

**Rab37 mediates trafficking and membrane presentation of PD-1 to sustain T cell
exhaustion in lung cancer**

Wan-Ting Kuo, I-Ying Kuo, Hung Chia Hsieh, Ssu-Ting Wu, Wu-Chou Su, and
Yi-Ching Wang

Supplemental Data

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Table S1. The plasmids and their characteristics used in the current study.

Plasmid	Target	Insert (bp)	Function	Source
pcDNA3.1-V5/His-vector	None	-- ^a	Vector control	Invitrogen
pCMV3- hRab37-WT-OFPSpark	Wild type human Rab37	672	Overexpression	Homemade ^b
pcDNA3.1-V5/His-hRab37-WT	Wild type human Rab37	672	Overexpression	Homemade ^b
pcDNA3.1-V5/His-hRab37-QL	Q89L human Rab37	672	Overexpression	Homemade ^b
pcDNA3.1-V5/His-hRab37-TN	T43N human Rab37	672	Overexpression	Homemade ^b
pcDNA3.1-V5/His-hPD-1-GFP	Wild type human PD-1	867	Overexpression	Sino Biological Inc
pcDNA3.1-V5/His -N49Q-PD-1-GFP	N49Q human PD-1	867	Overexpression	Homemade ^c
pcDNA3.1-V5/His -N58Q-PD-1-GFP	N58Q human PD-1	867	Overexpression	Homemade ^c
pcDNA3.1-V5/His -N74Q-PD-1-GFP	N74Q human PD-1	867	Overexpression	Homemade ^c
pcDNA3.1-V5/His-N116Q-PD-1-GFP	N116Q human PD-1	867	Overexpression	Homemade ^c
pcDNA3.1-V5/His -2NQ-PD-1-GFP	N49/58Q human PD-1	867	Overexpression	Homemade ^c
pcDNA3.1-V5/His -3NQ-PD-1-GFP	N49/58/74Q human PD-1	867	Overexpression	Homemade ^c
pGL4.30[luc2P/NFAT-RE/Hygro]	NFAT response element	90	Reporter vector	Promega

^a The plasmid is used as a backbone vector therefore there is no inserted DNA fragment.

^b Rab37-WT, Q89L or T43N was PCR-amplified with designated mutation at the primer sequences and cloned into pcDNA3.1-V5/His or pCMV3-OFPSpark expression vector to generate V5/His-tagged or RFP-tagged Rab37 expression vector.

^c PD-1-WT, N49Q, N58Q, N74Q, N116Q, 2NQ, 3NQ, were PCR-amplified with designated mutation at the primer sequences and cloned into pcDNA3.1-V5/His expression vector to generate GFP-tagged PD-1 expression vector.

Table S2. Antibodies and their reaction conditions used in the current study.

Target	KD	Raised in	Application	Dilution	Source	Catalog no.
Rab37	27	Rabbit	Western blot	1:1000	Proteintech	13051-1-AP
			Immunofluorescence	1:5000		
	-- ^a	Mouse	Immuno-EM	1:30	Homemade	
PD-1	32	Rabbit	Western blot	1:1000	Genetex	GTX31309
	-- ^a	Hamster	Flow cytometry (APC)	1:200	BD Bioscience	562671
	-- ^a	Mouse	Flow cytometry (BB515)	1:200	BD Bioscience	564494
PD-1	55	Rabbit	Western blot	1:1000	Cell Signaling Technology	86163
	-- ^a		Immuno-EM	1:20		
			Immunofluorescence	1:2000		
V5 tag	-- ^a	Mouse	Western blot	1:5000	Invitrogen	46-0705
Calnexin	68	Rabbit	Western blot	1:5000	Genetex	GTX109669
β-ACTIN	42	Mouse	Western blot	1:5000	Genetex	GTX26276
PERK	125	Rabbit	Western blot	1:1000	Genetex	GTX129275
Phospho PERK	125	Rabbit	Western blot	1:1000	Cell Signaling	3179S
eIF2α	38	Rabbit	Western blot	1:1000	Genetex	GTX101241
Phospho eIF2α	38	Rabbit	Western blot	1:1000	Cell Signaling	3398S

