ASPP1 deficiency promotes epithelial-mesenchymal transition, invasion and metastasis in colorectal cancer

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Abstract

The apoptosis-stimulating protein of p53 (ASPP) family of proteins can regulate apoptosis by interacting with the p53 family and have been identified to play an important role in cancer progression. Previously, we have demonstrated that ASPP2 downregulation can promote invasion and migration by controlling β-catenin-dependent regulation of ZEB1; however, the role of ASPP1 in colorectal cancer (CRC) remains unclear. We analyzed data from The Cancer Genome Atlas (TCGA) and coupled this to in vitro experiments in CRC cell lines as well as to experimental pulmonary metastasis in vivo. Tissue microarrays of CRC patients with information of clinical-pathological parameters were also used to investigate the expression and function of ASPP1 in CRC. Here, we report that loss of ASPP1 is capable of enhancing migration and invasion in CRC, both in vivo and in vitro. We demonstrate that depletion of ASPP1 could activate expression of Snail2 via the NF-κB pathway and in turn, induce EMT; and this process is further exacerbated in RAS-mutated CRC. ASPP1 could be a prognostic factor in CRC, and the use of NF-κB inhibitors may provide new strategies for therapy against metastasis in ASPP1-depleted CRC patients.

Introduction

The Apoptosis Stimulating Proteins of p53 (ASPP) family consists of three members (ASPP1, ASPP2 and iASPP), which have similar sequences in the C-termini including Ankyrin repeats, SH3 domain and Proline-rich region1–4. Functionally they bind with apoptosis regulating proteins, including p53, p63 and p73, to regulate cell apoptosis4. Studies have also linked the ASPP family to a number of other processes including autophagy, RAS-induced senescence, gestational trophoblastic disease and the development of cancer3,5–11.

A number of studies have identified a link between members of the ASPP family, epithelial-mesenchymal transition (EMT) and cancer progression. EMT is a cell plasticity program where epithelial cells lose their cell polarity and cell–cell adhesion to gain migratory and invasive properties in turn becoming mesenchymal cells12,13. It is widely regarded as being crucial for cancer progression14. EMT-Transcription Factors (EMT-TFs) including Snail1/2 (SNAI1/2), Twist-related protein 1/2 (TWIST1/2) and zinc-finger E-box-binding homeobox 1/2 (ZEB1/2) are able to activate the EMT program15,16.

The ASPP family have been implicated in cancer, ASPP1/2 are tumour suppressors, while iASPP is an oncogene4. High ASPP2/low iASPP expression in a number of human cancers has been evaluated and found to correlate with higher survival rates, improved curative effect and better prognosis17–22. Meanwhile, ASPP1 has been found to be downregulated in a variety of human cancers including acute lymphoblastic leukemia, breast
cancer, hepatitis B virus-positive hepatocellular carcinoma, clear cell renal cell carcinoma and colorectal cancer (CRC). Previously, we have demonstrated that ASPP2 downregulation can promote invasion and migration by controlling β-catenin-dependent regulation of ZEB1; however, the mechanisms underlying ASPP1’s functions in CRC remain unclear. Here, we show that reduced ASPP1 expression in CRC tissue correlates with more invasive disease. Further, we demonstrate that downregulation of ASPP1 can facilitate invasion and migration, via the NF-κB pathway, in turn activating Snail2, inducing EMT. This process is further exagger-ated in RAS-mutated CRC. This study could provide potential prognostic indicators and novel treatments for the treatment against metastasis in ASPP1-depleted CRC patients.

Results
Expression of ASPP1 is decreased in colorectal tissues, and correlates with both clinical and lymph node stages

To determine the role of ASPP1 in CRC, we first investigated the protein expression of ASPP1 in 86 paired CRC and adjacent normal tissues by IHC. We demonstrated that ASPP1 is expressed in the nucleus and cytoplasm, both in normal and CRC tissue. However, we determined that ASPP1 expression was significantly lower in both the nucleus (P = 0.0007) and cytoplasm (P < 0.0001) in CRC, compared to adjacent normal tissue (Supplementary Fig. S1).

