

The E3 Ubiquitin Ligase WWP1 Selectively Targets HER4 and Its Proteolytically Derived Signaling Isoforms for Degradation[∇]

Shu-Mang Feng,¹ Rebecca S. Muraoka-Cook,^{1,2} Debra Hunter,¹ Melissa A. Sandahl,¹
Laura S. Caskey,¹ Keiji Miyazawa,⁴ Azeddine Atfi,⁵ and H. Shelton Earp III^{1,3*}

UNC Lineberger Comprehensive Cancer Center,¹ Department of Genetics,² and Department of Medicine and Pharmacology,³
University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, North Carolina 27599; Department of
Molecular Pathology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan⁴; and INSERM U 482,
Hôpital St-Antoine, Paris, France⁵

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In general, epidermal growth factor receptor family members stimulate cell proliferation. In contrast, at least one HER4 isoform, JM-a/Cyt1, inhibits cell growth after undergoing a two-step proteolytic cleavage that first produces a membrane-anchored 80-kDa fragment (m80^{HER4}) and subsequently liberates a soluble 80-kDa fragment, s80^{HER4}. Here we report that s80^{HER4} Cyt1 action increased the expression of WWP1 (for WW domain-containing protein 1), an E3 ubiquitin ligase, but not other members of the Nedd4 E3 ligase family. The HER4 Cyt1 isoform contains three proline-rich tyrosine (PY) WW binding motifs, while Cyt2 has only two. WWP1 binds to all three Cyt1 PY motifs; the interaction with PY2 found exclusively in Cyt1 was strongest. WWP1 ubiquitinated and caused the degradation of HER4 but not of EGFR, HER2, or HER3. The HER4-WWP1 interaction also accelerated WWP1 degradation. Membrane HER4 (full length and m80^{HER4}, the product of the first proteolytic cleavage) were the preferred targets of WWP1, correlating with the membrane localization of WWP1. Conversely s80^{HER4}, a poorer WWP1 substrate, was found in the cell nucleus, while WWP1 was not. Deletion of the C2 membrane association domain of WWP1 allowed more efficient s80^{HER4} degradation, suggesting that WWP1 is normally part of a membrane complex that regulates HER4 membrane species levels, with a predilection for the growth-inhibitory Cyt1 isoform. Finally, WWP1 expression diminished HER4 biologic activity in MCF-7 cells. We previously showed that nuclear s80^{HER4} is ubiquitinated and degraded by the anaphase-promoting complex, suggesting that HER4 ubiquitination within specific cellular compartments helps regulate the unique HER4 signaling capabilities.

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) consists of EGFR/HER1/ErbB1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. All family members have a ligand-binding ectodomain, a single transmembrane segment, an intracellular tyrosine kinase domain, and a tyrosine-rich carboxy terminus. Ten ligands from two related ligand families, the epidermal growth factor (EGF) and heregulin/neuregulin families, bind to EGFR family RTKs. Ligand-dependent homo- and heterodimerization results in kinase activation and cross-phosphorylation. Although HER1 and HER4 are capable of both homo- and heterodimerization under most conditions, HER2 and HER3 are obligate heterodimeric partners, since HER2 does not bind any known ligand, and HER3 is devoid of intrinsic kinase activity. The activated receptor complexes exhibit multiple signaling capabilities including stimulation of canonical mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and/or STAT signaling pathways (10, 41, 47).

All four EGFR family members play a role in regulating cardiovascular, neuronal, and epithelial development. Null mutations of any of the EGFR family members result in embryonic lethality. Despite these similarities, HER4 exhibits

unique biological activities that set it apart from the rest of the EGFR family. Whereas EGFR, HER2, and HER3 stimulate cellular proliferation, survival, and migration (41), HER4 expression and activation often correlates with decreased cell growth and differentiation (13, 33, 34, 43). In studies of human breast cancer, aberrant expression or activity of EGFR, HER2, and HER3 appears to contribute to tumor progression and correlate with poorer patient outcomes (10, 17). In contrast, HER4 expression correlates with estrogen receptor positivity, lower tumor grade, and a more favorable prognosis in most studies (3, 21, 37, 46, 51, 52, 57); however, some studies report a poorer prognosis in subsets of HER4-positive breast cancers (4, 27). These paradoxical findings regarding HER4 expression in breast cancer may relate to the unique cellular biology of HER4; specifically, variant isoforms of HER4 are generated by alternative splicing that exhibit properties unique to HER4.

HER4 RNA is alternatively spliced to yield at least four isoforms that differ in the sequence encoding two specific regions of HER4. The first is the receptor juxtamembrane domain, in which alternative splicing gives rise to either the JM-a or the JM-b isoforms (12). Alternative splicing within the region encoding the midcytoplasmic domain results in the production of either the CYT-1 or CYT-2 HER4 isoform (11). Thus, four distinct HER4 isoforms have been observed: JM-a/Cyt1, JM-a/Cyt2, JM-b/Cyt1, and JM-b/Cyt2. Each isoform alters HER4 signaling in some manner affecting stability, turnover, cellular localization, and interaction with HER4 downstream elements (15, 19). Factors regulating “downregulation”

* Corresponding author. Mailing address: UNC Lineberger Comprehensive Cancer Center, University of North Carolina Chapel Hill, 102 Mason Farm Road, Chapel Hill, NC 27599. Phone: (919) 966-2335. Fax: (919) 966-3015. E-mail: hse@med.unc.edu.

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of the ligand binding or localization-specific stability of HER4 and its proteolytic products (see below) are not yet understood. However, it is clear that ligand binding does not lead to the well-studied clathrin-coated pit internalization mechanism characteristic of the EGFR.

One unique aspect of HER4 signaling among the EGFR family is the proteolytic cleavage that occurs upon activation, generating an 80-kDa fragment. Upon ligand binding or phorbol myristate acetate (PMA) treatment, HER4 is cleaved by a tumor necrosis factor-converting enzyme (TACE)-like activity, releasing the extracellular domain and leaving an 80-kDa membrane-associated HER4 (m80) (40, 54). This is followed by intramembranous HER4 cleavage by γ -secretase, releasing a soluble 80-kDa fragment, s80^{HER4}, into the cytoplasm (25, 34). The intracellular domain contains canonical nuclear localization and export sequences and in fact translocates to the nucleus in a manner requiring an active tyrosine kinase (33). Evidence is accumulating that the nuclear s80^{HER4} participates in gene transcriptional regulation and growth control (13, 33, 34, 42, 56). Alternative splicing has a direct influence on HER4 cleavage, because only the JM-a isoform, but not JM-b, is susceptible to proteolytic cleavage by TACE (8, 40).

Also unique to HER4 within this RTK family is the presence of protein-rich, WW-domain binding motifs known as PY sequences (PPXY, where X is any amino acid). WW domains are globular motifs consisting of 40 amino acids, two of which are highly conserved tryptophans (WW) (49). HER4 has been reported to interact with several WW containing proteins, including Itch (27), YAP (23), and WWOX (2). Again, alternative splicing of HER4 may directly affect the extent or strength of WW-domain binding. The HER4 Cyt1 isoform harbors 16 amino acids (SEIGHSPPPAYTPMSG), that are absent in the HER4Cyt2 isoform. This Cyt1-specific sequence harbors a WW-domain binding motif (PPAY), which also overlaps with a previously described phosphatidylinositol 3-kinase binding region (11). In all, HER4Cyt1 has three PY motifs in its cytoplasmic domain, whereas the HER4 Cyt2 isoform has only two PY motifs. The consequences of the differential in PY domains between Cyt1 and Cyt2, and the potential effects upon HER4 interaction with WW-domain containing proteins are not yet known but are profound since the Cyt1 isoform inhibits breast epithelial cell growth, while the Cyt2 isoform does not (R. S. Muraoka-Cook et al., unpublished data).

A full understanding of EGFR family biologic action must include knowledge regarding signal termination and, in the last decade, it has become apparent that receptor ubiquitination plays a general role in protein metabolism and a specific role in EGFR family signaling. Ubiquitination is a posttranslational modification that can (i) direct proteins for degradation by the 26S proteasome; (ii) target plasma membrane proteins for endocytosis, sorting, and destruction in the lysosome; or (iii) alter the function of proteins involved in signal transduction, transcription, or DNA repair. The ubiquitination requires three critical enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The E3 ubiquitin ligase provides substrate specificity and catalyzes isopeptide bond formation between ubiquitin and the substrate (16, 35). There are more than 500 E3 ligases and several target EGFR family members for degradation. Upon EGF stimulation, EGFR associates with an E3 ligase, Cbl, and

undergoes ubiquitination, which facilitates its lysosomal sorting and eventual degradation (26, 59). The E3 ligase CHIP (for carboxyl terminus Hsc70-interacting protein) associates with ErbB2 and induces ErbB2 ubiquitination and downregulation (58, 61). The E3 ligase Nrdp1 (for neuregulin receptor degrading protein 1) associates with ErbB3 and stimulates its ubiquitination and degradation by proteasomes (9, 39). Very recently, it has been reported that WW domain containing E3 ligase, Aip4/Itch, results in HER4 degradation (36).

Our previous work demonstrated that s80^{HER4} expression in multiple breast cancer cell lines translocates into nucleus, regulates the cell cycle (31, 48), inhibits cell proliferation (13), and induces differentiation (33). In the course of initial microarray gene expression studies, we noted a marked increase in the WWP1 transcript in HC11 mouse breast cell lines stably expressing GFP-s80^{HER4}Cyt1 compared to HC11 expressing green fluorescent protein (GFP) alone. In the experiments described here, we establish that HER4 and s80^{HER4} bind specifically to WWP1 via the HER4 cytoplasmic PY motifs. The E3 ligase activity specifically ubiquitinates HER4 with a preference for membrane-associated species and Cyt1 isoform leading to degradation; EGFR, HER2, and HER3 are not WWP1 targets.

MATERIALS AND METHODS

Cell culture. COS-7 cells were grown in Dulbecco modified Eagle medium (Gibco-BRL) supplemented with 10% fetal bovine serum. MCF-7 cells were grown in minimal essential medium with 10% fetal bovine serum plus 5 μ g of insulin (Gibco-BRL)/ml, 100 U of nonessential amino acids (Invitrogen)/ml, sodium pyruvate (1 mM), and antibiotics at 37°C with 5% CO₂. HC11 cells and their derivatives were cultured as previously described (13). Where indicated, the following factors were added: recombinant heregulin β (a gift from Genentech), MG132 (Peptide Institute, Inc., Minoh-shi Osaka, Japan), bafilomycin A1 (LC Laboratories, Woburn, MA), GM6001 (Calbiochem, San Diego, CA), and cycloheximide (CHX) and PMA (both from Sigma-Aldrich).

