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The E3 ubiquitin ligase HECW1 targets thyroid transcription factor 1 (TTF1/NKX2.1) for its degradation in the ubiquitin-proteasome system

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Abstract

Thyroid transcription factor 1 (TTF1/NKX2.1), is a nuclear protein member of the NKX2 family of homeodomain transcription factors. It plays a critical role in regulation of multiple organ functions by promoting gene expression, such as thyroid hormone in thyroid and surfactant proteins in the lung. However, molecular regulation of TTF1 has not been well investigated, especially regarding its protein degradation. Here we show that protein kinase C agonist, phorbol esters (PMA), reduces TTF1 protein levels in time- and dose-dependent manners, without altering TTF1 mRNA levels. TTF1 is ubiquitinated and degraded in the proteasome in response to PMA, suggesting that PMA induces TTF1 degradation in the ubiquitin-proteasome system. Furthermore, we demonstrate that an E3 ubiquitin ligase, named HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1 (HECW1), targets TTF1 for its ubiquitination and degradation, while downregulation of HECW1 attenuates PMA-induced TTF1 ubiquitination and degradation. A lysine residue lys151 was identified ubiquitin acceptor site within the TTF1. A lys151 to arginine mutant of TTF1 (TTF1K151R) is resistant to PMA- or HECW1-mediated ubiquitination and degradation. Further, we reveal that overexpression of TTF1 increases lung epithelial cell migration and proliferation, while the effects are reversed by HECW1. This study is the first to demonstrate that the E3 ubiquitin ligase HECW1 regulates TTF1 degradation by site-specific ubiquitination. This study will provide a new direction to clarify the molecular regulation of TTF1 in lung and its role in lung epithelial remodeling after injury.

Keywords

TTF1; HECW1; ubiquitination; proteasomal degradation

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7. CONFLICT OF INTEREST

The authors declare that they have no competing interest.

1. INTRODUCTION

TTF1, also known as NKX2.1, TITF1 or T/EBP (thyroid-specific-enhancer binding protein), is a nuclear protein member of the Nkx2 family of homeodomain transcription factors. TTF1 was first identified in the follicular epithelial cells of the thyroid. Thereafter it was found in other organs such as lung [1] and diencephalon [2, 3], pituitary [4], parathyroid gland [5]. TTF1 functions as transcriptional factor and plays a critical role in regulating gene expression in most tissues. Besides being identified a tissue-specific transcription factor in adult organs, TTF1 has been known to play roles in morphogenesis and differentiation [6–8]. The amino acid sequences of human, rat, and mouse of TTF-1 have 98% sequence similarity, with the complete conservation of the 60-amino-acid homeodomain.

In the lung, the expression of TTF1 is confined to the type II alveolar epithelial cells and the Clara cells. TTF1 promotes the transcription of the surfactant proteins A to D [9], and the Clara cell secretory protein [10] that are essential for lung stability and lung host defense. TTF1 has been considered as a biomarker to determine if a tumor arises from the lung [1, 11, 12]. For lung cancers, a great many of investigations have confirmed TTF1 is usually positive in adenocarcinomas and small cell carcinomas [13–15], while squamous cell carcinomas and large cell carcinomas are rarely positive. Considering the important roles that TTF1 plays, Russell PA et al. [16] have suggested that abnormal expression of TTF1 probably contributes to the pathogenesis of lung cancer. However, molecular mechanisms that control cell/tissue specific expression of TTF1 and TTF1 transcriptional activity are not fully understood. It has been shown that TTF1 gene expression is regulated by glucocorticoids, cAMP, and TGFβ in lung cells [17], however, the molecular regulation of protein stability of TTF1 has not been reported.

Ubiquitination is an enzymatic process that links an ubiquitin protein or an ubiquitin chain to a substrate protein [18, 19]. This has been referred as the molecular signal for protein degradation. The research on protein ubiquitination has been focused to investigate protein turnover in recent years. E3 ubiquitin ligases recognize a specific protein substrate, and assist or directly catalyze the transfer of ubiquitin to the protein substrate [20, 21]. HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1 (HECW1, also known as NEDL1) is a member of the E3 ligase HECT family [22]. It was first identified in brain tumors [23], while the biological functions of HECW1 have not been well studied. HECW1 has been shown to regulate p53-mediated apoptosis [24, 25]. Li Y et al. demonstrated that HECW1 enhances the p53-mediated apoptosis in its catalytic activity-independent manner [24], while the other study suggested that HECW1 interacts with another E3 ligase RNF43, which is associated transcriptional activity of P53 [25]. Recent study indicated the mutation of HECW1 may possess a significant role in the pathogenesis of transitional cell carcinoma. To our knowledge, two protein substrates of HECW1 has been reported [23, 26]. Protein turnover of mutant superoxide dismutase-1 [23] and ErB4 [26] are regulated by HECW1. Here we identify TTF1 a new substrate for HECW1.

This is the first study to investigate molecular regulation of protein stability of TTF1. We demonstrate that TTF1 degradation is mediated by the ubiquitin-proteasome system. E3

ligase HECW1 regulates TTF1 degradation through site-specific ubiquitination. Further, we reveal a new biological function of TTF1 in lung epithelial cells that TTF1 promotes lung epithelial cell remodeling after injury. This study provides a new insight into molecular regulation of TTF1 in lung epithelial cells.

2. MATIERIALS AND METHODS

2.1 Cell culture and reagents

Murine lung epithelial (MLE12) cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured with HITES medium containing 10% FBS at 37°C in 5% CO2 incubator. V5 tag antibody, mammalian expressional plasmid pcDNA3.1/His-V5 TOPO, and *Escherichia coli* Top 10 competent cells were from Invitrogen (Carlsbad, CA, USA). A549 (human lung carcinoma cell line) Beas2B (human lung bronchial epithelial cell line), HBE (human bronchial epithelial cells) and HEK293T (human embryonic kidney) were from ATCC. HECW1 siRNA, Leupeptin, and β-actin antibody were from Sigma Aldrich (St. Louis, MO, USA). MG-132 was from Calbiochem (La Jolla, CA, USA). Immunobilized protein A/G beads, ubiquitin, HA tag, TTF1, and control IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HECW1 antibody was from Sabbiotech (College Park, MD, USA). GAPDH antibody was from Proteintech (Rosemont, IL, USA). PMA was from Cayman Chemical Company (Ann Arbor, MI, USA). All materials in highest grades uses in the experiments are commercially available.

