

Supplementary Figure 1

PI value distribution of single-pass membrane proteins at the plasma membrane in immune cells and in total cells.

The PI values were measured for the first 10 amino acids in the cytoplasmic domains of human single-pass transmembrane proteins that localized at the plasma membrane. Source data are available in Source Data 3.

A

Species	Sequence	Acidic	Basic	Hydrophobic	pI
Mouse	TNSRRNRLLQSDYMMNMTPRRPGLTRKPYQPYAPARDFAAAYRP	4.8 %	21.4 %	33.3 %	11.27
Human	VR-K-S---H-----P--H-----P-----S	4.8 %	28.6 %	35.7 %	11.29
Rat	T-----H-----L-P--H-----P-----S	4.8 %	23.8 %	35.7 %	11.27
Cattle	MKNK---M-H-----P--RH-----S	4.8 %	26.2 %	35.7 %	11.29
Cat	MKTK-S---H-----P--RH-----S	4.8 %	26.2 %	35.7 %	11.29
Marmoset	MR---S---H-----C-P--RH-----R-----S	4.8 %	26.2 %	35.7 %	11.20
Sheep	MK-K---MHH-----P--RH-----T-----S	4.8 %	28.6 %	30.9 %	11.29
Macaque	MR-K-S---H-----P--H-----P-----S	4.8 %	28.6 %	35.7 %	11.29
Monkey	MR-K-S---H-----P--H-----P-----S	4.8 %	28.6 %	35.7 %	11.29
Hamster	K--K---H-----L-P--H-----S	4.8 %	23.8 %	35.7 %	10.66
Pig	MK-K-T-M-H-----L-P--H-----S	4.8 %	26.2 %	35.7 %	11.00
Giraffe	MK-K---M-H-----P--RH-----S	4.8 %	26.2 %	35.7 %	11.29
Horse	MRN---S-T-H-----P--H-----S	4.8 %	26.2 %	33.3 %	11.50
Ferret	MK---S-I-H-----P--RH-----S	4.8 %	26.2 %	35.7 %	11.50
Buffalo	MKNK---M-H-----P--RH-----S	4.8 %	26.2 %	35.7 %	11.29
Rhinoceros	MR-K-S-T-H-----P--H-----T-----S	4.8 %	26.2 %	30.9 %	11.29

Sequence
LOGO

B

Protein	Net charge
Human CD28 _{cd}	+6.97
Mouse CD28 _{cd}	+8.45

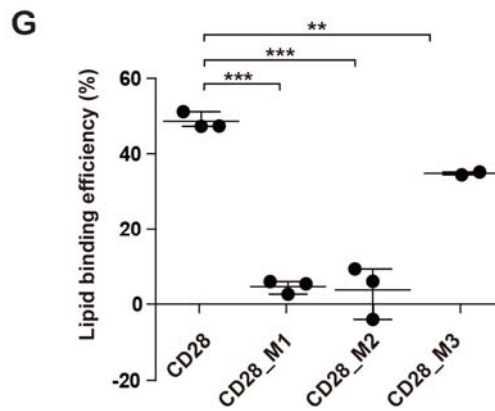
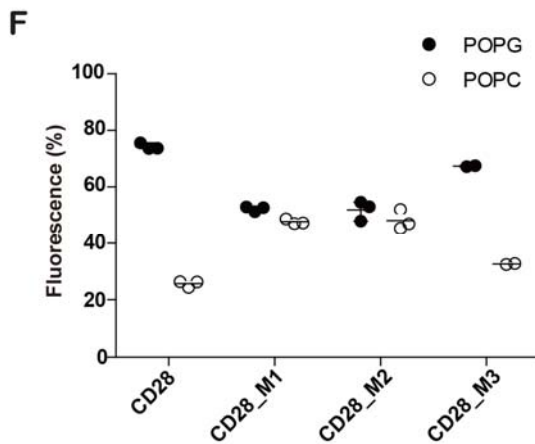
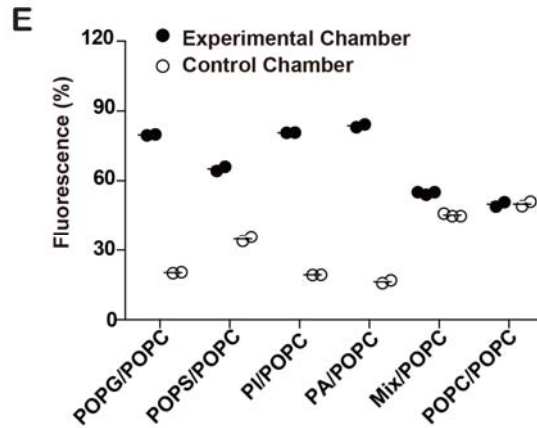
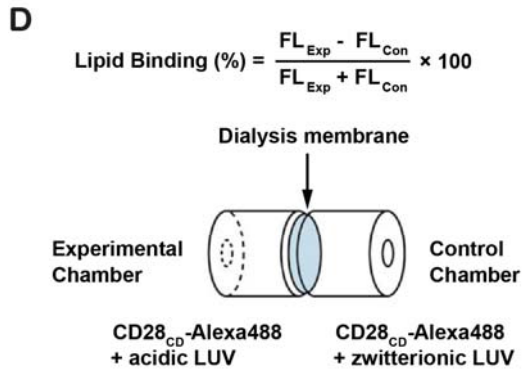
C

CD28_WT : TNSRRNRLLQSDYMMNMTPRRPGLTRKPYQPYAPARDFAAAYRP

CD28_M1 : ---AA-A-----

CD28_M2 : -----AA---AA-----

CD28_M3 : -----S-----S-----



Supplementary Figure 2

CD28 cytoplasmic domain specifically binds to acidic phospholipids.

(A) Alignment of CD28_{CD} from different species. Most residues are highly conserved, especially the two important YxxM and PYAP signaling motifs (marked in red) and basic residues (marked in green). The residue conservation is visualized by the sequence logo of CD28_{CD} generated from the Skyline web server <http://www.skyline.org>.

(B) Net charges of human and mouse CD28 cytoplasmic domains at pH 7.0.

(C) The protein sequences of mouse CD28_{CD} and three mutants used in (D-G). Two polybasic regions (PBR) are marked in red, and mutant sites are marked in blue.

