Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary Information

Development of green fluorescent protein-based cAMP indicators for covering a wide range of cAMP concentrations

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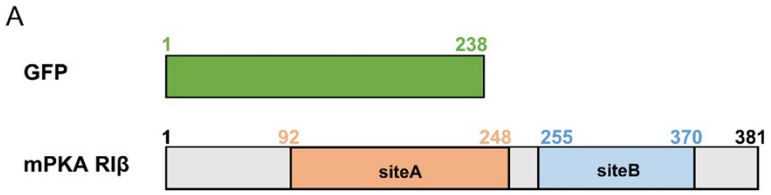
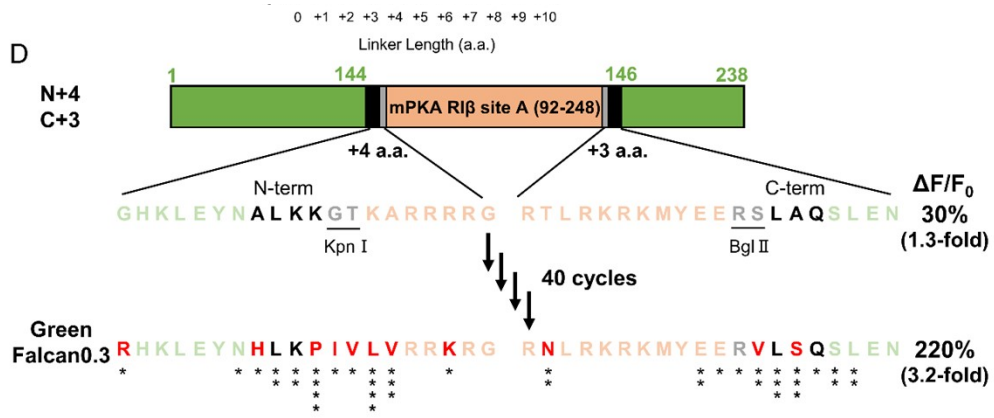


Table S1. Comparison of Single-FP based fluorescent cAMP indicator

Sensor name	Type	Ex/Em (nm)	Dynamic range (fold)	EC_{50} (μM)	Reference
Flamindo	Turn-off	506/521	2.0	2.1	11
Flamindo2	Turn-off	504/523	4.0	3.2	12
Pink Flamindo	Turn-on	567/590	4.2	7.2	13
cADDis	Turn-off	488/525*	1.4	NA	15
	Turn-on	488/525*	1.3	NA	
cAMPr	Turn-on	488/509	1.7	NA	16
gCarvi	Turn-on	504/523	2.0	1.5	17
G-Flamp1	Turn-on	490/510	13	2.2	18

