Supporting Information

Robust red FRET sensors using self-associating fluorescent domains

Laurens H. Lindenburg, Anne. M. Hessels, Eduard H.T.M. Ebberink, Remco Arts and Maarten Merkx*

*Corresponding author:
Laboratory of Chemical Biology, Department of Biomedical Engineering, Eindhoven University of Technology, P.O. Box 513, 5600 MB, Eindhoven, The Netherlands. M.MERKX@TUE.NL

Table of contents:

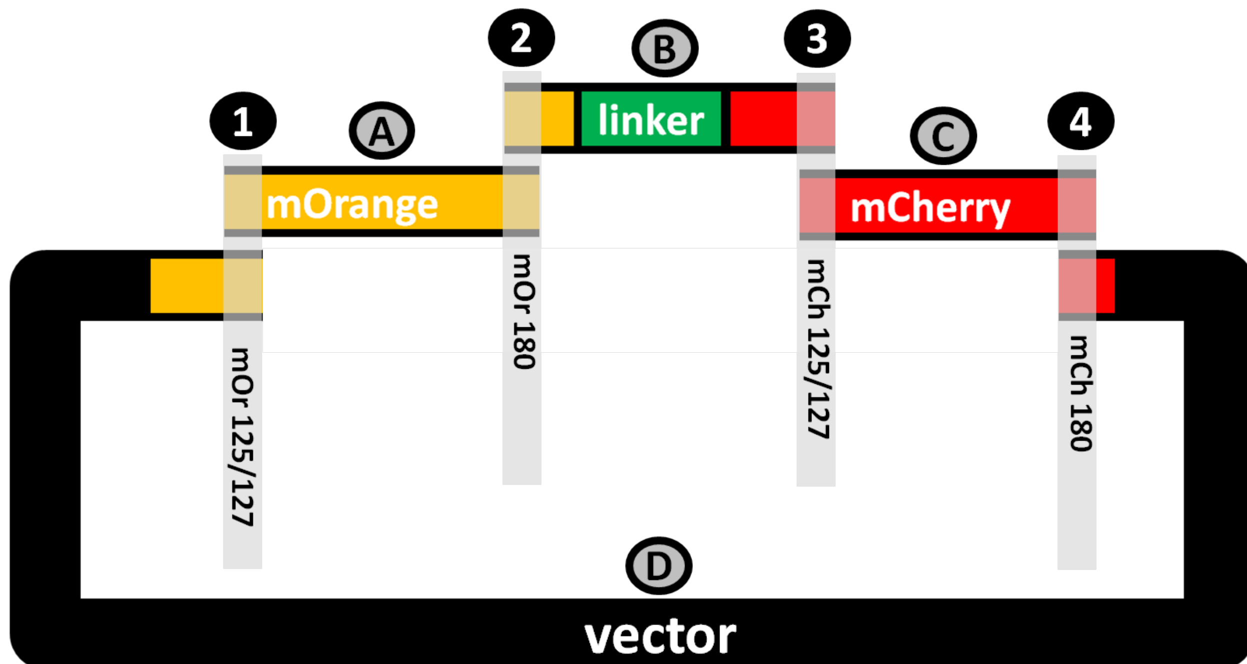
Supporting Methods	Description of the cloning and mutagenesis of genetic constructs used in this study Description of protein expression and purification	p. S2
Supporting Figure 1	Schematic overview of mutational CPEC strategy	p. S4
Supporting Table 1	Overview of primers used in mutational CPEC to generate library of mOrange-linker-mCherry variants	p. S5
Supporting Table 2	Overview of other primers used for cloning and site-directed mutagenesis	p. S6
Supporting Figure 2	Nucleotide and amino acid sequence of the mOrange-L9-mCherry ORF in bacterial expression vector pET28a	p. S7
Supporting Figure 3	Nucleotide and amino acid sequence of the redCALWY-1 ORF in bacterial expression vector pET28a	p. S9
Supporting Figure 4	Nucleotide and amino acid sequence of the redCALWY-4 ORF in mammalian expression vector pGen2.1	p. S11
Supporting Figure 5	Additional redCALWY measurements in HeLa cells	p. S13
Supporting Figure 6	Additional redCALWY measurements in HEK293 cells	p. S13