ATF6	110, 65	Rabbit	Western blot	1:1000	Genetex	GTX104820
XBP1s	50, 30	Rabbit	Western blot	1:1000	Cell Signaling	12782S
Rab11b	25	Rabbit	Western blot	1:1000	Genetex	GTX119095
			Immunofluorescence	1:200		
Rab8a	25	Rabbit	Western blot	1:1000	elabscience	EC8703
Rab3a	25	Rabbit	Western blot	1:1000	Proteintech	15029-1-AP
GAPDH	37	Mouse	Western blot	1:1000	Santa Cruz	Sc-32233
Na ⁺ /K ⁺ ATPase	113	Rabbit	Western blot	1:100000	Abcam	ab76020
EEA1	-- ^a	Rabbit	Immunofluorescence	1:200	Genetex	GTX109638
TGN46	-- ^a	Mouse	Immunofluorescence	1:200	Genetex	GTX22809
CD3	-- ^a	Mouse	Cell culture	1:1000	BD Bioscience	566685
CD28	-- ^a	Mouse	Cell culture	1:1000	BD Bioscience	555725
CD4	-- ^a	Rat	Flow cytometry (FITC)	1:200	BD Bioscience	553047
CD8	-- ^a	Rat	Flow cytometry (FITC)	1:200	BD Bioscience	553031
		Mouse	Flow cytometry (APC)	1:200	BD Bioscience	561952
		Rabbit	Immunofluorescence	1:3000	Abcam	ab217344
TIM3	-- ^a	Rabbit	Immunofluorescence	1:2000	Cell Signaling	45208T

	-- ^a	Human	Flow cytometry (BV421)	1:200	BD Bioscience	565562
Ki67	-- ^a	Rat	Flow cytometry (FITC)	1:200	eBioscience	2040334
		Mouse	Flow cytometry (BV510)	1:200	BD Bioscience	563462
CD107a/LAMP1	-- ^a	Mouse	Flow cytometry (PE-Cy7)	1:200	BD Bioscience	561348
	-- ^a	Rat	Flow cytometry (PerCP/Cy5.5)	1:200	BD Bioscience	121626
Granzyme B	-- ^a	Mouse	Flow cytometry (PE)	1:200	BioLegend	372207
TNF- α	-- ^a	Rat	Flow cytometry (BV421)	1:200	BD Bioscience	563387
CD25	-- ^a	Rat	Flow cytometry (APC)	1:200	BD Bioscience	557192
DAPI	-- ^a	- ^c	Immunofluorescence	- ^b	Genetex	GTX30920
Opal 520 Reagent	-- ^a	- ^c	Florescent immunohistochemistry	1:100	Akoya Biosciences	FP1013
Opal 570 Reagent	-- ^a	- ^c	Florescent immunohistochemistry	1:100	Akoya Biosciences	FP1014
Opal 620 Reagent	-- ^a	- ^c	Florescent immunohistochemistry	1:100	Akoya Biosciences	FP1495A
Opal 670 Reagent	-- ^a	- ^c	Florescent immunohistochemistry	1:100	Akoya Biosciences	FP1117

^a Molecular weight is not applicable to this antibody in such an application.

^b DAPI is a commercial product for nuclear staining.

^c Species is not applicable to this antibody in such an application.

Table S3. Characteristics of NSCLC patients and normal individuals for *ex vivo* assays in the current study.

	NSCLC patients	Normal individuals
Clinical features	N = 9	N = 3
Age: Median (range)	71 (46-83)	29 (28-30)
Sex		
Male	8	3
Female	1	0
Histology		
Adenocarcinoma	6	- ^a
Squamous cell carcinoma	2	
Others	1	
<i>EGFR</i> gene status		
Mutated	1	- ^a
Wild-type	7	
No data	1	
Stage		
I-III	0	- ^a
IV	9	

^a Not applicable.

