c-MYC-mediated TRIB3/P62\(^+\) aggresomes accumulation triggers paraptosis upon the combination of everolimus and ginsenoside Rh2

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Abstract  The mammalian target of rapamycin (mTOR) pathway is abnormally activated in lung cancer. However, the anti-lung cancer effect of mTOR inhibitors as monotherapy is modest. Here, we identified that ginsenoside Rh2, an active component of Panax ginseng C. A. Mey., enhanced the anti-cancer effect of the mTOR inhibitor everolimus both in vitro and in vivo. Moreover, ginsenoside Rh2 alleviated the hepatic fat accumulation caused by everolimus in xenograft nude mice models. The combination of everolimus and ginsenoside Rh2 (labeled Eve-Rh2) induced caspase-independent cell death and cytoplasmic vacuolation in lung cancer cells, indicating that Eve-Rh2 prevented tumor progression by triggering paraptosis. Eve-Rh2 up-regulated the expression of c-MYC in cancer cells as well as tumor tissues. The increased c-MYC mediated the accumulation of tribbles homolog 3 (TRIB3)/P62\(^+\) aggresomes and consequently triggered paraptosis, bypassing the classical c-MYC/MAX pathway. Our study offers a potential effective and safe strategy for the treatment of lung cancer. Moreover, we have identified a new mechanism of TRIB3/P62\(^+\) aggresomes-triggered paraptosis and revealed a unique function of c-MYC.
1. Introduction

The mammalian target of rapamycin (mTOR) functions as an essential downstream effector of multiple oncoproteins, including RAS and epidermal growth factor receptor (EGF)1,2. The mTOR signaling pathway is frequently mutated and abnormally activated in lung cancer3. Everolimus (Eve), a first-generation mTOR inhibitor, is approved by the U.S. Food and Drug Administration for the treatment of several solid tumors, including breast cancer, advanced renal cancer and pulmonary neuroendocrine tumors1. Currently, more than 30 clinical trials of Eve against lung cancer have been registered on ClinicalTrials.gov by the U.S. National Library of Medicine. However, the anti-lung cancer effect of the Eve monotherapy is modest1. The median overall survival and the median progression-free survival are 6.7 and 1.3 months, respectively, in the Eve treatment group (NCT00374140). The adverse events of Eve, including anemia (22.5%), metastasis and nutrition disorders (10%), neutropenia (32.5%), dyspnea and pneumonia (7.5%), frequently happen. High triglycerides or hypertriglyceridemia and hepatotoxicity or elevated liver enzymes in blood have also been observed in patients treated with Eve5, highlighting the necessity of improving the efficiency and reducing the toxicity of Eve.

Ginseng (Panax ginseng C. A. Mey.), one of the most famous tonic, is widely used as a cancer treatment option6 and has been registered as a complementary and alternative medicine by the National Center for Complementary and Integrative Health (China)7. Ginseng improves physical stamina, stimulates immune function, and relieves various other health problems, such as respiratory and cardiovascular disorders7. Ginsenosides are the main bioactive components of ginseng, many of which show anti-inflammatory and anti-cancer activities9,10. Ginsenoside Rh2 (Rh2) presents anti-cancer effects in various solid tumors, including lung cancer, breast cancer, ovarian cancer, prostate cancer and hematological tumors11. Rh2 has been recorded to restrain tumor growth12,13. Moreover, Rh2 acts on immune cells and regulates tumor microenvironment, thereby indirectly restraining tumor growth12,13.

Paraposis is a kind of programmed cell death characterized by cytoplasmic vacuolation, mitochondria and/or endoplasmic reticulum (ER) swelling, dependence on protein synthesis, caspase activation independence and lack of the typical hallmarks of apoptosis14–16. Paraposis frequently undergoes cellular stress responses, including ER stress and oxidative stress17,18. A large number of unfolded or misfolded proteins in the rapidly growing tumor cells causes ER stress19–21, suggesting that tumor cells may be more sensitive to agents that induce paraposis than normal cells. This study has verified that the combination of Eve and Rh2 (labeled Eve-Rh2) shows remarkable anti-cancer effect in vitro and in vivo, which is mediated by the Eve-Rh2-induced paraposis. The mechanical study suggests that Eve-Rh2 activates a unique function of c-MYC, through which Eve-Rh2 up-regulates the expression of tribbles homolog 3 (TRIB3), thereby mediating the accumulation of TRIB3/P62 aggresomes and ultimately triggering paraposis.

