Supplementary Material

G protein-coupled estrogen receptor activation by Bisphenol-A disrupts protection from apoptosis conferred by estrogen receptors ERα and ERβ in pancreatic beta cells

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Supplementary Table 1. List of siRNAs used in this study.

Code	Name	Distributor	Sequence (5'to 3')
siCTRL	Allstars Negative Control siRNA	Qiagen, Venlo, Netherlands	Sequence not provided
Rat si*Gper1*	Rn_Gpr30_1 FlexiTube siRNA (SI01518335)	Qiagen, Venlo, Netherlands	Sequence not provided
Rat si*Gper1*	Rn_Gpr30_2 FlexiTube siRNA (SI01518342)	Qiagen, Venlo, Netherlands	Sequence not provided
Rat si*Erα*	Esr1RSS302814 (3_RNAI)	Invitrogen, Pasley, UK	GCUACAAACCAAUGCACCAUCGAUA
Rat si*Erα*	Esr1RSS302815 (3_RNAI)	Invitrogen, Pasley, UK	GCUUAAUUCUGGAGUGUACACAUUU
Rat si*Erβ*	Esr2RSS303096	Invitrogen, Pasley, UK	CCCAAAUGUGCUAUGGCCAACUUCU
Rat si*Erβ*	Esr2RSS303097	Invitrogen, Pasley, UK	GCGUAGAAGGGAUUCUGGAAAUCUU
Human si*GPER1*	Hs_GPR30_1 FlexiTube siRNA (SI00430360)	Qiagen, Venlo, Netherlands	Sequence not provided
Human si*GPER1*	Hs_GPR30_1 FlexiTube siRNA (SI00430367)	Qiagen, Venlo, Netherlands	Sequence not provided
Human si*ERα*	HS ESR1 8 (SI02784101)	Qiagen, Venlo, Netherlands	Sequence not provided
Human si*ERβ*	HS ESR2 6 (SI03083269)	Qiagen, Venlo, Netherlands	Sequence not provided

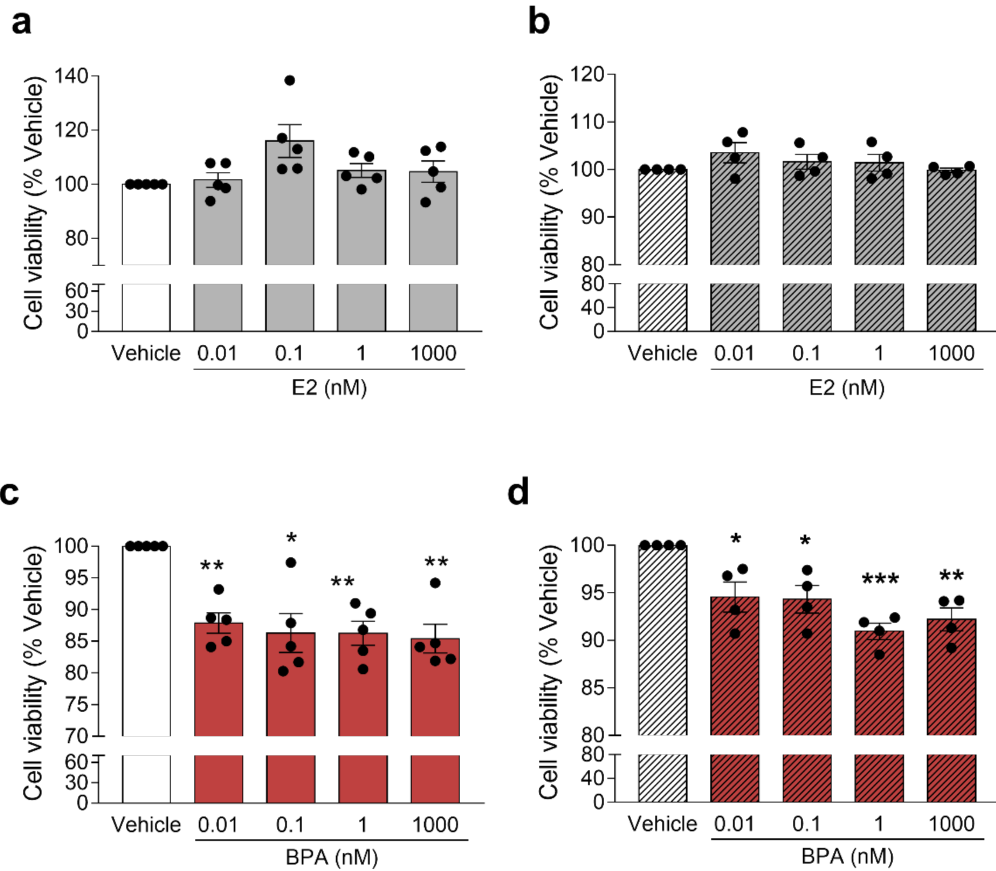
Supplementary Table 2. List of primers used in this study.

