Supplemental Information

GDC-0879, a $BRAF^{V600E}$ inhibitor, protects kidney podocytes from death

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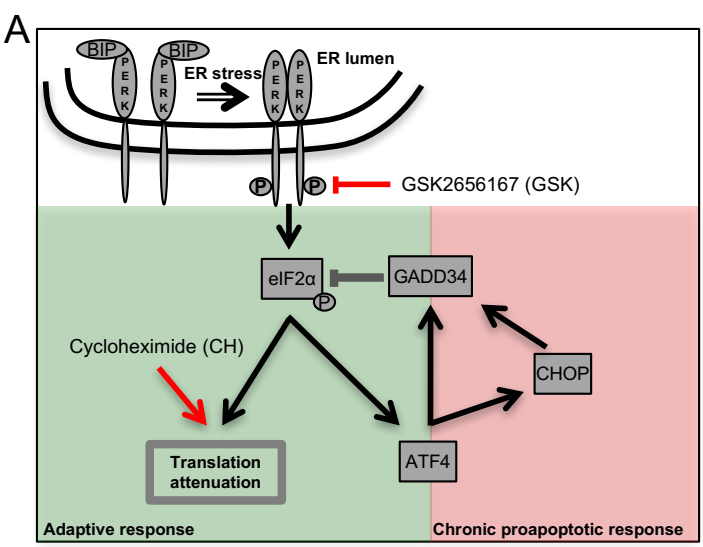
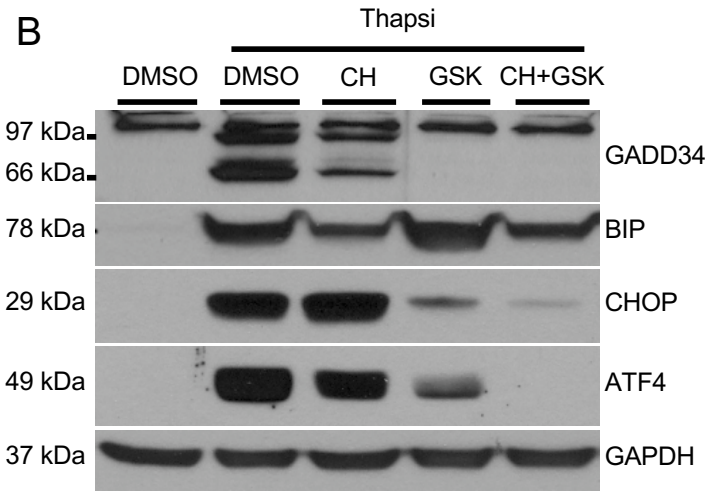
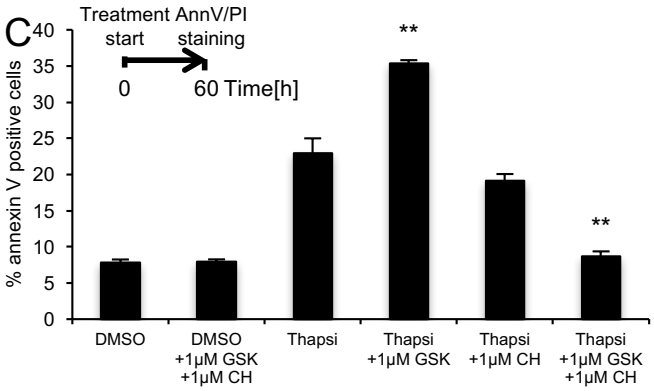
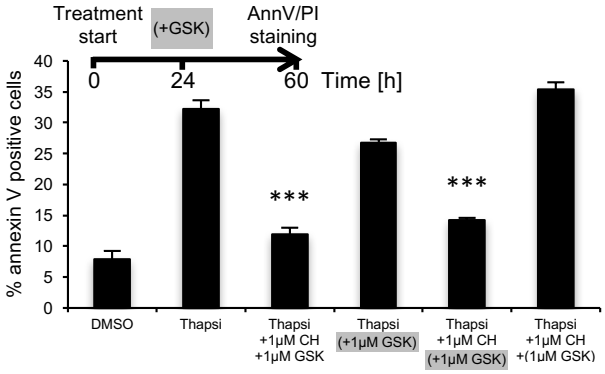
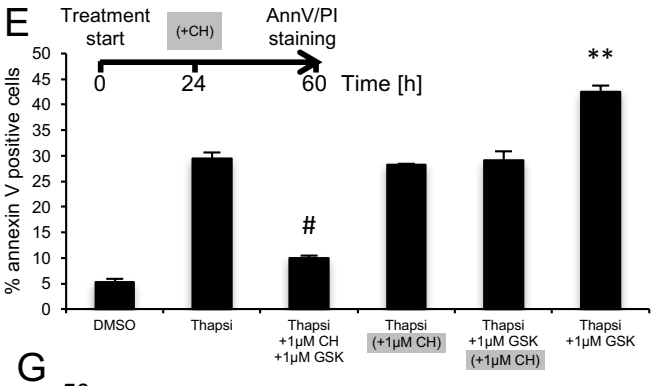
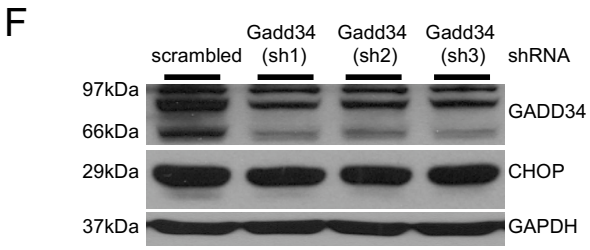

