**Biophysical Journal, Volume 116** 

# **Supplemental Information**

# Understanding the Fluorescence Change in Red Genetically Encoded

# **Calcium Ion Indicators**

Rosana S. Molina, Yong Qian, Jiahui Wu, Yi Shen, Robert E. Campbell, Mikhail Drobizhev, and Thomas E. Hughes

### Understanding the Fluorescence Change in Red Genetically Encoded Calcium Ion Indicators

Supporting Information

R.S. Molina<sup>1</sup>, Y. Qian<sup>2</sup>, J. Wu<sup>2,3</sup>, Y. Shen<sup>2</sup>, R.E. Campbell<sup>2,4</sup>, M. Drobizhev<sup>1</sup>, T.E. Hughes<sup>1</sup>

1. Department of Cell Biology & Neuroscience, Montana State University, Bozeman, Montana, USA

2. Department of Chemistry, University of Alberta, Edmonton, AB, Canada

3. Department of Pharmacology, Weill Cornell Medicine, New York, NY, USA

4. Department of Chemistry, The University of Tokyo, Tokyo, Japan

Section	Page
Supporting Materials and Methods	1-3
Table S1	4
Figures S1-S13	5-16
Supplemental References	17

Supporting Materials and Methods

## Protein purification

REX-GECO1 and jRGECO1a were cloned into the pUE backbone via ligation-independent cloning (In-Fusion, Clontech, Mountain View, CA). CAR-GECO1, K-GECO1, R-GECO1, R-GECO1.2, jRCaMP1a, and O-GECO1 were cloned into the pBAD backbone (Thermo Fisher Scientific, Waltham, MA) with restriction cloning. jREX-GECO1 was cloned into the modified pBAD backbone pTorPE with restriction cloning. All plasmids are available in Addgene. Plasmids were transformed into DH10B E. coli, which were grown for protein expression at 30°C for 2 days in Terrific Broth (BD, Sparks, MD) or Circlegrow (MP Biomedicals, Santa Ana, CA). To induce protein expression in the pBAD plasmids, 0.1% arabinose was added after growing approximately 5 hours. Bacteria pellets were lysed with BugBuster (EMD Millipore Corp., Burlington, MA) and the lysate was purified with His60 Ni Superflow Resin (Takara Bio, Mountain View, CA). Proteins were buffer-exchanged into pH 7.2 buffers containing 30 mM MOPS, 100 mM KCl, and 10 mM EGTA with or without 10 mM CaCl<sub>2</sub>. Protein characterization was done in these respective buffers, except where noted. Purified proteins were stored short term ( $\leq 2$  weeks) at 4°C and long term at -80°C. The Ca<sup>2+</sup>-free form of K-GECO1 exhibits photoswitching in room light; so the protein sample was stored covered in foil, and all of the photophysical measurements were done with minimal exposure to room light.

## Special corrections for pH titrations

For the titrations of the  $Ca^{2+}$ -saturated state of jREX-GECO1 and REX-GECO1 from pH 7.2 and higher, the OD at the peak of the anionic form absorbance was corrected for the absorbance of the neutral form at that position (Eq. S1):

$$OD_{A}^{A} = \frac{OD_{A+N}^{A} - OD_{A+N}^{N} \cdot (OD_{N}^{A}/OD_{N}^{N})}{1 - (OD_{N}^{A}/OD_{N}^{N}) \cdot (OD_{A}^{N}/OD_{A}^{N})}$$
(S1)

Here, the superscript indicates the spectral position of the OD value, either at the anionic peak position (<sup>A</sup>) or the neutral peak position (<sup>N</sup>). The subscript indicates the form of the chromophore that the OD belongs to, either anionic and neutral ( $_{A+N}$ ), only anionic ( $_A$ ), or only neutral ( $_N$ ). The fractions ( $OD_N^A/OD_N^N$ ) and ( $OD_A^N/OD_A^A$ ) were considered constant throughout the titration. The former was determined based on the spectrum at pH 7.2 (assuming 100% neutral form), and the latter was based on the spectrum at the pH where the anionic form reached its maximum.

# Special corrections for determination of the relative fraction of the excitable form of the chromophore

In the case of K-GECO1 and CAR-GECO1, the absorbance at pH 7.2 include significant absorption from other, non-red chromophore(s) which overlap with the absorption of the neutral form of the red chromophore. Their absorbance spectra were unmixed using excitation spectra in order to find the peak OD of the red neutral chromophore (Fig. S13).

For both the  $Ca^{2+}$ -free and  $Ca^{2+}$ -saturated spectra of K-GECO1, the red anionic form excitation spectrum (shifted to match the peak position if necessary) was fitted to the absorbance spectrum and subtracted, leaving only the absorption of the neutral chromophore and the unknown form (peaking near 400 nm). To this we fit the red neutral form excitation spectrum and took the peak value as the OD of the neutral chromophore (Fig. S13A and B). The anionic form excitation spectrum was measured with the  $Ca^{2+}$ -saturated sample with emission registration at 700 nm. The neutral form excitation spectrum was measured with the  $Ca^{2+}$ -free sample with emission registration at 530 nm.

