

Supplemental Figures

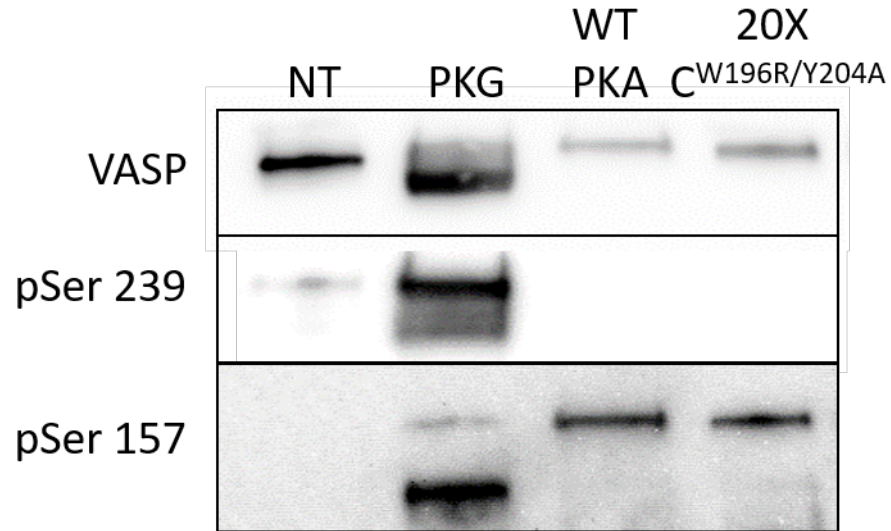


Figure S1. C^{W196R/Y204A} retains substrate specificity as revealed by selective phosphorylation of VASP S157. Related to Figure 4. VASP (80 ng) was incubated with or without 500 ng PKG or PKA in buffer (50 mM Tris + 100 NaCl + 10 mM MgCl₂ + 1 mM DTT + 1 mM EDTA + 1 mM ATP) with a final volume of 100 μ L at room temperature for 30 min. A 20-fold higher concentration of the C^{W196R/Y204A} was employed due to its' reduced activity relative to the wild type catalytic subunit of PKA. The double mutant retains the stringent VASP specificity displayed by PKA by only phosphorylating VASP at S157. (Note: only the region of the Western blot containing the bands of interest is shown. The rest of the blot has been cropped for clarity).

