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Hepatocyte Growth Factor-regulated Tyrosine Kinase Substrate (HRS) Interacts with PELP1 and Activates MAPK*

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PELP1 (proline-, glutamic acid-, and leucine-rich protein-1) (also known as the modulator of nongenomic activity of estrogen receptor) plays a role in genomic functions of the estrogen receptor via histone interactions and in nongenomic functions via its influence on the MAPK-Src pathway. However, recent studies have shown that differential compartmentalization of PELP1 could play a crucial role in modulating the status of nongenomic signaling by using molecular mechanisms that remain poorly understood. Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is an early endosomal protein that plays a role in regulating the trafficking of growth factor-receptor complexes through early endosomes. By using a yeast two-hybrid screen, we identified HRS as a novel PELP1-binding protein providing evidence of a physiologic interaction between HRS and PELP1. The noted HRS-PELP1 interaction was accompanied by inhibition of the basal coactivator function of PELP1 upon estrogen receptor transactivation. HRS was found to sequester PELP1 in the cytoplasm, leading to the activation of MAPK in a manner that is dependent on the epidermal growth factor receptor but independent of the estrogen receptor, Shc, and Src. In addition, stimulation of MAPK and the subsequent activation of its downstream effector pathway, Elk-1, by HRS or PELP1 were found to depend on the presence of endogenous PELP1 or HRS. Furthermore, HRS was overexpressed and correlated well with the cytoplasmic PELP1, increased MAPK, and EGFR status in breast tumors. These findings highlight a novel role of HRS in up-regulating MAPK, presumably involving interaction with PELP1.

PELP1 (proline-, glutamic acid-, and leucine-rich protein-1), a novel steroid receptor coregulator with 10 nuclear receptor L*XX*LL motifs, participates in both transcriptional regulation and nongenomic stimulation of Akt and MAPK[3] and localizes in both nuclear and cytoplasmic compartments (1–3). PELP1 is identical to the modulator of nongenomic activity of the estrogen receptor, which has been implicated in the nongenomic stimulation of the Src/MAPK pathways by the estrogen receptor (ER) (4). PELP1 is predominantly localized in the cytoplasm in a significant proportion of the human breast (5), endometrial (3), and salivary gland carcinomas (6), so PELP1-induced nongenomic signaling is expected to have physiological implications in breast cancer tumorigenesis. Although PELP1 has been implicated in the nongenomic stimulation of signaling kinases and interacts with Src, the nature of the upstream determinants of its nongenomic action remains unknown.

To understand better the cellular functions of PELP1 in breast cancer cells, we performed a yeast two-hybrid screen to clone PELP1-interacting proteins from the human mammary gland cDNA library. One of the several isolates was identified as the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), which was originally identified as a major tyrosine-phosphorylated protein in melanoma cells stimulated with hepatocyte growth factor. Subsequent studies established that HRS is ubiquitously expressed, and its phosphorylation is induced by a variety of polypeptide growth factors and cytokines (7, 8). HRS is an essential protein, and targeted disruption of HRS in mice embryos is lethal (9). HRS consists of a VHS domain, a clathrin box domain, a phosphatidylinositol 3-phosphate-interacting FYVE domain, two coiled-coiled domains, a proline domain, and a proline/glutamine-rich domain. HRS is localized in both cytosolic and membrane-associated compartments, and such targeting is presumably regulated by the intracellular level of phosphatidylinositol 3-phosphate, leading to phosphatidylinositol 3-phosphate interaction with the FYVE domain and membrane targeting (10). In addition, HRS also interacts with signaling and trafficking molecules, such as STAM, Smad2, EPS15, SNAP25, and clathrin (11–15). HRS is localized both in the early endosomes and cytosol, and it is widely believed to act as an integrator of vesicular trafficking and signaling (16, 17) and thus is implicated in endosomal EGFR sorting (18). In *Drosophila*, HRS promotes down-regulation of multiple signaling pathways, and *Drosophila* epithelial cells lacking HRS show elevated signaling components, because of a defect in sorting and degradation of receptors (19). More importantly, HRS-mediated receptor degradation in *Drosophila* was found to be ligand-independent (20). Furthermore, HRS has been also shown to play a role in interleukin-2-mediated c-*fos* induction of B cells (21). Although HRS plays an important role in signaling and protein routing, it remains unknown whether HRS could also influence the strength of the MAPK pathway in cancer cells.

In this study, we found that HRS directly interacts with PELP1 and impairs its nuclear ER transactivation function, and it plays an essential role in supporting the ability of PELP1 to stimulate MAPK in a manner independent of Src and Shc. HRS-mediated MAPK stimulation was accompanied by increased secondary genomic responses, such as Elk-1 activation. In addition, HRS was up-regulated in human breast tumors with increased MAPK, EGFR, and cytoplasmic PELP1. For the first time, these findings have exposed a novel connection among HRS, PELP1,

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; ER, estrogen receptor; EGFR, epidermal growth factor receptor; HRS, hepatocyte growth factor regulated tyrosine kinase substrate; EGF, epidermal growth factor; PBS, phosphate-buffered saline; siRNA, small interfering RNA; ERE, estrogen response element; DCC, dextran charcoal-stripped; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo-[3,4-*d*]pyrimidine.

and MAPK and revealed the existence of regulatory interactions between HRS and functions of PELP1 in breast cancer cells.

MATERIALS AND METHODS

Cell Cultures and Reagents—MCF-7, HeLa, COS-7, MDA-MB-435, MDA-MB-453, MDA-MB-468, ZR-75, and MCF-7/PELP1 clones were maintained in Dulbecco's modified Eagle's medium/F-12 (1:1) supplemented with 10% fetal bovine serum. Antibodies against vinculin, FLAG, epidermal growth factor (EGF), and the steroid hormone 17β-estradiol were purchased from Sigma. Antibodies against AKT, phospho-AKT, and phospho-p44/42(Thr-202/Tyr-204) were purchased from Cell Signaling (Beverly, MA). Antibodies for EGFR was from Neomarkers (Fremont, CA). Erk-1 and -2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for Src and the specific Src kinase inhibitor PP2 was purchased from Calbiochem. HRS antibody was purchased from Alexis Biochemicals (San Diego, CA). Antibody against T7 was purchased from Novagen. Charcoal-stripped serum (DCC serum) and ICI 182,780 was purchased from Sigma. Iressa (ZD1839) was from AstraZeneca (Macclesfield, UK).

Cell Extracts, Immunoblotting, and Immunoprecipitation—Cell lysates were prepared using Triton X-100 buffer (50 mM Tris-HCl, (pH 7.5), 100 mM NaCl, 0.5% Triton X-100, 1× protease inhibitor mixture, and 1 mM sodium vanadate). The lysates were centrifuged in an Eppendorf centrifuge at 4 °C for 15 min. Cell lysates containing equal amounts of protein (100 μg) were resolved on SDS-polyacrylamide gels (8% acrylamide), transferred to nitrocellulose membranes, probed with the appropriate antibodies, and developed using either the enhanced chemiluminescence method or the alkaline phosphatase-based color reaction method. Immunoprecipitation was performed for 4 h at 4 °C using 1 μg of antibody/mg of protein.

