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WWP1-dependent ubiquitination and degradation of the lung Krüppel-like factor, KLF2☆

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Abstract

The zinc-finger transcription factor Krüppel-like factor-2 plays an important role in pulmonary development, inhibition of adipocyte differentiation, and maintaining quiescence in single-positive T cells. KLF2 levels rapidly decrease during adipogenesis and activation of T cells, but the pathways involved remain unclear. Previously, we identified WWP1, a HECT-domain E3-ubiquitin ligase, as an interacting partner of KLF2. This led us to speculate that KLF2 may be targeted for ubiquitination. Here, we demonstrate that WWP1 interacts with KLF2 in vivo and mediates both poly-ubiquitination and proteasomal degradation of KLF2. Deleting the inhibitory domain of KLF2 abrogated KLF2–WWP1 interactions and abolished WWP1-mediated poly-ubiquitination and down-regulation of KLF2. Furthermore, lysine-121 in the inhibitory domain of KLF2 is critical for ubiquitin-conjugation. Finally, the catalytic cysteine of WWP1 is not required for KLF2-ubiquitination. Our experiments demonstrate for the first time that WWP1 promotes ubiquitination and degradation of KLF2 and is not involved in the ubiquitin-transfer reaction.

Keywords: Lung Krüppel-like factor; KLF2; E3-ubiquitin ligase; WWP1; Ubiquitination; Post-translational modification of Krüppel-like factors

KLF2, or lung Krüppel-like factor (LKLF), is a zinc-finger transcription factor that plays an important role in several tissues. Disruption of the KLF2 gene through gene targeting in embryonic stem cells results in embryonic lethality [1,2]. Homozygous null mice die in utero between 12.5 and 14.5 days of gestation due to severe hemorrhaging, defects in blood vessel morphology, and tunica media formation [1,2]. KLF2 plays an important role in programming the quiescent state of single-positive ($CD4^+$ or $CD8^+$) T cells and their survival in the peripheral lymphoid organs and blood [3]. KLF2 is known to regulate the signaling pathways involved in endothelial cell differentiation and the formation of the mature blood vessel walls [1]. In addition, a role for KLF2 as a negative regulator of adipogenesis has been suggested by recent studies where KLF2 was shown to directly inhibit PPAR-γ [4], a ligand-activated transcription factor known to regulate the adipogenic transcriptional cascade by binding the fat-selective enhancers of the αP2 and PEPCK genes in fat cells [5]. It is increasingly clear that although KLF2 was identified as a lung-specific transcription factor known to be critical for pulmonary development, it now appears to have a more complex role in diverse biological processes.

While the biological roles of KLF2 are relatively well defined, its activity, gene targets, and transcriptional regulation remain unclear. In an effort to identify factors that bind KLF2, we used a yeast two-hybrid screen and showed that WWP1, an E3 ubiquitin ligase, binds to the auto inhibitory domain of KLF2 [6]. This was interesting and expected because many transcription factors have short half-lives and their loss of activity is not completely explained by a reduced transcription rate. The changes in protein levels of several transcription factors are known to be regulated by mechanisms such as ubiquitin-mediated proteolysis [7]. Our prior studies

☆ *Abbreviations:* LKLF, lung Krüppel-like factor; KLF2, Krüppel-like factor-2; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; Ub, ubiquitin; MG132, N-CBZ-LEU-LEU-LEU-AL; E3s, E3 ubiquitin ligases.

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therefore suggest that WWP1 might have a role in regulating KLF2 levels. Since WWP1 is an E3 ligase, we reasoned that it might mediate formation of ubiquitinated KLF2.

Ubiquitination of a protein substrate involves a cascade of enzymatic reactions. First, Ubiquitin, a conserved 76-amino acid polypeptide, is activated by a ubiquitin-activating enzyme (E1), leading to an ATP-dependent formation of a thiol-ester bond between the C-terminus of ubiquitin and E1. The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). E2 mediates the transfer of ubiquitin to the target protein directly or through E3-ubiquitin ligase, which transfers ubiquitin to its substrate by covalent attachment between the ubiquitin and lysines on the target protein. A substrate may be repetitively ubiquitinated to form a poly-ubiquitinated chain, which marks it for recognition and eventual degradation by the 26 S proteasome [7,8].

There are multiple families of E3s, such as the RING finger ligases, the HECT domain ligases, and the U box proteins [9]. The HECT domain E3s form a thioester bond between an active cysteine and the ubiquitin C terminus after the binding of a charged E2. A sub-family of HECT domain E3s comprises of ligases that contain WW domains, so named because of the tryptophan residues spaced 22 or 23 residues apart. WWP1 belongs to this group along with WWP2, AIP4, NEDD4, E6-AP, Itch, and RPF1. These proteins have diverse functions. However, the direct functional consequences of WWP1 remain obscure, although its homolog in *Caenorhabditis elegans* appears to play a role in embryogenesis [10] and has been implicated in transcriptional regulation [11].

Prior to degradation, proteins may be post-translationally modified [12]. Earlier studies have shown that KLF2 degradation during T-cell development is preceded by an altered electrophoretic mobility of the protein [3]. This indicates that KLF2 protein may be modified, prior to being degraded. E3s are known to transfer ubiquitin moieties to lysine residues and sequence analysis shows that the inhibitory domain of KLF2 is rich in lysines. Taken together, the higher electrophoretic mobility of KLF2 prior to degradation suggests that it may be post-translationally modified; the presence of critical lysine residues in its inhibitory domain indicates the presence of potential ubiquitin transfer sites and the results of the yeast hybrid screen suggest an interaction with WWP1. However, studies that demonstrate in vivo interactions between E3s and Krüppel-like factors are lacking. Furthermore, the post-translational modifications of KLF2 and their consequences are unknown. In studies presented here, we demonstrate for the first time that WWP1 mediates ubiquitination and subsequent proteasomal degradation of KLF2. Lysine 121, located in the inhibitory domain of KLF2, is necessary for this reaction while the catalytic cysteine of WWP1 is dispensable.