For the Ca<sup>2+</sup>-saturated state of CAR-GECO1, the excitation spectrum of the immature anionic green chromophore was subtracted from the absorbance spectrum and then the red neutral form excitation spectrum was fitted under the difference spectrum to find the OD (Fig. S13C). For the Ca<sup>2+</sup>-free state, the red neutral form excitation spectrum was fitted to the unadulterated spectrum (Fig. S13D). The anionic green chromophore excitation spectrum was measured from Ca<sup>2+</sup>-saturated CAR-GECO1 with emission registration at 540 nm. The red neutral form excitation spectrum was measured scanning the Ca<sup>2+</sup>-free sample with emission registration at 540 nm.

## Emission spectra

The LS 55 Fluorescence Spectrometer (PerkinElmer, Waltham, MA) was used to collect emission spectra with 10 nm excitation slits and 5 nm emission slits. The emission spectra were corrected for spectral sensitivity of the detection system by applying a correction function created with the Spectral Fluorescence Standard Kit (Millipore-Sigma, Darmstadt, Germany).

### Structural analysis

The illustration of the K-GECO1 crystal structure in Fig. 2A and structural analyses from the discussion were performed with the UCSF Chimera package (University of California, San Francisco, CA). Chimera is developed by the UCSF Resource for Biocomputing, Visualization, and Informatics (supported by NIGMS P41-GM103311).

# Development and Ca<sup>2+</sup> K<sub>d</sub> determination of *jREX-GECO1*

To increase the Ca<sup>2+</sup> affinity of REX-GECO1 (1), the Q306D and M339F mutations were introduced using the QuikChange Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). These two mutations were reported to be responsible for the increased Ca<sup>2+</sup> affinity of jRGECO1a (2) relative to R-GECO1. The resulting variant was designated as jREX-GECO1. To determine the  $K_d$  of jREX-GECO1, Ca<sup>2+</sup> titrations were performed using EGTA-buffered Ca<sup>2+</sup> solutions as previously described (3). Briefly, purified proteins were diluted into a series of buffers with free Ca<sup>2+</sup> concentrations ranging from 0 nM to 39 µM at 25 °C. These buffered Ca<sup>2+</sup> solutions were prepared by mixing a CaEGTA buffer (30 mM MOPS, 100 mM KCl, 10 mM EGTA, 10 mM CaCl<sub>2</sub>) and an EGTA buffer (30 mM MOPS, 100 mM KCl, 10 mM EGTA) at appropriate ratios. Fluorescence intensities were measured and plotted against Ca<sup>2+</sup> concentrations and fitted by a sigmoidal binding function to extract the Hill coefficient and  $K_d$ for Ca<sup>2+</sup>.

**Table S1.** Additional biophysical properties of the red GECIs

Class	Protein	Ca <sup>2+</sup>	pK <sub>a</sub> (s), this work	p <i>K<sub>a</sub></i> , literature	<i>K<sub>d</sub></i> for Ca <sup>2+</sup> (nM)	Hill Coefficient	λ <sub>Rmax</sub> (nm)	$\frac{\varepsilon_{\rm e}(\lambda_{\rm Rmax})}{\pm 5\%}$ (mM <sup>-1</sup> cm <sup>-1</sup> )	$\sigma_{2,e} \\ (\lambda_{Rmax}) \\ \pm 17\% \\ (GM)$	$F_1(\lambda_{Rmax}) \pm 13\%$ (mM <sup>-1</sup> cm <sup>-1</sup> )	$F_2(\lambda_{Rmax}) \\ \pm 21\% \\ (GM)$
I	R-GECO1	-	$8.69 \pm 0.03$	$8.9^{\rm a}, 8.7^{\rm b}$	482 <sup>a</sup> ,	$2.06^{\rm a}, 2^{\rm b},$	516	27	16	0.23	0.14
		+	$5.21 \pm 0.02, \\ 7.1 \pm 0.1, \\ 10.33 \pm 0.05$	6.59 <sup>a</sup> , 6.4 <sup>b</sup>	337 <sup>b</sup> , 449 <sup>c</sup>	1.51°		32	26	5.3	4.4
	R-GECO1.2	-	$8.98\pm0.02$	8.93 <sup>d</sup>	1200 <sup>d</sup>	2.79 <sup>d</sup>	514	23	14	0.16	0.10
		+	$5.42 \pm 0.02,$ $8.0 \pm 0.3,$ $10.77 \pm 0.05$	5.99 <sup>d</sup>				30	28	7.3	7
	jRGECO1a	-	$8.63 \pm 0.03$	8.6 <sup>b</sup>	148 <sup>b</sup>	1.9 <sup>b</sup>	514	26	11	0.25	0.10
		+	$\begin{array}{c} 4.97 \pm 0.03, \\ 6.74 \pm 0.08, \\ 10.51 \pm 0.06 \end{array}$	6.3 <sup>b</sup>	-			33	26	5.9	5
	O-GECO1	-	$9.52 \pm 0.01$	9.44 <sup>d</sup>	1500 <sup>d</sup>	2.06 <sup>d</sup>	502	26	13	0.04	0.021
		+	$6.24 \pm 0.01,$ $11.25 \pm 0.09$	6.07 <sup>d</sup>	-			29	20	5.9	4.2
	CAR-	-	$8.96 \pm 0.02$	9.05 <sup>d</sup>	490 <sup>d</sup>	2.01 <sup>d</sup>	516	31	16	0.20	0.10
	GECO1	+	$5.29 \pm 0.01,$ $8.4 \pm 0.2,$ $10.5 \pm 0.1$	5.74 <sup>d</sup>				35	31	8.2	7
II	K-GECO1	-	$6.71 \pm 0.04,$ $8.23 \pm 0.04$		165 <sup>e</sup>	1.12 <sup>e</sup>	530	39	15	1.9	0.7
		+	$6.34\pm0.03$	-				44	22	16	8
	jRCaMP1a	-	$6.48\pm0.05$	5.6 <sup>b</sup>	214 <sup>b</sup>	0.86 <sup>b</sup>	518	27	17	4.3	2.7
		+	$6.20\pm0.03$	6.4 <sup>b</sup>	-			26	16	11	7
III	REX- GECO1	-	$4.6 \pm 0.2,$ $7.6 \pm 0.2,$ $10.0 \pm 0.1$	6.5 <sup>f</sup>	240 <sup>f</sup>	1.8 <sup>f</sup>	450	7.0	8.2	0.16	0.19
		+	$11.70 \pm 0.02$	-				25	33	5.2	7
	jREX- GECO1	-	$4.6 \pm 0.1,$ 7.80 ± 0.04, 9.67 ± 0.05		200	1.67	450	8.2	10	0.18	0.23
		+	$11.63 \pm 0.02$	-				26	36	5.4	7
Referen	ices:	+	$11.63 \pm 0.02$					26	36	5.4	



