Human Rhomboid Family-1 Suppresses Oxygen-Independent Degradation of Hypoxia-Inducible Factor-1α in Breast Cancer

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Abstract

Intermittent oxygen deficiency in cancers promotes prolonged inflammation, continuous angiogenesis, and increased drug resistance. Hypoxia-inducible factor-1 (HIF1) has a pivotal role in the regulation of cellular responses to oxygen deficiency. The α-subunit of HIF1 (HIF1α) is degraded in normoxia but stabilized in hypoxia. However, the molecular mechanism that controls oxygen-independent degradation of HIF1α has remained elusive. Human rhomboid family-1 (RHBDF1) is a member of a large family of nonprotease rhomboids whose function is basically unknown. We report here that RHBDF1 expression in breast cancer is highly elevated and is strongly correlated with escalated disease progression, metastasis, poor prognosis, and poor response to chemotherapy. We show that RHBDF1 interaction with the receptor of activated protein-C kinase-1 (RACK1) in breast cancer cells prevents RACK1-assisted, oxygen-independent HIF1α degradation. In addition, we show that the HIF1α-stabilizing activity of RHBDF1 diminishes when the phosphorylation of a tyrosine residue on the RHBDF1 molecule is inhibited. These findings are consistent with the view that RHBDF1 is a critical component of a molecular switch that regulates HIF1α stability in cancer cells in hypoxia and that RHBDF1 is of potential value as a new target for cancer treatment. Cancer Res; 74(10); 1–12. ©2014 AACR.

Introduction

Intermittent oxygen deficiency in cancer microenvironment promotes prolonged inflammation, continuous neovascularization, and escalated drug resistance. The transcription factor hypoxia-inducible factor-1 (HIF1) predominantly regulates cellular responses to oxygen deficiency, including the expression of genes required for tissue oxygen delivery and energy metabolism in developmental, physiologic, and pathologic conditions such as ischemic cardiovascular disease, stroke, and cancer (1). HIF1 is a heterodimer composed of α- and β-subunits. HIF1β level is maintained constitutively, whereas HIF1α under normoxic conditions is removed by oxygen-dependent prolyl hydroxylation, ubiquitination, and proteasomal degradation (2). In hypoxia, however, HIF1α level increases markedly as it is continuously synthesized (3), whereas its oxygen-dependent degradation is downregulated (2). An oxygen-independent mechanism has been proposed to account for the modulation of HIF1α in hypoxia (4), involving the receptor of activated protein C kinase-1 (RACK1) and HSP90 (5). HSP90 is a molecular chaperone that protects client proteins from misfolding and degradation (6). HSP90 binding stabilizes HIF1α. RACK1 competes with HSP90 for binding to HIF1α. RACK1 also binds to Elongin C and recruits other components of E3 ubiquitin ligases, thus facilitating HIF1α ubiquitination and degradation in an oxygen-independent manner (5). However, what this “molecular switch” consists of and how it operates remain unclear.

The human rhomboid family-1 gene product RHBDF1 is a protein found mainly within the endoplasmic reticulum and Golgi complex (7). Rhomboids are 6 or 7 transmembrane proteins that may be divided into two categories. Many rhomboids are serine proteases, conserved across all kingdoms of life, and regulate biologic processes as diverse as growth factor signaling, mitochondrial morphology, parasitic invasion, and bacterial protein translocation (8). The other category of rhomboid proteins, present in all sequenced metazoans, lack the known catalytic residues essential to the function of serine proteases (9). Few functions of these so-called "inactive rhomboids," including RHBDF1, are known. In Drosophila, noncatalytic rhomboids were shown to prevent the cleavage of the substrates of rhomboid
proteases by promoting their destabilization by endoplasmic reticulum–associated degradation (10). In human, RBHDF1 was shown to have a pivotal role in sustaining growth signals in epithelial cancers (11, 12). RBHDF1 mRNA level is significantly elevated in clinical specimens of invasive ductal carcinoma of the breast, and RBHDF1 gene silencing results in apoptosis or autophagy in breast cancer or head and neck cancer cells and inhibition of xenograft tumor growth (11). RBHDF1 was found to participate in the modulation of G-protein–coupled receptor-mediated transactivation of EGF receptor (12). These findings indicate that RBHDF1 may function as a regulatory protein involved in growth signal transduction.