Supporting Methods

Cloning and mutagenesis. A synthetic construct encoding for mOrange-linker-mCherry inserted in a pUC57 cloning vector was ordered (Genscript, USA). Circular polymerase extension cloning (CPEC)[1] was used to clone the DNA encoding for this construct from pUC57 to the bacterial expression vector pET28a (Novagen), creating pET28a-mOrange-L9-mCherry. Briefly, primers mOrange_F and Streptag_R (Supporting Table 2) were used to amplify mOrange-linker-mCherry, while primers Strptg_dwnsvec and mOrange_throm were used to amplify and linearize pET28a (Novagen), using high-fidelity DNA polymerase (Phusion, NEB). Primers were designed to incorporate overlapping ends in the PCR fragment, allowing the subsequent CPEC reaction. A CPEC-based mutational strategy was also used to introduce the various combinations of reversions R125I, V127T and T180I (numbering relative to DsRed) in one or both of the fluorescent domains. PCR fragments were generated using primers (Supporting Table 1) that introduced one or more of the desired reversions at the ends of fragments. Fragments were then combined and circularized using CPEC (Supporting Figure 1). To produce single mCherry domains for the intermolecular dimerization studies, site-directed deletion mutagenesis was carried out using a modification of a previously published mutagenesis technique, allowing a one-step 913 bp deletion of DNA encoding mOrange and linker[2]. To generate pET28a-mCherry, a primer pair (del_mOrange_R and del_mOrange_F) was designed with complementary 5'- and 3'-ends, ensuring amplification of the entire expression plasmid except the part encoding mOrange and linker. To produce the pET28a-LSSmOrange-L9-mCherry constructs, we introduced LSSmOrange-specific mutations[3] using a mutational CPEC strategy with primer pairs A44V_R & G196D_F, A44V_F & F83L_R, F83L_F & W143M_R, W143M_F & I161D_M163L_R and I161D_M163L_F & G196D. To produce the bacterial expression vectors pET28a-redCALWY-1 and pET28a-redCALWY-4, the ATOX-L9-WD4 insert was amplified from pET28a-eCALWY-1 or pET28a-eCALWY-4 using primers Ins_redeCalwy_F and Ins_redeCalwy_R. In parallel, pET28a-mOrange-L9-mCherry was PCR-linearized using primers Vec_redeCalwy_F and Vec_redeCalwy_R and insert and vector were combined using CPEC. To produce the pGen2.1-redCALWY-4 mammalian expression construct, we took as starting template pGen2.1-mOrange2(R125I)-linker-mCherry (R125I), a vector bearing a synthetic mammalian expression construct (Genscript), restricted it with *Kpn*I and *BspE*I and ligated it to an insert cut from peCALWY-4[4] with the same restriction enzymes. pGen2.1-redCALWY-

1 was generated by reverting the cysteine-to-serine mutation of the third cysteine of the WD4 domain in pGen2.1-redCALWY-4 back to a cysteine using primers WD4_S _to_C3_F and WD4_S_to_C3_R. Primers RCS_mOr2_I125R_F and RCS_mCh_I125R_F were used to introduce the I125R mutations in pGen2.1-redCALWY-4. All plasmids were confirmed by sequencing prior to use.

Protein expression and purification. *E. coli* BL21(DE3) transformed with expression plasmid was grown to OD_{600} in 500 mL (for the red protease constructs) or 2 L (for the redCALWY constructs) 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. 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 and were subsequently loaded onto a size exclusion column (Sephacryl S200, GE Healthcare). The SEC fractions were analyzed by SDS PAGE for correct size and purity, pooled and concentrated using 10 kDa MWCO centrifugation filters. The single domain mCherry constructs used for anisotropy studies were purified by Ni-NTA chromatography only, as SDS-PAGE revealed this single purification step to result in sufficient purity of these constructs. Since His-tags are known to readily bind zinc[5], they were removed from all Zn^{2+}-sensor 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 $CaCl_2$) using PD10 desalting columns (GE, Healthcare). Cleavage of the thrombin recognition site between the His-tag and the mOrange 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 4 °C 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. Elution of protein without a His-tag from the Ni-NTA required a buffer containing 30 mM imidazole, presumably due to a weak affinity of the WD4 and/or ATOX1 domain for Ni-NTA, as previously noted for Wilson disease proteins[6]. Uncleaved protein remained behind on the Ni-column at this imidazole concentration. The red Zn^{2+} sensor proteins were subsequently further purified by SEC chromatography. The SEC fractions were analyzed by SDS PAGE for correct size and purity, pooled and concentrated using 10 kDa MWCO centrifugation filters.



Supporting Figure 1. Schematic overview of mutational CPEC strategy. In order to introduce mutations at four different positions in the mOrange-linker-mCherry protease sensor simultaneously, a mutational CPEC strategy was followed. At each of the positions, mOrange 125/127, mOrange 180, mCherry 125/127 and mOrange 180 (labeled 1 to 4 respectively), primers were designed to allow amplification of four different fragments (labeled A, B, C and D). Primers were designed to incorporate different mutations into the ends of PCR fragments in such a way that both ends of each fragment overlapped with the ends of adjacent fragments, with a melting temperature of about 72 °C (calculated using Thermo Scientific's online Tm tool (http://www.thermoscientificbio.com/webtools/tmc/)). These fragments were subsequently recombined in a CPEC reaction, at equimolar concentration and 200 ng of vector fragment D. The primers used at positions 1 to 4, as well as the mutations they were designed to introduce, are listed in Table S1.

Supporting Table 1. Overview of primers used in mutational CPEC to generate small library of mOrange-linker-mCherry variants.

Each primer is designed to introduce a mutation at a location indicated schematically in Supporting Figure 1.

