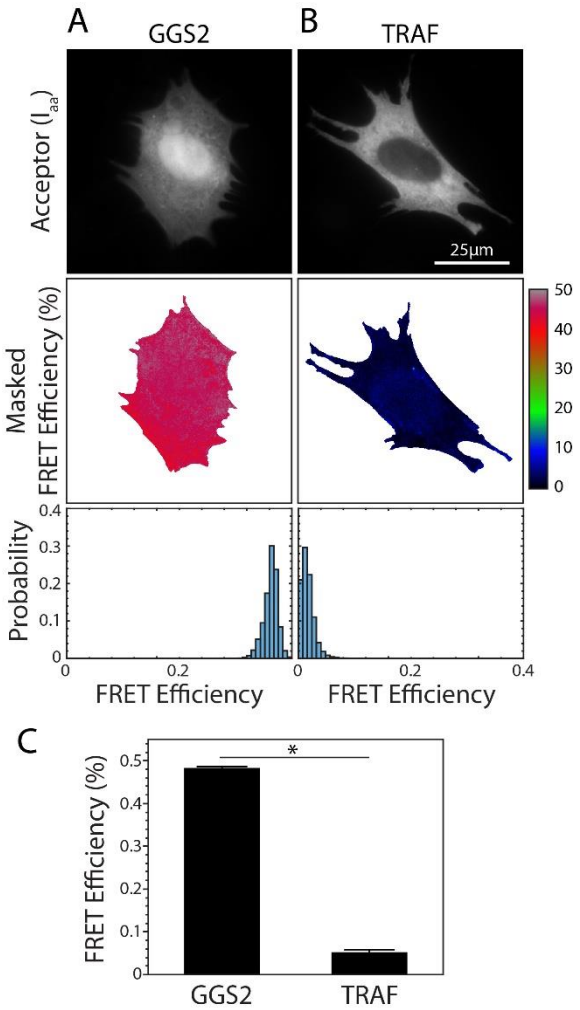
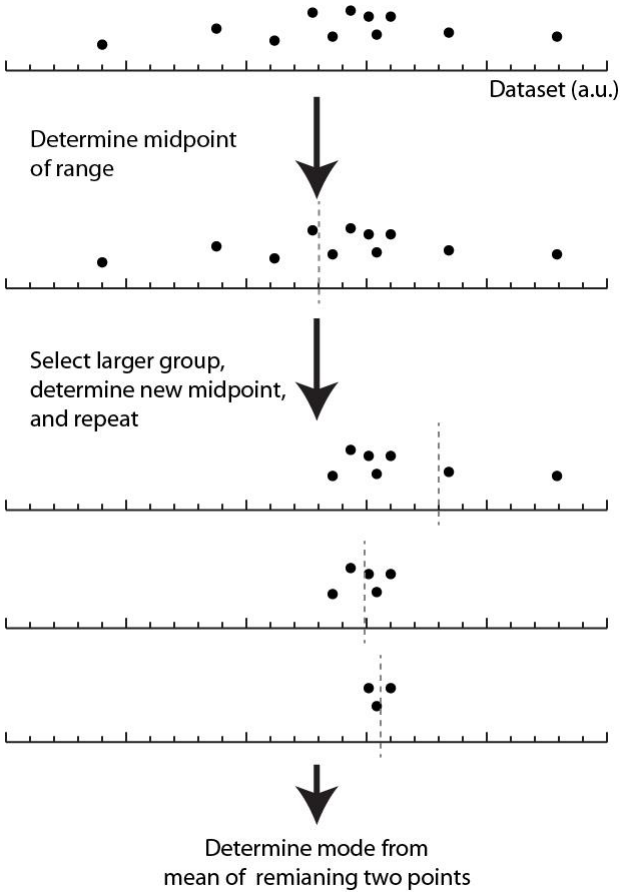
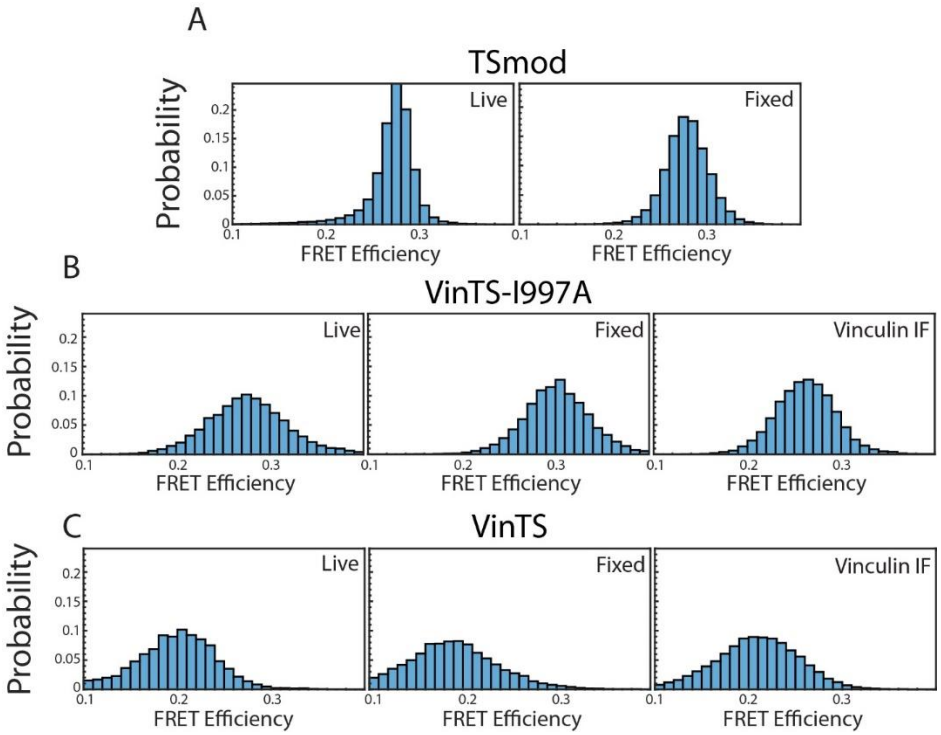
Supplement:



Supplementary Figure 1: Sample images, corresponding to **Fig. 1**, of acceptor and the calculated FRET efficiency of vinculin -/- MEFs expressing cytosolic (**A**) GGS2- and (**B**) TRAF-based constructs. The corresponding pixelwise FRET efficiency distributions are shown for each image. (**C**) Mean FRET efficiency for [D]/[A] filtered GGS2 (n = 200 cells, N = 4 experiments) and TRAF (n = 138, N = 4 experiments). Error bars are one standard error of the mean.



Supplementary Figure 2: An illustrative example of determining the mode using the half-range mode algorithm. First, the dataset is divided along the midpoint of the dataset's range. Then, the group with more points is selected. The process of selecting smaller intervals is repeated until only two points remain. The mean of the final two points is considered the mode.



Supplementary Figure 3: Pixelwise FRET efficiency histograms that correspond to each sample image shown in **Fig. 4** for (**A**) TSMod, (**B**) VinTS-I997A, and (**C**) VinTS.

Supplementary Table 1: Primers used in the construction of VinTS dark mTFP1 and VinTS dark Venus.

Gibson Assembly of VinTS dark mTFP1		
Fragment	Primer Name	Sequence (5’ to 3’)
Vector1	Ampicillin Reverse	AGGAGCTAACCGCTTTTTTGCACAA
Vector1	Dark mTFP1 Forward	GCGTTCGCCTTAGGCAACAG
Vector2	Ampicillin Forward	TTGTGCAAAAAAGCGGTTAGCTCCT
Vector2	Dark mTFP1 Reverse	CTGTTGCCTAAGGCGAACGC
Gibson Assembly of VinTS dark Venus A206K		
Fragment	Primer Name	Sequence (5’ to 3’)
Vector1	Ampicillin Reverse	AGGAGCTAACCGCTTTTTTGCACAA
Vector1	Dark Venus A206K Forward	CCTGGGCTTAGGCCTGCA
Vector2	Ampicillin Forward	TTGTGCAAAAAAGCGGTTAGCTCCT
Vector2	Dark Venus A206K Reverse	TGCAGGCCTAAGCCCAGG

Supplementary Table 2: Primers used in the construction of pcDNA3.1 mTFP1-TRAF-Venus.

Gibson Assembly of pcDNA3.1 mTFP1-TRAF-Venus		
Fragment	Primer Name	Sequence (5’ to 3’)
Vector1	Venus-TRAF Forward	CCGGACTCAGATCTATGAAGGGCGAGGAGCTGTTCACC
Vector1	Ampicillin Reverse	AGGAGCTAACCGCTTTTTTGCACAA
Vector2	Ampicillin Forward	TTGTGCAAAAAAGCGGTTAGCTCCT
Vector2	mTFP1-TRAF Reverse	TCTCCAGGCTCTCTCCGGAGCGGGCCACGGCG
Insert	mTFP1-TRAF Forward	TGGCCCGCTCCGGAGAGAGCCTGGAGAAGAAG
Insert	Venus-TRAF Reverse	TCCTCGCCCTTCATAGATCTGAGTCCGGAGAGCCCTGT

Supplementary Table 3: Glossary of symbols used in the calculation of FRET efficiency.

Symbol	**Definition**
F_c	Corrected FRET, defined as
I_f	Intensity in the FRET channel
I_{dd}	Intensity of donor channel
I_{aa}	Intensity of acceptor channel
abt	Acceptor bleedthrough
dbt	Donor bleedthrough
G	Calibration factor that represents a ratio of the sensitized acceptor emission to the amount of donor quenching due to FRET for single chain biosensors
k	Calibration factor that represents the fluorescence intensity ratio between the donor and acceptor in the absence of FRET for single chain biosensors
E	FRET efficiency
[D]	Donor concentration
[A]	Acceptor concentration
[D]/[A]	Ratio of donor-to-acceptor concentration