

Supplementary Information

Spatial encoding of cyclic AMP signalling specificity by GPCR endocytosis

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Supplementary Results

Supplementary Table 1. Beta2-adrenoceptor transcriptional response target genes.

Median-centered Log₂(Iso/No Drug) for each gene are shown from four replicate oligonucleotide microarray experiments. Replicates #1 and #2: cells treated with 1 μM isoproterenol; Replicates #3 and #4- cells treated with 10 nM isoproterenol.

Gene	Description	Replicate #1	Replicate #2	Replicate #3	Replicate #4
C6orf176	Chromosome 6 open reading frame 176	5.50	5.74	6.50	5.51
PCK1	Phosphoenolpyruvate carboxykinase 1	5.72	5.69	4.64	5.04
CGA	Glycoprotein hormones, alpha polypeptide	4.31	4.49	4.06	2.95
NR4A1	Nuclear receptor subfamily 4, group A, member 1	4.33	4.07	3.87	3.45
NR4A3	Nuclear receptor subfamily 4, group A, member 3	5.74	3.05	3.85	2.52
NR4A2	Nuclear receptor subfamily 4, group A, member 2	3.51	4.47	3.15	3.51
PDE4B	Phosphodiesterase 4B, cAMP-specific	2.99	2.45	2.72	2.35
PDE4D	Phosphodiesterase 4D, cAMP-specific	2.75	2.55	2.39	2.32
EST_AA481397	AA481397_Exon1_331	2.44	2.32	2.34	2.51
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	2.18	3.12	1.99	1.28
LOC387763	Hypothetical LOC387763	2.36	2.11	2.07	1.66
AREG	Amphiregulin (schwannoma-derived growth factor)	2.24	1.89	2.02	1.83
SNF1LK	SNF1-like kinase	2.01	2.48	1.31	1.84
ADAMTS5	ADAM metalloproteinase with thrombospondin type 1 motif, 5 (aggrecanase-2)	2.33	1.80	1.61	1.79
DACT2	Dapper, antagonist of beta-catenin, homolog 2	2.42	1.64	1.74	1.71
NTS	Neurotensin	1.99	1.97	2.03	1.17
AVPI1	Arginine vasopressin-induced protein 1	2.04	1.69	2.16	1.08
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	1.95	1.93	1.54	1.33
DUSP1	Dual specificity phosphatase 1	1.92	1.66	1.81	1.19
PTP4A1	Protein tyrosine phosphatase type IVA, member 1	1.68	1.56	1.44	1.83
IL11	Interleukin 11	2.25	1.46	1.65	0.96
IRX4	Iroquois homeobox protein 4	1.40	1.60	1.38	1.33
CASP9	Caspase 9, apoptosis-related cysteine peptidase	1.68	1.36	1.89	0.66
CD55	CD55 antigen, decay accelerating factor for complement	1.94	1.12	1.07	1.40
LYPD3	LY6/PLAUR domain containing 3	1.31	1.87	1.77	0.52
GEM	GTP binding protein overexpressed in skeletal muscle	1.55	1.36	1.30	1.16