Next, we analyzed the correlation between ASPP1 expressions (Fig. 1a) and clinical–pathological factors by categorizing tissues into high and low expression groups according to both clinical stage and lymph node stage. Low nuclear, but not cytoplasmic, expression of ASPP1 was significantly correlated with both lymph node metastasis and higher clinical stage (Fig. 1b; Tables 1 and 2), indicating that lower nuclear ASPP1 expression may promote invasion and migration in CRC.

ASPP1 inhibition promotes CRC invasion and migration in vivo and in vitro

Given the clinical findings implicating ASPP1, together previous studies demonstrating a role for the ASP family in migration and invasion; we sought to investigate whether ASPP1 is capable of facilitating invasion and migration in CRC. We stably expressed control or ASPP1 (PP311R13B) shRNA (shRNA1, shRNA2, shRNA3, Supplementary Fig. S2) in HCT116 cells and then utilized Transwell invasion and migration assays. In both Transwell migration and invasion assays, depletion of ASPP1 significantly promoted invasion and migration in HCT116 cells (Fig. 2). Similar results have been demonstrated in a MCF10A derivative cell line, MCF10A ER:HRASV12, which was engineered to express ER:HRAS. Addition of 4-hydroxytamoxifen (4-OHT) acutely activates the RAS pathway in this cell line. MCF10A is an immortalized human breast epithelial cell line, it maintains many features of normal breast epithelial cells, including the ability to form cell–cell junctions. These results show that when ASPP1 RNAi-transfected 4-OHT-treated MCF10A ER:HRASV12 cells are cultured in Matrigel, they have longer protrusions compared to those treated with 4-OHT alone (Supplementary Fig. S3), suggesting that ASPP1 inhibits RAS-induced invasion.

Next, we conducted experiments in a mouse model to examine whether ASPP1-depleted cells have more metastatic potential by using tail vein injection of firefly luciferase tagged HCT116 cells with ASPP1 shRNA or control shRNA. Lung fluorescence signals of mice from ASPP1 shRNA group were significantly more intense, suggesting more metastasis in mice lungs (P = 0.0254; Fig. 3a, b). The area of metastatic clusters present in the lungs of mice injected with ASPP1 shRNA/HCT116 cells was significantly higher than the group injected with control shRNA/HCT116 cells, as examined by hematoxylin and eosin (H/E) staining (P = 0.0069; Fig. 3c, d). These results indicate that ASPP1 may play a critical role in inhibiting invasion and metastasis of colorectal cancer cells.

ASPP1 depletion induces Snail2 expression and facilitates oncogenic RAS-induced EMT

Previously, we identified ASPP2 as a molecular switch of epithelial plasticity via the β-catenin-ZEB1 pathway. Given that the structure and function of ASPP1 is similar to ASPP2, we hypothesized they may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner. As such, we analyzed the expression of a number of EMT-TFs in HCT116 cells transfected by siRNA to ASPP1, we hypothesized that ASPP2 may act in a similar manner.
knockdown, but both are significantly increased; when both are used together levels of VIM double compared to RAS-activated (Fig. 5b). Taken together, this data suggest downregulation of ASPP1 induces EMT in CRC cells and this induction could be enhanced in the presence of active RAS.

ASPP1 inhibition induces expression of Snail2 via NF-κB pathway

The correlation between ASPP1 (PPP1R13B) and Snail2 (SNAI2) was further confirmed by analyzing TCGA data. Low ASPP1 (PPP1R13B) expression was significantly correlated with high levels of Snail2 (SNAI2) (Fig. 6a, b) in the colorectal adenocarcinoma RNASeq data (TCGA dataset, Nature 2012). In order to find how ASPP1 regulates Snail2 expression, we analysed data from TCGA project. Gene, RNASeq (IlluminaHiSeq) TCGA colorectal adenocarcinoma dataset were obtained from UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/). Significantly differential expressed genes between high and low ASPP1 (PPP1R13B) samples are shown in the heatmap in Fig. 6c. To demonstrate whether the significantly positive genes with ASPP1 (PPP1R13B) in TCGA dataset are involved in the same pathway, ToppGene Suite (https://toppgene.cchmc.org/) website was used to perform pathway analysis. We found both canonical and noncanonical NF-κB pathways are significantly enriched (Fig. 6d). These analyses suggested
that ASPP1 may regulate Snail2 expression via NF-κB pathway.

