**Supplementary documentation**

**Supplementary Materials and Methods**

**Data mining on the GEO database to identify downregulated transcripts in UBUCs**

To explore the clinical relevance of *TMCO1* mRNA level, data mining was first performed on the one dataset (GSE32894) from GEO dataset, analysis on 308 UBUCs using Illumina HumanHT-12 V3.0 Expression BeadChip (Sjodahl et al. 2012; *Clin Cancer Res* 18:3377-86). Another dataset (GSE31684) generated by using Affymetrix GeneChip® Human Genome U133 Plus 2.0 array (Riester et al. 2012; *Clin Cancer Res* 18:1323-33) was also analyzed to confirm the prognostic significance of *TMCO1* expression status. To computerize the expression level, raw files were imported into the Nexus Expression 3 software (BioDiscovery) as described earlier (Li et al. 2015; *Oncotarget* 6:9220-39).

**Branched DNA assay**

For *TMCO1* mRNA quantification, probes were design for *TMCO1* transcript and applied in QuantiGene Multiplex 2.0 assay systems (Panomics) according to the manufacturer’s instructions. In brief, oligonucleotide probes were mixed with the lysed paraffin sections, and the mixture was then added to a 96-well plate coated with capture probe oligonucleotides. Target RNAs were captured during an overnight incubation at 55°C. Unbound materials were removed by three-run washes with 300 μL of wash buffer followed by subsequent hybridization of DNA amplifier molecules. Three washes were performed following incubation. After the final wash, the dioxetane alkaline phosphatase substrate Lumi-Phos Plus (Lumingen) was added to the reaction wells. Next to a short incubation, luminescent signal was detected by using Luminex® 100™ system (Luminex). The detection level of *TMCO1* transcript was further normalized to the polymerase (RNA) II subunit A (*POLR2A*)level.

**Immunohistochemistry**

Slides were deparaffinized with xylene, rehydrated with ethanol, heated by microwave for retrieval of antigen epitopes in a 10 mM citrate buffer (pH 6) for 7 min. Endogenous peroxidase was quenched by 3% H2O2. Slides were washed with Tris buffered saline for 15 min and then incubated with a primary monoclonal antibody against TMCO1 (1:20; HPA014711, Sigma-Aldrich) and Ki-67 (1:200; EPR3610, Abcam) for 1 h, followed by antibody detection using a ChemMate EnVision™ kit (K5001; DAKO, Glostrup, Denmark). One expert pathologist (CF Li) blinded to clinicopathological information, patient outcomes and the xenograft group, interpreted the immunostainings. The immunointensity of membranous and cytosolic TMCO1 staining was scored by using H-score as described in our previous works (Chen et al. 2013; *Ann Surg Oncol* 20:4041-54) The median H-score of all cases was applied as the cutoff point to bisect the two groups, namely high expression (no less than median H-score) and low expression, respectively.

**Quantitative RT-PCR**

Total RNAs were extracted with TRIzol® reagent (Life Technologies) from cells and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies). The relative expression folds oftarget transcripts were given by 2-∆∆CT, where ∆∆CT = ∆CT (pTMCO1-HaloTag transfectant) - ∆CT (HUC or pHaloTag transfectant, control); ∆CT represented the CT of target subtracted the CT of the glycealdehyde-3-phosphate dehydrogenase(*GAPDH*) gene for cell lines. Only samples with CT values < 28 for *GAPDH* were considered to meet acceptable RNA quality standards and included in the analyses.

