Expanded View Figures

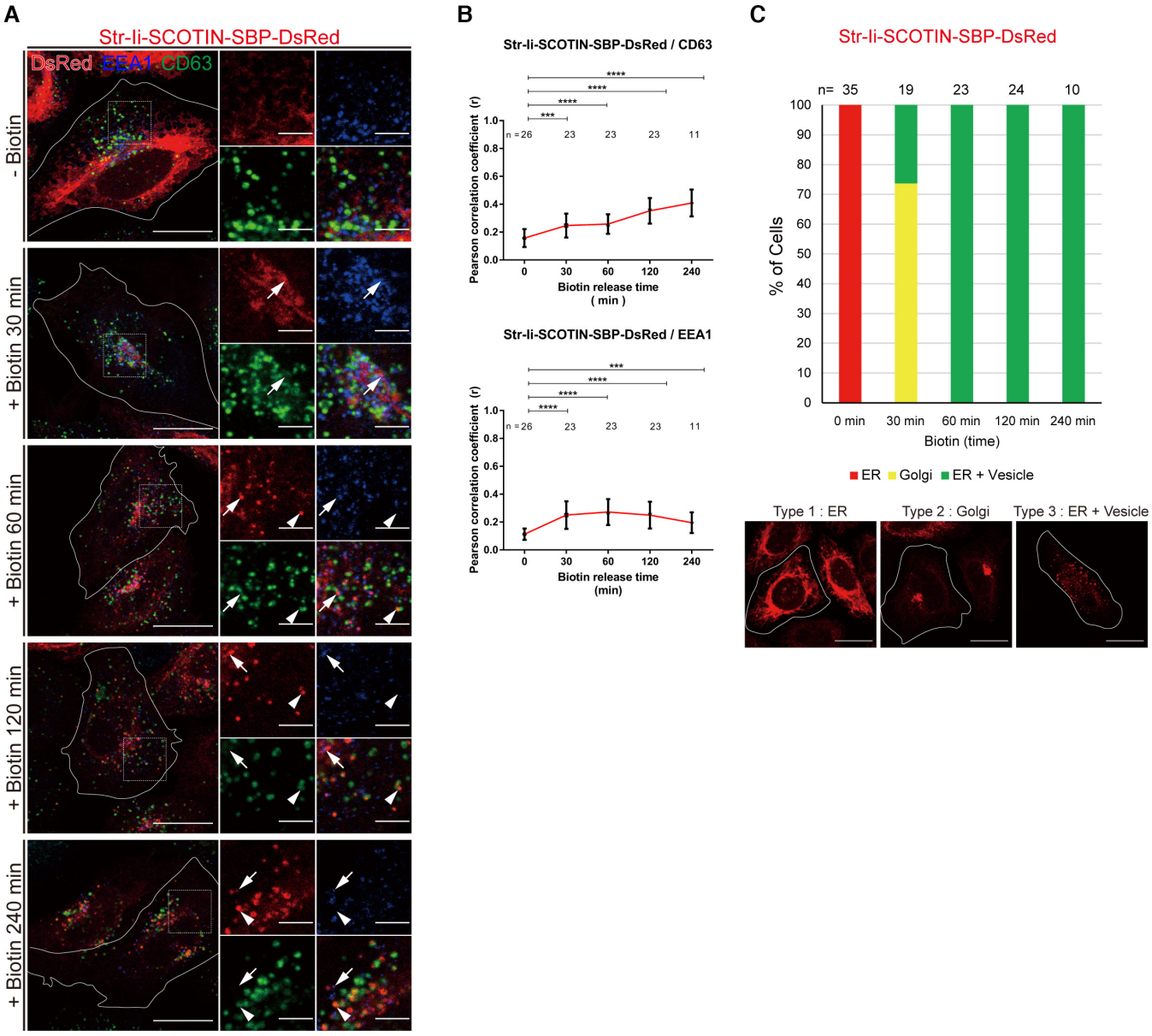

Figure EV1. SCOTIN is located on the ER and endosomal compartment.

