Supplemental Information

A coincidence reporter-gene system for high throughput screening

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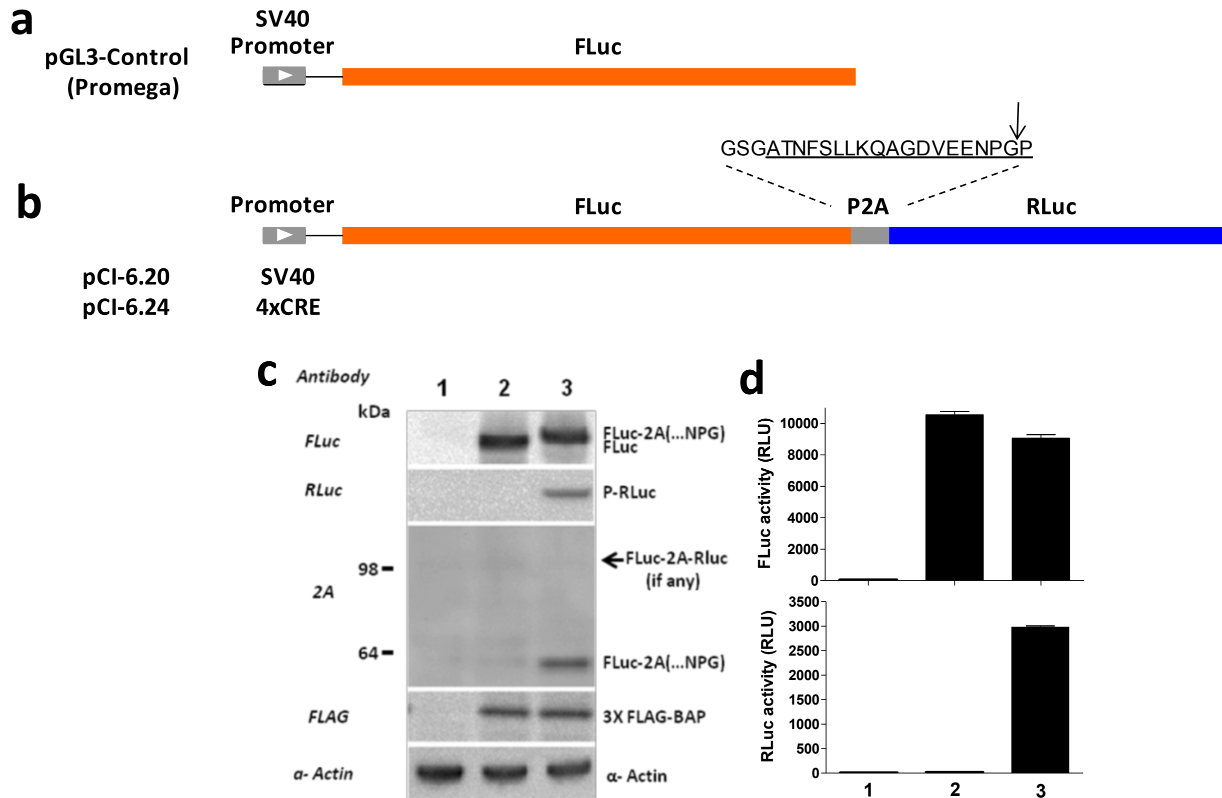
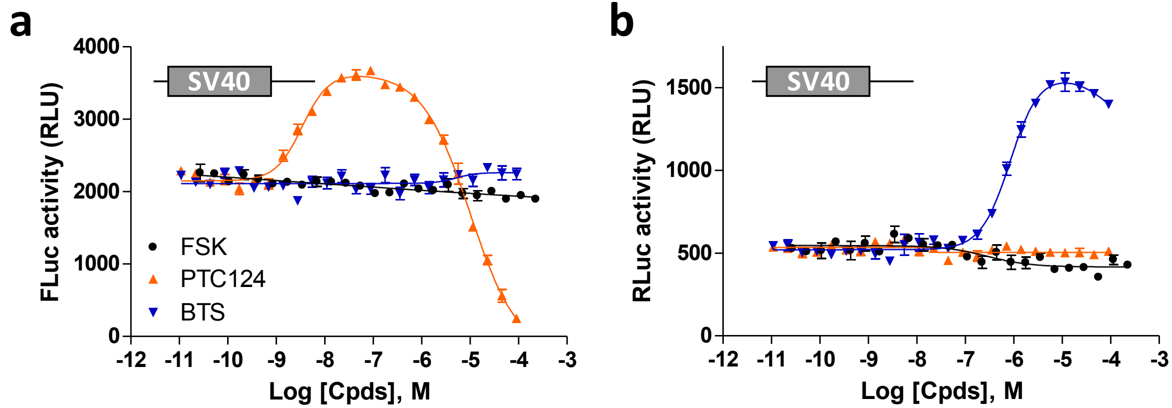
Supplementary Figure 1 | Design and characterization of FLuc-P2A-RLuc coincidence reporter biocircuit expression and function.



