Regulation of ERα Stability and Estrogen Signaling in Breast Cancer by HOIL-1

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Estrogen receptor α (ERα) is the major driver for breast tumor carcinogenesis and progression, while ERα positive breast cancer is the major subtype in breast malignancies, which account for 70% breast cancers in patients. The success of endocrine therapy such as tamoxifen is one of the biggest breakthroughs in breast cancer treatments. However, the endocrine therapy resistance is a headache problem in breast cancer. Further mechanisms need to be identiﬁed to the effect of ERα signaling in controlling breast cancer progression and drug resistance. HOIL-1 was ﬁrstly identiﬁed as the ERα transcriptional co-activator in modulating estrogen signaling in breast cancer. In our current study, we showed that HOIL-1, which was elevated in breast cancer, related to good prognosis in ERα positive breast cancer, but correlated with poor outcome in endocrine-treated patients. HOIL-1 was required for ERα positive breast cancer proliferation and clone formation, which effect could be rescued by further ERα overexpression. Further mechanism studies showed that HOIL-1 is required for ERα signaling activity in breast cancer cells. HOIL-1 could interact with ERα in the cytosol and modulate ERα stability via inhibiting ERα K48-linked poly-ubiquitination. Thus, our study demonstrated a novel post-translational modiﬁcation in ERα signaling, which could provide novel strategy for ERα-driven breast cancer therapy.

Keywords: HOIL-1, ERα, breast tumor, stability, ubiquitin

INTRODUCTION

The ubiquitination process functions to modulate the protein disposal and function in eukaryotic cell hemostasis (1, 2). E3 ubiquitin ligases promote the transfer of ubiquitin from the E2 ubiquitin conjugating enzymes to target protein substrates via their lysine residues (3). The ubiquitination can be classiﬁed as several types through the lysine residues on the ubiquitin proteins, including K63, K48, linear ubiquitination and mono-ubiquitination (2). The ubiquitination process was ﬁrstly discovered as a target for proteins degradations (4). However, the studies in recent years showed...
that the ubiquitin systems play important role in protein functions in a group of regulatory pathways, such as signaling transduction, DNA damage response and endocytosis (5, 6).

The ubiquitin process involves the coordinated reactions of E1 ubiquitin-activation enzyme, E2 ubiquitin enzymes and E3 ubiquitin ligases (4). It has been show that the E3 ubiquitin ligases are the key factors, which specifically interact with certain substrates and E2 enzymes for ubiquitin transferring. According to the functional domains of E3 ubiquitin ligases, they can be separated into four groups: HECT (Homologous to the E6-AP Carboxyl Terminus), RING, U-box and PHD-finger family (7). The RING family proteins are composed of more than 700 different proteins, most of which are not well investigated (8).

Based on current understanding of RING proteins, the RING family members are involved in several cell physiological functions, including cell proliferation, protein trafficking and DNA repair (9, 10). One of the most thoroughly studied proteins is BRCA1 (RNF53), which participates in DNA repair (9, 10). One of the most thoroughly studied proteins is BRCA1 (RNF53), which participates in DNA repair, gene functions, including cell proliferation, protein trafficking and DNA repair (9, 10).

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Recent studies showed that RING family proteins play important role in tumor carcinogenesis and progression (15). Several atypical ubiquitination manners, which modified quite a few nuclear receptors, exhibited regulatory functions in cancer signaling transductions. For example, RNF31 and RNF8 functioned quite a few different manners, most of which are not well investigated (8).

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Plasmids and siRNA
The FLAG-HOIL-1 plasmid is acquired from previous study (19). The HOIL-1 deletion constructs were acquired from the previous study (19). The ERα full and deletion constructs were described in previous study (20). The HA-K48 Ub, HA-K63 Ub and HA-Ub-KO plasmids were used in previous study (20). The Estrogen-Response-Element (ERE)-TK reporter and renilla plasmids were used in previous study and are transfected with Lipofectamin 2000 (1662298, Invitrogen) (21). For siRNA transfection, the HOIL-1 siRNA sequences are #1: 5-GCC UUC AGC UAC CAU UGC ATT-3, 5-UGC AAU GGU AGC UGA AGG CTT-3; #2: 5-CAC ACC UUC UGC AGG GAG UTT-3, 5-ACU CCC UGC AGA AGG UGU GTT-3. The siControl sequences are 5-UUC UCC GAA CGU GUC ACG UTT-3, 5-ACG UGA CAC GUU CG GAGA ATT-3.

