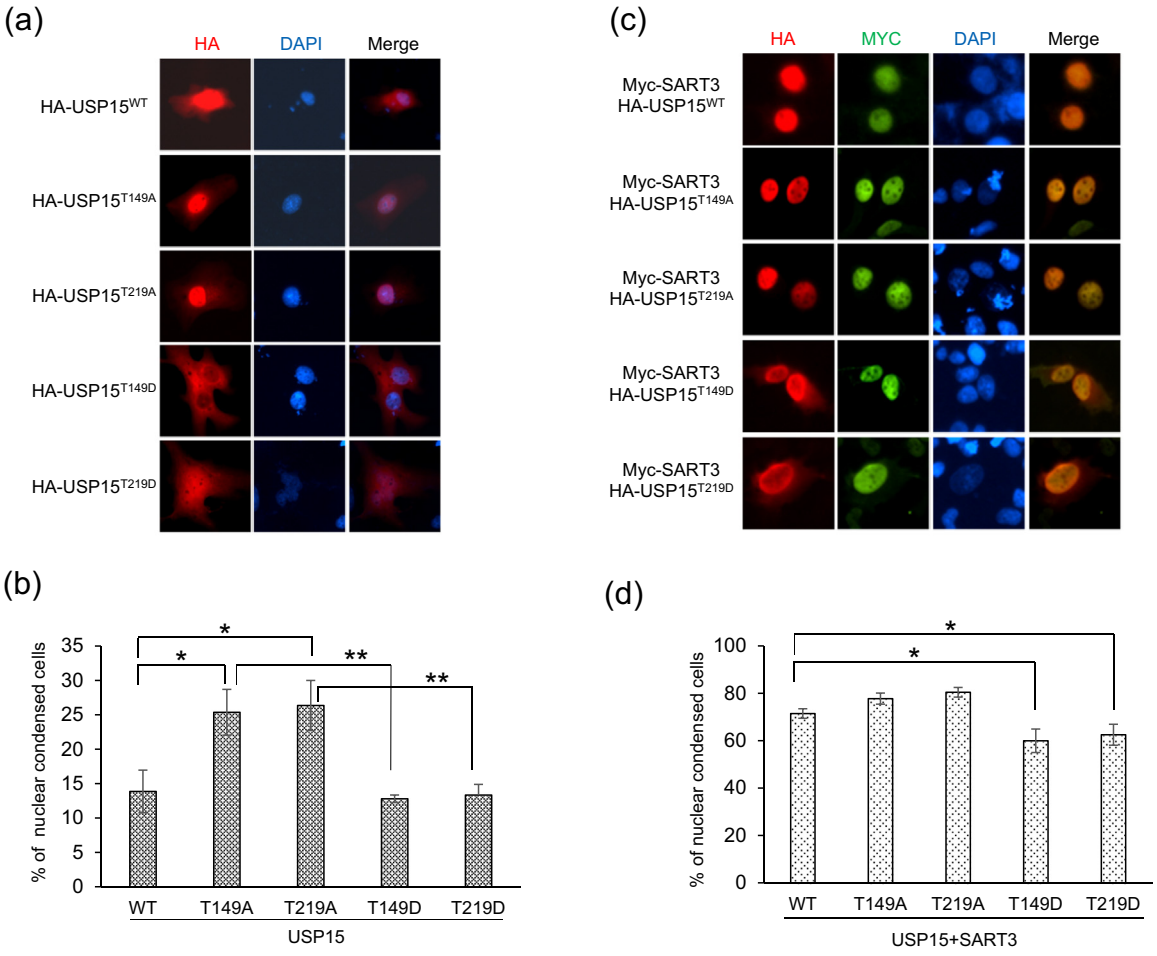
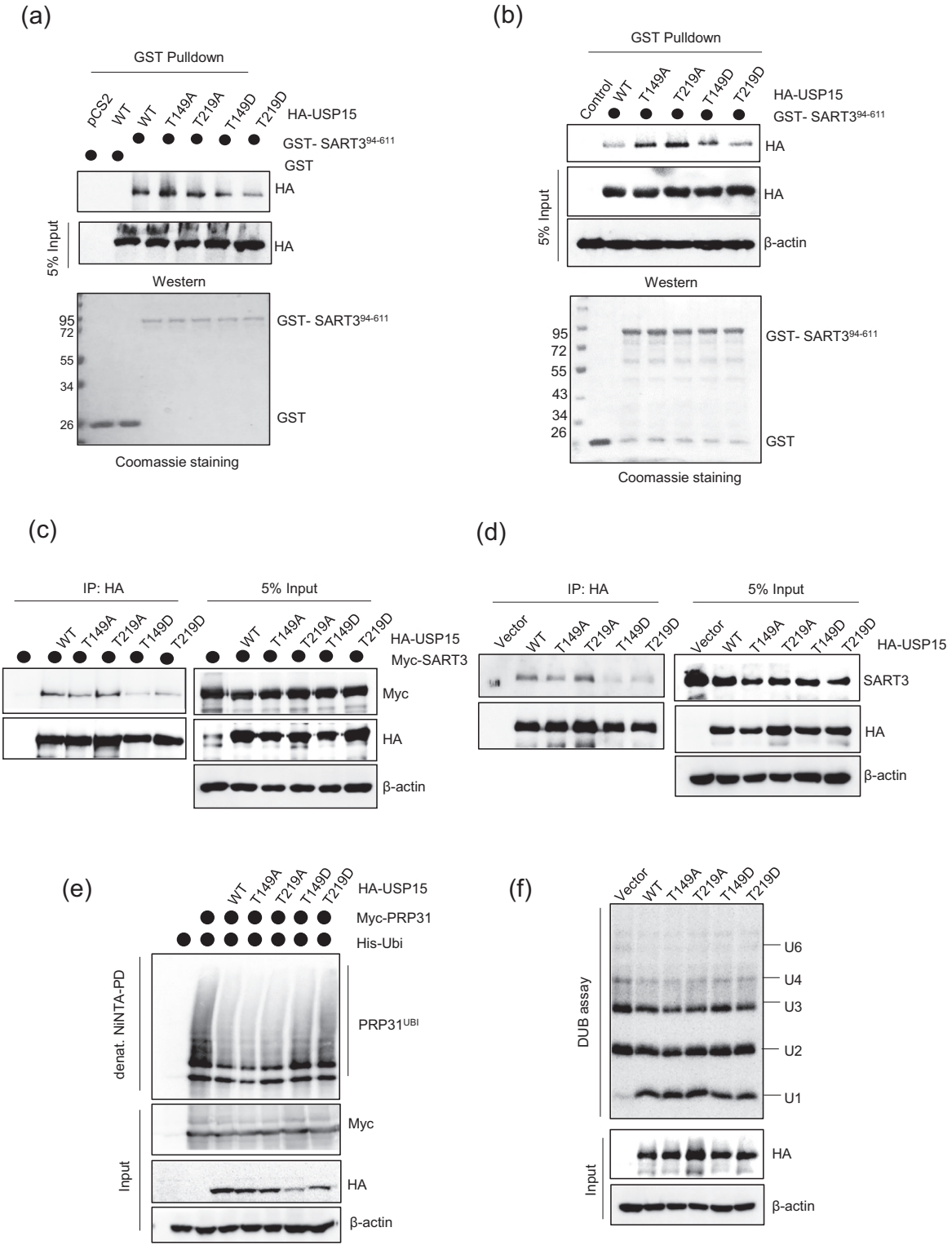


