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HER4 D-box sequences regulate mitotic progression and degradation of the nuclear HER4 cleavage product, s80^{HER4}

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Abstract

Heregulin-mediated activation of HER4 initiates receptor cleavage (releasing an 80-kDa HER4 intracellular domain, s80^{HER4}, containing nuclear localization sequences) and results in G2/M delay by unknown signaling mechanisms. We report herein that s80^{HER4} contains a functional cyclin B-like sequence known as a D-box, which targets proteins for degradation by APC/C, a multisubunit ubiquitin ligase. s80^{HER4} ubiquitination and ptoteosomal degradation occurred during mitosis but not during S-phase. Inhibition of an APC subunit (APC2) using siRNA knock-down impaired s80^{HER4} degradation. Mutation of the s80^{HER4} D-box sequence stabilized s80^{HER4} during mitosis, and s80^{HER4}-dependent growth inhibition via G2/M delay was significantly greater with the D-box mutant. Polyomvirus middle-T antigen-transformed HC11 cells expressing s80^{HER4} resulted in smaller, less proliferative, more differentiated tumors *in vivo* than those expressing kinase-dead s80^{HER4} or the empty vector. Cells expressing s80^{HER4} with a disrupted D-box did not form tumors, instead forming differentiated ductal structures. These results suggest that cell cycle-dependent degradation of s80^{HER4} limits its growth inhibitory action, and stabilization of s80^{HER4} enhances tumor suppression, thus providing a link between HER4-mediated growth inhibition and cell cycle control.

Keywords

HER4; mitosis; D-box; cell growth; cancer

Introduction

Cell cycle progression involves programming signals that drive proliferation and checkpoint mechanisms that limit cell cycle progression. Progression through mitotic checkpoints requires the proteolytic degradation of certain key regulatory proteins, mediated by the anaphase promoting complex/cyclosome (APC/C), a multisubunit ubiquitin ligase that

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catalyzes the attachment of polyubiquitin chains onto substrates (1,2), signaling their destruction by the 26S proteasome. Substrate specificity of APC/C is conferred by its association with coactivators, Cdc20 and Cdh1, each acting as a bridge between the substrate and APC/C. APC/C recognizes small motifs called D-boxes, A-boxes, or KEN boxes. The D-box bears the sequence RXXLXXXXN/D/E (with X being any amino acid), and is found in many proteins ubiquitinated by APC/C, e.g. cyclin B, polo-like kinase, and securin (3-6). To date, these motifs have not been reported in cell surface receptors. We report herein the presence of a functional D-box motif in the ErbB4/HER4 receptor tyrosine kinase (RTK).

The HER/ErbB family of RTKs consists of four members, HER1/ERBB1, HER2/ERBB2, HER3/ERBB3, and HER4/ERBB4. While HER1, -2, and -3 each contribute to aggressive tumor formation (7,8), HER4 signaling may impair cellular proliferation of human breast cells and promote differentiation (9-13). In breast cancers, HER4 expression correlates with more differentiated tumor grade, longer survival, and positive prognostic indicators [estrogen receptor expression, decreased BrdU incorporation (14-20)]. HER4 is required for heregulin-mediated growth inhibition in human breast cancer cell lines (9), via delayed progression of cells through late G2 or early mitosis (21).

Each of the ErbB receptors has been reported to translocate to the nucleus. For example, HER4 has been detected in the nuclei of breast cancer cells and normal human and mouse mammary tissue (15,18,22-25). Unlike HER1, -2, and −3, which translocate to the nucleus as full-length receptors, only a proteolytically-derived intracellular domain of HER4, with a constitutively active kinase domain, becomes nuclear (26-29). Recent work shows that one HER4 isoform, JMa, undergoes a ligand-dependent, sequential HER4 proteolytic process. First HER4 (JMa) is cleaved by tumor necrosis alpha-converting enzyme, followed by a transmembrane γ-secretase cleavage, which releases the soluble 80-kDa cytoplasmic domain ($s80^{HER4}$) into the cytosol (30-36). Once liberated, $s80^{HER4}$ exhibits nuclear-cytoplasmic shuttling.

In this report, we examine nuclear $s80^{HER4}$ regulation by demonstrating that proteolytic generation of $s80^{HER4}$ from full-length HER4 was required for HER4-mediated growth inhibition, and that expression of $s80^{HER4}$ induced growth inhibition via a delay in the G2/M phase of the cell cycle. We identified a D-box motif within $s80^{HER4}$ that directs APC/C-mediated $s80^{HER4}$ degradation during mitosis. Mutation of the HER4 D-box motif results in stabilization of $s80^{HER4}$ and greater growth inhibition in response to ligand-activated HER4 or experimentally expressed $s80^{HER4}$. Additionally, *in vivo* studies show that transformed HC11 cells expressing $s80^{HER4}$ or a mutated D-box have decreased tumorigenicity as compared to parental cells.

Methods

Plasmids

The pLXSN vectors expressing human HER4, $HER4^{KD}$, $HER4^{V675A}$, and the pcDNA4/TO vectors expressing $s80^{HER4}$ and $s80^{KD}$ are described elsewhere (21,37). D-box sequence point mutations in pcDNA4/TO-s80 and pLXSN-HER4 were done with the primer 5′-TATTCAGGGTGATGATC**c**TATGAAG**a**TTCCCAGTCCAAATGAC [lowercase underlined nucleotides represent incorporated mutations; Quick-change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA)].

Cell culture and transfection

HeLa-TRex cells (InVitrogen), referred to as HeLa-rtTA cells, were cultured according to manufacturer's protocol, transfected with Fugene6 (Roche) and selected with Zeocin (5 μg/

ml, InVitrogen) or G418 (400 μg/ml). Where indicated, cells were cultured in serum-free Dulbecco's Modified Eagle's Medium ± HRGβ_1 (10 ng/ml; Genentech), TET (2 μg/ml), NOC (50 ng/ml), or HU (10 mM) (each from Sigma-Aldrich, St. Louis MO). Cells synchronized in NOC were collected by shake-off, pelleted by centrifugation, resuspended in media containing 10% serum ± CHX, TET, or HRG, where indicated, then re-plated. Where indicated, cells were transiently transfected with siRNA sequences directed against the human APC2 gene using siRNA transfection Reagent (Santa Cruz Biotechnologies). HC11, MCF-10A, and MDA-MB-453 cells expressing GFP and GFP-tagged $s80^{HER4}$ have been described (Feng et al., submitted).

Western analysis and Immunoprecipitation

For membrane versus cytosolic separation, cells (10^8) were lysed in ice-cold buffer A [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 250 mM sucrose, 1 mM sodium vanadate, and 1× protease inhibitor cocktail (Roche)] using a 19-gauge needle. The samples were centrifuged at 1500 × *g*, 4°C for 10 min to pellet nuclei, which were lysed in ice-cold buffer B [5 mM Tris-HCl, pH 7.4, 450 mM NaCl, 5 mM EDTA, and 250 mM sucrose, 1 mM sodium vanadate, and 1× protease inhibitor cocktail]. Membranes were pelleted from buffer A supernatants by centrifugation for 1 h at 100,000 × *g* in a Beckman ultracentrifuge. The resultant supernatant represented cytosolic fractions. The pellets, representing membrane fractions, were resuspended in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM sodium vanadate and 1× protease inhibitor cocktail for 45 min at 4°C, then cleared by centrifugation. Whole cell extracts were prepared as described previously (21). HER4 was immunoprecipitated with α-HER4 Ab-132, or antibodies against cdc20, APC4 (Santa Cruz Biotechnologies), or cyclin B1 (Labvision Neomarkers, Fremont, CA), and protein A- or -A/G+ agarose beads (Santa Cruz Biotechnologies, Santa Cruz, CA). Immunoprecipitates were analyzed by immunoblotting using antibodies: GFP, APC4, APC2 (Santa Cruz Biotechnologies), Cyclin B or HER4 (Lab Vision Neomarkers) as previously described (21).

