

Conjugation of Complex Polyubiquitin Chains to WRNIP1

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Werner helicase interacting protein 1 (WRNIP1) is a ubiquitin-binding protein that undergoes extensive post-translational modification including ubiquitination, sumoylation, and phosphorylation. These post-translational modifications are expected to regulate the function of WRNIP1 in the DNA damage response. In this study, we use a denaturing tandem affinity purification technique along with mass spectrometry to show that, unlike most ubiquitin-binding proteins, WRNIP1 is polyubiquitinated. WRNIP1 polyubiquitination is reminiscent of the well-characterized phenomenon of the coupled monoubiquitination of ubiquitin-binding proteins in that this polyubiquitination is dependent on the presence of an intact ubiquitin-binding domain. The polyubiquitin chains conjugated to WRNIP1 are linked through lysines 11, 48, and 63. This study presents the first evidence for the conjugation of K11-K48-K63 polyubiquitin chains to a specific substrate *in vivo*. Polyubiquitination is likely to regulate WRNIP1's function in the DNA damage response, as UV radiation induces the hyperubiquitination of WRNIP1. Polyubiquitination with noncanonical intraubiquitin linkages may represent a unique mode of regulation of UBZ domain-containing proteins.

Keywords: WRNIP1 • UBZ • ubiquitin • mass spectrometry • proteomics • degradation • proteasome • post-translational modification • monoubiquitination • polyubiquitination

Introduction

The functional diversity of the proteome is exponentially expanded by the post-translational modification of proteins. One such modification, remarkable both for its striking evolutionary conservation and broad range of substrates, is ubiquitination. Ubiquitin is the eponymous member of a family of small proteins which can be conjugated through its carboxyl terminus to a lysine residue within a target protein.¹ The ubiquitin conjugation reaction requires a cascade of enzymes to activate the ubiquitin molecule, select the proper substrate, and determine the appropriate manner of ubiquitination.²

The conjugation of a single ubiquitin molecule to a substrate protein is known as monoubiquitination.³ Monoubiquitination that occurs at several sites within the same protein is called multiubiquitination.³ At least three classes of proteins are frequently monoubiquitinated: histones, transmembrane proteins, and proteins that contain ubiquitin-binding domains.^{4–6} Nearly all ubiquitin-binding proteins are monoubiquitinated.⁷ The ubiquitination of ubiquitin-binding proteins is referred to as coupled monoubiquitination, due to a requirement for the presence of an intact ubiquitin-binding domain.⁷ Coupled monoubiquitination can result in autoinhibition through an

intramolecular interaction between the conjugated ubiquitin and the ubiquitin-binding domain.⁸ However, given the diverse cellular functions of ubiquitin-binding proteins, it is likely that the role of coupled monoubiquitination is context-dependent.

The consequences of conjugation to polyubiquitin chains are quite distinct from those of monoubiquitination. Ubiquitin polymers are formed via ubiquitination of ubiquitin through one of its seven internal lysine residues.⁹ Polyubiquitin chains with different intraubiquitin linkages have different structural characteristics, and preferentially interact with different ubiquitin binding proteins.^{10,11} All seven possible ubiquitin chain linkages have been observed by mass spectrometry in yeast, and six of the seven linkages have been seen in humans.^{9,12,13} The seventh linkage, through lysine 27 (K27), can be formed through *in vitro* ubiquitination assays and is therefore also likely to be utilized *in vivo*.¹⁴

Of the seven types of polyubiquitin chains, only a few have been functionally characterized. K48 linkages are the canonical signal for proteasomal degradation.¹⁵ Conjugation to K29-linked chains can also lead to degradation by the proteasome.¹⁶ In contrast, K63-linked polyubiquitin chains have been shown to play a signaling role in processes such as inflammation and DNA repair, and do not target substrates to the proteasome.^{17,18} K6-linked polyubiquitin chains may also play a role in DNA repair-related signaling, but this function is poorly understood.¹⁹

Mass spectrometry has become a key technology in elucidating the functional significance of monoubiquitination and different types of polyubiquitin chains. Because mass spec-

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trometry permits the study of post-translational modifications on proteins obtained from their native environment, information about the type, site, and relative abundance of ubiquitination can be obtained without prior knowledge of the modification.¹² In this study, we use mass spectrometry and other techniques to characterize the ubiquitination of Werner helicase interacting protein 1 (WRNIP1), a protein that connects the ubiquitin-proteasome system to DNA replication and repair pathways.

WRNIP1 was originally identified through its interaction with Werner helicase, and has subsequently been shown to function in DNA replication and repair.^{20–22} WRNIP1 binds ubiquitin through its ubiquitin-binding zinc finger (UBZ) domain.²³ Interestingly, all known proteins which contain *bona fide* UBZ domains function in the postreplication repair of DNA damage.^{23,24} These ubiquitin-binding DNA repair proteins include DNA polymerase η , DNA polymerase κ , WRNIP1, and RAD18. In addition to binding ubiquitin, these UBZ family proteins are also ubiquitinated in a UBZ domain-dependent fashion.^{23–25} A deeper understanding of this dual role of the UBZ domain has the potential to shed light on the manner in which postreplication DNA repair is regulated by the ubiquitin-proteasome pathway.

In this study, we find that WRNIP1 is polyubiquitinated, in contrast to the classic paradigm of a ubiquitin binding protein that undergoes coupled monoubiquitination. Analysis by mass spectrometry of the polyubiquitin chains conjugated to WRNIP revealed the first *in vivo* example of mixed K11-K48-K63-linked polyubiquitin chains, which may target WRNIP1 to the proteasome. The finding that WRNIP1 is hyperubiquitinated in response to UV-induced DNA damage provides new insight into the myriad ways that the UBZ family of proteins is regulated by the ubiquitin-proteasome pathway.