2. Materials and methods

2.1. Reagents

Eve (purity >98%), z-VAD-FMK, MG-132, 10058-F4, and 10074-G5 were obtained from Selleck Chemicals (Houston, TX, USA). Rh2 (purity ≥98%) used in the cell models was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Rh2 (purity ≥98%) used in the animal models was purchased from Sichuan Victory Biological Technology Co., Ltd. (Chengdu, China). Chelerythrine (purity >98%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Actinomycin D (A-D), bafilomycin A1 and chloroquine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cycloheximide (CHX) was obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) test kits were provided by the Nanjing Jincheng Bioengineering Institute (Nanjing, China). The hematoxylin and eosin (H&E) staining kit, oil red O staining kit and lactate dehydrogenase (LDH) release assay kit were purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Cell culture

Human EGFR-mutant lung cancer cell lines NCI-H1975 and HCC827 were obtained from the American Type Culture Collection (Rockville, MD, USA). The osimertinib resistant NCI-H1975/OSIR cell line was established in our laboratory22. Cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco, Carlsbad, CA, USA). All cells were incubated under 5% CO2 at 37 °C.

2.3. Xenograft nude mice models

Five-week-old male BALB/c nude mice were provided by the specific-pathogen free Animal Facility of University of Macau and kept in the facility. All operations were approved by the Animal Ethics Committee of University of Macau. Human lung cancer NCI-H1975 (2 × 106) or HCC827 (3 × 106) cells were subcutaneously inoculated in the right back of each mouse. Five days after inoculation, the mice were randomly divided into four groups (n = 8) in accordance with the intragastric administration of the following treatments: G1, solvent control group; G2, Rh2 (5 mg/kg/day) group; G3, Eve (1 mg/kg/day) group; and G4, Eve-Rh2 group (1 mg/kg/day Eve + 5 mg/kg/day Rh2). All compounds were diluted in 0.9% saline solution. The dose of Eve was calculated according to the dosages used in clinical trials23, while that of Rh2 was determined by the preliminary study and relevant literature24. Tumor volume was calculated using Eq. (1):

\[
\text{Tumor volume} = \text{Length} \times \text{Width}^2 / 2
\]
After 28 days of administration, the levels of fasting blood glucose of four mice from each group in NCI-H1975 and HCC827 mice models were tested by using the OneTouch Ultra Easy Glucometer (LifeScan Inc., Milpitas, CA, USA). Serum was obtained from each mouse, and the levels of ALT and AST were determined. Afterwards, the mice were sacrificed, and the tumors as well as the organs (i.e., livers and kidneys) were dissected. The tumors were imaged. The pathologic changes in organs were observed using the H&E staining assay, the lipid droplets in the livers were observed using oil red O staining, and the expression level of c-MYC in tumors was analyzed using the immunohistochemical staining. The primary antibody against c-MYC (#ab32072) used in immunohistochemical staining was purchased from Abcam (Cambridge, UK).

2.4. MTT assay and LDH release assay

Cells were treated as indicated and incubated with the prepared 1 mg/mL tetrazolium salt MTT solution (Sigma–Aldrich). Afterwards, the MTT solution was discarded, and DMSO (Sigma–Aldrich) was added to dissolve the formazan crystals. The optical density at 570 nm was monitored. After treatment for 6 h, LDH release was determined using the LDH release assay kit according to the manufacturer’s instructions. Absorbance at 490 nm was measured by the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Cell morphology observation

Cells were treated as indicated, and the momentary cell morphology was captured using the Axiovert 200 inverted microscope (Zeiss, Oberkochen, Germany). The number of vacuolated cells was manually counted. The dynamic changes in the cell morphology after treatment were monitored using the IncuCyte S3 Live-Cell Analysis System (Essen BioScience Inc., Ann Arbor, MI, USA). Images were obtained every 20 min for 24 h.

2.6. Western blot

Cells were lysed using the RIPA lysis buffer (Beyotime Biotechnology). The dissolution agents for soluble and insoluble protein extraction were different. Cells were lysed by Triton-X100 in PBS and centrifuged to extract the soluble proteins in the supernatant. Sediments were dissolved in 1% sodium dodecyl sulfate to obtain the insoluble proteins. Equivalent protein samples were separated using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then proteins were transferred onto the polyvinylidene fluoride membranes. After blocking in 5% nonfat milk or BSA, the membranes were incubated with the primary antibodies overnight at 4 °C and further incubated with the relative secondary antibodies. Afterwards, the membranes were exposed using the ECL Select Western blot detection reagent (GE Healthcare, Uppsala, Sweden). The primary antibodies against Alix (#2171), ubiquitin (#3933), BIP (#3177), CHOP (#2895), ATF4 (#11815), IRE1α (#3294), XBP1s (#12782), PERK (#5683), P-eIF2α (#3398), eIF2α (#5324), ATF6 (#65880), P62 (#8025), c-MYC (#5605), MAX (#4739), HSP90 (#5087), β-actin (#3700), and GAPDH (#5174) as well as the anti-rabbit and the anti-mouse IgG HRP-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The primary antibody against TRIB3 (#ab137526) was purchased from Abcam (Cambridge, UK).

