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ORIGINAL ARTICLE

Yes-associated protein 1 mediates initial cell survival during lorlatinib treatment through AKT signaling in ROS1-rearranged lung cancer

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Abstract

Tyrosine kinase inhibitors (TKIs) that target the ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) gene have shown dramatic therapeutic effects in patients with ROS1-rearranged non-small-cell lung cancer (NSCLC). Nevertheless, advanced ROS1-rearranged NSCLC is rarely cured as a portion of the tumor cells can survive the initial stages of ROS1-TKI treatment, even after maximum tumor shrinkage. Therefore, understanding the mechanisms underlying initial cell survival during ROS1-TKI treatment is necessary to prevent cell survival and achieve a cure for ROS1-rearranged NSCLC. In this study, we clarified the initial survival mechanisms during treatment with lorlatinib, a ROS1 TKI. First, we established a patient-derived ezrin gene-ROS1-rearranged NSCLC cell line (KTOR71). Then, following proteomic analysis, we focused on yes-associated protein 1 (YAP1), which is a major mediator of the Hippo pathway, as a candidate factor involved in cell survival during early lorlatinib treatment. Yes-associated protein 1 was activated by short-term lorlatinib treatment both in vitro and in vivo. Genetic inhibition of *YAP1* using siRNA, or pharmacological inhibition of YAP1 function by the YAP1-inhibitor verteporfin, enhanced the sensitivity of KTOR71 cells to lorlatinib. In addition, the prosurvival effect of YAP1 was exerted through the reactivation of AKT. Finally, combined therapy with verteporfin and lorlatinib was found to achieve significantly sustained tumor remission compared with lorlatinib monotherapy in vivo. These results suggest that YAP1 could mediate initial cell resistance to lorlatinib in KTOR71 cells. Thus, combined therapy targeting both YAP1 and ROS1 could potentially improve the outcome of ROS1-rearranged NSCLC.

Abbreviations: AFP, α-fetoprotein; ALK, anaplastic lymphoma kinase; BP, biological process; COSMIC, Catalogue of Somatic Mutations in Cancer; CTGF, connective tissue growth factor; EGFR, epidermal growth factor receptor; EZR, ezrin gene; GAB2, GRB2-associated binding protein 2; GO, Gene Ontology; IGF1R, insulin-like growth factor 1 receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes; NSCLC, non-small-cell lung cancer; PAI-1, plasminogen activator inhibitor-1; Pik3cb, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase; TEAD, TEA domain transcription factor; TKI, tyrosine kinase inhibitor; YAP1, yes-associated protein 1.

KEYWORDS
initial survival, lung cancer, resistance, ROS1, YAP1

1 | INTRODUCTION

Molecular-targeted therapies for driver oncogenes, such as *ROS1* fusions, *EGFR* mutations, *ALK* fusions, and *BRAF* mutations, have dramatically improved the prognosis of advanced NSCLC.[1–5] ROS1 fusion genes have been identified in 1%–2% of patients with NSCLC.[1,6,7] As such, several ROS1 TKIs have been developed, including crizotinib, entrectinib, and lorlatinib. Crizotinib and entrectinib have been approved by the US FDA for the treatment of ROS1-rearranged lung cancer based on clinical trial results.[1,2] Lorlatinib, a third-generation ALK/ROS1 TKI, is characterized by high potency against ROS1 and ALK, as well as high blood–brain barrier permeability.[6,8,9] Although lorlatinib has been approved by the FDA as an ALK TKI, it has not been approved as a ROS1 TKI.[5] Meanwhile, in phase I and II clinical trials, lorlatinib has shown high response rates in patients with ROS1-rearranged NSCLC, including those with brain metastases.[3,4] Recently, in a phase III clinical trial of ALK-rearranged NSCLC, the receptor for which is highly homologous to the ROS1 receptor, lorlatinib showed a superior progression-free survival time and intracranial response to crizotinib (CROWN study) and has since been approved as a first-line option for patients with ALK-rearranged NSCLC.[1,6,10] Thus, lorlatinib is a promising therapeutic agent for patients with ROS1-rearranged NSCLC.

However, although these ROS1 TKIs exert dramatic effects in ROS1-rearranged NSCLC during the early phase of treatment, cures are generally not achievable, with tumors eventually acquiring resistance to ROS1 TKIs and patients experiencing clinical relapse.[1–4] As ROS1-rearranged lung cancer frequently develops at a younger age, there is an urgent need to establish a curative treatment.[11] Secondary mutations in the ROS1 kinase domain, such as G2032R, D2033N, S1986Y/F, L2026M, L1951R, and L2086F, impart resistance to ROS1 TKIs.[6,12,13] Next-generation ROS1 TKIs, such as entrectinib, are used for tumors with acquired resistance to crizotinib (possibly caused by secondary mutations); however, as the tumors will eventually gain resistance to these next-generation ROS1 TKIs due to other resistance mechanisms, their efficacy is limited.[3,4,12,14] The activation of bypass pathways, such as EGFR, human epidermal growth factor receptor 2 (HER2), KIT, and AXL, as well as phenotypic changes, including epithelial–mesenchymal transition, are also mechanisms of acquired resistance to ROS1 TKIs, which are known as off-target resistance mechanisms.[15–18] Considering the diverse nature of these off-target acquired resistance mechanisms, curative treatments have not succeeded in overcoming them. Moreover, tumor heterogeneity generally increases in the later phase of treatment, and the resistance mechanism becomes more complex, which also makes it harder to overcome acquired resistance.[19] Therefore, it is difficult to achieve a cure for ROS1-rearranged NSCLC by overcoming acquired resistance, prompting the need for novel treatment strategies.

Recently, a new curative strategy has been proposed for unresectable lung cancer that focuses on preventing the emergence of persistent cancer cells and eradicating the tumor before the acquisition of resistance.[20–22] This is because the persistent cancer cells can acquire resistance mechanisms—secondary mutations and bypass pathways—during prolonged treatment, then proliferate, resulting in clinical tumor recurrence (Figure 1A). Several research groups have reported the mechanisms and signaling pathways associated with initial cancer cell survival against molecular-targeted therapies in NSCLC harboring driver oncogenes.[23–27] For example, multiple factors, such as AXL and IGF1R, have been shown to play a key role in survival against EGFR TKIs in EGFR mutation-positive NSCLC.[23,24] Moreover, we previously showed that YAP1 mediates initial cell survival following ALK TKI treatment through antiapoptotic proteins in ALK-rearranged NSCLC.[25] However, the mechanism underlying early survival in ROS1-rearranged NSCLC has not yet been clarified.

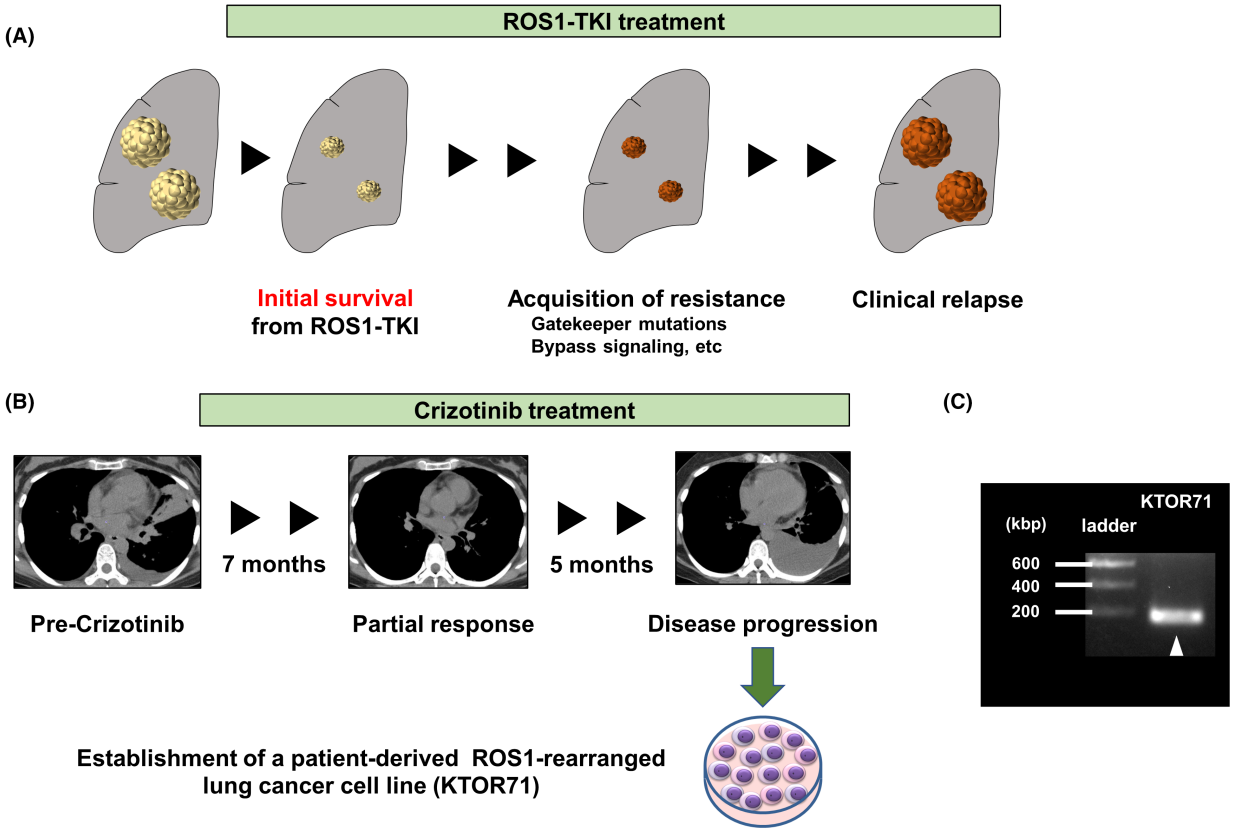
Therefore, the aim of this study is to elucidate the mechanisms of initial cell survival during treatment with the ROS1 TKI lorlatinib and to suggest new curative treatment strategies to prevent initial survival in ROS1-rearranged NSCLC.

