Chemotherapy resistance due to epithelial-to-mesenchymal transition is caused by abnormal lipid metabolic balance

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Abstract

Invasive cancer is defined by the loss of epithelial cell traits resulting from the ectopic expression of epithelial-mesenchymal transition (EMT)-related transcription factors such as Snail. Although EMT is known to impart chemoresistance to cancer cells, the precise molecular mechanisms remain elusive. We found that Snail expression confers chemoresistance by upregulating the cholesterol efflux pump ABCA1 as a countermeasure to the excess of cytotoxic free cholesterol relative to its major

interaction partner in cellular membranes, sphingomyelin. This imbalance is introduced by the transcriptional repression of enzymes involved in the biosynthesis of sphingomyelin by Snail. Inhibiting esterification of cholesterol, which renders it inert, selectively suppresses growth of a xenograft model of Snail-positive kidney cancer. Our findings offer a new perspective on lipid-targeting strategies for invasive cancer therapy.

Introduction

Metastatic cancer, characterized by the spread of tumor cells to distant organs, remains a major challenge in cancer treatment due to its high mortality rate. Metastasis involves a series of biological events, such as acquisition of cancer stem cell characteristics, tumor-microenvironment interactions, alterations in cell metabolism and resistance to chemotherapy, that are mediated by epithelial-mesenchymal transition (EMT) (Fischer *et al*, 2015; Lambert & Weinberg, 2021; Nieto *et al*, 2016; Zheng *et al*, 2015). Transcription factors driving EMT (EMT-TFs), including Snail, Slug and Twist, that are ectopically expressed in invasive cancers were initially thought to promote invasion and metastasis by suppressing the expression of adhesion molecules in cancer cells. However, since E-cadherin-mediated adhesion is essential for cell survival after metastasis (Padmanaban *et al*, 2019), invasive cancer cells do not undergo complete EMT but rather sustain a hybrid state with both epithelial and mesenchymal (hybrid E/M) characteristics. In a mouse model of pancreatic carcinoma and breast cancers where key EMT-TFs were deleted, the suppression of EMT did not alter the appearance of systemic dissemination or metastasis but rather contributed to increased sensitivity to

anticancer drugs (Fischer *et al.*, 2015; Zheng *et al.*, 2015). Therefore, in-depth understanding of the molecular mechanisms of resistance to anti-cancer drugs conferred by EMT-TFs is an essential prerequisite for devising effective therapeutic interventions (Davis *et al*, 2014). It was recently reported that expression of a Rho family GTPase, RHOJ, is upregulated in cancer cells undergoing EMT and that its activation enhances the DNA damage response, which enables tumor cells to efficiently repair chemotherapy-induced DNA damage (Debaugnies *et al*, 2023). Here, we investigated EMT-dependent acquisition of resistance to more common anticancer drugs that do not directly cause DNA damage, such as those targeting the ERK signaling pathway, and found that expression of EMT-TFs causes metabolic alterations of sphingomyelin, resulting in ectopic induction of drug efflux transporter expression and conferring chemotherapy resistance to cancer cells.

Results

Snail-induced ABCA1 expression confers chemoresistance to hybrid E/M cells

Renal cell carcinoma (RCC) is notorious for the lack of sensitivity to chemotherapy (Makhov *et al*, 2018), but the underlying mechanisms are not elucidated. Nitidine chloride (NC) holds promise as a drug treatment against RCC as it was shown to have a pro-apoptotic effect on RCC and inhibit tumor growth by suppressing the ERK signaling pathway in a xenograft model (Cui *et al*, 2020; Fang *et al*, 2014). However, in human lung adenocarcinoma cells, NC efficacy was inversely correlated with the expression level of the drug transporter ABCA1: cancer cells with

downregulated ABCA1 were more sensitive to NC treatment (Iwasaki *et al*, 2010). Therefore, we wondered whether ABCA1 could also confer NC resistance in RCC. We treated five different RCC cell lines with a combination of NC and an ABCA1 inhibitor cyclosporin A (CsA). Anti-proliferative effect of NC was significantly enhanced in the presence of CsA for three lines among five, Caki-1, 786-O and A498 (Fig. 1A). These cells consistently expressed higher levels of ABCA1 compared to the non-responsive cells (Fig. 1B), suggesting the acquisition of ABCA1-mediated drug resistance. To investigate clinical relevance of ABCA1 upregulation in cancers, we analyzed ABCA1 expression in cancer using the UCSC Xena platform. Comparative ABCA1 expression data of both normal tissue and primary tumors were available for 24 cancer subtypes in the GDC TCGA database. In addition to glioblastoma and head and neck cancer, all three types of renal cancers (clear cell carcinoma (ccRCC), chromophobe carcinoma and papillary cell carcinoma) exhibited significant ABCA1 upregulation in tumor (Fig. 1C and Fig. S1), indicating that ABCA1 upregulation frequently occurs in clinical renal cancers.

Regulatory mechanisms of ABCA1 expression in cancer cells is largely unknown. Interestingly, ABCA1 expression was positively correlated with expression of the EMT-TF Snail in the RCC cell lines (Fig. 1B). To examine whether the enrichment of ABCA1 protein in malignant cancer cells is detectable in clinical cases, we conducted immunohistochemical analysis on surgical specimens taken from patients with primary ccRCC. The findings showed that ABCA1 was upregulated at the lesion site of high-grade ccRCC (Fig. 1D). This clinical data aligns with our cellular experimental results, as Snail expression is known to be upregulated in high-grade ccRCC. Upon detailed comparison among grades, ABCA1 signal was more prominent

in the patients with the high-grade classification (Fig. S2), suggesting that ABCA1 could serve as a marker for high-grade ccRCC. Xena analysis also revealed significantly higher expression of Snail in ccRCC, also supporting the idea that ABCA1 upregulation is correlated with exogenous Snail expression (Fig. S1).

In order to clarify whether Snail expression alone can confer resistance to anti-cancer drugs in epithelial cells, Snail was ectopically expressed in normal epithelial cells and we investigated changes in resistance to anti-cancer drugs. Overexpression of Snail in a mouse mammary gland epithelial cell line EpH4 abolishes transcription of various genes involved in cell-cell adhesion including E-cadherin (Fig. 1E) (Batlle *et al*, 2000; Cano *et al*, 2000; Ikenouchi *et al*, 2003). EpH4-Snail cells exhibited coexistence of the mesenchyme-specific intermediate filament Vimentin and the epithelium-specific Cytokeratin-18 (Fig. 1F), which is a typical feature of a hybrid E/M state of cancer cells. The hybrid E/M EpH4-Snail cells were significantly more resistant to NC than parental EpH4 cells (Fig. 1G). Consistent with the results of kidney cancer cell lines, this resistance was cancelled by CsA (Fig. 1G), and upregulation of ABCA1 expression was also observed in EpH4-Snail cells (Fig. 1H). We also examined another model with a different cell line. In MDCK II, a canine kidney epithelial cell line, TGF-β treatment induced EMT under the expression of the oncogenic KRAS-G12V mutant (Arner *et al*, 2019). The upregulation of ABCA1 expression was also observed in this transient hybrid E/M model (Fig. 1H), suggesting that similar mechanisms to those in EpH4-Snail are also at play in kidney cell EMT. Notably, induction of ABCA1 expression requires a longer period of treatment with TGF-β (day 8) than Snail induction (day 3), indicating that ABCA1 is upregulated in response to cellular alteration by Snail but not induced directly by TGF-β signaling. Furthermore, in comparison between human

esophageal cancer cell lines, Snail-positive TE-8 cells exhibited higher expression of ABCA1 than Snail-negative TE-15 (Fig. 1I). These results suggest that the induction of ABCA1 by ectopic expression of Snail occurs regardless of cellular background.

Intracellular cholesterol accumulation drives ABCA1 expression in hybrid E/M cells

Then how does Snail induce ABCA1 expression, since as a transcriptional repressor it is incapable of doing so directly? Upregulation of ABCA1 was not observed in either E-cadherin or α-catenin KO cells (Fig. 1I), indicating that ABCA1 induction is driven as a specific response to a fundamental change in cellular state induced by Snail overexpression, not an ancillary effect of disrupting either cell-cell adhesion or apical-basal polarity. Generally, ABCA1 is a transporter for the efflux of cholesterol out of the cell when there is an excess of cholesterol in the cell and ABCA1 upregulation in response to excess loading of cholesterol is mediated by sterol-activated nuclear receptors, LXRs (Ignatova *et al*, 2013). Aside from the cholesterol-LXRs regulatory axis, it was recently reported that ABCA1 expression in epithelial cells is regulated via FOXO3a and c-Myc (Frechin *et al*, 2015). Activation of FAK/PI3K/Akt signaling depending on cell crowding state downregulates ABCA1 expression via phosphorylation of FOXO3a, a positive transcriptional regulator of ABCA1 (Frechin *et al.*, 2015). We found that phosphorylation level of FOXO3a at S453 is lower in EpH4-Snail cells than in wild-type EpH4 cells (Fig. S3A). However, since downregulation of Akt signaling by treatment with inhibitors of its activators GSK2334470 (PDK1), LY294002 (PI3K) and Wortmannin (PI3K) did not induce ABCA1 expression in wild-

type EpH4 cells (Fig. S3A), FOXO3a would not be responsible for ABCA1 expression in EpH4-Snail cells. Although ABCA1 expression is also downregulated by c-Myc in breast cancer, no remarkable difference of c-Myc expression was observed between EpH4 and EpH4-Snail cells, indicating that c-Myc is not likely involved in ABCA1 expression in EpH4-Snail cells (Fig. S3B). Therefore, we next pursued in detail the possibility that LXRs induce ABCA1 in EpH4-Snail cells. Immunofluorescence microscopy revealed that LXRs are highly accumulated in the nuclei of EpH4-Snail cells, suggesting that ABCA1 upregulation could be mediated by LXRs (Fig. 2A). Consistently, treatment with an LXR inhibitor GSK2033 abolished ABCA1 expression to an undetectable level in EpH4-Snail cells (Fig. 2B). Since transcriptional activity of LXRs are regulated by cellular cholesterol level, we examined whether ABCA1 is expressed in response to high cholesterol accumulation in the cells. Inhibition of cholesterol biosynthesis by simvastatin, an inhibitor of the rate-limiting enzyme of cholesterol biosynthesis 3-hydroxy-3-methylglutaryl-coenzyme A reductase, remarkably decreased ABCA1 expression in EpH4-Snail cells but residual ABCA1 was still detected (Fig. 2C, normal FBS). In contrast, treatment with simvastatin in the medium supplemented with lipid-free FBS completely abolished ABCA1 expression (Fig. 2C, lipid-free FBS). Together with the finding that incubation in lipid-free FBS medium alone did not markedly decrease ABCA1 expression, these results suggest that cholesterol biosynthesis is the primary source of excess accumulation of cholesterol in EpH4-Snail cells, but cholesterol uptake from media also significantly contribute to the accumulation as a secondary source.

