Supplemental Materials

Molecular Biology of the Cell

Mukherjee et al.

SUPPLEMENTAL INFORMATION

Rtn4a promotes exocytosis in mammalian cells

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Overexpression of Rtn4a, Rtn4b, and REEP5 changes ER morphology and decreases ER sheet volume. HeLa cells were transiently transfected with plasmids expressing GFP-NLS as a control, Rtn4a-GFP, GFP-Rtn4b, or mCherry-REEP5. **(A)** Cells were immunostained for Rtn4a. **(B)** Rtn4a immunofluorescence intensity was quantified for 47-51 cells per condition. **(C)** Whole cell lysates were immunoblotted for Rtn4a. It is typical for Rtn4a to run as a collection of bands ranging from 130 kDa to over 260 kDa (Li *et al.*, 2004; Osborne *et al.*, 2004). **(D)** Rtn4a band intensities were normalized to β-actin loading controls. Averages from three independent experiments are shown. **(E)** Whole cell lysates were immunoblotted for Rtn4. **(F)** Rtn4b band intensities were normalized to β-actin loading controls. Averages from three independent experiments are shown. **(G)** Cells were immunostained for REEP5. **(H)** REEP5 immunofluorescence intensity was quantified for 20-27 cells per condition. **(I)** Cells were immunostained for the total ER marker KDEL. **(J)** To quantify the distribution of KDEL staining, intensity values were measured along 25 μm long straight lines drawn from the nuclear envelope to the cell periphery. Pixel intensities along KDEL line scans were averaged for 10-12 cells per condition and 3 lines per cell. **(K)** Cells were immunostained for the ER sheet marker CLIMP63. **(L)** Quantitation of mean ER sheet volume based on CLIMP63 immunofluorescence from 3D reconstructed confocal z-stacks. 54-57 cells were quantified per condition.
Scale bars are 10 μm. Representative images are maximum intensity projections of confocal z-stacks. Error bars represent SD. **** $p \leq 0.0001$; *** $p \leq 0.001$.

Figure S2: Validation of Integrinβ1 and HLA-A surface staining (A-C). Overexpression of Rtn4a, Rtn4b, and REEP5 does not alter the total levels of Integrinβ1 and HLA-A (D-I). Rtn4 RNAi decreases Rtn4a and Rtn4b levels without affecting ER morphology (J-N). (A-C) HeLa cells were transiently transfected with plasmids expressing Rtn4a-GFP, GFP-Rtn4b, or mCherry-REEP5. Non-permeabilized cells were stained for surface-localized Integrinβ1 or HLA-A. Single confocal z-planes of transfected cells are shown from the same maximum intensity projected images in Fig. 1A-B. **(D-E)** HeLa cells were transiently transfected with plasmids expressing GFP-NLS as a control, Rtn4a-GFP, GFP-Rtn4b, or mCherry-REEP5. Whole cell lysates were immunoblotted for Integrinβ1 and HLA-A. Integrinβ1 and HLA-A band intensities were normalized to β-actin loading controls. Averages from three independent experiments are shown. **(F-I)** HeLa cells were transiently transfected with plasmids expressing GFP-NLS as a control or Rtn4a-GFP. Permeabilized cells were stained for total Integrinβ1 or HLA-A. Total Integrinβ1 fluorescence staining intensity was quantified for 39-49 cells per condition. Total HLA-A fluorescence staining intensity was quantified for 48-52 cells per condition. **(J-L)** HeLa cells were transiently co-transfected with siRNA against Rtn4 and Block-iT fluorescent control or with Block-iT alone. Whole cell lysates were immunoblotted for Rtn4. It is typical for Rtn4a to run as a collection of bands ranging from 130 kDa to over 260 kDa (Li *et al.*, 2004; Osborne *et al.*, 2004). Rtn4a and Rtn4b band intensities were normalized to β-actin loading controls. Averages from three independent experiments are shown. **(M-N)** HeLa cells were transiently co-transfected with siRNA against Rtn4 and Block-iT fluorescent control or with Block-iT alone. Cells were immunostained for the ER sheet marker CLIMP63. Mean ER sheet volume based on CLIMP63 immunofluorescence was quantified from 3D reconstructed confocal z-stacks. 35-57 cells were quantified per condition.
Scale bars are 10 μm. Unless otherwise noted, representative images are maximum intensity projections of confocal z-stacks. Error bars represent SD. ** p≤0.01; NS not significant.

Figure S3: Rtn4a overexpression increases cell surface localization, but not total levels, of Integrinβ1 and HLA-A in MRC-5 cells (A-H). Overexpression of Rtn4a

increases the secretion of endogenous FBLN5 and TSP1 (I-N), without causing ER stress (O-R). (**A-H**) MRC-5 cells were transiently transfected with plasmids expressing GFP-NLS as a control or Rtn4a-GFP. (**A-B**) Non-permeabilized cells were stained for surface-localized Integrinβ1 or HLA-A. (**C**) Integrinβ1 surface fluorescence staining intensity was quantified for 25-31 cells per condition. (**D**) HLA-A surface fluorescence staining intensity was quantified for 20-25 cells per condition. (**E-F**) Permeabilized cells were stained for total Integrinβ1 or HLA-A. (**G**) Total Integrinβ1 fluorescence staining intensity was quantified for 20-29 cells per condition. (**H**) Total HLA-A fluorescence staining intensity was quantified for 27-33 cells per condition. (**I-N**) HeLa cells were transiently nucleofected with plasmids expressing GFP-NLS as a control or Rtn4a-GFP. Media was collected and whole cell lysates were prepared 12 hours after transfection. Samples were subjected to sandwich ELISA. Data are presented from three independent experiments. **(I,L)** Amounts of secreted FBLN5 and TSP1 present in the media were quantified by ELISA and normalized to the number of live cells. **(J,M)** Amounts of intracellular FBLN5 and TSP1 present in whole cell lysates were quantified by ELISA and normalized to the number of live cells. **(K,N)** Total amounts of FBLN5 and TSP1 were calculated by summing the secreted and intracellular amounts of each protein per cell. (**O-R**) HeLa cells were transiently transfected with plasmids expressing GFP-NLS as a control or Rtn4a-GFP. Whole cell lysates were immunoblotted for ER stress-related chaperones calnexin, ERp72, and GRP78. Band intensities were normalized to β-actin loading controls, and data from three independent experiments are shown.
Scale bars are 10 μm. Representative images are maximum intensity projections of confocal z-stacks. Error bars represent SD. *** p≤0.001; ** p≤0.01; * p≤0.05; NS not significant.