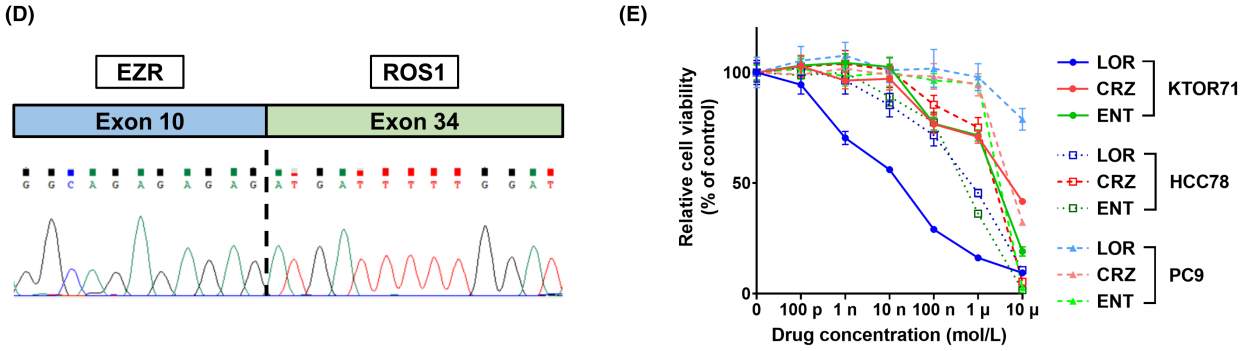
2 | MATERIALS AND METHODS

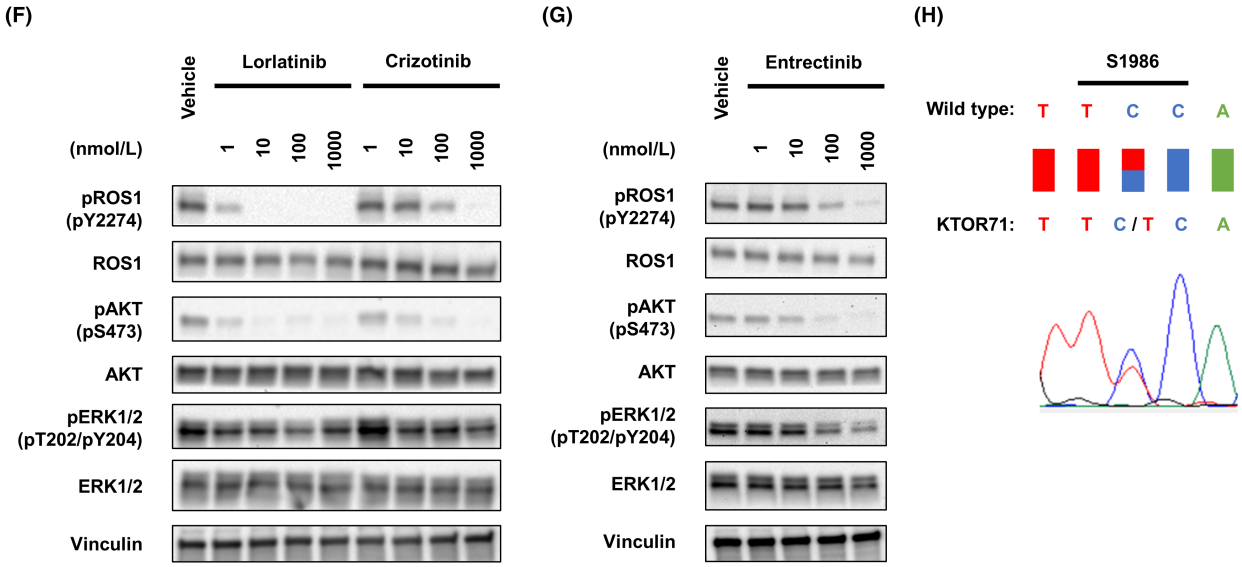
2.1 | Clinical information and procedures for obtaining informed consent

The study protocol was prepared in accordance with the Declaration of Helsinki and was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (certification nos. R0996, R2163). The patient provided written informed consent regarding their participation in this study.

FIGURE 1 ROS1-rearranged lung cancer patient-derived cell line KTOR71. (A) Schematic representation of initial survival from ROS tyrosine kinase inhibitors (TKIs) and clinical relapse in ROS1-rearranged lung cancer. (B) Computed tomography images of the clinical course of the patient during lorlatinib treatment and schematic representation of the establishment of KTOR71 cells. (C) Detection of mRNA for the ezrin (EZR)-ROS1 fusion protein. Gel electrophoresis of RT-PCR products using *EZR* and *ROS1* primers. (D) Sanger sequencing of the PCR product. (E) Cell viability assay of KTOR71, HCC78, and PC9 cells in the presence of lorlatinib (LOR), crizotinib (CRZ), and entrectinib (ENT). (F,G) KTOR71 cells were treated with indicated concentrations of lorlatinib, crizotinib (F), or entrectinib (G) for 6 h. Cell lysates were analyzed by immunoblotting with the indicated Abs. (H) Direct sequencing of the ROS1 tyrosine kinase-coding region, S1986. Error bars indicate ± SEM.







2.2 | Detection of EZR-ROS1 rearrangement

Total RNA was extracted from KTOR71 cells and purified using the PureLink RNA Mini Kit (Ambion). The expression of mRNA encoding the fusion protein EZR-ROS1 was examined using RT-PCR. Previously described primers[28] were used (Table S1). The PCR product was sequenced using Sanger's method with a 3130xl Genetic Analyzer (Applied Biosystems).

2.3 | Immunofluorescence staining in vitro

The cells were fixed with 4% paraformaldehyde/PBS (Nacalai Tesque) for 15 min then permeabilized with 0.2% Triton X-100/PBS for 15 min. Cells were blocked with PBS supplemented with 5% (w/v) normal donkey serum (Merck Millipore) and 1% (w/v) BSA (Sigma-Aldrich) for 30 min. Cells were then incubated with the primary Ab solution at 4°C overnight, followed by incubation with the secondary Ab solution for 30 min at room temperature. The Abs and their dilution factors are shown in Table S2.

2.4 | Yes-associated protein 1 nuclear localization assay

Immunofluorescent-stained cells were photographed with BZ-710 (Keyence), and the images were analyzed using ImageJ/FIJI (NIH). The YAP1 nuclear localization was quantified by colocalization analysis between YAP1-Alexa488 and Hoechst/DAPI. Colocalization analysis was undertaken by calculating Pearson's correlation coefficient using the Coloc2 plugin of ImageJ/FIJI.

2.5 | Xenograft models

Female BALB/c-nu mice (CAnN.Cg-Foxn1nu/CrlCrlj) were purchased at 6 weeks of age from Charles River Laboratories, Japan. To generate the xenograft model, KTOR71 cells (2×10^6) were suspended in Matrigel (Corning) and injected subcutaneously into the backs of the mice. The width and length of the tumors were measured every 3 days using electronic calipers. Tumor volume was calculated using the following formula: $(\text{length}\times\text{width}^2)\times0.52$. Treatment was initiated when the tumor volume reached 200 mm^3. All animal experiments were approved by the Animal Research Committee of Kyoto University (ID: MedKyo 19,294, 20,256, and 21,272) and conducted in accordance with ARRIVE guidelines.

2.6 | Genetic analysis

Genome DNA was extracted from KTOR71 cells and purified using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific). Whole exome sequencing and *TP53* mutation detection were carried out by Rhelixa Inc. The protocol for exome sequencing is described in Appendix S1. *TP53* mutations of HCC78 were investigated using the COSMIC database.

All other experimental procedures are detailed in Appendix S1.