**Immunoblotting analysis and antibodies**

Cell lysates were prepared with RadioImmunoPrecipitation Assay (RIPA) buffer (Upstate). Lysates containing equal amounts of protein were separated by SDS-PAGE and electroblotted onto Immobilon™-P Transfer Membrane (Millipore). The filters were individually probed with specific primary antibody. The primary antibodies and dilution ratios are listed in Supplementary materials and methods. Protein bands were detected by the Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer Life Sciences) with horseradish peroxide labeled anti-mouse or anti-rabbit secondary antibody as suggested by the manufacturer and visualized on a VersaDoc Image System (Bio-Rad).The intensity of bands was quantified by densitometry and normalized to GAPDH or actin, alpha 1, skeletal muscle (ACTA1) in each lane. The intensity of bands from immunoblotting assays were quantified by densitometry and normalized to that of GAPDH or ACTA1 in each lane. Anti-human TMCO1 (1:500, AV49429, Sigma-Aldrich), anti-DDK (1:1000, TA50011, Origene), anti-retinoblastoma 1 (RB1; 1:500, sc-102, Santa Cruz), anti-TP53 (1:1000, GTX102965, GeneTex), anti-phospho-TP53(Ser15) [pTP53(S15); 1:1000; #9286, Cell Signaling], anti-cyclin-dependent kinase inhibitor 1A (CDKN1A; 1:1000, #2947, Cell Signaling), anti-pCDKN1A(T145) (1:500, sc-20220-R, Santa Cruz), anti-CDKN1B (1:500, #3686, Cell Signaling), anti-pCDKN1B(T157) (1:3000, #AF1555, R&D Systems), anti-CDKN1C (1:500, sc-1040, Santa Cruz), anti-CDKN2A (1:500, sc-1207, Santa Cruz), anti-CDKN2B (1:500, sc-612, Santa Cruz), anti-CDKN2C (1:500, sc-865, Santa Cruz), anti-CDKN2D (1:500, sc-1063, Santa Cruz), anti-cyclin D1 (CCND1; 1:1000, #2926, Cell Signaling), anti-cyclin-dependent kinase 4 (CDK4; 1:500, sc-260, Santa Cruz), anti-CCNE1 (1:500, sc-198, Santa-Cruz), anti-CDK2 (1:500, sc-163, Santa-Cruz), anti-AKT serine/threonine kinase [AKT (pan); 1:10000, #4691, Cell Signaling], anti-pAKT1(S473) (1:10000, #4060, Cell Signaling), anti-MDM2 proto-oncogene, E3 ubiquitin protein ligase (MDM2; 1:1000, GTX110608, GeneTex), anti-pMDM2(S166) (1:1000; #3521, Cell Signaling), anti-poly (ADP-ribose) polymerase 1 (PARP1; 1:1000, #9542, Cell Signaling), anti-CD44 molecule, Indian blood group (CD44; 1:2500, 15675-1-AP, Proteintech), anti-vimentin (VIM; 1:1000, #3932, Cell Signaling), anti-PHLPP1 (1:1000, ab71972, Abcam), anti-PHLPP2 (1:1000, ab71973, Abcam), anti-gylcogen synthase kinase 3 alpha (GSK3A, 1:2500, #1742-1, Epitomics), anti-HaloTag® (1:1000, G9211, Promega), anti-GAPDH (1:5000, ab128915, Abcam) and anti-pan-actin (1:7000, MAB1501, Millipore) were used as primary antibodies for immunoblotting analysis. Protein bands were detected by Chemiluminescent Reagent Plus kit (Perkin-Elmer) with horseradish peroxidase-labeled secondary antibody.

**Lentivirus production and stable knockdown of the *TMCO1* gene**

Briefly, Phoenix-AMPHO cells (ATCC) were seeded in 6-cm tissue culture plate at a density of 1 × 106 in 5 mL medium with 10% FBS, 100 IU/mLpenicillin and 100 μg/mL streptomycin (Corning®) overnight. PolyJet™ (15 μL, #SL100688, *SignaGen® Laboratories)* was used to transfect the plasmid mixture [psPAX2 (2.25 μg, Addgene), PMD2.G (0.25 μg, Addgene) and 2.5 μg of shLuc (control), shTMCO1#3 or shTMCO1#5 plasmids]*, and t*he medium was changed after a 16 h incubation. Medium was collected and filtered (0.22 μm) at 40 h and 64 h post-transfection, aliquots of 1 mL were stored at -80°C. RT4 (1 × 106) and J82 cells (1 × 105) were next infected with media containing lentiviral particles with polybrene (8 μg/mL), and incubated for another 24 h at 37°C. Afterward, media containing 4 μg/mL puromycin (Sigma-Aldrich) were used to select positive cells for 7 d and subsequently maintained in media containing 2 μg/mL puromycin for further experiments.