2.2 Construction of plasmids

TTF1, HECW1, NEDD4L and hWWP1 cDNA were inserted into a pcDNA3.1D/V5-His vector or pcDNA3.1D/HA-His vector (Invitrogen, CA, USA) respectively. Site directed mutagenesis was performed to generate TTF1 lysine mutant according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA).

2.3 Immunoblotting and immunoprecipitation

Cells grown on 6-well plates or 100-mm dishes were washed with cold PBS and collected in cell lysis buffer containing 20 mM Tris HCl (PH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl2, 1% Trison X100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, and subjected to sonication and centrifugation. An equal amount of cell lysates (20 μg) was subjected to SDS-PAGE, electrotransferred to membranes and immunoblotted as standard protocol. For immunoprecipitation, equal amounts of cell lysates (1 mg) were incubated with a specific primary antibody overnight at 4°C, followed by the addition of 40 μ1 of protein A/G agarose and incubation for additional 2 h at 4°C. The immunoprecipitated complex was washed 3 times with cold phosphate-buffered saline and analyzed by immunoblotting with indicated antibodies.

2.4 Transfection of plasmids and siRNA

MLE12 cells were suspended in 120 μ1 of nucleofection buffer and mixed well with plasmid DNA or siRNA in an electroporation cuvette. Electroporation was performed in the

Nucleofection™ II System (Lonza, Gaithersburg, MD, USA), and the cells were cultured in complete HITES medium for 48 or 72 h.

2.5 In vivo ubiquitin assay

Cell were washed and collected with cold PBS. After centrifuging at 2000 rpm for 5 min, supernatant was removed, 1 μ1 of ubiquitin aldehyde and 1 μ1 of NEM were added into the cell pellet. 50-80 μ1 of 2% SDS lysis buffer was added according to the cell amount. Cell lysates were then sonicated on ice for 12 sec followed by boiling at 100°C for 10 min. Samples were diluted with TBS. The following steps were the same as normal IP.

2.6 RNA isolation, reverse transcription and quantitative PCR

Total RNA was isolated from cultured MLE cells using NucleoSpin RNA extraction kit (Clontech Laboratories, Inc.) according to the manufacturer's instructions, and RNA was quantified by spectrophotometry. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed to assess expression of TTF1 using primers designed based on mouse mRNA sequences. TTF1 primers: forward 5'-ACAGCCAAGCAAATTCAACCC-3' and reverse 5'-GCCTAAGCTTGGGAACCCATT-3'. Real-time PCR was performed using iQ SYBR Green Supermix and the iCycler real-time PCR detection system (Bio-Rad).

2.7 Cell migration assay

Cell migration was evaluated using a wound healing assay. In brief, transfected cells were cultured in six-well plates ($5\chi10^5$ cells per well). At 90-95% confluence, the monolayer of cells was scratched with using a sterile plastic micro pipette tip, and then cells were cultured with serum-free (blank) medium for 24 h. Cells were digitally photographed at 0 and 24 h after stimulation using an EVOS microscope (Thermo Fisher Scientific, USA). Quantitative analysis of the scratch area of closure was performed using Image J software. The percentage of wound closure was calculated as follows: [(pre-migration area – migration area)/pre-migration area] χ 100. Results are representative of three independent experiments performed in triplicate.

2.8 Cell proliferation assay

Cells were seeded at 5.0×10^5 cells per well in 6-well plates with serum-free (blank) medium at 24 h after transfection. The numbers of MLE12 cells were counted at 24, 48, and 72 h after the cells were seeded using the Countess II FL Automated Cell Counter (Invitrogen).

2.9 Statistical Analysis

All results were subjected to statistical analysis using one way or two way ANOVA, and, wherever appropriate, the data were analyzed by t-test and expressed as means ± SD. Data were collected from at least three independent experiments, and $p<0.05$ was considered significant (* $p<0.05$; ** $p<0.01$).

3. RESULTS

3.1 PMA induces TTF1 protein degradation by regulating protein stability

To investigate molecular regulation of TTF1 in lung epithelial cells, first, we examined TTF1 expression in different lung epithelial cell lines. MLE12 (Mouse type II alveolar epithelium), Beas2B (normal human bronchial epithelial cell line), and HBE (normal human bronchial epithelial cell line) cells, but not A549 (human non-small cell lung cancer) and 293T (human embryonic kidney) cells express TTF1 (Figure 1A). Further, we chose MLE12 cells in our study. PMA has been known to regulate phosphorylation of TTF1 [27]. We tested the effect of PMA on TTF1 protein levels. PMA reduced TTF1 protein levels in time-dependent (Figure 1B, 1C) and dose-dependent (Figure 1D, 1E) manners. PMA (0.1 μM) treatment for 8 h significantly diminished TTF1 protein. To examine whether the effect of PMA on the reduction of TTF1 protein mass is due to modulation of *TTF1* mRNA expression, we measured *TTF1* mRNA levels by RT- realtime PCR after PMA stimulation. KC was used as a positive control. As shown in Figure 1F, PMA increased *KC* expression, but it had no effect on *TTF1* mRNA expression, suggesting that PMA-induced TTF1 reduction was through the regulation of TTF1 protein stability.

3.2 PMA induces TTF1 degradation in the ubiquitin-proteasome system

The proteasome and lysosome systems are major pathways for intracellular protein degradation. In order to identify which pathway was involved in the degradation of TTF1, cells were treated with inhibitors of proteasomes (MG-132) or lysosomes (leupeptin) prior to administration of PMA. PMA-mediated TTF1 degradation was blocked by pretreatment with MG-132, but not leupeptin (Figure 2A, 2B). It suggested TTF1 degradation was mediated in the proteasome system, but not in the lysosome system. To examine if PMA induces TTF1 ubiquitination, cells were treated with PMA, *in vivo* ubiquitination assay revealed that PMA increased polyubiquitination of TTF1 (Figure 2C).