Materials and Methods

Constructs. pCGN-MAT, which contains a tandem hemagglutinin-metal affinity tag (HA-MAT) is derived from the pCGN vector.²⁶ The MAT tag was constructed by annealing the complementary phosphorylated oligonucleotides CTAGGCA-CAACCACGCCACAAGCACT and CTAGAGTGCTTGTGGCG-GTGGTTGTGC for 5 min at 95 °C, then allowing the DNA to cool to room temperature over the course of an hour. The annealed product was then ligated into pCGN digested with XbaI, yielding pCGN-MAT. pSV40TAg-CGN-MAT was constructed by PCR-subcloning of the SV40 large T antigen from the pT7-TAg vector²⁷ using the primers GGGTCTAGAATG-GATAAAAGTTAACAGAGAG and GGGGGATCCCTATGTTTCAG-GTTCTGGGGAGGTG. The PCR fragment was then digested with XbaI and BamHI, and ligated into a similarly digested pCGN-MAT vector. pFLAG-MAT2 was purchased from Sigma. pWRNIP1-FLAG-MAT2 has been previously described.²³ All constructs were verified by DNA sequencing and/or mass spectrometry of the resulting protein product.

Antibodies. Antibodies were purchased against total ubiquitin and beta-actin (Cell Signaling Technologies, clone P4D1 and catalog no. 4967, respectively), polyubiquitin (clone FK1, Biomol), and the FLAG epitope tag (clone M2, Sigma). The CSH2609 polyclonal antibody against human and mouse WRNIP1 has been previously described.²³ Agarose beads conjugated to antibodies that recognize the FLAG (clone M2) or HA (clone HA-7) epitope tags were purchased from Sigma.

Cell Culture. HEK293, IMR90, and 3T3 cells were cultured as recommended by American Type Culture Collection. Plas-

mid DNA was introduced into the cells by calcium phosphate transfection. The proteasome inhibitor MG132 (Z-Leu-Leu-Leu-CHO, Biomol) was reconstituted as a 4 mM stock in DMSO, and applied to the cells at a final concentration of 25 μ M.

Treatment Conditions. To screen for alterations in the post-translational modification of WRNIP1, the following treatments were applied to HEK293T cells: low serum (incubation in serum-free medium for 12 h), cycloheximide (30 μ g/mL, 12 h), vanillin (1 mM, 1 h), proteasome inhibitor (MG132, 10 μ M, 12 h), histone deacetylase inhibitor (trichostatin A, 0.1 μ g/mL, 12 h), hydroxyurea (25 mM, 12 h), camptothecin (1 μ M, 1 h), γ -irradiation (10 Gy, 1 h prior to harvest), UV irradiation (100 J/cm², 1 h prior to harvest), methyl methanesulfonate (MMS, 0.5 μ g/mL, 1 h), 4-nitroquinoline-1-oxide (4NQO, 1 μ g/mL, 1 h).

Cell Lysate Preparation. For mass spectrometry or gel-staining experiments, two 15 cm plates of near-confluent HEK293 cells were used for each purification. One 15 cm plate of cells was used for Western blotting experiments. To prepare lysates, the cells were washed twice with cold phosphate buffered saline, lysed in cold NP40 buffer (phosphate buffered saline, pH 7.5, 150 mM NaCl, 0.5% NP40, Roche Complete protease inhibitor tablet), sonicated in 0.5 s bursts for 20 s, and spun at 13 000 rpm in a microcentrifuge for 10 min to remove cell debris.

Denaturing Tandem Affinity Purification. Cleared cell lysates were incubated for 1 h on a rotator at 4 °C with 25 μ L of antibody-conjugated agarose beads. The beads were washed five times with cold NP40 buffer. For urea elution, 2 \times 200 μ L of DTAP buffer (8 M urea, 10 mM Tris-HCl, 115 mM NaH₂PO₄, 300 mM NaCl, and 0.1% NP40, pH adjusted to 8.0 with NaOH) was added directly to 25 μ L of antibody-conjugated beads, and the supernatant was removed following a 10 min incubation at room temperature. For peptide elution, the beads were incubated with 3XFLAG peptide (Sigma) at 100 μ g/mL in NP40 buffer. Solid urea was then added to the peptide eluate to a final concentration of 8 M. For both elution methods, the denatured eluate was incubated with 25 μ L of nickle(+2)-nitrilotriacetic acid beads (Ni-NTA, Qiagen) for 15 min at room temperature, and the beads were then washed five times with DTAP buffer.

Protein Analysis. SDS-PAGE was performed using precast gradient gels (NuPAGE Novex 4–12% Bis-Tris gel, 1 mm, Invitrogen) according to the manufacturer's instructions. For protein staining, gels were first fixed for 15 min in 50% methanol/7% acetic acid, and then stained with GelCode Blue (Pierce) according to the manufacturer's instructions.

Sample Preparation for Mass Spectrometry. For proteins eluted directly from affinity beads with trypsin, bead-bound proteins were washed an additional three times in 20 mM diammonium phosphate pH 8.0 (di-AP), and then incubated with 50 ng of sequencing grade modified trypsin (Promega) for 12 h at 37 °C. The supernatant was removed from the beads, reduced by boiling for 5 min with 10 mM Tris(2-carboxyethyl)phosphine (TCEP, Pierce), and alkylated with 15 mM iodoacetamide for 1 h in the dark. An equal volume of 5% formic acid was added prior to sample cleanup with C18 ZipTips (Millipore). Gel slices were reduced with 2.5 mM dithiothreitol for 30 min at 60 °C, alkylated by incubation with 200 mM iodoacetemide for 1 h in the dark, dehydrated by the addition of 100% methanol, rehydrated by the addition of 30% methanol, washed eight times in wash buffer (50% methanol, 20 mM di-AP, 0.01% NDSB) for 10–15 min each, washed in water for 10 min, and desiccated. The dried gel slices were then soaked in

10 μ L of 20 mM diAP containing 50 ng of trypsin, and left to digest at 37 °C overnight. An equal volume of 5% formic acid was added to the gel slices, which were then sonicated to release the peptides. The supernatant was then desalting with ZipTips according to the manufacturer's protocol. The samples used for mapping ubiquitin chain linkages were not subject to reduction and alkylation.