Plasmids, transfection, and small interfering RNA (siRNA). The following constructs were described previously: pLXSN-HER4 (JM-a, Cyt1) (43), pcDNA3-s80Cyt2 (33), pcDNA3-Flag-WWP1 and pcDNA3-Flag-WWP1(C890A) (22), a variety of WWP1 deletion constructs (44), CMV-HA-Yap2 (30), and pcDNA4-Flag-WWOX (28). pShuttle-CMV-GFP-CT^{HER4} (HER4 residues 989 to 1308) and pShuttle-CMV-GFP-s80^{HER4} (HER4 residues 676 to 1308) were generated from pMSCV-GFP-CT^{HER4} and pMSCV-GFP-s80^{HER4} (13), respectively. pShuttle-CMV-KDO^{HER4} expressing HER4 residues 676 to 995, was generated by removing the HindIII fragment from pShuttle-CMV-GFP-s80^{HER4}. An EcoRI/BamHI fragment from pShuttle-CMV-GFP-s80Cyt1 was used to replace the EcoRI/BamHI fragment in pcDNA3-s80Cyt2 to generate pcDNA3-s80Cyt1. A K751 to R point mutation to eliminate kinase activity of s80 was generated in pShuttle-CMV-GFP-s80Cyt1 by using GeneEditor (Promega, Madison, WI) and the primer 5'-CC TGTGGCTATTA~~CG~~GATTCTTAATGAG-3' (the mutated nucleotide is underlined), thus generating pShuttle-CMV-GFP-s80Cyt1KR^{HER4}. pcDNA3-m80Cyt1 expressing HER4 residues 632 to 1308, including the transmembrane domain, was constructed by replacing the AflII/KpnI fragment of pcDNA3-s80Cyt1 with the corresponding PCR amplified m80 fragment cDNA. To delete PY1 (1031-PPPIY-1035), thus generating pcDNA3-s80Cyt2 Δ PY1, two-step PCR-mediated mutagenesis was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and the following two sets of primers: primers F1 (5'-GGT ATT TGG GTA CCT GAA G-3' [the KpnI site is underlined]) and R1 (5'-GAG TCA ATT CTT GCT CTG GAA GTG ATG TTG AAA GCC TGA GGG AC-3') and primers F2 (5'-GTC CCT CAG GCT TTC AAC ATC ACT TCC AGA GCA AGA ATT GAC TC-3') and R2 (5'-CAC AAT AGG CCG GAT CCG CC-3' [the BamHI site is underlined]). The two overlapping PCR products were amplified by using the primers F1 and R2, and a KpnI/BamHI fragment was used to replace the corresponding fragment in pcDNA3-s80Cyt2. pcDNA3-s80Cyt2 Δ PY3 was generated by using PCR-mediated mutagenesis with *Pfu* DNA polymerase and the primers 5'-CCT TTG CCA ACA CCT TGG-3' and 5'-GCT CTA GAT TAC ACC ACA GTA TTC CCG TGT CTT AGC ACA GTG CCT GGC TTC AGG-3'; the BamHI/XbaI fragment was used to replace the corresponding fragment of pcDNA3-s80Cyt2. The KpnI/BamHI frag-

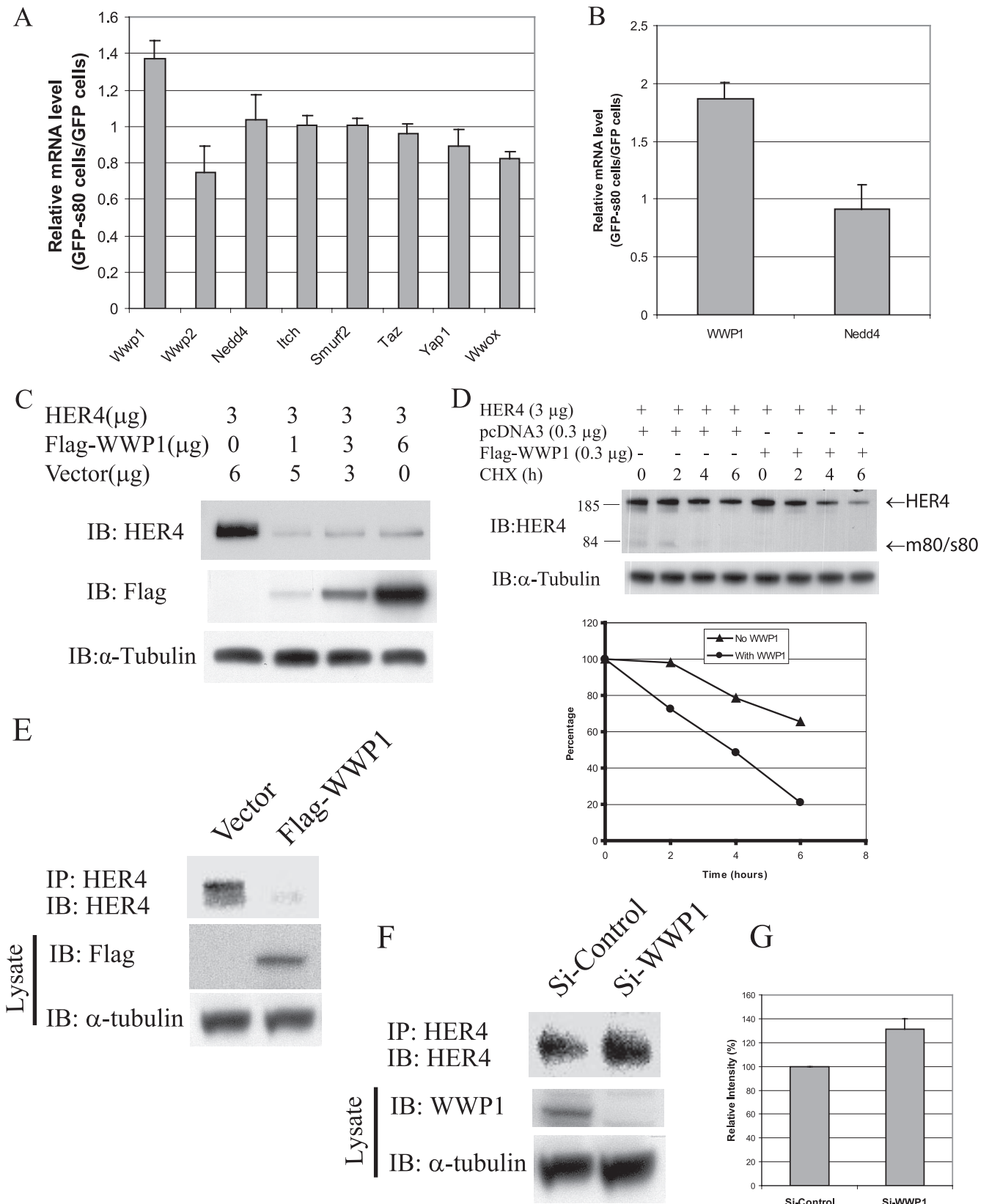


FIG. 1. HER4-induced expression of WWP1 results in degradation of HER4 and its cleavage product, m80/s80^{HER4}. (A) HC11 cells stably expressing GFP-s80 or GFP were used to extract total RNA for microarray analysis. Data mining for specific transcripts encoding the indicated WW-containing proteins was performed. The ratios of the mRNA levels in GFP-s80-expressing cells and those in GFP-expressing cells are shown. Experiments were performed in triplicate on three independent samples. Values represent the average ratio; the error bars represent the standard

ment of pcDNA3-s80Cyt2ΔPY3 was replaced with the corresponding fragment from pcDNA3-s80Cyt2ΔPY1 to generate pcDNA3-s80Cyt2ΔPY1,3 (both PPPY and PPPPY deleted). All constructs were verified by DNA sequencing. Transfection of COS-7 cells was performed by using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's protocol. Transfection of MCF-7 cells was performed by electroporation using reagents from Amaxa Biosystems (Gaithersburg, MD). The control siRNA (ON-TARGET_{plus} Non-Targeting Pool) and WWP1-specific siRNA (ON-TARGET_{plus} SMART Pool) were from Dharmacon RNA Technologies (Lafayette, CO). The control or WWP1 specific siRNA was electroporated into MCF-7 cells by using Amaxa Biosystems (Gaithersburg, MD) reagents. Luciferase assays were performed 48 h after transfection using 200 μg of protein (luciferase assay kit; Promega, Madison, WI) according to the manufacturer's instructions.

Cell fractionation, immunoprecipitation, and immunoblot analysis. Membrane and soluble fractions of cells were prepared by using ultracentrifugation as previously described (53). For immunoprecipitation and/or immunoblot analysis, cells were washed with cold phosphate-buffered saline and lysed in regular lysis buffer (RLB; 20 mM Tris-HCl [pH 7.5], 50 mM NaF, 10% glycerol, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 137 mM NaCl, 1 mM Na₃VO₄, and protease inhibitor cocktail [Roche]) or high-salt lysis buffer (20 mM HEPES, 50 mM NaF, 10% glycerol, 1% Triton, 5 mM EDTA, 500 mM NaCl, 1 mM Na₃VO₄, and protease inhibitors cocktail) and cleared by centrifugation (13,000 × g for 10 min at 4°C). Lysates were used for immunoprecipitation for 3 h or overnight at 4°C with protein A/G or protein A agarose beads (Santa Cruz Biotechnologies, Santa Cruz, CA) and mouse anti-FLAG antibody (Sigma, Louis, MO) or rabbit HER4 antibody (13). Immunocomplexes or protein lysates were separated on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were probed with antibodies against the following proteins: Flag (mouse anti-Flag antibody; Sigma); HER4 (HFR-1) and HER3 (Ab-1) from NeoMarkers (Fremont, CA); BRCA1, α-tubulin, and phosphotyrosine (PY20) from Santa Cruz Biotechnologies (Santa Cruz, CA); HER2 (clone 2F12; Upstate, Lake Placid, NY); GFP (Chemicon, Temecula, CA); WWP1 (44); or EGFR (C-22) (43). Western blots were developed by using enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL).

Microscopy and image acquisition. Cells grown on coverslips were fixed in 3.7% formaldehyde and stained with rabbit anti-HER4 antibody (Santa Cruz Biotechnologies), mouse anti-HER4 antibody (HFR-1), or mouse anti-Flag antibody, using the following secondary antibodies: fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (IgG), and rhodamine red-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole; 20 ng/ml). Slides were mounted with fluorescent mounting medium (DakoCytomation) and examined by using the Leica SP2 laser scanning confocal microscope with a ×63 oil NA 1.40 Plan Apo lens (Nikon). Images were acquired and processed by using Leica Confocal software. Minimal image processing was performed with Adobe Photoshop.

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (13). Briefly, total RNA was isolated from HC11 cells stably expressing GFP or GFP-s80^{HER4} by using an RNeasy kit (Qiagen) and was treated with RNase-free DNase (Ambion). WWP1 or Nedd4 primers and intervening fluorescent dye-labeled probes were designed by using Primer Express software (ABI/Perkin-Elmer). Total RNA (10 ng) isolated from each cell line was assayed by real-time fluorescence qRT-PCR using an ABI Prism 7900 instrument (PE Bio). The relative abundance of WWP1 or Nedd4 transcript was calculated by the formula: relative mRNA level = $e^{(40-Ct)}$.

