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Resveratrol Oligomers Trans-gnetin H Promotes Reactive Oxygen Species Generation and Rictor Degradation to Suppress Cancer Cell Viability

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The function of resveratrol as an antioxidant is to scavenge reactive oxygen species (ROS) in the body. The effects of resveratrol on cellular activity have been widely reported. Trans-gnetin H is a trimer of resveratrol that is found in seeds of peonies. However, there is not enough research on the cellular effects of trans-gnetin H, and it is not clear if it has the same physiological functions as resveratrol. This study tries to answer this question by investigating the effect of trans-gnetin H on ROS, mammalian target of rapamycin (mTOR), c ell viability, autophagy, and ferroptosis in several cancer cell lines. Trans-gnetin H was found to regulate cell viability, cell proliferation, and autophagy, but it did not affect ferroptosis. Molecular experiments showed that these effects were brought about via significant promotion of ROS generation and suppression of mTOR activation in an ROS-dependent manner. Further mechanistic experiments showed that trans-gnetin H inhibits mTOR activation by inducing FBXW7-mediated ubiquitination and degradation of Rictor, a key component of mTORC2 . Thus, trans-gnetin H appears to control tumor cell viability by inhibiting activation of the mTORC1 and mTORC2 pathways through a novel regulatory mechanism involving the ROS-Rictor signaling axis.

1.Introduction

Reactive oxygen species (ROS) are commonly found in the form of molecular oxygen derivatives such as O_2- and H_2O_2. There are many sources of intracellular ROS, which can be generated in the plasma membrane, cytoplasm, and organelles (including the endoplasmic reticulum, mitochondria, and peroxisomes)[1]. ROS production and ROS elimination need to be balanced for the maintenance of the intracellular redox state and several cell-, organ-, and organism-level physiological processes such as formation of the extracellular matrix, wound healing, and immunity[2-4]. ROS accumulation can lead to various diseases, including cancer, by inducing oxidative damage to proteins, DNA, and lipids. Interestingly, excessive ROS levels may also cause tumor cell death. Therefore, the target of numerous chemotherapeutic methods for the treatment o f tumors is ROS -induced cell death. As a result, there is considerable ongoing research about the mechanisms associated with ROS-induced cell death and related treatment targets that could be applied in cancer therapy.

Resveratrol is a commonly found polyphenolic compound with known antioxidant, anti-inflammatory, and immunomodulatory properties, among other benefits. Resveratrol has a broad spectrum of anti-tumor effects, showing significant anti-tumor activity in a variety of tumor cells. It can inhibit the growth, proliferation, invasion and metastasis of cancer cells through a variety of signaling pathways and regulate the expression of related proteins, induce cell apoptosis, and reverse chemotherapy resistance and enhance chemotherapy sensitivity. Trans-gnetin H[5] is a natural polyphenolic compound that is structurally composed of a trimer of resveratrol and one of the most important stilbenes in peony seeds. There is a large body of literature on t-he antitumor effects and mechanisms of resveratrol, but studies on the antitumor activity and mec-hanisms of trans-gnetin H ar -e few. In our previous study, we reported the antitumor potent -ial of trans-gnetin H[6] and found that trans-gnetin H controls tu -mor cell growth through regulation of autophagy[5]. In line with this observation, it is known that resveratrol, the compound th-at forms trans-gnetin H, plays a role in the initiation of autopha-gy[7-9]. In additi on, the antioxidant activity of resveratrol is one of its main functions[10-13], and it is also known to be involved in the induction of apoptosis[9, 14, 15] and cell necrosis[16]. In contras -t to these findings for resveratrol, the results of our previous st -udy indicated that trans-gnetin H did not play a role in regulati-ng apoptosis[5]. Further, it is not clear whether trans-gnetin H ha -s antioxidant capacity. Thus, although trans-gnetin H is compos -ed of resveratrol, it does not possess all the characteristics of r-esveratrol.

In our previous study, we also discovered that trans-gnetin H regulates autophagy via the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway. mTOR is an atypical s erine/threonine protein kinase that integrates a variety of ex tracellular signals, including amino acids, glucose, lipids, growth factors, and ROS, and is essential for autophagy[17]. It is primarily found in two intracellular complexes—mTORC1 an d mTORC2—which are sensitive and respond to different signals (including ROS) and, thus, exert different functions[18]. In our previous study, even though we discovered that trans- gnetin H can regulate the mTORC1 pathway, it was unclear w hether it affects the mTORC2 pathway. In the present study, we have tried to gain a better understanding of the antitumor activity and mechanisms of trans-gnetin H by investigating its effects on

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ROS levels and the mTOR pathway in several cancer cell lines. We believe that these findings will shed light on the functions of trans-gnetin H and the underlying autophagy-related mechanisms in tumor cells and also help identify potential therapeutic targets for antitumor treatment.

2. Materials and methods

2.1 Materials

Erastin (E7781), RSL3 (SML2234), cycloheximide (CHX), N-acetylcysteine (NAC, A7250), dichlorofluorescein diacetate (DCF H-DA, 35845), and secondary antibodies were obtained from Sigma-Aldrich (MO, USA). Antibodies for pT389-S6K (9234S/ L) , S6K (9202S), Rictor (9476), pS473-AKT (9271), AKT (9272), and SOD1 (37385) were obtained from Cell Signaling Technology (MA, USA). Actin (20536-1-AP), FBXW7α (28424-1-AP), Nrf2 (16396-1-AP), and HMOX1 (10701-1-AP) were obtained from Proteintech (Chicago, USA). NQO1 (ab80588) was obtained from Abcam (Cambridge, UK). β-mercaptoethanol, penicillin, fetal bovine serum (FBS) and streptomycin were purchased from Gibco (Grand Island, NY, USA). Phosphate-buffered sa line (PBS) and trypsin were purchased from HyClone (UT, USA). Trizol reagent, the PrimeScript RT reagent kit (RR047A), and the TB Green quantitative real-time quantitative polymerase chain reaction (qRT-PCR) kit (RR820A) were purchased fr om Takara (Dalian, China). Cell counting kit-8 (CCK8, K009) w as purchased from ZETA LIFE (CA, USA). BODIPY 581/591 C11 (D3861) was purchased from Thermo Fisher Scientific (MA, USA).

2.2 Origin and purity of trans-gnetin H

Trans-gnetin H (purity, >99%) was obtained from the seeds of th -e tree peony. For the cellular experiments, trans-gnetin H was dissolved in dimethyl sulfoxide to the required concentrations. The extraction and isolation of trans-gnetin H was conducted a-ccording to the published protocol[19]. Structural determination of isolated trans-gnetin H was elucidated by 1H - and 13C-NMR as below:

1H-NMR (500 MHz, CD3OD)δ (ppm): 7.19 [4H,dd, J = 2.0, 8.5 Hz, H-2(6), 2″ (6″)], 6.79 [4H, dd, J = 2.0,8.5 Hz, H-3(5), 3″ (5″)] , 6.69 (2H, d, J = 8.5 Hz, H-2′, 6′),6.51 (2H, d, J = 8.5 Hz, H-3′, 5 ′), 6.43 (1H, s, J = 8.5 Hz, H-12), 6.39 (2H, s, H-7′, 8′), 6.15 [6H, s, H-10(10″), 12(12″), 14(14″)], 5.41 (2H, d, J = 5.5 Hz, H-7, 7″), 4.41(2H, d, J = 5.5 Hz, H-8, 8″).

