# Supporting Information

## Ma et al. 10.1073/pnas.1202989109

### SI Materials and Methods

**Cell Culture and Transfection.** HEK293 cells (ATCC) were cultured in DMEM supplemented with 10% (vol/vol) FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin. MCF-7/WT (ATCC) and adriamycin-resistant human breast cancer cells (MCF-7/ADM) were cultured in RPMI supplemented with 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin. MCF-7/ADM cells were derived by treating MCF-7 cells with stepwise increasing concentrations of adriamycin over 8 mo. Transfection was done with Lipofectamine 2000 or by electroporation using Nucleofector II following the manufacturer's procedure.

**Plasmids, Antibodies, and siRNAs.** The dominant-negative construct of transient receptor potential channel isoform C5 (TRPC5-DN) was a kind gift from D. E. Clapham (Harvard University, Cambridge, MA) and was constructed by alanine replacement of a conserved LFW motif (amino acids 575–577) within the TRPC5 pore region. TRPC5-DN or EGFP cDNA were each subcloned into the lentiviral vector pRRL-cPPT-CMV-X-PRE-sin. Lentivirus carrying different constructs, including lenti-TRPC5-DN and lenti-GFP as control, were produced by cotransfecting HEK293 cells with respective lentiviral constructs and packaging vectors. Four isoforms of nuclear factor of activated T cells (NFATc1–4) were from Addgene. Primary antibodies anti-TRPC1 (ACC-010), anti-TRPC3 (ACC-016), anti-TRPC4 (ACC-018), and anti-TRPC6 (ACC-017) were from Alomone Labs. Anti-P-gp (P-glycoprotein) (SC-55510) was from Santa Cruz Biotechnology. Anti-TRPC5 (ab63151) used in immunohistochemistry experiments was from Abcam. The luciferase reporter vector carrying 5′ flanking 800-bp sequence of mdr1 gene (LR1028) (with promoter and enhancers) was from Panomics. Mutant with deletion at NFATc3 binding site (ΔTTTTCC, −542∼ −537 nt) was generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene). Mutagenic oligonucleotides were 5′-GTAAACAAATGAATTTCCATAAAGCTAATTTATCTTTATAATACTTATTACTTCAAATTCTTGTTACATTT-3′ and 5′-AAATGTAACAAGAATTTGAAGTAATAAGTATTATAAAGATAAATTAGCTTTATGGAAATTCATTTGTTTAC-3′.

siRNA sequences were as follows: siTRPC1 (sense strand, GGAUGUGCGGGAGGUGAAGtt, antisense strand, CUUCACCUCCCGCACAUCCtt, Ambion), siTRPC3 (sense strand, GCAGCAGCUCUUGACGAUCUGGUAU; antisense strand, AUACCAGAUCGUCAAGAGCUGCUGC, Invitrogen), siTRPC4 (sense strand, UGUCUAUGUUGGAGAUGCUCUAUUA; antisense strand, UAAUAGAGCAUCUCCAACAUAGACA, Invitrogen), siTRPC5 (BLOCK-iT in vivo siRNA from Invitrogen, sense strand, CCA AUG GAC UGA ACC AGC UUU ACU U; antisense strand, AAG UAA AGC UGG UUC AGU CCA UUG G), siTRPC6 (sense strand, CCCAAGGAAUAUUUGUUUGAGUUGU; antisense strand, ACAACUCAAACAAAUAUUCCUUGGG), siSTIM1 (sense strand, GCCUAUAUCCAGAACCGUUtt, antisense strand, AACGGUUCUGGAUAUAGGCaa, Ambion) and siOrai1 (sense strand, CGUGCACAAUCUCAACUCGtt; antisense strand, CGAGUUGAGAUUGUGCACGtt, Ambion).

**Preparation of TRPC5-Blocking Antibody T5E3 and Preimmune IgG.** T5E3 antibody was raised in rabbits using the strategy developed by others (1). Briefly, a peptide corresponding to TRPC5 putative pore-region (CYETRAIDEPNNCKG; E3 peptide) was synthesized and conjugated to keyhole limpet hemocyanin at Alpha Diagnostic International. The coupled T5E3 peptide (0.5 mg) was injected s.c. in the back of a rabbit at day 0, followed by two boost doses at day 21 and day 42, respectively. Antiserum was collected 4 wk after the second boost. IgG was purified from the T5E3 antiserum and the preimmune serum using a HiTrap protein G column (GE Healthcare). The T5E3 antibody was further purified from the IgG by an affinity column prepared with E3 peptide-conjugated SulfoLinked Coupling Resin (Thermo Scientific).

**Luciferase Activity Assays.** HEK293 cells stably expressing TRPC5 were seeded in 24-well plates. Cells were transiently cotransfected with 0.2 μg reporter plasmid (*mdr1*-luc or ΔTTTTCC-*mdr1*-luc), 0.2 μg expression plasmid (NFATc3), and 0.04 μg pRL-CMV, using Lipofectamine 2000 reagent. After 48 h, cells were harvested for luciferase reporter assay using the dual-luciferase reporter assay system (Promega), in which renilla luciferase activity of pRL-CMV was served as transfection control. Transcriptional activity of mdr1 promoter was expressed as fluorescence intensity increase in fold compared with that of empty vector-transfected cells.