A
BIP
PERK
ER stress
ER lumen
GSK2656167 (GSK)
eIF2α
GADD34
Cycloheximide (CH)
CHOP
Translation attenuation
ATF4
Adaptive response
Chronic proapoptotic response

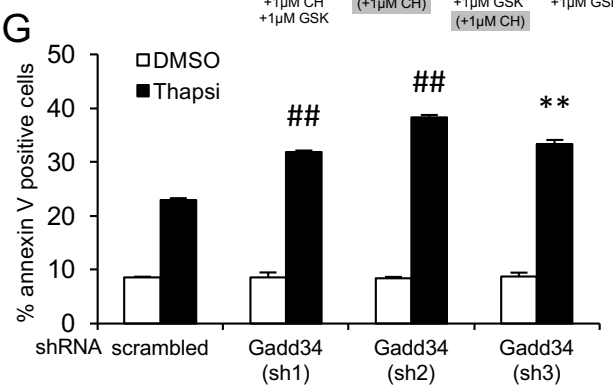

B
Thapsi
DMSO
DMSO
CH
GSK
CH+GSK
97 kDa
66 kDa
78 kDa
29 kDa
49 kDa
37 kDa
GADD34
BIP
CHOP
ATF4
GAPDH

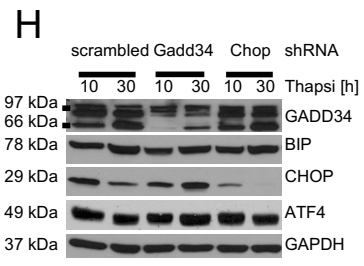

C
Treatment start
AnnV/PI staining
0
60 Time[h]
% annexin V positive cells
DMSO
DMSO +1μM GSK +1μM CH
Thapsi
Thapsi +1μM GSK
Thapsi +1μM CH
Thapsi +1μM GSK +1μM CH

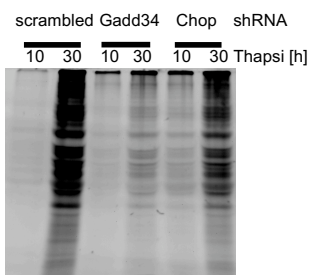

D
Treatment start
(+GSK)
AnnV/PI staining
0
24
60 Time [h]
% annexin V positive cells
DMSO
Thapsi
Thapsi +1μM CH +1μM GSK
Thapsi (+1μM GSK)
Thapsi +1μM CH (+1μM GSK)
Thapsi +1μM CH +(1μM GSK)

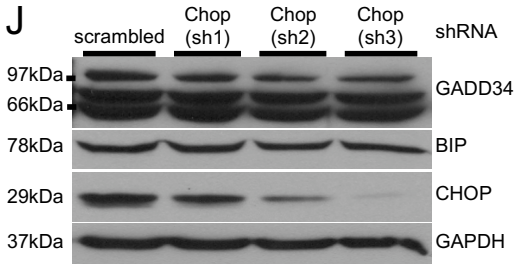

E
Treatment start
(+CH)
AnnV/PI staining
0
24
60 Time [h]
% annexin V positive cells
DMSO
Thapsi
Thapsi +1μM CH +1μM GSK
Thapsi (+1μM CH)
Thapsi +1μM GSK (+1μM CH)
Thapsi +1μM GSK


F
scrambled
Gadd34 (sh1)
Gadd34 (sh2)
Gadd34 (sh3)
shRNA
97kDa
66kDa
29kDa
37kDa
GADD34
CHOP
GAPDH


G
DMSO
Thapsi
% annexin V positive cells
shRNA scrambled
Gadd34 (sh1)
Gadd34 (sh2)
Gadd34 (sh3)


H
scrambled Gadd34
Chop
shRNA
10 30 10 30 10 30 Thapsi [h]
97 kDa
66 kDa
78 kDa
29 kDa
49 kDa
37 kDa
GADD34
BIP
CHOP
ATF4
GAPDH


I
scrambled Gadd34
Chop
shRNA
10 30 10 30 10 30 Thapsi [h]


