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 G_2-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 anaphase-promoting complex (APC)/cyclosome, a multisubunit ubiquitin ligase. s80^{HER4} ubiquitination and proteasomal degradation occurred during mitosis but not during S phase. Inhibition of an APC subunit (APC2) using short interfering RNA knockdown impaired s80^{HER4} degradation. Mutation of the s80^{HER4} D-box sequence stabilized s80^{HER4} during mitosis, and s80^{HER4}-dependent growth inhibition via G_2-M delay was significantly greater with the D-box mutant. Polyomavirus 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. [Cancer Res 2007;67(14):6582–90]

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 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. Whereas HER1, HER2, and HER3 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 (ER) expression and decreased bromodeoxyuridine 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 G_2 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, HER2, and HER3, 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, the extracellular domain of HER4 (JMa) is cleaved by tumor necrosis α-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 and show that proteolytic generation of s80^{HER4} from full-length HER4 was required for HER4-mediated growth inhibition, resulting in growth inhibition via a delay in the G_2-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 stabilized s80^{HER4} and increased growth inhibition in response to HER4 or s80^{HER4} both in cell culture and in tumor cells *in vivo*.

Materials and Methods

Plasmids. The pLXSN vectors expressing human HER4, HER4^{KD}, and 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 italicized nucleotides represent incorporated mutations; QuikChange Site-Directed Mutagenesis kit (Stratagene)].

Cell culture and transfection. HeLa-TRex cells (Invitrogen), referred to as HeLa-rtTA cells, were cultured according to the 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

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in serum-free DMEM ± heregulin β1 (HRG; 10 ng/mL; Genentech), tetracycline (2 μg/mL), nocodazole (50 ng/mL), or hydroxyurea (10 mmol/L; each from Sigma-Aldrich). Cells synchronized in nocodazole were collected by shake off, pelleted by centrifugation, resuspended in medium containing 10% serum ± cycloheximide (Sigma-Aldrich), tetracycline, or HRG, where indicated, and then replated. Where indicated, cells were transiently transfected with short interfering RNA (siRNA) sequences directed against the human *APC2* gene using siRNA transfection reagent (Santa Cruz Biotechnology). HC11, MCF-10A, and MDA-MB-453 cells expressing green fluorescent protein (GFP) and GFP-tagged s80^{HER4} have been described (38).

Western blot analysis and immunoprecipitation. Membrane and cytosolic extracts were prepared as described previously (37). Whole-cell extracts were prepared as described previously (21). HER4 was immunoprecipitated with α-HER4 Ab-132 or antibodies against Cdc20, APC4 (Santa Cruz Biotechnology), or cyclin B1 (Labvision NeoMarkers) and protein A-agarose or protein A/G+ agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were analyzed by immunoblotting using antibodies GFP, APC4, APC2 (Santa Cruz Biotechnology), cyclin B, or HER4 (Lab Vision NeoMarkers) as described previously (21).

Fluorescence imaging. Cells were fixed in methanol and incubated 1 h in antibody against α-tubulin (Sigma-Aldrich) or GFP (Santa Cruz