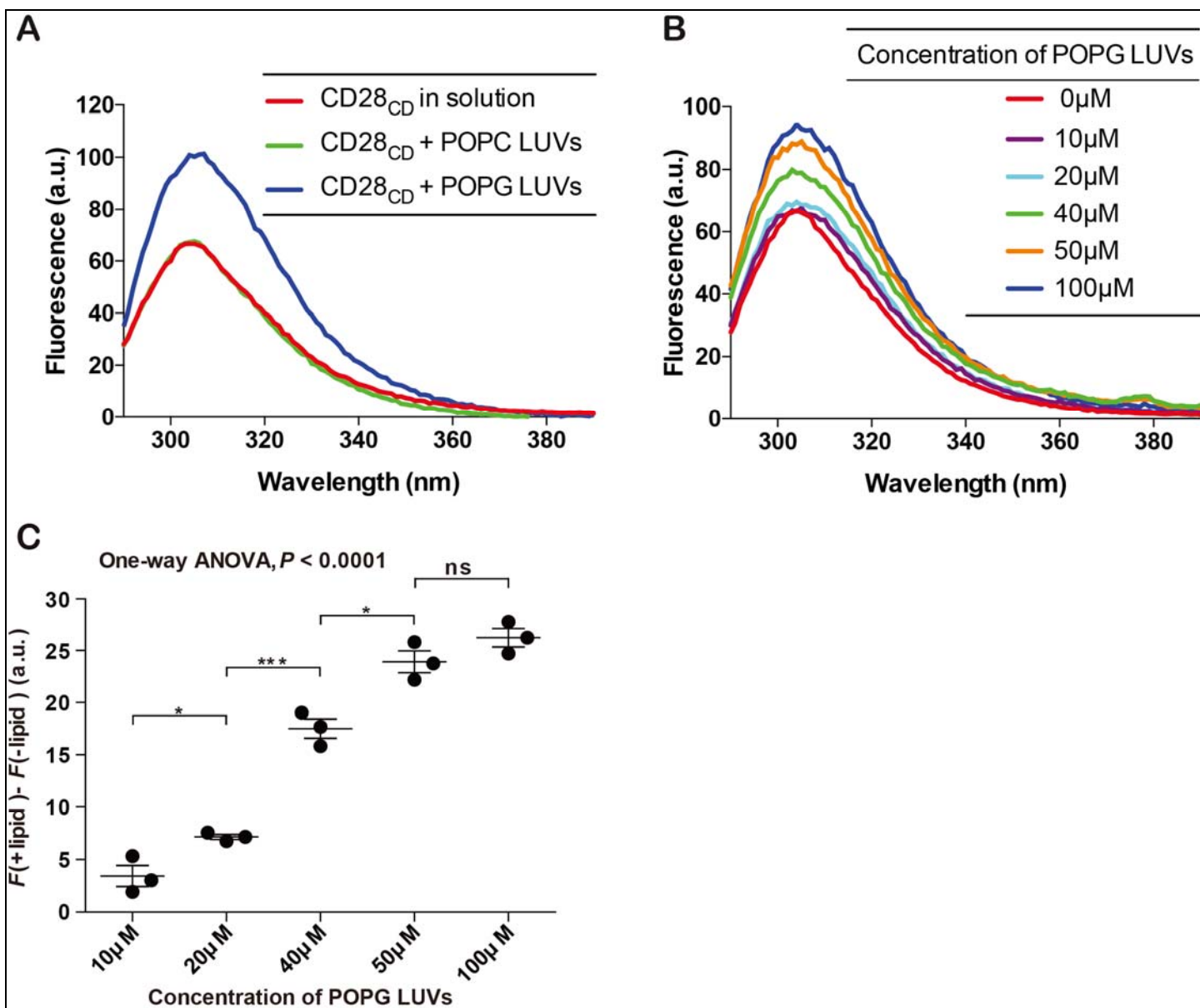
(D-G) Binding of CD28_{CD} WT and mutants to acidic phospholipids was measured by the equilibrium-based microdialysis assay.

(D) A cartoon illustration of the equilibrium-based microdialysis assay. See Online Methods for details.

(E) Binding of CD28_{CD} WT to different acidic phospholipids measured by the microdialysis assay. LUVs in the experimental chamber were composed of either 100% of the acidic phospholipids POPG, POPS, PI, PA, or a lipid mixture (40% POPC, 20% POPS, 20% POPE, 10% POPG and 10% PI). LUVs in the control chamber were composed of 100% of the zwitterionic lipid POPC. CD28_{CD}-Alexa 488 was used at a concentration of 10 nM and LUVs at 5 mM. Two to three independent samples were measured for each condition and the results are plotted as the percentage of the fluorescence intensity in the experimental and control chambers.

(F-G) Binding of CD28_{CD} WT and mutants to acidic phospholipids measured by the equilibrium-based microdialysis assay. Two to three independent samples were measured for each condition and the results are plotted as the percentage of the fluorescence intensity in the experimental and control chambers (F), and are further converted to the binding efficiencies of the mutants to POPG (G). In (G), data are representatives of two to three independent experiments, and were analyzed by unpaired *t*-test. ***P* < 0.01; ****P* < 0.001; ns: no significant difference.

The center value and error bar in **e-g** are detailed in Online Methods. Source data for **e-g** are available in Source Data 4.



Supplementary Figure 3

Measurement of membrane binding of CD28_{CD} by the aromatic fluorescence emission (AFE) assay.

