A functional analysis of the mammalian E3 ubiquitin ligase WWP1 in a yeast model

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"You cannot teach a man anything: you can only help him find it within himself."

—Galileo Galilei

... thank you for helping me find it within myself...
Abstract

A functional analysis of the mammalian E3 ubiquitin ligase WWP1 in a yeast model

A thesis presented to the Biology Department

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

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E3 ubiquitin ligases function in the ubiquitin proteasome pathway as a key component in the regulation of protein degradation. With their implications in a range of disease pathways, E3 ligases could be excellent drug targets. Previous research has shown that the mammalian E3 ligase, WWP1, degrades essential factors in skelctogcnnesis, making it a strong drug target for osteoporosis treatment. In previous studies, potential inhibitors of the catalytic HECT domain in WWP1 were identified and tested in vitro using polyubiquitination assays. In order to develop a high-throughput, physiologically relevant assay system for the inhibition of WWP1 activity by drugs, a yeast model with a single allele deletion of the yeast E3 ligase orthologue, Rsp5, was selected for an in vivo functional analysis of the WWP1 protein. We created domain deletion mutants and chimeric constructs of WWP1 and assayed their effect on yeast growth. Our results demonstrate that WWP1 is unable to functionally complement Rsp5 in the single RSP5 allele deletion yeast strain, and that expression of WWP1 in this yeast strain results in a toxic phenotype. Furthermore, our results indicate that there are potentially two mechanisms of toxicity mediated by WWP1 in this yeast model, as evidenced by the toxicity of various WWP1 domain mutants and chimeric constructs. The dual mechanisms of toxicity of WWP1 provide the possibility of a powerful drug screen with an internal control for drug specificity. Further studies should elucidate HECT domain binding activity in order to develop more efficient drug screens.
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List of Abbreviations

bp      Base pairs
C2      Calcium-binding domain
chA     Chimera A
chB     Chimera B
Dx      Dextrose
FRET    Fluorescence Resonance Energy Transfer
Gal     Galactose
GFP     Green fluorescent protein
HECT    Homologous to E6AP C-Terminus
L1      First linker region
L2      Second linker region
LB      Luria Broth
OD      Optical Density
PCR     Polymerase Chain Reaction
RING    Really Interesting New Gene
ura     Uracil
W1      First WW domain
W2      Second WW domain
W3      Third WW domain
W4      Fourth WW domain
WW      Protein interaction domain with two conserved tryptophan (W) residues
WWP1    WW domain-containing Protein 1
1. Introduction

Cells need to maintain a precarious balance of protein species and concentrations in order to function properly. As proteins are synthesized, they must also be degraded in a time-sensitive manner. Deficiencies in either of these processes lead to diseases ranging from cancer to neurodegenerative disorders to developmental and metabolic defects. The E3 ubiquitin ligases function in the ubiquitin/proteasome pathway as a key component in the regulation of protein degradation. Members of the E3 family of proteins have been implicated in pathways of retroviral particle release (Heidecker et al. 2007), bone cell formation (Jones et al. 2006), and the regulation of various tumour suppressors (Moren et al. 2005, Flasza et al. 2006, Komuro et al. 2004, Lainc and Ronai 2007). The roles of E3 ubiquitin ligases in a range of disease pathways make them a promising class of proteins to consider for drug targets.

1.1. E3 ubiquitin ligases

Ubiquitination regulates several processes, one of which is substrate modification for degradation by the 26S proteasome (Hochstrasser 2009). Substrates may be tagged with one or a few ubiquitin molecules for certain fates, but are usually marked with a polyubiquitin chain for complete proteasomal degradation. The process of ubiquitination, which results in the formation of a bond between the 76-amino acid ubiquitin polypeptide and a substrate, requires a sequential E1-E2-E3 enzyme cascade (Pickart 2001). The E1 enzyme activates ubiquitin via a thiol ester bond; then a conjugating E2 enzyme carries activated ubiquitin in the form of a thiol ester; and finally, an E3 ubiquitin-protein ligase facilitates the transfer of ubiquitin from the E2 enzyme to
the appropriate substrate. This cascade utilizes one E1 enzyme, a few E2s, and several E3 ligases. This hierarchy is important because substrate specificity for appropriate ubiquitin-protein modification is mediated almost entirely by the E3 enzymes (Pickart 2001).

The recognition of substrates for ubiquitination requires a signal on the substrate, in the form of a sequence or structure motif, which is recognized by E3 ubiquitin-protein ligases (Pickart 2001). These E3 enzymes not only catalyze the transfer of activated ubiquitin to a lysine residue on a substrate, but must also coordinate the binding and alignment of both the E2 ligase and substrate for this reaction to occur. Due in part to the diversity of known E3s, and in part to their complex catalytic function, the molecular mechanisms of E3 catalysis are poorly understood. There are two main classes of E3 ligases: the RING finger E3s and the HECT domain E3s. These two classes are not related in sequence or structure, but they function in the same step of the ubiquitination cascade. The RING (Really Interesting New Gene) finger E3s act as scaffolding proteins that bind the appropriate E2 and substrate, and catalyze ubiquitin transfer from the former to the latter. The HECT (Homologous to E6AP C-Terminus) domain E3s are unique in that they form an intermediate E3-ubiquitin thioester complex, rather than serving as scaffolding proteins to bring together E2 and substrate proteins (Kee and Huibregtse 2007). The importance of E3 ubiquitin-protein ligases in specificity of ubiquitination for protein degradation is recognized, but the mechanisms of E3 function remain to be elucidated. With their implications in many diseases, these proteins could be excellent drug targets for specific pathways, as they offer one of the few ways to upregulate the amount of one protein by inhibiting the action of another.
1.2. WWP1, a HECT domain E3 ubiquitin-protein ligase of interest

Of the approximately 50 known HECT E3 ligases in humans and five in *S. cerevisiae* (Kee and Huibregtse 2007), the best characterized subgroup, in terms of substrates and regulation, are those containing a C2-WW-HECT domain motif (Shearwin-Whyatt *et al.* 2006). One of these C2-WW-HECT E3 ligases, WWP1 (WW domain-containing Protein 1), is a mammalian E3 ligase that has been implicated in osteoblast formation (Jones *et al.* 2006), localization and regulation of p53 (Laine and Ronai 2007), and degradation of the tumour suppressor Smad4 (Moren *et al.* 2005), among other things. This 45-kDa, multidomain protein, first defined by its WW domains (Pirozzi *et al.* 1997), is a member of the Nedd4 (Neuronally Expressed, Developmentally Down-regulated protein 4) family of HECT domain E3 ligases, which is characterized by a C-terminal HECT domain and N-terminal WW domains (Kasanov *et al.* 2001). WWP1 contains a C2 domain, four class I WW domains, and a HECT domain (Flasza *et al.* 2002).

**Figure 1. Domain organization of WWP1**

![Domain organization diagram of WWP1](image)

Schematic diagram of the domains found in WWP1, a Nedd4-like E3 ubiquitin-protein ligase.

The ubiquitin transfer activity of WWP1 is performed by the HECT domain, which comprise the 376 amino acids at the C-terminus of the protein. This domain consists of a larger N-lobe that contains an E2 binding site, and a smaller C-lobe that contains the active site (Verdecia *et al.* 2003). The two lobes are connected by a linker region, whose conformational flexibility brings
together the two lobes to allow ubiquitin transfer. The catalytic activity of this domain requires an essential, conserved cysteine residue, which forms a thiol ester intermediate with ubiquitin before transferring it to the appropriate substrate for degradation. In addition to the catalytic cysteine residue, a phenylalanine residue located four amino acids from the end of the polypeptide chain is required for proper domain function, and truncation of the last five amino acids disrupts enzyme function (Verdecia et al. 2003).

