Deubiquitinase USP47/UBP64E Regulates β-Catenin Ubiquitination and Degradation and Plays a Positive Role in Wnt Signaling

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Wnt/β-catenin signaling plays an essential role in animal development and tumorigenesis (1, 2). In normal cells, β-catenin is sequentially phosphorylated by CKIα and GSK-3 in a protein complex containing the tumor suppressor proteins axin and APC (3). Phosphorylated β-catenin is recognized by the ubiquitin (Ub) ligase β-TrCP, an F-box and WD40 repeat protein (4). The WD40 repeat domain of β-TrCP binds β-catenin. β-TrCP also binds, via its F-box, to components of the ubiquitination machinery, including Skp1, cullin 1, ring box protein 1 (Rbx1), and ubiquitin–conjugating enzyme (E2). Ubiquitinated β-catenin is degraded via the 26S proteasome (4–7). Upon Wnt stimulation, and ubiquitin-conjugating enzyme (E2). Ubiquitinated β-catenin binds, via its F-box, to components of the ubiquitination protein complex containing the tumor suppressor proteins axin (3, 4), CKIα, and GSK-3 β, leading to β-catenin degradation through the proteasome. The phosphorylation and ubiquitination of β-catenin have been well characterized; however, it is unknown whether and how a deubiquitinase is involved. In this study, by screening RNA interference (RNAi) libraries, we identified USP47 as a deubiquitinase that prevents β-catenin ubiquitination. Inactivation of USP47 by RNAi increased β-catenin ubiquitination, attenuated Wnt signaling, and repressed cancer cell growth. Furthermore, USP47 deubiquitinates itself, whereas β-TrCP promotes USP47 ubiquitination through interaction with an atypical motif in USP47. Finally, in vivo studies in the Drosophila wing suggest that UBP64E, the USP47 counterpart in Drosophila, is required for Armadillo stabilization and plays a positive role in regulating Wnt target gene expression.

MATERIALS AND METHODS

Screening of an siRNA library for human deubiquitinases. The Dharmacon siGENOME RTF SMARTpool siRNA library for human deubiquitinating enzymes (H-004705; lot 08138) was used for screening for human deubiquitinase for USP47/UBP64E, that regulates β-catenin ubiquitination/degradation in vitro and in vivo.

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ciferase activities were analyzed with a luciferase assay system and the GloMax-Multi+ Microplate multimode reader (Promega).

RNAi, wing disc immunostaining, and generating transgenic lines in Drosophila. The RNAi lines that targeted each deubiquitinase in the Drosophila genome were obtained from the Vienna Drosophila RNAi Center (VDRC) (25). Wing-specific MS1096 Gal4 was used to assess for the induction of an adult wing phenotype. Upstream activation sequence (UAS)-Dicer was coexpressed with RNAi lines to enhance the RNAi effects. UBP64E RNAi lines (v26027 and v103743) were consistent in terms of the adult wing phenotypes and the effects on Armadillo (Arm) accumulation in wing discs. A standard protocol was used for the wing disc immunostaining. Briefly, wing discs from third-instar larvae with specific genotypes were dissected in phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde in PBS for 20 min. After permeabilization with PBS supplemented with 1% Triton X-100 (PBT), the discs were incubated with the indicated primary antibodies for 3 h and the corresponding secondary antibodies for 1 h sequentially and then washed with PBT three times, for 20 min per wash, following the incubations. The antibodies used in this study were mouse anti-Arm (DSHB; 1:10), anti-Wg (Developmental Studies Hybridoma Bank [DSHB]; 1:50), anti-Ptc (DSHB; 1:10), rabbit anti-Flag (ABR; 1:150), anti-Di (from Greece Bockhoff-Falk; 1:150), and guinea pig anti-Sens (from Hugo Bellen; 1:150).

Additional USP47 siRNA was or-directed from Qiagen (reference sequence, NM_017944; catalog number 30250) at 4°C overnight. The beads were washed with 1 ml PTU lysis buffer and then dialyzed against dialysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.2% NP-40, 1 µg/ml pepstatin, 3 µg/ml aprotinin, 10 mM NaF, and 2.5 µM MG132).

