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Supplementary Materials for

Ligand-tuned extracellular domain dynamics are correlated with signaling states in an adhesion GPCR

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Figs. S1 to S10

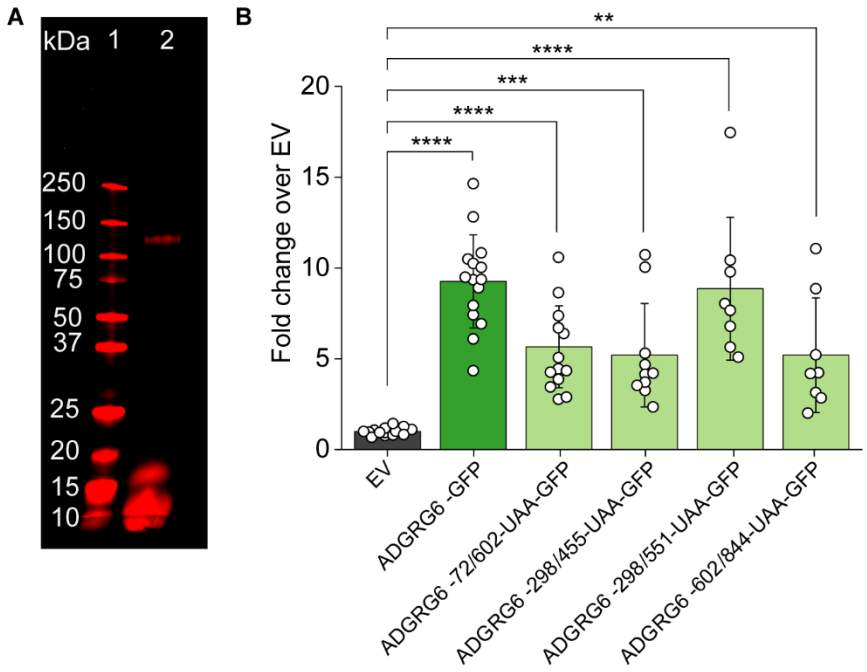


Fig. S1. Labeling specificity and signaling activity of ADGRG6 with unnatural amino acids.
(**A**) Image of gradient (4-20%) polyacrylamide gel electrophoresis of whole cell lysate from HEK293T cells expressing ADGRG6 with UAA sensor on CUB domain (position 72) and GAIN domain (position 602) labeled with Cy5-tetrazine dye. Lane 1: protein ladder. Lane 2: sample of cell lysate. The ~125 kDa-band represents the receptor, bands at low molecular weight correspond to free Cy5 dye and bromophenol blue dye from the sample buffer. Result represents an individual experiment. (**B**) Bar graph represents the signaling activity of ADGRG6-GFP-Flag and the receptor with dual-UAA in different domains measured by cAMP assay. Data show fold change relative to empty vector (EV), as mean ± SD from at least two independent experiments ($n > 8$). Statistical significance was determined by one-way ANOVA, Dunnett's post hoc test. $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.001$. Signaling data are not normalized to expression.