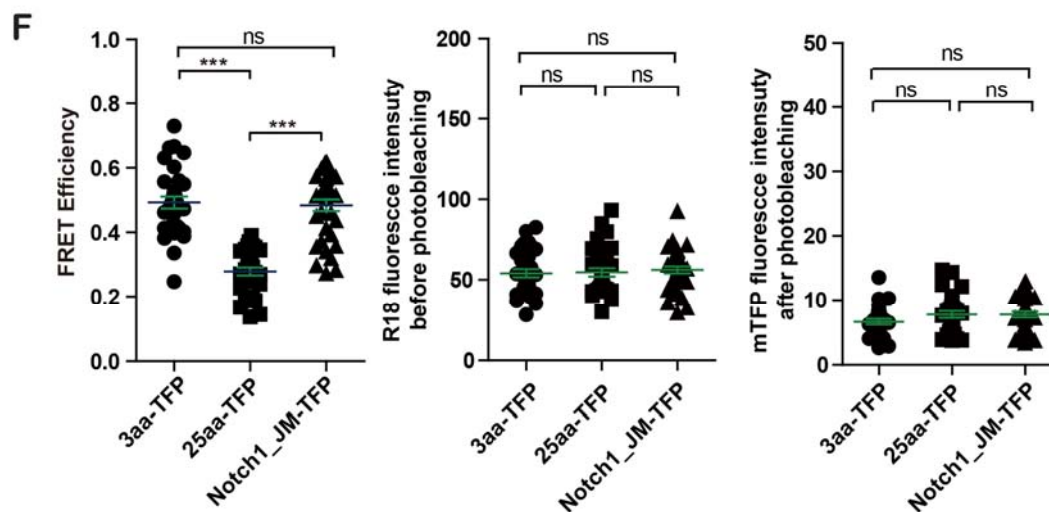
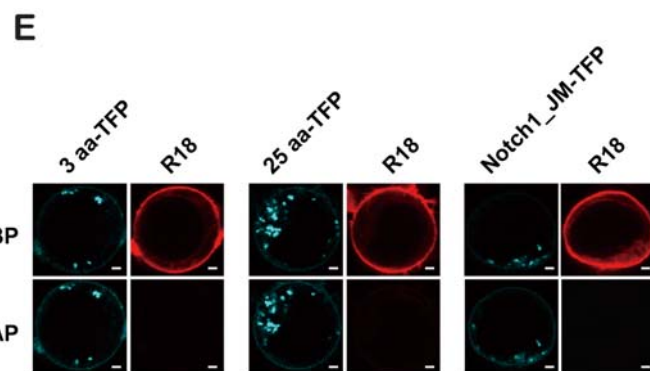
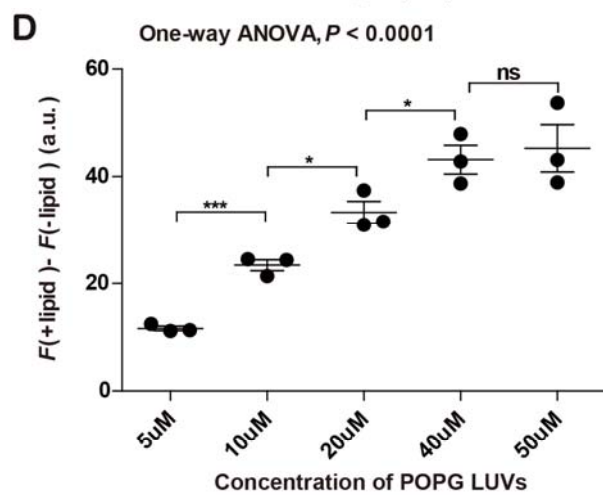
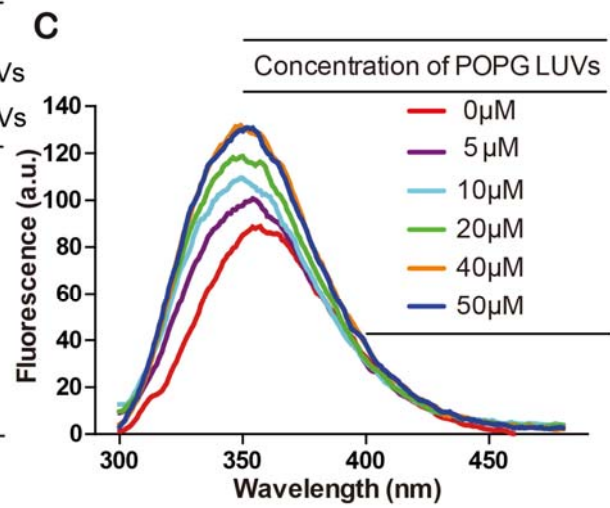
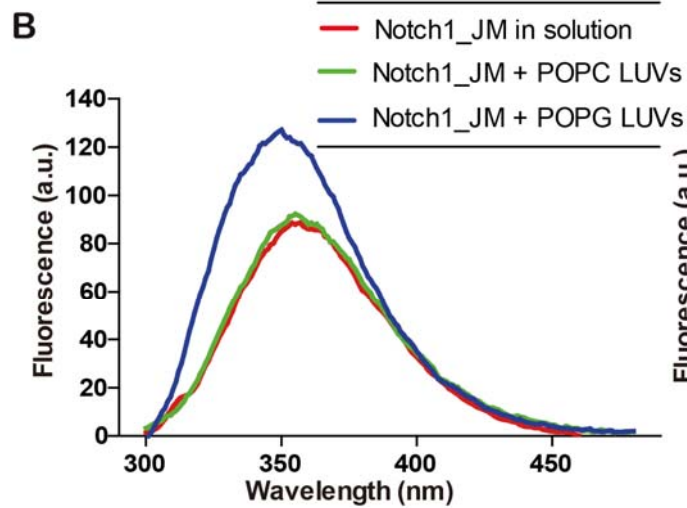
(A) Incubation of 2 μ M CD28_{CD} with 0.2 mM acidic POPG LUVs led to significant increase of AFE value but such an increase was not observed in that with 0.2 mM zwitterionic POPC LUVs.

(B-C) Titration of acidic POPG LUVs with the indicated concentrations into 2 μ M CD28_{CD} sample led to the gradient increase of AFE value (at 310nm).

For each condition, three independent samples were measured in one experiment. Three independent experiments were performed. Unpaired *t*-test was used for comparing each two groups. One-Way ANOVA was used to test whether POPG treatment could cause significant change of the AFE value, $P < 0.0001$ (C).

The center value and error bar in **c** are detailed in Online Methods. Source data are available in Source Data 5.

Protein	Sequence	Net charge
human Notch1_JM	SRKRRRQHGQLWFPECFKVS	12.01



Supplementary Figure 4

Membrane binding of Notch1 juxtamembrane polybasic region.