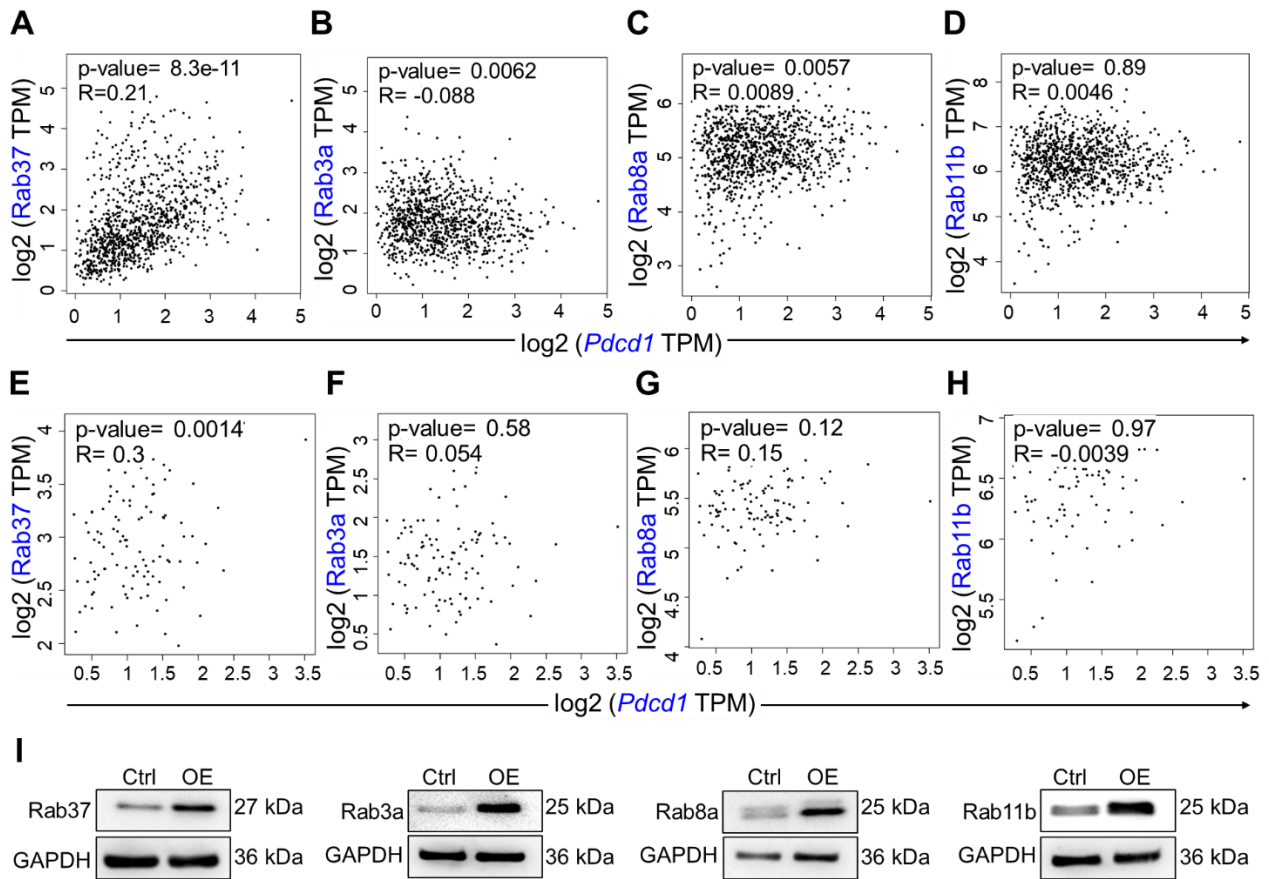


Figure S1. The expression of PD-1 and Rab37 exhibited positive correlation in T cells. The correlation of *Pcdcl1* mRNA expression with Rab37, Rab3a, Rab8a or Rab11b in patients with lung adenocarcinoma (n=483) and lung squamous cell carcinoma (n=486) (A-D) or in normal tissues (n=109) (E-H) from the GEPIA dataset. R square and P value by Pearson correlation coefficient are shown. (I) Jurkat T cells were transfected with Rab37, Rab3a, Rab8a, or Rab11b plasmid for 24h, respectively, and protein expression were detected by immunoblotting. OE: overexpression.