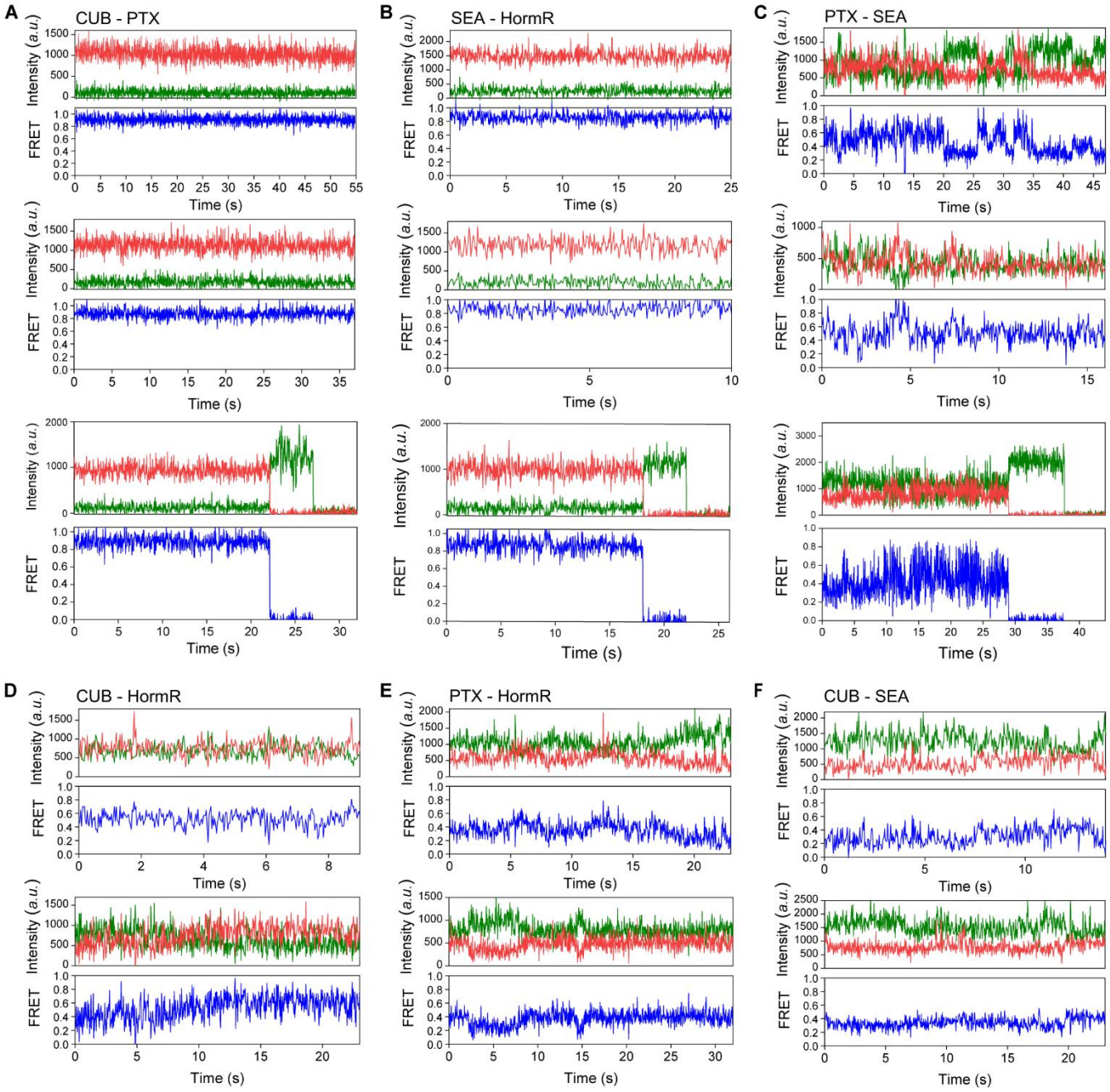


Fig. S2. Representative FRET traces.

(**A** to **F**) FRET traces from each different sensor between ECR domains of ADGRG6 in 5 mM Ca^{2+}. The donor signal is represented by a green line, acceptor signal by a red line, and the resulting FRET is displayed in blue.