* Wavelength used in the measurement

NA: Not applicable

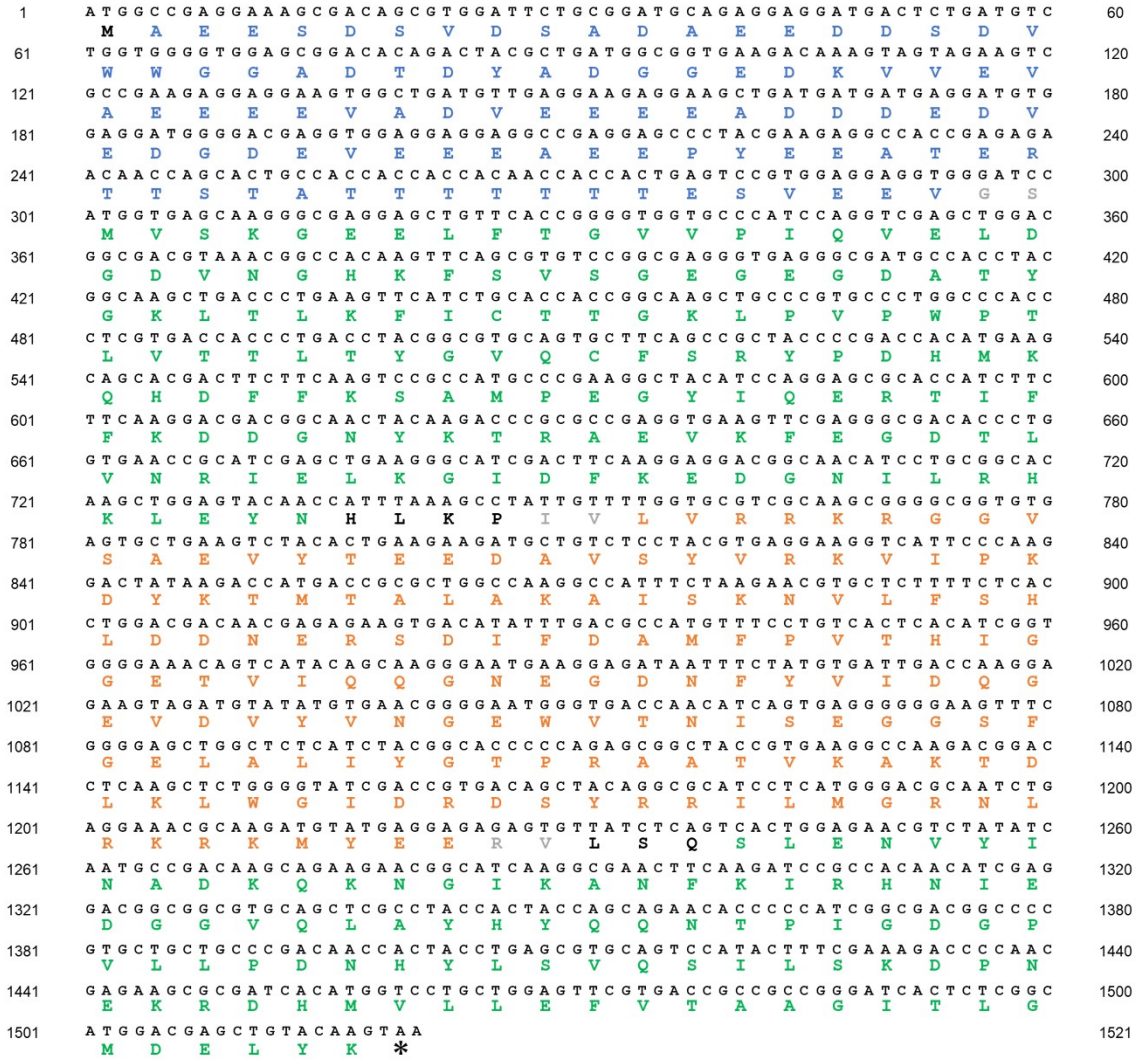


Supplementary Figures

Figure S1. Screening by the crude Green Falcan protein (A-C) Diagram for (A) GFP, mouse cAMP-dependent protein kinase (PKA) type I-beta regulatory subunit (mPKA RIβ), and the indicators under development, and the screening results of various constructs with modified linker lengths at (B) the C-terminus and (C) the N-terminus. Responses to 100 μM cAMP are shown as $\Delta F/F_0$. (D) The amino acid

sequences around the linker and introduced point mutations. Asterisks indicate where random mutations were performed, and red letters indicate mutated amino acids.

A **Green Falcan0.3**



B **Green Falcan1**

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1     ATGGCCGAGGAAAGCGACAGCGTGGATTCTGCGGATGCAGAGGAGGATGACTCTGATGTC   60
        M   A   E   E   S   D   S   V   D   S   A   D   A   E   E   D   D   S   D   V
61    TGGTGGGGTGGAGCGGACACAGACTACGCTGATGGCGGTGAAGACAAAGTAGTAGAAGTC  120
        W   W   G   G   A   D   T   D   Y   A   D   G   G   E   D   K   V   V   E   V
121   GCCGAAGAGGAGGAAGTGGCTGATGTTGAGGAAGAGGAAGCTGATGATGATGAGGATGTG  180
        A   E   E   E   E   V   A   D   V   E   E   E   E   A   D   D   D   E   D   V
181   GAGGATGGGGACGAGGTGGAGGAGGAGGCCGAGGAGCCCTACGAAGAGGCCACCGAGAGA  240
        E   D   G   D   E   V   E   E   E   A   E   E   P   Y   E   E   A   T   E   R
241   ACAACCAGCACTGCCACCACCACCACAACCACCACTGAGTCCGTGGAGGAGGTGGGATCC  300
        T   T   S   T   A   T   T   T   T   T   T   E   S   V   E   E   V   G   S
301   ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCAGGTCGAGCTGGAC  360
        M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   Q   V   E   L   D
361   GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGTGAGGGCGATGCCACCTAC  420
        G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D   A   T   Y
421   GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC  480
        G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P   W   P   T
481   CTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG  540
        L   V   T   T   L   T   Y   G   V   Q   C   F   S   R   Y   P   D   H   M   K
541   CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACATCCAGGAGCGCACCATCTTC  600
        Q   H   D   F   F   K   S   A   M   P   E   G   Y   I   Q   E   R   T   I   F
601   TTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTG  660