3 | RESULTS

3.1 | Establishment of a patient-derived ROS1-rearranged lung cancer cell line (KTOR71)

We established a cell line (KTOR71) using cells derived from a 28-year-old, female, crizotinib-refractory patient with ROS1-rearranged NSCLC. Chest computed tomography images demonstrated left pleural effusion and infiltrative shadows in the left lung (Figure 1B, left). The patient was clinically diagnosed with ROS1-rearranged lung cancer using FISH, and oral crizotinib (250 mg twice daily) was administered at Kyoto University Hospital. Crizotinib treatment was highly effective, and the left pleural effusion had disappeared at evaluation 7 months after crizotinib treatment initiation (Figure 1B, middle). However, 12 months after crizotinib initiation, disease progression was observed, with increased left pleural effusion (Figure 1B, right). We then established the ROS1-rearranged lung cancer cell line, KTOR71, from this re-established left pleural effusion. Total RNA was extracted from KTOR71 cells, and RT-PCR using *EZR-ROS1* detection primers indicated the mRNA for the EZR-ROS1 fusion protein in KTOR71 cells (Figure 1C). Sequencing of the PCR amplification products revealed fusion of the *EZR* and *ROS1* genes (*EZR*-Exon10 and *ROS1*-Exon34; Figure 1D).

3.2 | KTOR71 cells are sensitive to ROS1 TKI lorlatinib

Subsequently, to find an effective ROS1 inhibitor for KTOR71 cells, we investigated the sensitivity of KTOR71 cells to three ROS1 TKIs (lorlatinib, crizotinib, and entrectinib). PC9, an EGFR mutation-positive lung cancer cell line, was simultaneously used as a nonresponsive control. KTOR71 cells were resistant to crizotinib, concordantly with the clinical course of the patient, and entrectinib (Figure 1E and Table S3). In contrast, KTOR71 cells were sensitive to lorlatinib (Figure 1E and Table S3).

We also investigated whether KTOR71 cell survival was ROS1-dependent, as well as the effects of the ROS1 TKIs on ROS1 phosphorylation. Each ROS1 TKI was exposed in a stepwise concentration-dependent manner. Phosphorylation of ROS1, and its downstream growth signal molecules, ERK1/2 (pERK1/2) and AKT (pAKT), were then assessed by immunoblotting. pERK1/2 and pAKT are key molecules and activation markers of the MAPK and PI3K-AKT pathways, respectively. Phosphorylation of ROS1 was inhibited by lorlatinib at a low concentration (10 nmol/L), whereas crizotinib and entrectinib required a high concentration (1 μmol/L)

in KTOR71 cells (Figure 1F,G). Additionally, following inhibition of pROS1, pERK1/2 and pAKT were inhibited (Figure 1F,G). These results suggest that KTOR71 is ROS1-dependent and has developed a ROS1 secondary mutation that induces crizotinib and entrectinib resistance without causing lorlatinib resistance.

Next, we investigated secondary mutations in ROS1 tyrosine kinase-coding regions by exon sequencing using the previously described ROS1-sequencing primers in KTOR71[29] (Table S4). The S1986F mutation that leads to crizotinib resistance was found in exon 37 of the *ROS1* gene (Figure 1H). No other known ROS1 secondary mutations associated with crizotinib resistance (G2032R, D2033N, L2026M, or L1951R) were found in the coding region of the ROS1 tyrosine kinase domain (Figure S1). The ROS1 S1986F mutation is a solvent-front mutation that is reportedly resistant to crizotinib and entrectinib, but sensitive to lorlatinib,[6,12] which is consistent with our results.

In summary, KTOR71 cells were resistant to crizotinib due to the S1986F mutation in the coding region of the ROS1 tyrosine kinase domain, however, they were susceptible to lorlatinib. As the aim of this study was to investigate the mechanisms underlying initial cell survival during ROS1 TKI treatment, all subsequent experiments were carried out with lorlatinib due to the sensitivity of KTOR71 cells to this ROS1 TKI.

3.3 | Lorlatinib treatment induces spread of KTOR71 cells

To identify the mechanisms by which KTOR71 survives lorlatinib, protein expression was compared using proteome analysis between cells with and without lorlatinib exposure. A total of 3271 proteins were detected, of which 473 were upregulated in lorlatinib-treated cells (Appendix S2). Gene Ontology analysis was undertaken on the 473 upregulated proteins (Figure 2A). The top five BP terms with the highest enrichment scores are shown in Figure 2B and Appendix S3. We focused on three BP terms: "actin filament-based process" (GO:0030029), "regulation of cell-substrate adhesion" (GO:0010810), and "regulation of cell adhesion" (GO:0030155). Additionally, pathway enrichment analysis was carried out on the upregulated proteins using KEGG pathway analysis. As shown in Figure 2C and Appendix S4, the terms "regulation of actin cytoskeleton (hsa04810)" and "focal adhesion (hsa04510)" were annotated. KTOR71 cells that survived lorlatinib treatment for 72 h showed an extended morphology with an adherent area (Figure 2D). Furthermore, this extended morphology in lorlatinib-treated KTOR71 cells was confirmed by quantifying cell circularity using ImageJ/FIJI (Figure 2E). Previously, we found that ALK-rearranged lung cancer cells also show an extended morphology under ALK TKI treatment.[25] Thus, considering the similar cell morphology, and enriched GO terms and KEGG pathways on the cytoskeleton, KTOR71 cells might respond to molecular-targeted therapies in the same way as ALK-rearranged lung cancer cells. Accordingly, we hypothesized that YAP1, a transcriptional coactivator, functions as an initial survival factor in ROS1-rearranged lung cancer and ALK-rearranged lung cancer. We examined the proteomic analysis data on 21 proteins that are mapped as downstream targets of YAP1 in the KEGG Hippo signaling pathway (hsa04390) (Appendix S5). The proteomic analysis identified three proteins (AFP, PAI-1, and integrin subunit beta 2) among these 21 proteins. Of these three, AFP and PAI-1 were upregulated by lorlatinib exposure.

3.4 | Yes-associated protein 1 is activated by lorlatinib treatment in KTOR71 cells

Next, we evaluated whether lorlatinib treatment induces YAP1 activation. Yes-associated protein 1 activity is generally evaluated based on its nuclear localization as it translocates to the nucleus upon activation where it functions as a transcriptional coactivator.[30] In KTOR71, YAP1 translocated into the nuclei of cells exposed to lorlatinib for 72 h in vitro, whereas YAP1 was predominantly cytoplasmic in cells exposed to the vehicle (Figure 3A). Hence, YAP1 was activated by lorlatinib exposure in KTOR71 cells. To quantitatively assess the activity of YAP1, the colocalization values (Pearson's *r* value) for YAP1-Alexa488 and Hoechst were calculated using immunohistochemically stained cell images.[25,31] When exposed to stepwise concentrations of lorlatinib for 72 h, YAP1 tended to localize to the nucleus in a concentration-dependent manner (Figure 3B). Furthermore, nuclear translocation of YAP1 in response to lorlatinib occurred 12 h after exposure, and tended to increase over time (Figure 3C). Additionally, we undertook a luciferase assay using a YAP1/TAZ-responsive TEAD reporter (8XGTIIC-luciferase reporter) that contains eight YAP1/TEAD binding sites.[32] Lorlatinib treatment significantly increased the luciferase activity in KTOR71 cells compared to vehicle treatment (Figure 3D). This result supports the activation of YAP1 by lorlatinib exposure. Interestingly, *YAP1* knockdown reduced the luciferase activity in KTOR71 cells in the absence of lorlatinib (Figure 3D). This suggests that KTOR71 cells exhibit some YAP1 activity even without lorlatinib exposure. Subsequently, to clarify whether this activation of YAP1 by lorlatinib exposure in KTOR71 cells is due to ROS1 inhibition, we evaluated whether knockdown of *ROS1* with siRNA affects YAP1 activity. We confirmed that knockdown of *ROS1* using siRNA reduced ROS1 protein abundance (Figure 3E). Inhibition of *ROS1* with siRNA resulted in significant nuclear translocation of YAP1, that is, activation of YAP1 (Figure 3F, G). Moreover, both crizotinib and entrectinib treatments showed nuclear translocation of YAP1 at higher drug concentrations (>100 nmol/L), although nuclear translocation of YAP1 was not observed at lower drug concentrations (<10 nmol/L) (Figure S2A,B). These results indicate that YAP1 is activated by ROS1 inhibition in KTOR71 cells. Subsequently, YAP1 activation by lorlatinib treatment of KTOR71 cells was evaluated in vivo. Six KTOR71 xenografts in nude mice were treated with oral lorlatinib (2 mg/kg/day) or the vehicle for 4 days (Figure 3H). The lorlatinib-treated group showed YAP1 nuclear translocation (Figure 3I,J).