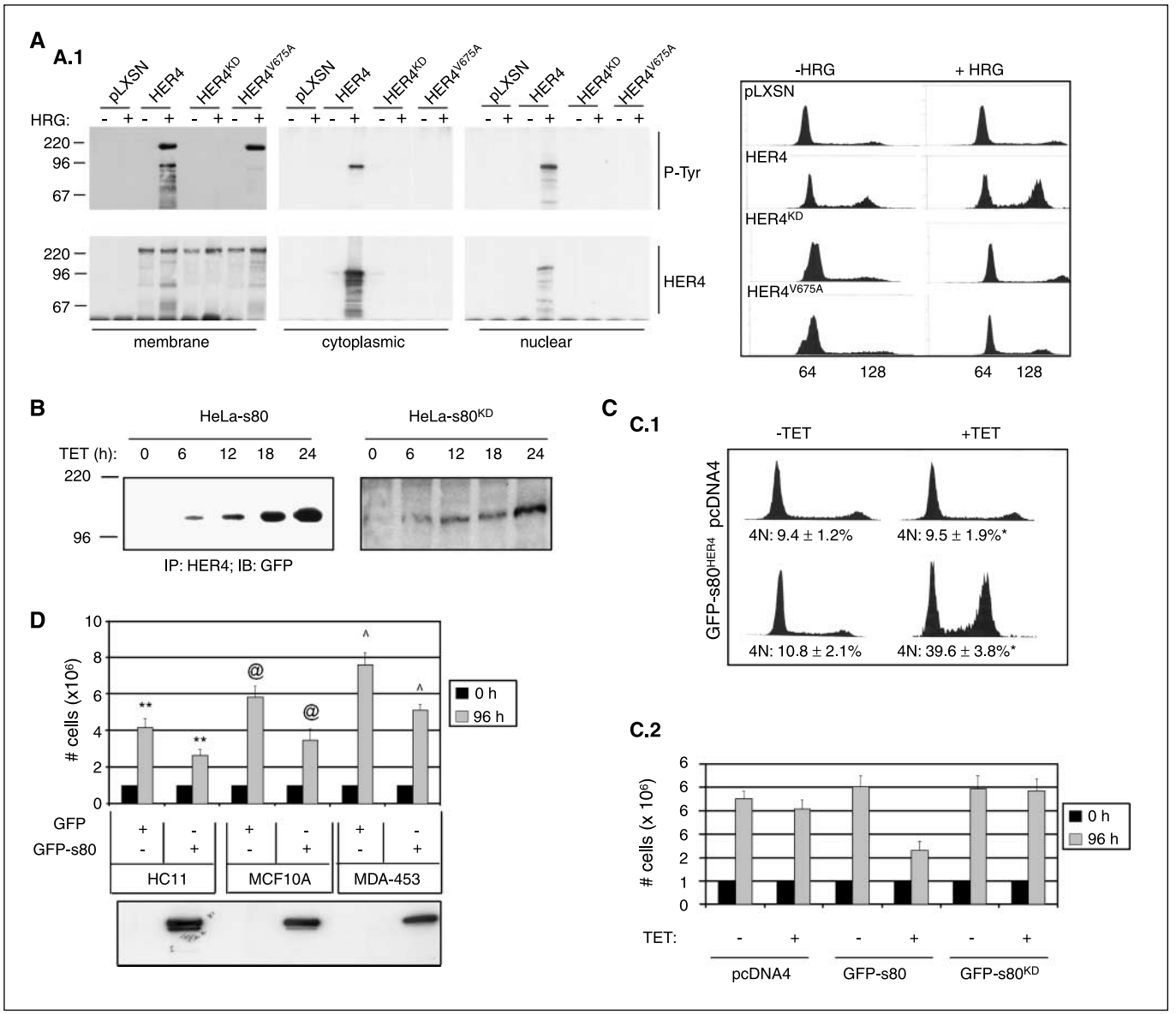

Figure 1. The intracellular domain of HER4, s80^{HER4}, inhibits growth via G_2-M delay. *A, A.1,* Western blot analysis to detect phosphorylated tyrosine (*P-Tyr*) residues or total HER4 expression in HER4 immunoprecipitates from membrane, cytoplasmic, or nuclear extracts from HeLa-rtTA cells expressing pLXSN, pLXSN-HER4, pLXSN-HER4^{KD}, or pLXSN-HER4^{VA}. Serum-starved cells were treated ± HRG (2 h) before separation into cellular compartments using biochemical methods. *Left,* molecular weights. *A.2,* cells grown in serum-free medium ± HRG (48 h) were stained with propidium iodide and >10,000 cells were analyzed by flow cytometry. Representative histograms. *B,* Western blot analysis to detect GFP in HER4 immunoprecipitates (*IP*) from HeLa-s80 and HeLa-s80^{KD} cells cultured + tetracycline (*TET*) for 0 to 24 h. *IB,* immunoblot. *C, C.1,* representative histograms of HeLa-pcDNA4 and HeLa-s80 cells grown in serum-free medium ± tetracycline (48 h) and stained with propidium iodide and >10,000 cells were 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 (24 h), and then treated for 0 or 96 h ± tetracycline in serum-free medium. *D,* equal numbers of HC11, MCF-10A, or MDA-453 cells expressing GFP-tagged s80 or GFP were plated, allowed to attach (24 h), and then cultured for 0 or 96 h in serum-free medium. Each sample was counted in duplicate; each experiment was done in triplicate. *Columns,* average number of cells; *bars,* SD. **, $P < 0.01$, Student's unpaired *t* test; @, $P < 0.02$, Student's unpaired *t* test; ^, $P < 0.01$, Student's unpaired *t* test. *Bottom,* Western blot analysis to detect s80^{HER4} in cell lysates.

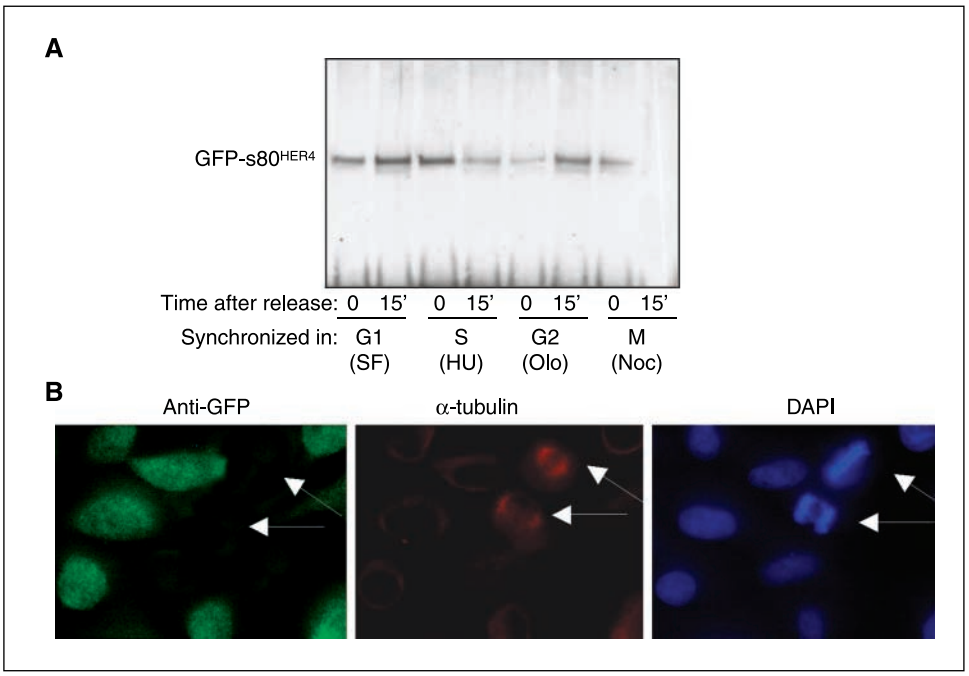

Figure 2. Cell cycle–specific expression of $s80^{HER4}$. *A,* HeLa-s80 cells were treated + tetracycline for 24 h to induce GFP-$s80^{HER4}$ followed by 16 h of treatment + tetracycline in serum-free (*SF*) medium, + tetracycline/ (*HU*) hydroxyurea, + tetracycline/+ olomoucine (*OLO*), or + tetracycline/+ nocodazole (*NOC*). Where indicated, cells were released into serum-containing medium for 15 min. Whole-cell extracts were examined by HER4 immunoprecipitation and GFP immunoblot. *B,* immunocytofluorescence of HeLa-s80 cells cultured + tetracycline (48 h). Cells were dual stained for GFP and α-tubulin. *Right,* DAPI images.