Structures are available for three HECT domains, including one HECT-E2 complex (Huang et al. 1999, Verdecia et al. 2003, Ogunjimi et al. 2005). There are significant differences between the known HECT domain (only) structures, as the N-terminal lobe region with the E2 binding site is only moderately conserved, and the lobes in each structure are also positioned with different relative orientations (Ingham et al. 2004). These differences among the limited examples of HECT domain structures provide some insight into E2 binding specificity, and the catalytic mechanism, of the HECT domain, but much remains unknown.

WWP1 has four WW domains, which mediate substrate binding. WW domains have been characterized as protein interaction modules (Sudol et al. 1995) that bind proline-rich peptide sequences (Zarrinpar and Lim 2000). These domains are made up of 30-40 amino acids folded in a triple-stranded, anti-parallel β-sheet whose ends are defined by two conserved tryptophan residues (Illesy et al. 2002). The aromatic grooves formed in the surface structure of these domains allow for highly specific binding of the unique backbone substitution pattern of X-P proline dipeptide units (Zarrinpar and Lim 2000). WW domains can also act as binding modules for phosphoserine and phosphothreonine (Lu et al. 1999).
Although these domains can be almost completely superimposed (Ilsley et al. 2002), they have high ligand specificity. There are six different classifications of WW domains, determined by the type of ligand sequence bound (Otte et al. 2002). Specificity in binding X-P units is mediated by the highly conserved aromatic residues in the β-sheets, while the surface loops and regions around the aromatic grooves confer additional specificity elements to create diverse binding preferences (Zarrinpar and Lim 2000). The WW domains in WWP1 are all class I domains, with experimentally demonstrated ligand specificity for the L/PPxY sequence (Kasanov et al. 2001). In some HECT domain E3 ligases containing class I WW domains, the HECT domain possesses the consensus sequence LxLPxY, which may be bound by the WW domains, providing a method of self-regulation by intramolecular binding. In fact, the first and fourth WW domains in WWP1 were shown to recognize the ligand sequence LKLPDY, which has a consensus sequence in the WWP1 HECT domain (Kasanov et al. 2001), suggesting that these domains may be involved in regulating WWP1 activity by autoinhibition of certain protein-protein interactions.

The N-terminal region of WWP1 consists of a C2 domain, which is calcium-regulated and localizes the protein to the plasma membrane (Malbert-Colas et al. 2003). WWP1 is known to regulate epithelial sodium channel degradation in certain tissues. In HECT E3 ligases, the C2 domain has been shown to have an autoinhibitory effect (Wiesner et al. 2003) in the Nedd4-like E3 ligase Smurf2. These autoinhibitory interactions are thought to help protect E3s and their substrates from excessive ubiquitination and futile degradation in cells.
1.3. **WWP1 as a promising drug target for osteoporosis**

Previous studies have shown that WWP1 is recruited by an adapter protein to degrade Runx2, an essential transcription factor in skeletogenesis (Jones et al. 2006). Jones et al. demonstrated that by knocking down WWP1 in mesenchymal stem cell cultures, there was an increase in osteoblast formation and, as a result, bone density. Therefore, WWP1 may be a promising target for the treatment of osteoporosis. Potential inhibitors of the WWP1 HECT domain were identified using virtual screening (W.R.P. Novak, Brandeis University; unpublished data), and the top hits were tested for inhibition of WWP1 activity using a polyubiquitination assay (Marc Wein, Harvard School of Public Health; unpublished data). These preliminary studies showed that some of the hits did prevent the ubiquitination activity of WWP1 (Fig. 2).

*In vitro* polyubiquitination assays have several limitations. If the substrates of the E3 ligase are not known, then the assay can only measure self-ubiquitination. Binding partners and regulatory pathways are omitted from these assays *in vitro*, so the data from these assays may not be representative of the protein's activity in a biological system. *In vitro* polyubiquitination assays also call for large amounts of purified protein and require expensive reagents such as ubiquitin-charged E2 enzymes. Enzyme activity, in terms of ubiquitination, can be measured by anti-ubiquitin antibody binding, and may be visualized by fluorescence resonance energy transfer (FRET) or electrochemiluminescence (Sun 2003). In order to more rigorously pursue WWP1 as a drug target, the development of an inexpensive, high-throughput, more physiologically relevant assay system is necessary.
1.4. Examining WWP1 in a yeast model

Yeast is widely used as a model organism in the study of eukaryotic cell processes. The presence of many conserved pathways can provide a strong in vivo system for testing genes of interest from higher eukaryotes, with the convenience of an organism that requires inexpensive growth media and relatively short doubling times. This unicellular model organism also provides the advantages of utilizing an in vivo biological system without the confounding factors of intercellular interactions and signalling. In addition, a sequenced genome and numerous technologies for genetic modification make yeast relatively easy to manipulate (Simon and Bedalov 2004). *Saccharomyces cerevisiae* encode a HECT E3 ubiquitin ligase, Rsp5, which functions in an E1-E2-E3 ubiquitination cascade and has a C2-WW-HECT domain arrangement (Wang et al. 1999). *RSP5* was found to be an essential gene, as a knockout of this gene in haploid yeast is inviable. In diploid yeast, knocking out a single copy of this gene causes sensitivity to environmental conditions such as temperature and pH, as well as toxicity from certain media. A diploid, single *RSP5* allele deletion strain of *S. cerevisiae* was recently used in the functional analysis of hNedd4 (Gajewska et al. 2003) by testing for complementation of Rsp5 function.

1.5. Statement of Thesis

In order to pursue the development of an inexpensive, high-throughput assay system for the inhibition of WWP1 activity, the FW1808 (single *RSP5* allele deletion) yeast model was selected for an in vivo functional analysis of the WWP1 protein. We created domain deletion mutants and
chimeric constructs of WWP1, cloned them into yeast expression vectors, and assayed their effect on yeast growth. Our results demonstrate that WWP1 is unable to functionally complement Rsp5 in the FW1808 yeast strain and that the expression of WWP1 in FW1808 yeast results in a toxic phenotype. Furthermore, our results indicate that there is potentially more than one mechanism of toxicity mediated by WWP1, as evidenced by the toxic phenotype observed from the expression of various WWP1 deletion mutant, point mutant, and chimeric proteins in FW1808 strains. This study has made substantial progress towards the development of a high-throughput, biologically relevant screen for the development of a novel class of drugs for osteoporosis treatment.
2. Materials and Methods