Supplementary Figure 1 | Design and characterization of FLuc-P2A-RLuc coincidence reporter biocircuit expression and function. Arrangement of elements in the (**a**) SV40-driven FLuc mono-reporter (pGL3-Control), and (**b**) the SV40-driven FLuc-P2A-RLuc dual reporter (pCI-6.20) and 4XCRE-driven FLuc-P2A-RLuc dual reporter (pCI-6.24). P2A amino acid sequence (underline) used in this construct is shown; arrow indicates ribosomal 'skipping' site. (**c**) Western blot analysis showing the efficient expression of non-tethered reporters, where lane 1 is non-transfection control (transfection reagent only); lane 2, SV40-driven FLuc mono-reporter (pGL3-Control); and lane 3, FLuc-P2A-RLuc dual reporter (pCI-6.20). Note that co-transfection of 3XFLAG-BAP is to demonstrate the transfection efficiency was similar. (**d**) Bioluminescent output from mono FLuc reporter and co-expressed FLuc and RLuc using Dual-Glo reagent; lanes are the same as in (**c**).

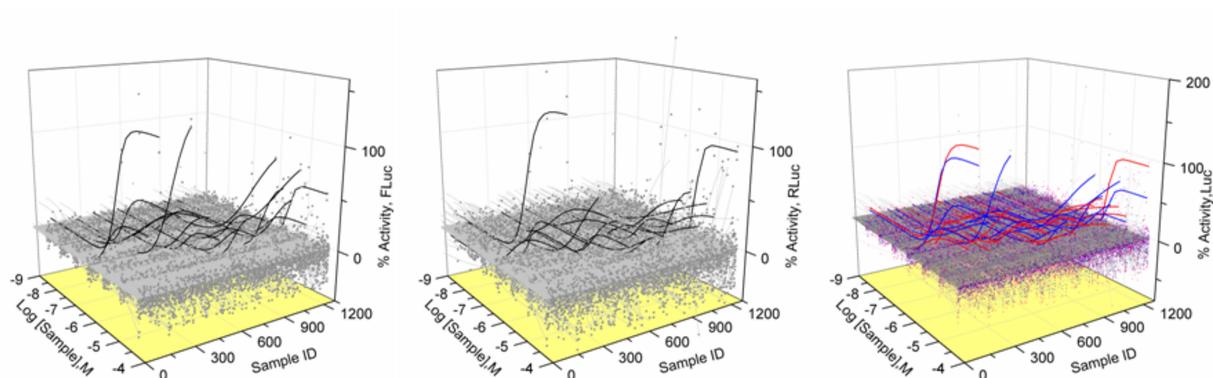
Supplementary Figure 2| Validation of reporter FLuc-2A-RLuc with SV40 element.



Supplementary Figure 2| Validation of reporter FLuc-P2A-RLuc with SV40 element. FLuc and RLuc are both sensitive and commonly used reporters with generally short half-lives and use different substrates and mechanisms to produce light. Response output from the FLuc (**a**) or the RLuc (**b**) reporter from transient transfection of the biocircuit plasmid in HEK293 cell variant, GripTite MSR in 384-well format. Cells were treated with FSK (●), PTC124 (▲) or BTS (▼). PTC124 and BTS are inhibitors of FLuc and RLuc, respectively, and act to increase the activity of the reporters by stabilizing their cellular half-life relative to non-treated control. Note that FSK is inactive in experiments where reporter expression is driven by the SV40 promoter, only displaying activity when the biocircuit is under control of 4XCRE (see **Fig. 1b**). FSK is forskolin.