Fig. 2. Phosphorylation at USP15^{Thr149} and USP15^{Thr219} affects USP15 localization. (a) HA-USP15 WT, phosphomutants, or phosphomimetics transfected HeLa cells were immunostained with anti-HA as primary and anti-mouse Alexa546 (red) as secondary antibody, while the nucleus was stained with DAPI. The intracellular localization of USP15 (red) was analyzed by fluorescence microscopy. (b) Localization of USP15 WT and phosphomutants in panel A was determined in at least 100 cells, and data from three independent experiments are shown in the graph (*$P < 0.05$ and **$P < 0.01$, two-tailed Student's *t*-test). (c) Myc-SART3 was co-transfected with HA-USP15 WT, phosphomutants, or phosphomimetics, and intracellular localization of SART3 (green) and USP15 (red) was examined by fluorescence microscopy followed by immunostaining. The nucleus was stained with DAPI. (d) Localization of USP15 in panel C was determined in at least 100 cells. Data from three independent experiments are shown in the graph (*$P < 0.05$, two-tailed Student's *t*-test).

Fig. 3. Phosphorylation of USP15 ^{Thr149} and USP15^{Thr219} impede the interaction and activity. (a) Control GST or GST-SART3$^{94–611}$ was immobilized on glutathione beads and incubated with *in vitro* transcribed and translated HA-USP15 WT, phosphomutants, or phosphomimetics. Bound proteins were separated in SDS-PAGE and detected by Western blotting and Coomassie staining. (b) Transfected HeLa cell lysates were incubated with control GST or GST-SART3$^{94–611}$, and immunoprecipitated proteins were separated in SDS-PAGE and detected by Western blotting or Coomassie staining. (c) HA-USP15 WT, phosphomutants, or phosphomimetics were co-expressed with Myc-SART3 in HeLa cells, purified on HA-agarose beads and co-precipitated SART3 was immunoblotted with anti-Myc antibody. (d) IP and immunoblot analysis of endogenous SART3 on overexpression of HA-USP15 WT, phosphomutants, or phosphomimetics in HeLa cells. (e) His-ubiquitin was co-expressed with Myc-PRP31 as well as USP15 WT, phosphomutants, or phosphomimetics; covalently modified proteins were purified on Ni-NTA-agarose under denaturing conditions; and ubiquitinated PRP31 was detected by the anti-Myc antibody. (f) HA-USP15 WT, phosphomutants, or phosphomimetics overexpressed HeLa cells were purified on anti-HA-agarose beads and incubated with DUB buffer containing K63-linked ubiquitin chains ($UB_{2–7}$, Boston Biochem) at 30 °C for 90 min. The reactions were stopped by boiling at 95 °C for 5 min in 2× SDS sample buffer and detected by anti-ubiquitin antibody after SDS-PAGE.