In vivo ubiquitination assays. Cells were pretreated with 25 μM MG-132 for 5 h and then lysed in RLB buffer containing 1% SDS. Protein samples were

diluted 1:10 and immunoprecipitated with anti-HER4 antibody. Immunoprecipitates were washed and prepared for immunoblot analysis.

RESULTS

Overexpression of s80^{HER4} increases WWP1 mRNA. HC11 mouse mammary cells stably expressing GFP, or GFP-tagged s80 HER4 (GFP-s80^{HER4}), were analyzed by cDNA microarray. The initial microarray showed a marked elevation of the transcript for WWP1 in the GFP-s80^{HER4} cell. Upon repeat in triplicate, transcript levels of WWP1, a member of the Nedd4 family of E3 ligases was still elevated in GFP-s80-expressing cells compared GFP-expressing cells, whereas the expression levels of other Nedd4 family members were similar in cells expressing GFP-s80 and GFP (Fig. 1A). Analysis by qRT-PCR confirmed that the levels of WWP1 mRNA levels, but not of Nedd4 mRNA, increased in GFP-s80 expressing HC11 cells over GFP-expressing cells (Fig. 1B). Thus, in cells expressing a HER4 Cyt1 isoform proteolytic product, s80^{HER4}, which slows HC11 cell growth (13), one member of an E3 ligase family exhibited increased steady-state mRNA levels.

WWP1 expression decreases HER4 stability. Based on the known role of WWP1 as an E3 ubiquitin ligase, which can target proteins for proteosomal degradation, we determined the effects of WWP1 expression on HER4 stability. COS-7 cells were cotransfected with pcDNA3-HER4 (JM-a, Cyt1) and increasing amounts of pcDNA3-Flag-WWP1. The expression of Flag-WWP1 substantially decreased HER4 protein levels (Fig. 1C). We examined the stability of HER4 protein by treating the cells with CHX, an inhibitor of new protein synthesis, for 0, 2, 4, or 6 h. Approximately 80% of the total HER4 was degraded in the presence 0.3 μg of Flag-WWP1 6 h after CHX treatment compared to 40% degradation in the absence of Flag-WWP1 (Fig. 1D).

To determine whether WWP1 affects HER4 levels in a cell in which HER4 exhibits biologic activity, we examined the effects of transient WWP1 overexpression on stability of endogenous HER4 in MCF7 breast cancer cells. Transient overexpression of Flag-WWP1 in MCF7 cells resulted in diminished levels of HER4 protein, compared to cells expressing empty vector (Fig. 1E). Using siRNAs to reduce endogenous WWP1 expression in MCF7 cells, we found that reduction of endogenous WWP1 correlated with an significant increase ($P = 0.034$ by using the Student *t* test) in endogenous MCF7 HER4 protein levels (Fig. 1F and G). These results demonstrate that WWP1 can regulate HER4 protein levels and that endogenous WWP1 may play a role in HER4 turnover in breast cells.

error of the mean (SEM). (B) Relative mRNA levels of *WWP1* and *Nedd4* as determined by qRT-PCR. HC11 cells stably expressing GFP, or GFP-s80^{HER4} were used to extract RNA. Experiments were performed in triplicate. Values are expressed as the average of the ratios of the mRNA levels of *WWP1* or *Nedd4* in GFP-s80-expressing cells versus GFP-expressing cells plus the SEM. (C) WWP1 promotes HER4 degradation. COS-7 cells transfected with HER4 and increasing amounts of WWP1 were lysed 40 h after transfection. Western analysis of lysates was performed using anti-HER4, anti-Flag antibody, and anti-α-tubulin antibody. (D) COS-7 cells transfected with HER4 and pcDNA3 or pcDNA3-Flag-WWP1. After 24 h, CHX was added for 0, 2, 4, or 6 h. Lysates were analyzed by Western blotting with the indicated antibodies. (E) MCF7 cells transfected with vector or Flag-WWP1 by electroporation were lysed 48 h after transfection. Lysates were analyzed by Western analysis with the indicated antibodies. (F) MCF7 cells were transfected with control or WWP1 siRNA by electroporation. After 48 h, cell lysates were analyzed by Western blotting with the indicated antibodies. (G) Densitometry of HER4 expression by Western blotting in MCF7 cells transfected with control or WWP1 siRNA by electroporation. The values are normalized with α-tubulin levels and the means of three experiments plus the SEM.

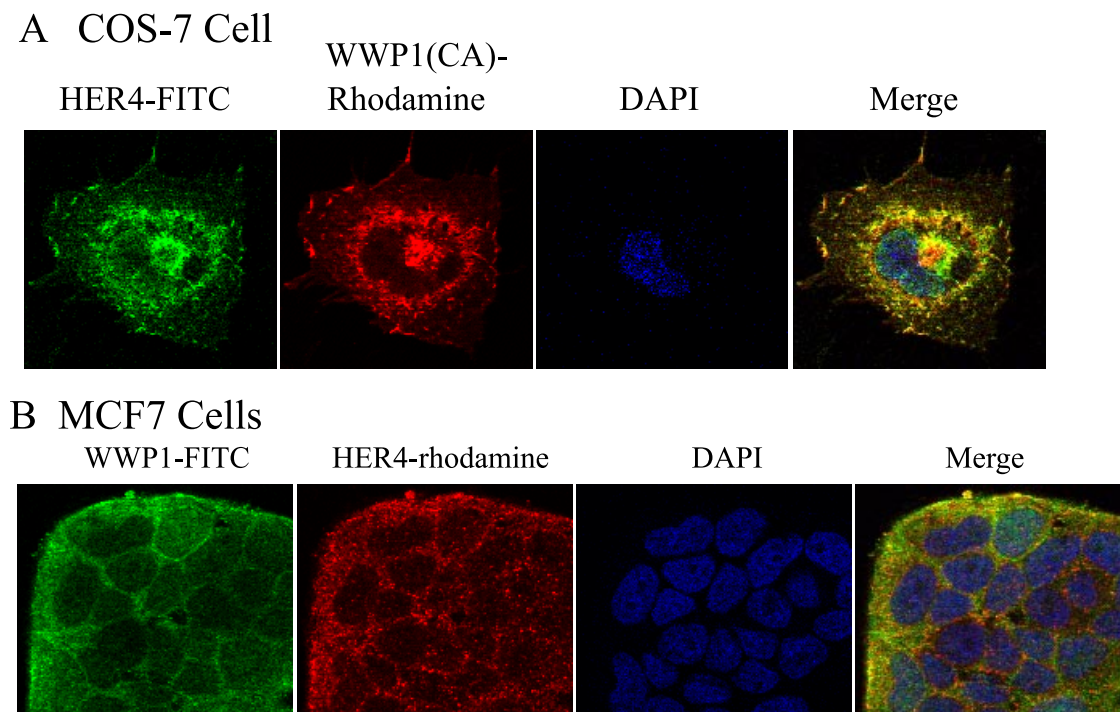


FIG. 2. Subcellular localization of HER4 and WWP1. (A) COS-7 cells were cotransfected with full-length HER4 and Flag-WWP1(CA). After 24 h, cells were immunostained with rabbit anti-HER4 antibody and mouse anti-Flag antibody, and fluorescence-conjugated secondary antibodies. (B) MCF7 cells were immunostained with rabbit anti-WWP1 antibody and mouse anti-HER4 antibody and then with fluorescence-conjugated secondary antibodies to detect localization of endogenous WWP1 and HER4.

Subcellular localization of HER4 and WWP1. Dual immunofluorescence of transfected COS-7 cells showed that overexpressed HER4 and WWP1(CA) colocalized in perinuclear area, on the cell plasma membrane, and in a punctate pattern throughout the cytoplasm (Fig. 2A). Using MCF-7 cells (human breast cancer-derived cells), which express endogenous HER4 and WWP1, we examined that subcellular localization of each endogenous protein. Dual immunofluorescence confirmed that both endogenous WWP1 and HER4 localized strongly at the cell periphery of MCF7 cells (Fig. 2B).

HER4 and WWP1: hierarchy of WW domain binding. HER4 and WWP1 form a physical complex, COS-7 cells were transfected with HER4 or Flag-WWP1 alone or cotransfected with HER4 and Flag-WWP1 or with HER4 and Flag-WWP1(CA), a site-directed mutant of WWP1 that allows WW domain interactions but that harbors a Cys890-to-Ala mutation abolishing ubiquitin ligase activity. Reciprocal immunoprecipitation demonstrated that HER4 antibodies precipitated both Flag-WWP1 and Flag-WWP1(CA) and anti-Flag antibodies precipitated HER4 (data not shown).

WWP1 is a E3 ubiquitin ligase containing a C2 domain, four WW domains, and a HECT catalytic domain. In a previous study, a series of WWP1 deletion constructs were generated to map the domain of WWP1 responsible for binding to TGIF (44). The series of deletion constructs included C2-WW1/4, WW1/4, and WW1/4-HECT, which retained their WW domains, and C2 and HECT, which contained no WW domain (Fig. 3). To determine whether the WW domains of WWP1 are responsible for binding HER4, COS-7 cells were cotransfected with HER4 and Flag-WWP1(CA) or the series of Flag-tagged

WWP1 deletion constructs (44), and their interactions were tested. Immunoprecipitation with anti-HER4 antibody precipitated WWP1(CA), C2-WW1/4, WW1/4, and WW1/4-HECT, in which WW domains were included, but not C2 and HECT in which the WW domains were deleted (Fig. 3). Similarly, in immunoprecipitation with anti-Flag antibody, only WWP1(CA), C2-WW1/4, WW1/4, and WW1/4-HECT, but not C2 and HECT, precipitated HER4 (data not shown). Thus, the WW domains of WWP1 are responsible for the interactions with HER4. Of particular interest was the construct WW1/4-HECT, which deleted phospholipid-binding domain C2 while retaining WW domains. This construct continued to bind to HER4.

