Supplementary Information for „A Dynamic FRET Reporter of Gene Expression Improved By Functional Screening“ by Schifferer & Griesbeck

Experimental Procedures, supplementary figures 1-8, supplementary table 1.

Experimental Procedures

cDNA cloning and gene construction.

All cloning experiments were performed using E. coli XL1 blue. For the initial peptide-aptamer FRET screen, all cDNAs encoding the different peptides were PCR-amplified without template using self-annealing primers that introduce a 5' *SphI* and a 3' *SacI* site. (5'-ATGGCCCGTGATCGTCGCCGTCGTGGCAGTCGCCCTAGTGGCGCCGAGCGTCGCCGTCG CCGTGCAGCTGCGGCGAATGCAGCAAAT-3'; 5'-CCCCCCCG GCATGCAA ATG GCC CGT GATCGTCGCCGTCGTGGCAGTCGCCCTAGTGGCGCCGAGCGTCG-3'; 5'-CCCCCCCGGAGCTCATTTGCTGCATTCGCCGCAGCTGCACGGCGACGGCGACGCTCGGC GCCACT-3'). The digested PCR-products were ligated between eCFP (Δ11 C-term) and cpCitrine of pRSETB-TnXL[16] using the introduced *SphI/SacI* restriction sites. Similarly, DNA encoding the aptamers was PCR-amplified while introducing *BamHI/EcoRI* restriction sites (5'-CGCGGATCC CGGCCGGGTCTGGGC GCAGCGCAAGCTGACGG-3'
5'-CCG GAA TTC CCG CGG GGCCTGTACCGTCAGCTTGCGCTGC-3') and subsequently subcloned into pRSETB. DNA-constructs encoding 5, 10 and 20 repeats of the RRE aptamer were synthesized (Eurofins) and cloned into a pcDNA3-mKO2 vector using *EcoRI/XbaI* sites.

In vitro transcription and RNA purification.

DNA templates for in vitro transcription were amplified by PCR using T7 forward and reverse primers and the corresponding template vectors encoding the aptamer variant. For in vitro transcription assays, T7 promoter variants yielding different mutations were introduced by PCR reaction. The RNA was in vitro transcribed using the T7 RiboMAX Express Large Scale

Production System (promega). Template DNA was removed by DNAseI digest and the RNA product was purified either by phenol-chloroform extraction or using the DNA-Free RNA kit (Zymed). RNA was heated to 95 °C for 5 min and cooled to room temperature for refolding. RNA concentration was measured based on UV absorbance at 260 nm using a Nanodrop 1000 spectrophotometer (Peqlab).

Protein expression

E. coli BL21 (DE3) cells were transformed with pRSETB and cultured overnight at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin, prior to induction with Isopropyl-β-D-thiogalactopyranosid (IPTG, 0.1 mM) at OD_{600}=0.6. Induced cells were grown for 4h at room temperature while shaking at 250 r.p.m.and harvested by centrifugation for 10 min at 6000 g. The cell pellet was resolved in 10 mL protein resuspension buffer (20 mM $NaPO_4$, pH 7.8, 300 mM NaCl) supplemented with protease inhibitors (1 mM PMSF, 5 µg/ml Pepstatin and 1 µg/µL Leupeptin). Bacteria were frozen at -80°C overnight, thawed on ice and lysed by addition of 1 mg lysozyme for 30 min. Cell membranes were solubilized by addition of Triton-X-100 (0.1%) and DNaseI (5 µg/mL, 50 µL) followed by ultransonification for 20 min and centrifugation (13000 rpm, 30 min, 4°C). Lysates bearing N- or C-terminal 6-His-tagged proteins were loaded onto an immobilized metal affinity column packed with 300 µL Ni-NT resin (Qiagen) (300 µL) for 2 h at 4°C. After washing recombinant proteins were eluted using elution buffer (20 mM Na_2HPO_4, 300 mM NaCl, 250 mM Imidazol, 10% Glycerol, pH 7.8). The concentration of purified fluorescent proteins was measured using the BCA Assay Kit (Pierce) and proteins were stored at -80 °C. For high yield protein expression and purification, a 600 mL E. coli culture was inoculated as described. For affinity chromatographic purification, a 5 mL HisTrap affinity column (GE) was used. Gel filtration was performed using a Superdex 200 (GE) coupled to an ÄKTA-FPLC device (GE Healthcare). The column was equilibrated with SEC buffer (40 mM Tris pH 7.5, 200

mM NaCl, 2 mM β-ME). For each run, 2 mL of sample was loaded and protein separation was performed at 0.3 ml/min.

