Human Rhomboid Family-1 (RHBDF1) 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. The molecular mechanism that controls oxygen-independent degradation of HIF1α has remained elusive, however. Human rhomboid family-1 (RHBDF1) is a member of a large family of non-protease 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. Additionally, 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.
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, physiological, and pathological 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) while 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 heat shock protein-90 (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 facilitates HIF1α ubiquitination and degradation in an oxygen-independent manner (5). What this “molecular switch” consists of and how it operates remain unclear, however.

The human rhomboid family-1 gene product RHBDF1 is a protein found mainly within the endoplasmic reticulum and Golgi complex (7). Rhomboids are six- or seven-transmembrane proteins that may be divided into two categories. Many rhomboids are serine proteases, conserved across all kingdoms of life, and regulate biological 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 non-catalytic 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, RHBDF1 was shown to have a pivotal role in sustaining growth signals in epithelial cancers (11, 12). RHBDF1 mRNA level is significantly elevated in clinical specimens of invasive ductal carcinoma of the breast, and RHBDF1 gene silencing results in apoptosis or autophagy in breast cancer or head and neck cancer cells, and inhibition of xenograft tumor growth (11). RHBDF1 was found to participate in the modulation of G-protein coupled receptor-mediated transactivation of epidermal growth factor receptor (12). These findings indicate that RHBDF1 may function as a regulatory protein involved in growth signal transduction.

In this study we discovered that RHBDF1 function is critical for the maintenance of HIF1α stability in breast cancer cells in hypoxia. We report here that elevated RHBDF1 expression in breast cancer strongly correlates with escalated disease progression, poor prognosis, and poor responses to chemotherapy. We show that RHBDF1 is an essential component of a “molecular switch”, which consists of also RACK1 and HSP90, that modulates oxygen-independent degradation of HIF1α. Additionally, we demonstrate that disrupting the phosphorylation of a tyrosine residue on the RHBDF1 molecule leads to diminished HIF1α stability. These findings define a new function for a non-protease 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 ATCC, Manassas, VA, USA) were maintained in DMEM (Lonza, Walkersville, MD), 10% fetal bovine serum, L-glutamate, penicillin and streptomycin. For hypoxic conditions the cells were cultured in a modular incubator chamber flushed with mixed gas consisting of 1% O$_2$, 5% CO$_2$ and 94% N$_2$, at 37°C. For normoxic conditions mixed gas consisting of 20% O$_2$, 5% CO$_2$, and 75% N$_2$ at 37°C were used.

**Reagents:** Plasmids pBabe-HA-HIF-1α and pBabe-HA-HIF1α (P402A/P564A) were gifts from Dr. W. G. Kaelin of Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA. FLAG-RHBDF1 and GFP-RHBDF1 plasmid were described previously (12). FLAG-RACK1 was constructed into pCI (Promega, Madison, WI, USA). RHBDF1 shRNA retroviral vector was derived from pSUPER.retro.puromycin (OligoEngine, Seattle, WA, USA) and contains a 19-nucleotide sequence against RHBDF1 as described previously (11, 12). 17-Allylamino-demethoxygeldamycin (17-AAG) and antibodies against β-actin, HA, and FLAG were purchased from Sigma (St Louis, MO, USA). MG132 was from Calbiochem (San Diego, CA, USA). Antibodies against RACK1, HSP90, HIF1α, p-Ser, PARP and Caspase-8 were from BD (Franklin Lakes, NJ, USA). Antibodies against caspase-3 and ubiquitin were from Cell Signaling Technology (Beverly, MA, USA). Elongin-C antibody and RACK1 siRNA, which contains three unique siRNA sequences (cat# sc-36354), were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Three siRNA molecules of human RACK1 were also synthesized, with the target sequences being 5’-GGGAUGAGAC CAACUAUGGtt (siRACK1 i), 5’-GGUAUGGAAC
CUGGCUAACtt (siRACK1 ii), and 5’-GGGAAAGAUC AUUGUAGAUtt (siRACK1 iii), respectively (13). The sequence of the RHBDF1 shRNA is (forward) GAGTGAGCAA GGACAGTGAT, as reported previously (RH1-3) (11). Four independent shRNA 29-mers against RHBDF1 and a scrambled shRNA sequence were purchased from OriGene Technologies (cat# TF302009; Rockville, MD, USA). The sequences of the 4 RHBDF1 shRNA 29-mers are (forward): GGCTTCTCGC AGCATGAGAC GGTGGACTC, ACCGCATAGC CATCATCTAC CTGCTGAGT, TGGCAGCGCA AGAGCATCCG TCACTGCAG, and CTGACCAGTT CTACCGCCTG TGGCTATCC, respectively. Antibody against phospho-tyrosine was purchased from Abcam (Cambridge, MA, USA).