J
scrambled
Chop (sh1)
Chop (sh2)
Chop (sh3)
shRNA
97kDa
66kDa
78kDa
29kDa
37kDa
GADD34
BIP
CHOP
GAPDH

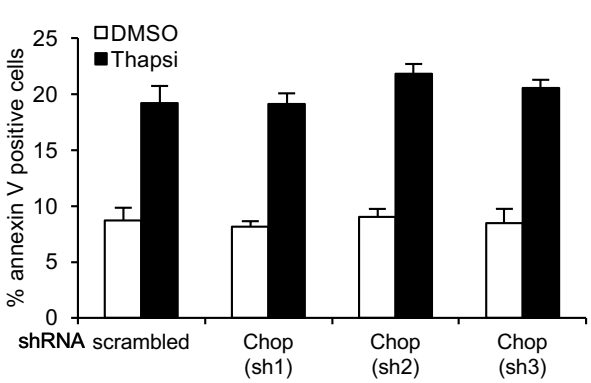

K
DMSO
Thapsi
% annexin V positive cells
shRNA scrambled
Chop (sh1)
Chop (sh2)
Chop (sh3)

Figure S1. Related to Figure 1. Dissecting the proapoptotic effects of thapsigargin in podocytes. A) Signaling model of the PERK branch of the UPR. During ER stress, the activation of PERK leads to the phosphorylation of eIF2α, which, by blocking general translation, induces ATF4. ATF4 upregulates CHOP and GADD34, and the latter is part of the negative feedback loop to restore translation. However, when ER stress persists, cells undergo apoptosis, linked to increased CHOP and GADD34. B) Western blot analysis of the UPR markers ATF4, CHOP, GADD34, and BIP after incubation of podocytes with 2.5μM thapsigargin in the presence or absence of CH and/or GSK for 15 hours. GAPDH served as a control. C-E) Podocyte cell death was determined at 60 hours of treatment with 2.5μM thapsigargin in the presence or absence of CH and/or GSK. The experiment was repeated with either GSK (C) or CH (D) applied 24 hours after initial drug treatment. Bar graphs represent mean percentages ± SD of annexin V positive cells (n=3). * $p < 0.05$; ** $p < 0.01$; # $p < 0.001$ (vs. thapsigargin, Bonferroni–corrected). F, J) Western blot analysis shows knockdown efficiency of several shRNAs against CHOP and GADD34. GAPDH served as a loading control. H, I) Western blot (GADD34, CHOP, ATF4) and de novo protein biosynthesis (see methods for details) analysis show the effect of GADD34– and CHOP–silencing on PERK activity. G, K) CHOP– and GADD34–depleted podocytes were treated in the presence or absence of 2.5μM thapsigargin. Cell death was assessed at 60h. Bar graphs represent mean percentages ± SD of annexin V positive cells (n=3). ** $p < 0.01$, ## $p < 0.0001$ (vs. scrambled thapsigargin, Bonferroni–corrected).