Materials and methods

Plasmids. The previously described full-length KLF2 cDNA [6] was cloned into both p3XFLAG-CMV 7.1 (Sigma, MO) and pRSGFP expression vectors (Clonetech, CA) to create a fusion protein of 3XFLAG-KLF2 and GFP-KLF2. KLF2 deletion mutants (GAL4-KLF2 1–267, GAL4-KLF2 1–110, and GFP-KLF2Δ111–267) were created by deletions utilizing the restriction enzyme sites (*Bss*HI, *Xma*III, respectively) in the coding region of KLF2 and the polylinker. After deletion of the 3′ end of KLF2 cDNA, the staggered ends generated from the restriction digests were blunted with T4 polymerase before ligation. Full-length Myc-WWP1 was kindly provided by Dr. Emery Bresnick and cloned into p3XFLAG-CMV 7.1 (Sigma, MO). The Site-directed Mutagenesis Kit (Stratagene, CA) was used to create lysine mutants in p3XFLAG-KLF2 and cysteine mutant C886S in PCMV-Myc-WWP1. All the mutations were confirmed by DNA sequencing.

Cell culture and transfection. Monkey kidney fibroblast (Cos-1) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and plated in 6-well plates at 1×10^5 per well. The next day, cells were transfected by Fugene 6 (Roche) with 0.25 μg KLF2 expression vector (KLF2 and its lysine or deletion mutants), 0.25–1.5 μg Myc-WWP1 (Wild type or its cysteine mutant as described in each figure). Vector DNA was added to keep the total DNA per well constant. For CAT assay, 0.25 μg of reporter HS2βCAT along with KLF2 or WWP1 expression plasmids and 0.1μg of pSV2LUC were co-transfected; GFP plasmid was co-transfected as internal control for Western blot. The cells were harvested 48 h after transfection for immunoprecipitation, Western blotting or CAT assays. In experiments using proteasome inhibitor, cells were treated with 10 μM MG132 for 16 h prior to harvest.

Chloramphenicol acetyltransferase assays. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described [6]. Briefly, the lysates were prepared by disrupting the cells with three cycles of freeze–thaw in 0.25 M Tris (pH 7.4) buffer to make protein extracts. An aliquot of the lysate was used for analysis of luciferase activity (Promega). This served as an internal control for normalizing transfection efficiency. The remaining extract was heat-inactivated at 65 °C for 10 min, and CAT assays were performed at 37 °C for 1 h. The samples were spotted on silica gel-coated; flexible, thin-layer chromatography plates (Whatman) and the plates were exposed to a PhosphorImager (Molecular Dynamics, CA) for quantitation. The values of CAT activity are based on percentage conversion of [^{14}C]chloramphenicol substrate to the acetylated forms and corrected for transfection efficiency by luciferase assay.

Immunoprecipitation. Cell transfections were carried out as described above. Cells were lysed with lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP-40, 1 mM DTT, and 0.5 mM PMSF) on ice for 10 min and centrifuged for 1 min at 4 °C. Protein concentration of the supernatant was detected by the BCA protein assay kit (Pierce). One fraction was used for immunoprecipitation and the remaining lysate for Western blotting. For co-immunoprecipitation, cell lysates were incubated with anti-Flag M2 (Sigma), anti-GAL4-DBD (Santa Cruz), anti GFP (Clontech), anti HA-Y11 (SantaCruz Biotechnology) antibodies or a control pre-immune serum. The protein–antibody complex was then precipitated by addition of protein G–Sepharose (Zymed Laboratories). After washing the absorbed beads in co-immunoprecipitation buffer, the precipitates were fractionated by 10% SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore). Western blotting was used to evaluate the association of the two proteins during the precipitation.

Western blot. Cell lysates were prepared as described earlier and electrophoresed on a 10% SDS–polyacrylamide gel. The proteins were transferred to a PVDF transfer membrane (Millipore). The membranes were probed with anti-Flag M2 (Sigma), anti-GFP (Clontech), anti-GAL4-DBD (Santa Cruz), anti-Myc-9E10 (Sigma), and anti-ubiquitin-P4D1 (Santa Cruz) primary antibodies. Protein–antibody interaction was visualized by chemiluminescence detection using the ECL Western blotting analysis system (Pierce).

Results

The inhibitory domain of KLF2 is required for in vivo interaction with WWP1

Our previous studies identified an interaction between WWP1 and the inhibitory domain of KLF2. To confirm this in vivo, KLF2-deficient Cos-1 cells were transfected with full-length GFP-KLF2 or the inhibitory domain deleted mutant (GFP-KLF2Δ111–267) (Fig. 1A), either with or without Flag-tagged WWP1. Forty-eight hours later, cell lysates were immunoprecipitated as described in Materials and methods, to pull down KLF2 protein. The immunoprecipitates were analyzed to detect any WWP1 that bound to KLF2. Full-length GFP-KLF2 bound to WWP1 significantly more than the mutant (Fig. 1B). These data are consistent with the yeast two hybrid screen and confirms KLF2–WWP1 interaction in vivo and the requirement of the inhibitory domain of KLF2 for this interaction.

