Functional Characterization of a WWP1/Tiul1 Tumor-derived Mutant Reveals a Paradigm of Its Constitutive Activation in Human Cancer*

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Background: WWP1/Tiul1 plays an instrumental role in cancer pathogenesis by restricting TGFβ cyostatic signaling.

Results: We identified a novel mechanism of inhibition of WWP1 that is disrupted by a tumor mutation found in prostate cancer.

Conclusion: Mutational activation of WWP1 contributes to the loss of TGFβ signaling in cancer.

Significance: Our data unveil a paradigm behind WWP1 hyperactivation in cancer.

Although E3 ubiquitin ligases are deemed to play key roles in normal cell function and homeostasis, whether their alterations contribute to cancer pathogenesis remains unclear. In this study, we sought to investigate potential mechanisms that govern WWP1/Tiul1 (WWP1) ubiquitin ligase activity, focusing on its ability to trigger degradation of TGFβ type I receptor (TβRI) in conjunction with Smad7. Our data reveal that the WWP1 protein is very stable at steady states because its autopolyubiquitination activity is silenced due to an intra-interaction between the C2 and/or WW and Hect domains that favors WWP1 monoubiquitination at the expense of its polyubiquitination activity, thereby driving its own degradation and that of TβRI as well. Intriguingly, a WWP1 point mutation found in human prostate cancer disrupts this regulatory mechanism by relieving the inhibitory effects of C2 and WW on Hect and thereby causing WWP1 hyperacitvation. That cancer-driven alteration of WWP1 culminates in excessive TβRI degradation and attenuated TGFβ cyostatic signaling, a consequence that could conceivably confer tumorigenic properties to WWP1.

The posttranslational modification known as ubiquitination involves the covalent attachment of a polypeptide ubiquitin to a protein substrate. In addition to its well established function in protein degradation, ubiquitination regulates a plethora of other biological processes, including endocytosis, DNA damage response, translation control, and subcellular localization, depending on the type of the ubiquitin chain linkage that is conjugated. Proteins can be monoubiquitinated or polyubiquitinated. Ubiquitin possesses a total of 7 inner lysine residues (lysines 6, 11, 27, 29, 33, 48, and 63); all of them behave as a potential attachment site for another ubiquitin moiety, resulting in different subsets of ubiquitin chains (1). Polyubiquitin chains might also be heterogeneous with two or more ubiquitin moieties linked to distinct internal lysine residues (2). Irrespective of the type of ubiquitin chains, the process of ubiquitination is carried out by the sequential action of three types of enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. The E1 activates ubiquitin by attaching to it in an ATP-dependent manner; activated ubiquitin is then transferred to an E2, which in concert with E3, transfers it to target proteins (1).

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3 The abbreviations used are: Hect, homologous to E6-AP C-terminal; TβRI, TGFβ type I receptor; Dox, doxycycline; Ub, ubiquitin.
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dpolypeptide hinge connecting the N and C lobes is essential for WWP1 ligase activity. This structural architecture is found throughout the Nedd4 family, which appears to add ubiquitin one by one from the catalytic cysteine to the lysine at the end of a growing chain, a process termed sequential addition mechanism.

Because of its alterations in numerous malignancies, several published data have postulated that WWP1 functions as an oncogenic protein (3, 5). For instance, the WWP1 gene has been found to be amplified in more than 30% of breast and prostate cancer tumors (7–10), and several functional studies have shown that WWP1 knockdown was sufficient to suppress cell proliferation in prostate and breast cancer cell lines (7–9, 11, 12). Moreover, WWP1 has also been shown to regulate the stability of several cancer-related proteins, prominent among them LATS1, EGF receptor, HER4, Runx2, JunB, p27, CXC4, KLF2, and KLF5 (5, 13–20). In other cases, some cancer-related proteins are also ubiquitinated by WWP1 without being degraded, although the significance of these mechanisms remains unclear (21–23). Finally, others and we have shown previously that WWP1 functions as a negative regulator of TGFβ signaling, which has widespread roles in cancer pathogenesis. WWP1 inhibits TGFβ signaling by triggering degradation of several active components of this pathway, including the activated TGFβ type I receptor (TβRI). This degradation requires association with the inhibitory Smad, Smad7, which functions as a bridging factor between WWP1 and TβRI (24, 25).

In our efforts to further characterize the modes of action and regulation of WWP1, we found that this E3 was only able to self-catalyze its monoubiquitination at steady states, and this was correlated with the silencing of its polyubiquitination activity. Mechanistically, we identified an autoinhibitory mechanism between C2 or WW and Hect, and its disruption upon binding to the Smad7-TβRI complex switches its monoubiquitination activity to polyubiquitination activity, culminating in degradation of WWP1 itself as well as TβRI. From a translational perspective, we provide proof-of-concept experiments demonstrating that this regulatory mechanism is disrupted by a tumor-derived point mutation in WWP1 found in human prostate cancer. Thus, by identifying a mechanism of negative regulation of WWP1 enzymatic activity and validating its clinical relevance, these findings yield tantalizing insights into the contribution of this oncogenic ubiquitin ligase to the pathogenesis of human malignancies.