2.1. Cloning

2.1.1. WWPI constructs. WWPI DNA from Mus musculus was a kind gift of Dr. Laurie Glimecher, Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts. Mutant constructs were made using gene-specific 5' and 3' primers (Operon Biotechnologies, Huntsville, AL) to amplify regions of the WWPI gene (Table 1).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward primer (N-terminus)</th>
<th>Reverse primer (C-terminus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWPI (full gene)</td>
<td>5'CGGGTGACCATGGGCACTGCTTCAAG</td>
<td>5'CGGGATCCCTATTTGTGCAATCCC</td>
</tr>
<tr>
<td>C2</td>
<td>5'CGGGATCCATGGGCACTGCTTCACCA</td>
<td>5'CGGGATCAATTTTCTGTCAATC</td>
</tr>
<tr>
<td>L1</td>
<td>5'GGGGTACCAGGAGCAAGAATATAAA</td>
<td>WWPI (full gene) C-terminus</td>
</tr>
<tr>
<td></td>
<td>CAAACTGC-3'</td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>5'GGGGTACCAGAACACAAAGGCTTGC</td>
<td>WWPI (full gene) C-terminus</td>
</tr>
<tr>
<td></td>
<td>CATCAG-3'</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>5'GGGGTACCAGGAGGACCAACCCCTG</td>
<td>WWPI (full gene) C-terminus</td>
</tr>
<tr>
<td></td>
<td>TACCTCC-3'</td>
<td></td>
</tr>
<tr>
<td>W3</td>
<td>5'GGGGATCCATGGTGGCCAGCCAGGG</td>
<td>WWPI (full gene) C-terminus</td>
</tr>
<tr>
<td></td>
<td>AAAAG-3'</td>
<td></td>
</tr>
<tr>
<td>W4</td>
<td>5'GGGGTACCAGAATGGAAGAACCCTGC</td>
<td>WWPI (full gene) C-terminus</td>
</tr>
<tr>
<td></td>
<td>CAGAAGG-3'</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>5'GGGGTACCAGCGAATGGGAATGTCC</td>
<td>WWPI (full gene) C-terminus</td>
</tr>
<tr>
<td></td>
<td>CTG-3'</td>
<td></td>
</tr>
<tr>
<td>ΔHECT</td>
<td>WWPI (full gene) N-terminus</td>
<td>5'CGGGATCTGCTTCCCATTGCGAGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCTTG-3'</td>
</tr>
</tbody>
</table>

Primers are listed 5' to 3' and restriction sites are underlined.
Constructs were made by standard PCR amplification of WWP1 template DNA. Amplified DNA was purified using the PCR Purification Kit (Qiagen Inc., Valencia, CA), digested with the appropriate restriction enzymes (New England Biolabs, Ipswich, MA) and ligated into the pYES2/CT yeast expression vector (Invitrogen Corporation, Carlsbad, CA). Constructs were verified by DNA sequencing (Genewiz Inc., South Plainfield, NJ).

The WWP1 C890A mutant in pYES2 was obtained from Dr. Walter Novak, Rosenstiel Basic Medical Research Center, Brandeis University, Waltham, Massachusetts.

2.1.2. WWP1-Rsp5 chimeric constructs. Chimeric constructs of WWP1 and Rsp5 were created using purified WWP1 DNA and genomic Rsp5 DNA as templates. Primers were designed in such a way that the C-terminal primer for the WW domain fragment would overlap with the N-terminal primer for the HECT domain. The following oligonucleotides were ordered (Operon Biotechnologies) to make the chimeras:

**Chimera A:**

**Rsp5 WW domain**

forward: 5'-GAATTCATGGGTCGTTTACCCCCCTGGTTGG-3'
reverse: 5'-CTTACGTCGAAGTCACGCTTTGATTGGG-3'

**WWP1 HECT domain**

forward: 5'-TACAAGCGTGACCTCAGACGTAAGCTTGCTCACTCCGTTATTTGTGCC-3'
reverse: 5'-GCGGCCCGCTCATTCTTGTCCAATCCCTC-3'
**Chimera B:**

*WWP1 WW domain*

forward: 5' - GAATTCA TGGAAACCTTGCCATCAGGGTG - 3'

reverse: 5' - CTTACGTCTGAAGTCACGCTTTGAAGCAATTTTGACC A CTTTAGTTAC - 3'

*Rsp5 HECT domain*

forward: 5' - TACAAGCGTGA TTTCA GACGTAAGGTTATTATTAT TCCAGG - 3'

reverse: 5' - GCGGCGC GTCATTCTTGACCAAACCCTATGG - 3'

For each chimeric construct, the individual fragments were amplified from their respective templates by PCR. The resulting PCR products were then used as templates in a second round of PCR, in which the overlapping regions of the two individual fragments primed each other, producing a chimera. The self-priming PCR was optimized by varying annealing temperatures and template concentrations. Samples were run on agarose gels to check for correct product length, and the appropriate band was extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, Orange, CA).

Chimeric gene products were inserted into a TA vector using the pCR2.1 TOPO TA cloning kit (Invitrogen Corp.) and transformed into OneShot Top10 competent cells (Invitrogen Corp.) following manufacturer's instructions. Cells were plated on LB agar plates containing 100 μg/ml ampicillin. Colonies were selected and grown up in liquid culture with ampicillin. DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen Inc.).
2.1.3. Cloning WWP1 and chimeric constructs into the pYES2/CT vector. Samples of all constructs (~500 ng - 1 μg DNA) were incubated at 37°C with 1 μl each of the following restriction enzymes (New England Biolabs) for double restriction digest overnight (Table 2).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Restriction Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWP1, L1, W1, W2, W4, L2, ΔHECT, WW+L</td>
<td>KpnI, EcoRI</td>
</tr>
<tr>
<td>C2</td>
<td>BamHI, XhoI</td>
</tr>
<tr>
<td>W3</td>
<td>BamHI, EcoRI</td>
</tr>
<tr>
<td>Chimera A, Chimera B</td>
<td>EcoRI, NotI</td>
</tr>
</tbody>
</table>

For each double restriction digest with a distinct pair of restriction enzymes, a corresponding double digest was set up for the pYES2/CT yeast expression vector (Invitrogen Corp.). Digested samples were run on agarose gels, and the appropriate bands were extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp.). Ligation reactions using T4 DNA ligase (Fisher Scientific, Pittsburg, PA) were set up for each construct with PCR product DNA to vector DNA ratios of 2:1, 1:1, and 1:2.

DNA from each construct ligation was transformed into OneShot Top10 chemically competent cells (Invitrogen Corp.) following manufacturer's instructions. Cells were plated on LB agar plates containing 100 μg/ml ampicillin and were incubated overnight at 37°C. Colonies were selected and grown up in liquid culture with ampicillin. Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen Inc.).

All constructs were verified by DNA sequencing (Genewiz Corporation). To ensure full sequence coverage of the chimeric genes, chimera constructs were sequenced from the N-
terminus, C-terminus, and also from a midpoint ~500 bp downstream of the start codon (See Appendix for full sequences).

2.2. Yeast growth assays

2.2.1. Yeast strains. Yeast strains FW1808 (isogenic Rsp5-1 mutant, ura3), FW1808 with Rsp5-pYES2, and FW1808 with pYES2 of S. cerevisiae were a kind gift of Dr. Jon Huibregtse, Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey. All WWP1 and chimeric constructs used in this study were transformed into a temperature-sensitive FW1808 strain of yeast. Successful transformants were selected using uracil dropout (-ura) media plates containing 6.7 g/L Difco Yeast Nitrogen Base without Amino Acids (Becton, Dickson and Company, Sparks, MD), 20 g/L Difco dextrose or galactose, 0.87 g/L ura- dropout supplement (Clontech Laboratories, Mountain View, CA), and 25 g/L agar.