Figure S4: Rtn4a overexpression and knockdown in Neuro-2a cells. (A-D) Undifferentiated Neuro-2a cells were transiently co-transfected with plasmids expressing Rtn4a-myc and mCherry or with mCherry only as a control. **(A)** Cells were immunostained for the myc epitope and Rtn4a. **(B)** Rtn4a immunofluorescence intensity was quantified for 36-39 transfected and non-transfected cells per condition. **(C)** Whole

cell lysates were immunoblotted for Rtn4a. **(D)** Rtn4a band intensities were normalized to β-actin loading controls. Averages from three independent experiments are shown. **(E-H)** Undifferentiated Neuro-2a cells were transiently co-transfected with siRNA against Rtn4a and Block-iT fluorescent control or with Block-iT alone. **(E)** Whole cell lysates were immunoblotted for Rtn4a. **(F)** Rtn4a band intensities were normalized to β-actin loading controls. Averages from three independent experiments are shown. **(G)** Cells were immunostained for Rtn4a. **(H)** Rtn4a immunofluorescence intensity was quantified for 29-36 transfected and non-transfected cells per condition.
Scale bars are 10 μm. Representative images are maximum intensity projections of confocal z-stacks. Error bars represent SD. **** $p \leq 0.0001$; * $p \leq 0.05$; NS not significant.

SUPPLEMENTAL REFERENCES

Li, W., Walus, L., Rabacchi, S.A., Jirik, A., Chang, E., Schauer, J., Zheng, B.H., Benedetti, N.J., Liu, B.P., Choi, E., Worley, D., Silvian, L., Mo, W., Mullen, C., Yang, W., Strittmatter, S.M., Sah, D.W., Pepinsky, B., and Lee, D.H. (2004). A neutralizing anti-Nogo66 receptor monoclonal antibody reverses inhibition of neurite outgrowth by central nervous system myelin. The Journal of biological chemistry *279*, 43780-43788.

Osborne, S.L., Corcoran, S.L., Prinjha, R.K., and Moore, S.E. (2004). Nogo A expression in the adult enteric nervous system. Neurogastroenterol Motil *16*, 465-474.

Figure S1

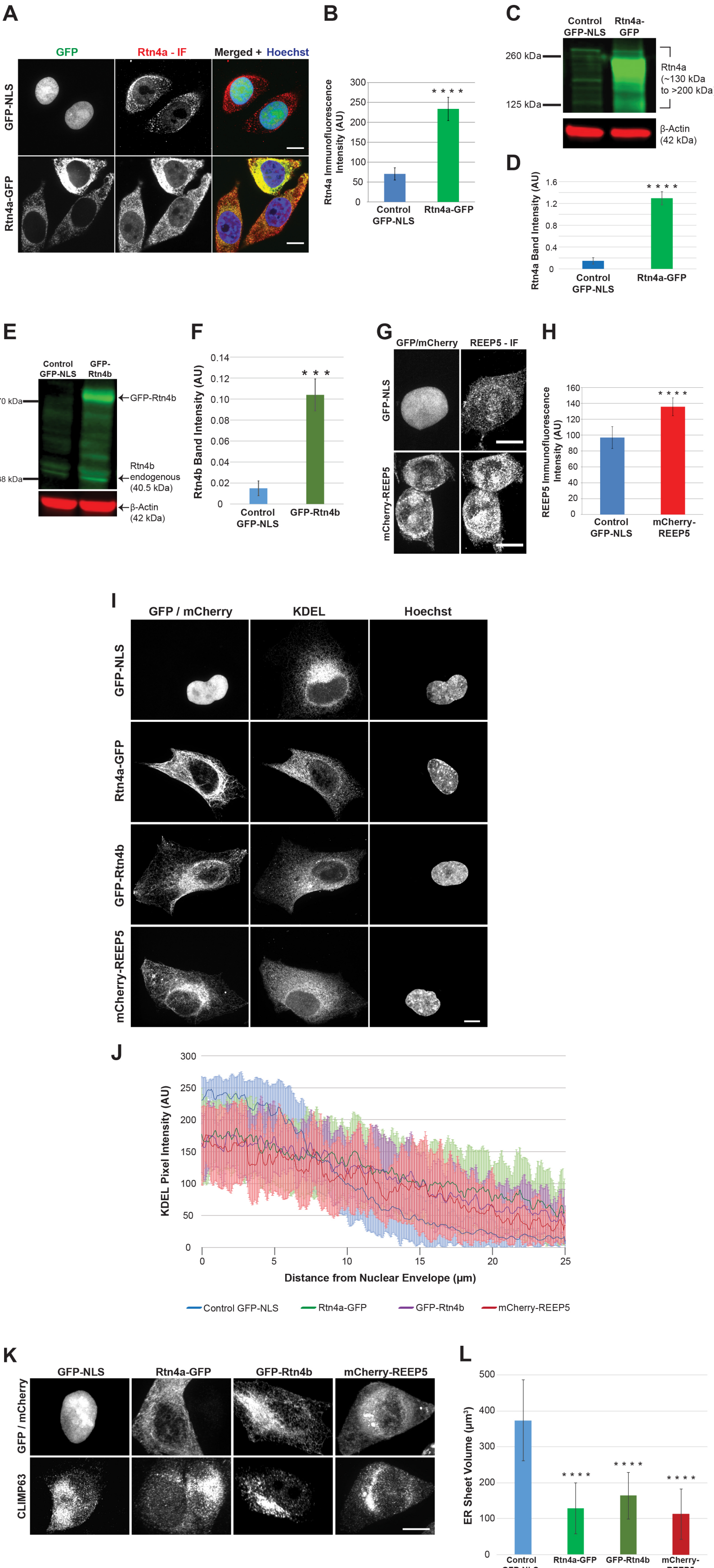
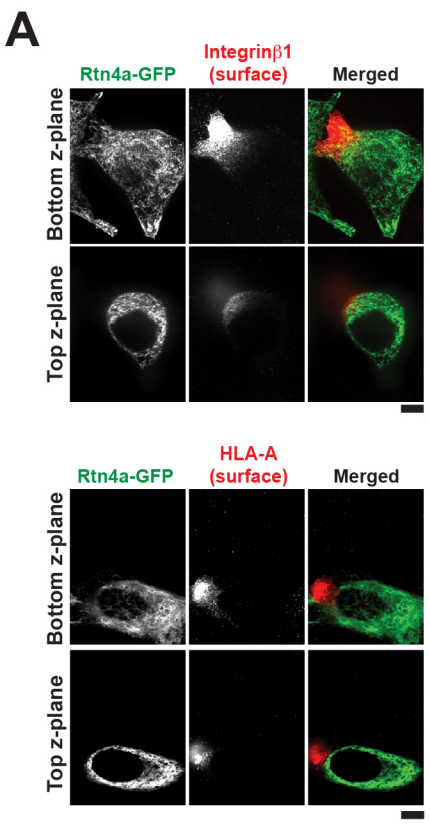
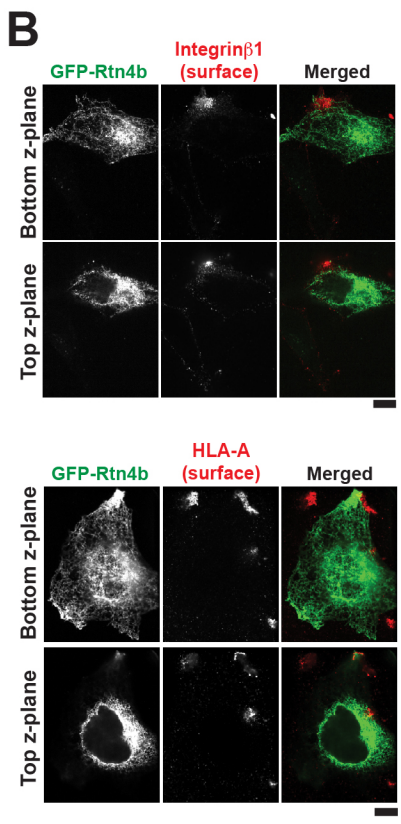
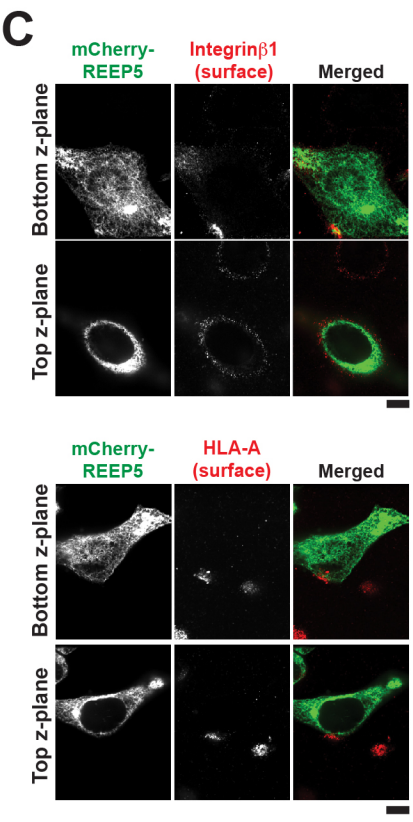
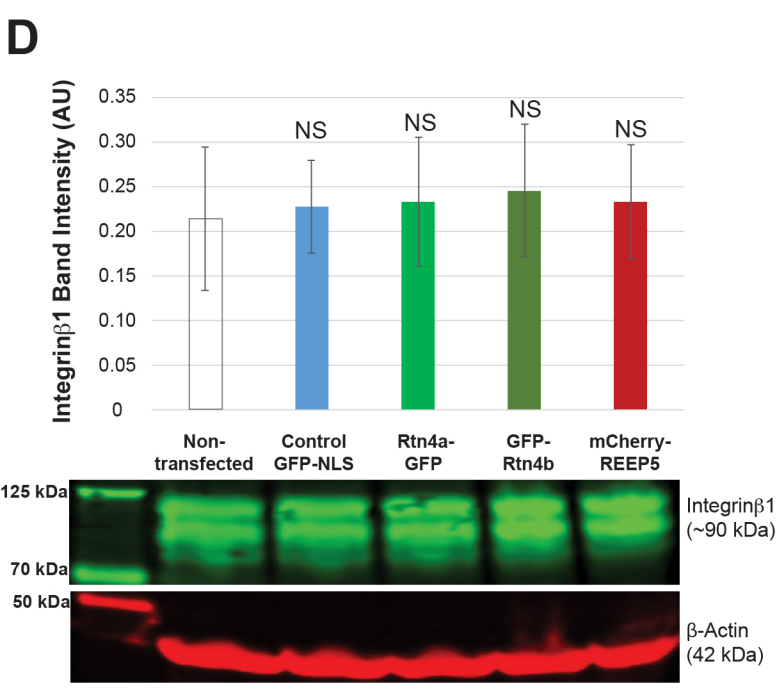