Mutation	Position/Fragment	Primer orientation	Sequence (5'-->3')	primer number
mOrange R125I	1/D	anti-sense	CGGAAAGTTAGTGCC**AAT**CAGCTTCAC	1
mOrange R125I/T127V	1/D	anti-sense	CGGAAAGTTA**AC**GCC**AAT**CAGCTTCAC	2
mOrange T127V	1/D	anti-sense	GGAAAGTTA**AC**GCCGCGCAGC	3
none	1/D	anti-sense	GGAAAGTTAGTGCCGCGCAGC	4
mOrange R125I	1/A	sense	GTGAAGCTG**ATT**GGCACTAACTTTCCG	5
mOrange R125I/T127V	1/A	sense	GTGAAGCTG**ATT**GGC**GT**TAACTTTCCG	6
mOrange T127V	1/A	sense	GCTGCGCGGC**GT**TAACTTTCC	7
none	1/A	sense	GCTGCGCGGCACTAACTTTCC	8
mOrange T180 (WT)	2/A	anti-sense	GGCTTTGTAGGTGGTTTTAACTTCAGAGG	9
mOrange T180I	2/A	anti-sense	GGCTTTGTAG**A**TGGTTTTAACTTCAGAGGTG	10
none	2/B	sense	CCTCTGAAGTTAAAACCACCTACAAAGCC	11
mOrange T180I	2/B	sense	CACCTCTGAAGTTAAAACCA**T**CTACAAAGCC	12
mCherry R125I	3/B	anti-sense	CAGACGGGAAGTTAGTACC**AAT**CAGTTTTACTTTATAG	13
mCherry R125I/T127V	3/B	anti-sense	CAGACGGGAAGTTA**AC**ACC**AAT**CAGTTTTACTTTATAG	14
mCherry T127V	3/B	anti-sense	CGGGAAGTTA**AC**ACCACGCAGTTTTACTTTATAG	15
mCherry T127V	3/B	anti-sense	CGGGAAGTTAGTACCACGCAGTTTTACTTTATAG	16
mCherry R125I	3/C	sense	CTATAAAGTAAAACTG**ATT**GGTACTAACTTCCCGTCTG	17
mCherry R125I/T127V	3/C	sense	CTATAAAGTAAAACTG**ATT**GGT**GT**TAACTTCCCGTCTG	18
mCherry T127V	3/C	sense	CTATAAAGTAAAACTGCGTGGT**GT**TAACTTCCCG	19
none	3/C	sense	CTATAAAGTAAAACTGCGTGGTACTAACTTCCCG	20
none	4/C	anti-sense	CGCTTTGTACGTGGTTTTCACTTCC	21
mCherry T180I	4/C	anti-sense	GCTTTGTA**GA**TGGTTTTCACTTCCGC	22
none	4/D	sense	GGAAGTGAAAACCACGTACAAAGCG	23
mCherry T180I	4/D	sense	GCGGAAGTGAAAACCA**TC**TACAAAGC	24

Supporting Table 2. Overview of other primers used for cloning and site-directed mutagenesis.