Expression of WWP1 induces ubiquitination and proteasomal degradation of KLF2 in vivo

Our laboratory has shown previously that WWP1 reduces the transcriptional activity of KLF2 [6]. While the reduced transactivation may be the result of WWP1 binding KLF2 and interfering with its activation domain, a more likely alternative is that WWP1, being an E3-ubiquitin ligase, promotes ubiquitination and possibly degradation of KLF2. To investigate this, we co-expressed Flag-KLF2 in the presence or absence of myc-WWP1 in Cos-1 cells. Before harvest, cells were treated with MG132, a proteasomal inhibitor. KLF2 was precipitated with anti-flag antibody and immunoblotted with anti-Ub antibody to probe for ubiquitinated KLF2. The results show that WWP1 induced the formation of poly-ubiquitinated KLF2 (Fig. 2A, Panel 1, compare lanes 1 and 3). The specificity of the anti-flag antibody was verified both by using an unrelated antibody, i.e., GFP and by untransfected cell lysates, in an immunoprecipitation assay (data not shown). It must be noted that MG132 treatment showed increased accumulation of poly-ubiquitinated KLF2 (Fig. 2A, Panel 1, lanes 2 and 4). This is an indication that the proteasomal pathway is involved in KLF2 regulation.

Next we determined if WWP1 expression caused the degradation of KLF2. We observed that co-transfection of KLF2 with WWP1 resulted in a significant reduction of KLF2 protein (Fig. 2A, Panel 2, lane 3). We also

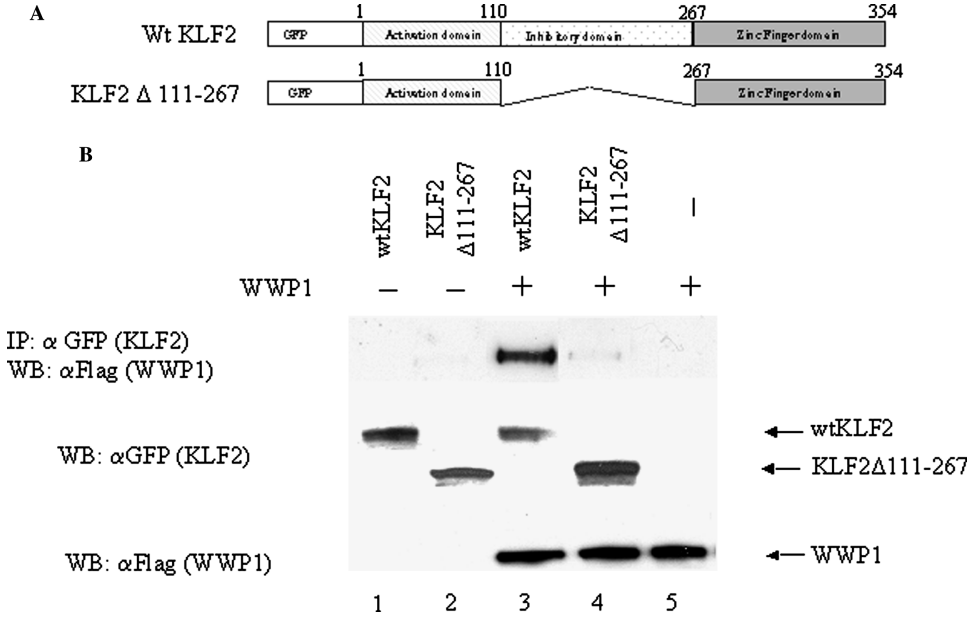

Fig. 1. The inhibitory domain of KLF2 is necessary for interaction with WWP1. (A) Schematic representation of GFP-conjugated wild-type KLF2 and mutant KLF2 where the inhibitory domain is deleted. (B) Cos-1 cells (1×10^5 per 6-well plate) were transfected either with 0.25 μg GFP-conjugated full-length KLF2 or mutant (Δ111–267) along with Flag tagged-WWP1. For control assays, wild-type GFP-KLF2, mutant KLF2 (Δ111–267), or Flag-WWP1 was transfected alone. Immunoprecipitation was performed with anti-GFP monoclonal antibody followed by Western blotting with anti-Flag M2 antibody. Western blots with anti-GFP mAb to detect KLF2 and anti-Flag mAb to detect WWP1 were also performed. Wild-type GFP-KLF2, mutant (GFP-KLF2 Δ111–267), and WWP1 are indicated by arrows.

