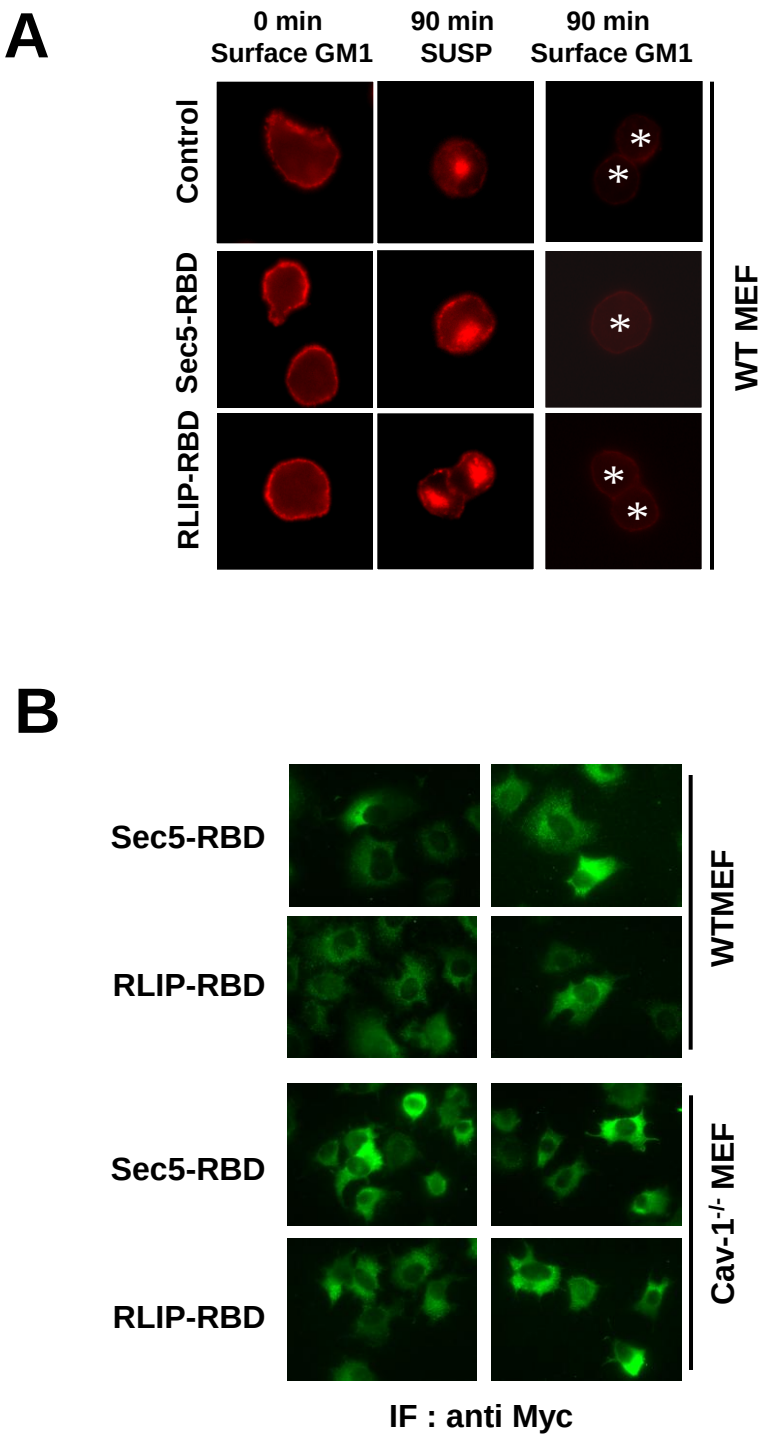
Figure S1


A
0 min
Surface GM1
90 min
SUSP
90 min
Surface GM1
Control
Sec5-RBD
RLIP-RBD
WT MEF
B
Sec5-RBD
RLIP-RBD
WTMEF
Sec5-RBD
RLIP-RBD
Cav-1-/- MEF
IF : anti Myc