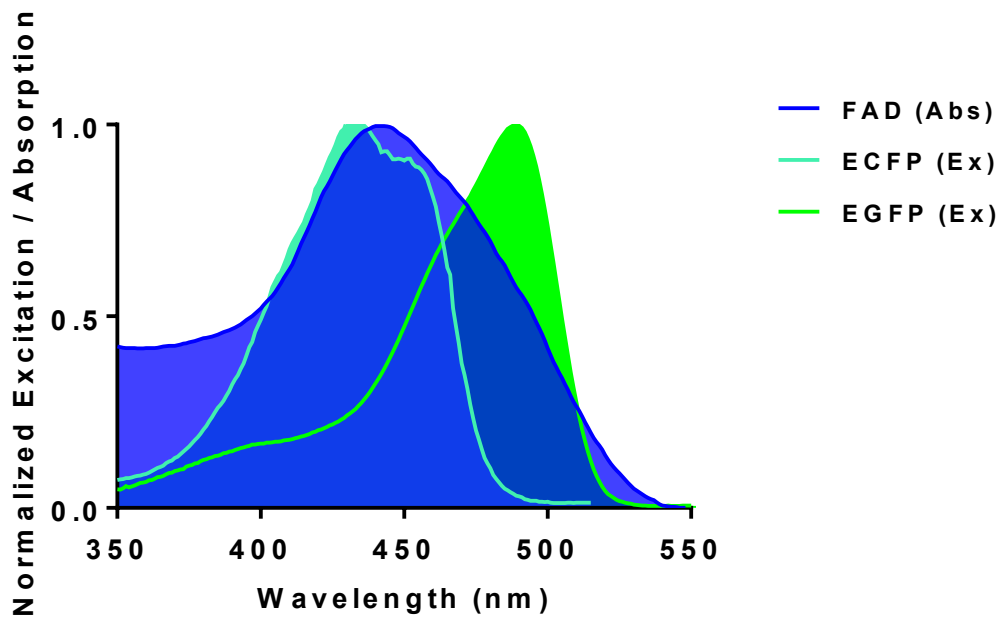


Figure S2. Related to Figure 3. Normalized excitation and absorption spectra for FAD (dark blue), Cry2's chromophore, ECFP (light blue), and EGFP (green). These spectra illustrate the spectral overlap between the three molecules. Spectral data was obtained from the University of Arizona Spectral Database at www.spectra.arizona.edu.

