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## Supplementary Information for

# iASPP suppresses Gp78-mediated TMCO1 degradation to maintain $Ca^{2+}$ homeostasis and control tumor growth and drug resistance

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## Supplementary materials and methods

### Cell lines and transfection

SW480, SW620, COLO 205, HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or HCT-15, HCT-116, H1299 cells were in RPMI-1640 medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Biological industries). All cells were grown in the humidified incubator (Thermo scientific) with 5% $CO_2$ at 37°C and free of mycoplasma contamination.

For transient transfection, the indicated plasmids or synthetic small interfere RNAs (siRNAs) (GenePharma, China) were introduced into the indicated cells by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. All transfections utilized either same amount of empty vector plasmid or si-scrambled RNA as controls. Cell were ready for the assays 48-72h after transfection. The siRNA sequences used in this study were listed in Supplementary Table 1.

To establish stable cell lines, the PLKO.1-GFP lentiviral plasmids expressing shiASPP, shTMCO1 or scrambled shRNA (shnone, Addgene #1864), together with pCMV.△8.9 and VSV-G plasmids, were introduced into virus packing line, 293T. 48-72h after transfection, virus was collected and used to infect target cells in the presence of polybrene (10μg/ml). Stable single clones were selected by sequential dilution. The knockdown (KD) efficiency of iASPP and TMCO1 in the clones was validated by WB. Those with high KD efficiency were used for future assays.

### RNA extraction and quantitative reverse transcription (qRT) PCR

According to the manufacturer's instructions, the total RNA was extracted by Trizol (Invitrogen, USA) and subsequently subjected to reverse transcription by GoScriptTM Reverse Transcription System (Promega, USA). Quantitative real-time PCR was carried out in the Vii7 real-time PCR (Applied Biosystems, USA) with SYBR Premix Ex Tag II (TaKaRa, Japan). The gene expression was analyzed by $2^{-\Delta\Delta}$CT method using GAPDH as an internal control. The primer sequences was summarized in Supplementary Table 2.

### Western blot (WB) assay

Total proteins of cancer tissue samples or cancer cell lines were lysed with urea buffer containing 2M Thiourea, 4% CHAPS, 40mM Tris-Base, 40mM DTT, 2% Pharmalyte and sonicated to crush DNA. The same amount of proteins was separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). After transferring protein samples from gel onto a PVDF membrane, samples were incubated with the corresponding primary and secondary antibodies. The protein signals were visualized by ECL and the images were captured by Image studio system (ECL, LI-COR, Lincoln, Georgia, USA). The quantification of proteins was analyzed using the Image J software.

**Measurement of $Ca^{2+}$ flux**

Intracellular $Ca^{2+}$ concentrations were measured by Fura-2 (1, 2) or Fluo-3 (3, 4) as described previously. To determine ER $Ca^{2+}$ content, cells were loaded with 5μM of Fura-2 AM (Invitrogen, F1221) and 0.1% pluronic F-127 (Invitrogen, P3000MP) in Hank's Balanced Salt Solution (HBSS) buffer [140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 20 mM HEPES (pH 7.3)] for 30 min at 37°C in the dark. After several washes with HBSS, cells were stayed for extra 10 min in HBSS buffer to allow de-esterification. After washes in HBSS-$Ca^{2+}$-free solutions ($Ca^{2+}$-free HBSS buffer containing 1mM EGTA), 1 μM ionomycin (Sigma) was applied to release $Ca^{2+}$ from ER in HBSS-$Ca^{2+}$-free solutions. The fluorescence images were recorded every 2s by the Nikon inverted microscope (Eclipse Ti) with a 40 ×magnification oil-immersion objective by alternatively excitation with 340 nm and 380 nm and the signal was processed by Metamorph software (version 7.0). The $Ca^{2+}$ level was determined by the 340/380 fluorescence ratio. ER $Ca^{2+}$ content was evaluated by the area under the curve (AUC) induced by ionomycin. To determine cytosolic $Ca^{2+}$, cells, after the indicated pro-apoptotic treatments, were incubated with 5 μM Fluo-3-AM (sigma, 39294) and 0.1% pluronic F-127 in HBSS buffer at 37°C for 30 min in the dark. The cells were then washed with HBSS buffer followed by immediate flow cytometry (BD) analysis. Fluo-3 was excited at the wavelength 488 nm, and the emission was collected at the wavelength 526 nm.

***In vitro* translation**

Protein was translated *in vitro* by Promega TNT T7 Quick coupled transcription/translation system (L1171, Promega) according to the manufacturer's instructions. Briefly, the mixture containing 1μg indicated plasmid, 1μL unlabeled methionine (1mM) and 40μL TNT® Quick Master Mix in a total volume 50μL was incubated at 30°C for 90 min. The *in vitro* translated proteins were ready for further in vitro immunoprecipitation (IP) assay, as described below.