Mass Spectrometry. For electrospray ionization mass spectrometry, microcapillary columns were constructed using PicoFrit columns (outer diameter 360 μ m, inner diameter 75 μ m, no coating, 15 μ m internal diameter tip). The capillary column was packed with 15 cm of 1.8 μ m Zorbax XDB C-18 particles using a homemade high-pressure column loader. The columns were equilibrated with 10% methanol/0.1% formic acid solution. The buffer solutions used to separate protein digests were 0.1% formic acid in water (buffer A) or in methanol (buffer B). A Micro-Tech splitless nano-HPLC was used at a flow rate of 500 nL/min. The tip of the pico-frit emitter was placed approximately 2 mm from the orifice of a Thermo Fisher LTQ mass spectrometer and electrospray was initiated by the application of 2.4 kV directly to the mobile phase. A cycle of one full scan (400–1700 *m/z*) followed by eight data-dependent MS/MS scans at 25% normalized collision energy was performed throughout the LC separation. The desalting sample was injected onto the microcapillary column at 10% buffer B. The samples were analyzed in 60 min runs, with a discontinuous gradient where buffer B increases to 25% over the course of 3 min and then to 55% over the next 40 min. Any remaining peptides were eluted by increasing the percentage of buffer B to 85% in 2 min.

LC-MALDI analysis of on-bead digests was performed using an Applied Biosystems 4800 mass spectrometer coupled with an Ultimate 3000 HPLC via a PROBOT target spotter. Briefly, the peptides were separated using an in-house packed column containing Jupiter Proteo Resin (15 cm \times 75 μ m). The gradient ranged from 5–50% acetonitrile in 0.1% TFA over a period of 90 min. Spots were acquired every 40 s at a flow rate of 300 nL/min using an LCPackings Probot. After the initial separation, the Probot applied 0.5 μ L of 2 mg/mL CHCA matrix onto the dried chromatography spots. The top 10 precursor ions from each spot were subjected to MS/MS analysis with 1500 laser shots and a laser intensity of 4100.

Data Analysis. RAW files from the LTQ were converted to mzXML files by ReadW (version 1.6). MGF files were created from the LC-MALDI data using TS2Mascot.exe (MatrixScience) with the mass range parameter set to a minimum of 60 Da and a maximum mass equal to that of the precursor ion. Monoisotopic peaks with a signal-to-noise ratio of at least 10-fold were included in the analysis.

All data files were searched against the Ensembl human protein database (v39) by the Global Proteome Machine interface to the X!Tandem algorithm (version 2006.06.01.2).²⁸ Searches were conducted using the following parameters: −2/+4 Da precursor ion mass error, 0.4 Da product ion mass error, complete carbamidomethylation of cysteines (+57 Da), partial oxidation of methionine (+16 Da), partial deamidation of asparagine and glutamine (−1 Da), and partial ubiquitination of lysine (+114.1 Da).

Results

Denaturing Tandem Affinity Purification (DTAP). The functional significance of the coupled monoubiquitination of ubiquitin-binding proteins is still a subject of debate. Our goal

is to further characterize the ubiquitination of the ubiquitin-binding protein WRNIP1. WRNIP1 binds polyubiquitin through its UBZ domain, and is ubiquitinated, sumoylated, and phosphorylated.^{23,29–32} We were interested in learning whether the ubiquitination of WRNIP1 fits the model of stable coupled monoubiquitination previously noted for other ubiquitin-binding proteins.⁷

The fact that WRNIP1 interacts both covalently and noncovalently with ubiquitin presents a purification challenge. At least one of the purification steps must take place under strongly denaturing conditions, in order to remove all noncovalently bound ubiquitin. The most commonly used strategy for denaturing purifications is the binding of a metal affinity tag (MAT) to a nickel-nitrilotriacetic acid (Ni-NTA) matrix in a buffer that contains a chaotropic agent such as urea or guanidinium chloride.^{9,33} However, as many endogenous proteins also have strong affinity for the Ni-NTA matrix, a single-step Ni-NTA purification protocol is usually insufficient to obtain the purity required for an in-depth analysis of the post-translational modifications of a single protein. We therefore developed a tandem affinity purification strategy with a strongly denaturing step to purify the protein of interest away from its noncovalently bound interaction partners.²³

Our denaturing tandem affinity purification (DTAP) protocol consists of two stages: a nondenaturing immunoprecipitation with an antibody against an epitope tag, followed by a denaturing purification with Ni-NTA beads (Figure 1A).²³ The second affinity step, which is performed under strongly denaturing conditions, eliminates protein partners which interact with the tagged protein. Prior to the incubation with the Ni-NTA beads, the protein complexes must be eluted from the antibody beads used in the first step. This elution can be accomplished either by the direct addition of the denaturing agent, or by incubation with a competitive peptide containing the epitope recognized by the antibody. The combination of a nondenaturing immunoaffinity step with a strongly denaturing chromatography step maximizes both purity and yield.

To test the efficiency of this protocol, we compared a single-step nondenaturing immunoprecipitation strategy versus the DTAP protocol. To demonstrate the utility of this technique with a variety of epitope tags and target proteins, we performed DTAP purifications from HEK293 cells transfected with either carboxy-terminal FLAG-MAT tagged WRNIP1, or SV40 large T antigen fused with an amino-terminal tandem tag containing the hemagglutinin epitope and the MAT sequence (HA-MAT). The single-step purification involved a nondenaturing immunoprecipitation with anti-FLAG or anti-HA beads as appropriate. For the DTAP protocol, parallel nondenaturing immunoprecipitations were performed, followed by one of two different strategies for eluting the proteins from the antibody bead. In the first elution method, the proteins are detached from the antibody bead by addition of DTAP buffer, which contains 8 M urea. The urea denatures the antibody and all of the proteins in the sample, releasing them from the bead. In the second variation, the proteins are eluted from the antibody bead with a competitive peptide, and then denatured by the addition of urea once the eluate has been removed from the antibody-conjugated beads. In both cases, the denatured eluate is then applied to the Ni-NTA beads for the second purification step (Figure 1A). For all purification strategies, the final step prior to SDS-PAGE was boiling the beads in Laemmli buffer or Laemmli buffer plus EDTA to elute all proteins.