In this study, we discovered that RBHDF1 function is critical for the maintenance of HIF1α stability in breast cancer cells in hypoxia. We report here that elevated RBHDF1 expression in breast cancer strongly correlates with escalated disease progression, poor prognosis, and poor responses to chemotherapy. We show that RBHDF1 is an essential component of a “molecular switch,” which also consists of RACK1 and HSP90 that modulate oxygen-independent degradation of HIF1α. In addition, we demonstrate that disrupting the phosphorylation of a tyrosine residue on the RBHDF1 molecule leads to diminished HIF1α stability. These findings define a new function for a nonprotease rhomboid gene and provide new insights into the mechanism underlying the modulation of HIF1 activity in hypoxia.

Materials and Methods

Cells

Human breast cancer cell lines MCF7, MDA-MB-231, T47D, and human kidney 293 cell (all from American Type Culture Collection) were maintained in Dulbecco’s Modified Eagle Medium (Lonza), 10% FBS, l-glutamate, penicillin and streptomycin. For hypoxic conditions, the cells were cultured in a modular incubator chamber flushed with mixed gas consisting of 20% O2, 5% CO2, and 75% N2 at 37°C and 5% CO2 for 16 hours at 4°C. RBHDF1 was found to participate in the modulation of G-protein-coupled receptor-mediated transactivation of EGF receptor (12). These findings indicate that RBHDF1 may function as a regulatory protein involved in growth signal transduction.

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Reagents

Plasmids pBabe-HA-HIF-1α and pBabe-HA-HIF1α (P402A/P564A) were gifts from Dr. W. G. Kaelin (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). FLAG-RBHDF1 and GFP-RBHDF1 plasmid were described previously (12). FLAG-RACK1 was constructed into pCI (Promega). RHBDF1 and GFP-RHBDF1 plasmid were described previously (12). FLAG-RACK1 was a gift from Dr. W. G. Kaelin (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). FLAG-RACK1 was a gift from Dr. W. G. Kaelin (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). FLAG-RACK1 was a gift from Dr. W. G. Kaelin (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). FLAG-RACK1 was a gift from Dr. W. G. Kaelin (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA).

Clinicopathological analysis of breast cancer specimens

The Ethics Committee of the Cancer Hospital of Tianjin Medical University (Tianjin, China) approved the use of human tissues for this study. Each patient signed an informed consent form for participation. Paraffin-embedded blocks of normal breast and breast cancer tissues diagnosed in 2003 were retrieved randomly from archives of Cancer Hospital of Tianjin Medical University. Patients were women 24 to 83 years of age (mean age 52.6 years). Specimens were collected before radiation or chemotherapy, or both. Histopathology was reviewed and diagnosis confirmed independently by two pathologists (LF and FFL) using the WHO criteria (14). For clinicopathologic analysis of breast cancer specimens, breast cancer specimens were retrieved from archives diagnosed during 2007 to 2008. All invasive breast cancer (IBC) specimens were obtained from patients who had completed preoperative neoadjuvant chemotherapy consisted of 4 to 6 cycles of anthracycline-based or anthracycline and taxane-based regimen before surgery. Response to chemotherapy was assessed according to Miller and Payne histologic grading system (15); grade 1, no change or some alteration to individual malignant cells but no reduction in overall cellularity; grade 2, minor loss (up to 30%) of cancer cells but overall cellularity remains high; grade 3, reduction of 30% to 90% of cancer cells; grade 4, more than 90% loss of cancer cells but small clusters or widely dispersed individual cancer cells remain; grade 5, no malignant cells identifiable in sections from the site of the tumor consisting of vascular fibroblastic stroma, often containing macrophages; however, ductal carcinoma in situ (DCIS) may be present. In this study, grades 3 to 5 were regarded as having a good response to chemotherapy, whereas grades 1 and 2 were regarded as having a poor response.