when SART3 was overexpressed with the phosphomutants, most of USP15 was condensed in the nucleus as expected. On the other hand, the cells overexpressing phosphomimetics, still showed cytoplasmic dispersion despite the co-expression with SART3 (Fig. 2c and d, Supplementary Fig. S2b). It is possible that there may be other factors, but these results suggest that



phosphorylation regulates SART3-mediated nuclear translocation of USP15.

Phosphorylation of USP15 alters the interaction with SART3 and deubiquitination of PRP31

We thought that the above phosphorylation-dependent changes of USP15 localization might be due to alterations in its interaction with SART3. To test this assumption, a pull-down assay was performed using GST-SART3$^{94–611}$ with *in vitro* transcribed and translated USP15 (Fig. 3a) or HA-tagged USP15 overexpressed cell lysates (Fig. 3b). As seen in Fig. 3a and b, the interaction between SART3 and USP15 phosphomimetics decreased with respect to the corresponding phosphomutants. To further confirm this in the cells, HA-tagged USP15 WT, phosphomutants, or phosphomimetics were overexpressed in HeLa cells and immunoprecipitated with anti HA agarose beads. In each case, the phosphomimetics showed a decreased interaction with the over-expressed or endogenous SART3 compared to the corresponding phosphomutants (Fig. 3c and d). These results indicate that the phosphorylation of USP15 regulates its interaction with SART3, which induces its accumulation in the nucleus. To examine whether a synergistic effect of the two phosphorylations exists in modulating the USP15 and SART3 interaction, both the Thr149 and Thr219 phosphorylation sites of USP15 were mutated. The double mutant, USP15$^{T149A/T219A}$, did not show any additional or synergistic effect on its interaction with SART3 compared to the single mutants (Supplementary Fig. S3), indicating that either each phosphorylation site was enough to modulate the interaction with SART3, or the effect was already saturated by the single mutants; therefore, no more additional effect was observed by the double mutant.

The observations of the altered interactions between SART3 and the phosphorylated USP15 at positions T149 and T219 prompted us to analyze the deubiquitinating activity toward PRP31 in the spliceosome. While the phosphomutants exhibited similar activity in the deubiquitination of PRP31 compared to the WT, the phosphomimetics exhibited a decreased activity (Fig. 3e). Consequently, we examined whether the altered substrate deubiquitination of the USP15-phosphomutants is caused by the abolished DUB enzymatic activity of USP15 itself. However, USP15 and its mutants similarly cleaved K63-linked ubiquitin chains, which were previously reported to be efficiently cleaved by USP15 [10] to produce an equal amount of mono and di-ubiquitin chains (Fig. 3f), suggesting that there was no difference in the USP15 enzymatic activity itself. Therefore, the reduced deubiquitination of PRP31 by USP15^{T149D} and USP15^{T219D} is most likely due to the altered interaction with SART3 by dissociating USP15 from the substrate.

CDKs are potential kinases for USP15 phosphorylation

Next, we searched for a potential kinase of USP15 phosphorylation. Because CDKs control various checkpoints during the cell cycle [16], we first examined the possible effect of CDKs by treating the cells with purvalanol A, a cell-permeable highly selective and potent CDK inhibitor. We also tested the effect of okadaic acid which is a potent inhibitor of protein phosphatase. The results showed an accumulation of USP15 in the nucleus when treated with purvalanol A (Fig. 4a and b). Furthermore, the purvalanol A treatment increased the interaction of USP15 with SART3 (Fig. 4c). On the other hand, okadaic acid did not show any significant effect on the USP15 and SART3 interaction (Fig. 4c). These results suggest that the non-phosphorylated USP15, as a result of CDK inhibition, is predominantly localized in the nucleus. In addition, purvalanol A treatment enhanced the deubiquitination of PRP31 by USP15 compared to the control or okadaic acid treated lysates (Fig. 4d). As expected, the effect of purvalanol A was similar to those of the phosphomutants, indicating that CDKs could be a possible candidate responsible for USP15 phosphorylation.

Because USP15 and USP4 share not only high sequence identities but also a common substrate targeting factor SART3 [12], we questioned whether USP4 also undergoes phosphorylation-dependent regulation. We tested the CDK-dependent phosphorylation effect on its localization, interaction, and deubiquitinating activity in the same manner. The treatment of cells with purvalanol A caused an increased nuclear accumulation of USP4 nuclear accumulation of USP4 (Supplementary Fig. S4a and b). Moreover, the USP4 interaction with SART3 (Supplementary Fig. S4c) and deubiquitination activity toward PRP3 (Supplementary Fig. S4d) were promoted by the purvalanol A treatment compared to the WT or okadaic acid-treated cells. These results suggest that CDKs might be a potential kinase for USP4 as well.

