学位論文

The role of YAP1 in small cell lung cancer
（肺小細胞癌における YAP1 の役割）

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1. Introduction

Lung cancer is the leading worldwide cause of cancer-related deaths [1]. It is histologically classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma [2]. ~~SCLC is an aggressive disease that accounts for approximately 14% of all lung cancers.~~ SCLC accounts for about 14% of all lung cancers and is a highly invasive disease. Despite numerous clinical trials, systemic ~~treatments~~ therapy for patients with SCLC have not significantly changed ~~in~~ over the past several decades [3]. SCLC is categorized as a subtype of neuroendocrine (NE) tumors; however, immunohistochemistry (IHC) for NE markers is not mandatory for its diagnosis [2]. ~~A previous study reported that~~ In s previous report, all NE markers may be negative in a subset of morphologically diagnosed SCLC [4]. Rudin et al. recently proposed a nomenclature to describe SCLC subtypes based on the dominant master regulator expressed, namely, achaete–scute complex homolog 1 (ASCL1), neurogenic differentiation factor 1, yes-associated protein 1 (YAP1), and POU class 2 homeobox 3, and classified the subtypes as SCLC-A, SCLC-N, SCLC-Y, and SCLC-P, respectively [5].

The Hippo pathway was originally identified as an evolutionarily-conserved signaling system that contributes to the ~~control~~ regulation of organ size [6]. This signaling pathway ~~was recently identified as a master regulator of the malignant progression of many cancers through its effects on cell proliferation and stem/progenitor cell expansion~~ has recently been shown to be a master regulator of

malignant transformation in many cancers, affecting cell proliferation and the growth of stem and progenitor cells [7]. YAP and transcriptional coactivator with PDZ-binding motif (TAZ, also known as WWTR1) are core downstream effectors of the Hippo pathway, which is involved in diverse biological processes [8]. Previous studies revealed that ~~the correlation between YAP and TAZ amplification and transcriptional activity is functionally relevant. After nuclear translocation,~~ YAP and TAZ mainly cooperate with the TEA domain family (TEAD1-TEAD4) of transcription factors to transactivate genes that regulate cell proliferation, migration, and epithelial–mesenchymal transition (EMT) [9].

The oncogenic effects of YAP and TAZ in NSCLC have recently been reported. The overexpression of YAP1 has frequently been observed in NSCLC, and is regarded as a poor prognostic factor [10]. The proliferation and migration of human lung NSCLC cell lines *in vitro* ~~was~~ were shown to depend on YAP [11]. ~~In SCLC, Horie et al. identified a unique SCLC subgroup characterized by the relatively high expression of YAP and TAZ. This subgroup displayed an adherent cell morphology and lower expression of ASCL1 and NE markers, which appeared to belong to SCLC-Y [5][12]. It was also revealed that YAP1-negative cases were more chemosensitive than YAP1-positive cases; therefore, YAP1 is a useful marker for stratifying SCLC into chemosensitive and chemoresistant groups [12].~~ However, the roles of YAP and TAZ in SCLC remain unclear. Therefore, the main aim of the present study was to elucidate the biological significance of YAP1 in SCLC. We examined YAP1 expression

in surgically resected SCLC tissues using IHC and in various human lung cell lines by Western blotting (WB) analysis. To modify YAP1 expression in SCLC cell lines, we used plasmid DNA transfection and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9). After an RNA sequence analysis of YAP1-knockout (KO) cells, various functional analyses were performed. YAP1-KO cells were implanted into immunocompromised mice, and the role of YAP1 in the growth and NE differentiation of tumors was investigated. We also performed a drug sensitivity analysis to reveal whether YAP1 contributes to drug resistance.

2. Materials and Methods

2.1. Cell lines

Seven SCLC cell lines (H69, H889, SBC1, H69AR, H1688, SBC3, and SBC5), three ADC cell lines (A549, H358, and H1975), and three SCC cell lines (H226, H2170, and H15) were used in the present study. H69, H889, H69AR, H1688, A549, H358, H1975, H226, and H2170 were purchased from ATCC (Manassas, VA, USA), and SBC1, SBC3, and SBC5 from the Japan Collection of Research Bioresources Cell Bank (Osaka, Japan). H15 was a generous gift from Dr. M. Suzuki (Department of Thoracic Surgery. Kumamoto University).

2.2. WB analysis

Cultured cells were prepared for the WB analysis, as previously described [12]. A list of the primary antibodies used is shown in Table 1. Membranes were washed and incubated with the respective secondary antibodies conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA) for 1 hour, and the immune complex was visualized with a chemiluminescence substrate (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.3. Tissue samples

Tissue samples of SCLC (n = 32) resected at the Department of Thoracic Surgery of Kumamoto University Hospital (Kumamoto, Japan) were used in the present study. Samples were histologically diagnosed according to the WHO criteria [2]. These sections were used for IHC. The present study followed the guidelines of the Ethics Committee of Kumamoto University (No.342).

2.4. IHC staining and evaluation

Formalin-fixed, paraffin-embedded specimens were cut into 4-μm-thick sections and mounted onto MAS-GP-coated slides (Matsunami Glass Ind., Osaka, Japan). After being deparaffinized and rehydrated, sections were heated in 0.01 mol/L citrate buffer (pH 7.0) using an autoclave for antigen retrieval. Sections were incubated with 0.3% H_2O_2 in absolute methanol for 15 min to block endogenous peroxidase activity. Sections were then incubated with skimmed milk for 30 min to block

non-specific staining. After this blocking step, sections were incubated with the primary antibodies (Table 1) at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer, Dako) and visualization with the Liquid DAB+Substrate Chromogen System (Dako). All slides were counterstained with hematoxylin for 30 s before being dehydrated and mounted. The specificity of the immunolabeling of each antibody was tested using normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (Santa Cruz Biotechnology). We evaluated IHC results based on staining intensity levels and the percentage of positively stained tumor cells. The percentage of positively stained tumor cells was divided into four groups: no staining, <5% of tumor cells are reactive, 5–25% reactive, and >50% reactive. Staining intensity levels were divided into three groups: negative, weak, and strong. We designed a table to allocate IHC scores to each specimen. IHC scores were classified into three groups: negative (0), weakly positive (1+), and positive (2+). We defined an IHC score of 2+ as significantly positive. Scoring was performed simultaneously by two independent researchers (H.S. and T.I.) who were blinded to patient details.

2.5. Transcriptome data of SCLC tissues

The transcriptome data of SCLC tissue samples from the GSE60052 (n = 79) RNA-sequence dataset [13] were used to examine the relationship between the expression levels of *YAP1* and insulinoma-

associated protein 1 (*INSM1)*.

2.6. YAP1-KO experiment using the H69AR and SBC5 cell line

Genome editing using CRISPR/Cas9 was used to KO the *YAP1* gene in the H69AR and SBC5 cell lines. pSpCas9(BB)-2A-Puro(px459) was obtained from Addgene (Cambridge, MA) [14]. The sgRNA target sequences of YAP1 were as follows: GCA CGA TCT GAT GCC CGG CG and GCA GCA GCC GCC GCC TCA AC. These plasmids were co-transfected with Lipofectamine 3000 (Thermo Fisher Scientific) into cells at subconfluency. After 48 hours, transfected cells were treated with 1 μg/mL puromycin (Clontech) for the selection of stably transfected cells.

2.7. Cell invasion assay

Mock-transfected H69AR cells and YAP1-KO H69AR cells were seeded at equivalent densities (5.0×10^5 cells/well) on 6-well plates. After 24, 48, 72, and 96 hours, cells were trypsinized and then counted. Experiments were repeated in triplicate to confirm reproducibility. Matrigel invasion assays were conducted in accordance with the standard protocol of the Corning Matrigel Invasion Chamber (Biocoat, Horsham, PA). Mock and YAP1-KO lines (5.0×10^4 cells) were plated onto each chamber in a serum-free growth medium. Chambers were placed in wells containing growth medium with 10% FBS. After a 24-hour incubation at 37°C in a CO_2 incubator, Matrigel membranes were collected and

stained with Diff-Quik (Sysmex, Hyogo, Japan). The numbers of stained cells in five randomly selected microscopic fields (×100) per filter were counted and the average number was calculated. Experiments were performed in triplicate.

2.8. Wound healing assay

Wound healing assay were conducted in accordance with the standard protocol of CytoSelect™ 24-Well Wound Healing Assay (Cell Biolabs, San Diego, CA). Mock and YAP1-KO cells were seeded at equivalent densities (1.0×10^6 cells/well) on 24-well plates. After cell suspension was added to the well with insert place, incubated overnight. The insert was removed from the well to begin the wound healing assay. The wounds were photographed with a light microscope at 0 and 48 hours after treatment. The numbers of migrated cells in five randomly selected microscopic fields (×200) per well were counted and the average number was calculated.

2.9. Cell counting assay

Mock and YAP1-KO cells were seeded at equivalent densities (5.0×10^5 cells/well) on 6-well plates. After 24, 48, 72, and 96 hours, cells were trypsinized and counted. Experiments were performed in triplicate to confirm reproducibility.

2.10. RNA sequence analysis

An RNA sequence analysis was performed by the Liaison Laboratory Research Promotion Center (LILA) (Kumamoto University) as follows. Total RNA was isolated from cultured cells, including ~~Mock~~ mock cells (p3×FLAG-CMV-14 and pSpCas9(BB)-2A-Puro(px459)) and YAP1-KO cells, using an RNeasy Mini Kit (Qiagen). All samples with an RNA integrity number >7.5 were used for sequencing. NextSeq 500 (Illumina, San Diego, CA) was used for analyses, the data were converted to Fastq files. Data quality control was performed by FastQC. Filtered reads were used to map to the UCSC hg19 genome reference genome using HISAT2 2.1.0. Fragments per kilobase of exon per million read~~s~~ mapped (FPKM) values were calculated using Cufflinks. Significant genes were extracted by cuffdiff (p <0.05).

2.11. GO analysis

GO annotation and classification were based on the following three categories: biological processes, molecular functions, and cellular components. The Database for Annotation, Visualization, and Integrated Discovery 6.8 (DAVID 6.8, https://david.ncifcrf.gov/) was used for the GO analysis [15]. The gene list contained significant genes in the RNA sequence analysis. The DAVID online database was used to visualize key molecular functions and biological processes. p <0.05 was used as the cut-off criterion for significance.

2.12. Tumor xenograft growth and histopathological evaluation

A total of 1.0×10^6 cells each of ~~Mock~~ mock and YAP1-KO cells were subcutaneously injected into the backs of $Rag2^{-/-}$:$Jak3^{-/-}$ mice (a generous gift from Prof. Seiji Okada Kumamoto University). Five animals were used in each group. Thirty days after the first injection, tumors were removed and measured. Samples were fixed with phosphate-buffered 4% paraformaldehyde solution and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE), and additional sections were used for IHC staining. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

2.13. Drug sensitivity analysis

H69, H69AR ~~Mock~~ mock, and H69AR YAP1-KO cells were treated with etoposide in triplicate. Cells (1.0×10^5 cells) were plated on the wells of 6-well culture plates. After 10 days, etoposide was added to the wells at the following final concentrations: 1, 2, 5, 10, 20, and 50 μM. Cells were removed using a treatment with trypsin/EDTA solution, stained with trypan blue, and then counted using the LUNA-II™ Automated Cell Counter (Logos Biosystems, Gyeonggi, South Korea). Results were plotted as cell viability versus $\log_{10}$ (concentration of reagents) and the IC_{50} value was calculated using the software GraphPad Prism v.7 (GraphPad Software, San Diego, CA).

2.14. Statistical analysis

All data were obtained from independent experiments and expressed as the mean ± SD of triplicate measurements. Differences in mean values between the two groups were statistically analyzed using the *t*-test. Prism v.7 software (GraphPad Software, San Diego, CA) was used for statistical analyses. $p < 0.05$ was considered to be significant.