Fluorescence and GFP imaging

For GFP visualization, paraformaldehyde-fixed cells were examined with the Nikon Microphot FXA Upright fluorescence microscope. For immunofluorescence, cells were fixed in methanol, incubated 1h in HER4 antibody (Labvision Neomarkers), α-tubulin (Sigma-Aldrich), phospho-Ser10 histone H3 (Cell Signaling), or GFP (Santa Cruz Biotechnologies), then with rhodamine-conjugated goat anti-mouse and Alexa488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Samples were mounted with Vectastain DAPI mounting media (Vector laboratories, Burlingame, VT).

Cell growth assays

Cells (0.1×10^6) were plated in 15-cm dishes at day 0 ± HRG_1 or TET. Three plates per cell type per condition were collected by trypsinization and manually counted daily in duplicate.

Cell cycle analysis

Cells were collected by trypsinization, fixed in 75% MeOH, then stained with propidium iodide + RNase A (Becton-Dickenson). 10,000 stained nuclei per sample were analyzed in a FACSCalibur™ flow cytometer (Becton Dickinson). DNA histograms were modeled using Modfit-LT Software (Verity).

Tumor studies

HC11P cells were generated by transfection of pcDNA3-PyVmT (a gift from Dr. Bruce Cuevos, University of North Carolina Chapel Hill), followed by selection with hygromycin.

Antibiotic-resistant clones were individually analyzed for PyVmT expression by northern analysis. A single clone, HC11P, was transfected with pcDNA4, pcDNA4-s80, pcDNA4-$s80^{KD}$, and pcDNA4-$s80^{db}$, selected with Zeocin, clonally expanded, and analyzed for expression. Pooled clones of cells (1×10^6) were injected into the inguinal mammary glands of 6-week old BALB/c female mice, using sterile surgical procedures. Mice were examined weekly by palpation. Mice were sacrificed 15 weeks post-tumor injection, and tumor-bearing mammary glands were immediately fixed in formalin, paraffin-embedded, and sectioned for analysis.

Results

HRG-induced cleavage of HER4 to produce $s80^{HER4}$ is required for HER4-mediated G2/M delay of HeLa cells

Ligand-mediated HER4 activation is known to result in HER4 cleavage and nuclear/cytoplasmic shuttling of the liberated 80 kDa intracellular domain. We demonstrated this in HeLa-rtTA cells (that do not express detectable HER4) transfected with a cDNA construct encoding wild-type human HER4 (Jma/Cyt1 splice isoform). Treatment of HeLa-HER4 cells with 10 ng/ml heregulin (HRG) for 2 hours (h) was followed by separation of the cells into membrane, cytoplasmic, and nuclear compartments. HER4 immunoprecipitates were analyzed for tyrosine phosphorylation and total HER4 expression by western analysis. HER4 immunoprecipitation from HRG-treated cells revealed ligand-dependent HER4 tyrosine-phosphorylation at 180 kDa in membranes from HeLa-HER4 cells, and the appearance of an 80-kD tyrosine-phopshorylated HER4 product in membrane, cytoplasmic, and nuclear extracts, but not in untreated, serum-starved HeLa-HER4 cells (Fig. 1A.1). We also examined the effects of HRG on cells expressing kinase-dead human HER4 ($HER4^{KD}$) and on cells expressing a HER4 mutant (V675 to A). This V675 to A mutation that abolishes the HER4 intramembranous, γ-secretase cleavage site (referred to herein as $HER4^{VA}$) (37). Tyrosine phosphorylation of membrane-bound, full-length $HER4^{VA}$ was detected in HRG-treated HeLa-$HER4^{VA}$ cells, as was a membrane-tethered 80 kDa $HER4^{VA}$ product, although to a lesser extent than what was observed for HRG-treated HeLa-HER4 cells. The 80 kDa $HER4^{VA}$ product was not detected in cytoplasmic or nuclear extracts from HRG-treated HeLa-$HER4^{VA}$ cells, suggesting that the initial HRG-dependent TACE-mediated cleavage of $HER4^{VA}$ occurred, but that the final intramembranous cleavage of $HER4^{VA}$ was not achieved, thus preventing the release of soluble $s80^{HER4}$. As previously shown, HRG-treatment of cells expressing kinase-dead $HER4^{KD}$ did not result in tyrosine phosphorylation of $HER4^{KD}$ (9) The 80 kD HER4 product was not observed in HRG-treated HeLa-$HER4^{KD}$ cells, consistent with previous reports that kinase activity of HER4 is required to initiate TACE-mediated cleavage of HER4 in its extracellular stalk domain (Fig. 1A.1).

HER4 is required for HRG-mediated growth inhibition in several breast cancer cells (9), resulting in an increased number of cells with 4N DNA (21). HeLa-pLXSN, -HER4, -$HER4^{KD}$, and $HER4^{VA}$ cells were treated ± HRG for 48 h in serum-free media, to examine the effects of HRG on cell cycle distribution. While exogenous HER4 expression conferred HRG-mediated G2/M delay to HeLa cells, expression of $HER4^{KD}$ and $HER4^{V675A}$ did not allow for any observable HRG-mediated increases in cells with 4N DNA (Fig. 1A.2), suggesting that kinase activity and $s80^{HER4}$ production are required for HRG-mediated cell cycle delay in HeLa-rtTA cells.

Expression of $s80^{HER4}$ decreases growth and G2/M transit of HeLa and breast cancer cells

Previous reports demonstrate that expression of $s80^{HER4}$ (the Cyt1 splice variant) results in growth inhibition of multiple breast and breast cancer cells (21,37) (Cook, et al, in preparation). We stably introduced into HeLa-rtTA cells a tetracycline (TET)-inducible

pcDNA4 construct expressing the Cyt1 isoform of $s80^{HER4}$ or kinase-dead $s80^{KD}$, each tagged at the NH_2-terminus with green fluorescent protein (GFP). Treatment of cells with TET induced target gene expression within 6-12 hours (h), reaching a maximum by 24 h (Fig. 1B). Induction of GFP-$s80^{HER4}$ expression increased the proportion of HeLa cells with 4N DNA as compared to HeLa-pcDNA4 control cells treated with TET (Fig. 1C.1). Expression of GFP-$s80^{HER4}$ for 96 h decreased the rate of total cell growth compared to TET-treated HeLa-pcDNA4 or HeLa-$s80^{KD}$ cells, expressing a GFP-tagged, kinase-dead variant of $s80^{HER4}$ harboring a lysine 751-to-arginine mutation (Fig. 1C.2). To demonstrate the effects of s80HER4 in breast epithelial cells, we stably expressed GFP-tagged $s80^{HER4}$ (or GFP alone) in HC11 cells (non-transformed mouse mammary cells), MCF10A cells (human non-transformed breast cells), and MDA-MB-453 cells (human breast cancer-derived cells). Cell numbers, measured 96 h after plating, revealed less cell growth in HC11, MCF10A, and MDA-MB-453 cells expressing GFP-$s80^{HER4}$ than in these cells expressing GFP alone (Fig. 1D). We have demonstrated that the GFP-$s80^{HER4}$-mediated decrease in overall cell growth was not due to increased apoptosis (Feng et al, in preparation). Taken together, these data suggest that $s80^{HER4}$ production is both necessary and sufficient to decrease growth of HeLa and breast-derived cells.

Nuclear-cytoplasmic shuttling of GFP-$s80^{HER4}$ is regulated with the cell cycle

After a 24 h TET treatment, we found GFP-$s80^{HER4}$ simultaneously in both the nucleus and cytoplasm of 46.8% of all cells (950 cells counted), while a smaller percentage of cells displayed GFP-$s80^{HER4}$ staining in only the nucleus (7.9%) or cytoplasm (30.0%); and some cells had no observable GFP-$s80^{HER4}$ fluorescence (15.3%) (Fig. 2A). Although these experiments were performed in pooled clones of GFP-$s80^{HER4}$-expressing cells, the heterogeneity of GFP-$s80^{HER4}$ localization was also observed in clonally-derived populations examined individually (data not shown), indicating that the nuclear-cytoplasmic shuttling is a property of GFP-$s80^{HER4}$ and not a property of different cell clones.