**Figure S1.** Analytical method to find correct fluorescence quantum yield, as described in Materials and Methods. Demonstrated in a mixture of 6.5  $\mu$ M 3,3'-Dioctadecyloxacarbocyanine perchlorate (DODOC) and 1.2  $\mu$ M Cresyl Violet (CV) in ethanol. (*A*) Absorbance spectrum of the mixture with respective spectra of CV and DODOC fitted underneath. Dashed lines indicate the excitation wavelengths used in the integrating sphere to get the quantum yield of CV. (*B*) Illustration of the "very short" (*E*) and "short" (*H*) fluorescence integrals for  $\lambda_{blue}$  and  $\lambda_{red}$  excitation of CV. The normalized absorption of CV is shown for reference.  $E_{blue}$  is shaded dark blue;  $E_{red}$  is dark red;  $H_{blue}$  is light blue; and  $H_{red}$  is pink.



**Figure S2.** Example plots with fitted linear slope used to determine the neutral and anionic extinction coefficients for some of the red GECIs, as explained in Materials and Methods. Shown is data from the CAR-GECO1 pH titrations.



**Figure S3.** Normalizing the Ca<sup>2+</sup>-saturated/free  $F_2$  ratio spectra to the ratios determined at three excitation wavelengths around the maximum. Shown are the ratio spectra normalized to the three wavelengths separately and then averaged for jRGECO1a, K-GECO1, and jREX-GECO1 as examples.



**Figure S4.** Illustration of the neutral and anionic forms of the chromophores in the red GECIs under study. (*A*) R-GECO1-type chromophore, same as Fig. 2D in main text (PDB ID: 4I2Y (4)). (*B*) O-GECO1-type chromophore (from mOrange structure, PDB ID: 2H5O (7)). (*C*) jRCaMP1a-type chromophore (from RCaMP structure, PDB ID: 3U0K (4)).

Figure S5 (continued on next page with legend). Protein sequence alignment of red GECIs under study.