3. Results

3.1. YAP1 is expressed at low levels in SCLC and negatively correlates with INSM1 expression

To examine YAP1 expression patterns, we performed a WB analysis of 13 lung cancer cell lines (7 SCLCs, 3 ADCs, and 3 SCCs). The results obtained revealed that the YAP1 protein was generally expressed at higher levels in NSCLC cell lines than in SCLC cell lines. ASCL1 is an important regulator of NE differentiation in lung cancer, and is specifically expressed in SCLC along with INSM1. The Notch1-Hes1 pathway represses NE differentiation by down-regulating the expression of NE-promoting transcription factors, such as ASCL1 and INSM1 [16]. YAP1 showed a similar expression pattern to NOTCH1 and HES1, and was mutually exclusive with NE markers, such ASCL1, INSM1, CHGA, and SYP (Fig. 1a). In the present study, the SCLC-A subtype cell lines H69, H889, SBC1, and H1688 were positive for ASCL1 and INSM1. In contrast, H69AR, ~~a~~ chemically induced

SCLC-Y subtype, and the SCLC-Y subtype cell lines ~~H69AR,~~ SBC3, and SBC5 were negative for ASCL1 and INSM1, but highly positive for YAP1. Thirty-two surgically resected SCLC tissues were immunohistochemically stained for YAP1 and INSM1. Typical expression patterns are shown (Fig. 1b); INSM1 was immunohistochemically positive in nuclei, and YAP1 in both nuclei and the cytoplasm. IHC revealed that INSM1 was expressed in most cases (28 out of 32 cases), while YAP1 was only expressed in four cases. All four cases were negative for INSM1 (Table 2). We confirmed that the percentages of YAP1 and INSM1 positivities were mutually exclusive. Two of the YAP1-positive patients had recurrences after surgical and medical treatment and died within two years of the first treatment. We also surveyed public datasets of gene expression profiles in human SCLC samples and evaluated their relationships. The RNA-seq dataset using the tumor samples of 79 SCLC patients confirmed the coordinated expression of YAP1 and INSM1 in human SCLC tissue samples (GSE60052: $\rho = -0.30681597$) (Fig. 1c) [13]. This result suggests that YAP1 negatively correlates with INSM1. We also found that YAP1 was positively correlated with NOTCH1 (GSE60052: $\rho = 0.289023181$) and WNT7B (GSE60052: $\rho = 0.241893866$) (Fig. S1). It was suggested that YAP1 could have crosstalk with Notch signaling and Wnt signaling.

3.2. YAP1 is a repressor gene in NE differentiation

To investigate the biological significance of YAP1 in SCLC cell lines, we performed the KO of the

YAP1 gene in H69AR cells, which is chemotherapy induced YAP1 positive SCLC cell lines ~~that are negative for ASCL1 and INSM1, but highly positive for YAP1~~. Besides, we performed *YAP1* gene knockout experiment in SBC5 cells, which are classified as the SCLC-Y subtype cell lines. We established H69AR, and SBC5 ~~Mock~~ mock cells using an empty vector as the control. The expression of CYR61, a downstream molecule of YAP1, was decreased in YAP1-KO cells of these cell lines (Figs. 2a, S2). Since the expression of NE markers, such as INSM1, SYP, and CHGA, was increased in YAP1-KO H69AR cells, these cells may be regarded as NE carcinoma cells (Figs. 2a, b). On the other hand, we found the increased expression of NOTCH1 and its downstream molecule HES1, both of which function in the suppression of NE (Fig. 2a). The YAP1-KO SBC5 cells expressed SYP (Fig. S2). We used ~~these~~ YAP1-KO H69AR cells in the RNA-seq analysis and compared expression by WB or heatmaps. In comparisons between ~~Mock~~ mock and YAP1-KO cells, the expression of the following genes ~~were~~ was found to be up-regulated: INSM1, ASCL1, CHGA, NCAM1, and SYN. These results suggest the importance of YAP1 in the suppression of NE (Fig. 2b). We used DAVID online bioinformatics resources 6.8 for the GO functional analysis and extracted the top ten enriched categories in biological processes and molecular functions. Biological processes comprise terms related to cell proliferation, such as cellular process, the positive regulation of biological regulation, and developmental process. Molecular functions include terms mainly associated with cell binding (Fig. 2c).

3.3. YAP1 regulates EMT in H69AR cells

A WB analysis of YAP1-KO cells was performed to investigate the role of YAP1 in EMT. In H69AR, reductions were detected in SLUG, VIMENTIN, and N-CADHERIN (Fig. 3a). In SBC5, SLUG was found to be decreased by *YAP1* gene KO (Fig. S2). These results suggest that YAP1 promoted EMT. To confirm this result, we performed invasion assays on ~~Mock~~ mock and YAP1-KO cells. The degree of cell invasion was markedly less in YAP1-KO cells than in ~~Mock~~ mock-cells (~~Mock~~ mock versus YAP1-KO, 39.4 ± 11.01 cell numbers/field versus 16.6 ± 9.61 cell numbers/field) (Fig. 3b). We also performed wound healing assay. The numbers of migrated cells was significantly lower in the YAP1-KO cells than mock (mock versus YAP1-KO, 120 ± 10.37 cell numbers/field versus 52.6 ± 7.23 cell numbers/field) (Fig. 3c).

3.4. YAP1 is involved in the proliferation of H69AR cells

To confirm the role of YAP1 in cell proliferation, we performed cell counting assays on ~~Mock~~ mock and YAP1-KO cells, and the results obtained revealed a reduction in H69AR YAP1-KO cell proliferation (Fig. 4a). In the WB analysis of YAP1-KO cells, no marked changes were observed in CASPASE 3, a molecule associated with apoptosis, whereas a decrease was detected in CYCLIN D1 (Fig. 4b). In further examinations, YAP1-KO and ~~Mock~~ mock cells were xenotransplanted into the

subcutaneous tissues of immunocompromised mice. Thirty days after xenotransplantation, tumors formed in subcutaneous tissues. These tumors were resected and measured. The size of xenotransplanted tumors from YAP1-KO cells was significantly smaller than those from ~~Mock~~ mock cells (Fig. 4c). A histological examination with H&E staining revealed no morphological differences between YAP1-KO and ~~Mock~~ mock cells. Using YAP1 and INSM1 IHC, the effects of the KO of YAP1 were reviewed in xenotransplanted tumors. Tumor tissues from YAP1-KO cells had lower expression levels of YAP1, but higher expression levels of INSM1. In addition, the Ki67-labeling index was higher in tumor tissues from YAP1-KO cells than those from ~~Mock~~ mock cells (Ki67-labeling index of tumors from mock-transfected versus YAP1-KO, 73. ± 4.12% versus 27.9 ± 3.71%) (Fig. 4d).

3.5. The KO of YAP1 induces drug sensitivity in H69AR cells

We examined the sensitivities of H69, H69AR, and H69AR YAP1-KO cell lines to etoposide. To assess IC_{50} values, cells were treated with increasing concentrations of etoposide from 1 to 50 μM. The sensitivity of each cell line to increasing doses of etoposide was demonstrated, with etoposide significantly inhibiting the proliferation of H69, H69AR, and YAP1-KO cells by 10 days. H69AR cells are a multidrug resistance variant of the SCLC cell line, H69 [17], and, thus, are more resistant to etoposide than H69 cells. The inhibition of YAP1-KO cell proliferation by etoposide at concentrations

between 10 and 50 μM was significantly greater ($p < 0.05$) than that of H69AR cells (Fig. 5a). Dose-response curves were generated and IC_{50} concentrations were calculated for the three cell lines (Fig. 5b). These results suggest that drug-resistant cell lines reacquire drug sensitivity with the KO of YAP1.

4. Discussion

Despite extensive research, the role of YAP1 in oncogenesis remains unclear because of the dual function of YAP1 as either an oncogene or tumor suppressor [18]. Recent studies reported the up-regulation of YAP1 in a number of human malignancies, such as breast cancer, melanoma, SCC, and lung cancer [19]. Furthermore, YAP1 has been reported to exert oncogenic effects on NSCLC [20]; However, its role in SCLC remains unclear ~~[12,13]~~ [21,22]. We herein demonstrated the importance of YAP1 in the NE differentiation, EMT, cell proliferation, and drug sensitivity of SCLC.

To elucidate the functional significance of YAP1 in SCLC, we performed the KO of YAP1 in the H69AR cell line. The WB analysis showed that NE markers, such as INSM1, SYP, and CHGA, were induced following the KO of the *YAP1* gene, and similar results were obtained in the RAN-seq analysis. Furthermore, IHC revealed that xenotransplanted tumors from YAP1-KO cells were completely negative for YAP1 and partially positive for INSM1. Ito et al. reported that VMRC-LCD, a YAP1-negative NSCLC cell line, showed the higher expression of NE markers at the protein level, suggesting a correlation between the loss of YAP1 and the expression of NE markers [22]. We generated a stable

H69AR YAP1-KO cell line that expresses NE markers ~~that were not originally expressed~~, suggesting that the SCLC-Y cell line may have phenotypically changed to SCLC-A. These results indicate that YAP1 is an important molecule in the heterogeneity of SCLC.

EMT, an important developmental process in cells, is evoked during tumor invasion and metastasis [23]. YAP1 has been shown to promote NSCLC tumorigenesis and metastasis by regulating the transcription of ~~Slug~~ SLUG; the direct target of YAP1/TEAD [24]. In the present study, we found that YAP1 was also associated with EMT and invasive capacity in SCLC, while its suppression exerted antitumor effects. Therefore, treatment strategies similar to those for NSCLC may be effective for tumors of the SCLC-Y subtype. It is possible that EMT is regulated in YAP1-positive SCLC by the same molecular mechanisms as in NSCLC, but the details remain unknown and are a subject of future studies. The size of xenotransplanted tumors from YAP1-KO cells was significantly smaller than those from ~~Mock~~ mock cells. These results suggest the potential of YAP1 as one of the most important therapeutic targets for subtypes of SCLC.

SCLC is a highly aggressive and metastatic malignancy that rapidly develops chemoresistance and has a high rate of recurrence [21]. The H69AR cell line is generated by the prolonged exposure of H69 cells to adriamycin and reflects clinical settings for SCLC, which may relapse after initial treatment and acquire drug resistance. In the present study, the KO of YAP1 in H69AR cells, which had acquired drug resistance, restored some drug sensitivity, which indicates the therapeutic potential of targeting

YAP1 in chemoresistant SCLC.

We previously reported that Notch1 controls cell invasion, metastasis, chemoresistance, and NE differentiation in SCLC [16,25,26]. The results of the WB analysis of 13 lung cancer cell lines showed similar expression patterns for YAP1 and Notch1, suggesting that one regulates the other (Fig. 1a). However, the KO of YAP1 in H69AR cells up-regulated the expression of NOTCH1 and HES1, while the KO of Notch1 in H69AR cells up-regulated that of YAP1 (Figs. 2a, ~~S1~~ S3). Although crosstalk between Notch signaling and YAP1 has been demonstrated in various organs, it has not yet been reported for SCLC. In SCLC-Y, YAP1 and NOTCH1 are complementary and may be involved in cell proliferation, EMT, drug resistance, and NE differentiation (Fig. 6).

5. Conclusion

In the present study, we performed a functional analysis of YAP1 in human cancer. The KO of YAP1 in H69AR cells, a chemically induced SCLC-Y subtype, reduced cell proliferation and invasion capacity and restored drug sensitivity. In addition, the expression of NE markers was up-regulated, indicating that YAP1 plays an important role in NE differentiation. YAP1 appears to be complementary to Notch1, both of which are important factors in therapeutic strategies for SCLC.

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Declarations

Conflict of interests The authors have no conflicts of interest to declare.

Ethical approval All studies using human pathological samples followed the guidelines of the Ethics Committee of Kumamoto University (No. 342). All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

Figure legends

Fig. 1 Expression of YAP1 and its interrelationship with INSM1. a A Western blotting (WB) analysis was conducted using lung cancer cell lines, including small cell lung carcinoma (SCLC), adenocarcinoma (ADC), and squamous cell carcinoma (SCC). The expression of YAP1, NOTCH1, HES1, INSM1, CHGA, SYP, and NCAM was examined in lung cancer cell lines. Three out of the seven SCLC cell lines simultaneously expressed YAP1 with NOTCH1 and HES1, and showed negative expression for INSM1. **b** Immunohistochemical staining for INSM1 and YAP1 was performed on 32 surgically resected SCLC specimens. Representative images of SCLC-A and SCLC-Y specimens are shown. INSM1 was positively immunostained in cell nuclei in an SCLC-A case. YAP1 was expressed in cell nuclei and in the cytoplasm of an SCLC-Y case. Scale bar = 50 μm. **c** Expression levels of *YAP1* and *INSM1* in the RNA-seq dataset of SCLC tissues. The GSE60052 (n = 79) dataset [13] was analyzed. A negative correlation was observed (ρ= -0.30681597, P=0.005920869).

Fig. 2 RNA sequence and GO analysis. a The WB analysis showed that the knockout of the *YAP1* gene in the H69AR cell line decreased phosphorylated (p)-YAP and CYR61. INSM1, SYP, and CHGA were induced by the knockout of the *YAP1* gene. In contrast, NOTCH1 and HES1 were also increased by the knockout of the *YAP1* gene. **b** RNA sequencing data are shown using a heatmap. Among

significantly up-regulated genes, INSM1, ASCL1, CHGA, NCAM, and SYN1 (SYP) appeared in KO YAP1 cell lines. **c** Gene ontology (GO) terms of biological processes or molecular functions sorted by combined scores. The top 10 categories in each of the terms are shown.