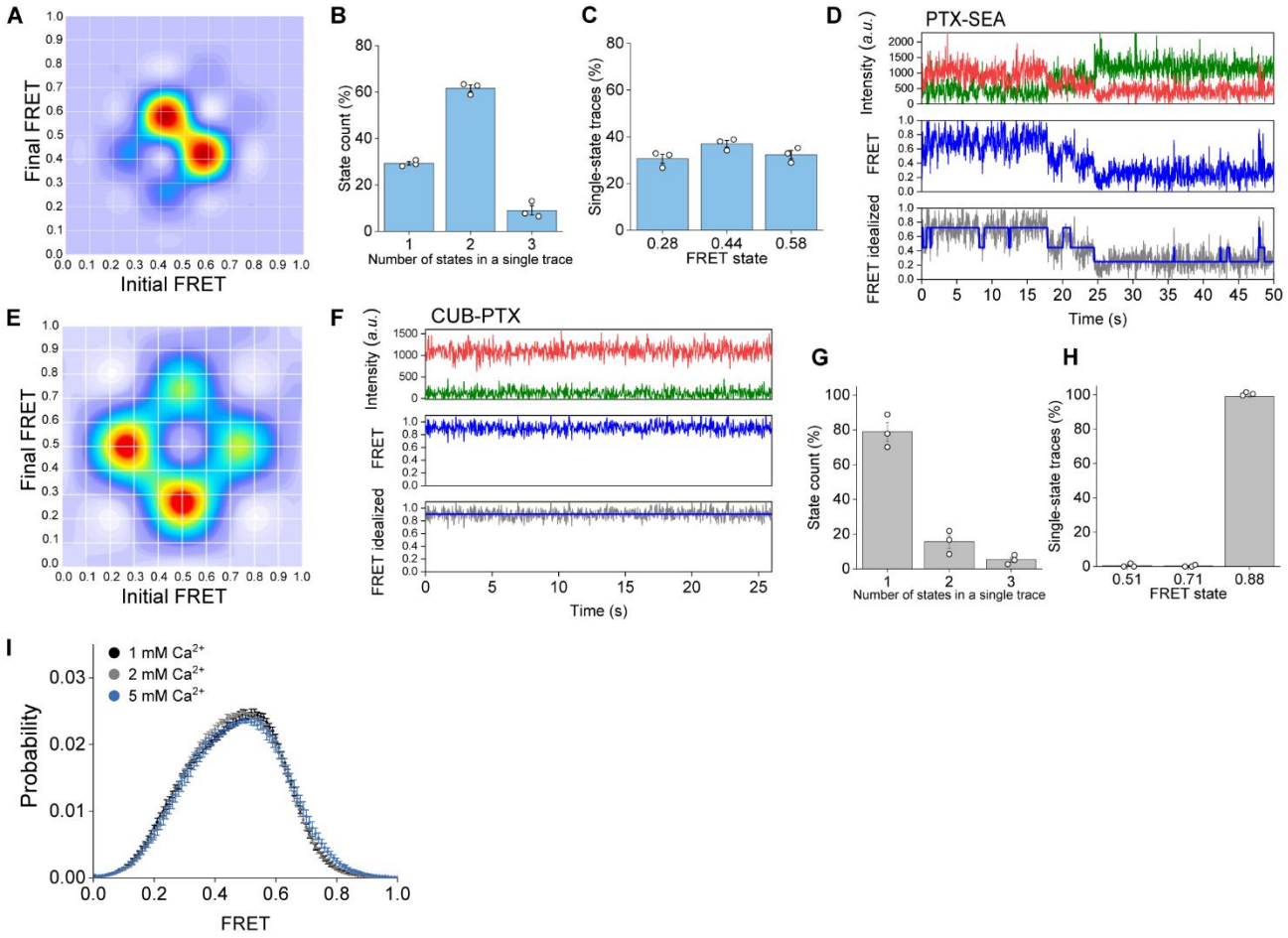


Fig. S3. FRET state occupancy and transition analysis of ADGRG6 ECR domains.
(**A**) Transition density plot of sensor on CUB – GAIN domains in 5 mM Ca^{2+}. Data from two independent biological replicates. (**B**) Number of states (%) detected in individual traces from CUB – GAIN sensor. (**C**) Occurrence of three different FRET states in population of single-state traces. (**D**) Representative FRET traces from sensor on PTX and SEA domain. The donor, acceptor and FRET signals are in green, red and blue. Grey signal with blue line represents raw and idealized FRET signals. (**E**) Transition density plot of sensor on PTX – SEA domains in 5 mM Ca^{2+}. Data from n=3 independent biological replicates. (**F**) sensor on CUB and PTX domain. The donor signal is represented by a green line, acceptor signal by a red line, and the resulting FRET is displayed in blue. Grey signal with blue line represents FRET signal fitting three idealized FRET states. (**G**) Number of states (%) detected in individual traces from CUB – PTX sensor and (**H**) occurrence of three different FRET states in population of single-state traces. (**I**) Population histogram of smFRET data obtained from CUB – GAIN sensor in the presence of 1 mM Ca^{2+}, 2 mM Ca^{2+}, or 5 mM Ca^{2+} conditions. (**B, C, G, H, I**) Data represent mean ±s.e.m. of n=3 independent biological replicates.

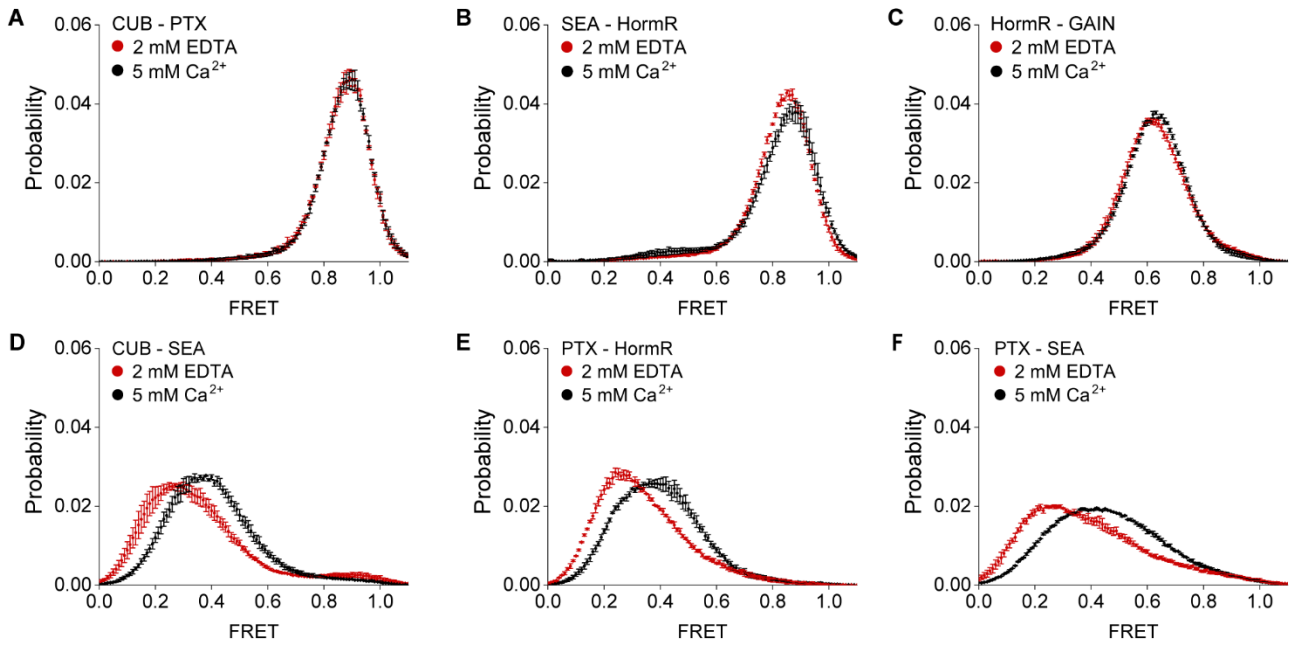


Fig. S4. Calcium-dependent conformation of ADGRG6 ECR.
(**A** to **F**) Population histograms of smFRET data from different sensors on ECR domains of ADGRG6 in presence of 2 mM EDTA or 5 mM Ca^{2+}. Data represent mean ±s.e.m, from three independent biological replicates.