Name	Sequence (5'-->3')
Strptg_dwnsvec	GTCCCATCCGCAGTTCGAGAAATAAGCTGAGCAATAACTAGCATAACCCCTTG
mOrange_F	ATGGTGAGCAAAGGTGAGGAAAAC
Streptag_R	TTATTTCTCGAACTGCGGATGGGAC
mOrange_throm	GTTTTCCTCACCTTTGCTCACCATATGGCTGCCGCGCGG
del_mCherry_F	CGAACTGTATAAATAAGCTGAGCAATAACTAGCATAACCCCTTGGGGCC
del_mOrange_R	GTCCTCTTCGCCCTTAGAAACCATATGGCTGCCGCGCG
del_mOrange_F	GTTTCTAAGGGCGAAGAGGACAATATGGCTATCATCAAAGAGTTCATGCG
del_mCherry_R	GTTATTGCTCAGCTTATTTATACAGTTCGTCCATACCGCCAGTAGAATGACG
A44V_F	TTTCCAGACTGTTAAACTGAAGGTGACCAAAGGCGG
A44V_R	ACCTTCAGTTTAACAGTCTGGAAGCCCTCATACGG
F83L_F	TCCCCGATTATTTAAAACTGTCATTCCCGGAAGGCTTCAAATG
F83L_R	GACAGTTTTAAATAATCGGGGATATCTGCAGGGTGTTTAACATACGCCTTG
W143M_F	ACTATGGGCATGGAAGCTTCCTCTGAACGTATGTATCCG
W143M_R	GAAGCTTCCATGCCCATAGTCTTTTTCTGCATAACCG
I161D&M160L_mOrF	GGTGAGGACAAACTGCGTCTGAAGCTGAAAGATGGC
I163D&M160L_mOrR	GACGCAGTTTGTCCTCACCTTTCAGCGCGCC
G196D_F	TGCTTACATTGTAGATATTAAACTGGATATCACGAGCCATAACGAG
G196D_R	CCAGTTTAATATCTACAATGTAAGCACCCGGCAGTTG
Vec_redeCalwy_F	TTCAGTCTCGGTACCGATGGTTTCTAAGGGCGAAGAGGACA
Vec_redeCalwy_R	GCATTCCGGATTTATACAGTTCGTCCATACCGCCAG
Ins_redeCalwy_F	GGACGAACTGTATAAATCCGGAATGCCGAAGCAC
Ins_redeCalwy_R	TTAGAAACCATCGGTACCGAGACTGAAGCCTCAAATC
WD4_S_to_C3_F	ATGACCTGTGCATCCTGTGTCCATTCCATTG
WD4_S_to_C3_R	CACAGGATGCACAGGTCATGCCGGCAATGG
RCS_mOr2_I125R_F	CTATAAAGTAAAACTGCGTGGTACGAATTTTCCCTCAGATGGACC
RCS_mOr2_I125R_R	CGTACCACGCAGTTTTACTTTATAGATGAACTCCCCATCCTG
RCS_mCh_I125R_F	CAAGGTGAAGCTTAGAGGCACAAACTTTCCAAGCGACG
RCS_mCh_I125R_R	GTGCCTCTAAGCTTCACCTTGTATATAAACTCGCCG

```
1     atgggcagcagccatcatcatcatcatcacagcagcggcctggtgccgcgcggcagccat  60
       M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H
61    atggtgagcaaaggtgaggaaaacaatatggcaatcattaaagagtttatgcgtttcaag  120
       M  V  S  K  G  E  E  N  N  M  A  I  I  K  E  F  M  R  F  K
121   gtccgtatggaaggttctgtgaacggtcacgagttcgagattgaaggcgagggtgagggt  180
       V  R  M  E  G  S  V  N  G  H  E  F  E  I  E  G  E  G  E  G
181   cgtccgtatgagggcttccagactgctaaactgaaggtgaccaaaggcggtcctctgccg  240
       R  P  Y  E  G  F  Q  T  A  K  L  K  V  T  K  G  G  P  L  P
241   ttcgcctgggatatcctgtctccgcaattcacatatgggtccaaggcgtatgttaaacac  300
       F  A  W  D  I  L  S  P  Q  F  T  Y  G  S  K  A  Y  V  K  H
301   cctgcggatatcccggattatttcaaactgtccttcccggaaggcttcaaatgggagcgt  360
       P  A  D  I  P  D  Y  F  K  L  S  F  P  E  G  F  K  W  E  R
361   gtaatgaacttcgaggacggcggcgtcgtaaccgttacccaggactcctctctgcaggac  420
       V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D  S  S  L  Q  D
421   ggtgagtttatttacaaagtgaagctgcgcggcactaactttccgtctgacggtccggtt  480
       G  E  F  I  Y  K  V  K  L  R  G  T  N  F  P  S  D  G  P  V
481   atgcagaaaaagactatgggctgggaagcttcctctgaacgtatgtatccggaagacggc  540
       M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M  Y  P  E  D  G
541   gcgctgaaaggtgagatcaaaatgcgtctgaagctgaaagatggcggtcattacacctct  600
       A  L  K  G  E  I  K  M  R  L  K  L  K  D  G  G  H  Y  T  S
601   gaagttaaaaccacctacaaagccaaaaagccggtacaactgccgggtgcttacattgtt  660
       E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P  G  A  Y  I  V
661   ggtattaaactggatatcacgagccataacgaggattacacgattgttgaacagtacgaa  720
       G  I  K  L  D  I  T  S  H  N  E  D  Y  T  I  V  E  Q  Y  E
721   cgtgccgagggccgtcattctactggcggtatggacgaactgtataaagaattcggcgga  780
       R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y  K  E  F  G  G
781   tccggcggaagcggcggatccggcggtagcggcggatccggcggctccggcggatccggc  840
       S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G
841   ggcagcggcggatccggtggaagcggtggatccggtggtagcggtggatccggtggaagc  900
       G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
901   ggtggatccggtggtagcggtggatccgggggtagtgaaaacctgtatttccagtccggt  960
       G  G  S  G  G  S  G  G  S  G  G  S  E  N  L  Y  F  Q  S  G
961   ggttctggtggttctatggtttctaagggcgaagaggacaatatggctatcatcaaagag  1020
       G  S  G  G  S  M  V  S  K  G  E  E  D  N  M  A  I  I  K  E
1021  ttcatgcgttttaaagtacacatggaaggctccgttaacggtcacgagtttgaaattgaa  1080
       F  M  R  F  K  V  H  M  E  G  S  V  N  G  H  E  F  E  I  E
1081  ggtgagggcgaaggtcgcccgtacgaaggcactcaaacggcgaagctgaaagtgaccaaa  1140
       G  E  G  E  G  R  P  Y  E  G  T  Q  T  A  K  L  K  V  T  K
1141  ggtggcccactgccgttcgcttgggacattctgtccccgcaattcatgtacggttctaaa  1200
       G  G  P  L  P  F  A  W  D  I  L  S  P  Q  F  M  Y  G  S  K
1201  gcgtacgtgaaacacccggcggatattccggattacctgaaactgtctttcccggaaggt  1260
       A  Y  V  K  H  P  A  D  I  P  D  Y  L  K  L  S  F  P  E  G
1261  ttcaaatgggaacgtgttatgaacttcgaagacggtggtgttgtaacggttactcaggac  1320
       F  K  W  E  R  V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D
1321  tctagcctgcaggacggcgaatttatctataaagtaaaactgcgtggtactaacttcccg  1380
       S  S  L  Q  D  G  E  F  I  Y  K  V  K  L  R  G  T  N  F  P
1381  tctgatggcccggttatgcaaaagaaaactatgggttgggaagcttctagcgaacgcatg  1440
       S  D  G  P  V  M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M
1441  tacccggaggacggtgccctgaaaggtgaaatcaaacaacgcctgaaactgaaggacggc  1500
       Y  P  E  D  G  A  L  K  G  E  I  K  Q  R  L  K  L  K  D  G
1501  ggccactacgatgcggaagtgaaaaccacgtacaaagcgaagaaaccagtacagctgcct  1560
       G  H  Y  D  A  E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P
1561  ggtgcctacaatgtgaatatcaaactggatattacctcccataacgaagactataccatc  1620
       G  A  Y  N  V  N  I  K  L  D  I  T  S  H  N  E  D  Y  T  I
1621  gtcgaacagtatgaacgtgctgaaggtcgccatagcaccggtggcatggatgagttatac  1680
       V  E  Q  Y  E  R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y
1681  aagtctagcggtctggttccacgcggtagccactggtcccatccgcagttcgagaaataa  1740
       K  S  S  G  L  V  P  R  G  S  H  W  S  H  P  Q  F  E  K  -
```