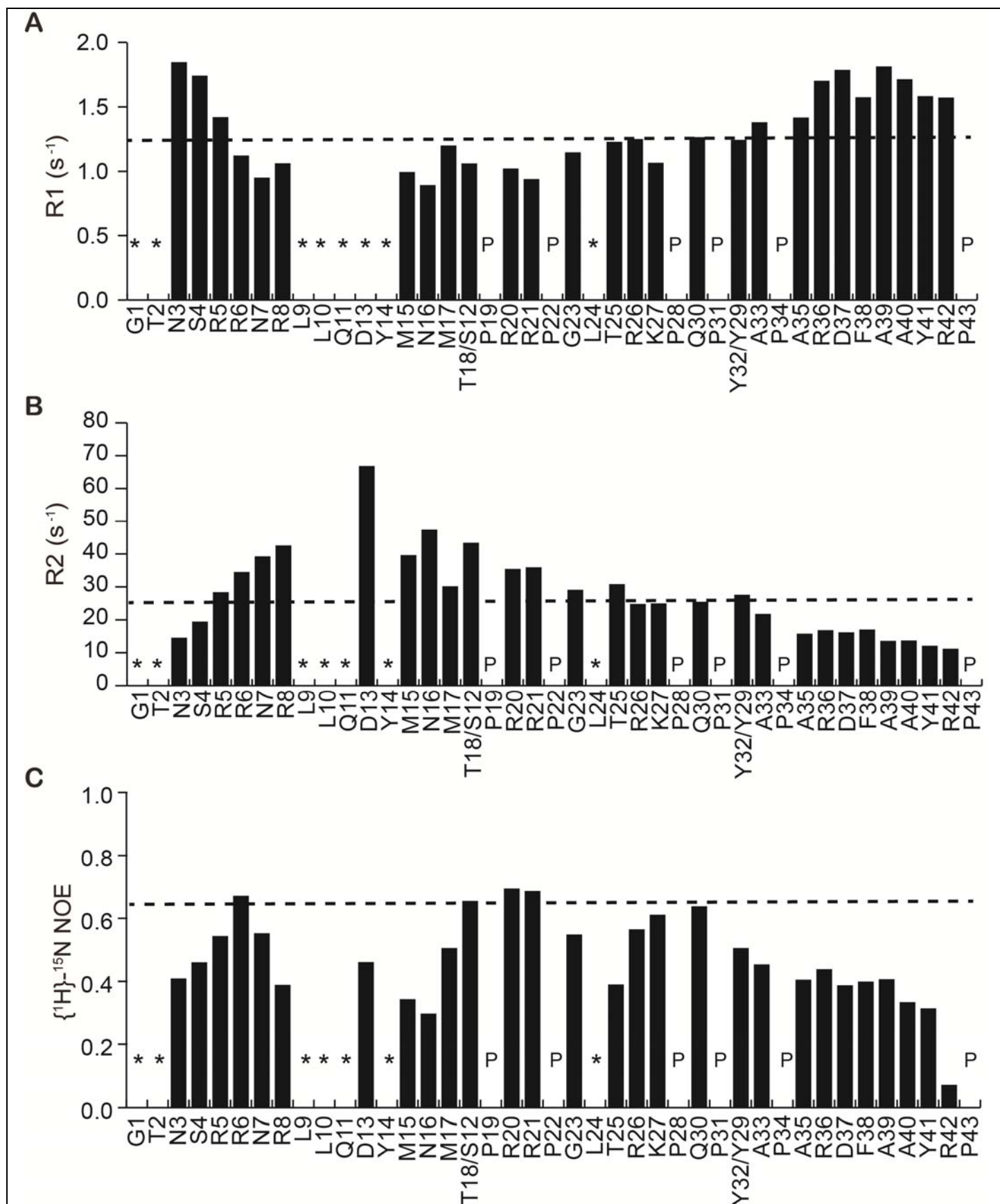
(A) Sequence information and charge property of human Notch1 juxtamembrane domain.

(B) Incubation of 2 μ M Notch1_JM with 0.1 mM acidic POPG LUVs led to significant increase of AFE value but such an increase was not observed in that with 0.1 mM zwitterionic POPC LUVs.

(C-D) Titration of acidic POPG LUVs with the indicated concentrations into 2 μ M Notch1_JM sample led to the gradient increase of AFE value (at approximate 350nm). Three independent samples were measured for each condition. Unpaired *t*-test was used for the statistical analysis of each two groups. One-Way ANOVA was used to test whether POPG treatment could cause significant change of the AFE value, $P < 0.0001$.

(E-F) The de-quenching FRET was used to measure the interaction of Notch1 juxtamembrane polybasic region with the plasma membrane in live Jurkat T cells. Representative pictures are shown in E. Bars in E represent 2 μ m. $N = 31, 32, 35$ for the three conditions (from left to right). Each dot represents the FRET value from one individual cell. One-Way ANOVA was used to analyze difference among three groups, the p-values of these three panels from left to right were $<0.0001, 0.2074$ and 0.1555 . Unpaired *t*-test was used to analyze difference between each two groups.

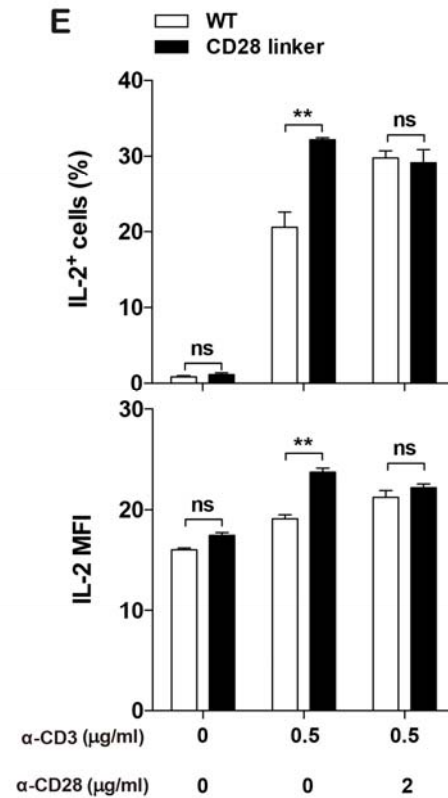
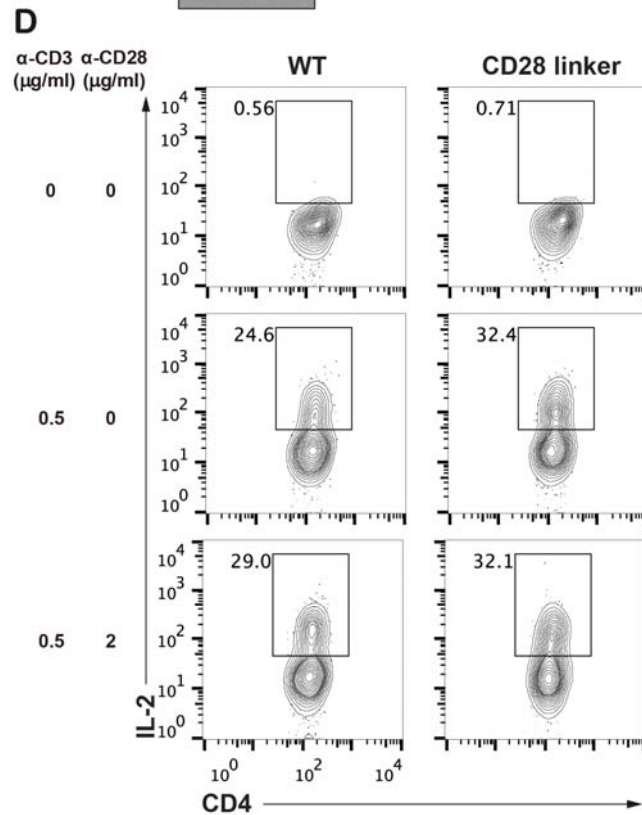
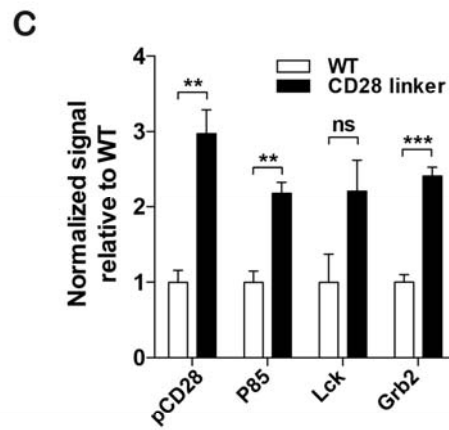
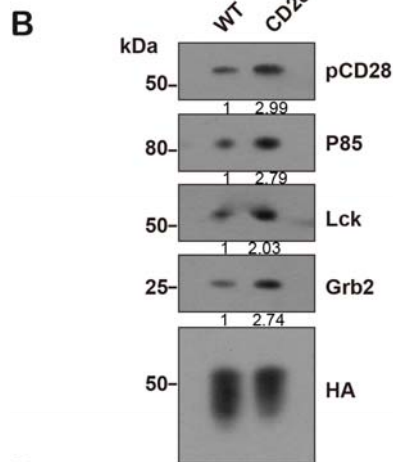
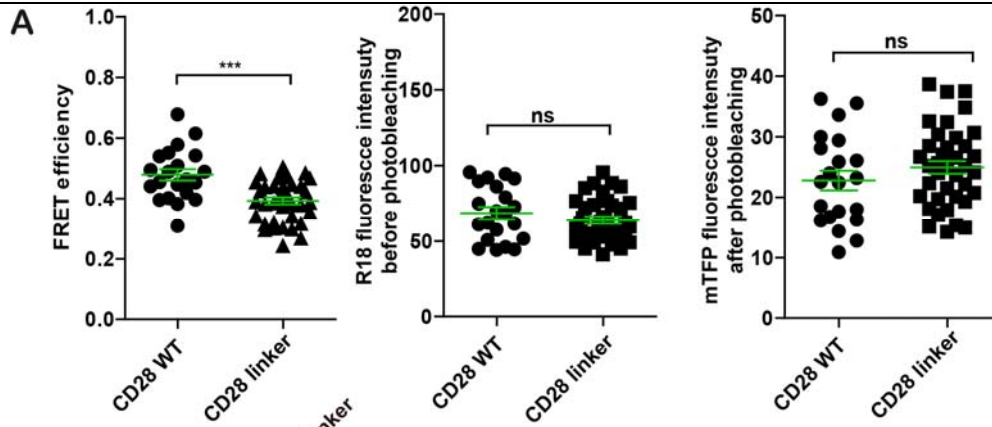
The center value and error bar in **d,f** are detailed in Online Methods. Source data for **b-d**, and **f** are available in Source Data 6.