Immunohistochemistry

Tissue sections (4 μm thickness) were deparaffinized and rehydrated. Antigen retrieval was performed at 121°C for 2 minutes, using citrate buffer (pH 6.0) for RBHDF1, ER, PR, C-erbB-2, or EDTA solution (pH 8.0) for HIF1α. After blocking with hydrogen peroxide and normal goat serum, the sections were incubated with primary monoclonal antibody against RBHDF1 (Abcam, cat# ab81342, 1:250 dilution) or HIF1α (Abcam, cat# ab8366, 1:250 dilution) for 16 hours at 4°C. The section was then washed and incubated with secondary antibody for 30 minutes. Signal was detected with 3,3′-diaminobenzidine (DAB) solution, hematoxylin counterstain, and dehydrated. A scoring system was calculated, including staining intensity (SI) and staining area (SA), with a total score of SI× SA.
sections were sequentially incubated with biotinylated goat anti-mouse immunoglobulin and peroxidase-conjugated streptavidin (DAKO), and the substrate 3,3′-diaminobenzidine tetrahydrochloride. Sections incubated with only PBS served as negative controls. RBHDF1 staining levels were classified using a modified scoring method (16) based on staining intensity (Supplementary Fig. S1A). Nuclear positivity of HIF1α was defined as the presence of perinuclear or diffuse stained nuclei (Supplementary Fig. S1B). A specimen was considered to contain HIF1α-positive nuclei when more than 0% of nuclei were positive (median value cut-off: 0%; ref. 17). There was a positive correlation between cytoplasmic and nuclear positivity of HIF1α in the 263 IBC specimens we analyzed (Supplementary Fig. S1C). Interpretation and scoring of ER, PR, and C-erbB-2 staining were described previously (18, 19).

Cell transfection
pSUPER-ShRBDF1, pSUPER-ShScramble and pBabe-HA-HIF1α (P402A/P564A) plasmids were each transfected into PT67 packing cells (Clontech). The transfected cell culture medium was then used to infect target cells. Cells were selected with puromycin (Invitrogen) for 2 weeks. Human RACK1 siRNA and control siRNA (Santa Cruz Biotechnology) were used for transient transfection, using Lipofectamine 2000 (Invitrogen).

Immunoprecipitation and Western blotting
Cell lysis buffer contained 50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 0.1% SDS, 0.5% NaDoc, 1% NP-40, and protease inhibitors (Roche). The lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. For coimmunoprecipitation (co-IP) studies, in which the extracts were incubated overnight at 4°C, then with protein G or protein α-Sepharose beads (Santa Cruz Biotechnology) for 2 hours at 4°C. The beads were washed exhaustively with the lysis buffer. Immobilized proteins were eluted with 2× Laemmli sample buffer and subjected to SDS-PAGE and Western blotting analysis.

Statistical analysis
Student two-tailed t test was used to analyze the statistical significance of differences in continuous variables between two groups. The Spearman correlation rank for nonparametric variables was used to assess the relationships between the categorical variables. Survival curves were calculated using the log-rank (Mantel–Kaplan) test. All statistical tests used a two-tailed significance level of P < 0.05.