Expression of GFP-$s80^{HER4}$ was induced with TET for 24 h. We then analyzed expression of GFP-$s80^{HER4}$ in nuclear extracts from cells that were randomly cycling for an additional 16 h, or that were synchronized for 16 h in G1 with serum-free media in the presence of TET, in S-phase with hydroxyurea (HU) + TET, or at the G2/M transition with olomoucine (OLO) + TET. In randomly cycling cells, cytoplasmic GFP-$s80^{HER4}$ was observed at greater levels than nuclear GFP-$s80^{HER4}$. We next examined whether relative levels of nuclear expression of GFP-$s80^{HER4}$ were enriched in specific phases of the cell cycle. Lower levels of nuclear GFP-$s80^{HER4}$ was observed in G1 and S-phase, as compared to the higher levels of nuclear GFP-$s80^{HER4}$ found in cells enriched for the G2/M transition (Fig. 2B). To examine the expression of $s80^{HER4}$ during mitosis (M), we used immunofluorescent staining of randomly cycling, TET-induced HeLa-s80 cells to detect the mitotic marker α-tubulin and GFP. Dual immunofluorescence revealed that of 47 cells harboring α-tubulin positive mitotic spindles, 0/47 cells simultaneously expressed GFP-$s80^{HER4}$ (Fig. 2C). Phospho-Ser10-histone H3, another mitosis-specific marker, was examined as a marker of mitotic cells. 0 of 81 phospho-Ser-10 histone H3-positive cells were HER4-positive. These results suggest that GFP-$s80^{HER4}$ expression decreases during mitosis.

Degradation of $s80^{HER4}$ in mitosis

We synchronized TET-treated HeLa-s80 cells in mitosis (M) with nocodazole (NOC) for 16 h, then upon release of cells from mitosis, examined GFP-$s80^{HER4}$ stability through the cell cycle by treating the cells with cycloheximide (CHX) to impair new protein synthesis at 0, 2, 4, 8, and 16 h after NOC release. Cells were harvested at 15-minute (min) intervals following CHX treatment and whole cell GFP-$s80^{HER4}$ was analyzed, as breakdown of the nuclear membrane under these circumstances precludes analysis of nuclear GFP-$s80^{HER4}$.

While GFP-s80^{HER4} expression was detected at T=0, it was undetectable within 15 min of CHX treatment/nocodazole release (Fig. 3A, lanes 1-4), indicating that s80^{HER4} was rapidly degraded upon release from NOC. By 4 h and 8 h after NOC release, GFP-s80^{HER4} showed increasing stability (lanes 10 and 14 show GFP-s80^{HER4} 15 min after release), and GFP-s80^{HER4} was stable through 45 min when examined 16 h after NOC release (lanes 17-20). While GFP-s80^{HER4} was rapidly degraded when cells were released in M, GFP-s80^{HER4} was highly stable in cells synchronized in S-phase with HU then released (Fig. 3B). This suggests that s80^{HER4} is more stable upon release from S-phase as compared to release from mitosis.

The APC/C directs proteasomal degradation of s80^{HER4} during mitosis

TET-treated HeLa-s80 cells were synchronized with NOC for 16 h, and treated with the proteasome inhibitor PS-341 for the final 4 h of synchronization. Cells were released from NOC in the presence of CHX at T=0, and stability of GFP-s80^{HER4} was measured as described above. Inhibition of the proteasome blocked degradation of s80^{HER4} through 45 minutes after release from NOC, and allowed for the accumulation of ubiquitinated GFP-s80^{HER4} (Fig. 3C), suggesting that s80^{HER4} is ubiquitinated during M and degraded by the proteasome.

The APC2 subunit, required for APC/C activity (38,39), was knocked down using transfection of short interfering RNAs (siRNAs) into HeLa-s80^{HER4} cells (Fig. 3D). Transient transfection of 20 nmol APC2 siRNA (Santa Cruz Biotechnologies) impaired expression of APC2 protein in HeLa-s80^{HER4} cells. APC2 siRNA-transfected cells were grown in 10% serum in the presence of TET for 48 h, adding NOC to the cells for the final 16 h of culture to synchronize the cells in M. Cells were released from NOC and treated with CHX at T=0. Expression of GFP-s80^{HER4} was analyzed at 15-min intervals following NOC release/CHX treatment. GFP-s80^{HER4} was detected through 45 min following NOC release in cells lacking APC2 expression. In contrast, cells transfected with a control siRNA sequence showed decreased levels of GFP-s80^{HER4} expression 15 min after NOC release, and did not harbor detectable levels of GFP-s80^{HER4} at 30 min following NOC release. Similarly, expression of cyclin B, a known target of the APC/C, was stabilized in cells lacking APC2 expression, but not in cells transfected with a control siRNA sequence. This suggests that s80^{HER4}, like cyclin B, is a target of the APC/C. This represents a novel regulatory mechanism of nuclear signaling for RTKs.

The D-box motif of s80^{HER4} is required for its APC/C-mediated degradation

Examination of the intracellular domain of human HER4 revealed a canonical D-box motif found in numerous proteins targeted by the APC/C for mitotic destruction, including cyclin B (Table 1). The D-box present in the HER4 intracellular domain is also seen in HER1/EGFR, but not in HER2 or HER3 (alignment per (40)). We introduced point mutations into the D-box motif of full-length HER4 and GFP-s80^{HER4} to eliminate the **R**XX**L**XXXX**D** consensus sequence, generating HER4^{db}/s80^{db} with sequences of **A**XX**A**XXXX**D** (Table 1). Expression constructs encoding GFP-tagged s80^{db} were used to generate stable TET-inducible HeLa-rtTA cell lines.

HeLa-s80^{HER4}, HeLa-s80^{db}, and HeLa-s80^{KD} cells were treated with TET for 24 h to induce expression of GFP-s80^{HER4}, GFP-s80^{KD}, and GFP-s80^{db}, respectively Constitutive tyrosine phosphorylation of GFP-s80^{HER4} and GFP-s80^{db} was observed (Fig. 4A.1), consistent with a recent report that s80^{HER4} is an active tyrosine kinase (41). Tyrosine phosphorylation of GFP-s80^{KD} was not observed (Fig. 4A.1), as expected. We synchronized TET-treated HeLa-s80^{db} cells in M with NOC for 16 h, then released the cells in the presence of CHX at T=0. Expression of GFP-s80^{db} was analyzed at 15 min intervals following NOC release/CHX

treatment (Fig. 4A.2). Strong GFP-s80^{db} expression was detected 45 min following CHX treatment after release into M, unlike GFP-s80^{HER4}, which was undetectable within 15 min following CHX treatment and release into M (see Fig. 3A). Cyclin B was readily degraded upon NOC release in HeLa-s80^{db} cells, suggesting that GFP-s80^{db} did not impair the APC/C or proteasomal machinery (Fig. 4A.2).

s80^{db} impairs growth of HeLa cells

Equal numbers of HeLa-s80 and HeLa-s80^{db} cells were plated ±TET and counted daily (Fig. 4B). Uninduced cells proliferated and cell number increased more than 4-fold in four days. TET-treated HeLa-s80 cells were less proliferative and only increased the cell number by 2.2-fold after 4 days in culture. TET-treated HeLa-s80^{db} cells were markedly growth inhibited; cell number increased by only 1.4-fold (P= 0.011 as compared to TET-treated HeLa-s80 cells, n=5). Moreover, HeLa-s80^{db} cells treated with TET for 48 h displayed a dramatic increase in the 4N-containing population compared to uninduced HeLa-s80^{db} cells (Fig. 4C).

