SUPPORTING INFORMATION

eZinCh-2: A Versatile, Genetically Encoded FRET Sensor for Cytosolic and Intraorganelle Zn^{2+} Imaging

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Supporting Methods

Cloning and mutagenesis. Plasmids encoding for Cerulean and Citrine, connected via a long $(GGSGGS)_9$ linker, containing different $(Cys)_4$ binding pockets were synthesized in previous research. A QuikChange Multi Site-Directed mutagenesis (Agilent Technologies) was used to simultaneously introduce different combinations of C144H and C206H mutants in pET28a-eZinCh-4 (primers 1&2 and 3&4 for mutating C144H and C206H, respectively, Supplementary Table 1), generation pET28a-eZinCh-2.1.

Vector pET28a-eZinCh-5 was used to introduce combinations of C206H and C208H mutations by using primer 5&6 and 7&8, respectively, yielding pET28a-eZinCh-2.2, pET28a-eZinCh-2.3 and pET28a-eZinCh-2.4.

To create the mammalian expression vector of one of the eZinCh mutants (pET28a-eZinCh-2), pET28a-eZinCh-2 was digested with *Nde* I and *Not* I to obtain a fragment encoding for eZinCh-2 that could be ligated into a *Nde* I/ *Not* I digested peCALWY-4 vector to yield the mammalian expression vector peZinCh-2[1]. For ER-targeting a pcDNA3.1 vector containing the Preproinsulin (PPI) signal peptide, cerulean and citrine fused by a nine GGSGGS linker and a four amino acid long retention sequence KDEL at the end of the C-terminus was ordered (Genscript, USA). For mitochondrial targeting a Not I restriction site was introduced in the pShuttle-mito-eCALWY-4 by site directed mutagenesis (Agilent Technologies). Next pET28a-eZinCh-2 was digested with *Age I* and *Not I* to obtain a part of the fragment encoding for eZinCh-2 that could be ligated into a *Age* I/ *Not* I digested pShuttle-mito-eCALWY-4 vector, yielding pShuttle-mito-eZinCh-2. To create the vesicle-targeted mammalian expression plasmid, pET28a-eZinCh-2 was digested with *Age* I and *Not* I, followed by ligation into a vesicle-targeted mammalian *Age I/Not I* digested expression vector. The correct open reading frame for all expression vectors was confirmed by DNA sequencing (BaseClear, Leiden, The Netherlands).

Protein expression and purification. *E. coli* BL21(DE3) transformed with expression plasmid was grown to OD600 in 500 mL Lysogeny Broth (LB) medium containing 50 μg/mL kanamycin in a shaking incubator at 37 °C. Following induction with 0.1 mM IPTG, the bacterial cultures were grown overnight at 25 °C in a shaking incubator at 250rpm. Cells were harvested by centrifugation and lysed using 10 mL BugBuster Protein Extraction reagent (Novagen) with 10 µL Benzonase. Proteins were purified by Ni-NTA affinity chromatography. Since His-tags are known to readily bind Zn^{2+}, they were removed from all constructs using thrombin cleavage. After elution from the Ni-NTA column, the buffer was exchanged to thrombin cleavage buffer (20 mM Tris-HCl (pH 8.4), 150 mM NaCl, 2.5 mM CaCl2) using PD10 desalting columns (GE, Healthcare). Cleavage of the thrombin recognition site between the His-tag and the Cerulean N-terminus was initiated by the addition of 0.3 U thrombin protease (Novagen) per mg target protein at a 0.2 mg/mL target protein concentration. Cleavage was carried out at RT for 20 hours, after which 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) was added to inactivate the thrombin enzyme. Proteins were then loaded onto Ni-NTA once again. To separate the cleaved and uncleaved protein and also the His-tags remained behind on the Ni-column. Subsequently the proteins were loaded onto a size exclusion column (Sephacryl S200, GE Healthcare), using a buffer containing 50 mM Tris, 100 mM NaCl, 4 M Urea and 5 mM DTT. The SEC fractions were analyzed by SDS PAGE for correct size and purity, pooled and concentrated using 10 kDa MWCO centrifugation filters.

Co-localization experiments. HeLa cells were seeded on coverslips and transfected with plasmids encoding for either ER-eZinCh-2 or mito-eZinCh-2 using Lipofectamine 2000. Proteins were allowed to over express for ~24 h. Cells were washed with HBSS buffer, and stained with 1 µM ER-Tracker Red (Life Technologies) or 300 nM MitoTracker Red (Life Technologies) for 30 min. Cells were imaged on the Leica, TCS SP5X, using the 63x water immersion objective. The expressed eZinCh-2 was excited using the 405 nm laser, followed by recording emission between 515-595 nm. The commercial available ER and Mito Tracker were excited around 587 nm and 581 nm, followed by recording the emission between 605 – 625 nm and 630 – 660 nm, respectively.