Results
Elevated RBHDF1 expression in breast cancer correlates with escalated disease progression
We compared RBHDF1 expression patterns in 343 clinical specimens of breast cancer of various stages and normal breast tissues by immunohistochemistry, using a four point grading scale (−, +, 2+, and 3+ for negative, weak, medium, and strong, respectively; Supplementary Fig. S1). RBHDF1 levels were mostly undetectable in normal breast tissues (n = 20), but steadily increased in the order of atypical ductal hyperplasia (ADH; n = 10; Z = −2.019; P = 0.043 by Mann–Whitney U test), DCIS (n = 30; Z = −2.032; P = 0.042), and IBC (n = 263; Z = −2.443; P = 0.015; Fig. 1A). RBHDF1 expression level in normal tissue adjacent to cancer (n = 20) was also higher compared with that in normal breast tissues (Z = −3.106; P = 0.002; Fig. 1A). The percentages of medium and strong RBHDF1 expression (2+/3+; n = 146) exhibited a poorer overall survival rate compared with patients with low RBHDF1 expression (−/++; n = 117; P = 0.0021; Fig. 1C). Progression-free survival was also markedly worse for patients with IBC with high RBHDF1 expression than for those with low RBHDF1 expression (P = 0.0003; Fig. 1D). Moreover, we examined the correlations between RBHDF1 expression levels and a range of clinicopathologic parameters in the IBC cases. The percentages of local recurrence in low and high RBHDF1 groups were 2.6% and 9%, respectively (P = 0.029; Fig. 1E). The percentages of cases in RBHDF1 low and high groups with metastasis to at least one positive lymph node were 53% and 66%, and those with 10 or more were 15% and 24%, respectively (P = 0.027; Fig. 1F). The percentages of distant metastasis in low and high RBHDF1 groups were 9.3% and 32.7%, respectively (P = 0.012; Fig. 1G). There were no statistically significant differences between RBHDF1 low and high groups with regard to age, tumor size, histologic grade, TNM stage, estrogen receptor expression, progesterone receptor expression, and C-erbB-2 levels (Supplementary Table S1). Furthermore, we studied RBHDF1 expression levels in the tumor specimens of 67 patients with breast cancer who had completed a full protocol of neoadjuvant chemotherapy before surgical excision of the tumor (refer to Supplemental Materials and Methods for treatment detail). We found that more than 80% of the cases in low RBHDF1 group had good responses in comparison with about 40% of good responders in high RBHDF1 group (Fig. 1H). These findings indicate that elevated RBHDF1 expression in breast cancer is strongly correlated with facilitated disease progression, local recurrence, lymph node and distant metastasis, poor prognosis, and poor responses to chemotherapy.

Raising RBHDF1 expression levels in cancer cells leads to enhanced HIF1α protein stability
To begin to investigate RBHDF1’s role in breast cancer progression, we compared RBHDF1 and HIF1α protein levels in the IBC specimens, and found that the expression patterns of the two are strikingly similar (rs = 0.593, P < 0.0001; Fig. 2A). Less than 30% of the patients in RBHDF1 low group exhibited high HIF1α (2+, 3+), whereas in sharp contrast, about 75% of the patients in RBHDF1-high group showed high HIF1t levels (Fig. 2B). In addition, we found that the percentage of poor responders to neoadjuvant chemotherapy was less than 20% when both RBHDF1 and HIF1t levels are low, whereas it reached about 70% when both RBHDF1 and HIF1t are high (Fig. 2C). We thus overexpressed RBHDF1 by cDNA transfection in breast cancer T47D cells, which exhibit constitutively
low expression of RHBDF1 (11). We found that raising RHBDF1 levels in these cells resulted in a marked increase in HIF1α levels in normoxia (20% O2; Fig. 2D) as well as in hypoxia (1% O2; Fig. 2E; the first two lanes). We cultured the cells in hypoxia for 4 hours, and then placed them in normoxia, and found a significant delay in HIF1α protein degradation in RHBDF1-overexpressing cells compared with vector-transfected control cells (Fig. 2E); the slower mobility species above the main HIF1α band were later identified as ubiquitinated HIF1α (see below). These findings indicate that not
only high RHBDF1 levels are strongly correlated with markedly enhanced HIF1α stability in breast cancer in clinical settings as well as in cancer cell cultures under either normoxic or hypoxic conditions, but RHBDF1 may have a role in the modulation of HIF1α degradation.