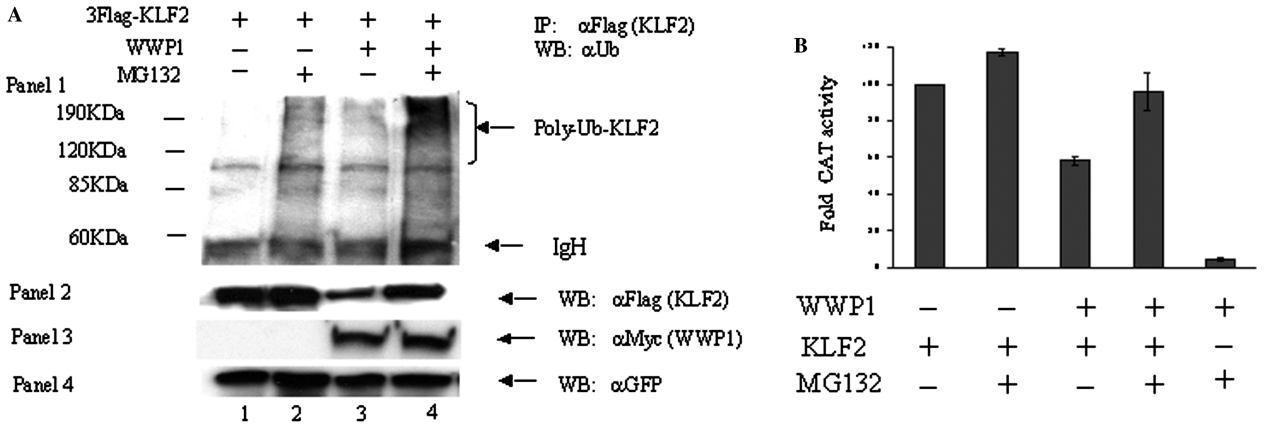

Fig. 2. WWP1 mediates the ubiquitination and proteasomal degradation of KLF2 in vivo. (A) Cos-1 cells (1×10^5 per 6-well plate) were transfected with 0.25 μg p3Xflag-CMV7.1-KLF2 either in the presence or absence of 1.0 μg pCMVmyc-WWP1 in the combinations indicated. Thirty-six hours after transfection, the cells were either mock-treated or treated with the proteasome inhibitor MG132 for 16 h and then harvested in lysis buffer. The lysates were incubated with anti-Flag antibody to immunoprecipitate KLF2. The immunoprecipitated protein was fractionated on a 10% SDS–PAGE and blotted with anti-ubiquitin mAb to detect ubiquitinated KLF2 (Panel 1). Poly-ubiquitinated KLF2 is indicated by an open parenthesis ([). The same lysates were analyzed with anti-flag to detect KLF2 (Panel 2), anti-myc antibody to detect the WWP1 protein (Panel 3), and anti-GFP which served as a loading control (Panel 4). (B) Cos-1 cells were transfected with 0.25 μg of the KLF2-responsive HS2βCAT reporter plasmid and 0.25 μg Flag-KLF2 with or without 1.0 μg pCMVmyc-WWP1. The total amount of DNA per transfection was kept constant by addition of vector DNA. The activation of KLF2 alone was set at 100. Results are presented as means ± SD ($n = 3$) of the luciferase normalized CAT activity.

probed the same membrane to confirm WWP1 expression (Fig. 2A, Panel 3) and GFP as an internal control to ascertain equal loading and transfection efficiency (Fig. 2A, Panel 4).

Several studies have shown that poly-ubiquitination of transcription factors can subsequently signal their destruction by proteasomes [12]. In our own study, we observed the increased accumulation of poly-ubiquitinated KLF2 in cells treated with MG132 (Fig. 2A, Panel 1, lane 2 and 4) indicating a role for the proteasomal pathway. In Cos-1 cell transfectants, treatment with MG132 blocked WWP1-mediated KLF2 degradation (Fig. 2A, Panel 2, compare lanes 3 and 4). Absence of MG132 treatment facilitated degradation, as expected and observed (Fig. 2A, Panel 2, lane 3). Furthermore, WWP1 attenuated the transactivational ability of KLF2 and addition of MG132 restored the normal transcriptional activity of KLF2 even in the presence of WWP1 (Fig. 2B). The above results clearly show that the proteasomal pathway is involved in degrading ubiquitinated KLF2 protein and this degradation also manifests as decreased KLF2-transactivation.

Role of the inhibitory domain in ubiquitination and degradation of KLF2

Given our earlier yeast two-hybrid data and the immunoprecipitation experiments in the present study (Fig. 1), it is clear that WWP1 interacts with the inhibitory domain of KLF2. We determined whether this domain is necessary for KLF2 ubiquitination and degradation. We used Gal4 fusion constructs containing the activation domain alone (Gal4 1–110) or one that had both the activation and inhibitory domains of KLF2 (Gal4 1–267) (Fig. 3A). We co-transfected the Gal4 fusion constructs along with WWP1 and GFP, as necessary, into Cos-1 cells. The cells were treated with MG132 to prevent proteasomal degradation of ubiquitinated KLF2. Poly-ubiquitination of KLF2 is only observed when the intact inhibitory domain is present (Fig. 3B). We used both un-transfected cell lysates and pre-immune rabbit serum to confirm the specificity of rabbit anti-GAL4 polyclonal antibody (data not shown). Subsequent to the ubiquitination reaction, we observed that KLF2 degradation by WWP1 required an intact inhibitory domain of KLF2 (Fig. 3C—Note that KLF2 degradation by WWP1 occurs only in KLF2 1–267, but not in KLF2 1–110). The GFP panel confirms equal loading. Collectively, these results show that WWP1-induced ubiquitination and degradation of KLF2 requires an intact inhibitory domain of KLF2.

Determination of ubiquitination site(s) in KLF2

Lysine residues in transcription factors frequently serve as sites for covalent modifications [12]. Interestingly, all the lysines of KLF2 are located in the inhibitory domain—a region known to be essential for WWP1-mediated ubiquitination. It was therefore reasonable to speculate that these lysines serve as sites for ubiquitin attachment. In order to determine the role of these lysines, if any, in ubiquitination, we created lysine to arginine mutants in single and multiple positions (Fig. 4A). These mutants and wild-type KLF2 along with a ubiquitin expression plasmid (HA-Ub) were