For immunocytochemical analysis, INS1(832/13) cells expressing VAMP2-eZinCh2 were fixed in paraformaldehyde 4% for 20 minutes, permeabilized with Triton X-100 (0.5%) for 25 minutes, and probed with primary antibody against insulin (1:100, DAKO, Cambridgeshire, U.K.), then visualised with Alexa Fluor 568 secondary antibodies (1:200; Life Technologies). Specimens were mounted on glass slides using Vectashield hard set (Vector Laboratories). Image acquisition was performed with a Zeiss Axiovert microscope coupled to a Nipkow spinning-disk head (Yokogawa CSU-10) using a 63x/NA1.4 objective. Two solid-state lasers (CrystaLaser) controlled by a laser-merge module (Spectral Applied Physics) provided wavelengths of 491 nm to excite VAMP2-eZinCh2.2 and 561 nm for insulin. Emitted light was filtered at 525/50 nm and at 630/50nm, respectively. Images were captured with a highly sensitive 16-bit, 512 × 512 pixel back-illuminated EM-CCD camera (ImageEM 9100-13; Hamamatsu).

Supporting Table 1: Sensor properties of the eZinCh mutants at pH 7.1.

Mutants	Cerulean	Citrine	K_d (pH 7.1)	Ratiometric change (%) (pH 7.1)
eZinCh-2.1	C144**H**, 206C	144C, 206C	3.7 nM	110%
eZinCh-2.2	C206**H**, 208C	C206**H**, 208C	1.0 nM	400%
eZinCh-2.3	C206**H**, 208C	206C, C208**H**	4.8 nM	109%

Supporting table 2: Free Zn^{2+} concentrations in various buffering systems at pH 6.0, 20 °C.[a]

Buffering system	0.1 mM Zn^{2+}	0.2 mM Zn^{2+}	0.3 mM Zn^{2+}	0.4 mM Zn^{2+}	0.5 mM Zn^{2+}	0.6 mM Zn^{2+}	0.7 mM Zn^{2+}	0.8 mM Zn^{2+}	0.9 mM Zn^{2+}
1 mM EGTA	$7.77 \cdot 10^{-8}$	$1.75 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$	$4.66 \cdot 10^{-7}$	$6.98 \cdot 10^{-7}$	$1.04 \cdot 10^{-6}$	$1.62 \cdot 10^{-6}$	$2.76 \cdot 10^{-6}$	$5.93 \cdot 10^{-6}$
5 mM EGTA	$1.42 \cdot 10^{-8}$	$2.92 \cdot 10^{-8}$	$4.47 \cdot 10^{-8}$	$6.09 \cdot 10^{-8}$	$7.78 \cdot 10^{-8}$	$9.54 \cdot 10^{-8}$	$1.13 \cdot 10^{-7}$	$1.33 \cdot 10^{-7}$	$1.54 \cdot 10^{-7}$
1 mM HEDTA	$3.31 \cdot 10^{-12}$	$7.45 \cdot 10^{-12}$	$1.27 \cdot 10^{-11}$	$1.99 \cdot 10^{-11}$	$2.98 \cdot 10^{-11}$	$4.47 \cdot 10^{-11}$	$6.96 \cdot 10^{-11}$	$1.19 \cdot 10^{-10}$	$2.69 \cdot 10^{-10}$

[a.] The free zinc concentrations were calculated using the program MaxChelator using the stability constants present within the program (http://www.stanford.edu/~cpatton/maxc.html/

Supporting table 3: Free Zn^{2+} concentrations in various buffering systems at pH 7.1, 20 °C. [a]

Buffering system	0.1 mM Zn^{2+}	0.2 mM Zn^{2+}	0.3 mM Zn^{2+}	0.4 mM Zn^{2+}	0.5 mM Zn^{2+}	0.6 mM Zn^{2+}	0.7 mM Zn^{2+}	0.8 mM Zn^{2+}	0.9 mM Zn^{2+}
1 mM EGTA	$5.5 \cdot 10^{-10}$	$1.2 \cdot 10^{-9}$	$2.1 \cdot 10^{-9}$	$3.3 \cdot 10^{-9}$	$4.9 \cdot 10^{-9}$	$7.4 \cdot 10^{-9}$	$1.2 \cdot 10^{-8}$	$2.0 \cdot 10^{-8}$	$4.5 \cdot 10^{-8}$
5 mM EGTA	$1.10 \cdot 10^{-10}$	$2.06 \cdot 10^{-10}$	$3.16 \cdot 10^{-10}$	$4.30 \cdot 10^{-10}$	$5.49 \cdot 10^{-10}$	$6.74 \cdot 10^{-10}$	$8.05 \cdot 10^{-10}$	$9.42 \cdot 10^{-10}$	$1.08 \cdot 10^{-9}$
1 mM HEDTA	$2.02 \cdot 10^{-13}$	$4.54 \cdot 10^{-13}$	$7.79 \cdot 10^{-13}$	$1.21 \cdot 10^{-12}$	$1.82 \cdot 10^{-12}$	$2.73 \cdot 10^{-12}$	$4.25 \cdot 10^{-12}$	$7.28 \cdot 10^{-12}$	$1.64 \cdot 10^{-11}$