**RHBDF1 gene silencing leads to HIF1α destabilization**

We determined the effect of RHBDF1 gene silencing on HIF1α stability, using breast cancer cell line MCF7 cells, which express high level of RHBDF1 (11). Transient transfection with a shRNA against RHBDF1 (shRHB) under hypoxic conditions caused a marked decline of HIF1α protein (Fig. 3A). To confirm this finding, we treated the cells with a mixture of 4 specific shRNA plasmids against RHBDF1. Western blotting analysis results indicated that silencing the RHBDF1 gene led to marked decline of HIF1α protein level in the cells under hypoxic conditions (Supplementary Fig. S5). We also found much faster HIF1α degradation during reoxygenation in shRHB-treated cells than that in shScr-treated cells (Fig. 3B). Treatment of the cells with shRHB in hypoxia led to a decreased nuclear accumulation of the HIF1α protein, which is consistent with a reduction of HIF1α stability (Fig. 3C). To find out whether shRHB inhibited HIF1 activity, we analyzed the secretion of VEGF, a target gene of HIF1α (20), by the cells. Treatment with
shRHB for 4 to 24 hours in hypoxia led to an approximately 50% decrease in VEGF secretion into the culture media determined by ELISA (Fig. 3D). A similar inhibition of hypoxia-induced VEGF production by shRHB treatment was observed when the experiment was repeated with human breast cancer cell line MDA-MB-231 (Fig. 3E). We found that HIF1α mRNA levels were not affected by RHBDF1 shRNA treatment of the cells; however, the transcription of GLUT1, a target gene of HIF1, diminished in RHBDF1-treated cells, consistent with decreased HIF1α activities (Supplementary Fig. S2). These findings indicate that RHBDF1 function is essential to the maintenance of HIF1α protein stability and HIF1 activity in breast cancer cells under hypoxic conditions.

RHBDF1 protects HIF1α from RACK1-facilitated oxygen-independent degradation

To investigate whether RHBDF1 modulation of HIF1α stability involved prolyl hydroxylase activity, we transfected MCF7 cells with a HA-tagged mutant HIF1α that had two proline-to-alanine substitutions (P402A/P564A), which confers irresponsiveness to prolyl hydroxylation (21). We found that HIF1α mRNA levels were not affected by RHBDF1 shRNA treatment of the cells; however, the transcription of GLUT1, a target gene of HIF1, diminished in RHBDF1-treated cells, consistent with decreased HIF1α activities (Supplementary Fig. S2). These findings indicate that RHBDF1 function is essential to the maintenance of HIF1α protein stability and HIF1 activity in breast cancer cells under hypoxic conditions.

RHBDF1-facilitated HIF1α stabilization. We first treated MCF7 cells with both RHBDF1 shRNA and RACK1 siRNA, and found that RACK1 siRNA treatment alone caused an accumulation of HIF1α in the cells; however, HIF1α diminished when the RHBDF1 gene was silenced at the same time (Fig. 4C and Supplementary Fig. S6A). In addition, we cotransfected T47D cells with RHBDF1 and the mutant HA-HIF1α, and treated the cells with RACK1 siRNA. RACK1 siRNA treatment of the RHBDF1-overexpressing cells significantly enhanced HIF1α stability (Fig. 4D and Supplementary Fig. S6B). To determine the involvement of HSP90, we simultaneously treated MCF7 cells with RHBDF1 shRNA and 17-AAG, which inhibit HSP90 activity (6), and found that RHBDF1-facilitated HIF1α stabilization was significantly abrogated by 17-AAG treatment (Fig. 4E). Moreover, we treated RHBDF1-overexpressing T47D cells with 17-AAG, and found that RHBDF1-facilitated HIF1α stabilization was significantly abrogated by 17-AAG treatment (Fig. 4E). These findings support the view that RHBDF1 activity has an impact on the actions of RACK1 and HSP90 on HIF1α stability.