**Purification of recombinant protein**

The corresponding sequence of the targeted genes were amplified by PCR from human genome. The PCR products were cloned into a pGEX-6p-1 expression vector. Proteins were expressed in the *E. coli* strain BL21 (DE3) after adding 0.1mM isopropyl β-d-1-thiogalactopyranoside (IPTG) to log-phase cultures followed by continued growth at 18°C overnight. After that, the bacteria were harvested and lysed in 10mL of NETN buffer per gram of cells. The resulting lysates were subjected to the analysis of SDS-PAGE and the expression of the induced protein was observed by comassie brilliant blue staining. The lysate with recombinant protein expression were mixed with 20 μl BeyoGold™ GST-tag Purification Resin (Beyotime，China). The target protein with GST tag was fully bound by slow shaking at 4°C for 3h in a shaker. The beads were washed twice by NETN buffer and eluted by elution buffer and the supernatant was the target protein with GST label. The purified proteins were ready for further in vitro IP assay, as described below.

**Immunoprecipitation (IP) assay**

Cells were lysed with NETN buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl,1% NP-40,1 mM EDTA) supplemented with Proteinase Inhibitor Cocktail (MedChemExpress, USA). For the in vitro IP assay, the protein were in vitro translated or purified and ready for the assay. Briefly, the lysates or protein mixtures were pre-cleaned by the protein G sepharose beads (GE Healthcare, USA) at 4°C. After that, the immunoprecipitation complex, containing protein lysates, blocked protein G sepharose and the corresponding antibodies or the control IgG came from the same species as the target antibody, were incubated at 4°C overnight，which needed to be done on a rotating wheel. The beads of binding immunoprecipitates were collected and washed four times with the cold NETN buffer, which is ready for WB analysis after boiled in SDS loading buffer for 5 min.

**Ubiquitination assay**

For the ubiquitination assay, the indicated plasmids were co-transfected into the indicate cells. 48h later, cells were treated with 20mM proteasome inhibitor MG132 for 6h before harvest. Cells were lysed with lysis buffer (2% SDS, 150mM NaCl, 10mM Tris-HCl, pH8.0) with freshly supplemented Proteinase Inhibitor Cocktail. The resulting lysates were subjected to IP and WB assay. For USP2cc treatment, the immunoprecipitates were washed with 1×PBS for three times and treated with in vitro purified USP2cc protein at 37°C for 1h and subsequently subjected to a WB assay.

**Apoptosis assay**

The apoptotic percentage was determined by Annexin V and propidium iodide (PI) staining according to the manufacturer's instructions (Sungene, Tianjin). Briefly, the cells were collected carefully after the indicated treatments. $5 \times 10^5$ cells were mixed with 5 μl Annexin V/FITC adequately for 10 min followed by 5 min incubation with 5 μl propidium iodide (PI) at room temperature. Cells were analyzed by flow cytometry (FCM) within 1h. The reactions were carried out in the dark.

**Caspase 3/7 activity assay**

The indicated cells were subjected to the caspase 3/7 activity assay by Caspase-Glo 3/7 Assay Systems (Promega) according to its manufacturer's instructions. Caspase 3/7 activity assay was repeated independently for three times, which was represented as a fold-increase of fluorescence calculated by comparing to untreated groups.

**Clonogenic survival assay**

The indicated cells were subjected to a typical clonogenic survival assay. Briefly, $1 \times 10^4$ cells were mixed with medium containing 0,7% agar followed by spread on the top of a bottom agar layer (1 % agar in DMEM full growth medium) in a 6-well plate in triplicates. Cells were cultured for about 2 weeks. Colonies were counted and photographs after stainde with 1 %

Crystal Violet.

***In vivo* Xenografted tumor model**

All animal procedures were performed according to protocols approved by the Rules for Animal Experiments published by the Chinese Government (Beijing, China) and approved by the Research Ethics Committee of Harbin Institute of Technology, China. The female nude mice between 4 and 5 weeks were purchased from Beijing HFK Bioscience Co., Ltd. The same number of cells ($1\times10^7$) were inoculated subcutaneously into either side of flank of the same female nude mouse. The tumor volumes and size and body weights of xenografts were measured every week. For the staurosporine treatment, the same numbers of shnone, shiASPP, shTMCO1, shiASPP+shTMCO1 HT-29 cells were inoculated into female nude mice (5/group), respectively. When the sizes of the tumor xenograft reached about 200 $mm^3$, the mice were treated with staurosporine (2 mg/kg, intraperitoneally, every other day for 4 weeks) with the shnone group treated with PBS as a control. After 4-5 weeks, the tumors were carefully removed, photographed, and weighed after the mice were anesthetized and culled. The obtained tumors immediately stored in liquid nitrogen and the isolated the protein to subject to western blotting and caspase 3/7 assays.

**Calpain activity assay**

Calpain activity was measured according to manufacturer's instructions (G8501; Promega). Briefly, the indicated cells were lysed in 10 mM HEPES (pH 7.2), 10 mM dithiothreitol (DTT), 1 mM EDTA, and 0.1% BSA. The resulting lysates were mixed with Calpain-Glo Buffer, Suc-LLVY-Glo Substrate, and Luciferin Detection Reagent. The luminescence was read with Multiscan Spectrum (BioTek Synergy2).