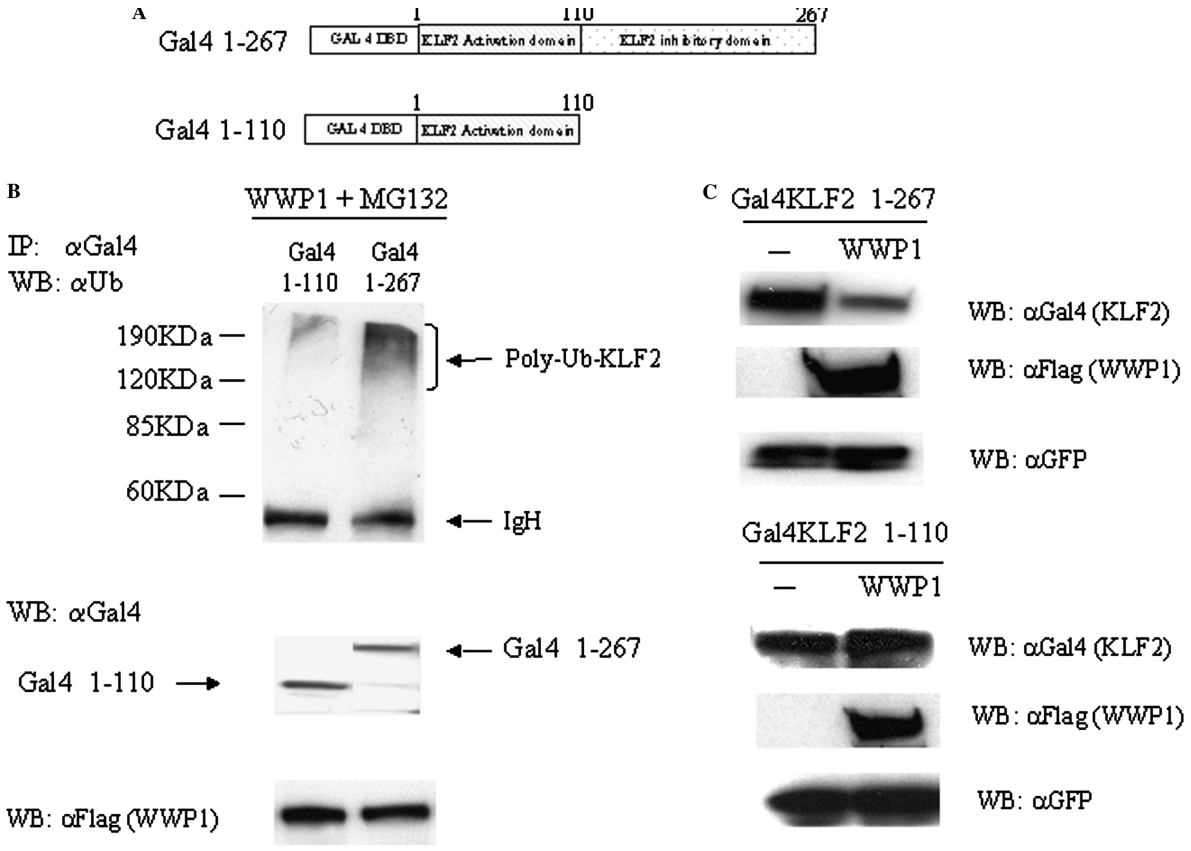

Fig. 3. The inhibitory domain of KLF2 is necessary for WWP1-mediated ubiquitination. (A) A schematic of Gal4 DNA binding domain fused to KLF2 containing both activation and inhibitory domains (Gal4 1–267) and a mutant containing the activation domain alone (Gal4 1–110). (B) Cos-1 cells were cotransfected with Gal4-KLF2 1–267 or Gal4-KLF2 1–110 and Flag-WWP1. After 36 h, the cells were treated with MG132 for 16 h before harvest. Cell lysates were immunoprecipitated with anti-Gal4 antibody. Ubiquitination of KLF2 was analyzed by Western blot with anti-ubiquitin antibody as described in Fig. 2. The same lysates were also analyzed with anti-Gal4 and anti-Flag antibodies to detect KLF2 and WWP1 proteins, respectively. The positions of Gal4-KLF2 1–110 and Gal4-KLF2 1–267 are indicated by arrows. (C) Gal4-KLF2 1–110 (top) or Gal4-KLF2 1–267 (bottom) were transfected into Cos-1 cells in the presence (+) or absence (−) of Flag-WWP1 and Western blot was performed with anti-Gal4 and anti-flag WWP1 antibodies. The same blot was re-probed with anti-GFP to assess transfection efficiency.

transfected into Cos-1 cells. Cell lysates were analyzed by immunoblotting to detect unconjugated and ubiquitinated wild type and mutant KLF2 proteins. Either wild-type KLF2 or HA-Ub plasmid was transfected alone (Fig. 4B lanes 1 and 8, respectively) to ensure that the mono-ubiquitinated KLF2 were not non-specific bands. We observed mono-ubiquitinated KLF2 only in cells transfected with wild type and 3KR (K255/257/266R) mutants and to a lesser extent, in K146R-KLF2 (Fig. 4B, upper Panel, lanes 2, 4, and 6), but not when K121R, K121/146R- or 5KR-KLF2 (121/146/255/257/266) mutants were transfected (Fig. 4B, upper Panel, lanes 3, 5, and 7). To further confirm that these proteins were indeed ubiquitinated KLF2, identical reaction mixtures were immunoprecipitated with anti-HA to pull down co-transfected HA-tagged ubiquitin and probed by Flag M2 mAb to identify the ubiquitinated wild type and lysine substituted KLF2. Again, the mono-ubiquitinated form was observed only in wild type-, K146R-, and 3KR-KLF2 (Fig. 4B, lower Panel). This shows that lysines in positions 146, 255, 257, and 266 do not affect ubiquitination of KLF2. However, mutating the lysine 121 abrogated ubiquitination, indicating that lysine 121 is the predominant site for ubiquitin-conjugation.

Lysine 121 of KLF2 controls WWP1-mediated poly-ubiquitination and degradation of KLF2

Our experiments have so far established that the lysines in positions 255, 257, and 266 do not appear to have a role in the ubiquitination of KLF2 and that lysines 121 and perhaps lysine 146 (to a lesser degree) were critical. Hence, we analyzed these lysines further to see their effect on WWP1-mediated poly-ubiquitination and degradation of KLF2. We used wild type-, K121R-, K146R-, and K121/146R-KLF2 (where lysines 121 and 146 are mutated) in Cos-1 cell transfection systems. The cell lysates were analyzed by Western blotting. In the presence of WWP1, mono-ubiquitinated KLF2 and the slow migrating, poly-ubiquitinated forms were observed with wild-type KLF2 and, to a lesser extent, in K146R mutant expressing cells (Fig. 4C, lanes 2 and 6).