Supporting Figure 2. Nucleotide and amino acid sequence of the mOrange-L9-mCherry ORF in bacterial expression vector pET28a. The DNA sequence is shown in lowercase, with the single letter amino acid code shown beneath each codon in uppercase. The His-tag is highlighted in dark yellow, the two thrombin cleavage sites (found in both the N- and the C-terminal regions) in green, mOrange in yellow, the linker (interrupted by a TEV cleavage site highlighted in turquoise) in bright green, mCherry in red, and the Strep-II tag in black. Positions that were subjected to mutagenesis to test for enhanced FP self-association, R125, T127 and T180, are highlighted in grey in both the mOrange and mCherry sequences. Positions that were mutated to convert mOrange to LSSmOrange, A44, F83, W143, I161, M163 and G196 are highlighted in dark blue.

```
1     atgggcagcagccatcatcatcatcatcacagcagcggcctggtgccgcgcggcagccat   60
       M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H
61    atggtgagcaaaggtgaggaaaacaatatggcaatcattaaagagtttatgcgtttcaag   120
       M  V  S  K  G  E  E  N  N  M  A  I  I  K  E  F  M  R  F  K
121   gtccgtatggaaggttctgtgaacggtcacgagttcgagattgaaggcgagggtgagggt   180
       V  R  M  E  G  S  V  N  G  H  E  F  E  I  E  G  E  G  E  G
181   cgtccgtatgagggcttccagactgctaaactgaaggtgaccaaaggcggtcctctgccg   240
       R  P  Y  E  G  F  Q  T  A  K  L  K  V  T  K  G  G  P  L  P
241   ttcgcctgggatatcctgtctccgcaattcacatatgggtccaaggcgtatgttaaacac   300
       F  A  W  D  I  L  S  P  Q  F  T  Y  G  S  K  A  Y  V  K  H
301   cctgcggatatcccggattatttcaaactgtccttcccggaaggcttcaaatgggagcgt   360
       P  A  D  I  P  D  Y  F  K  L  S  F  P  E  G  F  K  W  E  R
361   gtaatgaacttcgaggacggcggcgtcgtaaccgttacccaggactcctctctgcaggac   420
       V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D  S  S  L  Q  D
421   ggtgagtttatttacaaagtgaagctgattggcactaactttccgtctgacggtccggtt   480
       G  E  F  I  Y  K  V  K  L  I  G  T  N  F  P  S  D  G  P  V
481   atgcagaaaaagactatgggctgggaagcttcctctgaacgtatgtatccggaagacggc   540
       M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M  Y  P  E  D  G
541   gcgctgaaaggtgagatcaaaatgcgtctgaagctgaaagatggcggtcattacacctct   600
       A  L  K  G  E  I  K  M  R  L  K  L  K  D  G  G  H  Y  T  S
601   gaagttaaaaccacctacaaagccaaaaagccggtacaactgccgggtgcttacattgtt   660
       E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P  G  A  Y  I  V
661   ggtattaaactggatatcacgagccataacgaggattacacgattgttgaacagtacgaa   720
       G  I  K  L  D  I  T  S  H  N  E  D  Y  T  I  V  E  Q  Y  E
721   cgtgccgagggccgtcattctactggcggtatggacgaactgtataaatccggaatgccg   780
       R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y  K  S  G  M  P
781   aagcacgagttctctgtggacatgacctgtggaggctgtgctgaagctgtctctcgggtc   840
       K  H  E  F  S  V  D  M  T  C  G  G  C  A  E  A  V  S  R  V
841   ctcaataagcttggaggagttaagtatgacattgacctgcccaacaagaaggtctgcatt   900
       L  N  K  L  G  G  V  K  Y  D  I  D  L  P  N  K  K  V  C  I
901   gaatctgagcacagcatggacactctgcttgcaaccctgaagaaaacaggaaagactgtt   960
       E  S  E  H  S  M  D  T  L  L  A  T  L  K  K  T  G  K  T  V
961   tcctaccttggccttgagctcattcgtggcggatccggcggaagcggcggatccggcggt   1020
       S  Y  L  G  L  E  L  I  R  G  G  S  G  G  S  G  G  S  G  G
1021  agcggcggatccggcggctccggcggatccggcggcagcggcggatccggtggaagcggt   1080
       S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G
1081  ggatccggtggtagcggtggatccggtggaagcggtggatccggtggtagcggtggatcc   1140
       G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
1141  gggggtccgcggatgcagggcacatgcagtaccactctgattgccattgccggcatgacc   1200
       G  G  P  R  M  Q  G  T  C  S  T  T  L  I  A  I  A  G  M  T
1201  tgtgcatcctgtgtccattccattgaaggcatgatctcccaactggaaggggtgcagcaa   1260
       C  A  S  C  V  H  S  I  E  G  M  I  S  Q  L  E  G  V  Q  Q
1261  atatcggtgtctttggccgaagggactgcaacagttctttataatcccgctgtaattagc   1320
       I  S  V  S  L  A  E  G  T  A  T  V  L  Y  N  P  A  V  I  S
1321  ccagaagaactcagagctgctatagaagacatgggatttgaggcttcagtctcggtaccc   1380
       P  E  E  L  R  A  A  I  E  D  M  G  F  E  A  S  V  S  V  P
1381  atggtttctaagggcgaagaggacaatatggctatcatcaaagagttcatgcgttttaaa   1440
       M  V  S  K  G  E  E  D  N  M  A  I  I  K  E  F  M  R  F  K
1441  gtacacatggaaggctccgttaacggtcacgagtttgaaattgaaggtgagggcgaaggt   1500
       V  H  M  E  G  S  V  N  G  H  E  F  E  I  E  G  E  G  E  G
1501  cgcccgtacgaaggcactcaaacggcgaagctgaaagtgaccaaaggtggcccactgccg   1560
       R  P  Y  E  G  T  Q  T  A  K  L  K  V  T  K  G  G  P  L  P
1561  ttcgcttgggacattctgtccccgcaattcatgtacggttctaaagcgtacgtgaaacac   1620
       F  A  W  D  I  L  S  P  Q  F  M  Y  G  S  K  A  Y  V  K  H
1621  ccggcggatattccggattacctgaaactgtctttcccggaaggtttcaaatgggaacgt   1680
       P  A  D  I  P  D  Y  L  K  L  S  F  P  E  G  F  K  W  E  R
1681  gttatgaacttcgaagacggtggtgttgtaacggttactcaggactctagcctgcaggac   1740
       V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D  S  S  L  Q  D
1741  ggcgaatttatctataaagtaaaactgattggtactaacttcccgtctgatggcccggtt   1800
       G  E  F  I  Y  K  V  K  L  I  G  T  N  F  P  S  D  G  P  V
1801  atgcaaaagaaaactatgggttgggaagcttctagcgaacgcatgtacccggaggacggt   1860
       M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M  Y  P  E  D  G
1861  gccctgaaaggtgaaatcaaacaacgcctgaaactgaaggacggcggccactacgatgcg   1920
       A  L  K  G  E  I  K  Q  R  L  K  L  K  D  G  G  H  Y  D  A
1921  gaagtgaaaaccacgtacaaagcgaagaaaccagtacagctgcctggtgcctacaatgtg   1980
       E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P  G  A  Y  N  V
1981  aatatcaaactggatattacctcccataacgaagactataccatcgtcgaacagtatgaa   2040
       N  I  K  L  D  I  T  S  H  N  E  D  Y  T  I  V  E  Q  Y  E
2041  cgtgctgaaggtcgccatagcaccggtggcatggatgagttatacaagtctagcggtctg   2100
       R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y  K  S  S  G  L
2101  gttccacgcggtagccactggtcccatccgcagttcgagaaataa
       V  P  R  G  S  H  W  S  H  P  Q  F  E  K  -
```