**Mass spectrum**

Mass spectrum analysis was conducted as described previously(5). Briefly, samples in SDS-PAGE gel were extracted, digested resuspended in buffer A (5% ACN and 0.1% FA in water). The peptides were separated by an analytical C18 column (75μm*35cm*1.7μm, in-house) using the Ultimate3000 nano-HPLC instrument (Thermo Fisher Scientific, San Jose, CA). The gradient was run at 500 nL/min starting from 5 to 25% of buffer B (95%ACN, 0.1%FA) in 39 minutes, going up to 35% in 5 minutes. For the identification, peptides separated from nanoHPLC were subjected into the tandem mass spectrometry Orbitrap Fusion Lumos (Thermo Fisher Scientific, San Jose, CA) for DDA (data-dependent acquisition) detection by nano-electrospray ionization.

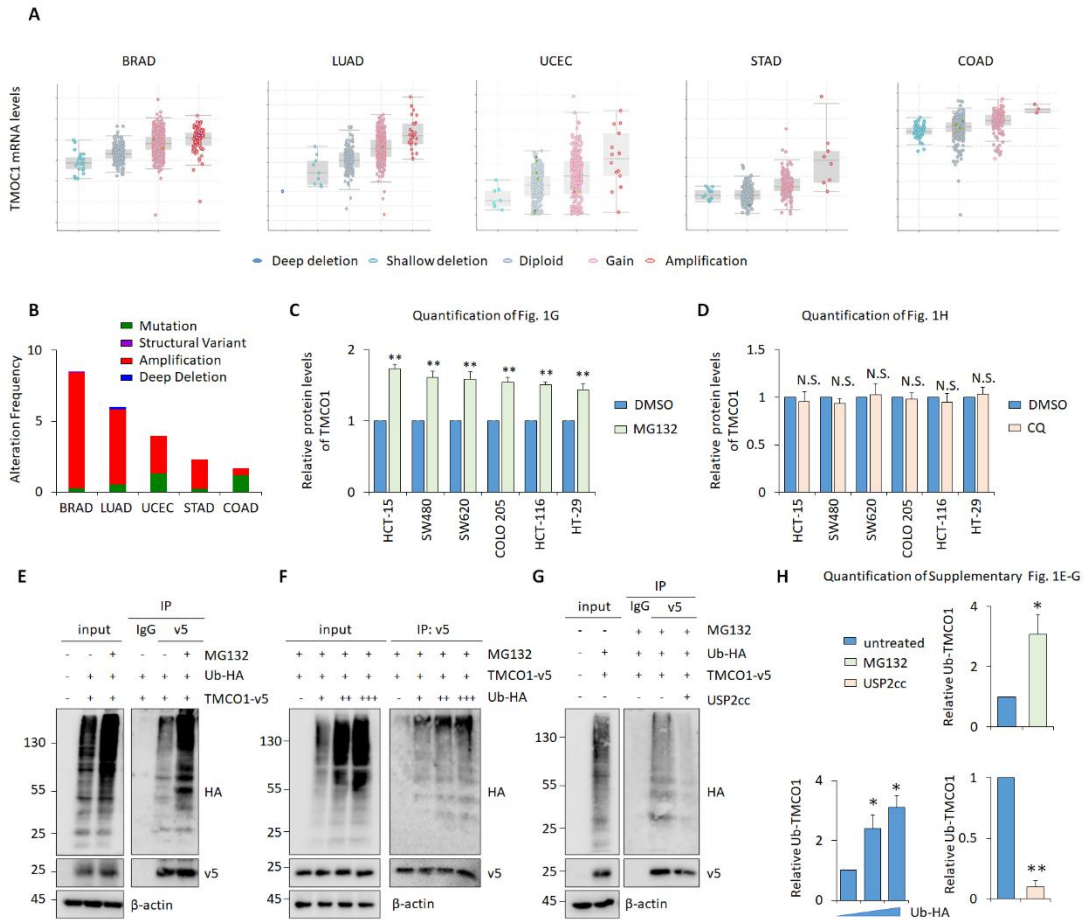
**Statistical analysis**

The data were showed as the means ± standard error of the means (SEM) or standard Deviation (SD). Statistical analysis was performed using the GraphPad software, version 5. Student t test was subjected to analyze the statistical significance and *P* value <0.05 were considered significant

## References


1. Wang Q-C*, et al.* (2016) TMCO1 Is an ER Ca(2+) Load-Activated Ca(2+) Channel. *Cell* 165(6):1454-1466.
2. Sun Z*, et al.* (2018) TMCO1 is essential for ovarian follicle development by regulating ER Ca store of granulosa cells. *Cell Death Differ* 25(9):1686-1701.
3. Assefa Z*, et al.* (2004) Caspase-3-induced truncation of type 1 inositol trisphosphate receptor accelerates apoptotic cell death and induces inositol trisphosphate-independent calcium release during apoptosis. *J Biol Chem* 279(41):43227-43236.
4. Wang Y, Bao X, Zhang Z, Sun Y, & Zhou X (2017) FGF2 promotes metastasis of uveal melanoma cells via store-operated calcium entry. *Onco Targets Ther* 10:5317-5328.
5. Zhang S*, et al.* (2013) Quantitative analysis of the human AKR family members in cancer cell lines using the mTRAQ/MRM approach. *J Proteome Res* 12(5):2022-2033.