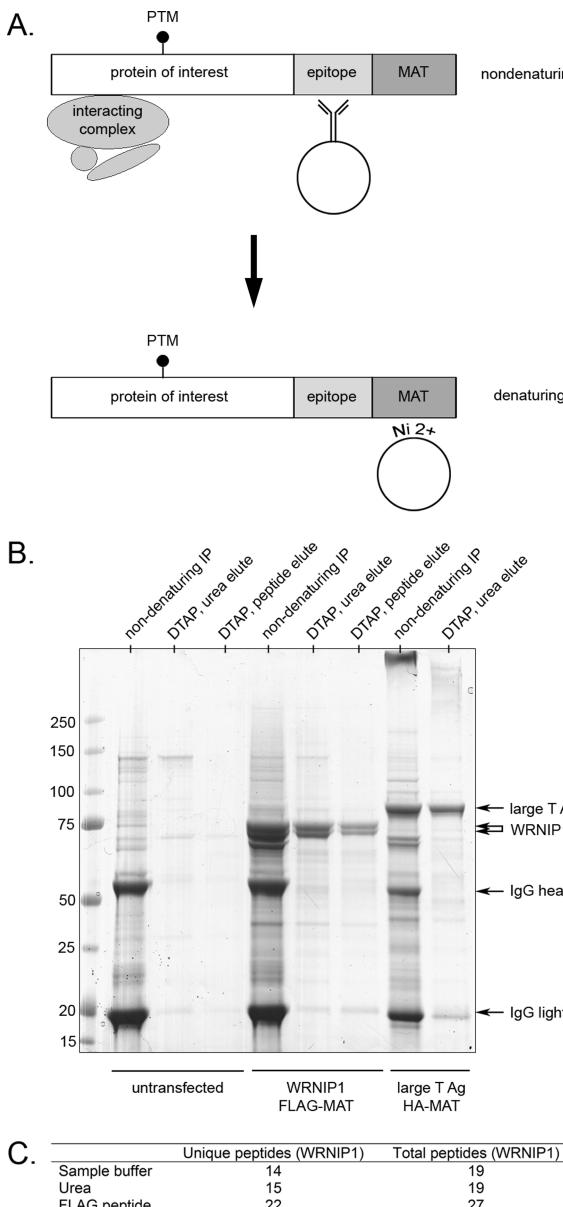


Figure 1. Denaturing tandem affinity purifications (DTAP). (A) The DTAP protocol consists of two steps: an immunoprecipitation against an epitope tag under non-denaturing conditions, followed by a denaturing purification using a Ni-NTA matrix to bind a metal affinity tag (MAT). (B) Test of the DTAP protocol with two different proteins (WRNIP1 and SV40 large T antigen) and two different tags (FLAG-MAT and HA-MAT). The lanes labeled “untransfected” were purified from cells that do not express an epitope-tagged protein. Each protein was purified by the method noted above the lane. Nondenaturing immunoprecipitations (IPs) consisted of a single-step affinity purification with anti-FLAG or anti-HA antibody conjugated beads. DTAP purifications consisted of a nondenaturing IP followed by elution with urea or the FLAG peptide, and subsequent affinity purification on Ni-NTA beads under denaturing conditions. In all lanes, the final elution prior to SDS-PAGE was accomplished by boiling in Laemmli buffer plus EDTA. (C) Table of the WRNIP1-derived peptides identified by LC-MALDI mass spectrometry from three different purification strategies: nondenaturing FLAG immunoprecipitation, DTAP purification eluted from the antibody beads with urea, or DTAP purification eluted from the antibody beads with the FLAG peptide. For all three purification strategies, the final elution step was incubation with trypsin to release peptides suitable for analysis by mass spectrometry.

Under nondenaturing conditions, numerous specific bands can be observed for both WRNIP1 and large T antigen, representing protein complexes in which WRNIP1 or large T antigen are members (Figure 1B). Both the specific bands and the nonspecific background are significantly reduced in the DTAP purifications. The peptide elution results in a lower nonspecific background than urea elution (Figure 1B). We subsequently subjected tryptic peptides from WRNIP1-FLAG-MAT purified by these three methods to analysis by LC-MALDI mass spectrometry. Despite the different total amounts of proteins in these samples, trypsin is not a limiting factor in the digestion, given the reaction conditions (Supplementary Figure 1). Purification by DTAP followed by elution with the FLAG peptide consistently yielded significantly more peptides from the bait protein than did a single-step purification or elution with urea (Figure 1C). Although examination of the stained gel reveals that the DTAP purifications yield slightly lower amounts of WRNIP1 protein than the nondenaturing immunoprecipitation, more peptides were identified by mass spectrometry from the DTAP sample because the proportion of WRNIP1 relative to other proteins in the sample has dramatically increased (Figure 1B and 1C). The resulting increase in the number of fragmentation events of peptides derived from the bait protein is advantageous for the search for novel protein post-translational modifications.

WRNIP1 Is Conjugated to Mixed Polyubiquitin Chains. WRNIP1 is a ubiquitin-binding protein, and might therefore be expected to undergo monoubiquitination. However, when WRNIP1 is purified from human cells and observed by Western blotting, the resulting smear runs from approximately 75 kDa (the predicted molecular mass of unmodified WRNIP1) to well over 250 kDa.²³ The same region of the blot also reacts with antibodies against ubiquitin.²³ This extensive ubiquitination does not seem likely to result solely from multiple monoubiquitination, so we chose to analyze the ubiquitination of WRNIP1 by mass spectrometry, looking for peptides indicative of the presence of polyubiquitin chains.

To further characterize the ubiquitination of WRNIP1, we purified WRNIP1 by the peptide elution variant of the DTAP protocol, to eliminate all noncovalently bound ubiquitin and other interacting proteins. The various species of WRNIP1 were then separated by SDS-PAGE and visualized by Coomassie blue staining (Figure 2A). Fourteen bands were excised, beginning at the top of the gel and continuing down to include the lowest WRNIP1 bands (Figure 2A). Each of these bands was then analyzed as a separate fraction by electrospray ionization mass spectrometry.

WRNIP1 and ubiquitin were readily detected in each of the 14 bands (Supplementary Table 1). In total, 204 peptides from ubiquitin were identified with a log(e) score of ≤ -3 . Fifty-three of the 204 peptides identified from ubiquitin contained the characteristic ubiquitination mass shift (+114.1 Da on lysine residues), and were therefore indicative of the polyubiquitin chain linkage. Peptides derived from ubiquitin were more likely to be found in slices from the higher molecular weight region of the gel, where polyubiquitination would be expected to be most prevalent (Figure 2B). In contrast, the slices closer to the 75 kDa molecular weight of WRNIP1 had a higher proportion of peptides derived from WRNIP1 relative to ubiquitin (Figure 2C).

