

Inhibition of heat shock protein 90 destabilizes receptor tyrosine kinase ROR1 in lung adenocarcinoma

Behnoush Khaledian^{1,2,3} | Ayumu Taguchi^{2,3} | Kazuo Shin-ya⁴ | Lisa Kondo-Ida^{1,5} | Noritaka Kagaya⁴ | Motoshi Suzuki^{1,6}  | Taisuke Kajino^{1,2,3} | Tomoya Yamaguchi^{1,7}  | Yukako Shimada^{1,2,3} | Takashi Takahashi^{1,8} 

¹Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan

²Division of Molecular Diagnostics, Aichi Cancer Center, Nagoya, Japan

³Division of Advanced Cancer Diagnostics, Department of Cancer Diagnostics and Therapeutics, Nagoya University Graduate School of Medicine, Nagoya, Japan

⁴National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

⁵Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Nagoya, Japan

⁶Division of Molecular Oncology, Fujita Health University School of Medicine, Toyooka, Japan

⁷Priority Organization for Innovation and Excellence, Kumamoto University, Kumamoto, Japan

⁸Aichi Cancer Center, Nagoya, Japan

Correspondence

Takashi Takahashi, Aichi Cancer Center, Nagoya, Japan.

Email: tak@aichi-cc.jp

Funding information

Japan Agency for Medical Research and Development; Japan Society for the Promotion of Science

Abstract

We have previously identified receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a direct transcriptional target of *TTF-1/NKX2-1*, a lineage-survival oncogene in lung adenocarcinoma. ROR1 sustains prosurvival signaling from multiple receptor tyrosine kinases including epidermal growth factor receptor, MET, and insulin-like growth factor 1 receptor in part by maintaining the caveolae structure as a scaffold protein of cavin-1 and caveolin-1. In this study, a high throughput screening of the natural product library containing 2560 compounds was undertaken using a cell-based FluoPPI assay detecting ROR1-cavin-1 interaction. As a result, geldanamycin (GA), a known inhibitor of heat shock protein 90 (HSP90), was identified as a potential inhibitor of ROR1. Geldanamycin, as well as two GA derivatives tested in the clinic, 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), decreased ROR1 protein expression. We found that ROR1 physically interacted with HSP90 α , but not with other HSP90 paralogs, HSP90 β or GRP94. Geldanamycin in turn destabilized and degraded ROR1 protein in a dose- and time-dependent manner through the ubiquitin/proteasome pathway, resulting in a significant suppression of cell proliferation in lung adenocarcinoma cell lines, for which the kinase domain of ROR1, but not its kinase activity or N-glycosylation, was required. Our findings indicate that HSP90 is required to sustain expression of ROR1 crucial for lung adenocarcinoma survival, suggesting that inhibition of HSP90 could be a promising therapeutic strategy in ROR1-positive lung adenocarcinoma.

KEYWORDS

geldanamycin, HSP90, lung adenocarcinoma, proteasomal degradation, ROR1

1 | INTRODUCTION

Lung cancer is the leading cause of cancer death in both men and women.¹ Adenocarcinoma is the most common histological subtype,

arising in the distal lung and accounting for ~40% and more than 50% of all lung cancer cases in the United States and in Japan, respectively.² Substantial progress in genomic profiling during the past decade enabled us to identify a subset of lung adenocarcinoma

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2020 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

patients who harbor key oncogenic driver mutations and benefit from molecular targeted therapies, but also revealed lung adenocarcinoma as a group of distinct diseases with widespread and complex molecular heterogeneity,^{3,4} suggesting the need for novel therapeutic targets and further personalized therapy based on the molecular features of the tumors.

The TTF-1/NKX2-1 homeodomain transcription factor is essential for lung morphogenesis and epithelial cell differentiation.⁵ It is amplified in 10%-15% of lung adenocarcinoma and plays a crucial role as a lineage-survival oncogene in lung adenocarcinoma.⁶⁻⁹ We previously reported that receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a direct transcriptional target of TTF-1/NKX2-1 that sustains epidermal growth factor receptor (EGFR)-mediated PI3K-AKT prosurvival signaling in kinase-dependent and independent manners.^{10,11} Intriguingly, ROR1 also possesses a kinase-independent function as a scaffold protein of cavin-1 and caveolin-1 to maintain caveolae structure, which is crucially involved in sustaining prosurvival signaling of multiple receptor tyrosine kinases (RTKs) including EGFR, MET, and insulin-like growth factor 1 receptor (IGF1R).¹² In addition, ROR1 is involved in caveolae-dependent endocytosis through binding to cavin-3, which in turn contributes to RTK-mediated prosurvival signaling.¹³ Expression levels of ROR1 in normal tissues are attenuated after birth,^{14,15} but ROR1 is significantly upregulated in a variety of cancers.¹⁴⁻¹⁸ The oncofetal expression pattern as well as multiple roles crucial to sustaining survival signaling in cancer point to a notion that ROR1 might be an effective drug target for cancer therapy development. In this regard, the involvement of ROR1 in sustaining signaling pathways activated by multiple tyrosine kinases, which can bypass signaling, conferring resistance to tyrosine kinase inhibitors, makes it an attractive therapeutic target to inhibit.¹⁹

In the present study, we aimed at identifying compounds that potentially target ROR1-cavin-1 interaction to inhibit the scaffold function of ROR1. A high throughput screening of the natural product library containing 2560 compounds using a cell-based fluorescent-based technology detecting protein-protein interactions (FluoPPI) assay resulted in the identification of geldanamycin (GA) as a potential inhibitor of ROR1-cavin-1 interaction. We report here that ROR1 interacts with heat shock protein 90 (HSP90) and GA destabilizes and degrades ROR1 protein through the ubiquitin/proteasome pathway, suggesting that HSP90 inhibition could be a promising therapeutic strategy in ROR1-positive lung adenocarcinoma.

2 | MATERIALS AND METHODS

2.1 | Reagents

Geldanamycin (G0334) was purchased from Tokyo Chemical Industry, and 17-AAG (100068) and 17-DMAG (100069) were purchased from Calbiochem. Cycloheximide (01810), MG132 (M7449), and gefitinib (SML1657) were purchased from Sigma-Aldrich. Recombinant HGF (100-39) was purchased from Peprotech. Chloroquine (catalog no.

tlrl-chq) was purchased from InvivoGen. Tunicamycin (202-08241) was purchased from Fujifilm Wako Pure Chemical Corporation.

2.2 | DNA constructs and transfection

Constructions of expression vectors of full-length human ROR1 cDNA (pCMVpuro-ROR1) and its various deletion mutants were carried out as previously described.^{10,12} pCMV-puro-HA-Ub plasmid was constructed as previously described.²⁰ Construction of hAG-ROR1-ICD and cavin-1-Ash plasmids was carried out as described for the FluoPPI assay. For transfection, 5×10^4 COS-7 cells were seeded in 6-well plates. After 24 hours, cells were transfected with plasmids at a concentration of 0.5 $\mu\text{g}/\text{mL}$ using FuGENE 6 (Promega) for 24 hours according to the manufacturer's instructions. For the ubiquitination assay, 24 hours after transfection with pCMV-puro-ROR1 or pCMV-puro-HA-Ub, COS-7 cells were treated with 10 $\mu\text{mol}/\text{L}$ MG132 with or without 0.5 $\mu\text{mol}/\text{L}$ GA for 3 hours.