Fig. 3 YAP1 is involved in EMT in H69AR cells. a Significant reduction~~s~~ in SLUG, VIMENTIN, and N-CADHERIN in YAP1-KO cells were detected by Western blotting (WB). However, a reduction in SNAIL was not evident. **b** The degree of cell invasion in YAP1-KO cells was less than that in ~~Mock~~ mock cells. **c** Representative images from wound healing assays using YAP1-KO cells and mock cells at 0, and 48 hours after treatment with the cell migration indexes shown. Scale bar = 200 μm. Data are shown as the mean ± SD (**b and c**). *** $p < 0.001$.

Fig. 4 Knockout of YAP1 suppressed cell proliferation. a Cell counting assays revealed that the knockout of YAP1 inhibited the proliferation of H69AR cells. **b** WB revealed that the knockout of YAP1 suppressed Cyclin D1. **c** Mock-transfected and YAP1-KO cells were subcutaneously injected into the backs of five Rag2 and Jak3 double gene-deficient mice. After 30 days, tumors were removed and measured. The size of xenotransplanted tumors from YAP1-KO cells was significantly smaller than those from ~~Mock~~ mock cells. In addition, tumors did not form in three out of five mice. Scale bar = 10 mm. **d** Hematoxylin and eosin (H&E) staining and immunostaining for YAP1, INSM1, and Ki67

in xenotransplanted tumors from ~~Mock~~ mock and YAP1-KO cells. IHC staining confirmed that tumor tissues from YAP1-KO cells had lower expression levels of YAP1, but higher expression levels of INSM1. Staining for Ki67 was lower in tumor tissues from YAP1-KO cells than in those from ~~Mock~~ mock cells. Scale bar = 50 μm. Data are shown as the mean ± SD (**a** and **c**). * $p < 0.05$. ** $p < 0.01$.

Fig. 5 a Relative cell survival of three cell lines: H69, H69AR, and H69AR YAP1-KO. H69 is an ASCL1-positive, INSM1-positive, and YAP1-negative small-cell lung cancer cell line. H69AR is an adriamycin-resistant H69 cell line that expresses YAP1. Cells were treated with etoposide at progressive concentrations (1, 2, 5, 10, 20, and 50 μM) for 10 days. **b** Dose–response curves of three cell lines: H69, H69AR, and H69AR YAP1-KO. The x-axis indicates the $\log_{10}$ (concentration of etoposide) and the y-axis indicates cell viability. * $p < 0.05$.

Fig. 6 The role of YAP1 in SCLC-Y. In the SCLC-Y subtype, YAP1 affects not only tumor cell proliferation, but also NE differentiation, the modulation of EMT, and drug sensitivity. YAP1 and NOTCH1 are complementary and each suppresses NE differentiation.

Fig. S1 Molecules that correlate with YAP1. The RNA-seq dataset using tumor samples from 79 SCLC patients identified NOTCH1 and WNT7B as molecules that positively correlate with YAP1 in

human SCLC tissues.

Fig. S2 YAP1-KO experiment in SBC5. The WB analysis showed that the knockout of the *YAP1* gene in SBC5 cells increased SYP and decreased SLUG.

Fig. S3 Expression of YAP1 and NOTCH1. The WB analysis showed that the knockout of the *Notch1* gene in H69AR cells reduced HES1. YAP1 was induced by the knockout of the *Notch1* gene.

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Table 1 Details of the primary antibodies used for WB, IHC analysis

Primary antibody	Manufacturer（location）	WB	IHC
YAP1 (H-125)	Santa Cruz Biotechnology (Santa Cruz, CA)	1:1000	1:100
p-YAP1 (s127)	Cell Signaling Technology (Danvers, MA, USA)	1:500	1:80
Cyr61 (A-10)	Santa Cruz Biotechnology	1:250	1:100
NOTCH1 (C44H11)	Cell Signaling	1:2000	
HES1	Gifted from T. Sudo, TORAY Industries, Yokohama	1:1000	
Cyclin D1 (RM-9104-S1)	Thermo Fisher Scientific (Waltham, MA, USA)	1:1000	
Caspase3 (9662)	Cell Signaling Technology	1:5000	
Cleaved Caspase3 (9661)	Cell Signaling Technology	1:100	
Slug (C19G7)	Cell Signaling Technology	1:500	
Snail (C15D3)	Cell Signaling Technology	1:500	
Vimentin (E-5)	Santa Cruz Biotechnology	1:2000	
E-cadherin (610181)	BD Biosciences (San Jose, CA)	1:1000	
N-cadherin (H-63)	Santa Cruz Biotechnology	1:500	
INSM1 (A-8)	Santa Cruz Biotechnology	1:500	
CHGA (H-300)	Santa Cruz Biotechnology	1:1000	
SYP (NCL-SYNAP-299)	Novocastra Laboratories Ltd (Newcastle upon Tyne, UK)	1:500	
NCAM (NCL-CD56-1B6)	Novocastra Laboratories Ltd	1:500	
Ki-67 (M7240)	Dako (Glostrup, Denmark)	1:10000	
β-actin (A-5441)	Sigma-Aldrich (Oakville, ON, Canada)		

Manufacturers, quantities, and working dilutions are indicated. YAP1, yes-associated protein 1; TAZ1, tafazzin 1; Hes1, hairy and enhancer of split1; NSM1, insulinoma-associated protein 1; SYP, synaptophysin; CHGA, chromogranin A. WB, Western blot; IHC, immunohistochemistry.

Table 2 Result of immunohistochemical staining of human SCLC.

Score	YAP1	INSM1
0, negative	28/32（87.5%）	4/32（12.6%）
1, weak positive	2/32（6.3%）	7/32（21.8%）
2, positive	2/32（6.3%）	21/32（65.6%）

Immunohistochemical staining for INSM1 and YAP1 was performed in 32 small cell lung carcinoma (SCLC) specimens that had been surgically resected.

Original Article

Title: The role of YAP1 in small cell lung cancer

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Abstract:

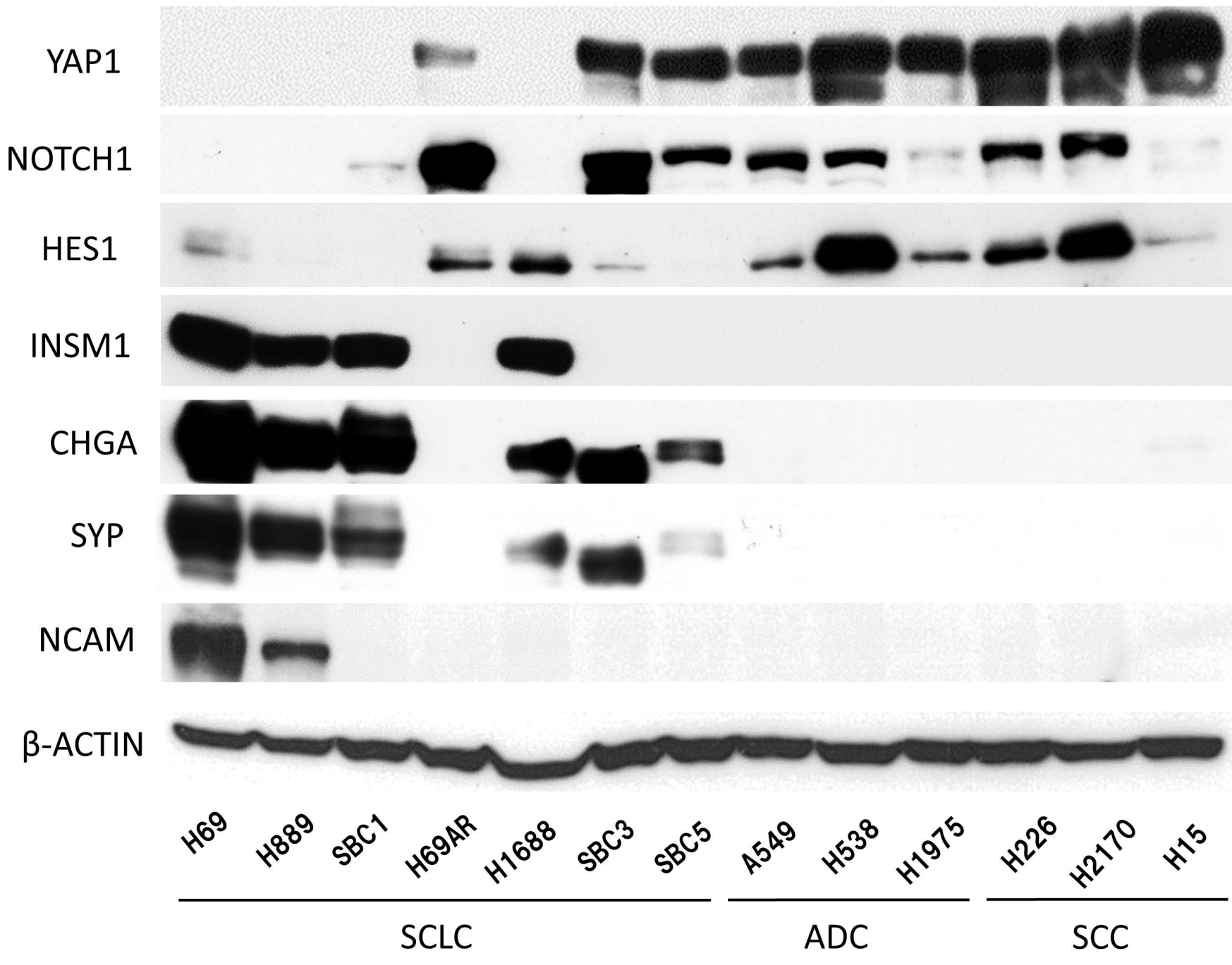
Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ, also known as WWTR1) are core downstream effectors of the Hippo pathway, which is involved in diverse biological processes. The oncogenic effects of YAP and TAZ in non-small cell lung cancer (NSCLC) have recently been reported; however, their roles in SCLC remain unclear. Immunohistochemistry (IHC) on lung cancer tissues and Western blotting (WB) on lung cancer cell lines were performed to

examine the expression of YAP1. Genome editing using CRISPR/Cas9 was then used to knockout the YAP1 gene in the H69AR cell line. An RNA sequence analysis, gene ontology (GO) analysis, WB, cell counting assay, invasion assays, and xenograft studies were conducted on these cells to investigate the biological roles of YAP1. IHC revealed that insulinoma-associated protein 1 was expressed in most cases (28 out of 32 cases), while only four cases expressed YAP1. The knockout of YAP1 in H69AR cells, ~~the~~ a chemically induced SCLC-Y subtype, reduced cell proliferation and invasion capacity and restored drug sensitivity. Xenograft assays revealed that the knockout of YAP1 suppressed cell proliferation. Tumor tissues showed the expression of neuroendocrine markers and a low ~~ki~~ Ki-67 index. In SCLC, YAP1 plays an important role in biological functions, such as cell proliferation, EMT, drug sensitivity, and neuroendocrine differentiation.