Indeed, ASPP1 has been previously demonstrated to bind with NF-κB, which is one of the key pathways that can directly bind the promoters of EMT-TFs and induce their expression. To further confirm the role of the NF-κB in these processes we utilized NF-κB reporter assays in HCT116 and HKe3 ER:HRAS V12 cells. Significantly, NF-κB activity in HCT116 transfected with ASPP1 siRNA increased nearly 3-fold compared to control siRNA group (Fig. 7a). In HKe3 ER:HRAS V12 cells, both ASPP1 knockdown and RAS-activation were sufficient to significantly increase NF-κB activity, however RAS-activation together with ASPP1 knockdown over doubled the effect of either treatment-alone (Fig. 7b). To investigate this further, we used an immunoprecipitation assay to try and determine whether ASPP1 could bind with NF-κB p65. In HCT116 cells, NF-κB p65 was immunoprecipitated with ASPP1 and NF-κB1 p50, but not NF-κB2 p52 (Fig. 7c). To further elucidate the role of these proteins, we knocked down ASPP1 in HCT116 cells and conducted a co-immunoprecipitation, we saw a significant increase in the binding between NF-κB1 p50 and p65 upon ASPP1 depletion (Fig. 7d). Finally, to confirm whether ASPP1 regulates Snail2 via NF-κB pathway, in HCT116 cells transfected with ASPP1 RNAi, we depleted Snail2 or p65 simultaneously. Knockdown of ASPP1 induced EMT in HCT116 cells, visible as decreased E-cadherin and increased Snail2 protein levels. The depletion of NF-κB p65 completely abolished the increase in Snail2, and restored E-cadherin expression (Fig. 7e). Depletion of Snail2 (SNAI2) was sufficient to restore the epithelial phenotype of HCT116 cells, demonstrated by the western blot of E-cadherin (Fig. 7e). Taken together, these findings suggest that ASPP1 knockdown induces EMT via NF-κB pathway mediated regulation of Snail2.

### Discussion

Colorectal cancer (CRC) has the third highest incidence of malignant tumors and was globally the second leading cause of cancer death in 2018. Development of...
CRC is a result of interactions between genetics and environmental factors. The RAS family (KRAS, HRAS, NRAS), are frequently mutated in human cancer, approximately 25% of human malignances harbor RAS mutations. In CRC, KRAS and NRAS mutations are found in approximately 44.7% and 7.5% of cases respectively, and RAS has not only been found to drive tumour progression but also is key in tumour maintenance. Lifestyle factors, including smoking, diet, lack of exercise, combined with an aging population all contribute to an increased risk of CRC. A number of advances have been made in recent years to both treatment and diagnosis; however, despite new treatments doubling survival for advanced disease, prognosis for metastatic disease is still poor. CRC frequently metastasizes to both the lungs and the liver, and this is a leading cause of treatment failure with up to 50% of CRC patients developing metastatic liver disease after resection of the primary tumour.

The role of ASPP1 in cancer has been reported in several studies. However, the relationship between ASPP1 and metastasis has never been reported in CRC. In our study, we found that nuclear ASPP1 is expressed at low levels in CRC patients and correlates with CRC patients TNM clinical stages (stages III + IV vs. I + II) and lymph node metastasis. CRC is typically staged based on a system established by the American Joint Committee on Cancer called the TNM staging system. The tumor is found in nearby lymph nodes from Stage III and distant organs from Stage IV. In the commercial available colorectal tissue microarray used in this study, there are only 3 cases of Stage IV CRC samples, which won’t allow us to directly check is the relationship between ASPP1 expression and distant metastasis. However, in mouse models, we demonstrated that loss of ASPP1 increased metastatic potential of CRC cells to the lungs. These corroborated the in vitro findings, which demonstrated, using Transwell migration and invasion assays that loss of ASPP1 increased invasion and migration in CRC.