2.3 | Cell culture

NCI-H1975, NCI-H441, NCI-H1299, NCI-H2228, HCC4006, and HeLa cells were purchased from ATCC, and PC-9 cells were obtained from Riken Cell Bank. All cell lines were cultured in RPMI-1640 medium with 10% FBS. Cell lines were authenticated by short tandem repeat DNA profiling and were free from mycoplasma contamination.

For treatment with the proteasomal inhibitor MG132 and the lysosomal inhibitor chloroquine, a total of 1×10^5 cells per well were seeded in 6-well plates and treated 24 hours later with GA, MG132, chloroquine, or DMSO at the indicated concentrations. The cells were harvested 8 hours after treatment for western blot analysis.

2.4 | Fluorescent-based technology detecting protein-protein interactions assay

The intracellular domains of ROR1 and full-length cavin-1 were subcloned into pHAG-MCL and pAsh-MCL plasmids, respectively. A FluoPPI assay was used by coinfecting expression constructs of the ROR1 intracellular domain fused with an N-terminal fluorescent protein tag (hAG-ROR1-ICD) and cavin-1 with a C-terminal assembly helper tag (cavin-1-Ash) in HeLa cells. For high throughput screening of a natural product library (~260 000 samples) containing 2560 compounds, HeLa clones stably expressing hAG-ROR1-ICD and cavin-1-Ash were seeded into 384-well plates at a density of 5×10^3 cells/well. After 4 hours, screening samples were added at 0.5% and incubation was continued for 24 hours. Cells were then fixed with a 10% formalin solution and stained with 1 $\mu\text{g}/\text{mL}$ Hoechst33342. Fluorescent images were acquired using an Opera Phenix High-Content Screening System (PerkinElmer) and analyzed with the Harmony software package (PerkinElmer). Formation

of foci was quantified by calculating the mean fluorescent intensity in foci divided by that intensity in cells. Hit criteria were set to under 3.0 of that value without inducing irregular cell shape or cytotoxicity.

2.5 | Western blot analysis, immunoprecipitation-western blot analysis, and protein ubiquitination assay

Western blot and immunoprecipitation-western blot analyses were carried out according to standard procedures using Immobilon-P filters (Millipore) and an Amersham ECL western blotting detection reagent (GE Healthcare). Ubiquitination assay was carried out as previously described.²¹ Antibodies against ROR1 (cat. no. 4102; Cell Signaling Technology), HSP90 α (cat. no. ab2928; Abcam), HSP90 β (cat. no. ab53497, Abcam), GRP94 (60012-1-Ig; Proteintech), p21 (cat. no. 2947; Cell Signaling Technology), LC3B (cat. no. 2775; Cell Signaling Technology), hemagglutinin (HA) (cat. no. M180-3; MBL), Azami-Green (hAG) (cat. no. PM011M; MBL), and cavin-1 (cat. no. ab135655, Abcam; cat. no. A301-271 A, Bethyl Laboratories) were used for western blot analysis. β -Actin (cat. no. A5441; Sigma) was used as a loading control. For immunoprecipitation, anti-goat IgG and immunoprecipitation-specific anti-ROR1 were used. Signal intensities of ROR1 protein bands were quantified using ImageJ (<https://imagej.nih.gov/ij/>) and normalized to β -actin signal intensities.

2.6 | RNA interference

Cells were transfected at a final concentration of 20 nmol/L siRNA using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions using the following siRNAs: siControl (AllStars Negative Control siRNA, Qiagen), siHSP90 α #1 (Hs-HSPCA-4; Qiagen), siHSP90 α #2 (Hs-HSPCA-5; Qiagen), siHSP90 β #1 (Hs-HSPCB-1; Qiagen), siHSP90 β #2 (Hs-HSPCB-5; Qiagen), and siGRP94 (M-006417-02-0005; Dharmacon). Cells were harvested 72 hours after transfection and cell lysates were subjected to western blot analysis. For double knockdown experiments, 10 nmol/L of each siRNA was mixed and transfected.

2.7 | Real-time quantitative PCR analysis

Total RNA was extracted using an miRNeasy mini kit (Qiagen). cDNA was prepared using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A quantitative PCR assay was carried out with a 7500 Fast Real-Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: ROR1 (forward, 5'-TTCTTCATTTGCGTCTGTCG-3'; reverse, 5'-GGCACACTCACCAATTCTT-3') and GAPDH (forward, 5'-TGGTATCGTGGAAGGACTCATGAC-3'; reverse, 5'-ATGCCAGT GAGCTTCCCGTTCAGC-3'). Each sample was run in triplicate. The

Ct values for each gene were calculated and normalized to Ct values for GAPDH (Δ Ct). The expression was estimated by $2^{-\Delta$ Ct}, and the ratio was calculated relative to control.

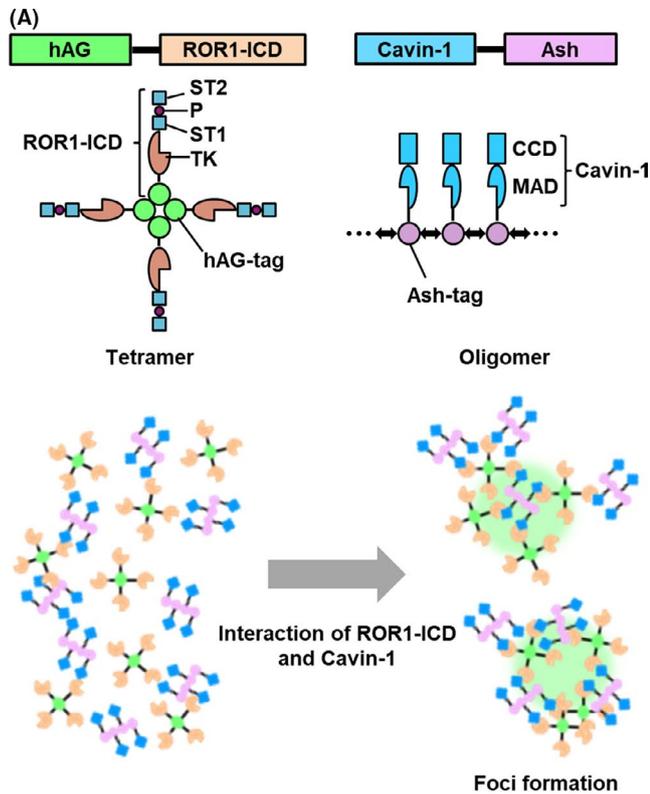
2.8 | Cell proliferation assay

For GA treatment, NCI-H1975, PC-9, NCI-H441, and HCC4006 cells were seeded at 1×10^5 cells/well, and NCI-H1299 and NCI-H2228 cells were seeded at 5×10^4 cells/well in 6-well plates. After 24 hours, cells were treated with different concentrations of GA for 96 hours. For treatment with gefitinib and hepatocyte growth factor (HGF), PC-9 cells were seeded at 1×10^5 cells/well in 6-well plates. After 24 hours, cells were treated with 0.5 μ mol/L GA, 1 μ mol/L gefitinib, and/or 50 ng/mL HGF for 96 hours. Cell proliferation was determined by colorimetric assay using CCK-8 (Dojindo Laboratories).