Biotechnology) and then with rhodamine-conjugated goat anti-mouse and Alexa Fluor 488–conjugated goat anti-rabbit antibody (Molecular Probes). Samples were mounted with Vectastain 4′,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories).

Cell growth assays. Cells (0.1×10^6) were plated in 15-cm dishes at day 0 ± HRG or tetracycline. 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% methanol, and then stained with propidium iodide + RNase A (Becton Dickinson). Stained nuclei per sample (10,000) 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, NC) followed by selection with hygromycin. Antibiotic-resistant clones were individually analyzed for polyomavirus middle T antigen (PyVmT) expression by Northern blot 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 after 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 G_2-M delay of HeLa cells. Ligand-mediated HER4 activation is known to result in HER4 cleavage to produce the liberated 80-kDa intracellular domain. Production of $s80^{HER4}$ was shown in HeLa-rtTA cells (that do not express detectable endogenous HER4) transfected with a cDNA construct encoding wild-type human HER4 (JMa/Cyt1 splice isoform). Treatment of HeLa-HER4 cells with 10 ng/mL HRG for 2 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 blot 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-kDa tyrosine-phosphorylated HER4 product in membrane, cytoplasmic, and nuclear extracts but not in untreated, serum-starved HeLa-HER4 cells (Fig. 1*A.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), which abolishes the HER4 intramembranous, γ-secretase cleavage site (referred to herein as $HER4^{VA}$; ref. 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 final intramembranous cleavage of $HER4^{VA}$ was not achieved, thus preventing the release of soluble $s80^{HER4}$. As previously shown, kinase-dead $HER4^{KD}$ was not phosphorylated and did not form the 80-kDa cleavage product, suggesting that kinase activity of HER4 is required to initiate HER4 cleavage (Fig. 1*A.1*).

HER4 is required for HRG-mediated growth inhibition and G_2-M delay in several breast cancer cells (9, 21). HeLa-pLXSN, HeLa-HER4, HeLa-$HER4^{KD}$, and HeLa-$HER4^{VA}$ cells were treated ± HRG for 48 h in serum-free medium to examine the effects of HRG on cell cycle distribution. Whereas exogenous HER4 expression conferred HRG-mediated G_2-M delay to HeLa cells, expression of $HER4^{KD}$ and $HER4^{VA}$ did not allow for any observable HRG-mediated G_2-M delay (Fig. 1*A.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 G_2-M transit of HeLa and breast cancer cells. We stably introduced into HeLa-rtTA cells a tetracycline-inducible pcDNA4 construct expressing the Cyt1 isoform of $s80^{HER4}$ or kinase-dead $s80^{KD}$, each tagged at the NH_2 terminus with GFP. Treatment of cells with tetracycline

induced target gene expression within 6 to 12 h, reaching a maximum by 24 h (Fig. 1*B*). Induction of GFP-s80^{HER4} expression increased the proportion of HeLa cells with 4N DNA compared with HeLa-pcDNA4 control cells treated with tetracycline (Fig. 1*C.1*). Expression of GFP-s80^{HER4} for 96 h decreased the rate of total cell growth compared with tetracycline-treated HeLa-pcDNA4 or HeLa-s80^{KD} cells (Fig. 1*C.2*). To show the effects of s80^{HER4} in breast epithelial cells, we stably expressed GFP-tagged s80^{HER4} (or GFP alone) in HC11 cells (nontransformed mouse mammary cells), MCF-10A cells (human nontransformed 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, MCF-10A, and MDA-MB-453 cells expressing GFP-s80^{HER4} than in these cells expressing GFP alone (Fig. 1*D*). Taken together, these data suggest that s80^{HER4} production is both necessary and sufficient to decrease growth of HeLa and breast-derived cells.

Expression of GFP-s80^{HER4} is regulated with the cell cycle. Expression of GFP-s80^{HER4} was induced with tetracycline for 24 h. We then analyzed expression of GFP-s80^{HER4} in extracts from cells that were synchronized for 16 h in G_1 with serum-free medium, in S phase with hydroxyurea, at the G_2-M transition with olomoucine, or in mitosis with nocodazole. Cells were then released from synchronization by feeding the cells with serum-containing medium, or in the case of nocodazole synchronization, the cells were collected by shake off and then resuspended and plated in medium with serum. Although expression of GFP-s80^{HER4} was detected in randomly cycling cells and in cells released into G_1, S, and G_2, we found that, 15 min after release of cells into mitosis, GFP-s80^{HER4} expression was undetectable (Fig. 2*A*). To further examine the expression of s80^{HER4} during mitosis, we used immunofluorescent staining of randomly cycling, tetracycline-induced HeLa-s80 cells to detect GFP-s80 and the mitotic spindle protein α-tubulin. Dual immunofluorescence revealed that, of 47 cells harboring α-tubulin–positive mitotic spindles, 0 of 47 cells simultaneously expressed GFP-s80^{HER4} (Fig. 2*B*). These results suggest that GFP-s80^{HER4} expression decreases during mitosis.