Gene	Forward	Reverse
	Sequence (5'-3')	**Sequence (5'-3')**
Rat *Esr1*	CTGACAATCGACGCCAGAA	TCGTTACACACAGCACAGTAG
Rat *Esr2*	TGGTCATGTGAAGGATGTAAGG	TTACGCCGGTTCTTGTCTATG
Rat *Gper1*	TCTACACCATCTTCCTCTTCCC	ACAGGTCTGGGATAGTCATCTT
Rat *Gapdh*	AGTTCAACGGCACAGTCAAG	TACTCAGCACCAGCATCACC
Human *ESR1*	CAGATGGTCAGTGCCTTGTT	GTTGGTCAGTAAGCCCATCAT
Human *ESR2*	TGGGCACCTTTCTCCTTTAG	AGGTGTGTTCTAGCGATCTTG
Human *GPER1*	GTCTCTAAACTGCGGTCAGATG	AGCAATTCTGTGTGAGGAGTG
Human β-actin	CTGTACGCCAACACAGTGCT	GCTCAGGAGGAGCAATGATC

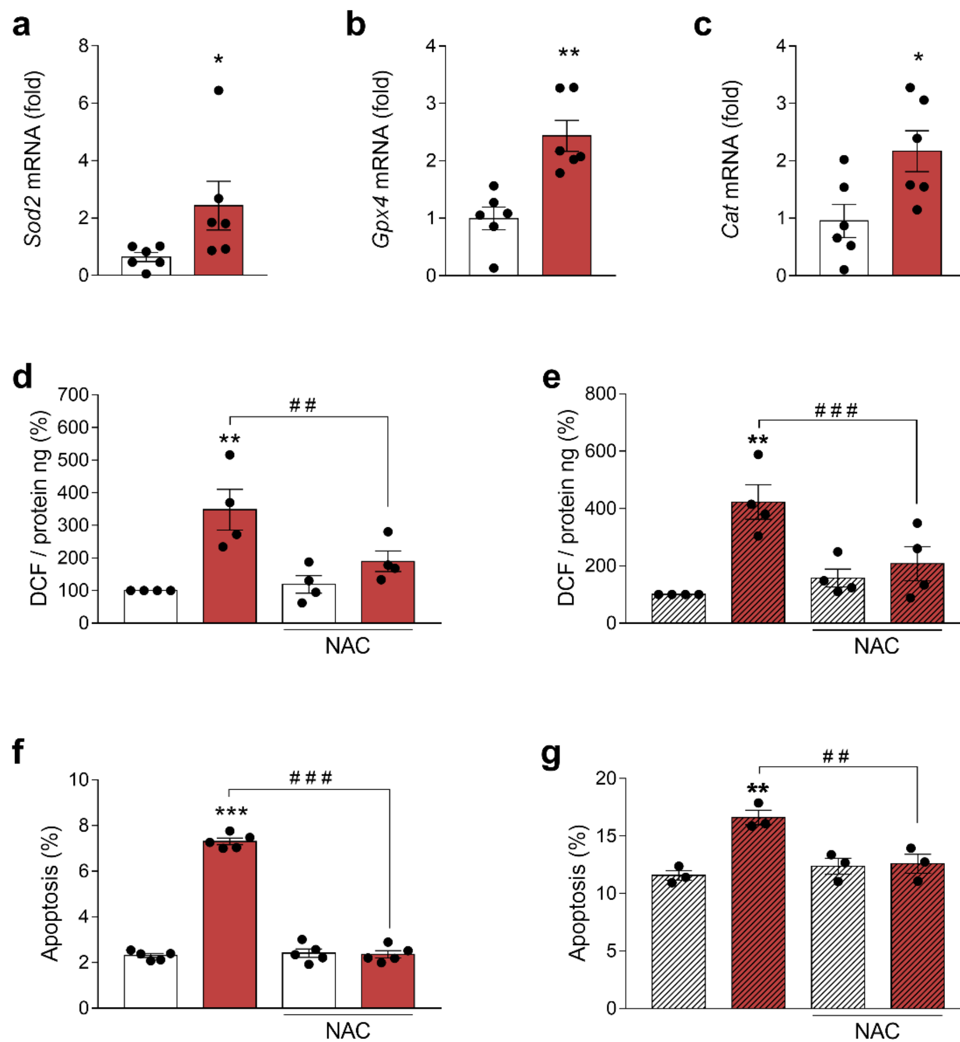
Supplementary Table 3. List of antibodies used in this study.