These findings imply dysregulation of cholesterol metabolism in EpH4-Snail. Filipin staining revealed that cholesterol was prominently accumulated in intracellular

structures in EpH4-Snail cells, whereas it localized primarily in the plasma membrane of wild-type EpH4 cells (Fig. 2D). Since excess cholesterol can be stored in lipid droplets, we stained cells with Lipi-Green to visualize lipid droplets. We found that lipid droplets were significantly enlarged in EpH4-Snail (Figs. 2, E and F). We next compared the total cholesterol levels in EpH4 cells and EpH4-Snail cells; the cellular amount of cholesterol slightly increased in EpH4-Snail cells, although the difference was not significant (Fig. 2G). These results suggest that the accumulation of cholesterol in lipid droplets in EpH4-Snail cells was not due to an increase in the amount of cholesterol, but to an abnormality in the subcellular distribution of cholesterol. Collectively, dysregulation of intracellular distribution of cholesterol activates LXR to induce ABCA1 expression in hybrid E/M cells.

Downregulation of VLCFA-SM synthesis induces Chol/SM imbalance in hybrid E/M cells

A large proportion (30-50%) of cellular cholesterol is associated with sphingomyelin (Das *et al*, 2014). Therefore, we next examined the possibility that sphingomyelin levels are altered in EpH4-Snail cells. We found that the sphingomyelin content relative to total phospholipids decreased in EpH4-Snail cells (Fig. 3A). To investigate the SM profile in detail, we next analyzed the fatty acid composition of SM using LC-MS. In EpH4-Snail cells, the fractions of VLCFA-SMs (22:1, 22:0 and 24:1) were significantly decreased whereas those of LCFA-SMs (14:0, 16:1 and 16:0) were rather increased (Fig. 3B), resulting in a prominent decrease in the total VLCFA-SM

fraction (Figs. 3, C and D). These results suggest that decrease in VLCFA-SMs are responsible for decrease in total SM content in EpH4-Snail cells.

Fatty acid composition of SM is mainly modulated by substrate specificity of two families of enzymes, Elovls and CerSs, that respectively catalyze the rate-limiting step of the fatty acid elongation cycle and the N-acylation of sphingoid bases to produce ceramides (Fig. 3E) (Levy & Futerman, 2010; Sassa & Kihara, 2014). Therefore, we hypothesized that transcriptional repression of genes from these families causes the decrease in VLCFA-SM content in EpH4-Snail cells. RT-PCR analyses identified ELOVL7 and CERS3 as genes which are completely repressed specifically in EpH4-Snail cells (Fig. 3F). Consistent with our hypothesis, Elovl7 and CerS3 control VLCFA-SM production (Fig. 3E). Collectively, exogenous expression of Snail represses transcription of VLCFA-SM-producing enzymes and results in a decrease in the amount of VLCFA-SM. Consequently, the ratio of cholesterol to SM (Chol/SM ratio) in the cells significantly increased in EpH4-Snail cells (Fig. 3H), suggesting that a substantial amount of cholesterol exist in a sphingomyelin-unbound form in EpH4-Snail.

Since ABCA1 expression is not aligned with either the loss of epithelial state (E-cadherin and α-catenin KO) or a non-epithelial basal state (fibroblasts), upregulation of ABCA1 is characteristic to the hybrid E/M cells expressing ectopic Snail (Fig. 3I). To investigate the mechanism underlying this difference in ABCA1 expression, we again compared Chol/SM ratio in EpH4-Snail and normal fibroblast cell lines L-929 and NIH3T3. Chol/SM ratio of these fibroblasts were slightly but not significantly higher than that of EpH4 (Fig. 3I). The cholesterol content in NIH-3T3 cells is significantly higher than in EpH4 cells (Fig. S2). Consequently, normal fibroblast, would synthesize more SMs to counteract the cholesterol load imposed by the decrease in VLCFA-SMs

and stabilize the Chol/SM ratio. By contrast, hybrid E/M cells are unable to respond appropriately to the harmful imbalance between cholesterol and SM due to the incomplete activation of the mesenchymal transcriptome.

To examine whether the above findings apply to human cancer cells, we used two esophageal squamous cell carcinoma cell lines, TE-15 and TE-8, that exhibit epithelial and hybrid-E/M phenotype, respectively (Fig. 3J) (Nishihira *et al*, 1993). As expected, expressions of ABCA1 and Snail were cooperatively upregulated in TE-8 cells (Fig. 3J). Consistent with our findings in the EpH4-Snail system, Chol/SM ratio was significantly higher in TE-8 cells than in TE-15 cells (Fig. 3K). Together with the results of mouse cell lines, normal epithelial and fibroblast cells maintain Chol/SM ratio around 1.0-1.5, and ABCA1 expression as a response to the excess Chol is induced when Chol/SM ratio exceed 1.5 (Fig. 3H). Notably, the difference in either cholesterol or SM contents between TE-15 and TE-8 cells, and cholesterol content between EpH4 and EpH4-Snail is not significant when each are compared alone (Fig. 2D and Fig. S4), indicating the importance of considering their functional cooperativity, i.e. to analyze the Chol/SM ratio.

Inhibitor of cholesterol esterification selectively inhibits growth of Snail-positive kidney cancer cells

Finally, we examined the possibility of targeting the imbalance of Chol/SM ratio in Snail-positive cancer cells to selectively inhibit their growth. As discussed above, hybrid E/M cells of high Chol/SM ratio must either export cholesterol out of cells or isolate it within cells in lipid droplets as cholesteryl esters to avoid the cytotoxic effects

of free cholesterol. Thus, inhibiting the means of clearing excess cholesterol could specifically target cells in the hybrid E/M state for self-extermination. Cholesterol efflux from cells is mainly dependent on ABCA1, and esterification of cholesterol is catalyzed by acyl-CoA:cholesterol acyl transferases (ACATs) (Fig. 4A) (Song *et al*, 2021). According to our findings above, hybrid E/M cells activate both cholesterol efflux and isolation system to handle excess cholesterol. Therefore, we examined the effect of ABCA1 inhibitor CsA and ACAT inhibitor TMP-153 on the EpH4-Snail model and renal carcinoma cells. While CsA non-selectively inhibited growth of all cell lines tested (Figs. 4B and 4D), TMP-153 selectively inhibited growth of Snail-positive cells (EpH4-Snail, 786-O, A498 and Caki-1; Figs. 4C and 4E). Thus, sequestration of cholesterol via esterification by ACATs is the primary mechanism for handling excess cholesterol to avoid cell death in these cells. Notably, TMP-153 treatment alone did not provide this selectivity between esophageal cancer cell lines, but combination of TMP-153 and cyclosporin A selectively inhibited growth of the Snail-positive TE-8 cells (Figs. S5, A-C). Collectively, these results suggest that inhibition of cholesterol efflux and cholesterol esterification selectively inhibit growth of cells stably in a Snail-induced hybrid E/M state.

We also investigated the potential of ACAT inhibitors as anti-tumor agents in vivo using xenograft models. We successfully transplanted the 786-O line into nude mice among Snail-positive ccRCC cell lines. Using this system, we found that TMP-153 treatment significantly reduced 786-O tumor volume in vivo (Figs. 4, F-H). Moreover, the combination of TMP-153 and cyclosporin A also inhibited the growth of TE-8 tumor xenografts (Figs. S5D-F). Together with the in vitro assays (Figs. 4, B-J and Figs. 5, A-C), we propose that cholesterol clearance systems, particularly ACATs,

can be used as molecular targets to selectively inhibit the growth of Snail-positive cancers.

Discussion

In recent years, it has been revealed that the expression of EMT-TFs, such as Snail, induces cancer cells to acquire mesenchymal characteristics while retaining epithelial traits, transforming them into so-called hybrid E/M cells. Hybrid E/M cells have been demonstrated to exhibit the highest plasticity and malignancy (Aiello *et al*, 2018; Pastushenko *et al*, 2018). Moreover, Snail expression contributes to cancer stemness and resistance to chemotherapy (Lambert & Weinberg, 2021). To date, molecular mechanisms connecting to EMT-TFs to chemoresistance remain to be established. Here we identify lipid metabolic imbalance-mediated ABCA1 expression as a mechanism of chemoresistance induced by Snail. We found reduced VLCFA-SM biosynthesis in Snail-positive cells with hybrid E/M phenotypes, resulting in high Chol/SM ratio. This should be detrimental in theory since it increases exposure to the toxic effect of free cholesterol but cancer cells deploy countermeasures: upregulating ABCA1 to move cholesterol out of cells and sequestering esterified cholesterol in the form of lipid droplets. Notably, it has been also reported that not only ABCA1 (Iwasaki *et al.*, 2010; Wang *et al*, 2021) but also enhanced lipid droplet formation triggers chemoresistance to various drugs (Cotte *et al*, 2018; Hultsch *et al*, 2018; Schlaepfer *et al*, 2012). Therefore, dietary or pharmacological control of cholesterol and also sphingolipids would be a new strategy to overcome resistance to chemotherapy.

The aberrant activation of EMT-TFs is implicated in multiple crucial steps in cancer progression, prompting efforts to explore therapeutic approaches aimed at suppressing EMT-TF functions in cancer cells (Davis *et al.*, 2014). However, as EMT-TFs are predominantly located in the nucleus, no effective treatment methods, including antibody drugs, have been established thus far. Therefore, instead of directly targeting EMT-TFs, focusing on the metabolic network vulnerabilities specific to hybrid E/M cells may prove to be an effective therapeutic strategy. In the present study, we found increased sensitivity of Snail-positive cells to toxicity of free-cholesterol accumulation induced by the ACAT inhibitor TMP-153. Notably, kidney cancer growth could be suppressed by inhibiting cholesterol esterification alone while esophageal cancer required the additional downregulation of ABCA1, indicating varying dependence on free cholesterol clearance strategies among cancers of different primary origin. Other cancers might employ alternative pathways to evade cytotoxic effects of excess cholesterol and identifying these traits will pave the way for the development of novel strategies in targeted molecular therapeutics against hybrid E/M cancers. An intriguing corollary here is the potential utility of ABCA1 expression as a diagnostic marker to identify cancers that would be sensitive to targeting the Chol/SM imbalance.