Western blot and binding assays. Bacterially expressed glutathione-S-transferase (GST) fusion proteins were purified with Glutathione Sepharose 4B beads as previously described (4). Cell fractionation, Western blotting, immunoprecipitation, and GST pulldown experiments were performed as previously described (16). Proteins were analyzed with the following antibodies: rabbit anti-USP47 (Bethyl; IHC-00235), rabbit anti-axin2 (Cell Signaling; 2151), rabbit anti-c-myc (Epitomics; 1472-1), rabbit anti-Sor9 (Abcam; ab185966), mouse anti-Flag (Sigma; F1804), rabbit antisurvivin (BioLegend; 614702), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Gentex; GTX627408).

RESULTS

Identification of USP47 as a positive regulator of Wnt signaling. The human genome has more than 100 deubiquitinase genes (31). In order to identify novel regulators of Wnt signaling, we generated a stable cell line that expresses the TOPFlash reporter. Using this cell line, we screened a human siRNA library and identified USP47 as a putative positive regulator of Wnt signaling.

To validate the function of USP47, we knocked down USP47 in mammalian cells with a different siRNA and analyzed Wnt signaling activity. USP47 siRNA strongly inhibited Wnt-mediated TOPFlash reporter activity (Fig. 1A). Two USP47 shRNAs that effectively knocked down USP47 expression in HEK293T cells also severely blocked the luciferase reporter activity (Fig. 1B). These data indicate that inactivation of USP47 by different approaches inhibits Wnt signaling, suggesting that USP47 is a novel positive regulator of Wnt signaling.

We wondered whether USP47 regulates β-catenin stability. We knocked down USP47 in HEK293T cells using shRNAs and analyzed the levels of β-catenin. We found that USP47 shRNAs significantly decreased the protein levels of nuclear β-catenin in HEK293T cells with or without Wnt treatment (Fig. 1C and D). To understand the biological functions of USP47, we analyzed the effects of USP47 shRNAs on A549 lung cancer cells and PC3 prostate cancer cells. Knocking down USP47 inhibited the expression of Wnt target genes and significantly repressed the proliferation of these cancer cells (Fig. 1E and F), suggesting that USP47 is re-
required for cancer cell growth. β-Catenin degradation is controlled by N-terminal S/T phosphorylation and K19/K49 ubiquitination; blocking phosphorylation or ubiquitination by mutating these residues may bypass its regulation by the deubiquitinase. Indeed, β-catenin with an S33 or K19 mutation is less sensitive to USP47 shRNA than wild-type β-catenin (Fig. 1G). These results suggest that USP47 regulates Wnt signaling through stabilizing β-catenin. USP47 directly interacts with β-catenin and regulates β-catenin ubiquitination. USP47 has a catalytic domain at the N terminus (Fig. 2A). Point mutations and truncations of USP47 were generated as indicated in Fig. 2A. We found that wild-type USP47, but not USP47m, a mutant form of USP47 that contains a C109S mutation at the enzyme activation site (Fig. 2A), rescued Wnt reporter inhibition by USP47 shRNA, suggesting that the catalytic activity of USP47 is required for Wnt signaling (Fig. 2B). To explore the mechanisms by which USP47 regulates β-catenin, we first determined whether USP47 acts in the same protein complex as β-catenin. Since HCT116 cells have high levels of β-catenin, we immunoprecipitated endogenous β-catenin from HCT116 cells and analyzed the physical interaction between β-catenin and USP47 with an anti-USP47 antibody. We found that endogenous USP47 bound endogenous β-catenin (Fig. 2C).
In the absence of Wnt, wild-type β-catenin is degraded through the ubiquitination/proteasome pathway. In our previous studies, when HEK293T cells were treated with MG132, a proteasome inhibitor, the levels of endogenous and overexpressed β-catenin, including ubiquitinated β-catenin, were increased (16). To precisely detect ubiquitinated β-catenin, His-Ub and Flag-tagged β-catenin (Flag-β-Cat) were cotransfected into HEK293T cells. The cells were treated with MG132 to stabilize the ubiquitinated β-catenin. Ubiquitinated proteins were pulled down by Ni-NTA beads under denaturing conditions and analyzed by Western blotting with the anti-Flag Ab. The error bars represent standard deviations. Masses of protein standards are given in kilodaltons.
lyzed by Western blotting (28). The mobility shift indicates the specific ubiquitination of the protein (Fig. 2D and E).