Target antigen	Antibody Name	Manufacturer and catalogue number (Cat no.)	Species raised in	Dilution	RRID
ERα	Estrogen Receptor alpha Monoclonal Antibody	Invitrogen; Cat no. MA5-13191	Mouse, monoclonal	1:1000 (WB)	AB_10986080
ERα	Estrogen Receptor alpha Monoclonal Antibody (SP1)	Thermo Fisher Scientific; Cat no. MA5-14501	Rabbit, monoclonal	1:200 (PLA)	AB_10981779
ERβ	Estrogen Receptor Beta Monoclonal Antibody	Invitrogen; Cat no. MA5-24807	Mouse, monoclonal	1:2000 (WB) and 2 mg/ml (IP)	AB_2717280
ERβ	Estrogen Receptor beta Monoclonal Antibody (14C8)	Thermo Fisher Scientific; Cat no. MA1-23217	Mouse, monoclonal	1:2000 (PLA)	AB_558839
GPER	Anti-G-protein coupled receptor 30 antibody	Abcam; Cat no. Ab-39742	Rabbit, polyclonal	1:1000 (WB)	AB_1141090
α-Tubulin	Monoclonal Anti-αTubulin antibody	Sigma; Cat no. T9026	Mouse, monoclonal	1:5000 (WB)	AB_477593
GAPDH	GAPDH (D16H11) XP Rabbit mAb antibody	Cell Signaling Technology; Cat no. 5174	Rabbit, monoclonal	1:1000 (WB)	AB_10622025
β-Actin	β-Actin (D6A8) Rabbit mAb antibody	Cell Signaling Technology; Cat no. 8457	Rabbit, monoclonal	1:1000 (WB)	AB_10950489
Goat anti-mouse IgG	Goat Anti-Mouse IgG (H+L) HRP Conjugate antibody	Bio-rad; Cat no. 170-6516	Goat, Polyclonal	1:5000 (WB)	AB_11125547