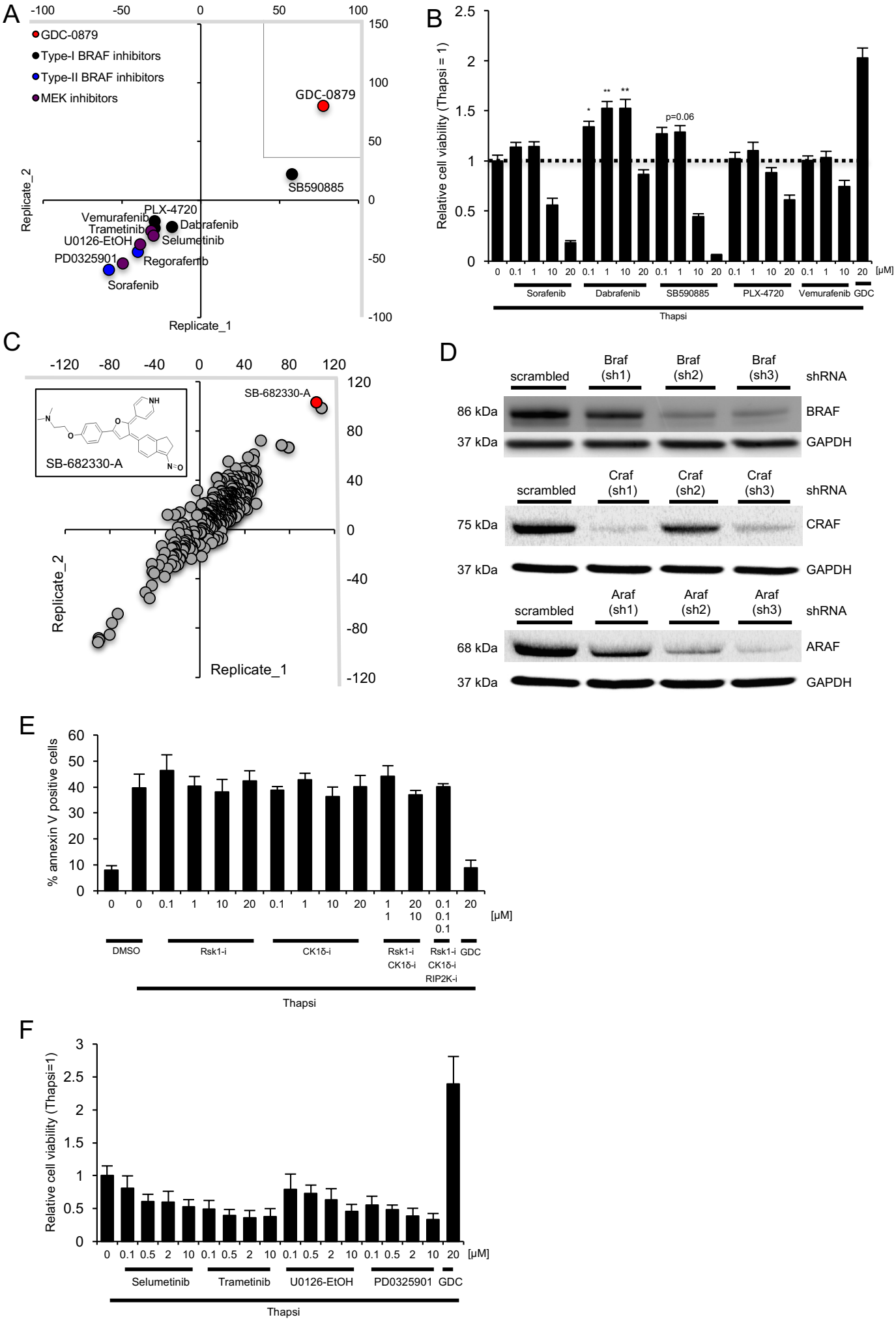

A
GDC-0879
Type-I BRAF inhibitors
Type-II BRAF inhibitors
MEK inhibitors
Replicate_1
Replicate_2
B
Relative cell viability (Thapsi = 1)
p=0.06
Sorafenib
Dabrafenib
SB590885
PLX-4720
Vemurafenib
GDC
Thapsi
C
SB-682330-A
Replicate_1
Replicate_2
D
scrambled
Braf (sh1)
Braf (sh2)
Braf (sh3)
shRNA
86 kDa
BRAF
37 kDa
GAPDH
Craf (sh1)
Craf (sh2)
Craf (sh3)
75 kDa
CRAF
Araf (sh1)
Araf (sh2)
Araf (sh3)
68 kDa
ARAF
E
% annexin V positive cells
DMSO
Rsk1-i
CK1δ-i
Rsk1-i CK1δ-i
Rsk1-i CK1δ-i RIP2K-i
GDC
Thapsi
F
Relative cell viability (Thapsi=1)
Selumetinib
Trametinib
U0126-EtOH
PD0325901
GDC
Thapsi

Figure S2. Related to Figure 1 and 2. The effect of BRAF and MEK inhibitors on podocyte survival. A) Data from the primary screen show GDC-0879 in relation to class I and class II BRAF and MEK inhibitors. SB590885 had a modest protective effect. B) A secondary screen of podocyte survival performed at 4 dose points (0.1 – 20μM) revealed statistically significant efficacy by Dabrafenib and a trend toward significance by SB590885. Bar graph represents relative cell viability (ATP levels) ± SD. Thapsigargin treatment was set to 1 (n=16); * $p<0.05$, ** $p<0.01$ (vs thapsi, Bonferroni-corrected). C) A screen of the GSK library consisting of 367 published kinase inhibitors (PKIS, 0.1μM and 1μM) identified the BRAF inhibitor SB-682330. Scatterplot between two replicates as % change of cell viability relative to thapsigargin–treated cells. D) Western blot analysis to verify BRAF, CRAF and ARAF knockdown. GAPDH served as a loading control. E) Podocytes were treated with thapsigargin in the presence or absence of inhibitors of CK1δ (IC 261), RSK (SL0101), and RIPK2 (Necrostatin-2), respectively, for 60 hours. Bar graphs represent mean percentages ± SD of annexin V positive cells (n=3). F) A secondary screen of podocyte survival performed at 4 dose points (0.1 – 20μM) of the indicated MEK inhibitors. Bar graph represents relative cell viability (ATP levels) ± SD. Thapsigargin treatment was set to 1 (n=16).