A Representative immunofluorescence image of Str-li-SCOTIN-SBP-DsRed in *SCOTIN*-KO cells treated with 40 μM D-biotin for the indicated times. Endogenous early endosomes (EEA1, blue) and late endosomes (CD63, green) were stained. The arrow indicates SCOTIN colocalized with EEA1. The arrowhead indicates SCOTIN with CD63.

B Colocalization of DsRed and EEA1 or CD63 in *SCOTIN*-KO cells was calculated as Pearson correlation coefficients from the experimental data in (A). The error bars indicate SEMs.

C Percent of Str-li-SCOTIN-SBP-DsRed-expressing cells depending on SCOTIN localization. Type 1: SCOTIN is mainly located on the ER; type 2: SCOTIN accumulates at the Golgi; type 3: SCOTIN is located on vesicles as well as on the ER.

Data information: All scale bars: 20 μm. Scale bars in the magnified images: 5 μm. *n*: number of cells analyzed. Data are shown as bars with dots and represent the means ± SEMs. *P*-values were determined using a two-tailed unpaired *t*-test. Significant differences are labeled, ***$P < 0.001$, ****$P < 0.0001$.

Figure EV2. Endogenous SCOTIN-FP$_{11}$ localizes at diverse endocytic vesicles.

A Schematic illustration of the proteinase K protection assay.

B Representative images of Rab5 Q79L-EGFP- and SCOTIN-DsRed-expressing cells with early endosomes (EEA1, white) and late endosomes (CD63, magenta). The arrowhead indicates the area of crowded small vesicles attached to enlarged endosomes. Scale bar: 30 μm. Scale bars in the magnified images: 1.5 μm.

C Schematic illustration of knock-in FP$_{11}$ to endogenous SCOTIN loci.

D Left: Representative high-resolution microscopy images of SCOTIN-FP$_{11}$ (green) and Sec61β, EEA1, CD63, TfR, and LAMP1 (red) in SCOTIN-FP$_{11}$ knock-in cells. Right: Plot analysis of SCOTIN-FP$_{11}$ relative to the organelle marker signal shown. The graph indicates the fluorescence intensity of each channel on the lines. Scale bars: 20 μm. Scale bars in the magnified images: 5 μm.

E Colocalization of SCOTIN-FP$_{11}$ (green) and EEA1, CD63, TfR, or LAMP1 was calculated as Pearson correlation coefficients.

Data information: *n*: number of cells analyzed. Data are shown as bars with dots and represent the means ± SEMs. *P*-values were determined using a two-tailed unpaired *t*-test. Significant differences are labeled, $*P < 0.05$, $***P < 0.001$, and $****P < 0.0001$.