To examine the progression of HeLa-s80, -s80^{db}, and -s80^{KD} cells through the cell cycle over a 24 h period, TET-treated cells in 10% serum were synchronized in S-phase with HU, then released into media containing 10% serum +TET at T=0 h. Cells were collected at 0, 2, 4, 8, 12, and 24 h following HU release, and were analyzed at each time point by flow cytometry to determine the proportion of cells in each phase of the cell cycle. Thus, we were able to observe the progression out of S-phase, through G2/M, and into G1 as a function of time. At T=0 h, all cells harbored DNA content between 2N and 4N, suggesting that HU-mediated synchronization in S-phase was complete for each cell line (Fig. 4D). An increase in the amount of 2N and 4N-containing populations was evident for each cell line by 2 h. However, by 8 h following HU release, HeLa-s80^{db} cells and HeLa-s80^{HER4} cells harbored a greater proportion of cells with 4N DNA as compared to HeLa-s80^{KD} cells, suggesting that HeLa-s80^{KD} cells progressed through G2/M more rapidly than did HeLa-s80 and HeLa-s80^{db} cells. By 12 h there was substantial differences in the relative cell populations with 4N DNA (HeLa-s80^{db} > HeLa-s80^{HER4} > HeLa-s80^{kd}), a trend that was continued through 24 h. These results suggest that expression of s80^{db} increases the time required for a cell to complete mitosis and return to G1.

Mutation of the D-box within full-length HER4 increases protein stability of proteolytically-derived s80^{HER4}

To determine the consequences of the D-box mutation in full-length HER4, we produced this site-directed mutant (HER4^{db}) and stably expressed HER4^{db} in He-La cells. HRG stimulation of HeLa HER4^{db} cells resulted in tyrosine phosphorylation of HER4^{db} and production of s80^{db} (Fig. 5A), similar to HeLa cells expressing wild-type HER4. This suggests that expression, kinase activity, and ligand-dependent proteolytic cleavage of HER4 are not impaired by mutation of the D-box. Immuno-localization of HER4 in HRG-treated HeLa-HER4^{db} cells revealed cells with nuclear HER4^{db} in the same microscopic field as other cells with strictly cytoplasmic HER4^{db} (Fig. 5B). Similarly, wild-type HER4 localized to nuclear compartments and cytoplasmic compartments differentially in cells within the same microscopic field of HRG-treated HeLa-HER4 cells (Fig. 5B). These results are similar to the subcellular distribution of s80^{HER4} in populations of TET-induced HeLa-s80^{HER4} cells (see Fig. 2A). This suggests that proteolytically-derived s80^{HER4} displays complex regulation of nuclear-cytoplasmic shuttling, similar to that seen for exogenously expressed GFP-s80^{HER4}. To determine if s80^{db} derived from full-length HER4^{db} displays greater stability than wild-type s80^{HER4} derived from full-length, wild-type HER4, HeLa-HER4 and HeLa-HER4^{db} cells were synchronized in M using NOC in 10% serum for 16 h, then serum-starved in the presence of NOC for an additional 16 h. Cells were treated with

HRG 1h prior to release. Finally, cells were released from NOC in the presence of HRG and CHX to examine the stability of proteolytically-derived $s80^{HER4}$ and $s80^{db}$ at 15 minute intervals. While $s80^{db}$ was detected through 45 min following release from NOC, $s80^{HER4}$ was not detected in cells released during M (Fig. 5C).

Expression of $s80^{db}$ prevents tumor formation *in vivo*

Transformation of HC11 cells by overexpression of human ErbB2 confers tumorigenic potential to HC11 cells *in vivo* (42). We used a similar approach, transfecting HC11 cells with a cDNA construct encoding the Polyomavirus middle T antigen (PyVmT). A clonal population of HC11-PyVmT (HC11P) cells was then transfected with pcDNA4-$s80^{HER4}$, -$s80^{KD}$, -$s80^{db}$, or empty pcDNA4. Antibiotic-selected, stably infected cell lines (1×10^6 cells) were injected into the inguinal mammary fatpads of 6-week old female BALB/c mice. Fifteen weeks following injection, the mammary gland was examined histologically. HC11P-pcDNA4 cells formed solid, poorly differentiated tumors (Fig. 6A). In contrast, tumors expressing $s80^{HER4}$ were smaller, harboring numerous differentiated epithelial structures organizationally similar to lobulo-alveolar structures often seen in mouse mammary glands during early pregnancy. HC11P-$s80^{KD}$ cells formed highly proliferative solid tumors resembling tumors formed by HC11P-pcDNA4 cells. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) revealed that HC11P-$s80^{KD}$ and HC11P-pcDNA4 tumors harbored similar ratios of PCNA+ cells ($P = 0.166$, Student's unpaired T-test, n=5 tumors and 3 fields per tumor; Fig. 6B), whereas HC11P-s80 tumors exhibited three-fold fewer PCNA+ cells than vector controls ($P < 0.001$). Implanted HC11P-$s80^{db}$ cells formed large cystic structures of single epithelial layers surrounding large lumens filled with eosinophilic proteins, suggesting a degree of lactogenic differentiation (Fig. 6A). These did not appear to have malignant elements, and these structures exhibited the lowest percentage of PCNA+ cells (9%, Fig. 6B), with a statistically significant decrease in PCNA staining compared to HC11-s80 tumors (12.5%, $P = 0.0371$). No statistically significant differences were detected in the rate of cell death between the four groups, as measured by TUNEL analysis (Fig. 6B). These results suggest that expression of $s80^{HER4}$ decreases growth of breast tumors in vivo, and mutation of the $s80^{HER4}$ D-box enhances tumor suppression by $s80^{HER4}$, perhaps by increasing the stability of $s8^{0HER4}$.

Discussion

Recent evidence shows that ligand-mediated activation of the JMa HER4 isoform results in HER4 cleavage and liberation of the intracellular domain, $s80^{HER4}$, which has the ability localize to the nucleus. Our studies demonstrate that nuclear-cytoplasmic shuttling of $s80^{HER4}$ is regulated, in part, by a cell cycle-dependent process, culminating in its degradation during M. The destruction of $s80^{HER4}$ during mitosis by the cell cycle machinery represents a novel regulatory mechanism for a receptor tyrosine kinase, and may relate directly to the ability of HER4 to slow cell growth by inhibiting progression through the G2/M transition or M.

Many proteins are degraded by the APC/C in a D-box-dependent manner. Of these, most have defined roles in regulating mitotic progression, including cyclin B, polo-like kinase, aurora A, and securin. Inhibition of APC/C-mediated degradation of these proteins results in delayed progression through M. For example, cyclin B1 in mammalian cells is degraded prior to anaphase (43) and expression of a nondegradable cyclin B1 harboring a mutation within the D-box delayed mitosis in mammalian cells and frog oocytes, by blocking the metaphase/anaphase transition (44,45). Similarly, mutation of the $s80^{HER4}$ D-box impaired its destruction and decreased the rate of G2/M progression, suggesting that destruction of the intracellular domain of HER4, like cyclin B, may be required for progression through the cell cycle in cells expressing nuclear HER4. The mechanism by which HER4 delays

progression through G2/M is currently under investigation. However, our results are consistent with the idea that the intracellular domain of HER4-Cyt1 may indeed be involved in regulating the G2/M transition or progression through early M.

The discovery of ligand-dependent JMa HER4 proteolysis and release of a nuclear localizing soluble tyrosine kinase has resulted in multiple studies investigating the function of $s80^{HER4}$. We and others have demonstrated $s80^{HER4}$ growth inhibitory action (21,33,37); and have shown that HRG-mediated HER4 activity, as well as de novo expression of s80HER4, slows the progression of cells through G2/M. The data presented herein suggests a physiologic mechanism for the regulation of $s80^{HER4}$ by APC-dependent ubiquitination and degradation during M-phase.