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 the 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 prior to radiation or chemotherapy, or both. Histopathology was reviewed and diagnosis confirmed independently by two pathologists (LF and FFL) using the WHO criteria (14). For pathological analysis of patient responses to chemotherapy, breast cancer specimens were retrieved from archives diagnosed during 2007–2008. All IBC specimens were obtained from patients who had completed preoperative neoadjuvant chemotherapy consisted of 4-6 cycles of anthracycline-based or anthracline and taxane-based regimen prior to surgery. Response to chemotherapy was assessed according to Miller and Payne histological 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, greater 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 3-5 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 RHBDF1, 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 RHBDF1 (Abcam, cat# ab81342, 1:250 dilution) or HIF1α (Abcam, cat# ab8366, 1:250 dilution) for 16 h at 4°C. The sections were sequentially incubated with biotinylated goat anti-mouse immunoglobulin and peroxidase-conjugated streptavidin (DAKO, Carpinteria, CA, USA), and the substrate 3, 3’-diaminobenzidine tetra-hydrochloride. Sections incubated with only PBS served as negative controls. RHBDF1 staining levels were classified using a modified scoring method (16) based on staining intensity (**Figure S1A**). Nuclear positivity of HIF1α was defined as the presence of perinecrotic or diffuse stained nuclei (**Figure S1B**). A specimen was considered to contain HIF1α-positive nuclei when greater than 0% of nuclei were positive (median value cut-off: 0%) (17). There was a positive correlation between cytoplasmic and nuclear positivity of HIF1α in the 263 IBC specimens we analyzed (**Figure**
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S1C). Interpretation and scoring of ER, PR, and C-erbB-2 staining were described previously (18, 19).

Cell transfection: pSUPER-ShRHBDF1, pSUPER-ShScramble and pBabe-HA-HIF1α (P402A/P564A) plasmids were each transfected into PT67 packing cells (Clonetech, Palo Alto, CA, USA). The transfected cell culture medium was then used to infect target cells. Cells were selected with puromycin (Invitrogen) for two 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 mM Tris, pH 8, 150 mM NaCl, 0.1% SDS, 0.5% NaDoc, 1% NP-40, and protease inhibitors (Roche, Indianapolis, IN, USA). The lysates were centrifuged at 14,000 rpm for 15 min at 4°C. For co-IP studies, in which the extracts were incubated overnight at 4°C, then with protein G or protein L-Sepharose beads (Santa Cruz) for 2 h at 4°C. The beads were washed exhaustively with the lysis buffer. Immobilized proteins were eluted with 2x Laemmli sample buffer and subjected to SDS-PAGE and Western blotting analysis.

Statistical Analysis: Student's two-tailed t-test was used to analyze the statistical significance of differences in continuous variables between two groups. The Spearman’s correlation rank for non-parametric variables was used to assess the relationships between the categorical variables. Survival curves were calculated using the Kaplan-Meier method, and the differences were estimated by using the log-rank (Mantel-Cox) test. All statistical tests used a two-tailed
significance level of p<0.05.
RESULTS

Elevated RHBDF1 expression in breast cancer correlates with escalated disease progression: We compared RHBDF1 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; Supplemental Figure S1). RHBDF1 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), ductal carcinoma in situ (DCIS; n=30) (Z=-2.032, p=0.042), and invasive breast cancer (IBC; n=263) (Z=-2.443, p=0.015) (Figure 1A). RHBDF1 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) (Figure 1A). The percentages of medium and strong RHBDF1 expression (2+/3+) in normal, ADH, DCIS, and IBC specimens were 0, 20, 24, and 55%, respectively (Figure 1B). In addition, IBC patients with high RHBDF1 expression (2+/3+; n=146) exhibited a poorer overall survival rate compared with patients with low RHBDF1 expression (−/+; n=117) (p=0.0021) (Figure 1C). Progression-free survival was also markedly worse for IBC patients with high RHBDF1 expression than for those with low RHBDF1 expression (p=0.0003) (Figure 1D). Moreover, we examined the correlations between RHBDF1 expression levels and a range of clinicopathological parameters in the IBC cases. The percentages of local recurrence in low and high RHBDF1 groups were 2.6% and 9%, respectively (p=0.029) (Figure 1E). The percentages of cases in RHBDF1 low and high groups with metastasis to at least one positive lymphnode were 53% and 66%, and those with 10 or more were 15% and 24%, respectively (p=0.027) (Figure 1F). The percentages of distant
metastasis in low and high RHBDF1 groups were 9.3% and 32.7%, respectively (p=0.012) (Figure 1G). There were no statistically significant difference between RHBDF1 low and high groups with regard to age, tumor size, histological grade, TNM stage, estrogen receptor expression, progesterone receptor expression, and C-erbB-2 levels (Supplemental Table 1). Furthermore, we studied RHBDF1 expression levels in the tumor specimens of 67 breast cancer patients who had completed a full protocol of neoadjuvant chemotherapy prior to surgical excision of the tumor (refer to Supplemental Materials for treatment detail). We found that greater than 80% of the cases in low RHBDF1 group had good responses in comparison with about 40% of good responders in high RHBDF1 group (Figure 1H). These findings indicate that elevated RHBDF1 expression in breast cancer is strongly correlated with facilitated disease progression, local recurrence, lymphnode and distant metastasis, poor prognosis, and poor responses to chemotherapy.