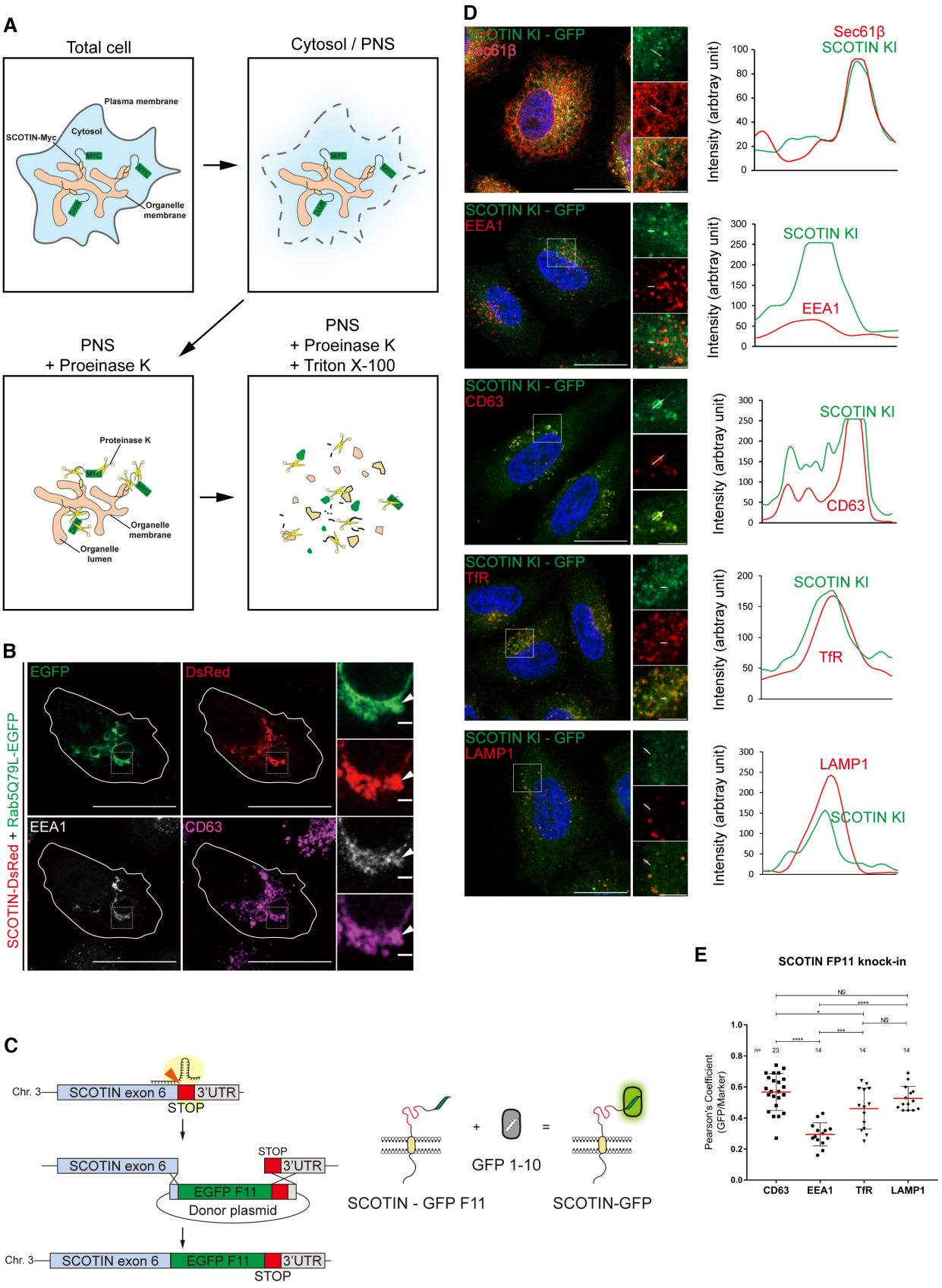
A
Total cell
Cytosol / PNS
Plasma membrane
SCOTIN-Myc
Cytosol
Organelle membrane
PNS + Proeinase K
PNS + Proeinase K + Triton X-100
Proteinase K
Organelle membrane
Organelle lumen
B
SCOTIN-DsRed + Rab5Q79L-EGFP
EGFP
DsRed
EEA1
CD63
C
Chr. 3
SCOTIN exon 6
3'UTR
STOP
EGFP F11
Donor plasmid
GFP 1-10
SCOTIN - GFP F11
SCOTIN-GFP
D
SCOTIN KI - GFP
Sec61β
EEA1
CD63
TfR
LAMP1
Intensity (arbtray unit)
SCOTIN KI
E
SCOTIN FP11 knock-in
Pearson's Coefficient (GFP/Marker)
CD63
EEA1
TfR
LAMP1

Figure EV2.

Figure EV3. SCOTIN specifically regulates ER and late endosome contact through the cytosolic PRD.

A Representative images from a PLA with RTN4 and Rab7 in WT and *SCOTIN*-KO cells. Only one primary antibody (anti-RTN4 alone or anti-Rab7 antibody alone) served as a negative control. The PLA color legends are inverted. All scale bars: 20 μm.

B The number of PLA signals in each cell was quantified by particle analysis with ImageJ.

C Schematic illustrating the SCOTIN domain.

D Representative images of VAPA-Rab7 PLA in WT and *SCOTIN*-KO cells. Cells were transfected with empty vector as a control, SCOTIN (FL)-Myc, or SCOTIN (ΔPRD)-Myc for 48 h. The PLA color legends are inverted. All scale bars: 30 μm.