RNA Extraction and qPCR Analysis
RNAeasy plus mini kits were used to extract total RNA (Qiagen). Real-time PCR was performed as previously described (18). 36B4 was used as internal control. Primer sequences for qPCR are provided: GREB1 F: CGT GTG GTG ACT GGA GTA GC, R: ACC TCT TCA AAG CTC GTC GT; HOIL-1 F: GCA GAT GAA CTG CAA GGA GTA TCA, R: TGC AGC ATC ACC TTC AGC AT; ER F: GCT ACG AAG TGG GAA TGA TGA AAG, R: TCT GGC GCT TGT GTT TCA AC; 36B4 F: GGC GAC CTG GAA GTC CA ACT, R: CCA TCA GCA CCA CAG CCT TC; PS2 (TFF1) F: TGG GCT TCA TGA GCT CCT TC, R: TTC ATA GTG AGA GAT GGC CGG.

Quantification of Cell Viability
MCF-7 and T47D cells were transfected with siHOIL-1 or siControl in 24-well plates. Twenty-four hours after transfection, the cells number was countered and 4,000 cells were seeded into 96-well plates. The relative cell viability was measured at indicated time points. Cell numbers were determined using the WST-1 cell proliferation reagent as previously described (22).

Western Blotting
Cells were harvested and lysed with RIPA buffer. Proteins were separated by electrophoresis on SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred to PVDF membrane. The antibodies used in this study were listed here: Anti-ERα (D8H8, 8644, Cell signaling Technology); Anti-ERα (SC-56833, Santa Cruz); Anti-GAPDH (Ab290, Abcam). Membranes were then washed with PBS for three times and incubated with secondary antibodies Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG. Fluorescent signals were visualized with ECL system (Amersham Imager 600, USA).

Luciferase Assay
The luciferase activity of estrogen signaling activity was measured at indicated time points. Cell numbers were determined using the WST-1 cell proliferation reagent as previously described (22).

MATERIALS AND METHODS

Cell Culture
MCF-7, T47D and HEK293 cells are originated form American Type Culture Collection (ATCC). T47D cells are cultured with RPMI-1640 (42401, Life Technologies) supplemented with 2 mM L-glutamine (25030, Life Technologies) and 10% FBS. MCF-7 and HEK293 are culture with Dulbecco’s Modified Eagle’s Medium that contains 4.5 g/L glucose and 4 mM L-glutamine (DMEM, 41965, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, 10270, Life Technologies). All cell lines are characterized by cell line authentication. The cell line authentication via Short Tandem Repeat (STR) is performed via PowerPlex 21 system. The STR data of MCF-7 and T47D cell lines are found consistent with STR data in ATCC.

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Luciferase Assay
The luciferase activity of estrogen signaling activity was performed using the Dual-Luciferase Reporter kit (Promega, Germany). The ERE luciferase reporter was transfected
together with the Renilla plasmid into the cells. Luciferase activity was measured after 24 h.

Co-Immunoprecipitation Assay
Immunoprecipitation was performed as described in previous study. The MCF-7 total cell lysates were pre-cleared with rabbit IgG for 2 h and subsequently immunoprecipitated with ERα antibody (D8H8, #8644) over night, while rabbit IgG (Santa Cruz) was used as the negative control. The bound protein was analyzed by Anti-HOIL-1 antibody (Ab108479). For the overexpression experiment, HEK293 cells were transfected with 5 μg FLAG-HOIL-1 (Full length or deletion domains) and ERα plasmid (Full length or deletion domains) in 10 cm dish. Cell lysates were pre-cleared with IgG and subsequently incubate with Flag (Ab49763) antibody, while rabbit IgG was used as the negative control. The bound proteins were analyzed by western blotting.

Poly-Ubiquitination Detection Assay
To directly detect the enriched overall ubiquitinated or K63-linked ERα protein level from the cell extracts, HEK293 cells were transfected with 4 μg Ub or 4 μg K63 Ubi plasmid, 2 μg ERα together with 0.5 μg Flag-HOIL-1 or Flag-vector. After 48 h, total protein was extracted and pre-cleared with 20 μl protein A (Santa cruz, SC-2001) for 2 h. The supernatant was collected and immunoprecipitated by ERα antibody. Western blot with HA antibody was performed to detect K48, K63 poly-ubiquitinated or mono-ubiquitinated ERα.