**Whole-Cell Patch Clamp.** Whole-cell current was measured with an EPC-9 patch clamp amplifier as described elsewhere (2). The holding potential was 0 mV, and current–voltage relationship was obtained using a ramp protocol from +100 mV to −100 mV, with 500-ms duration. The pipette solution contained (in mM) 130 Cs-aspartate, 2 $MgCl_2$, 5 $Na_2ATP$, 5.9 $CaCl_2$, 10 EGTA, and 10 Hepes (pH 7.2) with CsOH. Free $Ca^{2+}$ was ∼200 nM. The bath solution was the hypotonic solution containing (in mM) 65 $Na^+$-aspartate, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, and 10 glucose (pH 7.4 with NaOH), and calibrated with mannitol, ∼210 mOsm. If needed, 2-aminoethoxydiphenylborate (2-APB) (60 μM) was applied to the bath solution. Some cells were pretreated for 60 min with 20 μg/mL T5E3 or preimmune IgG as control. Some were transfected with TRPC5-DN (8 μg per well in six-well plates) or with empty vector as control, or with 200 nM TRPC5-siRNA (scrambled siRNA as control).

**$[Ca^{2+}]_i$ Measurement.** A $Ca^{2+}$-sensitive molecular construct, GECO1.2, was used (3). Normal physiological saline solution contained (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 Hepes (pH 7.4). GECO1.2 fluorescence signals were measured at room temperature (∼23 °C) using an Olympus fluorescence imaging system. Changes in $[Ca^{2+}]_i$ were displayed as a relative fluorescence intensity compared with the value before $La^{3+}$ application (100 μM, F1/F0). Genistein (50 μM) was included in the bath solution to potentiate TRPC5.

**Adriamycin Accumulation Assay.** Distribution of adriamycin in MCF-7/WT and MCF-7/ADM cells was determined using a Fluoview 1000 scanning confocal microscope (Olympus) with a ×60 oil immersion objective lens. The cells on coverslips were treated with adriamycin and then examined under confocal microscope. Adriamycin fluorescence was determined with excitation wavelength at 488 nm using an argon laser, and the emission was collected through a 530-nm long-pass filter.

**Real-Time PCR.** cDNAs from approximately $1 \times 10^5$ MCF-7/WT and MCF-7/ADM cells were obtained using an iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad) for 40 cycles of 95 °C for 15 s and 53 °C for 40 s. Data were analyzed with the iCycler Multicolor Real-time PCR Detection System (Bio-Rad) software. The primer pairs for P-gp were forward 5′-GCC GGG AGC AGT CAT CTG TGG T-3′, reverse 5′-GAT CCA TTC CGA CCT

CGC GCT-3′. The expected product size was 251 bp for P-gp. Relative mRNA concentrations (ng) were calculated with an internal standard curve. The internal standard is a pool consisting of equal amounts of each of the experimental samples, which were serially diluted from 200 to 12.5 ng for real-time PCR.

**NFATc3-GFP Translocation.** NFATc3-GFP levels in the cytosol and nucleus were measured using a Fluoview 1000 scanning confocal microscope (Olympus) with a ×60 oil immersion objective lens. Regions of interest of identical size were drawn in the cytosol and nucleus of each cell, and fluorescence was computed. The nuclear/cytosolic ratio of NFATc3-GFP fluorescence was calculated.

**Immunohistochemistry Staining.** Tissue slides were deparaffinized with xylene and rehydrated through a graded alcohol series. The endogenous peroxidase activity was blocked by incubation in a 3% hydrogen peroxide solution for 15 min. Antigen retrieval was carried out by immersing the slides in 10 mM sodium citrate buffer (pH 6.0) and maintaining at a subboiling temperature for 10 min. The slides were rinsed in PBS and incubated with 10% normal donkey serum (NDS) for 1 h. The slides were then incubated with the primary antibody [anti-P-gp (1:500) and anti-TRPC5 (1:500) diluted in 10% NDS and 0.4% sodium azide in PBS] at 4 °C in a humidified chamber. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG and biotinylated donkey anti-mouse IgG at a dilution of both 1:500 in PBS containing 5% NDS for 1 h. The sections were then incubated in ExtrAvidin peroxidase diluted 1:500 in PBS for 1 h. The sections were then processed according to the Dako liquid DAB$^+$ substrate chromogen system. All incubations were held at room temperature and separated by 3 × 5-min washes in PBS. Sections were lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped. As a negative control, an adjacent paraffin section of each sample was treated with 10% normal donkey serum in place of the primary antibody.

**In Vivo Tumorigenicity Assay.** MCF-7/ADM cells were s.c. injected into the flanks of female nude mice ($5 \times 10^6$ cells per mouse) and allowed to propagate for 4–8 wk. All mice were housed in an air-filtered pathogen-free condition. Tumor growth was monitored with digital calipers weekly. Tumor volumes were estimated using this formula: volume ($mm^3$) = $(\text{width})^2 \times \text{length}/2$. Tumor growth was plotted against time. When the tumors reached ~200 $mm^3$, nude mice bearing xenograft tumors derived from MCF-7/ADM cells were injected with adriamycin (3 mg/kg, i.p., once every 3 d). Animals were also injected once every 3 d at the tumor sites with T5E3 (2 or 4 μg) or preimmune IgG; or lenti-TRPC5-DN ($1.5 \times 10^7$ copies) or lenti-GFP as control; or TRPC5-siRNA (40 pmole) or scrambled siRNA as control; or 2-APB or 0.1% DMSO for control. The injection was scheduled 1 d before adriamycin injection. All animal experiments were performed in accordance with the laboratory animal guidelines and with approval from the Animal Experimentations Ethics Committee of the Chinese University of Hong Kong.