Experimental Procedures

Cell Culture and Transfection—HEK293, HeLa, and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). RWPE-1 cells were maintained in keratinocyte serum-free medium supplemented with 10% FCS (without tetracycline). To establish doxycycline (Dox)-inducible RWPE-1 cell lines, cells were infected with pLVX-Tet3G encoding the Dox transactivator and selected with G418 (500 μg/ml). Cells that express a high level of the transactivator were infected with pLVX-TRE3G-FLAG-WWP1.WT or pLVX-TRE3G-FLAG-WWP1.E798V, selected with puromycin (10 μg/ml), and maintained as a single population (RWPE-TR-FLAG-WWP1.WT cells and RWPE-TR-FLAG-WWP1.E798V cells). Lipofectamine reagent (Life Technologies) and DharmaFECT (GE Dharmacon) were used to transfect plasmids and siRNA, respectively, according to the manufacturers’ instructions. Cells were also cotransfected with GFP as a control of transfection efficiency.

Lentiviral Infections—To generate the lentiviruses producing the transactivator, pLVX-Tet3G was transfected into HEK293T cells along with the packaging mixture, and high titer lentiviruses were purified by centrifugation following the manufacturer’s guidelines (Thermo Scientific). A similar strategy was used to generate the lentiviruses pLVX-FLAG-WWP1.WT and pLVX-FLAG-WWP1.E798V.

For stable infection, RWPE cells were infected with the lentivirus pLVX-Tet3G in the presence of Polybrene (20 μg/ml) and selected with G418 (500 μg/ml) for 2 weeks. Then cells expressing the tetracycline transactivator were infected with pLVX-TRE3G, pLVX-TRE3G-FLAG-WWP1.WT, or pLVX-TRE3G-FLAG-WWP1.E798V in the presence Polybrene (20 μg/ml) and selected with puromycin (10 μg/ml) for 2 weeks. Resistant colonies were pooled and expanded as single populations.

Plasmids and Constructions—FLAG-WWP1, FLAG-WWP1.C890A, FLAG-Hect, FLAG-WWHect, and FLAG-Smurfl expression vectors were described previously (24). Expression vectors for HA-ubiquitin (Ub), HA-Ub.K48R, and HA-Ub.K63R were a gift from Dr. Ivan Dikic. FLAG-Smurfl2 was purchased from Addgene (ID 11746/Dr. Jeff Wrana’s laboratory). GST-Hect was generated by PCR using p3xFLAG-WWP1-Hect and subcloned into pGEX-4T3. FLAG-WWP1.E798V, FLAG-WWHect.E798V, FLAG-WPP1.E798V/C890A, FLAG-Hect.E798V, and FLAG-Hect.E798V/C890A were generated by substituting the glutamic acid (E) residue at amino acid position 798 for valine (V) using the QuikChange site-directed mutagenesis kit (Stratagene). HA-WWP1-C2 was generated by subcloning of the HindIII/BamHI insert from 3xFLAG-WWP1-C2 into HA-pCMV5, pLVX-TRE3G-FLAG-WWP1.WT and pLVX-TRE3G-FLAG-WWP1.E798V were generated by PCR using p3xFLAG-WWP1 and p3xFLAG-WWP1.E798V and subcloned into pLVX-TRE3G (Clontech).

Experiments—WW1–4 using the QuikChange site-directed mutagenesis kit (Stratagene). His-WWP1-C2 was generated by subcloning of the HindIII/BamHI insert from 3xFLAG-WWP1-C2 into HA-pCMV5, pLVX-TRE3G-FLAG-WWP1.WT and pLVX-TRE3G-FLAG-WWP1.E798V were generated by PCR using p3xFLAG-WWP1 and p3xFLAG-WWP1.E798V and subcloned into pLVX-TRE3G (Clontech).

WW1ΔWW1, WW1ΔWW2, WW1ΔWW3, and WW1ΔWW4 were generated by deleting individual WW domains or all four domains for WW1ΔWW1–4 using the QuikChange site-directed mutagenesis kit. His-WWP1-C2 was generated by subcloning of the HindIII/BamHI insert from HA-WWP1-C2-pCDNA3 into pET28 vector. We used ON-TARGET plus SMARTpool siRNAs (L-020068-00) from GE Dharmacon to target Smad7.