2.7. RNA extraction and qRT-PCR

After treatment, cells were lysed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted using chloroform and isopropanol. The complementary DNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The SYBR Green Kit (Roche, Basel, Switzerland) was used to prepare the samples, and qRT-PCR assay was conducted in the Viia 7 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Primers are listed in Supporting Information Table S1. GAPDH was used as the internal control, and the relative expression levels of indicated genes were calculated by $2^{-\Delta\Delta C_t}$.

2.8. Immunofluorescence

After treatment, cells were fixed using 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, blocked with 5% bovine serum albumin and incubated with indicated antibodies overnight at 4 °C. On the next day, the cells were incubated with the Alexa Fluor 594-conjugated or the Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) and further stained with Hoechst 33342. Then, the cells were imaged using the Leica TCS SP8 confocal microscope (Leica, Solms, Germany). The primary antibody against P62 (#18420-1-AP) was purchased from Proteintech Group, Inc. (Rosemont, IL, USA). The primary antibodies against TRIB3 and c-MYC were the same as those used in Western blot.

Aggresomes accumulation was detected using the Proteostat Aggresome Detection Kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer’s instructions. Briefly, cells were fixed using 4% paraformaldehyde and permeabilized using 0.5% Triton X-100. Then, the cells were incubated with the Proteostat Aggresome dye for 1.5 h in the dark at room temperature and imaged using the Leica TCS SP8 confocal microscope (Leica).

2.9. Proteasome-Glo chymotrypsin-like cell-based assay

The activity of the proteasome was determined by following the manufacturer’s instructions. Briefly, compound solutions were removed after treatment. New medium and the isopycnic Proteasome-Glo Cell-Based Reagents (Promega Corporation, Madison, WI, USA) were added to each well. The fluorescence intensity was measured using the PerkinElmer microplate reader (PerkinElmer, Wellesley, MA, USA). Relative activity of the proteasome was normalized to the control level.

2.10. RNA-Seq analysis

Cells were treated for 6 h, and the total RNA was extracted using the TRizol reagent. RNA-Seq was conducted by the Omigen (Hangzhou, China) through the Illumina HiSeq X Ten PE150 Sequencing System. Differentially expressed genes were identified through the differential gene expression algorithm in the Partek Genomics Suite. The genes showing more than two-fold changes and $P < 0.05$ were considered as differentially expressed genes. The top 100 differentiated genes sorting by $P$ value are
listed in Supporting Information Table S2. Gene Ontology analysis was performed to enrich the biological process in accordance with the database (http://www.geneontology.org/). Ingenuity Pathway Analysis (IPA) was performed to analyze the upstream regulators and the regulatory network on the basis of the Ingenuity Knowledge Base Library.

2.1. Luciferase assay

The pMYC-TA-luc plasmid (Beyotime Biotechnology) was used to determine the transcriptional activity of c-MYC, which contained several E-box DNA binding elements. The pMYC-TA-luc plasmid or the negative control plasmid and the TurboFect Transfection Reagent (Thermo Fisher Scientific) were transferred to the cells following the instructions. Cells were treated as indicated for 6 h and lysed using the lysis buffer. The Luciferase Assay Reagent II was added to the cell lysates by following the instructions of the Dual-Luciferase Reporter Assay System (Promega Corporation). The bioluminescence was measured using the 1420 Multilabel Counter Victor3 Microplate Reader (PerkinElmer).

2.12. Small interfering RNA (siRNA) transfection

The specific or scrambled siRNA was transfected into cells following the manufacturer’s instructions by using the Lipofectamine 2000 Transfection Reagent (Invitrogen). The sequences of siRNA are listed in Table S1. Two c-MYC specific siRNAs with different sequences were used together to silence the expression of c-MYC.

2.13. Transmission electron microscopy

After treatment, cells were fixed by using 2.5% glutaraldehyde at room temperature in dark. Transmission electron microscopy analysis was performed by Wuhan Servicebio Technology Co., Ltd. (Wuhan, China) through the HT7700 Transmission Electron Microscope (Hitachi High-Tech Corporation, Tokyo, Japan).

2.14. Statistical analysis

The in vitro data were presented as mean ± standard deviation (SD), whereas the in vivo data were presented as mean ± standard error of mean (SEM) due to the individual difference of animals. All presented in vitro experiments were repeated three times at least. Significance was analyzed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) through one-way ANOVA or two-tailed Student’s t test. *P < 0.05 and **P < 0.01 are considered statistically significant, whereas the ns stands for not statistically significant.