3.3 HECW1 diminishes TTF1 protein levels

To investigate which E3 ubiquitin ligase was associated with TTF1 degradation, we used an unbiased screening system and overexpressed several V5-tagged E3 ubiquitin ligases with different doses in MLE cells. Figure 3A and 3B showed that the ectopic expression of HECW1-V5 diminished endogenous TTF1 protein in a dose-dependent manner, while HECW1-V5 did not affect transcription factor-related proteins including IκB and E2F1. Overexpression of hWWP1-V5 moderately reduced TTF1 protein levels (Figure 3C, 3D), while overexpression of others including NEDD4L-V5 had no effect on TTF1 expression (Figure 3E, 3F). To further examine if the effect of HECW1-V5 on the reduction of TTF1 protein mass is due to modulation of *TTF1* mRNA expression, we measured *TTF1* mRNA levels by RT-realtime PCR (Figure 3G). HECW1-V5 had no effect on *TTF1* mRNA expression, suggesting that HECW1-mediated TTF1 reduction is through the down-regulation of TTF1 protein stability.

To examine if HECW1 regulates PMA-induced TTF1 degradation, HECW1 was downregulated by *HECW1* siRNA transfection prior to PMA treatment. As shown in Figure

3H and 3I, *HECW1* siRNA knockdown attenuated the effects of PMA on TTF1 degradation, suggesting that PMA induced TTF1 degradation is dependent on HECW1.

3.4 HECW1 targets TTF1 for ubiquitination

Next, we investigated if HECW1 is associated with TTF1. Immunoprecipitation (IP) with an antibody to TTF1 followed by Western blotting with an antibody to the V5 tag revealed that HECW1-V5 associates with TTF1 (Figure 4A). Further, endogenous TTF1 was detected in the HECW1-V5 immunoprecipitation complex (Figure 4B), suggesting that HECW1 is associated with TTF1. Interesting, the association of HECW1 and TTF1 was increased by PMA treatment (Figure 4C). To examine if HECW1 induced TTF1 ubiquitination, cells were transfected with HECW1-V5 plasmids. *In vivo* ubiquitination assay revealed that overexpression of HECW1 increased polyubiquitination of TTF1 (Figure 4D), while knockdown of HECW1 diminished PMA-induced TTF1 ubiquitination (Figure 4E).

3.5 Lys (K) 151 is a key ubiquitin acceptor site within TTF1

Lysine residues within a target protein are ubiquitin acceptor sites for linking mono-ubiquitin or poly-ubiquitin chain [28]. To investigate the molecular regulation of TTF1 ubiquitination, we screened the full length amino acid sequence of TTF1 and predicted the potential ubiquitination sites within TTF1 protein (UbPred: predictor of protein ubiquitination sites). The highest score for ubiquitination site is the lys151 (Figure 5A). To evaluate if the lys151 is the ubiquitin acceptor site, we substituted several candidate lys residues of TTF1 with arginine (R) to identify the putative ubiquitin acceptor site within TTF1 for HECW1. TTF1K151R-HA, not TTF1 wild type and TTF1K25/26R-HA, was resistant to HECW1-mediated degradation (Figure 5B-D). Meanwhile, compared to TTF1 wild type and TTF1K25/26R-HA, TTF1K151R-HA increased stability in the presence of PMA (Figure 5E-J). Further we compared the degree of ubiquitination of TTF1 wild type (TTF1-HA) and TTF1K151R-HA after treatment with PMA. As shown in Figure 5K, TTF1K151R-HA exhibited less ubiquitination, compared to TTF1-HA, suggesting that lys151 is a key ubiquitin acceptor site within TTF1. The structure analysis (completed by RaptorX structure prediction server) found that K151 site is located in the outer side of TTF1 and near the homeodomain under normal state (Figure 5L), which is also conducive to its combination with E3 ligase.

3.6 HECW1 attenuates TTF1- induced cell migration and proliferation

Cell migration and proliferation are essential for normal embryonic development, immune system function, and angiogenesis, as well as lung epithelial wound repair after injury. To access the effects of TTF1 and HECW1 on cell migration in lung epithelial cells, TTF1-overexpressed and TTF1+HECW1-V5 overexpressed MLE12 cells were scratched in cell culture dishes and cell migration were observed by measuring the area of wound closure. As shown in Figure 6A, TTF1 overexpression increased cell migration, while HECW1-V5 significantly reversed the effect of TTF1. Further, to examine the effect of TTF1 and HECW1 on regulating cell proliferation, MLE12 cells were transfected with TTF1 or TTF1+HECW1-V5 followed by cell proliferation assay. We found that TTF1 overexpression significantly increased cell proliferation, while co-overexpression with HECW1-V5 attenuated TTF1-induce cell proliferation (Figure 6B). These data indicate that TTF1

promotes lung epithelial cell remodeling after injury through increasing migration and proliferation. HECW1 negatively regulates the effect of TTF1.

4. DISCUSSION

TTF1 is a homeodomain-containing transcription factor, belonging to NKX2 family. It has been known that TTF1 plays key roles in the control of embryonic development and differentiation of lung [29, 30]. Abnormal changes of TTF1 expression contribute to the pathogenesis of human disorders including lung diseases. Reduction or absent of TTF1 levels are observed in regions of acute inflammation, bronchopulmonary dysplasia, and atelectasis in lungs of infants [31–34]. In contrast, TTF1 expression is increased in cells in regions of the lung that were undergoing regeneration. The role of TTF1 in lung diseases is possible due to its ability to regulate the expression of SP-A, SP-B, SP-C, and CCSP (Clara cell secretory protein) [35–38]. Despite of its importance in maintaining lung stability, little is known regarding the molecular regulation of TTF1 protein stability in normal lung or pulmonary diseases to date. In the present study, we demonstrate that TTF1 protein is degraded in the ubiquitin-proteasome system in alveolar epithelial cells. Further, here it is the first to demonstrate that the E3 ubiquitin ligase HECW1 is responsible for site-specific polyubiquitination and degradation of TTF1. This is of great significance for further investigating TTF1 stability in human disorders. We also reveal that increase in TTF1 levels may promote lung epithelial remodeling after injury through increasing cell migration and proliferation.