        F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G   D   T   L
661   GTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGCGGCAC  720
        V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I   L   R   H
721   AAGCTGGAGTACAACCATTTAAAGCCTATTGTTTTGGTGCGTCGCAAGCGGGGCGGTGTG  780
        K   L   E   Y   N   H   L   K   P   I   V   L   V   R   R   K   R   G   G   V
781   AGTGCTGAAGTCTACACTGAAGAAGATGCTGTCTCCTACGTGAGGAAGGTCATTCCCAAG  840
        S   A   E   V   Y   T   E   E   D   A   V   S   Y   V   R   K   V   I   P   K
841   GACTATAAGACCATGACCGCGCTGGCCAAGGCCATTTCTAAGAACGTGCTCTTTTCTCAC  900
        D   Y   K   T   M   T   A   L   A   K   A   I   S   K   N   V   L   F   S   H
901   CTGGACGACAACGAGAGAAGTGACATATTTGACGCCATGTTTCCTGTCACTCACATCGGT  960
        L   D   D   N   E   R   S   D   I   F   D   A   M   F   P   V   T   H   I   G
961   GGGGAAACAGTCATACAGCAAGGGAATGAAGGAGATAATTTCTATGTGATTGACCAAGGA 1020
        G   E   T   V   I   Q   Q   G   N   E   G   D   N   F   Y   V   I   D   Q   G
1021  GAAGTAGATGTATATGTGAACGGGGAATGGGTGACCAACATCAGTGAGGGGGGAAGTTTC 1080
        E   V   D   V   Y   V   N   G   E   W   V   T   N   I   S   E   G   G   S   F
1081  GGGGAGCTGGCTCTCATCTACGGCACCCCCAGACATGCTACCGTGAAGGCCAAGACGGAC 1140
        G   E   L   A   L   I   Y   G   T   P   R   H   A   T   V   K   A   K   T   D
1141  CTCAAGCTCTGGGGTATCGACCGTGACAGCTACAGGCGCATCCTCATGGGACGCAATCTG 1200
        L   K   L   W   G   I   D   R   D   S   Y   R   R   I   L   M   G   R   N   L
1201  AGGAAACGCAAGATGTATGAGGAGAGAGTGTTATCTCAGTCACTGGAGAACGTCTATATC 1260
        R   K   R   K   M   Y   E   E   R   V   L   S   Q   S   L   E   N   V   Y   I
1261  AATGCCGACAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACATCGAG 1320
        N   A   D   K   Q   K   N   G   I   K   A   N   F   K   I   R   H   N   I   E
1321  GACGGCGGCGTGCAGCTCGCCTACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCC 1380
        D   G   G   V   Q   L   A   Y   H   Y   Q   Q   N   T   P   I   G   D   G   P
1381  GTGCTGCTGCCCGACAACCACTACCTGAGCGTGCAGTCCATACTTTCGAAAGACCCCAAC 1440
        V   L   L   P   D   N   H   Y   L   S   V   Q   S   I   L   S   K   D   P   N
1441  GAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGC 1500
        E   K   R   D   H   M   V   L   L   E   F   V   T   A   A   G   I   T   L   G
1501  ATGGACGAGCTGTACAAGTAA                                          1521
        M   D   E   L   Y   K   *
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C **Green Falcan3**