Although various metabolic changes in cancer cells focusing on energy sources including sugars, amino acids and fatty acids have been intensely studied (Pavlova *et al*, 2022), alteration of sphingolipid homeostasis driven by EMT-TFs remains largely unknown. We found both quantitative and qualitative alteration of sphingomyelin profile: decrease in the total SM amount and the VLCFA-SM percentage. This alteration is attributed to transcriptional repression of enzymes for biosynthesis of VLCFA-SMs, CerS3 and Elovl7. Genes whose transcription is repressed by Snail

include those encoding proteins directly involved in epithelial adhesive structures, such as E-cadherin and Claudins. Hence, it is not surprising that VLCFA-SMs play important roles cooperatively with the adhesion molecules. Indeed, cholesterol accumulation is required for tight junction formation, and VLCFA-SMs are enriched in the junctional region (Shigetomi *et al*, 2018; Shigetomi *et al*, 2023). Studies focusing on the VLCFA-synthetic enzymes would provide a new layer of knowledge about mechanisms underlying the establishment of epithelial structures and functions. Such studies would also contribute to understanding how dysfunctions in lipid metabolisms promote cancer malignancy and tissue fibrosis as a consequence of EMT. The regulation of cell adhesion by fine-tuning of lipid composition should be reversible and therefore would explain the two conflicting events: the invasion and metastasis process and the viability process at the metastatic site.

Materials and methods

Cells and reagents

Cells were grown in DMEM supplemented with 10% FBS under 5% CO_2 at 37°C. Mouse Snail-expressing EpH4 cells (EpH4-Snail)(Ikenouchi *et al.*, 2003), E-cadherin KO and α-catenin KO EpH4 cells (Shigetomi *et al.*, 2018) were generated previously. EpH4-Snail cells were maintained in medium supplemented with 500 μg/ml G418 (Wako Pure Chemical). For KRAS G12V expression in MDCK II cells, pLVET-HA-K-RasG12V-IRES-GFP (Addgene Plasmid #107140) was transduced using the lentiviral system, and GFP-positive cells were collected using a cell sorter Sony SH-800. Nitidine chloride was purchased from Selleck Biotech, cyclosporin A from Wako, and TMP-153

from Cayman Chemical, filipin complex from *Streptomyces filipinensis* from Sigma-Aldrich, Lipi-Green from Dojindo Laboratories.

The following primary antibodies were used for immunoblotting, immunofluorescence microscopy and immunohistochemistry: Rabbit anti-ABCA1 mAb (96292S, IB) and rabbit anti-Snail mAb (3879S, IB) from Cell Signaling Technology; Rabbit anti-ABCA1 pAb (NB400-105, IHC) from Novus Biologicals; Rat anti-E-cadherin mAb (ECCD-2, IB) from TaKaRa; Goat anti-LXR alpha + LXR beta pAb (ab24362, IF) from Abcam; Mouse anti-α-tubulin (12G10, IB) produced in house.

The following secondary antibodies were used: HRP-conjugated anti-rat IgG (HAF005) and anti-mouse IgG (HAF007) from R&D Systems; HRP-conjugated anti-rabbit IgG (4030-05) from Southern Biotech; Cy3-conjugated anti-goat IgG (705-165-147) from Jackson Immunoresearch Laboratories.

RT-PCR

Total RNAs were prepared with RNeasy Mini Kit (Qiagen) and the retrotranscribed with Superscript III First-Strand Synthesis System (Invitrogen). RT-PCR was then performed using KOD FX (TOYOBO) following manufacturer's instructions. Primer sequences used are shown in Table S1.

Lipid analysis

Total lipids were extracted from cultured cells by Bligh-Dyer's method, dissolved in methanol/chloroform = 1/1 (v/v). Phospholipid content was determined by phosphate assay. SM and Chol content were determined using Sphingomyelin assay kit STA-601 (Cell Biolabs) and Amplex Red Cholesterol Assay Kit (Invitrogen), respectively,

according to the manufacturer's instructions. Fatty acid profile of SM was determined with LC-MS as described previously[32]. Briefly, electrospray ionization mass spectrometry analysis was performed on a 6420 triple-quadrupole liquid chromatography–mass spectrometer (Agilent Technologies) equipped with an HPLC system and an auto sampler (Infinity 1260; Agilent Technologies). The extracted lipids were directly subjected to electrospray ionization mass spectrometry analysis. The mobile phase composition was acetonitrile/methanol/water = 18:11:1 (0.1% ammonium formate). The flow rate was 4 µl/min. The mass range of the instrument was set at 650–950 *m/z*. *m/z* profiles of SM were extracted according to total ion chromatogram patterns, and all peaks corresponding to $[M+H]^+$ ions of d18:1-even chain length fatty acid-SMs were subjected to analyses.

Fluorescence microscopy

To visualize subcellular localization of cholesterol, cells cultured in glass-bottom dishes were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 50 µg/ml filipin prepared in PBS for 30 min. To visualize lipid droplets, live cells were washed once with HBSS and stained with Lipi-Green (Dojindo) for 20 min at 37°C. Cells were washed twice with HBSS and observed on a heating stage at 37°C. For immunofluorescence microscopy, cells cultured on coverslips were fixed with 4% paraformaldehyde prepared in PBS for 15 min at RT and permeabilized with 0.1% Triton X-100 prepared in PBS. Fixed cells were blocked with 1% BSA prepared in PBS for 1 h at RT. Cells were incubated with primary antibodies for 1 h at RT and secondary antibodies for 1 h at RT. Antibodies were prepared in the blocking solution. Fixed cells were observed at RT. All observation was performed with a confocal

microscope (Carl Zeiss LSM900) equipped with Plan-APO (63×/1.40 NA, oil immersion) objective. Images were acquired using Carl Zeiss Zen 3.4 software. Images were analyzed using ImageJ/Fiji.

Immunoblotting

Whole cells were lysed with SDS sample buffer and samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim-milk prepared in TBS-T, membranes were incubated with primary antibody for 1 h at RT or overnight at 4°C. Membranes were then washed and incubated with HRP-conjugated secondary antibody for 1 h at RT. Chemiluminescence signal was detected using a LAS4000mini imaging system (Fujifilm) and images were analyzed using ImageJ/Fiji.

Growth inhibition assay

Cells cultured in 96-well microtiter plates (2×10^3 cells/well, 48 h) were subjected to treatments with nitidine chloride, cyclosporin A, TMP-153 or their combination dissolved in DMSO for 24 h at 37°C. Final DMSO concentrations were less than 0.25%. After treatment, metabolically active cell number was determined using Cell Counting Kit-8 (Dojindo) according to manufacturer's instructions. Briefly, 10 µl of CCK-8 solution was directly added to each well containing 100 µl culture medium and cells were incubated 1 h at 37°C. Reaction was stopped by adding 10 µl of 1% sodium dodecyl sulfate prepared in MQ and plates were put at 4°C to avoid additional reaction. Absorbance of solution at 450 nm were measured using a spectrophotometer (Thermo GENESYS 10S UV-Vis). Data were expressed as relative to 0.25% DMSO control.

Immunohistochemistry

Formalin-fixed and paraffin-embedded surgical specimens were cut into 3 μm sections. These sections were immersed in antigen retrieval solution (pH 9.0) (Nichirei Bioscience Inc.) and heated in a pressure chamber. After that, the sections were incubated with the primary antibodies (rabbit anti-ABCA1 pAb, NB400-105) and later incubated with HRP-labeled goat anti-rabbit secondary antibodies (Nichirei Bioscience Inc.). Immunoreactivity was visualized by using 3–3 Diaminobenzidine Dab Substrate Kits (Nichirei Bioscience Inc.). Informed consent for experimental use of the samples was obtained from the patients in accordance with the hospital's ethical guidelines.

Tumor xenograft model

Four-week-old female athymic nude mice were purchased from Japan SLC and housed in a specific pathogen-free facility. The animal experiments were approved by the ethics committee of Aichi Medical University and performed according to the established guidelines.

To initiate xenografting of 786-O, 2×10^6 cells in 200 μL DMEM were subcutaneously injected into the back of mice by using a 27 G needle. After 7 days, when the inoculated tumor became visible, the mice were randomly divided into treatment and control groups, and treatment was initiated (day 0). TMP-153 (50 mg/kg body weight) or vehicle (0.5% hydroxypropyl methylcellulose with 0.1% polysorbate 80) was intraperitoneally administered to each mouse on days 0, 4, 7, 11, 14, and 18 to each mouse. The tumor volume was measured every week and calculated using the modified ellipsoid formula ($1/2 \times \text{length} \times \text{width}^2$). The xenografting of TE-8 was

performed with some modifications. For this, 1×10^7 cells were injected. A combination of TMP-153 and cyclosporin A (both at 10 mg/kg body weight) or vehicle was intraperitoneally administered to each mouse on days 0 and 4. Finally, the tumor volume was measured 36 days after the first administration.

Statistical analysis

Microsoft Excel for Microsoft 365 MSO 2306 and GraphPad Prism v8.4.1 were used for analyses and displays of quantitative data. Data are expressed as mean with each point or error bars representing standard deviations (SD). Unpaired two-tailed Student's *t*-test, Dunnett's multiple comparison test and Welch's *t*-test are performed using Prism. Unless otherwise noted, *n* denotes biological replicates.

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Author contributions

A.M. performed most of experiments and analyzed the data. A.I. designed and performed in vivo experiments with clinical specimens and tumor xenografts. W.H. made pathological diagnoses and provided clinical specimens. T.K. performed surgery and managed patient information. K.O. performed pathological analysis. A.M. and J.I. designed research and wrote the paper.

Declaration of interests

There are no competing interests to declare.