In vitro spectroscopy, pH titrations and in vitro transcription assay.

Fluorescent spectra were taken using a fluorescent spectrophotometer (Varian Eclipse). Purified proteins were diluted in 100 µL RNA binding buffer (20 mM HEPES (pH 7.25), 125 mM NaCl, 1 mM magnesium diacetate, 1 mM imidazole, 2 mM $CaCl_2$, 1 mM DTT, 2.5% (v/v) glycerol and 0.1% (w/v) Triton-X-100). FRET sensor proteins were excited at 432 nm (bandwidth 5 nm) and the emission was recorded from 450 nm to 600 nm (bandwidth 5 nm). An emission spectrum was taken before and after exposure of VAmPIRe to RNA in an equimolar ratio. To calculate the ΔR/R value elicited the following formula was used:

$$\Delta R/R \quad [\%] = \frac{\text{ratio}_{\text{after}} - \text{ratio}_{\text{before}}}{\text{ratio}_{\text{before}}} * 100$$

where ratio = $Peak_{YFP}/Peak_{CFP}$ (527nm/432nm).

RNA titrations of VAmPIRe variants were done in a cuvette in a fluorescence spectrophotometer in 100 µL RNA binding buffer. Small aliquots of RNA were added to the cuvette and afull fluorescence emission spectrum was taken after each addition. For pH titration, we used buffers containing 125 mM NaCl, 1 mM magnesium diacetate, and TRIS (pH 7.5, 7.75, 8.0, 8.25), MOPS (pH 6.5, 6.75, 7.0, 7.25, 7.5, 8.0), MES (5.5, 6.9, 6.5) or citrate (4.5, 5.0, 5.5).

DNA encoding 10 repeats of the RRE aptamer were amplified by PCR using primers that introduce a T7 promoter variant at the 5’ end (T7wt _10RRE_f 5'-

TAATACGACTCACTATAGGATCCTGTACACGGCCGGGTCTG-3', T7 _A8C_10RRE_f 5'-TAATACGCCTCACTATAGGATCCTGTACACGGCCGGGTCTG-3', T7_A8G_10RRE_f 5' TAATACGGCTCACTATAGGATCCTGTACACGGCCGGGTCTG-3'). 1µg of purified PCR product was used as a template for the vitro transcription reaction using the RiboMAX T7 Large Scale RNA Production System (promega). RNA generation was monitored by a fluorescence spectrophotometer as mentioned above. 1U RNAseI was added after 60 min in order to digest the synthesized RNA.

Sensor library generation and bacterial screening.

Different strategies for random mutagenesis were applied in order to create libraries with RNA peptides bearing amino acid substitutions, deletions and insertions. The DNA encoding the FRET sensor variant was subjected to an error-prone PCR (Gene Morph mutagenesis kit, Agilent) using CFP Chr for (5'- CTGACCTGGGGCGTGCAGTGCTCC-3') and cpCit Chr rev (5'- CGAAGCACCATCAGGCCGTAGCCG-3') primers. The mutation frequency ranging from 1-10 mutations per kb was controlled by the amount of template DNA. In order to increase the rate of single mutations, two sequential PCRs were performed using the purified product of the first one for the second reaction. The PCR product of the second reaction was digested *SphI/SacI* and ligated into a pRSFDuet-1-VAmPIRe vector (modified from Novagen). In another approach, degenerated primers (Eurofins) allowed the introduction of randomized amino acids at defined positions either within the Rsg peptide or at the N- and C-termini during normal PCR reaction.

The pRSFDuet sensor library was transformed into E. coli XI1 blue and plated on agar plates with kanamycin. The colonies were pooled and the sensor library DNA was retransformed into

E. coli Xl1 blue with 10 aptamer repeats (10xRRE) that are cloned into pMB133 (kind gift of Natalie Broude) using *HindIII/NotI*. Each colony expresses one FRET sensor variant and the pMB133-10xRRE vector yielding tetracyclin-inducible aptamers. The transformations were plated on a double (amp/kana) resistance agar plate, incubated over night at 37 °C and for 24 h at 4°C in order to get full repression of aptamer transcription.