Small Interfering RNA (siRNA)—HRS-specific, PELP1-specific, Shc-specific, and control nonspecific siRNA were purchased from Dharmacon (Lafayette, CO). siRNA transfections were performed using 100 nM of pooled siRNA duplexes and 4 μl of Oligofectamine (Invitrogen) according to the manufacturer's protocol in 6-well plates. After 72 h, cells were prepared for Western blotting and luciferase assay.

Confocal Microscopy and Immunohistochemistry—The cellular localization of various proteins was determined by indirect immunofluorescence, as described previously (1). Briefly, cells were grown on glass coverslips and fixed with 4% paraformaldehyde. Cells were incubated with the primary antibodies, washed three times in PBS, and then incubated with secondary antibodies conjugated with Alexa-546 (red) and Alexa-488 (green). Cells treated with only the secondary antibodies served as controls. Confocal scanning analysis was performed by using an Olympus FV300 laser-scanning confocal microscope according to established methods. Each section was examined for each stain at three excitations (488, 546, and 633 nm), and the data were compared pixel by pixel. Untransfected and HRS-transfected cells were examined for the reduction of nuclear PELP1. Cells were grouped into two groups, less than 50% reduction of nuclear PELP1 and more than 50% reduction of nuclear PELP1, compared with the intensity of the cells expressing the highest amount of nuclear PELP1 in that field. Counts were added and plotted in a bar graph.

Immunohistochemical detection of HRS, PELP1, EGFR, and phospho-MAPK was carried out in well studied breast cancer sections (2). Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized with xylene and rehydrated using graded ethanol solutions. Sections were incubated in 0.3% hydrogen peroxide and methanol for 30 min to inactivate the endogenous peroxidase. The sections were then boiled for 10 min in 0.01 M citrate buffer and cooled for 30 min at room temperature to expose antigenic epitopes. The sections were blocked with 2% normal goat serum in 1% bovine serum albumin and PBS for 30 min and then incubated overnight at room temperature with primary antibodies against HRS (1:250 dilution (Alexis), PELP1 (1:500), and EGFR (1:50 dilution (Neomarkers)) and phospho-MAPK (1:50 dilution (Cell Signaling)). Primary antibodies were diluted in diluent buffer (2% normal goat serum, 1% bovine serum albumin, and PBS). For HRS and PELP1, the sections were washed three times with 0.05% Tween in PBS for 10 min, incubated with horseradish peroxidase/secondary antibody at a 1:100 dilution (Amersham Biosciences) for 1 h, and then washed three times with 0.05% Tween in PBS for 10 min. For phospho-MAPK and EGFR, the sections were washed three times with 0.05% Tween in PBS for 10 min, incubated with a biotinylated secondary antibody at a 1:100 dilution (Vector Laboratories) for 1 h, then washed three times with 0.05% Tween in PBS for 10 min, incubated with a streptavidin horseradish peroxidase reagent (Dako Corp., Carpinteria, CA) for 15 min, and washed three times with 0.05% Tween in PBS for 10 min. The sections were then developed with diaminobenzidine/H_2O_2 and counterstained with Mayer's hematoxylin.

Reporter Gene Assays—For reporter gene transient transfections, cells were cultured for 48 h in minimal essential medium without phenol red, containing 5% DCC serum. Next, 200 ng of estrogen-response element-luciferase reporter constructs were cotransfected with or without 0.5 μg of HRS and PELP1 plasmid using the FuGENE 6 reagent according to the manufacturer's protocol (Roche Applied Science). Twenty four hours later, cells were treated with estrogen (10^{-9} M) for 24 h. Cells were then lysed with passive lysis buffer, and the luciferase assay was performed using a kit (Promega, Madison, WI). β-Galactosidase activity was used to normalize the transfection. Each transfection was performed in 6-well plates in triplicate. For treatment with EGF, cells were serum-starved for 48 h before being treated. Elk-1 luciferase assay was performed according to the kit protocol (Stratagene, La Jolla, CA).

RESULTS

PELP1 Interacts with HRS in Vitro and in Vivo—To gain insights about novel functions of PELP1, and a potential biochemical basis of its cytoplasmic localization, we performed a yeast two-hybrid screen to identify proteins that interact with PELP1. Screening of a mammary gland cDNA expression library with the N-terminal region of PELP1 as bait resulted in the isolation of several positive clones. One of the positive clone sequences matched HRS. The specificity of the HRS and PELP1 interaction was confirmed by the conventional survival assay in selection medium where transformed colonies showed the ability to grow in medium lacking adenosine, histidine, tryptophan, and leucine. Cells cotransformed with the control GBD vector did not grow. To map the region of PELP1 that interacts with HRS, we cotransformed GAD-HRS along with the GBD-PELP1 fusion constructs (amino acids 1–400, 400–600, 600–880, and 960–1130) into yeast cells, and the interaction was assessed by monitoring the growth of yeast cells on selective media. These results suggested that HRS interacts with PELP1 in the region of amino acids 1–400 (Fig. 1*A*).

To confirm the interaction between PELP1 and HRS, we next sought to determine *in vivo* interaction between HRS and PELP1. Currently available HRS and PELP1 (2) antibodies are not suitable for immunoprecipitation assays, so we transiently transfected HeLa cells with T7-HRS to verify the interaction between HRS and PELP1 in mammalian cells. The cell lysates were immunoprecipitated with T7 antibody and subjected to Western blotting with PELP1 or T7 antibodies (Fig. 1*B*). Similarly, cell lysates from well characterized MCF-7/T7-PELP1