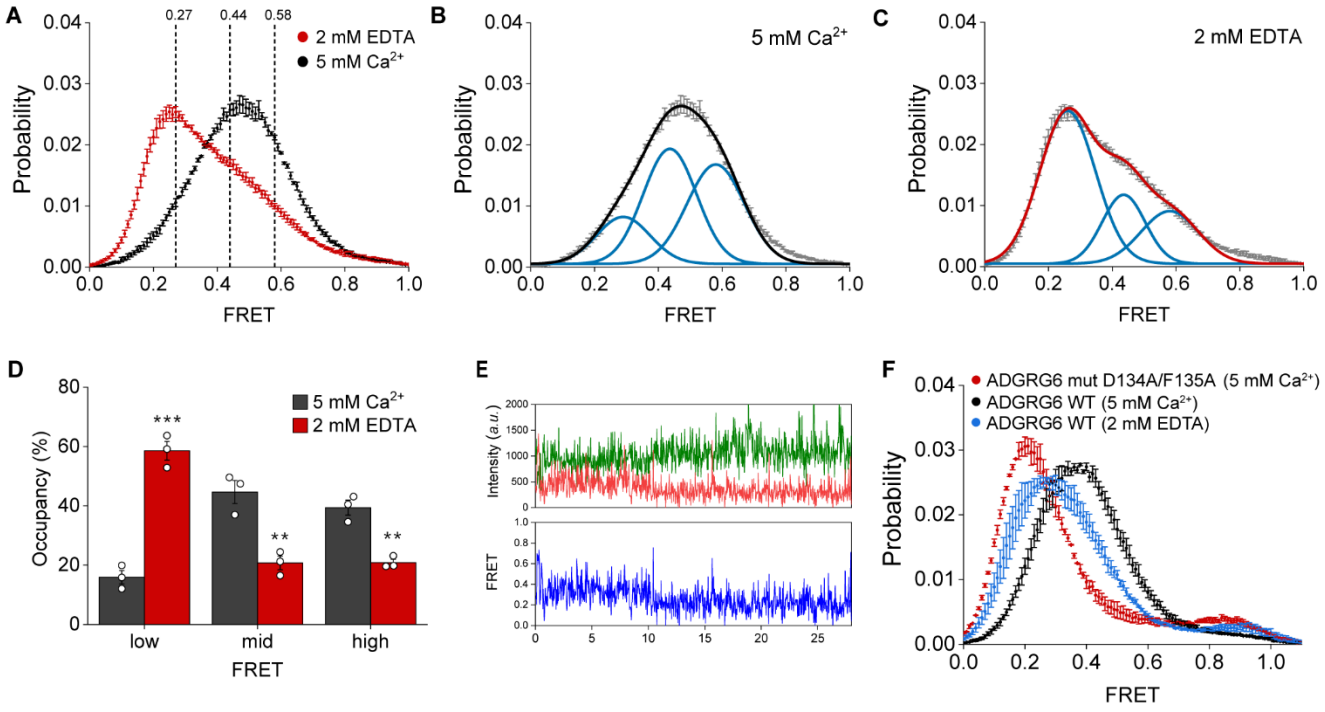


Fig. S5. FRET state occupancy analysis in WT receptor and conformation of mutant ADGRG6. (**A**) Population histogram of smFRET data obtained from CUB – HormR sensor in the presence of 2 mM EDTA or 5 mM Ca^{2+} conditions. Black dotted lines indicate the three FRET states: low (0.27), mid (0.44) and high (0.58). These FRET states were determined by fitting three gaussian distributions (blue) in (**B**) 5 mM Ca^{2+} and in (**C**) 2 mM EDTA. (**D**) Bar graph representing relative occupancy of the three conformational states. Statistical significance was determined by two-sample *t*-test (**p < 0.01, ***p < 0.001). (**A** to **D**) Data represent mean ±s.e.m, n = 3 independent biological replicates. (**E**) Representative FRET trace from sensor on CUB – SEA domains of ADGRG6 mut D134A/F135A in 5 mM Ca^{2+}. The donor signal is represented by a green line, acceptor signal by a red line, and the resulting FRET is displayed in blue. (**F**) smFRET population histograms (CUB – SEA sensor) of ADGRG6 mut D134A/F135A and ADGRG6 WT in different conditions. Data represent mean ± s.e.m, from at least two independent biological replicates.