TTF1 has been known to be phosphorylated by a variety of protein kinases including PKC [39]. Phosphorylation is an essential protein modification for protein degradation. Huang H et al. have demonstrated that activation of PKC by PMA caused reduction of TTF1 [40], however, they did not investigate if PMA affects TTF1 mRNA changes or protein stability. In this study, we discover that PMA induces TTF1 association with HECW1 and TTF1 ubiquitination and degradation, without altering TTF1 mRNA levels. These data reveals that abnormal changes of TTF1 protein stability result in alteration of TTF1 activity. This study builds a basis for investigating molecular regulation of TTF1 protein stability In the future study, we will examine if the phosphorylation by PMA is essential for TTF1 ubiquitination. TTF1 activity has been known to be negatively regulated by acetylation which is mediated by CBP, a histone acetyltransferase. The effect of acetylation on TTF1 ubiquitination and degradation will be future study focus. The ubiquitin-proteasome system is the major proteolytic pathway to degrade intracellular proteins to maintain the protein homeostasis. Our results indicate that PMA-induced TTF1 degradation is mediated in the ubiquitin-proteasome system, but not in the lysosome system. We also noticed that an investigation suggested the proteasome dysfunction inhibited SP-B gene expression via reducing expression of TTF-1 protein in papillary adenocarcinoma of the lung cells [41]. Though the authors did not discuss the mechanisms, it is possible that proteasome inhibitor affects TTF1 gene expression through regulation of its transcriptional factors expression. The detailed molecular mechanism remains to be further investigated.

We examined effects of several E3 ligases on TTF1 degradation and found only HECW1 significantly reduced TTF1 levels. WWP1 and Nedd4L belongs to the same E3 ligase family

with HECW1, while they did not exhibit effects on TTF1 stability. HECW1 is a relatively new discovered E3 ubiquitin ligase. HECW1 has shown to regulate ubiquitination and degradation of mutant SOD1 [23] and ErB4 [26]. We demonstrate that HECW1 is responsible for PMA-induced TTF1 ubiquitination and degradation in lung epithelial cells. This study reveals a new substrate for HECW1. There is no direct evidence showing that abnormal expression of HECW1 is involved in human disorders. Recently, a quantitative GWAS study for a heritable pathogenic defect in IgA nephropathy showed that an intronic SNP at the *HECW1* locus may be related to the diseases [42]. Future study may reveal if the SNP is associated with *HECW1* expression and TTF1 stability.

Ubiquitin links to the substrate on its lysine residue [43, 44]. We identified that lys151 is the ubiquitin acceptor site within TTF1. There are two nonsynonymous SNPs causing mutation of lys151 in *TTF1* have been have been identified [14, 45–47]. Lys151 can be replaced with asparaginev (N) or glutamic acid (E). Little is known about the mutation in human diseases. Based on our finding, these mutations may affect TTF1 biological function by increasing its protein stability.

This is the first evidence to support that TTF1 promotes cell migration and proliferation in normal lung epithelial cells, which suggests a potential protective effect of TTF1 in lung repair and remodeling after lung injury. In summary, the current study unveils that TTF1 is not stable in presence of PKC agonist and HECW1 targets TTF1 for its ubiquitination in lys151 and induces its proteasomal degradation. It provides a new direction to investigate the molecular mechanism of TTF1 turnover in lungs. A better understanding of molecular mechanisms, pathways and agents that control TTF1 levels in the lungs will be useful for the development of novel treatment strategies for lung diseases such as acute respiratory distress syndrome, pulmonary fibrosis, lung cancer, and others.

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ABBREVIATIONS

5.

TTF1	thyroid transcription factor 1
PMA	phorbol esters
HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1
CCSP	Clara cell secretory protein

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Highlights:

- TTF1/NKX2.1 stability is regulated by the ubiquitin-proteasome system.
- Lysine 151 is the ubiquitin acceptor site within TTF1/NKX2.1.
- The ubiquitin E3 ligase HECW1 catalyzes TTF1 ubiquitination.
- TTF1 promotes lung epithelial cell migration and proliferation.