To determine if USP47 controls β-catenin ubiquitination, we examined the levels of β-catenin ubiquitination when USP47 was inactivated. We knocked down USP47 in HEK293T cells using siRNA and found that the levels of ubiquitinated β-catenin were increased compared with the sample treated with control siRNA (Fig. 2F). Similarly, knocking down USP47 with shRNAs also increased the levels of β-catenin ubiquitination (Fig. 2G). Expression of wild-type USP47 decreased β-catenin ubiquitination (Fig. 2H). However, USP47m increased β-catenin ubiquitination (Fig. 2H). These results suggest that the enzymatic activity of USP47 is required for its function in regulating β-catenin ubiquitination. USP47m may act as a dominant-negative mutant. To further determine if USP47 directly catalyzes β-catenin deubiquitination, we performed an in vitro deubiquitination assay. Ubiquitinated β-catenin purified from HEK293T cells overexpressing Myc-β-catenin was incubated with the Flag-tagged USP47 catalytic domain (Flag-USP47CD) or its mutant (Flag-USP47CDm) purified separately from HEK293T cells. Compared with the control, USP47CDm reduced the levels of ubiquitinated β-catenin (Fig. 2I, top) and increased the levels of deubiquitinated β-catenin released into the supernatant from the Ni-NTA beads (Fig. 2I, middle), suggesting that USP47 is an enzyme that directly catalyzes β-catenin deubiquitination.

β-TrCP/USP47 regulate USP47 ubiquitination. β-Catenin ubiquitination is mediated by β-TrCP; it has been indicated that USP47 also interacts with β-TrCP (32), but the function of this interaction in the Wnt pathway is not clear. We found that the catalytic domain of USP47 was sufficient for β-TrCP binding in an immunoprecipitation assay carried out with HEK293T cells cotransfected with Flag-USP47CD and Myc-β-TrCP (Fig. 3A). We noted that the protein levels of USP47m were lower than the levels of wild-type USP47 (Fig. 2E). Also, in HEK293T cells transfected with USP47m and His-Ub, the protein levels of USP47m were decreased by ubiquitin overexpression and increased by MG132 treatment (Fig. 3B). As a control, the protein levels of green fluorescent protein (GFP) were not affected (Fig. 3B). These results indicate that USP47m is less stable than the wild-type USP47 and that the USP47m protein could be regulated by proteasome-mediated degradation.

Since USP47m contains a C109S mutation at the catalytic active site, we hypothesized that the wild-type USP47 could decrease its own ubiquitination and resist ubiquitination/degradation while USP47m lost the deubiquitinase activity and was more sensitive to proteasome degradation. To test this hypothesis, His-Ub and USP47m were cotransfected into HEK293T cells. The ubiquitinated proteins were pulled down with Ni-NTA beads, and USP47 was analyzed by Western blotting. We found that USP47m exhibited a mobility shift, indicative of ubiquitination (Fig. 3C). In contrast, wild-type USP47 never showed such a mobility shift (Fig. 3C). These data suggest that USP47 regulates its own ubiquitination.

To test if the ubiquitination of USP47 could be regulated by β-TrCP, we transfected His-ub, hemagglutinin-tagged USP47 (HA-USP47m), and Myc-β-TrCP into HEK293T cells and performed the Ni-NTA pulldown assay. We found that the overexpression of β-TrCP increased USP47m ubiquitination (Fig. 3D), indicating that β-TrCP is the ubiquitin ligase for USP47. To further test the enzymatic activity of USP47 in regulating its own ubiquitination, USP47CD was cotransfected with USP47m, His-ub, and β-TrCP into HEK293T cells. We found that both USP47 and USP47CD decreased the ubiquitination of USP47m (Fig. 3E), suggesting that the catalytic domain of USP47 is sufficient to catalyze the deubiquitination of USP47m. These results further indicate that USP47 could regulate its own ubiquitination and that USP47m lost the deubiquitinase activity with the consequent elevation of ubiquitination. To further validate the role of ubiquitination in USP47 degradation, we treated HEK293T cells with MG132 and cycloheximide. MG132 stabilized endogenous USP47 and expression of β-TrCP, and His-ub further enhanced USP47 degradation (Fig. 3F), suggesting that USP47 is degraded by the β-TrCP-mediated ubiquitination/proteasome pathway.