To determine whether RHBDF1 physically interacts with HIF1α or RACK1, we transfected human kidney 293 cell with FLAG-tagged RHBDF1 either alone or together with HA-tagged wild-type HIF1α. Co-IP analysis indicated that RHBDF1 was able to bind to RACK1 (Fig. 4G) and HIF1α (Fig. 4H), but not to HSP90 (data not shown).
with or without MG132 and analyzed co-IP of RHBDF1 with HIF1α and RACK1. The results indicated that RHBDF1, RACK1, and ubiquitinated HIF1α formed a complex (Fig. 4I). To determine the effect of oxygen on these interactions, we transfected T47D cells with FLAG-RHBDF1 and the P402A/P564A mutant HA-HIF1α, then treated the cells with either
siRACK1 or 17-AAG and carried out co-IP analysis. We found that siRACK1 treatment enhanced HIF1α binding to HSP90, whereas 17-AAG treatment led to more HIF1α binding to RACK1 (Fig. 4J). These findings suggest that RHBDF1 overexpression led to diminished RACK1–HIF1α interaction, thus protecting HIF1α from RACK1-induced, oxygen-independent degradation.

**RHBDF1 inhibits RACK1 binding to HIF1α**

To determine the effect of RHBDF1 gene silencing on RACK1 and HSP90 binding to HIF1α, we stably expressed shRHB in MCF7 cells, and treated the cells with MG132. Co-IP analysis revealed that shRHB inhibited HIF1α binding to HSP90 but, in sharp contrast, enhanced HIF1α binding to RACK1 (Fig. 5A). We then transfected the HA-tagged P402A/P564A mutant HIF1α into these cells and treated them with either 17-AAG or MG132, or both, in hypoxia. Ubiquitination of the HA-tagged mutant HIF1α and HIF1α or Elongin C binding to RACK1 were readily observed in shRHB-expressing cells when protein degradation was blocked with MG132, whereas HSP90 binding to HIF1α diminished; 17-AAG treatment caused a further decline of HSP90 binding to HIF1α; it is not immediately apparent to us, however, as to why there appeared to be a reduction in the ubiquitination of the mutant HIF1α upon treatment with 17-AAG and shRHB together compared with shRHB alone (Fig. 5B). To determine the effect of elevated RHBDF1 levels on the interaction between RACK1 and HIF1α, we transfected RHBDF1 cDNA into T47D cells that were engineered to over-express the P402A/P564A mutant HA-HIF1α, cultured the cells in hypoxia, and carried out co-IP analysis (Fig. 5C). We found that the mutant HIF1α became more stable and its binding to RACK1 declined when RHBDF1 level was raised. Inhibition of HSP90 activity with 17-AAG led to increased RACK1 or Elongin C binding to HIF1α; however, these effects diminished in RHBDF1-overexpressing cells. In addition, we carried out a “pull-down” experiment to determine RHBDF1 impact on RACK1 dimerization required for RACK1 binding to HIF1α (22). We overexpressed FLAG-RACK1 in human kidney 293 cell, isolated the protein by using an anti-FLAG antibody immobilized on agarose resin, and used the FLAG-RACK1–enriched resin to isolate endogenous RACK1 from MCF7 cell homogenates. The MCF7 cells were engineered to express either shRHB or shScr. We found that RACK1 dimerization became more prominent in RHBDF1 gene-silenced cells in normoxia and also in hypoxia in the presence of MG132 (Fig. 5D). Moreover, consistent with a previous report that RACK1 dimerization gives rise to phosphorylation of serine-146 on the RACK1 protein (22), we found that RHBDF1 gene silencing led to enhanced serine phosphorylation of RACK1 (Fig. 5E). These results indicate that RHBDF1 interacts with RACK1 prevents RACK1 dimerization, which in turn inhibits HIF1α binding to RACK1.