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1     ATGGCCGAGGAAAGCGACAGCGTGGATTCTGCGGATGCAGAGGAGGATGACTCTGATGTC   60
        M   A   E   E   S   D   S   V   D   S   A   D   A   E   E   D   D   S   D   V
61    TGGTGGGGTGGAGCGGACACAGACTACGCTGATGGCGGTGAAGACAAAGTAGTAGAAGTC  120
        W   W   G   G   A   D   T   D   Y   A   D   G   G   E   D   K   V   V   E   V
121   GCCGAAGAGGAGGAAGTGGCTGATGTTGAGGAAGAGGAAGCTGATGATGATGAGGATGTG  180
        A   E   E   E   E   V   A   D   V   E   E   E   E   A   D   D   D   E   D   V
181   GAGGATGGGGACGAGGTGGAGGAGGAGGCCGAGGAGCCCTACGAAGAGGCCACCGAGAGA  240
        E   D   G   D   E   V   E   E   E   A   E   E   P   Y   E   E   A   T   E   R
241   ACAACCAGCACTGCCACCACCACCACAACCACCACTGAGTCCGTGGAGGAGGTGGGATCC  300
        T   T   S   T   A   T   T   T   T   T   T   E   S   V   E   E   V   G   S
301   ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCAGGTCGAGCTGGAC  360
        M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   Q   V   E   L   D
361   GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGTGAGGGCGATGCCACCTAC  420
        G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D   A   T   Y
421   GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC  480
        G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P   W   P   T
481   CTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG  540
        L   V   T   T   L   T   Y   G   V   Q   C   F   S   R   Y   P   D   H   M   K
541   CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACATCCAGGAGCGCACCATCTTC  600
        Q   H   D   F   F   K   S   A   M   P   E   G   Y   I   Q   E   R   T   I   F
601   TTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTG  660
        F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G   D   T   L
661   GTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGCGGCAC  720
        V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I   L   R   H
721   AAGCTGGAGTACAACCATTTAAAGCCTATTGTTTTGGTGCGTCGCAAGCGGGGCGGTGTG  780
        K   L   E   Y   N   H   L   K   P   I   V   L   V   R   R   K   R   G   G   V
781   AGTGCTGAAGTCTACACTGAAGAAGATGCTGTCTCCTACGTGAGGAAGGTCATTCCCAAG  840
        S   A   E   V   Y   T   E   E   D   A   V   S   Y   V   R   K   V   I   P   K
841   GACTATAAGACCATGACCGCGCTGGCCAAGGCCATTTCTAAGAACGTGCTCTTTTCTCAC  900
        D   Y   K   T   M   T   A   L   A   K   A   I   S   K   N   V   L   F   S   H
901   CTGGACGACAACGAGAGAAGTGACATATTTGACGCCATGTTTCCTGTCACTCACATCGGT  960
        L   D   D   N   E   R   S   D   I   F   D   A   M   F   P   V   T   H   I   G
961   GGGGAAACAGTCATACAGCAAGGGAATGAAGGAGATAATTTCTATGTGATTGACCAAGGA 1020
        G   E   T   V   I   Q   Q   G   N   E   G   D   N   F   Y   V   I   D   Q   G
1021  GAAGTAGATGTATATGTGAACGGGGAATGGGTGACCAACATCAGTGAGGGGGGAAGTTTC 1080
        E   V   D   V   Y   V   N   G   E   W   V   T   N   I   S   E   G   G   S   F
1081  GGGGAGCTGGCTCTCATCTACGGCACCCCCAGACATGGTTCCGTGAAGGCCAAGACGGAC 1140
        G   E   L   A   L   I   Y   G   T   P   R   H   G   S   V   K   A   K   T   D
1141  CTCAAGCTCTGGGGTATCGACCGTGACAGCTACAGGCGCATCCTCATGGGACGCAATCTG 1200
        L   K   L   W   G   I   D   R   D   S   Y   R   R   I   L   M   G   R   N   L
1201  AGGAAACGCAAGATGTATGAGGAGAGAGTGTTATCTCAGTCACTGGAGAACGTCTATATC 1260
        R   K   R   K   M   Y   E   E   R   V   L   S   Q   S   L   E   N   V   Y   I
1261  AATGCCGACAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACATCGAG 1320
        N   A   D   K   Q   K   N   G   I   K   A   N   F   K   I   R   H   N   I   E
1321  GACGGCGGCGTGCAGCTCGCCTACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCC 1380
        D   G   G   V   Q   L   A   Y   H   Y   Q   Q   N   T   P   I   G   D   G   P
1381  GTGCTGCTGCCCGACAACCACTACCTGAGCGTGCAGTCCATACTTTCGAAAGACCCCAAC 1440
        V   L   L   P   D   N   H   Y   L   S   V   Q   S   I   L   S   K   D   P   N
1441  GAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGC 1500
        E   K   R   D   H   M   V   L   L   E   F   V   T   A   A   G   I   T   L   G
1501  ATGGACGAGCTGTACAAGTAA                                          1521
        M   D   E   L   Y   K   *
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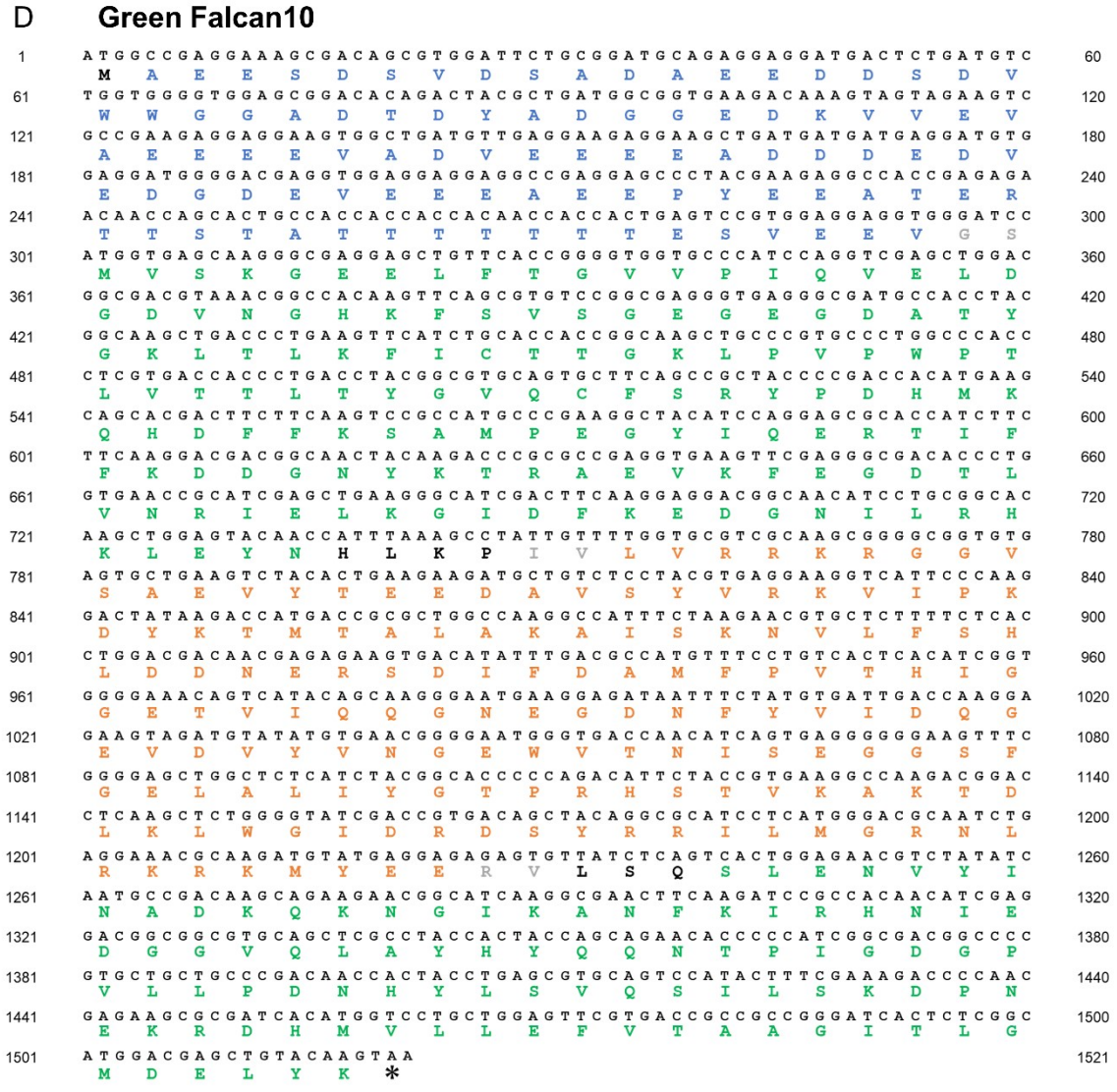