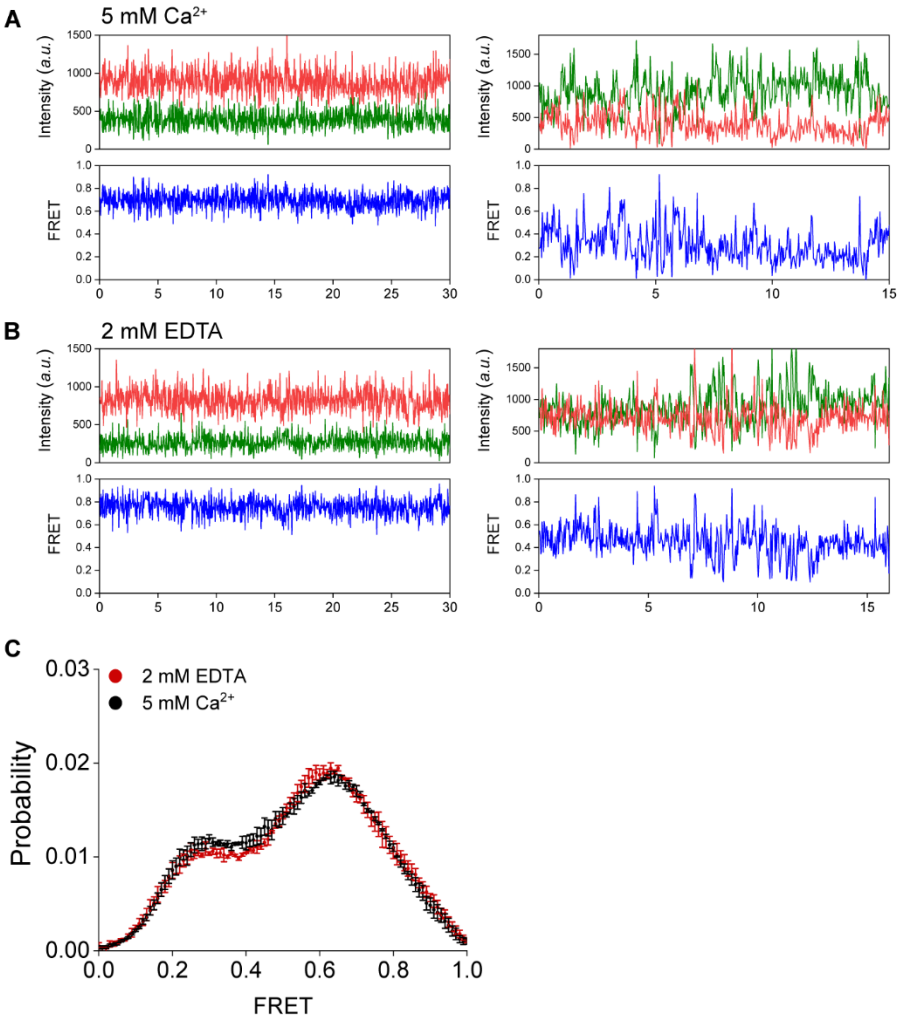


Fig. S6. smFRET analysis of the ADGRG6 mutant S136C/I586C.
Representative FRET traces from sensor on CUB – HormR domains sensor of ADGRG6 mutant S136C/I586C in (**A**) 5 mM Ca^{2+} or (**B**) 2 mM EDTA. (**A, B**) The donor signal is represented by a green line, acceptor signal by a red line, and the resulting FRET is displayed in blue. (**C**) Population histogram of smFRET of ADGRG6 mutant S136C/I586C. Data were obtained from CUB – HormR sensor in the presence of 2 mM EDTA or 5 mM Ca^{2+} conditions. Data represent mean ±s.e.m, n = 3 independent biological replicates.