**Cell-cycle, cell viability, proliferation and soft agar assays**

For cell cycle analysis, 1 × 106 cells were collected, washed with ice-cold PBS, fixed with 70% ethanol and stored at -20°C after stable transfection of pCMV6-Entry or pCMV6-TMCO1 plasmid, or infection with shTMCO1#3, shTMCO1#5, or shLuc lentiviral particles. Before analysis, fixed cells were washed with ice-cold PBS for three times and treatments with 200 μg/mL RNase A (#R6513, Sigma-Aldrich) and 20 μg/mL propidium iodide (#P4170, Sigma-Aldrich). A total of 10,000 events were analyzed; cell cycle distribution was analyzed by a flow cytometer (NovoCyte™ 2000, ACEA) and NovoExpression™ software, with respective wavelengths for excitation and emission of 488 and 530 nm.

To determine cell viability and proliferation upon alternation of *TMCO1* expression levels, 2 × 103 and 3 × 103 cells were seeded on 96-well microplates for MTT and BrdU assays, respectively. After removing the medium, 20 μL of MTT (5 mg/mL; Sigma-Aldrich) were added to each well and cells were incubated for another 4 h. At the end of incubation, the MTT solution was replaced by 100 μL of DMSO. Further, BrdU Cell Proliferation Assay Kit (QIA58, Calbiochem) was used to perform cell proliferation test. BrdU label (1:2000 dilution) was incubated for 24 h. Plates were then washed, stained with anti-BrdU antibody, and peroxidase-conjugated goat anti-mouse IgG. 3,3’,5,5’-tetramethylbenzidine substrate (0.1 mL in ethanol) was next added into the immunocomplex and the reaction was terminated via adding 100 μL of sulfuric acid (2.5 N). Absorbances were afterward measured at wavelengths of 570 and 490 nm for MTT and BrdU assays, correspondingly, using a Beckman Coulter PARADIGM™ Detection Platform (Fullerton). Percentages of viable cells (%) and proliferation rate (%) were calculated as 100 x [(ODindicated time after transfection 7d – OD7d after transfection)/OD7d after transfection].

CytoSelect™ 96-well in vitro tumor sensitivity assay (soft agar colony formation, CBA-150-5, CELL BIOLABS) was used to analyze whether stable expression and knockdown of *TMCO1* affected anchorage-independent cell growth. Briefly, 50 μL/well (in a 96-well sterile flat-bottom microplate) of the Base Agar Matrix Layer was prepared by mixing 1.25 mL of 2X DMEM/20% FBS medium, 1 mL of sterile water, 0.25 mL of melted 10X CytoSelect™ Agar Matrix Solution. Cell Suspension/Agar Matrix Layer under sterile conditions (75 μL/well) was made by mixing 1.75 mL of 2X DMEM/20% FBS medium, 1.375 mL of CytoSelect™ Matrix Diluent, 0.375 mL of melted 10X CytoSelect™ Agar Matrix Solution and 0.25 mL of Cell Suspension (5 × 103 cells), according to the manufactures’ instructions. The incubation periods were 7 days for both *TMCO1*-overexpressed TSGH8301 and *TMCO1*-knocked down RT4 and J82 cells. MTT assay was used to quantitate the anchorage-independent growth.