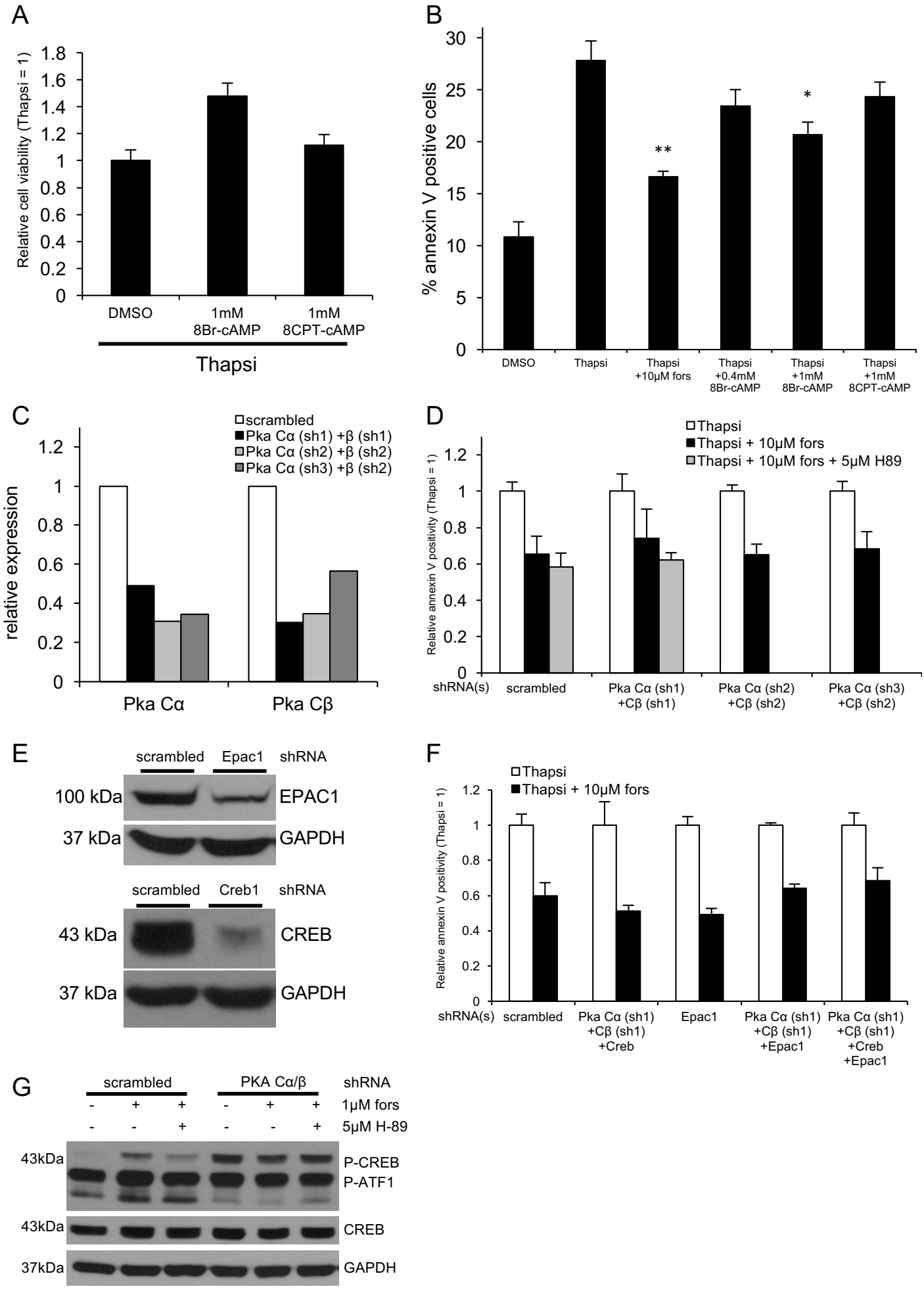

A
Relative cell viability (Thapsi = 1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
1mM 8Br-cAMP
1mM 8CPT-cAMP
Thapsi
B
% annexin V positive cells
30
25
20
15
10
5
0
**
*
DMSO
Thapsi
Thapsi +10μM fors
Thapsi +0.4mM 8Br-cAMP
Thapsi +1mM 8Br-cAMP
Thapsi +1mM 8CPT-cAMP
C
scrambled
Pka Cα (sh1) +β (sh1)
Pka Cα (sh2) +β (sh2)
Pka Cα (sh3) +β (sh2)
relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Pka Cα
Pka Cβ
D
Thapsi
Thapsi + 10μM fors
Thapsi + 10μM fors + 5μM H89
Relative annexin V positivity (Thapsi = 1)
1.2
1
0.8
0.6
0.4
0.2
0
shRNA(s)
scrambled
Pka Cα (sh1) +Cβ (sh1)
Pka Cα (sh2) +Cβ (sh2)
Pka Cα (sh3) +Cβ (sh2)
E
scrambled
Epac1
shRNA
100 kDa
EPAC1
37 kDa
GAPDH
scrambled
Creb1
shRNA
43 kDa
CREB
37 kDa
GAPDH
F
Thapsi
Thapsi + 10μM fors
Relative annexin V positivity (Thapsi = 1)
1.2
1
0.8
0.6
0.4
0.2
0
shRNA(s)
scrambled
Pka Cα (sh1) +Cβ (sh1) +Creb
Epac1
Pka Cα (sh1) +Cβ (sh1) +Epac1
Pka Cα (sh1) +Cβ (sh1) +Creb +Epac1
G
scrambled
PKA Cα/β
shRNA
- + + - + + 1μM fors
- - + - - + 5μM H-89
43kDa
P-CREB
P-ATF1
43kDa
CREB
37kDa
GAPDH