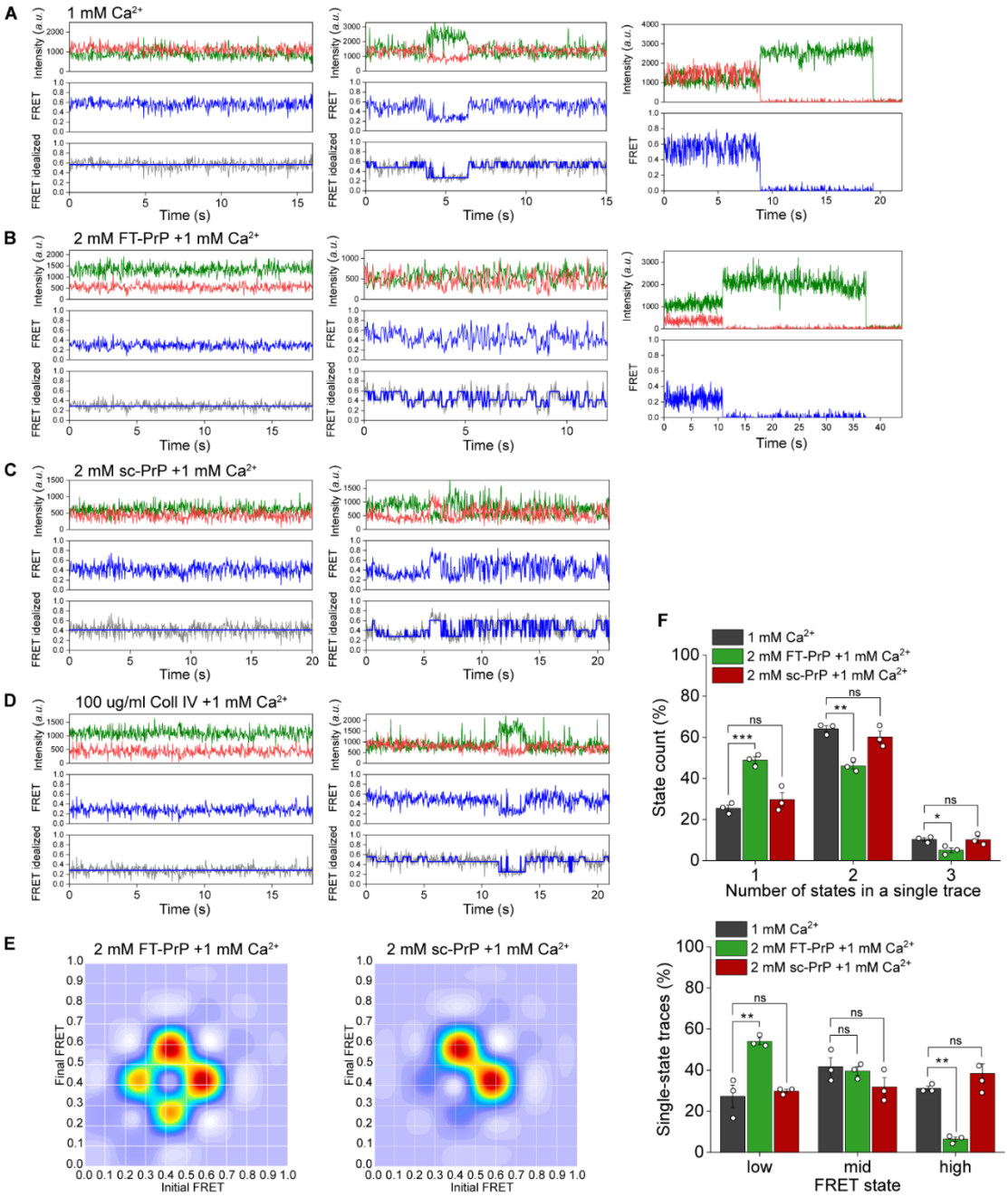


Fig. S7. Transitions and FRET state analysis of ADGRG6 ECR in the presence of ligands. Representative FRET trace from CUB – GAIN domains sensor of ADGRG6 in conditions (**A**) 1 mM Ca^{2+} (**B**) 2 mM FT-PrP +1 mM Ca^{2+} (**C**) 2 mM sc-PrP +1 mM Ca^{2+} and (**D**) 100 µg/ml Coll IV +1 mM Ca^{2+}. The donor, acceptor and FRET signals are in green, red and blue. (**E**) Transition density plot of sensor on CUB – GAIN domains in 2 mM FT-PrP +1 mM Ca^{2+}or 2 mM sc-PrP +1 mM Ca^{2+}. Data from three independent biological replicates. (**F**) Bar graphs represent number of states (%) detected in individual traces from CUB – GAIN sensor, and occurrence of three different FRET states in population of single-state traces. Data: mean ±s.e.m, n = 3 independent biological replicates. Statistical significance was calculated using one-way ANOVA, Dunnett's post hoc test. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

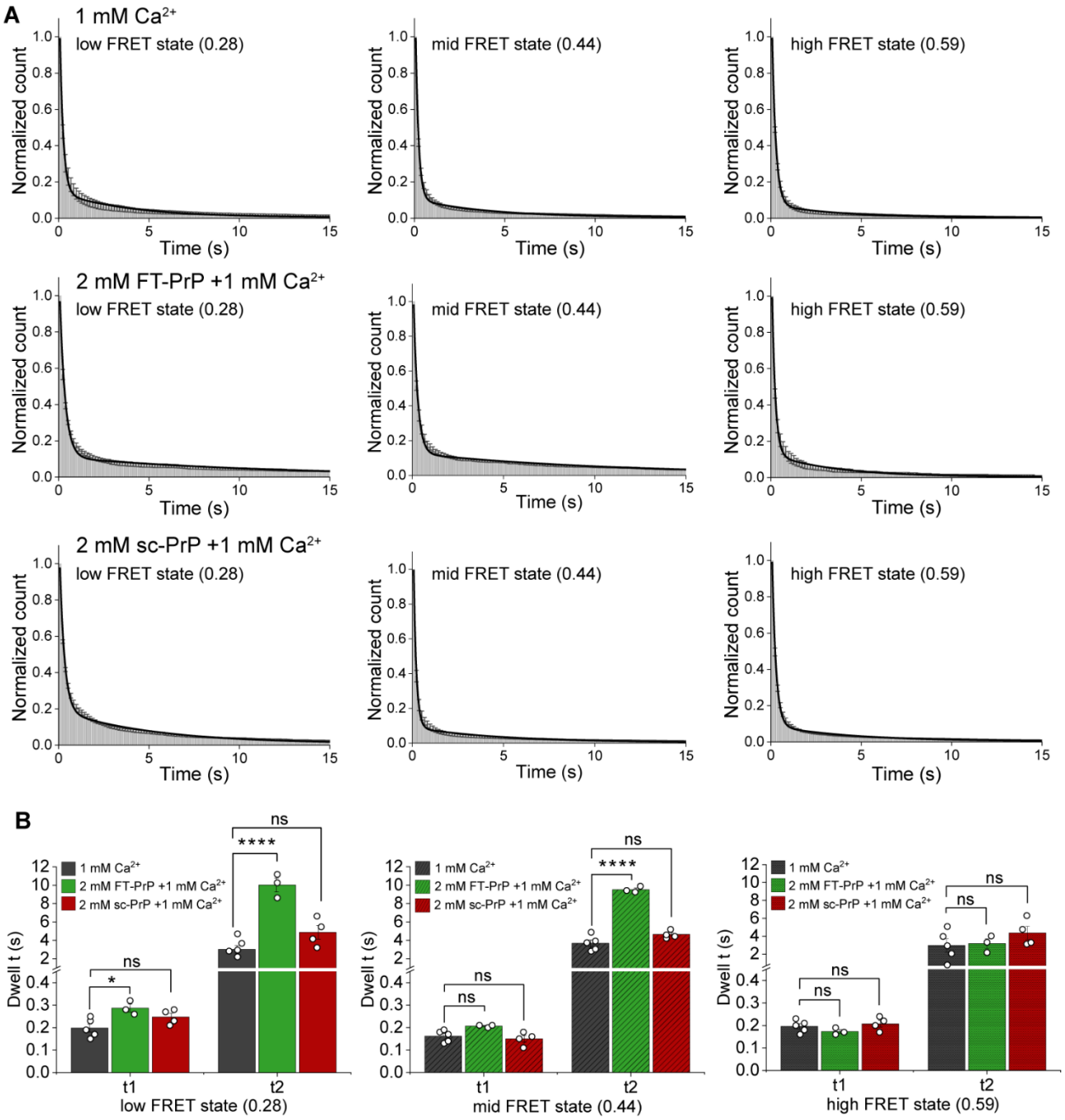


Fig. S8. Dwell time analysis.