To determine the HER4 domain responsible for interaction with WWP1, we generated a series of HER4 deletion constructs, each tagged with GFP. These constructs included the HER kinase domain only (GFP-KDO^{HER4}, amino acids 676 to 995), the C terminus of HER4 (GFP-CT^{HER4}, amino acids 989 to 1308), the entire intracellular cytoplasmic domain of HER4 (GFP-s80^{HER4}, amino acids 676 to 1308), and kinase-dead GFP-s80^{HER4} (single-amino-acid mutation K751R, GFP-s80KR^{HER4}) (Fig. 4A). COS-7 cells were cotransfected with WWP1(CA) and vectors expressing the HER4 deletion constructs. Immunoprecipitation with Flag antibodies demonstrated that the HER4 C terminus (GFP-CT^{HER4}) coprecipitated with Flag-WWP1(CA), as did the entire HER4 intracellular domain (GFP-s80^{HER4}). In contrast to the need for kinase activity for nuclear translocation, the WWP1-HER4 interaction was not dependent upon kinase activity or tyrosine phosphorylation of the HER4 intracellular domain, since the kinase inactive GFP-s80KR^{HER4} coprecipitated with Flag-WWP1(CA) (Fig. 4A). A construct consisting of

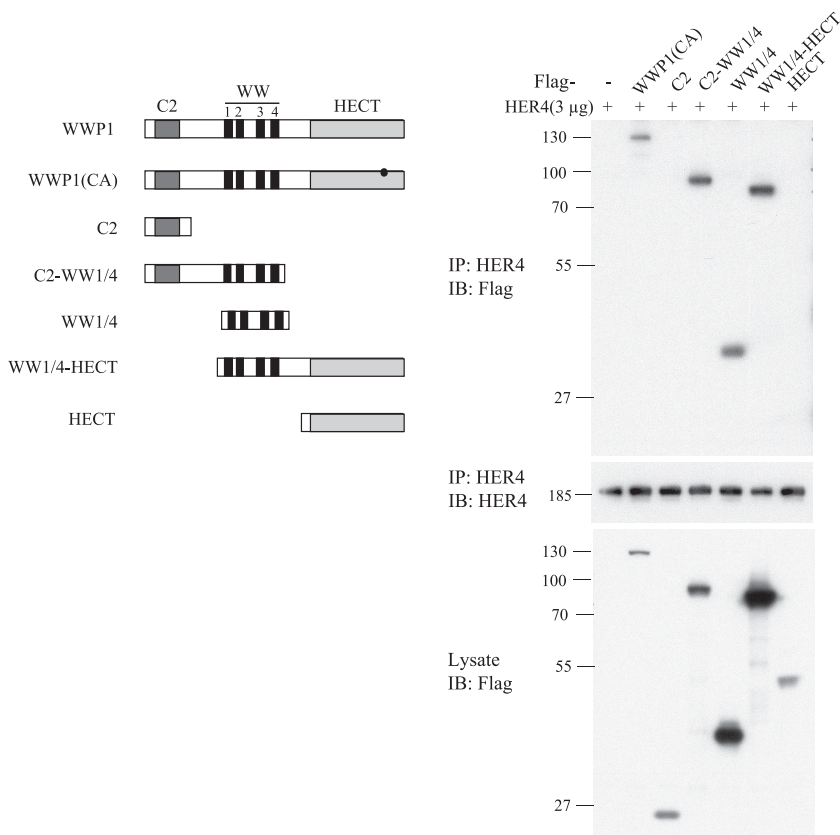


FIG. 3. WW domains of WWP1 are responsible for binding HER4. (Left panel) Schematic diagram of WWP1, mutated WWP1, and a series of WWP1 deletion constructs. (Right panel) COS-7 cells were cotransfected with HER4 (JMa-Cyt1) and different Flag-tagged WWP1 constructs as indicated. Cells were lysed 24 h after transfection. Cell lysates were immunoprecipitated with anti-HER4 antibody and analyzed by Western analysis with indicated antibodies. The lysates were also directly used for Western analysis to detect the expression levels of indicated proteins.

the HER4 kinase domain (GFP-KDO^{HER4}) without the C terminus did not coprecipitate with Flag-WWP1(CA). These data are consistent with the location of the three putative WW-domain binding regions of HER4, PY1, PY2, and PY3, each located within the C-terminal fragment of HER4 and absent in GFP-KDO^{HER4}.

Because one of the putative PY domains, PY2, resides within the 16-amino-acid region that is specific to s80Cyt1 (i.e., it is absent in s80Cyt2), we compared the coprecipitation of s80Cyt1 with Flag-WWP1(CA) to that of s80Cyt2. Both s80Cyt1 and s80Cyt2 were found in anti-Flag immunoprecipitates. However, s80Cyt1 was present in greater amounts in the coimmunoprecipitates, even though s80Cyt1 and s80Cyt2 were expressed at similar levels (Fig. 4B).

To determine whether the PY1 and PY3 domains are required for interactions with WWP1(CA), we introduced deletion mutations into s80Cyt2, such that s80Cyt2ΔPY1 lacks both PY1 and PY2, s80Cyt2ΔPY3 lacks both PY2 and PY3, and s80Cyt2ΔPY1,3 lacks all PY motifs (Fig. 4B). Deletion of the PY1 domain from s80Cyt2 did not greatly affect the presence of s80Cyt2 in Flag-WWP1 immunocomplexes. In contrast, deletion of the PY3 domain from s80Cyt2 substantially reduced the coprecipitation of s80 with Flag-WWP1(CA). Deletion of both PY1 and PY3 from s80Cyt2 completely abolished the interaction between s80Cyt2 and Flag-WWP1(CA) (Fig. 4B).

From these data, we may infer that the capability of each PY motif to bind WWP1 is PY2 \gg PY3 > PY1.

Previous reports described an interaction between the WW domain containing protein YAP and HER4 mediated through the PY3 motif of HER4; PY1 was dispensable for the HER4-YAP interactions (23). The authors of that study did not examine whether the PY2 motif, unique to s80Cyt1 and absent in s80Cyt2, mediated HER4 and YAP binding. To test the hierarchy of binding in another WW domain protein, we cotransfected s80Cyt1 and s80Cyt2 and hemagglutinin-tagged Yap2 (HA-Yap2), in COS-7 cells. Both s80Cyt1 and s80Cyt2 coprecipitated with HA-Yap2; however, s80Cyt1 coprecipitated with HA-Yap2 in greater abundance than s80Cyt2, even though both proteins were expressed at similar levels. This suggests that PY2 interacts more strongly in HER4-Yap2 interaction. We confirmed the previous reports that the PY3 motif is required for s80Cyt2-Yap2 interaction, since s80Cyt2ΔPY3 did not coprecipitate with HA-Yap2. The converse experiment, in which HER4 immunoprecipitates were analyzed by Western blotting with an anti-HA antibody, confirmed that loss of the PY2 domain alone reduced s80^{HER4} interaction with HA-Yap2 and that loss of both PY2 and PY3 together eliminated interaction between HA-Yap2 and s80^{HER4} (Fig. 4C).

We also used this set of HER4 constructs to examine binding between HER4 and Wwox, another WW domain-contain-

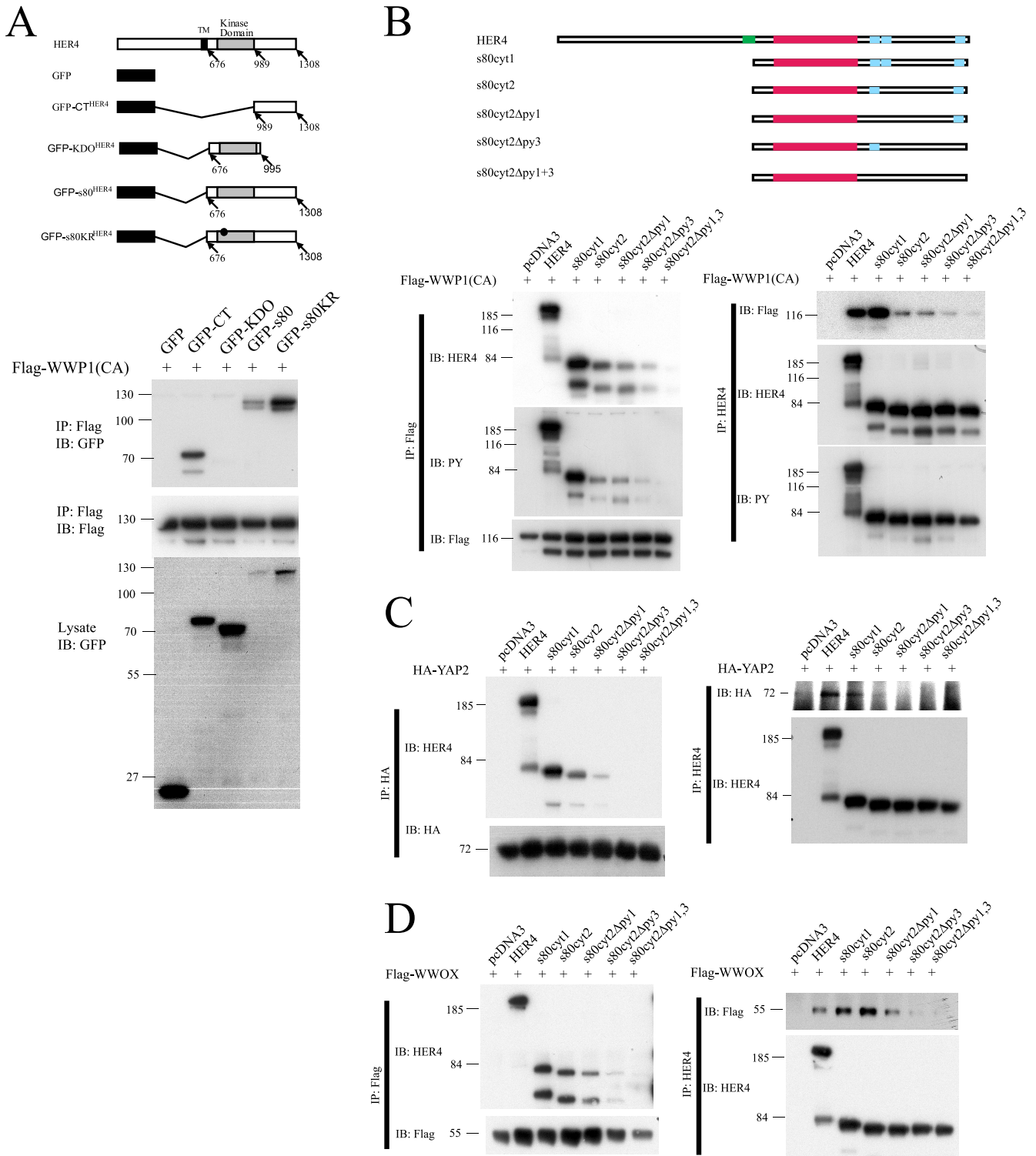


FIG. 4. WWP1 requires the PY2 and PY3 motifs in the C terminus of HER4 for interaction with HER4. (A) The upper panel shows a schematic diagram of HER4 and a series of HER4 deletion or mutation constructs used for expression in COS-7 cells. In the lower panel, COS-7 cells were cotransfected with Flag-WWP1(CA) (1 μ g) and different GFP-tagged construct (3 μ g of each) as indicated. Cells were lysed 24 h after transfection. Lysates were used for Western analysis where indicated, or were immunoprecipitated with anti-Flag antibody and analyzed by Western blotting. (B) In the upper panel, a series of deletion mutations were made within s80Cyt2 to delete the PY1 motif, the PY3 motif, or both. These were used for expression in COS-7 cells, as were expression constructs encoding s80Cyt1, s80Cyt2, and full-length HER4 (JM-a, Cyt1). In the lower panel, COS-7 cells were cotransfected with 1 μ g of Flag-WWP1(CA) and 3 μ g of empty vector, full-length HER4, s80Cyt1, s80Cyt2, s80Cyt2 Δ PY1, s80Cyt2 Δ PY3, or s80Cyt2 Δ PY1,3. The cells were lysed 24 h after transfection. Anti-Flag antibody and anti-HER4 antibody immunoprecipitates were analyzed by Western analysis as indicated. (C) Interaction of HER4 and Yap2. COS-7 cells were cotransfected with 2.5 μ g of HA-Yap2 and 3 μ g of empty vector, full-length HER4, s80Cyt1, s80Cyt2, s80Cyt2 Δ PY1, s80Cyt2 Δ PY3, or s80Cyt2 Δ PY1,3. After 24 h, HER4 and HA immunoprecipitates were analyzed by Western blotting with the indicated antibodies. (D) COS-7 cells were cotransfected with 2.5 μ g of Flag-Wwox and 3 μ g of empty vector, full-length HER4, s80Cyt1, s80Cyt2, s80Cyt2 Δ PY1, s80Cyt2 Δ PY3, or s80Cyt2 Δ PY1,3. After 24 h, HER4 and Flag immunoprecipitates were analyzed by Western blotting with the indicated antibodies.