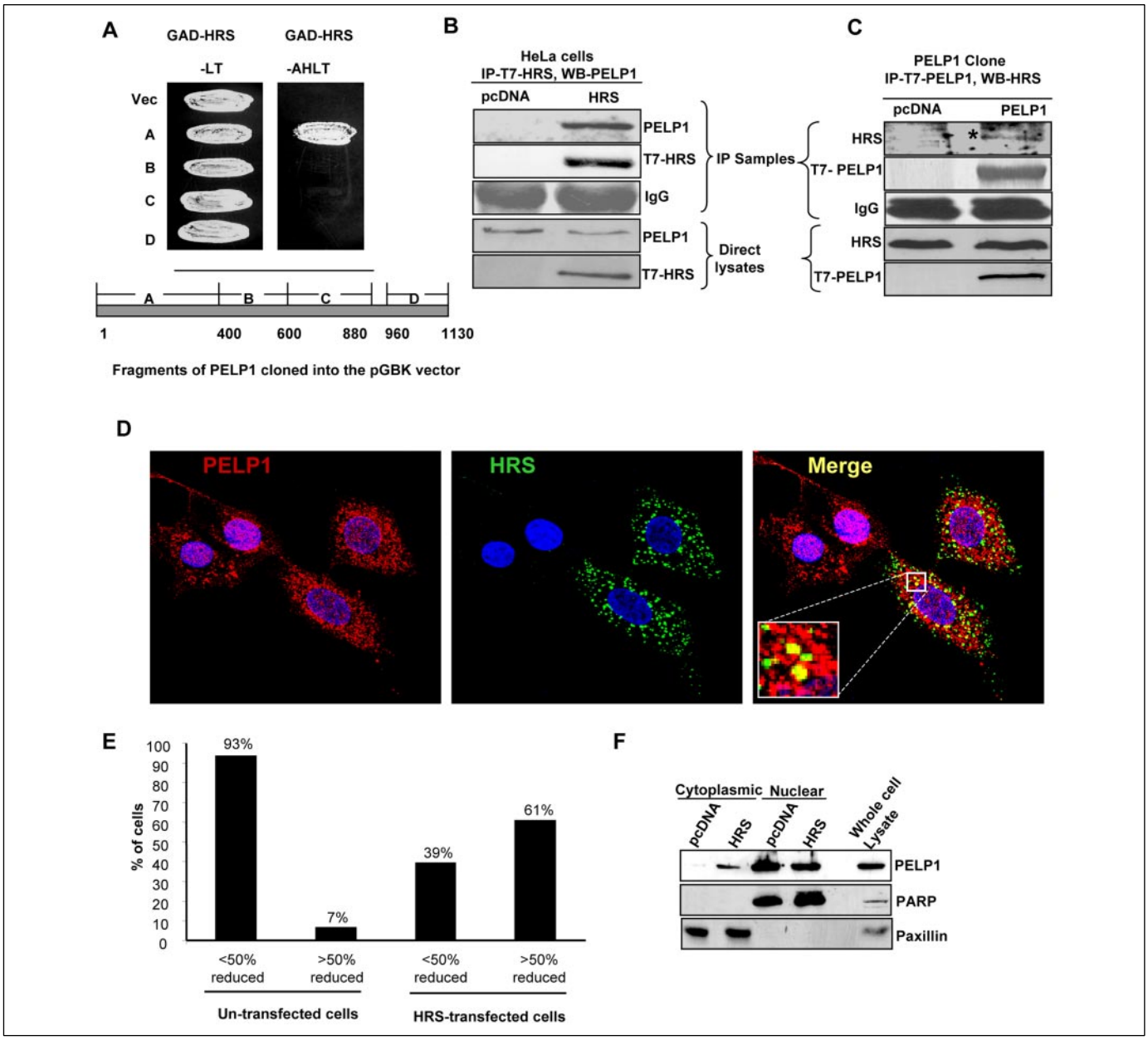


FIGURE 1. **PELP1 interacts with HRS.** *A,* yeast cells were cotransfected with a control Gal4 activation domain (*GAD*) vector or GAD-HRS. Gal4 activation domain fusions of various lengths of PELP1 were used to determine the PELP1 binding region in HRS. Growth was recorded after 72 h on selection plates lacking leucine and tryptophan (−*LT*) or adenosine, histidine, leucine, and tryptophan (−*AHLT*). Schematic representations of fragments of PELP1 cloned are shown in the *bottom panel*. *Vec*, vector. *B,* total cellular lysates from HeLa cells transfected with T7-HRS were immunoprecipitated (*IP*) with T7 antibody, after which Western analysis was performed for T7-HRS and PELP1. *C,* total cellular lysate from MCF-7 cells stably expressing pcDNA or T7-PELP1 were immunoprecipitated with a T7 antibody, after which Western analysis (*WB*) was performed for HRS and T7-PELP1. *D,* HRS sequesters PELP1 in the cytoplasm. MCF-7 cells were grown on coverslips and transiently transfected with myc-HRS. Cells were fixed with 4% paraformaldehyde. Coverslips were incubated with anti-PELP1 and anti-Myc antibodies and then incubated with secondary antibodies conjugated with Alexa-546 (*red*) and Alexa-488 (*green*), respectively. *Yellow* indicates colocalization. *E,* untransfected and HRS-transfected cells were examined for the reduction of nuclear PELP1. Cells were grouped into two groups, less than 50% reduction of nuclear PELP1 and more than 50% reduction of nuclear PELP1, compared with the intensity of the cells expressing the highest amount of nuclear PELP1 in that field. Counts were added up and plotted in a bar graph. *F,* nuclear and cytoplasmic extracts were prepared from MCF-7 cells transfected with pcDNA or HRS and Western blot analysis was performed for endogenous PELP1, paxillin, and poly(ADP-ribose) polymerase (*PARP*).

stable clones (5) were immunoprecipitated with T7 antibody and analyzed by Western blotting with HRS or T7 antibodies (Fig. 1*C*). Together, these results showed that PELP1 interacts with HRS *in vivo*.

HRS Sequesters PELP1 in the Cytoplasm—To confirm further the interaction between HRS and PELP1 *in situ*, we examined the subcellular localization of HRS and PELP1. Results from confocal microscopy of MCF-7 cells transfected with T7-tagged HRS showed that HRS was localized in the cytoplasm (Fig. 1*D*, *middle panel*). In the control cells, PELP1 was localized in the nuclear compartment and in the cytoplasm (Fig. 1*D*, *left panel*) as expected from the previous studies (2). However, when T7-HRS was overexpressed, most of the endogenous PELP1 was sequestered in the cytoplasm (Fig. 1*D*, *right panel*), shown by a reduced nuclear staining and a more intense cytoplasmic staining for PELP1. T7-tagged HRS also colocalized with PELP1 in the cytoplasm of the cell, visualized as *yellow* in Fig. 1*D*. To confirm the observed relocalization of PELP1 by HRS overexpression, we quantitated the reduction of nuclear PELP1 in multiple fields of HRS transfected as well as control vector-transfected cells. As shown in Fig. 1*E*, the number of cells with reduced

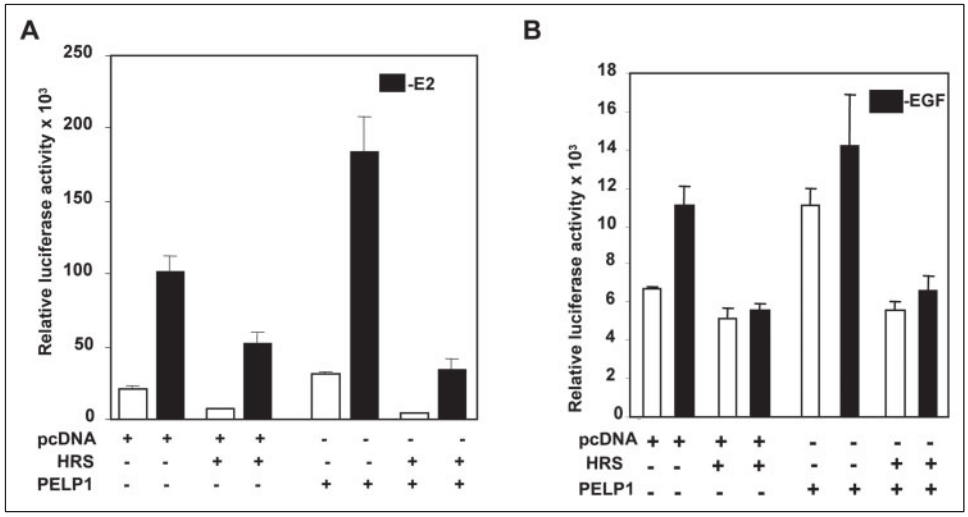


FIGURE 2. **HRS inhibits basal ER transcription functions of PELP1.** *A,* MCF-7 cells were cotransfected with the ERE-luciferase reporter with or without HRS and PELP1 in 5% DCC media. After 24 h, the cells were treated with estradiol (*E2*) (10^{-9} M) for 24 h, and then the ERE reporter activity was measured. *B,* MCF-7 cells were cotransfected with ERE-luciferase reporter gene along with or without HRS and PELP1. After 24 h, the cells were serum-starved and treated with EGF (100 ng/ml) for 8 h, and the ERE reporter activity was measured. The results shown are representative of three separate experiments.