Discussion

A number of DUBs have been reported to undergo various posttranslational modifications, which have vital roles in their regulation [17–19]. In particular, phosphorylation has been shown to regulate the catalytic activity, interaction with partner proteins, or localization of DUBs from different classes. For example, phosphorylation of human USP7 at Ser18 by the protein kinase CK2 is required for its own stability, which is involved in p53 downregulation by

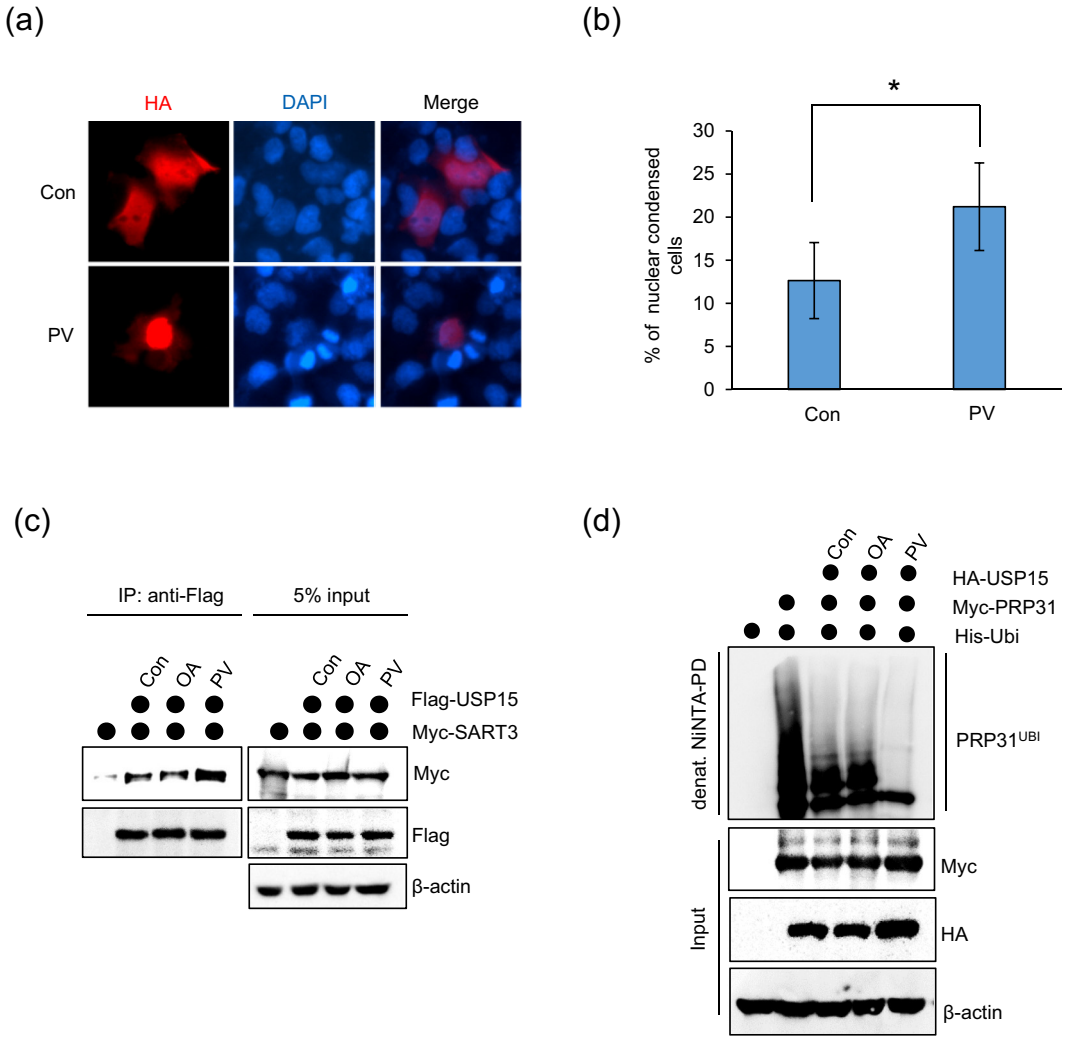


Fig. 4. Treatment with kinase inhibitor affects USP15 localization, interaction, and activity. (a) HeLa cells were transfected with HA-USP15 and treated with 10 μM purvalanol A (PV) for 2 h, and cells were immuno-stained with anti-HA antibody followed by fixation and permeabilization. Alexa546-conjugated anti-mouse was used as secondary antibody, while the nucleus was stained with DAPI. The intracellular localization of HA-USP15 (red) was analyzed by fluorescence microscopy. (b) The percentage of cells with prominent nuclear localization of USP15 was determined. Data are shown as mean ± S.D. from three independent experiments (n = 3; >100 cells per experiment; paired *t*-test $*P < 0.05$). (c) Flag-USP15 and Myc-SART3 were cotransfected into HeLa cells and treated with either 10 μM purvalanol A (PV) or okadaic acid (OA) for 2 h. Cell lysates were immunoprecipitated with anti-Flag-agarose, and co-precipitated Myc-SART3 was detected by Western blot. (d) Myc-PRP31 and HA-USP15 were co-expressed with His-ubiquitin, treated with either purvalanol A (PV) or okadaic acid (OA), and covalently modified proteins were purified on Ni-NTA-agarose under denaturing conditions. Ubiquitinated PRP31 was detected by the anti-Myc antibody.

