Supplemental material for the manuscript entitled

"Cell death induced by cationic amphiphilic drugs depends on lysosomal Ca^{2+} release and cyclic AMP"

- Table S1, List of Resources
- Supplemental figures S1-S6 with legends

Table S1. List of resources

Reagent / chemical	Source	Catalogue #; CAS #
Acetonitrile, ≥99.8% ofr HPLC	VWR	AXO145; 2114-00-3
Acetonitrile, for LC-MS	VWR	BJLC015; 2114-00-3
Astemizole	Sigma Aldrich	A2861; 68844-77-9
ATP	Sigma Aldrich	A2383; 34369-07-8
Bambuterol hydrochloride	Sigma Aldrich	B8684; 81732-46-9
BAPTA AM	Thermo Fisher Scientific	B-6769; 126150-97-8
Clarity™ Western ECL Substrate	Bio-Rad	170-5061; N/A (not applicable)
Dextran 10 kDa, Alexa Fluor® 594	Thermo Fisher Scientific	D22913; N/A
Dextran, 10 kDa, pHrodo™ Green	Thermo Fisher Scientific	P35368; N/A
Dextran 70 kDa, tetramethylrhodamine (TMR)	Thermo Fisher Scientific	D-1819; N/A
DiBAC4	Thermo Fisher Scientific	B438; 70363-83-6
Dibutyryl cAMP sodium salt	Sigma Aldrich	D0627; 16980-89-5
DMSO	VWR	VWRCN182, 67-68-5
DPIC	Sigma Aldrich	D2926; 4673-26-1
Ebastine	Cayman Chemical	15372, *90729-43-4*
Fluo-4-AM	Thermo Fisher Scientific	F14201, 273221-67-3
Formoterol fumarate dihydrate	Sigma Aldrich	F9552; 183814-30-4
Forskolin	Sigma Aldrich	93049; 66575-29-9
G418	Life Technologies	11811-031, 108321-42-2
Goat serum	DAKO	X0907; N/A
Glycylphenylalanine2-naphthylamide (GPN)	Santa Cruz	sc-252858, 21438-66-4
Hoechst 33258	Sigma Aldrich	861405; 23491-45-4
Hoechst 33342	Sigma Aldrich	B2261; 23491-52-3
IBMX	Sigma Aldrich	I5879; 28822-58-4
Lipofectamine® LTX with PLUS reagent	Thermo Fisher Scientific	15338100; N/A
Lipofectamine® RNAiMAX	Thermo Fisher Scientific	13778075; N/A
LLoMe	Santa Cruz	SC-285992, 16889-14-8
Loperamide hydrochloride	Alomone Labs	L-100, 34552-83-5
Loratadine	Sigma Aldrich	L9664, 79794-75-5
LysoTracker® Green DND-26	Molecular Probes	L-7526; N/A
LysoTracker® Red DND-99	Molecular Probes	L-7528; N/A
MLSA-1	Tocris	4746, 332382-54-4
Paraformaldehyde (PFA)	Ampliqon	432.261.000, 30525-89-4
Penfluridol	Sigma Aldrich	P3371; 26864-56-2
Phosphatase inhibitor cocktail	Roche	4906837001; N/A
Prolong Gold Antifade mountant	Life Technologies	P36934; N/A