[a.] The free zinc concentrations were calculated using the program MaxChelator using the stability constants present within the program (http://www.stanford.edu/~cpatton/maxc.html/

Supporting table 4: Free Zn^{2+} concentrations in various buffering systems at pH 7.8, 20 °C. [a]

Buffering system	0.1 mM Zn^{2+}	0.2 mM Zn^{2+}	0.3 mM Zn^{2+}	0.4 mM Zn^{2+}	0.5 mM Zn^{2+}	0.6 mM Zn^{2+}	0.7 mM Zn^{2+}	0.8 mM Zn^{2+}	0.9 mM Zn^{2+}
1 mM EGTA	$2.32 \cdot 10^{-11}$	$5.22 \cdot 10^{-11}$	$8.96 \cdot 10^{-11}$	$1.39 \cdot 10^{-10}$	$2.09 \cdot 10^{-10}$	$3.14 \cdot 10^{-10}$	$4.88 \cdot 10^{-10}$	$8.37 \cdot 10^{-10}$	$1.89 \cdot 10^{-9}$
1 mM HEDTA	$3.96 \cdot 10^{-14}$	$8.92 \cdot 10^{-14}$	$1.53 \cdot 10^{-13}$	$2.38 \cdot 10^{-13}$	$3.57 \cdot 10^{-13}$	$5.35 \cdot 10^{-13}$	$8.33 \cdot 10^{-13}$	$1.42 \cdot 10^{-12}$	$3.22 \cdot 10^{-12}$

[a.] The free zinc concentrations were calculated using the program MaxChelator using the stability constants present within the program (http://www.stanford.edu/~cpatton/maxc.html/

Supporting table 5: Free Zn^{2+} concentrations in various buffering systems at pH 8.0, 20 °C. [a]

Buffering system	0.1 mM Zn^{2+}	0.2 mM Zn^{2+}	0.3 mM Zn^{2+}	0.4 mM Zn^{2+}	0.5 mM Zn^{2+}	0.6 mM Zn^{2+}	0.7 mM Zn^{2+}	0.8 mM Zn^{2+}	0.9 mM Zn^{2+}
1 mM EGTA	$9.61 \cdot 10^{-12}$	$2.16 \cdot 10^{-11}$	$3.71 \cdot 10^{-11}$	$5.77 \cdot 10^{-11}$	$8.65 \cdot 10^{-11}$	$1.3 \cdot 10^{-11}$	$2.02 \cdot 10^{-10}$	$3.46 \cdot 10^{-10}$	$7.82 \cdot 10^{-10}$
1 mM HEDTA	$2.5 \cdot 10^{-14}$	$5.63 \cdot 10^{-14}$	$9.66 \cdot 10^{-14}$	$1.5 \cdot 10^{-13}$	$2.25 \cdot 10^{-13}$	$3.38 \cdot 10^{-13}$	$5.26 \cdot 10^{-13}$	$9.03 \cdot 10^{-13}$	$2.04 \cdot 10^{-12}$

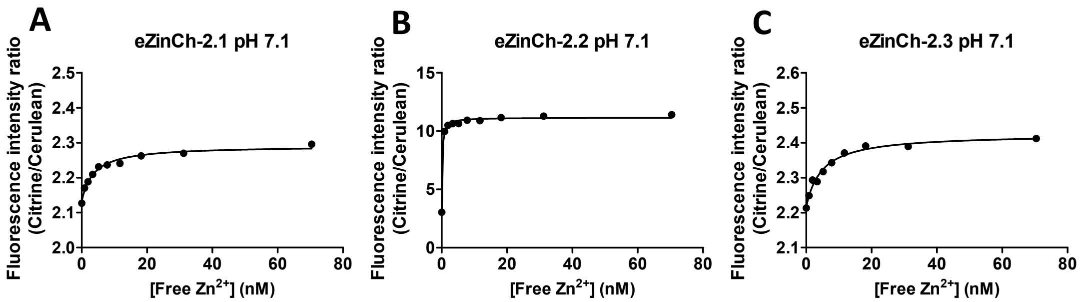
[a.] The free zinc concentrations were calculated using the program MaxChelator using the stability constants present within the program (http://www.stanford.edu/~cpatton/maxc.html/

Supporting Table 6: Cytosolic, ER and mitochondrial free Zn^{2+} concentrations measured in HeLa cells using different sensor variants