13C-NMR (125 MHz, CD3OD)δ (ppm):163.5 (C-11′, 13′), 160.6 [C-11(11″), 13(13″)], 159.0 (C-4, 4″), 158.8 (C-4′), 148.0 (C-9, 9″), 135.0 (C-9′), 134.6 (C-1″), 134.2 (C-1), 131.1 (C-1′), 129.14 (C-2′, 6′,8′), 128.6 [C-2(2″), 6(6″)], 123.0 (C-7′), 120.8 (C-10′, 14′), 116.8 [C-3(3″), 5(5″)], 116.6 (C-3′, 5′), 107.8 [C-10(10″), 1 4(14″)], 102.6 (C-12, 12″), 95.3 (C-7, 7″), 91.9 (C-12′), 59.4 (C-8, 8″).

The 1H- and 13C-NMR of trans-gnetin H only used for review because our related work haven't been published. The purity of trans-gnetin H was confirmed using HPLC. Evidence is presented in the supplementary material of our previously published articles.

2.3 Cell culture

HT29, H1299, MDA231, and HepG2 cells were purchased fro -m National Science & Technology Infrastructure (NSTI, Sha-nghai, China) and cultured according to the manufacturer's protocol. HT29 and H1299 cells were cultured in RPMI 1640 medium (Hyclone, USA), MDA231 and HepG2 cells were cult -ured in Dulbecco's modified Eagle medium (DMEM; Hyclon -e, USA) containing 10% FBS (Gibco, Grand Island, NY, USA) according to the ATCC guidelines.

2.4 Sample collection and preparation for lip idomic analysis

HT29 cells were treated with 10 μM trans-gnetin H for 2 ho-urs. Cells from both control and trans-gnetin H-treated grou -ps were then harvested. Six replicate samples per group (e ach contains 1 ×107 cells precisely counted via Cytometer, B -i-oRad, Hercules, USA) were flash-frozen in liquid nitrogen and stored at -80°C for lipid extraction and LC-MS analysis. Li-pids were extracted following Matyash [20] with modificati -ons. Briefly, samples were thawed at 4°C before adding 20 0 μ-L ice-cold distilled water. Homogenization was performe -d with 240 μL ice-cold methanol, followed by vortexing. Aft -er adding 800 μL methyl tert-butyl ether (MTBE), samples were vortexed, sonicated in an ice-water bath for 20 min, th -en incubated at room temperature for 30 min. Following ce -ntri-fugation (14,000 × g, 15 min, 10°C), the upper organic phase was collected and dried under nitrogen. For LC-MS an -alysis, dried lipids were reconstituted in 200 μL isopropanol, vortexed, centrifuged (1 4,000 × g, 15 min, 10°C), and the s-upernatant collected. A pooled quality control (QC) sample was generated by combining equal aliquots from each lipid extract.

2.5 LC-MS/MS analysis for lipids

Lipidomic analysis was performed according to the methods [21,22]. Briefly, samples were maintained at 10°C in an autosa - mpler, with 3 μL aliquots injected onto a reverse-phase CS-H C18 column (Waters ACQUITY UPLC CSH C18; 1.7 μm, 2.1 × 1 00 mm) via a UPLC system (SHIMADZU, Japan). The mobi -le phase comprised Solvent A (acetonitrile–water, 6:4 v/v, 0.1% f ormic acid, 0.1 mM ammonium formate) and Solvent B (acetonitrile–isopropanol, 1:9 v/v, 0.1% formic acid, 0.1 m -M ammonium formate), with a gradient starting at 30% Sol -vent B (30 0 μL/min), held for 2 min, linearly increased to 1 00% Solvent B over 23 min, and equilibrated at 5 % Solvent B for 10 min. S ample order was randomized to mitigate inst rumentation bia s. Post-separation, mass spectrometry was conducted on a Q-Exactive Plus (Thermo Scientific, USA) wit -h shared paramete rs (heater 300°C, sheath gas 45 arb, aux -iliary gas 15 arb, swe ep gas 1 arb, capillary 350°C); For posi -tive (ESI+) used 3.0 kV spray voltage, S-Lens RF 50%, and M -S1 scan 200–1800 m/z, while for positive (ESI−) used 2.5 kV, S-Lens RF 60%, and MS2 scan 250–1800 m/z. Quality contro -l samples were analyzed at each batch start and after every 5 samples to ensure data stability.

Raw data were processed using LipidSearch™ Software (v4.1, T -hermo Fisher Scientific, CA, USA) for peak alignment, retentio -n time correction, and peak area quantification. Analytical me -thod validation was performed with key parameters set as foll -ows: precursor tolerance = 5 ppm, product tolerance = 5 ppm, and product ion threshold = 5%. Lipid species exhibiting >30% relative standard deviation (RSD) or >50% missing values were excluded from the LipidSearch-extracted dataset. Following no -rmalization and integration via Pareto scaling, processed data were subjected to principal component analysis (PCA) using SI-MCA-P® software (v14.1, Umetrics, Umea, Sweden). Identified metabolites were annotated against the KEG G pathway datab -ase (https://www.genome.jp/kegg/pathway. html). Volcano p -lots were subsequently generated using R software.

2.6 siRNA knockdown

Non-specific control siRNA and siRNAs for FBXW7α were purch ased from GenePharma (Shanghai, China). Transfection of cells with the siRNA oligonucleotides was performed using Lipofec-tamine 2000 (Invitrogen, CA, USA) according to the manufact-urer's instructions. The siRNAs used are listed here:

si NC：5'-UUCUCCGAACGUGUCACGU-3'

si FBXW7α-1：5'-GCTCCCTAAAGAGTTGGCACTCTAT-3'

si FBXW7α-2：5'-ACAGGACAGUGUUUACAAATT-3'

2.7 Western blot analysis

The western blotting protocol used has been described by us p -reviously [23]. It can be briefly summarized as follows: the cells were washed with PBS, lysed with radio immune precipitation assay buffer for 30 min, and centrifuged at 12,000 rpm for 15 min (4°C). The supernatant was subjected to sodium dodecyl s -ulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for sepa -ration of proteins, and the proteins were transferred to nitroc -ellulose (NC) membranes (0.45 μm, GE). The NC membranes were then blocked with non-fat milk and incubated with prima -ry antibodies overnight at 4°C. Next, the membranes were wa -shed with PBS and incubated with secondary anti bodies (cont -aining horseradish peroxidase) for 1–2 h. Following this, the membranes were washed with PBST, and the target proteins w -ere detected with an imaging system from Bio- Rad (Hercules, CA, USA). Protein abundance was quantified using the ImageJ software.

2.8 Quantitative qRT-PCR analysis

Total RNA was isolated using TRIzol reagent and reverse transc -ribed with the PrimerScript RT reagent kit. qRT-PCR analysis w -as performed with the TB Green qRT-PCR kit. The comparativ-e Ct method was used to determine the relative quantity of th-e mRNA of the target gene, with the GAPDH gene as the intern -al control. The sequences of the primer pairs used are shown i -n Table 1.

Table 1 Primer sequences for qRT-PCR

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
HMOX1	TGCTCAACATCCAGCTCTTTGA	AACTGTCGCCACCAGAAAGC
SOD1	ACAAAGATGGTGTGGCCGAT	AACGACTTCCAGCGTTTCCT
NQO1	GAAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGG
Rictor	CGAGTACGAGGGCGGAAT	ATCTGGCCACATTTTGGAGA
GAPDH	CAACGAATTTGGCTACAGCA	AGGGGTCTACATGGCAACTG

2.9 Detection of lipid ROS

Cells were seeded in 6-well plates or 24-well plates and were treated with trans-gnetin H for 24 h. The cells were then inc-ubated with DCF-DA or 5 μM BODIPY C11 at 37°C for 30 min. The cells in the 6-well plates were washed, and fluorescence intensity was detected by flow cytometry (BD FACSAria III, US-A).