3. Results

3.1. Eve-Rh2 shows potential anti-lung cancer effect in vitro and in vivo

Our preliminary study showed that the combination of Eve (10 μmol/L) and Rh2 (10 μmol/L) remarkably triggered cell death in lung cancer cells. Afterwards, cancer cells were treated with the combination of Eve and Rh2 by using various treatment sequences, including pretreatment with Eve, pretreatment with Rh2, and concurrent treatment (Supporting Information Fig. S1A). The strongest anti-cancer effect in vitro was obtained in the group of co-treatment with Rh2 after the Eve pretreatment for 1 h (Fig. S1B), which was labeled as Eve-Rh2 and was chosen as the treatment manner in the following study. Herein, cell survival of the four human lung cancer cell lines (NCI-H1975, NCI-H1975/OSIR, HCC827, and A549) was tested under Rh2 (10 μmol/L), Eve (10 μmol/L), and Eve-Rh2 treatments for 24 h. Both Rh2 and Eve monotreatments induced a slight cytotoxicity in all tested cell lines (Fig. 1A), presumably because of the low concentration and short treatment time8,26. However, Eve-Rh2 triggered significant cell death within 24 h with cell inhibitory rates of 78.24 ± 8.74% in NCI-H1975, 66.56 ± 11.42% in NCI-H1975/OSIR, 54.80 ± 12.83% in HCC827 and 55.44 ± 4.73% in A549 (Fig. 1A). Meanwhile, the Eve-Rh2 treatment caused the formation of uneven-sized vacuoles in all tested cell lines (Fig. 1B). The anti-cancer effect of Eve-Rh2 was further evaluated in nude mice models by subcutaneously injecting NCI-H1975 or HCC827 cancer cells. Eve-Rh2 significantly suppressed tumor growth, and the anti-cancer effect of Eve-Rh2 treatment showed significant advantage over both Rh2 and Eve monotreatments (Fig. 1C–H). Eve monotreatment has been reported to cause hepatic steatosis in clinical case and hepatotoxicity in clinical trials17,28. In our study, about 30% livers in Eve treatment group exhibited pale spots in appearance (Supporting Information Fig. S2A and S2B), whereas the serum ALT and AST were not elevated after Eve treatment (Supporting Information Fig. S3). Nevertheless, small vacuoles were observed in the hepatocytes around the nucleus as shown by H&E staining (Fig. S2C and S2D), and lipid droplets in livers were demonstrated by oil red O staining (Fig. S2E and S2F), which was used for fat droplet identification29,30, indicating that Eve causes hepatic fat accumulation. In contrary, all livers in the Eve-Rh2-treated mice were healthy and as normal as untreated mice as evidenced by the appearance, H&E staining and oil red O staining (Fig. 1K, L and Fig. S2), suggesting that Rh2 attenuates Eve-caused hepatic fat accumulation. In addition, Eve-Rh2 treatment did not cause any injury to kidney, the primary organ of excretion31, as shown in histological sections (Fig. 1K and L), and the body weight of the mice in all groups had no difference after relative administration (Fig. 1I and J), further indicating the reliable safety of Eve-Rh2 treatment in vivo.

3.2. Eve-Rh2 induces paraptosis in lung cancer cells

Thereafter, the type of cell death induced by Eve-Rh2 treatment was clarified. As shown in Fig. 2A and B, Eve-Rh2 induced caspasess-independent cell death and did not trigger cell apoptosis (Supporting Information Fig. S4A). Bafilomycin A1 and chloroquine, which are autophagy inhibitors, did not reverse Eve-Rh2-induced cell death, even though the expression of LC3II was up-regulated by the Eve-Rh2 treatment (Fig. S4B–S4D). These data suggest that Eve-Rh2 caused neither apoptosis nor autophagic cell death. Then the IncuCyte S3 Live-Cell Analysis System was used to monitor the cell morphology regularly after Eve-Rh2 treatment (Fig. 2C). The formation of vacuoles was observed in cell cytosol after Eve-Rh2 treatment for 2 h. Increased cells vacuolized and ultimately died in a manner similar to balloon explosion. After Eve-Rh2...
treatment for 6 h, the rough ER lesions were diffused, and the ER lumens were significantly expanded and vacuolated (Fig. 2D). Cytoplasmic vacuolation and ER swelling are regarded as the characteristics of paraptosis, which can be reversed by protein synthesis inhibitors. In our study, both CHX (the translational inhibitor) and A-D (the transcriptional inhibitor) reversed the Eve-Rh2-mediated cell death (Fig. 2E). A similar phenomenon was also observed in A549 and HCC827 cells (Fig. 2H). Moreover, Eve-Rh2 reduced the expression level of Alix (Fig. 2I), serving as an inhibitory protein of paraptosis.

Although Rh2 and Eve monotreatments were reported to induce caspase-independent cell death in cancer cells, they did not induce cell death and cytoplasmic vacuolation alone in our tested cells (Fig. 1A and B), presumably because of the low concentration used here. In short, Eve-Rh2-induced paraptosis in lung cancer cells possess the following characteristics: independence of caspases activity, cytoplasmic vacuolation, antagonism by CHX or A-D, and down-regulation of Alix.