Identification of an atypical β-TrCP-binding motif in USP47. The consensus β-TrCP-binding site for β-catenin is DS GXXS (conserved amino acids are in boldface), where S is a phosphoserine or phosphothreonine (3). There is a consensus β-TrCP-binding site in the C terminus of USP47 (DSGTDS; amino acids aa 913 to 918) (Fig. 3F). However, deletion of the C terminus, which contains amino acids 913 to 918, did not affect β-TrCP binding (Fig. 4A). Since glutamic acid (E) can mimic phosphoserine/phosphothreonine, we identified two potential β-TrCP-binding sites in the catalytic domain of USP47 (DSG NEAE at amino acids 348 to 354 and DEGICLE at amino acids 373 to 379) (Fig. 4A). The latter site is conserved in UB6P64E, the Droso phila homolog of USP47. We mutated these sites and analyzed the interaction between each mutant and β-TrCP using an immunoprecipitation assay. We found that USP47CD with 348-349 mutations can still bind β-TrCP (Fig. 4B), whereas 373-374 mutations abolished the binding (Fig. 4B), indicating that 373-374 in USP47 is responsible for its interaction with β-TrCP. These findings suggest that β-TrCP mediates the ubiquitination of USP47 through optimized motifs.

To further characterize the role of these potential β-TrCP-binding sites in USP47 ubiquitination, we transfected wild-type or mutant forms of USP47 with His-ub and β-TrCP into HEK293T cells. USP47 ubiquitination was analyzed by Ni-NTA pulldown and Western blotting. Wild-type USP47 had a low level of ubiquitination even in the presence of β-TrCP (Fig. 4C). USP47m had a much higher level of ubiquitination (Fig. 4C). USP47 with 373-374 mutations had no detectable ubiquitination (Fig. 4C). These results suggest that the interaction between aa 373 to 379 of USP47 and β-TrCP is essential for β-TrCP-mediated ubiquitination of USP47.

Interactions of USP47 with β-catenin and β-TrCP. β-Catenin interacts with multiple proteins through its N-terminal destruction domain, central armadillo domain, and C-terminal transcription activation domain (Fig. 5A). USP47CD contains the N-terminal catalytic domain and is sufficient to interact with β-catenin (Fig. 2A and 5B). To determine which domain of β-catenin interacts with USP47, we generated several deletion mutants of β-catenin (Fig. 5A). We analyzed the binding between GST-USP47CD and the β-catenin mutants and found that the C terminus of β-catenin containing two armadillo repeats (Fig. 5A, Cat4) was sufficient for USP47 binding (Fig. 5C).

Since both β-catenin and USP47 bind β-TrCP, the proteins may compete with each other for the binding. To test this possibility, we carried out experiments to examine the interactions among the proteins. Myc-tagged β-TrCP was transfected into HEK293T cells. The cell lysates were incubated with purified GST
or GST-USP47CD protein from bacteria. The β-TrCP complex was immunoprecipitated with anti-Myc Ab, and -TrCP and USP47CD were analyzed by Western blotting with anti-Myc and anti-Flag Abs. (B) Stability of USP47m. HEK293T cells were transfected with HA-tagged USP47m and pEGFP, with or without His-Ub. Cells were treated or not with MG132 for 6 h before harvesting. The protein levels of USP47m were analyzed by Western blotting, with GFP as a control. (C) Ubiquitination of USP47. HEK293T cells were transfected with His-Ub, together with HA-tagged USP47m or USP47m constructs. Cells were treated with MG132 for 6 h before harvesting and then lysed in PTU buffer. Ubiquitin-conjugated proteins were pulled down by Ni-NTA. Ubiquitinated USP47 (top) and total USP47 (bottom) proteins were analyzed by Western blotting with an anti-HA Ab. (D) β-TrCP regulates USP47 ubiquitination. HEK293T cells were transfected with HA-tagged USP47m, together with or without His-Ub or Myc-tagged β-TrCP. The cells were treated with MG132 for 6 h before harvesting. The cells were lysed in PTU buffer, and ubiquitin-conjugated proteins were pulled down with Ni-NTA. Ubiquitinated USP47 (top) and total USP47 (middle) proteins were analyzed by Western blotting with an anti-HA Ab. (Bottom) β-TrCP was analyzed with an anti-Myc Ab. (E) The catalytic domain of USP47 is sufficient for deubiquitinating USP47. HEK293T cells were transfected with His-Ub, Myc-tagged β-TrCP, HA-tagged USP47, USP47m, and Flag-tagged USP47CD. The cells were treated with MG132 for 6 h and lysed in PTU buffer. Ubiquitin-conjugated proteins were pulled down by Ni-NTA. Ubiquitinated USP47 and total USP47 proteins were analyzed by Western blotting with an anti-HA Ab. (Bottom) β-TrCP proteins were analyzed with an anti-Myc Ab. USP47CD was analyzed with an anti-Flag Ab. (F) β-TrCP regulates USP47 degradation. HEK293T cells were transfected with control vector or Myc-β-TrCP plus His-Ub. After 36 h, cells were treated with CHX (40 μM) with or without MG132 (25 μM) for 6 h before harvesting. The protein levels of USP47 and myc-tagged β-TrCP were analyzed by Western blotting, with GAPDH as a loading control.