**Supplementary Fig.1**



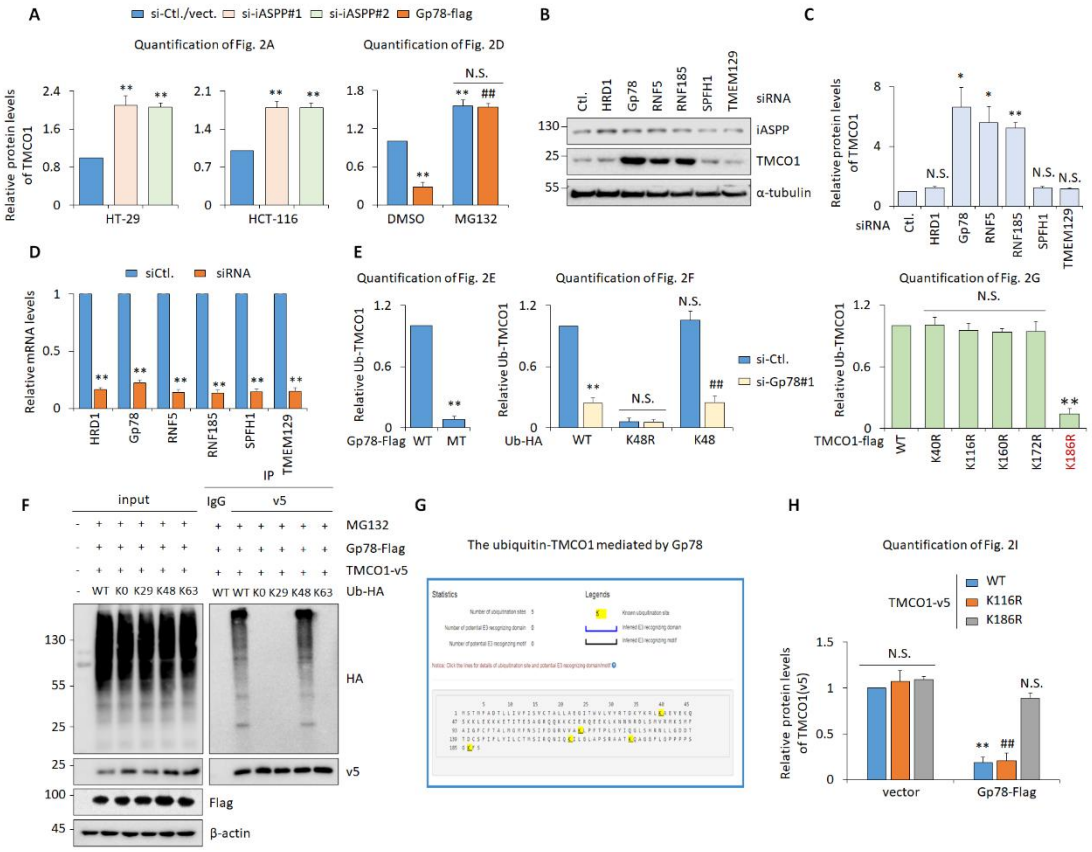

**Supplementary Fig.1. TMCO1 protein is overexpressed in human colon cancer tissues**

**(A-B)** The TMCO1 gene mutation, structural variant, amplification and deletion (B) and their association with TMCO1 mRNA expression (A) were analysed by cBioPortal database.

**(C-D)** TMCO1 bands shown in Fig 1G(C) or 1H (D) were quantified by image J. Data are derived from three independent experiments and represented as mean ± SEM in the bar graph.

**(E-H)** TMCO1 ubiquitination (Ub-TMCO1) was determined by IP of anti-v5 followed by WB with anti-HA after transfecting TMCO1-v5 and Ubiquitin (Ub)-HA in the presence of proteaseome inhibitor MG132 (20μM) (E), in the presence of increasing dose of Ub-HA (F) or after the treatment of USP2cc protein (G). β-actin was used as a loading control. Ub-TMCO1 signals in E-G were quantified by image J (H). Data are derived from three independent experiments and represented as mean ± SEM in the bar graph (H). Values in control cells were normalized to 1. *, $P$<0.05; **, $P$ < 0.01. (C, D, H)

**Supplementary Fig.2**




**Supplementary Fig. 2. TMCO1 is a substrate of Gp78**

**(A, E, H)** TMCO1 bands shown in Fig 2A and 2D (A), in Fig. 2D-F (E) or Ub-TMCO1 signal shown in Fig. 2I (H) were quantified by image J.