Plates were imaged at room temperature every 5 min and RNA aptamer transcription was induced by addition of 20 ng/µL anhydrotetracycline (Sigma). A Lambda LS light source (Sutter) was connected to a lambda 10-2 hardware (Sutter) by fiber optic light guides. The excitation filter was D436/40x and the emission filter for CFP was D480/40m and for cpCitrine HQ535/30m (all from Chroma). Images were detected by a CCD camera Coolsnap ES2 (Sutter) and processing was done in MetaFluor software (Meta Imaging series version 7.7). For automated colony ROI selection we used Cell profiler version 10415. Data were analysed by Origin 8.1 by plotting the ratio change 200 min after anhydrotetracycline addition over the starting FRET ratio. The selected colonies correspond to the ROIs with a ratio change higher and a starting ratio lower than the average of 6 control colonies with the wt sensor.

On average, 800 colonies were analysed per plate and 5-20 colonies (1500 in total) selected for minipreparation and subsequent in vitro validation. The pRSFDuet-1 vectors bearing the selected VAmPIRe variants were transformed into BL21 and grown in 5 mL LB medium overnight. The preculture was diluted in 12 mL LB medium supplemented with kanamycin and 0.1 mM IPTG for small-scale protein expression. After 4 h, cells were harvested and processed as described above but in a 1 mL volume format. For affinity purification, the lysates were incubated with 25 µL His Mag Sepharose Ni magnetic beads (GE), processed according to the manufacturer`s protocol and protein was eluted in 25 µL elution buffer. 1-5 µL of the 25 µL protein eluate were mixed with the RNA aptamer in 100 µL RNA binding buffer and FRET ratio change was detected in a fluorescence spectrophotometer as described. The variants with the

highest ratio change upon RNA aptamer addition were selected for sequencing and spectral analysis from larger culture volumes as described above.

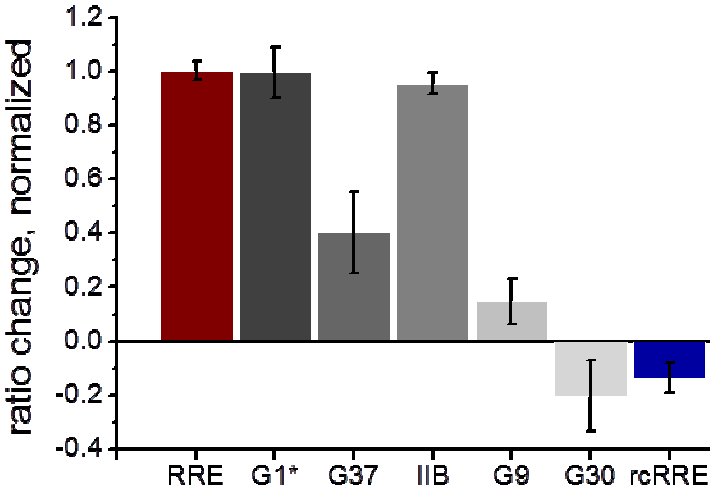
Cell culture, transfections and imaging.

The cDNAs encoding VAmPIRe or mKO2-20xRRE were cloned *BamHI/EcoRI* into pcDNA3. For imaging experiments, cells were plated on a 35-mm glassbottom dish (Mattek) in culture medium (Dulbecco`s modified Eagle`s medium containing 10% Fetal calf serum, 100 U/mL penicillin and 100 ug/mL streptomycin). Confluent NIH 3T3 fibroblasts cells were transfected with pcDNA3-VAmPIRe (0.5 µg) and pcDNA3-mKO2-20xRRE (2 µg) using Lipofectamine 2000 (Invitrogen). The fibroblast monolayer was scraped in a straight line using a scalpel to create a scratch and migrating cells were imaged 2 h afterwards. The VAmPIRe (0.25 µg) and pcDNA3-mKO2-20xRRE (1 µg) were transfected separately into HEK 293T cells. After 24 h, images were taken using a Zeiss VisiScope Cell Explorer System with a plan-apochromat 63x/1.4 oil objective (Zeiss) and FRET (436/20x, 535/30M), CFP (436/20x, 480/40M) and RFP (560/40x, 630/75M) filters (all from Chroma). Emitted light was detected by a Cool Snap HQ^2 CCD camera (Visitron). Cells were maintained at 37°C (Tempcontrol 37-2 digital, heating unit, Pecon) and the system was controlled and processed by MetaMorph software (Meta Imaging Series 7.7).