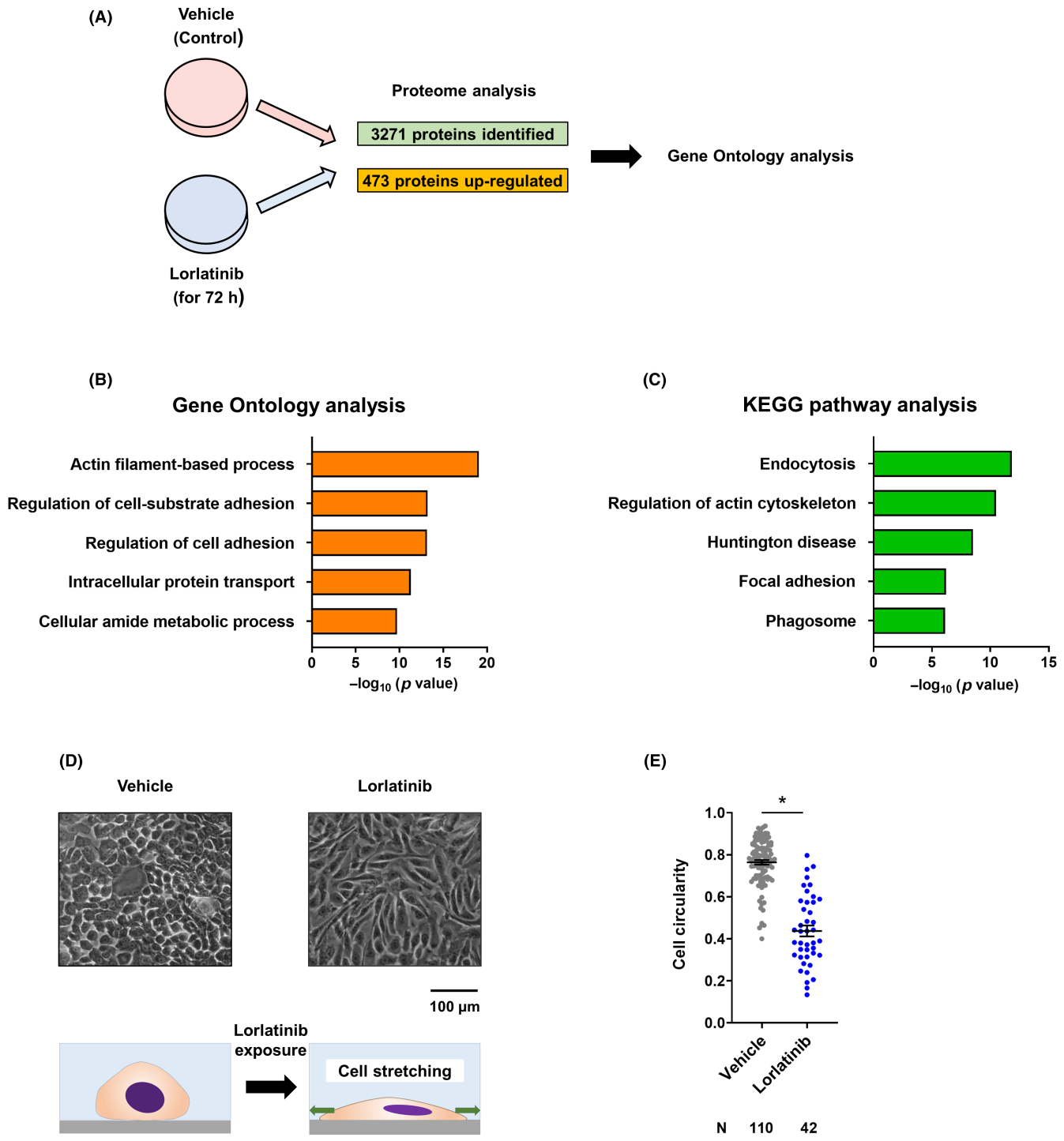


FIGURE 2 ROS1 tyrosine kinase inhibitor lorlatinib treatment induced morphological changes in KTOR71 cells. (A) Schematic representation of proteome and biological identification of the initial survival factor of lorlatinib. (B) Top five biological process terms with enrichment scores by Gene Ontology enrichment analysis of proteins upregulated by lorlatinib exposure for 72 h. (C) Top five terms with enrichment scores by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of proteins upregulated by lorlatinib exposure for 72 h. (D) Morphological changes in KTOR71 induced by lorlatinib exposure for 72 h. Optical microscopy image (upper panel). Schematic diagram (lower panel). (E) Cell circularity values of KTOR71cells exposed to vehicle or lorlatinib for 72 h. One dot represents the circularity of one cell. $^{*}p < 0.05$. Error bars indicate ± SEM.

Next, we evaluated the YAP1 activity states in HCC78, which is a commercially available ROS1-rearranged lung cancer cell line. The sensitivity of HCC78 cells to ROS1 TKIs (lorlatinib, crizotinib, and entrectinib) are shown in Figure 1E and Table S3. In HCC78 cells, YAP1 was localized in the nucleus with vehicle treatment, and remained localized in the nucleus with lorlatinib treatment; that is, YAP1 activation by lorlatinib treatment was not observed in HCC78 cells (Figure S2C,D). This suggests that YAP1 is not involved in the escape of ROS1-rearranged lung cancer cells from lorlatinib in HCC78 cells.