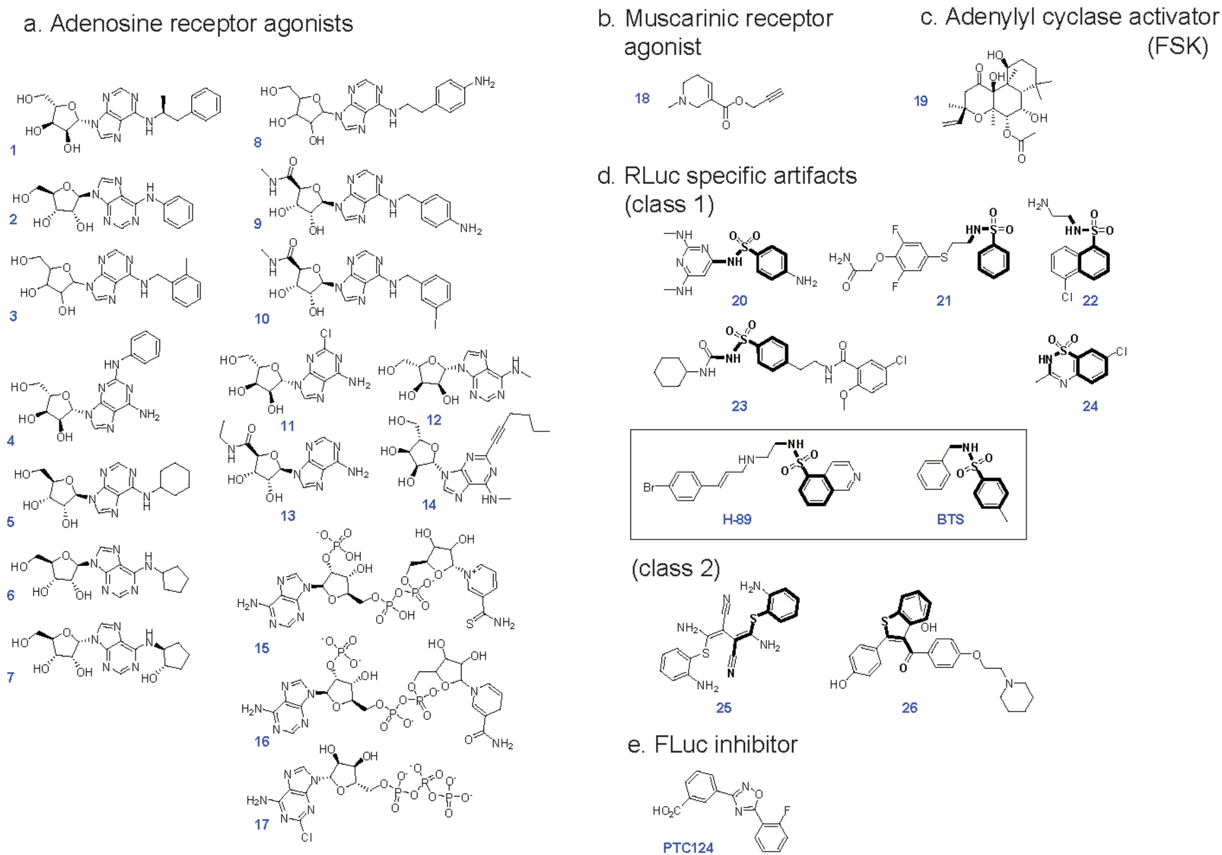
a **b** **c**

Supplementary Figure 3 | qHTS 3-axis plots for output from transiently transfected HEK MSR cells treated with the LOPAC library.