CCK	Cholecystokinin	1.37	1.70	1.13	1.14
RPRM	Reprimo, TP53 dependent G2 arrest mediator candidate	1.44	1.49	1.28	1.08
ELL2	Elongation factor, R polymerase II, 2	1.17	1.69	1.09	1.33
SYAP1	Synapse associated protein 1, SAP47 homolog	1.32	1.47	1.39	1.10
DKFZp686D0972	Similar to RIKEN cD 4732495G21 gene	1.74	0.84	1.41	1.19
DKK1	Dickkopf homolog 1	1.89	0.96	1.51	0.79
RPS9	Ribosomal protein S9	1.06	1.36	1.91	0.77
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	1.35	1.30	1.25	1.10
RHOB	Ras homolog gene family, member B	1.32	1.33	1.55	0.69
EN2	Engrailed homeobox 2	1.41	1.26	1.03	1.07
DUSP2	Dual specificity phosphatase 2	1.09	1.70	1.18	0.68
SEMA6A	Sema domain, transmembrane domain and cytoplasmic domain, (semaphorin) 6A	1.46	1.13	1.19	0.83
SLC2A14	Solute carrier family 2 (facilitated glucose transporter), member 14	1.11	1.13	1.31	1.00
PTX3	Pentraxin-related gene, rapidly induced by IL-1 beta	1.09	1.18	0.82	1.19
SOD1	Superoxide dismutase 1 (amyotrophic lateral sclerosis 1)	1.35	0.70	1.50	0.67
CFC1	Cripto, FRL-1, cryptic family 1	1.31	1.18	1.11	0.61
HSPA1A	Heat shock 70kDa protein 1A	1.22	1.41	0.58	1.00
TSC22D3	TSC22 domain family, member 3	1.39	1.05	1.13	0.63
CHMP1B	Chromatin modifying protein 1B	1.32	1.08	0.89	0.85
SGK	Serum/glucocorticoid regulated kinase	1.36	1.06	1.03	0.68
HSPB8	Heat shock 22kDa protein 8	1.31	0.78	1.19	0.84
FOSL2	FOS-like antigen 2	1.19	0.99	1.20	0.73
JUNB	Jun B proto-oncogene	1.24	1.49	0.65	0.72
MN1	Meningioma (disrupted in balanced translocation) 1	0.80	1.36	0.88	1.04
GABARAPL1	GABA(A) receptor-associated protein like 1	1.40	0.76	1.25	0.68
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	1.52	0.94	0.86	0.72
OLIG2	Oligodendrocyte lineage transcription factor 2	1.24	1.03	1.01	0.74
AXL	AXL receptor tyrosine kinase	1.05	0.99	0.98	0.50
ATP1B3	ATPase, +/K+ transporting, beta 3 polypeptide	0.83	1.71	0.78	0.70

Supplementary Table 2. Gene Ontology (GO) categories enriched among beta2-adrenoceptor transcriptional target genes. GO enrichment analysis was carried out with GeneTrail⁴⁰.

GO Category	<i>p</i> -value	Gene
transcription factor activity	0.013	NR4A1, NR4A3, NR4A2, FOSB, FOS, IRX4, EN2, TSC22D3, FOSL2, JUNB, OLIG2
response to cAMP	0.018	FOS, DUSP1, JUNB
glucose transmembrane transporter activity	0.018	SLC2A3, SLC2A14
steroid hormone receptor activity	0.022	NR4A1, NR4A3, NR4A2
3',5'-cyclic-AMP phosphodiesterase activity	0.022	PDE4B, PDE4D
receptor binding	0.022	CGA, AREG, ADAMTS5, NTS, IL11, CCK, DKK1, GABARAPL1
MAP kinase phosphatase activity	0.022	DUSP1, DUSP2
response to hormone stimulus	0.032	PCK1, CGA, NR4A3, FOS, DUSP1, JUNB
growth factor activity	0.036	AREG, IL11, DKK1
regulation of organ growth	0.038	CGA, SOD1
signal transduction	0.038	CGA, NR4A1, NR4A2, PDE4B, PDE4D, NTS, FOS, IL11, GEM, CCK, RHOB, DUSP2, SOD1, NEDD9
cell death	0.047	NR4A1, DUSP1, CASP9, CCK, RHOB, SEMA6A, SOD1, TSC22D3, HSPB8, FOSL2
multicellular organismal development	0.022	CGA, NR4A3, FOSB, NTS, FOS, PTP4A1, IL11, IRX4, CCK, DKK1, RHOB, EN2, SEMA6A, SLC2A14, SOD1, CFC1, JUNB, OLIG2
GDP binding	0.018	PCK1, GEM, RHOB
behavior	0.036	NR4A3, FOSB, FOS, CCK, SOD1
regulation of caspase activity	0.038	NR4A1, CASP9, CCK
regulation of membrane potential	0.044	CCK, SOD1, OLIG2

Supplementary Table 3. Endocytosis-dependent genes. Median-centered $\text{Log}_2(\text{Iso}/\text{No Drug})$ were averaged from $n = 2$ microarray experiments per condition. Annotated CREB targets are based on Zhang et al. ⁹.