2.10 Cell viability assay

For the determination of cell viability, cells were seeded in 6-well plates at a density of 10^4 cells/well and incubated with complete RPMI 1640 medium containing reagents. The me-dium was replaced with 100μL fresh medium containing 10% CCK8 reagent, and this was followed by incubation for 3 h at 37°C. The plate was then read with a Synergy HT microplate reader (Bio-Tek, USA) (absorbance, 450 nm).

2.11 Colony formation assay

Cells were seeded in 6-well plates at a density of 1000–2000 cells/well and treated with different combinations of regents. This was followed by culture in RPMI-1640 medium containing 10% FBS for 7 days. The colonies were then fixed with 4% paraformaldehyde, washed with PBS, and stained with crystal violet. All the assays were performed in triplicate.

2.12 Autophagy analysis

Autophagy was detected using a previously described proced-ure[24]. Briefly, GFP-LC3 plasmids were transfected into cells, which were then treated with trans-gnetin H. The cells were fixed with 4% paraformaldehyde for 30 min and 0.1 M NH4Cl for 10 min and subsequently treated with 0.1% TritonX-100 and incubated with bovine serum albumin. Next, DAPI staining was performed, and the number of GFP-LC3 puncta was counted with a laser confocal microscope.

2.13 Dead cell staining assay

The cytotoxicity of trans-gnetin H was determined using the dea -d cell staining assay test kit by treating cells with predetermine -d concentrations of trans-gnetin H for 24 h. Following this, the supernatant was discarded, and the cells was incubated with a working solution of propidium iodide (PI, 8 μM) for 30 min in th -e dark. The stained cells were then observed and imaged with a fluorescence microscope (OLYMPUS CKX53, Japan).

2.14 Ferroptosis analysis

Cells were grown in 96-well plates until they reached 50%–60% confluence and exposed to different concentrations of RSL 3 or erastin (for induction of ferroptosis), in combination with the in -dicated concentrations of trans-gnetin H. The effect of trans-gn -etin H on ferroptosis was determined by assessing cell viability with the CCK8 kit in combination with the Synergy HT microplat -e reader (Bio-Tek, USA).

2.15 Transmission electron microscopy

H1299 cell pellets were fixed in 2.5% electron microscopy-grade glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) at 4° C overnight. Cell sections were prepared by dehydration, embe- dding, and curing of the specimens, followed by 50-nm ultrathin section preparation and staining with uranyl acetate and lead ci -trate. These procedures followed previously described protocol -s[25] . The ultrathin specimens were examine d, and images wer e acquired with a transmission electron microscope (HT7800, Ja -pan).

2.16 Statistical analysis

The data distribution was assessed using the Shapiro-Wilk test i -n GraphPad Prism 9.0 for all datasets intended for parametric t -esting. Homogeneity of variances across groups was tested usi- ng Levene's test for all datasets intended for analysis via one-w- ay or two-way analysis of variance (ANOVA).Data were shown a -s mean ± SEM. Statistical tests included unpaired one-tailed or two-tailed Student's t-test and one-way or two- way analysis of variance. p value 0.05 was considered statistically significant. In the graphed data *, ** and *** denote p values of < 0.05, 0.01 and 0.001, respectively, ns, not significant.

3. Results

3.1 Trans-gnetin H promotes intracellular RO S generation

We first confirmed its growth-inhibitory effects in these models. Treatment dose-dependently reduced proliferation (Figure 1A, B), with peak efficacy at 10 μM. To dissect the anti-proliferative mechanism, we performed untargeted lipidomics in HT29 cells (10 μM, 2h). PCoA revealed profound metabolic reprogramming (Figure 1C), while volcano plots identified 395 dysregulated meta bolites (171 upregulated; 406 downregulated;Figure 1D). KEGG e -nrichment highlighted glutathione metabolism upregulation (Fi- gure 1E), suggesting oxidative stress.

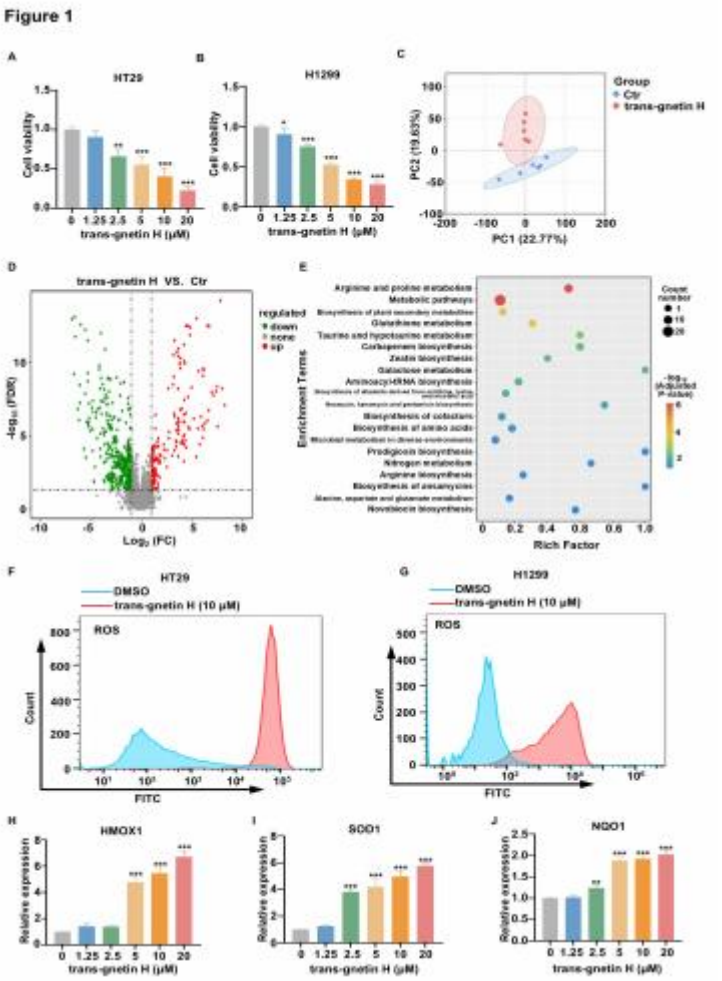

Figure 1. Trans-gnetin H promotes intracellular ROS generation. (A,B) HT29 (A) and H1299 (B) cells were treated with different concentrations of trans-gnetin H for 72 h, the viability of cells was detected by CCK8, n = 3, the number of technical or biological replicates performed. (C-E) Following 2h treatment with 10μM trans- gnetin H, HT29cells from treated and control groups (n=6 per group, equal cell numbers) were collected. Lipids were extracted from each group and analyzed using untargeted liquid chromatography-mass spectrometry (LC-MS) for lipid species identification. (C) Principal component analysis (PCA) score plots distinguish the trans -gnetin H and control groups. (D) The volcano plot reveals 171 significantly upregulated and 406 significantly do wnregulated metabolites. (E) KEGG pathway analysis was performed on these differential metabolites. (F, G) HT29 (F) and H1299 (G) cells were treated with trans-gnetin H (10μM) for 2h, and the ROS levels were detected by FACS. (H-J) HT29 cells were trea ted with different concentrations of trans -gnetin H for 2h, and the expression of HM OX1 (H), SOD1 (I), and NQO1 (J) were detected by qRT-PCR. Data were analyzed by on e-way ANOVA (A, B, H-J). *p < 0.05, **p < 0.01, ***p< 0.001.