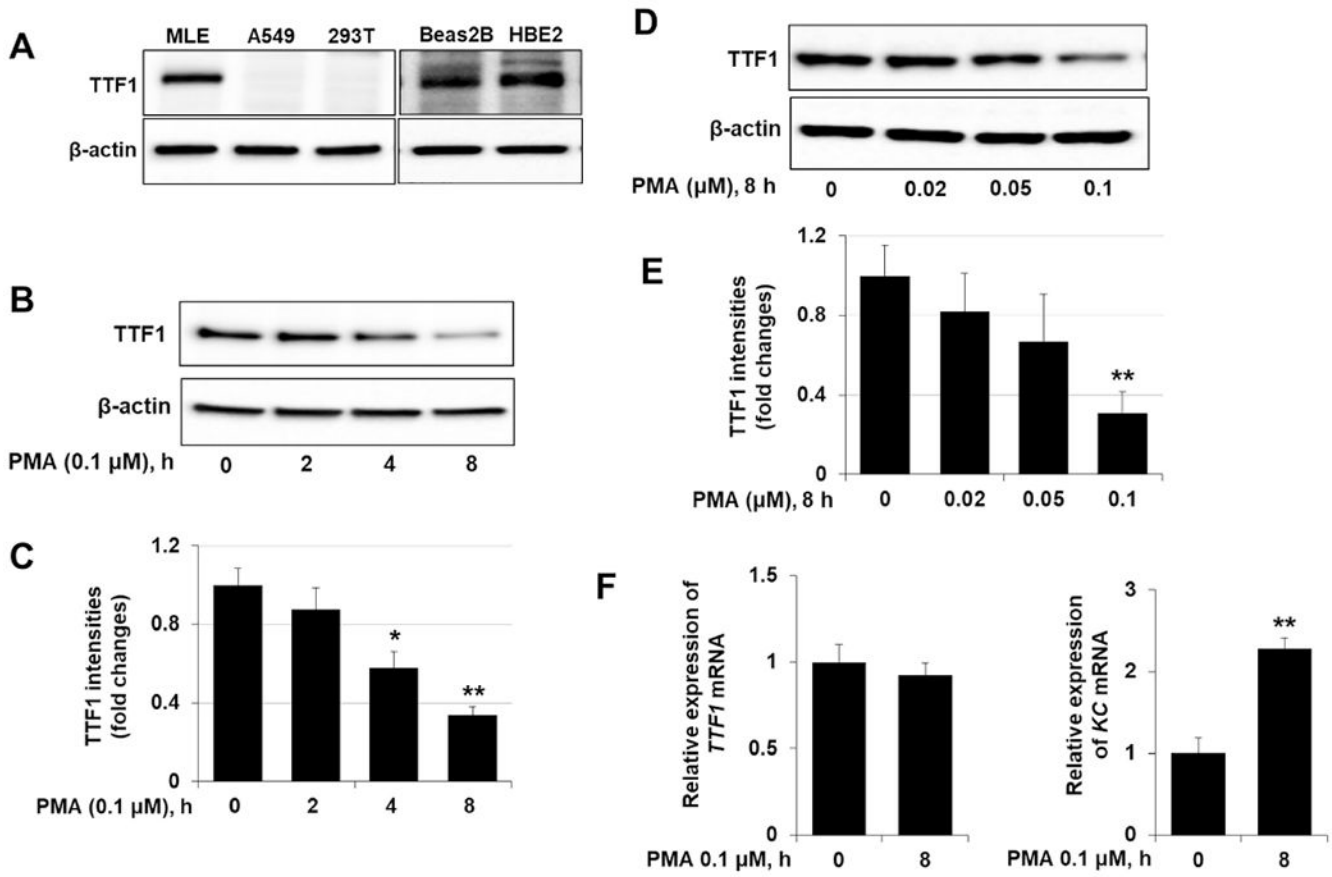

Figure 1. PMA induces TTF1 protein degradation by regulating protein stability.

A. MLE12, Beas2B, HBE2, A549 and 293T cells were detected for endogenous TTF1 respectively. Cell lysates were analyzed by immunoblotting with TTF1 and β-actin antibodies. **B.** MLE12 cells were treated with PMA (0.1 μM) for indicated incubation time. Cell lysates were analyzed by immunoblotting with TTF1 and β-actin antibodies. **C.** TTF1 levels from three independent PMA time-dependent experiments were quantified by image J software. * P<0.05; ** P<0.01. **D.** MLE12 cells were treated with indicated doses PMA for 8 h. Cell lysates were analyzed by immunoblotting with TTF1 and β-actin antibodies. **E.** TTF1 levels from three independent PMA dose-dependent experiments were quantified by image J software. ** P<0.01. **F.** Total RNA was extracted from vehicle or PMA (0.1 μM, 8 h) –treated MLE12 cells, TTF1 and KC mRNA levels were then examined by RT-real time PCR with specifically designed TTF1 and KC primers. * P<0.05; ** P<0.01. All the blots and images are representative of three independent experiments.

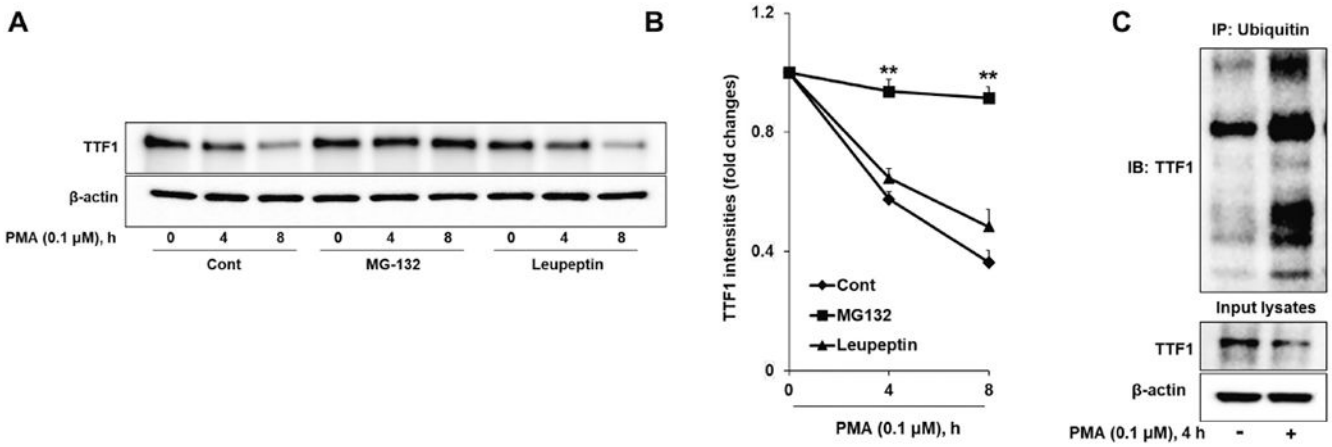

Figure 2. PMA induces TTF1 degradation in the ubiquitin-proteasome system.

A. MLE12 cells were treated with MG-132 (20 μM) or leupeptin (100 μM) 1 h prior to PMA (0.1 μM) treatment for indicated incubation time. Cell lysates were analyzed by immunoblotting with TTF1 and β-actin antibodies. **B.** TTF1 levels from three independent inhibitor blocking experiments were quantified by image J software. ** P<0.01. **C.** MLE12 cells were treated with vehicle or PMA (0.1 μM) for 4 h. Cell lysates were subject to immunoprecipitation with ubiquitin antibody, followed by TTF1 immunoblotting. Input lysates were analyzed by immunoblotting with TTF1 and β-actin antibodies. All the blots and images are representative of three independent experiments.