3 | RESULTS

To identify compounds that potentially inhibit ROR1-cavin-1 interaction, we used the cell-based FluopPI assay, in which protein-protein interaction can be evaluated as the formation of fluorescent foci in living cells (Figure 1A). A high throughput screening of an isolated natural product library containing 2560 compounds identified GA as a potential inhibitor of ROR1-cavin-1 interaction (Figure 1B). Geldanamycin is known as an HSP90 inhibitor that binds to ATP-binding site of HSP90,²² which is a molecular chaperone that plays a pivotal role in determining the stability and activity of client proteins.²³ However, ROR1 and cavin-1 are not known as HSP90 clients. To determine whether GA targets ROR1 or cavin-1 proteins themselves or physical interaction between ROR1 and cavin-1, we undertook immunoprecipitation-western blot analysis using ROR1 and cavin-1 antibodies. As shown in Figure 1C, GA decreased ROR1 protein expression but not cavin-1 protein expression. Interaction between ROR1 and cavin-1 remained 6 hours after GA treatment in NCI-H1975 cells, at which time point the total amount of ROR1 protein had already decreased markedly. Consistent with the findings observed in the FluopPI assay, treatment with GA for 24 hours almost completely diminished ROR1 protein as well as ROR1-cavin-1 interaction (Figure 1D), suggesting that ROR1 might be a client of HSP90 and degraded by GA treatment.

There are four major HSP90 paralogs, including HSP90 α and HSP90 β in cytoplasm, GRP94 in endoplasmic reticulum, and TRAP1 in mitochondria.²⁴ As endogenous ROR1 can be localized in cytoplasm or endoplasmic reticulum, we examined whether ROR1 can bind to HSP90 α , HSP90 β , or GRP94. Immunoprecipitation-western blot analysis showed interaction between ROR1 and HSP90 α , but not HSP90 β or GRP94 (Figure 2A). To determine whether HSP90 α is involved in ROR1 protein expression, we knocked down HSP90 α , HSP90 β , and GRP94 in two lung adenocarcinoma cell lines, PC-9 and NCI-H1975 cells. As shown in Figure 2B,C, knockdown of only HSP90 α reduced levels of



(B) HeLa (hAG-ROR1-ICD, Cavin-1-Ash)

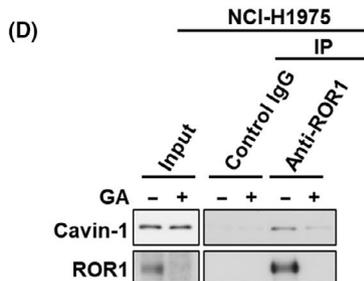
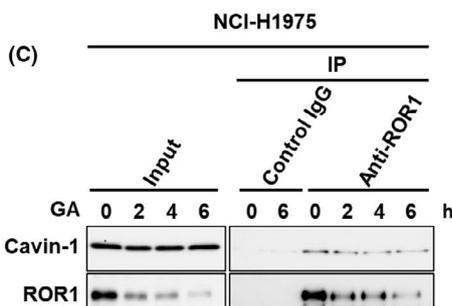
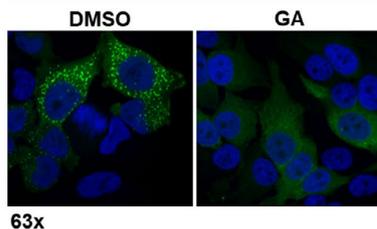


FIGURE 1 Identification of geldanamycin (GA) as a potential inhibitor of ROR1-Cavin-1 interaction. A, Schematic diagrams for constructs of humanized fluorescent Azami-Green (hAG)-ROR1-intracellular domain (ICD), cavin-1 assembly helper (Ash), and the fluorescent-based technology detecting protein-protein interactions assay. ROR1-ICD and cavin-1 were genetically fused with tetramerizing hAG-tag and oligomerizing Ash-tag, respectively. A tetramer of hAG-ROR1-ICD and an oligomer of cavin-1 can interact with multiple copies of each other, allowing the fluorescent proteins to form bright foci in cells. B, Fluorescent images of ROR1-ICD and cavin-1 interaction. Formation of fluorescent foci was suppressed after 24 h with 1 $\mu\text{mol/L}$ GA (right panel) compared to control (left panel) in HeLa cells stably expressing hAG-ROR1-ICD and cavin-1-Ash. C, D, Immunoprecipitation-western blot analyses using ROR1 Ab in NCI-H1975 cells treated with 0.5 $\mu\text{mol/L}$ GA for up to 6 h (C) and for 24 h (D). CCD, coiled-coil domain; IP, immunoprecipitation; MAD, membrane association domain; P, proline-rich domain; ST, serine/threonine-rich domain; TK, tyrosine kinase domain

ROR1 protein expression, suggesting ROR1 as a novel client protein of HSP90 α . mRNA expression levels of HSP90 α in lung adenocarcinoma was significantly increased in tumor tissues compared to adjacent normal lung but was not significantly associated with ROR1 mRNA expression levels in 574 lung adenocarcinoma RNA sequencing data from The Cancer Genome Atlas (TCGA) (<http://cancergenome.nih.gov/>) and in our previous microarray data (GSE13213)^{4,25} (Figure 2D,E).

We further examined the effects of GA on ROR1 expression. Levels of ROR1 protein decreased in both NCI-H1975 and PC-9 cells in a time-dependent manner, whereas mRNA expression levels of ROR1 were not obviously altered with GA treatment (Figure 3A), suggesting that GA regulated ROR1 at the protein level. Treatment with two GA derivatives, 17-AAG and 17-DMAG, also decreased ROR1 protein expression in NCI-H1975 and PC-9 cells (Figure 3B). As some other RTKs, including EGFR, MET, and IGF1R, have been known as HSP90 clients,²⁶⁻²⁸ we examined whether GA would reduce expression levels of these proteins in lung adenocarcinoma cell lines as well. Protein levels of EGFR, MET, and IGF1R, as well as ROR1, were decreased after 8 hours of treatment with GA in a dose-dependent manner (Figure 3C). Sensitivity of EGFR, MET, and IGF1R to GA was widely different among lung adenocarcinoma cell lines, but ROR1 was highly sensitive to GA in all six lung adenocarcinoma cell lines tested in this study. Interestingly, GA significantly decreased cell proliferation in these six cell lines (Figure 3D), suggesting the potential of HSP90 inhibitors in ROR1-positive lung adenocarcinoma. To determine whether GA can suppress activation of bypass signaling that potentially drives resistance to tyrosine kinase inhibitors, we treated gefitinib-sensitive PC-9 cells with HGF to activate MET signaling, which can compensate the inhibitory effect of gefitinib on EGFR.²⁹ Treatment with HGF restored cell proliferation reduced by gefitinib, and GA negated the effect of HGF (Figure 3E), suggesting that GA overcame HGF-mediated resistance to gefitinib in PC-9 cells.