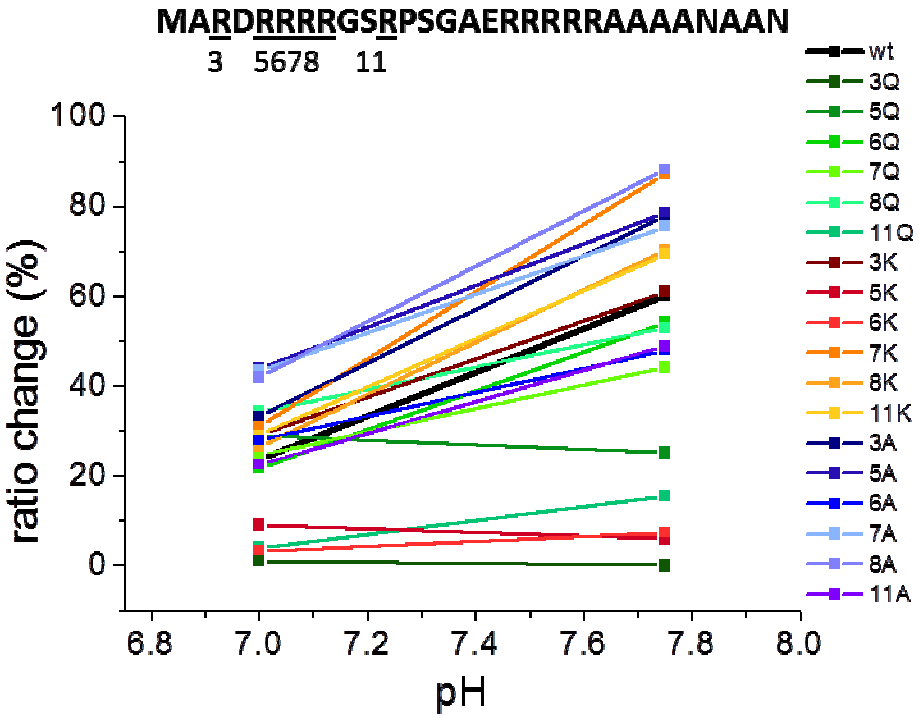
The T-REx HeLa cell line (Invitrogen) used for the Tet-ON experiments was expanded in culture medium supplemented with 5 µg/mL blasticidin (Invitrogen). For tetracycline-inducible expression of the aptamer fused to mKO2, mKO2-30xRRE was cloned *HindIII/XbaI* into pcDNA4/TO (Invitrogen). T-Rex HeLa cells were transfected with pcDNA3-VAmPIRe (0.25 µg) and pcDNA4/TO-mKO2-30xRRE (1 µg) using Lipofectamine and cultured for further 24 h. Before imaging, 0.1 µg/mL anhydrotetracycline was added to the medium to induce transcription from pcDNA4/TO. Images were taken every 10 min at the Zeiss VisiScope Cell Explorer System as described.

Image J software was used for image analysis. Background was corrected by subtracting the intensity of a region close to the cell of interest. FRET ratio was calculated by division of the YFP by the CFP channel. Fluorescence intensity and FRET of a region of interest were plotted.

Supplementary Figures



Supplementary Figure 1: Specificity of biosensor-aptamer interaction. Maximal ratio change of FR-Rsg1.2 at pH 8.0 with equal amounts of the RRE aptamer (wt) or RRE mutants with conserved stem loop structure but one or several nucleotide exchanges (G1 to G30). Mutant RREs G1 to G30 are described in Harada et al. (2008). *Nucleic Acids Symposium Series* No. 52: 13-14. rcRRE is a reverse RRE sequence.