Notably, despite structural similarity to the ROS antagonist resveratrol, trans-gnetin H exhibited opposing redox activity, prompting our hypothesis of ROS-dependent growth inhibition. Validation studies showed trans-gnetin H significantly increased intracellular ROS in both H1299 and HT29 cells(Figure 1F, G). This

pro-oxidant effect extended to breast (Figure S1A) and hepatocellular carcinoma (Figure S1B) models, suggesting potential broad-spectrum ROS induction across the colon, lung, breast, and hepa tocellular carcinoma. Critically, trans-gnetin H concentration-dependently upregulated antioxidant genes (HMOX1, SOD1, NQO1) , confirming ROS-mediated stress adaptation.

3.2 Trans-gnetin H upregulates ROS-associated proteins to suppress mTOR activation

Building upon initial findings, we assessed trans-gnetin H's impact on ROS-regulatory proteins. Treatment concentration-dependently upregulated both the redox sensor Nrf2 and its effector enzymes HMOX1/SOD1/ NQO1 at the protein level (Figure 2A & Figure S2A-D), aligning with prior transcriptional data.

To establish ROS-dependence of Nrf2 induction, we employed ROS scavenger N-acetylcysteine(NAC). NAC co-treatment abolished Nrf2 upregulation (Figure 2B & Figure S2B) and attenuated trans-gnetin H-induced expression of HMOX1/SOD1/ NQO1 mRN A (Figure 2C-E), with parallel effects on corresponding proteins (Figure 2B& Figure S2E-H).Thus, trans-gnetin H amplifies oxidative stress through coordinated Nrf2 pathway activation.

Given established mTORC1 modulation, we examined mTORC2 components. Trans-gnetin H dose-dependently reduced Rictor (mTORC2 core subunit) expression (Figure 2F, G) while suppressing mTORC2-mediated AKT Ser473 phosphorylation[26] and mTORC1-dependent S6K phosphorylation (Figure 2F, H & Figure S2I-L). Critically, NAC reversed all mTOR-related effects (Figure 2H & Figure S2K, L). Functionally, the mTORC1 activator MHY1485 rescued viability suppression in colony assays (Figure 2I, J). These data establish ROS-mediated dysregulation of both mTOR complexes by trans-gnetin H.

Figure 2

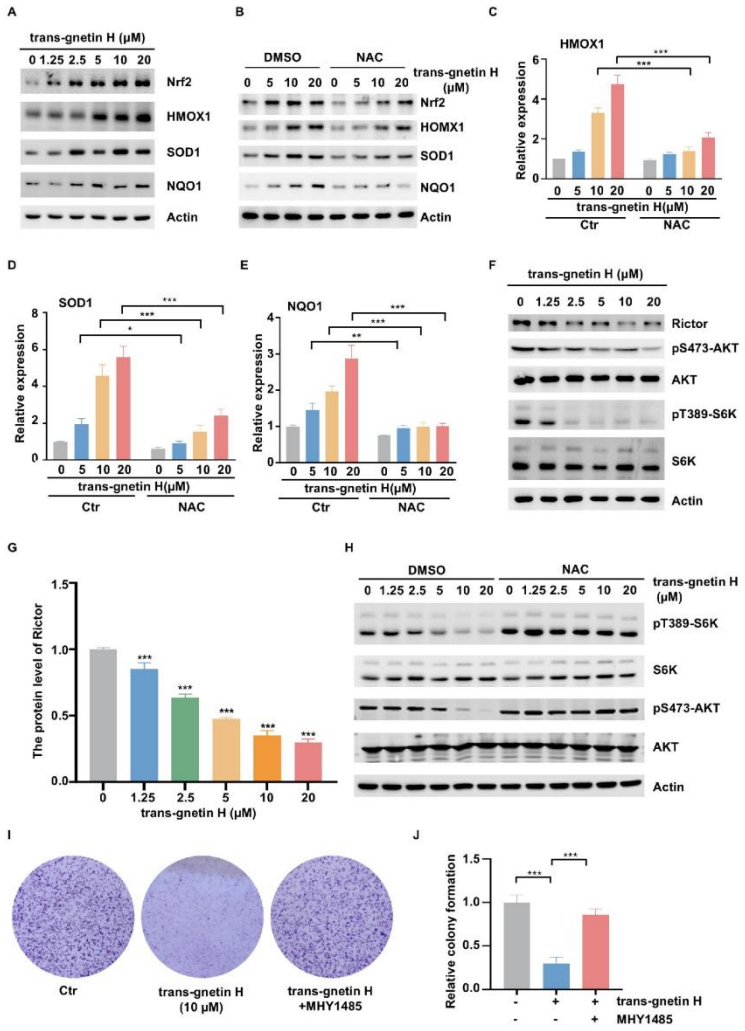

Figure 2. Trans-gnetin H upregulates ROS-associated proteins to suppress mTOR activation. (A) H1299 cells were treated with different concentrations of trans-gnetin H for 2h, and the indicated proteins were evaluated by western blot. (B) H1299 cells were treated with different concentrations of trans-gnetin H or NAC (2 mM) for 2h, and the indicated proteins were evaluated by western blot. (C-E) H1299 cells were treated with different concentrations of trans-gnetin H or NAC (2 mM) for 2h, and then the expression of HMOX1 (C), SOD1 (D), and NQO1 (E) were detected by qRT-PCR. (F, G) HT29 cells were treated with different concentrations of trans-gnetin H for 2h, and the indicated proteins were evaluated by western blot (F), quantitative for Rictor (G) is presented. (H) H1299 cells were treated with different concentrations of trans-gnetin H or NAC (2mM) for 2h, and the indicated proteins were evaluated by western blot. (I, J) HT29 cells were treated with trans-gnetin H (10 μM) or MHY1485 (10 nM), and the cell death was detected by colony formation assay (I), quantitative data are presented in (J). Data were analyzed by one-way ANOVA (G), two-way ANOVA (C-E, J). *p < 0.05, **p < 0.01, ***p< 0.001.

3.3 Trans-gnetin H promotes the degradation of Rictor via ROS

We next investigated the mechanism through which trans-gnetin H mediates the decrease in Rictor. However, we found that trans-gnetin H does not regulate Rictor expression at the transcriptional level (Figure 3A). To determine whether trans-gnetin H regulates Rictor at the post-translational level, we treated cells with CHX, an inhibitor of mRNA translation, and found that trans-gnetin H significantly shortens the half-life of Rictor (Figure 3B, C). Numerous previous studies have shown that the half-life of proteins is mainly regulated by ubiquitination[27-29]. Accordingly, our results showed that trans-gnetin H could significantly promote the ubiquitination of Rictor. Moreover, we found that the promotive effect of trans-gnetin H on Rictor degradation couldbe blocked by NAC treatment (Figure 3D, E), and the addition of NAC inhibited trans-gnetin-mediated ubiquitination of Rictor (Figure 3F).

It has been shown that ubiquitination of Rictor is mainly regulated by FBXW7α[30]. Therefore, we transfected cells with two siRNAs that specifically target FBXW7α and found that knockdown of FBXW7α blocked trans-gnetin H-mediated Rictor degradation (Figure 3G, H). These results indicate that trans-gnetin H facilitates FBXW7α-mediated ubiquitination of Rictor via ROS, which in turn, promotes Rictor degradation.