To determine the molecular mechanisms for GA-mediated regulation of ROR1 protein, we examined whether GA suppressed ROR1

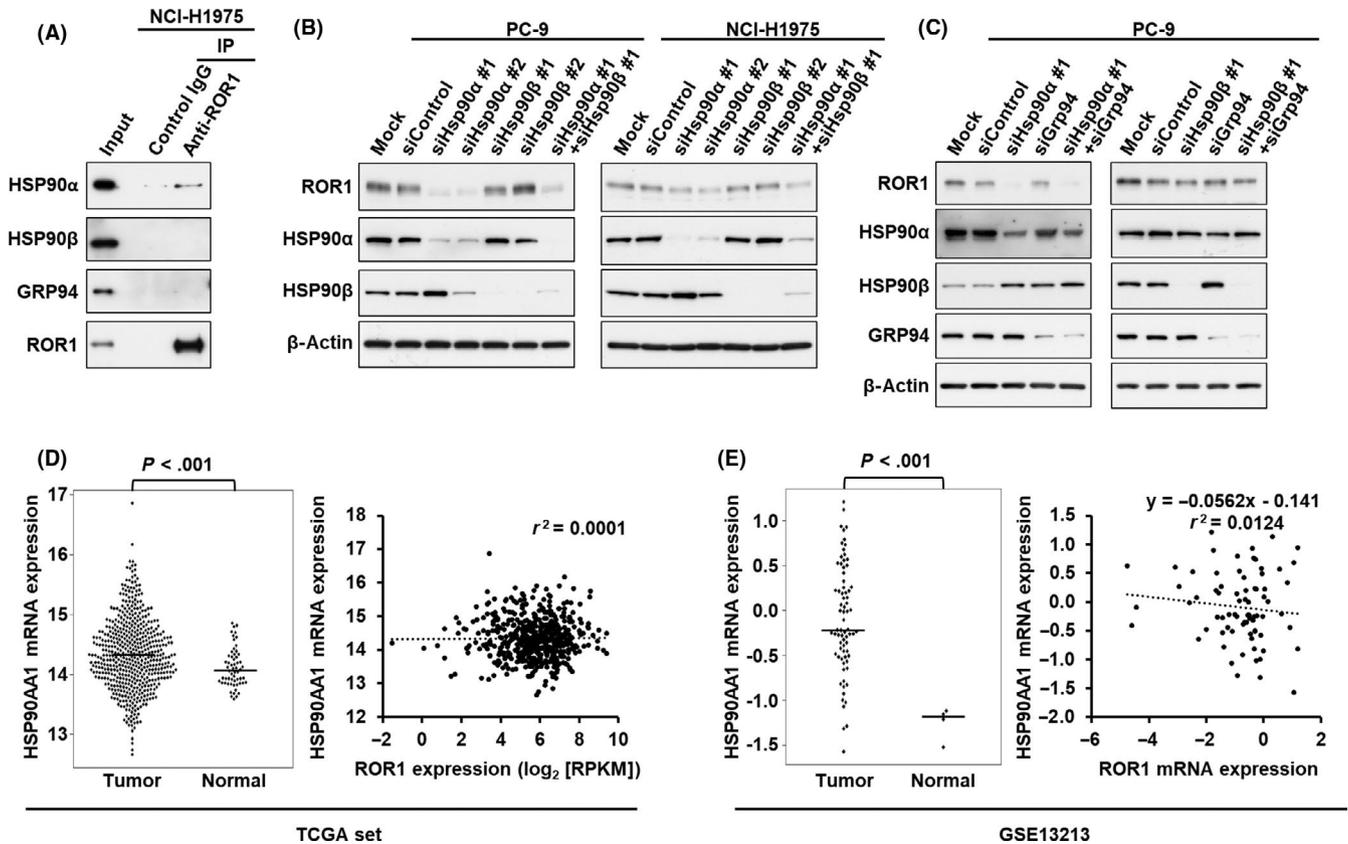


FIGURE 2 ROR1 interacts with heat shock protein (HSP)90 α . A, Immunoprecipitation (IP)-western blot analysis using ROR1 Ab in NCI-H1975 cells, showing physical interaction between ROR1 and HSP90 α , but not HSP90 β or GRP94. B, C, Levels of ROR1 protein expression were decreased in PC-9 and NCI-H1975 cells treated with siRNAs against HSP90 α , but not siRNAs against HSP90 β (B) or GRP94 (C). D, E, mRNA expression levels of HSP90AA1, which encodes HSP90 α protein, in lung adenocarcinoma tumors and adjacent normal tissues in The Cancer Genome Atlas (TCGA) dataset (tumor, N = 515; normal, N = 59) (D, left panel) and the GSE13213 dataset (tumor, N = 75; normal lung mixture, N = 4) (E, left panel). P values were calculated by unpaired t test. Correlation of mRNA expression levels of HSP90AA1 and ROR1 in tumor tissue in the TCGA dataset (D, right panel) and the GSE13213 dataset (E, right panel). Pearson correlation coefficient and P values were calculated

protein expression by attenuating protein stability or inhibiting protein synthesis. Although treatment with cycloheximide (CHX), an inhibitor of protein biosynthesis, did not largely affect expression levels of ROR1 protein, treatment with GA combined with CHX more rapidly decreased ROR1 protein than treatment with GA alone (Figures 3A and 4A, B), suggesting that GA reduced the stability of ROR1 protein. Next, we determined whether ROR1 protein is degraded through the ubiquitin/proteasome pathway or lysosomal degradation pathway.³⁰ NCI-H1975 and PC-9 cells were treated with GA by combining a proteasomal inhibitor MG132 or a lysosomal degradation inhibitor chloroquine (Figures 5A, B and S1). Protein expressions of p21 and LC3B were used as positive controls of MG132 and Chloroquine treatment, respectively. As shown in Figures 5A, B and S1, MG132, but not chloroquine, partially restored GA-mediated reduction of ROR1 protein levels. Degradation of ROR1 intracellular domain following GA treatment was also almost completely rescued by MG132 in HeLa cells expressing phAG-ROR1-ICD (Figure 5C). In addition, we showed that ROR1 protein was polyubiquitinated following GA treatment (Figure 5D), suggesting that ROR1 was degraded by GA through the ubiquitin/proteasome pathway.

We next determined the region of ROR1 protein responsible for GA-mediated proteasome degradation. The ROR1 WT and a

deletion mutant of the C-terminal region (Δ S/T1 + P+S/T2), but not a deletion mutant of the kinase domain (Δ TK), were degraded by GA (Figures 6A and S2). In addition, immunoprecipitation of ROR1 revealed that HSP90 α did not bind to Δ TK (Figure 6B). Our findings indicate that the kinase domain of ROR1 is responsible for binding with HSP90 α and GA-mediated proteasome degradation, which is consistent with a recent study that identified the amino acid sequence ELHHPNIV in the kinase domain of ROR1 as a binding motif of HSP90.³¹ Kinase activity was not involved in the interaction of ROR1 with HSP90 α , or proteasome degradation (Figure 6C, D). Although N-linked glycosylation of ROR1 could be associated with its subcellular localization and function,³² inhibition of N-linked glycosylation of ROR1 with tunicamycin did not clearly interfere with GA-mediated proteasome degradation (Figure 6E).