Figure S3. Related to Figure 1 and 3. The cAMP-mediated effect of forskolin is independent of PKA–CREB and EPAC signaling. A) The effect of 8Br-cAMP (1mM) and 8CPT-cAMP (1mM) on thapsigargin–treated podocytes at 96 hours. Bar graph represents relative cell viability (ATP levels) ± SD. Thapsigargin treatment was set to 1 (n=32). B) Annexin V in podocytes treated with thapsigargin in the presence or absence of 8Br-cAMP and 8CPT-cAMP at 48 hours. Bar graph shows mean percentages ± SD of annexin V positive cells (n=3). * $p < 0.05$, ** $p < 0.01$, ## $p < 0.0001$ (vs. thapsigargin, Bonferroni–corrected). C) PKA Cα and Cβ knockdown efficiency was determined by quantitative real–time PCR. Bar graph shows relative expression ± SD of PKA Cα and Pka Cβ. Data normalized to podocytes expressing a scrambled shRNA control. D) Podocytes silenced for PKA Cα and Cβ using multiple shRNAs were treated with thapsigargin in the presence or absence of forskolin (black) and H89 (grey). Bar graph represents relative apoptosis and necrosis (annexin V positive cells) ± SD. Thapsigargin alone treated cells served as a baseline control (n=3). E) Western blot analysis to verify EPAC and CREB knockdown. GAPDH served as a loading control. F) Podocytes depleted for PKA Cα/Cβ and/or EPAC and/or CREB were incubated with thapsigargin ± forskolin and cell death was assessed at 48 hours. Bar graph shows relative annexin V positive cells ± SD. Data normalized to thapsigargin (n≥3). G) PKA Cα/Cβ-silencing shows a compensatory increase in p-CREB abundance, which is insensitive to forskolin and H89.