10	20	30	40	50	60	70	
M V D S S R R K W N K A G H A	VRAIGRLSS-	P V V S E R	MYPEDGALKS	EIKKGLRLK	D G G H Y A A E V K	ТТҮКАККР	- V Q L
M V D S S R R K W N K A G H A	VRAIGRLSS-	P V V S E R	MYPEDGALKS	EIKKGLRLK	D G G H Y A A E V K	Т Т Ү К А К К Р – –	- V Q L
M V D S S R R K W N K A G H A	VRAIGRLSS-	P V V S E R	MYPEDGALKS	EIKKGLRLK	DGGHYAAEVK	Т Т Ү К А К К Р – –	- V Q L
M V D S S R R K W <mark>I</mark> K A G H A	VRAIGRLSS-	P V V S E R	MYPEDG <mark>V</mark> LKS	EIKKGLRLK	D G G H Y A A E V K	Т Т Ү К А К К Р – –	- V Q L
M V D S S R R K W N K A G H A	VRAIGRLSS-	P V V S E R	MYPEDGALKS	EIKKGLRLK	D G G H Y A A E V K	Т Т Ү К А К К Р – –	- V Q L
M V D S S R R K W N K A G H A	VRAIGRLSS-	<mark>R W</mark> V S E W	MYPEDGALKS	V I K <mark>E</mark> G L R L K	D G G H Y A A E V <mark>R</mark>	Т Т Ү К А К К Р – –	- V Q L
MVDSSRRKWNKAGHA	VRAIGRLSS-	<mark>RW</mark> VSEW	MYPEDGALKS	V I K <mark>E</mark> G L R L K	D	ТТҮКАККР	- V Q L
M V D S S R R K W N K T G H A	VRAIGRLSS-	AINCEM	MYPADGGLRG	YTHMALKVD	G G G H L S C S F V	TTYRSKKTVG	NIKM
MGSVKLIPSLTTVII	VKSMLRKRSF	GNPFKYNTET	LYPADGGLEG	ACDMALKLV	<mark>G        G        G        H        L        </mark>	T T Y <mark>R S</mark> K K P A T	NLKM
RS20/ckkap		L1 CPRF	P				
90	100	110	120	130	140	150	
AYIVDIKLDIVSHNE	DYTIVEQCER	A E G R H – <mark>S</mark> T G G	MDELYKGGTG	GSLVSKGEE	DNMAIIKEFM	RFKVHMEGSV	NGHE
AYIVDIKLDIVSHNE	DYTIVEQCER	A E G R H - <mark>S</mark> T G G	MDELYKGGTG	GSLVSKGEE	D N <mark>R</mark> A I <mark>V</mark> K E F M	R F K <mark>L</mark> H M E G S V	NGHE
AYIVDIKLDIVSHNE	EDYTIVEQCER	A E G R H - <mark>S</mark> T G G	MDELYKGGTG	GSLVSKGEE	DNMAIIKEFM	RFKVHMEGSV	NGHE
AYIVDIKLDIVSHNE	DYTIVEQCER	AEGRH - PTGG	R D E L Y K G G T G	GSLVSKGEE	DNMAIIKEFM	RFKVHMEGSV	NGHE
AYIVDIKLDIVSHNE	EDYTIVEQCER	: A E G R H - <mark>S</mark> T G G	MDELYKGGTG	G S L V S K G <mark>V</mark> E	D N M A I <mark>V</mark> K E F M	RFKTHIEGSV	NGHE
AYIVDIKLDIVSHNE	DYTIVEQCER	AEGRH - PTGG	MVGLYKGGTG	GSLVSKGEE	DNMAIIKEFM	RFKVHMEGSV	NGHE
AYIVDIKLDIVSHNE	EDYTIVEQCER	A E G R H - P T G G	MVGLYKGGTG	GSLVSKGEE	DNMAIIKEFM	RFKVHMEGSV	NGHE
IHSVSHRLERLEES	D N E M F V V Q R E H	AVAKFVGLGG		G S M N S	LIKENM	RMKVVLEGSV	NGHQ
						PMKIYMEGTV	NNHH
V Y N V D H R L E R I K E A I	0	A V A R Y V G L G G	<mark>G</mark> G G T G	G S V S E			
VYNVDHRLERIKEAI 170	180	A VA R Y V G L G G	200	210	220	230	
VYNVDHRLERIKEAI 170 IEGEGEGRPYEAFQT	180 AKLKVTKGGP	AVARYVGLGG 190 LPFAWDILSP	200 Q F M Y G S K A Y I	210 KHPADIPDY	220 FKLSFPEGFR	230 WERVMNFEDG	GIIH
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGERPYEAFQT	180 TAKLKVTKGGP	AVARYVGLGG 190 LPFAWDILSP	200 200 2 F MYGSKAYI Q L MYGSKAYI	210 KHPADIPDY KHPADIPDY	220 FKLSFPEGFR	230 WERVMNFEDG WERVMNFEDG	GIIH GIIH
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGERPYEAFQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP	200 QFMYGSKAYI QLMYGSKAYI QFMYGSKAYI QFMYGSKAYI	210 KHPADIPDY KHPADIPDY KHPADIPDY	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG	GIIH GIIH GIIH
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP	200 QFMYGSKAYI QLMYGSKAYI QFMYGSKAYI QFTYGSKAYI 0FTYGSKAYI	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG	GIIH GIIH GIIH GIIH GIIH
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP	200 QFMYGSKAYI QLMYGSKAYI QFMYGSKAYI QFTYGSKAYI QIMYGSKAYI QIMYGSKAYI OFMYGSKAYI	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNTEEDG	GIIH GIIH GIIH GIIH GIIH
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAWDILSL	200 QFMYGSKAYI QLMYGSKAYI QFMYGSKAYI QFTYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG	GIIH GIIH GIIH GIIH GIIH GIIH GIIH
VYNVDHRLERIKEAU 170 IEGEGEGERPYEAFQT IEGEGEGERPYEAFQT IEGEGEGERPYEAFQT IEGEGEGERPYEAFQT IEGEGEGERPYEAFQT IEGEGEGERPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAWDILSL LPFAFDILAT	200 QFMYGSKAYI QLMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI SFMYGSRTFI	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG	GIIH GIIH GIIH GIIH GIIH GIIH GIIH
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT CTGEGEGRPYEAFQT CTSEGEGKPYEGTQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAWDILSL LPFAFDILAT LPFAFDILAT	200 QFMYGSKAYI QLMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI QFMYGSKAYI SFMYGSRTFI SFMYGSRTFI	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDF	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTTYEDG	GIIH GIIH GIIH GIIH GIIH GIIH GVIT GVLT
VYNVDHRLERIKEAU 170 IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT CTGEGEGNPYMGTQT	180 TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP TAKLKVTKGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAWDILSL LPFAFDILAT LPFAFDILAT	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q T M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S R T F I S F M Y G S R T F I S F M Y G S R T F I	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTIYEDG	GIIH GIIH GIIH GIIH GIIH GIIH GVIT GVLT
VYNVDHRLERIKEAU 170 IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT CTGEGEGGNPYMGTQT CTSEGEGKPYEGTQT	180 AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP MRIKV IEGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAFDILAT LPFAFDILAT	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F T Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I S F M Y G S R T F I Chro	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDF	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTFEDG WERVTYEDG	GIIH GIIH GIIH GIIH GIIH GIIH GIIH GVIT GVLT
VYNVDHRLERIKEAU 170 IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGRPYEAFQT IEGEGEGRPYEAFQT 250	180 AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP AKLKVTKGGP MRIKVIEGGP MRIKVIEGGP	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAWDILSL LPFAWDILSL LPFAFDILAT	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I S F M Y G S R T F I Chro 280	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDF	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVTIYEDG WERVTTYEDG	GIIH GIIH GIIH GIIH GIIH GIIH GVIT GVLT
VYNVDHRLERIKEAU 170 IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT CTGEGEGGNPYEAFQT CTSEGEGGNPYEGTQT 250 0DSSL0DGVETYKY	180 A K L K V T K G G P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V T K C C A K L K V K K C K K C K K K K K K K K K K K K	190 LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSP LPFAWDILSL LPFAFDILAT LPFAFDILAT	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I Chro 280	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY CHPEKEAE	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT FKQSFPEGFT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTTYEDG WERVTTYEDG	GIIH GIIH GIIH GIIH GIIH GIIH GVIT GVLT