Propidium Iodide	Sigma Aldrich	P4864; 25535-16-4
Protease inhibitor cocktail	Roche	11697498001; N/A
Quinacrine	Sigma Aldrich	Q3251; 69-05-6
Salbutamol	Sigma Aldrich	S8260; 18559-94-9
Salmeterol	Tocris	4712, 94749-08-3
Siramesine	Gift from Christine Volbracht, H. Lundbeck A/S	N/A, 163630-79-3
SU11652	Enzo Life Sciences	BML-EI408-0001, 326914-10-7
Sunitinib malate	Selleck Chemicals	S1042, 341031-54-7
Terfenadine	Sigma Aldrich	T9652, 50679-08-8
Thapsigargin	Tocris	1138, 67526-95-8
Triton-X-100	Sigma Aldrich	T9284; 9002-93-1
Vinorelbine	Sigma Aldrich	V2264; 125317-39-7
Water, for LC-MS	VWR	BDH83545.400; N/A
Antibodies	**Source**	**Identifier**
ADCY1	Novus Biologicals	NBP1-19628
Anti GFP	Abcam	ab290
Beta actin HRP-conjugated	Abcam	ab20272
Cathepsin B	Ekkehard Weber, Martin Luther University Halle-Wittenberg	3E4+6D5, PubMed PMID: 25205719
CREB	Cell Signaling Technology	9197
CREB, P-Ser133	Cell Signaling Technology	9198S
Galectin 3	Sigma Aldrich	MABT51
GAPDH, HRP-conjugated	Thermo Fisher Scientific	MA5-15738-HRP
HSP90	BD Transduction Laboratories	610418
Lamin B, HRP-conjugated	Abcam	ab194109
LAMP1	Developmental Studies Hybridoma Bank	clone H4A3
LAMP2	Developmental Studies Hybridoma Bank	H4B4
P2RX4	Alomone Labs	APR-002
Mouse IgG (donkey) Alexa Fluor 488	Thermo Fisher Scientific	A-21202
Mouse IgG (donkey) Alexa Fluor 594	Thermo Fisher Scientific	A21203
Mouse IgG (rabbit) HRP-conjugated	Sigma Aldrich	A9044-2ML
Rabbit IgG (donkey) Alexa Fluor 488	Thermo Fisher Scientific	A-21206
Rabbit IgG (donkey) Alexa Fluor 594	Thermo Fisher Scientific	A21207
Rabbit IgG (goat) HRP-conjugated	Vector Laboratories	PI-1000
SERCA2	Abcam	ab2817
siRNA	**Source**	**Identifier**
ADCY1#1	Sigma Aldrich	SASI_Hs01_00226331

ADCY1#2	Sigma Aldrich	SASI_Hs01_00226332
ADCY1#3	Sigma Aldrich	SASI_Hs01_00226330
All Star non-targeting control siRNA	Qiagen	SI03650318
CALCR#1	Sigma Aldrich	SASI_Hs01_00077738
CALCR#2	Sigma Aldrich	SASI_Hs01_00077739
CALCR#3	Sigma Aldrich	SASI_Hs01_00077740
CXCR4#1	Sigma Aldrich	SASI_Hs01_00077740
CXCR4#2	Sigma Aldrich	SASI_Hs01_00077738
CXCR4#3	Sigma Aldrich	SASI_Hs01_00077739
GNAI#1	Sigma Aldrich	SASI_Hs01_00199944
GNAI#2	Sigma Aldrich	SASI_Hs01_00199945
GNAI#3	Sigma Aldrich	SASI_Hs01_00199946
P2RX4	Santa Cruz	sc-42569
Primers	**Source**	**Identifier**
ADCY1	Biomol	VHPS-168
Plasmids	**Source**	**Identifier**
ADCY1-GFP	OriGene	RG222738
Flamindo2	A gift from Tetsuya Kitaguchi	Addgene plasmid # 73938
LAMP1-mCherry	A gift from Michael Davidson	Addgene plasmid # 55073
ML1-CGamp3	A gift from Haoxing Xu	Ref. 41, Shen et al., 2012
P2X4-pHluorin123	A gift from Baljit Khakh	Addgene plasmid # 52926
KITS	**Source**	**Identifier**
HitHunter® cAMP XS+ cAMP assay Kit	DiscoverX	90-0075
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Venor®GeM Classic PCR kit	Minerva Biolabs	11-1100
Deposited Data	**Source**	**Identifier**
RNASeq: MCF7 control and MCF CAD resistant cells	GEO database, NCBI	accession number to be provided before publication
Software	**Source**	**Identifier**
CLC genomic workbench	Qiagen	www.qiagenbioinformatics.com
FlowJo	FLOWJO LLC	www.flowjo.com
ImageJ	Fiji	www.imagej.net/Fiji
MS Excel 2013	Microsoft	www.products.office.com/en/excel
Prism7	GraphPad	www.graphpad.com/scientific-software/prism/
Zeiss Zen	GraphPad	www.zeiss.com/microscopy/int/products/microscope-software/zen.html
Cell culture	**Source**	**Identifier**

A549, non-small cell lung carcinoma (male)	ATCC	ATCC® CCL-185™
Dulbecco's Modified Eagle's medium	Thermo Fisher Scientific	31966-021
Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+}/Mg^{2+}	Thermo Fisher Scientific	14040-117
Dulbecco's phosphate-buffered saline (DPBS) w/o Ca^{2+}/Mg^{2+}	Thermo Fisher Scientific	14190-094
HeLa, cervix carcinoma	ATCC	ATCC® CRM-CCL-2™
Fetal calf serum	Thermo Fisher Scientific	10270-106
Live Cell Imaging Solution	Thermo Fisher Scientific	A14291DJ
MCF7-S1, mammary adenocarcinoma (female)	Marja Jäättelä, Danish Cancer Society Research Center	PubMed PMID: 7540278
Penicillin/Streptomycin	Life Technologies	11811-031