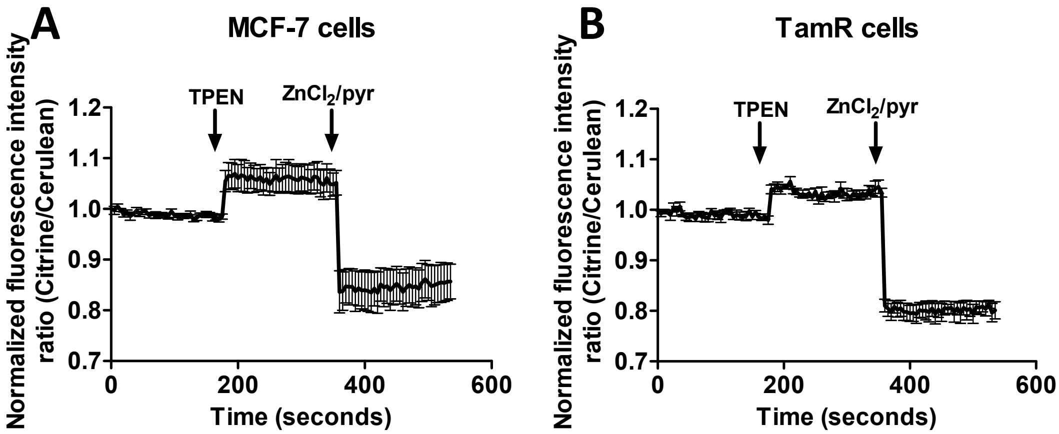
	Cytosolic Zn^{2+} concentration	ER Zn^{2+} concentration	Mitochondrial Zn^{2+} concentration
eCALWY-4	0.4[1]	>5 nM [2]	42 ± 28 pM
ZapCY-1	n.d.	0.9 ± 0.1 pM[3]	0.14 pM [4]
eZinCh-2	0.87 ± 0.1 nM	0.8 ± 0.6 nM	3.3 ± 1.2 pM

Supporting Table 7: Primers used for different cloning different eZinCh mutants

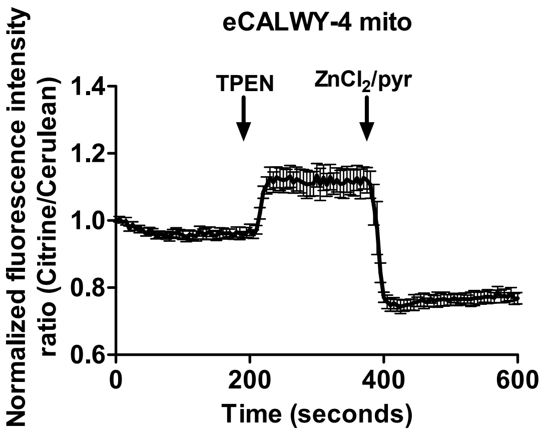
Primer	Sequence
1	5’-CACAAGCTGGAGTACCACGCCATCAGCGACAAC-3’
2	5’-CTGGGGCACAAGCTTGAGTACCACTACAACAGCCACAAC-3’
3	5’-CTGAGCACCCAGTCCCACCTGAGCAAAGACCCCAAC-3’
4	5’-CTGAGCTACCAGTCCCACCTGAGCAAAGACCCCAAC-3’
5	5’-CTGAGCACCCAGTCCTGCCTG**CA**CAAAGACCCCAACGAG-3’
6	5’-CTGAGCTACCAGTCCTGCCTG**CA**CAAAGACCCCAACGAG-3’
7	5’-CTGAGCACCCAGTCC**CA**CCTGTGCAAAGACCCCAACGAG-3’
8	5’-CTGAGCTACCAGTCC**CA**CCTGTGCAAAGACCCCAACGAG-3’



Supporting figure 1: Zinc binding properties of eZinCh mutants at pH 7.1. Emission ratio of eZinCh mutant as a function of Zn^{2+} concentration, EGTA was used as buffering system to obtain the desired free Zn^{2+} concentrations. Solid line represents a fit using a 1:1 binding model, yielding a K_d of ~1 nM at pH 7.1 for eZinCh-2.2. Titration measurements were performed using ~1 µM protein in 150 mM HEPES (pH 7.1), 100 mM NaCl, 10% (vol/vol) glycerol, 0.01% Tween and 1 mM dithiothreitol (DTT), pH 7.1 or 6.0 at 20 °C.



Supporting figure 2: Responses of MCF-7 (A), and TamR (B) cells expressing ER-eCALWY-4 to the addition of 50 µM TPEN, followed by the addition of excess 100 µM Zn^{2+}/ 5 µM pyrithione. Traces represents the average of at least four cells after normalization of the emission ratio at t=0. Error bars represent SEM.