Raising RHBDF1 expression levels in cancer cells leads to enhanced HIF1α protein stability: To begin to investigate RHBDF1’s role in breast cancer progression, we compared RHBDF1 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) (Figure 2A). Less than 30% of the patients in RHBDF1 low group exhibited high HIF1α (2+, 3+), whereas in sharp contrast about 75% of the patients in RHBDF1 high group showed high HIF1α levels (Figure 2B). In addition, we found that the percentage of poor responders to neoadjuvant chemotherapy was less than 20% when both RHBDF1 and HIF1α levels are low, whereas it reached about 70% when both RHBDF1 and HIF1α are high (Figure 2C). We thus overexpressed RHBDF1 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) (Figure 2D) as well as in hypoxia (1% O2) (Figure 2E; the first two lanes). We cultured the cells in hypoxia for 4 h, 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 (Figure 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 short hairpin RNA against RHBDF1 (shRHB) under hypoxic conditions caused a marked decline of HIF1α protein (Figure 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 (Figure S5). We also found much faster HIF1α degradation during re-oxygenation in shRHB-treated cells than that in shScr-treated cells (Figure 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 (Figure 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 4-24
h in hypoxia led to an approximately 50% decrease in VEGF secretion into the culture media determined by ELISA (Figure 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 (Figure 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 (Supplemental Figure 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 investigated 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 hydroxylase (21). We found that shRHB treatment facilitated the degradation of the mutant HIF1α in hypoxia, indicating an independence on prolyl hydroxylation (Figure 4A). We then co-transfected T47D cells with RHBDF1 cDNA and the mutant HIF1α, and found that raising RHBDF1 levels led to increased HIF1α stability in hypoxia (Figure 4B). We then examined whether RACK1 and HSP90 are involved in 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 (Figure 4C, and Figure S6A). In addition, we co-transfected 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 (Figure 4D, and Figure S6B). To determine the involvement of HSP90, we simultaneously treated MCF7 cells with RHBDF1 shRNA and 17-AAG, which inhibits HSP90 activity (6), and found that RHBDF1 gene silencing further accelerated HIF1α degradation caused by 17-AAG treatment (Figure 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 (Figure 4F). 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-immunoprecipitation (co-IP) analysis indicated that RHBDF1 was able to bind to RACK1 (Figure 4G) and HIF1α (Figure 4H), but not to HSP90 (data not shown). To determine whether RHBDF1-interacting HIF1α was ubiquitinated, we treated MCF7 cells 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 (Figure 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 (Figure 4J). These findings suggest that RHBDF1-overexpression lead to diminished RACK1-HIF1α interaction, thus protects 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 (Figure 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, while 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 the ubiquitination of the mutant HIF1α upon treatment with 17-AAG and shRHB together compared with shRHB alone (Figure 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 overexpress the P402A/P564A mutant HA-HIF1α, cultured the cells in hypoxia, and carried out co-IP analysis (Figure 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 (Figure 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 (Figure 5E). These results indicate that RHBDF1 interaction 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 (Supplemental Figure 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 in order to ensure entrance of the peptide into the cells (23) in case some of these potential phosphorylation sites were located inside the cells (Supplemental Table 2). 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 (Figure 6A) as well as the transcription of a number of HIF1-targeted genes, including VEGF, GLUT1, CA9 and ET1 (Supplemental Figure S4). VR56 treatment also facilitated HIF1α degradation (Figure 6B). A peptide identical to VR56 but without the tag (VH36), or the R11 tag itself (ER20), did not exhibit these activities. Additionally, we found that VR56 treatment of RHBDF1-overexpressing MCF7 cells in hypoxia led to a marked inhibition of RHBDF1-enhanced HIF1α stabilization (Figure 6C). VR56 inhibition of RHBDF1-enhanced HIF1α stabilization was dose-dependent (Figure 6D). VR56 also inhibited RHBDF1 tyrosine phosphorylation (Figure 6E). Treatment of MCF7 cells with VR56 resulted in
a marked decrease of cell viability (Supplemental Figure S4). We then treated RHBF1- and RACK1-overexpressing MCF7 cells with VR56 in hypoxia, and found that the treatment significantly inhibited RHBF1 binding to RACK1 (Figure 6F). When these experiments were carried out in the presence of MG132, VR56 treatment inhibited RHBF1 binding to RACK1 but enhanced HIF1α binding to RACK1 (Figure 6G). These findings indicate that disruption of RHBF1 tyrosine phosphorylation with VR56 leads to inhibition of RHBF1 binding to RACK1, enhancement of HIF1α binding to RACK1, and destabilization of HIF1α.
DISCUSSION