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Figure legends

Figure 1

Ectopic expression of Snail confers epithelial cells nitidine chloride resistance via induction of ABCA1 expression

(**A**) Enhancement of growth inhibitory effect of nitidine chloride (NC) on human renal carcinoma cells by co-treatment with an ABCA1 inhibitor cyclosporin A (CsA). Cells were grown with or without 20 μM NC and 10 μM CsA for 24 h, and cell number was determined with CCK-8. Fold change in cell number by NC was shown as relative to controls without NC. Results are shown as mean of at least four independent experiments ± SD (Tukey-Kramer's multiple comparison test: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

(**B**) Immunoblot analysis of whole cell lysate of human renal carcinoma cell lines.

(**C**) Comparison of ABCA1 expression between normal tissue and primary tumor of indicated subtypes of renal cancers analyzed using UCSC Xena. Bars indicate means (Welch's *t*-test: ***, $p<0.001$; ****, $p<0.0001$).

(**D**) Immunohistochemistry of a surgically extracted renal tissue from a patient with Fuhrman grade 3 primary ccRCC, indicating upregulation of ABCA1 in the lesion site. The ABCA1 staining was verified by the accumulation of known signals in renal tubules and glomeruli in normal tissue (Yang *et al*, 2010). Scale bars, 250 μm (left), 50 μm (right).

(**E**) Phase contrast images of EpH4 wild-type cells (top) and Snail-overexpression cells (bottom, EpH4-Snail). Scale bar, 50 μm.

(**F**) Immunofluorescence images of EpH4 and EpH4-Snail cells. Scale bars, 20 μm.

(**G**) Acquisition of NC resistance by exogenous expression of Snail in EpH4 cells and enhancement of NC effect on EpH4-Snail cells by co-treatment with CsA. Shown are means of three independent experiments ± SD (Tukey-Kramer's multiple comparison test: *, $p<0.05$; ****, $p<0.0001$).

(**H-J**) Immunoblot analyses of whole cell lysates of (H) EpH4, EpH4-Snail, E-cadherin KO and α-catenin KO EpH4 cells, (I) MDCK II cells expressing KRAS G12V treated with 5 μg/ml TGFβ for 0, 3 and 8 days and (J) human esophageal carcinoma cell lines TE-15 and TE-8. Phase contrast images are also shown on top (J). Scale bars, 50 μm.

Figure 2

Alteration of cellular cholesterol distribution induces ABCA1 expression in hybrid E/M cells

(**A**) Immunofluorescence images of cells stained with the LXRα+β antibody. Scale bars, 10 μm.

(**B**) Immunoblot analyses of whole cell lysates of EpH4-Snail cells treated with 1 μM GSK-2033 for 24 h.

(**C**) Immunoblot analyses of whole cell lysates of EpH4-Snail cells treated with indicated concentration of simvastatin or 0.1% DMSO ('0' μM) in DMEM supplemented with 10% normal or lipid-free fetal bovine serum (FBS) for 48 h.

(**D**) Subcellular distribution of Chol stained with filipin. Scale bar, 10 μm.

(**E**) Live cell images of lipid droplets (LDs) visualized by Lipi-Green dye.

(**F**) Quantification of LD signal per cell. The Lipi-Green signals of all optical sections were summed and divided by the number of cells counted using DAPI images. Shown are means of three different fields of view ±SD.

(**G**) Comparison of cholesterol (Chol) content relative to phospholipid concentration. Shown are means of three biological replicates ± SDs. (F,G) *p*-value of Student's *t*-test are shown.

Figure 3

Effects of Snail-induced EMT on sphingomyelin profile

(**A**) Comparison of sphingomyelin (SM) content relative to phospholipid content.

(**B**) Comparison of cholesterol (Chol)/SM ratio between EpH4 and EpH4-Snail cells.

(**C**) Fatty acid composition of SM determined using LC-MS. All peaks corresponding to d18:1-even fatty acid-SMs were included in the analysis and each peak value is expressed as a percentage. n.d., peak not detected.

(**D**) Pie charts of SM chain length profile from c. SMs were classified into long-chain fatty acid (LCFA, orange) (≤20) and very long-chain fatty acid (VLCFA, purple) (>20) SMs.

(**E**) Chain length specificity of Elovls and CerSs responsible for fatty acid profile of sphingolipids reported (Levy & Futerman, 2010; Sassa & Kihara, 2014). Elovls catalyze a rate-limiting step of fatty acid elongation cycle, and CerSs catalyze *N*-acylation of sphingoid bases to produce ceramides.

(**F**) Comparison of transcription levels of *ELOVL*s and *CERS*s. Notable decreases in expressions of *ELOVL7* and *CERS3* in EpH4-Snail cells are indicated by asterisks.

(**G**) Immunoblot analyses of whole cell lysates of mouse epithelial (MTD-1A, CSG) and fibroblast (L-929, NIH3T3) cells.

(**H**) Comparison of Chol/SM ratio of EpH4, EpH4-Snail and normal fibroblasts.

(**I**) Comparison of Chol/SM ratio between TE-15 and TE-8.

(**J**) Scatter plot of Chol content against SM content used in B, H and I. Dashed lines indicate Chol/SM = 1.0 (blue) and 1.5 (red). The region of Chol/SM > 1.5 is shown with red background.

(**K**) Possible mechanism of LD enlargement induction through decrease in SM. In EpH4, a certain amount of Chol is sequestered through interaction with SM. In EpH4-Snail, SM-unbound form of Chol is increased, detected by cellular cholesterol sensors, internalized and stored in LDs. In A, B, C, H and I, data are means of three independent experiments ± SDs (Student's *t*-test (A,B,C,I) or Dunnett's test (H): n.s., $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 4

Selective growth inhibition of Snail-positive cells by an ACAT inhibitor TMP-153

(**A**) Schematic image of molecular mechanisms for handling excess cholesterol to avoid cell death. Cells eliminate excess cholesterol through efflux mediated by transporters including ABCA1 or isolation into lipid droplets (LD) via esterification of cholesterol by acyl-CoA: cholesterol acyltransferases (ACATs) to avoid cell death. Cyclosporin A (CsA) and TMP-153 inhibits ABCA1 and ACATs, respectively.

(**B-E**)**,** Effects of treatments with cyclosporin A (B,D) or TMP-153 (C,E) to cellular growth of EpH4 and EpH4-Snail (B,C) or human kidney cancer cell lines (D,E). Cells were treated with drugs for 24 h and relative cell number to 0.1% DMSO control was determined by CCK-8. Results are shown as mean of at least three independent experiments ± SD.

(**F-H**) TMP-153 inhibits the growth of tumor xenografts composed of Snail-positive renal cancer 786-O cells. Experiments were performed as described in Materials and Methods. In brief, 50 mg/kg TMP-153 was administered intraperitoneally to nude mice bearing 786-O tumor xenografts. After 21 days of the first administration, the overall appearance (F) and the excised tumors (G) of control and treated mice were compared. (H) Tumor growth curve in the xenograft model. Results are shown as mean of four biological replicates ± SD (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$).