Supporting figure 3: Representative traces of mito-eCALWY-4 expressed in HeLa cells upon addition of 50 µM TPEN, followed by the addition of excess 100 µM Zn^{2+}/5 µM pyrithione. Trace represents the average of four cells after normalization of the emission ratio at t=0. Error bars represent SEM.

```
1       atgggcagcagccatcatcatcatcatcacagcagcggcctggtgccgcgcggcagccat    60
         M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H
61      atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggac    120
         M  V  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D
121     ggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctac    180
         G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y
181     ggcaagctgaccctgaagttcatctgcaccaccggtaagctgcccgtgccctggcccacc    240
         G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T
241     ctcgtgaccaccctgacctggggcgtgcagtgcttcgcccgctaccccgaccacatgaag    300
         L  V  T  T  L  T  W  G  V  Q  C  F  A  R  Y  P  D  H  M  K
301     cagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttc    360
         Q  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  F
361     ttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctg    420
         F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L
421     gtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcac    480
         V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H
481     aagctggagtacaacgccatcagcgacaacgtctatatcaccgccgacaagcagaagaac    540
         K  L  E  Y  N  A  I  S  D  N  V  Y  I  T  A  D  K  Q  K  N
541     ggcatcaaggccaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgcc    600
         G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A
601     gaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccac    660
         D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H
661     tacctgagcacccagtcccacctgtgcaaagaccccaacgagaagcgcgatcacatggtc    720
         Y  L  S  T  Q  S  H  L  C  K  D  P  N  E  K  R  D  H  M  V
721     ctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtcc    780
         L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  S
781     ggaggcggcgagctcattcgtggcggatccggcggaagcggcggatccggcggtagcggc    840
         G  G  G  E  L  I  R  G  G  S  G  G  S  G  G  S  G  G  S  G
841     ggatccggcggctccggcggatccggcggcagcggcggatccggtggaagcggtggatcc    900
         G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
901     ggtggtagcggtggatccggtggaagcggtggatccggtggtagcggtggatccgggggt    960
         G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G
961     ccgcggggctcggtacccatggtgagcaagggcgaggagctgttcaccggggtggtgccc    1020
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         I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G
1081    gagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctg    1140
         E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L
1141    cccgtgccctggcccaccctcgtgaccaccttcggctacggcctgatgtgcttcgcccgc    1200
         P  V  P  W  P  T  L  V  T  T  F  G  Y  G  L  M  C  F  A  R
1201    taccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtc    1260
         Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y  V
1261    caggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaag    1320
         Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K
1321    ttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggac    1380
         F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D
1381    ggcaacatcctggggcacaagcttgagtacaactacaacagccacaacgtctatatcatg    1440
         G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M
1441    gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggac    1500
         A  D  K  Q  K  N  G  I  K  V  N  F  K  I  R  H  N  I  E  D
1501    ggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtg    1560
         G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V
1561    ctgctgcccgacaaccactacctgagctaccagtcccacctgtgcaaagaccccaacgag    1620
         L  L  P  D  N  H  Y  L  S  Y  Q  S  H  L  C  K  D  P  N  E
1621    aagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatg    1680
         K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M
1681    gacgagctgtacaagtaaagcggccgcact                                  1711
         D  E  L  Y  K  -
```

Supporting figure 4: Nucleotide and amino acid sequence of eZinCh-2 for the bacterial expression vector pET28a. The His-tag and thrombin cleavage site are depicted in blue and magenta respectively. Cerulean and citrine are colored cyan, and yellow respectively. The flexible peptide linker is depicted in grey. The binding pocket of the sensor is depicted in red, (206H and 208C).

```
1         atataagcagagctggtttagtgaaccgtcagatccgctagcgccgccaccatgggccat     60
           I  -  A  E  L  V  -  -  T  V  R  S  A  S  A  A  T  M  G  H
61        atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggac     120
           M  V  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D
121       ggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctac     180
           G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y
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           G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T
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           L  V  T  T  L  T  W  G  V  Q  C  F  A  R  Y  P  D  H  M  K
301       cagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttc     360
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           V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H
481       aagctggagtacaacgccatcagcgacaacgtctatatcaccgccgacaagcagaagaac     540
           K  L  E  Y  N  A  I  S  D  N  V  Y  I  T  A  D  K  Q  K  N
541       ggcatcaaggccaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgcc     600
           G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A
601       gaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccac     660
           D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H
661       tacctgagcacccagtcccacctgtgcaaagaccccaacgagaagcgcgatcacatggtc     720
           Y  L  S  T  Q  S  H  L  C  K  D  P  N  E  K  R  D  H  M  V
721       ctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtcc     780
           L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  S
781       ggaggcggcgagctcattcgtggcggatccggcggaagcggcggatccggcggtagcggc     840
           G  G  G  E  L  I  R  G  G  S  G  G  S  G  G  S  G  G  S  G
841       ggatccggcggctccggcggatccggcggcagcggcggatccggtggaagcggtggatcc     900
           G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
901       ggtggtagcggtggatccggtggaagcggtggatccggtggtagcggtggatccgggggt     960
           G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G
961       ccgcggggctcggtacccatggtgagcaagggcgaggagctgttcaccggggtggtgccc     1020
           P  R  G  S  V  P  M  V  S  K  G  E  E  L  F  T  G  V  V  P
1021      atcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggc     1080
           I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G
1081      gagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctg     1140
           E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L
1141      cccgtgccctggcccaccctcgtgaccaccttcggctacggcctgatgtgcttcgcccgc     1200
           P  V  P  W  P  T  L  V  T  T  F  G  Y  G  L  M  C  F  A  R
1201      taccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtc     1260
           Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y  V
1261      caggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaag     1320
           Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K
1321      ttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggac     1380
           F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D
1381      ggcaacatcctggggcacaagcttgagtacaactacaacagccacaacgtctatatcatg     1440
           G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M
1441      gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggac     1500
           A  D  K  Q  K  N  G  I  K  V  N  F  K  I  R  H  N  I  E  D
1501      ggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtg     1560
           G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V
1561      ctgctgcccgacaaccactacctgagctaccagtcccacctgtgcaaagaccccaacgag     1620
           L  L  P  D  N  H  Y  L  S  Y  Q  S  H  L  C  K  D  P  N  E
1621      aagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatg     1680
           K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M
1681      gacgagctgtacaagtaaagcggccgcact                                   1711
           D  E  L  Y  K  -
```