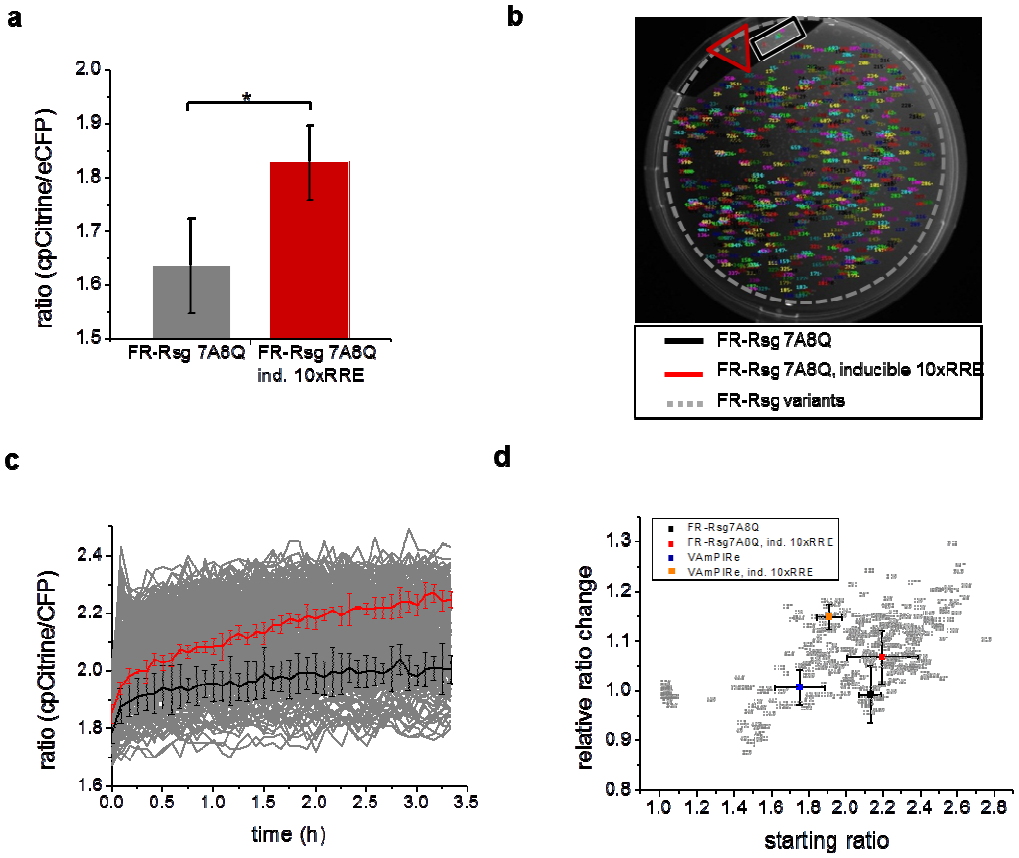
Supplementary Figure 2: Effects of N-terminal arginine substitutions within Rsg1.2 on pH-dependence of maximal FRET ratio change. Rsg1.2 sequence is shown with the Arg positions underlined that were subjected to mutagenesis to Gln, Lys or Ala. Performance of the sensor variants at two different pH values in the physiological range is plotted.

A

B

linker name	aa sequence
helical linkers	
H11	**KLYPYDVPDYA**
H16	**PDYAKLYPYDVPDYA**
H20	**LEYDVPDYAKLYPYDVPDYA**
flexible linkers	
FL2	**GS**
FL9	**LGGGGSAAA**
proline linkers	
1Pro	**P**
2Pro	**PP**
3Pro	**PPP**
4Pro	**PPPP**
5Pro	**PPPPP**
6Pro	**PPPPPP**
random linkers	
1N	**X**
2N	**XX**
3N	**XXX**
4N	**XXXX**

Supplementary Figure 3. Library design and linker variations**.**
(A) Schematics of the diversified indicator library. The Rsg1.2 peptide (Rsg, grey) was sandwiched between the donor fluorophore eCFP and the acceptor fluorophore cpCitrine (cpCit), connected via two diversified linker regions (Li). (**B**) Design and amino acid (aa) sequence of diversified linker regions. Initial attempts focused on rational design using α-helical linkers of differing lengths (H11-H20), flexible linkers (FL2, FL9) or series of prolines (1-6 prolines for each linker). As these rational designs were unsuccessful, random linker libraries of 1-4 random amino acids were designed. After a series of tests the final functional screen was performed with a library containing 4 random amino acids linkers on each side.