VYNVDHRLERIKEAU 170 IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT IEGEGEGGRPYEAFQT CTGEGEGGNPYEAFQT CTSEGEGGNPYEAFQT 250 QDSSLQDGVFIYKVF	180 A K L K V T K G G P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P C A K L K V T K G C P A K L K V T K G C P C A K L K V T K C C P C A K L K V T K C C A K L K V K K C C A K L K V K K C C A K L K V K K C C A K L K V K K C C A K L K V	190           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           PFAWDILSP           PFAWDILSP           PFAWDILSP           PFAFDILAT           PFAFDILAT           270           PVMQKKTMGW	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I Chro 280 280	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY CHPEKEAF 0 TAFEKEAF	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVTIYEDG WERVTTYEDG WERVTTYEDG MERVTTYEDG	GIIH GIIH GIIH GIIH GIIH GIIH GVIT RSLG RSLG
170           IEGEGEGERPYEAFQT           IEGEGEGEGERPYEAFQT           IEGEGEGEGERPYEAFQT           IEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEG	180 A K L K V T K G G P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V T K C P A K L K V K K C V K K C V K K C V K K C V K K K V K K K V K K K	190           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           PFAWDILSP           PFAWDILSP           PFAWDILSP           PFAWDILSP           PFAKDILSP           PFAKDILSP           PYAQKKTMGW           PVMQKKTMGW	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I Chro 280 280 280 280	210 KHPADIPDY KHPADI	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVTIYEDG WERVTTYEDG WERVTTYEDG MERVTTYEDG MERVTTYEDG	GIIH GIIH GIIH GIIH GIIH GVIT RSLG RSLG RSLG
170           IEGEGEGERPYEAFQT           IEGEGEGEGERPYEAFQT           IEGEGEGEGERPYEAFQT           IEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEG	180 A K L K V T K G G P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P P C A K L V V K C P P P P C A K L V V K C P P P P C A K L V V K C P P P P C A K L V	190           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           PFAWDILSP           PFAWDILSP           PFAWDILSP           PFAKDILSP           PFAKDILSP           PFAKDISS           PFAFDILAT           PVMQKKTMGW           PVMQKKTMGW           PVMQKKTMGW	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I Chro 280 280 280 280 280 280 280 280 280 280	210 KHPADIPDY KHPADI	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTTYEDG WERVTTYEDG WERVTTYEDG TTKELGTVM MTTKELGTVF ITTKELGTVF	GIIH GIIH GIIH GIIH GIIH GVIT RSLG RSLG RSLG RSLG
170           IEGEGEGERPYEAFQT           QCTSEGEGERPYEAFQT           250           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV	180 A K L K V T K G G P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P D G A K L K V T K C P P P P C A K L V Y K C P P P P C A K L V Y K C P P P P C A K L V Y K C P P P C A K L V Y	190 L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S L L P F A W D I L S L L P F A F D I L A T 270 P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F T Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S R T F I Chro 280 Z E A T R D Q L T E E E A T R D Q L T E E E A T R D Q L T E E E A T R D Q L T E E E A T R D Q L T E E	210 KHPADIPDY KHPADI	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTTYEDG WERVTTYEDG WERVTTYEDG TTKELGTVM ITTKELGTVF ITTKELGTVL	GIIH GIIH GIIH GIIH GIIH GVIT RSLG RSLG RSLG RSLG RSLG
170           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGRPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           IEGEGEGERPYEAFQT           QTSLQDGVFIYKVE           QDSSLQDGVFIYKVE           QDSSLQDGVFIYKVE           QDSSLQDGVFIYKVE           QDSSLQDGVFIYKVE           QDSSLQDGVFIYKVE           QDSSLQDGVFIYKVE	180 A K L K V T K G G P A K L K V T K G F P P D G K L R G T N F P P D G K L R	190           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           LPFAWDILSP           PFAWDILSP           PFAWDILSP           PFAWDILSP           PFAWDILSP           PFAFDILAT           PVMQKKTMGW           PVMQKKTMGW           PVMQKKTMGW           PVMQKKTMGW           PVMQKKTMGW	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I C F M Y G S K A Y I S F M Y G S K A Y I C	210 K H P A D I P D Y K H P A D I P D F Q I A E F K E A F	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTIYEDG WERVTTYEDG WERVTTYEDG TTKELGTVM ITTKELGTVM ITTKELGTVM	GIIH GIIH GIIH GIIH GIIH GVIT RSLG RSLG RSLG RSLG RSLG RSLG
170           IEGEGEGERPYEAFQT           QTSEGEGERPYEAFQT           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV           QDSSLQDGVFIYKV	180 A K L K V T K G G P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K G C P A K L K V T K C P P P G C K C V C P A K L K V T K C P P P G C K C V C P P P G C K C P P P G C K C P P P G C K C P P P G C K C P P P G C K C P P P C C K C P P P C C K C P P P C C K C P P P C C K C P P P C C K C P P P C C K C P P P C C K C P P P C C K C P P P C C K C P P	190 L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S I L P F A W D I L S I L P F A F D I L A T Z 70 P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I S F M Y G S K A Y I C H Y G S K A Y I C H Y G S K A Y I C H Y G S K A Y I D L T E E A T R D Q L T E E A T R D Q L T E E P T R D Q L T E	210 KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY KHPADIPDY QIAEFKEAF QIAEFKEAF QIAEFKEAF QIAEFKEAF QIAEFKEAF QIAEFKEAF QIAEFKEAF	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTIYEDG WERVTTYEDG WERVTTYEDG TTKELGTVM ITTKELGTVM ITTKELGTVM ITTKELGTVL ITTKELGTVL	GIIH GIIH GIH GIH GIH GIH GVIT GVIT RSLG RSLG RSLG RSLG RSLG RSLG RSLG
170           IEGEGEGERPYEAFQT           QTSLQDGVFIYKV           QDSSLQDGVFIYKV	180 A K L K V T K G G P A K L K V T K G F A K L K V T K F A K K V K	190 L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S P L P F A W D I L S I L P F A F D I L A T L P F A F D I L A T V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W P V M Q K K T M G W	200 Q F M Y G S K A Y I Q L M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I Q F M Y G S K A Y I S F M Y G S K A Y I C	210 KHPADIPDY KHPADI	220 FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKLSFPEGFR FKQSFPEGFT FKQSFPEGFT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT SLFDKDGDGT	230 WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMNFEDG WERVMIFEDG WERVMIFEDG WERVMIFEDG WERVTTYEDG WERVTTYEDG WERVTTYEDG TTKELGTVM ITTKELGTVM ITTKELGTVL ITTKELGTVL ITTKELGTVL	GIIH GIIH GIIH GIIH GIIH GVIT GVIT RSLG RSLG RSLG RSLG RSLG RSLG RSLG RSLG