Supplemental Fig 2

Figure S2

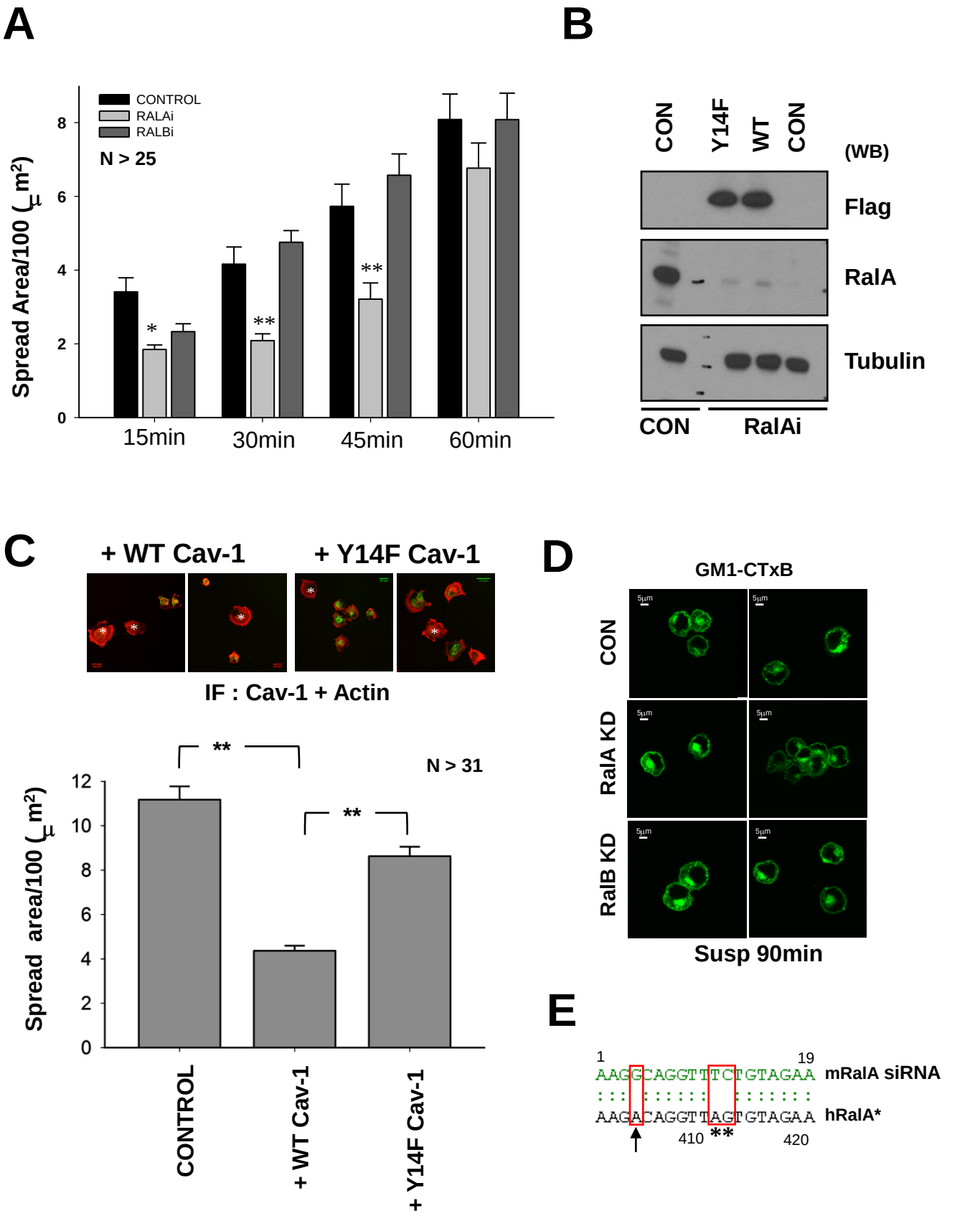


Figure S3