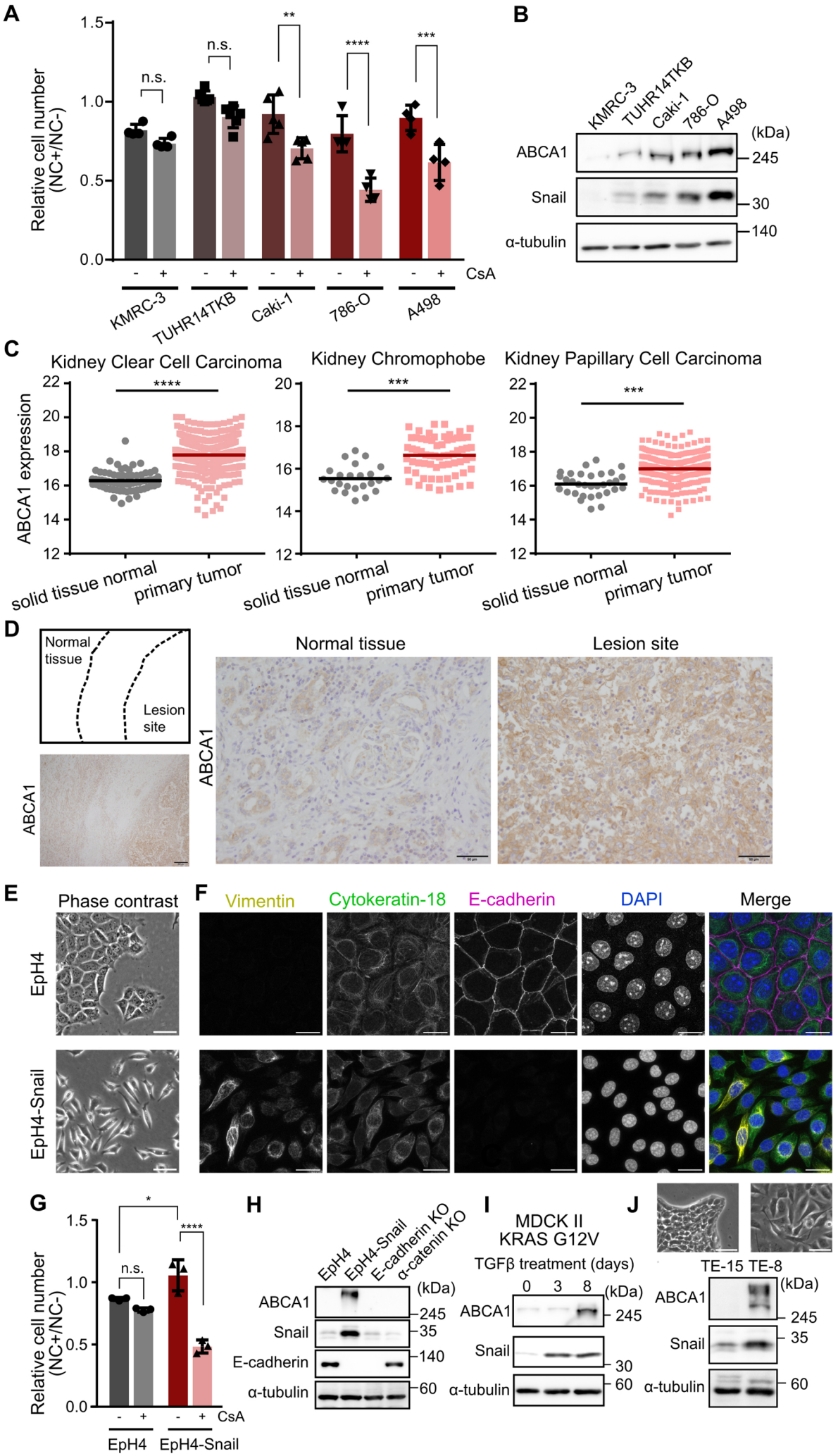

A
Relative cell number (NC+/NC-)
1.5
1.0
0.5
0.0
n.s.
n.s.
**
****
***
- + - + - + - + - + CsA
KMRC-3
TUHR14TKB
Caki-1
786-O
A498
B
KMRC-3
TUHR14TKB
Caki-1
786-O
A498
(kDa)
ABCA1
245
Snail
30
α-tubulin
140
C
Kidney Clear Cell Carcinoma
Kidney Chromophobe
Kidney Papillary Cell Carcinoma
ABCA1 expression
22
20
18
16
14
12
****
***
***
solid tissue normal
primary tumor
D
Normal tissue
Lesion site
ABCA1
Normal tissue
Lesion site
E
Phase contrast
EpH4
EpH4-Snail
F
Vimentin
Cytokeratin-18
E-cadherin
DAPI
Merge
G
Relative cell number (NC+/NC-)
1.5
1.0
0.5
0.0
*
****
n.s.
- + - + CsA
EpH4
EpH4-Snail
H
EpH4
EpH4-Snail
E-cadherin KO
α-catenin KO
(kDa)
ABCA1
245
Snail
35
E-cadherin
140
α-tubulin
60
I
MDCK II
KRAS G12V
TGFβ treatment (days)
0 3 8
(kDa)
ABCA1
245
Snail
30
α-tubulin
60
J
TE-15 TE-8
(kDa)
ABCA1
245
Snail
35
α-tubulin
60

Figure 1

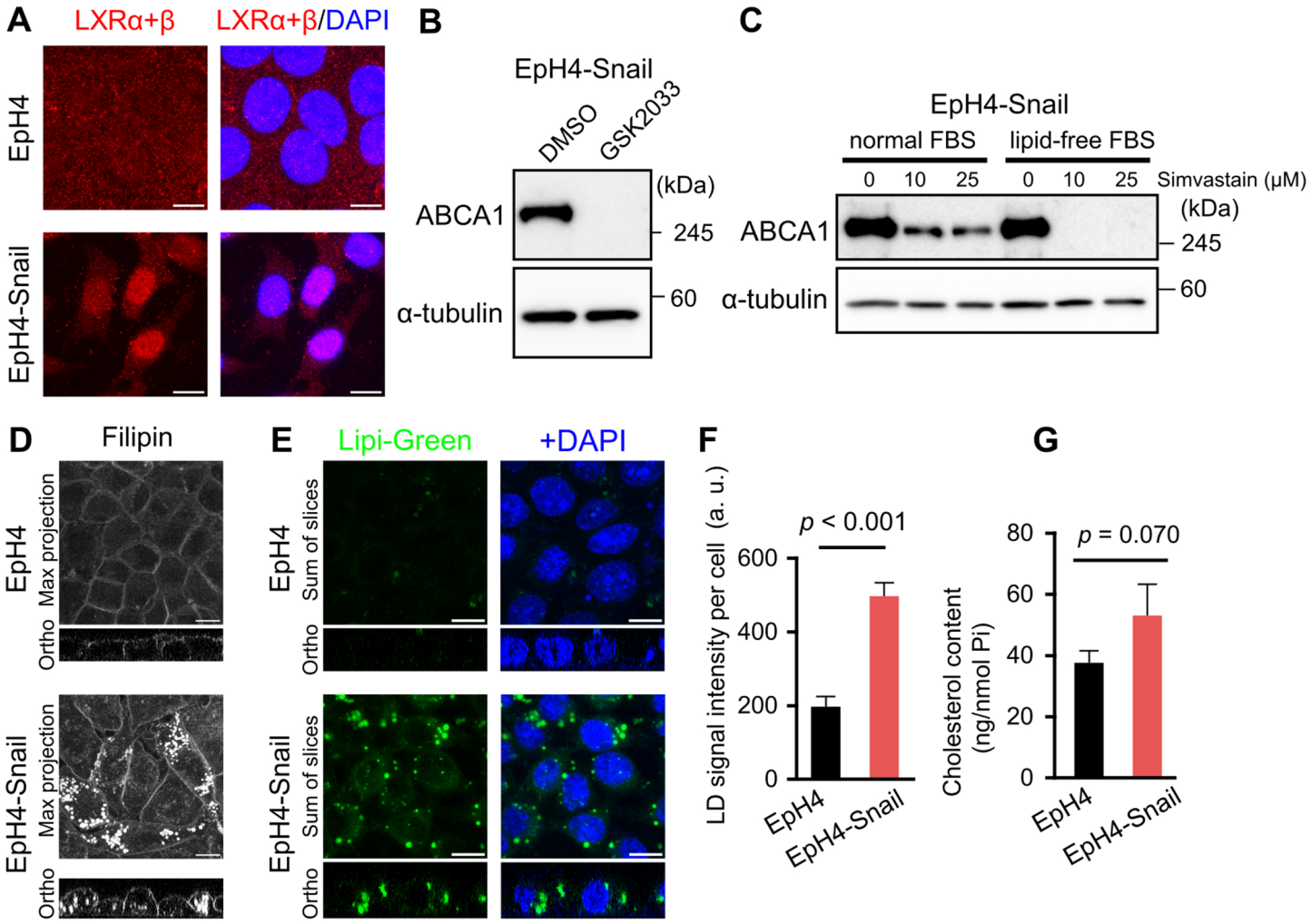

A
LXRα+β
LXRα+β/DAPI
EpH4
EpH4-Snail
B
EpH4-Snail
DMSO
GSK2033
(kDa)
ABCA1
245
α-tubulin
60
C
EpH4-Snail
normal FBS
lipid-free FBS
0
10
25
0
10
25
Simvastain (μM)
(kDa)
ABCA1
245
α-tubulin
60
D
Filipin
EpH4
Max projection
Ortho
EpH4-Snail
Max projection
Ortho
E
Lipi-Green
+DAPI
EpH4
Sum of slices
Ortho
EpH4-Snail
Sum of slices
Ortho
F
LD signal intensity per cell (a. u.)
p < 0.001
600
400
200
0
EpH4
EpH4-Snail
G
Cholesterol content (ng/nmol Pi)
p = 0.070
80
60
40
20
0
EpH4
EpH4-Snail