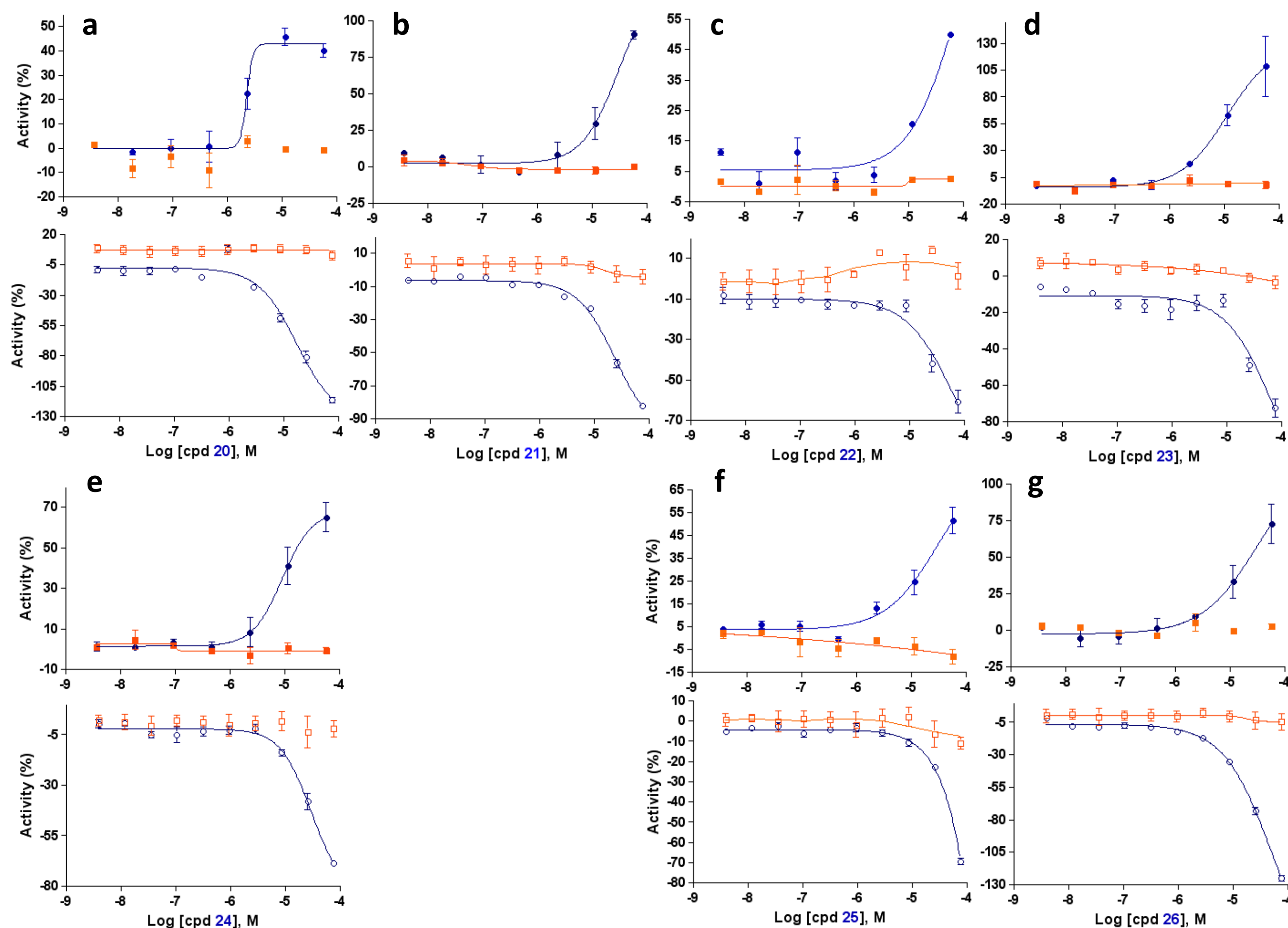
Supplementary Figure 3 | qHTS 3-axis plots for output from transiently transfected GripTite 293 MSR cells treated with the LOPAC library. (**a**) FLuc, (**b**) RLuc, and (**c**) both outputs (red, FLuc and blue, RLuc) plotted as a three-axis graphs for the entire LOPAC library where bold line fits are for compounds displaying qHTS curve classes of 1a, 1b or 2a in either replicate test. The following classification criteria define curve classes: Class 1 curves display two asymptotes, an inflection point, and r2 ≥0.9; subclasses 1a vs. 1b are differentiated by full (>80%) vs. partial (≤ 80%) response. Class 2 curves display a single left-hand asymptote and inflection point; subclasses 2a and 2b are differentiated by a max response and r^2, >80% and >0.9 or <80% and <0.9, respectively. Class 3 curves (second from right) have a single left-hand asymptote, no inflection point, and a response >3SD the mean activity of the sample field. Class 4 (far right) defines those samples showing no activity across the concentration range. The details of this classification scheme have been presented in Inglese et al.[3] The library compounds were ordered along the z-axis (Sample ID) for presentation clarity. Curve classes are also denoted as 1.1 (1a), 1.2 (1b), 2.1 (2a), and 2.2 (2b).

Supplementary Figure 4 | Structures of active compounds from CRE activation coincident reporter qHTS.



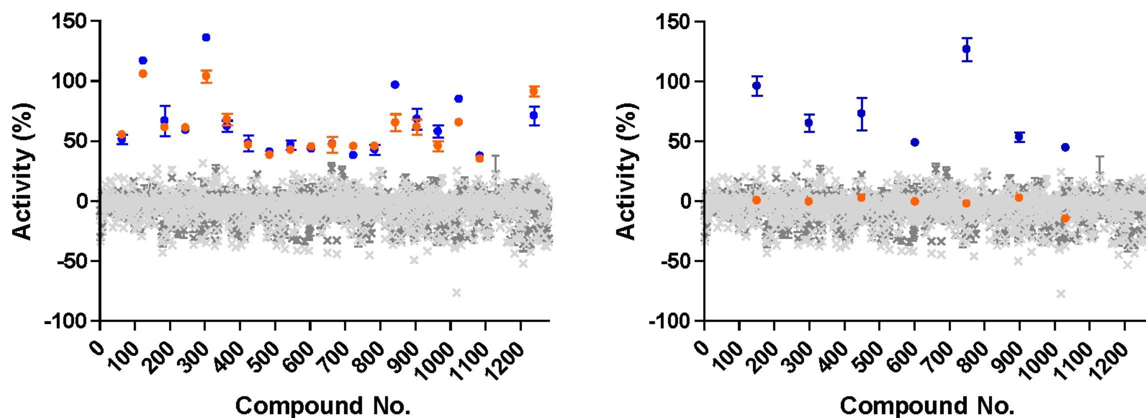
Supplementary Figure 4 | Structures of active compounds from CRE activation coincident reporter qHTS. (**a**) Compounds 1-17 are structurally related to adenosine and may function in the assay as adensine receptor agonists. (**b**) Compound 18 is Arecaidine propargyl ester a muscarinic agonist. Compound 18 was among 6 muscarinic receptor agonists in the LOPAC1280, but the only one active in the assay format used in this study. The other five were with product number(s) following chemical name: Bethanechol (C5259), OXA-22 (C011), Muscarine (M104), Oxotremorine (O100, O9126) and Pilocarpine (P6503, P6628) and were negative in both the FLuc and RLuc outputs. (**c**) Compound 19 is the adenylyl cyclase activator, forskolin (FSK). (**d**) RLuc specific artifacts include two chemical classes identified in this study. Class 1 are substituted aryl sulfonamide (bold substructure) inhibitors (cpds 20-24) of RLuc which belong to the general chemotype of previously identified RLuc inhibitors H-89[7] and BTS[8] and Class 2 a substituted aryl(vinyl) sulfane scaffold (cpds 25 and 26). (**e**) Structure of PTC124 a potent FLuc inhibitor.

