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HUWE1 and TRIP12 Collaborate in Degradation of
Ubiquitin-Fusion Proteins and Misframed Ubiquitin

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Abstract

In eukaryotic cells an uncleavable ubiquitin moiety conjugated to the N-terminus of a protein signals the degradation of the fusion protein via the proteasome-dependent ubiquitin fusion degradation (UFD) pathway. In yeast the molecular mechanism of the UFD pathway has been well characterized. Recently the human E3 ubiquitin-protein ligase TRIP12 was connected with the UFD pathway, but little is otherwise known about this system in mammalian cells. In the present work, we utilized high-throughput imaging on cells transfected with a targeted siRNA library to identify components involved in degradation of the UFD substrate Ub⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶⁶³
moiety, fused to the N-terminus of a protein, signals rapid degradation of the fusion protein. Elegant studies have defined the key components of this pathway in the budding yeast, *Saccharomyces cerevisiae* [7] [2]. Ubiquitylation is catalyzed by the HECT domain E3, called Ufd4 [9], while ubiquitin chain elongation is performed by the E4, Ufd2 [2]. In addition, the UFD pathway requires the AAA (ATPase associated with various cellular activities) ATPase, called Cdc48 in yeast and p97 or VCP in mammals, and the p97+ Cdc48 co-factors Ufd1 and Ufd3 [7] [10]. So far most studies on the UFD system have utilized artificial model substrates. However, more recently the human orthologue of budding yeast Ufd4, called TRIP12, was shown to participate in the UFD pathway and mediate degradation of UBB1, a physiological UFD substrate [11]. UBB1 is a frame shift mutant ubiquitin derived from a dinucleotide deletion in the mRNA of the ubiquitin B gene, leading to an unetable N-terminal ubiquitin moiety linked to a short C-terminal extension [12]. In patients with neurodegenerative disorders, including Alzheimer’s diseases and various polyglutaminopathies, UBB1 accumulates in brain tissue [13]. Studies have shown that high levels of UBB1 inhibits the UPS and leads to cell cycle arrest [14]. Thus, high concentrations of UBB1 will most likely contribute to an environment that favors accumulation of misfolded proteins and in this manner further aggravate the disease. However, at lower levels UBB1 is efficiently cleared via the UFD pathway [14–15] [11].

To determine if other cellular E3s collaborate with TRIP12 in the degradation of UFD substrates, we utilized a targeted siRNA screen to identify novel components involved in degradation of the ubiquitin fusion protein Ub1G76V-YFP. The most significant hits from the screen included the E3 ubiquitin-protein ligase HUWE1 and 26S proteasome subunits. Accordingly, knock down of HUWE1 led to a retarded degradation of the Ub1G76V-YFP and UBB1 substrates. Since we could co-precipitate HUWE1 with the substrate and the 26S proteasome, we propose that HUWE1 directly ubiquitylates UFD substrates at a late step during the degradation pathway.

Materials and Methods

Buffers, Plasmids and Antibodies

Buffer A, 25 mM Tris/HCl pH 7.5, 2 mM MgCl2, 2 mM ATP, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100.

Plasmids for expression of flag-tagged UBB1 were generously supplied by Prof. Yoon, Yonsei University, Korea.

The antibodies to p97 have been described previously [16]. Antibodies to human proteasomes were purchased from Enzo. The anti-GFP, anti-flag and anti-β-actin antibodies were purchased from Sigma. Antibodies to HUWE1 have been described previously [17] and were kindly supplied by Prof. Wing, McGill University, Canada. All secondary antibodies were purchased from Dako.

Cell Culture

MelJuso cells, stably transfected to express ubiquitin-G76V-YFP [18], were maintained in Dulbecco’s modified eagle’s media (DMEM) containing 10% fetal bovine serum (Biosera), and supplemented with glutamine, penicillin/streptomycin and 0.25 mg/ml geneticin at 37°C in a humidified atmosphere containing 7.5% CO2.