Based on these findings, we propose a “molecular switch” to explain RHBDF1 suppression of HIF1α degradation in hypoxia (Figure 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 since 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 clinicopathological 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 nodal 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. Since HIF1 directly promotes hypoxia-associated drug resistance by enhancing the expression of antiapoptotic proteins and diminishing the expression of proapoptotic proteins or by non-apoptotic 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 non-catalytic rhomboids, such as human RHBDF1, may function as regulatory proteins. Non-catalytic 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 anti-cancer therapeutics.
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REFERENCES

FIGURE LEGEND

Figure 1: Correlations between RHBDF1 expression, breast cancer disease progression, patient survival, and responses to chemotherapy. (A) RHBDF1 immunostaining of specimens of various disease states, including normal breast tissue, adjacent normal tissue, atypical duct hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive breast cancer (IBC). Magnification: 200×. (B) Percentage of specimens of various disease stages exhibiting different RHBDF1 levels (p<0.05, Kruskal-Wallis test and Mann-Whitney U-test); white, −; stripes, +; crosses, 2+; black, 3+. (C) Kaplan–Meier plots of overall survival rate of IBC patients exhibiting high (green, 2+/3+) or low (blue, −/+)) RHBDF1 levels (p=0.002, log-rank test). (D) Kaplan-Meier plots of progression-free survival of IBC patients exhibiting high (green, 2+/3+) or low (blue, −/+) RHBDF1 levels (p<0.001, log-rank test). (E) Percentages of recurrence in RHBDF1 low or high groups. (F) Percentages of distant metastasis in RHBDF1 low or high groups. (G) Percentages of lymphnode metastasis in RHBDF1 low or high groups; marks on bars indicate the number of cancer cell-positive lymphnodes per case: white, 0; stripes, 1-3; crosses, 4-9; black, 10 or more. (H) Percentages of poor (white) or good (stripes) responses to chemotherapy with regard to RHBDF1 levels. Numbers on bars indicate the number of cases. Spearman’s Rank-Correlation test, * p<0.05, ** p<0.01.

Figure 2: Positive correlation between RHBDF1 and HIF1α levels in breast cancer specimens and RHBDF1-transfected T47D cells. (A) RHBDF1 and HIF1α immunostaining of serial sections of IBC cases. Magnification: 200×; inset magnification: 400×. (B) Percentages of IBC specimens exhibiting various HIF1α levels (white, −; stripes, +; crosses, 2+; black, 3+) with...
regard to low (−/+ ) or high (2+/3+) RHBDF1 levels. (C) Responses of patients to chemotherapy with regard to levels of RHBDF1 and HIF1α; white, poor; stripes, good. (D) Impact of RHBDF1 (RHB) overexpression on HIF1α stabilization in T47D cells in normoxia compared with empty vector-transfection. (E) Impact of RHBDF1 overexpression on HIF1α protein stability in T47D cells during re-oxygenation; cells were cultured in 1% O2 in the absence or presence of MG132 (10 μM) for 4 h, then re-exposed to 20% O2 in the absence of MG132 for the indicated period of time (min). * p<0.01, ** p<0.001, Chi-square test, Kruskal-Wallis test, and Mann-Whitney U-test.