Immunoprecipitation and Immunoblotting—For immunoblotting, cells were lysed at 4 °C in TNMG buffer (24) followed by centrifugation at 13,000 rpm for 10 min at 4 °C. Lysates were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. For immunoprecipitation, cells were lysed at 4 °C in immunoprecipitation buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM NaF, 2 mM EGTA, 1 mM Na3PO4, 1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM sodium vanadate, and EDTA-free protease inhibitor mix-
ture (Roche Diagnostics) followed by sonication. Cell lysates were precleared using Sepharose-coupled protein G for 1 h and then centrifuged at 6,000 rpm for 10 min at 4 °C. Lysates were subjected to immunoprecipitation with the appropriate antibody for 2 h followed by adsorption to Sepharose-coupled protein G for 1 h. In all experiments involving ubiquitination, cells were incubated with MG-132 (10 μM) for 4 h before lysis. For His-Ub pulldown, cell lysates were incubated with nickel-nitri-
lotriacetic acid-agarose for 1 h, and the beads were washed three times with lysis buffer. Ubiquitinated proteins were then separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

The following antibodies were used for immunoblotting or immunoprecipitation: anti-FLAG M2-HRP (Sigma), anti-HA-HRP (Roche Applied Science), anti-Myc-HRP (Roche Applied Science), anti-ubiquitin (P4D1, Santa Cruz Biotechnology), anti-GAPDH (Abcam), anti-GFP-HRP (Abcam), anti-UbK63 (Enzo Life Sciences), anti-UbK48 (Millipore), anti-His6-HRP (Abcam), and anti-WWP1 (Abnova).

In Vitro Ubiquitination Assay—Immunoprecipitated FLAG-WWP1 or GST-Hect was incubated in a reaction mixture containing 100 ng of E1 (UBE1), 200 ng of E2 (UbcH7), 5 μg of Ub, and 1X energy regenerating solution containing ATP and MgCl2 (all reagents were obtained from Boston Biochem) and reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT). Reactions were incubated for 30 min at 37 °C. Reactions were stopped by the addition of Laemmli sample buffer and boiling and analyzed by Western blotting.

Reporters Gene Assays—Cells were seeded into 12-well plates and transfected 24 h later with expression vectors by Lipofectamine. Cells were subsequently treated with TGFβ1 (PeproTech) at 2 ng/ml in medium containing 0.5% FCS for 16 h. Luciferase assays were performed using the Dual-Luciferase reporter system (Promega). Luciferase activities were normalized to Renilla activities. Prior normalization was performed to obtain the equivalent amounts of wild-type WWP1 and WWP1.E798V.

Proliferation Assay—RWPE-TR-FLAG-WWP1.WT and RWPE-TR-FLAG-WWP1.E798V cells were plated in 6-well plates and treated with Dox (1 μg/ml) for 24 h prior to addition of TGFβ (5 ng/ml) in medium containing 0.5% FCS for 16 h. Luciferase assays were performed using the Dual-Luciferase reporter system (Promega). Luciferase activities were normalized to Renilla activities. Prior normalization was performed to obtain the equivalent amounts of wild-type WWP1 and WWP1.E798V.

Results

Mutational Activation of WWP1

WWP1 Undergoes Monoubiquitination at Steady States—Others and we have reported previously that WWP1 functions as a potent inhibitor of TGFβ signaling, functioning at least by triggering degradation of the activated TβRI in conjunction with Smad7 (24, 25). During the course of these previous investigations, we consistently noticed that the WWP1 band in Western blotting was accompanied with a slower migrating band whose molecular weight is consistent in size with monoubiquitinated WWP1. To further substantiate this observation, we initially performed an in vivo ubiquitination assay to assess formation of the WWP1 ubiquitin-conjugated products in the presence or absence of ubiquitin. We found that coexpressing ubiquitin with WWP1 resulted in a substantial increase in the abundance of the monoubiquitinated WWP1 species (Fig. 1A). An immunoprecipitation experiment using ubiquitin and WWP1 antibodies showed that endogenous WWP1 can undergo monoubiquitination (Fig. 1B). Extending our study to two other Hect E3 ubiquitin ligases, we found that Smurf2 was mainly monoubiquitinated, whereas Smurf1 is highly polyubiquitinated (Fig. 1A), attesting to the specificity of the monoubiquitination modification observed for WWP1 and Smurf2 and further indicating that the intrinsic ubiquitination of members of the Hect family of ubiquitin ligase is governed by distinct mechanisms despite the strong homology in their structures. During the course of these analyses, we also made use of a mutant WWP1.C890A in which the active cysteine in the Hect catalytic domain was mutated to alanine, rendering WWP1 catalytically inactive. In contrast to wild-type WWP1, WWP1.C890A was void of any monoubiquitination activity, suggesting that WWP1 catalyzes its monoubiquitination (Fig. 1, C and D). To test this directly, we performed an in vitro ubiquitination assay using purified WWP1, E1, E2, and ubiquitin in the presence of DTT, which disrupts disulfide bond between cysteines and thus enables exclusive detection of covalent attachments, such as those engaged in monoubiquitination or polyubiquitination (Fig. 1D). Under these stringent conditions, we mainly detected monoubiquitinated WWP1, providing further support to the notion that WWP1 undergoes primarily monoubiquitination at basal conditions in the absence of any interaction with molecular partners or any other ubiquitin ligases.