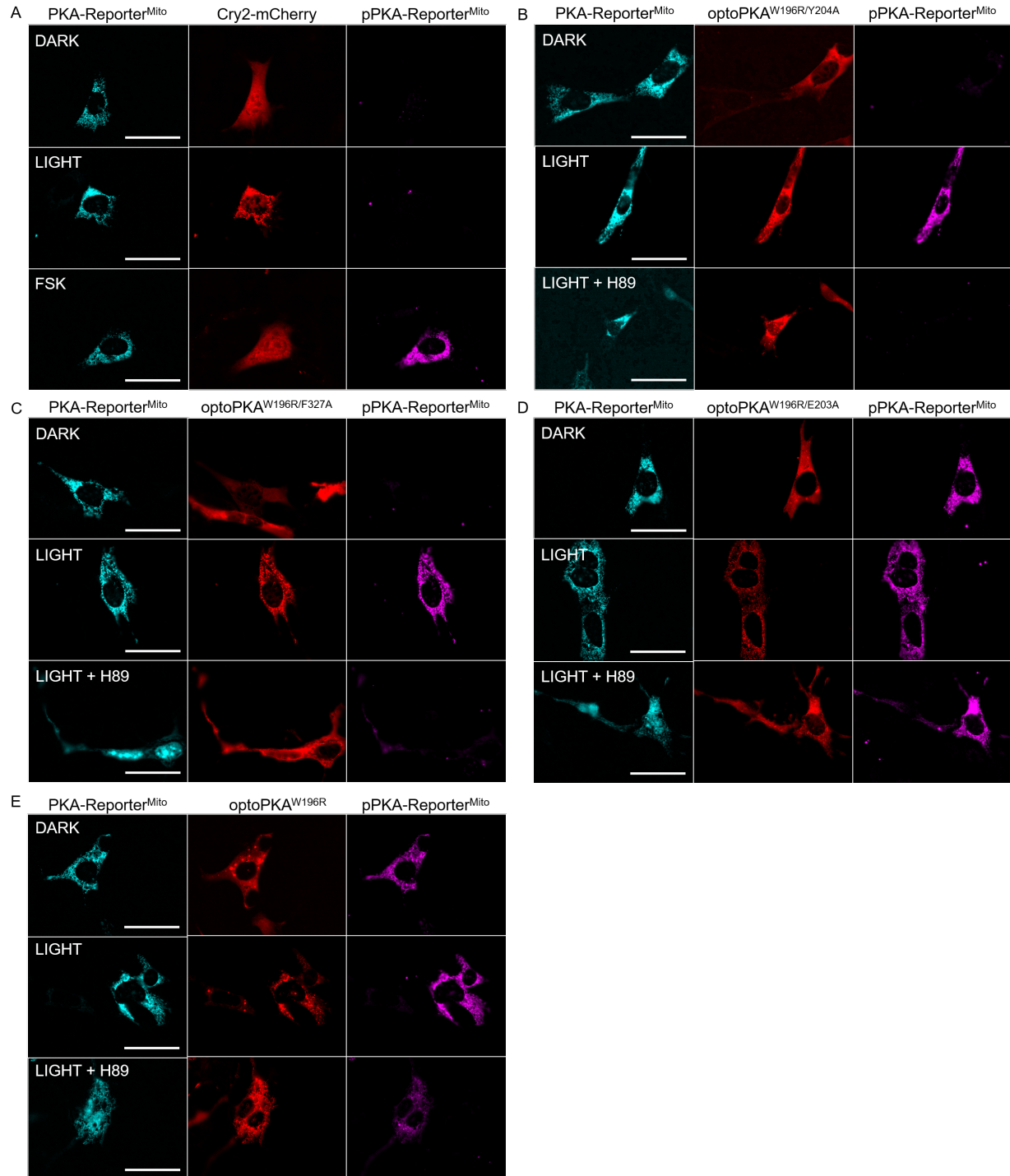


Figure S3. Related to Figure 4. Representative images of 1 min light activation of (A) Cry2-mCh, (B) optoPKA^{W196R/Y204A}, (C) optoPKA^{W196R/F327A}, (D) optoPKA^{W196R/E203A}, and (E) optoPKA^{W196R}. In the dark (top row), optoPKA is diffuse in the cytoplasm of cells and there is no detectable reporter phosphorylation. Upon stimulation with 1 min 470 nm LED light (middle row), the Cry2-mCh construct is recruited to the OMM. As expected, PKA-Reporter phosphorylation does not occur. By contrast, forskolin (FSK) does result in PKA-Reporter phosphorylation (bottom row). Scale bar = 50 μ m.

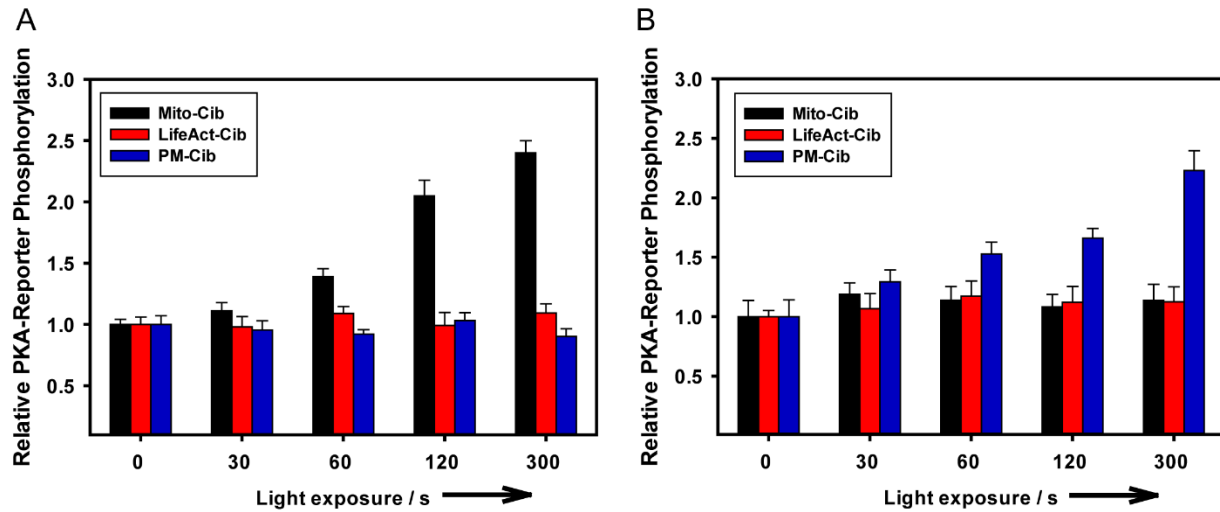


Figure S4. Light titration of PKA-Reporter phosphorylation as a function of the intracellular locations of the optoPKA constructs and PKA-Reporters. Related to Figure 6. (A) The PKA-Reporter positioned at the OMM is only phosphorylated by optoPKA^{W196R/F327A} recruited to the OMM. Recruitment of optoPKA^{W196R/F327A} to the PM or the cytoskeleton (LifeAct) fails to induce PKA-Reporter phosphorylation. (B) The PKA-Reporter positioned at the PM is only phosphorylated by optoPKA^{W196R/F327A} recruited to the PM. Recruitment of optoPKA^{W196R/F327A} to the OMM or the cytoskeleton (LifeAct) fails to induce PKA-Reporter phosphorylation. Data reported as mean +/- SEM n = 10 – 20 cells per group.