Electrophoresis and Blotting

Proteins were separated on 7 cm x 8 cm 12.5% or 8% acrylamide gels. Proteins were transferred to BA83 (Schleicher & Schuell) nitrocellulose membranes and probed with antibodies as indicated. Tissue samples from these experiments were analyzed by Western Blot.

HUWE1 and TRIP12 in Ubiquitin-Fusion Degradation

We interfered with RNA for human HUWE1, TRIP12 and p97 by using either siRNA #1 ON-TARGETplus SMARTpool from Thermo scientific (HUWE1: J-00718507, J-00718508, J-00718509, J-00718510, TRIP12: J-00718206, J-00718207, J-00718208, J-00718209, VCP/p97: D-00872705, D-00872706, D-00872707, D-00872708). Non-targeting pool: D-00872707 or siRNA #2 FlexiTube siRNA (SI04336718) from Qiagen. The siCONTROL siRNA #1 (Dharmacon) was used as an unspecific control. Trypsinized cells were incubated for 48 hours with a solution containing 5 nM siRNA, 0.001% lipofectamin RNAi MAX in 40% OptiMEM and 60% DMEM supplemented with 10% fetal bovine serum.

Co-precipitations

For precipitation experiments Mel-Juso cells, stably transfected to express Ub1G76V-YFP, were treated for 6 hours with 25 μM MG132 and then lysed by sonication in 4 volumes of buffer A. Cleared extracts were prepared by centrifugation (12000 g, 30 min). The cleared lysates were incubated at 4°C for 4 hours with gentle agitation with 0.25 μL anti-GFP (Sigma) or 1 μL anti-MCP20 (Enzo) and 20 μL Protein G Sepharose beads (GE Healthcare). As a negative control antibodies to 92-macroglobulin were used. The beads were then washed in 3 x 15 mL of buffer A before elution in SDS sample buffer. The eluted material was analyzed by SDS-PAGE and immunoblotting.

Protein Degradation Experiments

The stability of the Ub1G76V-YFP model substrate was followed by pulse-chase analysis on stably transfected cells expressing the substrate, as described previously [19]. The degradation of UBB1 was followed by cycloheximide decay assays as described [11].

Reverse Transcription PCR

The knock down efficiency of TRIP12 was determined by reverse transcription PCR. For this purpose mRNA was isolated using TRizol (Invitrogen) and Turbo DNA-free (Ambion). The RNA was reverse transcribed using Transcriptor first strand cDNA synthesis (Roche Applied Science), purified using GFXPCR DNA (GE Healthcare), and used for PCR. GAPDH was used as a negative control. The primers specific for GAPDH were: forward, AGCCTCCCGCTTCGCTCTCT, reverse, TGACCTTGGCCAGGGGTGCT. The primers specific for TRIP12 were: forward, CCGGGGGGCACACCAAGAC, reverse, TGGACGCTGAGGGAAACGC.

siRNA Screening

The siRNA library used was a customized Human Silencer siRNA Library from Applied Biosystems. The library consists of three individual siRNAs specific for 558 different gene products predicted to play a role in the ubiquitin-proteasome system. A list of all targets in the library can be found under supplementary materials (Table S1).

The automated screen was performed using the MicroLabSTAR liquid handling system (Hampton Robotics). MelJuso cells were reverse transfected with the customized siRNA library including individual positive and negative controls. In brief, 4 μL siRNA was added to 2.5 μL OptiMEM (Invitrogen) into each well of a 384-well plate (Corning) (Fig. S1). To this, a 6.4 μL OptiMEM/0.1 μL RNAiMAX (Invitrogen) mix was added and left for 15 min, after
which 27 μL of cells were added to give a total cell density of 2400 cells per well. The final concentration of siRNA was 50 nM. Cells were then incubated for 48 h or 72 h, followed by fixation (Gurr histological fixative, VWR) and Hoechst staining (Sigma-Aldrich). In addition, individual wells were treated with 25 μM MG132 4 hours prior to fixation. Plates were imaged on an IN Cell Analyzer 1000 (GE Healthcare) acquiring a minimum of 6 images per well using a 10× objective to count approximately 2000 cells per well.