Mdm2 stabilization [20]. In the human ubiquitin-specific protease CYLD, which is involved in cylindromatosis, phosphorylation at Ser418 affects its DUB activity negatively [21], while phosphorylation of the ovarian tumor domain (OTU)-containing protease A20 at Ser381 by kinase IKKβ leads to an enhancement of its DUB activity toward NEMO [22]. Phosphorylation of the human deubiquitinase DUBA (OTUD5) at a single residue, Ser177, is both necessary and sufficient to activate the enzyme [23]. DUB phosphorylation not only regulates the enzymatic activity but also controls the formation of protein complexes. In the case of USP1, UAF1, the cofactor of USP1, is required for the DUB activity and subcellular localization [24–26]. USP1 forms a complex with UAF1 in the cytoplasm, and then it is translocated into the nucleus. In addition, its interaction with UAF1 is regulated by phosphorylation of USP1 at Ser313 [26]. The translocation of DUBs is also regulated by phosphorylation. ATM-mediated phosphorylation of USP10 at Thr42 and Ser337 regulates the stability and translocation into the nucleus, which is critical for the activation of p53 [27].

Phosphorylation at Ser340 and Ser352 within the third ubiquitin-interacting motif of ataxin-3 by protein casein kinase 2 controls the nuclear localization and stability of ataxin-3 [28]. AKT-induced phosphorylation of USP4 promotes its subcellular distribution mostly in the membrane and cytoplasm, while the non-phosphorylated form is predominantly condensed into the nucleus [14]. However, despite the recent progress about the phosphorylation-dependent activity of different DUBs [23,29], the understanding of their regulation by post-translational modification, particularly phosphorylation, remains limited.

Here, we found that the two evolutionary conserved sites of USP15, namely, T149 and T219, are differentially phosphorylated in the cytoplasm. Both sites reside in its N-terminal region that is responsible for the interaction with SART3. But, unfortunately, the two sites are not defined in the crystal structure of the SART3–USP15$^{DUSP-UBL}$ complex reported earlier [13]. However, based on the corresponding sites in USP4 in the SART3–USP4$^{DUSP-UBL}$ complex structure [12] as well as the model obtained by superposing the unbound structures of USP15$^{DUSP-UBL}$ (PDB code: 3T9L; [30] and USP4$^{DUSP-UBL}$ (PDB code: 3JYU) onto the complex structure, the two sites are on the surface of USP15 (or USP4) about 30–40 Å away from the interface (Supplementary Fig. S5). The linker between the DUSP and UBL domains of both USP15 and USP4 plays a crucial role in the interaction with SART3, with the K_D being 0.19 and 0.88 μM, respectively [12]. In particular, the two residues in the linker are crucial for binding since the mutants, namely, F127A and F127A/V128A of USP4, showed no significant affinity for SART3, suggesting that the interactions between the two proteins are rather specific. Therefore, it is quite possible that the phosphorylation on T149 and T219 results in a conformational change in the linker region subsequently reducing the affinity for SART3 binding as seen in some of the kinases [31]. For example, phosphorylation of T149 in USP15 might involve conformational changes on the nearby residues such as E135, K137, R150, and R151, which in turn causes a compromised conformation for the linker region. Loss of phosphorylation by mutating T149 and T219 was found to increase the interaction with SART3 and the deubiquitination activity toward its substrate PRP31 (Fig. 3a–d). In addition, these phenomena in the loss of phosphorylation were similar to the treatment of CDK inhibitor (Fig. 4). Treatment with CDK inhibitor showed the same effect on the localization, interaction, and substrate deubiquitination of USP4 as shown in USP15 (Supplementary Fig. S4). Therefore, these results suggest that phosphorylation of USP15 and USP4 by CDKs may regulate the interaction with SART3, thereby controlling the deubiquitinating activity toward PRP31 and PRP3 in the nucleus.