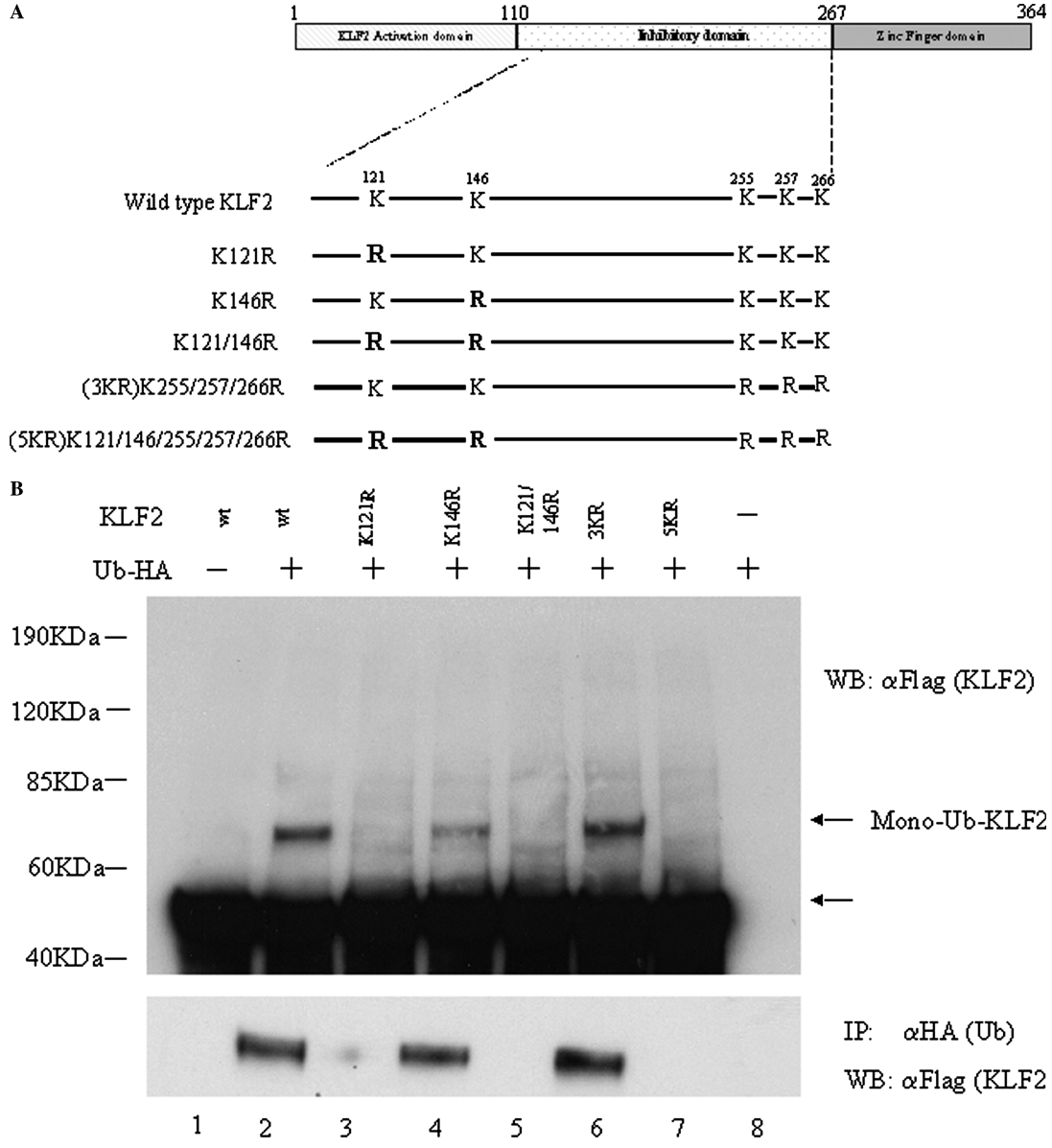

Fig. 4. Lysine 121 in the inhibitory domain of KLF2 is the target of WWP1-mediated ubiquitination and degradation. (A) Schematic representation of the lysine to arginine mutants in the inhibitory domain of KLF2. (B) Wild type or lysine to argenine mutants of KLF2 were co-expressed with HA-ubiquitin in Cos-1 cells. For control experiments, the cells were transfected with either wild-type KLF2 or HA-ubiquitin alone. Western blot was performed with anti-Flag monoclonal antibody. The unconjugated and mono-ubiquitinated forms of KLF2 are indicated by arrows. An aliquot of each cell lysate was immunoprecipitated with anti-HA antibody followed by Western blotting with anti-Flag M2 to detect mono-ubiquitinated KLF2 (bottom). (C) Wild-type KLF2 and the lysine to argenine mutants were transfected in the presence (+) or absence (−) of Myc-WWP1. Whole cell lysates were analyzed with anti-Flag M2 antibody as described in (B). Unconjugated KLF2 is indicated by an arrow. Mono-ubiquitinated forms are indicated by thick arrowheads and polyubiquitinated KLF2 is indicated by an arrow and open parenthesis (]). WWP1 expression was detected by anti-myc Western blot. (D) Aliquots of cell lysates from (C) were used to detect KLF2 and WWP1 levels using antiFlag and anti-Myc antibodies, respectively. Anti-GFP was used as a control for equal loading and transfection efficiency.