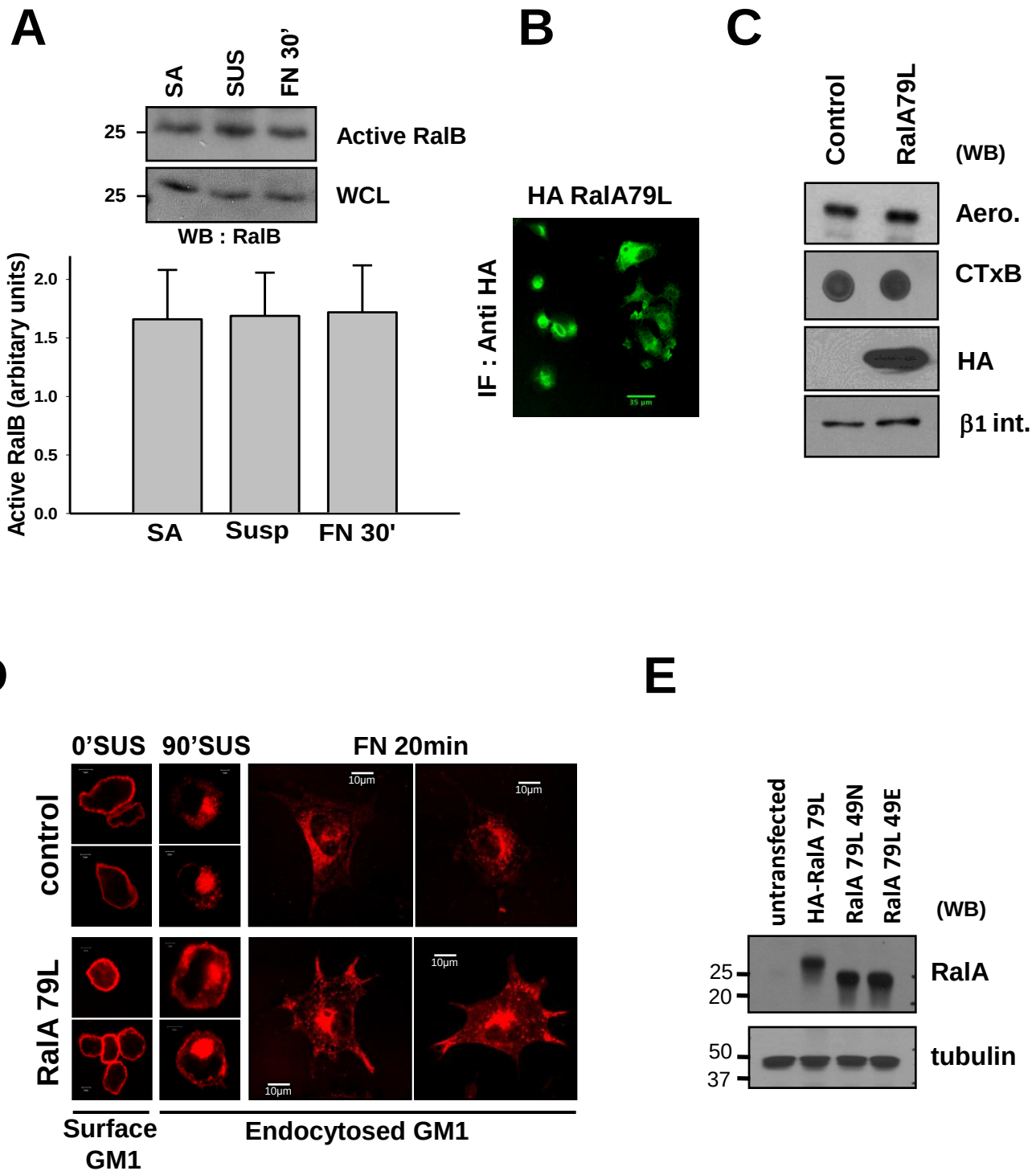
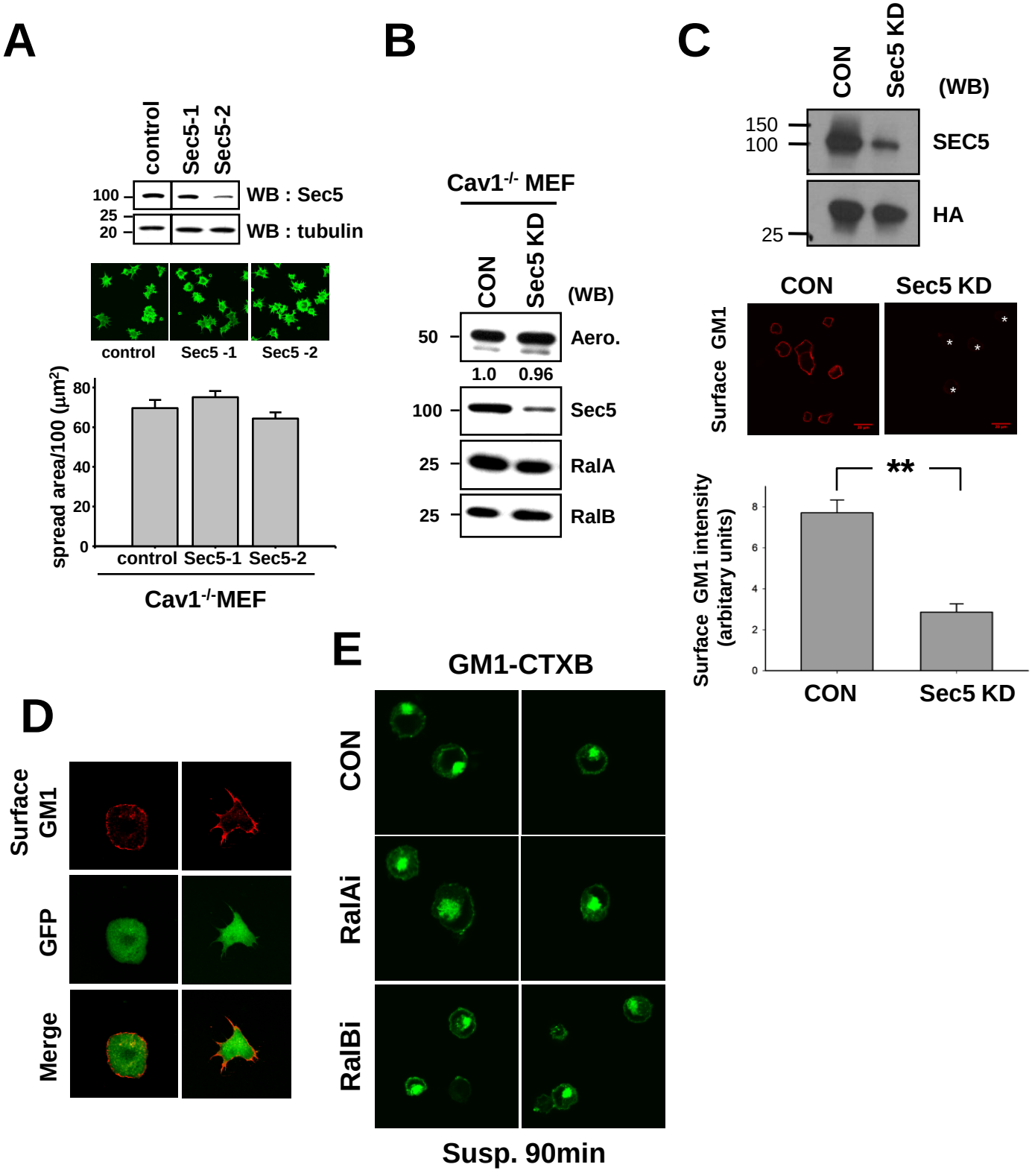