Figure S2. DNA and amino acid sequences of Green Falcans (A-D) DNA and amino acid sequences of Green Falcan0.3 (A), 1 (B), 3 (C), 10 (D). Blue, green, orange, black and gray letters correspond to amino acid from APP, GFP, PKA, linker and restriction enzyme site, respectively. Asterisks showed stop codon.

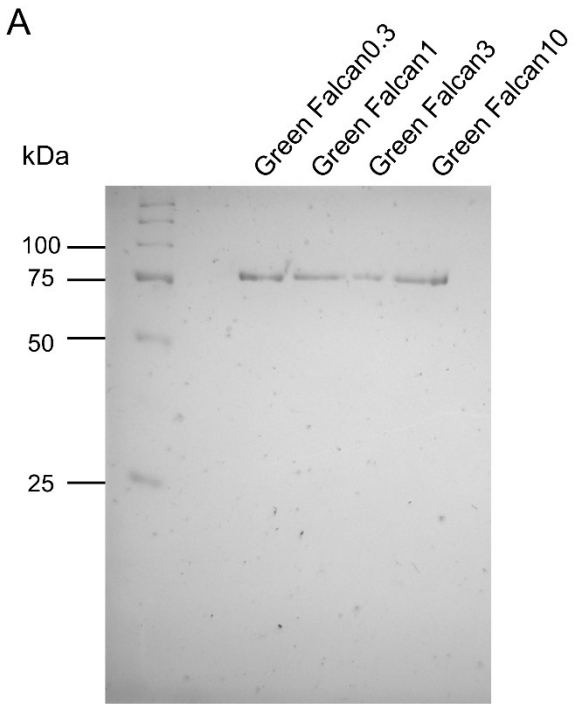

Figure S3. SDS-PAGE for purified Green Falcans protein (A) The image of SDS-PAGE for purified Green Falcans protein (1 μg).