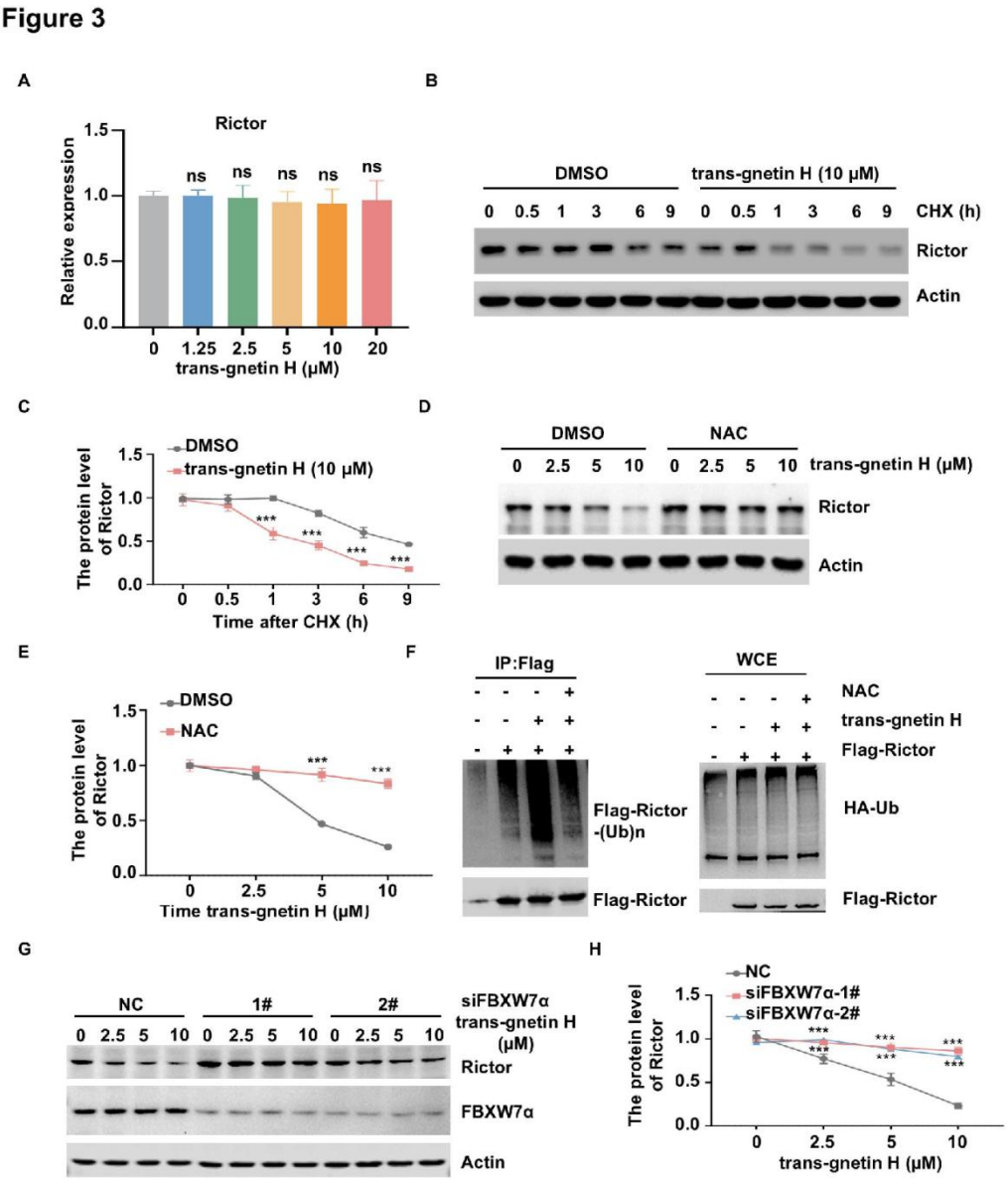

Figure 3. Trans-gnetin H promotes the degradation of Rictor via ROS. (A) HT29 cells were treated with different concentrations of trans-gnetin H for 2h, and the mRNA of Rictor were evaluated by qRT-PCR. (B,C) HT29 cells were treated with trans-gnetin H (10μM) or CHX (25mg/μL) for indicated time, and the indicated proteins were evaluated by western blot (B), quantitative data for Rictor are presented in (C). (D, E) HT29 cells were treated with different concentrations of trans-gnetin H or NAC (2mM) for 2h, and the indicated proteins were evaluated by western blot (D), quantitative data for Rictor are presented in (E). (F) HT29 cells were treated with trans-gnetin H (10μM) or NAC (2mM) for 2h, and the Rictor ubiquitination was analyzed by western blot. (G, H) FBXW7α-knockdown HT29 cells were treated with different concentrations of trans-gnetin H for 2h, and the indicated proteins were evaluated by western blot (G), quantitative data for Rictor are presented in (H). Data were analyzed by one-way ANOVA (A), two-way ANOVA (C, E, H). n = 3, the number of independent experiments performed. *p < 0.05, **p < 0.01, ***p< 0.001.

3.4 Trans-gnetin H does not affect cell ferroptosis

To determine whether trans-gnetin H can regulate cell ferroptosis, we investigated the regulatory effects of trans-gnetin H on lipid ROS production and mitochondrial morphology. Our results demonstrated that trans-gnetin H had no influence on intracellular lipid ROS generation (Figure 4A, B), even though it significantly promoted ROS generation. Further, trans-gnetin H did not affect mitochondrial morphological characteristics either (Figure 4C). In accordance with these results, trans-gnetin H did not play a role in sensitivity to erastin (a known inducer of cell ferroptosis) and RSL3-induced ferroptosis (Figure 4D, E). These results suggest that trans-gnetin H is not involved in cell ferroptosis.