4 | DISCUSSION

We and others have reported that cell survival of a subset of lung adenocarcinoma depends on TTF-1/NKX2-1 signaling, but TTF-1/NKX2-1 cannot be considered as a molecular target because of

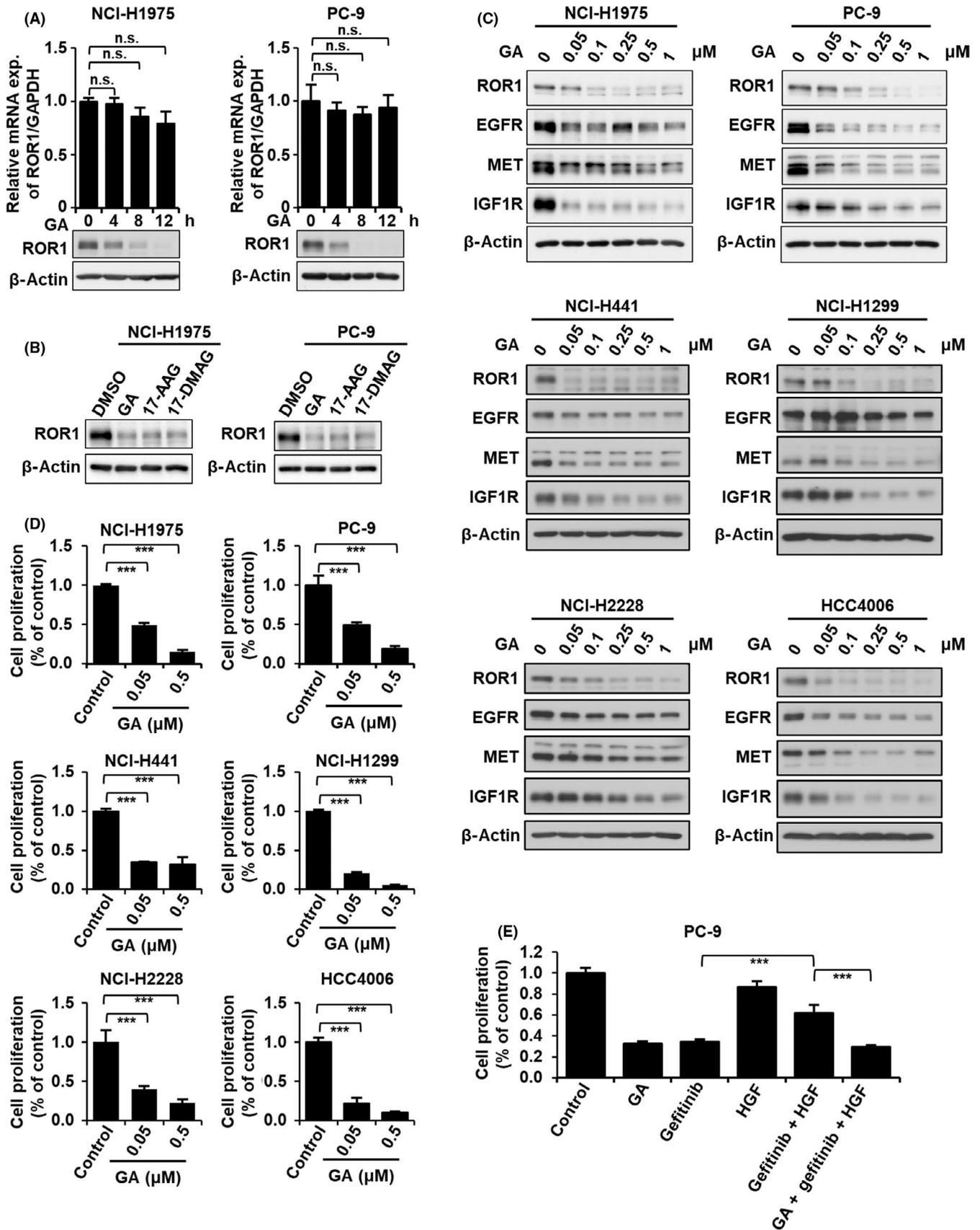


FIGURE 3 Geldanamycin (GA) decreases ROR1 protein and inhibits cell growth. A, Quantitative RT-PCR and western blot analyses of ROR1 in NCI-H1975 (left panel) and PC-9 (right panel) cells treated with 0.5 $\mu\text{mol/L}$ GA for the indicated time points. B, Western blot analysis of ROR1 protein in NCI-H1975 and PC-9 cells with 0.5 $\mu\text{mol/L}$ GA, 17-allylamino-17-demethoxygeldanamycin (17-AAG), and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG). C, Western blot analysis of ROR1, epidermal growth factor receptor (EGFR), MET, and insulin-like growth factor 1 receptor (IGF1R) in NCI-H1975, PC-9, NCI-H441, NCI-H1299, NCI-H2228, and HCC4006 cells treated with indicated concentrations of GA. β -Actin served as a loading control. D, Cell proliferation analysis of NCI-H1975, PC-9, NCI-H441, NCI-H1299, and HCC4006 cells treated with 0.05 or 0.5 $\mu\text{mol/L}$ GA using the colorimetric method. $***P < .01$. E, Cell proliferation analysis of PC-9 cells treated with 0.5 $\mu\text{mol/L}$ GA, 1 $\mu\text{mol/L}$ gefitinib, and/or 50 ng/mL hepatocyte growth factor (HGF) using the colorimetric method. In (A), (D), and (E), columns indicate the average of triplicate samples from a representative experiment and bars indicate SD. P values were calculated by unpaired t test. $***P < .01$. n.s., not significant