**Wound healing, transwell migration and transwell invasion assays**

For wound healing assay, *TMCO1*-overexpressed TSGH8301 and *TMCO1*-knocked down J82 cells were plated confluently in 6-cm dish overnight, treated with 5 μg/mL mitomycin C for 2 h and replaced with regular medium. A cell-free gap was created using a silicon Culture-Insert (ibidi GmbH) place on the Petri dishes. After removing the silicon insert from the surface, clean gap was formed. Cell migration into the clean region was recorded using Axiovert 40 CFL (Zeiss International) at 0 and 32 h, and the percentage of wound healing was determined via dividing the migrated distance by the scratched distance.

For transwell invasion assay, *TMCO1*-overexpressed TSGH8301 (3 × 105) and *TMCO1*-knocked down J82 cells (4 × 104) were starved in media (500 μL) containing 0.5% FBS at 37°C overnight, seeded in ECMatrix™-coated inserts in a 24-well plate. Literally 750 μL of media containing 10% FBS were added into the lower chambers and cells were cultured for another 24 h. The inserts were removed, placed into new lower chambers and the penetrated cells were detached with Cell Detachment Solution and lysed with Lysis Buffer/Dye Solution. The lysed mixtures were transferred to a 96-well plate for fluorescence measurement at wavelengths 480/520 nm using a Beckman Coulter PARADIGM™ Detection Platform. For transwell migration assay, ECMatrix™-coated inserts were replaced by BD Falcon Cell Culture Inserts (#353097).

**Coimmunoprecipitation (Co-IP)**

Cells were grown on coverslips, washed with PBS and fixed in ice methanol for 10 mim and 4% formaldehyde (in 1 × PBS). With each step forward, cells were washed with PBS for three times. Cells were next treated with 0.3% Triton X-100 and blocked overnight with PBS containing 1% bovine serum albumin at 4°C. anti-PHLPP1 (1:50, ab71972, abcam) and anti-PHLPP2 (1:50, ab71973, abcam) were applied, incubated at 4°C for 48 h; anti-TMCO1 antibody (1:200) was next added, incubated for another 3 h. Unbound antibodies were removed with PBS, and fluorescein isothiocyanate (FITC)-labeled antibody (1:100; #115-095-003, Jackson Laboratory) and goat anti-rabbit Texas Red® secondary antibody (1:200, ab6719, abcam) were loaded for 1 h at room temperature, rinsed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/mL) for 5 min. Following a series of washes, cells were mounted, viewed under a confocal microscope (IX-81, Olympus).

**Immunocytochemistry**

Briefly, 1 × 107 cells were seeded overnight and trypsinized. A total of 2,000 μL RIPA assay buffer containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was added into cell pellets and thoroughly blended with a Intelli Mixer (ELMI Laboratory) at 4°C for 15 min. The supernatant was next collected after centrifugation at 14,000 *×* g at 4°C for 15 min. Literal 200 μL of the supernatant was used as input (positive control) and the remaining 1,800 μL was equally divided and immunoprecipitated with 2 μg of normal rabbit IgG (NI01, Millipore), anti-PHLPP1 (ab71972, abcam) and anti-PHLPP2 (ab71973, abcam) antibodies pre-conjugated to protein G agarose beads (Thermo Scientific), respectively, overnight. Input and immunoprecipitates in Pierce™ IP Lysis Buffer (#87787, ThermoFisher Scientific) were subjected to SDS-PAGE and immunoblotted with anti-TMCO1 antibody as described in immunoblotting analysis.