**Inhibition of RHBDF1 tyrosine phosphorylation causes disruption of RHBDF1–RACK1 interaction and facilitates HIF1α degradation**

We carried out bioinformatics analysis on the primary sequence of RHBDF1 and discovered a number of potential phosphorylation sites (Supplementary Fig. S3). We synthesized a series of peptides with sequences corresponding to these sites. A poly-arginine tag (R11) was added to each of the peptides at the C-terminus to ensure entrance of the peptide into the cells (23) in case some of these potential phosphorylation sites were located inside the cells (Supplementary Table S2). We treated MDA-MB-231 cells with each of the peptides, and found that peptide VR56, which mimics the amino acid sequence flanking tyrosine-848 of RHBDF1, was able to substantially inhibit VEGF production by the cells in hypoxia (Fig. 6A) as well as the transcription of a number of HIF1-targeted genes, including VEGF, GLUT1, CA9, and ET1 (Supplementary Fig. S4). VR56 treatment also facilitated HIF1α degradation (Fig. 6B). A peptide identical to VR56 but without the tag (VIH36), or the R11 tag itself (ER20), did not exhibit these activities. In addition, we found that VR56 treatment of RHBDF1-overexpressing MCF7 cells in hypoxia led to a marked inhibition of RHBDF1–enhanced HIF1α stabilization (Fig. 6C). VR56 inhibition of RHBDF1–enhanced HIF1α stabilization was dose dependent (Fig. 6D). VR56 also inhibited RHBDF1 tyrosine phosphorylation (Fig. 6E). Treatment of MCF7 cells with VR56 resulted in a marked decrease of cell viability (Supplementary Fig. S4). We then treated RHBDF1- and RACK1-overexpressing MCF7 cells with VR56 in hypoxia, and found that the treatment significantly inhibited RHBDF1 binding to RACK1 (Fig. 6F). When these experiments were carried out in the presence of MG132, VR56 treatment inhibited RHBDF1 binding to RACK1 but enhanced HIF1α binding to RACK1 (Fig. 6G). These findings indicate that disruption of RHBDF1 tyrosine phosphorylation with VR56 leads to inhibition of RHBDF1 binding to RACK1, and destabilization of HIF1α.

**Discussion**

On the basis of these findings, we propose a “molecular switch” to explain RHBDF1 suppression of HIF1α degradation in hypoxia (Fig. 7). In this mechanism, RHBDF1 is in a position to control the competition of RACK1 and HSP90 for binding to HIF1α. RHBDF1 binding to RACK1 either takes the latter away from RACK1–HIF1α complex, or prevents RACK1 from binding to HIF1α. The latter scenario appears to be more likely, as RHBDF1 interaction with RACK1 results in an inhibition of the phosphorylation of the RACK1 protein and a disruption of RACK1 dimerization, which are necessary for RACK1 binding to HIF1α. Disruption of RACK1 binding to HIF1α by the action of RHBDF1 allows HIF1α to bind to HSP90 more readily instead of binding to RACK1 and being taken into the ubiquitin–Elongin C pathway for degradation. The action of RHBDF1 thus shifts the balance toward HIF1α stabilization.

It has been suggested that competition between HSP90 and RACK1 for binding to HIF1α may contribute to the establishment of the HIF1α “set point,” which is a given HIF1α protein level in a given type of cells (5). Influence of RHBDF1 on the competition may have a significant impact on such “set point.” Especially, considering that RHBDF1, RACK1, HSP90, and HIF1α protein levels are all significantly elevated in many cancer cells and tumor tissues (11, 24, 25), RHBDF1 influence
on the equilibrium between HIF1α-RACK1 and HIF1α-HSP90, may have a pivotal role in the maintenance of HIF1α stability under hypoxic conditions. It is plausible that this mechanism underlies the association of RHBDF1 with clinicopathologic parameters critical for the progression of the disease. As we have shown, RHBDF1 protein is nearly absent or present at low levels in normal mammary gland tissues, then changes to moderate levels in atypical ductal hyperplasia as well as in normal tissues adjacent to tumors, then increases markedly to high and very high levels in DCIS and IBC, respectively. Facilitation of HIF1 activity by RHBDF1 may explain the close correlation of high RHBDF1 protein levels with lymph node and distant metastasis of the cancer cells, local recurrence, and poorer overall and disease-free patient survival.