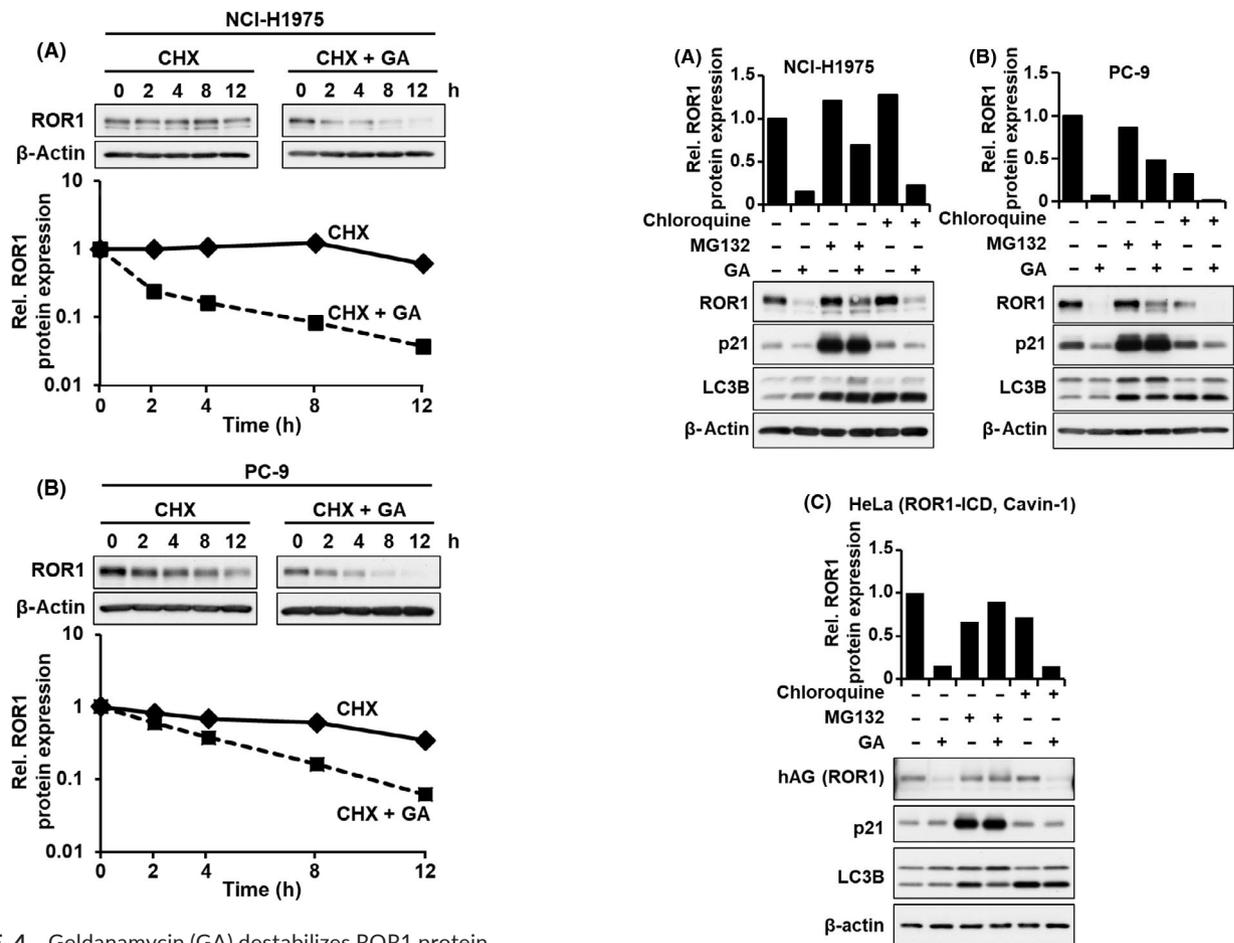


FIGURE 4 Geldanamycin (GA) destabilizes ROR1 protein. NCI-H1975 (A) and PC-9 (B) cells treated with 50 $\mu\text{g/mL}$ cycloheximide (CHX) in the presence or absence of 0.5 $\mu\text{mol/L}$ GA. Signal intensities of ROR1 protein bands were normalized to β -actin signal intensities

FIGURE 5 Geldanamycin (GA) degrades ROR1 protein through the ubiquitin/proteasome pathway. Western blot analysis of ROR1, p21, and LC3B in NCI-H1975 (A), PC-9 (B), and HeLa cells expressing humanized fluorescent Azami-Green-ROR1-intracellular domain (hAG-ROR1-ICD) (C), treated with proteasomal inhibitor MG132 (10 $\mu\text{mol/L}$) or lysosomal inhibitor chloroquine (10 $\mu\text{mol/L}$) for 8 h in the presence or absence of 0.5 $\mu\text{mol/L}$ GA. β -Actin served as a loading control. Columns indicate relative signal intensities of ROR1 protein. D, Immunoprecipitation (IP)-western blot analysis using ROR1 Ab in COS-7 cells transfected with HA-tagged ubiquitin (HA-Ub) and/or ROR1 in the presence or absence of 0.5 $\mu\text{mol/L}$ GA for 3 h