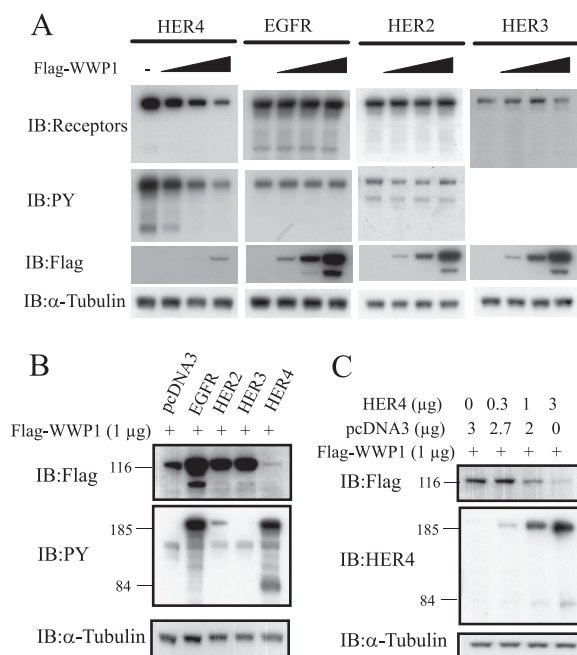


FIG. 5. WWP1 promotes degradation of HER4 but not EGFR, HER2, or HER3. (A) COS-7 cells were transfected with increasing amounts of Flag-WWP1 (0, 0.1, 0.3, or 1 μg) and HER4, EGFR, HER2, or HER3. After 40 h, lysates were analyzed by Western blotting with the indicated antibodies. (B) COS-7 cells were cotransfected with Flag-WWP1 and vector, EGFR, HER2, HER3 or HER4. After 40 h, lysates were analyzed by Western blotting with the indicated antibodies. (C) COS-7 cells were cotransfected with Flag-WWP1 and increasing amounts of HER4. After 40 h, lysates were analyzed by Western blotting with the indicated antibodies.

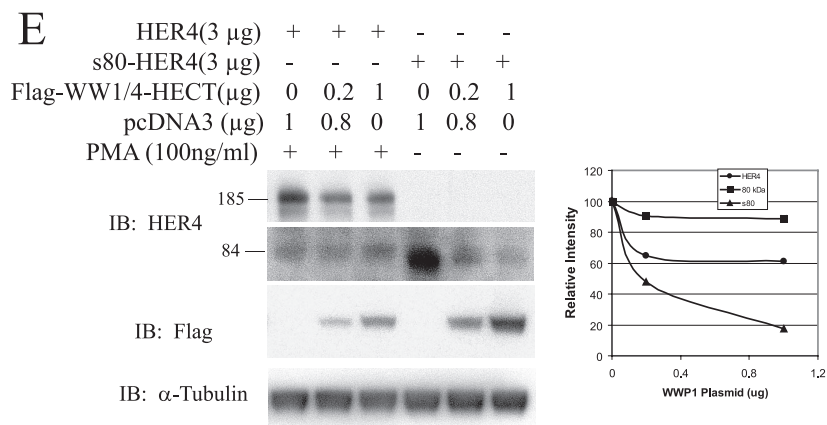
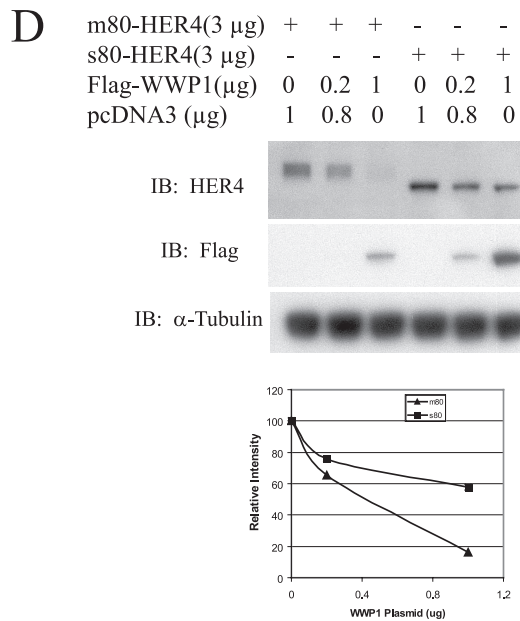
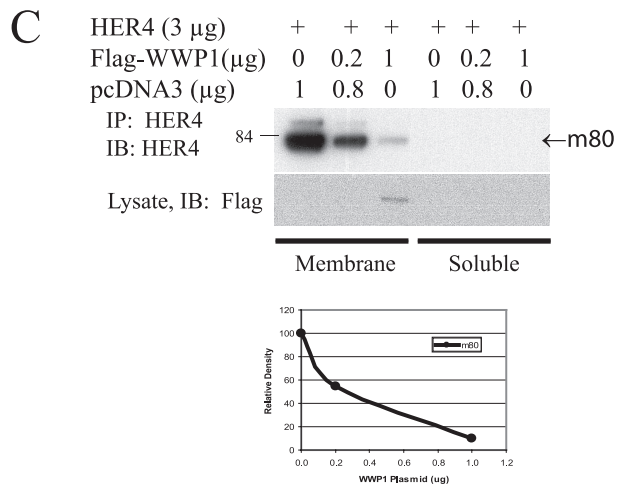
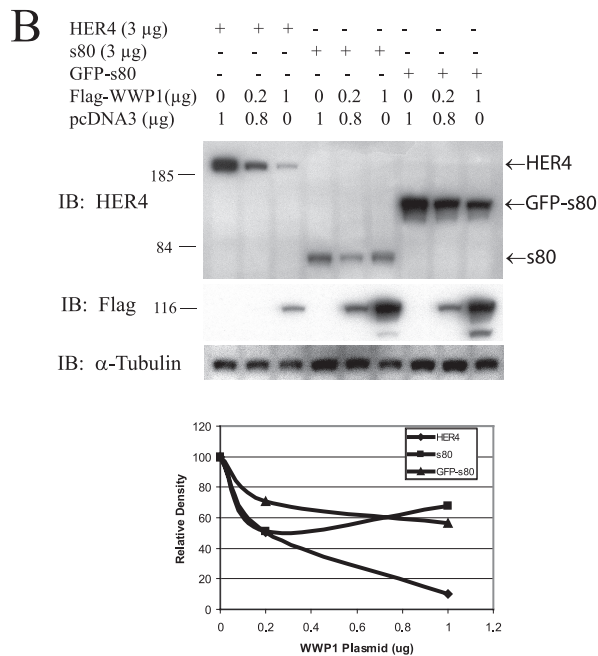
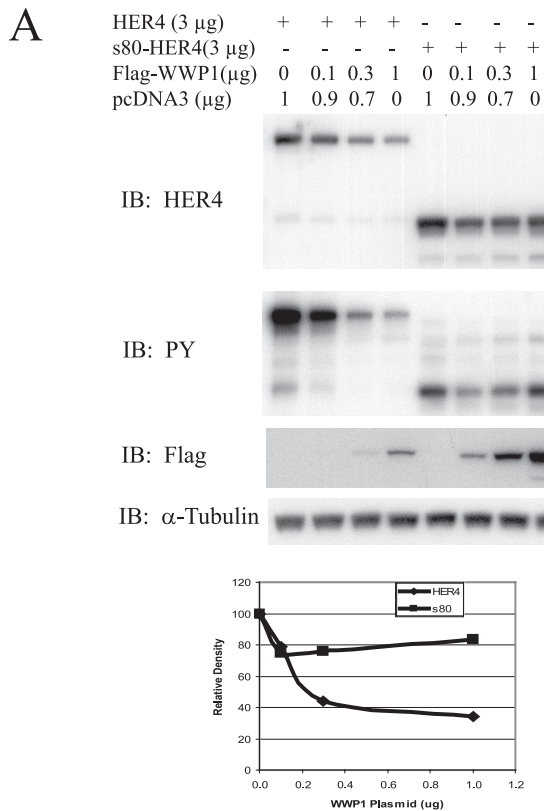
ing protein known to bind to HER4 (1, 2). Anti-Flag immunoprecipitates from COS-7 cells transfected with vectors expressed Flag-tagged Wwox (Flag-Wwox) were analyzed by Western blotting with HER4 antibodies. In contrast to WWP1 and Yap2, Flag-Wwox bound to full-length HER4Cyt1, s80Cyt1, and s80Cyt2 similarly. Binding between Flag-Wwox and HER4 diminished when PY1 and PY2 were deleted. Flag-Wwox and s80 binding was minimal if PY2 and PY3 were deleted and s80 and Wwox interaction was abolished upon the loss of all three PY motifs (Fig. 4D).

Taken together, these data indicate that the PY3 interacts with the WW domain of WWP1, YAP, and Wwox and that PY1 is less important for all three donor proteins. The fact that Wwox bound similarly to s80Cyt1 and s80Cyt2 indicates that binding to PY3 is the most important for WWOX. However, WWP1 and Yap2 displayed decreased binding to s80Cyt2 compared to s80Cyt1, suggesting that PY2 is more important for Yap2 or WWP1 HER4 interactions.