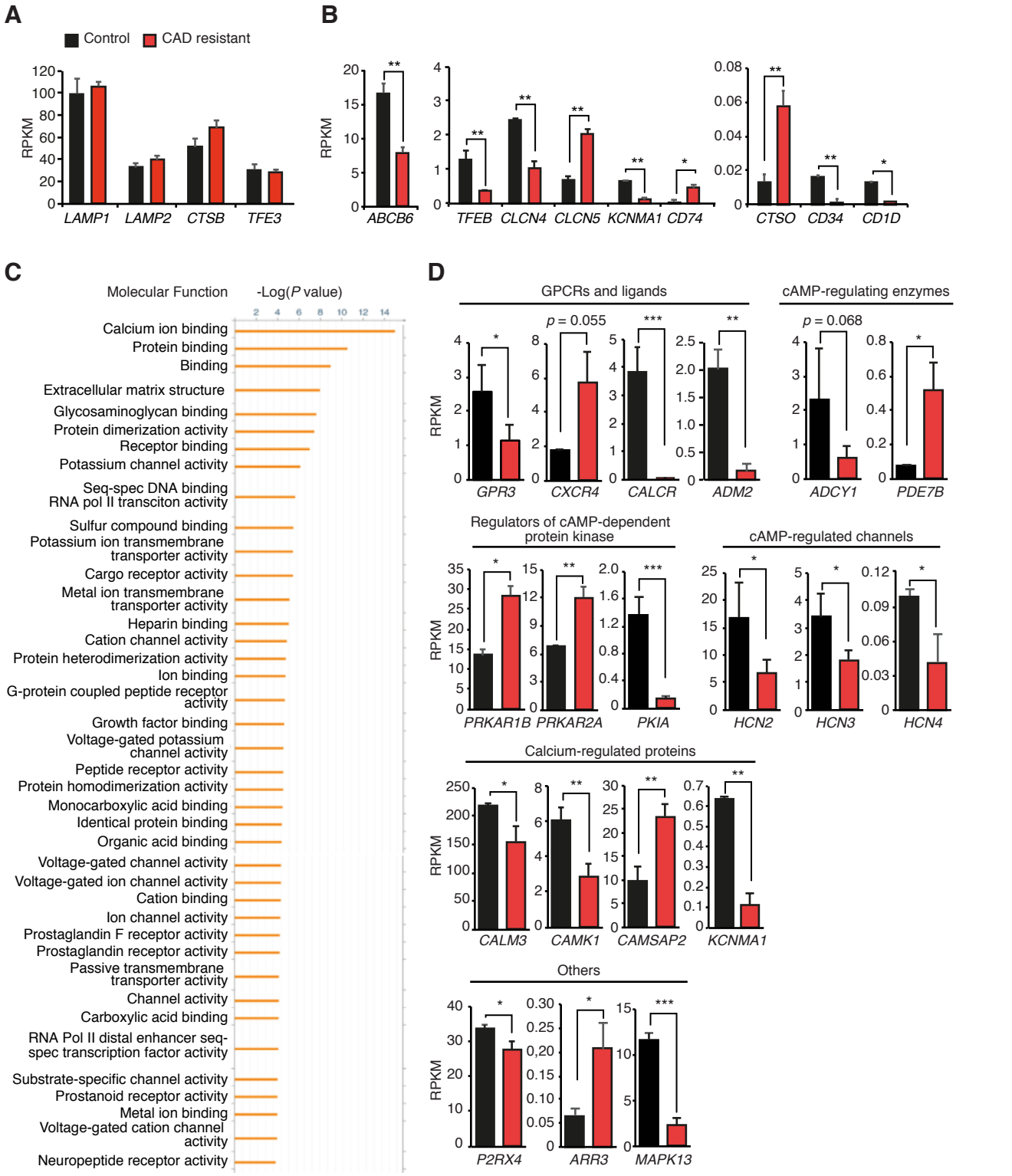

Figure S1. CAD resistance is associated with altered gene expression

Gene expression in two independent pools of control and four independent pools of CAD resistant MCF7 cells as analyzed by whole transcriptome sequencing (RNASeq).

(A) Mean expression of indicated lysosome-related genes in control (black) and CAD resistant (red) cells.

(B) Expression levels of lysosome-related genes whose expression was significantly altered (≥ 2-fold; P ≤ 0.05) iin control (black) and CAD resistant (red) cells.

(C) Gene ontology analysis comparing transcriptomes of control and CAD resistant cells performed by Metacore™ software (https://portal.genego.com/cgi/data_manager.cgi).