A
GFP
Rtn4a - IF
Merged + Hoechst
GFP-NLS
Rtn4a-GFP
B
Rtn4a Immunofluorescence Intensity (AU)
300
250
200
150
100
50
0
* * * *
Control GFP-NLS
Rtn4a-GFP
C
Control GFP-NLS
Rtn4a-GFP
260 kDa
125 kDa
Rtn4a (~130 kDa to >200 kDa)
β-Actin (42 kDa)
D
Rtn4a Band Intensity (AU)
1.6
1.2
0.8
0.4
0
* * * *
Control GFP-NLS
Rtn4a-GFP
E
Control GFP-NLS
GFP-Rtn4b
70 kDa
38 kDa
GFP-Rtn4b
Rtn4b endogenous (40.5 kDa)
β-Actin (42 kDa)
F
Rtn4b Band Intensity (AU)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
* * *
Control GFP-NLS
GFP-Rtn4b
G
GFP/mCherry
REEP5 - IF
GFP-NLS
mCherry-REEP5
H
REEP5 Immunofluorescence Intensity (AU)
160
140
120
100
80
60
40
20
0
* * * *
Control GFP-NLS
mCherry-REEP5
I
GFP / mCherry
KDEL
Hoechst
GFP-NLS
Rtn4a-GFP
GFP-Rtn4b
mCherry-REEP5
J
KDEL Pixel Intensity (AU)
300
250
200
150
100
50
0
0
5
10
15
20
25
Distance from Nuclear Envelope (µm)
Control GFP-NLS
Rtn4a-GFP
GFP-Rtn4b
mCherry-REEP5
K
GFP-NLS
Rtn4a-GFP
GFP-Rtn4b
mCherry-REEP5
GFP / mCherry
CLIMP63
L
ER Sheet Volume (µm³)
500
400
300
200
100
0
* * * *
* * * *
* * * *
Control GFP-NLS
Rtn4a-GFP
GFP-Rtn4b
mCherry-REEP5