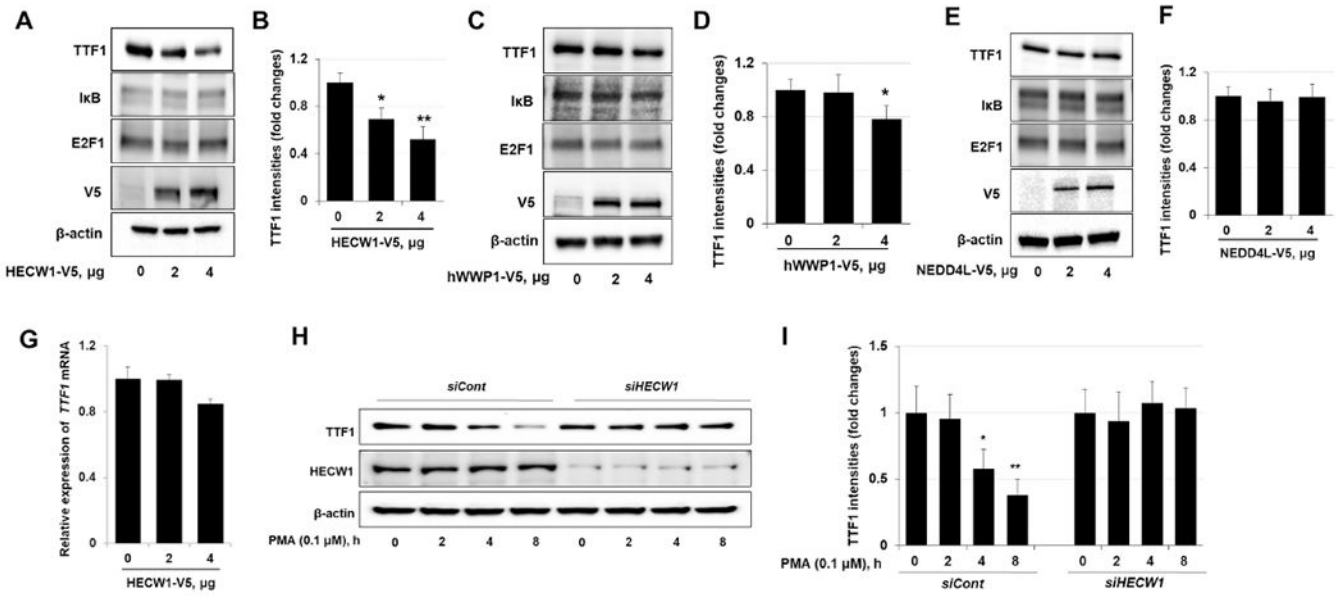

Figure 3. HECW1 diminishes TTF1 protein levels.

A. MLE12 cells were transfected HECW1-V5 plasmids in different doses for 48 h and then the cell lysates were analyzed by immunoblotting with TTF1, V5 tag, IκB, E2F1 and β-actin antibodies. **B.** TTF1 levels from three independent HECW1-V5 transfected experiments were quantified by image J software. * P<0.05; ** P<0.01. **C.** MLE12 cells were transfected hWWP1-V5 plasmids in different doses for 48 h and then the cell lysates were analyzed by immunoblotting with TTF1, V5 tag, IκB, E2F1 and β-actin antibodies. **D.** TTF1 levels from three independent hWWP1-V5 transfected experiments were quantified by image J software. * P<0.05. **E.** MLE12 cells were transfected NEDD4L-V5 plasmids in different doses for 48 h and then the cell lysates were analyzed by immunoblotting with TTF1, V5 tag, IκB, E2F1 and β-actin antibodies. **F.** TTF1 levels from three independent NEDD4L-V5 transfected experiments were quantified by image J software. **G.** Total RNA was extracted from empty vector or HECW1-V5 plasmid-transfected MLE12 cells. TTF1 mRNA levels were then examined by RT-real time PCR with specifically designed TTF1 primers. **H.** MLE12 cells were transfected with sicontrol RNA or HECW1 siRNA for 72 h followed by PMA (0.1 μM) treatment for indicated time. Then the cell lysates were analyzed by immunoblotting with TTF1, HECW1 and β-actin antibodies. **I.** TTF1 levels from three independent siHECW1 experiments were quantified by image J software. * P<0.05; ** P<0.01. All the blots and images are representative of three independent experiments.

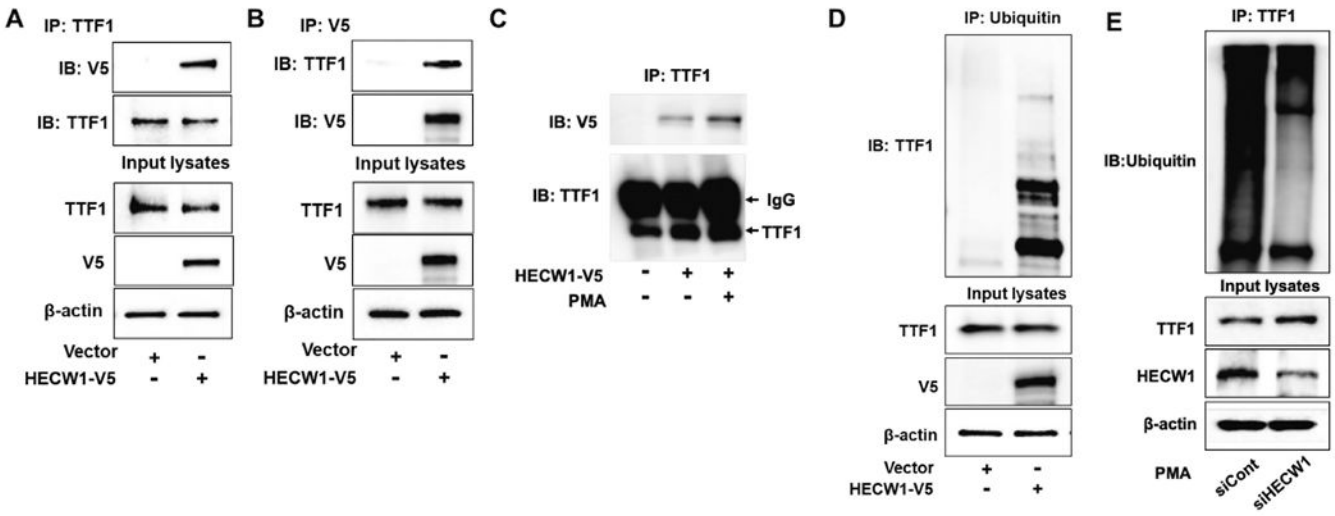

Figure 4. HECW1 targets TTF1 for ubiquitination.