nuclear PELP1 after HRS overexpression is dramatically increased as compared with untransfected cells. In addition, biochemical fractionation studies from MCF-7 cells transfected with HRS also showed an increased accumulation of PELP1 in the cytoplasm and a corresponding reduced nuclear PELP1 in HRS-transfected cells (Fig. 1*F*). Taken together, these data suggest an important role for HRS in the cytoplasmic sequestration of PELP1.

HRS Inhibits Basal ER Transactivation Functions of PELP1—Recent studies suggest that PELP1 acts as a coactivator of ER (2) and plays an important role in ER genomic functions by interacting with histones and chromatin remodeling (1). HRS sequesters PELP1 in the cytoplasm, so we next sought to determine the effect of its sequestration on nuclear functions of PELP1. To examine this effect, we determined the status of ER transactivation in cells with overexpressed HRS. Cotransfection of PELP1 with HRS significantly reduced basal ER transactivation function of PELP1. However, there was no significant effect of HRS on estradiol-induced fold induction of ER transactivation in the presence of PELP1 (Fig. 2*A*). Because it is known that PELP1 also promotes EGF-mediated ER transactivation (22), we next examined whether HRS had any effect on this activity. Cotransfection of PELP1 with HRS substantially reduced the basal ER transactivation of PELP1 (Fig. 2*B*). These findings suggested that HRS suppresses PELP1 nuclear functions, presumably by its cytoplasmic retention.

HRS Modulation of MAPK Signaling—Recent studies suggest that cytoplasmic PELP1 promotes activation of the MAPK and Akt pathways (5). Based on the finding that HRS sequesters PELP1 in the cytoplasm (Fig. 1*D*), we hypothesized that HRS might up-regulate MAPK and Akt pathways. Expression of FLAG-HRS in MCF-7 cells resulted in a clear up-regulation of phospho-MAPK without any change in the levels of phospho-Akt (Fig. 3*A*). To examine the contribution of endogenous HRS in MAPK activation in MCF-7 cells, we knocked down endogenous HRS by using HRS-specific siRNA. We found reduced levels of phosphorylated MAPK in cells with depleted HRS (Fig. 3*B*), suggesting a role for HRS in the stimulation of MAPK activation. Because we identified HRS as a PELP1-interacting protein, we next examined whether PELP1 and HRS cooperated in stimulating MAPK activity. As shown in Fig. 3*C*, HRS expression in MCF-7/PELP1 cells was accompanied by a significant up-regulation of phosphorylated MAPK. However, the extent of MAPK activation after the stimulation with the ligand was similar with or without HRS.

To validate these findings, we next knocked down HRS in MCF-7/PELP1 cells. Most interestingly, we noticed that depletion of HRS in MCF-7/PELP1 cells resulted in a significant reduction in the level of phospho-MAPK (Fig. 4*A*), suggesting a role of HRS in the earlier reported up-regulation of phospho-MAPK by PELP1. Consistent with this, we found that depletion of endogenous PELP1 in MCF-7 compromised the ability of HRS to stimulate MAPK activation (Fig. 4*B*).

The observed MAPK stimulation by HRS was independent of ER, as pure anti-estrogen ICI 182,780 had no effect on the levels of phospho-MAPK induced by HRS (Fig. 5*A*). HRS up-regulation of phospho-MAPK was also observed in ER-negative MDA-MB-435 and COS-7 cells, further confirming that it is ER-independent (Fig. 5*B*). These observations suggested that both PELP1 and HRS are required for the efficient stimulation of phospho-MAPK in cancer cells.

Receptor tyrosine kinases activate MAPK through an Shc-mediated pathway (23–25), and HRS up-regulates phospho-MAPK in exponentially growing breast cancer cells. These findings together raise the possibility that the growth factor pathway is involved in the up-regulation of phospho-MAPK by HRS. However, we found that serum deprivation had no effect on the ability of HRS to stimulate MAPK (Fig. 6*A*). Next, we examined the effect of depleting Shc on the levels of phospho-MAPK activation in cells with HRS up-regulation. As expected, Shc down-regulation was accompanied by reduced MAPK activation; however, the extent of MAPK activation by HRS was similar in cells with or without depleted Shc (Fig. 6*B*). PELP1 has been shown to activate MAPK via its interaction with Src kinase (4), and because HRS is a PELP1-interacting protein, so we sought to determine whether HRS-mediated MAPK stimulation requires Src kinase. Transfection of Syf cells (Src-null cells) with FLAG-HRS resulted in up-regulation of MAPK (Fig. 6*C*), suggesting that HRS-mediated MAPK up-regulation is independent of the ability of PELP1 to interact with Src kinase. This was further confirmed by using a specific Src kinase inhibitor (PP2) in MCF-7 cells transfected with HRS. PP2 reduced the basal level of phosphorylation, although there was no effect on HRS-induced MAPK activation (Fig. 6*D*). However, the observed HRS-mediated stimulation of MAPK was effectively blocked by UO126, a widely used inhibitor of MEK, which is an upstream activator of MAPK (Fig. 6*E*). These results suggest a role for direct upstream components of MAPK in its stimulation by HRS. PELP1 can interact with EGFR (22), and HRS has been shown to promote EGFR accumulation (18). We hypothesized that EGFR might be an essential component of the observed MAPK stimulation by HRS. To test this possibility, we examined the effect of HRS overexpression in EGFR-negative MDA-MB-453 cells (26). We found no evidence of MAPK stimulation by HRS in these cells (Fig. 6*F*), sug-