Monoubiquitination Is Correlated with Increased WWP1 Stability—We next sought to investigate the impact of monoubiquitination on WWP1 stability and activity, surmising that deleting specific WWP1 functional domains (Fig. 2A) might affect its stability and activity. In fact, a WWP1 construct that lacks its C2 and WW domains is less expressed than the full-length WWP1 (Fig. 2B). To determine whether this decrease is due to degradation, we performed a pulse-chase experiment in the presence of the protein synthesis inhibitor cycloheximide. In contrast to the Hect domain, full-length WWP1 exhibited a slow turnover rate, reinforcing the observation that removal of
C2 and WW yields an unstable protein (Fig. 2C). In ubiquitination experiments, WWP1 was barely polyubiquitinated, whereas Hect was highly polyubiquitinated (Fig. 2D). Of note, full-length WWP1 was primarily monoubiquitinated (Fig. 2D). Interestingly, the catalytically inactive mutant Hect.C890A was less polyubiquitinated (Fig. 2E) and degraded (Fig. 2F) than the wild-type Hect, indicating that Hect is mainly autopolyubiquitinated. An in vitro ubiquitination assay using purified GST-Hect produced in bacteria (and hence in the absence of any other E3 ubiquitin ligase source) confirmed the ability of WWP1 Hect to catalyze its own polyubiquitination (Fig. 2G). Altogether, these findings indicate that C2 and/or WW domains inhibit the polyubiquitination of the Hect domain.

Lys-48-linked ubiquitin chains usually target substrates for proteasomal degradation, whereas Lys-63-linked polyubiquitin chains are associated with lysosomal degradation (1). To determine which type of ubiquitin chains is catalyzed by WWP1, we conducted ubiquitination assays using mutant Ub.K48R or Ub.K63R. Consistent with the literature (26), we found that Hect preferentially catalyzed Lys-63-linked polyubiquitination (Fig. 2, H and I). However, we observed that Hect was also ubiquitinated through Lys-48-linked polyubiquitination chain although to a lesser extent as compared with Lys-63-linked polyubiquitination (Fig. 2J, bottom). Finally, we found that Hect was stabilized in cells exposed to MG-132 or leupeptin, which blocks protein clearance through the proteasome or lysosome, respectively (Fig. 2J). Collectively, these results strongly suggest that WWP1 is monoubiquitinated under steady state conditions and can be converted to Lys-63- or Lys-48-linked polyubiquitinated species upon removal of the C2 and WW domains, leading to WWP1 degradation.