Immunofluorescence Assay
MCF-7 cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked by 5% BSA in PBS for 1 h. A rabbit anti-HOIL-1 polyclonal antibody (Ab108479) and mouse anti-ERα monoclonal antibodies (SC-56833) were used, followed by Alexa Flour 647 (Invitrogen) anti-rabbit antibody and FITC-conjugated anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA). As negative controls, the samples were incubated with the secondary antibodies without primary antibodies. Images were acquired under conditions fulfilling the Nyquist criterion using Nikon A+ laser scanning confocal system with a 60× oil NA1.4 objective and pinhole size of 1.0 Airy Unit. The acquired pictures were further processed and assembled using ImageJ.

Statistics
Student’s t-test, Pearson correlation coefficient, and Cox regression analysis were used for comparisons. A P-value of <0.05 was considered to be significant.

RESULTS
HOIL-1 Is Elevated in Breast Cancer and Relates to Short Endocrine Treatment Outcome in Human Breast Cancer Tumors
We firstly analyzed the HOIL-1 expression level from public available database. The TCGA database (https://tcga-data.nci.nih.gov) showed that HOIL-1 was increased in breast cancer compared with breast tissues (Fold change = 1.35; P <0.001) (Figure 1A). In breast cancer subtype analysis, the data showed that HOIL-1 is all breast subtypes, including luminal type, HER2 positive type and triple negative type (Figure 1B). When we analyzed the HOIL-1 effect on breast cancer patient survival from KMPLOT database (https://kmplot.com), we observed that HOIL-1 related to longer progression survival in all patents and luminal type patients (Figures 1C, D). HOIL-1 expression also correlated with good prognosis in triple negative breast cancers (Figure S1A). However, HOIL-1 specially correlated with poor survival in endocrine-treated patients (Figure 1E). Besides, the gene expression analysis from the TCGA database showed that HOIL-1 was positively correlated with ERα target gene expression including GREB1 and TFF1 in breast tumors (P <0.01, R = 0.17; P = 0.008, R = 0.08 respectively) (Figures 1F, G). These clinical data showed the consistent trend with previous reports that HOIL-1 might promote ERα signaling and endocrine resistance (23, 24).

HOIL-1 Depletion Inhibits ERα Protein and ERα Signaling in Breast Cancer Cells
In order to uncover the role of HOIL-1 in ERα signaling in breast cancer cells, we depleted HOIP in MCF-7 cells. HOIL-1 depletion via two independent siRNA showed that HOIL-1 knocking-down decreases ERα protein level and ERα target gene expression, including PS2, GREB1 and PDZK1 (Figures 2A, B). Besides, HOIL-1 depletion could decrease ERα protein level in both vehicle and E2-treated condition in MCF-7 and T47D cells (Figures 2C, D). Consistent with this, HOIL-1 depletion also decreased ERα target gene expression, such as PS2, GREB1 and PDZK1 in both MCF7 and T47D cells (Figures 2E, F). In order to determine if HOIL-1 knocking down could affect ERα transcriptional activity, we measured estrogen response element (ERE) luciferase activity in both MCF-7 and T47D cells. It showed that HOIL-1 depletion decreases ERE reporter in both MCF7 and T47D cells (Figures 2G, H). All these data indicate HOIL-1 is required for ERα signaling in breast cancer cells. In addition, we also found HOIL-1 depletion facilitated PS2 protein level and its target gene expression (Figures S1B, C). We further investigated the function of HOIL-1 in ER negative breast cancer cells. In MDAMB231 cells, HOIL-1 depletion inhibited cell invasion (Figures S2A–C). Besides, HOIL-1 depletion also inhibited cell proliferation and migration in MDAMB231 cells (Figures S2D, E).