- R-GEC01
   R-GEC01.2
   jRGEC01a
   O-GEC01
   CAR-GEC01
   REX-GEC01
   jRCaMP1a
   K-GEC01

	330	340	350	360	370	380	390	400
1	PTEAELQDMINEVDA		FLTMMARKM	NDTDSEEEIRI	EAFRVFDKDGM	IGYIGAAELRI	HVMTDLGEKLT	DEEVD
2	PTEAELQDMINEVDA	DGDGTFDFPE	FLTMMARKM	NDTDSEEEIRI	EAFRVFDKDGM	IGYIGAAELRI	H V M T D L G E K <mark>I</mark> T	DEEVD
3	PTEAELQDMINEVDA	DGDGTFDFPE	FLTMMARKM	NDTDSEEEIRI	EAFRVFDKDGM	IGYIGAAELRI	H V M T D L G E K L T	DEEVD
4	PTEAELQDMINEVDA	DGDGTFDFPE	E F L T M M A R <mark>R</mark> M	N D T D S E <mark>V</mark> E I R I	E A F R V F D <mark>N</mark> D G M	I G Y I G A A E L R I	HVMTDLGEKLT	DEEVD
5	PTEAELQDMINEVDA	DGDGTFDFPE	FLTMMARKM	NDTDSEEEIRI	EAFRVFDKDGM	IGYIGAAELRI	HVMTDLGEKLT	DEEVD
6	PTEAELQDMINEVDA	DGDGTFDFPE	FLTMMARKM	N D <mark>S</mark> D S E E E I R I	EAFRVFDKDGM	I G Y I G A A E L R I	HVMTDLGEKLT	DEEVD
7	PTEAELQDMINEVDA	DGDGTFDFPE	FLTMMARKM	N D <mark>S</mark> D S E E E I R I	EAFRVFDKDGM	GYIGAAELRI	HVMTDLGEKLT	DEEVD
8	PTEAELQDMINEVDA	A D G D G T <mark>I</mark> D F P E	E F L <mark>I</mark> M M A R K M	<mark>K Y</mark> T D S E E E I R I	E A F <mark>G</mark> V F D K D G N	I G Y I <mark>S</mark> A A E L R I	H V M T <mark>N</mark> L G E K L T	DEEVD
9	PTEAELQDMINEVDA		FLTMMARKM	<mark>SYRVT</mark> EEEIRI	EAFRVFDKDGM	I G Y I G A A E L R I	HVMTDLGEKLT	DEEVD
	410	420						
1	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	ĸĸ					
2	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	K					
3	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	κ					
4	EMIRVADIDGDGQVN	1 Y E E F V Q M M T <i>A</i>	κ					
5	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	K					
6	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	κ					
7	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	K					
8	E	IYEEFVQMMT#	к					
9	EMIRVADIDGDGQVN	I Y E E F V Q M M T A	K					
1. ⊢ 2. ⊢	R-GECOI							
<b>3.</b> j	RGECO1a							
4. 0	D-GECO1							
6. F	REX-GECO1							
<b>7.</b> j	REX-GECO1							
8. j 9. k	KCaMP1a (-GECO1							