The mechanisms underlying metastasis are complicated, from epithelial cell invading locally through surrounding extracellular matrix (ECM) and stromal cell layers, to resuming their proliferative programs at metastatic sites. These complex biological processes are regulated by molecular pathways in cancer cells and cell non-autonomous interactions between carcinoma cells and non-neoplastic stromal cells. EMT is a dynamic process, where epithelial cells convert to a mesenchymal phenotype and this process has been widely implicated in cancer progression. EMT can be induced in a number of ways, including by pleiotropically acting EMT-TFs.
including SNAIL1/2 (Snail1/2), ZEB1/2 and TWIST1/2\textsuperscript{15}, these have been shown to have a role in cancer. Snail2 (SNAI2), in particular, has been reported to have a key role in the invasion and migration of cancer\textsuperscript{16,45,46} and we have previously demonstrated it can promote EMT in RAS-active colorectal cancer cells\textsuperscript{27}.

The NF-κB pathway consists of five transcription factors and these regulate a number of cellular processes, such as proliferation and apoptosis\textsuperscript{47}. Constitutive NF-κB activity has been reported in human cancers\textsuperscript{29,47,48}, as a result of inflammatory microenvironment and oncogenic mutations\textsuperscript{47}, and has been linked to cancer progression and promoting an invasive phenotype\textsuperscript{49–52}. Further it has been shown to mediate EMT-TFs, ZEB1 and TWIST1, to regulate EMT\textsuperscript{53}. Although ASPP1 has been shown to bind with NF-κB\textsuperscript{30–32,54}, these mechanisms haven’t been previously described in cancer. Here we show using analysis of CRC TCGA datasets that reduced ASPP1 expression correlates with increased SNAI2 expression and pathway analysis showed that low ASPP1 expression resulted in NF-κB activation. We observed that downregulating ASPP1 induces NF-κB activation, potentially by enhancing the complex formation between NF-κB1 p50 and NF-κB p65. Together, these data suggest that ASPP1 inhibition induces expression of Snail2 via NF-κB pathway (Fig. 7f).

Taken together, we demonstrated that ASPP1 expression was reduced in CRC and low ASPP1 expression correlates with higher clinical stage. Our study also identifies a new mechanism showing that downregulated ASPP1, could increase Snail2 to promote invasion and migration in colorectal cancer via active NF-κB. Further, downregulated ASPP1 could cooperate with active RAS could facilitate EMT, invasion and migration via upregulating expression of Snail2. Our research provides a valuable indication that ASPP1 could be a prognostic
indicator of CRC and patients with low ASPP1 levels could be more suitable for therapy with NF-κB inhibitors\textsuperscript{55,56}. These results may offer new information for the development of novel drugs in the treatment of CRC.

Methods

Colorectal cancer tissue microarray and immunohistochemistry (IHC)

A colorectal tissue microarray (HColA180Su11; Shanghai Outdo Biotech, Shanghai, China) with 86 matched pairs of primary CRC samples and adjacent normal tissues were purchased for IHC analysis. All procedures were approved by the Ethical Committee of Tongji Hospital. Informed consent was obtained from all subjects. The tissue section was de-waxed, rehydrated and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity for 30 min. After microwave antigen retrieval, sections were blocked and then incubated overnight at 4°C with a primary antibody against ASPP1 (1:100, HPA006394, Sigma). Sections were then incubated with secondary antibodies for 60 min at room temperature. After washing with TBS three times, the slides were stained with 3,3-diaminobenzidine and counterstained with hematoxylin. Counterstained by haematoxylin, the slides were dehydrated in graded alcohol and mounted. For each section, five random non-overlapping fields containing at least 200 cells per field were observed and scored based on the percentage of positively stained cells (0–100%) and the staining intensity (score 0 for negative, 1 for weak staining, 2 for moderate staining, and 3 for strong staining). The immunoreactive score (IRS) was calculated with the formula, the percentage of positive cells × the staining intensity × 100, to produce a value between 0 and 300. Patients were divided into low and high expression groups according to the median IRSs.