**C2 and WW Domains Interfere with WWP1 Polyubiquitination**—The results outlined so far suggest that the C2 and/or WW domains play a crucial role in ensuring WWP1 stability. To elucidate how these two domains fulfill their function, we took advantage of published data reporting an intramolecular interaction between the C2 and Hect domains of Smurf2 that prevents its autoubiquitination (27). As WWP1 and Smurf2 are related C2-WW-Hect E3 ubiquitin ligases, we hypothesized that the C2 domain of WWP1 might also bind to and stabilize the Hect domain. As shown in Fig. 3A, immunoprecipitation of lysates from transfected HEK293 cells with anti-HA directed against the C2 domain of Smurf2 revealed the presence of FLAG-Hect (Fig. 3A), underscoring the existence of an intramolecular association between the C2 and Hect domains. Using GST-Hect and His-C2 produced in bacteria in an in vitro interaction assay then performed at 37 °C for 30 min. Ubiquitinated WWP1 was visualized by immunoblotting with anti-FLAG antibody. Mr, molecular weight marker. C, HEK293 cells were transfected with His-Ub together with FLAG-WWP1 or FLAG-WWP1.C890A. Cell lysates were pulled down with nickel-agarose and immunoblotted with anti-FLAG antibody. Of note, deletion of individual WW domains had little or no effect on WWP1 polyubiquitination (Fig. 3F), underscoring a requirement for all WW domains in WWP1 stabilization. In several attempts, we were not able to detect an interaction between the
FIGURE 2. Full-length WWP1 is more stable than the Hect domain. A, Schematic representation of the functional domains of WWP1. B and C, HEK293 cells were transfected with FLAG-F-WWP1 or FLAG-Hect. B, cell lysates were immunoblotted with anti-FLAG antibody to detect the expression of full-length WWP1 (WWP1.FL) or Hect and with anti-GFP antibody to assess transfection efficiency. C, cells were treated with cycloheximide (CHX) and harvested at the indicated time points for immunoblotting analysis to assess the expression levels of FLAG-WWP1 or FLAG-Hect. D, cells were transfected with His-Ub together with FLAG-WWP1 or FLAG-Hect. Cell lysates were pulled down with nickel-agarose and immunoblotted with anti-FLAG antibody. E, HEK293 cells were transfected with His-Ub together with FLAG-Hect or the catalytically inactive mutant FLAG-Hect.C890A. Cell lysates were pulled down with nickel-agarose and immunoblotted with anti-FLAG antibody. F, HEK293 cells were transfected with FLAG-Hect or FLAG-Hect.C890A and treated with MG-132 (10 μM) for 4 h. Cell lysates were subjected to immunoblotting with anti-FLAG antibody to assess the expression levels of FLAG-Hect or FLAG-Hect.C890A. G, in vitro ubiquitination reaction using bacterially expressed GST-Hect or GST alone was performed in the presence of Ub, E1, E2, and ATP. Ubiquitinated Hect was visualized by immunoblotting using anti-ubiquitin antibody. The blot was stained with Ponceau S prior to immunoblotting to visualize the amount of GST proteins loaded. H and I, expression vectors encoding FLAG-Hect together with HA-Ub.WT or HA-Ub.K48R or HA-Ub.K63R mutant were transfected into HEK293 cells, and cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody before being analyzed by immunoblotting with anti-FLAG antibody (H, top) or immunoprecipitated with anti-FLAG antibody before being analyzed by immunoblotting with anti-UbK48 antibody that specifically recognizes Lys-48-linked polyubiquitination chains (H, bottom) or anti-UbK63 antibody that specifically recognizes Lys-63-linked polyubiquitination chains (I, top). J, HEK293 cells were transfected with FLAG-WWP1 or FLAG-Hect and treated with MG-132 (10 μM) for 4 h or with leupeptin (100 μg/ml) for 4 h. Cell lysates were subjected to immunoblotting with anti-FLAG antibody to assess the expression levels of FLAG-WWP1 and FLAG-Hect. WB, Western blotting.
WW and Hect domains (Fig. 3A), suggesting that the WW domains may stabilize WWP1 by a mechanism that does not rely on an intramolecular interaction. Collectively, these results indicate that both C2 and WW domains play important roles in promoting WWP1 stability, most likely by inhibiting WWP1 polyubiquitination.

Binding to Smad7 Relieves WWP1 Autoinhibition—Having shown that both C2 and WW domains of WWP1 exert a negative influence on Hect polyubiquitination activity, we next wondered whether the interaction of WWP1 with Smad7 plays a role in regulating WWP1 stability and activity. Because we have shown previously that Smad7 is required for WWP1-induced polyubiquitination and degradation of the activated TβRI (24, 25), we speculated that binding of Smad7 to WWP1 might relieve the autoinhibitory interplay between C2 or WW and Hect. Indeed, overexpressing Smad7 resulted in a marked

**FIGURE 3. C2 and WW domains inhibit WWP1 autopolyubiquitination.** A, HA-C2, HA-WW, and FLAG-Hect were transfected into HEK293 cells, and cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody before being analyzed by immunoblotting with anti-FLAG antibody. B, glutathione-Sepharose-immobilized GST or GST-Hect (GST PD) were incubated with clarified bacterial lysates in which His-C2 expression had been induced or not with isopropyl-β-D-thiogalactoside (IPTG). C2 domain binding to the Hect domain was detected by immunoblotting with anti-His antibody. Equivalent GST, GST-Hect, and His-C2 were confirmed by Ponceau S staining and immunoblotting with anti-His antibody, respectively. C, HEK293 cells were transfected with FLAG (F)-Hect together with His-Ub and increasing amounts of HA-C2. Cell lysates were pulled down with nickel-agarose and immunoblotted with anti-FLAG antibody. D, HEK293 cells were transfected with FLAG-Hect together with increasing amounts of FLAG-C2. Cell lysates were immunoblotted with anti-FLAG antibody to detect the expression of Hect and C2. E, full-length WWP1 or the indicated fragments of WWP1 were transfected into HEK293 cells together with His-Ub. Cell lysates were pulled down with nickel-agarose and immunoblotted with anti-FLAG antibody. F, full-length WWP1 or the indicated WW1,WW mutants were transfected into HEK293 cells together with His-Ub. Cell lysates were pulled down with nickel-agarose and immunoblotted with anti-FLAG antibody. WB, Western blotting.
decrease in WWP1 abundance (Fig. 4A, top), whereas overexpressing Smad1, which failed to interact with WWP1 (24), had no effect (Fig. 4A, bottom). Silencing of Smad7 by siRNA resulted in increased expression of endogenous WWP1 (Fig. 4B), providing further evidence that the interaction of WWP1 with Smad7 affects WWP1 stability. Silencing Smad7 in two other cell lines (i.e., MCF-7 and HeLa) also increased WWP1 expression, indicating that the Smad7-mediated WWP1 destabilization can occur in several cell types (Fig. 4B). The ability of Smad7 to destabilize WWP1 likely depends on WWP1 enzymatic activity because Smad7 had little or no effect on the expression levels of the catalytically inactive mutant WWP1.C890A (Fig. 4A). Corroborating this finding, Smad7 expression induced WWP1 polyubiquitination (Fig. 4C). In our efforts to delineate the underlying mechanisms, we found that Smad7 was able to inhibit the association between C2 and Hect domains (Fig. 4D). Likewise, expression of Smad7 dampened the ability of WW domains to block Hect polyubiquitination (Fig. 4E). Collectively, these findings strongly suggest that a physiological function of Smad7 is to disable the autoinhibitory mechanism between C2 or WW and Hect domains and thereby enforce WWP1 polyubiquitination and degradation.