**Figure S5** (*continued from previous page*). Protein sequence alignment of red GECIs under study. Residues that differ from the consensus sequence are highlighted in pink. The colored bars underneath the sequence indicate the respective parts of the protein: the CaM-binding peptide RS20 or ckkap is yellow; linkers 1 and 2 are blue; the circularly-permuted red FP is red with the chromophore sequence in gold; and calmodulin is aqua.



**Figure S6.** Excitation (one-photon) spectra (ex) and emission spectra (em) of the  $Ca^{2+}$ -free and  $Ca^{2+}$ -saturated states for each protein. All spectra are normalized to 1. The excitation spectra were scanned while collecting the entire integrated fluorescence spectrum by setting the emission grating to the 0-th diffraction order. The excitation wavelength of light used when scanning the emission spectrum is indicated on each plot.



**Figure S7.** Plotted as in Fig. 4 of the main text and Fig. S8. Titrations from neutral to acidic pH and neutral to alkaline pH were done separately and then combined. The figures are normalized to the observed maximal absorbance of the anionic form. (*A-F*) Absorbance pH titrations, measured in either Ca<sup>2+</sup>-free or Ca<sup>2+</sup>-saturated buffer as noted. Each spectrum was measured at the pH value designated in the legends. The final spectrum (red dashed line) belongs to the denatured chromophore. (*G-I*) Apparent p $K_a$  curves, showing the OD of the anionic form as a function of pH. The p $K_a$  values are indicated on the fitted curve.





**Figure S8.** Plotted as in Fig. 4 of the main text and Fig. S7. Titrations from neutral to acidic pH and neutral to alkaline pH were done separately and then combined. The figures are normalized to the observed maximal absorbance of the anionic form. (*A-F*) Absorbance pH titrations, measured in either  $Ca^{2+}$ -free or  $Ca^{2+}$ -saturated buffer as noted. Each spectrum was measured at the pH value designated in the legends. The final spectrum (red dashed line) belongs to the denatured chromophore. (*G-I*) Apparent p $K_a$  curves, showing the OD of the anionic form as a function of pH. The p $K_a$  values are indicated on the fitted curve. (*H*) Only part of the jRCaMP1a titrations could be fitted because of protein precipitation at pH values below those displayed and early denaturation of the anionic form at higher pH values.



**Figure S9.** Spectral analysis of R-GECO1 (row 1), R-GECO1.2 (row 2), and O-GECO1 (row 3). Plots are set up as in Fig. 5 of main text and Fig. S10. The vertical dashed line on each plot indicates the transition wavelength of excitation ( $\lambda_{Rmax}$ ) where the Ca<sup>2+</sup>-saturated/free F<sub>1</sub> and F<sub>2</sub> ratios are approximately maximum. (*A-C*) Ca<sup>2+</sup>saturated/free (Sat/Free) F<sub>1</sub> (dotted line) and F<sub>2</sub> (solid line) ratios as a function of excitation wavelength, measured directly by taking the ratio of the integrated fluorescence signal normalized to the known relative protein concentration between the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated samples. (*D-F*) Spectra of the one-photon brightness (F<sub>1</sub>, dotted lines, left and bottom axes) and two-photon brightness (F<sub>2</sub>, solid lines, right and top axes) of the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated states. (*G-I*) One-photon absorption (1PA) and two-photon absorption (2PA) of the excitable form of the chromophore, shown in values of  $\varepsilon_e$  (left and bottom axes) and  $\sigma_{2,e}$  (right and top axes), respectively, for the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated states.