Figure 2

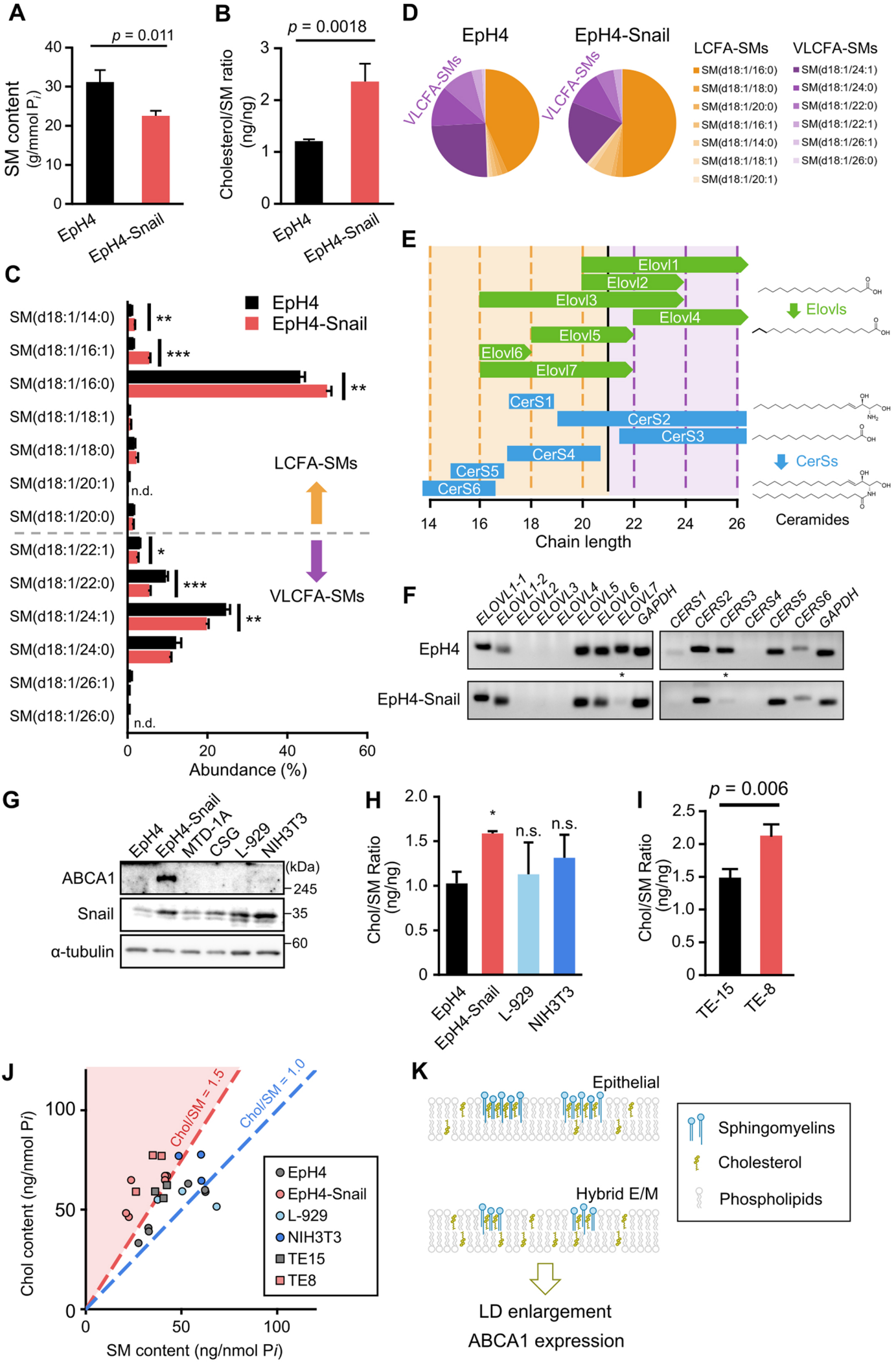

A
p = 0.011
SM content (g/mmol Pi)
EpH4
EpH4-Snail
B
p = 0.0018
Cholesterol/SM ratio (ng/ng)
EpH4
EpH4-Snail
D
EpH4
EpH4-Snail
VLCFA-SMs
LCFA-SMs
VLCFA-SMs
SM(d18:1/16:0)
SM(d18:1/18:0)
SM(d18:1/20:0)
SM(d18:1/16:1)
SM(d18:1/14:0)
SM(d18:1/18:1)
SM(d18:1/20:1)
SM(d18:1/24:1)
SM(d18:1/24:0)
SM(d18:1/22:1)
SM(d18:1/22:0)
SM(d18:1/26:1)
SM(d18:1/26:0)
C
EpH4
EpH4-Snail
SM(d18:1/14:0)
SM(d18:1/16:1)
SM(d18:1/16:0)
SM(d18:1/18:1)
SM(d18:1/18:0)
SM(d18:1/20:1)
SM(d18:1/20:0)
SM(d18:1/22:1)
SM(d18:1/22:0)
SM(d18:1/24:1)
SM(d18:1/24:0)
SM(d18:1/26:1)
SM(d18:1/26:0)
n.d.
LCFA-SMs
VLCFA-SMs
Abundance (%)
E
Elovl1
Elovl2
Elovl3
Elovl4
Elovl5
Elovl6
Elovl7
CerS1
CerS2
CerS3
CerS4
CerS5
CerS6
Elovls
CerSs
Ceramides
Chain length
F
ELOVL1-1
ELOVL1-2
ELOVL2
ELOVL3
ELOVL4
ELOVL5
ELOVL6
ELOVL7
GAPDH
CERS1
CERS2
CERS3
CERS4
CERS5
CERS6
GAPDH
EpH4
EpH4-Snail
G
EpH4
EpH4-Snail
MTD-1A
CSG
L-929
NIH3T3
ABCA1
Snail
α-tubulin
(kDa)
245
35
60
H
Chol/SM Ratio (ng/ng)
n.s.
n.s.
EpH4
EpH4-Snail
L-929
NIH3T3
I
p = 0.006
Chol/SM Ratio (ng/ng)
TE-15
TE-8
J
Chol/SM = 1.5
Chol/SM = 1.0
Chol content (ng/nmol Pi)
SM content (ng/nmol Pi)
EpH4
EpH4-Snail
L-929
NIH3T3
TE15
TE8
K
Epithelial
Hybrid E/M
Sphingomyelins
Cholesterol
Phospholipids
LD enlargement
ABCA1 expression

Figure 3

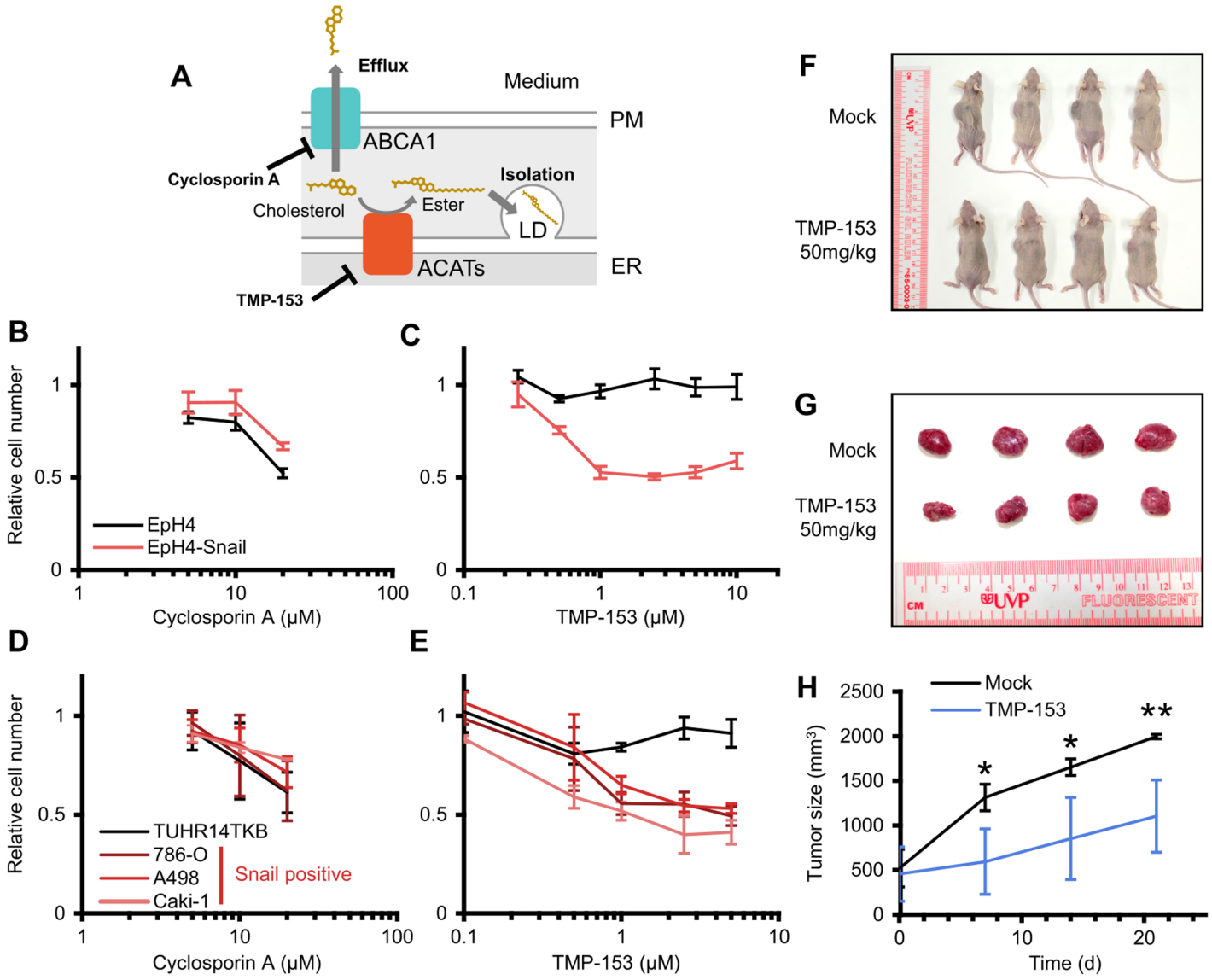
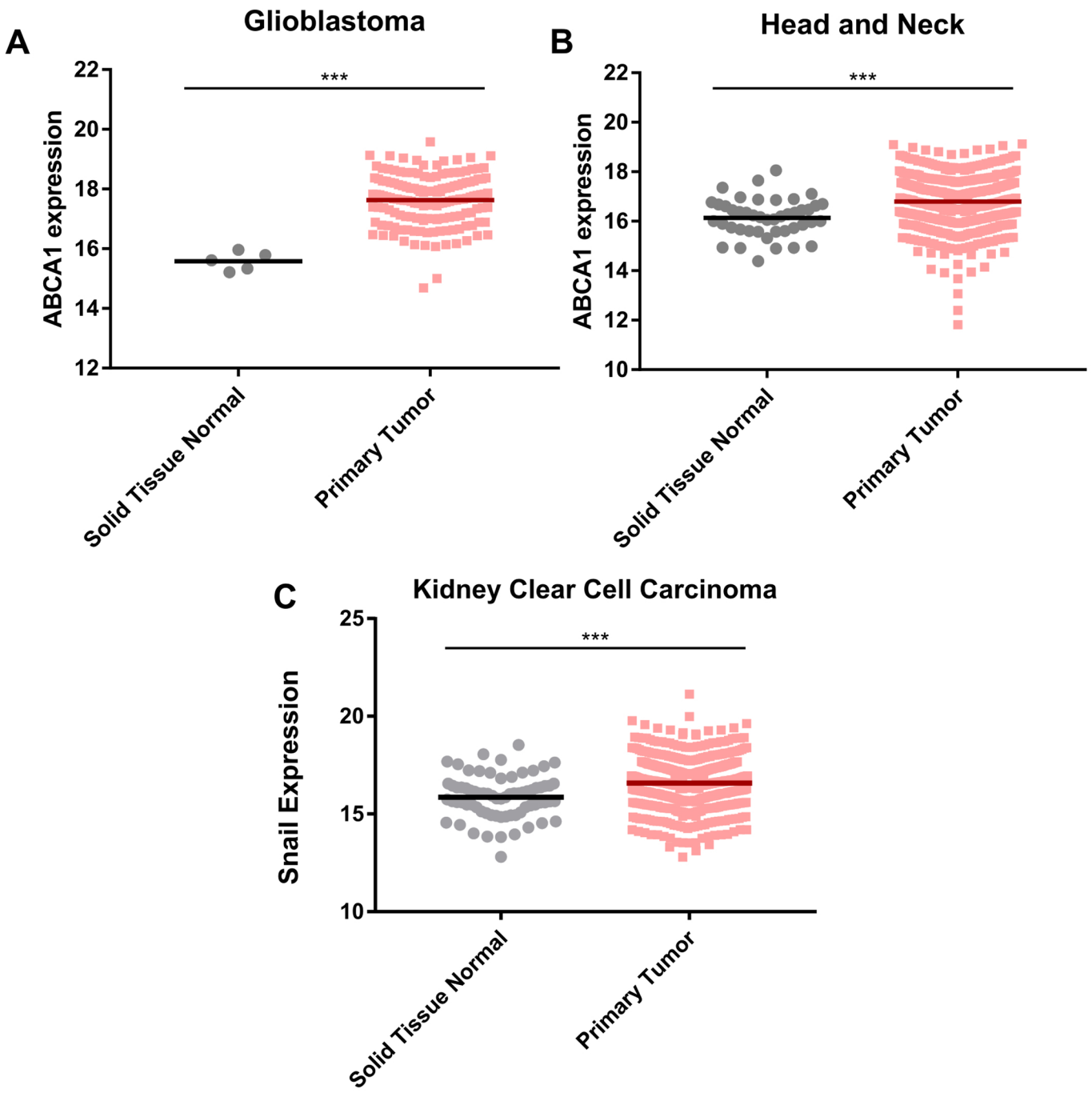

A
Efflux
Medium
PM
ABCA1
Cyclosporin A
Isolation
Cholesterol
Ester
LD
ACATs
ER
TMP-153
B
Relative cell number
EpH4
EpH4-Snail
Cyclosporin A (μM)
C
TMP-153 (μM)
D
TUHR14TKB
786-O
A498
Caki-1
Snail positive
E
F
Mock
TMP-153
50mg/kg
G
H
Mock
TMP-153
Tumor size (mm³)
Time (d)