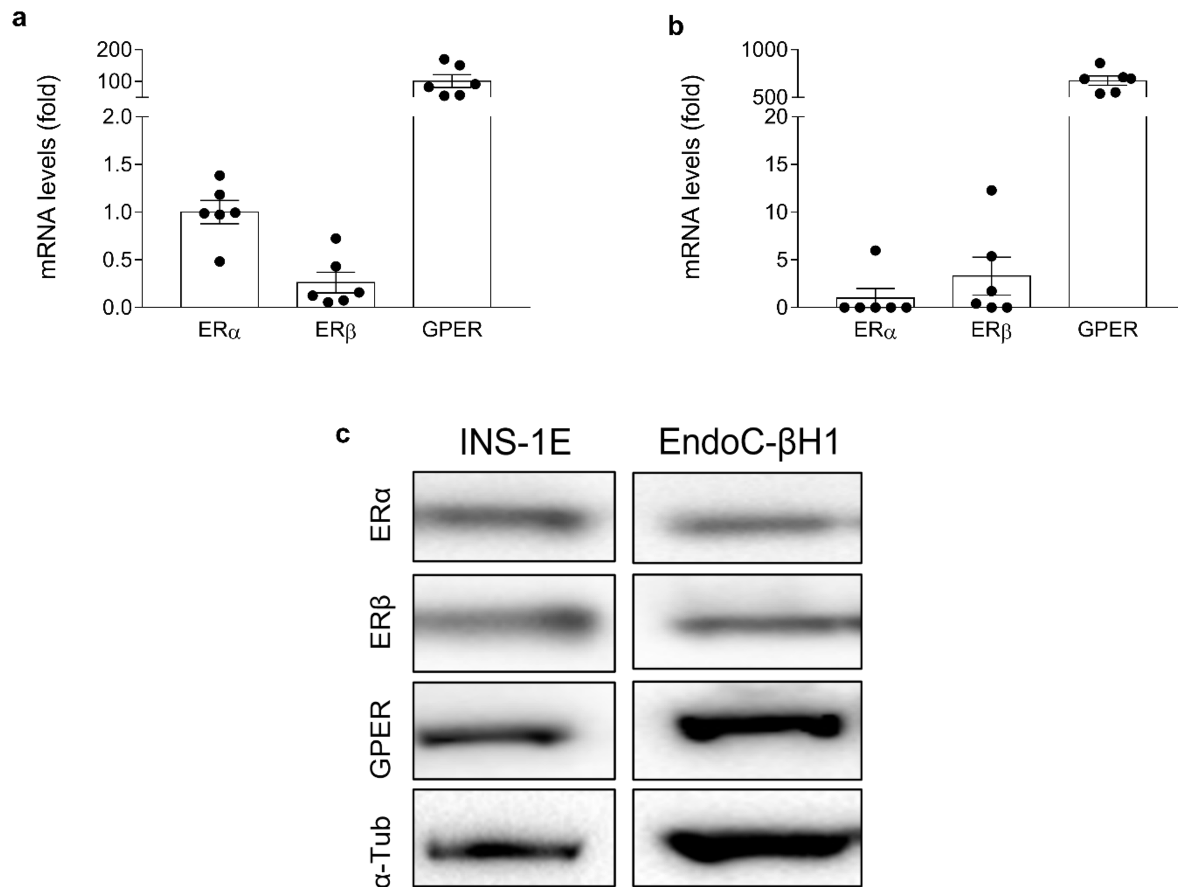
Goat anti-rabbit IgG	Goat Anti-Rabbit IgG (H+L) HRP Conjugate antibody	Bio-rad; Cat no. 170-6515	Goat, Polyclonal	1:5000 (WB)	AB_11125142
Goat anti-rabbit IgG	Normal Rabbit IgG antibody	Cell Signaling Technology; Cat no. 2729	Goat, Polyclonal	Used for IP	AB_1031062



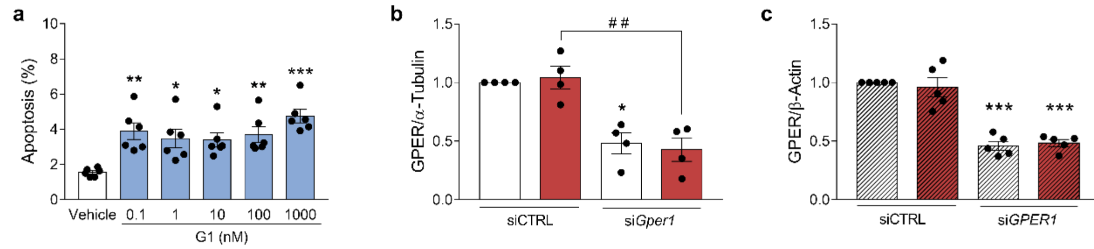
Supplementary Fig. 1 E2 and BPA have different effects on beta cell viability. INS-1E (**a,c**) and EndoC-βH1 cells (**b,d**) were treated with vehicle (white bars), E2 (grey bars) or BPA (red bars) for 48 h. Cell viability was evaluated by MTT assay. Data are shown as means ± SEM of 5 independent experiments. *p≤0.05, **p≤0.01 and ***p≤0.001 vs Vehicle. One-way ANOVA.