Degradation of s80^{HER4} in mitosis. We synchronized tetracycline-treated HeLa-s80 cells in mitosis with nocodazole for 16 h and then, on release of cells from mitosis, examined GFP-s80^{HER4} stability through the cell cycle by treating the cells with cycloheximide to impair new protein synthesis at 0, 2, 4, 8, and 16 h after nocodazole release. Cells were harvested at 15-min intervals following cycloheximide treatment and expression of GFP-s80^{HER4} was analyzed. Although GFP-s80^{HER4} expression was detected at time (T) = 0, it was undetectable within 15 min of cycloheximide treatment/nocodazole release (Fig. 3*A*, *lanes 1–4*), indicating that s80^{HER4} was rapidly degraded on release into mitosis. By 4 and 8 h after nocodazole release, GFP-s80^{HER4} showed increasing stability (Fig. 3*A*, *lanes 10* and *14* show GFP-s80^{HER4} 15 min after release), and GFP-s80^{HER4} was stable through 45 min

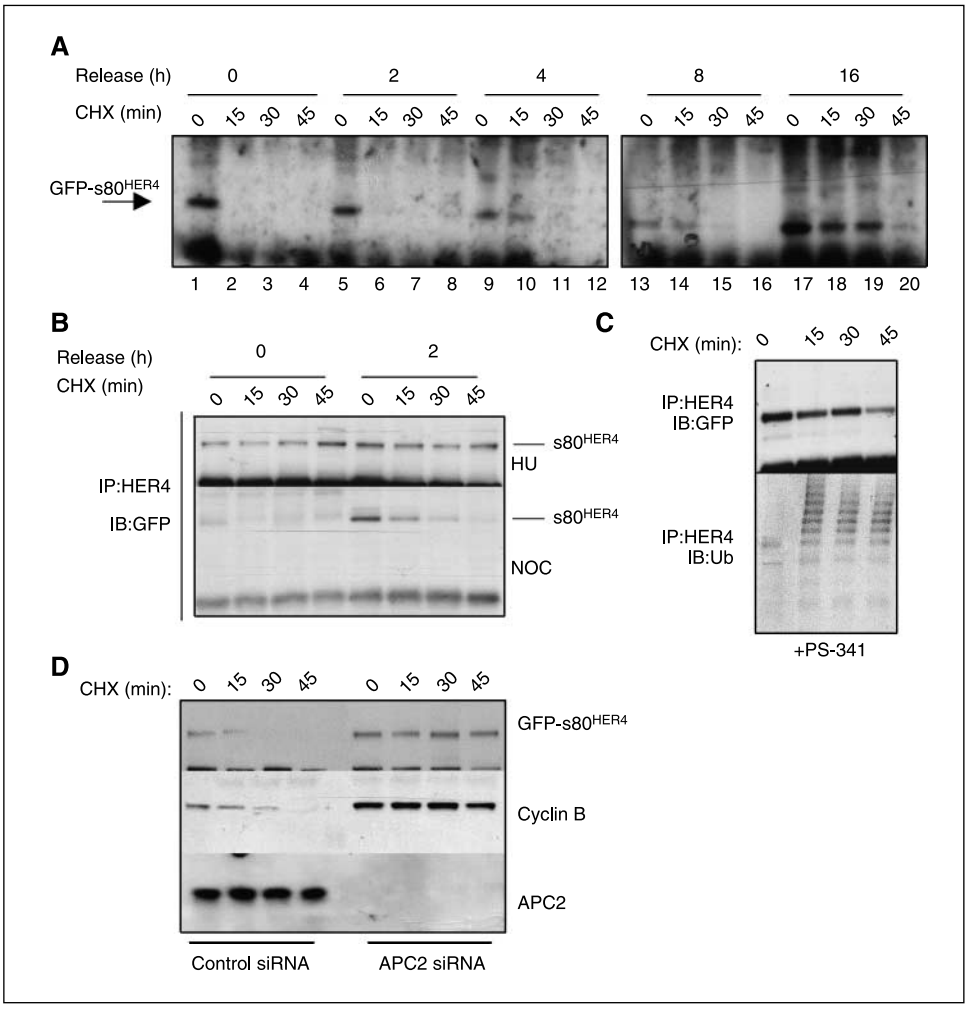

Figure 3. Degradation of s80^{HER4} during mitosis directed by APC/C. *A,* HeLa-s80 cells were cultured + nocodazole/+ tetracycline for 16 h. Cells were released at T = 0 and treated + cycloheximide at T = 0, 2, 4, 8, and 16 h after release. Expression of GFP-s80^{HER4} was analyzed at 15-min intervals beginning at the time of cycloheximide (*CHX*) treatment by HER4 immunoprecipitates and GFP immunoblot. *B,* HeLa-s80 cells were synchronized + hydroxyurea/+ tetracycline (*top*) or + nocodazole/+ tetracycline (*bottom*) for 16 h and then released + tetracycline at T = 0. Cells were treated + cycloheximide at T = 0 and 2 h. Expression of GFP-s80^{HER4} was analyzed as described above. *C,* cells were cultured + nocodazole/+ tetracycline for 16 h, with PS-341 added for the final 4 h. Cells were released + tetracycline/+ PS-341 at T = 0. Cycloheximide was added at T = 0. Expression of GFP-s80^{HER4} was analyzed as described above; blots were stripped and then probed for ubiquitin (*bottom*). *D,* HeLa-s80^{HER4} cells transfected with 10 nmol siRNA sequences targeting luciferase or APC2 were treated + nocodazole/+ tetracycline for 16 h. Cells were released + tetracycline/+ cycloheximide. Expression of GFP-s80^{HER4} and cyclin B was analyzed at 15-min intervals beginning at the time of cycloheximide treatment by immunoblot of anti-HER4 or anti-cyclin B immunoprecipitates using GFP or cyclin B antibodies, respectively. APC2 was analyzed by immunoblot.

when examined 16 h after nocodazole release (Fig. 3*A, lanes 17–20*). Although GFP-s80^{HER4} was rapidly degraded when cells were released in mitosis, it was highly stable in cells synchronized in S phase with hydroxyurea and then released (Fig. 3*B*). This suggests that s80^{HER4} is more stable on release from S phase compared with release from mitosis.