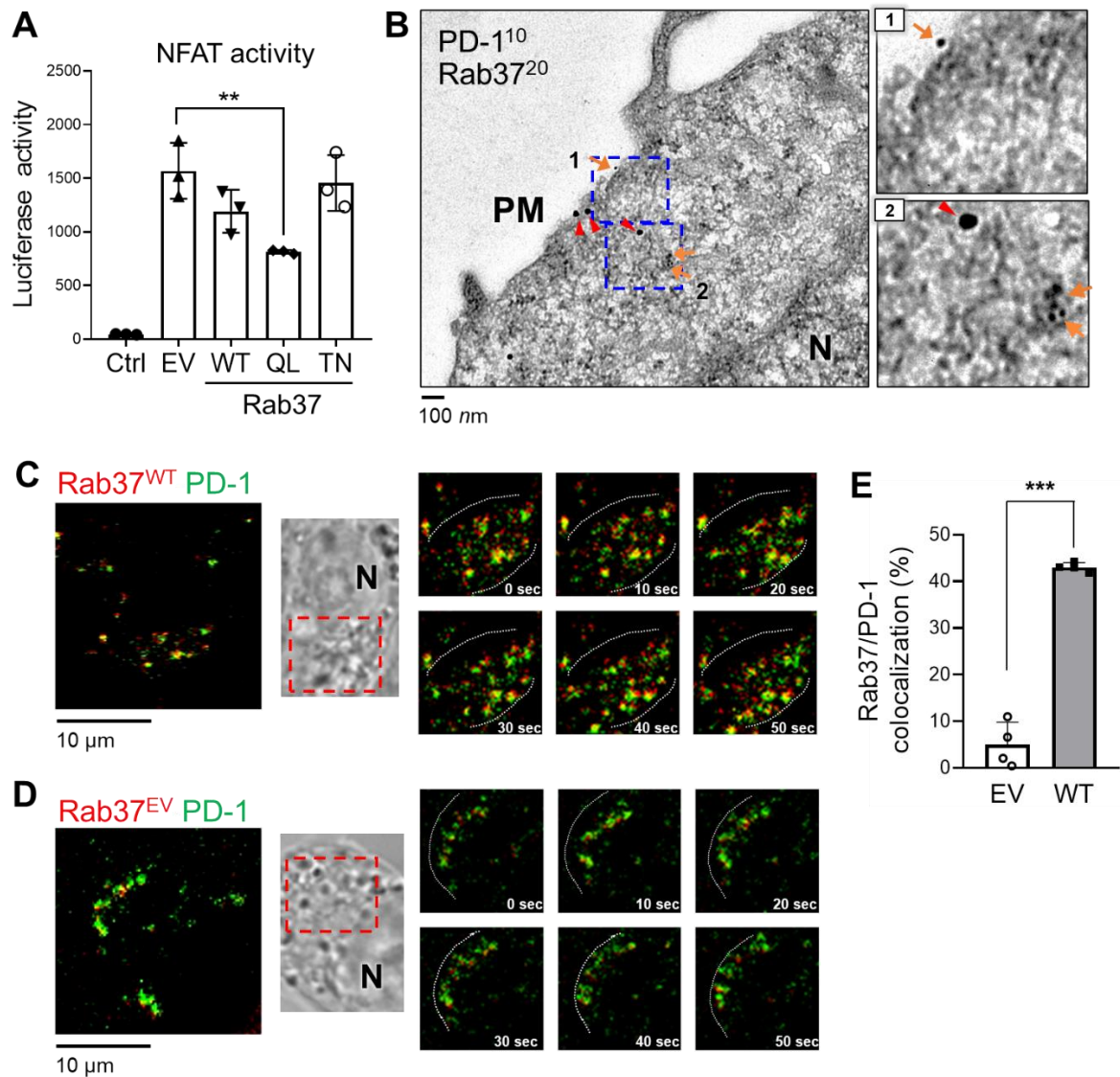


Figure S2. Rab37 mediates PD-1 membrane trafficking. (A) The NFAT activity was determined by luciferase reporter assay in Jurkat T cells overexpressing EV, Rab37-WT, Rab37-Q89L, or Rab37-T43N. (B) Ultrastructural localization of Rab37 (20 nm of gold, red triangle) and PD-1 (10 nm of gold, orange arrow) illustrated by immune-EM images. PM: plasma membrane, N: nucleus. Scale bars: 100 nm. (C-D) The dynamics of Rab37-regulated PD-1 trafficking by real-time confocal live images. Selected frames from time-lapse images Jurkat cells expressing RFP-Rab37 and GFP-PD-1 (C) or GFP-PD-1 with RFP-EV (D). Enlarged images of the boxed areas from [movies S3-S4](#) with time intervals in seconds are shown. Colocalized RFP-Rab37/GFP-PD-1 puncta are shown in yellow. Scale bar: 10 μm. (E) Quantification data of the colocalization between GFP-PD-1 and RFP-Rab37-WT in the intracellular cytoplasm. The data are shown as the mean ± S.D. P values were determined by two-tailed Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