HOIL-1 Is Mainly Localized in the Cytoplasm and Modulates ERα Stability
In order to investigate the role of HOIL-1 in breast cancer cells, we depleted HOIL-1 in both MCF-7 and T47D cells. WST assay showed that HOIL-1 depletion significantly decreased breast cancer cell proliferation in MCF-7 and T47D cells In MTT assay (Figures 3A, B). Besides, the EdU (5-ethynyl-2-doxuridine) incorporation assay showed that HOIL-1 depletion significantly decreased the EdU positive cells in MCF-7 and T47D cells (Figures 3C, D). In the wound-healing...
FIGURE 1 | HOIL-1 is elevated in breast cancer and relates to short endocrine treatment outcome in human breast cancer tumors. (A) HOIL-1 mRNA level is elevated in breast cancer samples compared with breast tissues from TCGA database (https://tcga-data.nci.nih.gov). (B) HOIL-1 mRNA level is elevated in all subtypes of breast cancer compared with normal breast tissues from TCGA database (https://tcga-data.nci.nih.gov). (C) HOIL-1 mRNA level correlates with good prognosis in breast cancer patients. These clinical data are acquired from KMPLOT database (http://kmplot.com/analysis/). (D) HOIL-1 mRNA level correlates with good prognosis in ER positive breast cancer patients. These clinical data are acquired from KMPLOT database (http://kmplot.com/analysis/). (E) HOIL-1 mRNA level correlates with poor prognosis in endocrine-treated breast cancer patients. These clinical data are acquired from KMPLOT database (http://kmplot.com/analysis/). (F) HOIL-1 mRNA level correlates with GREB1 expression in human breast cancer samples. These clinical data are acquired from TCGA database (https://tcga-data.nci.nih.gov). (G) HOIL-1 mRNA level correlates with TFF1 expression in human breast cancer samples. These clinical data are acquired from TCGA database (https://tcga-data.nci.nih.gov).
FIGURE 2 | HOIL-1 depletion inhibits ERα protein and ERα signaling in breast cancer cells. (A) HOIL-1 depletion effect on ERα protein level by two different siRNA oligos. MCF-7 cells were transfected with two independent HOIL-1 siRNAs or siControl. HOIL-1 and ERα protein levels were determined by Western blot analysis. Tubulin was used as internal control. (B) HOIL-1 depletion decreases ERα target genes using two different siRNA oligos. MCF-7 cells were transfected with siHOIL-1 or siControl. After 48 h, total RNA was prepared and the expression of the endogenous ERα target genes, PS2, GREB1, and PDZK1 were determined by qPCR. Shown are the results from three experiments. *P <0.05; **P <0.01; ***P <0.001 for target gene expression comparison. (C) HOIL-1 depletion effect on ERα protein level. MCF-7 cells were transfected with siHOIL-1 or siControl. After 48 h, cells were treated with ethanol or 10 nM estradiol for 6 h. HOIL-1 and ERα protein levels were determined by Western blot analysis. Tubulin was used as internal control. (D) HOIL-1 depletion effect on ERα protein level. T47D cells were transfected with siHOIL-1 or siControl. After 48 h, cells were treated with ethanol or 10 nM estradiol for 6 h. HOIL-1 and ERα protein levels were determined by Western blot analysis. Tubulin was used as internal control. (E) HOIL-1 depletion decreases ERα target genes. MCF-7 cells were transfected with siHOIL-1 or siControl. After 48 h, cells were treated with ethanol or 10 nM estradiol for 6 h. Total RNA was prepared and the expression of the endogenous ERα target genes, PS2, GREB1, and PDZK1 were determined by qPCR. Shown are the results from three experiments. *P <0.05; **P <0.01; ***P <0.001 for target gene expression comparison. (F) HOIL-1 depletion decreases ERα target genes. T47D cells were transfected with siHOIL-1 or siControl. After 48 h, cells were treated with ethanol or 10 nM estradiol for 6 h. Total RNA was prepared and the expression of the endogenous ERα target genes, PS2, GREB1, and PDZK1 were determined by qPCR. Shown are the results from three experiments. *P <0.05; **P <0.01; ***P <0.001 for target gene