Supplementary Figure 5: Enzyme inhibition CRC for RLuc and FLuc from RLuc cell-based activators.



Supplementary Figure 5 | Enzyme inhibition concentration response curves for RLuc and FLuc from RLuc cell-based activators. (**a-g**) CRCs for cpds 20-26 where cpds 20-24 are the class 1 aryl sulfonamide and cpds 25 and 26 are the aryl(vinyl) sulfanes (structures in **Supplementary Fig. 4**). For each set of curves the top plot shows the CRCs from the qHTS for the FLuc (solid orange squares) and RLuc (solid blue circles) reporters. The bottom CRCs are from enzymatic assays using either purified FLuc (open orange squares) or purified RLuc (open blue circles). Note that in each case the cell-based activation response mirrors the enzymatic inhibition on the respective reporter.

Supplementary Figure 6: Cross-section scatter plot analysis from the 57 µM concentration of the 7-pt. qHTS.



Supplementary Figure 6: Cross-section scatter plot analysis from the 57 µM concentration of the 7-pt. qHTS. Highlighted are the agonists having a coincident FLuc (●) and RLuc (●) response (left) and therefore activating reporter gene transcription via CRE-responsive signaling pathways, compared to the RLuc-specific (●) activators; response from FLuc (●), right plot. None of these RLuc-specific activators are annotated to be involved in CRE-dependent signaling. All data are plotted as average of replicate (*n*=2) determinations where error bars represent s.d.

Supplementary Table 1| All qHTS data for replicate measurements on FLuc and RLuc (Excel file)

Notes: Data is arranged as follows: Column A = NCGC sample ID, Column B = SMILE, Column C = common name, Columns D, H, L and P = Curve class (CC) for respective reporters and experimental runs, Columns E, I, M and Q = LogEC50 values as determined from the respective reporter output for a given experimental run, Columns F, J, N and R = EC50 values as determined from the respective reporter output for a given experimental run, and Columns G, L,O and S = Efficacy values as determined from the respective reporter output for a given experimental run.

All adenosine receptor agonists are colored green and muscarinic receptor agonists are colored blue. Forskolin is colored red.

In this assay format a substantial activity required a curve fit that yielded a CC of positive high quality curve classes (i.e., 1.1, 1.2 , 2.1 or 2.2) in either of the reporter outputs.

Supplementary Table 2| Detailed analysis of qHTS actives (**a**) coincident actives (**b**) RLuc-specific actives.

Table S2a| Detailed analysis of qHTS coincident actives

Categroy	cpd #	SID	LOPAC ID	FLuc EC_{50} (μM)	RLuc EC_{50} (μM)	Ratio F/R	Sample Name	Description
1	13	NCGC00025260-05	Lopac-E-2397	0.30	0.54	0.56	5'-N-Ethylcarboxamidoadenosine	adenosine receptor agonist with wqual affinity at A_1 and A_2 receptors
1	5	NCGC00093771-04	Lopac-C-9901	16.94	25.12	0.67	N6-Cyclohexyladenosine	selective A_1 adenosine receptor agonist
1	10	NCGC00024978-05	Lopac-I-146	5.29	7.57	0.70	IB-MECA	A_3 adenosine receptor agonist
1	6	NCGC00023909-06	Lopac-C-8031	0.95	1.26	0.75	N6-Cyclopentyladenosine	selective A_1 adenosine receptor agonist
1	16	NCGC00162286-02	Lopac-N-7505	18.20	22.39	0.81	NADPH tetrasodium	a ubiquitous cofactor and biological reducing agent
1	7	NCGC00162105-02	Lopac-G-5794	2.69	3.16	0.85	GR 79236X	A_1 adenosine receptor agonist
1	2	NCGC00023481-04	Lopac-P-108	12.73	14.62	0.87	N6-Phenyladenosine	A_1 adenosine receptor agonist
1	15	NCGC00162362-02	Lopac-T-5515	2.39	2.51	0.95	Thio-NADP sodium	blocks nicotinate adenine dinucleotide phosphate (NAADP)-induced Ca^{2+} release
1	4	NCGC00025270-03	Lopac-P-101	10.69	11.22	0.95	2-Phenylaminoadenosine	selective A_2 adenosine receptor agonist
1	11	NCGC00021540-06	Lopac-C-5134	0.43	0.38	1.13	2-Chloroadenosine	adenosine receptor agonist with selectivity for A_1 over A_2
1	12	NCGC00162241-04	Lopac-M-5501	16.67	13.27	1.26	N6-Methyladenosine	selective A_1 adenosine receptor agonist
1	8	NCGC00015017-05	Lopac-A-202	1.45	0.93	1.56	N6-2-(4-Aminophenyl)ethyladenosine	non-selective A_3 adenosine receptor agonist
1	14	NCGC00025218-02	Lopac-H-3288	2.69	1.51	1.78	HEMADO	selective A_3 adenosine receptor agonist
1	3	NCGC00015640-04	Lopac-M-225	1.34	0.61	2.20	Metrifudil	adenosine receptor agonist which displays some selectivity for the A_2 receptor type
1	17	NCGC00162130-02	Lopac-C-145	11.50	3.89	2.96	2-Chloroadenosine triphosphate tetrasodium	P2Y purinoceptor agonist
1	1	NCGC00162295-03	Lopac-P-4532	9.37	2.82	3.32	R(-)-N6-(2-Phenylisopropyl)adenosine	A_1 adenosine receptor agonist
1	9	NCGC00162075-03	Lopac-A-236	1.51	0.43	3.51	AB-MECA	A_3 adenosine receptor agonist
2	18	NCGC00015006-04	Lopac-A-140	3.59	3.09	1.16	Arecaidine propargyl ester hydrobromide (APE)	muscarinic acetylcholine receptor agonist exhibiting slight selectivity for M_2 receptor
3	19	NCGC00015445-05	Lopac-F-6886	1.32	1.47	0.90	Forkskolin	adenylyl cyclase activator