Figure 4

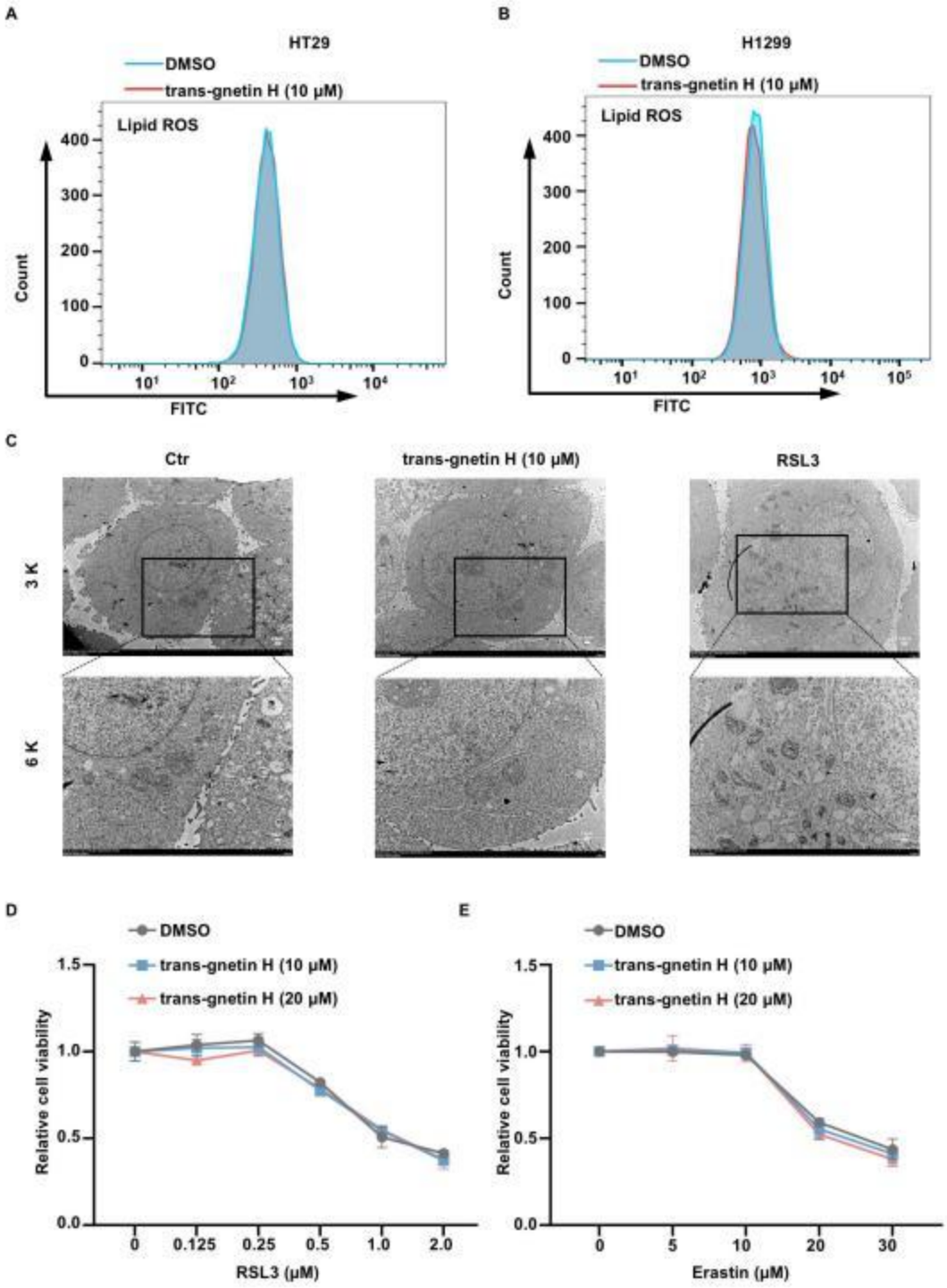

Figure 4. Trans-gnetin H does not affect cell ferroptosis. (A,B) HT29(A) and H1299(B) cells were treated with the trans-gnetin H (10μM) for 2h, and the lipid ROS was analyzed by FACS. (C) H1299 cells were treated with the trans-gnetin H (10μM) or RSL3 (μM) for 12h, and analyze the ultrastructure of mitochondria with transmission electron microscopy. (D,E) H1299 cells were treated with different concentrations of trans-gnetin H, RSL3(D) for 24h or erastin(E) for 24h, and the viability of indicated cells was examined using CCK8.

3.5 Trans-gnetin H regulates cell viability via ROS

The results of our PI staining experiments on H1299 cells demonstrated that trans-gnetin H significantly inhibited cell growth, a-nd this effect could be blocked by NAC (Figure 5A). Consistent results were obtained with the CCK8 assay (Figure 5B). Moreover, we found that knockdown of FBXW7α blocked the inhibitory effect of trans-gnetin H on cell viability (Figure 5C). In our previous study, we found that trans-gnetin H could regulate cellular autophagy[5] . Consistent with the previous findings, trans-gnetin H demonstrated the ability to significantly promote autophagy (Figure 5D, E). Further, the addition of NAC could block trans-gn-etin H-induced autophagy (Figure 5D, E), and knockdown of FBXW7α inhibited the induction of autophagy by trans-gnetin H (Figure 5F, G). Thus, these results suggest that trans-gnetin H has the ability to inhibit cell viability and induce autophagy through pathways that involve ROS and FBXW7α (which mediates ubiquitination of Rictor).

Figure 5

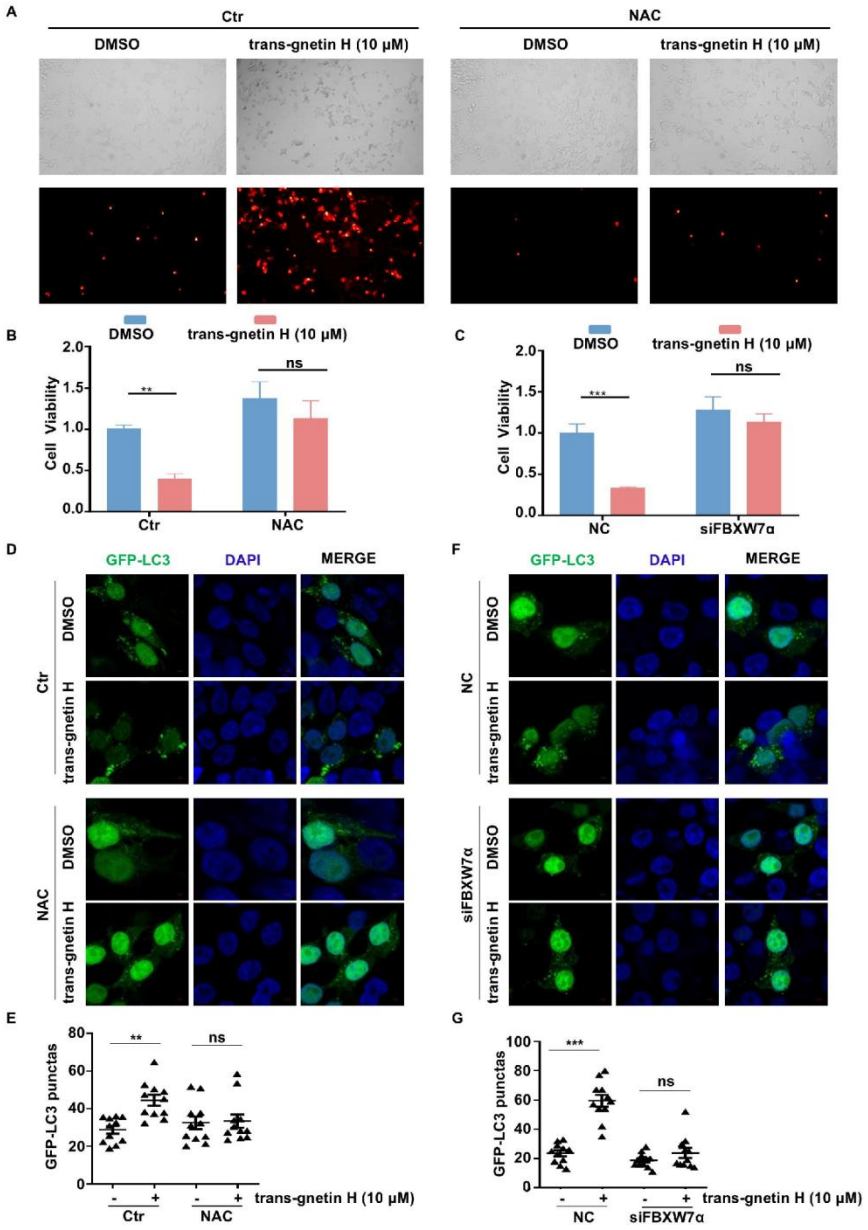

Figure 5. Trans-gnetin H regulates cell viability via ROS. (A) H1299 cells were treated with the trans-gnetin H (10μM) for 2h, and the dead cell was detected by staining assay. (B) HT29 cells were treated with the trans-gnetin H (1 μM) or NAC for 2h, and the cell viability was detected by CCK8. (C) FBXW7α-knockdown HT29 cells were treated with trans-gnetin H for 2h, and the cell viability was detected by CCK8. (D,E) HT29 cells were treated with the trans-gnetin H (10μM) or NAC (2mM) for 2h, and autophagy was detected by GFP-LC3 puncta (D), quantitative data for GFP-LC3 puncta are presented in (E). (F,G) FBXW7α-knockdown HT29 cells were treated with trans-gnetin H (10μM) for 2h, and autopha-gy was detected by GFP-LC3 puncta (F), quantitative data for GFP-LC3 puncta are presented in (G). n = 3, the number of technical or biological replicates performed. *p < 0.05, **p < 0.01, ***p< 0.001.