**Primers for TMCO1 mutagenesis**

Primers 5’-ACCTGGGTCCTGGTTTACAGGGCCGACAAGTACAAGAGACTGAAG-3’ and 5’-CTTCAGTCTCTTGTACTTGTCGGCCCTGTAAACCAGGACCCAGGT-3 were used to construct pTMCO1(T33A)-HaloTag plasmid. Primers 5’-AGGAAACAATAACAGAGGCAGCTGGTCGACAACAG -3’ and 5’-CTGTTGTCGACCAGCTGCCTCTGTTATTGTTTCCT-3’ were used to construct pTMCO1(S60A)-HaloTag plasmid. Primers 5’-ACTGAAGAATAACAACAGAGATCTAGCCATGGTTCGAATGAAATC-3’ and

5’-GATTTCATTCGAACCATGGCTAGATCTCTGTTGTTATTCTTCAGT-3’ were used to construct pTMCO1(S84A)-HaloTag plasmid.

**Determination of TMCO1 gene promoter methylation using pyrosequencing**

Promoter methylation of the *TMCO1* gene was quantified by pyrosequencing in RT4, TSGH8301, T24 and J82 cell lines and tissues from non-tumor urothelium (*n* = 8), UBUC with high TMCO1 protein level (*n* = 8) and UBUC with low TMCO1 protein level (*n* = 8). After bisulfate treatment and cleanup of DNA by using EpiTect 96 Bisulfite Kit (Qiagen), PCR amplification and sequencing were performed by using Hs\_TMCO1\_01\_PM and Hs\_TMCO1\_02\_PM PyroMark CpG assays (PM00089061 & PM00089068, Qiagene) following our previous protocol (Wang et al. 2015; *Oncotarget* 6:31069-84). Finally, PyroMark Q24 software (Qiagen) was used to quantify cytosine methylation. Methylation was called when the average methylation percentage of all CpG islands tested was higher than 3.74%, which was the mean +3SD of the average methylation percentage of CpG islands from 8 nontumor urothelium as described previously (Chanudet et al. 2010; *Leukemia* 24:483-87).

**Table S1.** *TMCO1* is identified as a differentially expressed transcript with molecular function of growth factor activity (GO:0008083) and low expression levels shows positive correlations to cancer invasiveness and metastasis in the transcriptome of urothelial carcinoma of urinary bladder (GSE31684)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Probe | pT2-T4 vs. pTa | | pT2-T4 vs. pT1 | | Gene Symbol |
| log ratio | *P* value | log ratio | *P* value |
| ILMN\_1793829 | -0.2629 | **0.0006\*** | -0.1972 | **0.0174\*** | *TMCO1* |

**Table S2.** Correlations between the TMCO1 protein level and important clinicopathological parameters in urothelial carcinomas

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameter** | **Category** | ***n*** | **TMCO1 level** | | ***P* value** |
|  |  |  | High | Low |  |
| **Gender** |  |  |  |  | 0.376 |
|  | Male | 216 | 105 | 111 |  |
|  | Female | 79 | 43 | 36 |  |
| **Age (years)** |  |  |  |  | 0.587 |
|  | < 65 | 121 | 63 | 58 |  |
|  | ≥ 65 | 174 | 85 | 89 |  |
| **Primary tumor (T)** |  |  |  |  | **< 0.001\*** |
|  | Ta | 84 | 57 | 27 |  |
|  | T1 | 88 | 50 | 38 |  |
|  | T2-T4 | 123 | 41 | 82 |  |
| **Nodal metastasis** |  |  |  |  | 0.165 |
|  | Negative (N0) | 266 | 137 | 129 |  |
|  | Positive (N1-N2) | 29 | 11 | 18 |  |
| **Histological grade** |  |  |  |  | **0.003\*** |
|  | Low | 56 | 38 | 18 |  |
|  | High | 239 | 110 | 129 |  |
| **Vascular invasion** |  |  |  |  | **0.003\*** |
|  | Absent | 246 | 133 | 113 |  |
|  | Present | 49 | 15 | 34 |  |
| **Perineural invasion** |  |  |  |  | 0.160 |
|  | Absent | 275 | 141 | 134 |  |
|  | Present | 20 | 7 | 13 |  |
| **Mitotic rate (per 10 high power fields)** |  |  |  |  |  |
|  | < 10 | 139 | 79 | 60 | **0.031\*** |
|  | ≥ 10 | 156 | 19 | 87 |  |