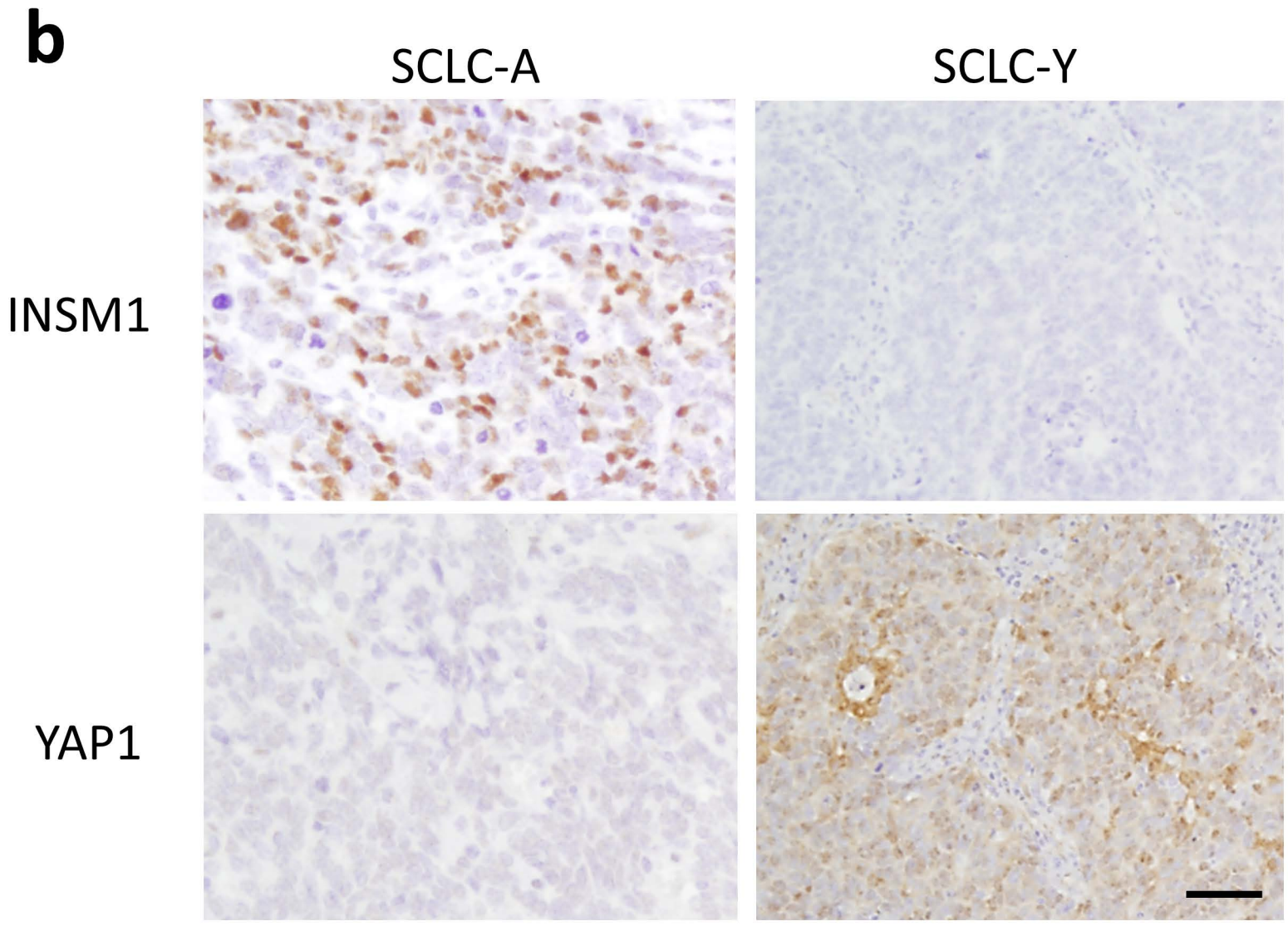
Keywords:

YAP1, small cell lung carcinoma (SCLC), neuroendocrine (NE) differentiation, epithelial–mesenchymal transition (EMT), drug sensitivity

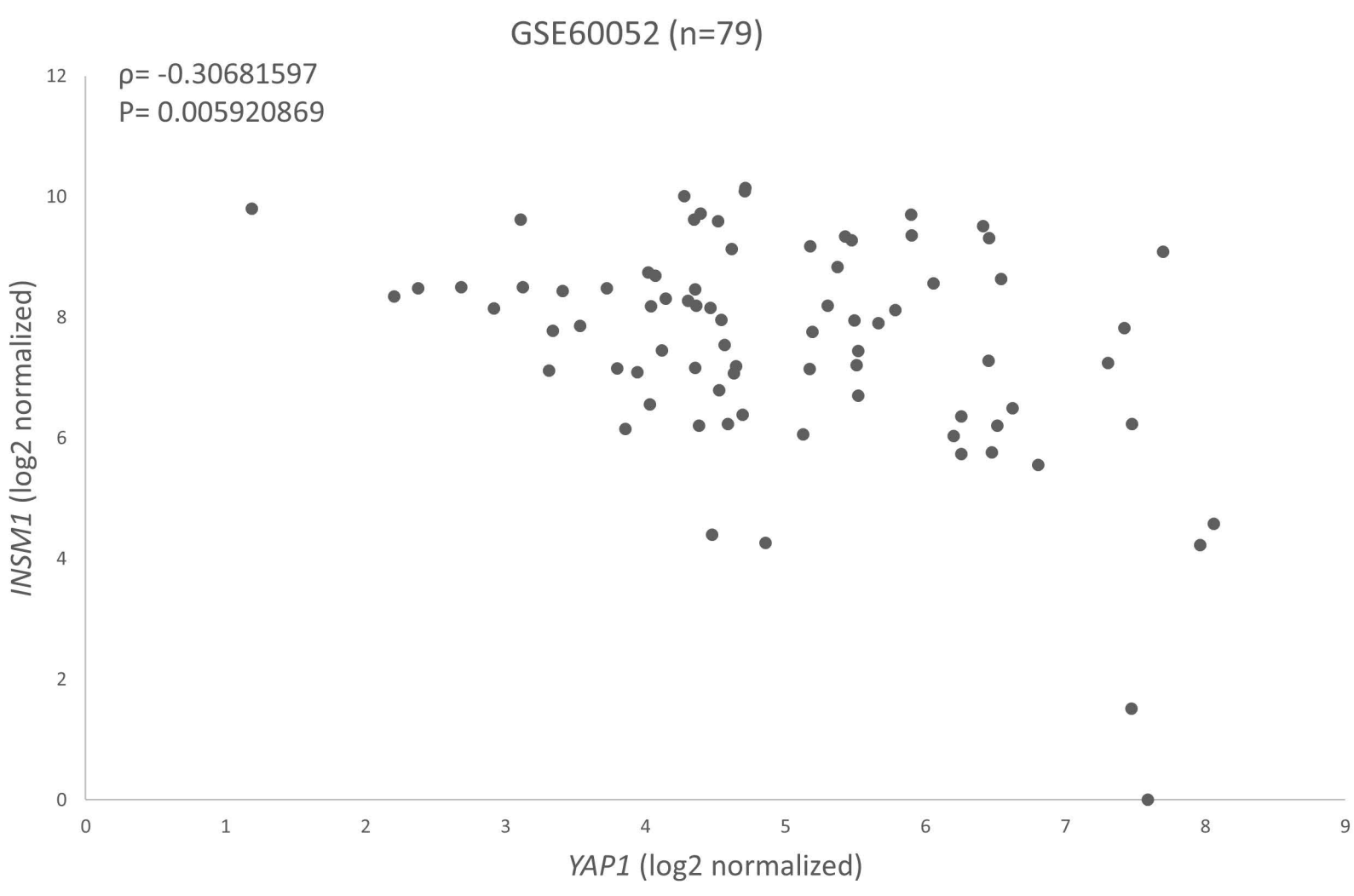
a


YAP1
NOTCH1
HES1
INSM1
CHGA
SYP
NCAM
β-ACTIN
H69
H889
SBC1
H69AR
H1688
SBC3
SBC5
A549
H538
H1975
H226
H2170
H15
SCLC
ADC
SCC

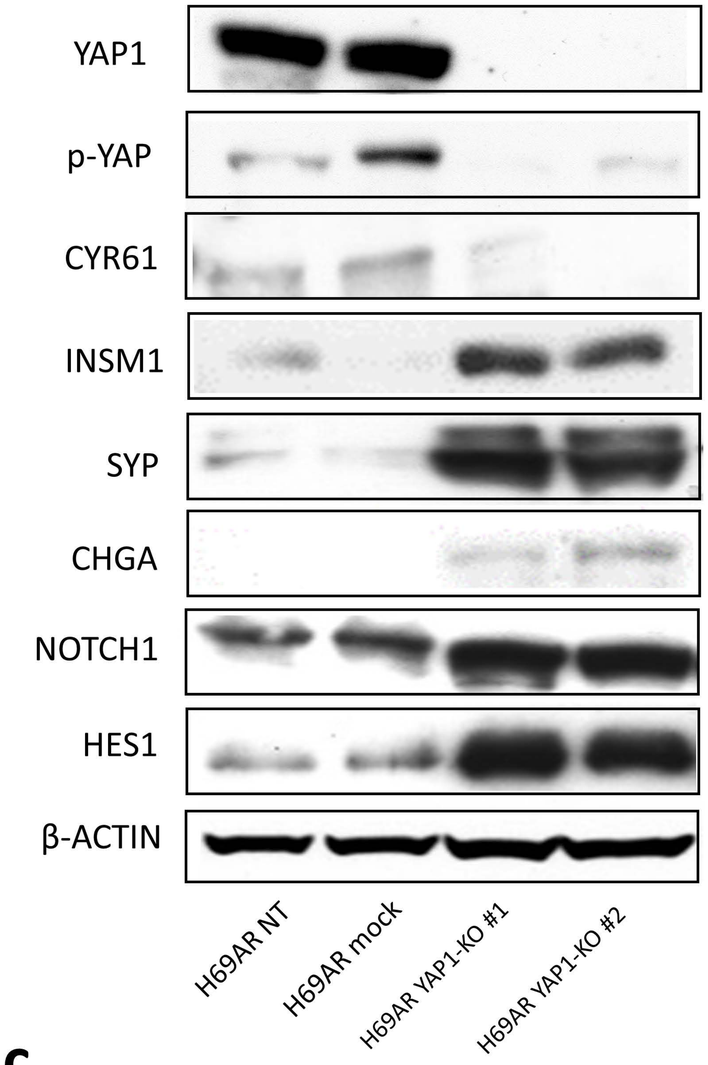
b


SCLC-A
SCLC-Y
INSM1
YAP1

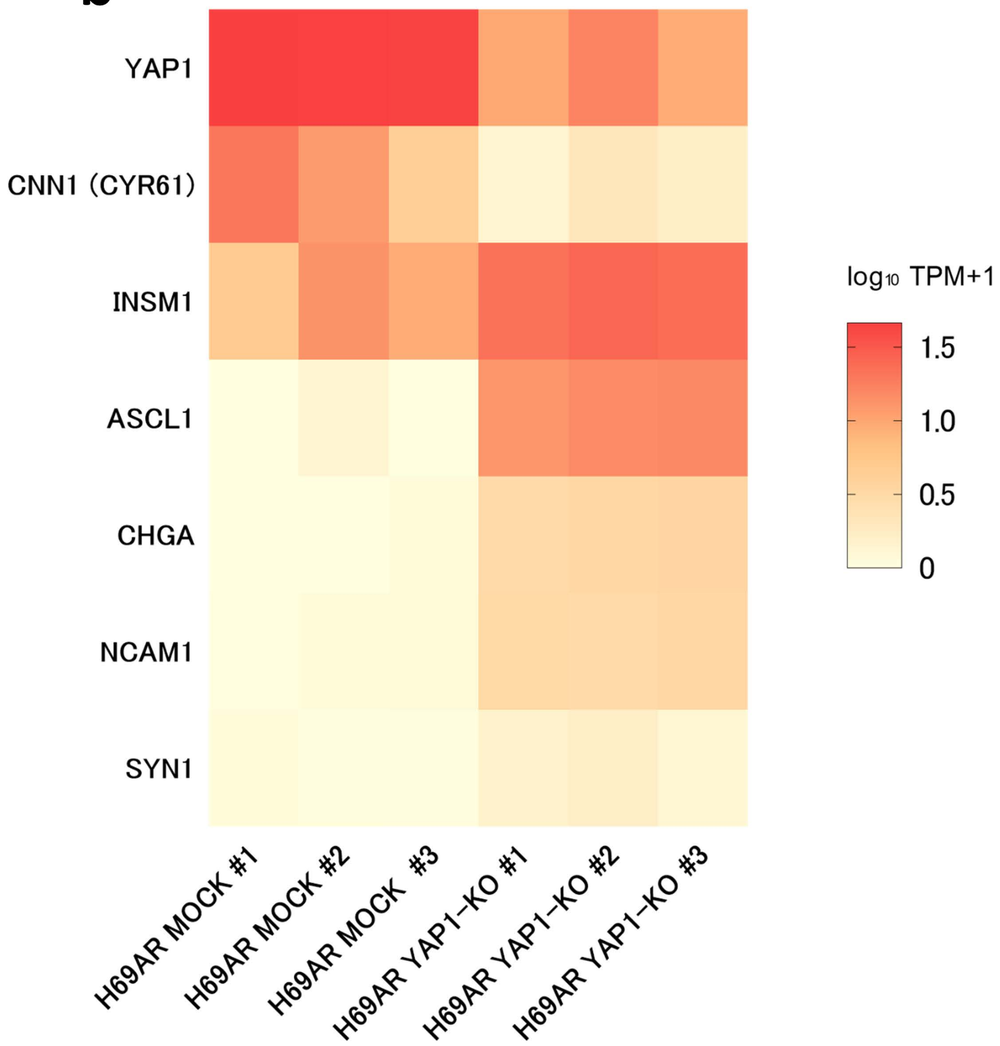
c


GSE60052 (n=79)
ρ= -0.30681597
P= 0.005920869
INSM1 (log2 normalized)
YAP1 (log2 normalized)
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
9