(D) Mean expression of of indicated genes related to GPRC signaling pathways in in control (black) and CAD resistant (red) cells.

Error bars, SD of two control MCF7 pools and three or four CAD resistant MCF7 pools.

* P < 0.05, ** P < 0.01, *** P < 0.001 as analyzed by 2-tailed, homoscedastic student's t-test.

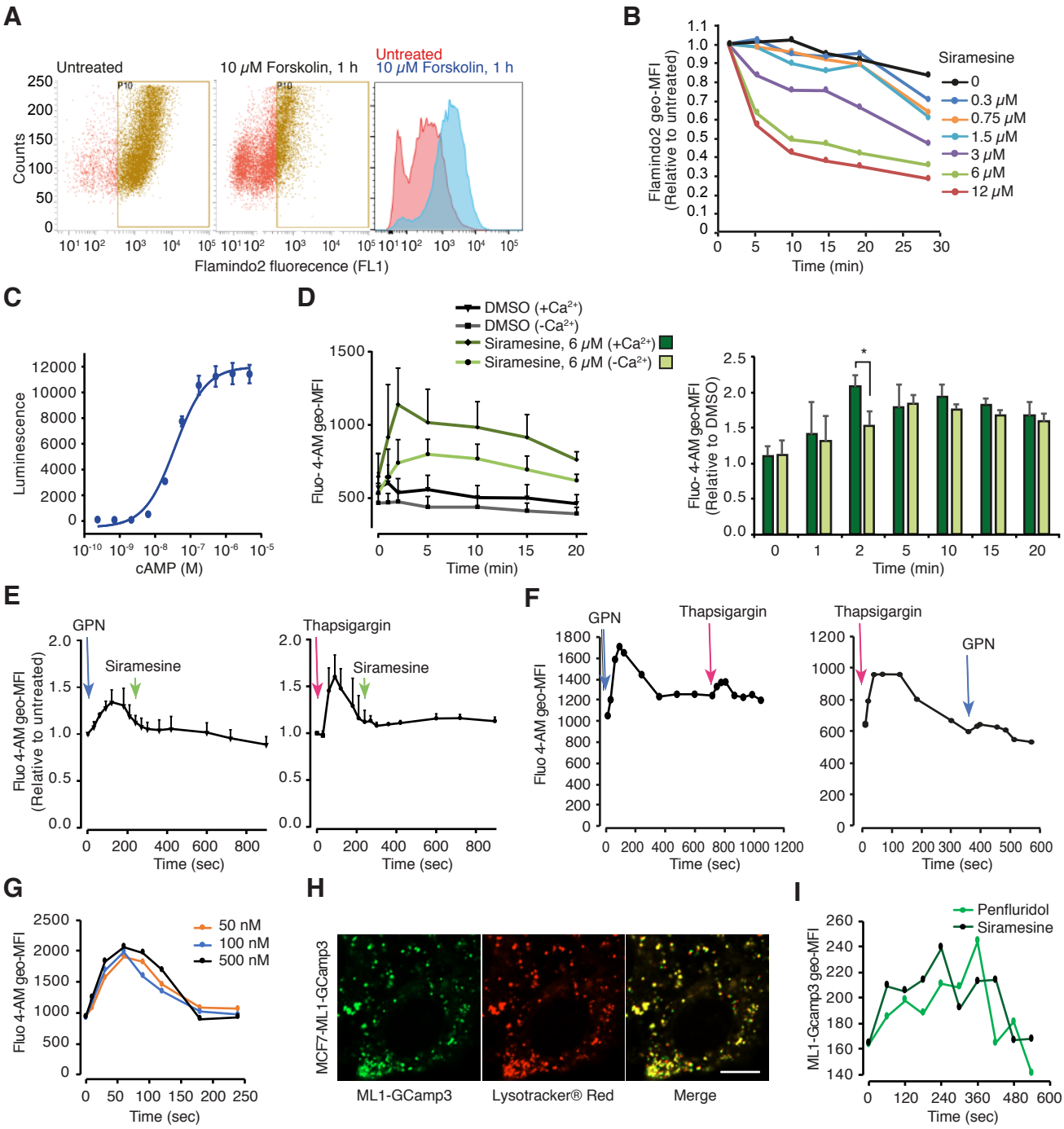

Figure S2. CAD resistance is associated with reduced intracellular cAMP and Ca^{2+} levels

(A) Representative flow cytometer profiles showing Flamindo2 fluorescence (FL1) in MCF7-Flamindo2 cells left untreated or treated with 10 μM forskolin in the absence of extracellular Ca^{2+} for 1 h.