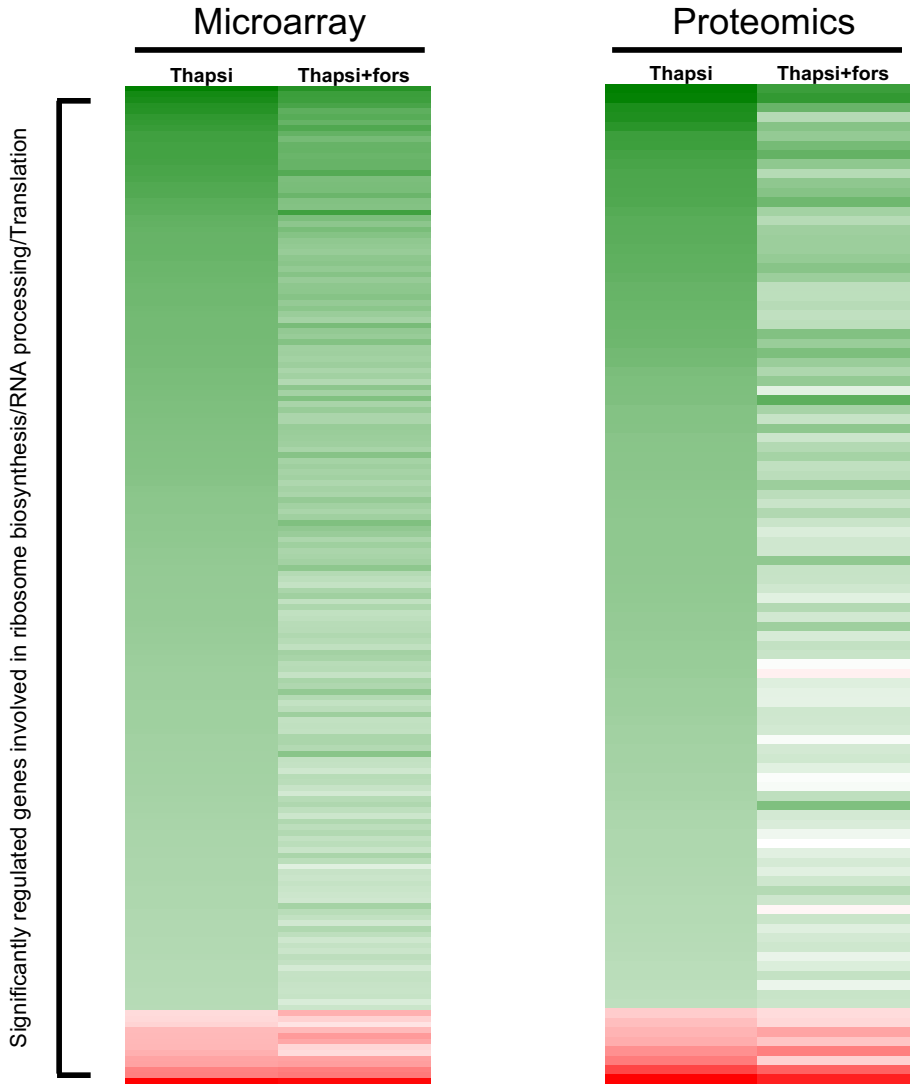


Figure S4. Related to Figure 3. Forskolin attenuates the thapsigargin-mediated upregulation of signaling programs related to protein biosynthesis. Heat maps show significantly regulated genes (microarray) or proteins (MS proteomics) linked with protein biosynthesis that contributed to the enriched GO gene sets in Figure 3A.