The APC/C directs proteasomal degradation of s80^{HER4} during mitosis. Tetracycline-treated HeLa-s80 cells were synchronized with nocodazole for 16 h and treated with the proteasome inhibitor PS-341 for the final 4 h of synchronization. Cells were released from nocodazole in the presence of cycloheximide 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 min after release from nocodazole and allowed for the accumulation of ubiquitinated GFP-s80^{HER4} (Fig. 3*C*), suggesting that s80^{HER4} is ubiquitinated during mitosis and degraded by the proteasome.

The APC2 subunit, required for APC/C activity (39, 40), was knocked down using transfection of siRNAs into HeLa-s80^{HER4} cells (Fig. 3*D*). Transient transfection of 20 nmol APC2 siRNA (Santa Cruz Biotechnology) impaired expression of APC2 protein in HeLa-s80^{HER4} cells. APC2 siRNA–transfected cells were grown in the presence of tetracycline for 48 h, adding nocodazole to the cells for the final 16 h of culture to synchronize the cells in mitosis. Cells were released from nocodazole and treated with cycloheximide at $T = 0$. Expression of GFP-s80^{HER4} was analyzed at 15-min intervals following nocodazole release/cycloheximide treatment. GFP-s80^{HER4} was detected through 45 min following nocodazole 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 nocodazole release and did not harbor detectable levels of GFP-s80^{HER4} at 30 min following nocodazole 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 amino acid sequence of human HER4 revealed a canonical D-box, a motif found in numerous proteins targeted by the APC/C for mitotic destruction, including cyclin B (Table 1). Examination of sequences revealed that the D-box present in the HER4 intracellular domain was also seen in HER1/epidermal growth factor receptor (EGFR) but not in HER2 or HER3 (alignment according to ref. 41). To test the functionality of this motif, 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 tetracycline-inducible HeLa-rtTA cell lines.

HeLa-s80^{HER4}, HeLa-s80^{db}, and HeLa-s80^{KD} cells were treated with tetracycline 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. 4*A.1*), consistent with a recent report that s80^{HER4} is an active tyrosine kinase (42). Tyrosine phosphorylation of GFP-s80^{KD} was not observed (Fig. 4*A.1*) as expected. We synchronized tetracycline-treated HeLa-s80^{db} cells in mitosis with nocodazole for 16 h and then released the cells in the presence of cycloheximide at $T = 0$. Expression of GFP-s80^{db} was analyzed at 15-min intervals following nocodazole release/cycloheximide treatment (Fig. 4*A.2*). Strong GFP-s80^{db} expression was detected 45 min following cycloheximide treatment after release into mitosis, unlike GFP-s80^{HER4}, which was undetectable within 15 min following cycloheximide treatment and release into mitosis (see Fig. 3*A*). Cyclin B was readily degraded on nocodazole release in HeLa-s80^{db} cells, suggesting that GFP-s80^{db} did not impair the APC/C or proteasomal machinery (Fig. 4*A.2*).

Table 1. Protein sequence of human cyclin B1 (National Center for Biotechnology Information accession number NM_031966) aligned with that of human HER4/s80^{HER4}, human HER1, human HER2, and human HER3

Protein name	Amino acid no.*	Sequence
Cyclin B1	40–60	RP***R****TA****L****GDIG****N***KVSEQLQAKM
HER4/s80^{HER4}	990–1,010	DD***R****MK****L****PSPN****D***SKFFQNLLDE
HER4^{db}/s80^{db}	990–1,010	DD***a****MK****a****PSPN****D***SKFFQNLLDE
HER1/EGFR	960–980	DE***R****MH****L****PSPT****D***SNFYRALMDE
HER2/ErbB2	992–1,012	ED-LGP-ASPLDSTFYRSLLED
HER3/ErbB3	930–950	SGPGIAPGPEPHGLTNKKLEE

NOTE: Residues defining the D-box are bolded and italicized. Site-directed mutagenesis was done to generate R-to-A and L-to-A mutations, shown in lowercase.
*Alignment of ErbB sequences according to ref. 40.

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

To examine the progression of HeLa-s80, HeLa-s80^{db}, and HeLa-s80^{KD} cells through the cell cycle over a 24-h period, tetracycline-treated cells were synchronized in S phase with hydroxyurea and then released into medium + tetracycline at $T = 0$ h. Cells were collected at 0, 2, 4, 8, 12, and 24 h following hydroxyurea release and 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 G_2-M, and into G_1 as a function of time. At $T = 0$ h, all cells harbored DNA content between 2N and 4N, suggesting that hydroxyurea-mediated synchronization in S phase was complete for each cell line (Fig. 4*D*). 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 hydroxyurea release, HeLa-s80^{db} cells and HeLa-s80^{HER4} cells harbored a greater proportion of cells with 4N DNA compared with HeLa-s80^{KD} cells, suggesting that HeLa-s80^{KD} cells progressed through G_2-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} (57.8 ± 3.1%) > HeLa-s80^{HER4} (14.6 ± 2.6%) > HeLa-s80^{KD} (4.8 ± 1.2%)], 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 G_1.