Supplementary Figure 5

Intrinsic dynamics of membrane-bound CD28_{CD}.

¹⁵N backbone spin relaxation measurements of CD28_{CD} in POPG bicelles. (A) ¹⁵N longitudinal relaxation rates (R1), (B) ¹⁵N transverse relaxation rates (R2) and (C) the heteronuclear ¹H-¹⁵N NOEs. Disappeared or overlapped residues are marked by asterisks. Prolines that lack backbone amides are marked by P.



Supplementary Figure 6

Disrupting membrane binding of CD28_{CD} promoted its basal signaling.

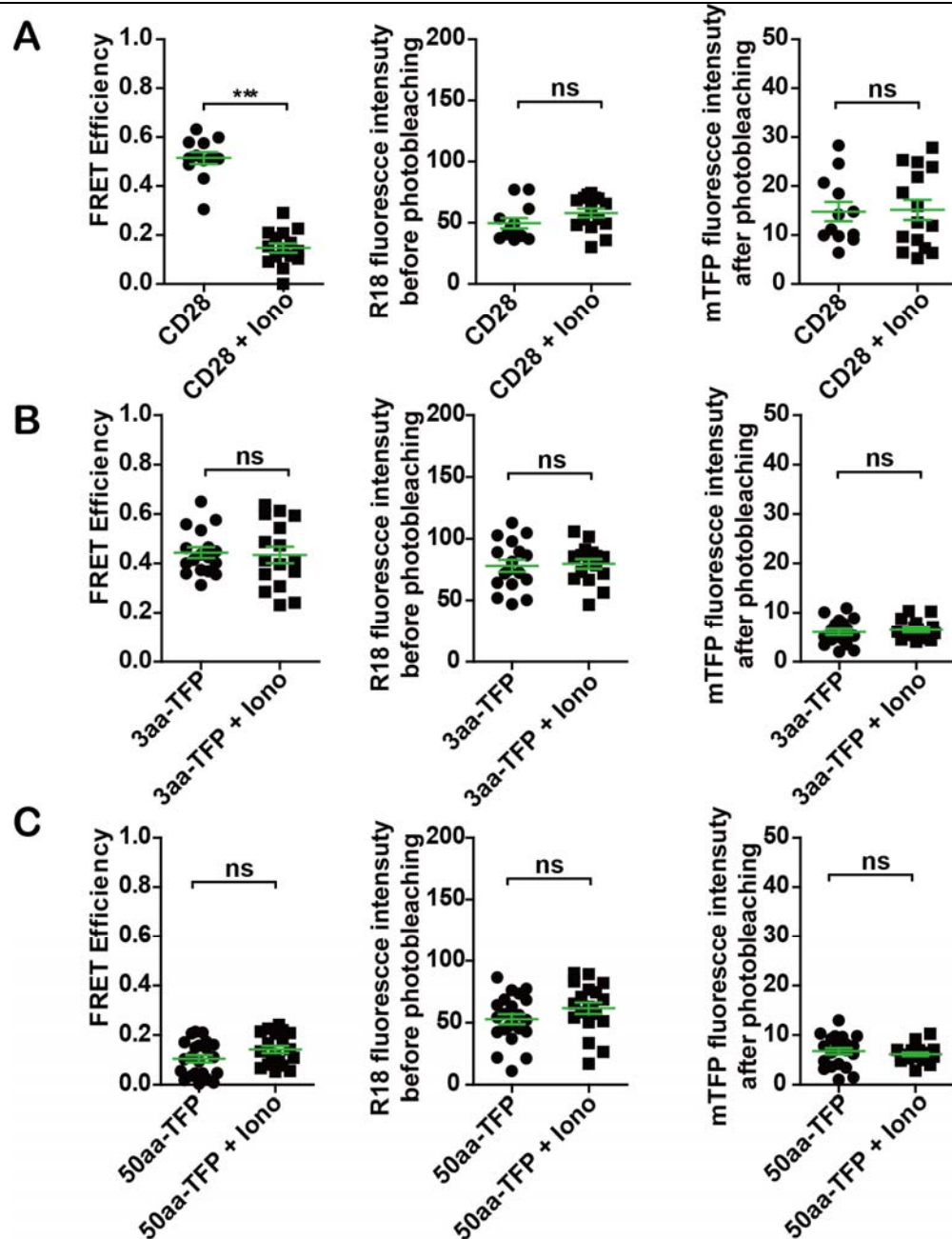
(A) The de-quenching FRET was used to measure the membrane binding of CD28 WT and CD28 linker mutant (n = 21, 38 (from left to right), each dot represents the FRET value from one individual cell).