**MTT Assay.** MTT was used to assess the viability of cells in 96-well plates according to the MTT Kit protocol (Roche Applied Science). Briefly, for experiments studying protective effect of TRPC5 antagonists, cells were preincubated with 2-APB (100 μM) or T5E3 (20 μg/mL) or TRPC5-DN (8 μg per well in six-well plates), followed by various concentrations of adriamycin for 48 h. After the treatments, 10 μL MTT was added to form formazan crystal, which was later dissolved in solubilization solution. Absorbance was read at 570 nm.


1. Xu SZ, et al. (2005a) Generation of functional ion-channel tools by E3 targeting. *Nat Biotechnol* 23:1289–1293.
2. Shen B, et al. (2008) Epinephrine-induced $Ca^{2+}$ influx in vascular endothelial cells is mediated by CNGA2 channels. *J Mol Cell Cardiol* 45:437–445.
3. Zhao Y, et al. (2011) An expanded palette of genetically encoded $Ca^{2+}$ indicators. *Science* 333:1888–1891.


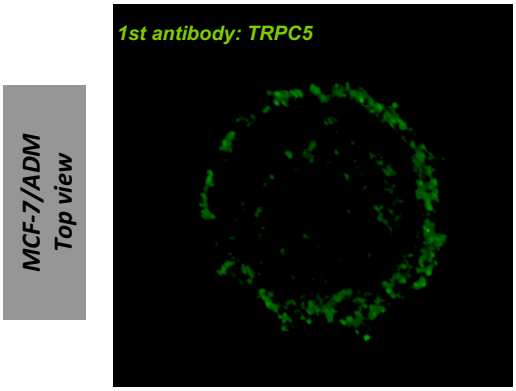


**Fig. S1.** Representative immunostaining image showing subcellular distribution of TRPC5 in an MCF-7/ADM cell. Fluorescent image of a representative cell was taken using a confocal laser scanning microscope. The cells were maintained under 10 μg/mL adriamycin. *n* = 4–6 experiments.

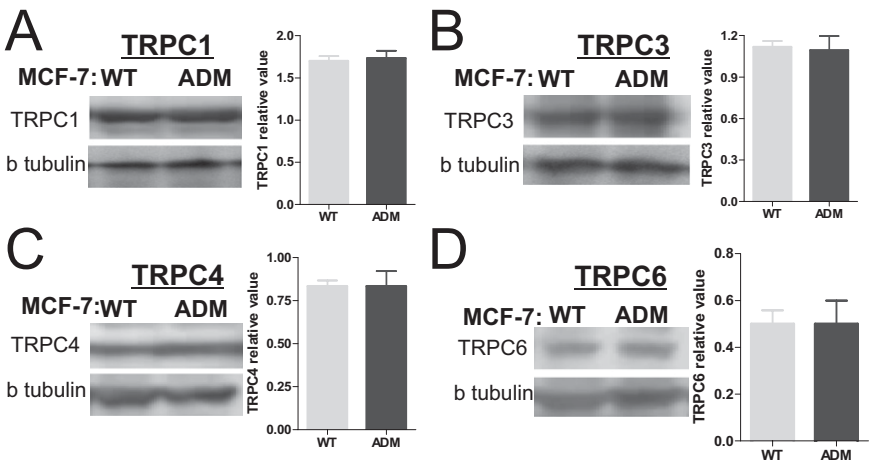


**Fig. S2.** Comparison of TRPC1, TRPC3, TRPC4, and TRPC6 expression between MCF-7/WT and MCF-7/ADM cells. Shown are representative immunoblots and data summary for TRPC1 (*A*), TRPC3 (*B*), TRPC4 (*C*), and TRPC6 (*D*). In representative immunoblots, left lane is samples from MCF-7/WT (WT) cells, whereas the right lane illustrates the samples from MCF-7/ADM cells (ADM). The lower panel in each set of immunoblots was β-tubulin control. In data summary, relative expression is shown, in which intensity of bands for TRPC and P-gp was divided by corresponding internal control (β-tubulin). Values are means ± SE of three experiments.

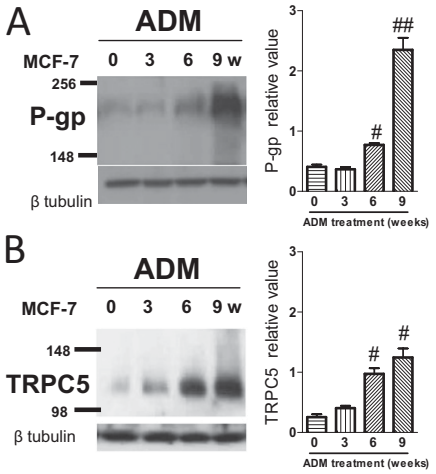


**Fig. S3.** Time course (0–9 wk) of TRPC5 and P-gp expression in MCF-7 cells treated with adriamycin. (*A* and *B*) Representative immunoblots (*Left*) and data summary (*Right*) of P-gp (*A*) and TRPC5 expression (*B*). In data summary, relative expression is shown, in which intensity of bands for TRPC5 and P-gp was divided by corresponding internal control (β-tubulin). Values are means ± SE of four experiments. $^{\#}P < 0.05$ vs. time 0; $^{\#\#}P < 0.01$ vs. time 0.