The Tumor-derived Mutant WWP1.E798V Is Constitutively Active—Given the prominent role of WWP1 in cancer pathogenesis, we were curious whether alterations of WWP1 in human cancer could affect WWP1 stability and activity. To probe this possibility, we turned our attention on a mutant detected in a prostate cancer that harbors a single base substitution that replaces a glutamate by a valine at position 798 (WWP1.E798V) (7). Crucially, WWP1.E798V displayed a dramatic increase in polyubiquitination as compared with the wild-type counterpart (Fig. 5A). To rule out the possibility that the WWP1.E798V mutant affects the folding of WWP1, which could conceivably lead to its constitutive polyubiquitination and degradation, we introduced the mutation C890A into WWP1.E798V, creating a catalytically inactive mutant,
WWP1.E798V/C890A. As shown in Fig. 5B, the double mutant was defective in its polyubiquitination, suggesting that the constitutive polyubiquitination of WWP1.E798V may stem from its autopolyubiquitination activity rather than the activity of other ubiquitin ligases that govern clearance of unfolded proteins. Introducing the mutation C890A into Hect.E798V also resulted in protein stabilization, confirming that Hect.E798V catalyzes its own polyubiquitination (Fig. 5C).

We then attempted to elucidate the mechanism underlying the constitutive autopolyubiquitination activity of WWP1.E798V, focusing here again on the interaction between the C2 and Hect domains. When compared with the wild-type Hect, the Hect.E798V displayed reduced affinity for the C2 domain (Fig. 5D). Likewise, E798V also compromised the ability of the WW domain to oppose Hect autopolyubiquitination (Fig. 5E). Overall, these findings provide a molecular basis that could account for the constitutive autopolyubiquitin activity of the tumor-derived mutant WWP1.E798V.

Next, we sought to investigate whether the increased autopolyubiquitination activity of WWP1.E798V mutant is associated with increased ubiquitin ligase activity toward TβRI in the presence of Smad7, which is known to mediate the association between WWP1 and TβRI (24, 25). Because WWP1.E798V is expressed at a lower level than wild-type WWP1 due to its constitutive polyubiquitination and degradation, we first performed titration experiments to determine the concentration of WWP1.E798V plasmid that yields the same expression level as wild-type WWP1. As anticipated, coexpression of Smad7 with WWP1 decreased the expression of the activated TβRI (Fig. 6A). More crucially, this effect was more pronounced when WWP1.E798V was used instead of wild-type WWP1, in particular with small amounts that were not sufficient for wild-type WWP1 to induce degradation of the activated TβRI. We also found that WWP1.E798V was more potent than wild-type WWP1 at inducing accumulation of polyubiquitin-conjugated TβRI (Fig. 6B). This scenario is physiologically meaningful because WWP1.E798V, which is expressed at an even lower level, was more effective than wild-type WWP1 at suppressing TGFβ-induced transcriptional responses as gauged by a TGFβ-sensitive reporter gene (Fig. 6C). To further investigate...
the functional relevance of the tumor-derived mutant WWP1.E798V, we generated populations of normal human prostatic epithelial RWPE cells expressing Dox-inducible WWP1.WT or WWP1.E798V. Upon Dox treatment, we observed that the expression level of WWP1.E798V was much lower than that of wild-type WWP1 (Fig. 6D). Exposure of these cells to MG-132 increased expression of WWP1.E798V to a level approaching that of wild-type WWP1 (Fig. 6E), confirming the constitutive autopolyubiquitination and degradation of WWP1.E798V (Fig. 5A). The results are expressed as mean ± S.D. (error bars) of triplicates from a representative experiment performed three times. Student’s t test was performed, and significant differences between wild-type WWP1 and WWP1.E798V are indicated (**, p < 0.01; ***, p < 0.001).