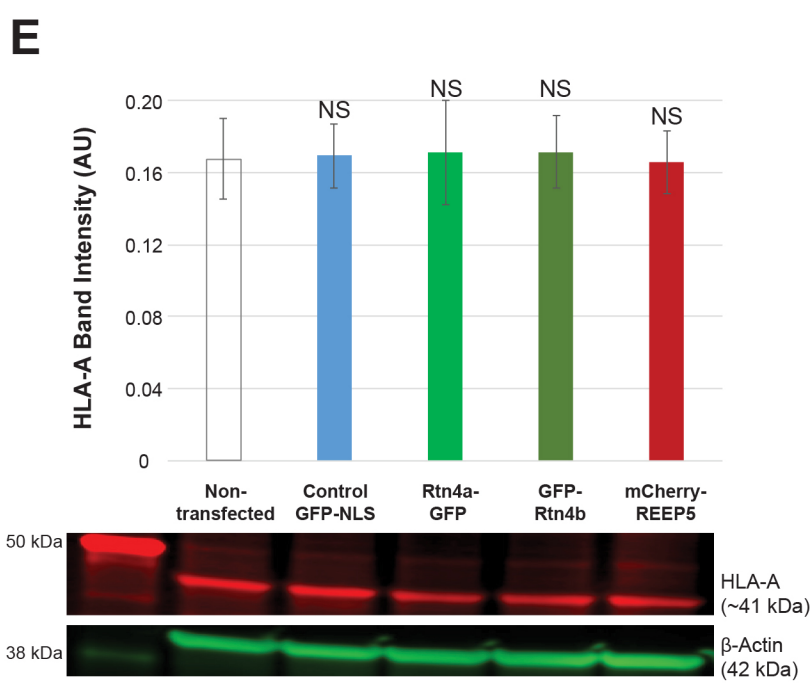
Figure S2

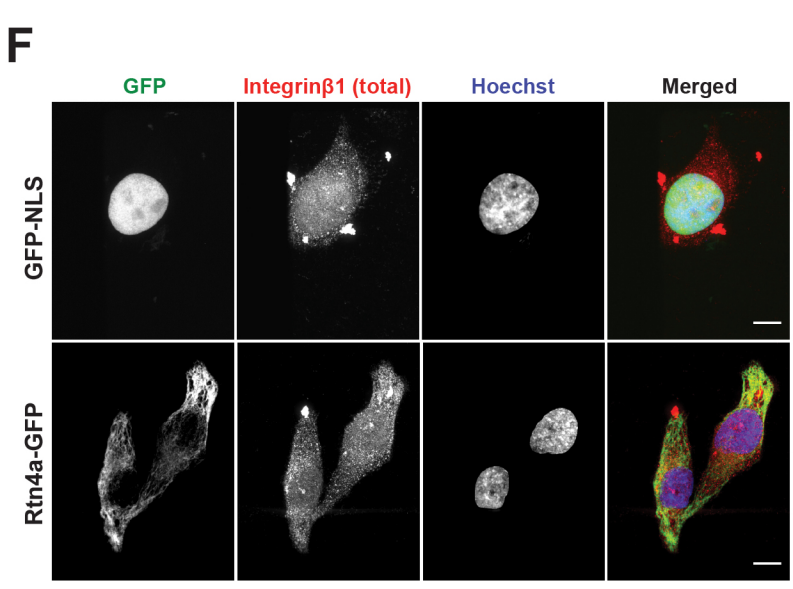

A
Rtn4a-GFP
Integrinβ1 (surface)
Merged
Bottom z-plane
Top z-plane
HLA-A (surface)

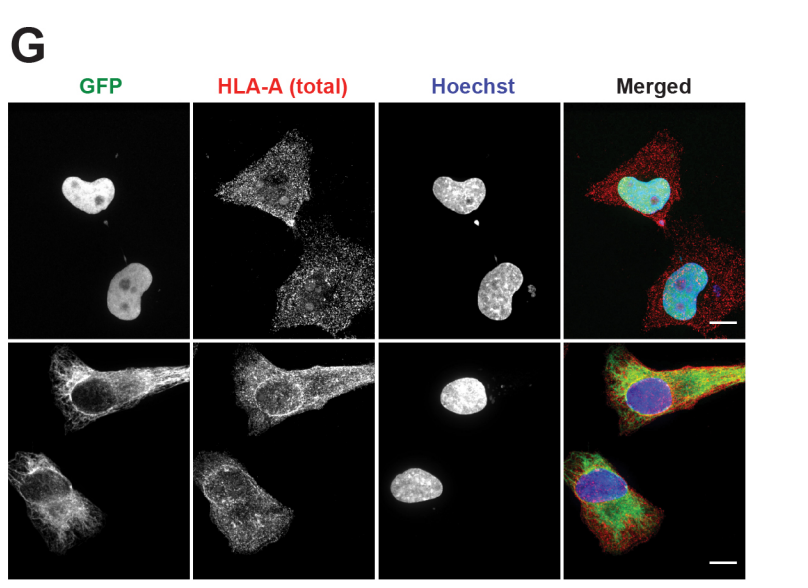

B
GFP-Rtn4b
Integrinβ1 (surface)
Merged
Bottom z-plane
Top z-plane
HLA-A (surface)

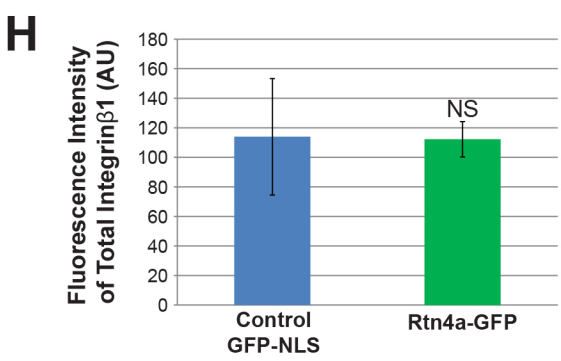

C
mCherry-REEP5
Integrinβ1 (surface)
Merged
Bottom z-plane
Top z-plane
HLA-A (surface)

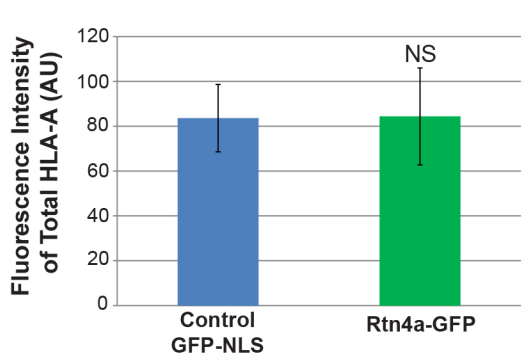

D
Integrinβ1 Band Intensity (AU)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
NS
Non-transfected
Control GFP-NLS
Rtn4a-GFP
GFP-Rtn4b
mCherry-REEP5
125 kDa
70 kDa
50 kDa
Integrinβ1 (~90 kDa)
β-Actin (42 kDa)

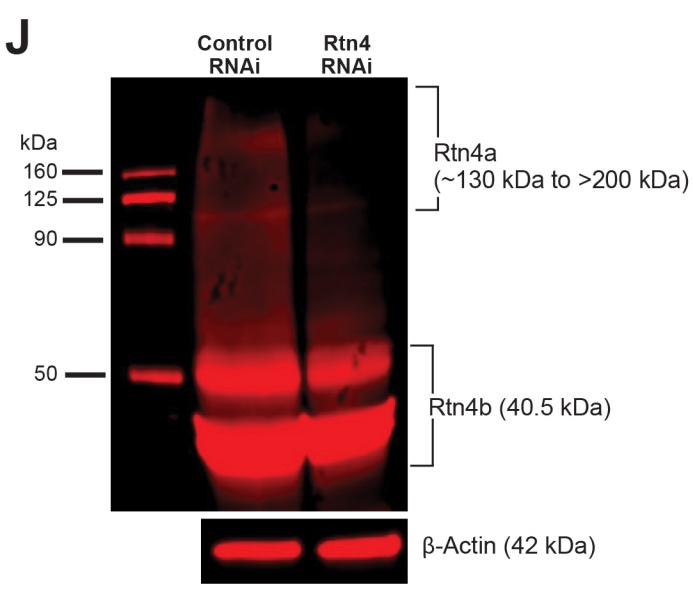

E
HLA-A Band Intensity (AU)
0.20
0.16
0.12
0.08
0.04
0
NS
Non-transfected
Control GFP-NLS
Rtn4a-GFP
GFP-Rtn4b
mCherry-REEP5
50 kDa
38 kDa
HLA-A (~41 kDa)
β-Actin (42 kDa)