Table S2b| Detailed analysis of qHTS RLuc-specific actives

Class	Cpd #	SID	LOPAC ID	FLuc EC_{50} (μM)	RLuc EC_{50} (μM)	Ratio F/R	Sample Name	Description
1	20	NCGC00015885-04	Lopac-R-140	N/A	2.05	N/A	Ro 04-6790 hydrochloride	selective 5-HT_6 serotonin receptor antagonist
1	24	NCGC00015380-12	Lopac-D-9035	N/A	9.15	N/A	Diazoxide	selective AMPA ionotropic glutamate receptor agonist
1	22	NCGC00024555-06	Lopac-A-1980	N/A	15.85	N/A	A3 hydrochloride	selective estrogen receptor modulator
1	21	NCGC00015379-04	Lopac-D-8941	N/A	21.44	N/A	2,6-Difluoro-4-[2-(phenylsulfonylamino) ethylthio]phenoxyacetamide	non-selective casein kinase (CK) inhibitor
1	23	NCGC00015467-16	Lopac-G-0639	N/A	30.35	N/A	Glybenclamide	selective inhibitor of both MEK1 and MEK2
2	25	NCGC00094462-03	Lopac-U-120	N/A	8.49	N/A	U0126	selectively blocks ATP-sensitive K^+ channels
2	26	NCGC00015889-07	Lopac-R-1402	N/A	12.00	N/A	Raloxifene hydrochloride	selective ATP-sensitive K+ channels activator

Notes: Activators identified from LOPAC qHTS. (**a**) Agonists from LOPAC qHTS acting through enhanced gene transcription via the CRE element. (**b**) Apparent agonists from LOPAC qHTS acting through RLuc-dependent stabilization of reporter half-life. Compound annotations were taken from the LOPAC Navigator: http://www.sigmaaldrich.com/chemistry/drug-discovery/validation-libraries/lopac1280-navigator.html. Category headings refer to chemotypes and pharmacology: for **Supplementary Table 2a** Category 1 are purinergic Y2 receptor agonists, Category 2 muscarinic agonist, and Category 3 general AC activator. In **Supplementary Table 2b** Class 1 are substituted aryl sulfonamide inhibitors of RLuc which belong to the general chemotype of previously identified RLuc inhibitors H-89 and BTS (see **SI Figure 4**) and Class 2 an apparent substituted aryl(vinyl) sulfane scaffold. Light grey shading Table 3a indicates FLuc:RLuc EC_{50} ratios between 0.5 and 1.0; medium grey, >1.0 and 2.0; and dark grey >2.0 and 4.0. For **Supplementary Table 2b** light grey shading indicates RLuc EC_{50} values between 1 and 10 μM; medium grey between 10 and 25 μM, and dark grey >25 μM.

Supplementary Table 3| Oligonucleotide sequences used in pCI construction.