WWP1 is specific for HER4. We examined the stability of EGFR family members in the presence or absence of WWP1. Although transient coexpression of HER4 with increasing amounts of WWP1 reduced HER4 protein levels in a dose-dependent manner, the protein expression levels of EGFR, HER2, and HER3 were not affected by WWP1 coexpression (Fig. 5A). This selectivity indicates that WWP1 is potentially one of the factors that regulate HER4 biologic action, as distinct from other family members. In addition, HER4 receptor

tyrosine phosphorylation correlated directly with total receptor expression, suggesting that WWP1 expression affects the total levels of HER4 receptor expression but does not impair HER4 tyrosine kinase activity or tyrosine phosphorylation (Fig. 5A). Interestingly, when Flag-WWP1 was coexpressed with HER4, WWP1 protein was markedly diminished compared to WWP1 levels after coexpression with EGFR, HER2, and HER3 (Fig. 5B). Increasing amounts of HER4 protein expression decreased the WWP1 protein level in a dose-dependent manner (Fig. 5C), suggesting that HER4 and WWP1 are both degraded following their interaction. This type of codegradation has been observed for other E3 ligase substrate pairs (62). The physiologic significance of this feedback or autodegradation is thought to allow the cells to reaccumulate the substrate.

WWP1-dependent degradation is greater for membrane-associated full-length and 80-kDa HER4 than for soluble 80-kDa HER4. HER4 JM-a isoforms, but not JM-b isoforms, can be cleaved by TACE into m80^{HER4} (40, 54). An unknown proportion of m80^{HER4} is further cleaved into s80^{HER4} by γ-secretase (34). To determine whether WWP1 induces degradation of s80^{HER4}, we transfected COS-7 cells with increasing amounts of Flag-WWP1 and HER4 or s80^{HER4}. Western blotting showed that WWP1 more effectively induced degradation of full-length HER4 than s80^{HER4} (Fig. 6A). To determine whether WWP1 induces degradation of GFP-s80^{HER4}. We transfected COS-7 cells with increasing amounts of Flag-WWP1 and HER4, s80^{HER4}, or GFP-s80^{HER4}. Western blotting showed that WWP1 more effectively induced degradation of full-length HER4 than s80^{HER4} or GFP-s80^{HER4} (Fig. 6B). Next, we transfected COS-7 cells with HER4 and increasing amounts of Flag-WWP1. After 40 h, the cells were treated with 100 ng of PMA/ml for 1 h to increase the formation of m80^{HER4}. The cells were then lysed and separated into membrane and soluble fractions by ultracentrifugation. Western blot showed that membrane-bound 80-kDa HER4 was susceptible to WWP1-induced degradation (Fig. 6C). s80^{HER4} was not observed, presumably because it is made in small amounts under these conditions. WWP1 was found predominantly in the membrane fraction (Fig. 6C). There is a C2 phospholipid binding domain at the N terminus of WWP1 (14), which may explain the propensity of WWP1 to distribute to the membrane fraction. This localization might also indicate why s80^{HER4} is less susceptible to WWP1-induced degradation, although when overexpressed by transfection the two entities can bind to each other (Fig. 4). To test the specificity of WWP1-mediated degradation for m80^{HER4}, we made an m80 construct (HER4 residues 632 to 1308, including the transmembrane domain). We transfected COS-7 cells with increasing amounts of full-length Flag-WWP1 and m80 or s80. WWP1 again more effectively promoted the degradation of m80 compared to s80 (Fig. 6D). To explore this further, increasing amounts of Flag-WWP1/4-HECT, a WWP1 fragment without C2 domain but with HER4 binding and ligase capability, were transfected into COS-7 cells with HER4 or s80. As shown in Fig. 6E, Flag-WWP1/4-HECT (without C2) more efficiently promoted s80 degradation than full-length HER4 or PMA induced m80 HER4 degradation. This suggests that the specificity for membrane bound HER4 (full length and m80) is dictated by the membrane association of WWP1 (produced by the C2 domain). Overexpression of a soluble (C2-deleted WWP1) will



allow s80 degradation, but this is not the normal state of the cell. Many of our experiments point to an efficient membrane complex in breast cells that enhance WWP1 and regulates HER4 levels.

WWP1 expression directs proteosomal degradation of membrane bound 80-kDa HER4, and full-length HER4 degradation is less dependent on the proteasome. To determine the mechanism of WWP1-dependent decreased HER4 stability, we coexpressed full-length HER4 (JM-a, Cyt1) and increasing amounts of Flag-WWP1 in COS-7 cells. After 24 h cells were treated or not treated with the proteasome inhibitor MG132. Proteasome inhibition had little effect on WWP1-dependent HER4 loss but markedly increased accumulation of 80-kDa HER4 species (on a gel one cannot distinguish the membrane-bound m80, which had not undergone the γ -secretase cleavage or the soluble s80, which has undergone the second cleavage). However, while proteosomal inhibition markedly alternated 80-kDa HER4 degradation, it did not completely block this WWP1-induced effect (Fig. 7A). Interestingly, proteosomal inhibition increased the accumulation of m80/s80 HER4 even in the absence of exogenous WWP1 expression, suggesting that, at least in COS-7 cells, the HER4 cleavage product (presumably m80) is made constitutively at some rate and is the target of proteosomal degradation. Whether this is the result of endogenous E3 ubiquitin ligases, such as WWP1, is not yet known.

We next examined the effects of the lysosome inhibitor bafilomycin A1 on HER4 stability. Although bafilomycin A1 increased m80/s80HER4 accumulation to some extent, MG132 and bafilomycin A1 together resulted in substantially increased m80/s80 accumulation (Fig. 7B). These data suggest that the proteasomal and lysosomal degradation pathways may each participate in m80/s80HER4 destruction.

Metalloprotease activity, i.e., TACE, is involved in the cleavage of HER4 to form m80^{HER4} (40, 54). To determine whether metalloprotease cleavage of HER4 was required for WWP1-induced HER4 degradation, we coexpressed HER4 (JM-a, Cyt1) with increasing levels of Flag-WWP1, treating the cells with or without the metalloprotease inhibitor GM6001. As expected, GM6001 decreased the accumulation of m80/s80 HER4, resulting in the persistence of full-length HER4 (Fig. 7C). However, GM6001 did not block WWP1-induced HER4 degradation, suggesting that cleavage of full-length HER4 by TACE is not a prerequisite to WWP1-induced HER4 degradation.

To determine whether tyrosine phosphorylation of HER4

affects WWP1-induced HER4 degradation, we expressed Flag-WWP1 with wild-type or kinase-dead HER4. WWP1 promoted the degradation of both wild-type and kinase-dead HER4 similarly (Fig. 7D).

Previous reports demonstrated that PMA can activate TACE in the absence of HER4 activity. Thus, PMA treatment bypasses the requirement for HER4-induced TACE activation, and results in the generation of m80 independently of HER4 kinase activity (54). In agreement with previous work, PMA induced the formation of m80 HER4 from kinase-dead HER4. However, this m80^{HER4} product was degraded in a WWP1-dependent manner. As the opposite of GM6001, PMA accelerated WWP1-induced degradation of full-length HER4 (kinase active or kinase inactive) (Fig. 7D and data not shown).

Taken together, these data indicate that proteosomal and lysosomal pathways contribute to the degradation of m80/s80^{HER4} but are less involved in full-length HER4 degradation. This full-length HER4 is degraded in response to WWP1 by other unknown mechanisms that do not require HER4 tyrosine kinase activation or TACE-mediated HER4 cleavage.

WWP1 ubiquitinates HER4 and causes HER4 degradation.

Because WWP1 is an E3 ubiquitin ligase and associates with HER4, we used transient expression of HER4 and WWP1 in COS-7 cells to determine whether their coexpression results in the ubiquitination of HER4. Immunoprecipitation of HER4 from MG132-treated cells revealed ubiquitinated HER4 when expressed in the presence of Flag-WWP1 but not in the presence of Flag-WWP1(CA), the mutant isoform of WWP1 that binds to HER4 but lacks E3 ubiquitin ligase activity (Fig. 8A). We used WWP1(CA) expression to determine whether ubiquitin ligase activity of WWP1 is required for WWP1-mediated HER4 degradation. Although Flag-WWP1 resulted in the decreased presence of HER4 in COS-7 cells, increasing levels of exogenous Flag-WWP1(CA) expression resulted in the accumulation of full-length HER4 and 80-kDa HER4 (Fig. 8B). These results demonstrate that WWP1-mediated ubiquitination is required for HER4 degradation.

Expression of exogenous WWP1 downregulates HER4-induced functions. Previous studies have shown that STAT5-dependent promoters, such as the β -casein promoter, are stimulated by heregulin (HRG)-dependent activation of HER4 (33). To determine whether WWP1 overexpression affects biological consequences of HER4 activation, MCF7 cells were cotransfected with a β -casein promoter-luciferase reporter plasmid (33) and either pcDNA3-WWP1 or empty pcDNA3. Transfected cells were treated with or without heregulin for

FIG. 6. Soluble 80-kDa HER4 is less susceptible to WWP1-induced degradation than full-length HER4 and membrane-bound 80-kDa HER4. (A) COS-7 cells were transfected with increasing amounts of Flag-WWP1 (0, 0.1, 0.3, and 1 μ g) and 3 μ g of HER4 or s80^{HER4}. After 40 h, lysates were analyzed by Western blotting with the indicated antibodies. (B) COS-7 cells were transfected with increasing amounts of Flag-WWP1 (0, 0.2, and 1 μ g) and 3 μ g of HER4, s80^{HER4}, or GFP-s80^{HER4}. After 40 h, lysates were analyzed by Western blotting with the indicated antibodies. (C) COS-7 cells were transfected with HER4 and increasing amounts of Flag-WWP1 (0, 0.2, and 1 μ g). After 40 h, the cells were treated with 100 ng of PMA/ml for 1 h to increase the formation of 80-kDa membrane-associated HER4. The cells were then lysed, and the lysates were separated into membrane and soluble fractions by ultracentrifugation. Western blot analysis was performed with anti-Flag antibody. Membrane and soluble fractions were further lysed with 1% Triton X-100, immunoprecipitated, and analyzed by Western blotting with an anti-HER4 antibody. (D) COS-7 cells were transfected with increasing amounts of Flag-WWP1 (0, 0.2, and 1 μ g) and 3 μ g of m80^{HER4} or s80^{HER4}. After 40 h, the cells were lysed and lysates were analyzed by Western blotting with the indicated antibodies. (E) COS-7 cells were transfected with increasing amounts of Flag-WWP1/4-HECT lacking the C2 membrane association domain (0, 0.2, and 1 μ g) and 3 μ g of HER4 or s80^{HER4}. After 40 h, the cells transfected with HER4 were treated with 100 ng of PMA/ml for 1 h to increase the formation of 80-kDa membrane-associated HER4. All of the cells were then lysed, and lysates were analyzed by Western blotting with the indicated antibodies.