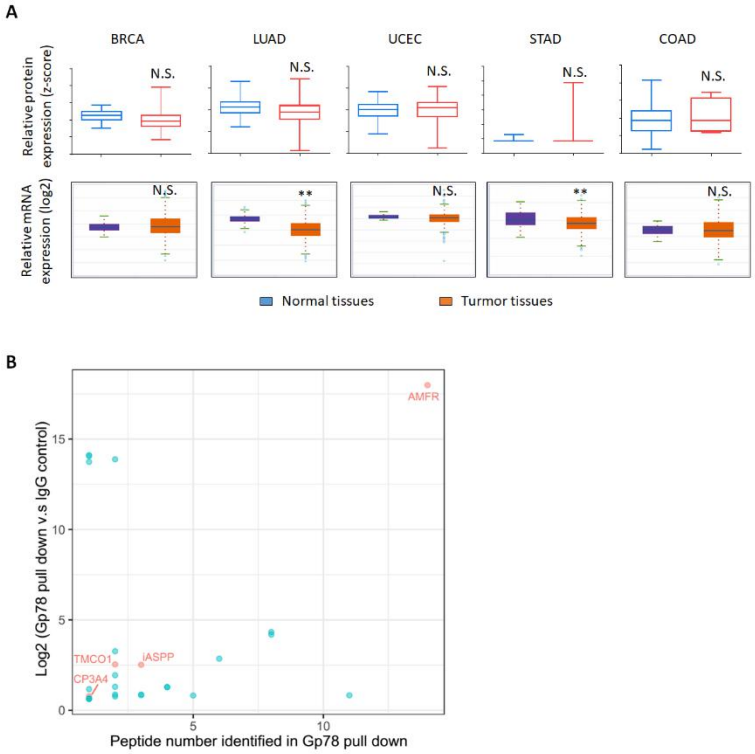
**(B-D)** The protein levels of iASPP and TMCO1 were determined by WB after knockdown (KD) of ER-resident E3 ligase candidates (HRD1, Gp78, RNF5, RNF185, SPFH1 and TMEM129) (B). TMCO1 bands shown were quantified by image J (C). The KD efficiency were confirmed by qRT-PCR(D).

**(F)** Ub-TMCO1 was determined by IP/WB in HT-29 cells after the indicated treatments.

**(G)** The potential ubiquitination sites in TMCO1 protein catalysed by Gp78 E3 ligase were predicted by a bioinformatics tool, ubibrowser http://ubibrowser.ncpsb.org/ubibrowser/.

The data derived from three independent experiments are presented as mean ± SEM in the bar graphs(A, C, D, E, H). Values in control cells were normalized to 1. *, $P$<0.05; **, $P$ < 0.01; N.S., not significant(A, C-E, H), ##, $P$ < 0.01, compared with Gp78-flag (B) or K48 (E) or K116R (H) control.

**Supplementary Fig.3**

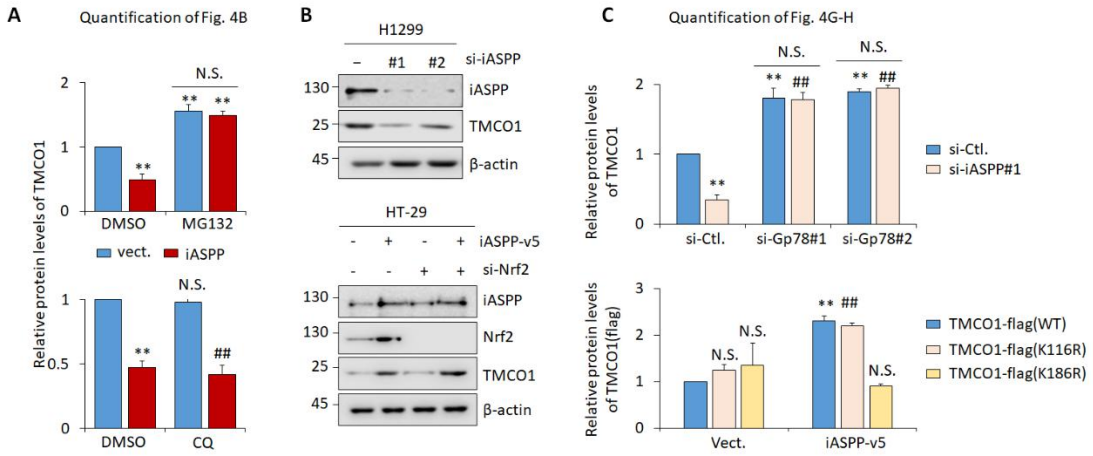



**Supplementary Fig. 3. Gp78 protein and mRNA expression in human cancers**

**(A)** Gp78 protein and mRNA levels were obtained in BRCA, LUAD, UCEC, STAD and COAD and normal control tissues by analysing CPTAC (upper panels) and TCGA (lower panels) databases, respectively.

**(B)** the potential protein was showed as a Gp78 interacting protein by mass spectrum following Gp78 pull-down. The log2 value of the abundance ratio (Gp78 pull-down versus IgG control) is plotted against the number of peptides identified from Gp78 pull-down.