(B) Geometric mean fluorescence intensities (geo-MFIs) of MCF7-Flamindo2 cells treated with indicted concentrations of siramesine in the absence of extracellular Ca^{2+} for indicated times were analyzed by flow cytometry.

(C) Standard curve for intracellular cAMP measurements presented in Figure 3D.

(D) Actual (left) and normalized (right) Fluo-4-AM geo-MFIs reflecting free $[Ca^{2+}]c$ in MCF7 cells treated as indicated in the presence or absence of extracellular Ca^{2+}.

(E) Normalized Fluo-4-AM geo-MFIs in MCF7 cells treated with 500 μM GPN (left) or 500 nM thapsigargin (right) at 0 sec followed by 6 μM siramesine at 240 sec in the absence of extracellular Ca^{2+}.

(F) Fluo-4-AM geometric MFIs in MCF7 cells treated with 500 μM GPN at 0 sec followed by 500 nM thapsigargin at 720 sec (left) or 500 nM thapsigargin at 0 sec followed by 500 μM GPN at 360 sec (right) in the absence of extracellular Ca^{2+}.

(G) Fluo-4-AM geo-MFIs of MCF7 cells treated with indicated concentrations of thapsigargin in the absence of extracellular Ca^{2+}.

(H) Representative images of MCF7 cells transfected with ML1-Gcamp3 for 72 h and loaded with Lysotracker® Red for 16 h followed by a 4 h chase in the absence of Lysotracker® Red 52 h after the transfection. Scale bar, 10 μM.

(I) ML1-Gcamp3 geo-MFIs in HeLa cells transfected with ML1-Gcamp3 72 h prior to the treatment with 10 μM penfluridol or siramesine in the absence of extracellular Ca^{2+}.

Error bars, SD of ≥ 3 independent experiments.

* $P < 0.05$ as analyzed by 2-tailed, homoscedastic student's t-test.