3.3. Eve-Rh2 mediates aggresomes accumulation and ER stress

The Eve-Rh2-induced paraptosis could be attenuated by the co-treatment with CHX or A-D, whereas Eve-Rh2 suppressed the de novo protein synthesis instead of increasing it (Supporting Information Fig. S5A). Moreover, the cap-dependent translation was suppressed by Eve and Eve-Rh2 treatments (Fig. S5B) owing to the inhibitory effect of Eve on the mTOR signaling pathway (Fig. S5C). Apparently, Eve-Rh2 induced paraptosis independent of the protein synthesis. Alix can interact with the endosomal sorting complex required for transport III (ESCRT-III), resulting in the regulation of protein sorting. Down-regulation of Alix may disturb the proteostasis and cause ER swelling by interfering with the function of the ESCRT-III. Further study showed that Eve-Rh2 decreased the protein level of Alix without reducing its transcription level (Supporting Information Fig. S6A and S6B). The overexpression of Alix delayed but did not reverse Eve-Rh2-mediated paraptosis (Fig. S6C–S6F). The proteasome inhibitor, MG-132, reversed the Eve-Rh2-decreased Alix protein.
level (Fig. S6C) but dramatically enhanced the cytotoxicity of Eve-Rh2 treatment. Eve-Rh2 with MG-132 pretreatment induced significant cell death within 6 h (Fig. 3A and B, Supporting Information Fig. S7), indicating that Alix played a marginal role in the Eve-Rh2-induced paraptosis. Besides, Eve-Rh2 increased the level of ubiquitin (Fig. 3C). Eve-Rh2 caused the
accumulation of protein aggresomes similar with the positive control MG-132 (Fig. 3D), and the phenomenon could be reversed by co-treatment of CHX or A-D (Supporting Information Fig. S8A). These results suggest that Eve-Rh2 induced paraptosis by increasing the accumulation of protein aggresomes. It is well known that MG-132 induces aggresomes accumulation through proteasome inhibition. However, Eve-Rh2 displayed almost no inhibitory effect on the proteasomes (Fig. 3E), implying that Eve-Rh2 induced aggresomes accumulation through a different mechanism. The Gene Ontology analysis suggested that ER stress or unfolded protein response was the main biological process affected by Eve-Rh2 treatment (Fig. 3F), which was highly correlated with the protein aggregation.

Figure 3  Eve-Rh2 caused the accumulation of protein aggresomes in lung cancer cells. Cells were pretreated with MG-132 (5 μmol/L) and Eve (10 μmol/L) for 1 h and treated with Rh2 (10 μmol/L). (A) Cell viability was determined using MTT assay after treatment for 6 h. Data represent mean ± SD, n = 3; **P < 0.01. (B) Cell morphology was imaged after treatment for 6 h (scale bar = 100 μm). (C) Expression level of ubiquitin was detected using Western blot after treatment with Rh2 (10 μmol/L), Eve (10 μmol/L), or Eve-Rh2 for 6 h in NCI-H1975 cells. (D) Proteostat Aggresome Detection Kit was used to detect the aggresomes in NCI-H1975 and HCC827 cells after Eve-Rh2 or MG-132 (5 μmol/L) treatment for 6 h. (E) Activity of proteasome was detected using the Proteasome-Glo chymotrypsin-like cell based assay in NCI-H1975 cells after treatment with Rh2 (10 μmol/L), Eve (10 μmol/L), Eve-Rh2, or MG-132 (5 μmol/L) for 6 h. Data are shown as mean ± SD, n = 3; **P < 0.01 between the tagged group and ctrl group. (F) Gene Ontology analysis was performed on the RNA-Seq data to enrich biological processes after Eve-Rh2 treatment for 6 h. (G) Expression levels of genes related to ER stress were displayed using a heatmap. The color depth represented the expression level. (H) Protein levels of ER stress-related factors were detected using Western blot in NCI-H1975 cells after treatment with Rh2 (10 μmol/L), Eve (10 μmol/L), or Eve-Rh2 for 6 h.

3.4  Eve-Rh2 induces the accumulation of P62\(^{\text{+}}\) aggresomes leading to paraptosis

Protein aggresomes can be ubiquitinated and recruited to P62 for clearance. Without increased expression of P62 (Fig. 4A and B), Eve-Rh2 triggered the colocalization of P62\(^{\text{+}}\) puncta (green) with aggresomes (red) in cancer cells (Fig. 4C). By contrast, P62 was scattered in untreated cells, which had minimal aggresomes. After Eve-Rh2 treatment, the level of P62 in the Triton-X100-insoluble fraction increased, whereas that in the Triton-X100-soluble fraction decreased (Fig. 4D). These data suggest that Eve-Rh2 induced the accumulation of P62\(^{\text{+}}\) aggresomes by altering the function...
and/or the soluble-insoluble form of P62. Thereafter, knockdown of P62 interfered with the accumulation of insoluble P62 (Fig. 4E). As shown in Fig. 4F, the interference with P62 accumulation partially reversed Eve-Rh2-induced ER stress, protein aggregation, cell death, and cytoplasmic vacuolation, indicating that the Eve-Rh2-mediated accumulation of P62+ aggresomes triggered paraptosis.