Supplementary Figure 4: Optimizing ratio change through evolutionary linker extension and bacterial colony screening. (a) Average ratio of bacterial colonies expressing FR-Rsg 7A8Q alone (grey) or with the 10xRRE. n= 100, P<0.05 in a one-way ANOVA followed by Tukey`s post test) (b) bacterial plate as used for colony pre-screening (cpCitrine emission). Colonies are automatically assigned a region of interest. Up to 1000 regions corresponding to diversified indicator variants are imaged simultaneously. For comparisons and calibration colonies expressing exclusively the parental sensor FR-Rsg 7A8Q alone (black rectangle) or in combination with the inducible aptamer (red triangle) are imaged together with the library. (c) Time course of 10xRRE RNA induction. Ratio increases in control colonies expressing the sensor FR-Rsg 7A8Q and the inducible 10xRRE (red) but not in control colonies without any aptamer (black). Ratios of colonies expressing diversified sensors and the inducible 10xRRE are shown in grey. (d) The performance of different variants from the sensor library of one plate (grey numbers) is shown by plotting the ratio before anhydrotetracycline addition against the relative ratio change after 3 h. Average values of 4 control colonies expressing FR-Rsg 7A8Q or VAmPIRe with (black, blue, respectively) and without (red, orange, respectively) the inducible 10xRRE are shown.

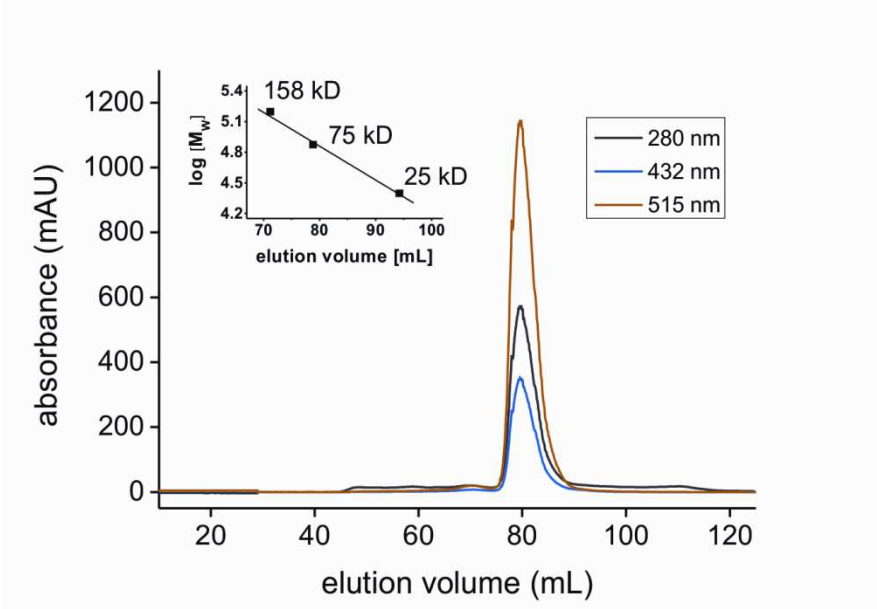
A

GGATCCCGCGGCCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGA
GCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGCGGCGAGGGCGAGGGCGATGCCACCAACGGCAA
GCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACC
TGGGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCG
AAGGCTACGTCCAGGAGCGTACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGG
GCACAAGCTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGG
CCCACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCC
CATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCC
AACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGCGCATGCAATCCCATCGTGATGATC
GTCGCGCACAAGGCAGTCGCCCTAGTGGCGCCGAGCGTCGCCGTCGCCGTGCAGCTGCGGCGATGGATAGGC
GTGAGCTCATGGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGC
TGCTGCCCGACAACCACTACCTGAGCTACCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCACAT
GGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGCGGTACCGG
CGGCAGCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGA
CGTAAACGGCCACAAGTTCAGCGTGCGCGGCGAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAA
GTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGATG
TGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA
GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC
CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
ACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATC
CGCCACAACATCGAGGACTAAGAATTC