Supporting figure 5: Nucleotide and amino acid sequence of eZinCh-2 in a mammalian expression vector.Cerulean and citrine are colored cyan, and yellow respectively. The flexible peptide linker is depicted in grey. The binding pocket of the sensor is depicted in red, (206H and 208C) .

```
1       tcgaaattaatacgactcactatagggagacccaagctggctagcatggccctgtggatg      60
        S  K  L  I  R  L  T  I  G  R  P  K  L  A  S  M  A  L  W  M
61      cgcctcctgcccctgctggcgctgctggccctctggggacctgacccagccgcagccttc      120
         R  L  L  P  L  L  A  L  L  A  L  W  G  P  D  P  A  A  A  F
121     atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggac      180
         M  V  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D
181     ggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctac      240
         G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y
241     ggcaagctgaccctgaagttcatctgcaccaccggtaagctgcccgtgccctggcccacc      300
         G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T
301     ctcgtgaccaccctgacctggggcgtgcagtgcttcgcccgctaccccgaccacatgaag      360
         L  V  T  T  L  T  W  G  V  Q  C  F  A  R  Y  P  D  H  M  K
361     cagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttc      420
         Q  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  F
421     ttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctg      480
         F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L
481     gtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcac      540
         V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H
541     aagctggagtacaacgccatcagcgacaacgtctatatcaccgccgacaagcagaagaac      600
         K  L  E  Y  N  A  I  S  D  N  V  Y  I  T  A  D  K  Q  K  N
601     ggcatcaaggccaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgcc      660
         G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A
661     gaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccac      720
         D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H
721     tacctgagcacccagtcccacctgtgcaaagaccccaacgagaagcgcgatcacatggtc      780
         Y  L  S  T  Q  S  H  L  C  K  D  P  N  E  K  R  D  H  M  V
781     ctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtcc      840
         L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  S
841     ggaggcggcgagctcattcgtggcggatccggcggaagcggcggatccggcggtagcggc      900
         G  G  G  E  L  I  R  G  G  S  G  G  S  G  G  S  G  G  S  G
901     ggatccggcggctccggcggatccggcggcagcggcggatccggtggaagcggtggatcc      960
         G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
961     ggtggtagcggtggatccggtggaagcggtggatccggtggtagcggtggatccgggggt      1020
         G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G
1021    ccgcggggctcggtacccatggtgagcaagggcgaggagctgttcaccggggtggtgccc      1080
         P  R  G  S  V  P  M  V  S  K  G  E  E  L  F  T  G  V  V  P
1081    atcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggc      1140
         I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G
1141    gagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctg      1200
         E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L
1201    cccgtgccctggcccaccctcgtgaccaccttcggctacggcctgatgtgcttcgcccgc      1260
         P  V  P  W  P  T  L  V  T  T  F  G  Y  G  L  M  C  F  A  R
1261    taccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtc      1320
         Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y  V
1321    caggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaag      1380
         Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K
1381    ttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggac      1440
         F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D
1441    ggcaacatcctggggcacaagcttgagtacaactacaacagccacaacgtctatatcatg      1500
         G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M
1501    gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggac      1560
         A  D  K  Q  K  N  G  I  K  V  N  F  K  I  R  H  N  I  E  D
1561    ggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtg      1620
         G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V
1621    ctgctgcccgacaaccactacctgagctaccagtcccacctgtgcaaagaccccaacgag      1680
         L  L  P  D  N  H  Y  L  S  Y  Q  S  H  L  C  K  D  P  N  E
1681    aagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatg      1740
         K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M
1741    gacgagctgtacaaggtcgacaaggacgagctgtaagcggccgcact                   1788
         D  E  L  Y  K  V  D  K  D  E  L  -
```