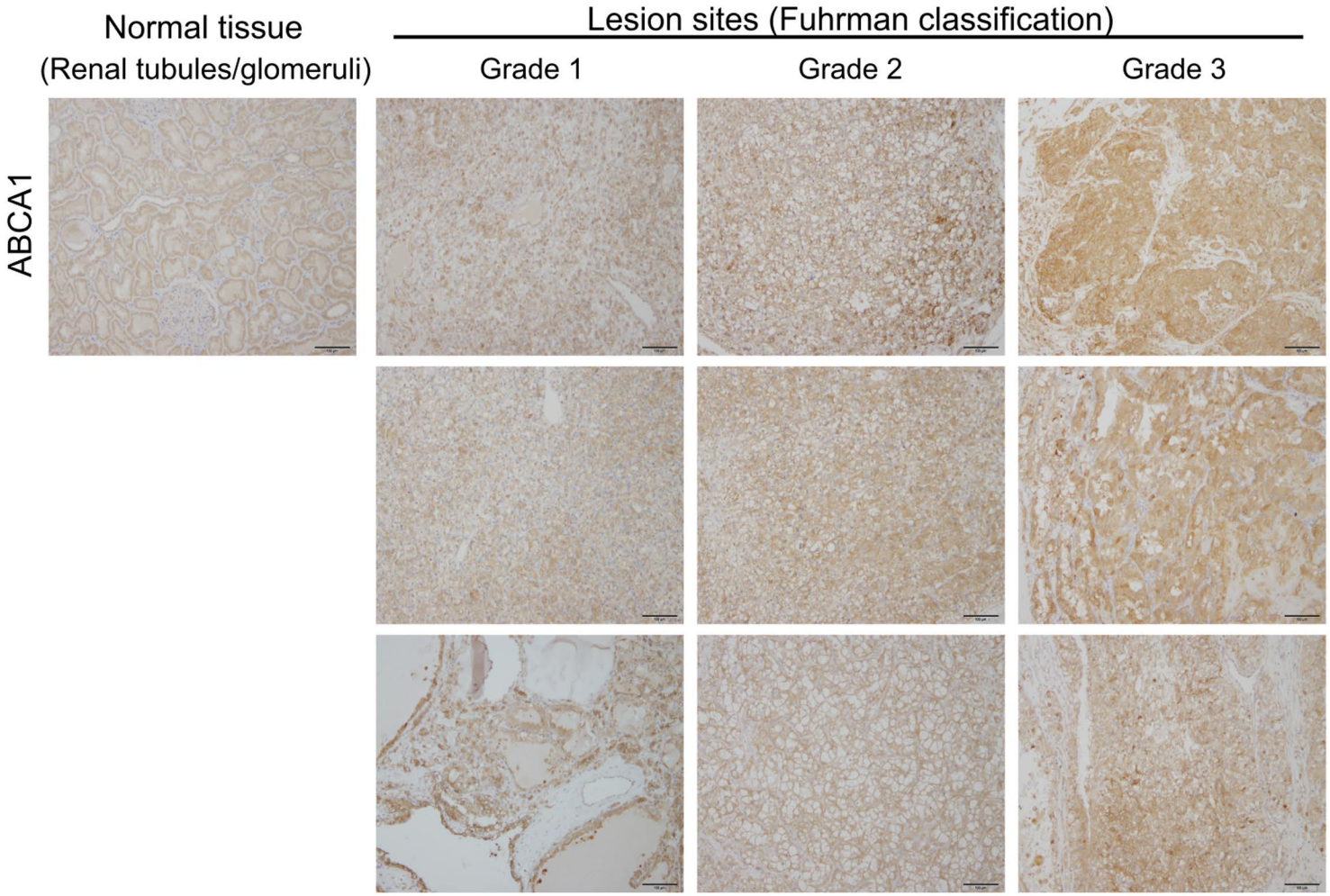
Figure 4



Supplementary Figure 1

Cancer types that exhibit tumor specific upregulation of ABCA1 transcription

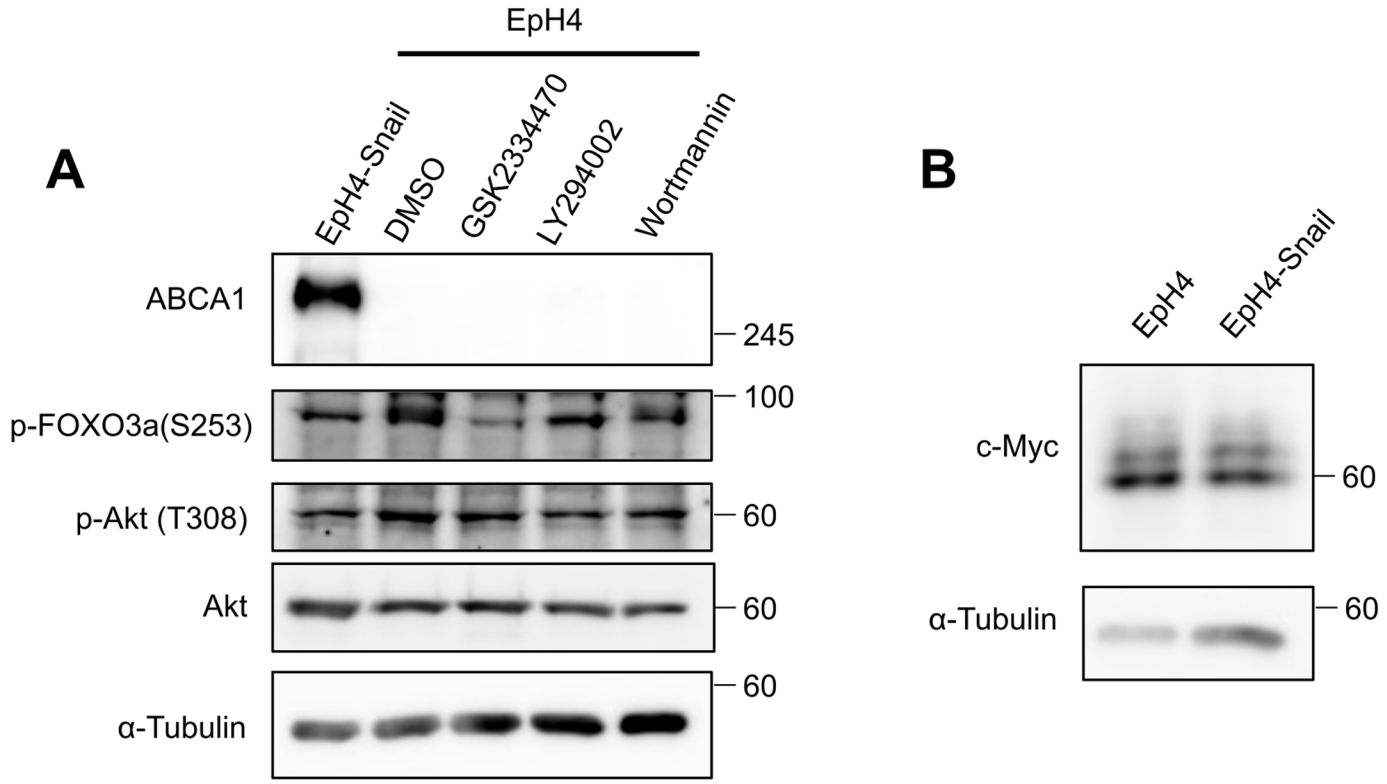
(**A-C**) Comparison of mRNA transcription of ABCA1 (A,B) and Snail (C) in different cancer types. Expression data from GDC TCGA database were analyzed using USCS Xena platform. Significant differences (***, $p < 0.001$) by Welch's *t*-tests are shown.



Supplementary Figure 2

Comparison of ABCA1 expression in different progression grades of kidney cancer cases

Immunohistochemistry of surgically extracted renal tissues from patients with Fuhrman grade 13 primary ccRCC, indicating elevation of ABCA1 signal at the lesion site of higher-grade ccRCC. All samples were collected from independent patients. The ABCA1 staining was verified by the accumulation of known signals in renal tubules and glomeruli in normal tissues (32). Scale bars, 100 μm.