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 HER4^{db} by site-directed mutagenesis and stably expressed HER4^{db} in HeLa cells. HRG stimulation of HeLa HER4^{db} cells resulted in tyrosine phosphorylation of HER4^{db} and production of s80^{db} (Fig. 5*A*), 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. Immunolocalization 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} similar to full-length, wild-type HER4 (Fig. 5*B*). 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

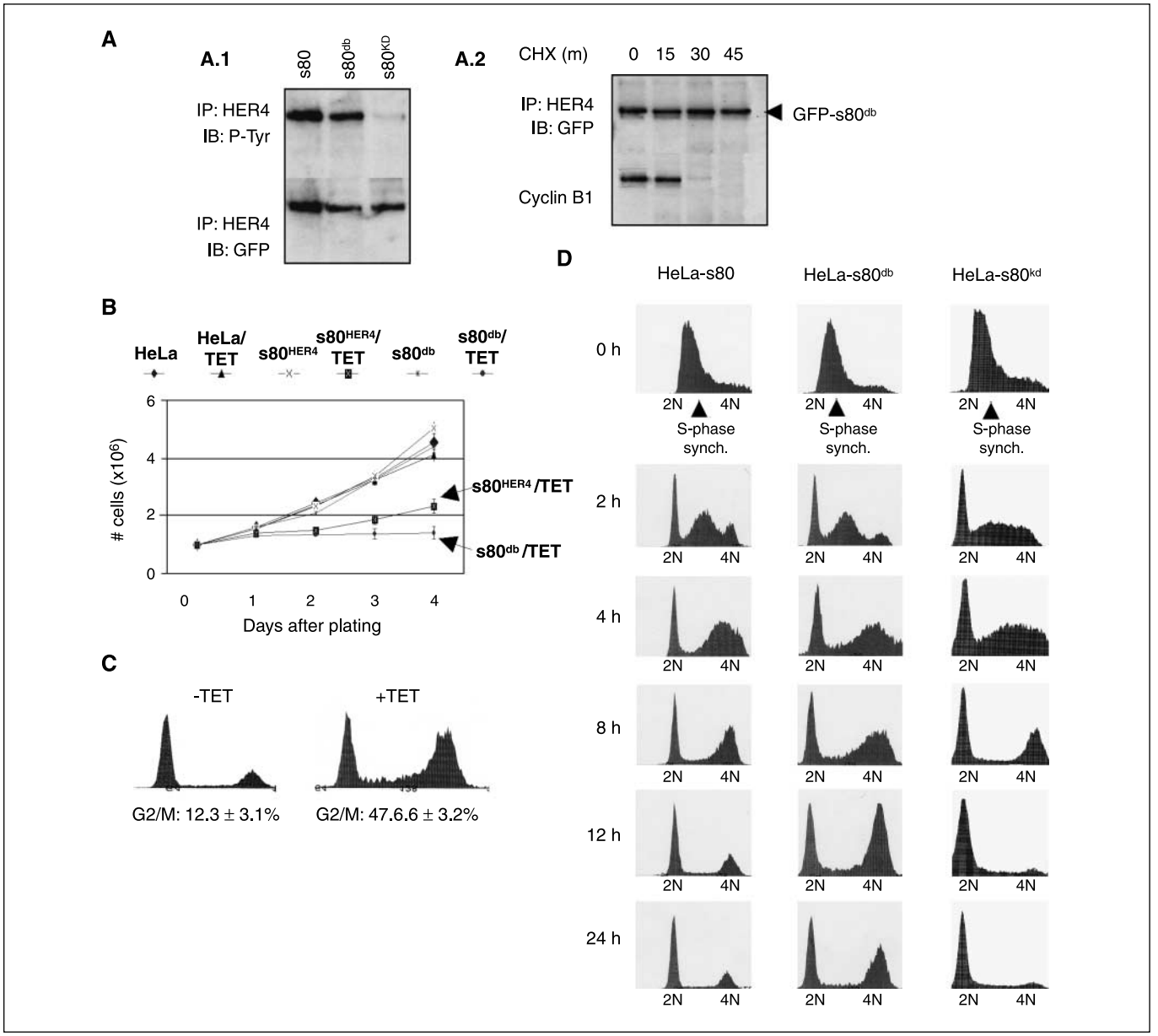

Figure 4. Mutation of the s80^{HER4} D-box eliminates mitotic degradation of s80^{HER4} and enhances s80^{HER4}-mediated growth inhibition. *A,* mutation of the s80^{HER4} D-box stabilized s80^{HER4} during mitosis. *A.1,* Western blot analysis of HER4 immunoprecipitates from HeLa-s80, HeLa-s80^{db}, and HeLa-s80^{KD} cells cultured 24 h + tetracycline. Immunoprecipitates were analyzed for phosphorylated tyrosine or GFP. *A.2,* HeLa-s80^{db} cells were cultured + nocodazole/+ tetracycline (16 h). Cells were released + tetracycline/+ cycloheximide at $T = 0$, and extracts were 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 ± tetracycline. Cells were counted at 1-d intervals through 4 d. $P = 0.011$, comparing cell number of HeLa-s80^{HER4} + tetracycline at day 4 with cell number of HeLa-s80^{db} + tetracycline at day 4, $n = 5$, analyzed in triplicate. *C,* cell cycle analysis of HeLa-s80^{db} cells cultured ± tetracycline (48 h). Nuclei (>10,000) were analyzed per condition ($n = 3$). Representative histograms. *D,* cells were cultured + hydroxyurea/+ tetracycline for 16 h to synchronize in S phase. Cells were released + tetracycline at $T = 0$ h. Cells were collected at 0, 2, 4, 8, 12, and 24 h following hydroxyurea release. Cells were stained with propidium iodide and analyzed by flow cytometry as described above.

mitosis using nocodazole for 16 h and then serum starved in the presence of nocodazole for an additional 16 h. Cells were treated with HRG 1 h before release. Finally, cells were released from nocodazole in the presence of HRG and cycloheximide to examine the stability of proteolytically derived $s80^{HER4}$ and $s80^{db}$ at 15-min intervals. Whereas $s80^{db}$ was detected through 45 min following release from nocodazole, $s80^{HER4}$ was not detected in cells released during mitosis (Fig. 5*C*).