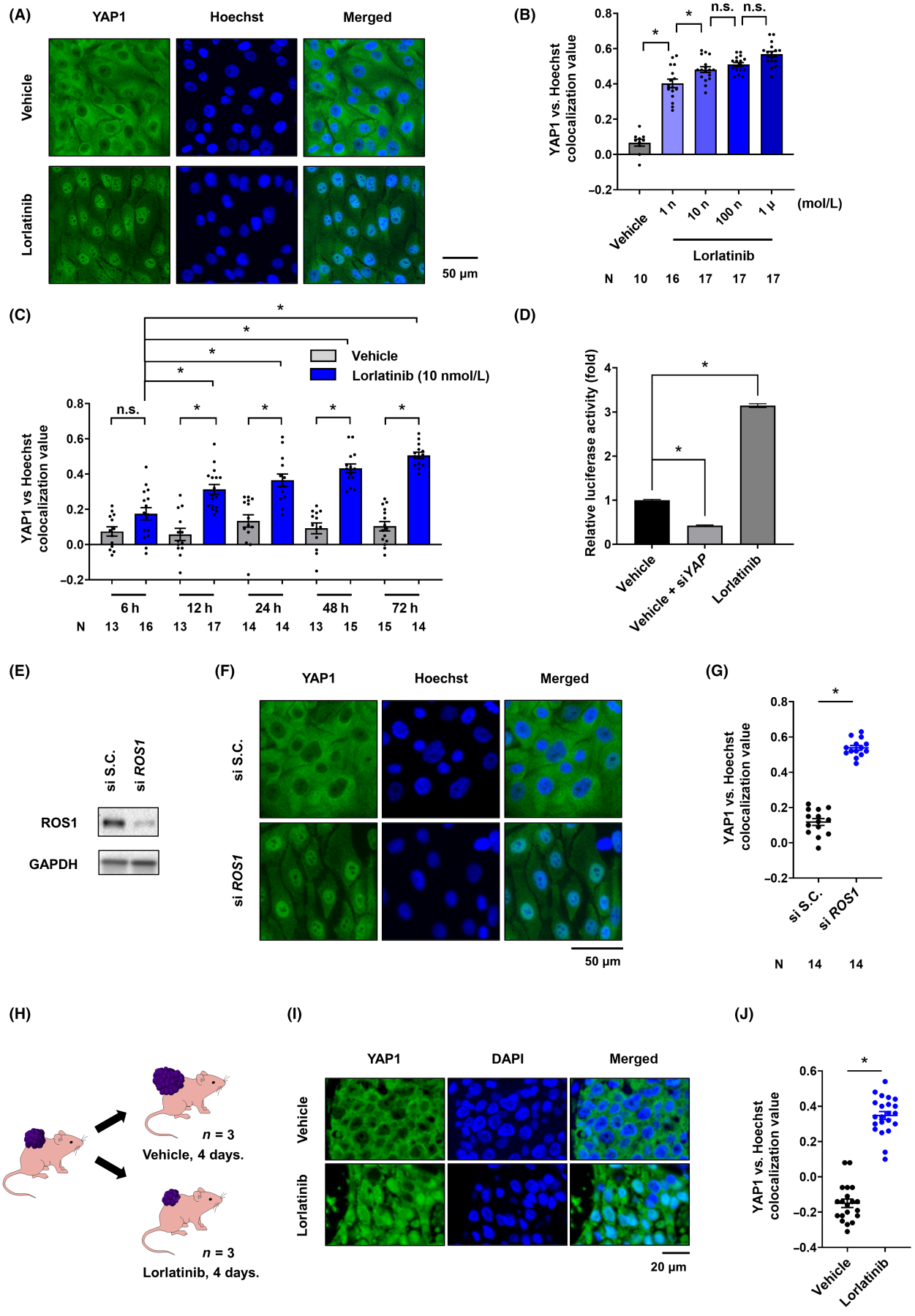


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FIGURE 3 Yes-associated protein 1 (YAP1) was activated by lorlatinib treatment in KTOR71 cells. (A) Immunofluorescence staining of KTOR71 cells exposed to vehicle or lorlatinib (10 nmol/L) for 72 h in vitro. Cells were stained with YAP1 Abs (green). All nuclei were labeled with Hoechst (blue). (B) YAP1 nuclear localization assay in KTOR71 cells exposed to vehicle or stepwise concentrations of lorlatinib for 72 h. (C) YAP1 nuclear localization assay in KTOR71 cells exposed to vehicle or lorlatinib (10 nmol/L) for various times (6–72 h). (D) TEA domain transcription factor luciferase activity in KTOR71 cells treated with vehicle or lorlatinib (10 nmol/L) for 24 h after transfection with *YAP1* siRNA or negative control siRNA (si). (E) ROS1 protein expression was detected by immunoblotting following siRNA knockdown for 72 h in KTOR71. (F) Immunofluorescence staining of KTOR71 cells following siRNA knockdown of *ROS1* for 72 h. (G) YAP1 nuclear localization assay in KTOR71 cells following siRNA knockdown of *ROS1* for 72 h. (H) Experimental design of the xenograft study. (I) Immunofluorescence staining of KTOR71 xenograft cells treated with vehicle or lorlatinib (2 mg/kg/day) for 4 days. Xenografts were stained with YAP1 Abs (green). All nuclei were stained with DAPI (blue). (J) YAP1 nuclear localization assay of KTOR71 xenografts treated with vehicle or lorlatinib for 4 days. Error bars indicate ± SEM. In Figures B, C, G, and J, one dot indicates the *r* value of cells in a single field of view. $^{*}p < 0.05$. S.C., scrambled control.

3.5 | Inhibition of YAP1 enhances sensitivity to lorlatinib and induces apoptosis in KTOR71 cells

To evaluate the function of YAP1 in cell survival following lorlatinib treatment in KTOR71 cells, YAP1 was inhibited using siRNA, resulting in reduced *YAP1* gene expression and protein abundance (Figure S3A,B). To assess YAP1 transcriptional activation, expression of *CTGF* was investigated. *CTGF* is a direct target gene of YAP1/TEAD[33] and is widely used to monitor YAP1 transcriptional activation.[34,35] Predictably, the expression of *CTGF* was increased by lorlatinib treatment and reduced by genetic inhibition of YAP1 (Figure 4A). In the cell viability assay, *YAP1* inhibition by siRNA enhanced the sensitivity of KTOR71 cells to lorlatinib (Figure 4B). Compared to the noninhibition of *YAP1*, inhibition of *YAP1* with siRNA during lorlatinib treatment significantly increased caspase 3/7, suggesting an increase in apoptosis of KTOR71 cells (Figure 4C). Similarly, verteporfin, a YAP1 inhibitor, increased sensitivity to lorlatinib (Figure 4D). Verteporfin inhibits the binding of YAP1 to TEAD and suppresses the transcription of various factors through the cotranscription complex YAP1/TEAD.[36] We confirmed that combined therapy with verteporfin and lorlatinib reduced the expression of *CTGF* compared to lorlatinib monotherapy, as in the case of *YAP1* inhibition by siRNA (Figure 4E). Next, to elucidate the role of YAP1 in KTOR71 cell survival under ROS1 inhibition, we undertook a cell viability assay with dual knockdown of *ROS1* and *YAP1*. Knockdown of *ROS1* alone resulted in a 30% inhibition of KTOR71 cell viability, and dual knockdown of *ROS1* and *YAP1* further decreased it by 50% (Figure 4F). These results confirmed that coinhibition of ROS1 and YAP1 results in lower cell viability in KTOR71 cells. Conversely, in HCC78 cells, inhibition of *YAP1* using siRNA did not improve susceptibility to lorlatinib (Figure S3C–E). These results suggest that ROS1-rearranged NSCLC contains a subtype, such as KTOR71, in which YAP1 is involved in initial cell survival during ROS1 TKI treatment, as well as a subtype such as HCC78, in which it is not.

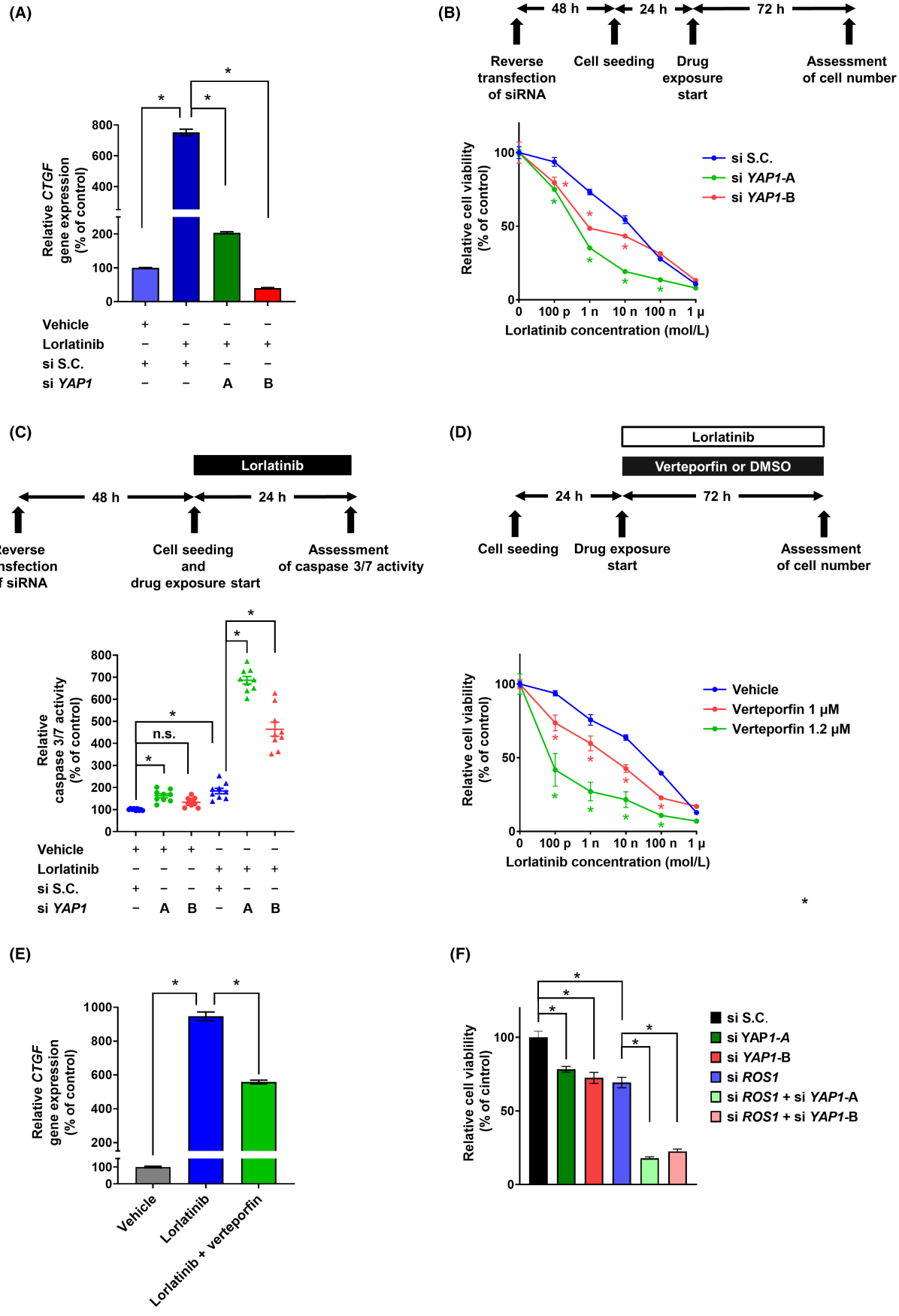
3.6 | Combination therapy with YAP1 inhibitor verteporfin and lorlatinib suppresses tumor regrowth in vivo

Considering the efficacy of the combined verteporfin and lorlatinib treatment in vitro in KTOR71 cells, we evaluated whether this combination therapy is also effective in vivo. Xenograft models using KTOR71 cells were established and randomized into four groups: vehicle, verteporfin monotherapy, lorlatinib monotherapy, and combined therapy with lorlatinib and verteporfin (Figure 5A). Each group received the assigned treatment for a week and was tracked for tumor size during and after treatment (Figure 5A). The dose of lorlatinib was 2 mg/kg/day based on the human dose (100 mg/day/body), and the dose of verteporfin was set to 25 mg/kg twice a week based on previous reports.[3,4,25,37] Lorlatinib monotherapy (2 mg/kg/day) for 7 days exerted a remarkable effect on KTOR71 growth, however, it caused tumor regrowth within a few days after treatment (Figure 5B). Vehicle and verteporfin monotherapy groups were observed for only 15 days to avoid unnecessary animal suffering.[38] Combined therapy with lorlatinib and verteporfin for 1 week suppressed tumor regrowth more significantly than lorlatinib monotherapy (Figure 5B,C). Hence, YAP1 mediates initial cell survival in response to lorlatinib in KTOR71. Moreover, short-term combination therapy with verteporfin and lorlatinib did not result in weight loss and was considered tolerable (Figure 5D).