A. MLE12 cells were transfected with empty vector or HECW1-V5 plasmids. Cell lysates were subjected to immunoprecipitation with TTF1 antibody, followed by V5 tag immunoblotting. Input lysates were analyzed by immunoblotting with TTF1, V5 tag and β-actin antibodies. **B.** MLE12 cells were transfected with empty vector or HECW1-V5 plasmids. Cell lysates were subjected to immunoprecipitation with V5 tag antibody, followed by TTF1 immunoblotting. Input lysates were analyzed by immunoblotting with TTF1, V5 tag and β-actin antibodies. **C.** MLE12 cells were transfected with empty vector or HECW1-V5 plasmids, and then cells were treated with PMA (0.1 μM) treatment for 4 h. **D.** MLE12 cells were transfected with empty vector or HECW1-V5 plasmids. Cell lysates were subjected to immunoprecipitation with an ubiquitin antibody, followed by TTF1 immunoblotting. Input lysates were analyzed by immunoblotting with TTF1, V5 tag and β-actin antibodies. **E.** MLE12 cells were transfected with sicontrol RNA or HECW1 siRNA for 72 h followed by PMA (0.1 μM) treatment for 4 h. Cell lysates were subjected to immunoprecipitation with TTF1 antibody, followed by ubiquitin antibody immunoblotting. Input lysates were analyzed by immunoblotting with TTF1, HECW1 and β-actin antibodies. All the blots and images are representative of three independent experiments.

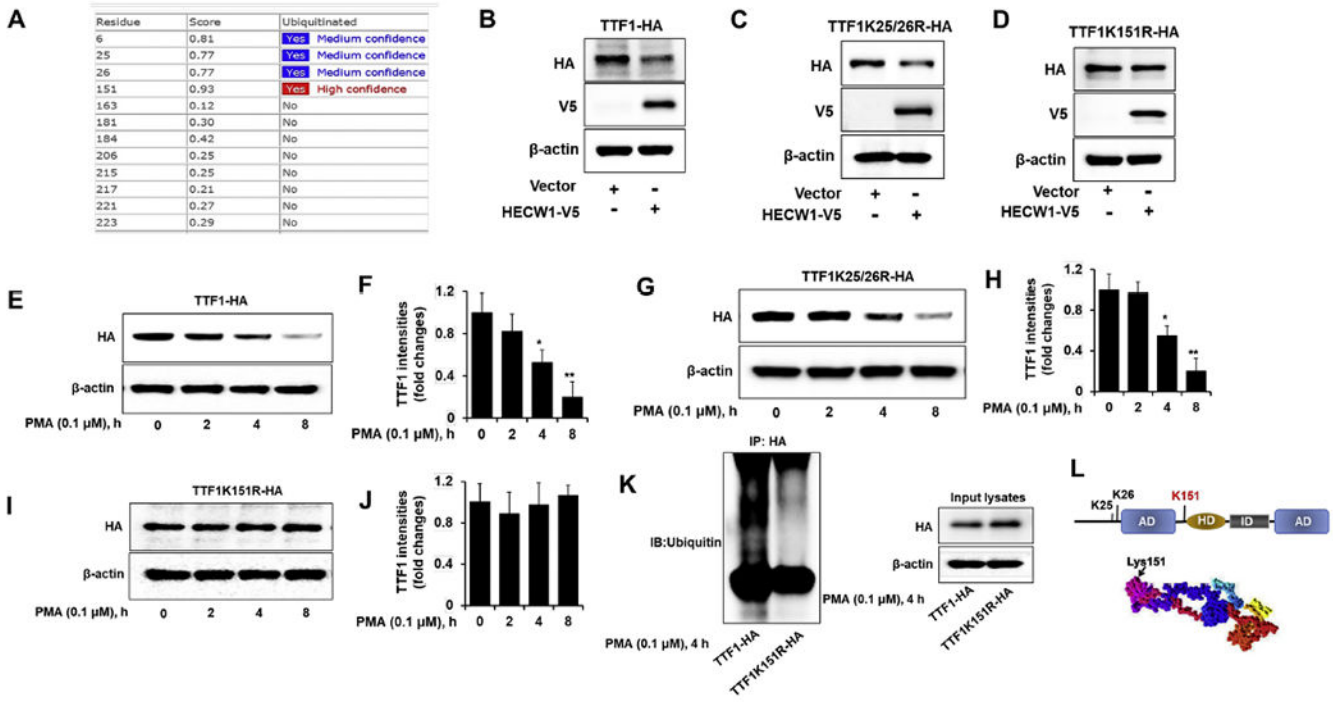

Figure 5. Lys (K) 151 is a key ubiquitin acceptor site within TTF1.

A. Predicted potential ubiquitination sites within TTF1 protein. (Used by UbPred: predictor of protein ubiquitination sites). High confidence ubiquitinated: red; Medium confidence ubiquitinated: blue. **B.** MLE12 cells were co-transfected with TTF1-HA wild type and vector or HECW1-V5 plasmids for 48 h as indicated. Cell lysates were analyzed by immunoblotting with antibodies to HA tag, V5 tag and β-actin. **C.** MLE12 cells were co-transfected with TTF1K25/26R-HA and vector or HECW1-V5 plasmids for 48h as indicated. Cell lysates were analyzed by immunoblotting with antibodies to HA tag, V5 tag and β-actin. **D.** MLE12 cells were co-transfected with TTF1K151R-HA and vector or HECW1-V5 plasmids for 48h as indicated. Cell lysates were analyzed by immunoblotting with antibodies to HA tag, V5 tag and β-actin. **E.** MLE12 cells were transfected with TTF1-HA wild type plasmids for 48 h followed by PMA (0.1 μM) treatment for indicated time. Then the cell lysates were analyzed by immunoblotting with HA tag and β-actin antibodies. **F.** TTF1 levels from three independent TTF1-HA wild type transfected experiments were quantified by image J software. * P<0.05; ** P<0.01. **G.** MLE12 cells were transfected with TTF1K25/26R-HA plasmids for 48 h followed by PMA (0.1 μM) treatment for indicated time. Then the cell lysates were analyzed by immunoblotting with HA tag and β-actin antibodies. **H.** TTF1 levels from three independent TTF1K25/26R-HA transfected experiments were quantified by image J software. * P<0.05; ** P<0.01. **I.** MLE12 cells were transfected with TTF1K151R-HA plasmids for 48 h followed by PMA (0.1 μM) treatment for indicated time. Then the cell lysates were analyzed by immunoblotting with HA tag and β-actin antibodies. **J.** TTF1 levels from three independent TTF1K151R-HA transfected experiments were quantified by image J software. **K.** MLE12 cells were transfected with TTF1-HA wild type or TTF1K151R-HA for 48 h followed by PMA (0.1 μM) treatment for 4 h. Cell lysates were subjected to immunoprecipitation with HA tag antibody, followed by