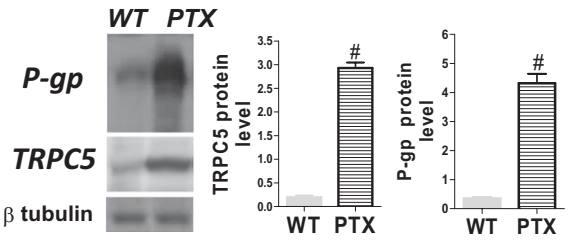


**Fig. S4.** Up-regulation of TRPC5 and P-gp in MCF-7/PTX cells (a paclitaxel-resistant MCF-7 cell line). Representative immunoblots (*Left*) and data summary (*Center* and *Right*) of TRPC5 and P-gp expression. In representative blots, left lane is samples from MCF-7/WT cells (WT), whereas the right lane illustrates the samples from MCF-7/PTX cells (PTX). The lower panel in each set of immunoblots was β-tubulin control. In data summary, relative expression is shown, in which intensity of bands for TRPC5 and P-gp was divided by corresponding internal control (β-tubulin). Values are means ± SE of four experiments. $^{\#}P < 0.05$ vs. WT.

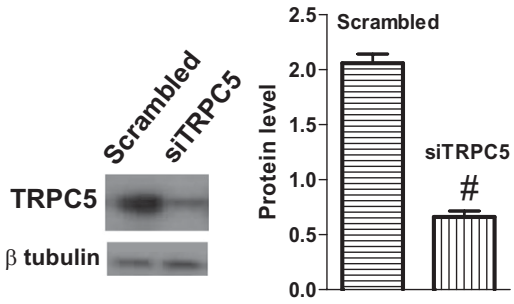


**Fig. S5.** Knocking-down effect of TRPC5-siRNA on TRPC5 expression in MCF-7/ADM cells. Shown are representative immunoblots and summary of data. In data summary, relative expression is shown, in which intensity of bands for TRPC5 was divided by corresponding internal control (β-tubulin). Values are means ± SE of three experiments. $^{\#}P < 0.05$ vs. scrambled.

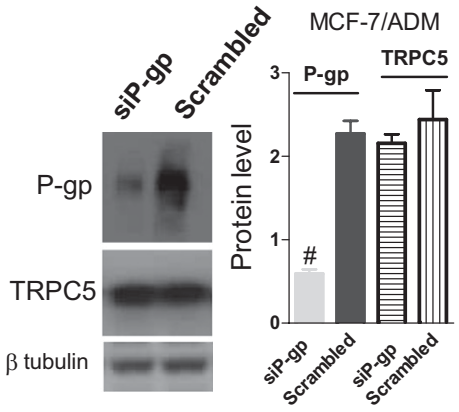


**Fig. S6.** Lack of effect for P-gp-siRNA on TRPC5 expression in MCF-7/ADM cells. Shown are representative immunoblots (*Left*) and summary of data (*Right*). In data summary, relative expression is shown, in which intensity of bands for P-gp and TRPC5 was divided by corresponding internal control (β-tubulin). Values are means ± SE of three experiments. $^{\#}P < 0.05$ vs. scrambled.