There have been several reports revealing the cellular localization of USP4 and USP15. In earlier reports, there were contradictory findings regarding the cellular localization of USP4, which was initially described as nuclear [32] or cytoplasmic [33]. Later reports showed USP4 as a nucleocytoplasmic shuttling protein with varying extents of nuclear or cytoplasmic localization depending on the cell types; however, USP15 is generally cytonuclear in most cell types including HeLa cells [34]. Other reports also showed USP4 and USP15 as cytonuclear proteins in HeLa cells [11,12,14,35]. The cytonuclear shuttling of USP4 is reported to be mediated by NES1 (^{102}LIDELDYVLV^{111}) and NES2 (^{133}VEVYLLELKL^{142}) in the N terminus and by NLS1 (^{413}KKKP^{416}) and NLS2 (^{766}QPQKKKK^{772}) in the C terminus [34]. In addition, the NLS of SART3 binds importin-α in a bipartite manner, and SART3 interaction with USP4 and USP15 induces translocation of USP4 and USP15 into the nucleus [12]. Although NLS and NES trigger the shuttling of proteins between the nucleus and the cytoplasm, the sub-cellular distribution of certain proteins may also, in part, be regulated by modifications such as phosphorylation. For example, AKT-induced phosphorylation at Ser445 of USP4, which resides in neither NES nor NLS, triggers its localization from the nucleus to the cytoplasm, thereby reaching the cell membrane for deubiquitination of the TGF-β receptor I [14]. Similarly, a sequence alignment showed that USP15 has NES1 (^{83}LIDELDYVLV^{92}), NES2 (^{129}VEVYLTELKL^{138}), NLS1 (^{372}KKPY^{375}), and NLS2 (^{749}YKPPKK^{754}) corresponding to USP4. Although our predicted phosphorylation sites, T149 and T219, are not located on the proposed NES or NLS of USP15, the alteration of USP15 localization showed in our current study is triggered by phosphorylation. It is possible that localization of USP15 and USP4 may be regulated by phosphorylation or a nuclear signal such as NLS and NES depending on the cellular conditions.

USP15 and USP4 are required for the spliceosome dynamics by deubiquitinating PRP31 and PRP3, components of U4 snRNP. The interaction of PRP31 or PRP3 and the U5 snRNP component PRP8 is reported to be regulated by the ubiquitination and deubiquitination status of PRP31 and PRP3, which is required for efficient RNA splicing of chromosome segregation related genes, probably by stabilizing the U4/U6.U5 tri-snRNP complex [10]. Normally, USP15 and USP4 interact with SART3, which contribute to RNA splicing through deubiquitination of PRP31 and PRP3. However, if the CDK activity increases under some conditions such as in mitosis, USP15 and USP4 may get phosphorylated at Thr149 and Thr219, thereby dissociating from SART3, which will eventually result in a decrease of RNA splicing. This molecular mechanism then could contribute to

the suppression of RNA splicing during mitosis. In fact, RNA splicing is regulated by the cell cycle, and there are several indications that RNA splicing is repressed during mitosis [36]. SRp38, a representative mitosis-specific repressor of the RNA splicing machinery, is phosphorylated in interphase but dephosphorylated in mitosis, and the dephosphorylated SRp38 tightly interacts with U1 snRNP, thereby inhibiting the interaction between U1 snRNP and the 5′ end-splice site of pre-mRNA required for the splicing initiation [37,38]. It is quite possible that other factors, such as the phosphorylation status of USP15 and USP4, may well regulate RNA splicing by cell cycle depending on their modification status.

Cell cycle-dependent phosphorylation of USP15 isoform 1 has been reported previously. In interphase, USP15 isoforms 1 and 2 are mostly localized in the cytoplasm; however, both isoforms become accumulated in the nucleus at prophase where isoform1 is phosphorylated at its S229 residue, which is located in the 28-amino-acid serine-rich cassette of USP15 isoform1 only but not in isoform 2, during mitotic entry. Phosphorylation at S229 selectively abrogates the USP15 function in maintaining TOP2A-mediated genomic stability in a cell cycle-dependent manner [39]. The absence of the S229 residue by alternative splicing in isoform 2 further strengthens the possibility that other sites might be phosphorylated to regulate this cell cycle-dependent nuclear accumulation predominantly at mitosis. [39]. In light of our previous study revealing that USP15 is required for proper RNA splicing by regulating the spliceosome dynamics [10], it may be speculated from our current study that USP15 phosphorylation at T149 and T219 may regulate proper RNA splicing by fine-tuning its localization, interaction with SART3, and activity toward PRP31. However, our current and previous studies were performed using only USP15 isoform 2. Therefore, we cannot be sure whether our proposed phosphorylation-dependent regulation of USP15 is isoform-specific. Nevertheless, further studies are needed to elucidate the regulation of RNA splicing according to the cell cycle by reversible phosphorylation of USP15 or USP4.

It should be noted that in addition to the interaction with SART3, the misregulation of phosphorylation of USP15 could be closely related to diseases. The COSMIC database shows that Thr at position 219 of USP15 is mutated to Ala or Ile in blastoma (COSMIC catalog of somatic mutations in cancer; https://cancer.sanger.ac.uk/cosmic). In addition, the same site is found to be mutated in liver and colorectal cancer (International Cancer Genome Consortium, https://icgc.org/). Although the frequency of this mutation is not very high, it obviously indicates that the disrupted modification of this specific site may alter its function and regulation and therefore is involved in human cancer.