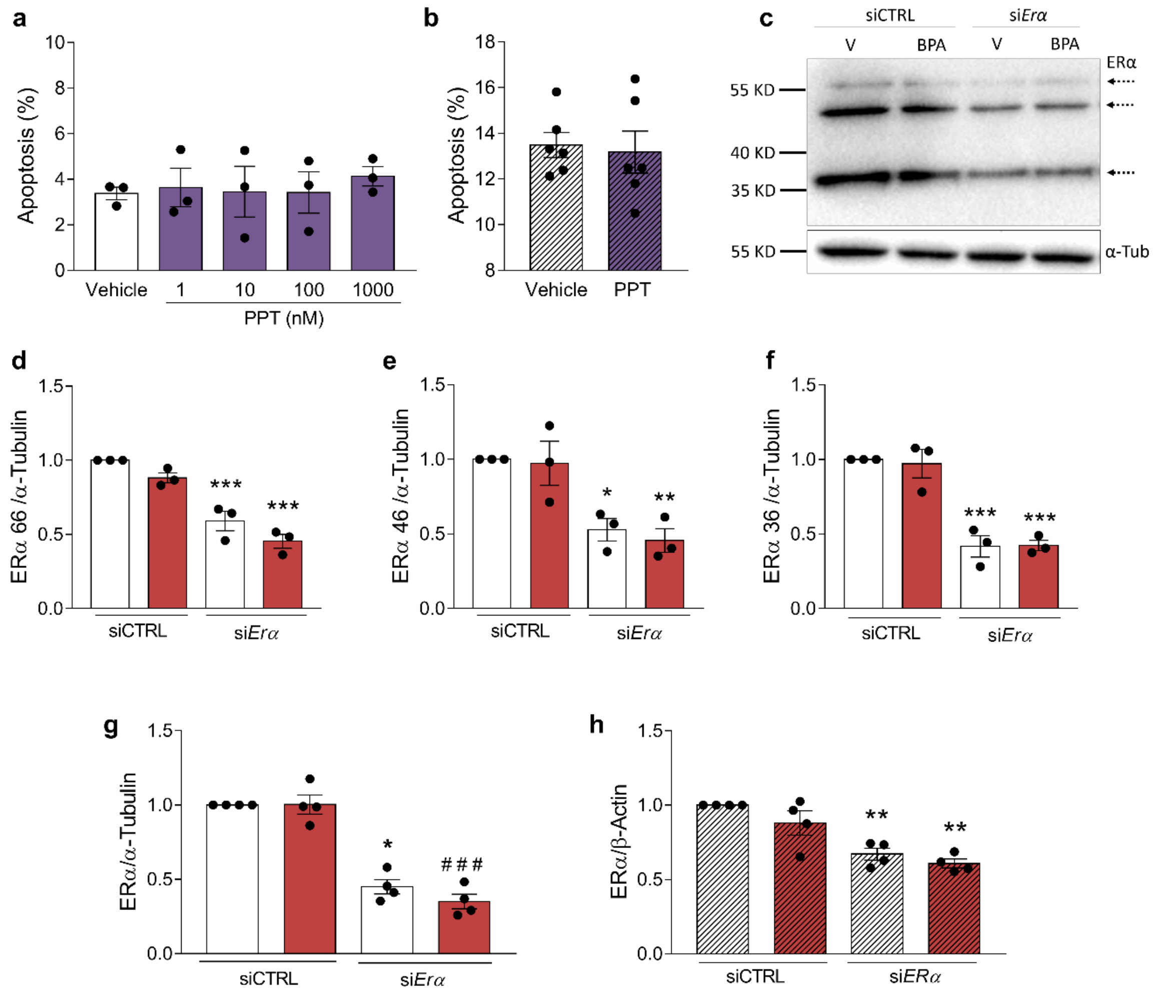
Supplementary Fig. 2 Involvement of oxidative stress in BPA-induced apoptosis. (**a-c**) mRNA expression of *Sod2* (**a**), *Gpx4* (**b**), and *Cat* (**c**) in INS-1E cells treated with vehicle (white bars) or BPA 1 nmol/l (red bars) for 24 h. mRNA expression was measured by qRT-PCR and normalised to the housekeeping gene *Gapdh,* and it is shown as fold vs vehicle. INS-1E (**d**) and EndoC-βH1 (**e**) cells were treated with vehicle (white bars) or BPA 1 nmol/l (red bars) in the absence or presence of N-acetylcysteine (NAC; 3 mmol/l) for 24 h. Oxidative stress was measured by oxidation of the fluorescent probe DCF and normalized by total protein. INS-1E (**f**) and EndoC-βH1 (**g**) cells were treated with vehicle (white bars) or BPA 1 nmol/l (red bars) in the absence or presence of NAC 3 mM for 24 h. Apoptosis was evaluated using Hoechst 33342 and propidium iodide staining. Data are shown as means ± SEM of 3-6 independent experiments. (**a-c**) *$p \leq 0.05$ and **$p \leq 0.01$, by two-tailed Student's *t* test. (**d-g**) **$p \leq 0.01$ and ***$p \leq 0.001$ vs the respective vehicle; $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$ as indicated by bars. Two-way ANOVA.