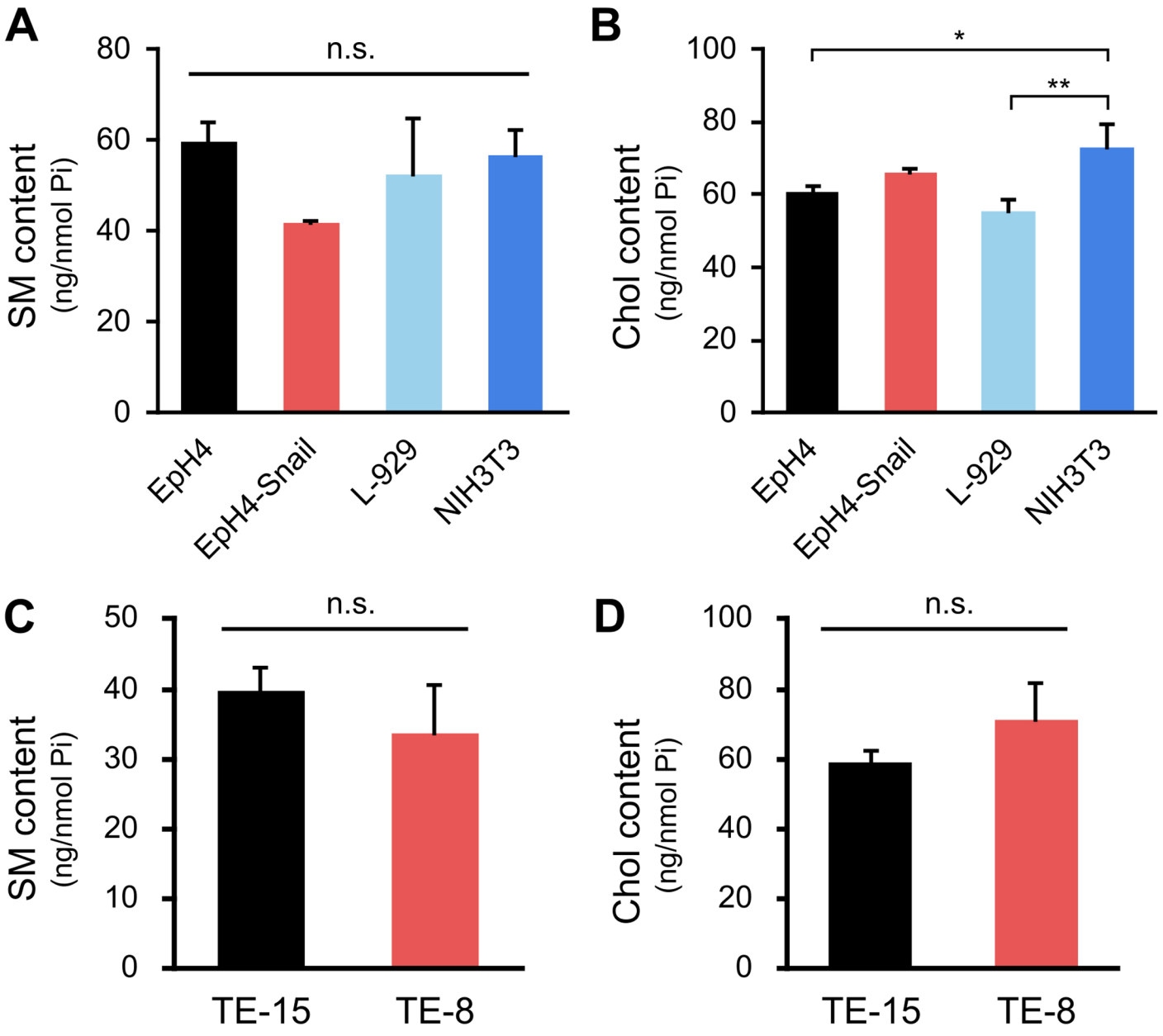
Supplementary Figure 3

Contribution of transcription factors involved in ABCA1 transcription in epithelial cells

(**A**) Immunoblot analysis of whole cell lysates of EpH4-Snail (without treatment) and EpH4 cells treated with inhibitors of PI3K-Akt pathway (5 μM GSK2334470: PDK1, 50 μM LY294002: PI3K, or 1 μM Wortmannin: PI3K for 24 h).

(**B**) Immunoblot analysis of whole cell lysates of EpH4 and EpH4-Snail cells.

Figure S3



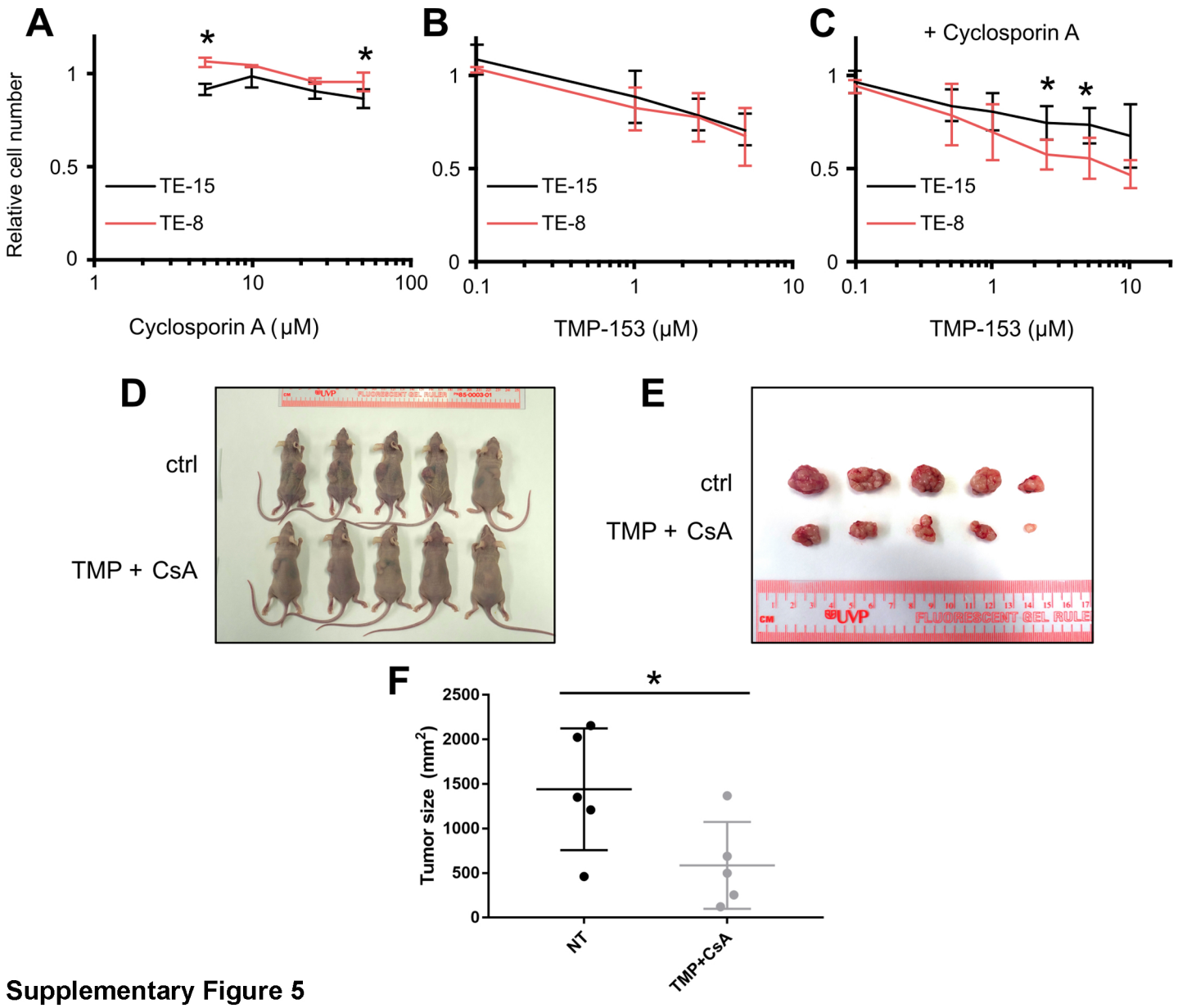
Supplementary Figure 4

Comparison of SM and cholesterol content

(**A,B**) Comparison among EpH4, EpH4-Snail, and fibroblasts.

(**C,D**) Comparison between esophageal cancer cell lines TE-15 (Snail-negative) and TE-8 (Snail-positive). $n = 3$, ** $p < 0.01$, * $p < 0.05$, n.s., $p \geq 0.05$ by Tukey's multiple comparison test (A,B) and Student's *t*-test (C,D).

Figure S4



Supplementary Figure 5

Selective growth inhibition of a Snail positive esophageal cancer cell line by combined treatment of cyclosporin A and TMP-153

(**A-C**) Effects of treatments with cyclosporin A (**a**), TMP-153 (**b**) or combination of 50 μM cyclosporin A and indicated concentration of TMP-153 (**c**) to cellular growth of human esophageal cancer cell lines TE-15 and TE-8. Cells were treated with drugs for 24 h and relative cell number to 0.1% DMSO control was determined by CCK-8. n ≥ 3, error bars, SDs. Significant differences between TE-15 and TE-8 by unpaired one-sided Student's *t*-test are shown (*, $p<0.05$).

(D-F), Combined treatment of cyclosporin and TMP-153 inhibits the growth of TE-8 tumor xenografts. Experiments were performed as described in Materials and Methods. In brief, 10 mg/kg cyclosporin A and 10 mg/kg TMP-153 were simultaneously administered intraperitoneally to nude mice bearing TE-8 tumor xenografts. After 36 days of the first administration, the overall appearance (D), the excised tumors (E), and the tumor size (F) of control and treated mice were compared. $n = 5$, error bars, SDs. Significant differences by unpaired one-sided Student's *t*-test are shown (*, $p < 0.05$).

Table S1 Primer sequences used in this study

Gene		Primer sequence	
CerS isozymes			
mouse	CerS1	forward	5'-CTGTACATTGTGGCTTTCGC-3'
		reverse	5'-GGCTGTCTGAGCTTCCAGAG-3'
mouse	CerS2	forward	5'-CCCTGCTCTTTCTCGTCATTC-3'
		reverse	5'-CGTGACAAAAGGTCTACCTCC-3'
mouse	CerS3	forward	5'-TTTCAAGCATTCCACAAGCAAAC-3'
		reverse	5'-ATCGCCAGCAAGACTCCT-3'
mouse	CerS4	forward	5'-GGCTGTGCGAATTGTCTT-3'
		reverse	5'-CGGGTTGGGCTTTATCTTT-3'
mouse	CerS5	forward	5'-AGTAAGGTTATTGGCTCTGTGTT-3'
		reverse	5'-GCTCAGAAGTGACCAGACAAT-3'
mouse	CerS6	forward	5'-CCGGAGTGGACAAAGCAAGAT-3'
		reverse	5'-GCCGTTCTTACCTCTCTGGAAGATG-3'
Elovl isozymes			
mouse	Elovl1 variant1	forward	5'-TGGAGGCTGTTGTGAACTTG-3'
		reverse	5'-ATGAAGCCTCGAAGTTGGAA-3'
mouse	Elovl1 variant2	forward	5'-GATCCCTTTG AACCCTTCAC-3'
		reverse	5'-TCAGAAACCGCATGCTTCAT-3'
mouse	Elovl2	forward	5'-GATCCCTTTGAACCCTTCAC-3'
		reverse	5'-GAGAATCTCGTGGTCCAAACA-3'
mouse	Elovl3	forward	5'-CGGGTTAAAAATGGACCTGA-3'
		reverse	5'-CCAACAACGATGAGCAACAG-3'
mouse	Elovl4	forward	5'-ACCGTGGAGTTCTATCGCTG-3'
		reverse	5'-TGCTTATGCTTATCGTTGGC-3'
mouse	Elovl5	forward	5'-CTTGCACATCCTCCTGCTC-3'
		reverse	5'-CTGAGTGACGCATCGAAATG-3'
mouse	Elovl6	forward	5'-CCAATGGATGCAGGAAAACT-3'
		reverse	5'-AACTTGGCTCGCTTGTTCAT-3'
mouse	Elovl7	forward	5'-GGAAAATGGCGTTCAGTGAT-3'
		reverse	5'-CATAGAGGCCCAGGATGATG-3'