\*, statistically significant

**Table S3.** *TMCO1* promoter methylation detected by pyrosequencing in urothelial cancer cells and tissues

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Hs\_TMCO1\_01\_PM PyroMark CpG assay@ | | | | Hs\_TMCO1\_02\_PM PyroMark CpG assay$ | | | |
| Site 1 (%) | Site 2 (%) | Site 3 (%) | Site 4 (%) | Site 1 (%) | Site 2 (%) | Site 3 (%) | Site 4 (%) |
| RT4 cell line | 3 | 1 | 2 | 4 | 2 | 1 | 6 | 5 |
| T24 cell line | 2 | 1 | 1 | 1 | 1 | 2 | 4 | 4 |
| J82 cell line | 4 | 1 | 1 | 2 | 2 | 1 | 7 | 3 |
| Non-tumor urothelium(*n* = 8)**#** | 1.125 | 1.250 | 1.375 | 1.375 | 1.375 | 1.500 | 3.375 | 4.500 |
| UBUC with high TMCO1 protein level (*n* = 7)**#** | 1.286 | 1.429 | 1.571 | 2.286 | 1.571 | 1.571 | 4.000 | 4.571 |
| UBUC with low TMCO1 protein level (*n* = 7)**#** | 1.429 | 1.286 | 1.714 | 2.143 | 1.429 | 1.286 | 4.143 | 4.286 |

#, average methylation percentage of each CpG site is presented

@, Cat No. PM00089061; $, Cat No. PM00089068

**Figure S1.** High-resolution oligonucleotide-based array comparative genomic hybridization shows frequent DNA copy number gain at loci spanning *TMCO1* gene at 1q24.1 (9/40, 22.5%; including 1 exhibited high-level gain). The lack of deletion involving *TMCO1* gene locus suggested genome deletion is less likely the inactivation mechanism of *TMCO1*.

**Figure S2.** Alteration the TMCO1 levels changes the ratios of pCDKN1A(T145) (inactive form)/CDKN1A and CDN1B(T157) (inactive)/CDKN1B but *TP53* mRNA levels were changed inconsistently. Overexpression of the *TMCO1* gene in BFTC905 cells **(A)** notably downregulated phospho/inactive CDKN1A and CDKN1B to ~25%, yet was not able to induce *TP53* mRNA levels **(B). C**o-transfection of pTMCO1-HaloTag and a constitutive active AKT1 plasmid, pHRIG-AKT1, recovered phospho/inactive CDKN1A and CDKN1B to ~66% and 49%, respectively **(A)**. Knockdown of the *TMCO1* gene with 2 distinct shRNAi clones **(C)** downregulated phospho/inactive CDKN1A and CDKN1B to ~70 to 92%, however, upregulated *TP53* mRNA levels. **(D)** Overexpression pTMCO1-HaloTag carrying wild-type (WT) or another 3 mutants T33A, S60A and S84A, were not able to alter the *TP53* mRNA levels.

**Figure S3.** Stable knockdown of the *TMCO1* gene with two distinct shRNAi clones in J82 cells downregulated *TMCO1* mRNA and protein levels **(A);** decreased and increased cells in G1 and S phases, respectively **(B);** enhanced cell viability **(C)**, cell proliferation **(D),** colony formation/anchorage-independent cell growth **(E, F).** Protein levels of RB1, TP53, pTP53(S15), CDKN1A, CDKN1B, CDK4 were downregulated; CCND1, CCNE1, CDK2, pAKT1(S473), MDM2, pMDM2(S166) protein levels and pCDKN1A(T145)/CDKN1A and pCDKN1B(T157)/CDKN1B ratios were notably upregulated **(G)**. All experiments were performed in triplicate and results are expressed as the mean ± SEM. For immunoblotting analysis, on representative image is shown. Pan-actin served as a loading control. Statistical significance: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**Figure S4.** Immunoblotting analyses on a series of cyclin-dependent kinase inhibitors after stable *TMCO1* overexpression and knockdown showed that CDKN1C, CDKN2B, CDKN2A, CDKN2C and CDKN2D protein levels were not able to be induced **(A)** and suppressed **(B)** in TSGH8301 and RT4 cells, respectively. All experiments were conducted in triplicated and one representative immunoblotting image is shown. GAPDH served as a loading control.