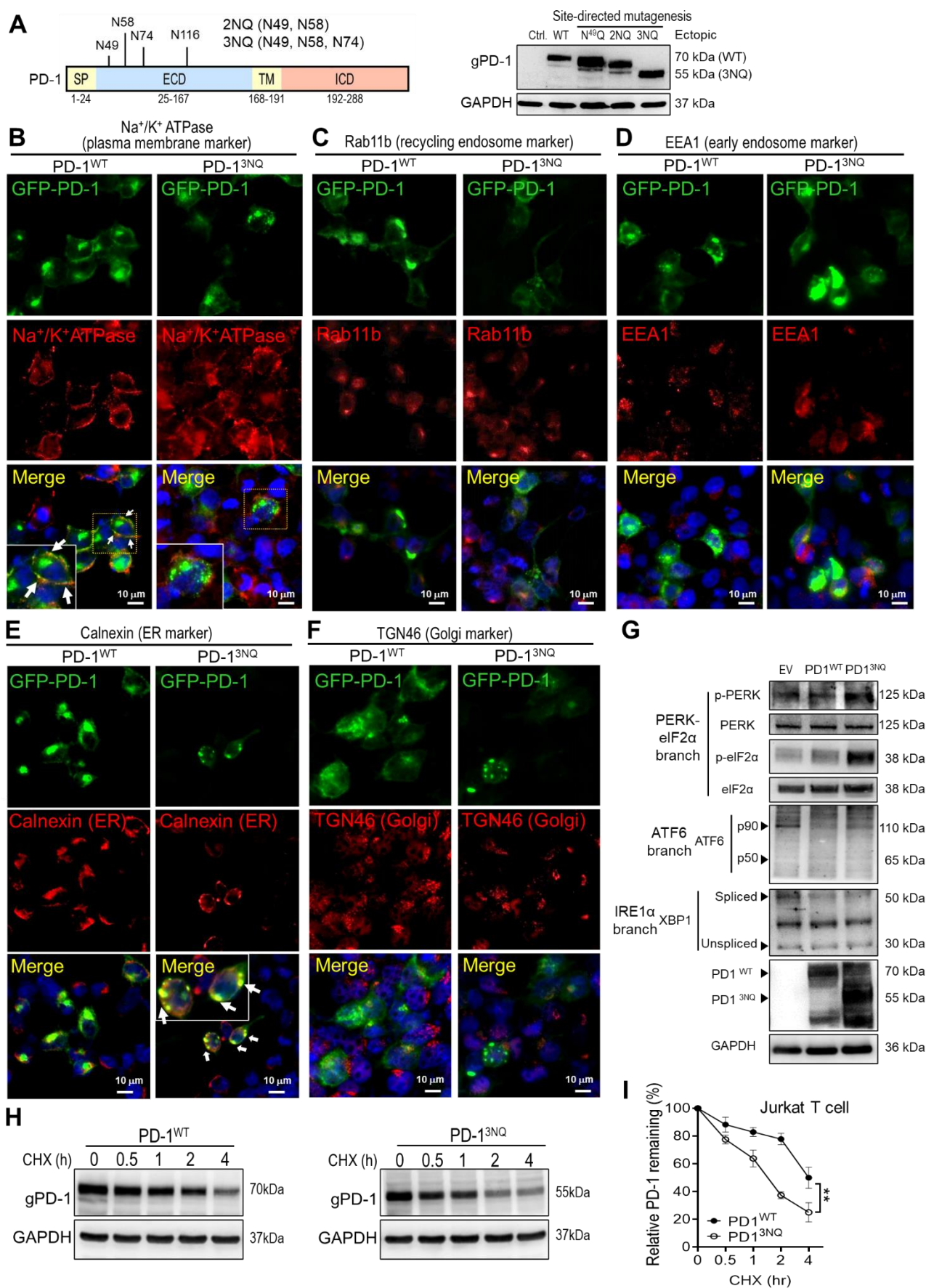


Figure S3. Glycosylation on PD-1 promotes protein stability and transport to the PM. (A) Schematic diagram of full-length PD-1. SP, signal peptide; ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain. Four putative Asn-X-Ser/Thr motifs in the ECD domain are labeled in red. The numbers indicate the amino acid positions. We established