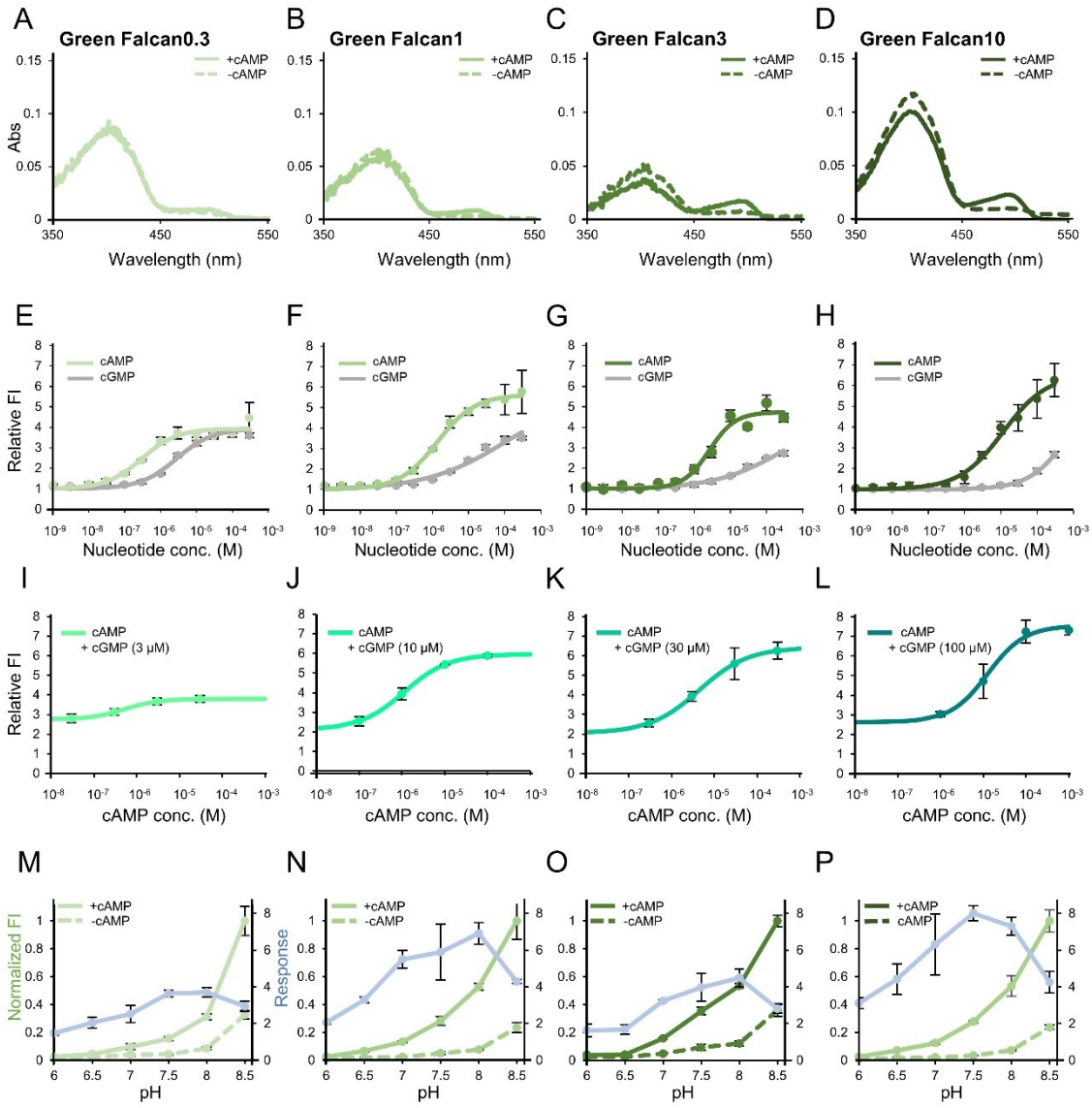

Figure S4. Property evaluation of Green Falcans (A-D) Absorption spectra of purified Green Falcan0.3, 1, 3, and 10 in the presence (solid line) and absence (dashed line) of 100 μM cAMP. (E-H) Dose-response curves of purified Green Falcan0.3, 1, 3, and10 for cAMP (green line) and cGMP (gray line). The FI was measured with excitation at 480 nm. The normalized FI was calculated by dividing by the FI of the peak in the absence of cAMP, and the minimum FI in fitting was normalized to 1. The data represent the means ± standard deviation (n=3). (I-L) Dose-response curves of purified Green Falcan0.3, 1, 3, and10 for cAMP in the presence of cGMP (10-times of the EC_{50} value for cAMP). The FI was measured with excitation at 480 nm. The Relative FI was calculated by dividing by the FI of the peak before the addition of cAMP.

The data represent the means ± standard deviation (n=3). (M-P) pH sensitivity of purified Green Falcan0.3, 1, 3, 10. The FI was measured with excitation at 480 nm. The normalized FI was calculated by dividing the FI in all conditions by the FI of each Green Falcan after the addition of cAMP in pH 8.5. Response was calculated by dividing the FI with cAMP by the FI without cAMP for each pH condition. The data represent the means ± standard deviation (n=3).

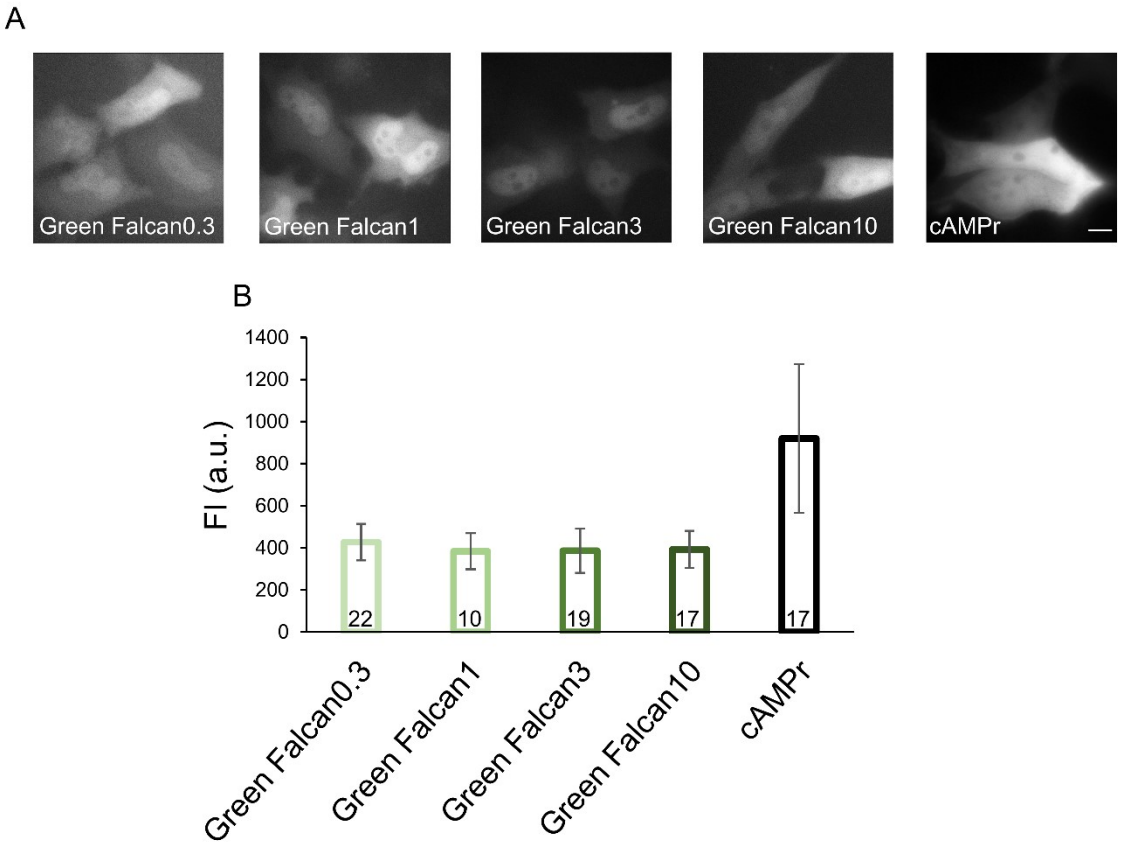

Figure S5. Basal fluorescence intensity of Green Falcans and cAMPr (A) Image of Green Falcans and cAMPr expressing HeLa cells. Scale bar represents 10 μm. (B) Comparison of basal fluorescence intensity. The data represent the means ± standard deviation. The numbers in bar graphs represent the number of cells showed 1.5 times higher than background, analyzed from three independent experiments.