**FIGURE 6.** WWP1.E798V is more active than wild-type WWP1. **A**, HEK293 cells were transfected with the activated TβRI (HA-TβRIP) and Smad7 together with FLAG-F-WWP1.WT or FLAG-F-WWP1.E798V. Cell lysates were immunoblotted with anti-HA antibody to detect the expression levels of activated TβRI. **B**, HEK293 cells were transfected with His-Ub together with Smad7 and either increasing amounts of either FLAG-F-WWP1.WT or FLAG-F-WWP1.E798V and treated with TGFβ for 16 h. The results are expressed as mean ± S.D. (error bars) of triplicates from a representative experiment performed three times. Student’s t test was performed, and significant differences between wild-type WWP1 and WWP1.E798V are indicated (**, p < 0.01; ***, p < 0.001). D, RWPE-TR-FLAG-F-WWP1.WT and RWPE-TR-FLAG-F-WWP1.E798V cells were treated with Dox for 24 h. Cell lysates were immunoblotted with anti-FLAG antibody to detect WWP1.WT and WWP1.E798V. E, RWPE-TR-FLAG-F-WWP1.WT and RWPE-TR-FLAG-F-WWP1.E798V cells were treated with Dox for 24 h and then cultured with or without TGFβ (5 ng/ml) and Dox for 3 or 6 days. The rate of cell proliferation was assessed by determining the cell numbers using an automatic cell counter and expressed as a percentage of control. The results are expressed as mean ± S.D. (error bars) of triplicates from a representative experiment performed three times. Student’s t test was performed, and significant differences between WWP1 and control as well as between wild-type WWP1 and WWP1.E798V are reported (**, p < 0.01; ***, p < 0.001). IP, immunoprecipitation; WB, Western blotting.
conditions in which wild-type WWP1 elicited only a weak response, expression of WWP1.E798V almost completely blocked TGFβ-induced growth arrest (Fig. 6F). Together, these findings provide compelling evidence that the tumor-derived mutant WWP1.E798V displays elevated ubiquitin ligase activity against itself as well as TβRI, unveiling a potential molecular mechanism that could account for the ability of WWP1 to elicit its oncogenic function during the course of human cancer pathogenesis.

Discussion

Despite the prominent roles of E3 ubiquitin ligases in regulating biological processes that contribute to the pathogenesis of human cancer and other diseases, the physiological mechanisms that govern their abundance or enzymatic activity remain unclear. Here, we present evidence that the WWP1 activity is maintained in a silent conformation under steady state conditions because of an autoinhibitory mechanism involving either C2 and/or WW and Hect domains. Interestingly, this autoinhibitory mechanism correlates with WWP1 autmonoubiquitination instead of autopolyubiquitination. A potential mechanism could be that both C2 and WW domains exert a steric pressure on the Hect domain, which in turn favors monoubiquitination at the expense of polyubiquitination. Based on our findings, we propose a model in which engagement of WWP1 with the Smad7-TβRI complex subsequent to activation of TGFβ signaling disrupts the molecular interplay between the C2 or WW and Hect domains, causing a decrease in WWP1 monoubiquitination but increasing its autopolyubiquitination and polyubiquitination of the activated TβRI. The physiological relevance of this regulatory mechanism was ascertained by demonstrating an ability of the tumor-derived point mutant of WWP1.E798V to disrupt the functional interaction between C2 or WW and Hect, a process that culminates in increased WWP1 autopolyubiquitination and degradation. Such increase in autopolyubiquitination likely recapitulates a general increase in WWP1.E798V enzymatic activity as gauged by the enhanced activity against the activated TβRI and attendant suppression of TGFβ signaling. Thus, our findings provide key insights into the mode of regulation of a representative member of the large family of Hect E3 ubiquitin ligases and further unveil a paradigm behind constitutive activation of WWP1 in human malignancies.