glycosylated mutants of PD1 by site-directed mutagenesis. Immunoblot analysis of glycosylated PD-1 (gPD-1) protein expression in WT-PD-1 and NQ-PD-1 Jurkat T cells after transfection for 18 h. **(B-F)** Transfection with GFP-WT-PD-1 and GFP-3NQ-PD-1 in 293T cells for 18 h followed by immunostaining with Na⁺/K⁺ ATPase, Rab11b, EEA1, Calnexin, and TGN46. **(G)** Western blot analysis of ER stress signaling proteins in COS-1 cells overexpressing GFP-WT-PD1 or GFP-3NQ-PD1. **(H-I)** Jurkat T cells were transfected with GFP-WT-PD1 and GFP-3NQ-PD1 for 18h followed by cycloheximide (CHX, 50 µg/ml) treatment. The quantification of remaining PD-1 protein is shown. The data are shown as the mean ± S.D. P values were determined by two-tailed Student's t test. ** *P*<0.01

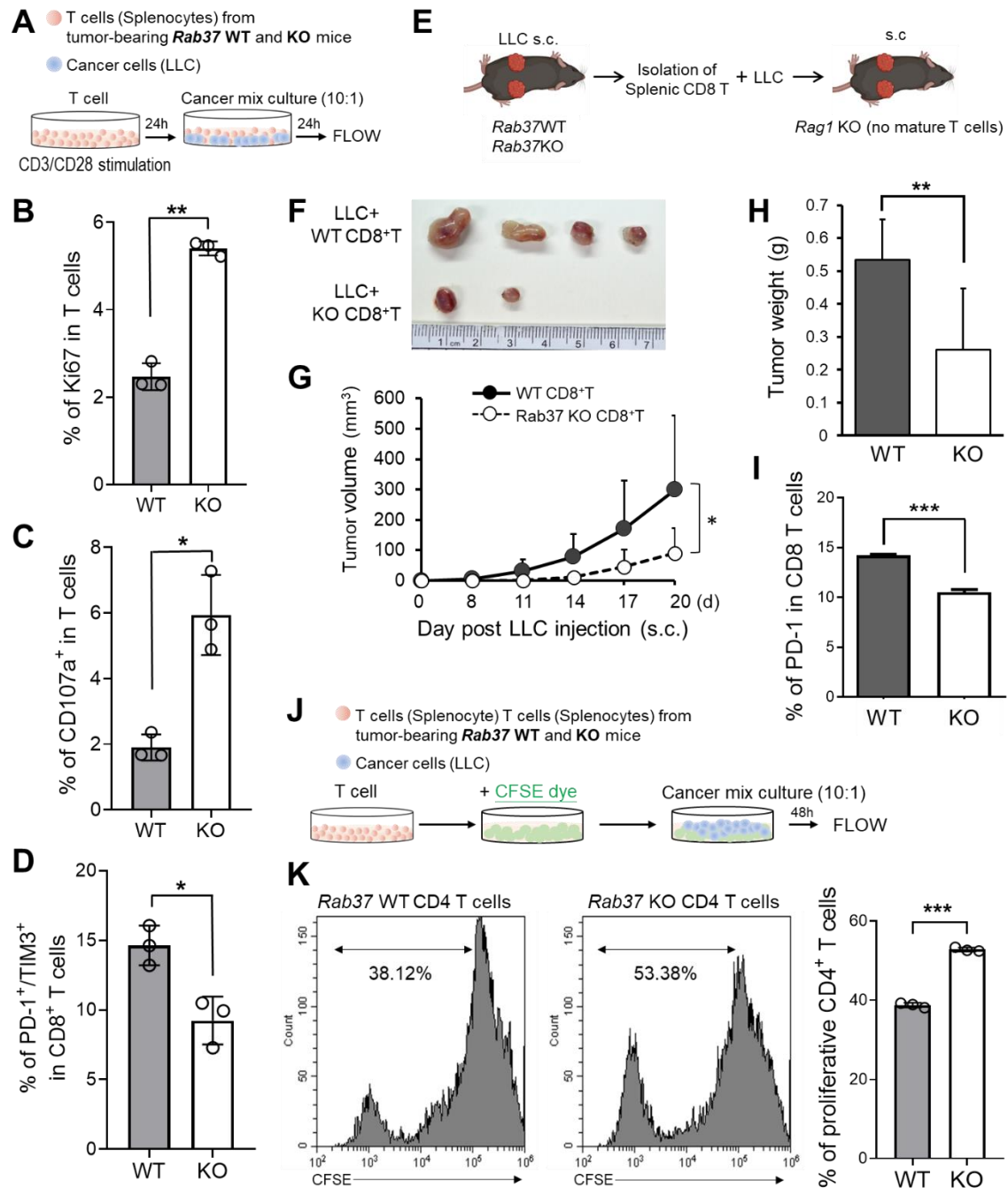


Figure S4. Rab37-mediated PD-1 PM presentation reduces T cell function. (A-D) Splenocytes of LLC tumor-bearing *Rab37* KO and WT mice were stimulated for 24 h and/or co-cultured with LLC for another 24 h (A) to analyze the percentage of Ki67⁺ cells (B), cytotoxic-activity marker CD107a expression (C), and PD-1⁺TIM3⁺ expression (D) by flow cytometry. (E-I) Splenic CD8 T cells were isolated from LLC tumor-bearing mice from WT or *Rab37* KO background, mixed with LLC, and then injected subcutaneously (s.c.) into *Rag1* KO mice (E). The tumor size and volume (F-G) and tumor weight (H) were analyzed. The percentages of PD-1⁺ in CD8 T cells derived from WT or *Rab37* KO mice were determined by flow cytometry (I). (J-K) Histograms and quantification showing intensity of CFSE on CD4⁺ cells of splenocytes from tumor-bearing *Rab37* WT or KO mice upon stimulation by PMA/Io analyzing for cell proliferation by flow cytometry. The data are shown as the mean ± S.D. P values were determined by two-tailed Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