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* (43). We used a similar approach, transfecting HC11 cells with a cDNA construct encoding the PyVmT. A clonal population of HC11-PyVmT (HC11P) cells was then transfected with pcDNA4-$s80^{HER4}$, pcDNA4-$s80^{KD}$, pcDNA4-$s80^{db}$, or empty pcDNA4. Cells (1×10^6) were injected into the inguinal mammary fat pads of 6-week-old female BALB/c mice. Fifteen weeks following injection, the injected mammary glands were examined histologically. HC11P-pcDNA4 cells formed solid, poorly differentiated tumors (Fig. 6*A*). In contrast, tumors expressing $s80^{HER4}$ were smaller, harboring numerous differentiated epithelial structures organizationally similar to lobuloalveolar 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. 6*B*), whereas HC11P-s80 tumors exhibited 3-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. 6*A*). These did not seem to have malignant elements, and these structures exhibited the lowest percentage of $PCNA^+$ cells (9%; Fig. 6*B*), with a statistically significant decrease in PCNA staining compared with 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 terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis (Fig. 6*B*). 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 $s80^{HER4}$.

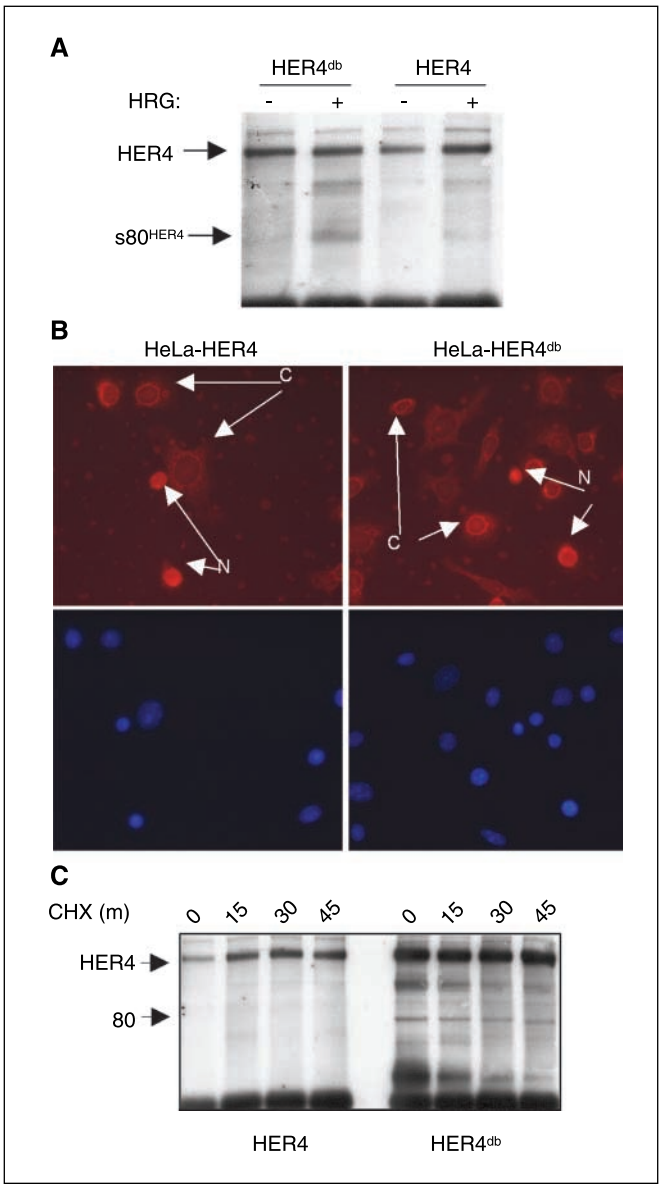

Figure 5. Mutation of the D-box within full-length HER4 does not reduce $s80^{HER4}$ production but does stabilize $s80^{HER4}$. *A,* HeLa cells stably transfected with pLXSN-HER4 or pLXSN-$HER4^{db}$ were serum starved 16 h and then treated + HRG (1 h). HER4 immunoprecipitates were analyzed for total HER4. *B,* cells were serum starved (16 h) and treated + HRG (1 h). Cells were analyzed for HER4 localization by immunocytofluorescence. *Bottom,* DAPI staining. *N,* nuclear localization of HER4; *C,* cytoplasmic localization of HER4. *C,* HeLa-HER4 and HeLa-$HER4^{db}$ cells were cultured in 10% serum + nocodazole (16 h) and then in serum-free medium + nocodazole (16 h), adding HRG for the final hour. Cells were released into serum-free medium + HRG/+ cycloheximide at $T = 0$ and harvested at $T = 0$, 15, 30, and 45 min. HER4 immunoprecipitates were analyzed as described above.

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 to localize to the nucleus. Our studies show the destruction of $s80^{HER4}$ during mitosis by the cell cycle machinery, representing a novel regulatory mechanism for a RTK, and may relate directly to the ability of HER4 to slow cell growth by inhibiting progression through G_2 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 mitosis. For example, cyclin B1 in mammalian cells is degraded before anaphase (44) 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 or cell division (45, 46). Similarly, mutation of the $s80^{HER4}$ D-box impaired its destruction and decreased the rate of G_2-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 G_2-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 G_2-M transition or progression through early mitosis.