Supporting figure 6: Nucleotide and amino acid sequence of ER-eZinCh-2. The N-terminal PPI signal peptide and C-terminal retention sequence KDEL are depicted in green. Cerulean and citrine are colored cyan, and yellow, respectively. The flexible peptide linker is depicted in grey. The binding pocket of the sensor is depicted in red (206H and 208C).

```
1      cggcggctcccagtgccgcgcgccaagatccattcgttggggatctcatgtccgtcctg        60
        R  R  L  P  V  P  R  A  K  I  H  S  L  G  D  L  M  S  V  L
61     acgccgctgctgctgcggggcttgacaggctcggcccggcggctcccagtgccgcgcgcc       120
        T  P  L  L  L  R  G  L  T  G  S  A  R  R  L  P  V  P  R  A
121    aagatccattcgttgccgccggagggagatctcaagagctcgagcgccaccatgggccat       180
        K  I  H  S  L  P  P  E  G  D  L  K  S  S  S  A  T  M  G  H
181    atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggac       240
         M  V  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D
241     ggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctac      300
         G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y
301     ggcaagctgaccctgaagttcatctgcaccaccggtaagctgcccgtgccctggcccacc      360
         G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T
361     ctcgtgaccaccctgacctggggcgtgcagtgcttcgcccgctaccccgaccacatgaag      420
         L  V  T  T  L  T  W  G  V  Q  C  F  A  R  Y  P  D  H  M  K
421     cagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttc      480
         Q  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  F
481     ttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctg      540
         F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L
541     gtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcac      600
         V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H
601     aagctggagtacaacgccatcagcgacaacgtctatatcaccgccgacaagcagaagaac      660
         K  L  E  Y  N  A  I  S  D  N  V  Y  I  T  A  D  K  Q  K  N
661     ggcatcaaggccaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgcc      720
         G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A
721    gaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccac       780
         D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H
781     tacctgagcacccagtcccacctgtgcaaagaccccaacgagaagcgcgatcacatggtc      840
         Y  L  S  T  Q  S  H  L  C  K  D  P  N  E  K  R  D  H  M  V
841     ctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtcc      900
         L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  S
901     ggaggcggcgagctcattcgtggcggatccggcggaagcggcggatccggcggtagcggc      960
         G  G  G  E  L  I  R  G  G  S  G  G  S  G  G  S  G  G  S  G
961     ggatccggcggctccggcggatccggcggcagcggcggatccggtggaagcggtggatcc      1020
         G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
1021    ggtggtagcggtggatccggtggaagcggtggatccggtggtagcggtggatccgggggt      1080
         G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G
1081    ccgcggggctcggtacccatggtgagcaagggcgaggagctgttcaccggggtggtgccc      1140
         P  R  G  S  V  P  M  V  S  K  G  E  E  L  F  T  G  V  V  P
1141    atcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggc      1200
         I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G
1201    gagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctg      1260
         E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L
1261    cccgtgccctggcccaccctcgtgaccaccttcggctacggcctgatgtgcttcgcccgc      1320
         P  V  P  W  P  T  L  V  T  T  F  G  Y  G  L  M  C  F  A  R
1321    taccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtc      1380
         Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y  V
1381    caggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaag      1440
         Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K
1441    ttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggac      1500
         F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D
1501    ggcaacatcctggggcacaagcttgagtacaactacaacagccacaacgtctatatcatg      1560
         G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M
1561    gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggac      1620
         A  D  K  Q  K  N  G  I  K  V  N  F  K  I  R  H  N  I  E  D
1621    ggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtg      1680
         G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V
1681    ctgctgcccgacaaccactacctgagctaccagtcccacctgtgcaaagaccccaacgag      1740
         L  L  P  D  N  H  Y  L  S  Y  Q  S  H  L  C  K  D  P  N  E
1741    aagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatg      1800
         K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M
1801   gacgagctgtacaagtaagcggccgcact                                      1830
         D  E  L  Y  K  -
```