Other recent reports have suggested specific transcriptional roles for nuclear $s80^{HER4}$ complexed with transcription factors or co-factors. These include a positive effect on estrogen receptor-mediated gene transcription in estrogen receptor positive breast cancer cells (46), on STAT5A-depdendent gene expression in differentiating mammary epithelial cells, and repression of astrocyte-specific gene transcription in astrocyte progenitor cells in the developing mouse brain (47). In these studies, $s80^{HER4}$ colocalized in complexes on specific DNA elements, for example, on the progesterone receptor promoter with the estrogen receptor (ER) or the GFAP promoter with TAB2 and N-Cor (46,47). Additionally, $s80^{HER4}$ may enhance transcription factor activity through kinase-mediated phosphorylation. For example, $s80^{HER4}$ phosphorylates the transcription factor, STAT5A, and enhances STAT5A-dependent transcription (12,37). $s80^{HER4}$ also facilitates movement of the transcriptional complex from cytoplasm to nucleus. Whether our postulated functional interaction with G2/M checkpoint cell cycle control mechanisms requires transcriptional control at specific promoters or tyrosine phosphorylation of other regulatory elements, remains to be determined. It will be of interest to determine whether altering $s80^{HER4}$ mitotic destruction via the D-box mutation changes transcriptional modulators to address whether growth inhibition and transcriptional control by $s80^{HER4}$ are distinct mechanistically.

The findings reported here may have clinical implications. Most clinical correlative studies of human breast cancer agree that nuclear HER4 correlates with positive prognostic indicators. We have shown the HER4 activation (9) or $s80^{HER4}$ expression (21) (and see Fig. 1) inhibited breast cancer cell growth by decreasing the progression of cells through G2/M (21). Furthermore, we have demonstrated (Fig. 6) that $s80^{HER4}$ slowed transformed breast cell proliferation when expressed in PyVmT-expressing HC11 cells. Ligand-mediated activation of HER4 results in $s80^{HER4}$ release, nuclear-cytoplasmic shuttling, and growth inhibition; interference with proteolytic cleavage ($HER4^{V675A}$, see Fig. 1) abrogates this action. Thus, $s80^{HER4}$ production is an obligatory step for HER4-mediated growth inhibition. Moreover, $s80^{HER4}$ expression is sufficient to confer growth inhibition to HeLa cells, or breast cancer cells, in the absence of full-length HER4 or ligand stimulation (21). The growth inhibitory signal of $s80^{HER4}$ was strengthened by D-box mutation. Since the D-box is required for degradation during M, it is possible that $s80^{HER4}$ impairs growth of cells by delaying the cell cycle in M, such that degradation of $s80^{HER4}$ is required for mitotic progression. Although this hypothesis requires further investigation, these data demonstrate the potential link between nuclear HER4 signaling and regulation of mitotic progression. The suppression of PyVmT-induced tumor cell proliferation *in vivo* by $s80^{HER4}$ and the increased effectiveness of the $s80^{HER4}$ D-box mutant highlight the need for mechanistic studies, which in turn will help elucidate the therapeutic implications of $s80^{HER4}$ expression in breast cancer.

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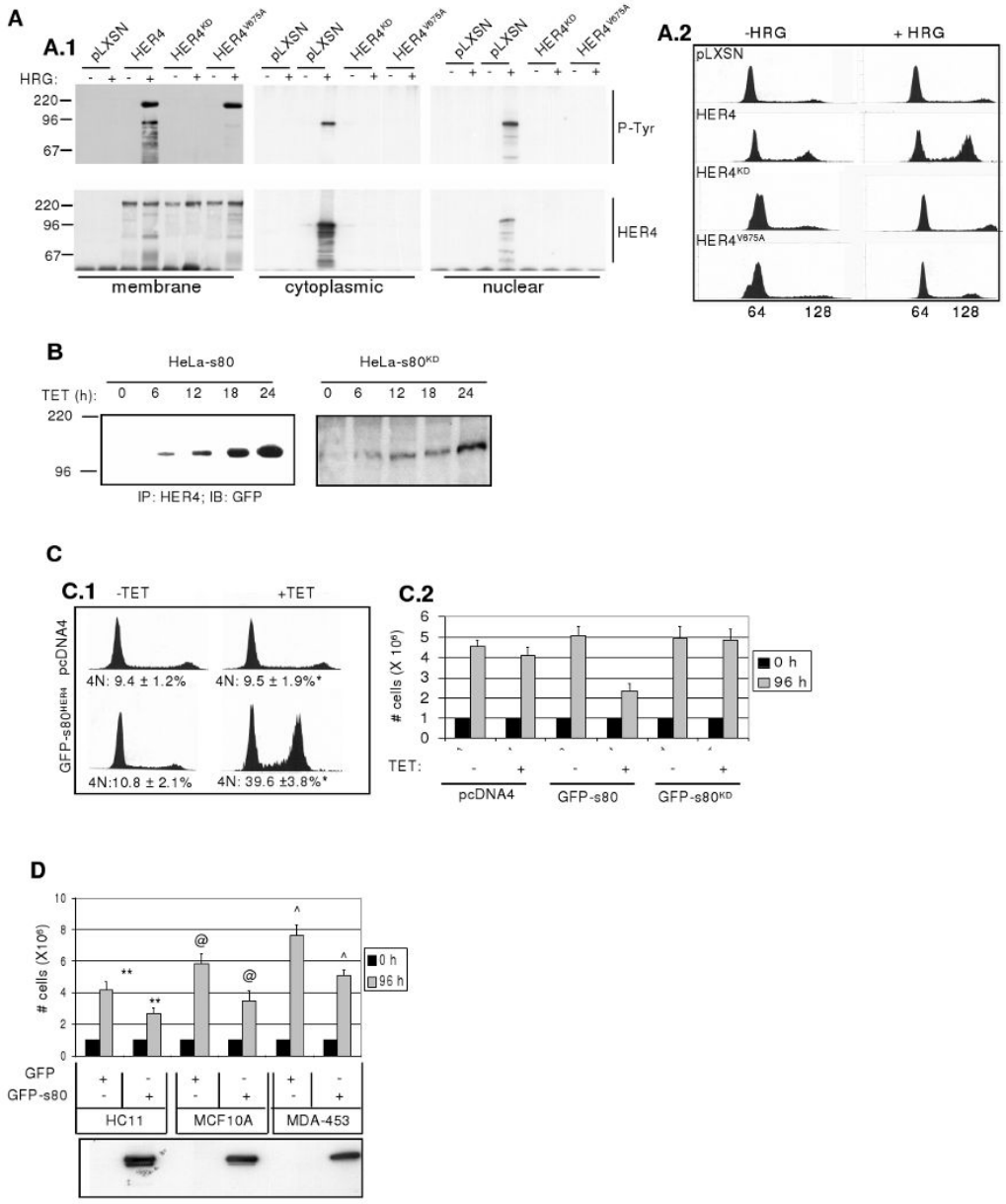

Figure 1. The intracellular domain of HER4, s80HER4, inhibits growth via G2/M delay in HaLa-rtTA cells A

Mutation of the HER4 γ-secretase cleavage site inhibits formation of s80^{HER4} and interferes with HER4-mediated growth inhibition: **A.1** Western analysis to detect phospho-tyrosine residues or total HER4 expression in HER4 immunoprecipitates (IPs) from membrane, cytoplasmic, or nuclear extracts from HeLa-rtTA cells expressing pLXSN, pLXSN-HER4, pLXSN-HER4^{KD}, or pLXSN-HER4^{VA}. Serum-starved cells were treated ± HRG (2h) before separation into cellular compartments using biochemical methods. Molecular weights are indicated at left. **A.2**: Cells grown in SF media ± HRG (48h) were stained with propidium iodide (PI) and analyzed by flow cytometry. Representative histograms are shown. **B.** Western analysis to detect GFP in HER4 IPs from HeLa-s80 and HeLa-s80^{KD} cells cultured +TET for 0-24 h. **C.** Growth inhibition and G2/M delay in cells expressing GFP-s80^{HER4}, but not in cells expressing GFP-s80^{KD}. **C.1:** Representative histograms of

HeLa-pcDNA4 and HeLa-s80 cells grown in SF media ±TET (48h), stained with PI and analyzed by flow cytometry. *P < 0.002, Student's unpaired T-test. **C.2** Equal numbers of HeLa-pcDNA4, HeLa-s80, and HeLa-$s80^{KD}$ cells were plated, allowed to attach (24h), then treated for 0 or 96h ±TET in serum-free (SF) media. **D.** Equal numbers of HC11, MCF-10A, or MDA-453 cells expressing GFP-tagged s80 or GFP, were plated, allowed to attach (24h), then cultured for 0 or 96h in SF media. Each sample was counted in duplicate; each experiment was performed in triplicate. Values represent the average number of cells ± S.D. **P < 0.01; @P < 0.02; ^P < 0.01; each calculated using Student's unpaired T-test Western analysis to detect $s80^{HER4}$ in cell lysates is shown in lower panel.