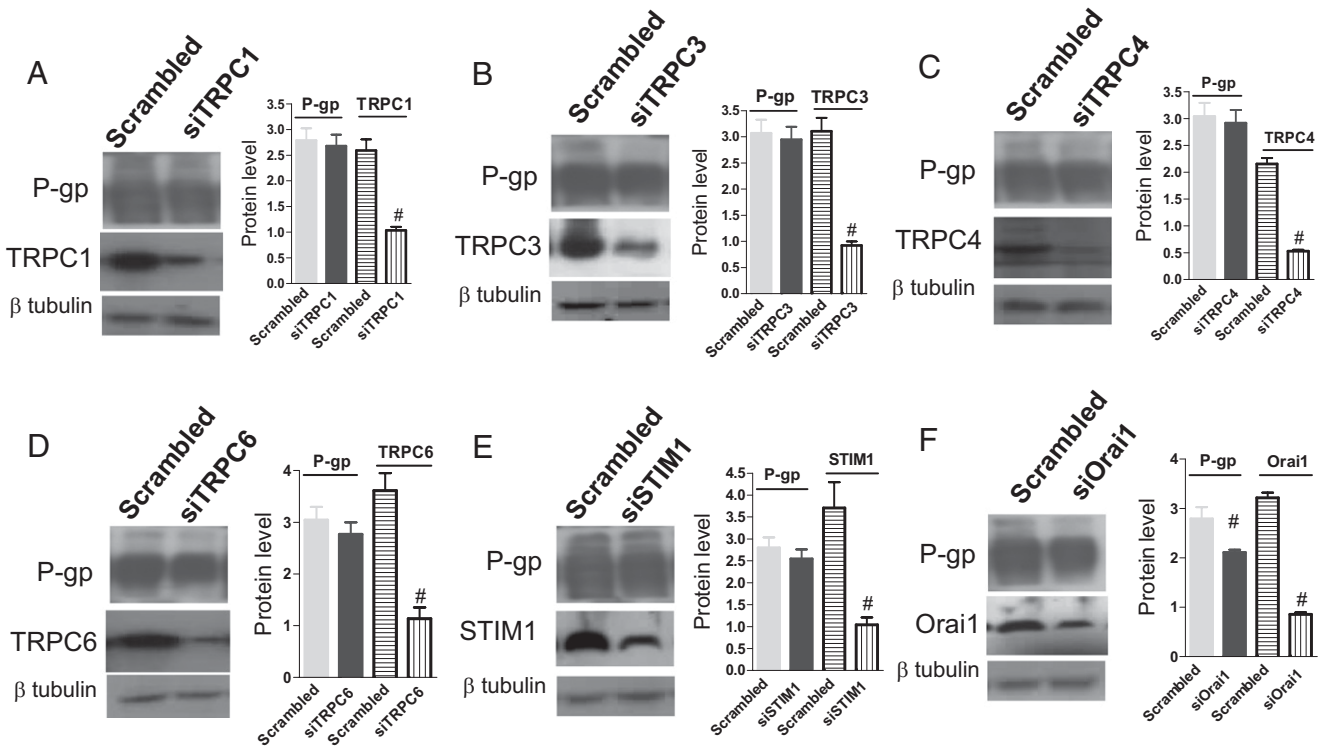


**Fig. S7.** Effect of siRNAs targeted against TRPCs/Orail/STIM1 on P-gp expression in MCF-7/ADM cells. (*A–F*) Representative immunoblots (*Left*) and summary of data (*Right*). In data summary, relative expression is shown, in which intensity of bands for TRPCs/Orai1/STIM1 and P-gp was divided by corresponding internal control (β-tubulin). Values are means ± SE of four experiments. $^{\#}P < 0.05$ vs. scrambled.

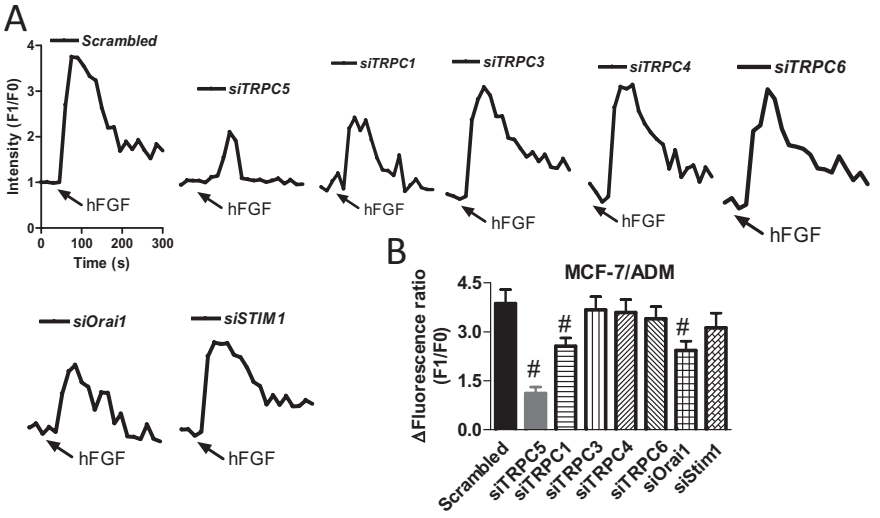


**Fig. S8.** Human fibroblast growth factor (hFGF)-induced $[Ca^{2+}]_i$ rise was markedly reduced in MCF-7/ADM cells that were treated with TRPC5-siRNA. Shown are representative traces of cytosolic $Ca^{2+}$ response to hFGF (*A*) and summary of data (*B*) in cells treated with different siRNAs. MCF-7/ADM cells were treated with TRPCs/Orail/STIM1 siRNAs (200 nM each). hFGF, 10 ng/mL. Values are means ± SE of three-to five experiments. $^{\#}P < 0.05$ vs. scrambled.