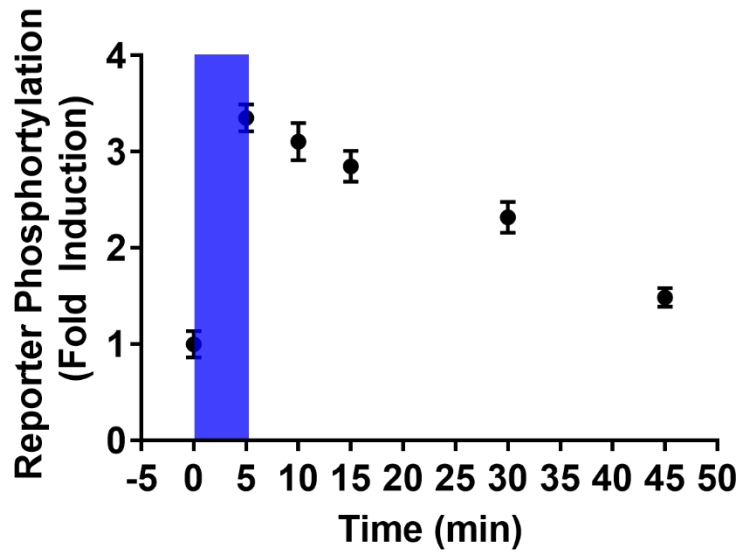


Figure S5. *optoPKA^{W196R/Y204A}* activation is reversible. Related to Figure 6. The PKA-Reporter at the OMM is maximally phosphorylated after 5 min stimulation with blue light (blue box) and decays nearly to baseline over 45 min.

Supplementary Table S1. The *in vitro* activities of the wild type (WT) and W196R mutant catalytic (C) subunits in the presence of regulatory (R) subunit, with and without cAMP. (S. E. Builder, J. A. Beavo, E. G. Krebs, *J. Biol. Chem.* 1980, 255, 3514-9) Related to Figure 1. Addition of cAMP to the WT C (10 nM) + R (50 nM) subunits enhances catalytic activity, thereby exhibiting the cAMP-dependence characteristic of PKA. Note that, even in the absence of added cAMP, WT C + R subunits display some catalytic activity. This is due to the fact that purified R subunit contains some bound cAMP (only treatment of the R subunit with high concentrations of urea is able to completely remove bound cAMP). By contrast, the corresponding experiment using the W196R mutated C subunit, reveals cAMP-independence (i.e. the same activity in the absence and presence of cAMP). Although the same concentration of WT and mutant C subunits was used in all experiments, bacterial expression of these two species could result in different amounts of properly folded protein. Consequently, it may not be appropriate to compare the relative activities of the mutant and WT C subunits.

C subunit	Activity (s⁻¹) +R subunit	Activity (s⁻¹) +R subunit/+cAMP
WT	5.0 ± 0.7	15.0 ± 1.4
W196R	9.4 ± 1.1	9.9 ± 0.5

Supplementary Table S2. Related to Figure 1. The *in vitro* activities of the wild type (WT) and double mutant catalytic (C) subunits.

C subunit	Activity (s⁻¹)	% Activity
WT	17 ± 3	100 ± 16
W196R + E203A	7.4 ± 1.0	43 ± 6
W196R + Y204A	0.95 ± 0.20	5.6 ± 1.3
W196R + F327A	0.21 ± 0.02	1.2 ± 0.1

Supplemental Table S3. Related to Figure 2. Association times for optoPKAs to achieve maximal subcellular localization (OMM, cytoskeleton, and PM) and $t_{1/2}$ dissociation times. Data expressed as mean +/- SEM. N = 3 cells per group.