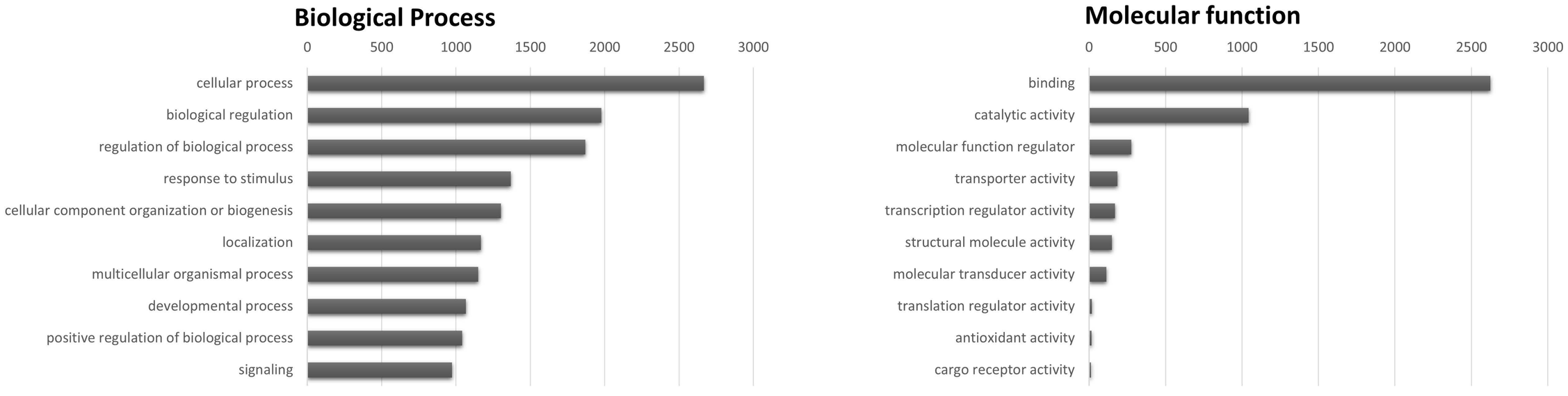
a


YAP1
p-YAP
CYR61
INSM1
SYP
CHGA
NOTCH1
HES1
β-ACTIN
H69AR NT
H69AR mock
H69AR YAP1-KO #1
H69AR YAP1-KO #2

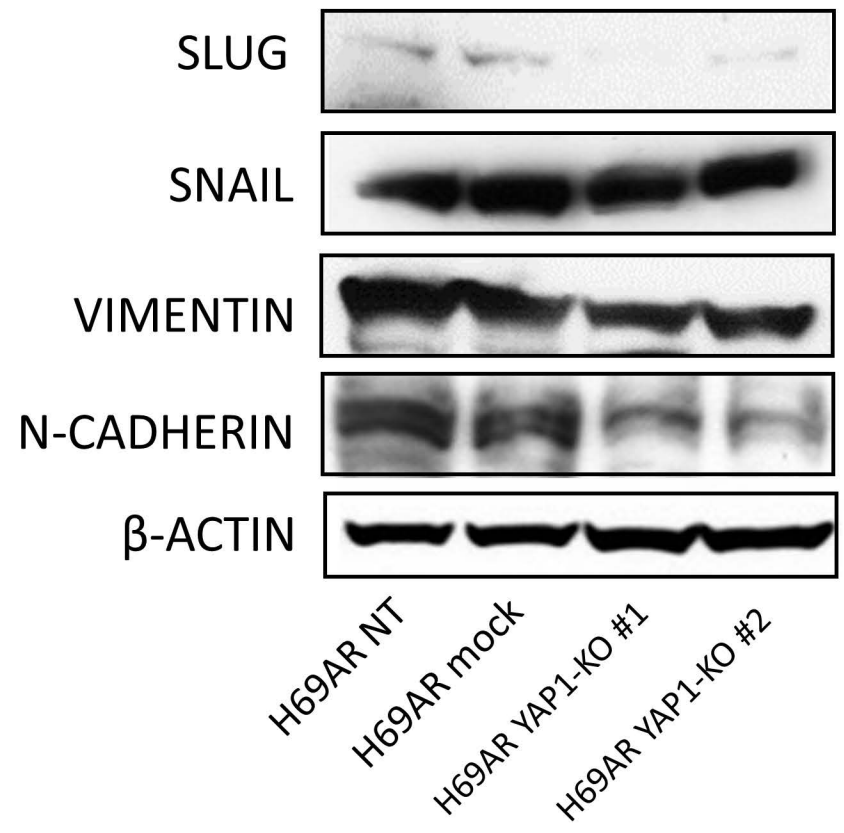
b


YAP1
CNN1 (CYR61)
INSM1
ASCL1
CHGA
NCAM1
SYN1
H69AR MOCK #1
H69AR MOCK #2
H69AR MOCK #3
H69AR YAP1−KO #1
H69AR YAP1−KO #2
H69AR YAP1−KO #3
log10 TPM+1
1.5
1.0
0.5
0

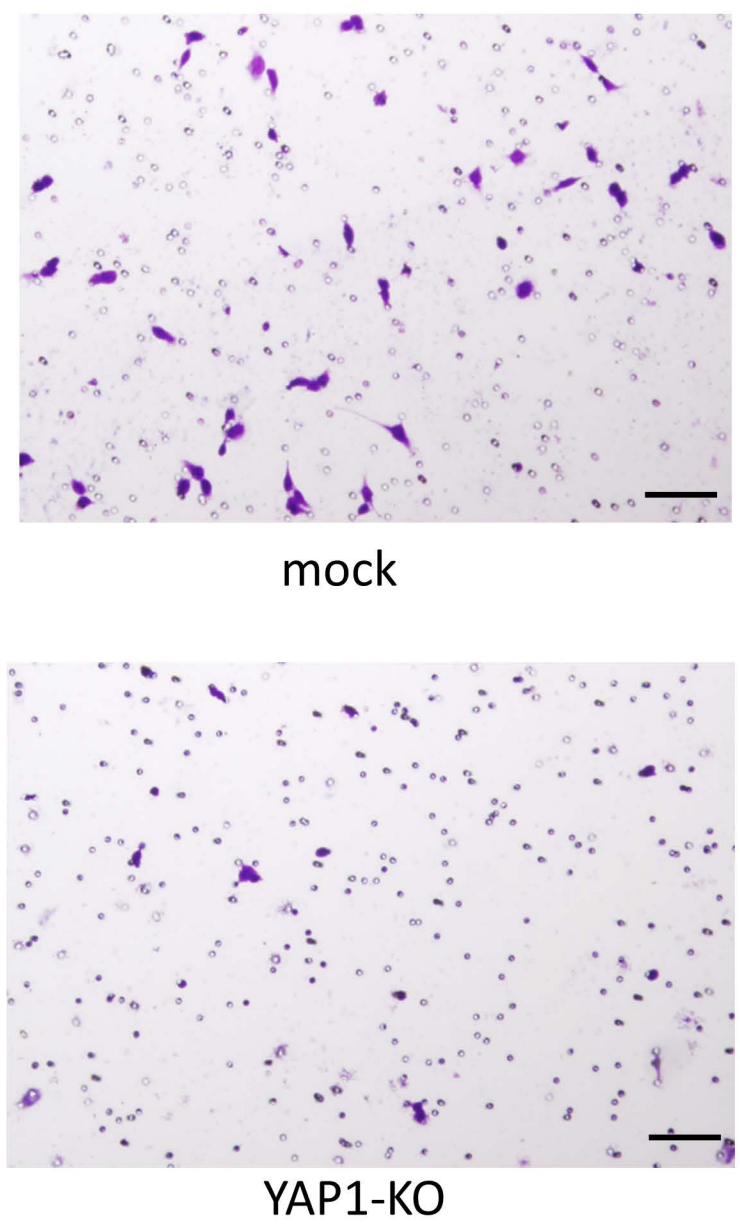
c

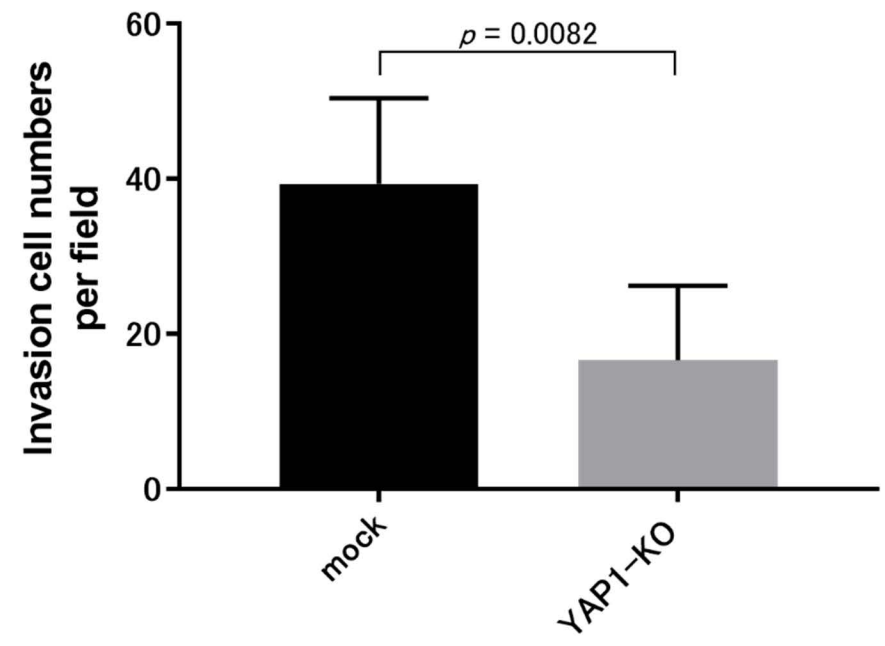

H69AR mock vs H69AR KO-YAP1
Biological Process
0
500
1000
1500
2000
2500
3000
cellular process
biological regulation
regulation of biological process
response to stimulus
cellular component organization or biogenesis
localization
multicellular organismal process
developmental process
positive regulation of biological process
signaling
Molecular function
0
500
1000
1500
2000
2500
3000
binding
catalytic activity
molecular function regulator
transporter activity
transcription regulator activity
structural molecule activity
molecular transducer activity
translation regulator activity
antioxidant activity
cargo receptor activity