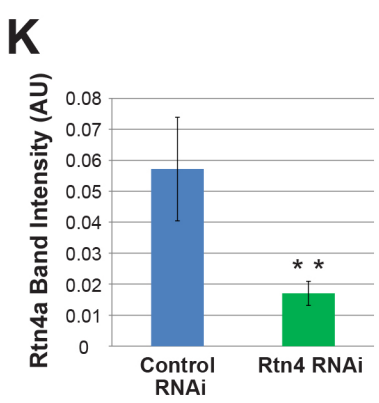

F
GFP
Integrinβ1 (total)
Hoechst
Merged
GFP-NLS
Rtn4a-GFP

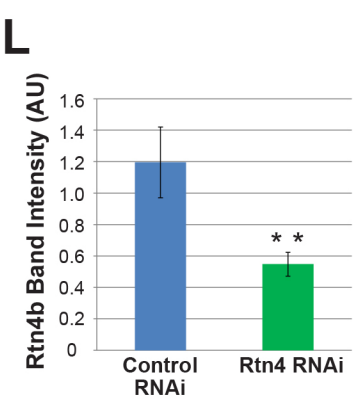

G
GFP
HLA-A (total)
Hoechst
Merged
GFP-NLS
Rtn4a-GFP

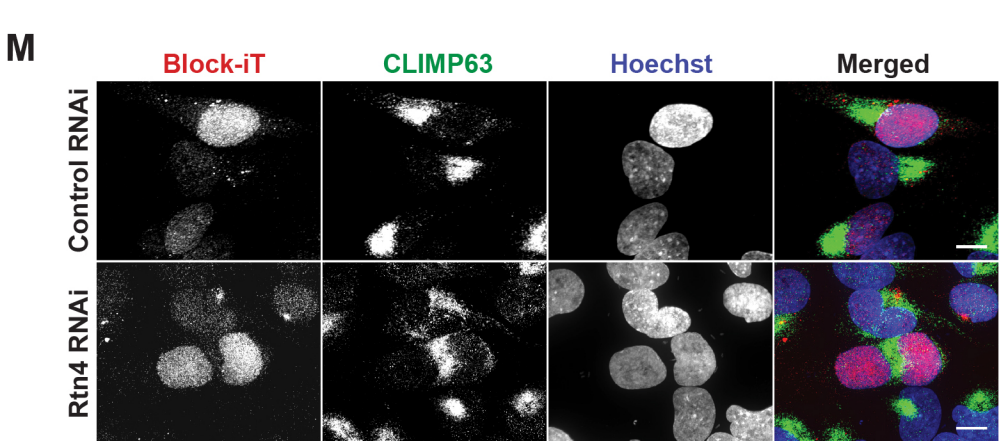

H
Fluorescence Intensity of Total Integrinβ1 (AU)
180
160
140
120
100
80
60
40
20
0
NS
Control GFP-NLS
Rtn4a-GFP


I
Fluorescence Intensity of Total HLA-A (AU)
120
100
80
60
40
20
0
NS
Control GFP-NLS
Rtn4a-GFP


J
Control RNAi
Rtn4 RNAi
kDa
160
125
90
50
Rtn4a (~130 kDa to >200 kDa)
Rtn4b (40.5 kDa)
β-Actin (42 kDa)


K
Rtn4a Band Intensity (AU)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
* *
Control RNAi
Rtn4 RNAi


L
Rtn4b Band Intensity (AU)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
* *
Control RNAi
Rtn4 RNAi


M
Block-iT
CLIMP63
Hoechst
Merged
Control RNAi
Rtn4 RNAi

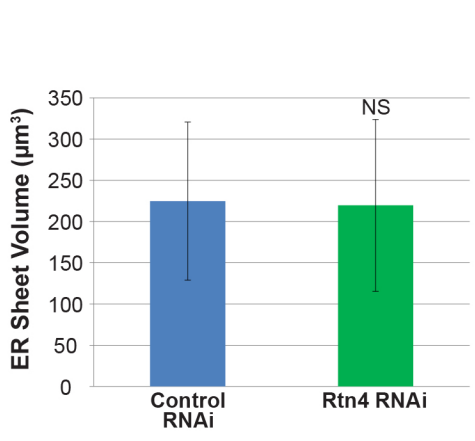
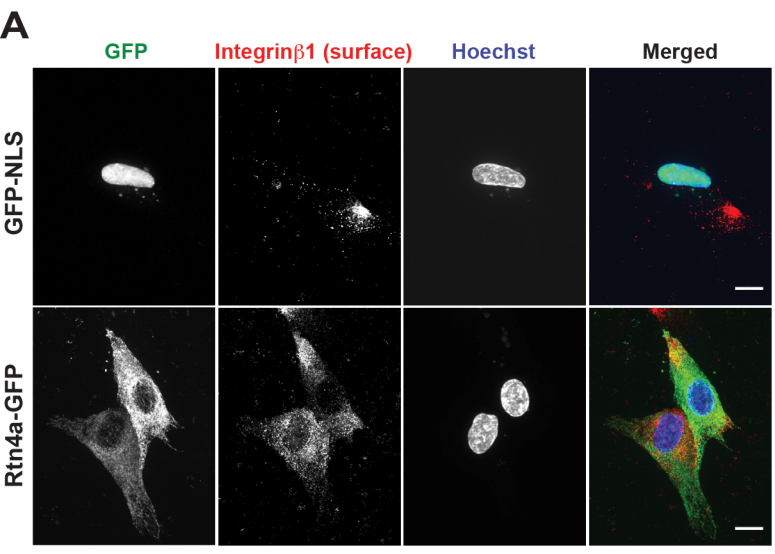
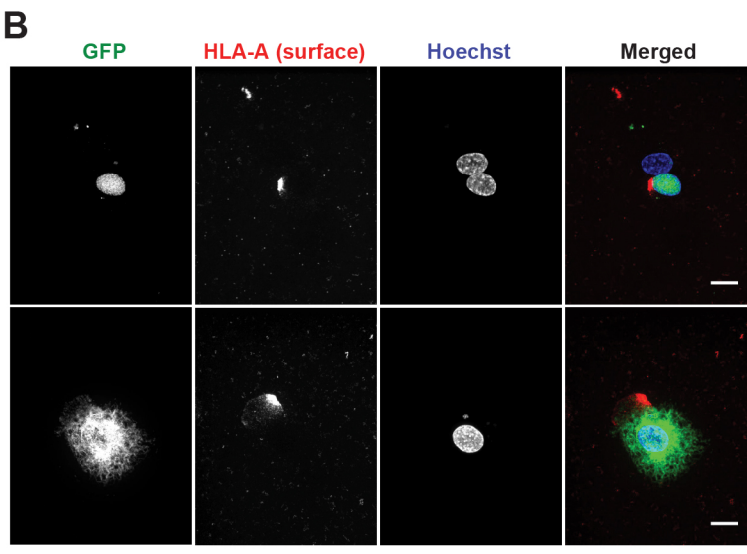
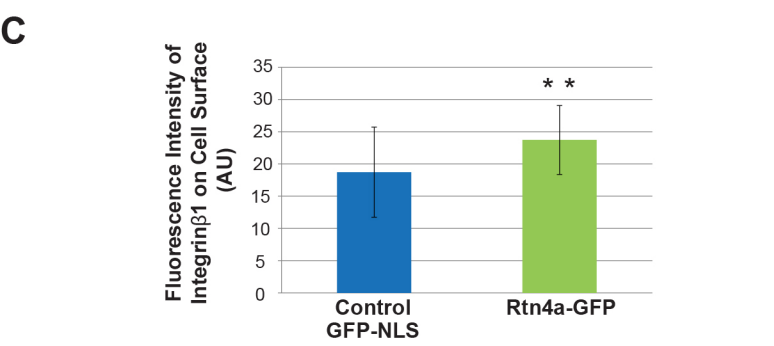
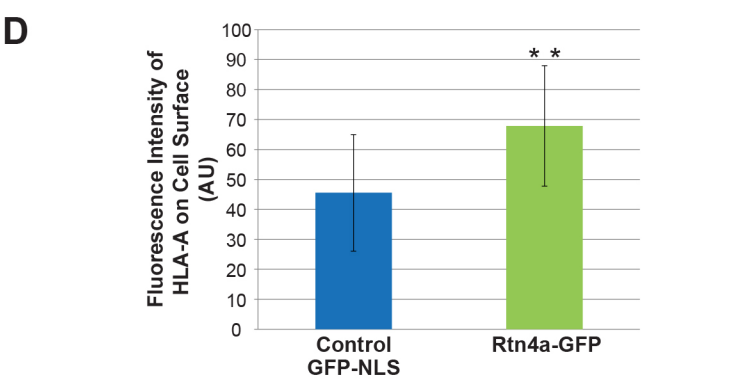