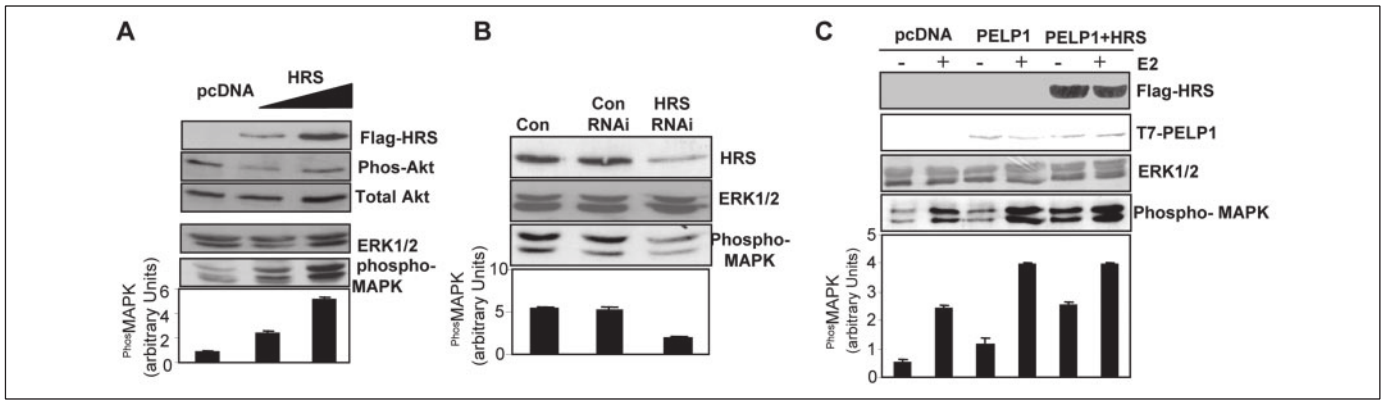
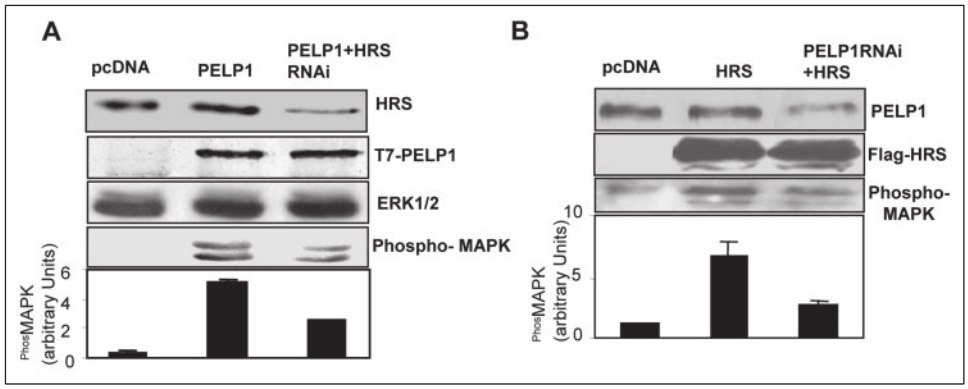


FIGURE 3. **HRS selectively modulates MAPK activation.** *A,* exponentially growing MCF-7 cells in 10% serum were transfected with or without HRS, and total lysates were immunoblotted with a phospho-MAPK-specific antibody. *B,* MCF-7 cells were transfected with control or HRS-specific siRNA. After 48 h, cell lysates were immunoblotted with a phospho-MAPK-specific antibody. *C,* MCF-7 cells stably expressing pcDNA or T7-PELP1 were transfected with or without FLAG-HRS and treated with estradiol (*E2*) for 10 min, and MAPK activation was analyzed by Western blotting analysis using a phospho-specific antibody. The results shown are representative of three separate experiments. *Con*, control; *RNAi*, RNA interference.

FIGURE 4. **HRS requirement for MAPK activation.** *A,* MCF-7 cells stably expressing pcDNA or PELP1 were transfected with control or HRS siRNA. After 48 h, MAPK activation was analyzed by Western blotting. *B,* MCF-7 cells transfected with or without FLAG-HRS were again transfected with the control or PELP1-specific siRNA, and total lysates were immunoblotted with a phospho-MAPK-specific antibody. The results shown are the representative of three separate experiments. *RNAi*, RNA interference.



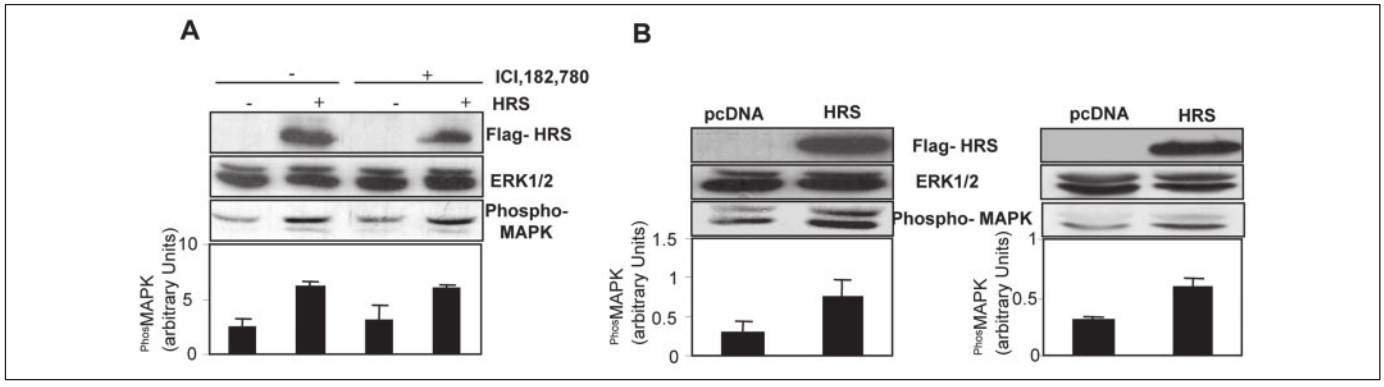


FIGURE 5. **HRS regulation of MAPK is ER-independent.** *A,* MCF-7 cells transfected with FLAG-HRS were treated with or without ICI 182,780 (10^{-8} M) for 48 h. Total cell lysates were subjected to Western blotting using phospho-MAPK antibody. *B,* MDA-MB-435 and COS-7 cells were transfected with or without FLAG-HRS, and total lysates were immunoblotted with a phospho-MAPK-specific antibody.

gesting a role for EGFR in HRS stimulation of MAPK. Consistent with a role for EGFR in HRS stimulation of MAPK, the activation of MAPK was suppressed by the EGFR inhibitor Iressa in EGFR-positive MDA-MB-468 cells (Fig. 6*G*), suggesting that EGFR is required for the HRS-induced stimulation of MAPK in breast cancer cells.

HRS Interaction with PELP1 Is Essential for HRS-induced MAPK Activation—To provide more insight to the significance of the HRS-PELP1 interaction, we mapped the regions of HRS that interact with PELP1 in glutathione *S*-transferase pulldown assay. Results showed that 391–600 amino acids of HRS interact with PELP1 (Fig. 7*A*). To investigate whether HRS interaction with PELP1 is required to activate MAPK by HRS, we transfected MCF-7 cells with HRS deletion constructs (HRS amino acids 1–390 and 391–600) that do or do not bind to PELP1, and the MAPK activation was analyzed by Western blotting. Results showed that HRS amino acids 1–390 that do not bind to PELP1 cannot activate MAPK, whereas the region that binds to PELP1(HRS amino acids 391–600) activate MAPK (Fig. 7*B*). These findings implicate the role of HRS-PELP1 interaction in MAPK activation.