a

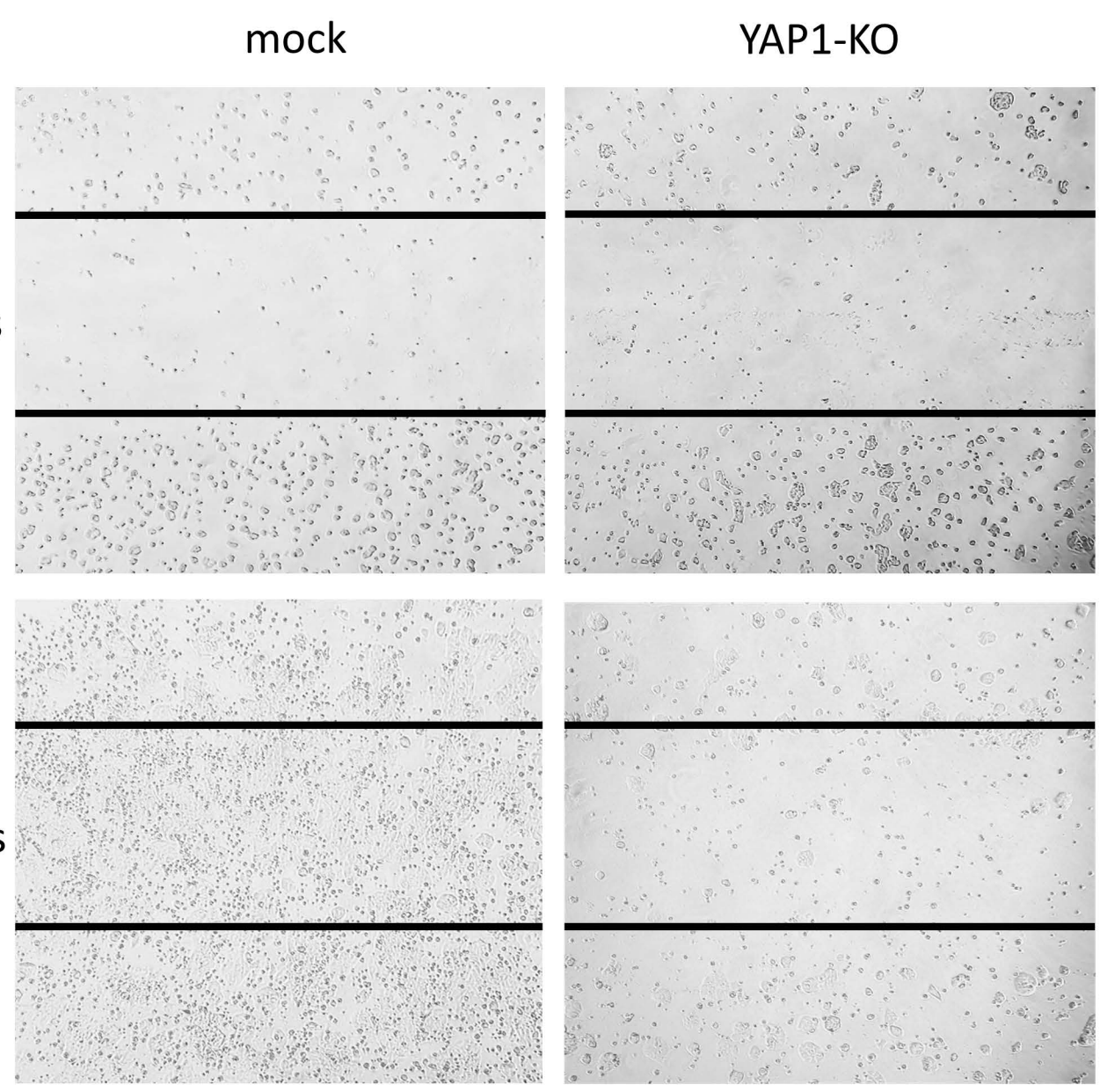

SLUG
SNAIL
VIMENTIN
N-CADHERIN
β-ACTIN
H69AR NT
H69AR mock
H69AR YAP1-KO #1
H69AR YAP1-KO #2

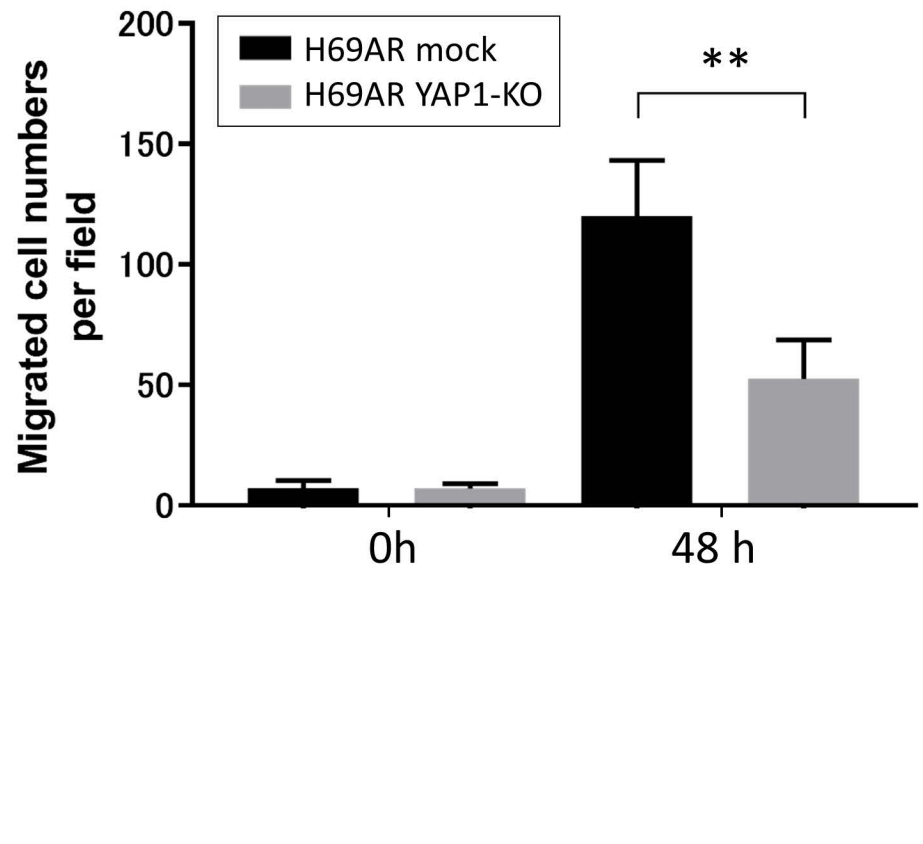
b


mock
YAP1-KO

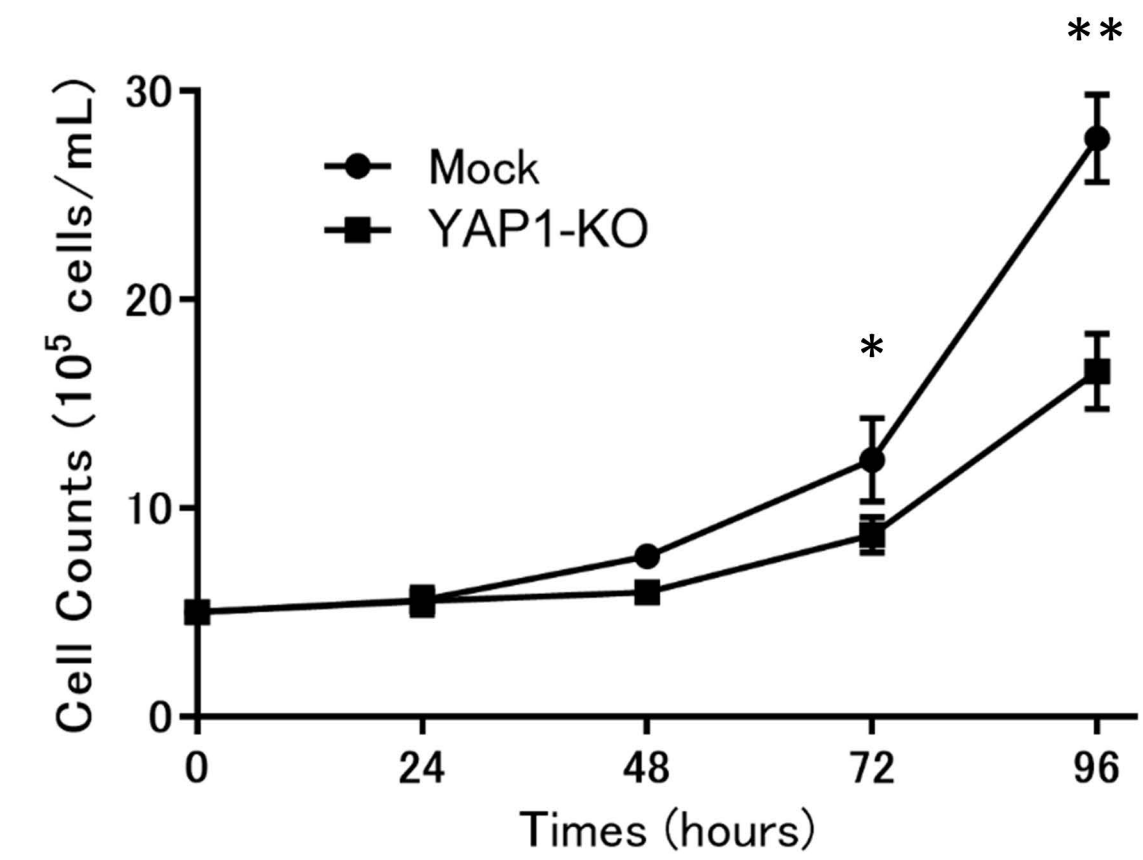

p = 0.0082
Invasion cell numbers per field
60
40
20
0
mock
YAP1-KO

c

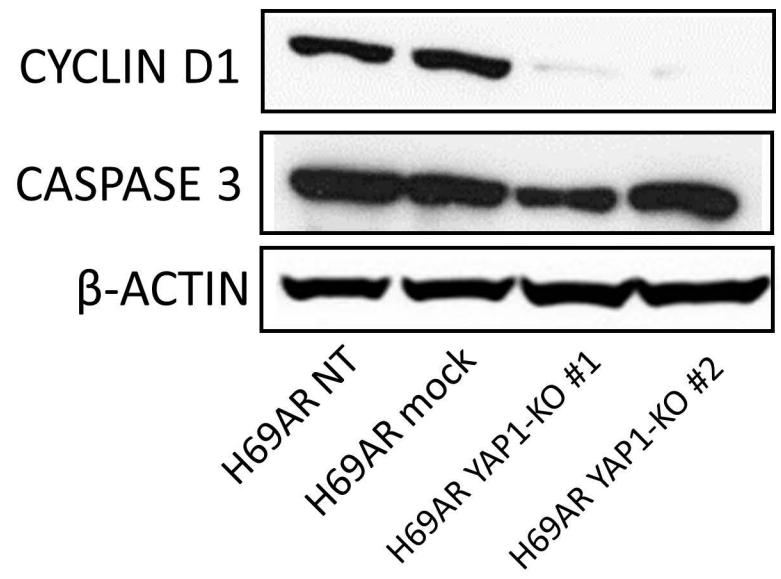

mock
YAP1-KO
0 hours
48 hours


H69AR mock
H69AR YAP1-KO
**
Migrated cell numbers per field
200
150
100
50
0
0h
48 h

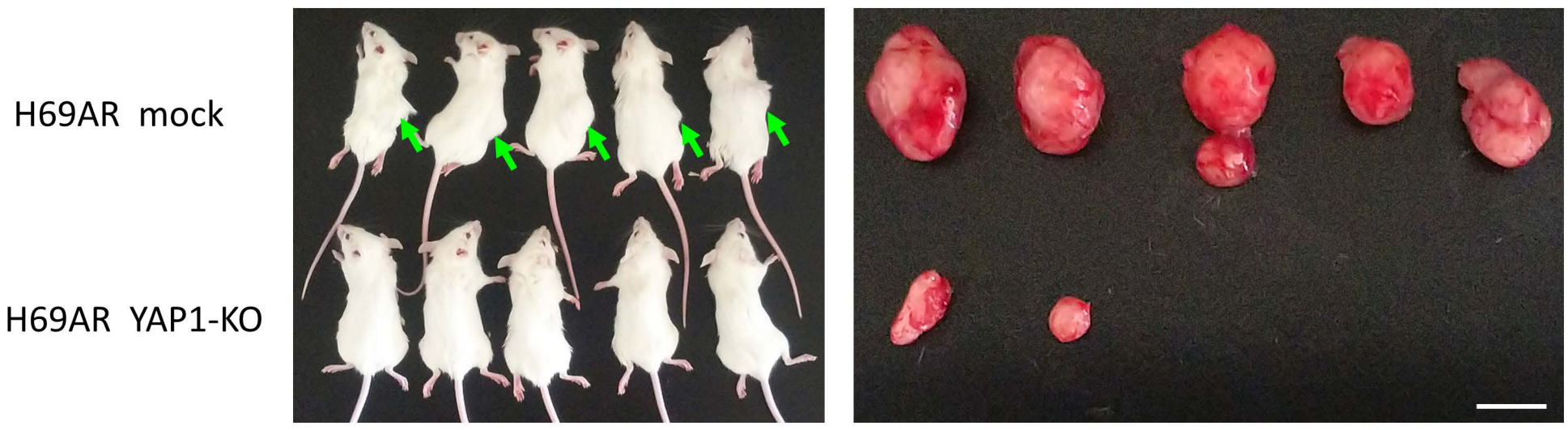
a

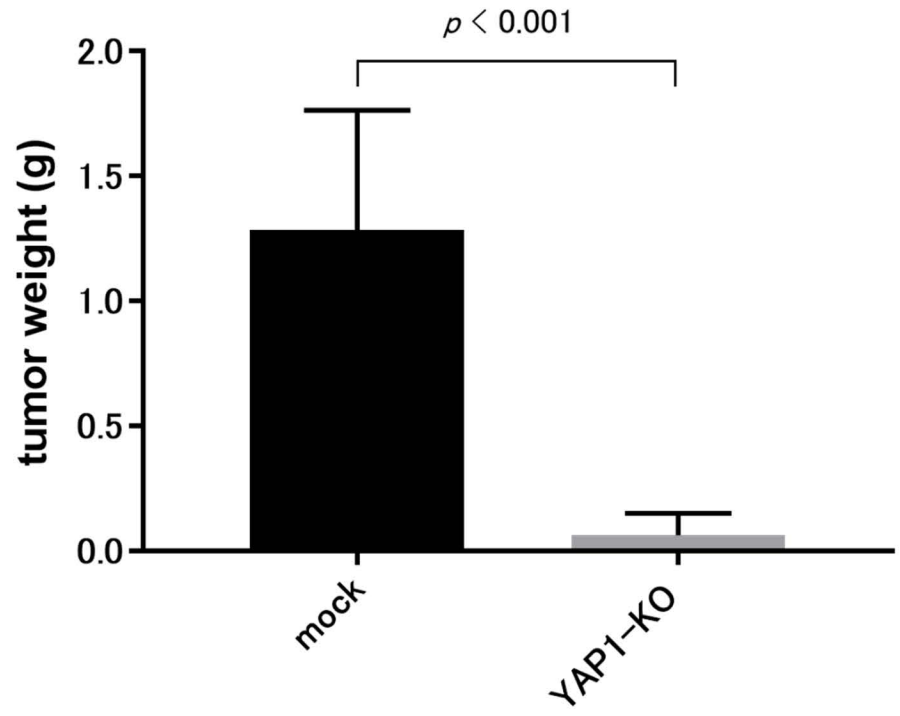

Mock
YAP1-KO
Cell Counts (10^5 cells/mL)
30
20
10
0
*
**
0
24
48
72
96
Times (hours)