**Supplementary Fig.4**



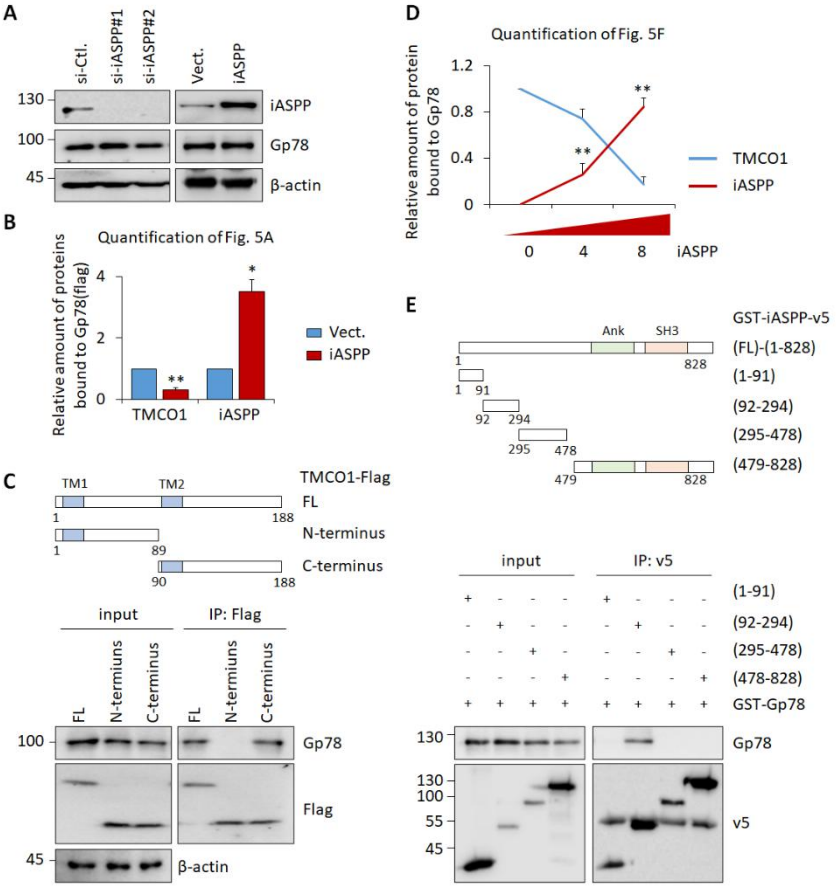

**Supplementary Fig. 4. iASPP affects the TMCO1 levels independently of Nrf2 or p53**

**(A, C)** TMCO1 bands shown in Fig 4B (A), Fig. 4G-H (C) were quantified by image J.

**(B)** Representative WB of TMCO1 protein expression in p53 null H1299 (top) and Nrf2 KD HT-29 cells (bottom). β-actin was used as a loading control.

The data derived from three independent experiments are presented as mean ± SEM in the bar graphs (A, C). Values in control cells were normalized to 1.**, $P < 0.01$; N.S., not significant.(A, C) ##, $P < 0.01$, compared with CQ treated vect.(A) or iASPP KD (C, top) or TMCO1-flag(K116R) (C, bottom) control.

**Supplementary Fig.5**




**Supplementary Fig. 5. iASPP promotes TMCO1 stabilization by competitive binding to Gp78**

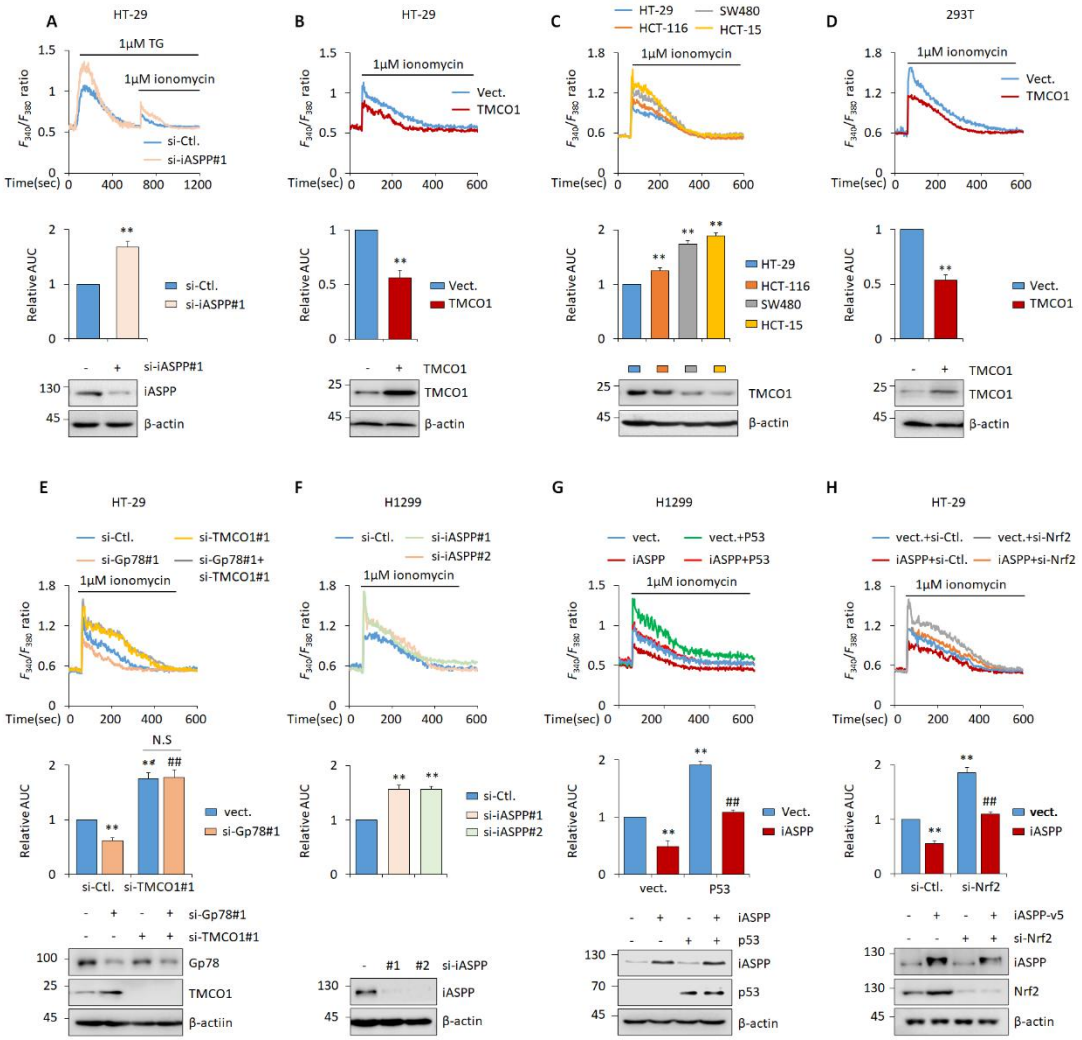
**(A)** The representative WB images of Gp78 in iASPP KD or OE in HT-29 cells. iASPP KD and OE efficiency was confirmed and β-actin was used as a loading control. The representative image of HT29 iASPP OE blot is same as the one presented in Fig. 4A.