Polyubiquitin chains can be formed from any one of seven different lysines in ubiquitin. Of these seven lysines, we identified ubiquitination sites on three of them: K11, K48, and

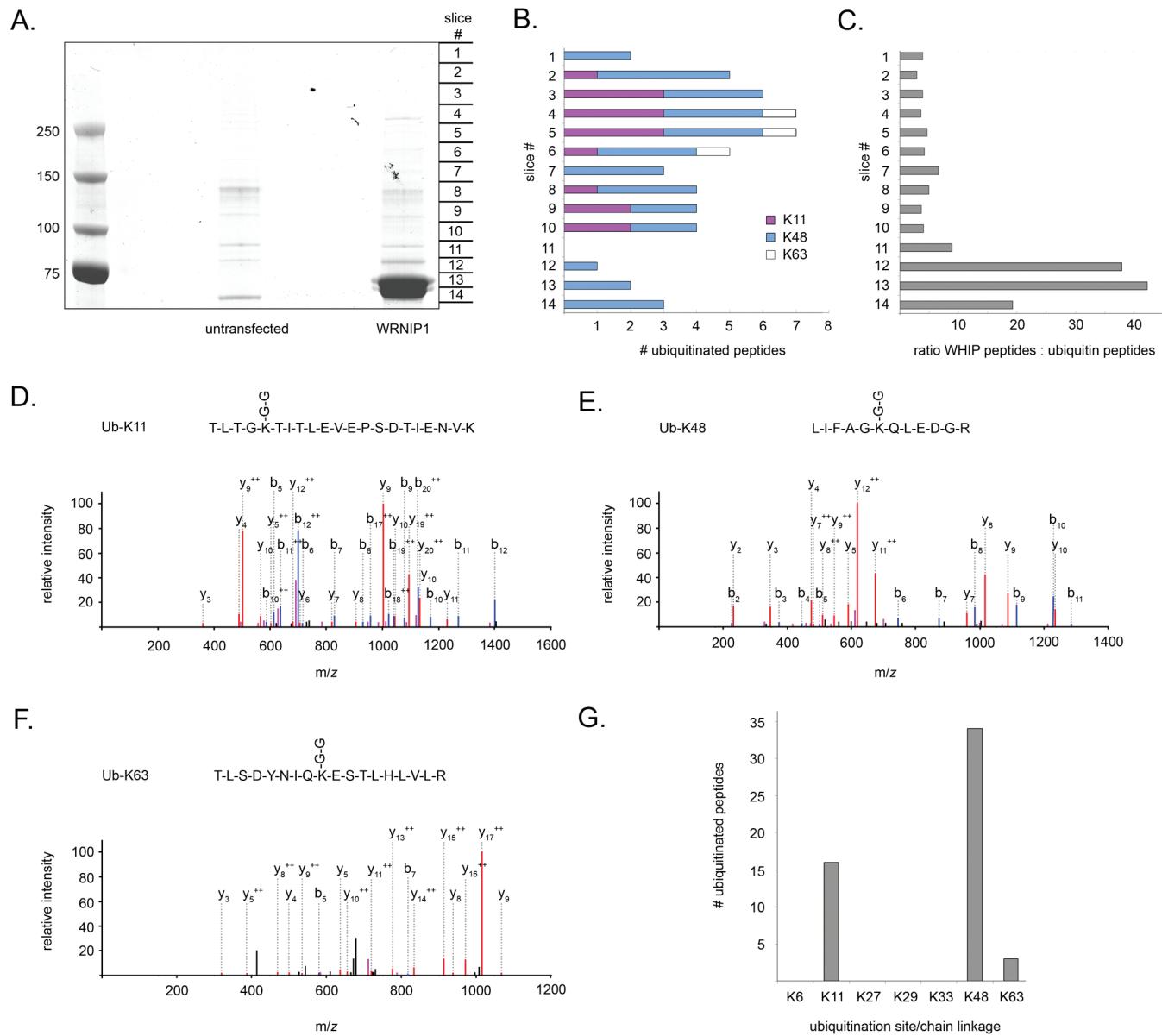


Figure 2. Conjugation of WRNIP1 to K11-K48-K63-linked polyubiquitin chains. (A) DTAP-purified WRNIP1-FLAG-MAT was separated by SDS-PAGE and stained with GelCode Blue to visualize proteins. The lane marked “untransfected” is a DTAP purification from cells that do not express an epitope-tagged protein. The WRNIP1 lane of the gel was cut into 14 slices as indicated, and each slice was subjected to LC-MS/MS analysis on a Thermo LTQ mass spectrometer. (B) The number and type of ubiquitinated peptides from ubiquitin identified in each gel slice from part A. (C) Ratio of the number of WRNIP peptides to the number of ubiquitin peptides identified in each gel slice from part A. (D) Representative diagnostic spectrum from a K11-linked polyubiquitin chain (only y- and b-ions are noted). The x-axis represents the m/z value of the fragment ions, and the y-axis notes the relative intensity. The amino acid sequence of the peptide is noted above the spectrum. (E) Representative diagnostic spectrum from a K48-linked polyubiquitin chain. (F) Representative diagnostic spectrum from a K63-linked polyubiquitin chain. (G) Number of intraubiquitin chain linkages identified for each lysine in ubiquitin.

K63 (Figure 2D-F). In repetitions of this experiment, not a single ubiquitination site on K6, K27, K29, or K33 of ubiquitin has ever been observed. K48-ubiquitinated peptides were the most frequently identified, followed by K11, with three peptides observed that were indicative of K63-linked ubiquitination (Figure 2G).

Confirmation of WRNIP1 Polyubiquitination. Ubiquitin-binding proteins are generally thought to be monoubiquitinated, but mass spectrometry of the post-translational modifications of WRNIP1 demonstrates that WRNIP1 is covalently conjugated to polyubiquitin chains. To confirm this result, and to further characterize the extent of WRNIP1

polyubiquitination, we used the monoclonal antibody FK1, which binds polyubiquitin, but does not react with free monoubiquitin or monoubiquitinated proteins.³⁴ DTAP-purified WRNIP1 was split into two aliquots, and blotted with monoclonal antibodies against total ubiquitin (clone P4D1) and polyubiquitin (clone FK1). WRNIP1 reacted with both antibodies, indicating that WRNIP1 is polyubiquitinated. However, WRNIP1 carrying a mutation in a conserved residue of the UBZ domain reacted with neither antibody (Figure 3). The smear on the P4D1 total ubiquitin blot begins at a lower molecular mass than the FK1 polyubiquitin smear, suggesting that WRNIP1 is both monoubiquitinated and