(B-E) TFP-T2A-HA-CD28 WT or linker mutant construct was transduced into mouse CD28^{-/-} T cells by retrovirus, respectively. After protein translation of the fusion construct, TFP and HA-CD28 were separated due to the self-cleaving property of T2A. Basal CD28 signaling was measured in transduced cells without stimulation. IL-2 production was measured in transduced cells under TCR and/or CD28 stimulation.

(B-C) The cells without stimulation were lysed and the immunoprecipitation assay was performed to detect basal phosphorylation of CD28 and its interactions with signaling proteins. The bands were quantitated by ImageJ. The pCD28/HA, P85/HA, Lck/HA and Grb2/HA ratios were obtained and further normalized to the value of WT condition of each strip. Average results of 4 independent samples are shown in panel C.

(D-E) The cells were stimulated for 4 hours with plated-bound α -CD3 (0.5 μ g/ml) alone or α -CD3 (0.5 μ g/ml) + α -CD28 (2 μ g/ml), and IL-2 production was measured by intracellular staining and flow cytometric analysis. CD4⁺ TFP⁺ cells were gated for the analysis of IL-2 level. TFP levels of CD28 WT cells and CD28 linker cells were matched, reflecting the comparable expression level of CD28 WT and CD28 linker. Percentage of IL-2 positive cells and median fluorescence intensity (MFI) of all cells are shown (n = 3).

Data are representatives of three (A, C) or four (E) independent experiments, and were analyzed by unpaired *t*-test. The center value and error bar in **c,e** are detailed in Online Methods. The original gel image of **b** can be found in **Supplementary Data Set 2**. Source data for **a, c** and **e** are available in Source Data 7.

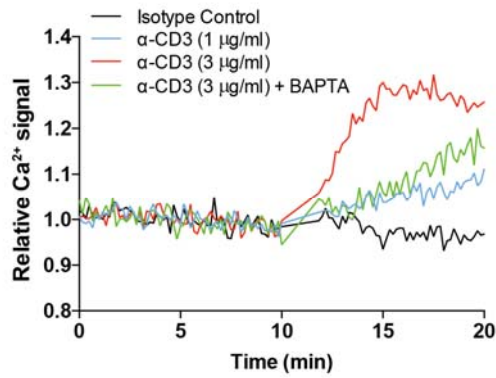
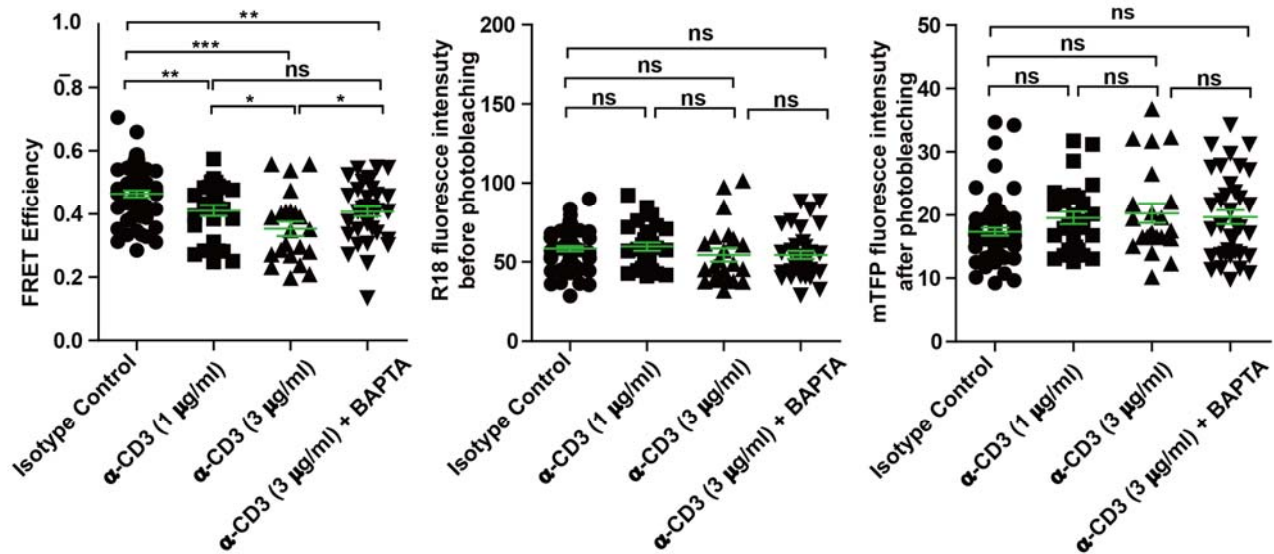
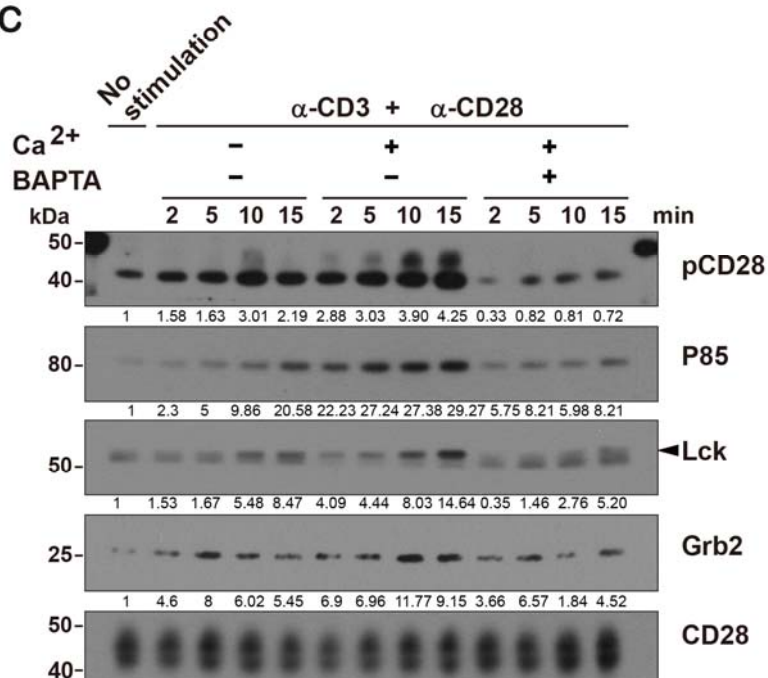


Supplementary Figure 7

Ionomycin-induced Ca^{2+} influx enhances the openness of CD28.