Cell culture, reagents and transfections

HKe3 ER:HRAS V12, HCT116 and firefly luciferase tagged HCT116 cells were cultured in DMEM (Fisher Scientific UK, 11594446) supplemented with 10% FBS (Invitrogen, 10270106). MCF10A ER: HRAS V12 cells were maintained in a 1:1 mixture of DMEM and Ham’s F12 medium (Fisher Scientific UK, 11524436) supplemented with 5% horse serum (Gibco, 26050088), 20 ng/ml EGF (Bio-techne, 236-EG), 100 ng/ml cholera toxin (Sigma, C8052), 10 μg/ml insulin (Sigma, I1882), 500 ng/ml hydrocortisone (Sigma, H0888) and antibiotics. Cells were kept in a humidified 37°C incubator with 5% CO\textsubscript{2} and 95% air. For 3D acini cultures, MCF10A ER:HRAS V12 cells were cultured as previously described\textsuperscript{57} on growth factor-reduced Matrigel (BD Biosciences, 354230). 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma-Aldrich (H6278). No mycoplasma contamination was detected in the cell lines used.

MCF10A ER:HRAS V12 cells were transfected with the indicated siRNA oligos at a final concentration of 37.5 nM using Lullaby (OZ Biosciences, LL71000), according to the manufacturer’s instructions. All other cell lines were transfected with the indicated siRNA oligos at a final concentration of 35 nM using Dharmafect 1 reagent (Dharmacon, T-2001–03). Short interfering RNA (siRNA) oligos against ASPP1 (MQ-010492-01-0002) were purchased from Dharmacon (Lafayette, CO, USA). Sequences are available from Dharmacon or upon request. As a negative control, we used siGENOME RISC Free siRNA (Dharmacon).

Stable knockdown of human ASPP1 was carried out using pLVX-shRNA-mCherry-Hygro lentiviral expression plasmid. The ASPP1 short hairpin RNA (shRNA) plasmid

![Fig. 4 ASPP1 depletion induces Snail2 expression. a Relative fold change in mRNA levels of SNAI1 (Snail1), SNAI2 (Snail2), ZEB1, ZEB2, TWIST1 in HCT116 cells transfected with ASPP1 siRNA and control siRNA. ***P < 0.001. Scores above the bars are relative levels when compared with control cells. b Protein expression of ZEB1, ZEB2, Snail2, Snail1, ASPP2 and ASPP1 in HCT116 cells with indicated treatment. β-tubulin was used as a loading control.](image-url)
was designed to target the three independent shRNA constructs. Target sequences are:

shRNA-1, 5′-GCAACGAACTCAGAGAAATGT-3′;
shRNA-2, 5′-GGTTGGGAATCCACGTGTTGA-3′;
shRNA-3, 5′-GCAATCTGTCTGCTGAAATAG-3′.

Western blot analysis

Western blot analysis was performed with lysates from cells or tissues with urea buffer (8 m urea, 1 m thiourea, 0.5% CHAPS, 50 mm DTT and 24 mm spermine). For immunoprecipitations, the cells were lysed for 30 min at 4 °C in pNAS buffer [50 mm Tris/HCl (pH 7.5), 120 mm NaCl, 1 mm EDTA and 0.1% Nonidet P-40], with protease inhibitors. Indicated antibodies and immunoglobulin G (IgG) agarose were added to the lysate for 16 h at 4 °C. Immunoprecipitates were washed four times with cold PBS followed by the addition of SDS sample buffer. The bound proteins were separated on SDS
polyacrylamide gels and subjected to immunoblotting with the indicated antibodies. Primary antibodies were from: Sigma (ASPP1, HPA006394; ASPP2, HPA021603), Santa Cruz Biotechnology (ZEB1, sc-25388; E-cadherin, sc-21791; Snail2, sc-166476), Abcam (β-tubulin, ab6046; NF-κB1 p50, ab32360; NF-κB2 p52, ab174482; ZEB2, ab138222), Cell Signaling Technology (phospho-AKT, 9271; AKT, 4685; Snail1, 3879; Snail2, 9585; NF-κB p65, 8242), BD Transduction Laboratories (E-cadherin, 610405). Signals were detected using an ECL detection system (GE Healthcare) (Chicago, IL, USA) or an Odyssey imaging system (LI-COR), and evaluated by ImageJ 1.42q software (National Institutes of Health) (Berhesda, MD, USA).