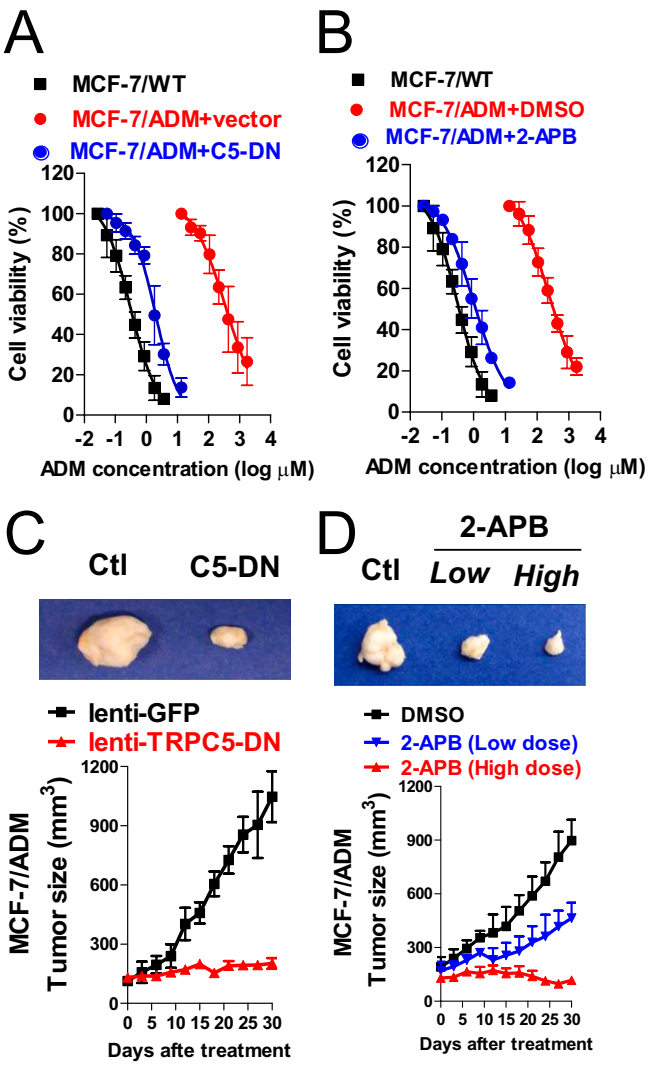


**Fig. S9.** Inhibition of TRPC5 reverses the adriamycin resistance in MCF-7/ADM cells by MTT assay and reduces the growth of human breast tumor xenografts in athymic nude mice. (*A* and *B*) MTT assay. MCF-7/ADM cells were treated with TRPC5-DN (8 μg per well, 48 h; *A*) or 2-APB (100 μM, overnight; *B*), followed by ADM incubation at different concentrations for 48 h. Empty plasmid and 0.1% DMSO were used as respective controls. In MTT assay, cell viability was measured and expressed as percentage of no-adriamycin control. (*C* and *D*) Representative photographs (*Upper*) of harvested tumors 30 d after lenti-TRPC5-DN (*C*) or 2-APB (*D*) and corresponding tumor growth curves (*Lower*) measured at indicated time points. Female nude mice bearing xenograft tumors derived from MCF-7/ADM were injected at the tumor sites with lenti-TRPC5-DN (lenti-GFP as control), or 2-APB [12 (low dose) or 24 nmol (high dose) or 0.1% DMSO as control]. Values are means ± SE of three to eight experiments.

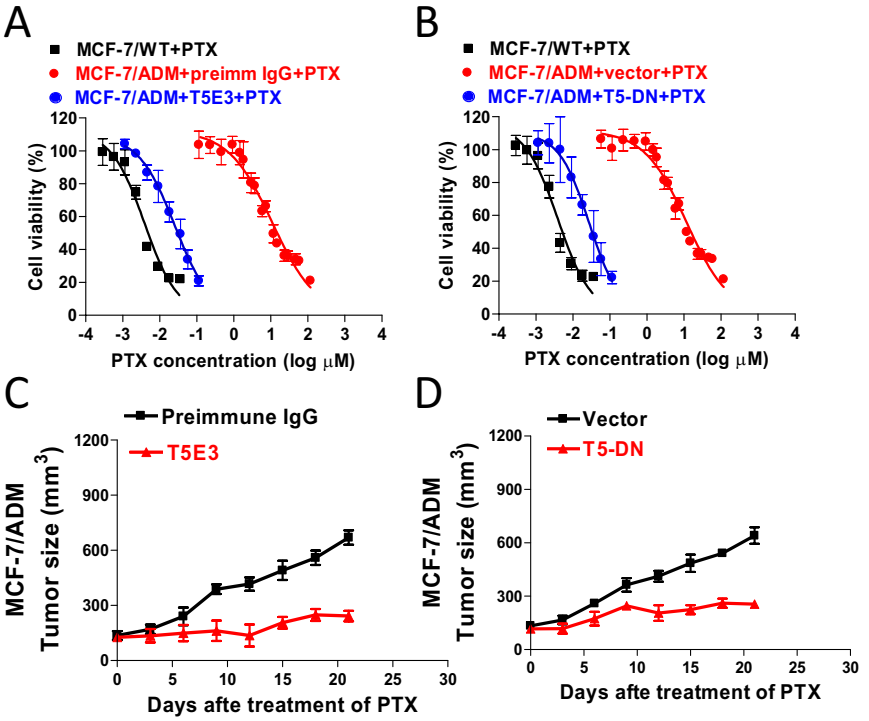


**Fig. S10.** Inhibition of TRPC5 reverses the paclitaxel (PTX) resistance of MCF-7/ADM cells in MTT cell death assay and reduces the growth of human breast tumor xenografts in athymic nude mice. (*A* and *B*) MTT assay. MCF-7/ADM cells were treated with T5E3 (20 μg/mL; *A*) or TRPC5-DN (8 μg per well; *B*), followed by paclitaxel incubation at different concentrations for 48 h. Preimmune IgG (*A*) and empty plasmid (*B*) were used as respective controls. Cell viability was then measured by MTT assay and expressed as percentage of no-paclitaxel control. (*C* and *D*) Effect of T5E3 (*C*) or lenti-TRPC5-DN (*D*) treatment on tumor growth curves. Female nude mice bearing xenograft tumors derived from MCF-7/ADM were injected with or without T5E3 [2 μg (low dose) or 4 μg (high dose) per tumor per 3 d; or preimmune IgG as control] or lenti-TRPC5-DN ($1.5 \times 10^7$ copies per tumor per 3 d, or lenti-GFP as control) at the tumor sites. Paclitaxel (0.2 mg/kg body weight) was also i.v. injected once every 3 d, scheduled 1 d after 2-APB/T5E3/lenti-TRPC5-DN injection. Values are means ± SE of three to eight experiments.