2.2.2. Bioscreen growth assay. FW1808 strains were grown up in dextrose -ura liquid media overnight at 30°C. Cells were pelleted via centrifugation at 10,000 rpm for 4 minutes, resuspended in both fresh dextrose -ura and galactose -ura liquid media, and diluted to an optical density at 600 nm (OD_{600}) of 0.2 in the appropriate -ura media. 96-well bioscreen plates were prepared with 125 µl samples in triplicate, and incubated at either 30°C or 37°C for 3 days with continuous shaking and OD readings at 600 nm every 15 minutes. Data were collected using EZExperiment software (Oy Growth Curves Ab Ltd., Piscataway, New Jersey).
2.2.3. Plate drop assay. FW1808 strains were grown up in dextrose -ura liquid media overnight at 30°C. Cells were pelleted via centrifugation at 10,000 rpm for 4 minutes, and resuspended in sterile water to an OD<sub>600</sub> of 1.0. Samples were then diluted 10<sup>-1</sup>, 100<sup>-1</sup>, and 1000-fold in sterile water. The dilution series were plated in 3 µl drops on two sets of dextrose -ura and galactose -ura dropout media plates. One set of plates was incubated at 30°C, and the other set at 37°C, for 3 days.

2.2.4. Streak plates. All FW1808, W303, and BY4743 strains were grown in dextrose -ura liquid media overnight at 30°C. Cells were pelleted via centrifugation at 10,000 rpm for 4 minutes, and resuspended in sterile water. Inoculation loops were used to pick up and streak samples on dextrose -ura and galactose -ura dropout media plates in duplicate. One set of plates was incubated at 30°C, and the other set at 37°C, for 3 days.

2.3. Domain comparison of WWPI and Rsp5

2.3.1. Sequence comparison. Protein sequences of the domains in Rsp5 (accession version U18916.2, GI number 7677630) and WWPI (accession version NM007013.3, GI number 33946331) were compared for sequence percent identity using BLAST alignment (Altschul <i>et al.</i> 1993; NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.2. ClustalW2 alignment. The WW domain sequences of Rsp5 and WWPI were aligned and compared using ClustalW2 (Lopez and Lloyd, 1997; EMBL-EBI, http://www.ebi.ac.uk/Tools/clustalw2/index.html).
2.3.3. Visualizing conserved residues in domain structure. An image of the PDB file for the X-ray crystal structure of the WWPI HECT domain (PDB ID 1ND7; Verdecia et al. 2003) was produced using the UCSF Chimera package (Pettersen et al. 2004; Resource for Biocomputing, Visualization, and Informatics, UCSF; http://www.cgl.ucsf.edu/chimera, supported by NIH P41 RR-01081). Using a ClustalW2 alignment of the WWPI and Rsp5 HECT domains, the conserved residues between the two domains were visualized on the WWPI HECT domain structure.
3. Results

3.1. Preliminary results from WWP1 drug screens

Potential inhibitors of the WWP1 HECT domain were identified using virtual screening (W.R.P. Novak, Brandeis University; unpublished data), and the top hits were tested for inhibition of WWP1 activity using a polyubiquitination assay (Marc Wein, Harvard School of Public Health; unpublished data). These preliminary studies showed that some of the hits prevented the polyubiquitination activity of WWP1 in vitro (Fig. 2).

Figure 2. In vitro polyubiquitination assay of the WWP1 HECT domain with drugs

Western blots measuring ubiquitination of 200 ng WWP1 HECT domain incubated with 150 μM potential inhibitor drugs in vitro. Assays were done using biotin-ubiquitin, and visualized using standard horse radish peroxidase conjugated to streptavidin. Drug compounds incubated with the reaction are indicated above each lane. Incubation times are listed to the left of each blot. Left: Compounds were assayed at 150 μM concentrations. Right: Varying concentrations of compound NCI 13.10 were used in the same assay. All blots show distinct bands along each lane, indicating the attachment of one, a few, or several ubiquitins. (Marc Wein, Harvard School of Public Health; unpublished data)
The FW1808 (single RSP5 allele deletion) strain of yeast was used to test the effect of WWP1 inhibitors on yeast cell growth in the presence and absence of WWP1 expression (Fig. 3; W.R.P. Novak, Brandeis University; unpublished data). WWP1, a WWP1 C890A catalytic mutant, and Rsp5 were expressed in FW1808 yeast strains when grown in galactose media, but repressed in dextrose media.

FW1808 strains expressing the WWP1 protein grew after approximately 70 hours in liquid media, at a severely lagging pace as compared to strains of FW1808 expressing Rsp5 or containing the empty pYES2 vector. Drugs were thus tested in liquid culture, in order to examine the effect of these inhibitors on yeast growth.

Since expression of WWP1 and the WWP1 C890A mutant slowed yeast growth even in the absence of inhibitors, the growth patterns observed in the presence of inhibitors could not be definitively attributed to effective inhibition of the WWP1 active site. In addition, inhibitors that slowed yeast growth significantly in the presence of WWP1 expression were also found to have the same effect if the WWP1 C890A catalytic mutant was expressed, implicating inhibition of something other than the catalytic cysteine residue in WT WWP1. Thus, a functional analysis of WWP1 in FW1808 yeast was needed in order to more accurately determine the effect of inhibitors on the WWP1 protein in this yeast model.
Figure 3. The effect of drugs on the growth of yeast expressing/suppressing WWP1

Drug Dependency of Yeast Growth Expressing/Suppressing WWP1 Homologs

Growth of FW1808 yeast in liquid culture with WWP1 HECT inhibitor drugs after 70 hours of incubation at 30°C. Yeast strains are described by the vector they contain. pyes, pYES2 yeast expression vector. rsp5, Rsp5-pYES2. wwp1, WWP1-pYES2. c2a, WWP1 C890A mutant. (Walter Novak, Brandeis University; unpublished data)
3.2. Testing for complementation of Rsp5 function with WWP1

FW1808 Rsp5-1, a ura3- diploid strain of S. cerevisiae with one copy of the RSP5 gene deleted (Δrsp5-1), was used to test for complementation of Rsp5 function. The single allele deletion strain of RSP5 results in a temperature sensitive yeast strain; it survives at 30°C but dies at 37°C. Genes were transformed into the FW1808 yeast strain in the pYES2/CT yeast expression vector under a Gal1 promoter and with a URA3 selection marker.

Separate FW1808 strains containing Rsp5-pYES2, WWP1-pYES2, and pYES2 (empty vector) were created. These strains were grown in -ura liquid media containing dextrose (Dx) or galactose (Gal) to test for complementation. A bioscreen-based growth curve assay was performed using these strains at 37°C, using OD₆₀₀ readings to measure yeast growth (Fig. 4).
Figure 4. Bioscreen of FW1808 strains at 37°C

FW1808 strains Rsp5-pYES2, pYES2, and WWP1-pYES2 were grown up in Dx-ura liquid media overnight to equal densities, pelleted, and resuspended in Dx-ura or Gal-ura liquid media to an OD$_{600}$ of 0.1. Samples for the bioscreen were prepared with 125 µl/well, in triplicate, and grown at 37°C with shaking. OD$_{600}$ readings shown here are averages of three samples after 26 hours of growth. Error bars indicate one standard deviation from the mean.

The bioscreen assay at 37°C demonstrated that the FW1808 strains are temperature-sensitive, as they did not grow in Dx-ura media, when the plasmid gene expression was repressed by dextrose. All sample strains had OD$_{600}$ readings of approximately 0.1, comparable to that of the media alone. Using Gal-ura media at 37°C, only the FW1808 Rsp5-pYES2 strain was able to rescue the temperature-sensitive phenotype of the FW1808 parent yeast strain. In contrast, overexpression of the WWP1 protein (FW1808 WWP1-pYES2) and vector control failed to rescue yeast viability at 37°C.
3.3. *WWP1 toxicity at 30°C*

In addition to the assays performed at 37°C, FW1808 strains containing the Rsp5-pYES2, WWP1-pYES2, and pYES2 plasmids were streaked onto Dx-ura and Gal-ura agar plates, and incubated at 30°C for 3 days (Fig. 5) as controls in the complementation test.