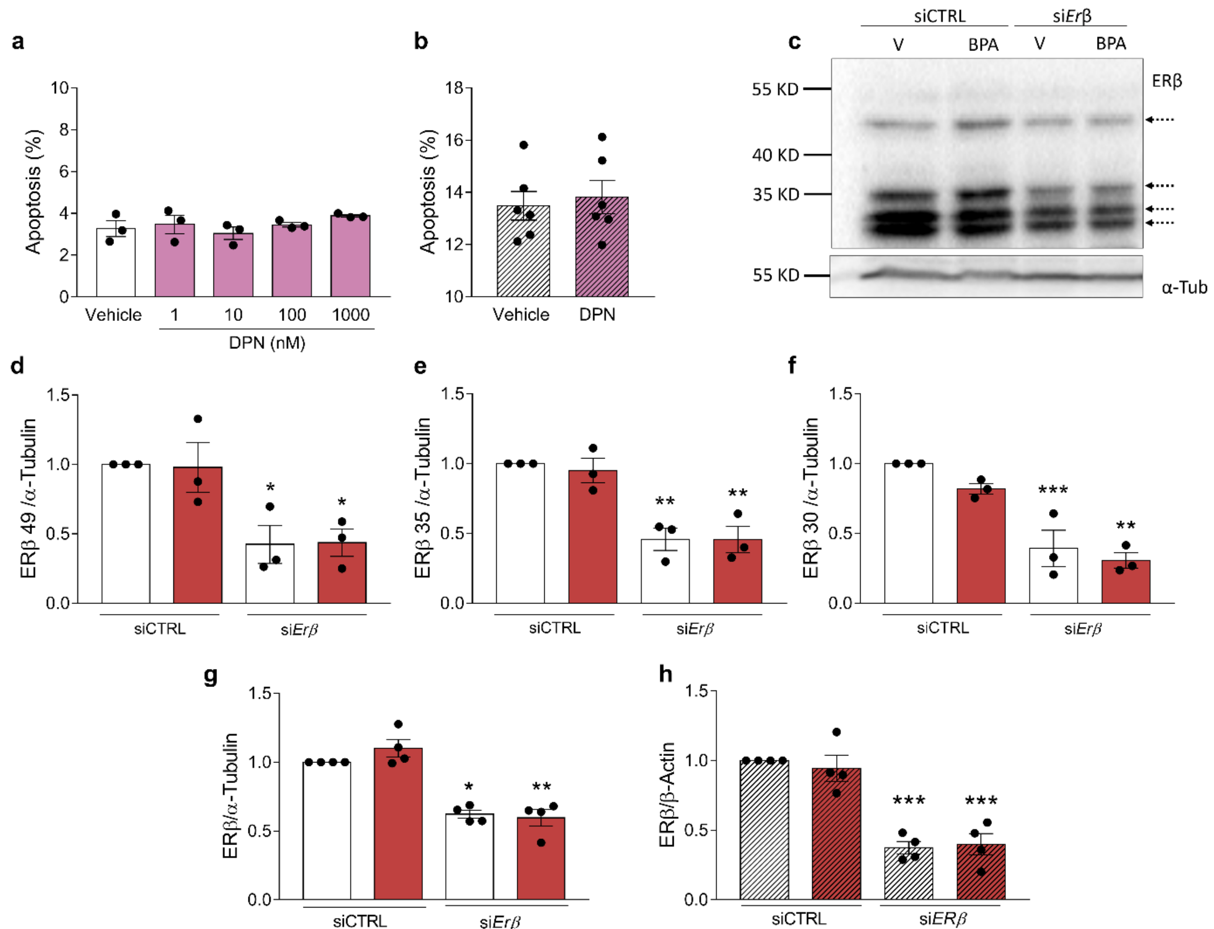
Supplementary Fig. 3 ERα, ERβ and GPER expression in INS-1E and EndoC-βH1 cells. mRNA expression of ERα, ERβ and GPER in INS-1E (**a**) and EndoC-βH1 cells (**b**). mRNA expression was measured by qRT-PCR and normalised to the housekeeping genes *Gapdh* (**a**) and β-actin (**b**). Data are shown as fold-change of ERα expression (considered as 1). (**c**) Protein expression of ERα, ERβ and GPER in INS-1E (left panel) and EndoC-βH1 cells (right panel) was measured by western blot.