As expected, the appearance of poly-ubiquinated KLF2 was dramatically decreased when lysine 121 was mutated (Fig. 4C, lane 4) and virtually absent when lysines 121 and 146 were altered (Fig. 4C, lane 8). This clearly emphasized the importance of lysine 121 in WWP1-mediated poly-ubiquitination of KLF2. WWP1 expression was confirmed by Western blotting (Fig. 4C, lower Panel). Similar to the ubiquitin-conjugation of p53, the small proportion of KLF2 that accumulates as ubiquitinated products (Fig. 4C) is probably due to intracellular removal of ubiquitin by C-terminal hydrolases [13]. Parallel to the results of ubiquitination, only wild type and K146R–KLF2 protein levels were decreased when co-expressed with WWP1 (Fig. 4D, upper Panel, lanes 2 and 6). Mutating lysine 121 of KLF2 abolished WWP1-mediated degradation of KLF2 (Fig. 4D upper Panel,

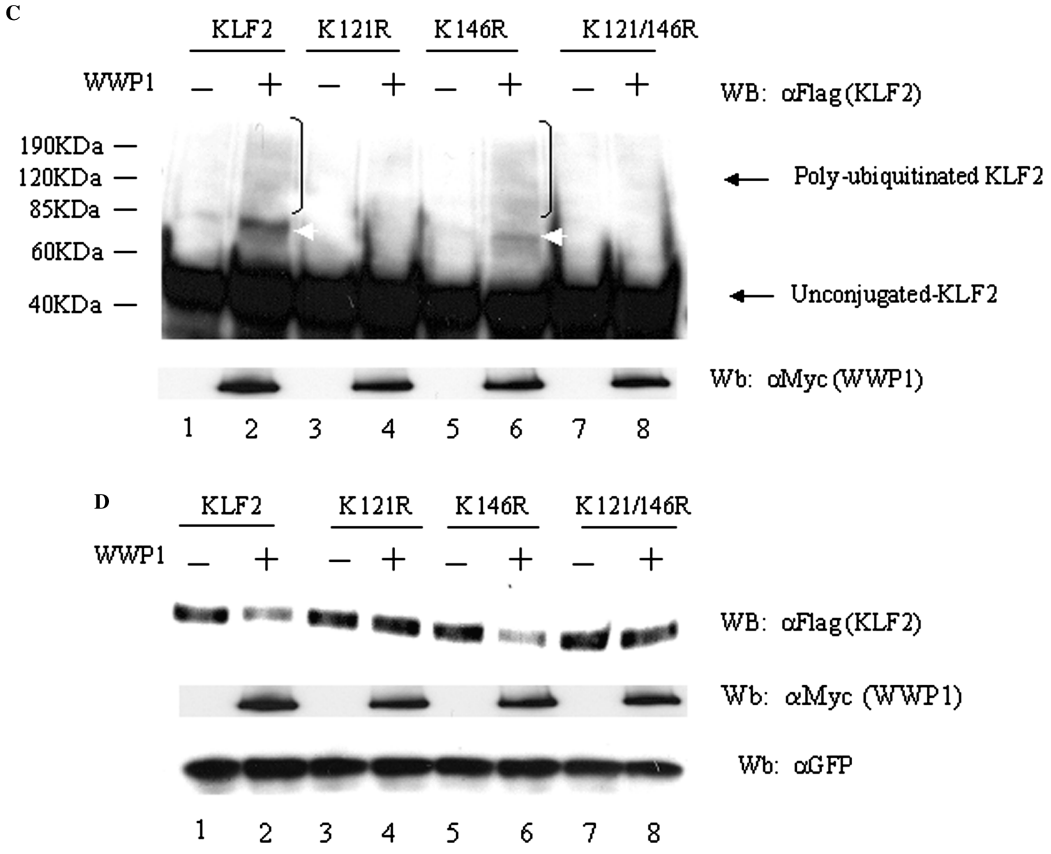

Fig. 4. (*continued*)

lane 4), thereby, emphasizing the importance of lysine 121. WWP1 expression was confirmed by Western blotting and GFP levels ascertained equal loading (Fig. 4D). We also noted that poly-ubiquitination of KLF2 required WWP1 (Compare Figs. 4B and C).

The catalytic cysteine of WWP1 is not required for ubiquitination of KLF2

E3 ligases of the HECT domain family share a region of homology at the C-terminus, which contains an E2 binding site and a cysteine that catalytically transfers Ub to its substrate [14]. To evaluate whether this catalytic cysteine of WWP1 is necessary for KLF2 ubiquitination, we generated a construct in which the catalytically active cysteine 886 was mutated to serine (C886S) and examined whether this mutation affected WWP1-mediated ubiquitination and degradation of KLF2. Quite contrary to our expectations, the mutant WWP1 (C886S) acted similar to wild type and mediated near-identical levels of KLF2 poly-ubiquitination (Fig. 5A compare lanes 4 and 6) and degradation (Fig. 5B, upper Panel, compare lanes 3 and 5). Further, a HECT domain deletion mutant of WWP1 behaved similarly (data not shown). Taken together, these data demonstrate that while WWP1 is necessary for the ubiquitination and degradation of KLF2, its catalytic cysteine does not seem to be involved in carrying out this reaction.

Discussion

The results presented here show that the loss of KLF2 transcriptional activity correlates with its ubiquitination and degradation by WWP1. In addition, we have identified the domain of KLF2 required for in vivo interaction with WWP1 and the critical residues essential for WWP1-mediated degradation. While this represents another example of ubiquitin-mediated proteolysis negatively affecting a transcription factor [13,15–17], it is the first evidence of a Krüppel-like factor interacting with and being post-translationally modified by an E3 ligase.

WWP1 is known to interact with proline-rich regions or phosphoserine and phosphothreonine-containing elements within its binding partner [18–21]. KLF2 is a proline-rich protein and contains a PEST sequence [22,23] (rich in proline, glutamic acid, serine, and threonine). This makes it a very attractive candidate for interacting with WWP1. Furthermore, the PEST sequence resides in the inhibitory domain (aa 164–184) of KLF2, which is critical for proteasomal degradation.