b

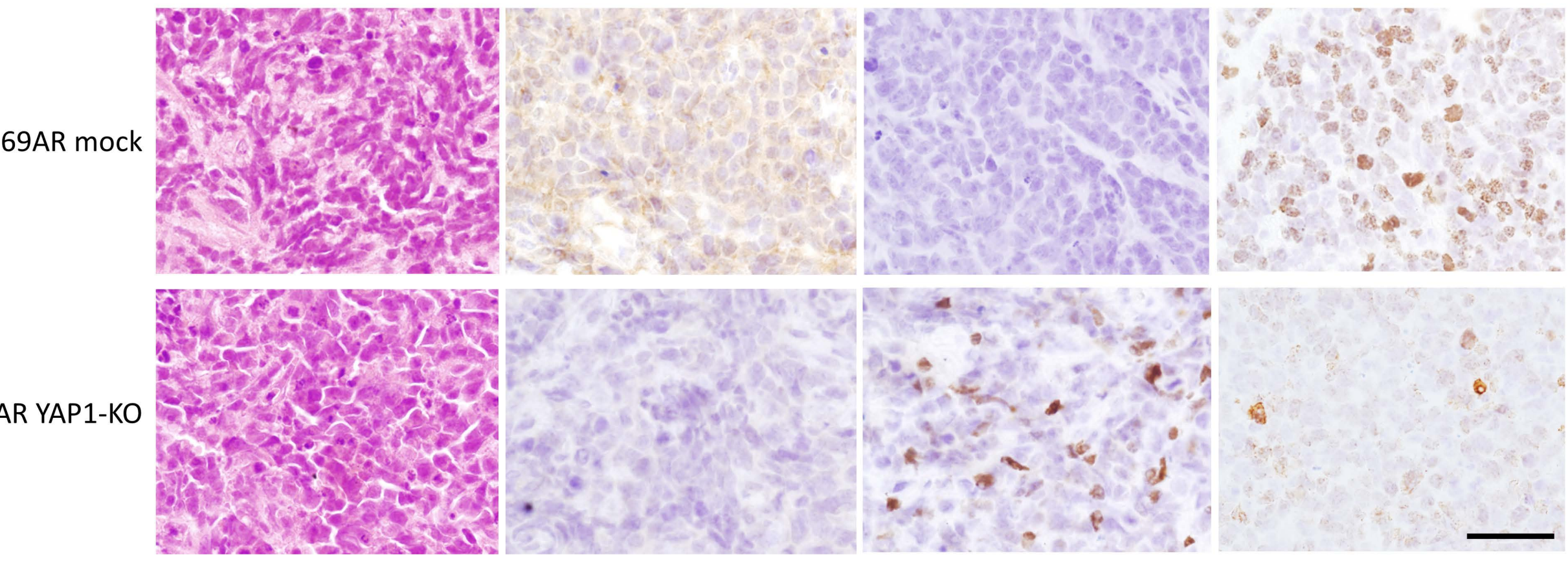

CYCLIN D1
CASPASE 3
β-ACTIN
H69AR NT
H69AR mock
H69AR YAP1-KO #1
H69AR YAP1-KO #2

c

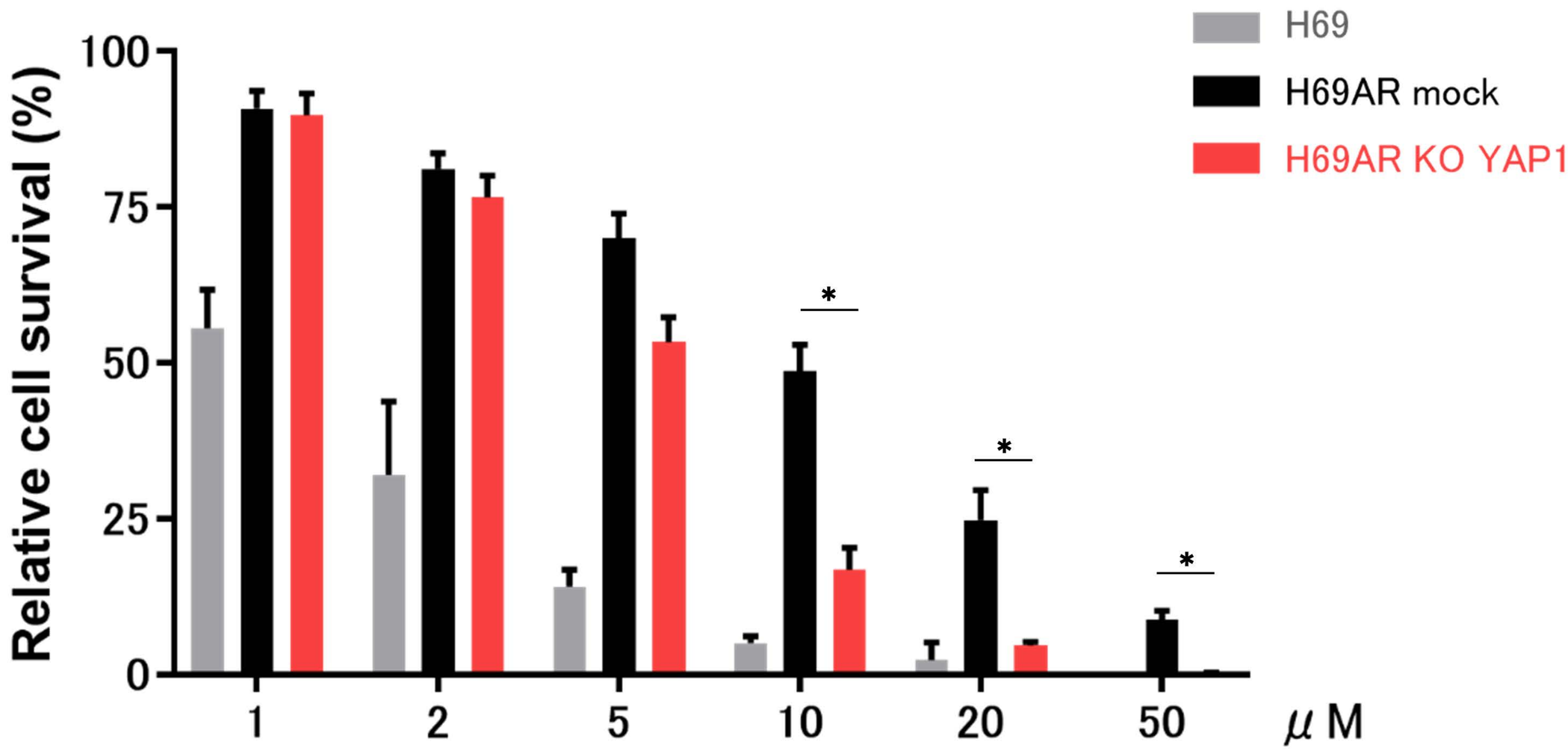

H69AR mock
H69AR YAP1-KO


$p < 0.001$
tumor weight (g)
2.0
1.5
1.0
0.5
0.0
mock
YAP1-KO

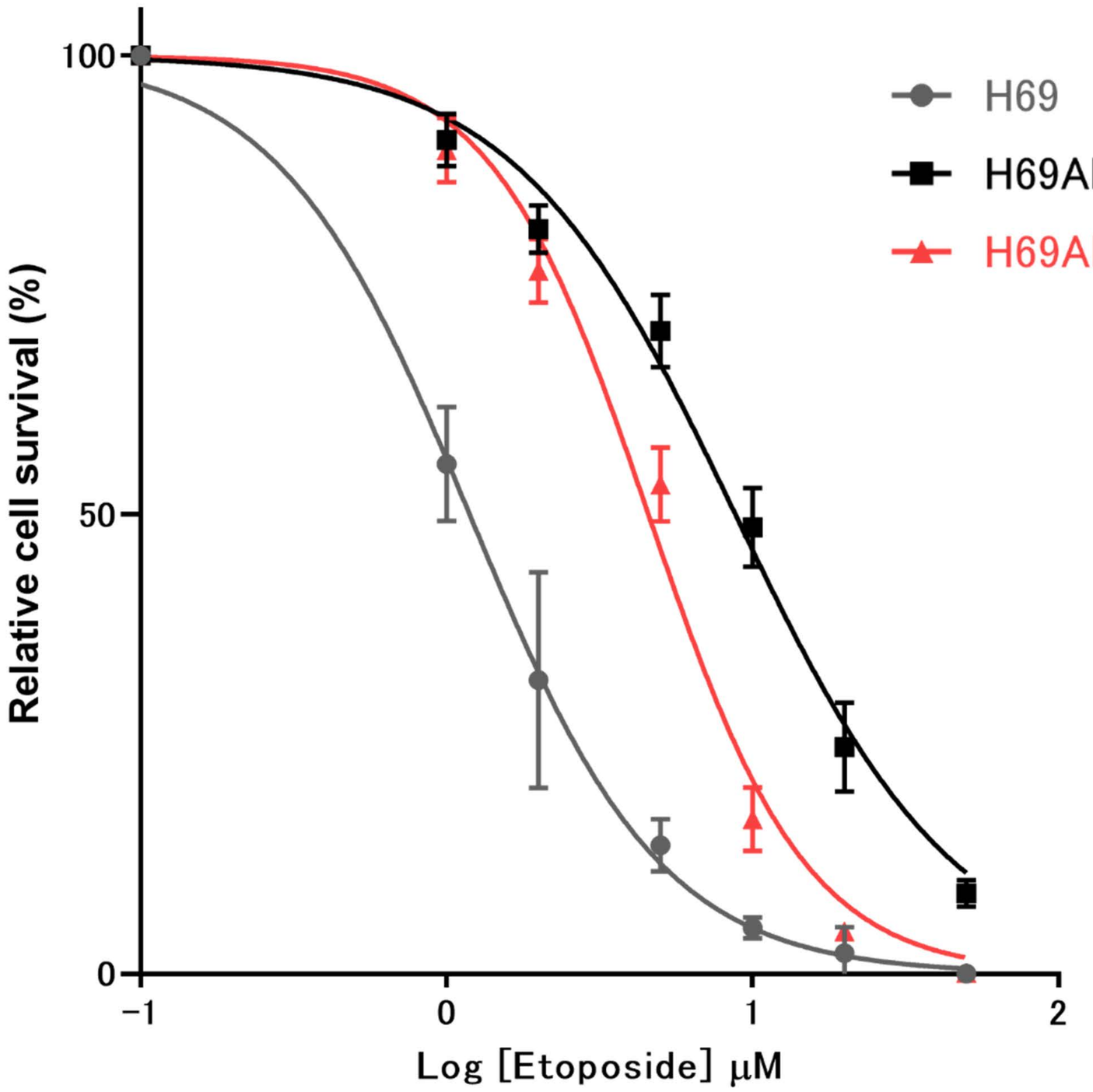
d

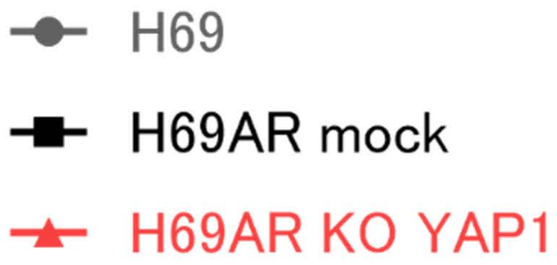

H&E
YAP1
INSM1
Ki67
H69AR mock
H69AR YAP1-KO

a



b



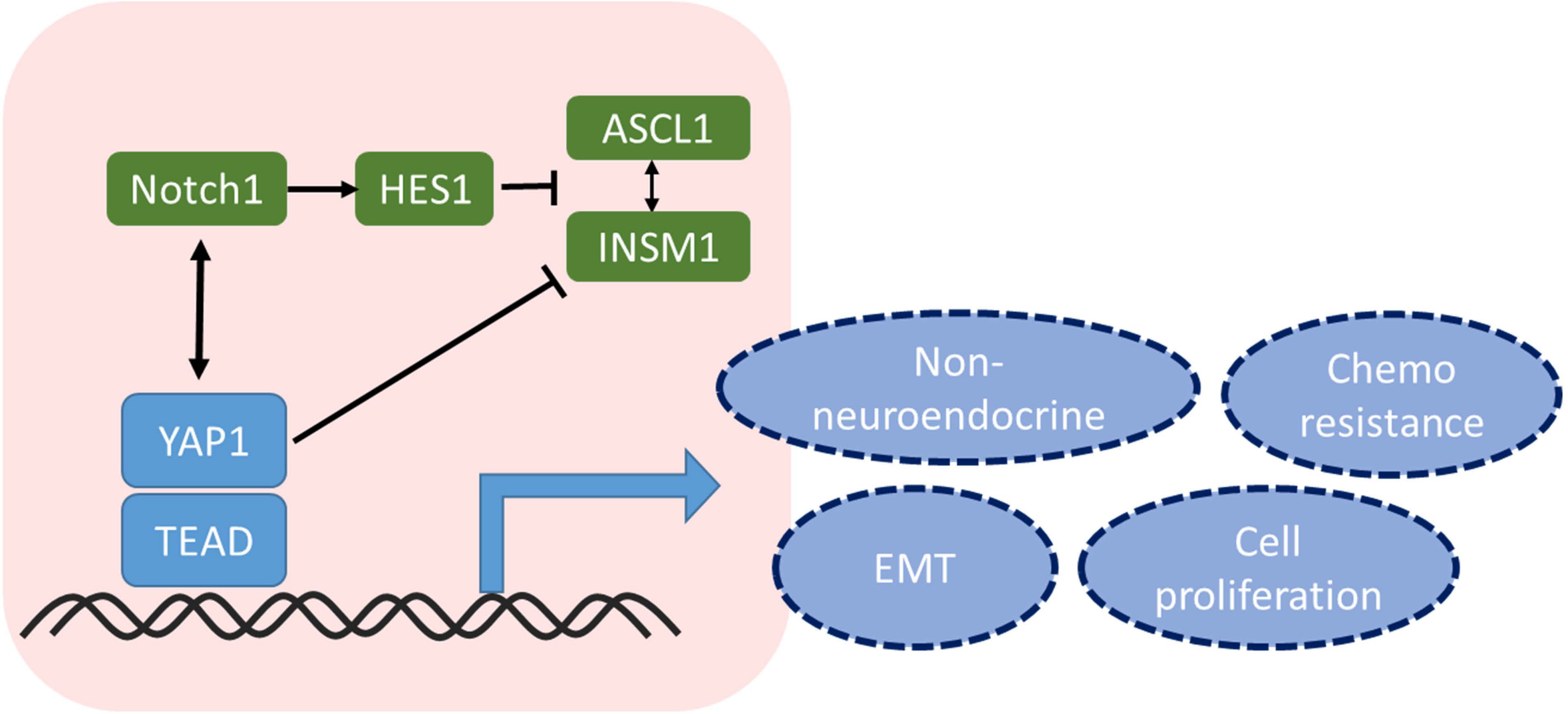


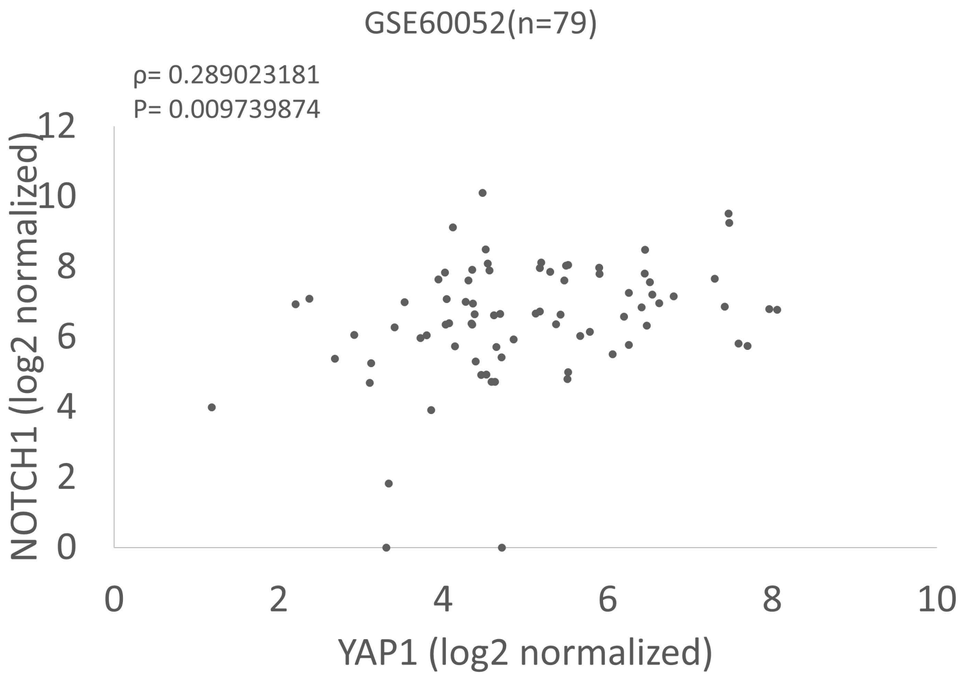
$LogIC_{50}$ =0.07601μM

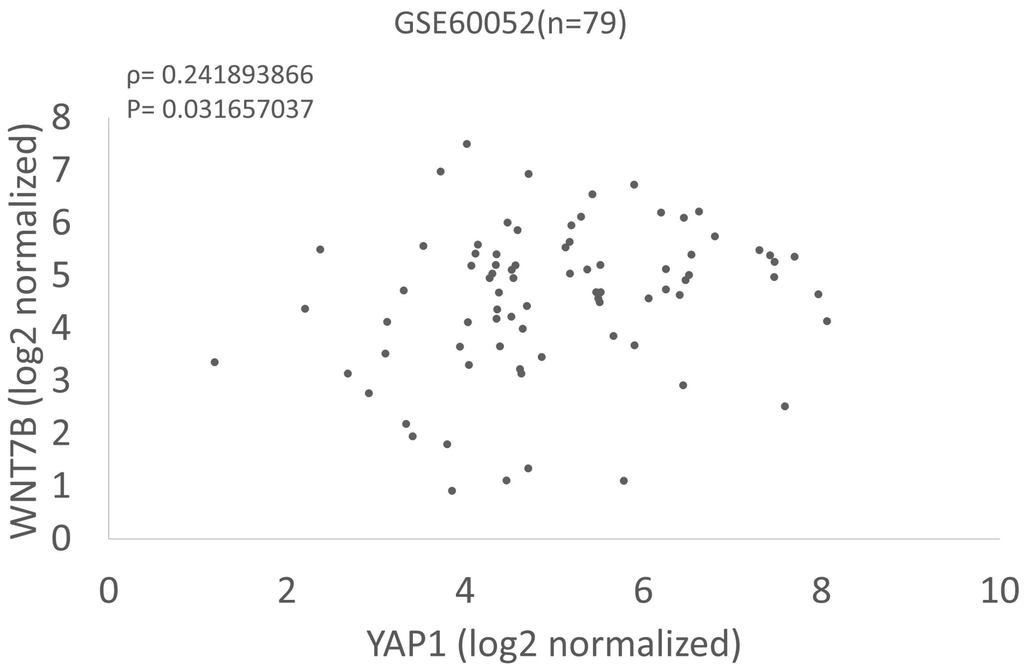