**Figure 5. Growth of FW1808 strains at 30°C**

FW1808 strains on Dx-ura and Gal-ura agar plates at 30°C. *(A)* This template indicates the position of each strain streaked on the uracil dropout plates. *(B)* The Dx-ura (left) and Gal-ura (right) plates were streaked with the strains as indicated in the template, and incubated at 30°C for 3 days. The section circled in red indicates the FW1808 strain containing WWP1-pYES2.
After three days of incubation at 30°C, all FW1808 strains showed growth on the Dx-ura plate, but only the Rsp5-pYES2 and pYES2 strains showed growth on the Gal-ura plate. This result suggests that overexpression of WWP1 protein is toxic to the Rsp5 deficient strain of yeast.

3.4. **WWP1-pYES2 does not kill generic yeast strains**

Two “generic” strains of yeast, W303 and BY4743, were used to test whether WWP1 is toxic to yeast possessing normal levels of the Rsp5 protein. Yeast strains were considered generic if they had no mutations other than those used to accommodate plasmid selectivity markers, such as *ura-, his-, trp-*, etc. These strains were transformed with pYES2 vector only and WWP1-pYES2, were streaked onto Dx-ura and Gal-ura plates, and incubated at 30°C for 3 days (Fig. 6).
Figure 6. Growth of generic yeast strains with WWP1

Growth of generic yeast strains with WWP1 at 30°C after 3 days of incubation. (A) Templates of streak plates, specifying the plasmid contained in each streaked sample. The yeast strain used is indicated above each template. FW1808 is missing one copy of RSP5. W303 and BY4743 are used as "generic" yeast strains. (B) FW1808 strains. The WWP1-pYES2 strain is circled in red for each plate. (C) W303 strains. The WWP1-pYES2 strain is circled in yellow. (D) BY4743 strains. The WWP1-pYES2 strain is circled in yellow.

The W303 and BY4743 strains streaked on Gal-ura plates showed growth comparable to the strains on the Dx-ura plates, but the FW1808 WWP1-pYES2 showed significantly less growth on Gal-ura than Dx-ura media (Fig. 6), indicating that overexpression of the WWP1 protein is not toxic in yeast strains containing two copies of RSP5.

3.5. Catalytic domain mutant of WWP1 is toxic at 30°C
The essential catalytic cysteine residue in the HECT domain of WWP1 was mutated to an alanine residue. The HECT domain requires Cys890 in order to catalyze the protein's ubiquitin transfer activity. The C890A mutant was tested for growth in FW1808 yeast cells in order to determine whether the catalytic activity of the WWP1 HECT domain was causing toxicity of the full protein in yeast.

Figure 7. Growth of FW1808 with WWP1 C890A catalytic domain mutant

A  Streak plate
    template

B

Dx-ura  Gal-ura

Growth of FW1808 strain containing the WWP1 C890A mutant on Dx-ura and Gal-ura agar plates at 30°C. (A) This template indicates the position of each strain streaked on the uracil dropout plates. (B) The Dx-ura (left) and Gal-ura (right) plates were streaked with the strains as indicated in the template, and incubated at 30°C for 3 days. WWP1-pYES2 strains are indicated by red circles, and WWP1 C890A-pYES2 strains are indicated by yellow circles.
At 30°C, the FW1808 WWP1 C890A strain grew on Dx-ura media but not on Gal-ura media, suggesting that WWP1 toxicity is not mediated solely by HECT domain catalytic activity.

3.6. Minimal domain requirement for WWP1 toxicity

Mutant constructs of WWP1 were created in order to probe which domain(s) mediate toxicity in the FW1808 yeast strain. The mutations made were progressive truncations of domains (Fig. 8).

**Figure 8. WWP1 Δ-domain mutant constructs**

<table>
<thead>
<tr>
<th>WWP1</th>
<th>1-922</th>
<th>[Diagram of WWP1 Δ-domain mutant constructs]</th>
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<tr>
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<tr>
<td>L1</td>
<td>145-922</td>
<td>[Diagram of WWP1 Δ-domain mutant constructs]</td>
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</tr>
<tr>
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<td>528-922</td>
<td>[Diagram of WWP1 Δ-domain mutant constructs]</td>
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<tr>
<td>ΔHECT</td>
<td>1-544</td>
<td>[Diagram of WWP1 Δ-domain mutant constructs]</td>
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Schematic of WWP1 Δ-domain mutant constructs. (Numbers indicate the amino acids of the full protein retained in the mutants.)

All the WWP1 Δ-domain gene constructs were cloned into the pYES2 vector. FW1808 yeast cells were transformed with the mutant constructs and the strains were assayed for growth at 30°C. Strains were grown with Dx-ura and Gal-ura media, both in liquid culture (Fig. 9) and on agar plates (Fig. 10).
Figure 9. Bioscreen assay of WW1 Δ-domain mutants in FW1808 yeast

FW1808 strains of WW1 Δ-domain mutant constructs in pYES2 were grown up in Dx-ura liquid media overnight to equal densities, pelleted, and resuspended in Dx-ura or Gal-ura liquid media to an OD$_{600}$ of 0.1. Samples for the bioscreen were prepared with 125 μl/well, in triplicate, and grown at 30°C with shaking. OD$_{600}$ readings shown are averages of three samples after 24 hours of growth. Error bars indicate one standard deviation from the mean.

After 24 hours of growth at 30°C in the bioscreen, the FW1808 WW1 Δ-domain strains and controls in Dx-ura media all grew to comparable densities, with OD$_{600}$ readings of approximately 0.75. The Rsp5-pYES2 and pYES2 control strains grew robustly in Gal-ura media, to higher densities than these same strains in Dx-ura media. The WW1 construct strains in Gal-ura media showed varied levels of growth. The C2 strain grew as well as the abovementioned control strains. WW1 and L1 strains showed no growth in Gal-ura media, but there was a progressive increase in growth from strain W1 to W2 to W3. The W4, L2, and ΔHECT strains all showed no growth, indicating that these constructs are toxic when expressed in FW1808 yeast.
The same WWP1 Δ-domain mutant strains were used in a drop assay on Dx-ura and Gal-ura agar plates. A drop assay allows us to examine the extent of growth on solid media more precisely than streak plating. All strains were plated in serial dilution and incubated at 30°C for 3 days (Fig. 10).

After 3 days of incubation, the FW1808 WWP1 Δ-domain strains and controls on Dx-ura drop assay plates all showed relatively equal growth across all dilutions. On the Gal-ura drop assay plates, the Rsp5-pYES2 and pYES2 controls showed growth in all but the lowest concentration of cells plated. The difference in the growth of these strains on Dx-ura and Gal-ura plate is due to a lag seen in yeast growth when galactose is the carbon source in agar plates. The WWP1 constructs on Gal-ura media showed varied levels of growth. The C2 strain showed robust growth, comparable to the Rsp5-pYES2 and pYES2 controls. WWP1 and L1 strains showed no growth on Gal-ura, indicating that the L1 construct, like the full length protein, is toxic when expressed in FW1808 yeast. The W1, W2 and W3 strains showed partial growth, with W1<W2<W3. Strains containing the W4, L2, or ΔHECT construct all showed no growth, indicating that these constructs are toxic when expressed in FW1808 yeast.