**Figure S5.** Wound healing, transwell migration and transwell invasion assays demonstrated that knockdown of the *TMCO1* gene in RT4 cells enhanced cell migration **(A, B)** and invasion **(B)**.

**Figure S6.** BFTC905 cells (2 × 105) were seeded overnight, starved (0.5% FBS) for 2 h and treated with a pan-PHLPP inhibitor, NSC117079 for 35 min with different concentrations. Immunoblot analysis identified 15 μM of NSC117079 profoundly upregulated pAKT1(S473) protein levels.

**Figure S7.** Immunocytochemistry (ICC) shows that TMCO1 and PHLPP1 proteins were only partially colocalized in BFTC905 and RT4 cells. Blue: DAPI (nuclear); red: TMCO1 (cell membrane & cytoplasma), green: PHLPP1 (membrane). One representative ICC image is shown for each cell line.

**Figure S8.** Tumor growth curve and relative volumes of SCID/NOD mice bearing BFTC905 cell xenografts. Tumor growth curve of mice stably carrying pHaloTag (control), wild-type TMCO1 [pTMCO1(WT)-HaloTag], and another three mutants: pTMCO1(T33A)-HaloTag, pTMCO1(S60A)-HaloTag and pTMCO1(S84A) plasmids.

**Figure S9.** In vitro and in vivo studies show that *TMCO1* is a tumor suppressor in HTB-33 epithelial cells. Stable overexpression of the *TMCO1* gene in HTB-33 cells (cervix, derived from metastatic site, omentum) **(A)** induced the expression of TMCO1-DDK (Flag) protein, **(B)** induce G1 cell cycle arrest, reduced cells in the S phase;suppressed cell viability **(C)**, cell proliferation **(D),** colony formation **(E)**, anchorage-independent cell growth **(F)**; altered the expression levels of several cell cycle regulators, downregulated phospho/inactive CDKN1A and CDKN1B ratios **(G)**, downregulated pAKT1(S473), MDM2 and pMDM2(S166) protein level **(H)**, upregulated nuclear CDKN1A, both nuclear and cytosolic CDKN1B **(I)**, yet did not change the *TP53* mRNA level **(J)**, enhanced cell migration and invasion **(K, L)**, accompanied with CD44 and VIM downregulation (M). Exogenous TMCO1 expression was not able to upregulate PHLPP1 and PHLPP2 protein levels **(N)**. However, one pan-PHLPP inhibitor, NSC117079, restored TMCO1-suppressed pAKT1(S473) level **(O)**. Immunocytochemistry further indicated that TMCO1 and PHLPP2 are colocalized in cytoplasm and cell membrane **(P)**. Co-transfection of the pTMCO1-HaloTag and pcDNA3-HA-PHLPP2 plasmids, notably downregulated pAKT1(S473) **(R)** and AKT kinase activity **(S)** compared to transfection with the pTMCO1-HaloTag or the pcDNA3-HA-PHLPP2 plasmid alone. *TMCO1*-overexpressed HTB-33 cells along with SCID/NOD mice models show that *TMCO1* suppresses tumor growth in vivo **(T, U)**. Mice were sacrificed on day 28, control xenografts (cells carrying pHaloTag) displayed a carcinoma with high cellularity, while the *TMCO1*-overexpressing group (pTMCO1-HaloTag) showed large areas of necrosis and stromal hyalinization and a much low percentage of cancer components.