In summary, we showed that Thr149 and Thr219 of USP15 are differentially phosphorylated in the cytoplasm and nucleus, and the phosphorylation status is important in its interaction with SART3, and consequently in its localization and regulation of the DUB activity in the spliceosome. The same applies to USP4. We further showed that the phosphorylation of both USP15 and USP4 is regulated by the activity of CDKs, although a question remains on which CDK, or whether more than one CDK, is required for this phosphorylation. Collectively, our findings reveal a novel phosphorylation-dependent regulation of USP15 and USP4 in their interaction, localization, and substrate targeting.

Materials and Methods

Cloning

Information on USP15 WT (isoform 2), SART3, and PRP31 genes is the same as that described in our previous study [10]. The phosphomutants of USP15 were constructed by mutating USP15 threonine (ACT and ACA) at positions 149 and 219 into alanine (GCT and GCA), respectively, using a site-directed mutagenesis kit (Stratagene) followed by PCR amplification. Taking $USP15^{T149A}$ as a template, the double mutant $USP15^{T149A/T219A}$ was made by inserting a primer targeted alanine mutation at T219 site. For making phosphomimetics of the corresponding sites, each threonine residues were substituted with aspartic acid (GAT). All the mutated sequences were confirmed by DNA sequencing.

Cell culture and transfections

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (10 U/mL), and streptomycin (100 μg/mL). All cells were incubated at 37 °C in 5% CO_2. Plasmids were transfected into HeLa cells using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions.

Antibodies

The following antibodies were purchased and used for immunostaining and Western blotting: antibodies against USP15 (Bethyl Laboratories Inc.), USP4 (Bethyl Laboratories Inc.), SART3 (Bethyl Laboratories Inc.), β-actin (Santa Cruz Biotechnology Inc.), Myc (Santa Cruz Biotechnology), HA (Santa Cruz Biotechnology), Flag (Sigma-Aldrich), PRP31 (Sigma-Aldrich), Actin (AB frontier), and Alexa-488 or Alexa-546 conjugated secondary antibodies (Invitrogen).

Nuclear-cytoplasmic extraction

HeLa cells overexpressed with Flag-USP15 were harvested by trypsinization after 36 h of transfection. Harvested cells were centrifuged at 7000 rpm for 5 min and washed by resuspending the cell pellet in 1× DPBS. Cytoplasmic and nuclear fractions were separated by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacturer's instructions. Extracted fractions were subjected to immunoprecipitation (IP) with anti-Flag agarose beads and elute were separated by SDS-PAGE and detected by Western blot. Immunoprecipitated USP15 were excised and requested to the ProteomTech Inc. for in-gel digestion and mass spectrometry analysis. For USP15 analysis in the nuclear fraction, we collected several gel pieces and then analyzed by mass spectrometry.

In-gel digestion with trypsin and extraction of peptides

The procedure for the in-gel digestion of protein spots from Coomassie blue-stained gels was carried out as previously described [40]. Briefly, protein spots were excised from stained gels and cut into pieces. The gel pieces were washed for 1 h at room temperature in 25 mM ammonium bicarbonate buffer (pH 7.8) containing 50% (v/v) acetonitrile (ACN). Following the dehydration of the gel pieces in a centrifugal vacuum concentrator for 10 min, the gel pieces were rehydrated in 50 ng of sequencing grade trypsin solution (Promega, Madison, WI, USA). After incubation in 25 mM ammonium bicarbonate buffer (pH 7.8) at 37 °C overnight, the tryptic peptides were extracted with 5 µL of 0.5% formic acid containing 50% (v/v) ACN for 40 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator. Prior to mass spectrometric analysis, the peptide solution was subjected to a desalting process using a reversed-phase column [41]. Briefly, after an equilibration step with 10 µL of 5% (v/v) formic acid, the peptide solution was loaded onto the column and washed with 10 µL of 5% (v/v) formic acid. The bound peptides were eluted with 5 µL of 70% ACN with 5% (v/v) formic acid.

Identification of proteins by LC–MS/MS

After desalting, eluted tryptic peptides were separated and analyzed using a nano ACQUITY UPLC (Waters, Milford, MA, USA) directly coupled to a Finnigan LCQ DECA ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA). Briefly, the peptides were bound to the ACQUITY UPLC peptide BEH C18 column (1.7 µm in size, 130 Å in pore size, 100 µm × 100 mm) with distilled water containing 0.1% (v/v) formic acid, and the bound peptides were eluted with a 40-min gradient of 0–90% (v/v) ACN containing 0.1% (v/v) formic acid at a flow rate of 0.4 µL/min. For tandem mass spectrometry, the full-mass scan range mode was m/z = 400–2000 Da. After determination of the charge states of the ion on zoom scans, product ion spectra were acquired in the MS/MS mode with a relative collision energy of 55%. The individual spectra from the MS/MS were processed using the SEQUEST software (Thermo Quest, San Jose, CA, USA), and the generated peak lists were used to query the NCBI database using the MASCOT program (Matrix Science Ltd., London, UK). We set the modifications of methionine, cysteine, methylation of arginine, and phosphorylation of serine, threonine, and tyrosine for MS analysis, and tolerance of peptide mass was 10 ppm. MS/MS ion mass tolerance was 0.8 Da, allowance of missed cleavage was 1, and charge states (+2, +3) were taken into account for data analysis. We took only significant hits as defined by MASCOT probability analysis.