Hypoxia-associated drug resistance is a major clinical issue. Consistent with RHBDF1-facilitation of HIF1 activity, we found that patients with low RHBDF1 protein levels responded better to the new adjuvant chemotherapy regimen. Because HIF1

Figure 5. Involvement of RHBDF1, RACK1, HSP90, and Elongin C in oxygen-independent HIF1α ubiquitination and degradation. A, co-IP of HIF1α, HSP90, and RACK1 from MCF7 cells stably expressing shRHB or ShScr. B, co-IP of HA-P402A/P564A-HIF1α (HA-HIF1α) and RACK1 or RACK1 and Elongin C from MCF7 cells stably expressing HA-HIF1α and shRHB in the presence or absence of 17-AAG (0.5 mmol/L, 24 hours) or MG132 (10 μmol/L, 4 hours), or both, in hypoxia. C, co-IP of HA-HIF1α, RACK1, and Elongin C from T47D cells overexpressing HA-HIF1α and FLAG-RHBDF1 and treated with 17-AAG in hypoxia. D, "pull-down" analysis of RACK1 dimerization in MCF7 cells stably expressing shRHB or shScr, transfected with FLAG-RACK1, and cultured in normoxia or hypoxia. E, analysis of RACK1 serine-phosphorylation and Elongin C-binding in MCF7 cells stably expressing shRHB or shScr and cultured in normoxia or hypoxia. Each experiment was repeated two times and representative results from one experiment are shown.
directly promotes hypoxia-associated drug resistance by enhancing the expression of antiapoptotic proteins and diminishing the expression of proapoptotic proteins or by nonapoptotic mechanisms (26, 27), inhibition of RHBDF1-facilitated HIF1α stabilization may potentially be beneficial not only to suppress tumor angiogenesis, but also to curb cancer cell resistance to apoptosis-inducing chemotherapies. In this regard, our study has demonstrated that by using the peptide mimic VR56, we may intervene RHBDF1-facilitated stabilization of HIF1α by inhibiting RHBDF1 tyrosine phosphorylation. This suggests that RHBDF1 may serve as a target for cancer drug development.
It is of interest that noncatalytic rhomboids, such as human RHBDF1, may function as regulatory proteins. Noncatalytic rhomboids are considered to have evolved from rhomboid proteases that lost their catalytic activity but retained their location in the protein synthesis apparatus (10), and plausibly maintained their abilities to bind to what were once their substrates. New functions as regulatory proteins may be acquired by taking advantages of the expression pattern, subcellular location, and substrate-binding capacity. Consistent with this notion is the wide range of inactive cognates of many enzymes (28). That RHBDF1 may have evolved from a membrane-bound protease into a regulatory protein may exemplify this evolutionary route.

In summary, our experimental data are consistent with the view that RHBDF1, RACK1, and HSP90 form a "molecular switch" that controls oxygen-independent degradation of HIF1α. In this mechanism, RHBDF1 facilitates HIF1α stability by preventing RACK1 binding to HIF1α, thus attenuating ubiquitin-mediated HIF1α proteasomal degradation and shifting HIF1α binding toward HSP90. RHBDF1 is therefore an essential component of cell survival mechanism underlying cellular responses to oxygen deficiency. In addition, our findings illustrate that intervention of RHBDF1 activity by gene silencing or by specific inhibition of tyrosine phosphorylation of the RHBDF1 protein presents a potentially new approach to develop anticancer therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: F. Liu, F. Shu, L. Fu, L.-Y. Li
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