Biologic Effect of the HRS-induced MAPK Activation—To provide evidence that the HRS-MAPK pathway is biologically relevant in breast cancer cells, we evaluated the role of MAPK in the activation of Elk-1, a downstream target transcriptional factor that is phosphorylated and activated by MAPK (27). To study the effect of HRS on the MAPK-Elk-1 pathway, we used a well characterized reporter system using a pFA2-

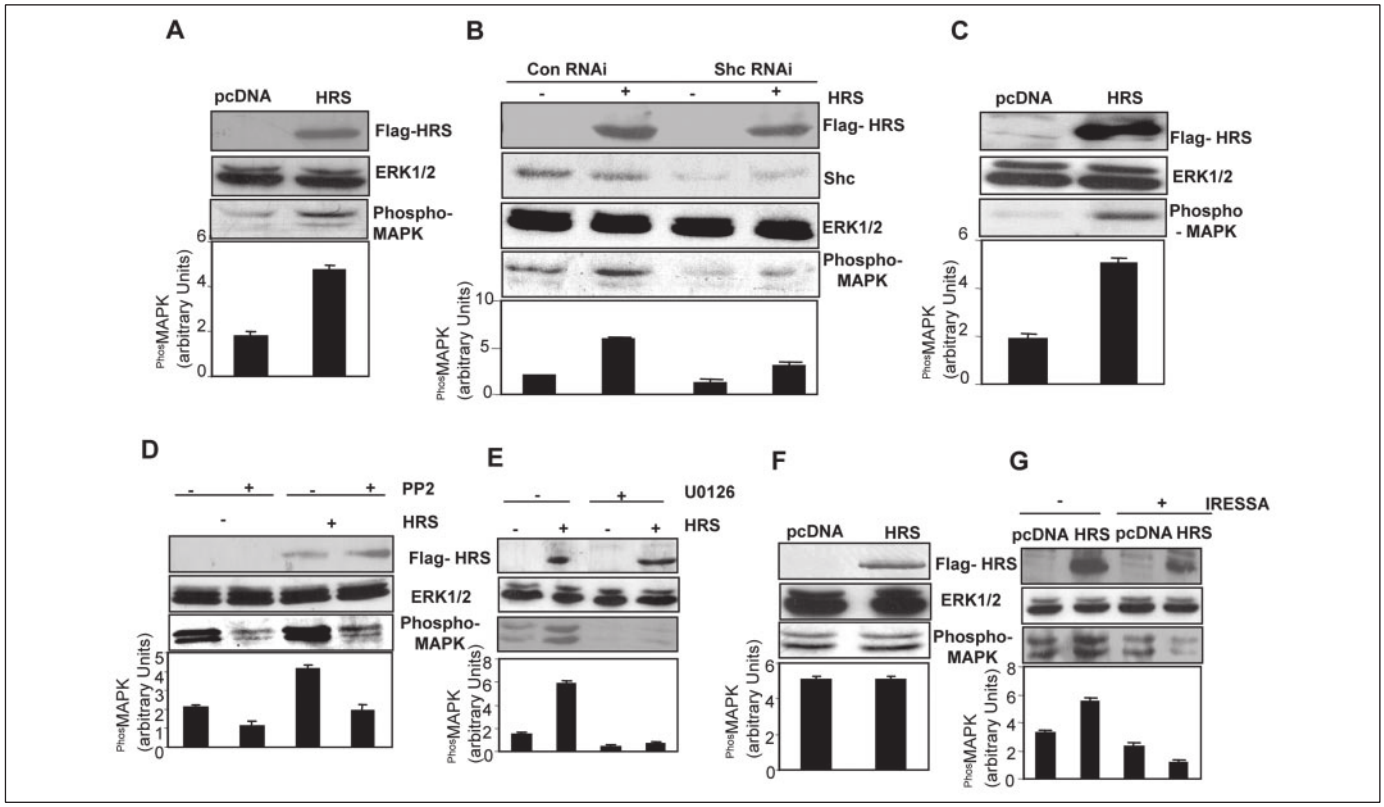


FIGURE 6. **HRS regulates MAPK activation independent of Shc and Src pathway.** *A,* MCF-7 cells were transfected with or without HRS and serum-starved for 48 h, and total lysates were immunoblotted with a phospho-MAPK-specific antibody. *B,* MCF-7 cells transfected with or without FLAG-HRS were transfected with the control or Shc-specific siRNA, and total lysates were immunoblotted with a phospho-MAPK-specific antibody. *Con*, control; *RNAi*, RNA interference. *C,* murine fibroblast cells deficient of src kinase (SYF cells) were transfected with or without HRS, and MAPK phosphorylation was analyzed by immunoblotting. *D,* MCF-7 cells transfected with or without HRS were treated with Src kinase inhibitor PP2 for 30 min. Activation status of MAPK was analyzed by Western blotting. *E,* MCF-7 cells were transfected with HRS and treated with or without the MEK inhibitor UO126 for 1 h, and MAPK activation was analyzed by Western blotting. *F,* MDA-MB-453 cells negative for EGFR were transfected with or without HRS, and MAPK activation was analyzed by Western blotting. *G,* EGFR-positive MDA-MB-468 cells were transfected with or without HRS and treated with Iressa for 1 h, and MAPK activation was analyzed by Western blotting. The results shown are the representative of three separate experiments.

Elk-1 plasmid and pFR-luciferase along with HRS in MCF-7 and ZR-75 cells. We found that HRS increased both basal and EGF-induced Elk-1 activation (Fig. 8*A*). Similar results were observed in ER-negative but PELP1-positive MDA-MB-435 cells (Fig. 8*B*). Collectively, the results from Fig. 5, *A* and *B,* suggest that Elk-1 luciferase activity can be rapidly stimulated by HRS in different breast cancer cell lines. The noted variability of the basal and fold activation among different panels was because of the use of different cell lines. To test the contribution of endogenous HRS to basal Elk-1 activation, we knocked down HRS in MCF-7 cells by using HRS-specific siRNA. Results showed a significant decrease in the levels of Elk-1 activation in cells with HRS-siRNA compared with cells treated with control siRNA (Fig. 8*C*). Similarly, we found that eliminating HRS in MCF-7/PELP1 cells also resulted in reduced basal and growth factor-induced Elk-1 activation (Fig. 8*D*). Depletion of PELP1 in MDA-MB-435 cells compromised the ability of HRS to stimulate Elk-1 activation (data not shown). We also found that there is no effect of HRS on Elk-1 activation in EGFR-negative MDA-MB-453 cells (data not shown). These findings suggest that HRS-induced MAPK may have functional implications in breast cancer cells.