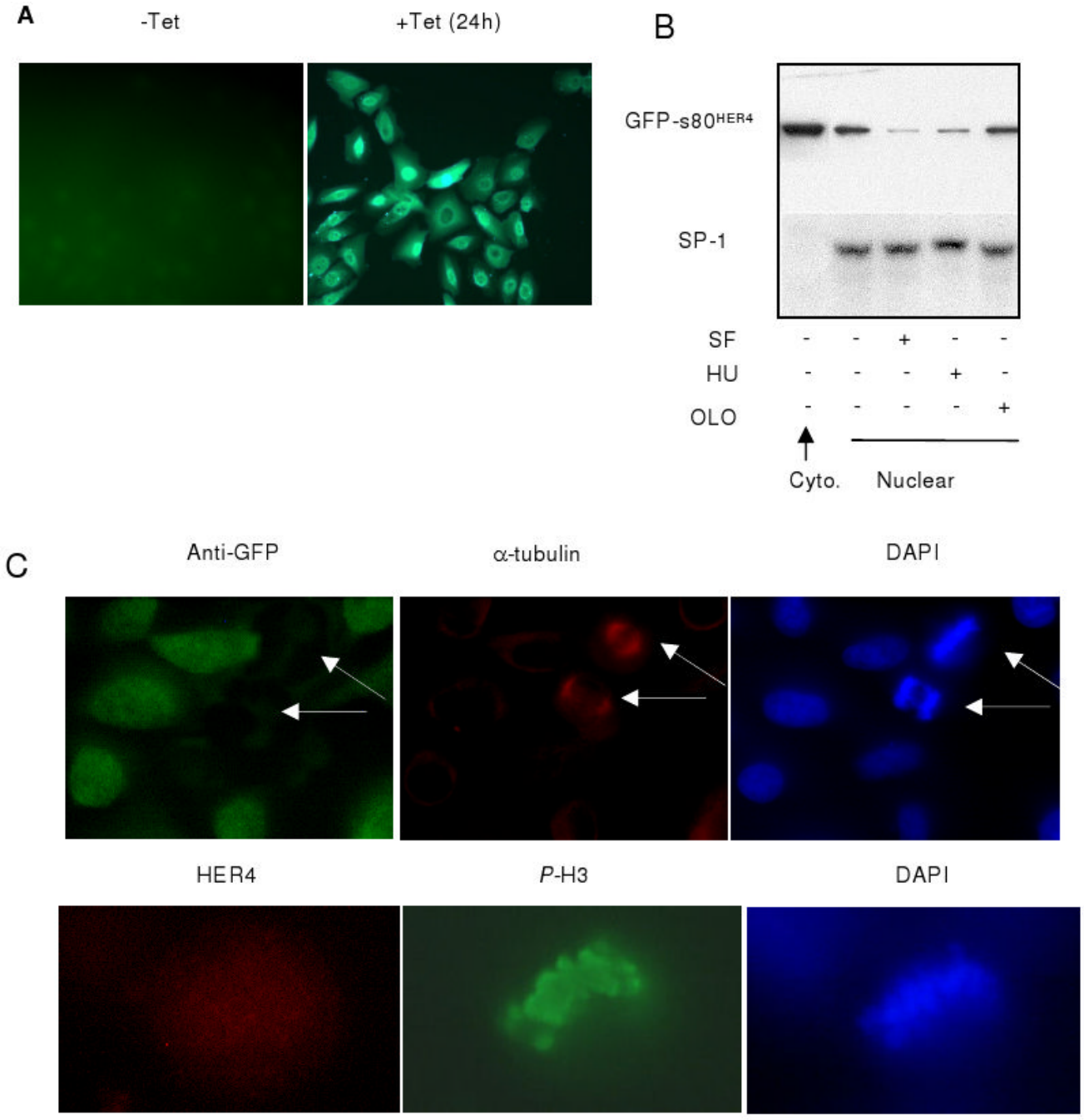

Figure 2. Cell-cycle-specific expression of s80^{HER4}. A
GFP imaging of HeLa-s80 cells cultured 24 h ± TET. **B.** HeLA-s80 cells were treated +TET for 24 h to induce expression of GFP-s80^{HER4}, followed by 16 h treatment +TET in SF media, or +TET in 10% serum +HU, or +OLO. Cytoplasmic and nuclear extracts were examined by western analysis, using HER4 IP followed by immunoblotting for GFP (upper panels) or by analysis of extracts by immunoblotting for SP-1 (lower panel). **C.** Immunocytofluorescence of HeLa-s80 cells cultured +TET (48 h). Upper: Cells were dual-stained for GFP and α-tubulin. DAPI images shown at right. Lower: Immunocytofluoresence HER4 and phospho-Ser10 histone H3. DAPI staining shown at right.