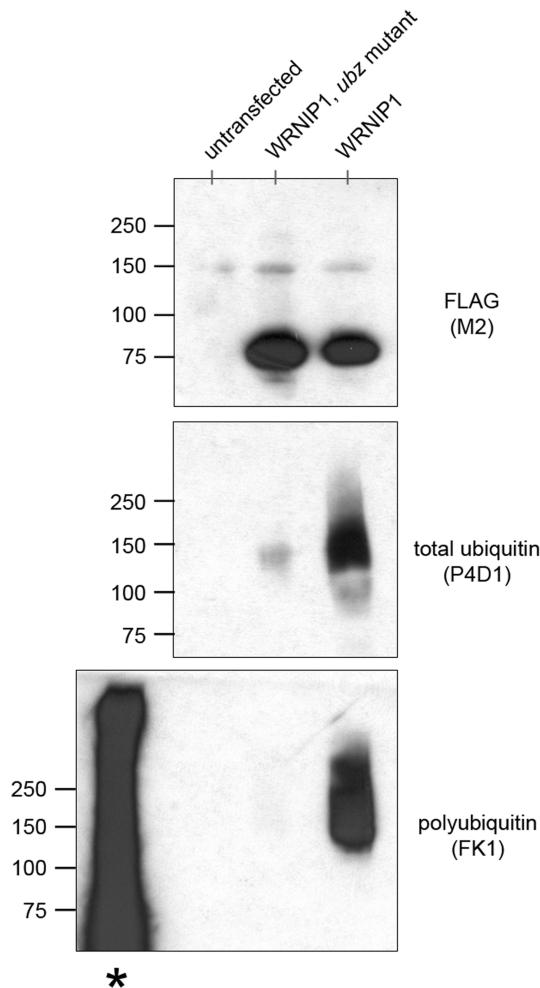


Figure 3. WRNIP1 is polyubiquitinated. WRNIP1-FLAG-MAT was purified by the DTAP protocol, and blotted with antibodies against FLAG, total ubiquitin (clone P4D1), or polyubiquitin (clone FK1). The *ubz* mutant WRNIP1 construct in the middle lane contains the D37A mutation which abolishes ubiquitin binding. The asterisk (*) indicates loading of 200 ng of polyubiquitin (K48-linked polyubiquitin chains 1–7, Boston Biochem) as a positive control for the FK1 antibody.

polyubiquitinated (Figure 3). The inclusion of a WRNIP1 construct with a mutation in the UBZ domain (D37A) confirms that this polyubiquitination is dependent on the presence of a functional ubiquitin-binding domain, and can therefore be considered a form of coupled ubiquitination.²³

WRNIP1 Is Degraded by the Proteasome. The conjugation of complex K11-K48-K63 polyubiquitin chains to cyclin B1 *in vitro* results in its degradation by the proteasome.³⁵ We tested whether WRNIP1 may also be subjected to proteasomal degradation. Mouse and human fibroblast cell lines (3T3 and IMR90, respectively) were treated with the proteasome inhibitor MG132, and lysates from these cells were then subjected to Western blotting with an antibody that recognizes endogenous human and murine WRNIP1. WRNIP1 can be detected as multiple bands in a Western blot with the CSH2609 polyclonal antibody.²³ WRNIP1 levels were observed to increase after 10 h in both mouse and human cells, indicating that WRNIP1 may be degraded by the proteasome (Figure 4A).

WRNIP1 Is Hyperubiquitinated in Response to UV-Induced DNA Damage. As WRNIP1 is heavily modified with ubiquitin and other post-translational modifications, we screened for

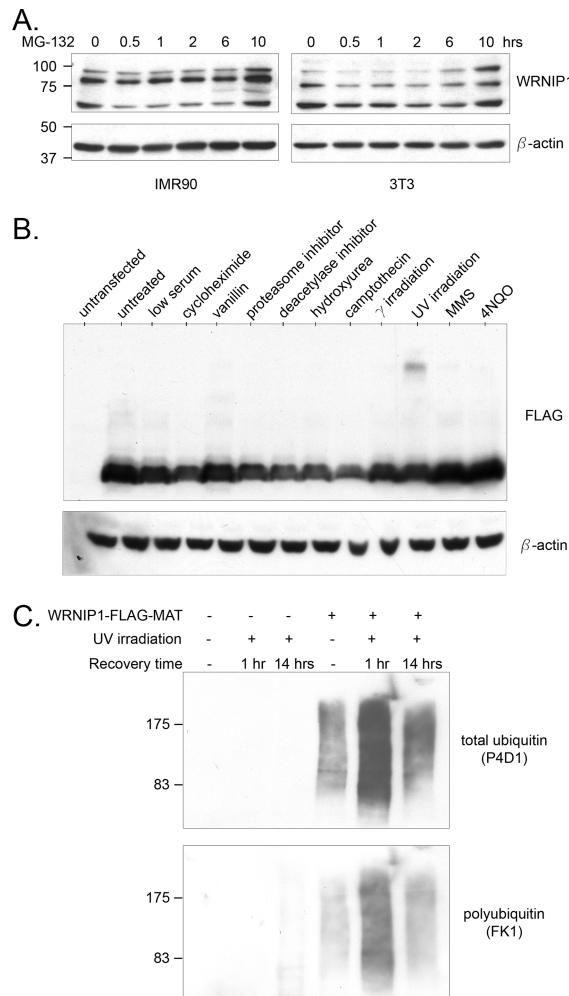


Figure 4. WRNIP1 polyubiquitination and degradation. (A) WRNIP1 is degraded by the proteasome. Human (IMR90) or murine (3T3) fibroblasts were incubated with the proteasome inhibitor MG132 for the indicated period of time. Cell lysates were blotted with antibodies against WRNIP1 or β -actin. (B) WRNIP1 is hyperubiquitinated after UV-induced DNA damage. HEK293 cells expressing WRNIP1-FLAG-MAT were mock treated, or exposed to 10 J/m² UV irradiation and allowed to recover for the indicated period prior to harvesting of cell lysates. WRNIP1 was then purified according to the DTAP protocol, and the resulting immunoprecipitates were blotted with antibodies against total ubiquitin (P4D1) or polyubiquitin (FK1). (C) The post-translational modifications of WRNIP1 are altered upon treatment of cells with UV irradiation. HEK293 cells expressing WRNIP1-FLAG-MAT were exposed to a variety of treatments (see Materials and Methods for details). Lysates from these cells were blotted with antibodies against the FLAG epitope and β -actin.