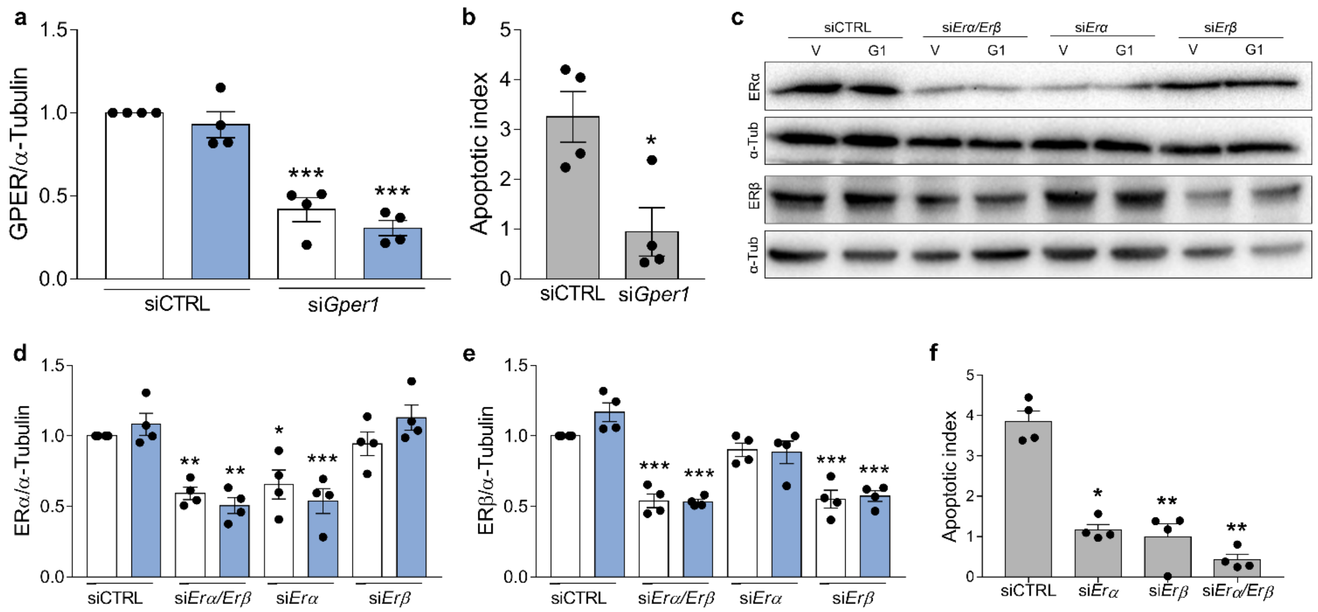
Supplementary Fig. 4 GPER activation induces apoptosis. (**a**) INS-1E cells were treated with vehicle (white bars) or GPER agonist G1 (blue bars) for 24 h. Apoptosis was evaluated using Hoechst 33342 and propidium iodide staining. (**b,c**) Densitometry analysis of immunoblots shown in Fig. 2e (**b**) and Fig. 2h (**c**). Values were normalised by α-tubulin (**b**) or β-actin (**c**) and then by the value of siCTRL-transfected vehicle-treated cells of each experiment (considered as 1). Data are shown as means ± SEM of 4-6 independent experiments. (**a**) $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ vs Vehicle, by one-way ANOVA. (**b,c**) $^*p \leq 0.05$ and $^{***}p \leq 0.001$ vs the respective siCTRL. $^{\#\#}p \leq 0.01$ as indicated by bars. Two-way ANOVA.