Figure 1. The proteasome and p97 are required for UbG76V-YFP degradation. (a) The steady state level of the UbG76V-YFP substrate was determined by blotting extracts from cells treated with the proteasome inhibitor MG132 and siRNA to p97. Actin served as a loading control. (b) Fluorescence microscopy of UbG76V-YFP (green) in cells treated with the proteasome inhibitor MG132 or siRNA to p97. Hoechst staining (blue) was used to mark the nuclei. (c) Pulse-chase analyses of UbG76V-YFP degradation in cells transfected with control siRNA or siRNA specific for p97. doi:10.1371/journal.pone.0050548.g001

Image Analysis and Statistics
Acquired images were analyzed using the IN Cell Analyzer Workstation 3.6 software (GE Healthcare) and percentage of ubiquitinG76V-YFP positive cells scored based on the overall nuclear and cellular intensity. This data was then normalized to the median sample score of each plate (excluding controls) and a gene-based hit list was generated using the statistical method “redundant siRNA activity” (RSA) analysis [20]. The RSA method first ranked individual siRNAs according to their
Results

p97 is Required for Degradation of Ub\(^{G76V}\)-YFP

To study the UFD pathway, cells stably transfected to express the ubiquitin-G76V-YFP fusion protein (Ub\(^{G76V}\)-YFP) were used [18]. In these cells, the steady-state level of the Ub\(^{G76V}\)-YFP substrate was very low. By Western blotting using antibodies to GFP, we were unable to detect the Ub\(^{G76V}\)-YFP protein in unperturbed cells (Fig. 1a). However, upon addition of the proteasome inhibitor MG132, a clear signal at about 35 kDa, corresponding to ubiquitin (\(\approx 8\) kDa) and YFP (\(\approx 25\) kDa), was obtained (Fig. 1a). This suggests that the low cellular level of the Ub\(^{G76V}\)-YFP protein was due to rapid proteasomal degradation. By fluorescence microscopy, the Ub\(^{G76V}\)-YFP protein was also undetectable in unperturbed cells, but again the level increased dramatically upon treatment with MG132 (Fig. 1b). In accordance with previous results [18], the fluorescent signal of Ub\(^{G76V}\)-YFP appeared somewhat stronger in the nucleus than in the cytosol (Fig. 1b).

Since the AAA-type ATPase p97 is critical for degradation of many proteasome substrates, we also tested if knock down of p97 led to an increased level of the Ub\(^{G76V}\)-YFP substrate protein. Indeed, we observed a robust increase in the Ub\(^{G76V}\)-YFP level upon p97 knock down, both by Western blotting and by fluorescence microscopy (Fig. 1a and 1b). To determine the degradation kinetics of the UFD substrate, we performed pulse-chase analyses on cells transfected with siRNA to p97. In cells transfected with control siRNA we found that Ub\(^{G76V}\)-YFP was rapidly degraded (Fig. 1c). As expected, p97 knock down strongly
inhibited degradation (Fig. 1c), showing that p97 is critical for degradation of UbG76V-YFP.

**Identification of Novel UFD Pathway Components by siRNA Screening**

Since knock down of p97 led to a strong increase in the UbG76V-YFP level, it was ideally suited as a positive control for a targeted siRNA screen. A customized siRNA library consisting of three individual siRNAs to each of 558 different gene products, predicted to play a role in the ubiquitin system (Table S1), was therefore reverse transfected into the reporter cell line. An siRNA targeting p97 and MG132 treatment were included as positive controls, whilst scrambled siRNA served as a negative control. After 48 or 72 hours the cells were fixed and the percentage of cells showing a YFP signal was monitored for each transfection by automated fluorescence microscopy. By subsequent image analysis, the percentage of cells displaying YFP signal was determined and plate-wise normalized. We observed no significant differences between the 48 and 72 hour transfection periods (Fig. S2). To minimize the risk of false scoring based on off-target effects of individual siRNAs, we computed a statistical score that modeled the probability of a gene 'hit' based on the collective activities of all three siRNAs per gene using the statistical method ‘redundant siRNA activity’ (RSA) analysis [20]. Thus, RSA analysis scored genes based on a combined p-value of all siRNAs per gene. Three individual screens were then further combined to a common p-value based on Fisher’s probability test (Fig. 2a and Table S1). In total 19 genes, corresponding to about 3% of the library, scored significantly (Table 1). In addition to the p97 transfection control (Fig. S2), proteasome subunits PSMD7 and PSMD14 scored highly significant (Fig. 2b), suggesting that the screening approach worked. Another significant hit was the E3 ubiquitin-protein ligase HUWE1 (Fig. 2b).