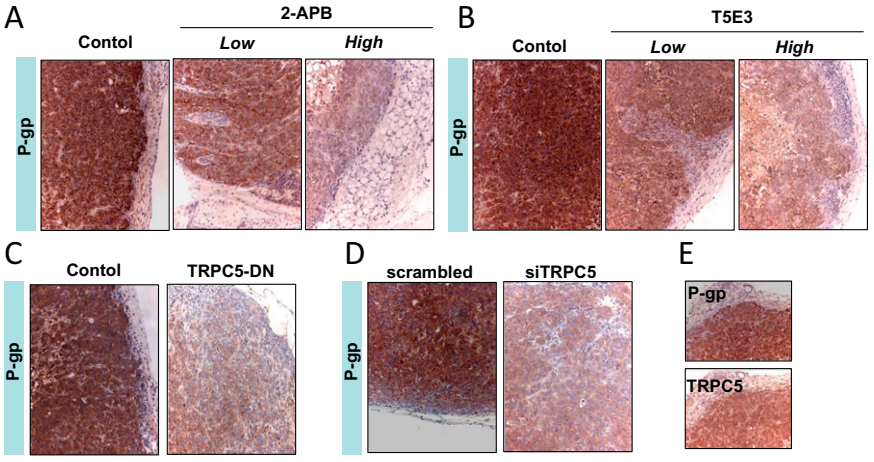


**Fig. S11.** P-gp expression was substantially reduced in 2-APB-, T5E3-, lenti-TRPC5-DN-, or TRPC5-siRNA–treated tumor xenografts. (*A*–*D*) Immunohistochemical staining for P-gp in sections of tumor xenografts before (control) and after 2-APB (*A*), T5E3 (*B*), lenti-TRPC5-DN (*C*), or TRPC5-siRNA (*D*). (*E*) Coexistence of TRPC5 and P-gp in sections of tumor xenografts. Female nude mice bearing xenograft tumors derived from MCF-7/ADM were injected with or without 2-APB [12 (low dose) or 24 nmol (high dose) per tumor per 3 d; or 0.1% DMSO for control] or T5E3 [2 μg (low dose) or 4 μg (high dose) per tumor per 3 d; or preimmune IgG as control] or lenti-TRPC5-DN ($1.5 \times 10^7$ copies per tumor per 3 d, or lenti-GFP as control) or TRPC5-siRNA (40 pmol per tumor per 3 d; or scrambled siRNA as control) at the tumor sites. Adriamycin (4 mg/kg body weight) was also i.v. injected once every 3 d, scheduled 1 d after 2-APB/T5E3/lenti-TRPC5-DN injection. Values are means ± SE of three to eight experiments.

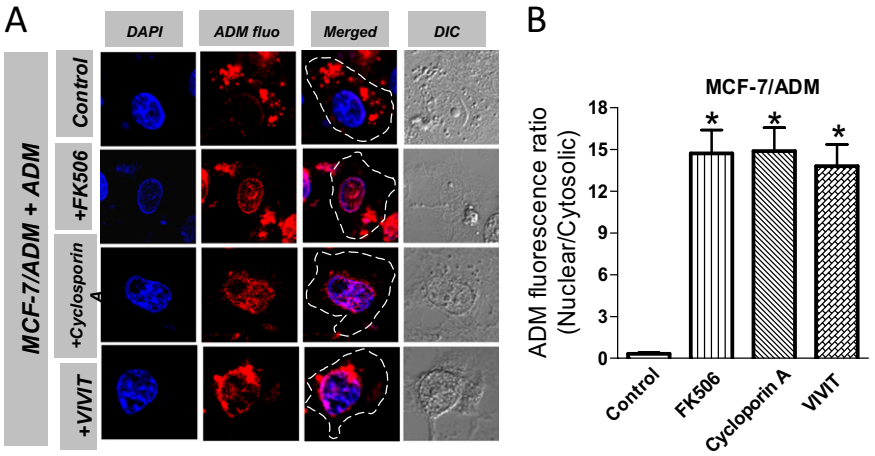


**Fig. S12.** Inhibition of NFAT causes adriamycin reaccumulation in cell nucleus. (*A*) Representative images demonstrating the effect of FK506 (10 μM) or cyclosporin A (15 μM) or VIVIT (2 μM) on adriamycin (ADM) redistribution at the subcellular level. Shown are representative nuclear DAPI staining (*Left*, blue), adriamycin autofluorescence (*Center Left*, red), merged images (*Center Right*), and bright-field images (*Right*). Fluorescent images were taken from a confocal laser scanning microscope. Sketched lines in merged images represent the cell boundary, which could be visualized at higher magnification in differential interference contrast mode. The cells were maintained under 10 μg/mL adriamycin. (*B*) Summary data of adriamycin subcellular distribution ratio (nuclear/cytosolic) in *A*. $n = 4$ experiments.