expression comparison. (G) HOIL-1 depletion affects ERE-luciferase activity in MCF-7 cells. MCF-7 cells were transfected with siHOIL-1 or siControl together with ERE luciferase reporter plasmid. Cells were treated with 10 nM estradiol or vehicle. Luciferase activity was measured 48 h after transfection. Shown are the results from three experiments. *P <0.05; **P <0.01; ***P <0.001 for luciferase activity comparison. (H) HOIL-1 depletion affects ERE-luciferase activity in T47D cells. T47D cells were transfected with siHOIL-1 or siControl together with ERE luciferase reporter plasmid. Cells were treated with 10 nM estradiol or vehicle. Luciferase activity was measured 48 h after transfection. Shown are the results from three experiments. *P <0.05; **P <0.01; ***P <0.001 for luciferase activity comparison.
FIGURE 3 | HOIL-1 is required for breast cancer progression in ER positive breast cancer cells. (A) HOIL-1 depletion inhibits the cell proliferation in breast cancer cells. MCF-7 cells were transfected with 50 nM HOIL-1 siRNA (mix of #1 and #2) or 50 nM control siRNA. After 24 h, the WST assay was used to determine the cell metabolic activity at indicated time points after transfection. Experiments were done in triplicates. *P <0.05; **P <0.01; ***P <0.001 for cell growth comparison. (B) HOIL-1 depletion inhibits the cell proliferation in breast cancer cells. T47D cells were transfected with 50 nM HOIL-1 siRNA (mix of #1 and #2) or 50 nM control siRNA. After 24 h, the WST assay was used to determine the cell metabolic activity at indicated time points after transfection. Experiments were done in triplicates. *P <0.05; **P <0.01; ***P <0.001 for cell growth comparison. (C) HOIL-1 depletion decreased the EdU positive cells in MCF-7 cell. MCF-7 cells were transfected with 50 nM HOIL-1 siRNA (mix of #1 and #2) or 50 nM control siRNA. After 24 h, the EdU reagents were added into the cell culture medium for 2 h. After that, the cells were fixed and the EdU positive cells were counted. Experiments were done in triplicates. *P <0.05; **P <0.01; ***P <0.001 for comparison. (D) HOIL-1 depletion decreased the EdU positive cells in T47D cell. T47D cells were transfected with 50 nM HOIL-1 siRNA (mix of #1 and #2) or 50 nM control siRNA. After 24 h, the EdU reagents were added into the cell culture medium for 2 h. After that, the cells were fixed and the EdU positive cells were counted. Experiments were done in triplicates. *P <0.05; **P <0.01; ***P <0.001 for comparison. (E) Wound-healing assay of MCF-7 cells were transfected with siControl or siHOIL-1. Quantification of wound closure at the indicated time points. Data are presented as ± SD. **P<0.01, ***P < 0.001. * means the P value is less than 0.05 but more than 0.01.
FIGURE 4 | HOIL-1 is mainly localized in the cytoplasm and modulates ERα stability. (A) HOIL-1 protein mainly locates in the cytosol. The subcellular protein fractionation kit (Thermo scientific, 78840) was used for cytoplasm and nuclear separation. Tubulin and Histone-3 were used for cytoplasm and nuclear control. (B) Intracellular localization analysis of HOIL-1 and ERα by immunofluorescence assay. MCF7 cells were cultured in normal medium before fixation. Intracellular localization of HOIL-1 (green) and ERα (red) were shown. Nuclei (blue) were stained with 4',6-diamidino-2-phenylindole (DAPI). (C) In the presence of the proteasome inhibitor MG132, the stabilization effect of HOIL-1 on ERα did not further increase ERα protein levels. HEK293 cells were transfected with 2 µg ERα plasmid and 0.5 µg Flag-tag or Flag-HOIL-1 plasmids. After 24 h, cells were treated with 10 µM MG132/vehicle for 6 h. Cell lysates were prepared for Western blot analysis. The results are representative for three independent experiments. ** means the P value is less than 0.01, but more than 0.001. *** means P value is less than 0.001.
assay, we found that HOIL-1 was required for breast cancer cell migration in MCF-7 cells (Figure 3E).