3.7 | Yes-associated protein 1 mediates initial cell survival in response to lorlatinib through AKT signaling in KTOR71 cells

Finally, we clarified the mechanism by which YAP1 promotes cell survival following lorlatinib treatment. Specifically, we investigated how the activities of AKT and ERK, which are survival signals, change over time following lorlatinib exposure. Six hours after

FIGURE 4 Yes-associated protein 1 (YAP1) inhibition enhanced sensitivity to lorlatinib in KTOR71 cell in vitro. (A) Relative gene expression of *CTGF* normalized to *GAPDH* in KTOR71 cells transfected with *YAP1* siRNA (si) or negative control siRNA in combination with lorlatinib (10 nmol/L) or vehicle exposure for 48 h. (B) Cell viability assay of KTOR71 cells transfected with *YAP1* siRNA or negative control siRNA and treated with lorlatinib for 72 h following 48 h of transfection. (C) Apoptosis assay using Caspase-Glo in KTOR71 cells exposed to lorlatinib (10 nmol/L) or vehicle, in combination with *YAP1* knockdown. (D) Cell viability assay after lorlatinib exposure in KTOR71 cells in the presence of verteporfin for 72 h. (E) Relative gene expression of *CTGF* normalized to *GAPDH* in KTOR71 cells treated with vehicle, lorlatinib (10 nmol/L), or lorlatinib plus verteporfin (1 μmol/L) for 48 h. (F) Cell viability assay of KTOR71 cells transfected with *ROS1* siRNA and/or YAP1 siRNA for 72 h. Error bars indicate ± SEM. $^{*}p < 0.05$. n.s., not significant; S.C., scrambled control



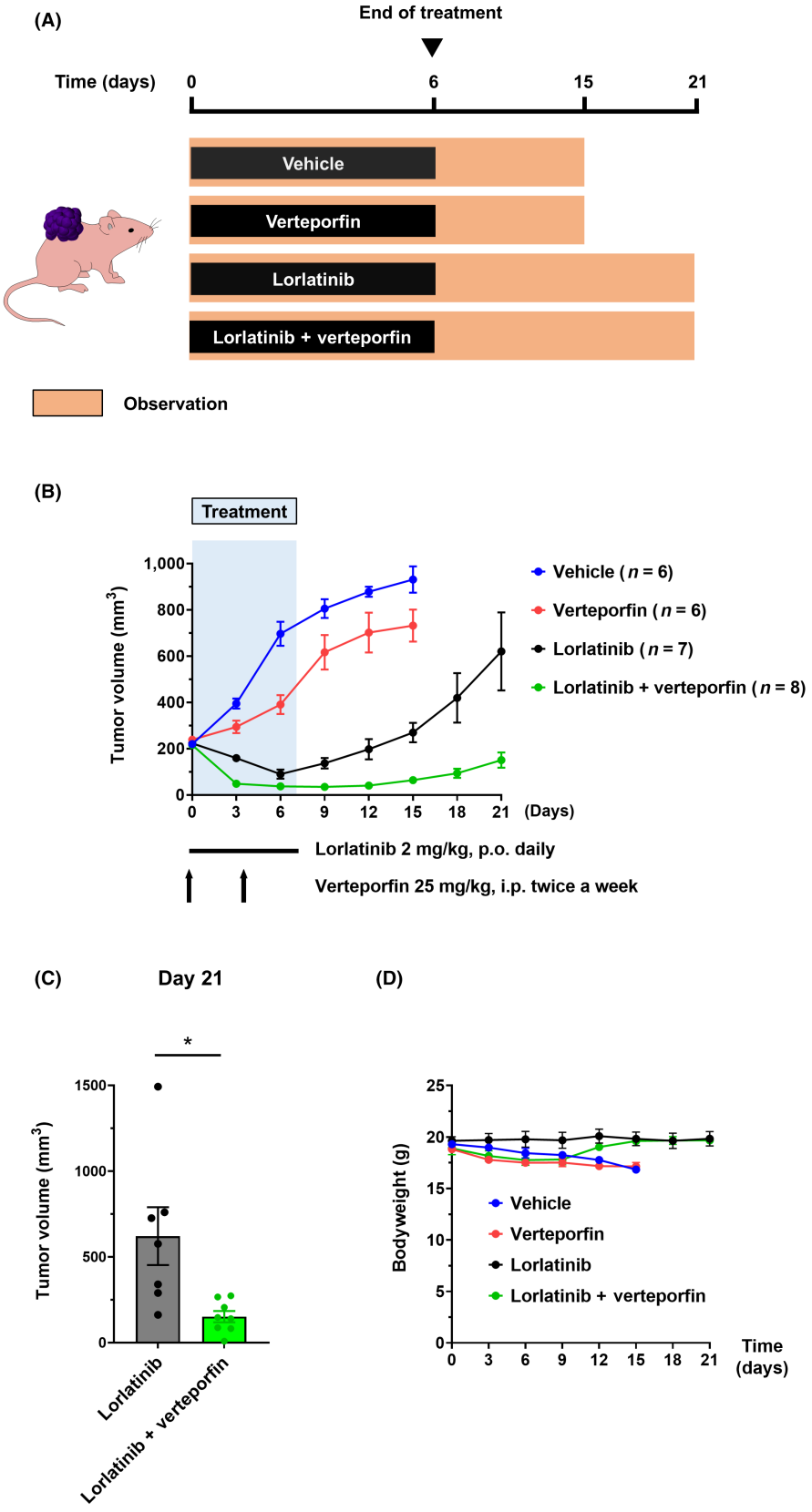


FIGURE 5 Combined therapy with lorlatinib and verteporfin significantly suppressed tumor regrowth compared to lorlatinib monotherapy in KTOR71 xenograft models. (A) KTOR71 xenograft tumors were treated with vehicle, verteporfin (25 mg/kg, i.p., twice a week), lorlatinib (2 mg/kg, orally, daily), or lorlatinib and verteporfin for 7 days. (B) Tumor volume curve following vehicle treatment, verteporfin monotherapy, lorlatinib monotherapy, or combination therapy with lorlatinib and verteporfin. (C) Tumor volumes on day 21 in lorlatinib monotherapy and lorlatinib plus verteporfin groups. (D) Bodyweight changes in KTOR71 xenografts according to the treatment regimen. Error bars indicate ± SEM. $^{*}p < 0.05$.

lorlatinib exposure, AKT and ERK activity was inhibited (Figure 6A). However, after 24 h, AKT was reactivated, whereas reactivation of ERK was not observed (Figure 6A). Therefore, we hypothesized that YAP1 activation leads to the reactivation of AKT. Genetic inhibition of *YAP1* by siRNA suppressed the reactivation of AKT 72 h after lorlatinib exposure (Figure 6B). Pharmacological inhibition of YAP1 function by verteporfin also suppressed AKT reactivation following lorlatinib exposure (Figure 6C).

Subsequently, we confirmed the involvement of AKT in the survival of KTOR71 cells. AKT has three isoforms: AKT1, AKT2, and