As seen in the bioscreen assay (Fig. 9) and drop assay (Fig. 10) data, growth of FW1808 yeast strains increased as the N-terminal domains of WWP1 were deleted through the second WW domain, but deletion of the third WW domain (W4 construct) resulted in no growth on Gal-ura plates at 30°C. Deletion of the catalytic HECT domain also prevented yeast growth. This suggests that the toxicity exhibited by the overexpression of WWP1 in FW1808 yeast might be
caused by more than one domain, specifically the WW containing domain and the catalytic HECT domain.

Figure 10. Drop assay of FW1808 strains with WWP1 Δ-domain constructs

FW1808 strains of WWP1 Δ-domain constructs in pYES2 were grown up overnight in Dx-ura liquid media to equal densities. Cells were pelleted, resuspended in water to an OD$_{600}$ of 1.0, and a series of 10-fold dilutions were made. Strains were plated in serial dilution and incubated at 30°C for 3 days. (Black triangles show low to high concentration of cells.) Plates are Dx-ura (left) and Gal-ura (right), with strains labelled by row. (vector), pYES2 empty vector. Strains WWP1, L1, W4, L2, and ΔHECT on Gal-ura plates are boxed in red to indicate no growth. Strains W1, W2 and W3 on Gal-ura plates are boxed in blue to indicate partial growth.
3.7. Chimeric constructs of WWPI and Rsp5 domains

To further investigate the two modes of toxicity seen from the WWPI Δ-domain mutant constructs in FW1808, we created chimeric constructs of WWPI and Rsp5. The constructs joined Rsp5 WW domains with the WWPI HECT domain, and alternatively, the WWPI WW domains with the Rsp5 HECT domain (Fig. 11).

Figure 11. Chimeric constructs of WWPI and Rsp5

Schematic of chimeric constructs. Chimera A: Rsp5 WW domains and a WWPI HECT domain. Chimera B: WWPI WW domains and an Rsp5 HECT domain.

Both chimeric gene constructs were cloned into the pYES2 vector, and used to transform FW1808 yeast cells. Strains were assayed using a bioscreen at 30°C (Fig. 12) and a drop assay on Dx-ura and Gal-ura agar plates (Fig. 13).
FW1808 strains of WWP1-Rsp5 chimeric constructs in pYES2 were grown up in Dx-ura liquid media overnight to equal densities, pelleted, and resuspended in Dx-ura or Gal-ura liquid media to an OD<sub>600</sub> of 0.1. Samples for the bioscreen were prepared with 125 ul/well, in triplicate, and grown at 30°C with shaking. OD<sub>600</sub> readings shown are averages of three samples after 26 hours of growth. Error bars indicate one standard deviation from the mean.

After 26 hours of growth, the FW1808 chimeric strains and controls all grew to comparable densities in Dx-ura liquid media, with OD<sub>600</sub> readings of approximately 0.9. In Gal-ura liquid media, Rsp5-pYES2 and pYES2 control strains grew to almost the densities of the corresponding Dx-ura cultures, and the WWP1-pYES negative control strain showed an OD<sub>600</sub> of 0.1. The chimera A strain grew to an OD<sub>600</sub> of 0.18, slightly higher than the WWP1-pYES2 strain, suggesting that the chimera A expression protein may be toxic. The chimera B strain in Gal-ura media, however, showed growth that was comparable to the growth of the Rsp5-pYES2 and pYES2 controls.
The same chimeric construct strains were used in a drop assay on Dx-ura and Gal-ura agar plates to corroborate the data from liquid media in the bioscreen. All strains were incubated at 30°C for 3 days (Fig. 13).

**Figure 13. Drop assay of FW1808 yeast with chimeric constructs**

FW1808 strains containing WWP1-Rsp5 chimeric constructs in pYES2 were grown up overnight in Dx-ura liquid media to equal densities. Cells were pelleted, resuspended in water to an OD$_{600}$ of 1.0, and a series of 10-fold dilutions were made. Strains were plated in serial dilution and incubated at 30°C for 3 days. (Black triangles show high to low concentration of cells.) Strains plated are labelled by row. (vector), pYES2 empty vector, chA, chimera A, chB, chimera B. The chimera A strain on Gal-ura media is boxed in red to indicate no growth. The chimera B strain on Gal-ura media is boxed in blue to indicate growth.

All the FW1808 strains showed relatively equal growth on Dx-ura at 30°C after 3 days of incubation. On Gal-ura, the Rsp5-pYES2 and pYES2 positive control strains grew, and the negative control WWP1-pYES2 strain prevented yeast growth. Chimera A, comprised of Rsp5 WW domains and the WWP1 HECT domain, showed no growth (Fig. 13, red box), suggesting that this construct is toxic when expressed in FW1808 yeast. Chimera B showed growth (Fig. 13, blue box) comparable to the Rsp5-pYES2 and pYES2 strains, which indicates that this construct is not toxic in the same yeast strain.
3.8. Sequence alignments of WWP1 and Rsp5 domains

The sequence conservation between the domains of WWP1 and Rsp5 was evaluated using NCBI BLAST (Fig. 14). This allowed us to compare the sequence percent identity between specific domains of these proteins.

Figure 14. Sequence identity between WWP1 and Rsp5 domains

<table>
<thead>
<tr>
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<tr>
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<td>WW1</td>
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<td>WW1</td>
<td>80%</td>
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<td>WW2</td>
<td>78%</td>
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<td>HECT</td>
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Protein sequence percent identities between the homologous domains of WWP1 and Rsp5.

In order to assess the similarities between the WW domains of Rsp5 and WWP1, the peptide sequences of these WW domains were aligned using ClustalW2. This program produces biologically meaningful alignments of divergent protein sequences and shows the evolutionary relationship between them. Our ClustalW2 alignment (Fig. 15a) provided a cladogram showing the evolutionary relationships between the Rsp5 and WWP1 WW domains (Fig. 15b). This cladogram shows that Rsp5 WW 1 and WWP1 WW 2 are most closely aligned to each other. Rsp5 WW 3 and WWP1 WW 3 are also evolutionarily closest to each other than to any other WW domain in this comparison. The WWP1 WW 1 domain is most recently related to the Rsp5 WW 1 and WWP1 WW 2. The WWP1 WW 4 and Rsp5 WW 2 domains are not closely aligned with any of the other WW domains shown above. This analysis of WW domain similarities shows that the second and third WW domains of WWP1 are very closely aligned with Rsp5 WW
domains, but the first and fourth WW domains of this protein are relatively distant from the other domains shown.

**Figure 15. Alignment of WWP1 and Rsp5 WW domains**

(A) Cladogram showing the relationship between WW domains as determined by a ClustalW2 alignment of the WW domains in WWP1 and Rsp5. (B) Sequence alignment of the WWP1 and Rsp5 WW domains in ClustalW2. A (*) indicates conserved amino acids. A (:) indicates similar amino acids.