Based on the significant impact in breast cancer cell phenotype by HOIL-1, we further investigated the potential mechanism. Nuclear and cytoplasm separation showed that HOIL-1 is mainly localized in the cytoplasm, while ERα is primarily localized in the nuclear (Figure 4A). Immuno-staining showed the same trend that ERα is located mainly in the nuclear, while HOIL-1 is mainly in the cytoplasm (Figure 4B). Then we investigated the potential role of HOIL-1 on ERα stability. Since ERα could regulate its own expression in MCF-7 cells, which make it difficult to identify the direct effect of HOIL-1 on ERα protein or mRNA (25). We utilize HEK293 cells to measure the protein stability of ERα via co-transfection with HOIL-1. In the protein stability assay, HOIL-1 could stabilize ERα. However, with the presence of a proteasome inhibitor MG132, the stabilization effect on ERα protein level could not further been increased (Figure 4C). With the inhibition of protein synthesis cycloheximde, HOIL-1 could significantly increases ERα stability in HEK293 cells (Figures 4D, E). All these data indicate that HOIL-1 could prolong ERα stability.

**HOIL-1 Associates With ERα AF1 Domain Through Its RING Domain and Stabilizes ERα Possibly by Promoting Mono-Ubiquitination**

ERα is composed of three functional domains: AF1 domain, DNA binding domain and AF2 domain (Figure 5A). HOIL-1 is composed of three function domains, including UBL domain, NZF domain and RBR domain (Figure 5B). Endogenous immuno-precipitation shows that HOIL-1 could associate with ERα in MCF-7 cells (Figures 5C, D). Then the full length of ERα or ERα deletion constructs is transfected together with HOIL-1 in HEK293 cells. Co-IP indicates that HOIL-1 associates with ERα AF1 domain (Figures 5E, F). Besides, the full length of HOIL-1 or HOIL-1 deletion constructs is transfected together with ERα full length. Co-IP shows that ERα associates with HOIL-1 RBR domain (Figure 5G). Further experiments are carried out to measure ERα ubiquitination. Ubiquitin-based immuno-precipitation assay show that HOIL-1 could inhibit ERα overall ubiquitination (Figure 6A). K48-linked ubiquitin assay shows that HOIL-1 could inhibit ERα K48-linked ubiquitination (Figure 6B).
In this study, we identified the RING family E3 ubiquitin ligase HOIL-1, which was highly expressed in human breast cancer samples, facilitated ERα signaling and breast cancer progression via post-translational modification. HOIL-1 associated with ERα and inhibits ERα poly-ubiquitination and degradation (Figure 6C).

ERα was firstly cloned from MCF-7 cell in 1985 (26). About 70% of breast cancers are ERα positive, while the risk of breast cancer is also correlated with the ERα expression level in breast tissue (27). Higher levels of ERα expression in breast cancer cell can lead to increased estrogen-independent activity of ERα (28). ERα-positive cancer depends on ERα signaling for cell growth, which makes ERα a suitable target for breast cancer therapy. For ERα positive breast cancer patients, selective estrogen receptor modulators, such as tamoxifen, are standard endocrine treatment. However, endocrine resistance is one important issue in breast cancer therapy. Interesting, still most of the endocrine resistant breast cancer are ERα positive, which might indicate that ERα might play important role in mediating tamoxifen resistance. Modulating ERα protein stability could be one plausible strategy to overcome endocrine resistance.

There are about 500–1,000 different E3 ubiquitin ligases. Among these families, RING family is the largest. RING-In-Between-RING (RBR) E3 ligase is a subfamily of the RING family (29). One of the important functions of RBRs is the modulation of NFKB signaling and nuclear receptors (30–32). Recent studies reveal that several RBRs are necessary for ERα signaling activation and breast cancer proliferation. For example, RNF8 could associate with ERα and function as a co-activator for ERα target genes. Besides, RNF8 could also mono-ubiquitinate ERα and promote ERα protein stability (17). Our previous work focused on several E3 ubiquitin ligases, which were able to enhance ERα signaling activity either via genomic regulation or post-translational modifications, including RNF31, RNF168 and SMURF1 (9, 20). Here, we identifies HOIL-1, which is one interaction protein with RNF31 could modulate ERα stability via inhibiting ERα K48-linked poly-ubiquitination.