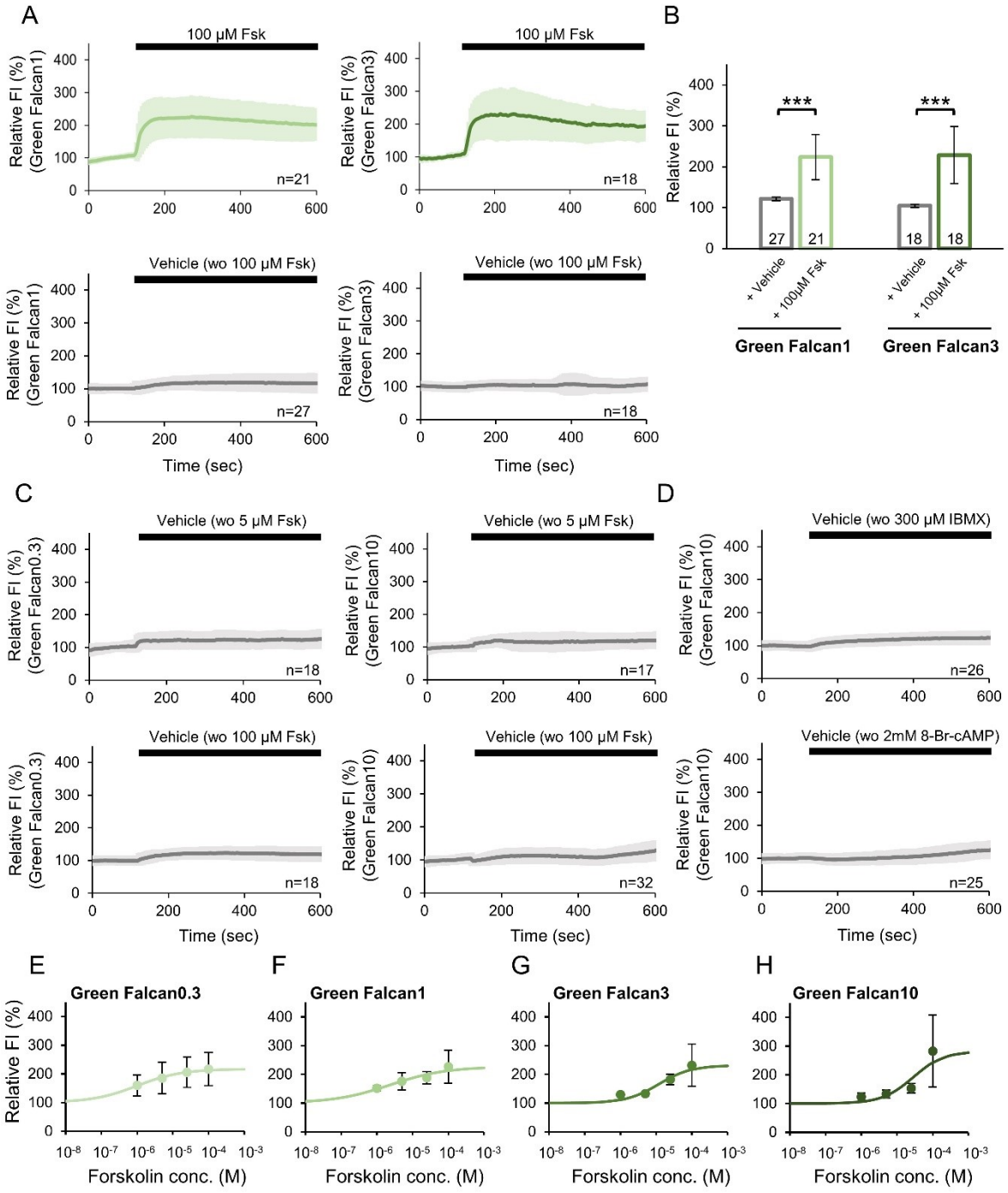

Figure S6. Live-cell imaging using Green Falcans (A) Time courses of fluorescence intensity (FI) in HeLa cells expressing Green Falcan1 and 3 in response to 100 μM forskolin. Relative FI was calculated by dividing by the mean of the FI during the 2 min prior to administration. The data represent the means ± standard deviation. The numbers in the lower right corner of the graphs represent the number of cells

analyzed from three independent experiments. (B) Comparison of relative FI at 2 min after the administration of DMSO (vehicle) or 100 μM forskolin to HeLa cells expressing Green Falcan1 and 3. The data represent the means ± standard deviation. The numbers in bar graphs represent the number of cells analyzed from three independent experiments. ***$p<0.001$. (C) Time courses of fluorescence intensity (FI) in HeLa cells expressing Green Falcan0.3 and 10 in response to vehicle (wo 5, 100 μM forskolin). Relative FI was calculated by dividing by the mean of the FI during the 2 min prior to administration. (D) Time courses of fluorescence intensity (FI) in HeLa cells expressing Green Falcan10 in response to vehicle (300 μM IBMX or 2 mM 8-Br-cAMP). Relative FI was calculated by dividing by the mean of the FI during the 2 min prior to administration. (E-H) Dose-response curves of all Green Falcans for the administration of forskolin. Each plot showed the maximum relative FI during imaging (10 min) after the administration of forskolin. For curve fitting of all Green Falcans, the minimum relative FI was normalized to 100, and maximum relative FI to maximum value. For Green Falcan10 fitting, the hill coefficient was fixed to 1. The data represent the means ± standard deviation (n=3).