4. Discussion

In the present study, we provide confirmatory evidence for t he effect of trans-gnetin H on the cell viability of cancer cells. Further, we demonstrate that the underlying mechanisms in volve the inhibition of mTORC1 and mTORC2 activity via the ROS-Rictor signaling axis. Specifically, our results show that trans-gnetin H-induced ROS can regulate mTORC1 activity through the FBXW7 α-Rictor-mTORC2-AKT signaling axis: that is, ROS can enhance FBXW7 α-mediated ubiquitination and degradation of Rictor, which in turn inhibits mTORC2 and mTORC1 activity. This is a novel mechanism of trans-gnetin H that has not been reported so far, so the findings have significant implications in terms of the future therapeutic potential of trans-gnetin H.

As trans-gnetin H is a trimer of resveratrol, it seems logical that it would have stronger scavenging ability than resveratrol. In co-ntrast, we found that trans-gnetin H promotes the production of cellular ROS and enhances the expression of the Nrf 2 protein, which is an important effector protein that plays a role in the re-sponse to ROS and is an important indicator of intracellular RO S changes[31] . In addition, we also found the trans-gnetin H upregulates the expression of the downstream Nrf2 antioxidant proteins HMOX1, SOD1, and NQO1. Further, when the cells were pretreated with NAC before exposure to trans-gnetin H, this effect was inhibited. This suggests that contrary to the previously r-eported ROS clearance function of resveratrol, trans-gnetin H s-ignificantly promotes intracellular ROS production while enhan-cing intracellular antioxidant protein expression.Thus, although trans-gnetin H is composed of resveratrol, it does not possess a-ll the characteristics of resveratrol.

Resveratrol trimers exhibit a broad spectrum of bioactivities att-ributed to their structural diversity. Miyabenol C demonstrates notable multi-target pharmacological activities: inducing anti-pr-oliferative and apoptotic effects in tumor cells; inhibiting prote-in kinase C (PKC); antagonizing the human serotonin (5-HT) rec-eptor; mediating estrogen-like effects via estrogen receptor bi-nding; and exhibiting antagonistic activity toward ecdysone in D-rosophila cell models. Significantly, Miyabenol C has been iden-tified as a potent inhibitor of β-secretase (B ACE1). It effectively suppresses the pathological production o f β-amyloid (Aβ) in bo-th in vitro and in vivo model systems, strongly suggesting its th-erapeutic potential for central nervous system neurodegenerat-ive disorders such as Alzheimer's di sease[32]. Further research r-evealed that the antitumor effects of another trimer, pauciflor-ol B, are primarily mediated through activation of the p53 signal-ing pathway, thereby regulating cellular apoptosis and senesce-nce[33]. In contrast, the trimer α-viniferin displays selective anti-bacterial activity, exhibiting potent inhibition against Staphyloc-occus aureus and Escherichia coli, while demonstrating compar-atively weaker activity against Salmonella Paratyphi[34]. These e-xamples highlight the functional heterogeneity among resverat-rol oligomers and underscore the unique ROS-promoting and mTOR-inhibitory properties of trans-gnetin H identified in our st-udy.

In our next set of experiments, we tried to elucidate the potential mechanism underlying intracellular ROS production in-duced by trans-gnetin H. First, we tried to detect changes in the function of the four complexes in the mitochondrial respiratory chain, but we found that trans-gnetin H did not affe-ct this mechanism of ROS production. Studies have shown t-hat lipid overload-induced toxicity and insulin resistance is o-ne of the mechanisms that drives ROS production, and cholesterol and oxidized sterols can also cause mitochondrial dys-function and ROS production[35]. However, our non-targeted lipidomics analysis of trans-gnetin H-treated cells showed th-at there was no increase in cholesterol and fatty acids after trans-gnetin H treatment. Thus, the mitochondrial and lipid overload mechanisms of intracellular ROS production were r-uled out as the ROS induction pathways of trans-gnetin H.

We next analyzed the molecular structure of trans-gnetin H and found a large number of hydroxyl groups (-OH). Based on this observation, we speculated that hydroxyl radicals released from trans-gnetin H may account for the increase in ROS observed in cells treated with trans-gnetin H. In addition, we analyzed the phenotype at the cellular level, and our results showed that the expression of superoxide dismutase and cata lase was significantly inhibited by trans-gnetin H. Superoxide dismutase binds to the superoxide by-products of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. Thus, changes in these enzymes may also be an important factor in the promotion of ROS production by trans-gnetin H.

In our previous studies, trans-gnetin H was found to inhibit mTORC1 activity by activating the AMPK pathway[5]. In this st-udy, we also wanted to explore whether trans-gnetin H has an effect on mTORC2. Our results show that trans-gnetin H si-gnificantly suppressed the phosphorylation of AKT at Ser47-3, which is a conserved site for mTORC2-mediated phosphor-ylation on AKT[26]. Thus, our results suggest that trans-gnetin H c an also significantly inhibit mTORC2 activity. We wante-d to learn more about how trans-gnetin H performs this fun-ction. Our previous study showed that vitamin C-induced R-OS regulated mTORC2 activation by promoting the degradati-on of Rictor[36], which is an important component protein of mTORC2[37]. Building upon these findings, we further investi-gated the effect of trans-gnetin H on Rictor. Our data demo-nstrate that trans-gnetin H inhibits mTORC2 activity by induc-ing intracellular ROS generation, which activates the ubiquit-in ligase FBX W7α . This activation triggers ubiquitination an-d subsequent proteasomal degradation of the mTORC2 com-ponent Rictor. Notably, although trans-gnetin H potently in-duced FBXW7α- mediated Rictor ubiquitination and degrada-tion, it failed to enhance the FBXW7α-Rictor physical intera-ction in co-immun oprecipitation assays (Figure S3A). This fin-ding suggests that trans-gnetin H/ ROS facilitates Rictor ubi-quitination through mechanisms independent of strengtheni-ng the FBXW7α-Rict or binding affinity. While this dissociati-on between binding affinity and degradation efficiency warr-ants further investigati on, it is well-established that mTORC 2 regulates mTORC1 activity via AKT phosphorylation at Ser-473. This established link aligns with our prior observation of trans-gnetin H-mediated mTORC1 inhibition. Therefore, coll-ectively, our findings indicate that trans-gnetin H suppresse

-s mTORC1 activity through dual pathways: AMPK activation an d mTORC2 inhibition.