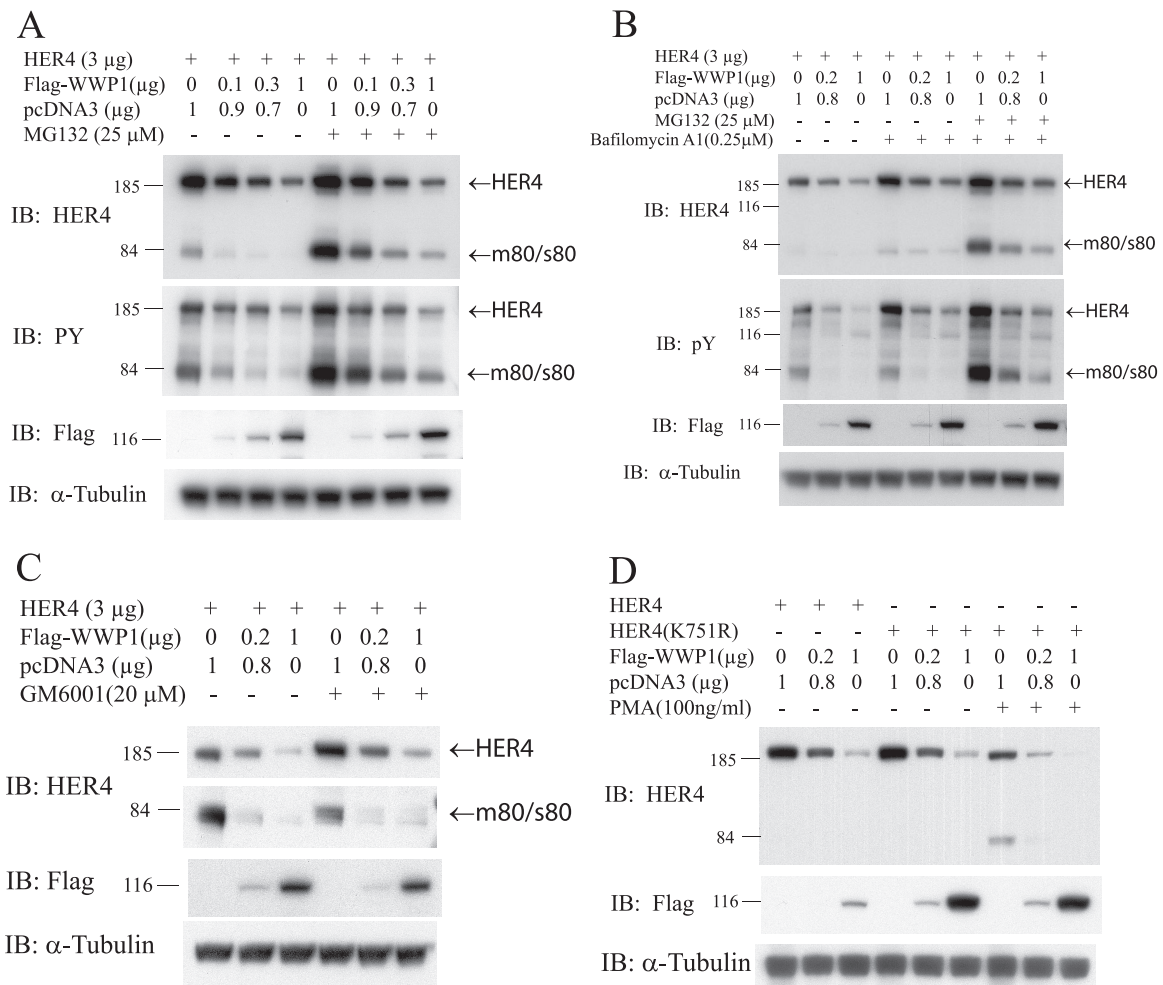


FIG. 7. Proteasomal and lysosomal degradation of m80/s80 HER4. (A and B) COS-7 cells were transfected with HER4 and increasing amounts of Flag-WWP1. After 24 h, the cells were treated with or without proteasome inhibitor MG132 for 5 h (A) or treated with or without the lysosome inhibitor bafilomycin A1 or bafilomycin A1+MG132 for 5 h (B). Lysates were analyzed by Western blotting with the indicated antibodies. (C) COS-7 cells were transfected with HER4 and increasing amounts of Flag-WWP1. After 40 h, cells were treated with or without GM6001 for 5 h. Lysates were analyzed by Western blotting with the indicated antibodies. (D) COS-7 cells were transfected with increasing amounts of Flag-WWP1 and HER4 or kinase-dead HER4. After 40 h, the cells transfected with kinase-dead HER4 were treated with or without 100 ng of PMA/ml for 30 min (where indicated). The cells were then lysed, and the lysates were processed as described in panel C.

48 h, which has been previously shown to induce HER4-dependent activation of this promoter (33). HRG-treated cells transfected with pcDNA3 displayed a 19-fold increase in luciferase activity versus untreated cells (Fig. 9A), a finding consistent with previous reports (33). However, cells overexpressing WWP1 responded to HRG with only an eightfold increase in luciferase activity (Fig. 9A). Because previous studies have shown that HRG-induced activation of HER4 results in activation of this promoter and because increased WWP1 expression results in the targeted degradation of HER4 in these cells, the results presented here are consistent with the idea that WWP1 expression antagonizes the biological consequences of HER4 signaling, likely through its ability to decrease total HER4 levels.

Previous studies suggest that heregulin induces the expression of BRCA1 mRNA and protein in breast cancer cells in a HER4-dependent, but HER2-independent manner (31). Because this heregulin-induced effect is specifically dependent on

HER4, we were interested to determine whether increased expression of WWP1 impaired the induction of BRCA1 in response to ligand activation of HER4. We generated MCF-7 cells stably expressing WWP1 (Fig. 9B). The cells were treated with or without heregulin for 24 h. Expression of WWP1 was not affected by treatment with heregulin for 24 h. While heregulin increased BRCA1 protein levels in parental MCF7 cells, BRCA1 levels were unchanged in response to heregulin in the cells overexpressing WWP1 (Fig. 9B). To determine whether eliminating endogenous WWP1 enhances the induction of BRCA1 in response to ligand-activated HER4, we "knocked down" WWP1 by using an siRNA approach. MCF7 cells were transfected by electroporation with control siRNA oligonucleotides or WWP1 specific siRNA oligonucleotides. Decreased WWP1 protein was seen in MCF7 cells transfected with WWP1-specific sequences but not in cells transfected with control siRNA sequences. Elimination of WWP1 correlated with a 30% increase in HER4 protein levels (see Fig. 1F and

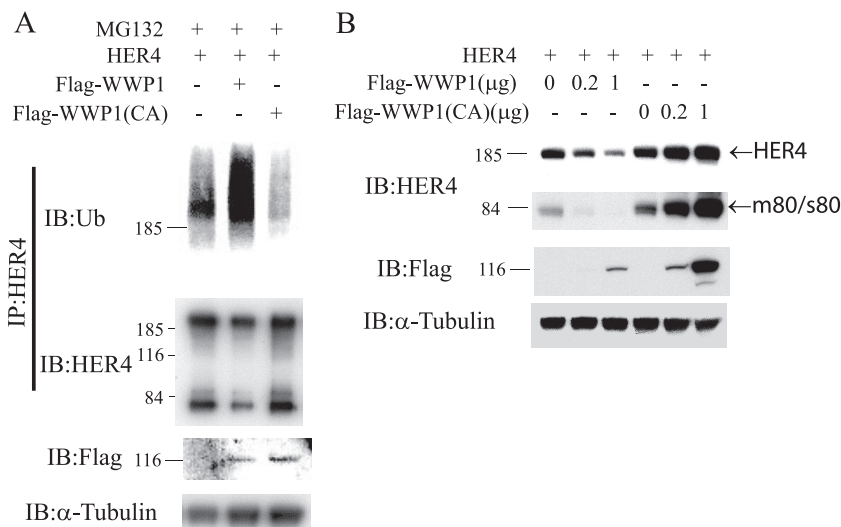


FIG. 8. WWP1 ubiquitinates HER4 and causes HER4 degradation. (A) COS-7 cells were transfected with HER4, WWP1, and WWP1(CA). At 24 h after transfection, the cells were treated with MG132 for 5 h and then lysed in RLB buffer containing 1% SDS. Lysates were used for Western analysis, or were diluted 1:10, and immunoprecipitated with anti-HER4 antibody, and immunocomplexes were analyzed with the indicated antibodies. (B) COS-7 cells were transfected with 3 μg of HER4 and increasing concentrations (0, 0.2, 1 μg) of vectors expressing Flag-WWP1 or Flag-WWP1(CA). After 24 h, the lysates were used for Western analysis as shown or were immunoprecipitated with the indicated antibodies and analyzed by Western blotting.

G). However, the heregulin-dependent elevation of BRCA1 was similar in the presence or absence of WWP1 (Fig. 9C). Therefore, the 30% increase in HER4 expression may not be sufficient to further increase HRG-induced BRCA1 induction. In contrast, overexpression of exogenous WWP1 eliminated HER4 expression in MCF7 cells (Fig. 1E) and eliminated the HER4-dependent induction of BRCA1 expression in response to heregulin. Alterations in WWP1 signaling may impact protein levels of BRCA1 by alternate signaling pathways, a possibility that is not ruled out by the results presented here. However, because the heregulin-induced endpoints are specifically HER4 dependent and because basal levels of BRCA1 expression and β-casein promoter activation were unaffected in untreated WWP1-expressing cells compared to untreated parental cells, it is clear that HER4-directed signaling is attenuated by WWP1.

DISCUSSION

We have shown here that the E3 ligase, WWP1, ubiquitinates HER4, targeting it for degradation through the proteosomal and lysosomal pathways. Although other E3 ligases are known to target EGFR family members for degradation, WWP1 demonstrates specificity within the EGFR family for HER4. This specificity is driven by three PY motifs, or WW-domain binding motifs, which are present in HER4 but are absent in other EGFR members.