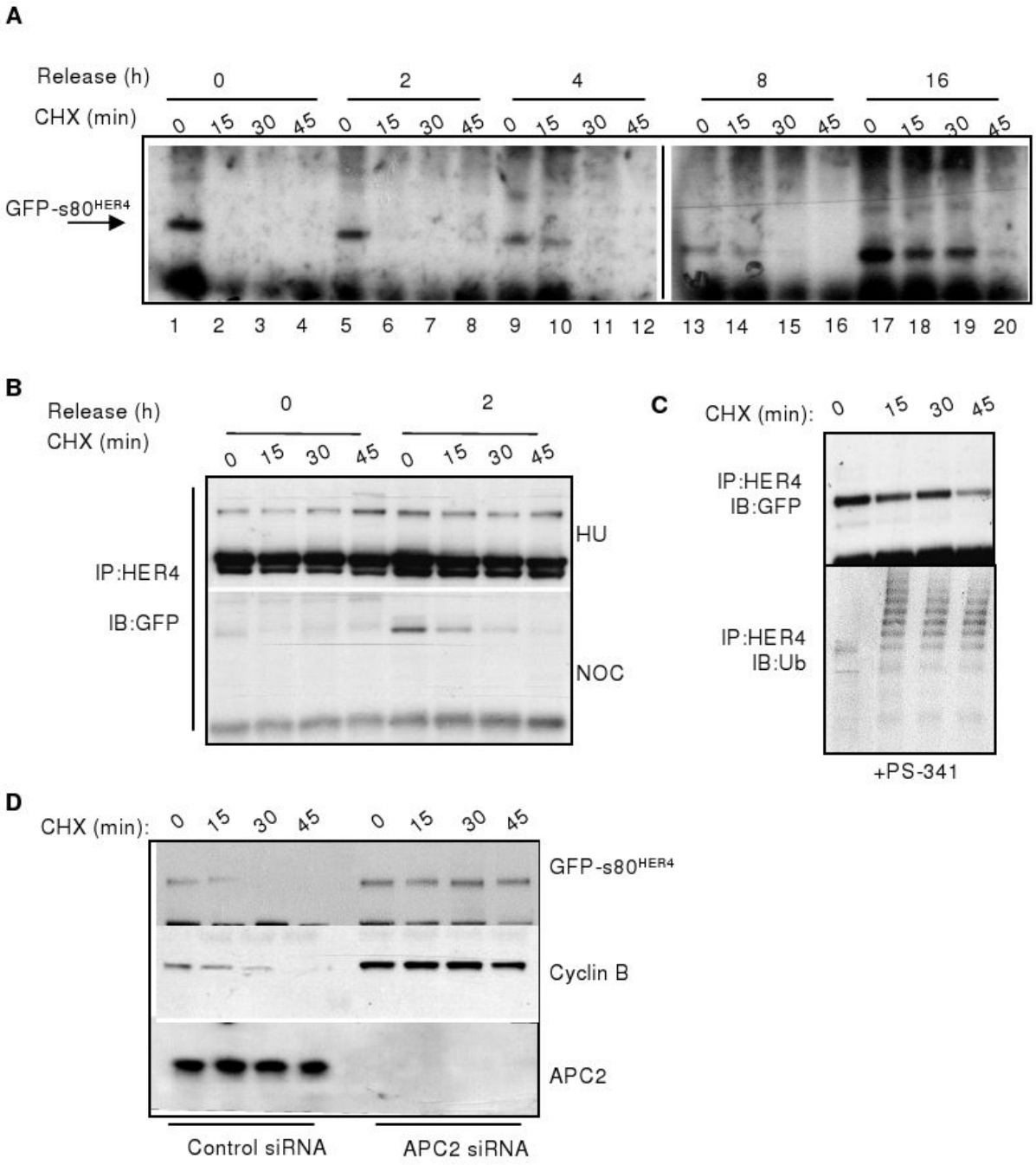

Figure 3. Degradation of s80^{HER4} during mitosis directed by APC/C. A

HeLa-s80 cells were cultured +NOC/+TET in 10% serum for 16 h. Cells were released into 10% serum +TET at time (T) =0, and treated +CHX at T=0, 2, 4, 8, and 16h after NOC release. Expression of GFP-s80^{HER4} was analyzed at 15 min intervals beginning at the time of CHX treatment, by HER4 IP followed by GFP immunoblot (IB). **B.** HeLa-s80 cells were synchronized +HU/+TET (upper panel) or +NOC/+TET (lower panel) in 10% serum for 16 h, then released into 10% serum +TET at T=0. Cells were treated +CHX at T=0 and 2h. Expression of GFP-s80^{HER4} was analyzed as described above. **C.** Cells were cultured +NOC/+TET in 10% serum for 16 h, with PS-341 added for the final 4 h. Cells were released into 10% serum +TET/+PS-341 at T=0. CHX was added at T=0. Expression of

GFP-s80^{HER4} was analyzed as described above; blots were stripped then probed for ubiquitin (lower panel). **D.** HeLa-s80^{HER4} cells transfected with 10 nmol siRNA sequences targeting luciferase or APC2 were treated +NOC/+TET +10% serum for 16 h. Cells were released into 10% serum +TET/+CHX. Expression of GFP-s80^{HER4} and cyclin B were analyzed at 15 min intervals beginning at the time of CHX treatment, by IB of anti-HER4 or anti-cyclin B IP's using GFP or cyclin B antibodies, respectively. APC2 was analyzed by IB.

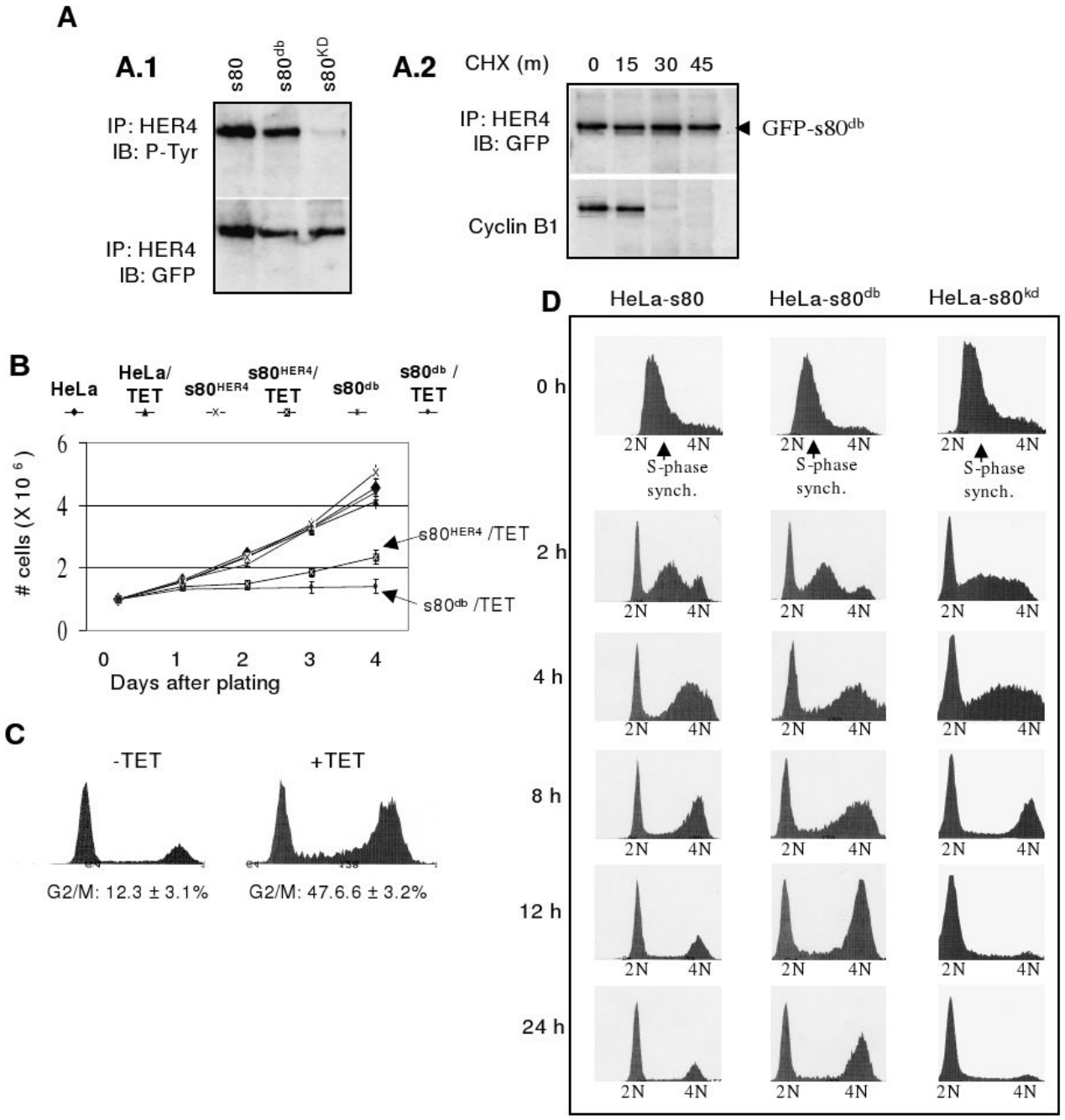

Figure 4. Mutation of the s80^{HER4} D-box eliminates mitotic degradation of s80^{HER4} and enhances s80^{HER4}-mediated growth inhibition. A

Mutation of the s80^{HER4} D-box stabilized s80^{HER4} during mitosis. **A.1** Western analysis of HER4 IP's from HeLa-s80, -s80^{db}, and -s80^{KD} cells cultured 24 h +TET. IP's were analyzed for phospho-tyrosine or GFP. **A.2:** HeLa-s80^{db} cells were cultured +NOC/+TET in 10% serum (16 h). Cells were released into 10% serum +TET/+CHX at T=0, and extracts collected at 15 min intervals. Expression of s80^{db} and cyclin B was analyzed as described in Fig. 3. **B.** Equal numbers of cells were plated at day 0 ±TET. Cells were counted at 1-day intervals through 4 days. P= 0.011 comparing cell number of HeLa-s80^{HER4} +TET at day 4 to cell number of HeLa-s80^{db} + TET at day 4, n=5, analyzed in triplicate. **C.** Cell cycle

analysis of HeLa-s80^{db} cells cultured ±TET (48h). > 10,000 nuclei were analyzed per condition; n=3; representative histograms shown. **D.** Cells were in 10% serum +HU/+TET for 16 h to synchronize in S-phase. Cells were released into 10% serum +TET at T=0 h. Cells were collected at 0, 2, 4, 8, 12, and 24 h following HU release. Cells were stained with propidium iodide and analyzed by flow cytometry as described above.