**(B, D)** Grp78 bands shown in Fig 5A (B), Fig. 5F (D) were quantified by image J. The data derived from three independent experiments are presented as mean ± SEM in the graphs. **, $P < 0.01$; *, $P<0.05$.

**(C)** The interactions of Gp78 protein with full length (FL) TMCO1, N-terminal or C-terminal TMCO1 fragments, as indicated in the diagram (top), were compared by IP assay (bottom) in HT-29 cells. β-actin was used as a loading control.

**(E)** The interactions of purified Gp78 with purified iASPP truncates, as indicated in the diagram (top), were compared by in vitro IP assay (bottom).

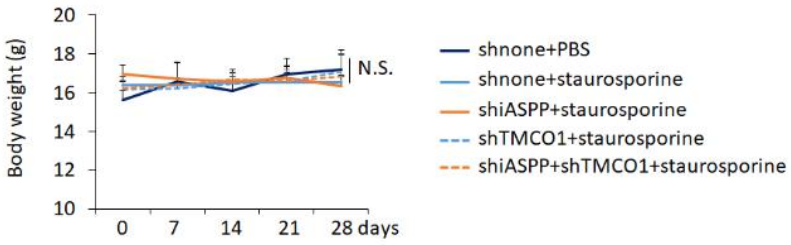
**Supplementary Fig.6**




**Supplementary Fig. 6. iASPP lowers ER $Ca^{2+}$ store in p53- and Nrf2- independent manners**

**(A-H)** Representative WB images showing the protein overexpression or KD efficiency of iASPP (A,F-H), TMCO1(B-E), p53 (G) or Nrf2 (H) in the indicated cancer cell lines or non-malignant HEK293T cells. β-actin was used as a loading control (bottom). ER $Ca^{2+}$ store was measured by loading with 5μM Fura-2 AM following TG (1μM) followed by added ionomycin (1μM) (A) or ionomycin (1μM)(B-H) in the presence of $Ca^{2+}$-free medium (n=20). The representative images shown on the top. The quantification of area under curve (AUC) derived from at least three independent experiments are presented as mean ± SEM in the bar graph in the middle (A-H). Values in control cells were normalized to 1. *, $P$<0.05; **, $P$ < 0.01; N.S., not significant. ##, $P$<0.01, compared with Gp78 KD (E), p53 overexpression (G) or Nrf2 KD (H) control.