**Figure S10.** Spectral analysis of CAR-GECO1 (row 1), jRCaMP1a (row 2), and REX-GECO1 (row 3). Plots are set up as in Figure 5 of main text and Figure S9. The vertical dashed line on each plot indicates the transition wavelength of excitation ( $\lambda_{Rmax}$ ) where the Ca<sup>2+</sup>-saturated/free F<sub>1</sub> and F<sub>2</sub> ratios are approximately maximum. (*A-C*) Ca<sup>2+</sup>-saturated/free (Sat/Free) F<sub>1</sub> (dotted line) and F<sub>2</sub> (solid line) ratios as a function of excitation wavelength, measured directly by taking the ratio of the integrated fluorescence signal normalized to the known relative protein concentration between the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated samples. (*D-F*) Spectra of the one-photon brightness (F<sub>1</sub>, dotted lines, left and bottom axes) and two-photon brightness (F<sub>2</sub>, solid lines, right and top axes) of the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated states. (*G-I*) One-photon absorption (1PA) and two-photon absorption (2PA) of the excitable form of the chromophore, shown in values of  $\varepsilon_e$  (left and bottom axes) and  $\sigma_{2,e}$  (right and top axes), respectively, for the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-saturated states.



**Figure S11.** Two-photon absorption spectra plotted according to two-photon brightness. The scale for the  $Ca^{2+}$ -saturated spectra is on the left, and the scale for the  $Ca^{2+}$ -free spectra is on the right.



**Figure S12.** Plotted as in Fig. 6 of main text.  $Ca^{2+}$ -saturated/free ratios (Sat/Free) for  $\rho_e$ ,  $\varphi_e$ ,  $\varepsilon_e(\lambda_{Rmax})$ , and  $\sigma_{2,e}(\lambda_{Rmax})$ . Insets:  $Ca^{2+}$ -saturated/free F<sub>1</sub> and F<sub>2</sub> ratios, both calculated from the independent measurements of  $\rho_e$ ,  $\varphi_e$ ,  $\varepsilon_e$ , and  $\sigma_{2,e}$  (tan) and measured directly (green) as described in Fig. 5 of main text. The horizontal dashed line marks a Sat/Free ratio of 1 (no change) for each plot. Error bars represent estimated standard deviations.



**Figure S13.** Separating the components of the absorbance spectra of K-GECO1 and CAR-GECO1 using excitation spectra as described in Supporting Materials and Methods.

### Supporting References

- Wu, J., A.S. Abdelfattah, L.S. Miraucourt, E. Kutsarova, A. Ruangkittisakul, H. Zhou, K. Ballanyi, G. Wicks, M. Drobizhev, A. Rebane, E.S. Ruthazer, and R.E. Campbell. 2014. A long Stokes shift red fluorescent Ca<sup>2+</sup> indicator protein for two-photon and ratiometric imaging. Nat. Commun. 5: 5262.
- Dana, H., B. Mohar, Y. Sun, S. Narayan, A. Gordus, J.P. Hasseman, G. Tsegaye, G.T. Holt, A. Hu, D. Walpita, R. Patel, J.J. Macklin, C.I. Bargmann, M.B. Ahrens, E.R. Schreiter, V. Jayaraman, L.L. Looger, K. Svoboda, and D.S. Kim. 2016. Sensitive red protein calcium indicators for imaging neural activity. Elife. 5.
- Zhao, Y., S. Araki, J. Wu, T. Teramoto, Y.-F. Chang, M. Nakano, A.S. Abdelfattah, M. Fujiwara, T. Ishihara, T. Nagai, and R.E. Campbell. 2011. An expanded palette of genetically encoded Ca<sup>2+</sup> indicators. Science. 333: 1888–1891.
- Akerboom, J., N. Carreras Calderón, L. Tian, S. Wabnig, M. Prigge, J. Tolö, A. Gordus, M.B. Orger, K.E. Severi, J.J. Macklin, R. Patel, S.R. Pulver, T.J. Wardill, E. Fischer, C. Schüler, T.-W. Chen, K.S. Sarkisyan, J.S. Marvin, C.I. Bargmann, D.S. Kim, S. Kügler, L. Lagnado, P. Hegemann, A. Gottschalk, E.R. Schreiter, and L.L. Looger. 2013. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. Front. Mol. Neurosci. 6: 2.
- Wu, J., L. Liu, T. Matsuda, Y. Zhao, A. Rebane, M. Drobizhev, Y.-F. Chang, S. Araki, Y. Arai, K. March, T.E. Hughes, K. Sagou, T. Miyata, T. Nagai, W.-H. Li, and R.E. Campbell. 2013. Improved orange and red Ca<sup>2+</sup> indicators and photophysical considerations for optogenetic applications. ACS Chem. Neurosci. 4: 963–972.
- Shen, Y., H. Dana, A.S. Abdelfattah, R. Patel, J. Shea, R.S. Molina, B. Rawal, V. Rancic, Y.-F. Chang, L. Wu, Y. Chen, Y. Qian, M.D. Wiens, N. Hambleton, K. Ballanyi, T.E. Hughes, M. Drobizhev, D.S. Kim, M. Koyama, E.R. Schreiter, and R.E. Campbell. 2018. A genetically encoded Ca<sup>2+</sup> indicator based on circularly permutated sea anemone red fluorescent protein eqFP578. BMC Biol. 16: 9.
- 7. Shu, X., N.C. Shaner, C.A. Yarbrough, R.Y. Tsien, and S.J. Remington. 2006. Novel chromophores and buried charges control color in mFruits. Biochemistry. 45: 9639–9647.