N
ER Sheet Volume (μm³)
350
300
250
200
150
100
50
0
NS
Control RNAi
Rtn4 RNAi

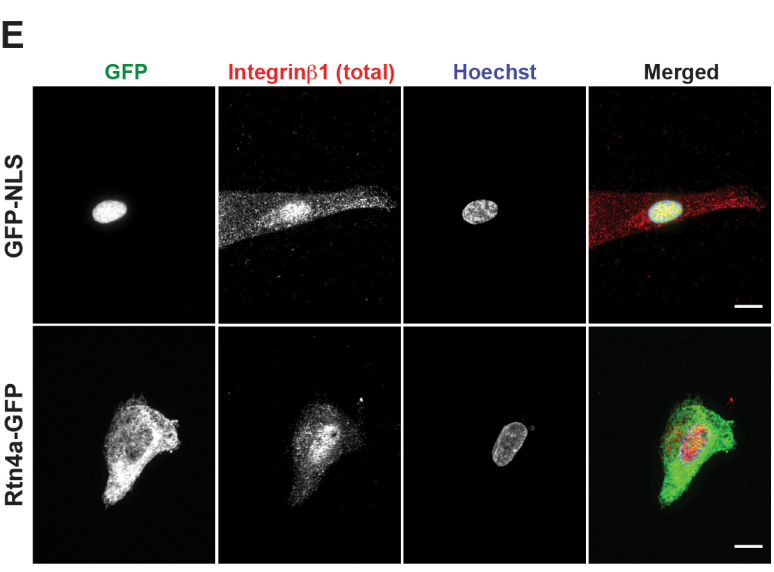
Figure S3

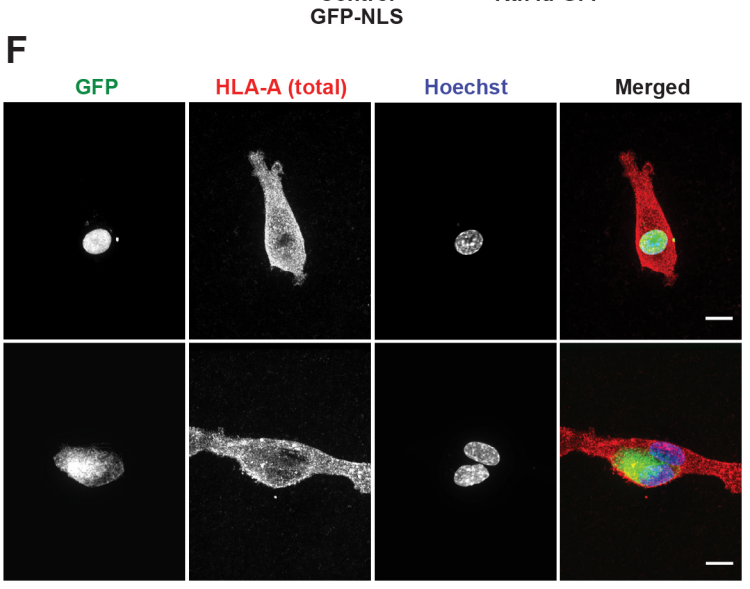

A
GFP
Integrinβ1 (surface)
Hoechst
Merged
GFP-NLS
Rtn4a-GFP

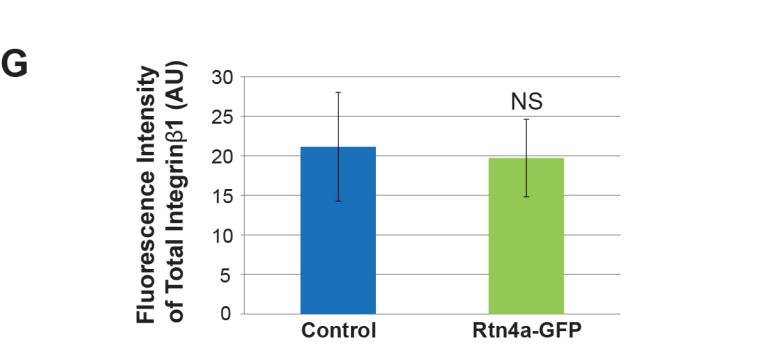

B
GFP
HLA-A (surface)
Hoechst
Merged

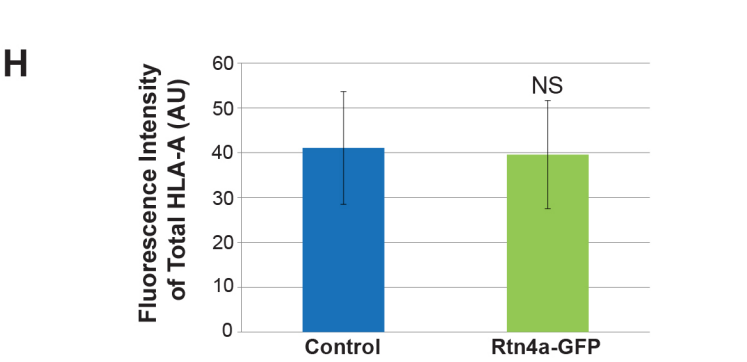

C
Fluorescence Intensity of Integrinβ1 on Cell Surface (AU)
35
30
25
20
15
10
5
0
* *
Control GFP-NLS
Rtn4a-GFP