qRT-PCR

The real-time RT-PCR was carried out using genespecific primers (QuantiTect Primer Assays, Qiagen) for SNAI1, SNAI2, ZEB1, ZEB2, TWIST1, CDH1, VIM, GAPDH or ACTN with QuantiNova SYBR Green RT-PCR kits (Qiagen). Relative transcript levels of target genes were normalised to GAPDH or ACTN mRNA level.

Transwell migration and Matrigel invasion assays

For the Transwell migration assay, Transwell membranes (8-μm pore size, 6.5-mm diameter; Corning Costar, 3422) were used. The bottom chambers of the Transwell were filled with migration-inducing medium (with 30% FBS). The top chambers were seeded.
with $1.5 \times 10^5$ live serum-starved control or ASPP1 shRNA HCT116 cells per well. After 48 h, the filters were fixed with 4% paraformaldehyde for 10 min at room temperature; subsequently, the cells on the upper side of the membrane were scraped with a cotton swab. Similar inserts coated with Matrigel (Corning, 354480) were used to determine invasive potential in invasion assays. Filters were stained with crystal violet for light microscopy. Images were taken using an Olympus inverted microscope and migratory cells were evaluated by ImageJ 1.42q software (National Institutes of Health, United States).

Fig. 7 ASPP1 represses Snail2 expression via inhibiting NF-κB pathway. Fold changes in NF-κB reporter activity in HCT116 (a) or HKe3 ER:HRAS V12 cells (b) with the indicated treatments. NF-κB reporter luciferase readings normalized to Renilla in control cells were used to set the baseline value at unity. Data are mean ± s.d. $n = 3$ samples per group. **$P < 0.01$, ***$P < 0.001$. c Total cell lysates from HCT116 cells were immunoprecipitated (IP) with an anti-p65 antibody or control IgG. ASPP1, p65, NF-κB1 p50 and NF-κB2 p52 levels are indicated. d HCT116 cells transfected with control or ASPP1 RNAi were immunoprecipitated with an anti-p65 antibody or control IgG. NF-κB1 p50, p50 and ASPP1 levels are indicated. β-tubulin was used as a loading control. Scores under the bands are relative levels when compared with control cells (1.0). e Protein expression of E-cadherin, Snail2, p65 and ASPP1 in HCT116 cells with indicated treatment. β-tubulin was used as a loading control. f Diagram showing ASPP1 represses EMT via inhibiting NF-κB-Snail2 pathway in CRC.
Immunofluorescence microscopy

Cells were fixed in 4% PBS (Fisher Scientific UK, 12579099)-paraformaldehyde for 15 min, incubated in 0.1% Triton-X-100 (Fisher Scientific UK, 11471632) for 5 min on ice, then in 0.2% fish skin gelatin (Sigma, G7041) in PBS for 1 h and stained for 1 h with an anti-E-cadherin (1:100, Santa Cruz sc-21791, mouse monoclonal 67A4) antibody. Protein expression was detected using Alexa Fluor 488 (1:400; Fisher Scientific UK) for 20 min. TO-PRO-3 (Invitrogen, T3605: 1:1000) was used to stain nucleic acids. For immunofluorescence staining of 3D cultures from MCF10A ER:HRAS V12 cells, acini were fixed with 4% paraformaldehyde for 40 min, permeabilized in 0.5% Triton X-100 for 10 min on ice and stained with Rhodamine-phalloidin (Molecular Probes, R415) for 1 h at room temperature. Acini were counterstained with DAPI. Samples were observed using a confocal microscope system (Carl Zeiss LSM 510 or LSM 710, or Leica SP8). Acquired images were analyzed using Photoshop (Adobe Systems, United States) according to the guidelines of the journal.