HOIL-1 was firstly identified in a yeast two-hybrid screen as a PKC interaction protein (33). The C-terminal part of the protein contains the RBR domain, while the N-terminal contains UBL domain and RZF domain (34). The RBR domain was regarded as
functional domain for ubiquitin ligation, while the UBL domain could interact with 26 proteasome (35). Previous studies showed that HOIL-1 could be an important marker for poor tamoxifen response (24). Nina et al. showed HOIL-1 could promote ERα gene expression and also co-located with ERα at ERα target gene promoter regions (23). However, our study confirms that HOIL-1 is a positive modulator for ERα signaling, but through different mechanisms. Our immuno-staining indicates that HOIL-1 is mainly localized in the cytosol, not in the nuclear in MCF-7 cells. Since ERα is mostly degraded in the cytosol, HOIL-1 could exert its dual function in ERα signaling. When HOIL-1 is in the cytosol, it co-activates ERα gene expression, while HOIL-1 is in the cytosol, it associates with ERα and enhances ERα stability.

Our study identifies that the RBR family protein HOIL-1 could modulate ERα signaling and breast cancer progression through a post-translational manner. Our study strengthens the critical role of HOIL-1 in both genomic regulation and post-translational regulation of ERα pathway. As such an important regulator of ERα signaling, HOIL-1 could be an important target for ERα positive breast cancer therapy.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

JD performed most of the bench work. PK supervised the process of the study and performed the manuscript writing. JD participated in western blot and real-time PCR work. PK performed the prognosis data analysis. All authors contributed to the article and approved the submitted version.

**REFERENCES**

1. Sens D, Qi J, Ronai ZA. Ubiquitin Ligases in Oncogenic Transformation and Cancer Therapy. Nat Rev Cancer (2018) 18(2):69–88. doi: 10.1038/nrc.2017.105
2. Han ZJ, Feng YH, Gu BH, Li YM, Chen H. The Post-Translational Modification, SUMOylation, and Cancer (Review). Int J Oncol (2018) 52(4):1081–94. doi: 10.3892/ijo.2018.4280
3. Song L, Luo ZQ. Post-Translational Regulation of Ubiquitin Signaling. J Cell Biol (2019) 218(6):1776–86. doi: 10.1083/jcb.201902074
4. Varshavsky A. The Ubiquitin System, Autophagy, and Regulated Protein Degradation. Annu Rev Biochem (2017) 86:123–8. doi: 10.1146/annurev-biochem-061516-044859
5. Rona G, Pagano M. Mixed Ubiquitin Chains Regulate DNA Repair. Genes Dev (2019) 33(23-24):1615–6. doi: 10.1101/gad.334383.119
6. Kahlhofer J, Leon S, Teis D, Schmidt O. The Alpha-Arrestin Family of Ubiquitin Ligase Adaptors Links Metabolism With Selective Endocytosis. Biol Cell (2020) 113(4):183–196. doi: 10.1111/boc.202000137
7. Zheng N, Shabek N. Ubiquitin Ligases: Structure, Function, and Regulation. Annu Rev Biochem (2017) 86:129–57. doi: 10.1146/annurev-biochem-060815-014922
8. Dove KK, Klevit RE. RING-Between-RING E3 Ligases: Emerging Themes Amid the Variations. J Mol Biol (2017) 429(22):3363–75. doi: 10.1016/j.jmb.2017.08.008
9. Zhu J, Zhao C, Kharman-Biz A, Zhuang T, Jonsson P, Liang N, et al. The Atypical Ubiquitin Ligase RNF31 Stabilizes Estrogen Receptor Alpha and Modulates Estrogen-Stimulated Breast Cancer Cell Proliferation. Oncogene (2014) 33(34):4340–51. doi: 10.1038/onc.2013.573
10. Zong D, Adam S, Wang Y, Sasanuma H, Callen E, Murga M, et al. BRCA1 Haploinsufficiency Is Masked by RNF168-Mediated Chromatin

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.664689/full#supplementary-material

**Supplementary Figure 1** | (A) HOIL-1 mRNA level correlates with good prognosis in triple negative breast cancer patients. These clinical data are acquired from KMPLOT database (http://kmplot.com/analysis/). (B) HOIL-1 depletion effect on P53 protein level. MCF-7 cells were transfected with shHOIL-1 or siControl. After 48 h, HOIL-1 and P53 protein levels were determined by Western blot analysis. Actin was used as internal control. (C) HOIL-1 depletion increases P53 target genes. MCF-7 cells were transfected with shHOIL-1 or siControl. After 48 h, total RNA was prepared and the expression of the endogenous P53 target genes, P21, BTF2 and P53INP1 were determined by qPCR. Shown are the results from three experiments. *P<0.05; ** P<0.01; ***P<0.001 for target gene expression comparison.