1 μM isoproterenol with or without Dyngo:

Gene	Description	Average 1 μM Iso	Average 1 μM Iso +Dyngo	CREB Target?
PCK1	Phosphoenolpyruvate carboxykinase 1	5.70	2.15	Yes
C6orf176	Chromosome 6 open reading frame 176	5.62	4.20	Yes
NR4A1	Nuclear receptor subfamily 4, group A, member 1	4.20	2.41	Yes
CGA	Glycoprotein hormones, alpha polypeptide	4.40	1.62	Yes
NR4A2	Nuclear receptor subfamily 4, group A, member 2	3.99	1.94	Yes
PDE4B	Phosphodiesterase 4B, cAMP-specific	2.72	0.83	Yes
PDE4D	Phosphodiesterase 4D, cAMP-specific	2.65	0.93	Yes
EST_AA481397	AA481397_Exon1_331	2.38	0.81	NA
DACT2	Dapper, antagonist of beta-catenin, homolog 2	2.03	0.15	NA
LOC387763	Hypothetical LOC387763	2.24	1.07	NA
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	2.06	0.27	Yes
AVPI1	Arginine vasopressin-induced 1	1.86	0.52	NA
SNF1LK	SNF1-like kinase	2.24	1.00	Yes
NTS	Neurotensin	1.98	0.56	Yes
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	1.94	0.55	Yes
PTP4A1	Protein tyrosine phosphatase type IVA, member 1	1.62	0.60	Yes
CASP9	Caspase 9, apoptosis-related cysteine peptidase	1.52	0.16	Yes
EN2	Engrailed homeobox 2	1.33	0.29	Yes
IRX4	Iroquois homeobox protein 4	1.50	0.49	Yes
TSC22D3	TSC22 domain family, member 3	1.22	0.36	NA
CCK	Cholecystokinin	1.53	0.76	Yes
SGK	Serum/glucocorticoid regulated kinase	1.21	0.13	Yes
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	1.32	0.56	NA
RHOB	Ras homolog gene family, member B	1.33	0.32	NA
CHMP1B	Chromatin modifying protein 1B	1.20	0.51	NA
OLIG2	Oligodendrocyte lineage transcription factor 2	1.14	0.18	NA
JUNB	Jun B proto-oncogene	1.36	0.66	Yes

AXL	AXL receptor tyrosine kinase	1.02	-0.01	NA
FOSL2	FOS-like antigen 2	1.09	0.23	Yes
SLC2A14	Solute carrier family 2 (facilitated glucose transporter), member 14	1.12	0.56	NA

10 nM isoproterenol with or without Dyngo:

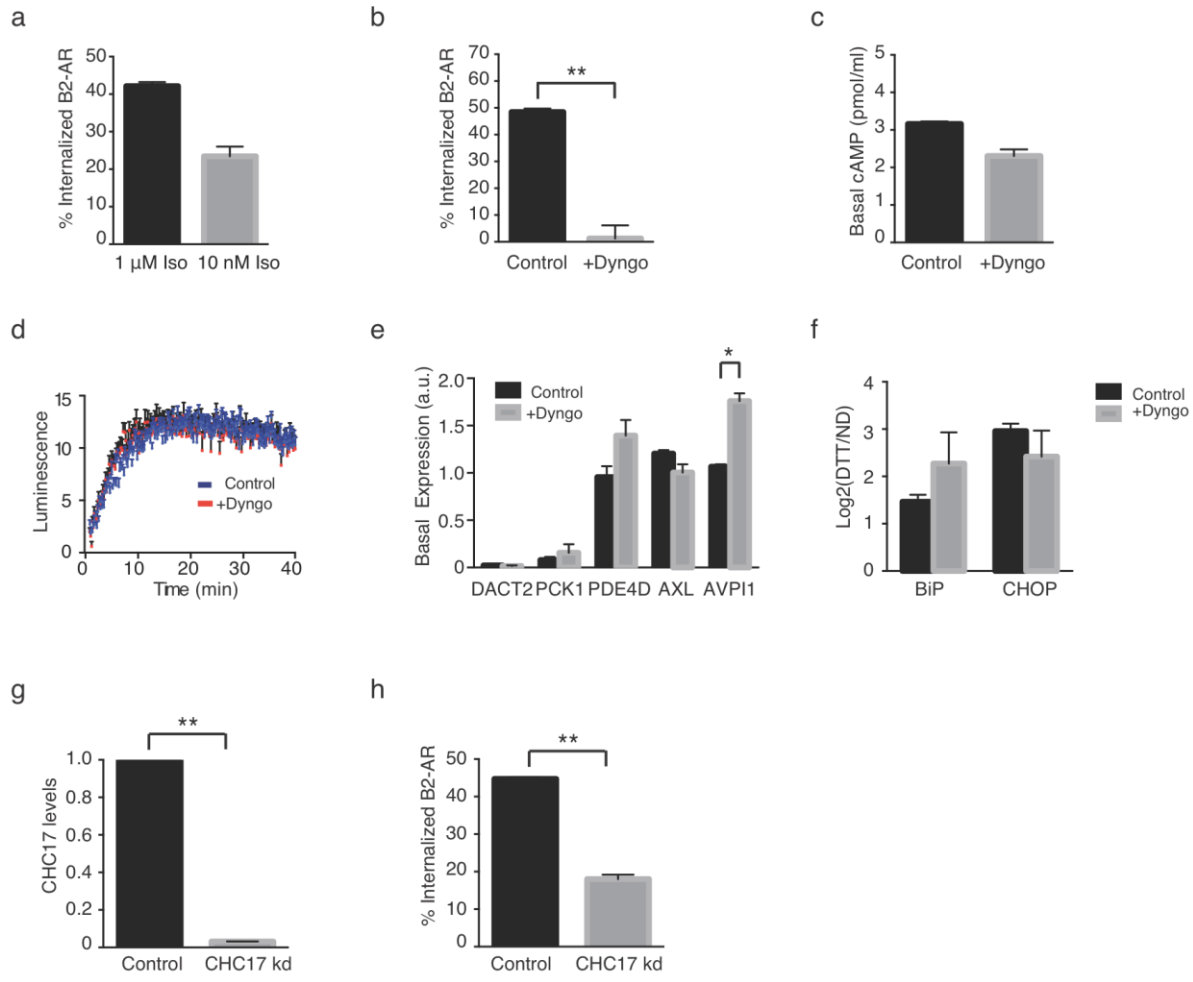
Gene	Description	Average 10nM Iso	Average 10nM Iso +Dyngo	CREB Target?
PCK1	Phosphoenolpyruvate carboxykinase 1	4.84	1.97	Yes
NR4A1	Nuclear receptor subfamily 4, group A, member 1	3.66	0.84	Yes
NR4A2	Nuclear receptor subfamily 4, group A, member 2	3.33	0.87	Yes
PDE4B	Phosphodiesterase 4B, cAMP-specific	2.53	1.09	Yes
EST_AA481397	AA481397_Exon1_331	2.43	0.93	NA
PDE4D	Phosphodiesterase 4D, cAMP-specific	2.35	0.98	Yes
AREG	Amphiregulin (schwannoma-derived growth factor)	1.93	0.87	Yes
LOC387763	Hypothetical LOC387763	1.86	0.74	NA
DACT2	Dapper, antagonist of beta-catenin, homolog 2	1.72	-0.01	NA
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	1.70	0.39	Yes
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	1.64	0.25	Yes
PTP4A1	Protein tyrosine phosphatase type IVA, member 1	1.63	0.70	Yes
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	1.43	0.16	Yes
IRX4	Iroquois homeobox protein 4	1.36	0.59	Yes
DKFZp686D0972	Similar to RIKEN cDNA 4732495G21 gene	1.30	0.39	NA
GEM	GTP binding protein overexpressed in skeletal muscle	1.23	0.26	Yes
ELL2	Elongation factor, RNA polymerase II, 2	1.21	0.31	Yes
RPRM	Reprimo, TP53 dependent G2 arrest mediator candidate	1.18	0.24	NA
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	1.18	0.29	NA
SLC2A14	Solute carrier family 2 (facilitated glucose transporter), member 14	1.16	0.13	NA
CCK	Cholecystokinin	1.13	0.50	Yes
EN2	Engrailed homeobox 2	1.05	0.43	Yes
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	0.79	0.16	NA

1 μ M isoproterenol with Dyngo vs 10 nM isoproterenol without Dyngo:

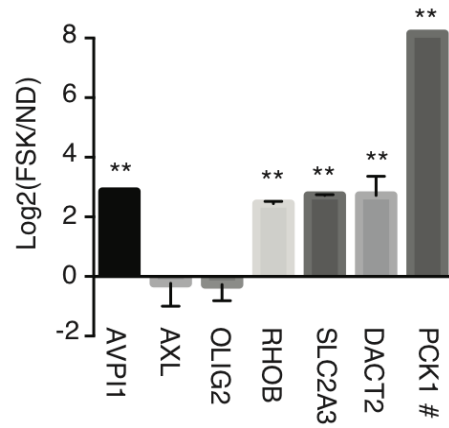
Gene	Description	Average 10nM Iso	Average 1 μ M Iso +Dyngo	CREB Target?
PCK1	Phosphoenolpyruvate carboxykinase 1	4.84	2.15	Yes
NR4A1	Nuclear receptor subfamily 4, group A, member 1	3.66	2.41	Yes
NR4A2	Nuclear receptor subfamily 4, group A, member 2	3.33	1.94	Yes
EST_AA481397	AA481397_Exon1_331	2.43	0.81	NA
PDE4D	Phosphodiesterase 4D, cAMP-specific	2.35	0.93	Yes
ADAMTS5	ADAM metalloproteinase with thrombospondin type 1 motif, 5 (aggrecanase-2)	1.70	0.27	Yes
PTP4A1	Protein tyrosine phosphatase type IVA, member 1	1.63	0.60	Yes
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	1.43	0.55	Yes
IRX4	Iroquois homeobox protein 4	1.36	0.49	Yes
DKFZp686D0972	Similar to RIKEN cDNA 4732495G21 gene	1.30	0.50	NA
ELL2	Elongation factor, RNA polymerase II, 2	1.21	0.71	Yes
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	1.18	0.56	NA
EN2	Engrailed homeobox 2	1.05	0.29	Yes
CHMP1B	Chromatin modifying protein 1B	0.87	0.51	NA

Supplementary Table 4. Primer sequences used in SYBR Green qPCR analysis.