ubiquitin antibody immunoblotting. Input lysates were analyzed by immunoblotting with HA tag and β-actin antibodies. **L.** The amino acid structure of TTF1 protein under normal state (1-264 residues). K151 site is indicated by the arrows. All the blots and images are representative of three independent experiments.

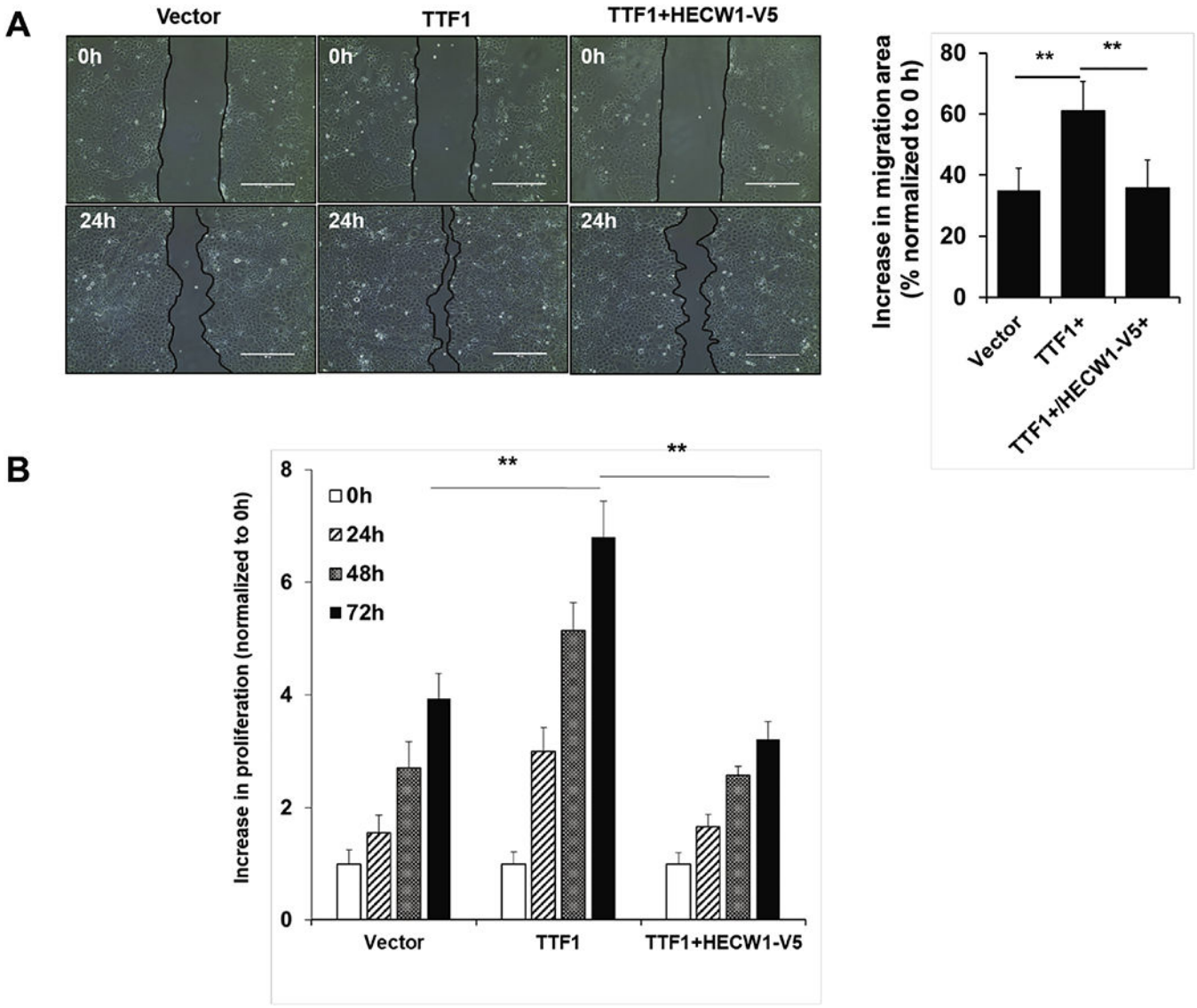

Figure 6. HECW1 attenuates TTF1- induced cell migration and proliferation.

A. MLE12 cells were transfected with empty vector, TTF1 or TTT1+HECW1-V5 plasmids for 48 h until 95-100% confluence. Monolayers were scratched using a sterile 10 μ1 pipette tip. Cells were digitally photographed at 0 and 24 h. The extent of cell migration was quantified using image J software. The percentage of wound closure was calculated as follows: [(pre-migration area – migration area)/pre-migration area] × 100. ** P<0.01. **B.** MLE12 cells were transfected with empty vector, TTF1 or TTT1+HECW1-V5 plasmids for 24 h. MLE12 cells were seeded into 6-well plates in triplicates at a density of 5.0 χ 10^5 cells per well. After 12 h of culture, the medium was replaced with serum-free (blank) medium, and then the cells were cultured for 0-72 h. The numbers of MLE12 cells were counted at 0, 24, 48 and 72 h using the Countess II FL Automated Cell Counter (Invitrogen) respectively. ** P<0.01. All results are representative of three independent experiments performed in triplicate.