**Supplementary Figure 2** | (A) HOIL-1 depletion effect on MDAMB231 cells by two different siRNA oligos. MDAMB231 cells were transfected with two independent HOIL-1 siRNAs or siControl. HOIL-1 protein levels were determined by Western blot analysis. Actin was used as internal control. (B-C) HOIL-1 depletion inhibited cancer cell invasion by trans-well assay in MDAMB231 cells. (D) HOIL-1 depletion inhibits the cell proliferation in breast cancer cells. MDAMB231 cells were transfected with 50nM HOIL-1 siRNA (mix of #1 and #2) or 50nM control siRNA. After 24 hours, the WST assay was used to determine the cellular metabolic activity at indicated time points after transfection. Experiments were done in triplicates. *P<0.05; ** P<0.01; ***P<0.001 for cell growth comparison. (E-F) Wound-healing assay of MDAMB231 cells were transfected with siControl or si-HOIL-1. Quantification of wound closure at the indicated time points. Data are presented as ± SD. **, P<0.01, ***P<0.001.
Ubiquitylation. *Mol Cell* (2019) 73(6):1267–1281 e1267. doi: 10.1016/j.molcel.2018.12.010

26. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, et al. Cloning of the Human Estrogen Receptor cDNA. *Proc Natl Acad Sci USA* (1985) 82(24):7976–80. doi: 10.1073/pnas.82.24.7976

27. Shaaban AM, Sloane JP, West CR, Foster CS. Breast Cancer Risk in Usual Ductal Hyperplasia is Defined by Estrogen Receptor-Alpha and Ki-67 Expression. *Am J Pathol* (2002) 160(2):597–604. doi: 10.1016/S0002-9440(10)64879-1

28. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential Response of Estrogen Receptor Alpha and Estrogen Receptor Beta to Partial Estrogen Agonists/Antagonists. *Mol Pharmacol* (1998) 54(1):105–12. doi: 10.1124/mol.54.1.105

29. Eisenhaber B, Chumak N, Eisenhaber F, Hauser MT. The Ring Between Ring Fingers (RBR) Protein Family. *Gene* (2007) 382(2):209. doi: 10.1016/j.ygeno.2007.6-3-209

30. Tian Y, Zhang Y, Zhang B, Wang YY, Diao FC, Wang RP, et al. RBCK1 Negatively Regulates Tumor Necrosis Factor- and Interleukin-1-Triggered NF-KappaB Activation by Targeting TAB2/3 for Degradation. *J Biol Chem* (2007) 282(23):16776–82. doi: 10.1074/jbc.M701913200

31. Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, et al. SHARPIN Forms a Linear Ubiquitin Ligase Complex Regulating NF-KappaB Activity and Apoptosis. *Nature* (2011) 471(7340):637–41. doi: 10.1038/nature09814

32. Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, Sakata S, et al. SHARPIN is a Component of the NF-KappaB-Activating Linear Ubiquitin Chain Assembly Complex. *Nature* (2011) 471(7340):633–6. doi: 10.1038/nature09815

33. Tatematsu K, Tokunaga C, Nakagawa N, Tanizawa K, Kuroda S, Kikkawa U. Transcriptional Activity of RBCK1 Protein (RBCC Protein Interacting With Tubulin). *Biochem Biophys Res Commun* (1998) 247(2):392–6. doi: 10.1006/bbrc.1998.7895

34. Tatematsu K, Yoshimoto N, Koyanagi T, Tokunaga C, Tachibana T, Yoneda Y, et al. Nuclear-Cytoplasmic Shuttling of a RING-IBR Protein RBCK1 and Its Functional Interaction With Nuclear Body Proteins. *J Biol Chem* (2005) 280(24):22937–44. doi: 10.1074/jbc.M413476200

35. Yoshimoto N, Tatematsu K, Koyanagi T, Okajima T, Tanizawa K, Kuroda S. Cytoplasmic Tethering of a RING Protein RBCK1 by Its Splice Variant Lacking the RING Domain. *Biochem Biophys Res Commun* (2005) 335(2):550–7. doi: 10.1016/j.bbrc.2005.07.104

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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