**Slowed UbG76V-YFP Degradation upon Knock Down of HUWE1**

If HUWE1 identified from the siRNA screening indeed partakes in the degradation of the UFD substrate, one would expect that the degradation kinetics of the UbG76V-YFP protein are slowed when HUWE1 is knocked down. To test this and thus verify the result of the high-throughput cellular imaging, pulse-chase experiments were performed.

Upon knock down of HUWE1, we observed a clear reduction in the cellular level of the HUWE1 protein (Fig. 3a). Since we were unable to obtain functional antibodies to TRIP12, successful depletion of this component was analyzed by RT-PCR (Fig. 3a). When expression of HUWE1, or as controls TRIP12 and p97, were knocked-down the steady-state level of the UbG76V-YFP protein was increased (Fig. 3b). Accordingly, we observed that UbG76V-YFP degradation upon knock down of HUWE1 was slowed (Fig. 3c). HUWE1, like TRIP12, is a HECT-type E3 ubiquitin-ligase. However, when HUWE1 expression was knocked down, ubiquitylation of the UFD substrate was still apparent (Fig. 3c), suggesting that other cellular E3s also target the substrate. We therefore decided to compare the effect of HUWE1 with that of TRIP12.

Knock down of TRIP12 also led to a stabilization of the UbG76V-YFP substrate (Fig. 3d). Again, the ubiquitylation pattern of the UFD substrate did not appear to be affected. In double knock down cells, lacking both HUWE1 and TRIP12, the stability of the substrate was further increased (Fig. 3e), suggesting that HUWE1 and TRIP12 function in parallel. However, since ubiquitylation of the UFD substrate was still apparent (Fig. 3e), the function of HUWE1 and TRIP12 must overlap with yet other E3s.

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**Table 1. Significantly scoring genes.**

<table>
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<tr>
<th>Gene</th>
<th>Protein</th>
<th>Fisher’s combined p-values</th>
</tr>
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<tr>
<td>PSMD14</td>
<td>proteasome 26S subunit, non-ATPase 14</td>
<td>2.1E-14</td>
</tr>
<tr>
<td>HUWE1</td>
<td>HECT, UBA and WWE domain containing 1</td>
<td>2.7E-06</td>
</tr>
<tr>
<td>PSMD7</td>
<td>proteasome 26S subunit, non-ATPase 7</td>
<td>0.0013</td>
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<tr>
<td>USP9X</td>
<td>ubiquitin specific protease 9, X-linked</td>
<td>0.0014</td>
</tr>
<tr>
<td>UBE2L3</td>
<td>ubiquitin-conjugating enzyme, E2L3</td>
<td>0.0014</td>
</tr>
<tr>
<td>WSB1</td>
<td>WD repeat and SOCS box-containing 1</td>
<td>0.012</td>
</tr>
<tr>
<td>FBXL21</td>
<td>F-box and leucine-rich repeat protein 21</td>
<td>0.014</td>
</tr>
<tr>
<td>TRAF3</td>
<td>TNF receptor-associated factor 3</td>
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</tr>
<tr>
<td>BRCC2</td>
<td>BRCC2</td>
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</tr>
<tr>
<td>RNF187</td>
<td>ring finger protein 187</td>
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<tr>
<td>RNF169</td>
<td>ring finger protein 169</td>
<td>0.026</td>
</tr>
<tr>
<td>C13orf7</td>
<td>chromosome 13 open reading frame 7</td>
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</tr>
<tr>
<td>USP41</td>
<td>ubiquitin specific protease 41</td>
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<tr>
<td>MGC4832</td>
<td>membrane-associated ring finger (C3HCA)3</td>
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<td>HECTD1</td>
<td>HECT domain containing 1</td>
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<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
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<tr>
<td>FBXO42</td>
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<td>CBL1</td>
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<tr>
<td>RNF19</td>
<td>ring finger protein 19</td>
<td>0.043</td>
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</table>