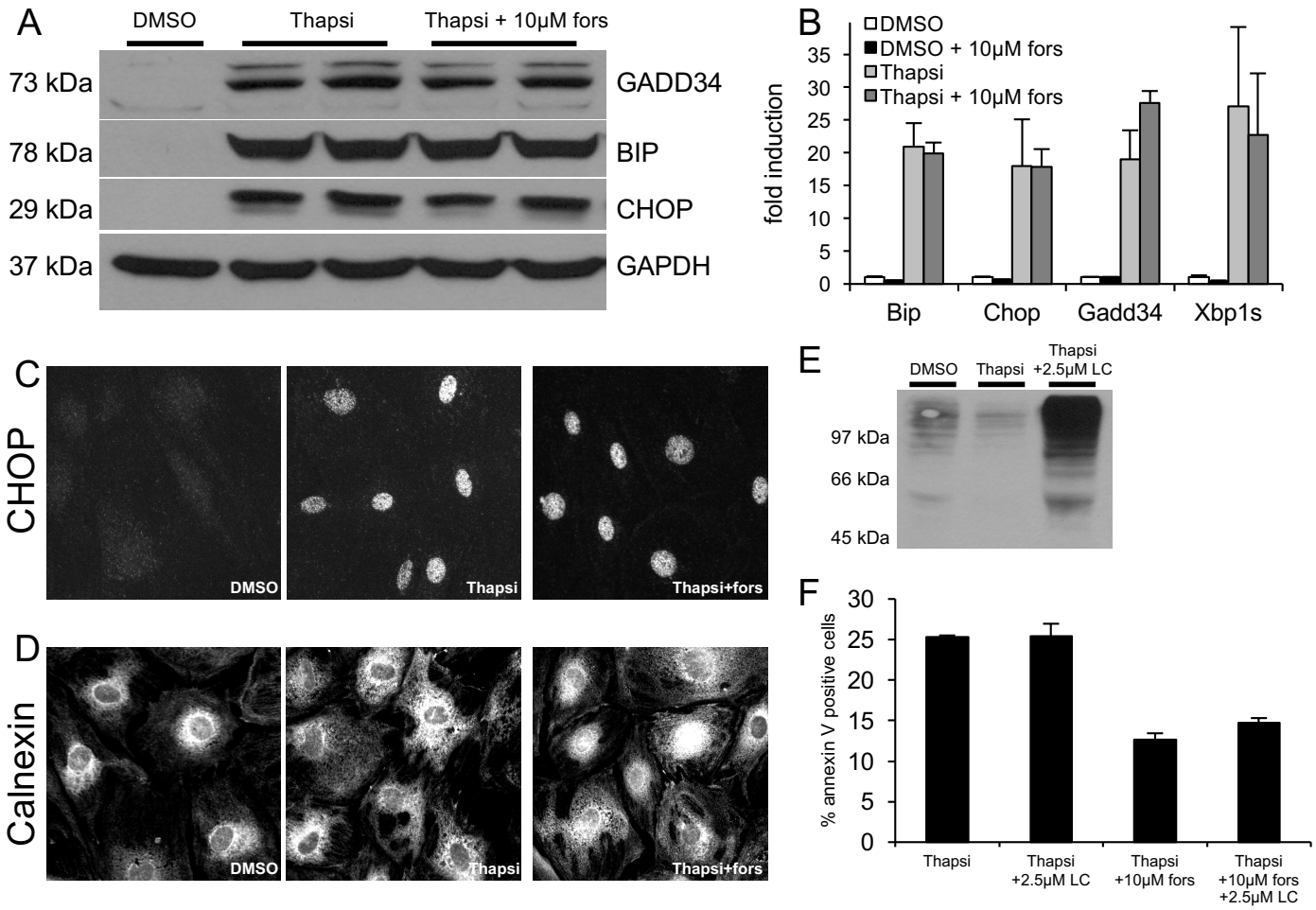


Figure S5. Related to Figure 4. Forskolin attenuates podocyte death in a UPR-independent manner. A) Western blot analysis of GADD34, BIP, and CHOP in whole cell lysates of podocytes treated with thapsigargin in the presence or absence of forskolin for 15 hours. GAPDH served as a loading control. B) Relative mRNA levels of Gadd34, Bip, Chop, as well as the spliced variant of Xbp1 (Xbp1s) in podocytes treated with thapsigargin ± forskolin for 8 hours were evaluated by quantitative real–time PCR. Bar graph represents relative mRNA expression ± SD. Vehicle (DMSO) treatment served as a baseline control (n=3). C, D) Immunocytochemistry of CHOP (C) and Calnexin (D) in podocytes treated with thapsigargin ± forskolin for 15 hours. Representative images are shown. E) Western blot analysis of ubiquitination in whole cell lysates of podocytes treated with thapsigargin ± lactacystin (LC) for 15 hours. F) Podocytes were incubated with thapsigargin ± forskolin and/or LC and cell death was measured at 60 hours. Bar graph shows mean percentages ± SD of annexin V positive cells (n=3).

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primers (5'-3'); F: Forward; R: Reverse		
Gapdh F-CTGCACCACCAACTGCTTAGC R-GGCATGGACTGTGGTCATGAG	(Sieber *et al.*, 2013)	N/A
Pkaca F-GAATACAGCCCAGTTGGATCA R-CTCGCCACCAGCTACATACTC	(Watson *et al.*, 2006)	N/A
Pkacb F-GGGGAACACTGCGATCGCCAA R-CCAGGGACGTATTCCATAACC	(Watson *et al.*, 2006)	N/A
Bip F-CACCAGGATGCGGACATTGA R-AGGGCCTCCACTTCCATAGA	(Cunha *et al.*, 2009)	N/A
Chop F-CAACAGAGGTCACACGCACA R-GGCACTGACCACTCTGTTTC	(Cunha *et al.*, 2009)	N/A
Gadd34 F-AGAGAAGACCAAGGGACGTG R-AACAATCTGAGCCGCCTCTG	(Cunha *et al.*, 2009)	N/A
Xbp1s F-TGCTGAGTCCGCAGCAGGTG R-ACTAGCAGACTCTGGGGAAGG	(Van Schadewijk *et al.*, 2012)	N/A
shRNAs (5'-3'; PLKO.1 vector)	Mission shRNA bacterial glycerol stocks	
Pkaca sh1: CCCGAGATTATCCTGAGCAAA sh2: CCACTTCAGCTCTGACTTGAA sh3: CACGAGTAACTTTGACGACTA	Sigma-Aldrich	SHCLNG-NM_008854
Pkacb sh1: CCTCAGCAAGGGTTACAATAA sh2: GTGGATCTGACAAAGCGATTC	Sigma-Aldrich	SHCLNG-NM_011100
Epac1 GCTACTCAGGAAGTTCATCAA	Sigma-Aldrich	
Creb1 GCCTGAAAGCAACTACAGAAT	Sigma-Aldrich	SHCLNG-NM_133828
Chop sh1: GCGGGCTCTGATCGACCGCAT sh2: GAAACGAAGAGGAAGAATCAA sh3: GATTCCAGTCAGAGTTCTATG	Sigma-Aldrich	SHCLNG-NM_007837
Gadd34 sh1: CTGAGAAAGTCACAGTCCATT sh2: TTCCAGGTGGCCTTCTATTTA sh3: GCGGGCTCTGATCGACCGCAT	Sigma-Aldrich	SHCLNG-NM_008654
Araf sh1: CAGGCTCATCAAAGGAAGAAA sh2: GCATGAGTGTCTATGACTCTT sh3: CCTAAAGTCCAACAATATCTT	Sigma-Aldrich	SHCLNG-NM_009703
Braf sh1: GCAGATGAAGATCATCGCAAT sh2: CCACATCATTGAGACCAAATT sh3: CGAGGATACCTATCTCCAGAT	Sigma-Aldrich	SHCLNG-XM_355754
Raf1 (Craf) sh1: GCTTTGGTACTACAGAACTTT sh2: TCCAGATGTTCCAGCTAATTG sh3: CAAGCAATACTATCCGGGTTT	Sigma-Aldrich	SHCLNG-NM_029780
Scrambled CAACAAGATGAAGAGCACCAA	Sigma-Aldrich	SHC002

Table S1. Related to Key Resources Table. Primer and shRNA sequences.