Oligo Name	Sequence
KC026	GAAGGGCGGAAAGATCGCCGTGGAATTCTAGAGTCGGGGCGGCCGG
KC027	CCGGCCGCCCCGACTCTAGAATTCCACGGCGATCTTTCCGCCCTTC
KC028	CCCGGCGTCTTGAATTCGGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCTATGACTTCGAA AGTTTATGATCCAGAAC
KC029	CCCGGCGTCTTGAATTCTTATTGTTCATTTTTGAGAACTCGCACAACG
KC030	AGCTTGCTCGAGATCTGCGATCTAAGAGCCTGACGTCAGAGAGCCTGACGTCAGAGAGCCTGACGTCAGAGAGCCTGACGTCAGAGGAATTCA GACACTAGAGGGTATATAATGGAAGCTCGACTTCCAGCTTGGCATTCCGGTACTGTTGGTAAAGA
KC031	AGCTTAACTTTACCAACAGTACCGGAATGCCAAGCTGGAAGTCGAGCTTCCATTATATACCCTCTAGTGTCTGAATTCCTCTGACGTCAGGCTCTC TGACGTCAGGCTCTCTGACGTCAGGCTCTCTGACGTCAGGCTCTTAGATCGCAGATCTCGAGCA

Supplementary Methods

Generation of FLuc-P2A-RLuc constructs All DNA oligonucleotides used and constructs generated are listed and depicted in **Supplementary Table 3**. Here we used the high 'cleavage' efficiency 2A sequence from porcine teschovirus-1 (P2A) to which we also added nucleotides encoding Gly-Ser-Gly to the 5' end of the P2A peptide.[1,2] The pGL3-Control vector (Promega) was used as the backbone to generate the SV40-driven FLuc-P2A-RLuc construct (pCI-6.20). First, oligonucleotides KC026 and KC027 (Integrated DNA Technologies) were used to remove the stop codon and add an EcoRI site by QuickChange II Site-Direct Mutagenesis Kit (Agilent Technologies) to create the construct pCI-6.17. Second, by using pRL-CMV vector (Promega) as the template, Gly-Ser-Gly-P2A-RLuc fragment was generated by PCR using a 5' primer (KC028) with an EcoRI site plus the Gly-Ser-Gly-P2A sequence[1,2] and a 3' primer (KC029) with a EcoRI site identical in reading frame to that found at the start codon of FLuc. The PCR product was then cut by EcoRI-HF (New England Biolabs) and cloned into EcoRI site of pCI-6.17 to make the final pCI-6.20 construct.

To create the 4XCRE-driven FLuc-P2A-RLuc construct (pCI-6.24), we first generated the promoterless FLuc-P2A-RLuc construct (pCI-6.22) using the pGL3-Enhancer vector (Promega) as the backbone. pCI-6.22 made exactly the same way as pCI-6.20 as described above. The oligonucleotides KC030 and KC031 containing 4XCRE plus a minimal promoter sequences and HindIII sites at both ends were annealed and cloned into the HindIII site of pCI-6.22. The resulting construct was termed pCI-6.24.

Cell culture and transfection The GripTite 293 MSR cell line was obtained from Life Technologies. Cells were maintained in DMEM-GlutaMAX (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 units/ml Penicillin and 100 μg/ml Streptomycin (Life Technologies). Transient transfection of plasmids into GripTite 293 MSR cells was performed using

Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions.

Sequential single-well FLuc-RLuc reporter assay and compound test This protocol measures bioluminescence derived from both FLuc and RLuc expression from a single assay (see table below for stepwise protocol. Purified DNA constructs pGL3-Control and pCI-6.20 were co-transfected with p3XFLAG-CMV-7-BAP control plasmid (Sigma) into GripTite 293 MSR cells (Life Technologies). Sixteen hr after transfection, cells were trypsinized and then dispensed at 2,000 cells/20 μL/well in 384-well tissue culture treated white/solid bottom plates (Greiner Bio-One North America). The assay plates were incubated at 37°C for 10 hr before adding the Dual-Glo detection reagent (Promega). Luminescence from luciferase activity was detected by using a ViewLux plate reader (PerkinElmer).

For compound test, forskolin, PTC124 and BTS were prepared in a 24-point intraplate titration format and pre-diluted in the cell culture medium. Purified pCI-6.24 construct was transfected into GripTite 293 MSR cells (Life Technologies). Sixteen hr post transfection, cells were trypsinized and then dispensed at 2,000 cells/15 μL/well in 384-well tissue culture treated white/solid bottom plates (Greiner Bio-One North America). Five μL of pre-diluted compound was transferred into assay plates, resulting in the final concentration ranged from 0.027 nM to 227 μM (forskolin) and 0.011 nM to 91 μM (PTC124 and BTS). The assay plates were incubated at 37°C for 10 hr. FLuc and RLuc activity were then detected using Dual-Glo reagent (Promega) and a ViewLux plate reader (PerkinElmer). Concentration-response curves and concentrations of half-maximal activity (EC_{50}) for each compound were generated by using Prism 4 (GraphPad Software, Inc.).