doi:10.1371/journal.pone.0050548.t001
HUWE1 Associates with both the UFD Substrate and the 26S Proteasome

If HUWE1 directly regulates the degradation of UbG76V-YFP, the two components should at least transiently form an enzyme-substrate complex. To test this prediction, we performed immunoprecipitation experiments with the UbG76V-YFP substrate from cells treated with MG132. The precipitates were resolved by SDS-PAGE and analyzed by Western blotting. Probing the blots with antibodies to GFP revealed that the UbG76V-YFP substrate was precipitated, but not present in the negative controls (Fig. 4a). During the course of these experiments, we also tested if 26S proteasomes associate with HUWE1. When immuno-precipitating the 26S proteasome, we observed that HUWE1 specifically co-precipitated (Fig. 4b).

HUWE1 Stimulates Degradation of UBB+1

So far, the ubiquitin mutant UBB+1 constitutes the only physiologically relevant substrate of the UFD pathway [11]. In order to determine if HUWE1 also plays a role in UBB+1 degradation, the turnover of flag-tagged UBB+1 was followed in cultures treated with the translation inhibitor cycloheximide (CHX). UBB+1 was rapidly degraded in cells transfected with control siRNA and stabilized upon knock down of HUWE1 and TRIP12 (Fig. 5), revealing that HUWE1 also partakes in the degradation of UBB+1.

Figure 3. HUWE1 and TRIP12 are required for UbG76V-YFP degradation. (a) Efficient knock down of HUWE1 was validated by Western blotting, while for TRIP12 knock down was validated by RT-PCR. Actin and GAPDH served as loading controls. (b) The steady state level of the UbG76V-YFP substrate was determined in cultures by blotting with antibodies to GFP. Actin served as a loading control. (c) Pulse-chase analyses of UbG76V-YFP degradation in cells transfected with control siRNA or siRNA specific for HUWE1. (d) Pulse-chase analyses of UbG76V-YFP degradation in cells transfected with control siRNA or siRNA specific for TRIP12. (e) Pulse-chase analyses of UbG76V-YFP degradation in cells transfected with control siRNA or siRNA specific for TRIP12 and HUWE1.

doi:10.1371/journal.pone.0050548.g003
Discussion

Bioinformatic analyses have shown that a significant fraction of the eukaryotic genome is dedicated to the UPS, in some species occupying as much as 3% of the genome [21]. This large number of genes of the UPS is mainly attributed to the many different E3 ubiquitin-protein ligases [21] that are the major factors in determining the specificity in conjugation of ubiquitin to target proteins. Since the human genome is predicted to encode more than 600 distinct E3s, the substrate specificity of the system should at least be of a similar diversity. A major challenge is to link the E3s with their respective substrate classes. This is not a trivial task, which is further complicated by a significant amount of cross talk between the degradation pathways. Thus, several proteins are ubiquitylated by different E3s. For instance, p53 is ubiquitylated by multiple E3s, including Mdm2, Hrd1/Synoviolin and CHIP [22].

In the present study, we utilized high-throughput cellular imaging on cells transfected with a targeted siRNA library to screen for components involved in degradation of ubiquitin fusion proteins. This is a powerful tool for studying gene function and mapping biochemical pathways, which has recently been successfully applied to study various aspects of ubiquitin-dependent protein degradation, including identification of protein quality control factors [23].