E The number of PLA signals in each cell was quantified by particle analysis with ImageJ.

F–H Cellular localization of endogenous RTN4 (red) and CD63 (magenta) in (F) SCOTIN-, (G) TMPRD-, or (H) PRD-Myc (green)-reconstituted *SCOTIN*-KO cells. The graph indicates the fluorescence intensity of each channel on the lines.

Data information: *n*: number of cells analyzed. Data are shown as bars with dots and represent the means ± SEMs. *P*-values were determined using a two-tailed unpaired *t*-test. Significant differences are labeled, $^{****}P < 0.0001$.

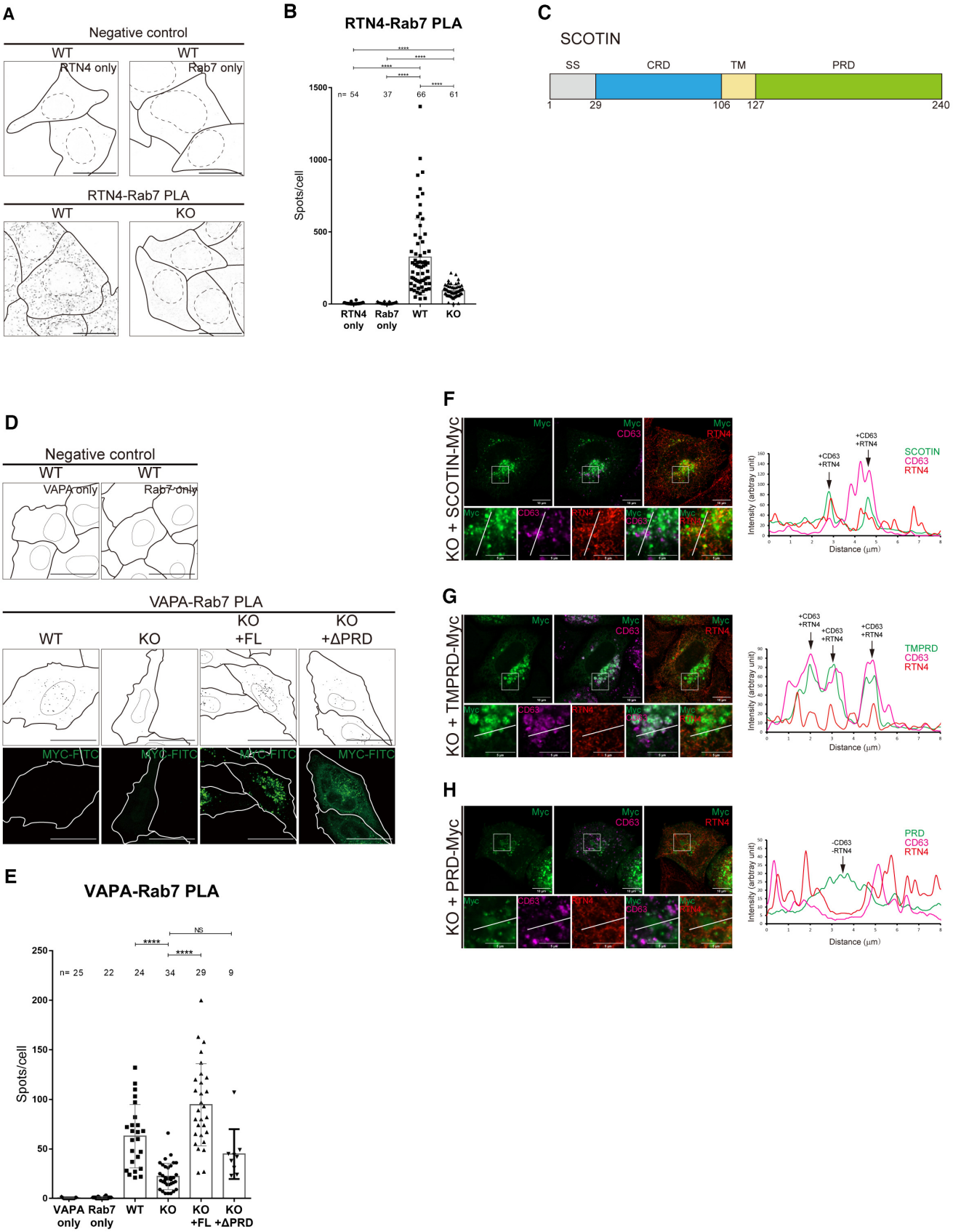
A
Negative control
WT
WT
RTN4 only
Rab7 only
RTN4-Rab7 PLA
WT
KO
B
RTN4-Rab7 PLA
Spots/cell
n= 54 37 66 61
RTN4 only
Rab7 only
WT
KO
C
SCOTIN
SS
CRD
TM
PRD
1 29 106 127 240
D
Negative control
VAPA only
Rab7 only
VAPA-Rab7 PLA
WT
KO
KO +FL
KO +ΔPRD
MYC-FITC
E
VAPA-Rab7 PLA
Spots/cell
n= 25 22 24 34 29 9
VAPA only
Rab7 only
WT
KO
KO +FL
KO +ΔPRD
F
KO + SCOTIN-Myc
Myc
CD63
RTN4
SCOTIN
Intensity (arbtrary unit)
Distance (μm)
G
KO + TMPRD-Myc
TMPRD
H
KO + PRD-Myc
PRD