Supporting Figure 3. Nucleotide and amino acid sequence of the redCALWY-1 ORF in bacterial expression vector pET28a. The DNA sequence is shown in lowercase, with the single letter amino acid code shown beneath each codon in uppercase. The His-tag

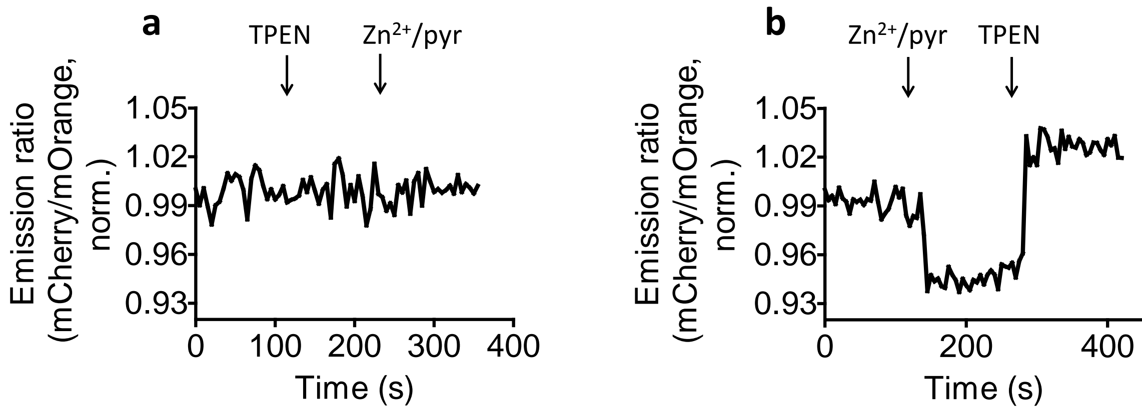
is highlighted in dark yellow, the two thrombin cleavage sites (at the N- and at the C-terminus) in green, mOrange in yellow, the ATOX1 domain in turquoise, the linker in bright green, the WD4 domain in dark blue, mCherry in red, and the Strep-II tag in black. Reversion R125I is highlighted in grey in both the mOrange and mCherry sequences. Finally, the third cysteine residue located in WD4, mutated to serine in redCALWY-4, is highlighted in dark red.

```
1    atggattacaaggatgacgacgataaggcggccgcgatggtttctaaaggcgaggagaat    60
      M  D  Y  K  D  D  D  D  K  A  A  A  M  V  S  K  G  E  E  N
61   aatatggcaatcattaaagagtttatgagattcaaggtcagaatggaaggaagcgtgaat    120
      N  M  A  I  I  K  E  F  M  R  F  K  V  R  M  E  G  S  V  N
```