$LogIC_{50}$ =0.9445μM

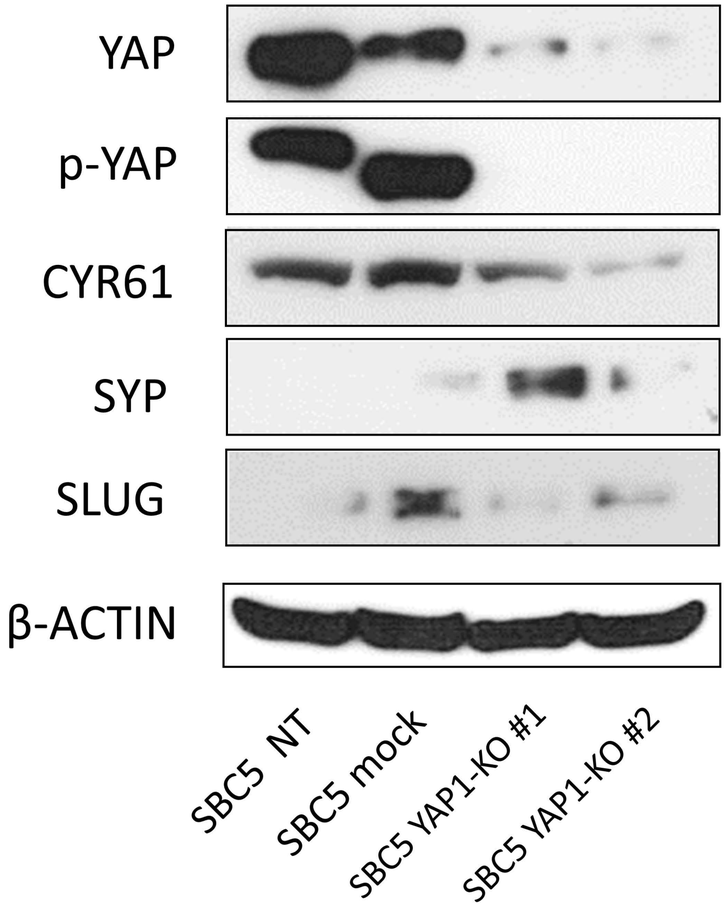
$LogIC_{50}$ =0.6604μM

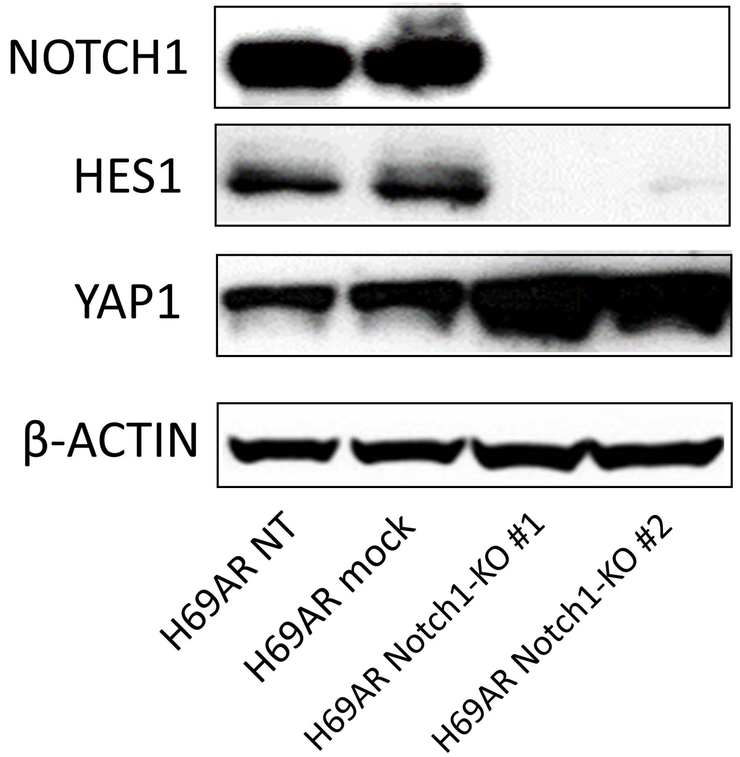
SCLC-Y subtype




GSE60052(n=79)
ρ= 0.289023181
P= 0.009739874
NOTCH1 (log2 normalized)
YAP1 (log2 normalized)
0
2
4
6
8
10
12


GSE60052(n=79)
ρ= 0.241893866
P= 0.031657037
WNT7B (log2 normalized)
YAP1 (log2 normalized)
0
1
2
3
4
5
6
7
8
10


YAP
p-YAP
CYR61
SYP
SLUG
β-ACTIN
SBC5 NT
SBC5 mock
SBC5 YAP1-KO #1
SBC5 YAP1-KO #2


NOTCH1
HES1
YAP1
β-ACTIN
H69AR NT
H69AR mock
H69AR Notch1-KO #1
H69AR Notch1-KO #2