Figure EV3.

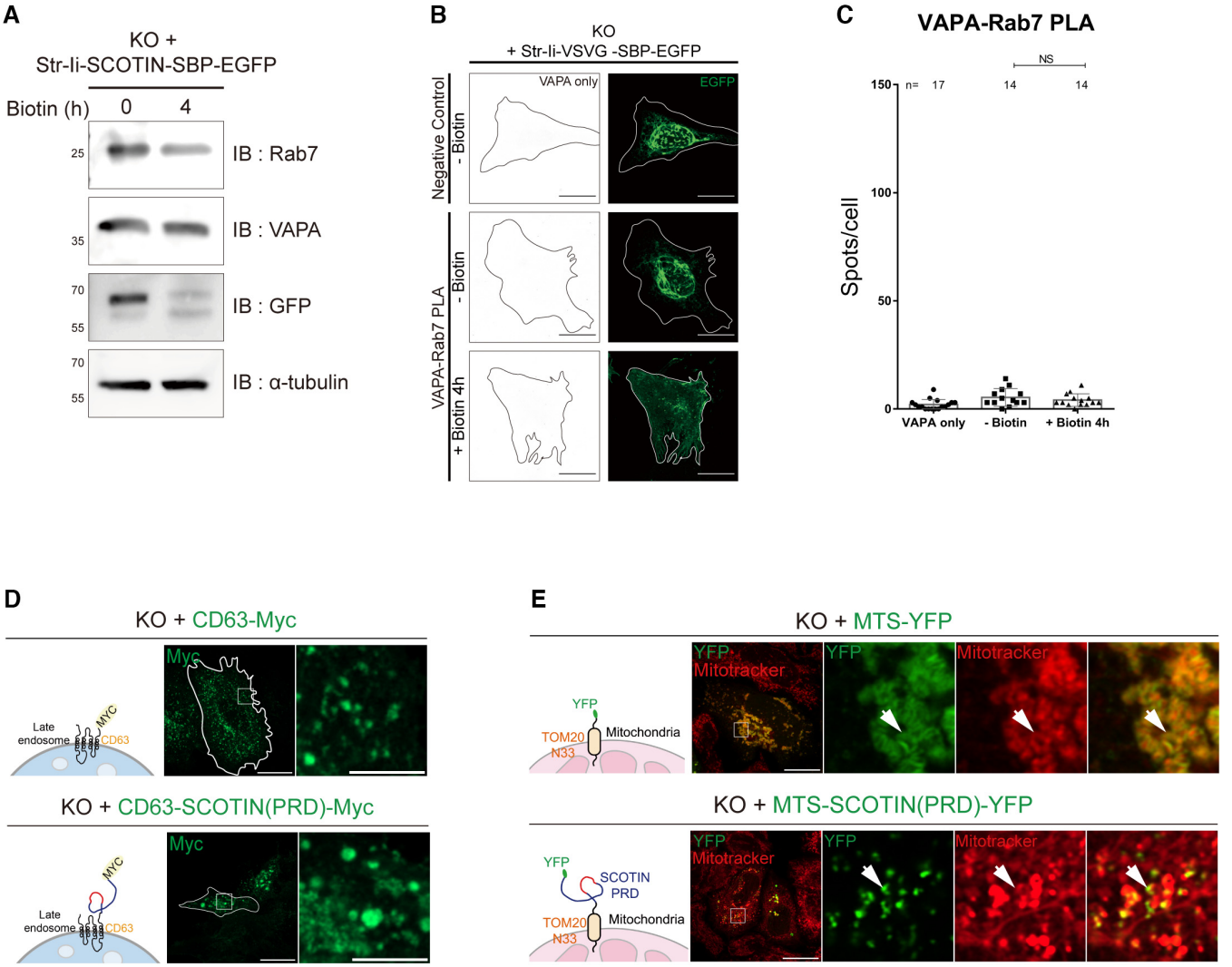

Figure EV4. Expression pattern of membrane-targeted PRD.

A Immunoblot analysis of Str-li-SCOTIN-SBP-EGFP-transfected *SCOTIN*-KO HeLa cell lysates with or without biotin treatment. VAPA, Rab7, GFP, and α-tubulin were immunoblotted.

B *SCOTIN*-KO cells were transfected with li-Str-VSVG-SBP-EGFP, and contact between the ER and endosomes was tested. Representative images of VAPA-Rab7 PLA are shown, along with images of negative controls incubated with antibody against VAPA alone. The PLA color legends are inverted. All scale bars: 20 μm.

C The PLA signals in the indicated samples were quantified by particle analysis with ImageJ. *n*: number of cells analyzed. Data are shown as bars with dots and represent the means ± SEMs. *P*-values were determined using a two-tailed unpaired *t*-test.

D Representative images of *SCOTIN*-KO cells expressing CD63 or CD63-PRD-Myc (green). Scale bars: 20 μm. Scale bars in the magnified images: 5 μm.

E Representative images of MitoTracker (red) with MTS- or MTS-PRD-YFP (green)-expressing *SCOTIN*-KO cells. The arrow indicates mitochondria membrane-targeted MTS or MTS-PRD. Scale bars: 20 μm. Scale bars in the magnified images: 5 μm.

Figure EV5. SCOTIN (PRD Δ150-177) could not proximate the membrane in vitro and in cells.