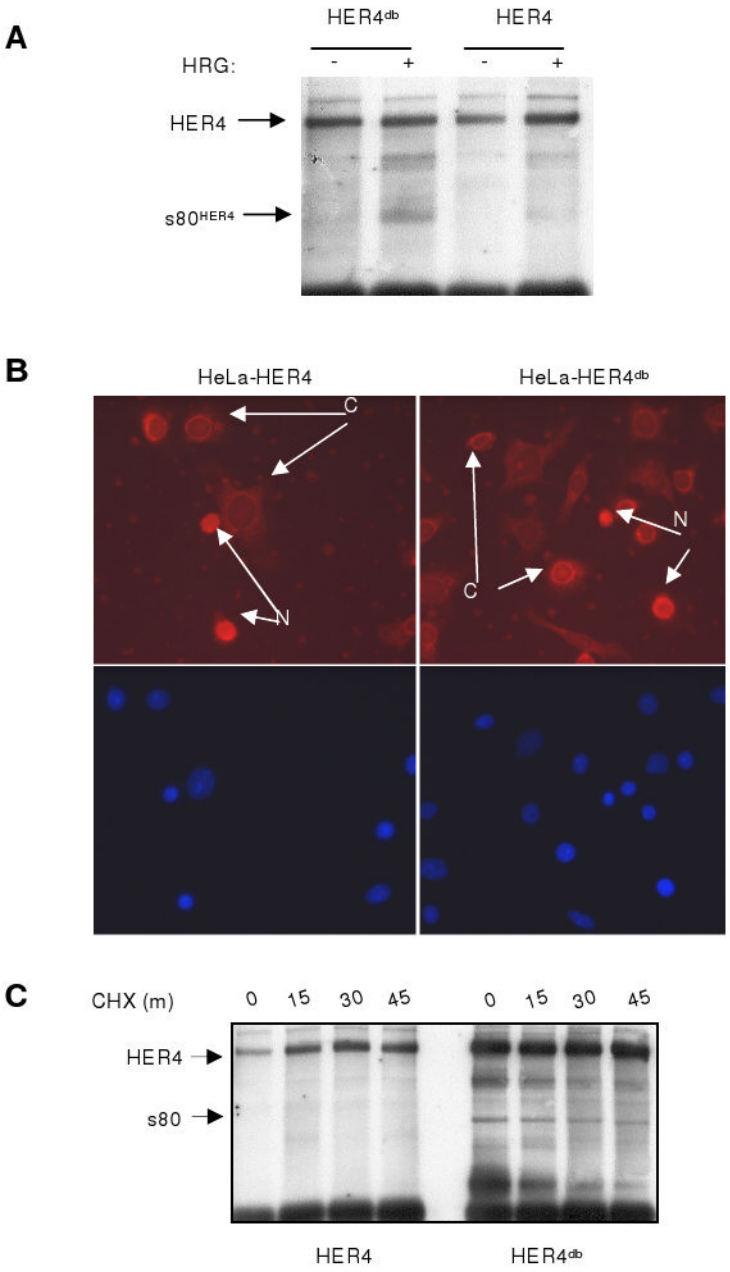

Figure 5. Mutation of the D-box within full length HER4 does not reduce s80^{HER4} production but does stabilize s80^{HER4}. A

HeLa cells stably transfected with pLXSN-HER4 or -HER4^{db} were serum-starved 16 h then treated +HRG (1h). HER4 IP's were analyzed for total HER4. **B.** Cells were serum-starved (16 h), treated +HRG (1h). Cells were analyzed for HER4 localization by immunocytofluorescence. DAPI staining is shown at bottom. N= nuclear localization of HER4. C= cytoplasmic localization of HER4. **C.** HeLa-HER4 and HeLa-HER4^{db} cells were cultured in 10% serum +NOC (16 h), then in SF media +NOC (16 h), adding HRG for the final hour. Cells were released into SF media +HRG/+CHX at T=0 and cells were harvested at T=0, 15, 30, and 45 min. HER4 IPs were analyzed as described above.

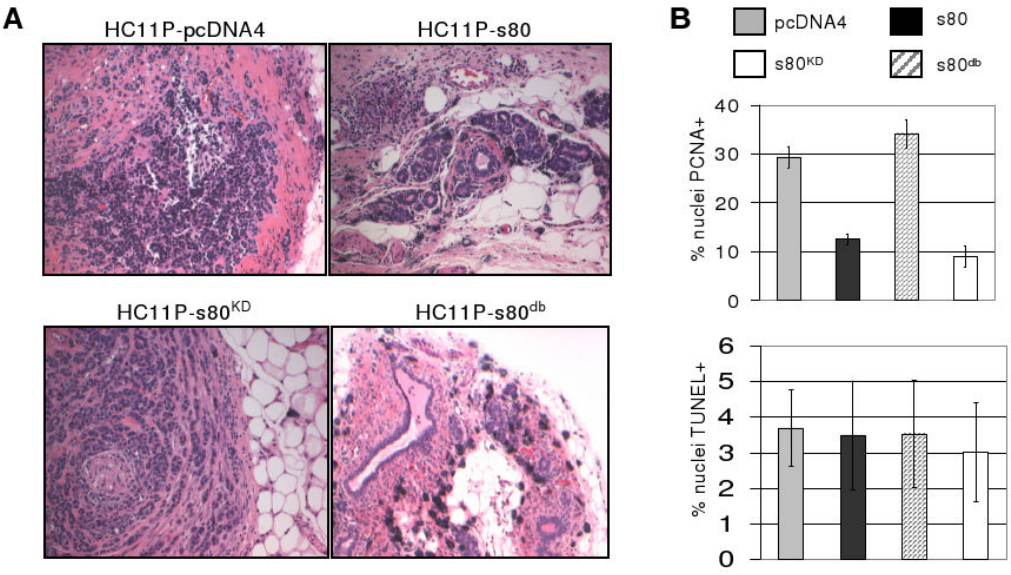

Figure 6. Decreased tumor formation in PyVmT-transformed HC11 cells expressing s80^{db}. A HC11P cells expressing s80^{HER4}, s80^{KD}, s80^{db}, or the empty pcDNA4 vector were injected into the inguinal mammary fatpads of female BALB/c mice. Fifteen weeks post-implantation of cells, mice were euthanized and the mammary glands analyzed histologically; representative images are shown. **B.** Five tumor samples per group were analyzed by immunohistochemistry. PCNA positive (upper panel) or TUNEL positive nuclei (lower panel) were scored in 3 randomly chosen 400× fields per tumor section, for a total of 15 fields counted. HC11P-pcDNA4 and HC11P-s80^{KD} had equivalent percentages of PCNA-positive nuclei (29.4±2.2 and 34.1±2.9, respectively). HC11P-s80 had a statistically significantly lower percentage of PCNA-positive cells (12.5±1.1, $p<0.0001$) as compared to vector alone. While HC11P-s80^{db} percentage of PCNA-positive cells was the lowest of all groups (9.0±2.2), and was also significantly decreased when compared to HC11P-s80 ($p<0.037$).

Table 1

Protein sequence of human cyclin B1 (NCBI accession number NM_031966) aligned with that of human HER4/s80^{HER4}, human HER1, human HER2, and human HER3. Residues defining the D-box are bolded and underlined. Site-directed mutagenesis was performed to generate R-to-A and L-to-A mutations, shown in lowercase. * Alignment of ErbB sequences according to Plowman et al., 1993.

Protein name	Amino acid #*	Sequence
Cyclin B1	40-60	RP**R**TA**L**GDIG**N**KVSEQLQAKM
HER4/s80^{HER4}	990-1010	
DD**R**MK**L**PSPN**D**SKFFQNLLDE		
HER4^{db}/s80^{db}	990-1010	
DD***a***MK***a***PSPN**D**SKFFQNLLDE		
HER1/EGFR	960-980	DE**R**MH**L**PSPT**D**SNFYRALMDE
HER2/ErbB2	992-1012	ED - LGP-ASPLDSTFYRSLLED
HER3/ErbB3	930-950	SGPGIAPGPEPHGLTNKKLEE