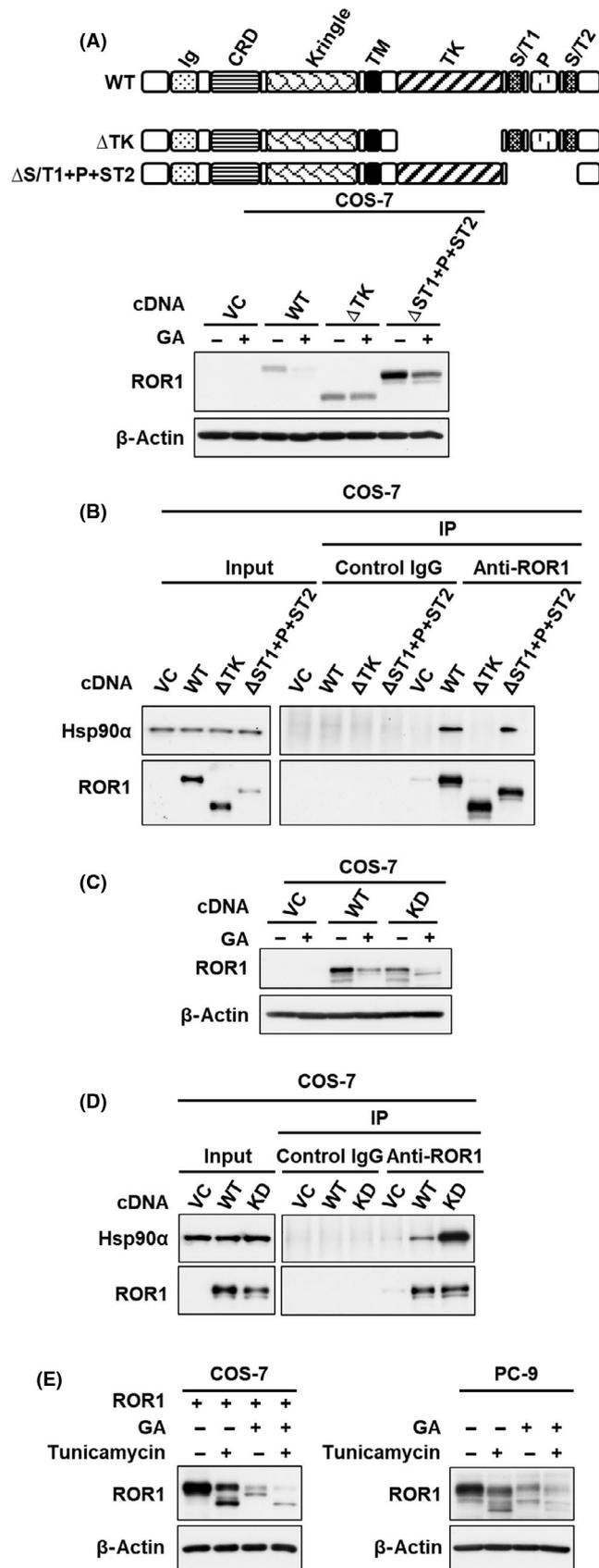


FIGURE 6 Kinase domain of ROR1 is required for geldanamycin (GA)-induced proteasome degradation. Western blot analysis of ROR1 in COS-7 cells transfected with ROR1 WT, (A) a deletion mutant of entire kinase domain (ROR1- Δ TK) and the C-terminal region (ROR1- Δ ST1 + P+ST2), and (C) a kinase-dead mutant (ROR1 K558R), in the presence or absence of 0.5 μ mol/L GA for 24 h. Immunoprecipitation-western blot analysis using ROR1 Ab in COS-7 cells transfected with ROR1 WT, (B) ROR1- Δ TK and ROR1- Δ ST1 + P+ST2, and (D) a kinase-dead mutant (ROR1 K558R). E, Western blot analysis of ROR1 in COS-7 cells with overexpressing ROR1 (left panel) and PC-9 cells (right panel), treated with 0.5 μ g/mL tunicamycin for 12 h in the presence or absence of 0.5 μ mol/L GA. In (A) to (E), β -actin served as a loading control. CRD, cysteine-rich domain; Ig, Ig-like domain; P, proline-rich domain; ST, serine/threonine-rich domain; TK, tyrosine kinase domain; VC, vector control

prosurvival signaling in TTF-1/NKX2-1-positive lung adenocarcinoma cell lines.¹⁰ Furthermore, ROR1 was shown to act as a scaffold protein of cavin-1 and caveolin-1, two essential structural components of caveolae, sustaining caveolae formation and pro-survival signaling through multiple additional RTKs, such as MET and IGF1R.¹² As bypass signaling through diverse RTKs confers EGFR-TKI resistance,^{29,34-36} the scaffold function of ROR1 appears to be an attractive target for overcoming EGFR-TKI resistance due to bypass signaling. It is also of note that, given its broad expression in common solid tumors and hematologic malignancies with minimal expression in healthy adult tissues,¹⁴⁻¹⁸ ROR1-targeted therapies using CAR-T cells,³⁷ mAbs,³⁸ and small-molecule inhibitors³⁹ are also being developed in a variety of cancer.

In the present study, we showed direct interaction between ROR1 and HSP90 α , but not with its paralog, HSP90 β ; both are localized in the cytoplasm and their functions are mostly overlapping, but some paralog-specific functions have been reported.²² Heat shock protein 90 α is induced by cellular stimuli such as heat shock, whereas HSP90 β is constitutively expressed. It is also of note that HSP90 α and HSP90 β have variable affinities for client proteins as well as small-molecule inhibitors including GA⁴⁰; expression of Hsp90 β alone could not rescue yeast cells from the HSP90 inhibitor radicicol, in contrast to the conferral of marked resistance by comparable expression of Hsp90 α alone.⁴¹ Geldanamycin, a benzoquinone ansamycin derived from *Streptomyces hygroscopicus*, is the first established inhibitor of HSP90 that interferes with the ATP-binding site of HSP90.²⁴ Despite its potent cytotoxic effects, its phase I clinical trial was suspended due to liver toxicity, metabolic instability, and poor solubility.⁴² Geldanamycin derivatives with improved efficacy and side-effects, including 17-AAG and 17-DMAG, were then developed. In addition to IPI-504, a reduced form of 17-AAG, derivatives of another natural HSP90 inhibitor, radicicol (such as ganetespib), purine analogues, and some other compounds have been tested in multiple clinical trials for advanced solid tumors and hematologic malignancies, including lung cancer.^{43,44}

Epidermal growth factor receptor, MET, and IGF1R are known clients of HSP90,²⁶⁻²⁸ and GA indeed reduced those RTK proteins

its crucial role for maintaining physiological functions of normal lung.³³ In this connection, we previously found that ROR1 is a transcriptional target for TTF-1/NKX2-1 and sustains EGFR-mediated

in the six lung adenocarcinoma cell lines examined in the present study. Furthermore, it is interesting to note that the concentrations of GA required to decrease each RTK protein were noticeably different among the cell lines. In contrast, ROR1 protein was consistently decreased in all of the lung adenocarcinoma cell lines treated with GA at a concentration lower than that required for significant reduction of other RTK proteins, which was accompanied by significant cell growth inhibition (Figure 3C,D). ROR1 itself has been shown to sustain signaling of multiple RTKs in both kinase-dependent and independent manners,^{10,12,13} thus it is difficult to determine the contributions of ROR1 or other RTKs to the inhibitory effects of GA on cell growth. Nevertheless, these findings suggest a critical role for ROR1 in lung adenocarcinoma cell growth.

In conclusion, we have shown that ROR1 is a novel client protein of HSP90 with specific binding to HSP90 α isoform. Our present findings also suggest promising potential of HSP90 inhibitors targeting ROR1 and its scaffold function to overcome resistance caused by bypass signaling through activation of RTKs.

ACKNOWLEDGMENTS

We thank Ms Arisa Tamura for her technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (A) (grant number: 16H02468) from the Japan Society for the Promotion of Science and the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) (project number: 18cm0106107h9903) from the Japan Agency for Medical Research and Development.

CONFLICT OF INTEREST

The authors declare no competing interests to disclose.