Supporting figure 7: Nucleotide and amino acid sequence of mito-eZinCh-2. The N-terminal signal sequence cytochrome *c* oxidase subunit VIII depicted in purple. Cerulean and citrine are colored cyan, and yellow, respectively. The flexible peptide linker is depicted in grey. The binding pocket of the sensor is depicted in red (206H and 208C).

```
1      gctggtttagtgaccgtcagatccgctagtaccatgggcatgtcggctaccgctgccacc   60
        A  G  L  V  T  V  R  S  A  S  T  M  G  M  S  A  T  A  A  T
61     gtcccgcctgccgccccggccggcgagggtggcccccctgcacctcctccaaatcttacc   120
        V  P  P  A  A  P  A  G  E  G  G  P  P  A  P  P  P  N  L  T
121    agtaacaggagactgcagcagacccaggcccaggtggatgaggtggtggacatcatgagg   180
        S  N  R  R  L  Q  Q  T  Q  A  Q  V  D  E  V  V  D  I  M  R
181    gtgaatgtggacaaggtcctggagcgagaccagaagctatcggaactggatgatcgcgca   240
        V  N  V  D  K  V  L  E  R  D  Q  K  L  S  E  L  D  D  R  A
241    gatgccctccaggcaggggcctcccagtttgaaacaagtgcagccaagctcaagcgcaaa   300
        D  A  L  Q  A  G  A  S  Q  F  E  T  S  A  A  K  L  K  R  K
301    tactggtggaaaaacctcaagatgatgatcatcttgggagtgatttgcgccatcatcctc   360
        Y  W  W  K  N  L  K  M  M  I  I  L  G  V  I  C  A  I  I  L
361    atcatcatcatcgtttacttcagcactggaggctccgctagcgccgccaccatgggccat   420
        I  I  I  I  V  Y  F  S  T  G  G  S  A  S  A  A  T  M  G  H
421    atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggac   480
        M  V  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D
481    ggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctac   540
        G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y
541    ggcaagctgaccctgaagttcatctgcaccaccggtaagctgcccgtgccctggcccacc   600
        G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T
601    ctcgtgaccaccctgacctggggcgtgcagtgcttcgcccgctaccccgaccacatgaag   660
        L  V  T  T  L  T  W  G  V  Q  C  F  A  R  Y  P  D  H  M  K
661    cagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttc   720
        Q  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  F
721    ttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctg   780
        F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L
781    gtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcac   840
        V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H
841    aagctggagtacaacgccatcagcgacaacgtctatatcaccgccgacaagcagaagaac   900
        K  L  E  Y  N  A  I  S  D  N  V  Y  I  T  A  D  K  Q  K  N
901    ggcatcaaggccaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgcc   960
        G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A
961    gaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccac   1020
        D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H
1021   tacctgagcacccagtcccacctgtgcaaagaccccaacgagaagcgcgatcacatggtc   1080
        Y  L  S  T  Q  S  H  L  C  K  D  P  N  E  K  R  D  H  M  V
1081   ctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtcc   1140
        L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  S
1141   ggaggcggcgagctcattcgtggcggatccggcggaagcggcggatccggcggtagcggc   1200
        G  G  G  E  L  I  R  G  G  S  G  G  S  G  G  S  G  G  S  G
1201   ggatccggcggctccggcggatccggcggcagcggcggatccggtggaagcggtggatcc   1260
        G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
1261   ggtggtagcggtggatccggtggaagcggtggatccggtggtagcggtggatccgggggt   1320
        G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G
1321   ccgcggggctcggtacccatggtgagcaagggcgaggagctgttcaccggggtggtgccc   1380
        P  R  G  S  V  P  M  V  S  K  G  E  E  L  F  T  G  V  V  P
1381   atcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggc   1440
        I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G
1441   gagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctg   1500
        E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L
1501   cccgtgccctggcccaccctcgtgaccaccttcggctacggcctgatgtgcttcgcccgc   1560
        P  V  P  W  P  T  L  V  T  T  F  G  Y  G  L  M  C  F  A  R
1561   taccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtc   1620
        Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y  V
1621   caggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaag   1680
        Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K
1681   ttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggac   1740
        F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D
1741   ggcaacatcctggggcacaagcttgagtacaactacaacagccacaacgtctatatcatg   1800
        G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M
1801   gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggac   1860
        A  D  K  Q  K  N  G  I  K  V  N  F  K  I  R  H  N  I  E  D
1861   ggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtg   1920
        G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V
1921   ctgctgcccgacaaccactacctgagctaccagtcccacctgtgcaaagaccccaacgag   1980
        L  L  P  D  N  H  Y  L  S  Y  Q  S  H  L  C  K  D  P  N  E
1981   aagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatg   2040
        K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M
2041   gacgagctgtacaagtaagcggccgcact                                  2070
        D  E  L  Y  K  -
```

Supporting figure 8: Nucleotide and amino acid sequence of VAMP2-eZinCh-2. VAMP2, cerulean, and citrine are colored army green, cyan and yellow respectively. The flexible peptide linker is depicted in grey. The binding pocket for Zn^{2+} is depicted in red (206H and 208C).

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