A VLXT, VSL2, VL3, and IUPred2 assigned scores of disordered tendencies between 0 and 1 to the sequences are shown.
B Differential interference contrast (DIC) images of purified proteins (10 μM) in assembly buffer.
C Coomassie blue-stained gels and immunoblot analysis showing the purified 12His-PRDΔ150-177 proteins used in the reconstitution study.
D Coomassie blue-stained gels and immunoblot analysis of the cosedimentation assay. SUV-bound 12His-PRDΔ150-177 were verified.
E PRDΔ150-177-SUVs and GUVs were mixed and subjected to confocal microscopy.
F Representative images from a PLA with RTN4 and Rab7 in *SCOTIN*-KO HeLa cells reconstituted with FL SCOTIN or SCOTIN(Δ150-177)-DsRed. The PLA color legends are inverted. Asterisks indicate transfected cells. All scale bars: 20 μm.
G The number of PLA signals in each cell was quantified by particle analysis with ImageJ. Data are shown as bars with dots and represent the means ± SEMs. *P*-values were determined using a two-tailed unpaired *t*-test. Significant differences are labeled, $*P < 0.05$ and $****P < 0.0001$. (F) and (G) are derived from three independent experiments.
H Immunoblot analysis of Ii-SCOTIN(Δ150-177)-SBP-EGFP-transfected *SCOTIN*-KO HeLa cell lysates with or without biotin treatment. VAPA, Rab7, GFP, and α-tubulin were immunoblotted.