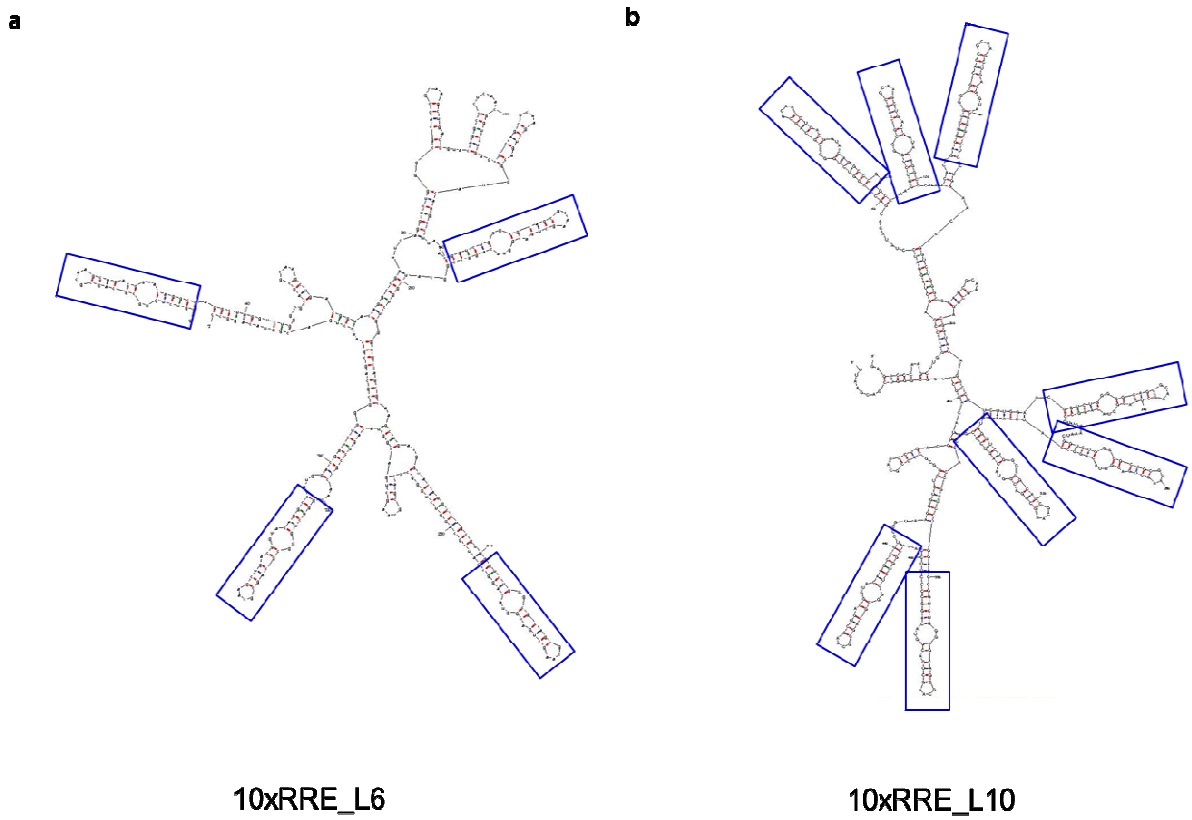
B

FR-Rsg1.2	M A R D R R R R G S R **P S G A E R R R R R A A A A** N A A N
FR-Rsg7A8Q	M A R D R R A Q G S R **P S G A E R R R R R A A A A** N A A N
#95	F A H S D R R A Q G S R **P S G A E R R R R R A A A A A** L V R H
VAmPIRe	S H R D D R R A Q G S R **P S G A E R R R R R A A A A** M D R R