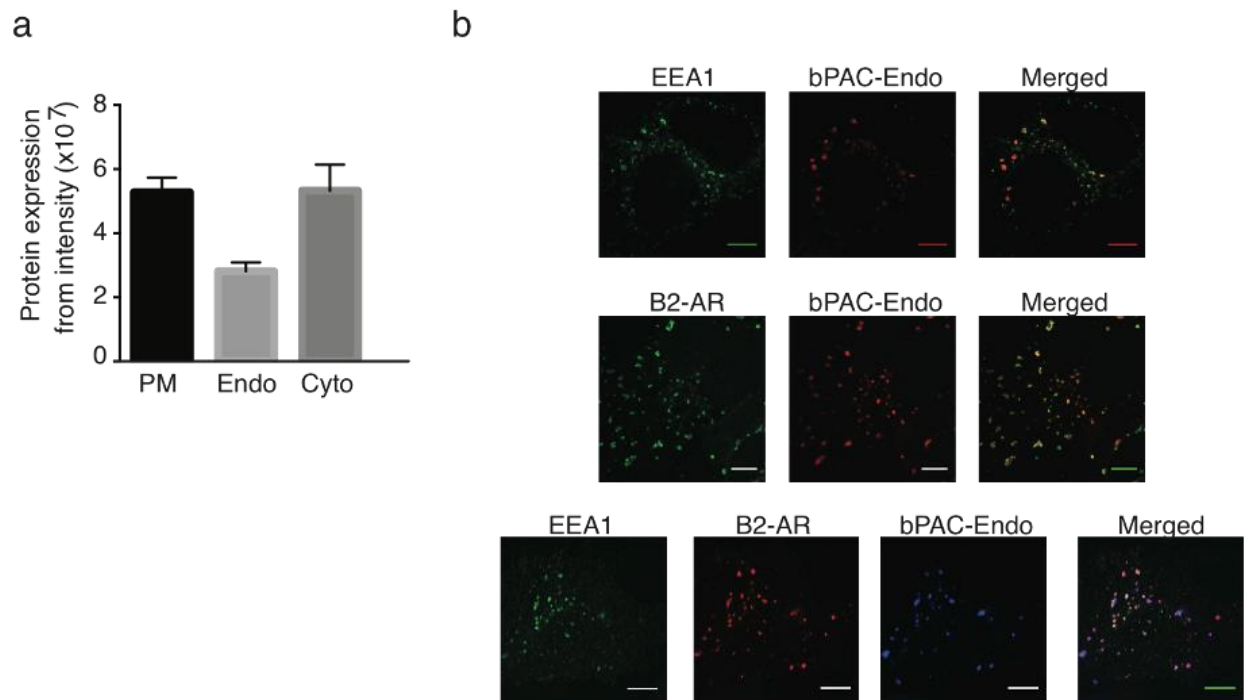
Gene	Primers (Forward/Reverse)	
<i>PCK1</i>	F: 5'-CTGCCCAAGATCTTCCATGT-3'	R: 5'-CAGCACCTGGAGTTCTCTC-3'
<i>ACTA</i>	F: 5'-CTGAGCGTGGCTACTCCTTC-3'	R: 5'-GCCATCTCGTTCTCGAAGTC-3'
<i>GAPDH</i>	F: 5'-CAATGACCCCTTCATTGACC-3'	R: 5'-GACAAGCTTCCCGTTCTCAG-3'
<i>CHC17</i>	F: 5'-ACTTAGCCGGTGCTGAAGAA-3'	R: 5'-AACCGACGGATAGTGTCTGG-3'
<i>RHOB</i>	F: 5'-ACATTGAGGTGGACGGCAAGCA-3'	R: 5'-CTGTCCACCGAGAAGCACATGA-3'
<i>AVP11</i>	F: 5'-GCCACTCGCTACCACTGCA-3'	R: 5'-CCTGGCACTTTTCTTCCTAGAGT-3'
<i>OLIG2</i>	F: 5'-ATGCACGACCTCAACATCGCCA-3'	R: 5'-ACCAGTCGCTCCATCTCCTCCA-3'
<i>SLC2A3</i>	F: 5'-TGCCTTTGGCACTCTCAACCAG-3'	R: 5'-GCCATAGCTCTTCAGACCCAAG-3'
<i>AXL</i>	F: 5'-GTTTGGAGCTGTGATGGAAGGC-3'	R: 5'-CGTTCACTCAGGAAATCCTCC-3'