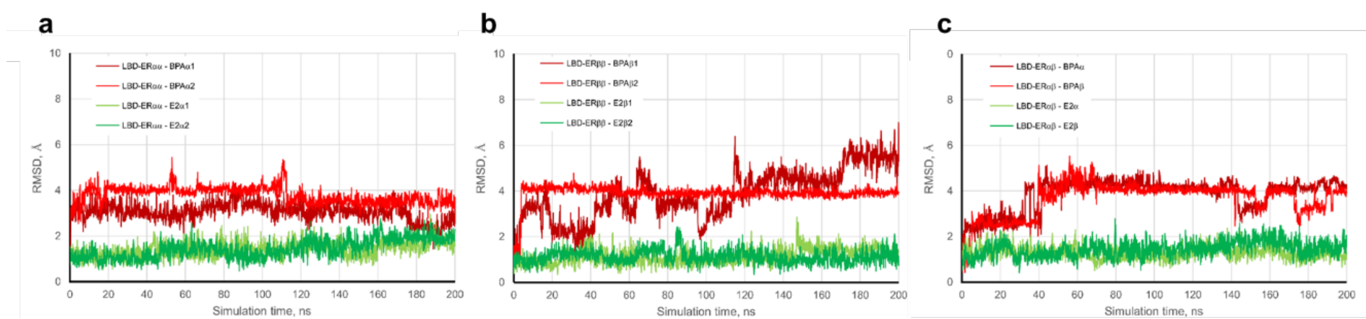
Supplementary Fig. 5 PPT effect on viability and confirmation of ERα knockdown. INS-1E (**a**) and EndoC-βH1 cells (**b**) were treated with vehicle or ERα agonist PPT (1 nmol/l in **b**) for 24 h. Apoptosis was evaluated using Hoechst 33342 and propidium iodide staining. (**c-f**) INS-1E cells were transfected with siCTRL or with a siRNA targeting ERα (si*Erα*). Cells were treated with vehicle (white bars) or BPA 1 nmol/l (red bars) for 24 h. Protein expression was measured by western blot. Representative images of three independent experiments are shown (**c**) and densitometry results are presented for different ERα variants, namely ERα 66 (**d**), ERα 46 (**e**) and ERα 36 (**f**). Values were normalised by α-tubulin (α-tub) and then by the value of siCTRL-transfected vehicle-treated cells of each experiment (considered as 1). (**g,h**) Densitometry analysis of immunoblots shown in Fig. 3d (**g**) and Fig. 3g (**h**). Values were normalised by α-tubulin or β-actin and then by the value of siCTRL-transfected vehicle-treated cells of each experiment (considered as 1). Data are shown as means ± SEM of 3-4 independent experiments. (**d-h**) $*p\leq0.05$, $**p\leq0.01$, $***p\leq0.001$ vs the respective siCTRL, by two-way ANOVA. PPT, propylpyrazoletriol.



Supplementary Fig. 6 DPN effect on viability and confirmation of ERβ knockdown. INS-1E (**a**) and EndoC-βH1 cells (**b**) were treated with vehicle or ERβ agonist DPN (1 nmol/l in **b**) for 24 h. Apoptosis was evaluated using Hoechst 33342 and propidium iodide staining. (**c-f**) INS-1E cells were transfected with siCTRL or with a siRNA targeting ERα (si*Erβ*). Cells were treated with vehicle (white bars) or BPA 1 nmol/l (red bars) for 24 h. Protein expression was measured by western blot. Representative images of three independent experiments are shown (**c**) and densitometry results are presented for different ERβ variants, namely ERβ 49 (**d**), ERβ 35 (**e**) and ERβ 30 (**f**). Values were normalised by α-tubulin (α-tub) and then by the value of siCTRL-transfected vehicle-treated cells of each experiment (considered as 1). (**g,h**) Densitometry analysis of immunoblots shown in Fig. 4c (**g**) and Fig. 4f (**h**). Values were normalised by α-tubulin or β-actin and then by the value of siCTRL-transfected vehicle-treated cells of each experiment (considered as 1). Data are shown as means ± SEM of 3-6 independent experiments: (d-h) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ vs the respective siCTRL, by two-way ANOVA. DPN, diarylpropionitrile.



Supplementary Fig. 7 GPER requires ERα and ERβ to induce apoptosis. (**a**) Densitometry analysis of immunoblots shown in Fig. 5c. Values were normalised by α-tubulin and then by the value of siCTRL-transfected vehicle-treated cells of each experiment (considered as 1). (**b**) G1-induced apoptosis data from Fig. 5d are presented as apoptotic index. (**c-e**) Related to Fig. 5i. Protein expression was measured by western blot. Representative images of four independent experiments are shown (**c**) and densitometry results are presented for ERα (**d**) or ERβ (**e**). (**f**) G1-induced apoptosis data from Fig. 5i are presented as apoptotic index. Data are shown as means ± SEM of 4 independent experiments. (**a,d,e**) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ vs the respective siCTRL, by two-way ANOVA. (**b**) *$p \leq 0.05$, by two-tailed Student's *t* test. (**f**) *$p \leq 0.05$ and **$p \leq 0.01$ vs siCTRL, by one-way ANOVA.



Supplementary Fig. 8 Molecular dynamics simulation of homo- and heterodimers of hLBD-ER. Analysis of the trajectory of the ligands bound to the closed cavity of the LBD-ER for homodimers α/α (**a**), homodimers β/β (**b**) and heterodimers α/β (**c**).