3.5. TRIB3 mediates the Eve-Rh2-induced accumulation of P62+ aggresomes

Generally, P62 binds to LC3 in the membranes of autophagosome, thereby mediating the clearance of P62 cargo, containing protein aggresomes43. However, the interaction between TRIB3 and P62 leads to the accumulation of P62, and the autophagy-lysosome-dependent degradation is attenuated44. The expression level of TRIB3 was significantly increased after Eve-Rh2 treatment, as indicated in the RNA-Seq results (data not shown). Here, Eve-Rh2 increased the protein and mRNA levels of TRIB3 (Fig. 5A and B). TRIB3 and aggresomes were colocalized in the cytosol of Eve-Rh2-treated cells (Fig. 5C). The levels of soluble and insoluble TRIB3 significantly increased after the Eve-Rh2 treatment (Fig. 5D). Interference with the accumulation of insoluble P62 suppressed Eve-Rh2-mediated increase of insoluble TRIB3 (Fig. 5E), and knockdown of TRIB3 reversed Eve-Rh2-induced insoluble P62 accumulation, ER stress, and protein aggregation (Fig. 5F–H). These results suggest that the interaction between TRIB3 and P62 mediated the Eve-Rh2-induced aggresomes accumulation. Furthermore, the knockdown of TRIB3 inhibited Eve-Rh2-caused cell death and cytoplasmic vacuolation (Fig. 5I and J), indicating that the TRIB3-mediated accumulation of aggresomes triggered Eve-Rh2-induced paraptosis.

3.6. c-MYC mediates the accumulation of TRIB3/P62+ aggresomes via up-regulating the TRIB3 expression

The IPA was performed on the RNA-Seq data to determine the upstream regulators of TRIB3/P62+ aggresomes accumulation in response to Eve-Rh2 treatment. The top 20 upstream regulators were sorted using the P value. Ten transcription regulators, including NUPR1, ATF4, JUN, NFKB1, MYC, HIC1, XBP1, EGR1, KLF6 and RELB, were enriched (Fig. 6A). The over-expression of MYC is usually associated with poor prognosis45, whereas cells with the higher expression of inducible MYC seem
more sensitive to the Eve-Rh2 treatment (data not shown). Some studies have reported that c-MYC mediates apoptosis in the absence of growth-stimulating signals. However, whether c-MYC is involved in the regulatory network of paraptosis remains unclear. The IPA network analysis suggested that MYC might be an important modulator of the Eve-Rh2 treatment (Fig. 6B). Eve-Rh2 up-regulated the protein and mRNA levels of c-MYC (Fig. 6C and D), and the increased c-MYC located in the nucleus (Fig. 6E). Moreover, the expression level of c-MYC was increased after Eve-Rh2 treatment in tumor tissues, and this finding was consistent with the results of in vitro experiments (Fig. 6F). Silencing of c-MYC inhibited the Eve-Rh2-provoked insoluble P62 accumulation, ER stress, protein aggregation, cell death, and cytoplasmic vacuolation (Fig. 6G–K). Knockdown of c-MYC also suppressed the Eve-Rh2-up-regulated TRIB3 expression level (Fig. 6L and M), whereas knockdown of TRIB3 did not affect the up-regulation of c-MYC after the Eve-Rh2 treatment (Fig. 6N), indicating that c-MYC was the upstream regulator of TRIB3. These results demonstrate that the Eve-Rh2-induced c-MYC up-regulation mediated the accumulation of TRIB3/P62\(^+\) aggregates by increasing the transcriptional level of TRIB3 and consequently triggered paraptosis.