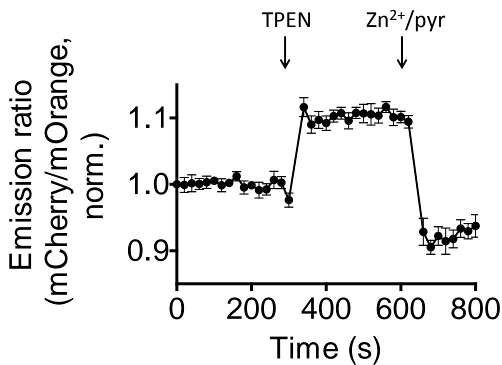
```
121   ggtcatgagttcgaaattgaaggcgagggcgagggaaggccttatgaggggtttcaaaca   180
       G  H  E  F  E  I  E  G  E  G  E  G  R  P  Y  E  G  F  Q  T
181   gcaaaactcaaggttactaagggcgggccccttccatttgcctgggatatactgtctccg   240
       A  K  L  K  V  T  K  G  G  P  L  P  F  A  W  D  I  L  S  P
241   cacttcacctatggatctaaggcttacgtgaaacaccctgccgatattccagattatttt   300
       H  F  T  Y  G  S  K  A  Y  V  K  H  P  A  D  I  P  D  Y  F
301   aaactgtccttccccgaagggttcaagtgggagagagtaatgaactatgaggacggcggc   360
       K  L  S  F  P  E  G  F  K  W  E  R  V  M  N  Y  E  D  G  G
361   gttgtgacagttactcaggattcatcgttacaggatggggagttcatctataaagtaaaa   420
       V  V  T  V  T  Q  D  S  S  L  Q  D  G  E  F  I  Y  K  V  K
421   ctgattggtacgaattttccctcagatggacctgtcatgcagaagaagaccatgggttgg   480
       L  I  G  T  N  F  P  S  D  G  P  V  M  Q  K  K  T  M  G  W
481   gaggcaagttcagagagaatgtaccccgaggacggcgcgctaaagggtaaaatcaagatg   540
       E  A  S  S  E  R  M  Y  P  E  D  G  A  L  K  G  K  I  K  M
541   aggctgaagctcaaggacggcgggcattacacttctgaagtaaaaacaacttataaggct   600
       R  L  K  L  K  D  G  G  H  Y  T  S  E  V  K  T  T  Y  K  A
601   aaaaaaccagtgcaattaccaggggcttacatagtggacatcaagctcgacatcacaagc   660
       K  K  P  V  Q  L  P  G  A  Y  I  V  D  I  K  L  D  I  T  S
661   cataatgaggactacactattgtggagcagtatgagcgtgctgaaggtcgccacagtaca   720
       H  N  E  D  Y  T  I  V  E  Q  Y  E  R  A  E  G  R  H  S  T
721   ggaggtatggacgaactgtacaagtccggaatgccgaagcacgagttctctgtggacatg   780
       G  G  M  D  E  L  Y  K  S  G  M  P  K  H  E  F  S  V  D  M
781   acctgtggaggctgtgctgaagctgtctctcgggtcctcaataagcttggaggagttaag   840
       T  C  G  G  C  A  E  A  V  S  R  V  L  N  K  L  G  G  V  K
841   tatgacattgacctgcccaacaagaaggtctgcattgaatctgagcacagcatggacact   900
       Y  D  I  D  L  P  N  K  K  V  C  I  E  S  E  H  S  M  D  T
901   ctgcttgcaaccctgaagaaaacaggaaagactgtttcctaccttggccttgagctcatt   960
       L  L  A  T  L  K  K  T  G  K  T  V  S  Y  L  G  L  E  L  I
961   cgtggcggatccggcggaagcggcggatccggcggtagcggcggatccggcggctccggc   1020
       R  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G
1021  ggatccggcggcagcggcggatccggtggaagcggtggatccggtggtagcggtggatcc   1080
       G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S  G  G  S
1081  ggtggaagcggtggatccggtggtagcggtggatccgggggtccgcggatgcagggcaca   1140
       G  G  S  G  G  S  G  G  S  G  G  S  G  G  P  R  M  Q  G  T
1141  tgcagtaccactctgattgccattgccggcatgacctgtgcatccagtgtccattccatt   1200
       C  S  T  T  L  I  A  I  A  G  M  T  C  A  S  S  V  H  S  I
1201  gaaggcatgatctcccaactggaaggggtgcagcaaatatcggtgtctttggccgaaggg   1260
       E  G  M  I  S  Q  L  E  G  V  Q  Q  I  S  V  S  L  A  E  G
1261  actgcaacagttctttataatcccgctgtaattagcccagaagaactcagagctgctata   1320
       T  A  T  V  L  Y  N  P  A  V  I  S  P  E  E  L  R  A  A  I
1321  gaagacatgggatttgaggcttcagtctcggtacccatggtgagcaagggtgaagaagac   1380
       E  D  M  G  F  E  A  S  V  S  V  P  M  V  S  K  G  E  E  D
1381  aacatggccattattaaagaattcatgcgctttaaggtgcatatggaagggtccgtgaat   1440
       N  M  A  I  I  K  E  F  M  R  F  K  V  H  M  E  G  S  V  N
1441  gggcacgaattcgaaatagagggagaaggggaaggacggccttacgagggaacccagaca   1500
       G  H  E  F  E  I  E  G  E  G  E  G  R  P  Y  E  G  T  Q  T
1501  gcaaagctcaaagtgaccaagggaggaccccttccctttgcatgggatattttgtctcca   1560
       A  K  L  K  V  T  K  G  G  P  L  P  F  A  W  D  I  L  S  P
1561  cagttcatgtacggcagcaaagcttacgtgaagcaccccgcagacatccccgattactta   1620
       Q  F  M  Y  G  S  K  A  Y  V  K  H  P  A  D  I  P  D  Y  L
1621  aaactgagtttccctgaaggcttcaagtgggaacgcgtgatgaactttgaagatggagga   1680
       K  L  S  F  P  E  G  F  K  W  E  R  V  M  N  F  E  D  G  G
1681  gtcgtgaccgtcacccaggattcatccctccaagacggcgagtttatatacaaggtgaag   1740
       V  V  T  V  T  Q  D  S  S  L  Q  D  G  E  F  I  Y  K  V  K
1741  cttatcggcacaaactttccaagcgacggccccgtgatgcaaaaaaagaccatgggctgg   1800
       L  I  G  T  N  F  P  S  D  G  P  V  M  Q  K  K  T  M  G  W
1801  gaagcaagcagtgaacgaatgtacccagaggatggtgcgctgaagggagaaataaaacag   1860
       E  A  S  S  E  R  M  Y  P  E  D  G  A  L  K  G  E  I  K  Q
1861  agactaaaactgaaagacggggggacactacgacgccgaggtgaagacgacatacaaagcg   1920
       R  L  K  L  K  D  G  G  H  Y  D  A  E  V  K  T  T  Y  K  A
1921  aagaagcccgtccagctgcctggcgcctataatgtgaatatcaaactggacatcacctcc   1980
       K  K  P  V  Q  L  P  G  A  Y  N  V  N  I  K  L  D  I  T  S
1981  cacaacgaggactacacaatagtggaacagtatgagcgagccgaaggaaggcattccacc   2040
       H  N  E  D  Y  T  I  V  E  Q  Y  E  R  A  E  G  R  H  S  T
2041  gggggcatggatgagttatataaataa
       G  G  M  D  E  L  Y  K  -
```