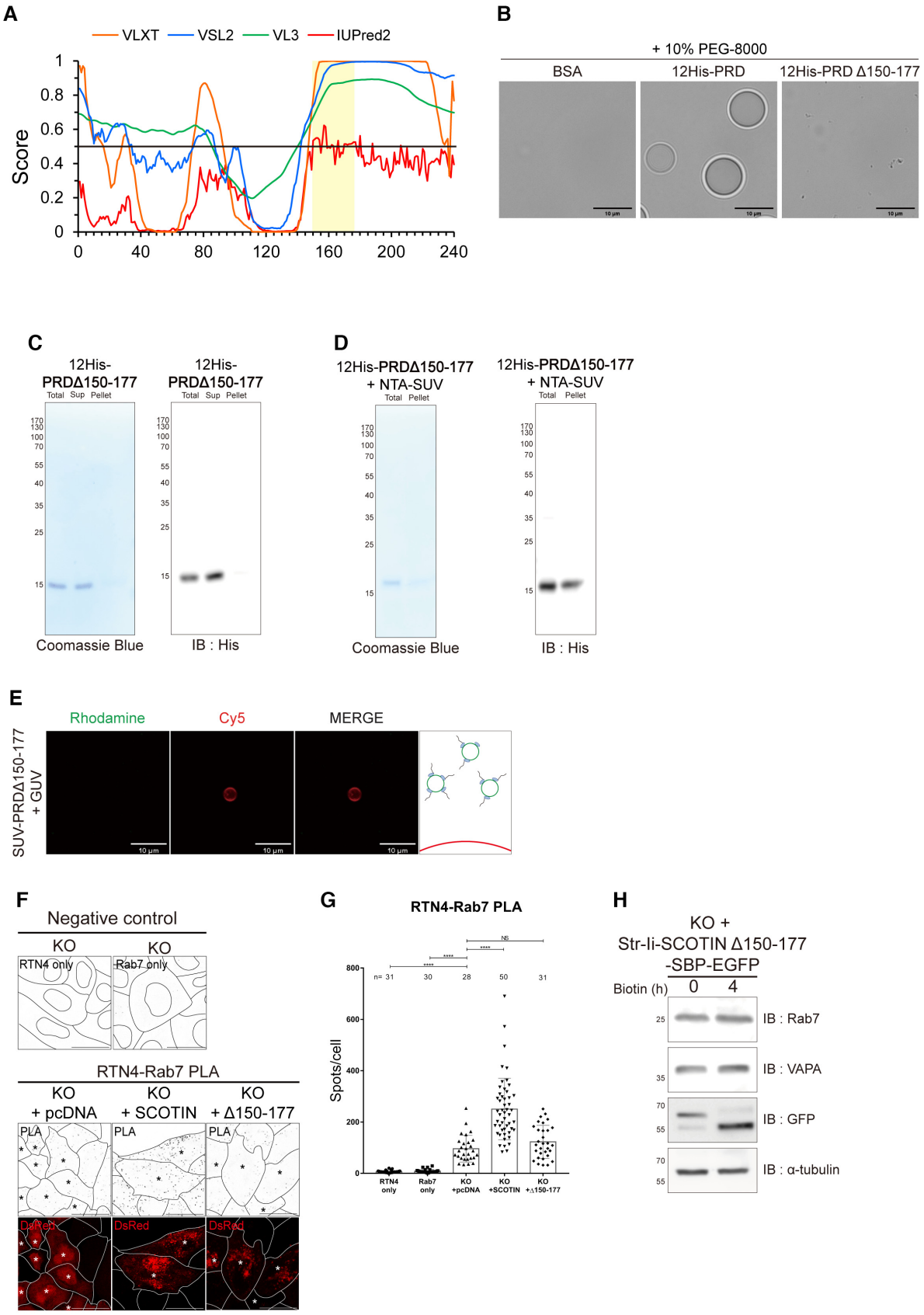

Figure EV5.