HRS Expression in Human Breast Tumors—Because HRS was identified as a PELP1-interacting protein and PELP1 overexpression and cytoplasmic localization have been observed in some breast tumors (5), we next evaluated the status of HRS in breast tumors and the correlation between among HRS, EGFR, cytoplasmic PELP1, and phospho-MAPK in breast cancer samples. To provide proof-of-principle evidence in support of this possibility, we used a set of breast tumors previously characterized for their PELP1 status (2). Examination of 10 paired samples of human breast tumors and adjacent normal tissue showed higher expression of HRS in 8 of 10 tumors than in normal tissues (Fig. 9*A*). Immunohistochemical examination of representative tumors expressing increased HRS revealed intense nuclear phospho-MAPK staining (6 of 7 samples), strong cytoplasmic PELP1 staining (5 of 7 samples), strong EGFR staining (5 of 7 samples), and HRS overexpression along with cytoplasmic PELP1 (5 of 7 samples). Two representative immunohistochemical images are shown in Fig. 9*B*. There was no correlation of the noted changes with the ER status of these tumors. These results suggested that HRS up-regulation might contribute to the up-regulation of MAPK in breast tumors and could be closely related to the cytoplasmic location of PELP1.

DISCUSSION

Previous studies have shown that differential compartmentalization of the ER coactivator PELP1 promotes nongenomic ER functions, such as activation of MAPK, Akt, Src, and phosphatidylinositol 3-phosphate kinase (5). It is believed that subcellular distribution of PELP1 might alter the balance between genomic and nongenomic signaling in breast cancer cells. However, molecular mechanisms of intracellular localization of PELP1 and how trafficking proteins affect its localization remain unknown. In this study, we discovered that HRS interacts with PELP1 both *in vitro* and *in vivo* and sequesters PELP1 in the cytoplasm, leading to suppression of basal ER transactivation functions of PELP1 and activation of MAPK. This altered localization by sequestration with HRS

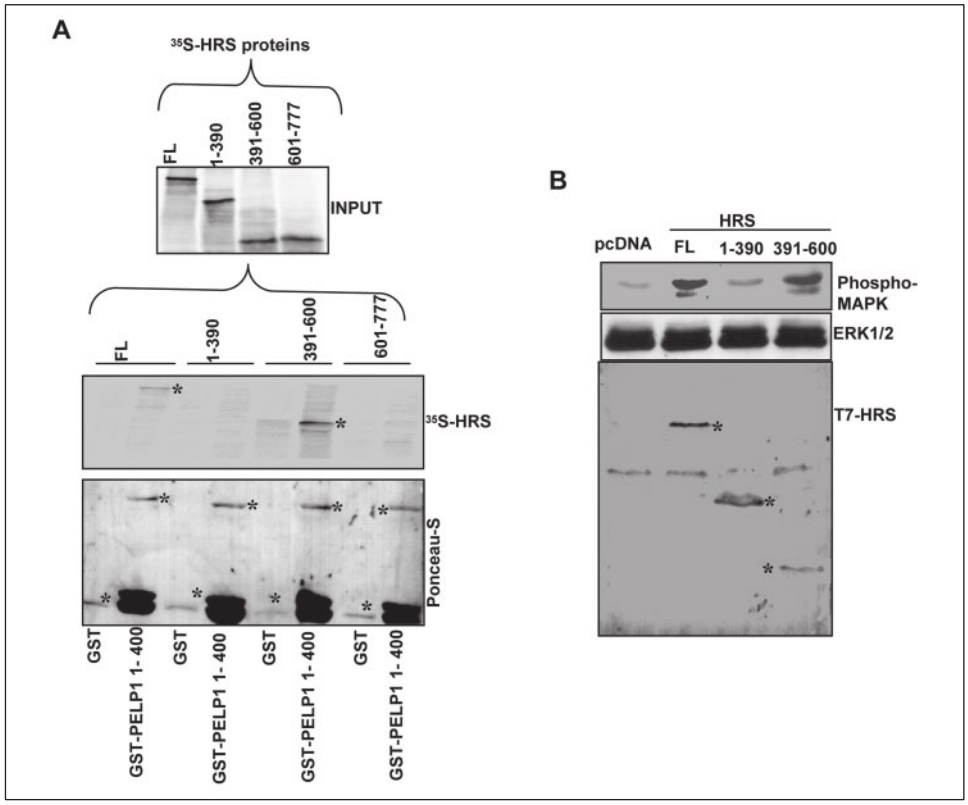


FIGURE 7. **HRS interaction with PELP1 is essential for HRS-induced MAPK activation.** *A,* glutathione *S*-transferase pulldown assay showing the association of the GST-PELP1 1–400-amino acid construct with the *in vitro*-translated ^{35}S-HRS constructs. HRS interacts with PELP1 using 391–600 amino acids. *B,* exponentially growing MCF-7 cells in 10% serum were transfected with pcDNA, FL-HRS, HRS 1–390 amino acids, or HRS 391–600 amino acids, and total lysates were immunoblotted with a phospho-MAPK-specific antibody.