The HECT domains of WWP1 and Rsp5 were also aligned using ClustalW2. The aligned residues were visualized on the structure of the WWP1 HECT domain using Chimera (Fig. 16) in order to visualize conservation between the two HECT domains in three dimensional space. This model of the WWP1 HECT domain shows the sections conserved between WWP1 and Rsp5. Conserved residues in the ribbon model are shown in red, and the rest of the protein in blue. The structure of the domain around the catalytic Cys890 is mostly coloured blue in this model, indicating that there may be significant differences in structure, binding, and function between these two homologous HECT domains.
Figure 16. Conserved sequences between the Rsp5 and WW1 HECT domains

The HECT domain sequences of WW1 and Rsp5 were aligned using ClustalW2, and the conserved residues were visualized on a ribbon model of the structure of the WW1 HECT domain. Red indicates conserved residues, blue indicates non-conserved residues. The catalytic Cys890 residue is shown in ball-and-stick form, with the sulphur atom indicated in yellow.
4. Discussion

4.1. A Drug Screen for WWPI

WWPI, a HECT E3 ubiquitin ligase, has been implicated as a possible drug target for treatment of osteoporosis (Jones et al. 2007) and several types of cancer (Chen & Matesic 2007). There are in vitro ubiquitin assays for E3 ligase function that can be used to screen for small molecule inhibitors, but they are expensive and time-intensive (Sun 2003). Since yeast possess a homologue of the WWPI protein, and since some WWPI homologues can functionally complement for Rsp5 in yeast, we asked whether WWPI could also complement Rsp5. Further, we assessed whether WWPI function in a temperature-sensitive Arsp5-l yeast model might yield a rapid, inexpensive in vivo drug screen. Initial results demonstrated that WWPI does not complement Rsp5 function in an isogenic Arsp5-l strain of yeast (Fig. 4), but rather, WWPI is toxic when expressed in this strain (Fig. 5).

Despite the lack of complementation, the toxic phenotype exhibited by the overexpression of WWPI in FW1808 yeast has the potential for a powerful drug screen. However, the validity of the screen relies on understanding the mechanism of toxicity. By determining the domain responsible for toxicity, a screen for a drug inhibiting that domain would show a rescue of the toxic phenotype in this yeast system. However, if it is not known whether the inhibitor is targeting the domain causing toxicity of this protein in this system, then a rescue of toxic phenotype in the presence of a drug would not indicate an effective inhibitor. Therefore, we investigated the mechanism of WWPI toxicity in yeast in order to learn more about this protein as well as to potentially develop an efficient drug screen.
4.2. **WWP1 is toxic in yeast with a single RSP5 allele deletion**

WWP1 was found to be toxic in FW1808 yeast, a strain which is *Arsp5-l* and consequently temperature sensitive at 37°C (Fig. 5). In order to find out whether WWP1 is toxic in all yeast, we transformed generic yeast W303 and BY4743, each containing two copies of *RSP5*, with the same WWP1 construct in pYES2 yeast expression vector. The expression of WWP1 in these generic yeast strains did not prevent yeast growth (Fig. 6), indicating that overexpression of this protein is not toxic in all yeast strains. This result suggests that the toxicity of this protein is related to E3 ubiquitin ligase function in our *Arsp5-l* strain of yeast.

The active site of WWP1 is in the HECT domain at the C-terminus and contains a critical Cys890 that is required for the protein's catalytic activity (Verdecia *et al.* 2003). In order to determine whether the toxicity of WWP1 is mediated by its catalytic activity, we tested a C890A mutant for growth in FW1808 yeast. Mutation of the critical catalytic residue in the HECT domain did not prevent toxicity, as this construct still prevented yeast growth (Fig. 7). Thus, the toxicity we observe is not solely due to the catalytic activity of WWP1, although it may be due to some binding property of the catalytic domain.

4.3. **Mechanisms of WWP1 toxicity**

We wanted to elucidate the domains that are involved in WWP1 toxicity. Testing Δ-domain constructs of WWP1 (Fig. 8) for growth in FW1808 yeast indicated that there may be two mechanisms of toxicity. The HECT domain alone seems to be toxic, suggesting one mechanism.
Another mechanism involves the N-terminal linkers and WW domains, which, even in the absence of the HECT domain, also mediate some toxic effect.

Our results from growth assays of WWP1 Δ-domain constructs in FW1808 yeast show that as we progressively delete the N-terminal domains of the protein, retaining the HECT domain, there is less toxicity, up to the deletion of the third WW domain. By removing the first linker region (W1 construct), followed by the first and second WW domains, respectively, there is an increase in growth compared to the full WWP1 strain. However, the strains do not reach full growth as compared to pYES2 and Rsp5-pYES2 positive controls (Figs. 9 and 10). When the HECT domain is attached to just a linker region or the most C-terminal WW domain (W4 and L2 constructs), we see toxicity comparable to that of the full protein. Improper localization of the protein by the WW domains may allow the HECT domain to ubiquitinate proteins incorrectly, so that species needing proteasomal degradation do not get tagged or that species needed for cell function get mistakenly degraded. This would suggest that the WWP1 HECT domain is active in FW1808 yeast, and the differences in WW domains among our various constructs would modulate toxicity by localizing the toxic HECT activity in the cell. Another possibility is that this E3 ligase does not have the appropriate E2 binding partners in yeast (Ingham et al. 2004). Thus, without the correct ubiquitin transfer from charged E2 ligases, the WWP1 HECT domain may bind various proteins without processing them in the ubiquitin-proteasome pathway, resulting in protein complexes that are deleterious in the cell.

The WWP1 Δ-domain growth assays showed that deleting the HECT domain still produces a toxic phenotype. The ΔHECT construct prevents yeast growth to the same extent as the full
protein and the W4 and L2 constructs (Figs. 9 and 10). These data implicate the linker regions and the WW domains in a mechanism of toxicity separate from the WWP1 HECT domain. Perhaps these N-terminal domains are binding to other proteins or structures in the cell and, by sequestering them, preventing their cellular processes from occurring. WW domains are known to have high specificity in binding (Zarrinpar and Lim 2000), and have been reported to recognize peptide sequences found in HECT domains (Kasanov et al. 2001). Particularly, binding up Rsp5 in the cell would prevent the native yeast E3 ligase from functioning properly, and cause toxicity due to an ineffective ubiquitin-proteasome pathway.

Since the results from the Δ-domain constructs suggested two possible mechanisms of toxicity within the WWP1 protein, we made chimeric constructs using domains from WWP1 and Rsp5 (Fig. 11) to further elucidate this mechanism. Chimera A (chA), which contains Rsp5 WW domains and the WWP1 HECT domain, prevented yeast growth, indicating that it is toxic. Chimera B (chB), which contains WWP1 WW domains and the Rsp5 HECT domain, showed full growth, and thus is not toxic (Figs. 12 and 13). These results show that the WWP1 WW domains are not sufficient to cause toxicity alone because, when attached to the Rsp5 HECT domain, their presence alone does not prevent cell growth. It is possible that the WWP1 WW domains are not properly localizing the chimeric protein in the cell, in which case the mislocalization of the Rsp5 HECT domain is not seen to cause toxicity in FW1808 cells. These results are consistent with previous studies, which show that the Rsp5 HECT domain expressed by itself is not sufficient for rescue of temperature sensitivity in Δrsp5-1 yeast but is also not toxic when expressed at 30°C (Wang et al. 1999). The WWP1 HECT domain, however, may mediate toxicity. Even when this domain is attached to Rsp5 WW domains and should be
properly localized to carry out E3 ubiquitin ligase activity in yeast, we see no cell growth. Our results suggest that this domain's toxicity may not be due to active ubiquitylation of the wrong substrates in the cell, but rather to a lack of proper E3 ligase activity. The data from these WWP1-Rsp5 chimeric constructs show that the WWP1 HECT domain is sufficient to mediate toxicity in FW1808 yeast.