### 3.7. Disruption of c-MYC/MAX interaction enhances Eve-Rh2-induced paraptosis

The pMYC-TA-luc plasmid containing several reported c-MYC binding sites (E-box element) was used to determine the classical transcriptional activity of c-MYC after Eve-Rh2 treatment. As shown in Fig. 7A, Eve-Rh2 did not increase the relative luciferase activity of c-MYC, indicating that the E-box binding sites-dependent transcriptional activity of c-MYC was constant after Eve-Rh2 treatment. The c-MYC/MAX interaction inhibitor, 10058-F4, did not reverse the cell death induced by Eve-Rh2 treatment. Instead, 10058-F4 enhanced Eve-Rh2-induced cell death and cytoplasmic vacuolation (Fig. 7B and C), and it exaggerated the Eve-Rh2-mediated accumulation of insoluble TRIB3 and P62 as well as the up-regulation of the TRIB3 mRNA level (Fig. 7D and E). 10074-G5, another inhibitor of c-MYC/MAX interaction, enhanced the Eve-Rh2-induced cytoplasmic vacuolation (Fig. 7F). Eve-Rh2 did not affect the expression level of MAX (Fig. 7G). Knockdown of MAX also enhanced the Eve-Rh2-induced cytoplasmic vacuolation (Fig. 7H and I), which was similar to the phenomenon of Eve-Rh2 plus 10058-F4 or Eve-Rh2 plus 10074-G5 co-treatment. These results indicated that c-MYC
Figure 6  c-MYC mediated the accumulation of TRIB3/P62+ aggresomes by up-regulating the TRIB3 expression. (A) Enriched upstream regulators with different expression levels in Ingenuity Pathway Analysis (IPA) were sorted using the P value (Eve-Rh2 vs. Ctrl). The top 20 regulators were identified, and 10 transcription regulators (green dots) and the other factors (blue dots) were pointed out. (B) Results of IPA network analysis (Eve-Rh2 vs. Ctrl). After Rh2 (10 μmol/L), Eve (10 μmol/L), or Eve-Rh2 treatment for 6 h, the expression level of c-MYC was determined using (C) Western blot and (D) qRT-PCR in NCI-H1975 cells. Data represent mean ± SD, n = 3; *P < 0.05 and **P < 0.01 between the tagged group and ctrl group. (E) Localization of c-MYC was detected using the immunofluorescence after Eve-Rh2 treatment for 6 h. (F) Expression level of c-MYC in tumor tissues was detected using immunohistochemical staining. (G) After the silencing of c-MYC, cells were treated with Eve-Rh2. (H) Protein levels of insoluble and soluble P62, BIP and CHOP were determined using Western blot after 6 h of Eve-Rh2 treatment. (I) Aggresomes accumulation was detected using the immunofluorescence after cells were transfected with c-MYC siRNA and treated with Eve-Rh2 for 6 h. (J) Cell viability was detected using MTT assay after Eve-Rh2 treatment for 24 h in cells with silenced c-MYC. Data represent mean ± SD, n = 3; **P < 0.01. Expression level of c-MYC was determined using (L) qRT-PCR and (M) Western blot in NCI-H1975 cells with silenced c-MYC and Eve-Rh2 treatment for 6 h. Data represent mean ± SD, n = 3; *P < 0.05. (N) Protein level of c-MYC was determined using Western blot in NCI-H1975 cells with silenced TRIB3 and Eve-Rh2 treatment for 6 h.
mediated the TRIB3 up-regulation and triggered the Eve-Rh2-induced paraptosis through an unclassical c-MYC regulatory network.

4. Discussion

On the grounds of The Cancer Genome Atlas Database, the mTOR pathway is highly active in patients with lung cancer. mTOR is an essential molecule that regulates cell growth, metabolism and survival and a downstream regulator of the EGFR signaling pathway. In addition, the hyperactivated mTOR pathway is a feature of KRAS mutant lung adenocarcinoma after chemotherapy. In this study, we have demonstrated that the combination of Eve (the mTOR inhibitor) and Rh2 exhibits significant anti-lung cancer effect in vitro and in vivo, not only in EGFR-mutant but also KRAS-mutant lung cancer cells. Eve-Rh2 is more effective in inhibiting tumor growth compared with either Eve monotherapy or Rh2 monotherapy. Moreover, about 30% of the cells exhibit vacuolated morphology, indicating the occurrence of paraptosis.
mice in the Eve group suffer from mild hepatic fat accumulation, which is one of the major risk factors of nonalcoholic fatty liver disease\(^{45}\). In contrary, the mice in the Eve-Rh2 group have no evident liver lesion, indicating that Rh2 attenuates Eve-caused hepatic fat accumulation. Chronic mTOR complex 1 inhibition may lead to hepatic steatosis through persistent induction of the gluconeogenic program in liver\(^{51\text{-}53}\). The hepatic steatosis and hepatotoxicity of Eve has been reported in the clinical case, clinical trials and preclinical study with certain incidence, which may be correlated to Eve-induced glucose and insulin intolerance\(^{27\text{-}29\text{,}54}\). Rh2 has been reported to decrease the plasma glucose\(^{49\text{-}56}\). In our preliminary study, Rh2 reduced Eve-increased fasting blood glucose level (Supporting Information Fig. S9), suggesting that Rh2 attenuated Eve-caused hepatic fat accumulation might partially through improving the glucose metabolism. Rh2 is the main component of ginseng, which is a tonic and has a long history of clinical application. Rh2 enhances the anti-lung cancer effect of Eve and attenuates its adverse effects, suggesting that Eve-Rh2 is a potential anti-lung cancer candidate and showing the feasibility of incorporating natural products into clinical cancer treatment.