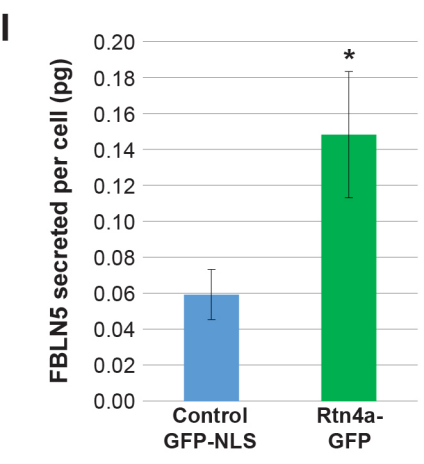

D
Fluorescence Intensity of HLA-A on Cell Surface (AU)
100
90
80
70
60
50
40
30
20
10
0
* *
Control GFP-NLS
Rtn4a-GFP

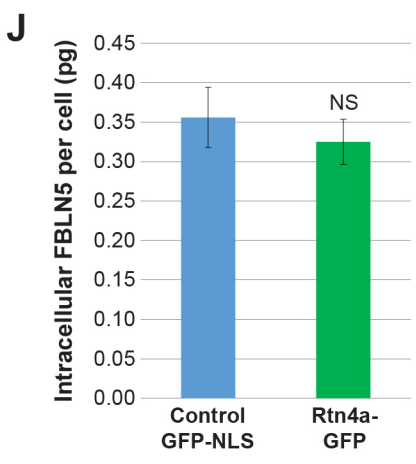

E
GFP
Integrinβ1 (total)
Hoechst
Merged
GFP-NLS
Rtn4a-GFP

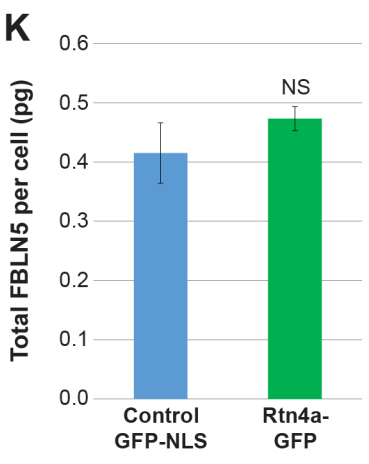

F
GFP
HLA-A (total)
Hoechst
Merged

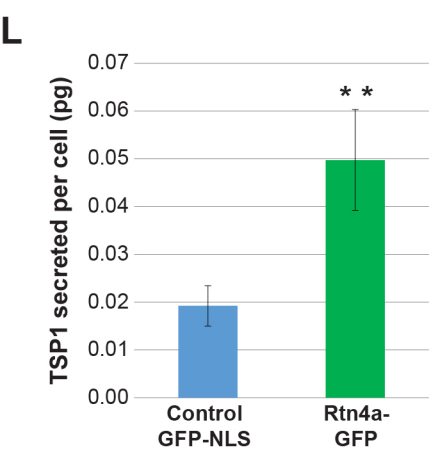

G
Fluorescence Intensity of Total Integrinβ1 (AU)
30
25
20
15
10
5
0
NS
Control GFP-NLS
Rtn4a-GFP

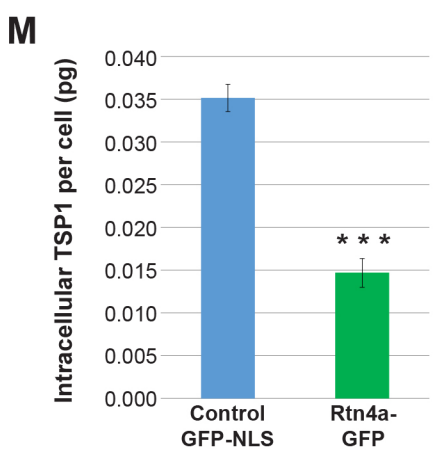

H
Fluorescence Intensity of Total HLA-A (AU)
60
50
40
30
20
10
0
NS
Control GFP-NLS
Rtn4a-GFP

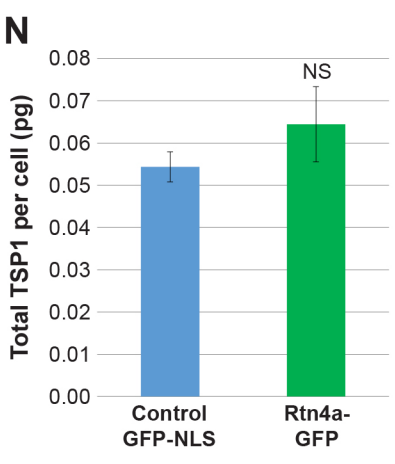

I
FBLN5 secreted per cell (pg)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Control GFP-NLS
Rtn4a-GFP

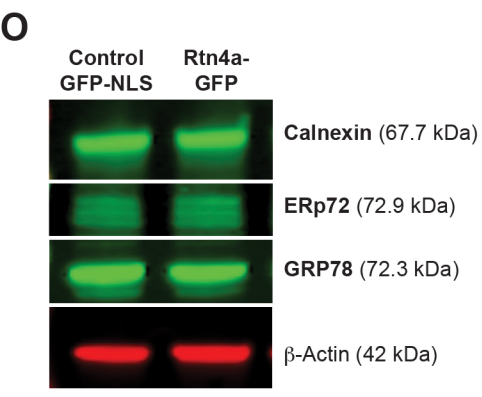

J
Intracellular FBLN5 per cell (pg)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
NS
Control GFP-NLS
Rtn4a-GFP

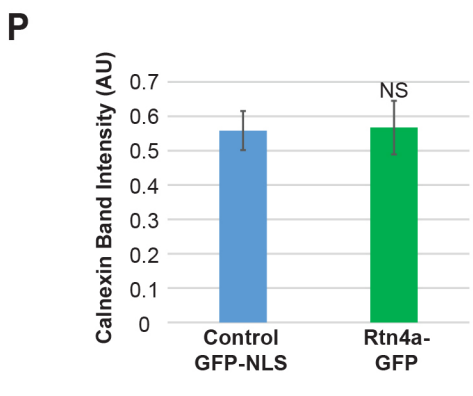

K
Total FBLN5 per cell (pg)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NS
Control GFP-NLS
Rtn4a-GFP