The de-quenching FRET method was used to measure the effect of Ca^{2+} on CD28_{CD}-membrane binding in live Jurkat T cells. Ionomycin (1 μM) was used to trigger Ca^{2+} influx in T cells. Ca^{2+} influx led to the significant decrease of the FRET value of CD28-TFP (A), indicating the dissociation of CD28_{CD} from the membrane. In contrast, the FRET values of the 3aa-TFP and 50aa-TFP control constructs were insensitive to Ca^{2+} influx (B, C). The FRET efficiency was measured and plotted as mean \pm s.e.m. ($n = 12, 15$ in (A), $n = 17, 16$ in (B), $n = 22, 19$ in (C) (from left to right), each dot represents the FRET value from one individual cell). The surface mTFP level and R18 level were comparable.

Data are representatives of two independent experiments. All the data were analyzed by unpaired *t*-test except the left sub-panels in (A) and (C) where Mann-Whitney test were used because the FRET efficiency data sets of the CD28 group and the 50aa-TFP group do not fit the normal distribution. The center value and error bar are detailed in Online Methods. Source data are available in Source Data 8.

A**B****C**

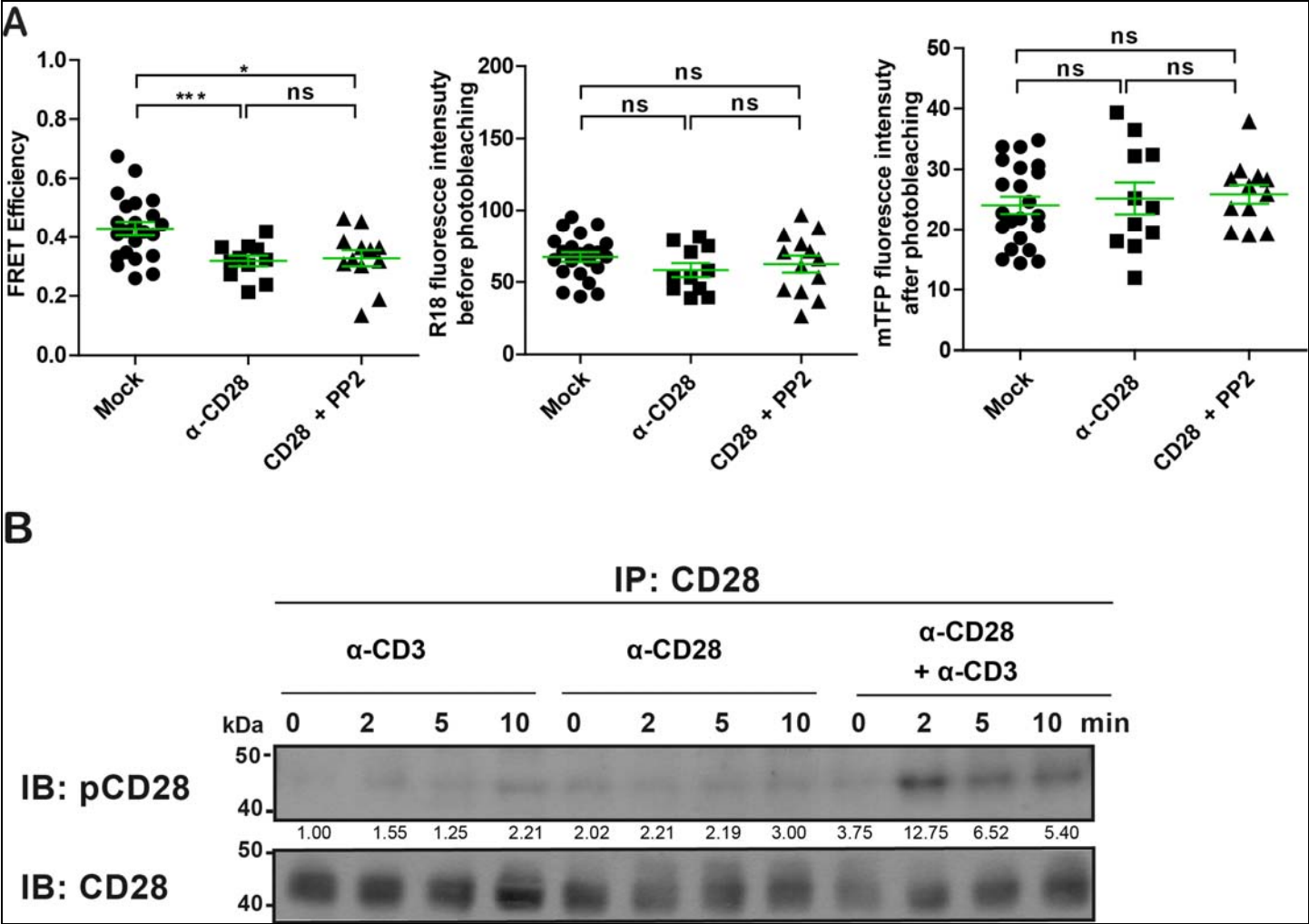
Supplementary Figure 8

Ca²⁺ influx enhances the openness of CD28 and its signaling.

(A-B) To further demonstrate the effect of Ca²⁺ on CD28_{CD}-membrane binding, we use different concentrations of α-CD3 to generate different levels of Ca²⁺ influx. We also used BAPTA pre-treatment to chelate the intracellular Ca²⁺ (A). The de-quenching FRET was used to detect the effect of TCR-induced Ca²⁺ influx on the CD28_{CD}-membrane interaction. Different doses of α-CD3 induced different levels of Ca²⁺ influx (A). Treatment of 10 μM BAPTA led to partial chelation of intracellular Ca²⁺. N = 64, 28, 22, 36 (from left to right) for each condition. Each dot represents the FRET value from one individual cell. Mouse IgG (3 μg/ml) was used as the isotype control antibody for α-CD3.

(C) To exam the effect of Ca²⁺ influx on CD28 signaling in primary T cell, the mouse CTL were generated and stimulated with α-CD3 (0.5 μg/ml) + α-CD28 (2 μg/ml) with indicated times in the Ca²⁺/Mg²⁺-free Ringer's buffer containing 1 mM Ca²⁺ or not at 37 °C. After stimulation, cells were lysed for immunoprecipitation and immunoblotting. The bands were quantitated by ImageJ. The pCD28/CD28, P85/CD28, Lck/CD28 and Grb2/CD28 ratios were obtained and further normalized to the value of no stimulation condition of each strip.