optoConstruct + OMM-Cib	Time to Max Association ± SEM (s)	Dissociation $t_{1/2}$ ± SEM (s)
mCh	190 ± 35	420 ± 49
W196R	210 ± 27	470 ± 28
W196R/E203A	140 ± 22	390 ± 50
W196R/Y204A	100 ± 9	380 ± 28
W196R/F327A	167 ± 15	397 ± 54

optoConstruct +Cytoskeleton-Cib	Time to Max Association ± SEM (s)	Dissociation $t_{1/2}$ ± SEM (s)
mCh	143 ± 22	420 ± 17
W196R/Y204A	145 ± 30	450 ± 47
W196R/F327A	177 ± 20	470 ± 33

optoConstruct + PM-Cib	Time to Max Association ± SEM (s)	Dissociation $t_{1/2}$ ± SEM (s)
mCh	90 ± 9	270 ± 18
W196R/Y204A	140 ± 44	310 ± 56
W196R/F327A	140 ± 30	375 ± 20

Table S5. Related to Figure 7. Examples of known PKA substrates/phosphorylation sites that are phosphorylated upon optoPKA recruitment to the OMM or PM.

Protein	Protein name	AA	L/D Ratio	Sequence window
PM Recruitment				
P35222	β -catenin	S ⁶⁷⁵	1.4	FRMS \underline{S} EDKPQDYKKRLSVELTSSLFRTEPMAW
P35222	β -catenin	S ⁵⁵²	1.5	VQLLVRAHQDTQRRTS \underline{M} GGTQQQFVEGVRME
P15056	BRAF	S ⁴⁴⁷	17.6	SEDRNRMKTLGRRDS \underline{S} DDWEIPDGQITVGQR
P06241	FYN	S ²¹	9.2	CKDKEATKLTEERDGS \underline{L} NQSSGYRYGTDPTP
OMM Recruitment				
Q92934	BAD	S ¹¹⁸	2.9	NLWAAQRYGRELRRMS \underline{D} EFVDSFKKGLPRPK
P04049	RAF1	S ²⁵⁹	2.9	SPSSEGSLSQRQRST \underline{S} TPNVHMOVSTTLPVDS
P49840	GSK3A	S ²¹	2.5	PSGGGPGGSGRARTS \underline{S} FAEPGGGGGGGGGGP
Q00536	CDK16	S ¹²	1.4	MDRMKKIKRQLS \underline{M} TLRGGRGIDKTNGA

Table S6. Related to Figure 7. Examples of proteins containing the canonical PKA consensus sequence that is phosphorylated upon optoPKA recruitment to the PM and OMM, where proteins highlighted in green are phosphorylated at R-R-X-S-X sequence and proteins highlighted in yellow are phosphorylated at a R-X-X-S-X sequence.

Protein	Protein Name	AA	L/D Ratio
PM Recruitment			
O75152	Zinc finger CCCH domain-containing protein 11A	S ⁷⁵⁸	46.1
Q5VTL8	Pre-mRNA-splicing factor 38B	S ⁵²⁷	25.5
Q9BXB4	Oxysterol-binding protein-related protein 11	S ¹⁸⁹	24.4
Q96D71	RalBP1-associated Eps domain-containing protein 1	S ²⁷²	11.1
P43243	Matrin-3	S ¹⁸⁸	3.7
Q9NYF3	Protein FAM53C	S ²³²	3.2
P42356	Phosphatidylinositol 4-kinase alpha	S ²³⁰	2.2
Q6P6C2	RNA demethylase ALKBH5	S ³⁶¹	1.8
Q9H2G2	STE20-like serine/threonine-protein kinase	S ¹⁸⁹	1.8
Q96Q42	Alsin	S ⁴⁸³	1.6
Q12756	Kinesin-like protein KIF1A	S ¹³⁷⁰	36.4
Q5VTL8	Pre-mRNA-splicing factor 38B	S ⁵²⁹	31.1
P46937	Transcriptional coactivator YAP1	S ¹⁰⁹	21.1
O75592	E3 ubiquitin-protein ligase MYCBP2	S ³⁴⁶⁷	14.1
Q9HB09	Bcl-2-like protein 12	S ²⁴²	10.5
Q4G0J3	La-related protein 7	S ²⁵⁸	8.8
Q86YR5	G-protein-signaling modulator 1	S ⁴⁹²	8.3
Q15596	Nuclear receptor coactivator 2	S ⁷⁷¹	5.8
Q86W92	Liprin-beta-1	S ⁶⁰¹	5.6
O43379	WD repeat-containing protein 62	S ³²	3.2
Q13098	COP9 signalosome complex subunit 1	S ⁴⁷⁴	2.8
Q96D71	RalBP1-associated Eps domain-containing protein 1	S ⁷⁰⁹	2.8
P43243	Matrin-3	S ⁵⁹⁸	1.9
Q9UDY2	Tight junction protein ZO-2	S ²⁴⁴	1.8
OMM Recruitment			
Q6ZNB6	NF-X1-type zinc finger protein NFXL1	S ⁸³⁵	136.1
O75152	Zinc finger CCCH domain-containing protein 11A	S ²⁹⁰	47.0
O15027	Protein transport protein Sec16A	S ¹¹⁹¹	15.8
Q13428	Treacle protein	S ¹³⁵⁰	13.6
Q6GYQ0	Ral GTPase-activating protein subunit alpha-1	S ⁸⁶⁰	6.6
Q8IW50	Protein FAM219A	S ⁶⁴	6.2
Q9UNF1	Melanoma-associated antigen D2	S ²¹⁸	5.7
Q03001	Dystonin	S ⁷⁵¹⁰	5.2