Further supporting this proposed mechanism, our supplementar -y experiments revealed that trans-gnetin H significantly upregu -lates FBXW7 protein expression (Figure S3B). This finding indica -tes that trans-gnetin H not only facilitates FBXW7-m ediated ub -iquitination and degradation of Rictor but may also potentiate FBXW7's regulatory effect on Rictor by directly enhancing FBXW -7 expression. Within cellular systems, FBXW7 exists as three dis -tinct isoforms: the α isoform localizes predominantly to the nu-cleus, the β isoform exhibits cytoplasmic distribution, and the γ isoform primarily resides within the nucleolus[38]. Given this bro -ad subcellular distribution of FBXW7, examining only its subcell -ular localization may be insufficient to elucidate the precise me -chanism by which trans-gne tin H or ROS regulate FBXW7-medi -ated Rictor degradation. It is extensively documented that GSK 3β-mediated phosphoryl ation of Rictor triggers FBXW7-depend -ent degradation[30]. A lthough this study did not directly assess FBXW7 phosphorylation status, our functional analyses demons trate that trans-gnetin H-induced ROS promotes Rictor degradat -ion specifically through FBXW7α—highlighting a central finding of this work. Therefore, post-translational modifications (PTMs) of FBX W7α, such as phosphorylation, represent a plausible reg-ulatory mechanism through which ROS may modulate its activit-y, constituting a significant direction for future investigations.

Autophagy is a highly conserved self-degrading system induced primarily by nutritional deprivation or stress[35] . It contributes t -o the maintenance of cellular homeostasis by promoting the de -gradation of intracellular protein aggregates, organelles, and ot -her macromolecules[39]. Abnormalities in autophagy may induc-e the initiation phase of tumorigenesis. In the later stages of tu-mor development, autophagy is essential for obtaining nutrient-s and dealing with the unfavorable tumor microenvironment[40]. Our previous study showed that trans-gnetin H can promote aut -ophagy via inhibition of the mTORC1-TFEB signaling axis to facil -itate the localization of TFEB within the nucleus[5]. TFEB belong -s to a class of transcription factors that have been reported to r -egulate lysosomal biogenesis and autophagic gene expression[4 1]. In the present study, we have expanded on the related mech-anisms by reporting that trans-gnetin H-promoted autophagy is highly dependent on ROS. Further, we found that the promotive effect of ROS on autophagy was brough about via suppression of the mTOR pathway. By combining our present findings with our previous findings, we can deduce that trans-gnetin H promo -tes autophagy through the mTOR/AMPK/ ROS axis. In the futur -e, it would be interesting to explore the mechanisms involved a -s a way of identifying potential treatment targets.

Elevated ROS is an important feature of cell ferroptosis, and lipi -d ROS and mitochondrial crinkling represent the gold standard for detecting cell ferroptosis[42]. In this study, we tried to find ev -idence that trans-gnetin H regulates ferroptosis in cells. As sho-wn in Figure 4, although trans-gnetin H did result in a significant increase in the generation of intracellular ROS and had a broad spectrum of other effects, it did not induce the production of in -tracellular lipid ROS (elevated lipid ROS is an important conditi-on for ferroptosis). In addition, our experimental results show t-hat trans-gnetin H has no obvious destructive effect on the mito -chondria in the cell. Importantly, our ferroptosis assay showed that trans-gnetin H did not cause ferroptosis in cells. In line with the present findings, in our previous study, too, we ruled ou -t the ability of trans-gnetin H to cause apoptosis. In summar -y, we believe that the production of intracellular ROS induc-ed by trans-gnetin H inhibits the activity of mTOR and activat -es autophagy, similar to our previous study.

In this paragraph, we will highlight some of the questions th-at are still to be answered in the future and some limitations of our study. The selection of cancer cell lines in this study w -as strategically aligned with global cancer burden data. The four cell lines investigated—NCI-H1299 (lung cancer), HT29 (colorectal cancer), HepG2 (liver cancer), and MDA-MB-231 (breast cancer)—represent malignancies ranked highest in gl-obal incidence and mortality: lung cancer ranks first in morta -lity, colorectal cancer third in incidence, liver cancer second in mortality, and breast cancer first in incidence[43]. These ca -ncers collectively span major physiological systems (respirat ory, digestive, and reproductive), enhancing the translationa -l relevance of our findings across diverse human cancers.Ho -wever, it is important to note that while our initial screenin-g included MDA-MB-231 and HepG2 cells, our subsequent de -tailed mechanistic investigations focused primarily on H129 9 and HT29models. Consequently, the generalizability of the specific ROS-Rictor-mTOR signaling axis identified in this stu-dy to breast cancer (MDA-MB-231) and liver cancer (HepG2) cells remains to be fully characterized and constitutes a limit -ation of this work. Additionally, the experiments were cond-ucted at the cellular level and need to be verified through in vivo studies in the future, especially for evaluating the side e -ffects of trans-gnetin H.our cellular models inherently overl-ook tumor microenvironment complexity and interpatient h-eterogeneity within each cancer type. For instance, while M DA-MB-231 represents triple-negative breast cancer, other molecular subtypes (e.g., HER2+, ER+) may respond different ly[44]. Similarly, the reliance on single cell lines per cancer typ -e limits extrapolati on to intratumoral genomic diversity. Fu -ture studies incorporating primary patient-derived cells, co-culture systems, and in vivo models will be essential to confi-rm pathophysiological relevance. At the level of mechanism analysis, our previous study showed that mTORC1 can be inh -ibited by trans-gnetin H[5], and in our present study, trans-gnetin H was found to have the ability to inhibit both mTORC 1 and mTORC2 activity via ROS, as well as the viability of the cancer cell lines examine d. While these findings indicate the potential anti-tumor effects of trans-gnetin H, it is not clear which upstream signaling molecule of mTOR is targeted by tr -ans-gnetin H-induced ROS. Current studies on the upstream signaling molecules of mTORC1 focus on amino acids, growt -h factors, and glucose[45], so they might shed light on the po -tential targets of trans-gnetin H-induced ROS. In the future, this line of investigation might help identify therapeutic targ-ets for cancer treatment. In our previous study, we also foun -d that trans-gnetin H inhibited mTORC1 activity through AM PK[5], but in this study, we did not investigate the relationshi-p between ROS and the AMPK pathway. Thus, it is not clear whether ROS can regulate mTOR through the AMPK pathway, or whether the regulation of the ROS-FBXW7α-Rictor axis is dependent on AMPK signaling. Another limitation was that al -though we found that ROS can promote FBXW7α-mediated ubiquitination and degradation of Rictor, we did not clarify t-he mechanism of ROS regulation of FBXW7α . Thus, whether ROS promotes the expression of FBXW7α or regulates its sta-

bility is a topic that warrants investigating. Another shortcoming of the present study is that we have not explored whether trans-gnetin H can cause pyroptosis and necrotic apoptosis, which is a topic we hope to explore in subsequent studies.

In summary, the present findings reveal that trans-gnetin H inhibits tumor cell viability through the following pathway : trans-gnetin H-induced increase in ROS production leads to FB XW7α-mediated ubiquitination degradation of Rictor; this further leads to inhibition of mTORC2 activity that, in turn, inhibits mTORC1 activity via inhibition of AKT phosphorylation. With regard to the underlying mechanism, we were able to add to the literature by demonstrating that trans-gnetin H can regulate cell viability and autophagy through its effects on ROS and FBXW7α .

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Conflict of interest statement:

The authors declare that the y have no conflict of interest.

Data Availability Statement:

The data that suppo rt the findings ofthis study are available from the corresponding author upon reas onable request.

Ethical statement:

We promise that the results of this study will be reported in an honest, objective, and responsible manner without any modifica tion or manipulation that may mislead the reader or compromise the integrity oft he study.

SUPPORTING INFORMATION

Additional supplementary information is available for downloa-d and review in the supplementary information section located on the right-hand side of this article's HTML page.

Figure S1.
Figure S2.
Figure S3.