In vitro translation and GST pull-down assay

GST proteins were immobilized by incubating purified control GST or GST-SART3$^{94–611}$ proteins with glutathione beads (Sigma) in MBP coupling buffer [10 mM Hepes (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 0.1% Tween-20, and 1× BSA] for 2 h at 4 °C with rotation. USP15 WT, phosphomutants, or phosphomimetics were synthesized with TnT® Quick Coupled Transcription/Translation System (Promega) and incubated with the above GST proteins coupled beads for 4 h at 4 °C while rotating. Beads were washed with buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM KCl, and 0.1% Tween-20, eluted with 2× SDS sample buffer, separated by SDS-PAGE, and detected by Western blot and Coomassie staining.

Immunoprecipitation

Transfected HeLa cells were harvested and lysed with IP buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM KCl, 0.1% Tween-20, 2 mM DTT, and protease inhibitor cocktail (Roche)]. Lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, and supernatants were incubated with anti-Flag M2 (Sigma) or anti-HA (Sigma) agarose beads for 4 h at 4 °C. Beads were washed with buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM KCl, 0.1% Tween-20, and 2 mM DTT. Beads were incubated with 100 mg/mL 3× FLAG peptide (Sigma) in lysis buffer and eluted with the FLAG peptide or eluted with 2 × SDS sample buffer. Samples were analyzed and detected by Western blotting.

Immunofluorescence analysis

HeLa cells were seeded on coverslips and transfected with indicated WT, phosphomutants, or phosphomimetics plasmids. After 48 h, cells were

fixed with 4% paraformaldehyde, permeabilized, and incubated with anti-HA (Santa Cruz Biotechnology) or anti-Myc (Santa Cruz Biotechnology) antibody for 1 h at room temperature. Goat anti-rabbit coupled to Alexa488 (Invitrogen) and goat anti-mouse coupled to Alexa546 (Invitrogen) were used as secondary antibodies. The nucleus was stained with DAPI (Invitrogen). Coverslips were mounted and fluorescence was visualized with 40× magnification on a confocal laser scanning microscope (Carl Zeiss, Inc.), and images were analyzed with the NIS-element Software (NES). Nuclear or cytoplasmic condensed USP15 were counted at 40× magnification, and results were shown as the mean ± S.D. from three sets of independent experiments.

His-ubiquitin pull-down assay

HeLa cells were co-transfected with pCS2-His-ubiquitin and other plasmids as indicated. After 36 h of transfection, cells were resuspended in urea lysis buffer [8 M urea, 0.3 M NaCl, 0.5 M Na_2HPO_4, 0.05 M Tris, 0.001 M PMSF, 0.01 M imidazole (pH 8.0)] and sonicated for 4 min. Cell lysates were added to 50 μL of equilibrated Ni-NTA agarose and incubated for 4 h at room temperature. Beads were then washed five times with urea wash buffer [8 M Urea, 0.3 M NaCl, 0.5 M Na_2HPO_4, 0.05 M Tris, 0.001 M PMSF, 0.02 M imidazole (pH 6.5)]. Conjugated proteins were eluted with 40 μL 2× Laemmli/Imidazole (200 mM imidazole) and boiled at 95 °C for 10 min. Eluted proteins were then analyzed by Western blotting.

Deubiquitination assay

Transfected HeLa cells were harvested and lysed with IP buffer. After a 20-min incubation on ice, lysates were centrifuged at 13,000 rpm for 30 min, and supernatants were incubated with 20 μL equilibrated Flag bead with rotating at 4 °C for 4 h. Bound beads were washed, eluted with flag peptide in 40 μL DUB buffer (20 mM Hepes, 100 mM NaCl, 5 mM DTT), and incubated each elutes with 2 μL of K63-linked polyubiquitin chain (100 ng/μL, Boston Biochem) for 1 h at 30 °C. Reactions were terminated by adding 6× SDS sample buffer. After boiling at 95 °C for 10 min, samples were separated in 15% SDS-PAGE and detected by the anti-ubiquitin antibody.

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Appendix A. Supplementary data

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Abbreviations used:
USP15, ubiquitin-specific protease 15; SART3, squamous cell carcinoma antigen recognized by T cells 3; DUB, deubiquitinating enzyme; NLS, nuclear localization sequence; CDK, cyclin-dependent kinase; WT, wild type; IP, immunoprecipitation; ACN, acetonitrile; USP4, ubiquitin-specific protease 4; PTM, post translational modification; NES, nuclear export signal.

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