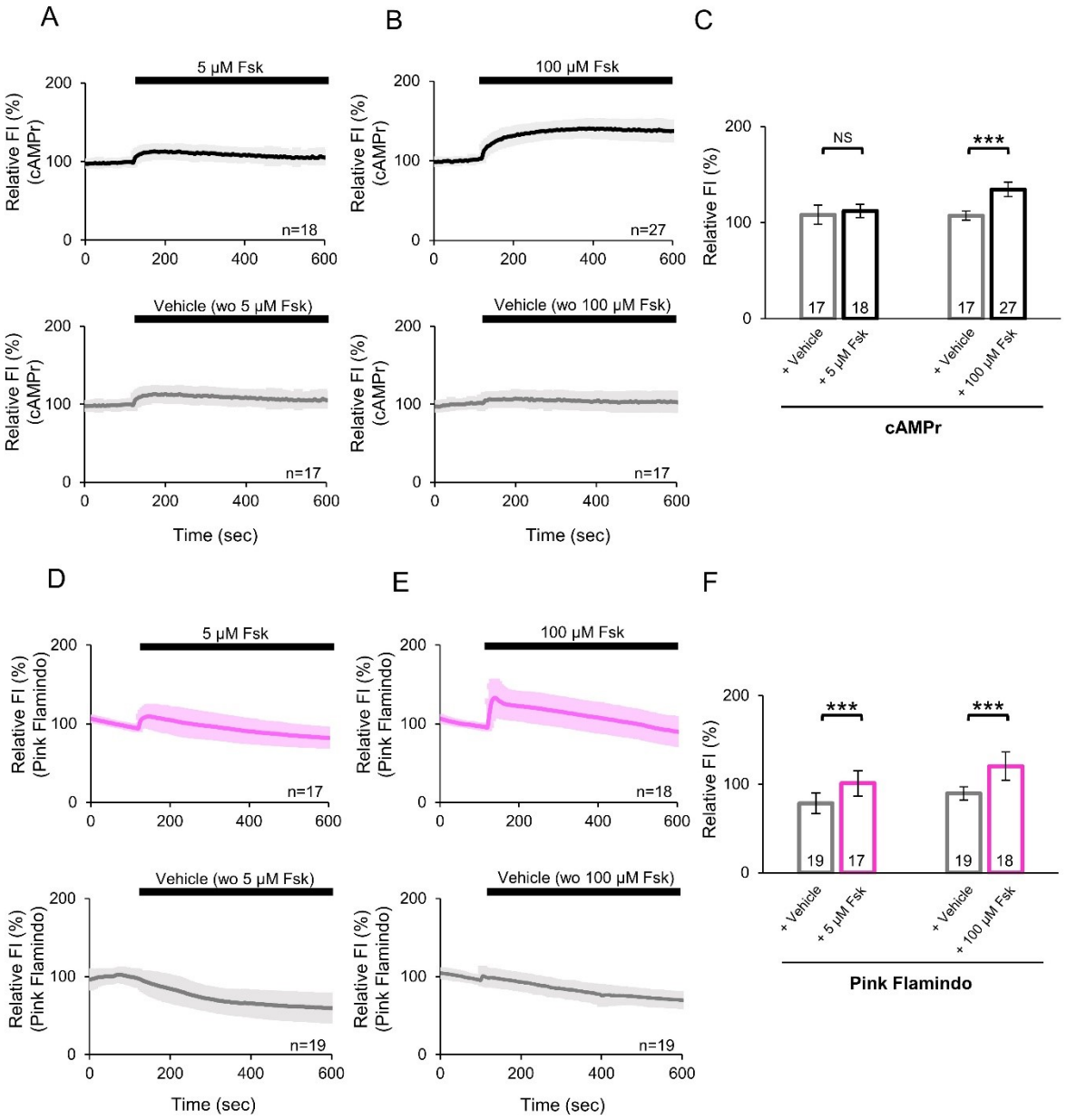

Figure S7. Live-cell imaging using other cAMP indicators (A, B) Time course of FI in HeLa cells expressing cAMPr in response to 5 μM forskolin and DMSO (A), or 100 μM foskolin and DMSO (B). The data represent the means ± standard deviation. The numbers in the lower right corner of the graphs represent the number of cells analyzed from three independent experiments. (C) Comparison of relative FI at 2 min after the administration of DMSO (vehicle) or 5 μM forskolin, and after that of DMSO (vehicle) or 100 μM forskolin to HeLa cells expressing cAMPr. The data represent the means ± standard deviation. The numbers in bar graphs represent the number of cells analyzed from three independent experiments.

***p<0.001. (D, E) Time course of FI in HeLa cells expressing Pink Flamindo in response to 5 μM forskolin and DMSO (D), or 100 μM forskolin and DMSO (E). The data represent the means ± standard deviation. The numbers in the lower right corner of the graphs represent the number of cells analyzed from three independent experiments. (F) Comparison of relative FI at 2 min after the administration of DMSO (vehicle) or 5 μM forskolin, and after that of DMSO (vehicle) or 100 μM forskolin to HeLa cells expressing Pink Flamindo. The data represent the means ± standard deviation. The numbers in bar graphs represent the number of cells analyzed from three independent experiments. ***p<0.001.