Data are representatives of three independent experiments. In (B), for the left sub-panel, One-Way ANOVA was used to analyze difference among four groups (*P*<0.0001) and unpaired *t*-test was used to analyze difference between two groups. For the middle and right sub-panels, the R18 level of the α-CD3 (3μg/ml) group and the mTFP level of the isotype control group do not fit the normal distribution. To compare each two groups, Mann Whitney test was used for data sets that do not fit the normal distribution and unpaired *t*-test was used for the rest. The center value and error bar in **d,f** are detailed in Online Methods. The original gel image of **b** can be found in **Supplementary Data Set 2**. Source data for **b** are available in Source Data 9.



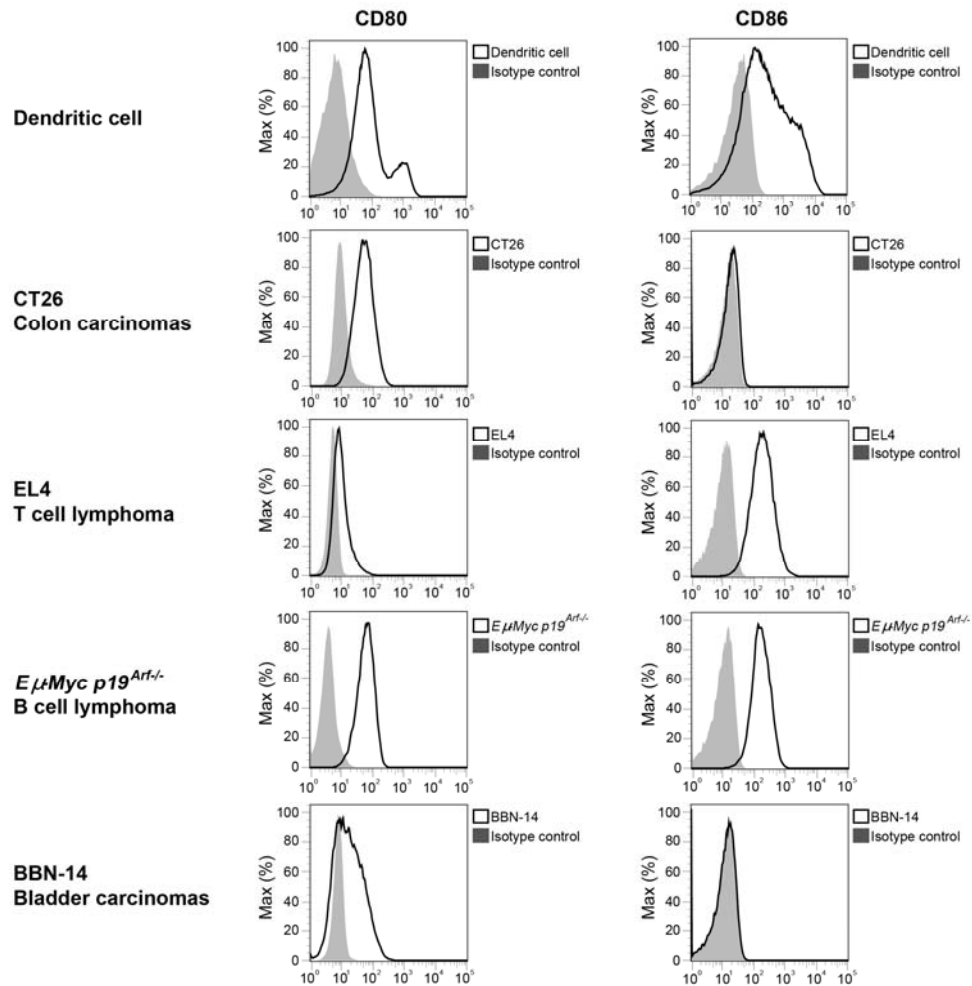
Supplementary Figure 9

CD28 ligation caused the dissociation of CD28 cytoplasmic domain from the membrane but was insufficient to induce CD28 phosphorylation.

(A) The de-quenching FRET method was used to measure whether CD28 ligation could induce CD28 cytoplasmic domain to dissociate from the membrane in live Jurkat T cells. The stimulating buffer was $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Ringer's buffer. Compared with mock treatment (no antibody), addition of 5 $\mu\text{g}/\text{ml}$ $\alpha\text{-CD28}$ induced dissociation of CD28_{CD} from the membrane. To test whether this induction was dependent on the phosphorylation of CD28, 100 μM PP2 was pre-incubated with T cells for 10 min to inhibit Src family kinase and then the cells were stimulated by 5 $\mu\text{g}/\text{ml}$ $\alpha\text{-CD28}$. The result indicated CD28 phosphorylation was not required for the dissociation of CD28_{CD} from the membrane. $N = 22, 11, 12$ for each condition (from left to right). Each dot represents the FRET value from one individual cell. Data are representatives of three independent experiments. Unpaired t -test was used to analyze difference between two groups.

(B) CD28-deficient Jurkat T cells expressing HA-mCD28 were stimulated by 1 $\mu\text{g}/\text{ml}$ $\alpha\text{-CD3}$ alone, 2 $\mu\text{g}/\text{ml}$ $\alpha\text{-CD28}$ alone or 2 $\mu\text{g}/\text{ml}$ $\alpha\text{-CD28}$ and 1 $\mu\text{g}/\text{ml}$ $\alpha\text{-CD3}$ in normal Ringer's buffer (containing 1 mM Ca^{2+}) for the indicated time at 37 °C. Either CD3 ligation alone or CD28 ligation alone could not trigger CD28 phosphorylation, but CD28 ligation and TCR ligation together could trigger CD28 phosphorylation. The bands were quantitated by Image J. The pCD28 band intensity was divided by the corresponding CD28 band intensity to obtain the pCD28/CD28 ratio, which were further normalized to the value of 0 min of $\alpha\text{-CD3}$ condition.

The center value and error bar in **a** are detailed in Online Methods. The original gel image of **b** can be found in **Supplementary Data Set 2**. Source data for **a** are available in Source Data 10.



Supplementary Figure 10

Low surface expression of CD28 ligands in tumor cells.

CD80 and CD86 surface levels were detected on CT26 murine colon carcinoma cell line, EL4 murine lymphoma cell line, *EμMyc p19^{Arf}-/-* mouse B cell lymphoma cell line and BBN-14 murine bladder carcinoma cell line. Activated dendritic cell (activated by 100 ng/ml LPS at 37 °C for 24 h) was used as a positive control.