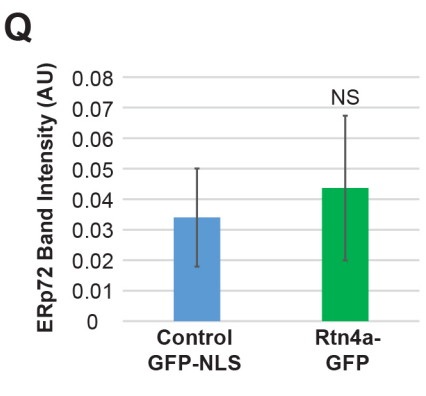

L
TSP1 secreted per cell (pg)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
* *
Control GFP-NLS
Rtn4a-GFP


M
Intracellular TSP1 per cell (pg)
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
* * *
Control GFP-NLS
Rtn4a-GFP


N
Total TSP1 per cell (pg)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
NS
Control GFP-NLS
Rtn4a-GFP


O
Control GFP-NLS
Rtn4a-GFP
Calnexin (67.7 kDa)
ERp72 (72.9 kDa)
GRP78 (72.3 kDa)
β-Actin (42 kDa)


P
Calnexin Band Intensity (AU)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NS
Control GFP-NLS
Rtn4a-GFP


Q
ERp72 Band Intensity (AU)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
NS
Control GFP-NLS
Rtn4a-GFP

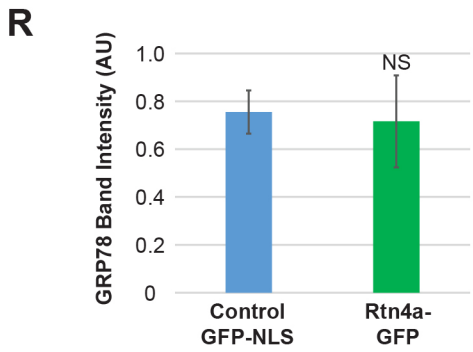
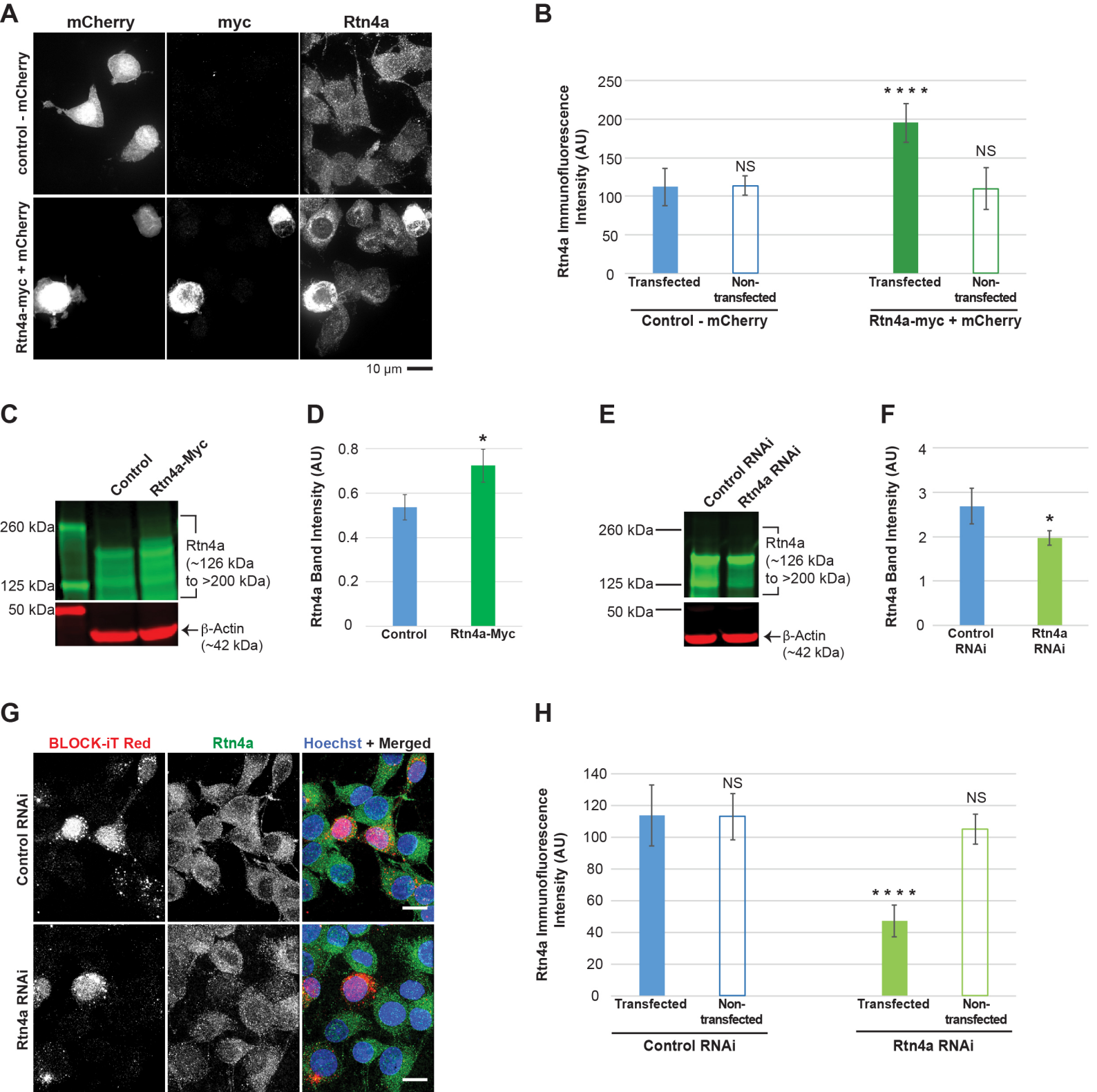

R
GRP78 Band Intensity (AU)
1.0
0.8
0.6
0.4
0.2
0
NS
Control GFP-NLS
Rtn4a-GFP

Figure S4


A
mCherry
myc
Rtn4a
control - mCherry
Rtn4a-myc + mCherry
10 µm
B
Rtn4a Immunofluorescence Intensity (AU)
250
200
150
100
50
0
* * * *
NS
NS
Transfected
Non-transfected
Transfected
Non-transfected
Control - mCherry
Rtn4a-myc + mCherry
C
Control
Rtn4a-Myc
260 kDa
125 kDa
50 kDa
Rtn4a (~126 kDa to >200 kDa)
β-Actin (~42 kDa)
D
Rtn4a Band Intensity (AU)
0.8
0.6
0.4
0.2
0
*
Control
Rtn4a-Myc
E
Control RNAi
Rtn4a RNAi
260 kDa
125 kDa
50 kDa
Rtn4a (~126 kDa to >200 kDa)
β-Actin (~42 kDa)
F
Rtn4a Band Intensity (AU)
4
3
2
1
0
*
Control RNAi
Rtn4a RNAi
G
BLOCK-iT Red
Rtn4a
Hoechst + Merged
Control RNAi
Rtn4a RNAi
H
Rtn4a Immunofluorescence Intensity (AU)
140
120
100
80
60
40
20
0
NS
NS
* * * *
Transfected
Non-transfected
Transfected
Non-transfected
Control RNAi
Rtn4a RNAi