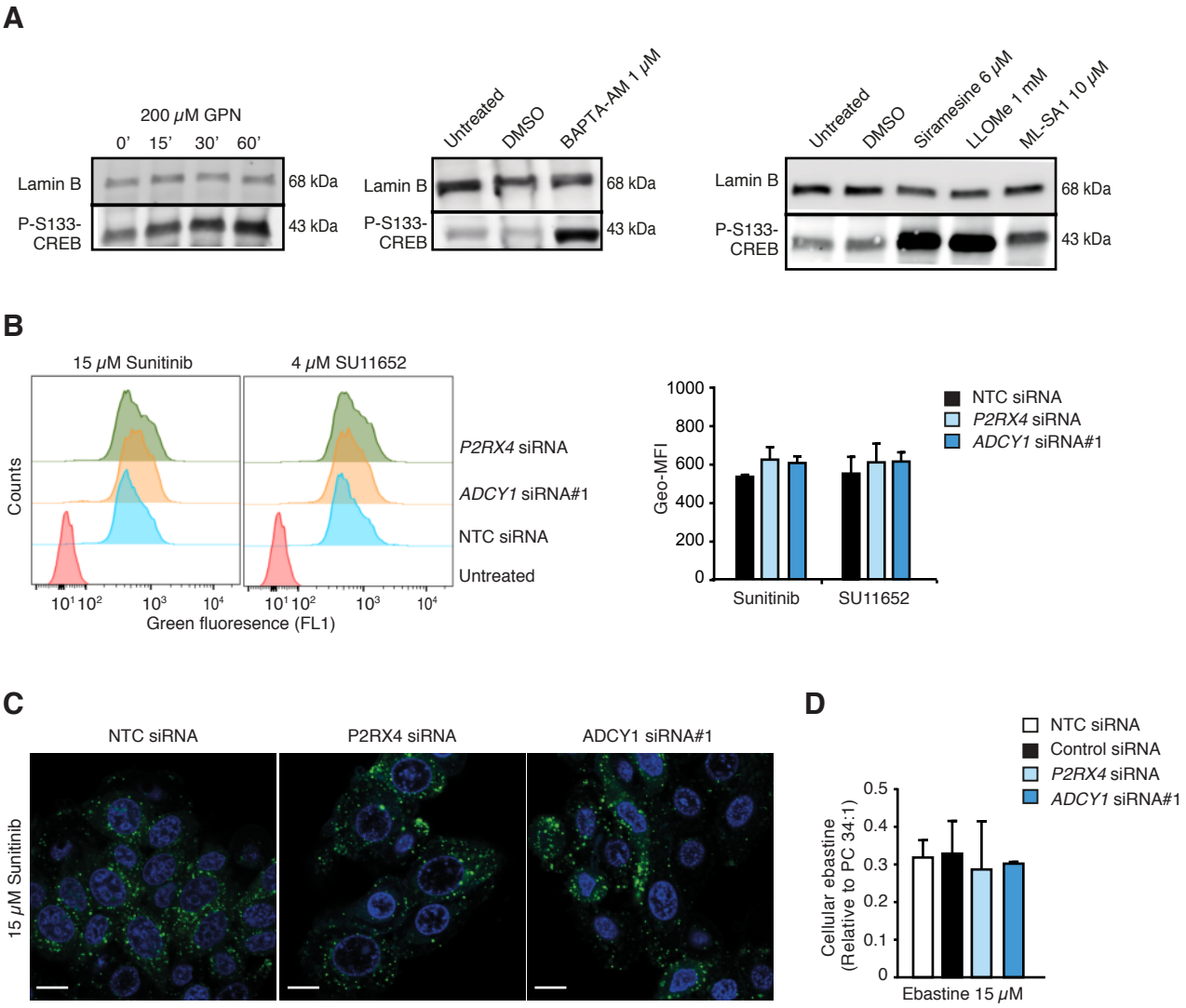

Figure S3. Ca^{2+}-induced CREB phosphorylation (A) and CAD uptake in siRNA treated MCF7 cells (B-D)
(A) Representative immunoblots of P-S133-CREB and Lamin B (loading control) in lysates of MCF7 cells treated as indicated for indicated times (left) or for 2 h (middle and right)
(B) Representative flow cytometer profiles (left) and quantification of geometric MFIs (right) in MCF7 cells treated with indicated siRNAs for 48 h and with 15 μM sunitinib or 4 μM SU11652 for the last 2 h. Untreated cells serve as a negative control.
(C) Representative confocal images of MCF7 cells treated with indicated siRNAs for 48 h and with 15 μM sunitinib for the last 2 h. Images were taken with 63x magnification using Zeiss LSM700 confocal microscope. The optimal slice thickness (approximately 350 nm) was defined by the Zeiss zen software. Scale bar, 10 μm.
(D) Ebastine levels in lipid extracts of MCF7 cells treated with indicated siRNAs for 48 h and with 15 μM ebastine for the last 2 h were determined by mass spectrometry. The values are presented relative to phosphatidylcholine (PC) 34:1.
Error bars, SD of 3 independent experiments.