Q00537	Cyclin-dependent kinase 17	S ⁹	3.5
O60293	Zinc finger C3H1 domain-containing protein	S ³⁵²	3.1
Q5VZ89	DENN domain-containing protein 4C	S ¹⁰⁴²	2.2
Q9UKV3	Apoptotic chromatin condensation inducer in the nucleus	S ⁸²⁵	1.8
Q7Z333	Probable helicase senataxin	S ¹³⁶⁶	1.8
Q01813	ATP-dependent 6-phosphofructokinase, platelet type	S ³⁸⁶	21.0
P11171	Protein 4.1	S ⁸⁴	8.3
P42684	Abelson tyrosine-protein kinase 2	S ⁶⁷¹	7.3
Q02543	60S ribosomal protein L18a	S ¹²³	7.0
Q7Z2K8	G protein-regulated inducer of neurite outgrowth 1	S ⁷⁵	4.8
Q86VR2	Protein FAM134C	S ²⁶	4.7
Q15884	Protein FAM189A2	S ²⁷⁵	3.5
P11166	Solute carrier family 2, facilitated glucose transporter member 1	S ²²⁶	3.5
Q5JSZ5	Protein PRRC2B	S ¹⁴²²	3.3
O60343	TBC1 domain family member 4	S ⁵⁸⁸	3.0
Q8NHM5	Lysine-specific demethylase 2B	S ⁹⁵¹	2.9
O75044	SLIT-ROBO Rho GTPase-activating protein 2	S ⁹⁰⁹	2.8
Q96AV8	Transcription factor E2F7	S ⁴¹⁰	2.5
O43524	Forkhead box protein O3	S ²⁵³	2.3
Q9Y485	DmX-like protein 1	S ⁵⁷⁴	2.2
Q9UHR4	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1	S ³³¹	2.0
P55196	Afadin	S ²¹⁶	1.9
Q96N67	Dedicator of cytokinesis protein 7	S ¹³⁸³	1.9
P15924	NF-X1-type zinc finger protein NFXL1	S ²²	1.8
Q9HAU0	Zinc finger CCCH domain-containing protein 11A	S ⁴¹⁰	1.7
Q9BXF6	Protein transport protein Sec16A	S ³⁰⁷	1.6
Q9H4Z3	Treacle protein	S ¹¹⁶	1.5
Q86X29	Ral GTPase-activating protein subunit alpha-1	S ⁴³²	1.5

Methods S1: DNA sequences of all constructs generated for this study.

pMCSG11-C subunit:

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optoPKA^{W196R}:

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OMM-Reporter

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PM-Reporter

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