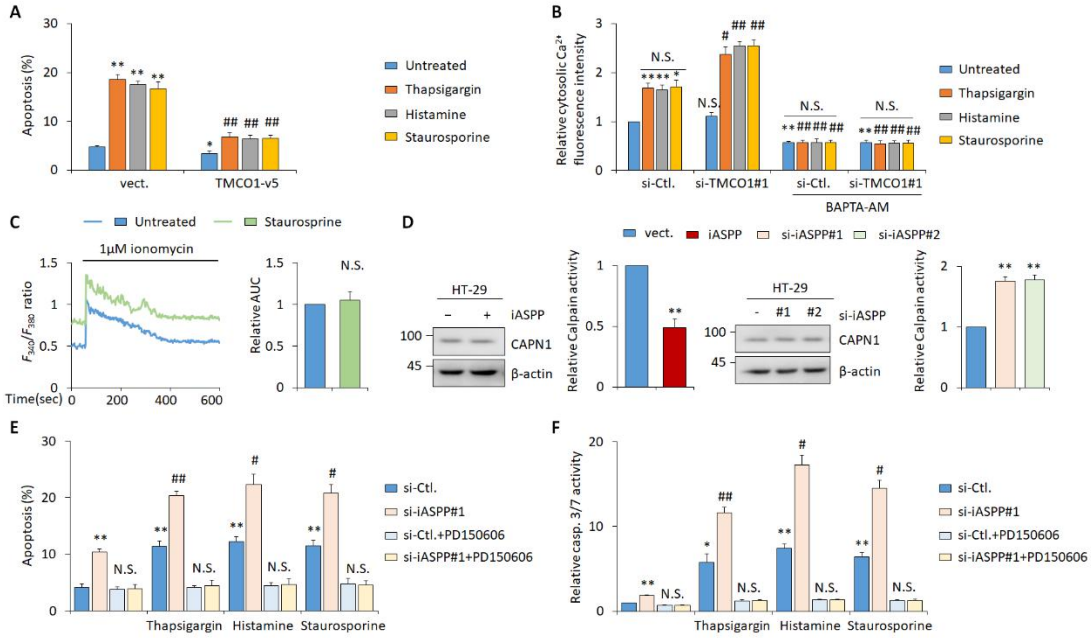
**Supplementary Fig.7**




**Supplementary Fig. 7. The body weight of the nude mice**

Body weight the indicated nude mice were presented. The average values are present in the graphs (means ± SD) (n = 5 for each group). N.S. not significant.

**Supplementary Fig.8**




**Supplementary Fig. 8. Inhibition of iASPP/TMCO1 promotes cytosolic $Ca^{2+}$-induced apoptosis.**

**(A)** The apoptosis levels were revealed by Annexin V/PI staining after TMCO1 overexpression in the presence of thapsigargin (1μM), histamine (100μM), staurosporine (100nM) or etoposide (10μM) in HT-29 cells.

**(B)** The Cytosolic $Ca^{2+}$ concentrations were measured by loading with 5μM Fluo-3 A after Thapsigargin (1μM), histamine (100μM) or staurosporine (100nM) treatments in the control and TMCO1 KD cells in the presence or absence of BAPTA-AM (50 μM).

**(C) The** ER $Ca^{2+}$ store was measured by loading with 5μM Fura-2 AM following ionomycin (1μM) treatment in $Ca^{2+}$-free medium in HT-29 cells(n=20) after staurosporine (100nM) treatment.

**(D)** The calpain activity was measured by calpain activity kit (G8501, Promega) in iASPP overexpression or KD HT-29 cells.

**(E-F)** The apoptosis levels were revealed by Annexin V/PI staining (D) and caspase 3/7 activity assay (E) after iASPP KD with or without calpain inhibitor (PD150606) in HT-29 cells.

The data derived from three independent experiments are presented as mean ± SEM in the bar graphs (A-B, D-F). Values in control cells were normalized to 1. **, $P < 0.01$; *, $P<0.05$; **, $P < 0.01$; N.S. not significant; $^{\#}$, $P < 0.05$, $^{\#\#}$, $P < 0.01$, compared with drug treated control (A-F).

**Supplementary Table 1**

| | | siRNA sequence(5'-3') |
|---|---|---|
| Control | GenePharma | UUUUCCGAACGUGUCACGUTT |
| iASPP#1 | GenePharma | ACUACUCUAUCGUGGAUUU |
| iASPP#2 | GenePharma | GGCTGACAGAAACAAGCAT |
| Nrf2 | GenePharma | CCGGCATTTCACTAAACACAA |
| p53 | GenePharma | GCACAGAGGAAGAGAAUCU |
| TMCO1 | GenePharma | GGACAGACAAGUACAAGAGTT |
| Gp78#1 | GenePharma | GACGGAUUCAAGUACCUUU |
| Gp78#2 | GenePharma | CATGCAGAATGTCTCTTAA |
| HRD1 | GenePharma | UCAUCAAGGUUCUGCUGUA |
| RNF5 | GenePharma | CGGCAAGAGTGTCCAGTAT |
| RNF185 | GenePharma | GAUAUUUGCCACAGCAUUU |
| SPFH1 | GenePharma | TCCCAGAAGCCATAAGAAG |
| TMEM129 | GenePharma | UGACAAGUUUGCCACCGGU |

**Supplementary Table 2**

| Real-time RT-PCR primer(5'-3') | | |
|---|---|---|
| *TMCO1* | Comate Bioscience | Forward:ATTCCATATTTGATGGTAGAGTGG<br>Reverse: AACAGTCTGTGGTGTCATCTCCC |
| *HRD1* | Comate Bioscience | Forward: AGAGATGGAGCACCTTCTGGAA<br>Reverse: GCTGCGTTCCATAAAGTCCACA |
| *Gp78* | Comate Bioscience | Forward: TGAGAGACAGCATCTCAAAGAC<br>Reverse: CCATCAGGTGCAGAAAGACAAG |
| *RNF5* | Comate Bioscience | Forward: GAGAGCAGAGGGGGATTCCA<br>Reverse: AGATCCACACCTGTACCCCG |
| *RNF185* | Comate Bioscience | Forward: CAGCCGAGACAAGGTCATCC<br>Reverse: ATCCCCCTCTATTCTCCGGC |
| *SPFH1* | Comate Bioscience | Forward: ACGGGACTGAGGGTCCTTTT<br>Reverse: TGGTCCACTGGGGCTAGTTA |
| *TMEM129* | Comate Bioscience | Forward: TAACTTTTCAGCTCTGGGATGC<br>Reverse: ACTACAAAAGCCTAGAAAGGGAA |
| *GAPDH* | Comate Bioscience | Forward: CGACCACTTTGTCAAGCTCA<br>Reverse: ACTGAGTGTGGCAGGGACTC |