4.4. Similarities between WWP1 and Rsp5 domains

We wanted to compare the sequences of the Rsp5 and WWP1 WW domains, as differences between these domains may provide insight into differences in their function. This was done by aligning the three Rsp5 WW and four WWP1 WW domains using ClustalW2. The alignment (Fig. 15) showed that, of all the WW domains in Rsp5 and WWP1, the third Rsp5 WW domain is most similar to the third WWP1 WW domain. The third Rsp5 WW domain was shown to be the minimum necessary WW domain for Rsp5 function (Wang et al. 1999). Taken together, these data indicate that the WWP1 W3 construct may have shown the best growth because it had the closest correct localization with minimal interference from other WW domains N-terminal to them. The alignment shows that the second WWP1 WW and the first Rsp5 WW domains are most closely related to each other. The first WWP1 WW domain is somewhat related to the first Rsp5 WW domain, and the fourth WWP1 WW domain is not closely aligned with any of the other WW domains in the two proteins. These alignment differences may provide insight into the reason for the mitigated toxicity seen in some of the WWP1 Δ-domain constructs. Some WW-HECT E3 ligase WW domains are known to have intramolecular autoinhibitory effects on the HECT domain, but this is not seen in Rsp5 (Kee and Huibregtse 2007). The diversity of these
intramolecular interactions, and resulting autoinhibitory effects, may be mediated by the differences in WW domains, such as those seen between Rsp5 and WWP1 in our ClustalW2 alignment.

The HECT domains of Rsp5 and WWP1 were also compared using ClustalW2. The residues in WWP1 conserved from Rsp5 were visualized on a ribbon structure of the WWP1 HECT domain structure, using UCSF Chimera (Fig. 16). The residues forming the folded structure around the catalytic cysteine in the HECT domain are not conserved. Based on these data, it is possible that the binding partners and interactions of this region may differ between WWP1 and Rsp5 (Ingham et al. 2004).

4.5. Model of WWP1 toxicity in FW1808 yeast

We propose that the mechanism of WWP1 toxicity is two-fold, as shown by the yeast growth data from our Δ-domain and chimeric constructs in FW1808 yeast (Fig. 17). The first mechanism is mediated by the HECT domain. This toxicity may be due to overactivity of the HECT domain, whereby it ubiquitylates excessively and may non-specifically tag proteins for degradation. It is also possible that the WWP1 HECT domain is catalytically inactive, but binds various proteins or structures in the cell, interfering with native yeast cellular processes, and thus preventing growth. Particularly, binding Rsp5 in this way may lead to a deficiency in protein degradation that causes toxicity in FW1808 yeast but not in generic yeast strains.
The second mechanism of WWP1 toxicity proposed involves its WW domains. E3 ligases with C2 and WW domains are thought to have autoinhibitory effects on their own catalytic activity (Kasanov et al. 2001, Wiesner et al. 2007) by intramolecularly binding their own HECT domains and keeping the protein in a closed conformation. The toxicity observed by expression of a construct that lacks the HECT domain and retains various WW and linker regions may be due to such an autoinhibitory effect acting in trans so that the WWP1 construct blocks either the activity of the native Rsp5 HECT domain or some other HECT domain required for cellular function in yeast.

Figure 17. Proposed mechanisms of WWP1 toxicity in FW1808 yeast

(A) Schematic of the full WWP1 protein with all domains labelled. (B) Toxicity mediated by the WWP1 HECT domain. Top: Overubiquitination of substrates. Ub, ubiquitin. Bottom: Binding and sequestering of cellular proteins without ubiquitination activity. (C) Toxicity mediated by WWP1 WW domains binding and sequestering cellular proteins.
4.6. Future Perspectives

Our results from a functional analysis of WWP1 in a single RSP5 allele deletion yeast model suggest that the toxicity of this protein may be mediated by two distinct mechanisms. One mechanism involves the HECT domain. In order to determine whether the WWP1 HECT domain is overactive or inactive in our FW1808 yeast strains, we would like to create a catalytic mutant of chimera A (containing Rsp5 WW domains and the WWP1 HECT domain) by site-directed mutagenesis of the catalytic cysteine residue to an alanine residue. If this construct rescues growth, then that would indicate that the toxicity is due to an overactive HECT domain, making chimera A a good model against which to test drugs that inhibit the catalytic activity of this HECT domain. However, if this chimeric catalytic mutant construct is also toxic, it would then indicate that the HECT domain is binding up various cellular components but not ubiquitinating them. In this case, the binding partners could be determined using a yeast-2-hybrid screen. It would be of particular interest to determine whether the WWP1 HECT domain binds Rsp5, and whether overexpressing both WWP1 and Rsp5 in the same yeast strain allows the cells to live.

The other potential mechanism of toxicity is mediated by the WW domains of WWP1. We would like to visualize the localization of our constructs in yeast cells in order to better understand the function of WW domains, and observe how these differ between WWP1 and Rsp5. This can be done by using GFP fusion tags for each of our constructs and visualizing their localization in yeast cells by confocal microscopy. It would also be useful to further investigate the binding specificity of these WW domains, and the nature of their interactions both within and between WWP1 and Rsp5.
The dual mechanisms of toxicity of WWP1 provide the possibility of a powerful drug screen. In our current yeast model, overexpression of certain WWP1 constructs gives a toxic phenotype. Therefore, drugs that effectively target the domain(s) responsible for toxicity should block the toxic phenotype and rescue yeast growth. The availability of constructs that are toxic because of either the WW domains or the HECT domain of WWP1 allows us to screen for drugs that effectively target different parts of this protein. Furthermore, testing drugs simultaneously against both the construct containing the HECT domain alone, and the construct containing all the WW domains without the HECT domain, provides an internal control for drug specificity. In order to be an effective inhibitor of a particular part of WWP1, a drug should only block the toxic phenotype and allow growth for one of the two constructs, but not both. Drugs that rescue growth in the presence of either construct would, then, be targeting something other than the overexpressed WWP1 construct in the yeast cell. However, in such a case, determining the protein or pathway that is actually targeted by that drug would offer further insight into the mechanism of WWP1 toxicity.

4.7. Concluding Remarks

E3 ubiquitin ligases are abundant in mammalian and yeast cells, but much of their binding and functional specificity remains unknown. This makes them difficult to assay as drug targets, even though many E3 ligases have been implicated in a range of diseases. Our results demonstrate that expression of WWP1 in Δsp5-1 yeast causes toxicity by two potential mechanisms involving separate domains of the protein. Having a model in which WWP1 toxicity can be mediated by
different domains provides the possibility of targeting specific parts of this protein with drugs. Moreover, the dual mechanism of toxicity allows for a powerful drug screen with an internal control for drug target specificity, ensuring that it is the overexpressed WWP1 construct that is being affected by the drug.

Our results further indicate that there are significant functional differences between the mammalian WWP1 HECT E3 ligase and its yeast orthologue, Rsp5, despite their domain and sequence homology. Further studies should be performed to elucidate the differences in HECT domain binding activity, WW domain specificity, and regulatory modulation between E3 ligases. A better understanding of this class of proteins, whose members have been identified as potential drug targets, would be beneficial not only in the understanding of disease pathways, but also in developing efficient drug screens.
References


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