TCGA data mining and pathway analysis

Colorectal adenocarcinoma (TCGA, Nature 2012), RNASeq, RPKM, mRNA expression was obtained from the cBioPortal for Cancer Genomics website (http://www.cbioportal.org/). The colorectal adenocarcinoma samples were separated into high and low PPP1R13B (ASPP1), mRNA expression, by selecting the top and bottom 20% of samples for high and low ASPP1 expression, respectively. Then an unpaired t-test was performed to find the significantly different (P < 0.05) mRNAs between high and low ASPP1 sample groups (all done using R, version 3.4.4).

To explore the pathways represented in the colorectal adenocarcinoma TCGA mRNA data analysis, ToppGene Suite (https://toppgene.cchmc.org/) was used. In TCGA mRNA data, 4319 mRNAs were significantly upregulated in high ASPP1 sample group compared to the low ASPP1 group. The identified 4319 mRNAs in TCGA gene data were analysed with ToppGene Suite website to detect functional enrichment of the genes on pathway analysis. The identified NF-κB related pathways in pathway analysis with the number of shared mRNAs and P-value were plotted with histogram plot in GraphPad Prism V8.2.1.

GraphPad Prism V8.2.1 was used to plot bar plot to identify mRNA expression of SNAI2 between high and low ASPP1 groups in colorectal adenocarcinoma (TCGA, Nature 2012) dataset. Moreover, the correlation between SNAI2 and ASPP1 mRNA expression within all colorectal adenocarcinoma samples was analysed by Pearson’s correlation analysis in GraphPad Prism V8.2.1.

Luciferase reporter assay

HCT116 and HKe3 ER:HRAS V12 cells were transfected with the indicated siRNAs for 48 h in 24-well plates, followed by 24 h transfection with 250 ng of NF-κB reporter and 10 ng of phRL-CMV (Promega, E2261), which constitutively expresses the Renilla luciferase reporter. One day before the measurement of luciferase activity 100 nM 4-OHT was added. Finally, the transcription assay was carried out using the Dual-luciferase® reporter assay system (Promega, E1960) following the manufacturer’s protocol.

Tumorigenicity and metastasis experiment

For lung metastasis, 1 × 10^6 cells were injected into the tail vein of 8 weeks old male nude mice anesthetized with 2% isoflurane gas. Two weeks later, Images of Lung fluorescence signals were acquired by IVIS Lumina LT series III (Caliper, MA) with the excitation (640 nm) and emission wavelength (710 nm 845 nm). The mice were injected with D-luciferin, anesthetized with isoflurane, and imaged 5 min after luciferin injection. Fluorescence intensity within specific regions of individual animals was quantified using the region of the interest (ROI) tools in the Live Image 4.5 software (PerkinElmer). Four weeks after injection, mice were euthanized by CO2 followed by cervical dislocation. Lungs were removed and fixed in 10% buffer formalin and paraffin embedded. Sections (5 μm) of lungs were stained with hematoxylin and eosin to visualize the pulmonary metastases under a microscope. All animal experiments were performed in accordance with a protocol approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China.

Blinding, randomization, statistical analysis and repeatability of experiments

IHC scoring was conducted with the researcher blind to the treatment. The animal studies were randomized and blinded according to the protocol approved by Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. The sample size was estimated in light of a retrospective analysis of our previous studies. Each experiment was repeated at least twice. Unless otherwise noted, data are presented as mean ± s.d., and a two-tailed, unpaired Student’s t-test was used to compare two groups for independent samples. Chi-square test or Fisher exact test were used to evaluate the relationship of ASPP1 expression and clinical parameters of CRC. Correlation between the expression of ASPP1 and SnaI2 was analysed using Pearson’s correlation. Statistical analysis was conducted using SPSS version 19.0 (Endicott, NY, USA). Unpaired t-test was performed for TCGA data analysis in R version
3.4.4 or in GraphPad prism V8.2.1. P < 0.05 was considered statistically significant.

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Conflict of interest
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