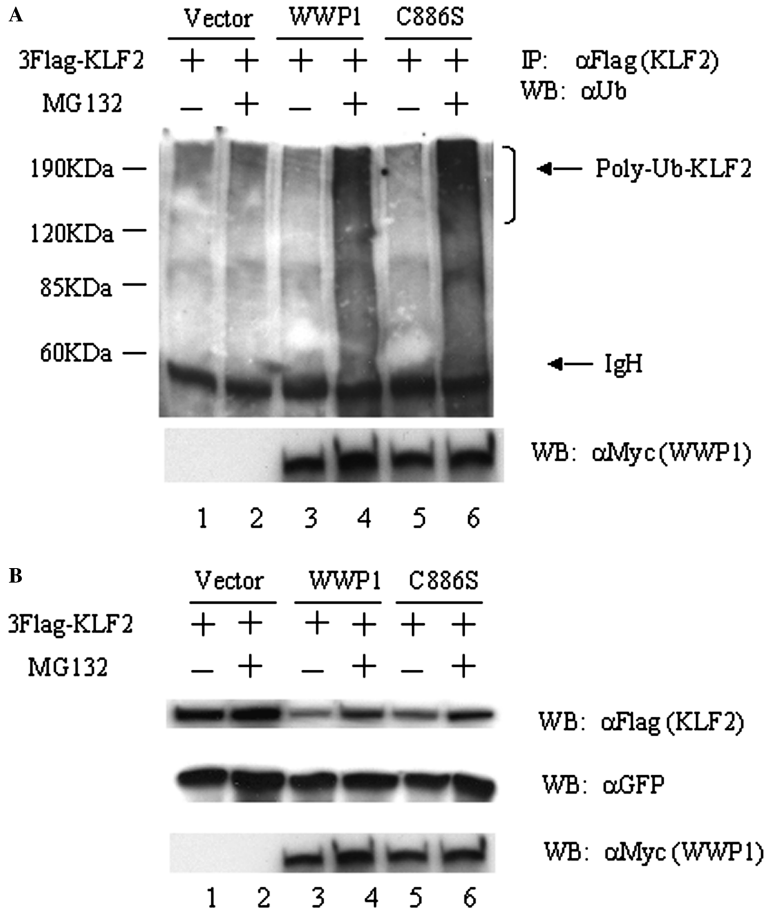

Fig. 5. The catalytic Cysteine 886 of WWP1 is not required for the ubiquitination of KLF2. (A) The transfection, immunoprecipitation, Western blotting, and MG132 treatments are the same as described in Fig. 2. In addition, cells were transfected with KLF2 in the presence or absence of C886S-WWP1. Poly-ubiquitinated KLF2 is indicated by an open parenthesis (]). (B) Aliquots of cell lysates from (A) were analyzed by Western blot with indicated antibodies to detect KLF2, GFP, and WWP1.

This further validates the hypothesis that it interacts with WWP1 and is targeted for ubiquitination and degradation. Consistent with our hypothesis, our present experiments clearly demonstrate that WWP1 interacts with the inhibitory domain of KLF2 in vivo and that the inhibitory domain of KLF2 plays a key role not only as a site for ubiquitin-targeting but also in substrate recognition by WWP1 and ultimately, by the proteasomes.

The accepted model of ubiquitination of transcription factors is that ubiquitin moieties are attached onto specific motifs on the factor and recognized by the ubiquitin machinery. The addition of long, homopolymeric chains of ubiquitin invariably serves as a signal for degradation by the 26S proteasome [12]. Our results show beyond doubt that basal levels of monoubiquitination occur even in the absence of exogenous WWP1. However, poly-ubiquitination and degradation definitely require WWP1. Our findings represent one of the first reports that indicate a defined role for WWP1 in mediating the ubiquitination of a specific protein.

Many studies have shown that ubiquitin modification involves one or more specific lysine residues in the target protein [24–27]. We demonstrated here that lysine 121 is the major ubiquitin targeting site and lysine 146 may be used as an alternative site for ubiquitin conjugation. Both of these lysines are located in the KLF2 inhibitory domain, which we know is critical for WWP1-mediated alteration of the transcriptional activity of KLF2. Since the conserved cysteines in the HECT domain of E3s are known to be involved in ubiquitin transfer, we expected this to be the case for WWP1. Surprisingly, we observed that altering this catalytic cysteine (C886) did not affect WWP1's ability to mediate ubiquitin-modification and proteasomal degradation of KLF2. Furthermore, mutagenesis studies showed that the seven additional cysteines in the HECT domain of WWP1 are not involved in mediating KLF2 degradation either (data not shown). Based on these observations, we speculate that WWP1 may function as a cofactor/adaptor, rather than the catalytic E3-ubiquitin ligase and promotes the ubiquitination of KLF2 through another E3. Our studies therefore suggest that the regulation of KLF2 is

complex and involves more than a direct factor–ligase interaction.

There are several other reports that provide evidence for highly intricate pathways governing the ubiquitination of proteins and therefore support our conclusions. Genetic analysis of various transgenic SLI-1 mutants in *C. elegans* showed that the SLI-1 RING-defective mutant was still competent in down-regulating LET-23, indicating the involvement of another E3 [28]. Mammalian Nedd4 and its budding yeast ortholog Rsp5 are HECT-domain E3 ligases and their WW domains interact with other cofactors to mediate ubiquitin/proteasome-dependent proteolysis [29]. Furthermore, E3s of the RING and HECT families are known to cooperate in mediating Notch ubiquitination [30]. Courbard et al. [31] have provided direct evidence that Cbl-C (RING domain E3) and AIP4 (HECT domain E3) interact and cooperate to down-regulate EGFR signaling. Thus, our studies and those described above suggest a cooperation of E3-ubiquitin ligases with each other or additional factors. In such cases, one can expect that certain HECT domain E3s may not have a direct catalytic function in ubiquitination.

In conclusion, we have shown that WWP1, a HECT domain-containing E3 ubiquitin ligase, interacts in vivo and mediates the ubiquitination and proteasome-dependent degradation of KLF2, which consequently down-regulates the transcriptional activity of KLF2. We have identified the region and the specific residues of KLF2 that are essential for these post-translational processes. Our data also suggest that a cofactor of E3-ligase may be recruited, or that a residue other than the known catalytic cysteine of WWP1 is involved, in the ubiquitination and degradation of KLF2.

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