conditions under which the post-translational modifications of WRNIP1 are visibly altered. Gross changes in protein post-translational modification can often be visualized as an alteration in electrophoretic mobility during SDS-PAGE. To screen for conditions under which an alteration of WRNIP1's post-translational modifications could indicate the regulation of protein function, we transfected pFLAG-MAT2-WRNIP1 into HEK293 cells, and performed a Western blot against the FLAG epitope on cell lysates harvested after a variety of treatments (Figure 4B). The treatments included six DNA damaging agents (hydroxyurea, camptothecin, ionizing radiation, UV irradiation, methyl methanesulfonate, and 4-nitroquinoline-1-oxide), as

well as a variety of other conditions. In the cells treated with UV irradiation, significantly more signal could be observed from the high molecular weight region of the blot, indicating a specific increase in post-translational modification for this type of DNA damage (Figure 4B). Interestingly, WRNIP1 and the rest of the UBZ family of ubiquitin-binding proteins are known to participate in the response to UV-induced DNA damage.^{21,36,37}

To test whether this alteration in the electrophoretic mobility of WRNIP1 after UV irradiation can be attributed to an increase in ubiquitination, we performed DTAP purifications of WRNIP1 from untreated or UV-treated cells, and then blotted with antibodies against total ubiquitin and polyubiquitin (Figure 4C). Total levels of both ubiquitinated WRNIP1 and polyubiquitinated WRNIP1 rose 1 h after exposure to UV irradiation, and returned to normal levels by 14 h later (Figure 4C), indicating that the post-translational modification of WRNIP1 with ubiquitin is specifically regulated in response to UV-induced DNA damage.

Conclusions

Exception of UBZ Family Proteins from Coupled Monoubiquitination. In this study, we use mass spectrometry and other techniques to demonstrate that the ubiquitin-binding protein WRNIP1 is polyubiquitinated. Most ubiquitin-binding proteins are thought to be monoubiquitinated. The monoubiquitination of ubiquitin-binding proteins is referred to as coupled monoubiquitination, because the modification is invariably dependent on the presence of an intact ubiquitin-binding domain. The polyubiquitination of WRNIP1 stands as an apparent contradiction to the theory of coupled monoubiquitination. However, this unusual modification of WRNIP1 does show features reminiscent of coupled monoubiquitination. A significant fraction of WRNIP1 appears to be monoubiquitinated, and both the mono- and polyubiquitination are strictly dependent on the UBZ domain. Furthermore, the degradation of WRNIP1 by the proteasome appears to be slow, consistent with the observations that WRNIP1 is a relatively stable protein.^{22,23}

Interestingly, another UBZ domain-containing protein, Rad18, is also polyubiquitinated and degraded.³⁸ As with WRNIP1, both the mono- and polyubiquitination of Rad18 require the presence of a functional UBZ domain, and the two proteins are degraded at a similarly slow rate.²⁴ Proteins which contain UBZ domains appear to share many properties, including specific noncovalent interactions with ubiquitin, participation in the same postreplication DNA repair pathway, and now a unique propensity among ubiquitin-binding proteins to undergo polyubiquitination and degradation.^{23,24,38} Two other UBZ domain-containing proteins also undergo UBZ domain-dependent ubiquitination: the Y-family DNA polymerases η and κ .²⁴ While these modifications were reported as monoubiquitination, the possibility that these two proteins also undergo polyubiquitination and degradation has not yet been investigated.²⁴

K11-K48-K63-Linked Polyubiquitin Chains in Multiple Biological Contexts. We have used mass spectrometry to determine that the polyubiquitin chains covalently conjugated to WRNIP1 are linked through lysines 11, 48, and 63 of ubiquitin. While the mass spectrometry data clearly demonstrate that polyubiquitin chains linked through these lysine residues are conjugated to WRNIP1, the configuration of these chains remains entirely unknown. Three possible chain configurations are illustrated in Figure 5. In one scenario, each molecule of polyubiquitinated WRNIP1 is conjugated to pure

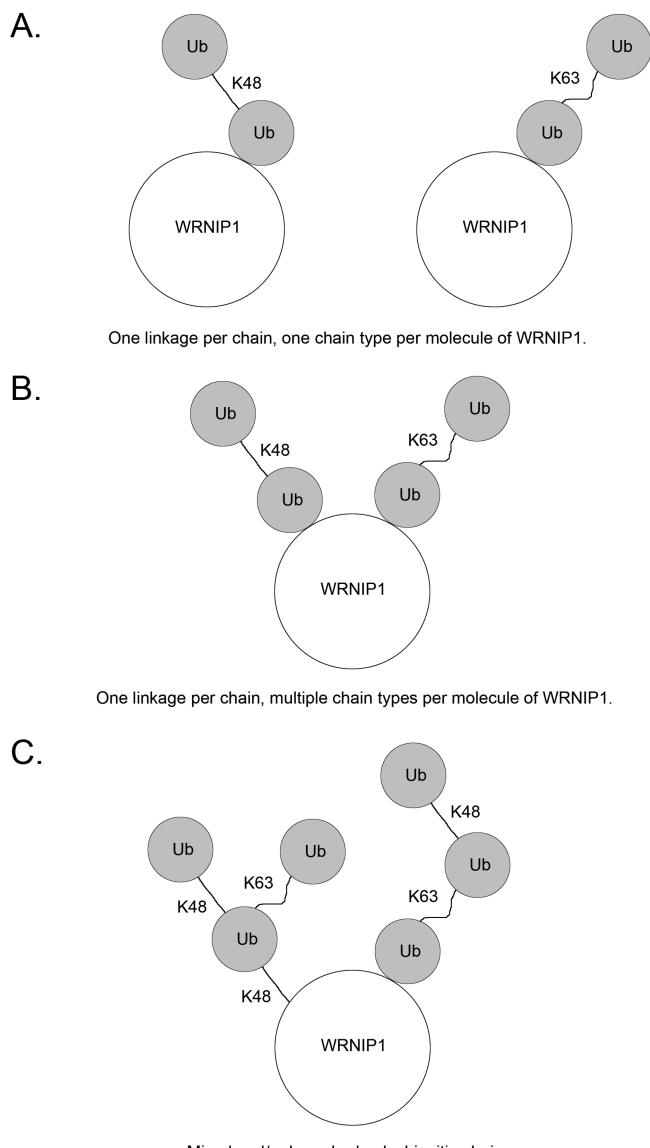


Figure 5. Possible conformations of polyubiquitin chains conjugated to WRNIP1. WRNIP1 is conjugated to polyubiquitin chains linked through lysines 11, 48, and 63. Several possible configurations of these complex polyubiquitin chains are illustrated in this diagram. (A) Pure polyubiquitin chains. Each molecule of WRNIP1 is conjugated only to a single chain type. (B) Pure polyubiquitin chains. Each molecule of WRNIP1 can be conjugated to multiple polyubiquitin chain types. (C) Mixed and/or branched polyubiquitin chains.