The success of the screen was readily apparent since two proteasome subunits PSMD7 and PSMD14 appeared as highly significant hits. Our targeted siRNA library did not include any other proteasome subunits, but presumably knockdown of most proteasome subunits would interfere with the UFD pathway. The second best scoring hit was the HECT-type E3 ubiquitin-protein ligase HUWE1 (also known as ARF-BP1, Mule, HECTH9, E3Histone, LASU1 and UREB1), recently connected with X-linked mental retardation [21]. Since we could co-precipitate HUWE1 with the UbG76V-YFP model substrate, we suggest that HUWE1 directly ubiquitylates this protein for subsequent degradation. Several HUWE1 targets, including signaling proteins [25–26] and core histones [27], have previously been identified, but this is the first demonstration of HUWE1 functioning in the UFD pathway.

The E2 called UBE2L3 was another significant hit in our screen. This is noteworthy since UBE2L3 (also known as UbcH7) is an E2 enzyme which displays specificity for HECT type E3s and has been reported to associate with HUWE1 [25] [28–29]. Studies have shown that UBE2L3 functions as the cognate E2 for HUWE1 catalyzed ubiquitylation of the anti-apoptotic Mcl-1 protein [25]. It is therefore possible that UBE2L3 functions as E2 enzyme in the UFD pathway.

In pulse-chase analyses, knock down of HUWE1 and the human orthologue of Ufd4, TRIP12, led to a comparable stabilization of the UFD substrate. In addition to the C-terminal HECT domains, both HUWE1 and TRIP12 also contain WWE domains [3], but do otherwise not share sequence similarity. However, since studies have shown that the TRIP12 HECT domain alone can mediate degradation of UFD substrates [11] it is possible that other HECT-type E3s will function in a similar fashion. TRIP12 was not included in our siRNA library, but indeed we note that another HECT domain E3, HECTD1 (Fig. 2b), was a significant hit in our screen. However, various RING-type E3s also scored significantly, while several other HECT domain E3s, included in the siRNA library, failed to score.

Since the UFD pathway is phylogenetically conserved in eukaryotes [8], it is surprising that UBB+1 so far is the only

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**Figure 4.** HUWE1 is associated with UbG76V-YFP and the 26S proteasome. (a) Immunoprecipitates (IP) of the UbG76V-YFP substrate using either control antibodies or antibodies to GFP from cultures transfected to express UbG76V-YFP (left panel) or as a control mock transfectants (right panel). The precipitates were analyzed by blotting with antibodies to HUWE1 and GFP. (b) 26S proteasomes were immunoprecipitated from MelJuSo cells using antibodies to Rpn10 and analyzed by blotting with antibodies to HUWE1 and the 26S proteasome subunits Rpn10 and Rpt2.

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**Figure 5.** HUWE1 is required for UBB+1 degradation. The degradation of flag tagged UBB+1 was followed by blotting of cultures treated with cycloheximide (CHX) for the indicated times. Actin served as a loading control.
known UFD substrate, which is physiologically relevant. However, HUWE1 has recently been shown to catalyze N-terminal (α-amino) ubiquitylation, thus forming linear ubiquitin fusion proteins [26]. It is therefore conceivable that HUWE1 and other E3 ligases in this manner generate natural UFD substrates.

Previous studies have shown that TRIP12 mediates ubiquitylation and degradation of UBR1 [11], and our data show that this function is shared with HUWE1. However, in comparison with TRIP12 the effect of HUWE1 shown here appears more moderate. Previously, a proteomics study found that HUWE1 associates with 26S proteasomes from brain tissue [30]. We also found that HUWE1 co-precipitates with 26S proteasomes, suggesting it might function at a rather late step in the degradation pathway, where perhaps several degradation pathways converge.

Given that HUWE1 contains several ubiquitin binding domains it is possible that it may display specificity for already ubiquitylated substrates and could therefore function in ubiquitin chain elongation similar to the E4 Ufd2 [2].

Supporting Information

Figure S1 siRNA Library Overview. The figure depicts the layout of the siRNA library plates utilized.

References