Further study demonstrates that Eve-Rh2 increases the expression level of c-MYC in the process of triggering lung cancer cell death. The sensitivity to Eve-Rh2 treatment seems to be related to the inducible expression level of c-MYC, implying that tumors with highly inducible expressed c-MYC is more likely to benefit from the Eve-Rh2 treatment. As a renowned oncogene, MYC is often activated in various cancers, including lung cancer, breast cancer, colon cancer and glioblastomas. Generally, c-MYC functions as a transcription factor, which forms a heterodimer with MAX through a basic-helix-loop-helix leucine-zipper motif and binds to the E-box elements of targeted genes, thereby mediating multiple cellular processes, including cell growth, proliferation, loss of differentiation and apoptosis\(^{57}\). In this study, c-MYC mediates the up-regulation of the TRIB3 mRNA level, leading to the aggresomes accumulation-triggered paraptosis. However, the classical transcriptional activity of c-MYC, which relies on c-MYC/MAX interaction, is not enhanced by the Eve-Rh2 treatment. The inhibitors of c-MYC/MAX interaction and the knockdown of MAX strengthen the Eve-Rh2-induced paraptosis. Therefore, c-MYC up-regulates the TRIB3 transcription level and triggers the Eve-Rh2-induced paraptosis in a new way. The c-MYC/MAX-dependent transcription and tumor malignant development are not activated by the Eve-Rh2-elevated c-MYC when Eve-Rh2 is used for lung cancer treatment. Moreover, the inhibitors of c-MYC/MAX interaction can be added in the regimen of the Eve-Rh2 treatment to further enhance the anti-lung cancer effect of Eve-Rh2. This study provides a treatment strategy for lung cancer, as well as the other cancers with highly inducible expressed MYC, through the overactivation of the MYC oncogene.

Paraptosis is first described as a non-apoptotic form of programmed cell death in 2000\(^{48}\), and is characterized by cytoplasmic vacuolation as well as caspase independence and can be reversed by de novo protein synthesis inhibitors\(^{15}\). The inhibitory effect of protein synthesis inhibitors is often used to determine paraptosis, whereas seldom research has proven the explicit increase in new protein synthesis after treatment with paraptosis inducers. In this study, CHX and A-D reverse the paraptotic morphology induced by the Eve-Rh2 treatment, but Eve-Rh2 does not enhance protein synthesis. Meanwhile, Eve-Rh2 does not affect the proteasome activity, even though the proteasome inhibition is reported to trigger paraptosis\(^{55\text{-}61}\). Eve-Rh2 causes protein accumulation through mediating the TRIB3/P62\(^{42}\) aggresomes formation, which blocks the clearance of the existing unfolded or misfolded proteins, thereby leading to ER stress and ultimately triggering paraptosis. Thus, increased protein synthesis, proteasome inhibition, or aggresomes accumulation eventually leads to protein accumulation, which may be the true executioner of paraptosis.

Furthermore, CHX and A-D suppress the up-regulation of c-MYC and TRIB3 induced by the Eve-Rh2 treatment (Fig. S8B), suggesting that CHX and A-D reverse the Eve-Rh2-induced paraptosis presumably by blocking the up-regulation of c-MYC. Conceivably, c-MYC is the core molecule that mediates the Eve-Rh2-induced paraptosis. As shown in Fig. 7A, Eve-Rh2 does not affect the c-MYC/MAX-dependent transcription, indicating that the elevated c-MYC may collaborate with another uncertain cofactor to induce paraptosis, but the cofactor remains unclear. The two c-MYC inhibitors used in this study (i.e., 10058-F4 and 10074-G5) bind to the basic-helix-loop-helix leucine-zipper domain of c-MYC (the domain to which MAX binds)\(^{42}\), which enhances the anti-tumor effect of Eve-Rh2. This finding indicates that the inhibitors block the interaction between c-MYC and MAX while do not affect the binding of c-MYC to the cofactor. Therefore, the binding site of the cofactor and c-MYC is presumably different from that of MAX, and the dissociative c-MYC may actively collaborate with this cofactor. Moreover, 10058-F4 alone up-regulated the expression level of TRIB3 and induced the accumulation of insoluble TRIB3 and P62 (Fig. 7D and E), further implying that mediation of TRIB3 expression through collaborating with the cofactor is a novel inherent function of c-MYC. Several cofactors are reported to combine with c-MYC and mediate cellular processes, such as MIZ-1, IN1 and HSFI\(^{46}\). HSFI is a pivotal cofactor of c-MYC in tumorigenesis and positively correlated with the c-MYC expression in hepatocellular carcinoma\(^{46}\). Moreover, HSFI exhibited the potential in regulating TRIB3 expression\(^{46}\), implying that HSFI may be involved in the Eve-Rh2-induced paraptosis. The exact cofactor, which cooperates with c-MYC and up-regulates the TRIB3 expression, thereby mediating the accumulation of TRIB3/P62\(^{47}\) aggresomes and eventually triggering paraptosis (Fig. 7J), remains to be further explored.

5. Conclusions

Our study indicates for the first time that the protein accumulation is the true executioner of paraptosis. We demonstrate that c-MYC triggers paraptosis by mediating the TRIB3/P62\(^{42}\) aggresomes accumulation in an uncanny way. Importantly, dual therapy combining Rh2 with Eve attenuates the hepatic fat accumulation caused by Eve and enhances its anti-cancer effect, shedding new light for the treatment of lung cancer.

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Author contributions

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Conflicts of interest

The authors declare no conflict of interest, financial or otherwise.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.09.014.

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