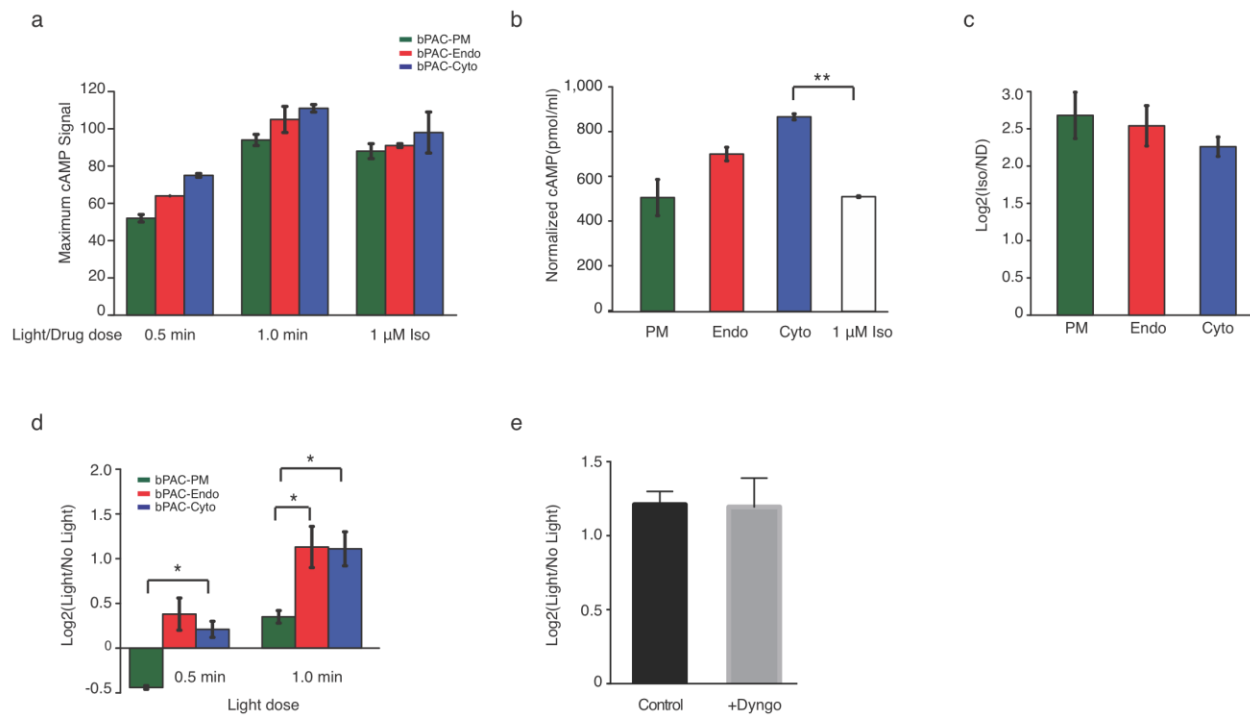
Supplementary Figure 1. Pharmacological and genetic inhibition of endocytosis. (a-b) Isoproterenol-induced effects on surface β 2-AR number in cells stably expressing a FLAG epitope-tagged receptor **(a)** after treatment with 1 μ M or 10 nM isoproterenol or **(b)** in the presence of 30 μ M Dyngo or vehicle (DMSO) and 1 μ M isoproterenol as analyzed by flow cytometry. Data = mean surface fluorescence from $n = 2$, 10,000 cells/condition, each time point in quadruplicate. **(c)** Basal cAMP levels in cells pre-treated with DMSO or Dyngo were measured using cAMP ELISA kit (Enzo Life Sciences) and normalized to total protein concentration per sample. Data from $n = 2$. **(d)** Dyngo treatment does not affect intracellular accumulation of the membrane permeable cAMP analog, 8-bromo-cAMP, added at a final concentration of 300 μ M. Real-time cAMP measurements were carried out using pGLO-20F (Promega). Data = average from $n = 3$ experiments. **(e)** Effects of Dyngo on basal gene expression for five β 2-AR transcriptional targets. qPCR measurements for each gene were normalized to expression levels for a housekeeping gene. Data = average from $n = 2-9$. **(f)** Dyngo does not block activation of the unfolded protein response genes BiP and CHOP. Cells were pre-treated with 30 μ M Dyngo or vehicle (DMSO) for 15 min, then 1mM DTT was added for 90 min. Data = average from $n = 2$ experiments. **(g-h)** Clathrin heavy chain siRNA-mediated knockdown. Cells stably expressing FLAG epitope-tagged β 2-AR were transfected with 20 nM *CHC17* or control siRNA for 72 hrs. **(g)** qRT-PCR analysis of siRNA mediated knockdown of *CHC17*. *CHC17* levels in control-transfected cells were adjusted to 1 for comparison. siRNA knockdown of *CHC17* diminished the mRNA levels by >90%. **(h)** Isoproterenol-induced effects on surface β 2-AR number in *CHC17* or control siRNA transfected cells as analyzed by flow cytometry. Data = mean surface fluorescence for $n = 2$, 10,000 cells/condition, each time point in quadruplicate. ND= no drug; Iso = isoproterenol. ** $p < 0.005$, * $p < 0.05$ by unpaired t-test; error bars = \pm s.e.m.