By targeting HER4 for degradation, WWP1 expression has a negative impact on the biologic consequences of HER4 activity, such as lactogenic differentiation (as measured by β-casein promoter activity [Fig. 9A]), or regulation of BRCA1 expression, which we have postulated is important in HER4-dependent growth inhibition (31) (Fig. 9B). Increased differentiation and decreased growth of breast cells can occur in

response to HER4 Cyt1 activity (13, 33, 43) and are consistent with the observations that HER4 expression in breast cancers generally correlates with a more favorable prognosis (3, 21, 37, 46, 51, 52, 57). The results presented here suggest that increased expression of WWP1 may counteract the potential tumor suppressor effects of HER4 in breast cancers (see reference 48). Interestingly, not all studies of HER4 in breast cancer suggest that HER4 correlates with a favorable prognosis (4, 27). The reason for this discrepancy is unknown but may relate to alternative HER4 splicing. In fact, some studies have shown altered signaling potential and increased stability of the HER4Cyt2 isoform compared to HER4Cyt1 (50). Interestingly, the PY2 motif of HER4 is only present in the Cyt1 splice variant. The absence of PY2 in the HER4Cyt2 variant appears to decrease interaction with WWP1, rendering HER4Cyt2 less susceptible to downregulation by WWP1. Decreased interaction between HER4Cyt2 and WWP1 may explain in part the enhanced stability of HER4Cyt2 over HER4Cyt1. Likewise, the different biologic outcomes between Cyt1 and Cyt2 HER4 may also be due to a decreased association of the Cyt2 isoform with YAP as our data show for the first time (Fig. 4C). The PY3 motif of HER4 (present in both Cyt1 and Cyt2) supported interaction between each of the WW-domain containing proteins tested, including Wwox (Fig. 4), but Wwox interacted equally with HER4Cyt1 or HER4Cyt2. Thus, Wwox may play a similar role in the action of both HER4 isoforms. These data underscore the specificity underlying the overlapping but distinct functions of these (and perhaps other) WW domain-containing proteins in HER4 signaling.

WWP1 is a member of Nedd4 family of related E3 ligases, which also includes Nedd4-1, Nedd4-2, WWP1/Tiul1, WWP2, Aip4/Itch, Smurf1, Smurf2, HecW1/NedL1, and HecW2/NedL2 (18). Among the family members, Nedd4, WWP1, Smurf1, and Smurf2 have been reported to be overexpressed in

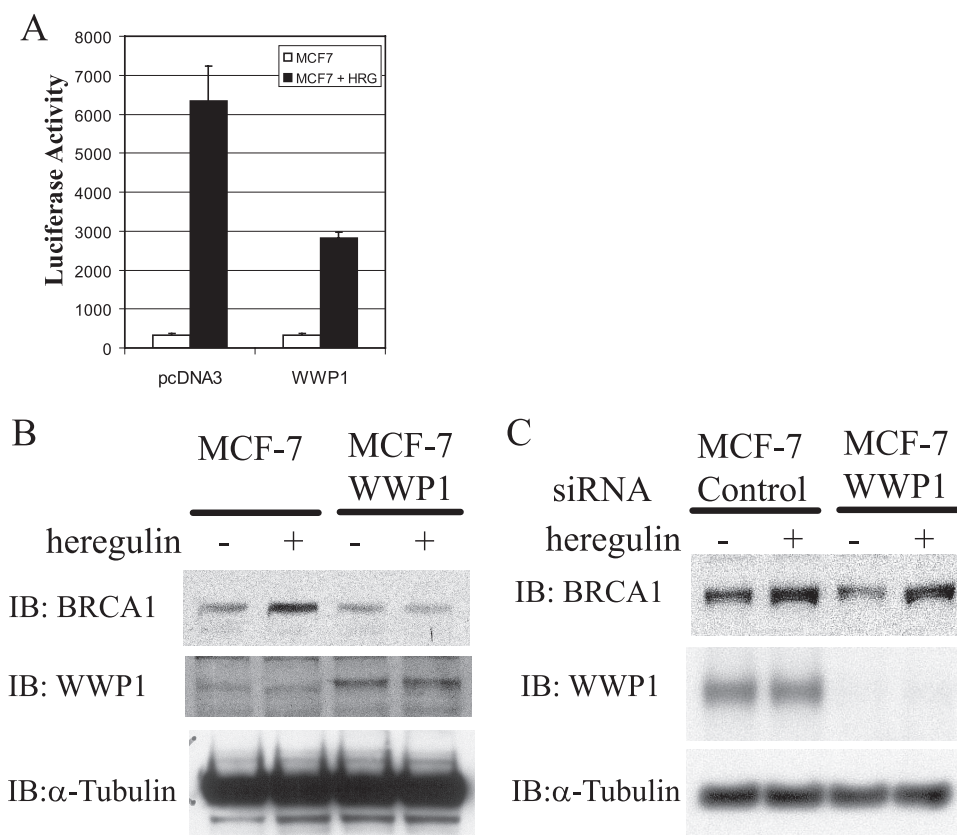


FIG. 9. Expression of exogenous WWP1 decreases biological consequences of HER4 activity. (A) MCF7 cells were transfected with pcDNA3 or pcDNA3-Flag-WWP1 and β -casein promoter-luciferase reporter plasmid and then treated or not treated with HRG. Luciferase activity was measured 48 h after transfection and is expressed as relative light units per milligram of total protein. Experiments were performed in triplicate. Values represent the averages plus the SEM. (B) Parental and Flag-WWP1-expressing MCF7 cells were treated (+) or not treated (-) with HRG and lysed, and the lysates were analyzed by Western blotting with the indicated antibodies. (C) MCF7 cells were transfected by electroporation with control siRNA oligonucleotides or WWP1 specific siRNA oligonucleotides. The cells were then treated with HRG or not treated with HRG and lysed, and the lysates were analyzed by Western blotting with the indicated antibodies.

several human cancers (6, 7, 22, 29, 55). It is possible that overexpression of these factors may contribute to tumorigenesis through a negative impact on the balance of tumor suppressors or proteins that promote differentiation, such as pTEN or p53, through ubiquitination-directed degradation. This scenario has been proposed for Nedd4, which ubiquitinates the tumor suppressor pTEN, targeting it for degradation (55). Because pTEN plays a central role in the growth and survival of prostate and other cancer cells, Nedd4 expression levels could influence cellular decisions along the pathway toward malignancy. Similarly, WWP1 and Smurfs target Smads of growth-inhibitory transforming growth factor β (TGF- β) signaling pathway for degradation (5). While TGF- β has the ability to inhibit growth of some tumor cells in a Smad-dependent manner, TGF- β signaling also promotes motility and survival of tumor cells in a Smad-independent manner, thus contributing to malignancy (32). The degradation of Smads as directed by WWP1 and Smurfs may tilt the balance of the TGF- β signaling pathway toward Smad-independent mechanisms that promote survival and motility and remain unchecked by growth control (29, 44). It has also been reported that WWP1 ubiquitinates p53 to prevent its translocation to nucleus and decrease its transcriptional activities (24).

Consistent with the observation that WWP1 is often overexpressed in human cancers and may promote tumorigenesis, the gene for WWP1 is located at 8q21, a region frequently amplified in human prostate and breast cancer. WWP1 overexpression promotes prostate and breast cell proliferation and survival (6, 7), perhaps due to the degradation of one or more of the WWP1 substrates described above or those unidentified. Our report that WWP1 is an E3 ligase for HER4, is a new addition to our understanding on the WWP1's role in cancer, especially in breast cancer. HER4 signaling decreases cellular proliferation of human breast cells and promotes differentiation (13, 33, 38, 43). Our work indicates that this is due to the action of the Cyt1 isoform (13, 31, 33), the isoform most likely to be affected by WWP1. In breast cancers, HER4 expression correlates with the presence of estrogen receptor, a more differentiated tumor grade, and longer survival (37, 51, 57). The studies cited did not have the reagents to distinguish whether HER4 isoform expressed as Cyt1 or Cyt2. However, degradation of the HER4 Cyt1 signal caused by WWP1 overexpression could be another important mechanism of enhanced tumorigenesis.

Initial reports of m80^{HER4} production from full-length HER4 described the rapid ubiquitination and degradation of

m80^{HER4}. The observation was made that m80 was far more susceptible to proteosomal degradation than was full-length HER4 (54). This is similar to our findings that demonstrate proteosomal/lysosomal degradation of m80 HER4, but unknown mechanisms of full-length HER4 degradation (Fig. 7). Most recently, Omerovic et al. screened a brain cDNA phage library with HER4 peptides and found that Itch could interact with HER4 and then demonstrated that Itch could ubiquitinate and target HER4 for degradation (36). It is very likely that both WWP1 and Itch regulate HER4 protein levels via ubiquitination. Functional redundancy seems to be common among members of the Nedd4 E3 ligase family. For example, Smurf1 and Smurf2 can target Smad1, Smad2, and Smad5 for degradation, and WWP1 targets Smad2 for degradation (5). Smurf1, Smurf2, and WWP1 each target RUNX2 for ubiquitin-mediated degradation (20, 45, 60). Consistent with the idea of functional redundancy between WWP1 and Itch, it has been described (unpublished data referred to in reference 5) that combined loss of both WWP1 and Itch in genetically engineered mice resulted in postnatal lethality within 72 h of birth due to lung hemorrhage, but that single loss of either was compatible with fetal development. The fact that WWP1 knockdown by siRNA in MCF7 cells increases endogenous HER4 suggests that, at least in the MCF-7 breast cell, WWP1 may be the relevant E3 ligase and that the Itch and WWP1 may have tissue or cell-type-specific functions.

Our data do not fully elucidate the “downregulation” mechanism involved in HER4 signaling. Our previous results show that ligand-dependent HER4 signaling in cells can be prolonged (33). The data presented here indicate that WWP1 binds to HER4 and is most effective in degrading the membrane-associated species HER4 and m80^{HER4}. WWP1 binds to s80^{HER4} but does not appear to promote degradation at the same rate as the membrane-associated forms. Previous data suggest that HER4 Cyt1 growth inhibition and differentiation requires the action of the intracellular s80^{HER4} derived from m80^{HER4} (33, 34). Perhaps one major action of WWP1 is to act at the membrane to prevent the m80^{HER4} conversion to s80^{HER4} by causing its rapid degradation through a largely proteasome-dependent process. The elimination of the C2-membrane association domain of WWP1 (Fig. 6E) reverses the membrane-soluble HER4 specificity of WWP1. The overexpression of soluble WWP1 (without C2) and soluble 80-kDa HER4 Cyt1 shows that under these conditions the WWP1-s80^{HER4} complex can access the degradation machinery efficiently. Under the “normal” circumstance in which WWP1 is membrane associated via its C2 domain, it appears that a more efficient degradation of m80^{HER4} is achieved, presumably through a membrane complex. What triggers WWP1 activity toward m80, preventing its prolonged signaling (and subsequent stochastic, γ -secretase-dependent release of the potent differentiation and growth inhibitory fragment s80^{HER4}), is unknown. The mechanism of the WWP1's effect on full-length HER4 is at least in these experiments less well defined. However, the fact that s80^{HER4} expression stimulates WWP1 RNA accumulation does suggest that a cellular feedback mechanism exists whereby s80 action would result in more WWP1 (presumably sent to the membrane) to downmodulate the signal.

In summary, we have demonstrated that WWP1 binds, ubiquitinates, and promotes the degradation of HER4, but not

other members of the EGFR family. Given that HER4 is the unique member of the EGFR family that decreases growth and promotes differentiation of breast cells, it will be important to understand the relationship between HER4 and WWP1, as well as other WW domain-containing E3 ligases, in breast cancer. It is conceivable that inhibition of WWP1 activity could result in the stabilization of HER4 and its cleavage products, resulting in decreased growth and differentiation of breast cancer cells.

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