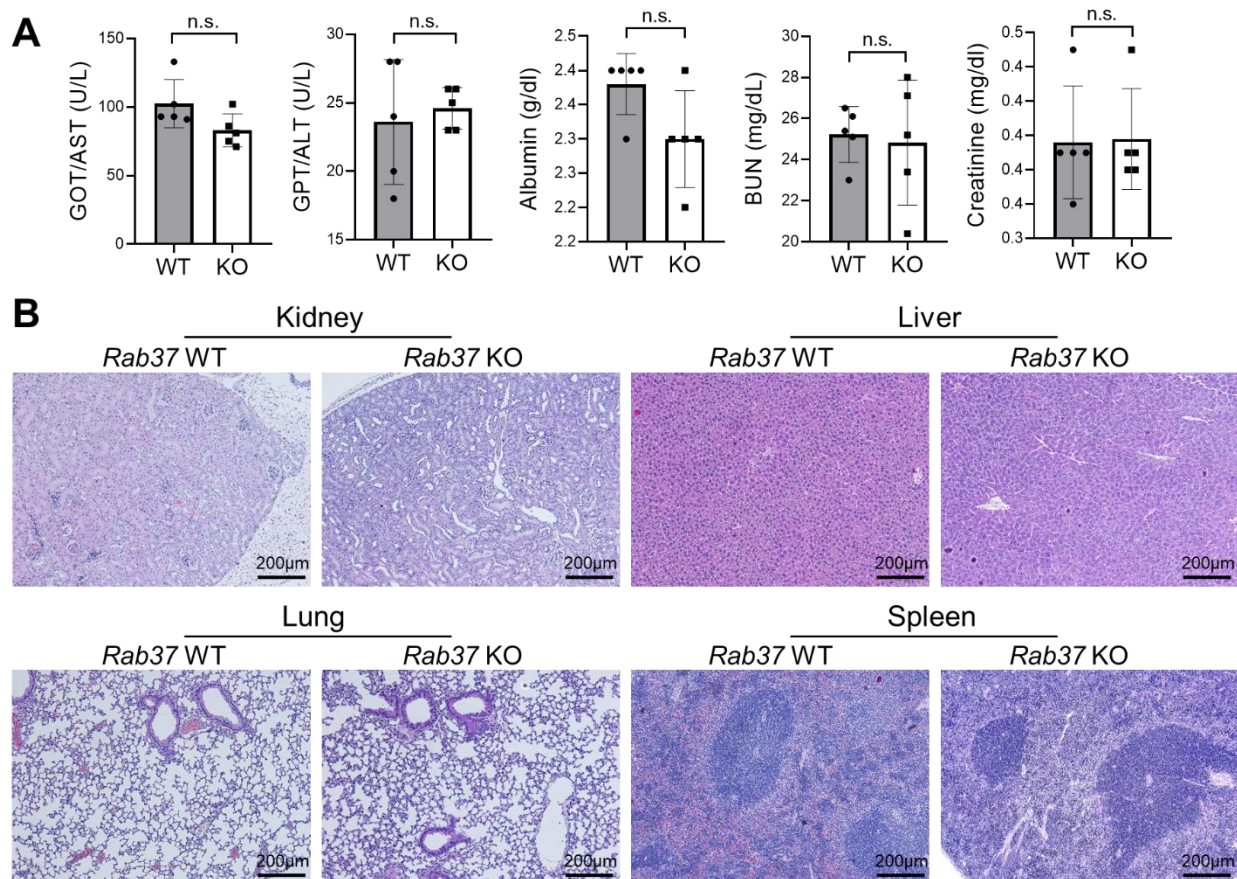


Figure S5. The serum biochemical parameters and major organ histology in LLC tumor-bearing *Rab37* KO and WT mice. (A-B) Serum biochemical markers (A) and major organ histology (B) of treated mice examinations revealed no significant adverse effects between *Rab37* KO and WT mice. Scale bar: 200 μm. The data are shown as the mean ± S.D. P values were determined by two-tailed Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

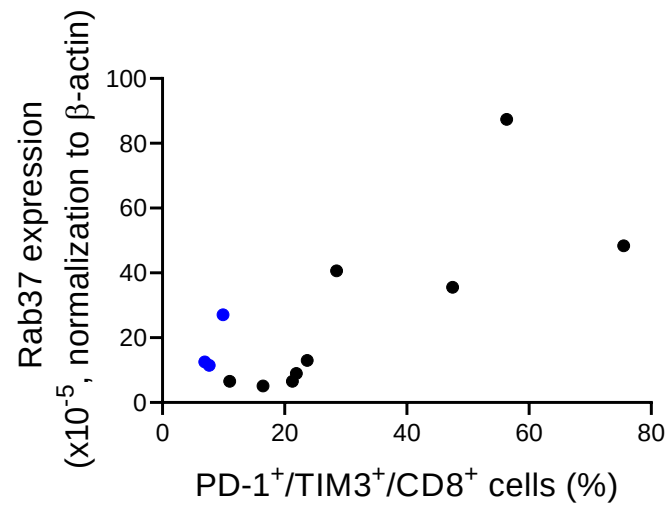


Figure S6. The relationship between Rab37 expression and population of PD-1/TIM3/CD8 cells derived from PBMCs treated with CD3 and CD28 antibodies. Blue dot represents PBMCs from healthy donor (N=3). Black dot represents PBMCs from lung cancer patients (N=9).

Movie legends

Movie S1. Time-lapse movie of TIRF images in 293T cells expressing GFP-PD-1 and RFP-tagged Rab37. Images were captured with TIRF microscope at 491 and 561 nm laser every 6 s over a period of 18 s. Time intervals in minutes and seconds are shown. Stills presenting in 00:01 to 00:04 of this movie correspond to 00:00 to 00:18 in **Figure 2F** (RFP-Rab37/GFP-PD-1). Scale bars: 20 μm .

Movie S2. Time-lapse movie of TIRF images in 293T cells expressing GFP-PD-1 with RFP-tagged EV. Images were captured with TIRF microscope at 491 and 561 nm laser every 10 s over a period of 30 s. Time intervals in minutes and seconds are shown. Stills presenting in 00:01 to 00:08 of this movie correspond to 00:00 to 00:30 in **Figure 2G** (RFP-EV/GFP-PD-1). Scale bars: 20 μm .

Movie S3. Time-lapse movie of confocal images in 293T cells expressing GFP-PD-1 and RFP-tagged Rab37. Images were captured with live confocal fluorescence microscope at 491 and 561 nm laser every 10 s over a period of 50 s. Time intervals in minutes and seconds are shown in **Figure S2C** (RFP-Rab37/GFP-PD-1). Scale bars: 10 μm .

Movie S4. Time-lapse movie of confocal images in 293T cells expressing GFP-PD-1 with RFP-tagged EV. Images were captured with live confocal fluorescence microscope at 491 and 561 nm laser every 10 s over a period of 50 s. Time intervals in minutes and seconds are shown in **Figure S2D** (RFP-EV/GFP-PD-1). Scale bars: 10 μm .