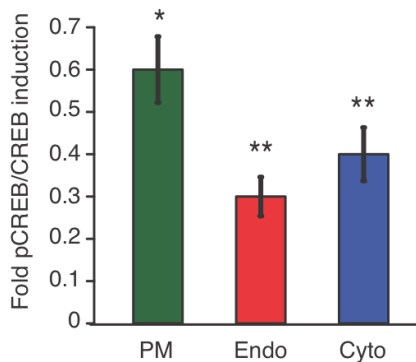
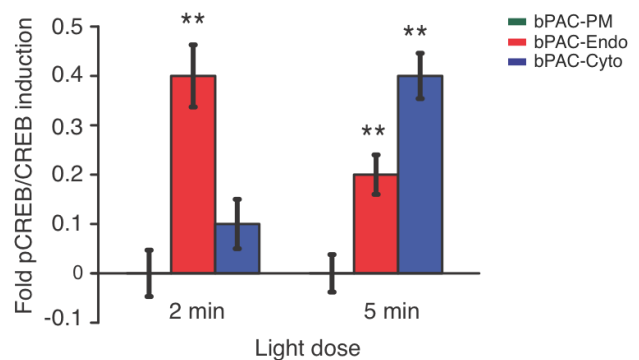
Supplementary Figure 2. cAMP-dependent transcription of β 2-AR target genes. Cells were treated with 5 μ M forskolin (FSK) for 2 hrs and the expression of seven endocytosis-dependent isoproterenol target genes was evaluated by qRT-PCR. Data = averaged log2 ratios (FSK/DMSO) of n = 2-3 experiments \pm s.e.m. ND = no drug. #- *PCK1* (a known CREB target) is included as positive control. ** $p < 0.005$ by unpaired t-test comparing FSK-induced and basal gene expression levels.



Supplementary Figure 3. Expression of bPAC constructs in HEK293 cells. (a) Protein expression from bPAC constructs quantified from immunofluorescence staining. Data = average of $n = 57$ cells total from 3-4 independent transfections. (b) Co-localization of bPAC-Endo with early endosome marker EEA1 or $\beta 2$ -AR (after treatment with $10 \mu\text{M}$ isoproterenol for 15 min) visualized by spinning disk confocal microscopy. Endogenous EEA1 protein was stained with anti-EEA1 antibody, while FLAG-tagged receptor and myc-tagged bPAC-Endo constructs were transiently expressed and stained with Alexa-conjugated antibodies. Scale bar = $10 \mu\text{m}$. “Cyto” – cytosolic bPAC, “PM” – Lyn-bPAC, “Endo” – 2xFYVE-bPAC; error bars = \pm s.e.m.



Supplementary Figure 4. bPAC-dependent signaling. (a-b) bPAC-transfected cells were exposed to lower doses of light than shown in Figure 4 to generate cAMP levels comparable to 1 μ M isoproterenol. (a) Maximum cAMP signal in response to low doses of light or bath application of 1 μ M isoproterenol was measured using the enzyme-based biosensor pGLO-20F (Promega). Data = average from $n = 2$ experiments, each experiment in triplicate wells of 10,000 cells each. (b) Absolute cAMP levels were measured 5 min after addition of 1 μ M isoproterenol or after 30 sec of light using cAMP ELISA kit (Enzo Life Sciences) and normalized to total protein concentration. Data = average from $n = 2$. (c) GAPDH-normalized *PCK1* expression was measured by qRT-PCR before or after treatment of bPAC-transfected cells with 10 nM isoproterenol for 30 min. Data are average of $n = 3$. (d) *PCK1* expression levels were measured by qPCR at indicated doses of light and normalized to *GAPDH*. Data = average from $n = 2$ (for 0.5 min light) or $n = 3-4$ (for 1 min light). (e) Pre-treatment of cells with Dyngo does not affect bPAC-Endo-dependent induction of *PCK1* expression at a light dose of 5 min. Data = average from $n = 2$ experiments. “PM” – Lyn-bPAC, “Endo” – 2xFYVE-bPAC, “Cyto” – cytosolic bPAC, Iso = isoproterenol, ND = no drug. ** $p < 0.005$, * $p < 0.05$ by unpaired t-test; error bars = \pm s.e.m.

a**b**

Supplementary Figure 5. Phosphorylation of CREB. (a-b) Phosphorylation of CREB was evaluated as a fraction of total CREB by immunofluorescence staining using CREB and phospho-CREB specific antibodies. The ratio of phospho-CREB/CREB in bPAC-transfected cells not exposed to light was set to zero. Cells were (a) treated with 10 μ M forskolin for 10 min ($n = 37-41$ cells from 2 independent transfections) or (b) exposed to light at indicated doses ($n = 40-66$ cells from 2 independent transfections). “PM” – Lyn-bPAC, “Endo” – 2xFYVE-bPAC, “Cyto” – cytosolic bPAC. ** $p < 0.005$, * $p < 0.05$ by unpaired t-test comparing light-induced and basal pCREB levels; error bars = \pm s.e.m.