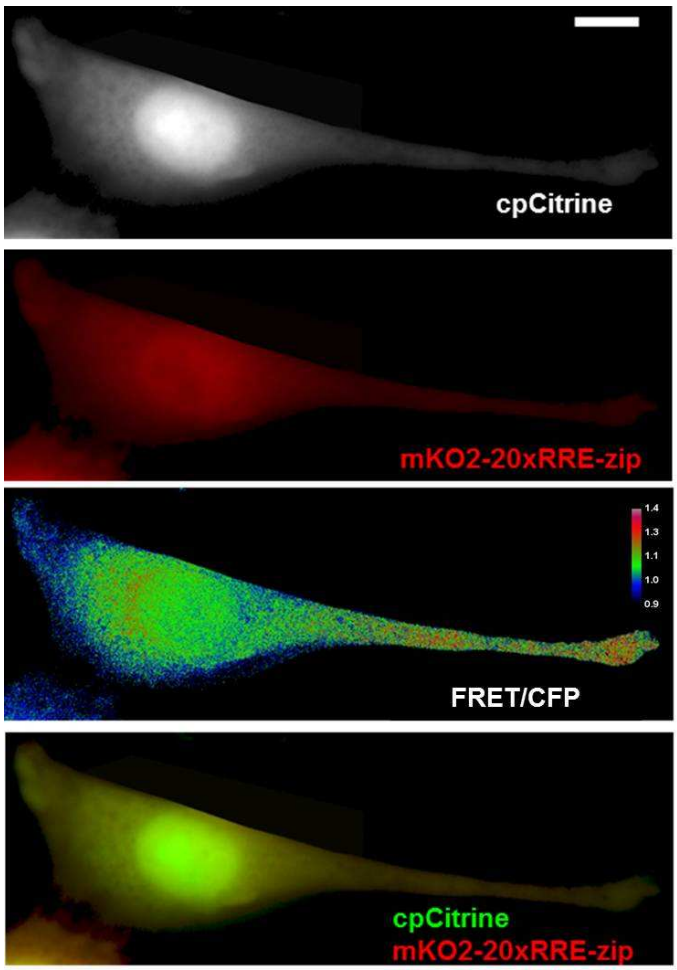
Supplementary Figure 5. Sequence information.
(**A)** VAmPIRe nucleotide sequence**.** Highlighted in color are the cloning restriction sites BamHI, SphI, SacI and EcoRI (purple), linkers (green), eCFP (blue), cpCitrine174 (yellow), and engineered mutations within the Rsg1.2 peptide (red) **(B)** Amino acid sequences of the aptamer binding Rsg1.2 peptide moiety in 4 sensor variants (for reference see also Figure 1c). Aptamer-contacting amino acids are in bold, linkers of the original parental construct in grey, mutations rationally engineered into the Rsg1.2 peptide (R7A, R8Q) are in red, improved linkers within VAmPIRe and the sensor variant #95 identified in the functional screen are highlighted in green.



Supplementary Figure 6. Elution profile of recombinant purified VAmPIRe in size exclusion chromatography using Superdex 200 (16/60, prep grade, GE Healthcare). Graphs show the absorbance (in 10^3 arbitrary units, mAU) at the indicated wavelength: 280 nm for protein, 432 nm for eCFP and 515 nm for cpCitrine with a maximum for elution volume of monomeric VAmPIRe. The inlay shows the calibration standard.



Supplementary Figure 7: Multimerization of RRE and optimization of RRE aptamer folding through inter-aptamer linker variation. Lowest energy structures of two aptamers with either 10 copies of RRE and a six nucleotide linker (10xRRE_L6 dG = -218.08 kcal/mol) (a) or 10 copies of RRE and a 10 nucleotide linker are shown (10xRRE_L10 dG = -257.92 kcal/mol) (b). The predicted correctly folded RRE stem-loops are highlighted with blue boxes. Structures were modeled according to the programme mFold (Zuker et al., 2003).



Supplementary Figure 8: Subcellular mRNA distribution imaged with VAmPIRe in NIH 3T3 fibroblasts. Cells were co-transformed with VAmPIRe and mKO2-20xRRE fused to the zip-code sequence of ß-actin (mKO2-20xRRE-zip). Note increased cpCitrine/CFP ratio in the leading edge of a moving filopodium. Scale bar, 20 µm.

peptide	aptamer	K_D (nM)	FRET ratio change (%)
BIV tat	BIV tar	0.5	30
HIV-1 Rev	RRE	6	-
Rsg 1.2	RRE	40	40
HTLV Rex	HTLV apt	6	-
P22	boxB	100	-23
Φ 21	boxB	80	-23
λ N21	boxB	20	-

Supplementary Table 1: Ratio change in FRET biosensor constructs employing selected arginine rich RNA binding peptides as sensing moiety. Peptides were sandwiched between the fluorescent proteins CFP and cpCitrine. The maximal ratio change after binding the corresponding aptamer is shown. Positive values indicate increased energy transfer from donor to acceptor, a negative value decreased energy transfer from donor to acceptor. Kd values are collected from the literature.