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 shown s80^{HER4} growth-inhibitory action (21, 33, 37) and that HRG-mediated HER4 activity, as well as *de novo* expression of s80^{HER4}, slows the progression of cells through G_2-M. The data presented herein suggest 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 cofactors. These include a positive effect on ER-mediated gene transcription in ER-positive breast cancer cells (47), on signal transducers and activators of transcription 5A (STAT5A)-dependent gene expression in differentiating mammary epithelial cells, and on repression of astrocyte-specific gene transcription in astrocyte progenitor cells in the developing mouse brain (48). In these studies, s80^{HER4} colocalized in complexes on specific DNA elements [e.g., on the progesterone receptor promoter with the ER or the glial fibrillary acidic protein promoter with TAB2 and N-Cor (47, 48)]. Additionally, s80^{HER4} may enhance transcription factor activity through kinase-mediated phosphorylation of target proteins. 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 G_2-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 HER4 correlates with positive prognostic indicators. We have shown the HER4 activation (9) or s80^{HER4} expression (see Fig. 1; ref. 21) inhibited breast cancer cell growth by decreasing the progression of cells through G_2-M (21). Furthermore, we have shown (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^{VA}; 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. Because the D-box is required for degradation during mitosis, it is possible that s80^{HER4} impairs growth of cells by delaying the cell cycle in mitosis such that degradation of s80^{HER4} is required for mitotic progression. Although this hypothesis requires further investigation, these data show the potential link between nuclear

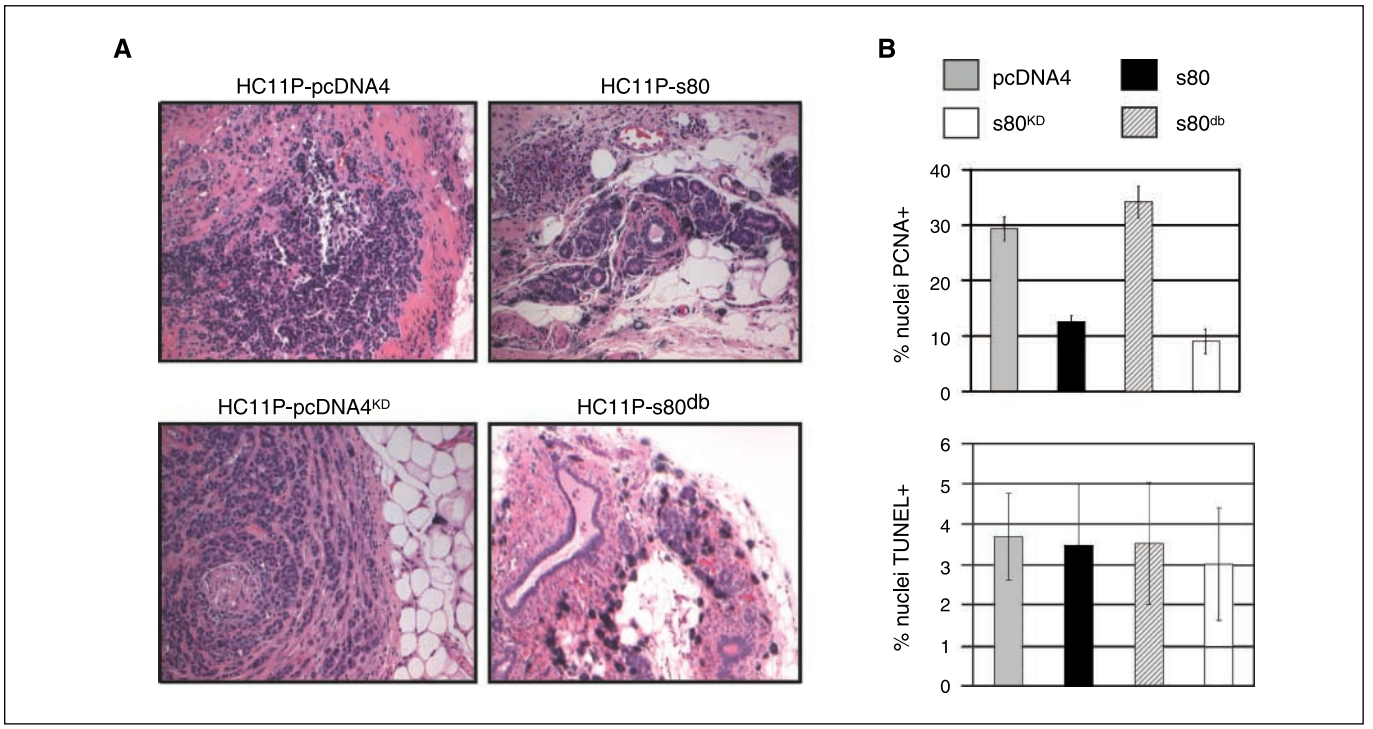

Figure 6. Decreased tumor formation in PyVmT-transformed HC11 cells expressing s80^{db}. *A,* HC11P cells expressing s80^{HER4}, s80^{KD}, s80^{db}, or the empty pcDNA4 vector were injected into the inguinal mammary fat pads of female BALB/c mice. Fifteen weeks after implantation of cells, mice were euthanized and the mammary glands were analyzed histologically. Representative images. *B,* five tumor samples per group were analyzed by immunohistochemistry. PCNA-positive (*top*) or TUNEL-positive (*bottom*) nuclei were scored in three 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; $P < 0.0001$) compared with vector alone, whereas 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 with HC11P-s80 ($P < 0.037$).

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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HER4 D-Box Sequences Regulate Mitotic Progression and Degradation of the Nuclear HER4 Cleavage Product s80 HER4

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