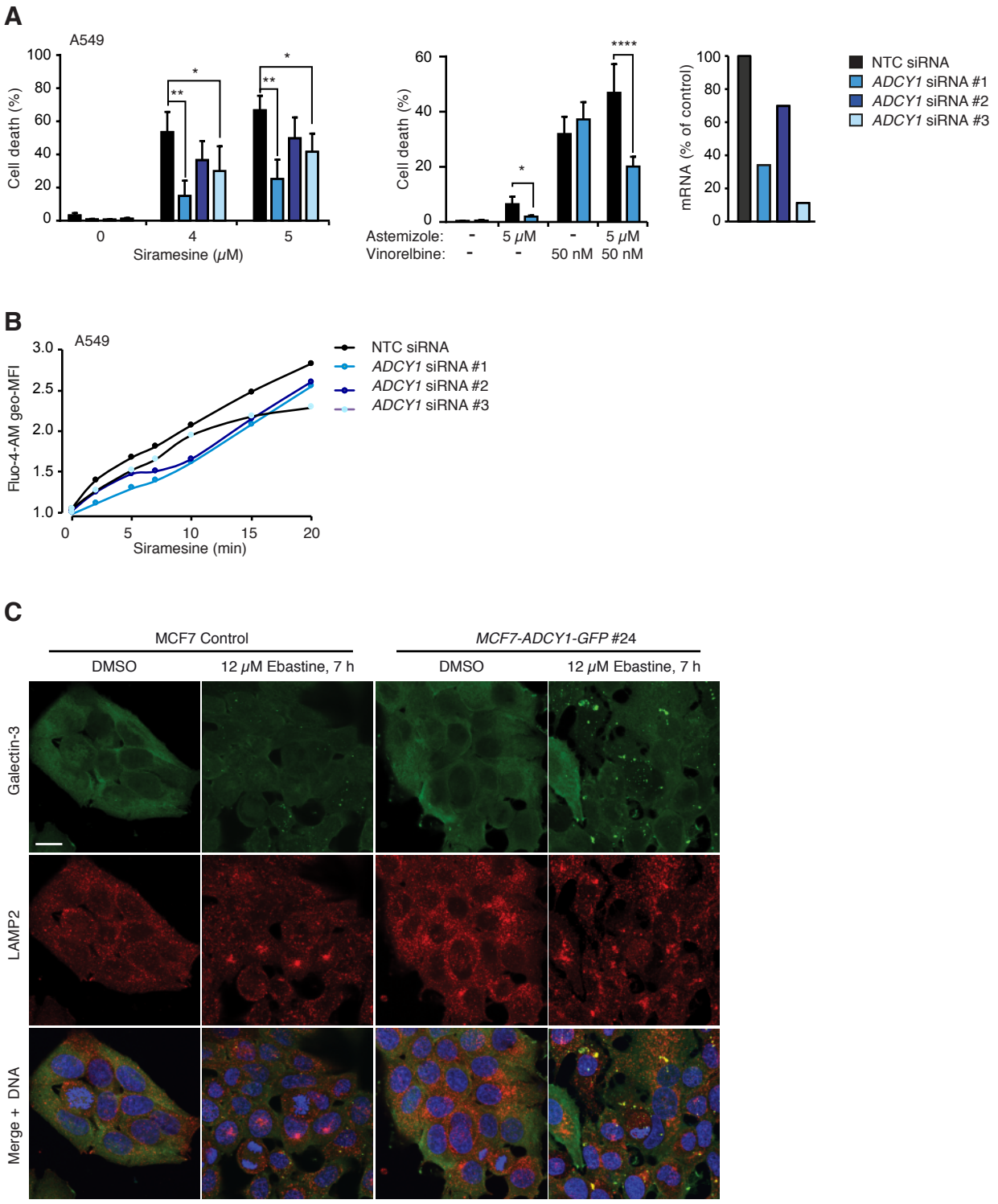

Figure S4. ADCY1 mediates CAD-induced cAMP response and cell death

(A) Cell death of A549 cells treated with indicated siRNAs for 72 h and with indicated concentrations of drugs for the last 48 h (left). Error bars, SD of ≥ 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ as analyzed by 2-tailed, homoscedastic student's t-test. The efficacy of siRNAs was analyzed by qPCR (right).

(B) Fluo-4-AM geometric MFIs in A549 cells treated with indicated siRNAs for 72 h and with 10 μM siramesine for the last 20 min.

(C) Representative confocal images of MCF7-ADCY-GFP #24 cells stained with Lysotracker® Red and Hoechst 33342. Images were taken with 63x magnification using Zeiss LSM700 confocal microscope. The optimal slice thickness (approximately 350 nm) was defined by the Zeiss zen software.