Figure 3: Inhibitory Effect of RHBDF1 gene silencing in MCF7 cells on HIF1α stability and VEGF secretion. (A) Effect of RHBDF1 shRNA (shRHB) or scrambled shRNA (shScr) on HIF1α degradation in MCF7 cells in hypoxia. (B) Effect of shRHB or shScr on HIF1α protein stability in MCF7 cells during re-oxygenation. (C) Nuclear translocation of HIF1α in shSrc- or shRHB-treated MCF7 cells; α-Tubulin and Myc are cytoplasmic and nuclear protein controls, respectively. (D) Changes of VEGF concentrations in the condition media of shRHB (white bars)- or shScr (black bars)-treated MCF7 cells under normoxic or hypoxic conditions as a function of time (h). (E) Changes of VEGF concentrations in the condition media of shRHB (white bars)- or shScr (black bars)-treated MDA-MB-231 cells under normoxic or hypoxic conditions as a function of time (h). Each experiment was repeated two times. * p<0.05, Student t-test.

Figure 4: Involvement of RHBDF1, RACK1 and HSP90 in oxygen-independent HIF1α degradation. (A) Effect of RHBDF1 gene silencing in MCF7 cells on the stability of HA-tagged
P402A/P564A mutant HIF1α (HA-HIF1α) in hypoxia. (B) Effect of RHBDF1 overexpression in T47D cells on the stability of the mutant HIF1α in hypoxia. (C) Effect of RACK1 gene silencing on HIF1α stability in MCF7 cells in hypoxia. (D) Effect of FLAG-RHBDF1 overexpression in T47D cells on the stability of the mutant HIF1α in hypoxia. (E) Effect of 17-AAG treatment (0.5 mM, 24 h) of RHBDF1 gene-silenced MCF7 cells on HIF1α stability in hypoxia. (F) Effect of 17-AAG treatment (0.5 mM, 24 h) of RHBDF1-overexpressing T47D cells on HIF1α in hypoxia. (G) Co-IP of RHBDF1 and RACK1 from MCF7 cells transfected with FLAG-RHBDF1. (H) Co-IP of RHBDF1 and HIF1α from MCF7 cells co-transfected with HA-tagged wild type HIF1α. (I) Co-IP of RHBDF1 with HIF1α and RACK1 from MCF7 cells in the absence or presence of MG132 (10 μM, 4 h) in normoxia or hypoxia. (J) Co-IP of HIF1α with HSP90, and of RHBDF1 with RACK1 from MCF7 cells co-transfected with FLAG-RHBDF1 and the mutant HIF1α upon treatment with either control siScr, RACK1 siRNA (siRACK1), or 17-AAG (0.5 mM). Each experiment was repeated two times and representative Western blotting analysis or co-IP results from one experiment are shown.

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 mM, 24 h) or MG132 (10 μM, 4 h), 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 shSrc,
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.

**Figure 6:** Interruption of RHBDF1 tyrosine phosphorylation causes an inhibition of RHBDF1-facilitated HIF1α stabilization and disruption of RHBDF1 with RACK1. (A) ELISA measurement of VEGF secretion by MDA-MB-231 cells in hypoxia treated with various peptides (20 μM, 24 h). (B) HIF1α levels in MDA-MB-231 cells treated with the indicated peptides for 24 h, followed by 6 h culture in hypoxia. (C) HIF1α stability in RHBDF1- or empty vector-transfected MCF7 cells treated with VR56 or ER20 in hypoxia. (D) HIF1α stability in RHBDF1-overexpressing MCF7 cells in response to various VR56 doses. (E) Inhibition of RHBDF1 tyrosine phosphorylation by VR56 in RHBDF1-overexpressing MCF7 cells. (F) Disruption of RHBDF1-RACK1 interaction by VR56 in MCF7 cells co-transfected with RHBDF1 and RACK1; the cells were treated with vehicle, ER20, or VR56 for 48 h in normoxia, followed 6 h culture in hypoxia. (G) RACK1 binding to RHBDF1 or HIF1α in MCF7 cells co-transfected with RHBDF1 and RACK1 in response to VR56 or ER20 treatment; the cells were treated with the peptides for 24 h in normoxia, followed by 6 h culture in hypoxia in the presence of MG132. Each experiment was repeated two times and representative results from one experiment are shown.

**Figure 7:** A schematic representation of a hypothesized mechanism of RHBDF1-facilitated HIF1α stabilization.
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Human Rhomboid Family-1 (RHBDF1) Suppresses Oxygen-Independent Degradation of Hypoxia-Inducible Factor-1 α in Breast Cancer

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