ORCID

Motoshi Suzuki  <https://orcid.org/0000-0003-0682-5006>

Tomoya Yamaguchi  <https://orcid.org/0000-0001-6933-543X>

Takashi Takahashi  <https://orcid.org/0000-0003-0615-7001>

REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
- Cheng TY, Cramb SM, Baade PD, Youlden DR, Nwogu C, Reid ME. The International Epidemiology of Lung Cancer: Latest Trends, Disparities, and Tumor Characteristics. *J Thorac Oncol*. 2016;11:1653-1671.
- Devarakonda S, Morgensztern D, Govindan R. Genomic alterations in lung adenocarcinoma. *Lancet Oncol*. 2015;16:e342-e351.
- Takeuchi T, Tomida S, Yatabe Y, et al. Expression profile-defined classification of lung adenocarcinoma shows close relationship with underlying major genetic changes and clinicopathologic behaviors. *J Clin Oncol*. 2006;24:1679-1688.
- Maeda Y, Dave V, Whitsett JA. Transcriptional control of lung morphogenesis. *Physiol Rev*. 2007;87:219-244.
- Tanaka H, Yanagisawa K, Shinjo K, et al. Lineage-specific dependency of lung adenocarcinomas on the lung development regulator TTF-1. *Cancer Res*. 2007;67:6007-6011.
- Weir BA, Woo MS, Getz G, et al. Characterizing the cancer genome in lung adenocarcinoma. *Nature*. 2007;450:893-898.
- Kendall J, Liu Q, Bakleh A, et al. Oncogenic cooperation and co-amplification of developmental transcription factor genes in lung cancer. *Proc Natl Acad Sci U S A*. 2007;104:16663-16668.
- Kwei KA, Kim YH, Girard L, et al. Genomic profiling identifies TTF1 as a lineage-specific oncogene amplified in lung cancer. *Oncogene*. 2008;27:3635-3640.
- Yamaguchi T, Yanagisawa K, Sugiyama R, et al. NKX2-1/TTF1/TTF-1-Induced ROR1 is required to sustain EGFR survival signaling in lung adenocarcinoma. *Cancer Cell*. 2012;21:348-361.
- Ida L, Yamaguchi T, Yanagisawa K, et al. Receptor tyrosine kinase-like orphan receptor 1, a target of NKX2-1/TTF-1 lineage-survival oncogene, inhibits apoptosis signal-regulating kinase 1-mediated pro-apoptotic signaling in lung adenocarcinoma. *Cancer Sci*. 2016;107:155-161.
- Yamaguchi T, Lu C, Ida L, et al. ROR1 sustains caveolae and survival signalling as a scaffold of cavin-1 and caveolin-1. *Nat Commun*. 2016;7:10060.
- Yamaguchi T, Hayashi M, Ida L, et al. ROR1-CAVIN3 interaction required for caveolae-dependent endocytosis and pro-survival signaling in lung adenocarcinoma. *Oncogene*. 2019;38:5142-5157. <https://doi.org/10.1038/s41388-019-0785-7>.
- Fukuda T, Chen L, Endo T, et al. Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. *Proc Natl Acad Sci USA*. 2008;105:3047-3052.
- Zhang S, Chen L, Wang-Rodriguez J, et al. The onco-embryonic antigen ROR1 is expressed by a variety of human cancers. *Am J Pathol*. 2012;181:1903-1910.
- Bicocca VT, Chang BH, Masouleh BK, et al. Crosstalk between ROR1 and the Pre-B cell receptor promotes survival of t(1;19) acute lymphoblastic leukemia. *Cancer Cell*. 2012;22:656-667.
- Daneshmanesh AH, Porwit A, Hojjat-Farsangi M, et al. Orphan receptor tyrosine kinases ROR1 and ROR2 in hematological malignancies. *Leuk Lymphoma*. 2013;54:843-850.
- Balakrishnan A, Goodpaster T, Randolph-Habecker J, et al. Analysis of ROR1 Protein Expression in Human Cancer and Normal Tissues. *Clin Cancer Res*. 2017;23:3061-3071.
- Rotow J, Bivona TG. Understanding and targeting resistance mechanisms in NSCLC. *Nat Rev Cancer*. 2017;17:637-658.
- Liu Z, Yanagisawa K, Griesing S, et al. TTF-1/NKX2-1 binds to DDB1 and confers replication stress resistance to lung adenocarcinomas. *Oncogene*. 2017;36:3740-3748.
- Xiong H, Wang D, Chen L, et al. Parkin, PINK1, and DJ-1 form a ubiquitin E3 ligase complex promoting unfolded protein degradation. *J Clin Investig*. 2009;119:650-660.
- Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat Rev Mol Cell Biol*. 2010;11:515-528.
- Schopf FH, Biebl MM, Buchner J. The HSP90 chaperone machinery. *Nat Rev Mol Cell Biol*. 2017;18:345-360.
- Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer*. 2005;5:761-772.
- Tomida S, Takeuchi T, Shimada Y, et al. Relapse-related molecular signature in lung adenocarcinomas identifies patients with dismal prognosis. *J Clin Oncol*. 2009;27:2793-2799.
- Shimamura T, Lowell AM, Engelman JA, Shapiro GI. Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. *Cancer Res*. 2005;65:6401-6408.
- Webb CP, Hose CD, Koochekpour S, et al. The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network. *Cancer Res*. 2000;60:342-349.

28. Martins AS, Ordonez JL, Garcia-Sanchez A, et al. A pivotal role for heat shock protein 90 in Ewing sarcoma resistance to anti-in-sulin-like growth factor 1 receptor treatment: in vitro and in vivo study. *Cancer Res.* 2008;68:6260-6270.
29. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res.* 2008;68:9479-9487.
30. Pedersen NM, Madshus IH, Haslekas C, Stang E. Geldanamycin-induced down-regulation of ErbB2 from the plasma membrane is clathrin dependent but proteasomal activity independent. *Mol Cancer Res.* 2008;6:491-500.
31. Liu Z, Liu J, Zhang T, et al. Destabilization of ROR1 enhances activity of Ibrutinib against chronic lymphocytic leukemia in vivo. *Pharmacol Res.* 2020;151:104512.
32. Kaucka M, Krejci P, Plevova K, et al. Post-translational modifications regulate signalling by Ror1. *Acta Physiol (Oxf).* 2011;203:351-362.
33. Yamaguchi T, Hosono Y, Yanagisawa K, Takahashi T. NKX2-1/TTF-1: an enigmatic oncogene that functions as a double-edged sword for cancer cell survival and progression. *Cancer Cell.* 2013;23:718-723.
34. Guix M, Faber AC, Wang SE, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. *J Clin Invest.* 2008;118:2609-2619.
35. Turke AB, Zejnullahu K, Wu YL, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell.* 2010;17:77-88.
36. Wilson TR, Fridlyand J, Yan Y, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature.* 2012;487:505-509.
37. Hudecek M, Lupo-Stanghellini MT, Kosasih PL, et al. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin Cancer Res.* 2013;19:3153-3164.
38. Choi MY, Widhopf GF 2nd, Ghia EM, et al. Phase I Trial: Cirmtuzumab Inhibits ROR1 Signaling and Stemness Signatures in Patients with Chronic Lymphocytic Leukemia. *Cell Stem Cell.* 2018;22(951-9):e3.
39. Hojjat-Farsangi M, Daneshmanesh AH, Khan AS, et al. First-in-class oral small molecule inhibitor of the tyrosine kinase ROR1 (KAN0439834) induced significant apoptosis of chronic lymphocytic leukemia cells. *Leukemia.* 2018;32:2291-2295.
40. Prince TL, Kijima T, Tatokoro M, et al. Client Proteins and Small Molecule Inhibitors Display Distinct Binding Preferences for Constitutive and Stress-Induced HSP90 Isoforms and Their Conformationally Restricted Mutants. *PLoS One.* 2015;10:e0141786.
41. Millson SH, Truman AW, Racz A, et al. Expressed as the sole Hsp90 of yeast, the alpha and beta isoforms of human Hsp90 differ with regard to their capacities for activation of certain client proteins, whereas only Hsp90beta generates sensitivity to the Hsp90 inhibitor radicicol. *FEBS J.* 2007;274:4453-4463.
42. Supko JG, Hickman RL, Grever MR, Malspeis L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother Pharmacol.* 1995;36:305-315.
43. Garcia-Carbonero R, Carnero A, Paz-Ares L. Inhibition of HSP90 molecular chaperones: moving into the clinic. *Lancet Oncol.* 2013;14:e358-e369.
44. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer.* 2010;10:537-549.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Khaledian B, Taguchi A, Shin-ya K, et al. Inhibition of heat shock protein 90 destabilizes receptor tyrosine kinase ROR1 in lung adenocarcinoma. *Cancer Sci.* 2021;00:1-10. <https://doi.org/10.1111/cas.14786>