(**A**) Dwell times of the three FRET states 0.28, 0.44 and 0.59 (CUB – GAIN sensor). Data shows the mean ±s.e.m from at least 3 independent measurements. A double exponential decay function was fitted to the data to calculate the dwell times for each condition (1 mM Ca^{2+} or 2 mM FT-PrP +1 mM Ca^{2+} or 2 mM sc-PrP +1 mM Ca^{2+}). (**B**) Bar graph representing the mean ±s.e.m of t1 and t2 dwell times of each FRET state (0.28, 0.44 and 0.59). Statistical significance was calculated using one-way ANOVA, Dunnett's post hoc test. $^{ns}p > 0.05$, $*p < 0.05$, $****p < 0.0001$.

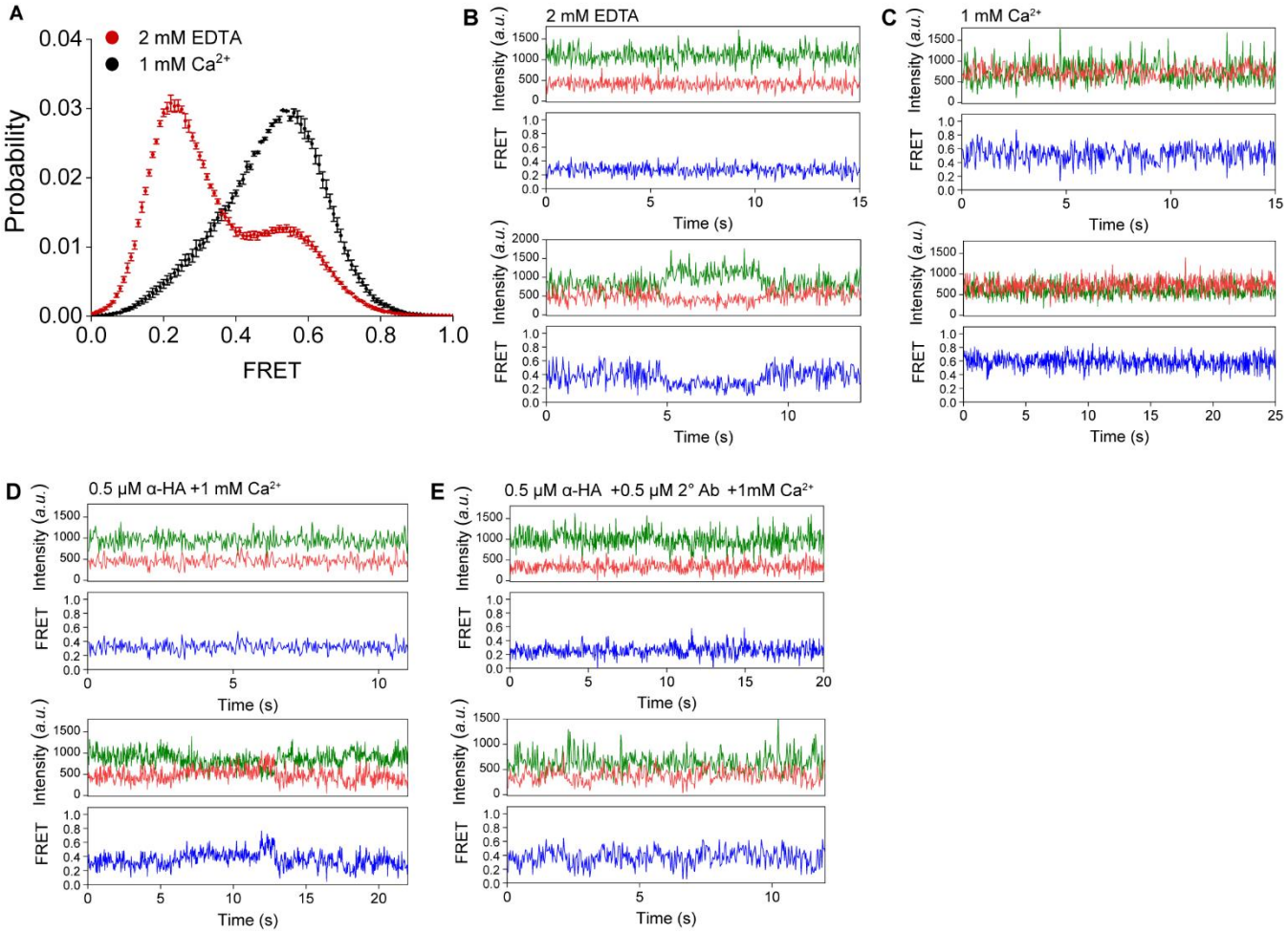


Fig. S9. smFRET analysis of HA-ADGRG6.

(A) smFRET population histograms of HA-ADGRG6. Data were obtained from CUB – GAIN sensor in the presence of 2 mM EDTA or 1 mM Ca^{2+}. Data represent mean ± s.e.m, n = 3 independent biological replicates. Representative FRET traces from sensor on CUB – GAIN domains sensor of HA-ADGRG6 in conditions **(B)** 2 mM EDTA (**C**) 1 mM Ca^{2+} (**D**) 0.5 μM anti-HA-tag antibody and 1 mM Ca^{2+} (α-HA) and (**E**) 0.5 μM anti-HA-tag antibody + 0.5 μM secondary antibody (2° Ab) and 1 mM Ca^{2+}. The donor, acceptor and FRET signals are in green, red and blue.