Sequential single-well FLuc-RLuc reporter assay (384- or 1536-well plate format)			
Step	**Parameter**	**Value**	**Description**
1	Reagent	20 μL *or* 4 μL	~2000 / ~500 cells into white/solid bottom plates
2	Incubation time	1 hr	37°C cell incubator
3	Compounds	5 μL *or* 25 nL	Pipette or Pin tool delivery
4	Incubation time	10 hr	37°C cell incubator
5	Reagent	20 μL *or* 3.5 μL	Dual-Glo luciferase reagent
6	Time	10 min	Cell lysis
7	Assay read 1	550-570 nm	ViewLux plate reader
8	Reagent	20 μL *or* 3.5 μL	Dual-Glo Stop & Glo reagent
9	Time	10 min	
10	Assay read 2	550-570 nm	ViewLux plate reader
Step	*Notes*		
5	See manufacturer's instructions for details: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/0/Dual%20Glo%20Luciferase%20Assay%20System%20Protocol.pdf		

Preparation of whole-cell extracts and Western blot analysis Cells were rinsed with phosphate-buffered saline (PBS [Life Technologies]) and lysed in iced-cold M-PER mammalian protein extraction reagent (Thermo Scientific) supplemented with complete Mini protease inhibitor cocktail tablet (Roche) 24 hr post-transfection. Each lysate was subject to SDS-polyacrylamide gradient gel (4-12% NuPAGE, Life Technologies) electrophoresis and transferred to PVDF membrane (Life Technologies). For Western blot analysis, primary antibodies used were goat polyclonal anti-FLuc (1:1000, Promega), mouse monoclonal 5B11.2 anti-RLuc (1:1000, Millipore), rabbit polyclonal anti-2A peptide (1:1000, Millipore), mouse monoclonal anti-α-actin (1:1000, Sigma), and HRP-conjugated mouse monoclonal M2 anti-FLAG (1:4000, Sigma). Secondary

antibodies were goat anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnology), donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology), and goat anti-rabbit IgG-HRP (1:2000, Santa Cruz Biotechnology). The bound antibodies were detected using Novex ECL chemiluminescent substrate reagent kit (Life Technologies) and visualized by ChemiDoc XRS+ System (Bio-Rad).

LOPAC1280 qHTS screening The coincident biocircuit encoding FLuc and RLuc driven by a CRE array was used to identify compounds capable of eliciting an agonistic response in a HEK293 cell line derivative using quantitative HTS (qHTS). qHTS measures the pharmacological activity of each library compound by determining concentration response profiles of all library members[3]. This was accomplished here as follows, purified DNA construct pCI-6.24 was transiently transfected into GripTite 293 MSR cells (Life Technologies). Sixteen hrs after transfection, cells were trypsinized and then dispensed at 500 cells/4 μL/well in 1,536-well tissue culture treated white/solid bottom plates (Greiner Bio-One North America) using a multidrop combi dispenser (Thermo Fisher Scientific). Compounds from Library of Pharmacological Active Compounds (LOPAC), obtained from Sigma, were prepared as interplate titrations of seven dilutions.[4] Twenty three nL of compound from LOPAC was pin-transferred into the assay plates by a pin tool array (V&P Scientific)[5] manipulated by an automated pin transfer station (Kalypsys)[6]. This resulted in a 174-fold dilution and the final compound concentration in the 4 μL assay ranged from ~4 nM to 57 μM. The assay plates were incubated at 37°C for 10 hr before adding the Dual-Glo detection reagent (3.5 μL + 3.5 μL for each well) (Promega). Luminescence from luciferase activity was detected by using ViewLux (PerkinElmer). Note that each experimental plate contained forskolin as a positive control and DMSO as a negative control. Percentage activity was defined as the percentage signal relative to forskolin (100%) and DMSO (0%). The assay performed well with signal-to-background ratios (S/B) of 3.37 for FLuc and 4.30 for RLuc , with additional parameters as follows:

Readout	Assay Format	Z' factor	S:B ratio	CV	Intraplate Forskolin Control (μM)	
					Mean	s.d.
FLuc	**1536 interplate**	0.40	3.37	23.87	0.86	0.36
RLuc		0.45	4.30	19.66	0.88	0.43

FLuc and RLuc enzymatic assays To determine compound potency against purified luciferase enzymes, 3 μL of luciferase substrate was dispensed to each well of 1536-well white/solid bottom plates (Greiner Bio-One North America) using the BioRaptor FRD (Beckman Coulter), for a final concentration of 5 μM coelenterazine-H (Promega) or 10 μM D-luciferin (Sigma) and 10 μM ATP. Twenty-three nL of compounds were transferred using a 1536-pin tool (Wako) into assay wells, resulting in final concentrations ranged from ~3 nM to 57 μM with 11 titration points. One μL of purified luciferase was the dispensed into each well for final concentrations 10 nM *P. pyralis* (FLuc) or 1 nM *Renilla* luciferase (RLuc). The bioluminescence outputs were measured by an Envision reader (PerkinElmer).

References

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