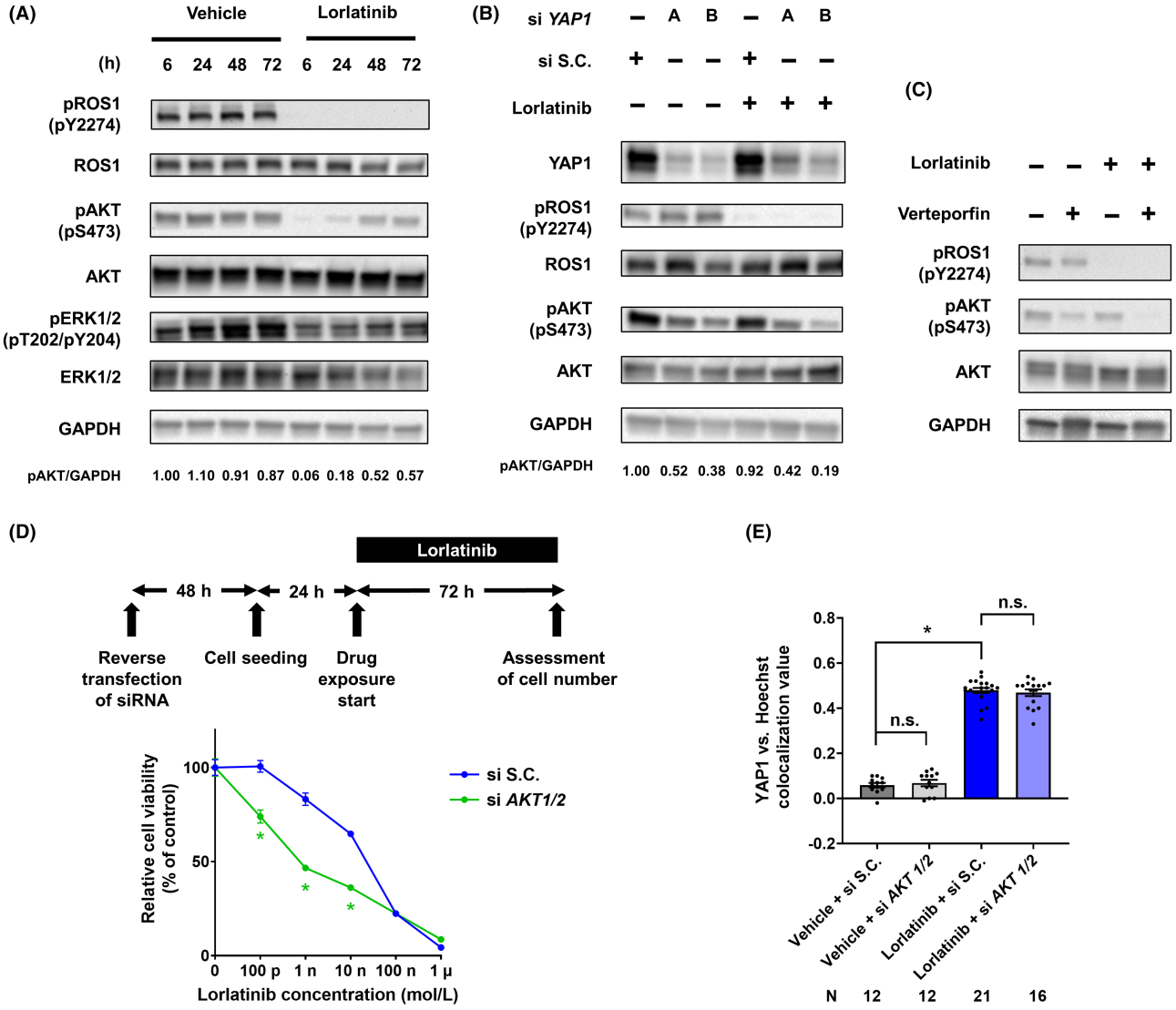


FIGURE 6 Legend on next page

FIGURE 6 Yes-associated protein 1 (YAP1) induced KTOR71 cell survival against lorlatinib by mediating AKT activation. (A) KTOR71 cells were treated with lorlatinib (10 nmol/L) or vehicle for 6–72 h. Phosphorylated protein expression levels of AKT and ERK1/2 were evaluated by immunoblotting. (B) KTOR71 cells transfected with *YAP1* siRNA (si) or negative control siRNA were treated with lorlatinib (10 nmol/L) or vehicle for 72 h. Phosphorylated protein expression levels of AKT were evaluated by immunoblotting. (C) KTOR71 cells treated with lorlatinib (10 nmol/L) and verteporfin (1 μmol/L) for 72 h. Phosphorylated AKT expression was measured using immunoblotting. (D) Cell viability assay of KTOR71 cells transfected with *AKT1/2* siRNA and negative control siRNA and treated with lorlatinib for 72 h following 48 h of transfection. (E) YAP1 nuclear localization assay in KTOR71 cells transfected with *AKT1/2* siRNA or negative control siRNA and treated with lorlatinib (10 nmol/L) or vehicle for 72 h. (F) Schematic explanation of the initial survival mechanisms against lorlatinib in KTOR71 cells. Error bars indicate ± SEM. $^{*}p < 0.05$. n.s., not significant; S.C., scrambled control.

AKT3.[39] However, as AKT3 is expressed at low levels in all tissues, except brain and endocrine tissues,[39] we focused on AKT1/2 function in KTOR71 cells. *AKT1/2* inhibition with siRNA markedly suppressed total AKT protein abundance (Figure S4A). Moreover, in the cell viability assay, genetic inhibition of *AKT1/2* with siRNA increased sensitivity to lorlatinib (Figure 6D). In contrast, the nuclear localization of YAP1 induced by lorlatinib was not altered by the genetic inhibition of *AKT1/2* (Figure 6E). These results indicated that YAP1 mediated initial cell survival in response to lorlatinib by activating AKT (Figure 6F). Moreover, *AKT1/2* inhibition also enhanced sensitivity to other ROS1 TKIs (crizotinib and entrectinib) in KTOR71 cells (Figure S4B). Considering that YAP1 is also activated by exposure to high concentrations of crizotinib and entrectinib, this result supports the hypothesis that the YAP1-AKT signaling pathway plays an important role in cell survival in KTOR71 cells.

The function of AKT in cell survival was also examined in HCC78 cells. *AKT1/2* inhibition enhanced sensitivity to lorlatinib (Figure S4C,D). *AKT1/2* inhibition also enhanced the sensitivity of crizotinib and entrectinib (Figure S4E). These results suggest that AKT also plays an important role in HCC78 cell survival. However, in HCC78 cells, knockdown of *YAP1* did not reduce AKT activity (Figure S4F). This is consistent with the result that *YAP1* knockdown did not increase sensitivity to lorlatinib in HCC78 cells (Figure S3E).

Three mechanisms by which YAP1 regulates AKT activity have been previously reported.[40–42] One mechanism is the negative regulation of PTEN protein expression, and the others occur through positive regulation of *GAB2* or *Pi3kcb* expression, respectively. However, in KTOR71 cells, PTEN protein expression was not altered by *YAP1* knockdown under lorlatinib exposure, and neither *GAB2* nor *Pik3cb* gene expression were downregulated by *YAP1* knockdown under lorlatinib exposure (Figure S5A–C). In other words, YAP1 regulates AKT activity in KTOR71 cells by some mechanism other than those previously reported, such as PTEN, GAB2, or PIK3CB regulation.

3.8 | Yes-associated protein 1 activity is regulated by a different mechanism in KTOR71 cells than in ALK-rearranged lung cancer

Previously, we reported the involvement of Lats1 and Ajuba as upstream factors regulating YAP1 activity in ALK-rearranged lung cancer.[25] Therefore, Lats1 and Ajuba were evaluated for possible candidate factors regulating YAP1 in KTOR71. As shown in Figure S5D, Lats1 phosphorylation (pLats1) expression was not changed by lorlatinib treatment. In addition, although *Ajuba* expression was slightly increased by lorlatinib treatment, knockdown of *Ajuba* did not inhibit the activation of YAP1 in KTOR71 (Figure S5E–G). These results suggest that YAP1 activation does not occur through Lats1 or Ajuba in KTOR71, unlike in ALK-rearranged lung cancer.

3.9 | TP53 mutations could affect the activation state of YAP1 in ROS1-rearranged lung cancer

KTOR71 and HCC78 cells showed different YAP1 activity states in the absence of lorlatinib, with the former having YAP1 predominantly in the cytoplasm, and the latter predominantly in the nucleus. *TP53* mutations in KTOR71 and HCC78 cells were examined because an association between *TP53* mutations and high YAP1 activity has been reported.[43,44] The HCC78 cell line has a *TP53* mutation (S241F) according to the COSMIC database. The S241F mutation is reported to be a potential loss-of-function mutation.[45] Next, we evaluated *TP53* mutations in KTOR71 cells using whole exome sequencing. In KTOR71 cells, there was no *TP53* mutation in the exome region, although two *TP53* mutations were detected in the intron regions (Appendix S6). To the best of our knowledge, these two mutations in the intron region likely have no clear effect on TP53 function. Therefore, *TP53* mutations could potentially be involved in the difference in YAP1 activity in the absence of lorlatinib.

4 | DISCUSSION

Here, we clarified that YAP1 plays a crucial role in cell survival during the initial phase of treatment with the ROS1 TKI lorlatinib in a patient-derived ROS1-rearranged lung cancer cell line. Yes-associated protein 1 activated by lorlatinib protected cancer cells against lorlatinib through AKT signaling. To our knowledge, this study is the first to report the significance of YAP1 in the escape of ROS1-rearranged lung cancer from ROS1 inhibitor treatment.