USP47/UBP64E regulates Wnt signaling in Drosophila. To examine the physiological function of USP47 in vivo, we turned to the Drosophila wing model. We performed both loss- and gain-of-function studies in Drosophila wings to examine the regulation of β-catenin/Arm by USP47/UBP64E. We found that the expression of UBP64E RNAi by MS1096 Gal4 induced a wing margin bristle phenotype, which is a partial loss of the Wingless/Wnt phenotype (Fig. 6, compare panel D with wild-type wings in panels A to C). The coexpression of Dicer with UBP64E RNAi dramatically enhanced this phenotype and severely disrupted the wing morphology (Fig. 6, compare panels E and F with wild-type wings in panels A and G, respectively). The wing margin phenotype induced by UBP64E RNAi is unlikely to be due to an off-target effect because another transgenic RNAi line (v103743) targeting a nonoverlap-
The USP47 proteins were immunoprecipitated with an anti-HA Ab, and the interacting Arm in wing discs. As shown in Fig. 6O, the expression of UBP64E and total USP47 (bottom) proteins were analyzed by Western blotting with an anti-HA Ab. With MG132 for 6 h before harvesting and lysed in PTU. Ubiquitin-conjugated proteins were pulled down by Ni-NTA. Ubiquitinated USP47 (top) and total USP47 (middle) and bottom) The expression levels of USP47 (middle) and potential -TrCP binding. HEK293T cells were transfected with His-Ub, together with different HA-tagged USP47 constructs. The cells were treated with MG132 for 6 h before harvesting and lysed in PTU. Ubiquitin-conjugated proteins were pulled down by Ni-NTA. Ubiquitinated USP47 (top) and total USP47 (bottom) proteins were analyzed by Western blotting with an anti-HA Ab.

To examine the role of UBP64E in regulating Wnt target genes, UBP64E RNAi was coexpressed with Dicer in wing discs by MS1096 RNAi was coexpressed with Dicer in wing discs by Engrailed (en)-Gal4. We found that the inactivation of UBP64E abolished the expression of the Wnt Distalless (Dll) target gene (Fig. 6, compare panel M with panels K and L) but did not affect the expression of Patched (Ptc), which is a target gene in Hedgehog signaling (Fig. 6N), suggesting the pathway specificity of UBP64E.

We next examined whether UBP64E regulates the stability of Arm in wing discs. As shown in Fig. 6O, the expression of UBP64E RNAi by the posterior cell-specific Engrailed (en)-Gal4 inhibited Arm accumulation in these cells (Fig. 6O, left), leading to the attenuated expression of Senseless (Sens) (Fig. 6O, middle), and Wingless (Fig. 6P, left). We also found that the overexpression of UBP64EC >S blocked the accumulation of Arm and attenuated the expression of Sens (Fig. 6Q). The efficiency of UBP64E RNAi was analyzed by quantitative PCR (qPCR) (Fig. 6R). These data suggest that UBP64E is required for Wingless/Wnt signal transduction in Drosophila wing discs. The levels of Arm accumulation in wing discs correlated with the adult wing phenotypes when UBP64E RNAi or UBP64EC >S was expressed, suggesting that the adult wing phenotypes were caused, at least in part, by UBP64E-mediated regulation of Arm.

DISCUSSION

β-Catenin degradation is tightly regulated by Wnt signaling in normal cells. Mutations in the Wnt pathway are associated with human cancers and many other diseases. β-Catenin is degraded by a ubiquitin/proteasome pathway that is mediated by the ubiquitin protein ligase β-TrCP. Our collaborative efforts led to the identification of a new regulator for β-catenin ubiquitination. We found that the deubiquitinase USP47/UBP64E interacts with and regulates β-catenin ubiquitination and degradation. The ubiquitination of USP47 is also regulated by β-TrCP. Our collaborative efforts led to the identification of a new regulator for β-catenin ubiquitination. USP47/UBP64E regulates the accumulation of β-catenin and the expression of Wnt target genes. Taken together, the data show that USP47/UBP64E directly deubiquitinates β-catenin and is a novel positive regulator for β-catenin stabilization.