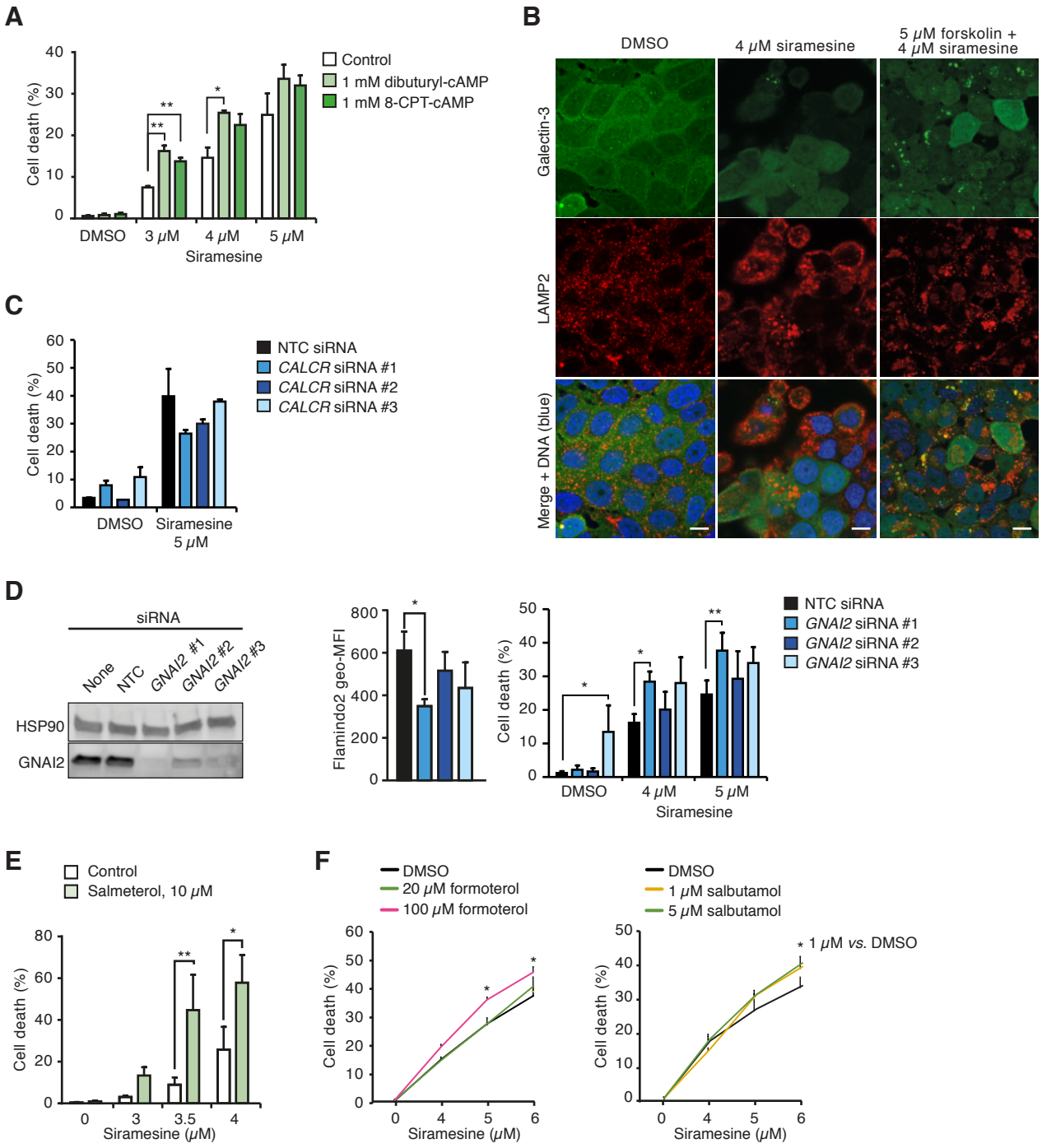

Figure S5. cAMP inducers sensitize cancer cells to CAD-induced cytotoxicity

(A) Cell death of MCF7 cells treated as indicated for 48 h.

(B) Representative confocal images of MCF cells treated as indicated for 16 h and stained for galectin-3 and LAMP2. Scale bar, 10 μm. Images were taken with 63x magnification using Zeiss LSM700 confocal microscope. The optimal slice thickness (approximately 350 nm) was defined by the Zeiss zen software.

(C) Death of MCF7 cells transfected with NTC or CALCR siRNAs for 72 h, and treated as indicated for the last 48 h.

(D) Representative immunoblot of indicated proteins (left), Flamindo2 geometric MFIs (middle) and cell death (right) of MCF7-Flamindo2 cells treated with NTC or GNAI2 siRNAs for 72 h. For cell death measurements, cells were treated with indicated concentrations of siramesine for the last 48 h.

(E and F) Death of A549 (E) and MCF7 (F) cells treated as indicated for 48 h.

Error bars, SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ as analyzed by 2-tailed, homoscedastic student's t-test.

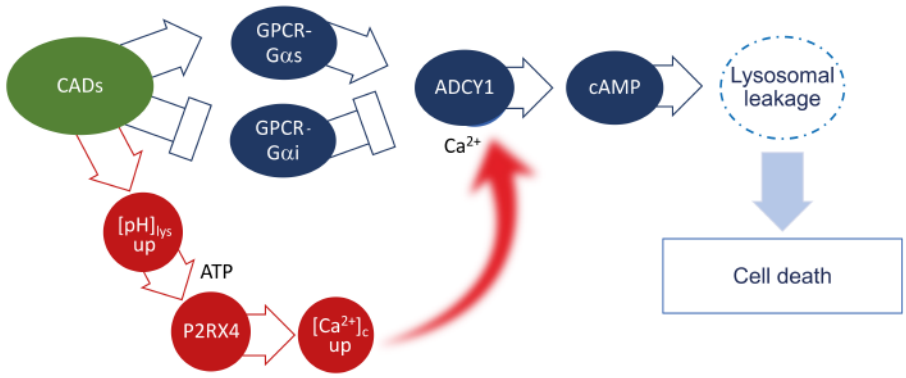

Figure S6. Schematic presentation of CAD-induced signaling that promotes lysosome-dependent cell death
The accumulation of CADs in lysosomes increases lysosomal pH, which results in the ATP-mediated activation of P2RX4 and lysosomal Ca^{2+} release. Ca^{2+} enhances the ADCY1-dependent synthesis of cAMP, which is essential for the CAD-induced lysosomal membrane permeabilization and lysosome-dependent cell death.