Although members of the C2-WW-Hect subclass of E3 ubiquitin ligases share strong structural homologies, it is becoming increasingly clear that their mode of regulation can vary among members. Along these lines, our study reveals that WWP1 is only able to catalyze its own monoubiquitination at steady states. Comparative experiments showed that this process is not restricted to WWP1 as Smurf2 also displays mainly autmonoubiquitination in unstimulated cells. In contrast, Smurf1 undergoes mainly polyubiquitination under the same experimental conditions, supporting the hypothesis that the catalytic activity of the Hect E3 ubiquitin ligases might be governed by distinct mechanisms despite the strong homology in their structures. In further support of this notion, the WWP1 and Smurf2 catalytic activities do not seem to be regulated exactly by the same mechanisms as Wiesner et al. (27) have shown that removal of the C2 domain of Smurf2 is sufficient to alleviate the autoinhibition, whereas our study showed that the C2 domain exerted only partial inhibition, and full autoinhibition requires the presence of WW domains. A similar scenario has been observed for Nedd4-2 and Itch apparently involving an intramolecular interaction between the WW domains and a PPXY motif within the Hect domain (28, 29). Of note, the PPXY motif is also conserved in the WWP1 Hect domain; however, we were not able to detect any interaction between WW and Hect domains of WWP1. Although we cannot rule out the possibility that our experimental conditions preclude the detection of such intramolecular interaction, our finding is in agreement with published observations on Smurfl, which also possesses a PPXY motif that does not seem to interact with Hect (27). Given that these E3 ubiquitin ligases possess various numbers of WW domains (i.e. WWP1 has four WW domains, Smurf1 has two WW domains, and Smurf2 has three WW domains), one would speculate that these molecular features might enable engagement of the ubiquitin ligases with different partners and/or substrates, which cooperate together to fine-tune the ubiquitin ligase catalytic activity.

So far, there is no consensus for the type of ubiquitin linkages that WWP1 is able to conjugate on its substrates and the consequence of this ubiquitination on substrate outcome. Indeed, many published studies have described various consequences of the WWP1-induced ubiquitination depending on the substrates, including proteasomal degradation, lysosomal degradation, subcellular redistribution, and transcriptional activity (5, 22, 23, 30, 31). Here, we found that WWP1 conjugated its polyubiquitination mainly through Lys-63 polyubiquitin chains, although we could clearly detect Lys-48-linked polyubiquitination. In light of the abundant literature that Lys-63 and Lys-48 polyubiquitination linkages are associated with lysosomal and proteasomal degradation, respectively, we suggest that WWP1 may trigger its degradation through the proteasome and/or lysosomal pathways. Consistent with this hypothesis, degradation of the WWP1 Hect domain was blocked by both MG-132 and leupeptin, which inhibit the proteasome and lysosome, respectively. Because modification of substrates by Lys-63 polyubiquitin chains plays other roles unrelated to protein degradation (1), we cannot rule out the possibility that the Hect domain of WWP1 might also regulate its activity by coordinating other mechanisms, such as subcellular redistribution or interaction with substrates or partners.

A wealth of data postulates that WWP1 functions as an oncogenic ubiquitin ligase likely because of its genomic amplification and/or overexpression in prostate and breast cancers (3, 5). Intriguingly, a previous study revealed the presence of a point mutation in WWP1 in human prostate cancer, raising the intriguing question of whether mutational alteration of WWP1 could contribute to human cancer pathogenesis (7). It is in this respect that our present findings provide insights into the impact of WWP1 mutational alteration on cancer development. Of particular importance, we found that the tumor-derived mutation WWP1.E798V displayed increased activity, which ultimately disrupts the TGFβ cytostatic signaling, as evidenced using the human normal prostatic cell line RWPE. In light of these findings, it would be interesting to determine
whether the mutant WWP1.E798V displays constitutive ubiquitin ligase activity toward cancer-related genes other than TβRI, a finding that will likely improve our understanding of the oncogenic role of WWP1 in human cancer. Likewise, it would also be interesting to determine whether a similar prostate cancer-derived mutation occurs in other human malignancies. Therefore, our findings open up attractive molecular frameworks that could be exploited to unravel mechanistic paradigms of human malignancies and apply these concepts to implement accurate and safe therapeutic strategies.

To the best of our knowledge, our findings reveal for the first time that a point mutation in a Hect ubiquitin ligase culminates in a constitutive ubiquitin ligase activity. Many point mutations in other families of signaling proteins, such as kinases, GTPases, phosphatases, and transcription factors, have been described and extensively studied, although these alterations usually affect a specific cellular process. As such, our functional characterization of a tumor-derived mutation in a representative member of the Hect superfamily emphasizes another important layer of complexity to the etiology of human cancer that deserves particular attention as protein degradation represents a universal system that simultaneously keeps in check the abundance of many proteins, including the most prominent oncogenes and tumor suppressor genes. We anticipate that targeting Hect ubiquitin ligases, such as WWP1, could represent an innovative strategy to curb aberrations in many signaling pathways that typically accumulate over time during the course of cancer development and progression.

Author Contributions—T. C. and N. F. designed, performed, and analyzed most of the experiments. A. E. and S. K. designed, performed, and analyzed the experiments shown in Fig. 6, D, E, and F. L. L. and O. F. contributed to the management and design of the project. C. P. and A. A. designed the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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