USP47 was first identified as a β-TrCP-binding protein that regulates Cdc25A expression and cell survival (32). The effects of USP47 on other β-TrCP substrates, such as IκBα and β-catenin, are not clear. Since the majority of β-catenin proteins are localized on the cell membrane and only cytoplasmic and nuclear β-catenins are sensitive to degradation, it is difficult to examine the change in total β-catenin levels. We found that USP47 RNAi decreased the levels of nuclear β-catenin. More importantly, USP47/UBP64E RNAi decreased Arm levels in Drosophila, suggesting that USP47 regulates β-catenin stability both in vitro and in vivo.

Previous studies did not detect the effects of β-TrCP on USP47 (32). In our study, HEK293T cells were transfected with His-Ub, followed by immunoprecipitation with Ni-NTA beads, which was likely a more sensitive and reliable approach. Our results clearly demonstrated that β-TrCP regulates USP47 ubiquitination, which may lead to USP47 degradation. Since wild-type USP47 has deubiquitinase activity, it is difficult to detect the ubiquitination of wild-type USP47. It is much easier to detect the ubiquitination of mutant USP47, which loses the deubiquitinase activity.

In some cases, deubiquitinase binds its substrate through a ubiquitin ligase. For example, Mdm2 mediates the binding of HAUSP and P53 (33). The binding between USP47 and β-catenin is not β-TrCP dependent, because β-TrCP binds the N terminus...
of β-catenin and USP47 binds the C terminus of β-catenin. We found that USP47 has a novel β-TrCP recognition motif (DEGX\(XXE\)). This is not a typical β-TrCP-binding motif (DSGX/XXS) found previously (3). Several nontypical β-TrCP-binding motifs have been reported. For example, β-TrCP binds a DDGX\(XXD\) motif in Cdc25A and Cdc25B (34), suggesting that acidic residues can replace phosphoserine/phosphothreonine in the consensus β-TrCP-binding site. The β-TrCP-binding motif is located in a predicted loop of USP47 (35) and is thus likely to be surface exposed and poised to mediate protein interactions.

The Wnt pathway is conserved from invertebrates to vertebrates (2). The role of Wnt/Wingless signaling is well established in Drosophila. Many key components of the Wnt pathway, such as Wingless (Wg), Armadillo (β-catenin), Zeste-white 3 (GSK-3), and Slimb (β-TrCP), were identified or characterized in Drosophila (36–40). In Drosophila, Wingless promotes the accumulation and nuclear translocation of Armadillo, leading to the expression of its target genes, such as \(w^{g}\), \(dll\), and sens (41, 42). Our previous work has demonstrated that CK\(1α\) regulates Drosophila embryonic development by regulating β-catenin phosphorylation and degradation (3). We have also demonstrated that PP2A (Twins) regulates Drosophila wing development by regulating β-catenin dephosphorylation (18). The Drosophila model provides a powerful tool to study Wnt/Wg signaling in vivo. In this study, in vivo screening identified several RNAi lines, including USP8, causing wing phenotypes, such as small wing, sick wing, or abnormal margin bristles. However, only UBPE46 RNAi gave the most obvious Wnt phenotype. It has been reported that the expression of UBPE46 RNAi under the ubiquitous Gal4 or panneuronal Gal4 caused a drooping-wing phenotype (43). In our study, to examine the wingless phenotype, we used the wing-specific \(MS1096\) Gal4. We analyzed the levels of Arm and the expression of Wnt target genes,
and only UBP64E RNAi decreased the levels of both Arm and its targets (Fig. 6).

In conclusion, we have identified a β-catenin deubiquitase that regulates Wnt signaling in vitro and in vivo. Recently, it has been reported that the RNF220/USP7 complex can also deubiquitinate β-catenin (44). USP7 is closely related to USP47; it will be interesting to investigate whether they play redundant roles in β-catenin regulation. Although our studies suggest that USP47 regulates β-catenin deubiquitination, we cannot rule out the possibility that USP47 also regulates other components in the Wnt pathway, and the precise mechanism of β-catenin regulation by USP47 needs further study. In addition, USP47 has been reported to be a deubiquitinating enzyme for DNA polymerase γ that regulates DNA repair and genome integrity (45). In Drosophila, USP47 also controls cell fate by regulating the transcriptional repressor tramtrack (46). As demonstrated in Fig. 1E and F, knockdown of USP47 inhibited cancer cell proliferation, probably by affecting multiple targets of USP47. Given the roles of USP47 in Wnt signaling, cell survival, and DNA repair, USP47 may play an important role in cancer biology and may become a novel therapeutic target for anticancer agents.

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REFERENCES