Supporting Figure 4. Nucleotide and amino acid sequence of the redCALWY-4 ORF in mammalian expression vector pGen2.1. The DNA sequence is shown in lowercase, with the single letter amino acid code shown beneath each codon in uppercase. The FLAG-tag is highlighted in dark yellow, mOrange2 in yellow, the ATOX1 domain in turquoise, the linker in bright green, the WD4

domain in dark blue and mCherry in red. Reversion R125I is highlighted in grey in both the mOrange and mCherry sequences. Finally, redCALWY-4's cysteine to serine mutation in WD4 is highlighted in dark red.



Supporting Figure 5. Additional redCALWY measurements in HeLa cells. (a) HeLa cell transfected with redCALWY-4 with wild-type mOrange and mCherry domains (lacking the R125I mutations) were exposed first to 50 μM TPEN followed by 100 μM $ZnCl_2$ and 10 μM pyrithione. (b) HeLa cell transfected with redCALWY-4 were exposed first to 10 μM $ZnCl_2$ and 1 μM pyrithione, followed by 50 μM TPEN.



Supporting Figure 6. Response of redCALWY-4 in HEK293 cells. Cells transfected with redCALWY-4 were exposed first to 50 μM TPEN, followed by 100 μM $ZnCl_2$ and 5 μM pyrithione. The trace represents the average of four cells after normalization of the emission ratio at t=0. Error bars represent SEM.

Supporting references

1. Quan, J., and Tian, J. (2009) Circular polymerase extension cloning of complex gene libraries and pathways, *PLoS One 4*, e6441.
2. Liu, H., and Naismith, J. H. (2008) An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol, *BMC Biotechnol. 8*, 91.
3. Shcherbakova, D. M., Hink, M. A., Joosen, L., Gadella, T. W., and Verkhusha, V. V. (2012) An orange fluorescent protein with a large Stokes shift for single-excitation multicolor FCCS and FRET imaging, *J. Am. Chem. Soc. 134*, 7913-7923.
4. Vinkenborg, J. L., Nicolson, T. J., Bellomo, E. A., Koay, M. S., Rutter, G. A., and Merkx, M. (2009) Genetically encoded FRET sensors to monitor intracellular Zn2+ homeostasis, *Nat. Methods 6*, 737-740.
5. Evers, T. H., Appelhof, M. A., Meijer, E. W., and Merkx, M. (2008) His-tags as Zn(II) binding motifs in a protein-based fluorescent sensor, *Protein Eng. Des. Sel. 21*, 529-536.
6. Wernimont, A. K., Yatsunyk, L. A., and Rosenzweig, A. C. (2004) Binding of copper(I) by the Wilson disease protein and its copper chaperone, *J. Biol. Chem. 279*, 12269-12276.