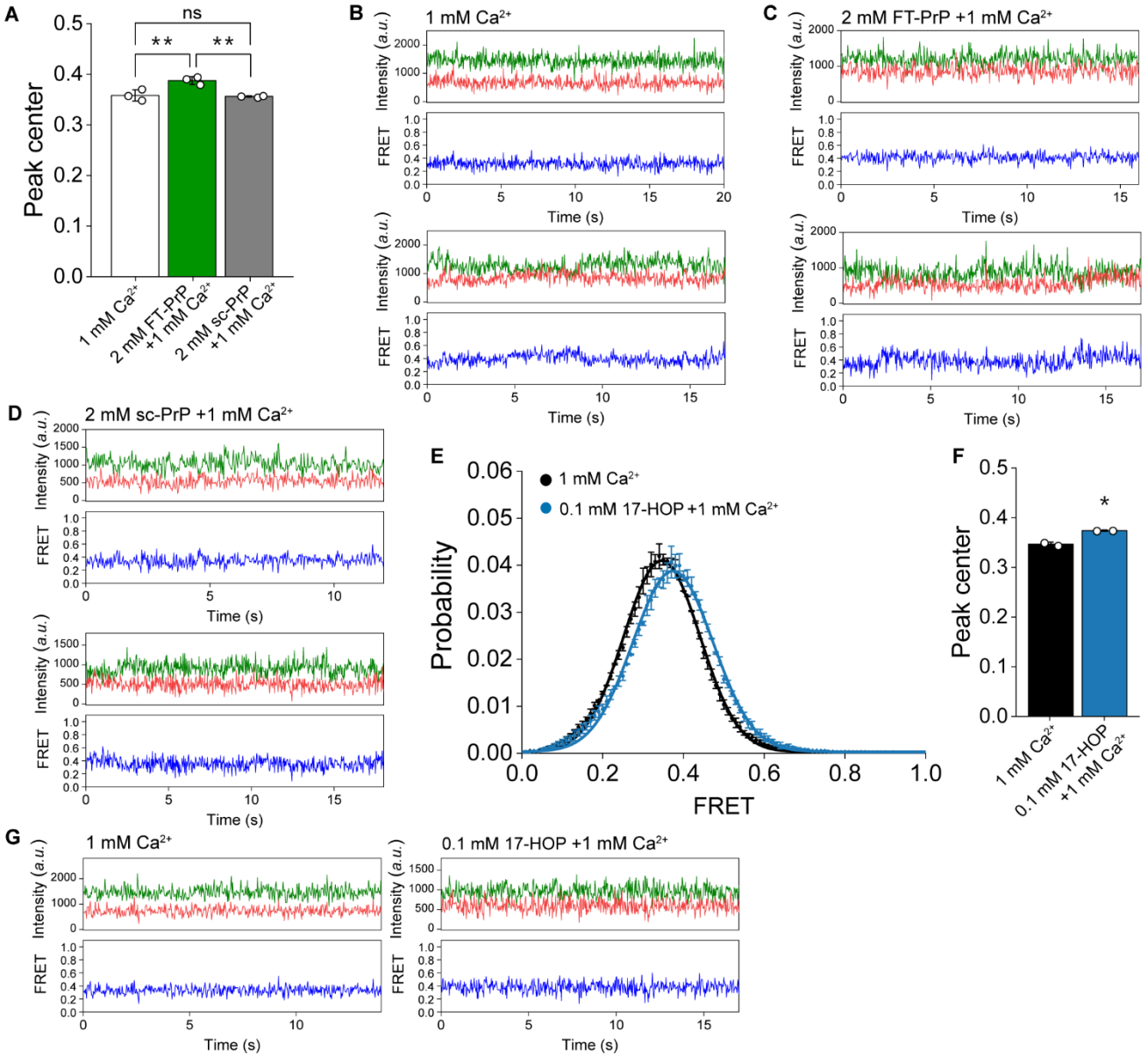


Fig. S10. Analysis of GAIN – 7TMD dynamics.

(A) Bar graph represents center of fitted single gaussian distribution peak to smFRET histograms (sensor GAIN – 7TMD) in 1 mM Ca^2, 2 mM FT-PrP +1 mM Ca^{2+} or 2 mM sc-PrP +1 mM Ca^{2+}. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test. $^{ns}p > 0.05$; $^{**}p < 0.01$. Data represent mean ± SD, n = 3 independent biological replicates. Representative FRET traces from ADGRG6 (sensor GAIN – 7TMD) in (**B**) 1 mM Ca^{2+}, (**C**) 2 mM FT-PrP +1 mM Ca^{2+}or (**D**) 2 mM sc-PrP +1 mM Ca^{2+}. (**E**) smFRET population histogram representing data collected from GAIN – 7TMD sensor in 1 mM Ca^{2+} +1% DMSO and with or without 0.1 mM 17α-HOP. Data represent mean ± s.e.m, n = 2 independent biological replicates. (**F**) Bar graphs represent center of a fitted single gaussian distribution (sensor GAIN – 7TMD). Statistical significance was determined by two-sample t-test. $^{*}p < 0.05$. Data represent mean ± SD, n = 2 independent biological replicates. (**G**) Representative FRET traces (sensor GAIN – 7TMD) in 1 mM Ca^2 +1% DMSO or 0.1 mM 17-HOP +1 mM Ca^{2+} +1% DMSO. (**B, C, D, G**) The donor, acceptor and FRET signals are in green, red and blue.