polyubiquitin chains containing a single chain linkage (Figure 5A). In this case, the total cellular pool of ubiquitinated WRNIP1 would be subdivided into at least three distinct fractions, one for each polyubiquitin chain linkage. (A separate pool of WRNIP1 which is sumoylated but not ubiquitinated has already been shown to exist.)²³ Given that pure K48- and K63-linked chains are already known to confer dramatically different fates on their protein partners, the scenario presented in Figure 5A raises the interesting possibility that distinct pools of WRNIP1 within the cell are regulated by different ubiquitin ligases. If this configuration for WRNIP1's polyubiquitin chains holds true, then the hyperubiquitination of WRNIP1 after UV-induced DNA damage may in fact represent the specific upregulation of only one type of polyubiquitin chain. Instances where K63-

linked polyubiquitination is upregulated in response to DNA damage are well-documented, suggesting that the K63-linked pool of WRNIP1 could play a repair-specific role.^{39,40} WRNIP1 hyperubiquitination may therefore represent the augmentation of the repair-capable pool of WRNIP1 in response to newly detected DNA lesions.

A second possible scenario still involves pure polyubiquitin chains (each chain containing a single type of intraubiquitin linkage), but would allow for multiple types of polyubiquitin chains on a single molecule of WRNIP1 (Figure 5B). This scenario is feasible, because 12 ubiquitination sites on WRNIP1 have been previously identified, some of which are known to be ubiquitinated simultaneously.²³ The significance of such a ubiquitination scheme is unclear. At least one other protein, receptor interacting protein (RIP), can be modified with both K48- and K63-linked polyubiquitin chains on a single molecule, although in this case the two chain types occur sequentially and not contemporaneously.⁴¹

A third possible configuration of WRNIP1's polyubiquitination is the presence of mixed or branched chains (Figure 5C). A mixed chain results when a single polyubiquitin chain has intraubiquitin linkages through more than one lysine at different points throughout the length of the chain (Figure 5C, right-hand chain). Branched polyubiquitin chains are a subset of mixed chains in which a single ubiquitin moiety within a polyubiquitin chain is connected to the carboxy-termini of two or more donor ubiquitin molecules, creating a branch point in the chain (Figure 5C, left-hand chain). Mixed and branched chains have been observed as products of *in vitro* ubiquitination reactions, but their relevance in a physiological setting remains to be determined.⁴²

This latter scenario of WRNIP1 conjugated to mixed or branched polyubiquitin chains brings up the question of the biological function of this novel polyubiquitin configuration. The current prevailing hypothesis is that K48-linked polyubiquitin chains of at least four units in length are the predominant signal for degradation by the proteasome. Most of the data supporting this hypothesis were obtained using ubiquitin constructs containing various lysine mutations, or synthetic polyubiquitin chains. Advances in mass spectrometry, along with the development of specialized purification protocols such as DTAP, permit the direct analysis of ubiquitination and other post-translational modifications on a protein that was modified *in vivo*. Data from this study and two other recent reports converge into a new model for how proteins can be marked with ubiquitin as a signal for degradation by the proteasome.^{35,43} In this model, K11-K48-K63-linked polyubiquitin chains can also serve as a signal for degradation by the proteasome.

Kirkpatrick et al. recorded the first example of mixed K11-K48-K63-linked polyubiquitin chains.³⁵ These chains were formed in an *in vitro* ubiquitination reaction on cyclin B1, a known substrate for the proteasome.⁴⁴ The finding that both cyclin B1 and WRNIP1 are both conjugated to the same type of complex polyubiquitin chains, and are both targeted to the proteasome, supports a model in which these K11-K48-K63-linked chains are a novel signal for proteasomal degradation.

A second key aspect of the model proposed by Kirkpatrick et al. also holds true for WRNIP1. Cyclin B1 is first monoubiquitinated on numerous sites, and the chains are subsequently extended through the K11-K48-K63 linkages. The authors postulate that the signal for recognition by the proteasome can be a relatively dense population of short K11-K48-K63-linked polyubiquitin chains, instead of the canonical single long K48-

linked chain. WRNIP1 is ubiquitinated on at least 12 distinct lysines, while nine ubiquitination sites have been observed on cyclin B1. WRNIP1's ubiquitination sites occur in several clusters along the length of the protein, which would allow the ubiquitin receptor of the proteasome to sense the presence of multiple short polyubiquitin chains. Thus, our data regarding WRNIP1 suggest that this novel model of proteasomal recognition of short, dense, complex polyubiquitin chains could occur *in vivo*, and is more generally applicable than just to cyclin B1. Another study shows that the absolute abundance of K11, K48, and K63 linked polyubiquitin chains increased in a variety of situations where proteasome function was compromised, implying that these polyubiquitin chains are normally substrates for the proteasome.⁴³ These results suggest that a significant proportion of the K11, K48, and K63 linked polyubiquitin formed within the cell is normally degraded.⁴³

In this study, we have shown that WRNIP1 is the first example of the conjugation of these chains *in vivo* to a specific substrate. Several lines of evidence point to the importance of this novel post-translational modification to the regulation of the function of WRNIP1 and perhaps other UBZ family proteins in DNA repair. A deeper understanding of the purpose and mechanism of these complex polyubiquitin chains has the potential to illuminate the process by which the cell maintains genomic stability.

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Supporting Information Available: Supplementary Table 1, mass spectrometry statistics for WRNIP1 and ubiquitin, and Supplementary Figure 1, on-bead digestion of affinity-purified proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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