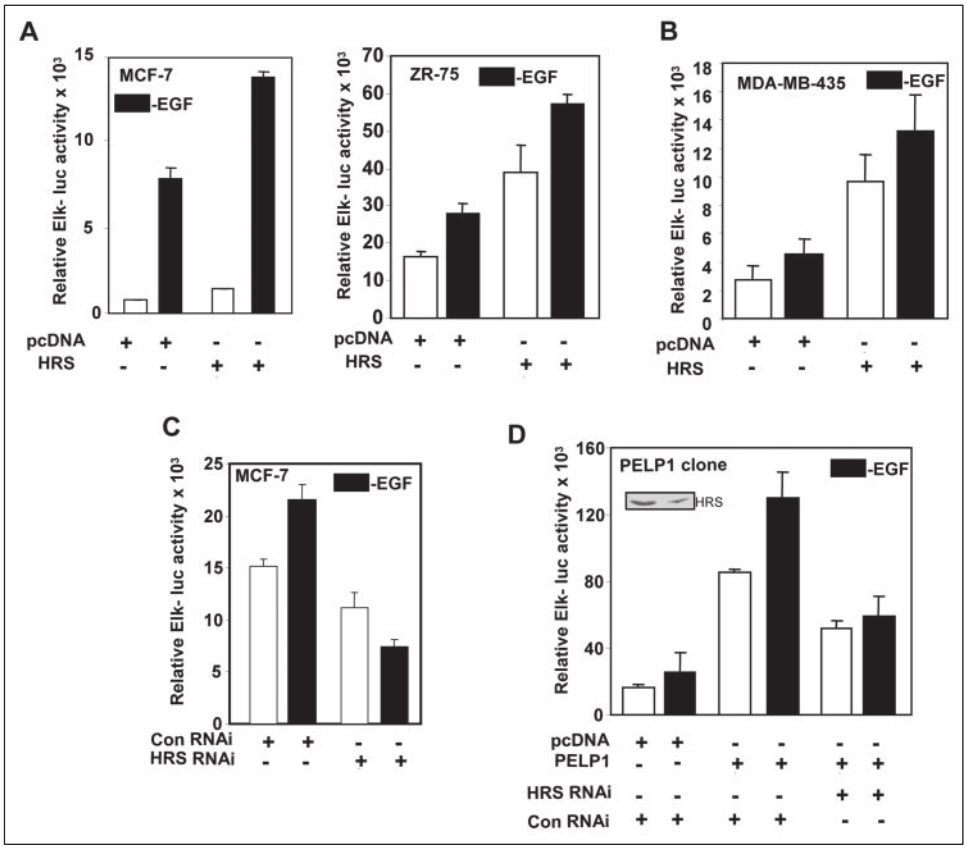


FIGURE 8. **HRS-stimulated MAPK substrate Elk-1 activation.** *A* and *B*, MCF-7, ZR-75, and MDA-MB-435 cells were cotransfected with Elk-1 luciferase reporter along with or without HRS. After 24 h, the cells were serum-starved and treated with or without EGF (100 ng/ml) for 8 h, and then the Elk-1 reporter activity was measured. *C,* MCF-7 cells were transfected with control or HRS-specific siRNA and then transfected with the Elk-1 luciferase reporter gene. After 24 h, the cells were serum-starved for an additional 24 h and then treated with EGF for 8 h, and then the Elk-1 reporter activity was measured. *Con*, control; *RNAi*, RNA interference. *D,* MCF-7 cells stably expressing pcDNA or PELP1 were transfected with control or HRS-specific siRNA and then transfected with Elk-1 luciferase reporter gene. After 24 h, the cells were serum-starved for an additional 24 h and then treated with EGF for 8 h, and then the Elk-1 reporter activity was measured. The results shown are representative of three separate experiments.

apparently mimics the recently reported cytoplasmic localization of PELP1 in breast tumors (5) and is expected to have physiologic significance.

The biological relevance of the sequestration of PELP1 by HRS is inferred from the demonstration that HRS activates MAPK, which can be significantly compromised by depleting endogenous PELP1, indicat-

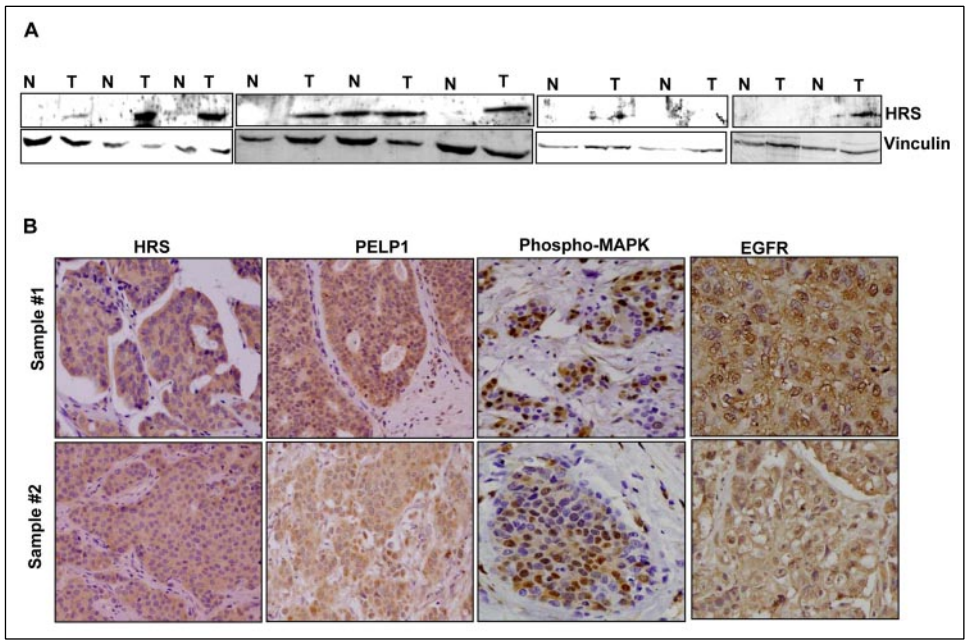


FIGURE 9. **HRS expression is deregulated in human breast tumors.** *A*, breast tumor lysates were analyzed for HRS expression by Western blot analysis, and the blots were reprobed with vinculin antibody. *B*, paraffin-embedded tumor tissue sections from above were analyzed by immunostaining with antibodies against HRS, PELP1, phospho-MAPK, and EGFR.

ing that this interaction is essential for MAPK activation by HRS. Most interestingly, there was no change in the status of Akt activation by HRS, suggesting that HRS-PELP1 interaction might influence the MAPK pathway only. We also found no evidence of MAPK activation after HRS overexpression in EGFR-negative cells, indicating that this activation depends on the status of EGFR. However, unlike in the classical pathway (28), this HRS up-regulation of MAPK was independent of Shc and Src, two major upstream regulators of the MAPK pathway and dependent on its interaction with PELP1.

The ability of HRS to stimulate Elk-1 activation, one of the downstream effectors of MAPK, is another notable finding of this study. Elk-1 activation was abolished in EGFR-negative cells, indicating that EGFR plays a role in this pathway. The extent of Elk-1 activation was compromised by the endogenous levels of both HRS and PELP1. The differential behavior of various promoters in response to MAPK activation is regulated by various MAPK substrates that were found in both cytoplasmic and nuclear compartments. For example, ERK1/2 phosphorylates and alters the properties of the AP-1 family of transcription factors (29, 30). The Ets domain proteins, such as Elk-1, mediate transcription from serum-response elements contained in the promoters of c-*fos* and other serum-induced genes.

Overexpression of HRS has been shown to promote accumulation of EGFR on early endosomes, resulting in an extended half-life of EGFR, leading to prolonged EGFR signaling from endosomes (31). HRS has been also shown to interact with various molecules implicated in protein trafficking through endosomes such as STAM, eps15, and SNX1 (11, 15, 32). HRS overexpression in mammalian cells has been shown to trap and inhibit the functions of these proteins, resulting in accumulation of EGF/EGFR complexes (15, 32). On the basis of these studies, it has been hypothesized that HRS regulates the sorting of growth factor receptors on early endosomes between recycling to the cell surface (not ligand-bound) and delivery to lysosomes for degradation (ligand-bound) (18). Because PELP1 is shown to interact with EGFR upon EGF stimulation (22) and because HRS is able to sequester PELP1 (this study), it is possible that HRS deregulation enhances the interaction of PELP1 with the components of the growth factor receptor signaling pathway. In this context, our findings suggest a working model wherein HRS-PELP1-EGFR interactions are involved in the activation of MAPK by PELP1.

Our observation of HRS deregulation in breast tumors with increased MAPK activation and cytoplasmic PELP1 is novel, and it provides clues about the physiological significance of the noted HRS-PELP1 interaction in cancer cells. In addition, these findings also raise the possibility that part of the cytoplasmic PELP1 signaling might be regulated by the status of HRS in breast cancer cells. Because the MAPK activation stimulated by HRS was sufficient to cause secondary Elk-1 stimulation, our results also suggest the overall functionality of HRS/PELP1-associated MAPK in nuclear functions, although the nuclear function appears to be suppressed. In summary, the present study shows for the first time that HRS, a component of endosome and trafficking pathways, is up-regulated in breast tumors, interacts with PELP1, and participates in its extranuclear activities in cancer cells.

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