Specifically, we showed that YAP1 was activated during the initial phase of lorlatinib treatment in ROS1-rearranged NSCLC and was involved in initial cell survival against lorlatinib. Several preclinical studies have aimed to eradicate lung cancer cells harboring driver oncogenes by targeting initial survival.[23–27,46] In EGFR mutation-positive NSCLC, AXL and IGF1R are thought to be crucial for initial cell survival against the EGFR-TKI osimertinib in subtypes with high

AXL expression and low AXL expression, respectively.[23,24] Regarding ALK-rearranged NSCLC, our group previously revealed that YAP1 is involved in cell survival in response to ALK TKIs through the antiapoptotic proteins BCL-XL and MCL-1.[25] In addition, c-Jun N-terminal kinase/c-Jun signaling and HER3 signaling might also be involved in resistance to ALK TKIs.[26,27] However, there is only one report on survival mechanisms during the initial phase of ROS1 TKI treatment in ROS1-rearranged NSCLC.[46] Vaishnavi et al. indicated that EGFR-mediated responses to the ROS1 TKIs crizotinib and TAE684 occur through three mechanisms: classical bypass signaling, EGFR transactivation of ROS1 fusion kinase, and adapter switching using growth factor receptor-bound protein 2 (GRB2).[46] Herein, we clarified for the first time that YAP1 is a key factor in cell escape from ROS1 TKI.

A major downstream effector of the Hippo pathway, YAP1 functions as a transcriptional coactivator and preferentially binds to TEAD.[33] The activated YAP1-TEAD complex promotes the expression of several genes, such as *SOX2*, *BCL2L1*, *MCL1*, *BIRC5*, *SNAI2*, and *ZEB1*, which induce cell growth, antiapoptosis, and epithelial–mesenchymal transition.[25,47,48] Consequently, YAP1 activation is involved in the acquired resistance mechanisms for molecular-targeted therapies in various cancer types.[49] When activated, YAP1 translocates from the cytoplasm to the nucleus.[47,48] Additionally, YAP1 acts as a mechanical sensor and its activation is regulated by the actin cytoskeleton and cell tension.[32,50] Accordingly, YAP1 is activated and localized in the nucleus of spread cells and cells attached to large areas of the ECM.[47,50] In the present study, GO analysis of proteins upregulated by lorlatinib exposure in KTOR71 cells extracted three GO terms and two KEGG pathways involved in cytoskeleton and morphology changes: "actin filament-based process" (GO:0030029), "regulation of cell-substrate adhesion" (GO:0010810), and "regulation of cell adhesion" (GO:0030155), and "regulation of actin cytoskeleton (hsa04810)" and "focal adhesion (hsa04510)". Lorlatinib exposure induced changes in cell morphology, and cell spread change was observed in KTOR71 cells. This change in morphology led us to focus on YAP1 as a possible initial survival factor for lorlatinib. Similarly, we previously found that exposure to ALK TKIs also results in the spread of ALK lung cancer cells and that YAP1 is involved in the initial survival of ALK TKIs.[25] Thus, it is reasonable to infer that YAP1 is a common initial survival factor in both ROS1- and ALK-rearranged NSCLC, as ROS1- and ALK-rearranged NSCLC have certain common TKIs due to the high homology of the ROS1 and ALK kinase domains and have clinical commonalities in being predominant in young individuals with no history of smoking.[11] However, the proteomic analysis did not specifically extract YAP1 or the Hippo signaling pathway in the present study on ROS1-rearranged NSCLC. Therefore, the possible involvement of factors other than YAP1 in the initial survival from lorlatinib requires further study.

Yes-associated protein 1 regulates cell survival after lorlatinib treatment by positively regulating the activity of the serine/threonine protein kinase AKT in ROS1-rearranged NSCLC. AKT is an essential component of the PI3K pathway and is activated by phosphorylation.[39] AKT promotes cell survival and growth by regulating downstream factors, such as BAD, glycogen synthase kinase (GSK)-3, the tuberous sclerosis complex, and MDM2.[39] Interactions between YAP1 and the PI3K-AKT pathway have been reported in previous studies.[40–42] According to a previous study on endometrial cancer, YAP1 positively regulates PI3K-AKT pathway activity through GAB2, which acts as a scaffold linking growth factor receptors to PI3K pathways.[41] Moreover, Tumaneng et al. indicated that YAP1 induced AKT phosphorylation by suppressing PTEN, a key antagonist of PI3K, through microRNA-29 in the human mammary epithelial cell line MCF10A.[40] Furthermore, Lin et al. reported that YAP1 activated AKT by directly activating the expression of *Pik3cb* in rat hearts.[42] However, in KTOR71 cells, YAP1 did not regulate AKT by these three mechanisms. Therefore, elucidating the molecular mechanisms by which YAP1 regulates AKT in KTOR71 cells requires further investigation.

In this study, we proposed a curative treatment using lorlatinib as a ROS1 inhibitor. Lorlatinib, which is a high-affinity ALK/ROS1 TKI, has recently become a key first-line drug for ALK-rearranged NSCLC based on the CROWN study,[8,10] with increasing expectations for its future clinical application to ROS1-rearranged NSCLC treatment. In addition, lorlatinib is a promising drug for the treatment of ROS1 TKI after first-line therapies. One of the clinical problems with ROS1-rearranged NSCLC is that there are few options for other ROS1 TKIs after the development of resistance to first-line ROS1 TKI. Presently, crizotinib and entrectinib are the only ROS1 TKIs available in normal clinical settings; however, entrectinib treatment is less effective after the development of resistance to crizotinib.[5,14] In a preclinical study, lorlatinib showed activity against several crizotinib-resistant ROS1 mutations, including L2026M, S1986F, and D2033N,[6] indicating its potential as a therapeutic option after crizotinib resistance.

This study has some limitations. First, the research was carried out using only two ROS1 lung cancer cell lines. Unfortunately, basic research on ROS1-rearranged lung cancer frequently uses only a small number of cell lines because ROS1 rearrangements are rare events in lung cancer and only one commercially available ROS1-rearranged lung cancer cell line (HCC78) has been developed.[7] We suspect that ROS1-rearranged lung cancer might have a subtype in which YAP1 is an initial survival factor, such as KTOR71, and a subtype in which YAP1 is not, such as HCC78. The diversity of initial survival factors among lung cancers with the same driver oncogene has also been observed in EGFR mutation-positive NSCLC. Epidermal growth factor receptor mutation-positive NSCLC has been classified into two subtypes: AXL-mediated and IGF1R-mediated initial survival mechanisms.[23,24] However, evaluation using more cell lines is required to confirm that ROS1-rearranged lung cancer can be classified into two subtypes based on YAP1 function and to understand the frequency of each subtype. Second, KTOR71 was established after the acquisition of crizotinib resistance but not before initiating ROS1 TKI treatment. As the S1986F mutation in the coding region of ROS1 tyrosine kinase served as the crizotinib resistance mechanism in KTOR71, KTOR71 maintained ROS1-dependency and sensitivity to lorlatinib, which supports the scientific validity of this study on initial cell survival in response to lorlatinib. However, crizotinib treatment could have

affected the results regarding YAP1 function. Third, the mechanism of YAP1 activation in KTOR71 was found to be different from that in ALK-rearranged lung cancer, but the specific mechanism could not be determined. Fourth, it was not experimentally clear that AKT regulation by YAP1 is enhanced by treatment with lorlatinib. However, we clarified that: (1) YAP1 is activated by lorlatinib exposure, (2) with the ROS1-AKT pathway inhibited by lorlatinib, AKT reactivation occurs almost in parallel with YAP1 activation, and (3) inhibition of YAP1 suppresses this AKT reactivation. These results suggest that YAP1 considerably mediates AKT activity under lorlatinib exposure and that coinhibition of ROS1 and YAP1 is important for suppressing AKT signaling.

In conclusion, this study revealed the significance of YAP1 in ROS1-rearranged NSCLC treated with the ROS1 TKI lorlatinib. YAP1 activation during lorlatinib treatment mediates initial cell survival in response to lorlatinib through AKT signaling. Thus, combined treatments against both YAP1 and ROS1 could be an effective curative strategy for ROS1-rearranged NSCLC.

AUTHOR CONTRIBUTIONS

Conception and design: M. Yamazoe, H. Ozasa, T. Tsuji. Development of methodology: M. Yamazoe, H. Ozasa, T. Tsuji, W. Aoki. Acquisition of data: M. Yamazoe, H. Ozasa, W. Aoki. Analysis and interpretation of data: M. Yamazoe, H. Ozasa, W. Aoki. Writing, review, and/or revision of the manuscript: M. Yamazoe, H. Ozasa, T. Tsuji, T. Funazo, H. Yoshida, K. Hashimoto, K. Hosoya, T. Ogimoto, H. Ajimizu, H. Yoshida, R. Itotani, Y. Sakamori, K. Kuninaga, W. Aoki, T. Hirai. Administrative, technical, or material support: M. Yamazoe, H. Ozasa, T. Tsuji, T. Funazo. Study supervision: H. Ozasa, Y. Sakamori, T. Hirai.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data generated in this study are available upon request from the corresponding author.

ETHICAL APPROVAL

The study protocol was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee (certification nos. R0996, R2163).

INFORMED CONSENT

The patient provided written informed consent regarding their participation in this study.

ANIMAL STUDIES

All animal experiments were approved by the Animal Research Committee of Kyoto University (ID: MedKyo 19,294, 20,256, and 21,272) and conducted in accordance with ARRIVE guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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