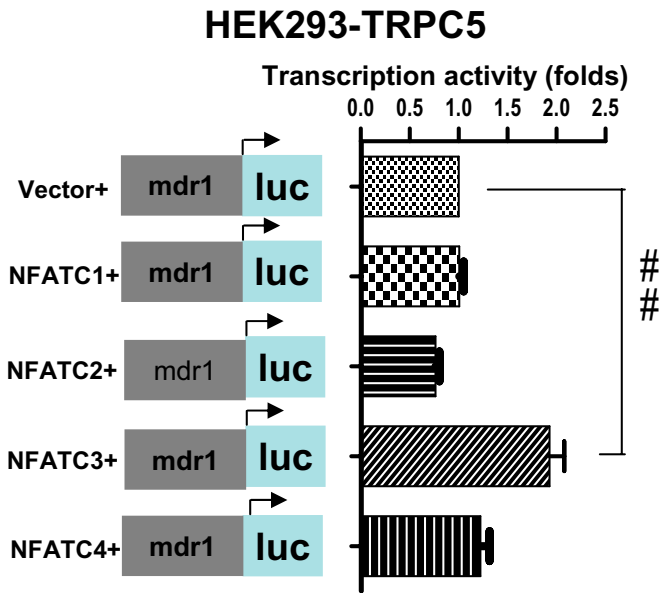


**Fig. S13.** Ability of different NFAT isoforms in stimulating the transcriptional activity of *mdr1* promoter. HEK293 cells were cotransfected with TRPC5, the indicated NFAT isoform, and a luciferase reporter vector carrying the 5′ flanking 800-bp sequence of *mdr1* gene. The cells were incubated with carbachol (100 μM). Horizontal axes indicate relative transcriptional activity by luciferase assay in HEK293 cells overexpressing the indicated constructs. Activity of the cells carrying vector + mdr1-luc + TRPC5 was normalized to 1. Values are means ± SE of four experiments. $^{\#\#}P < 0.05$ vs. vector.

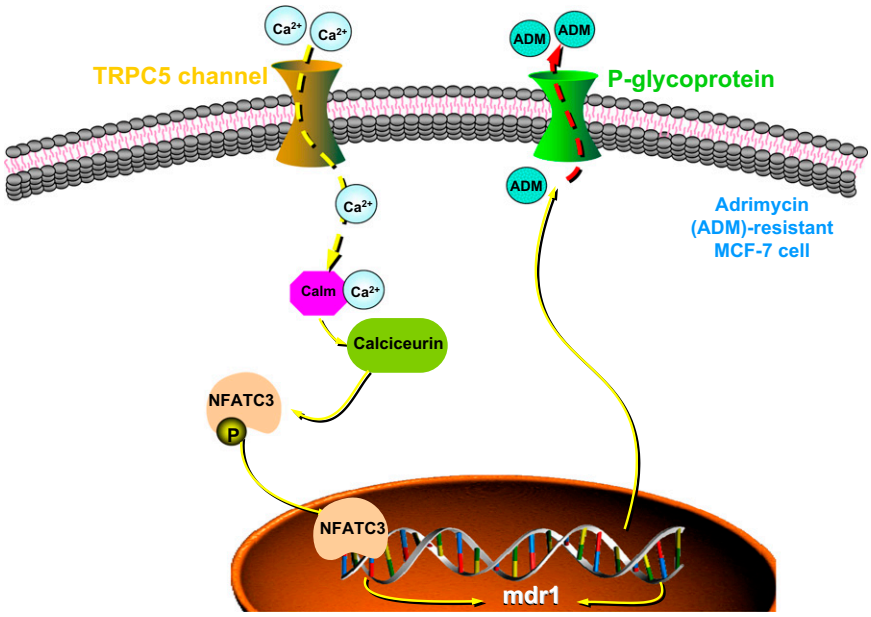


**Fig. S14.** Schematic illustration showing the role of TRPC5 in P-gp production in drug-resistant cancer cells. $Ca^{2+}$ entry through TRPC5 channels, via calmodulin-calcineurin dependent NFATc3 signal pathway, stimulates P-gp overproduction in adriamycin-resistant MCF-7/ADM breast cancer cells. Inhibiting/suppressing TRPC5 could reverse the adriamycin resistance of these cells.

**Table S1. Cross-resistance of MCF-7/ADM cells to other chemotherapeutic drugs**

| Drugs | MCF7/ADM resistance factor |
|---|---|
| Adriamycin | 630-fold increase |
| Paclitaxel | 146-fold increase |
| Epirubicin | 56-fold increase |
| Vincristine | 4.9-fold increase |
| Mitoxantrone | 127-fold increase |

MTT assay showed that MCF-7/ADM cells displayed a 630-fold higher resistance to adriamycin than MCF-7/WT, and 146-fold, 56-fold, 4.9-fold, and 127-fold resistance to paclitaxel, epirubicin, vincristine, and mitoxantrone, respectively. Values are from three to four experiments.