A
SA
SUS
FN 30'
25
Active RalB
25
WCL
WB : RalB
Active RalB (arbitary units)
2.0
1.5
1.0
0.5
0.0
SA
Susp
FN 30'
B
HA RalA79L
IF : Anti HA
C
Control
RalA79L
(WB)
Aero.
CTxB
HA
β1 int.
D
0'SUS
90'SUS
FN 20min
control
RalA 79L
10μm
Surface GM1
Endocytosed GM1
E
untransfected
HA-RalA 79L
RalA 79L 49N
RalA 79L 49E
(WB)
25
20
RalA
50
37
tubulin

Figure S4


A
control
Sec5-1
Sec5-2
100
25
20
WB : Sec5
WB : tubulin
control
Sec5 -1
Sec5 -2
spread area/100 (μm²)
80
60
40
20
0
control Sec5-1 Sec5-2
Cav1-/-MEF
B
Cav1-/- MEF
CON
Sec5 KD
(WB)
50
Aero.
1.0 0.96
100
Sec5
25
RalA
25
RalB
C
CON
Sec5 KD
(WB)
150
100
SEC5
25
HA
CON
Sec5 KD
Surface GM1
Surface GM1 intensity (arbitary units)
**
8
6
4
2
0
CON
Sec5 KD
D
Surface GM1
GFP
Merge
E
GM1-CTXB
CON
RalAi
RalBi
Susp. 90min

Inventory of Supplemental information.

All supplemental text is in a single document. It includes:
1. Materials and Methods
2. List of supplemental figures
3. Supplemental figure legends

SUPPLEMENTAL DATA (Balasubramanian et. al.).

MATERIALS AND METHODS.

Reagents.

Cholera toxin subunit B (CTxB) labeled with Alexa 594 (C22843) or Alexa 488 (C22841) was from Molecular Probes. Purified unlabelled Cholera toxin subunit B (CTxB) and anti Cholera toxin subunit B (CTxB) antibody was obtained from Calbiochem. Anti RalA and anti-phospho-caveolin-1 (Tyr14) antibodies were purchased from Transduction Labs. Anti RalB antibody was from R&D Biosystems and anti-HA antibody from Covance. Anti Myc (9E10) and anti-beta tubulin (E7) antibodies were from the Developmental Studies Hybridoma Bank. Anti p44/42 MAPK, Phospho-p44/p42 MAPK (Thr202/Tyr204) (E10), phospho-Akt (Ser473) antibodies were from Cell Signaling. Anti-Akt, anti-caveolin-1 and anti-GFP antibodies were from Santa Cruz Biotechnology. Purified proaerolysin and anti-aerolysin antibody were from Protox Biotech. Myc-Sec5 RBD, Myc-RLIP76 RBD, HA-RalA 79L, Myc WT hSec5, Myc T11A hSec5 and anti-sec5 antibody were obtained from Michael White (University of Texas Health Sciences Center, Dallas TX). WT Sec5-GFP was obtained from Charles Yaeman (Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa, Iowa City). The monoclonal anti-Arf6 was as described [1]. RNA interference sequence used for knockdown studies were as described,

RalA : 5'-AAGGCAGGTTTCTGTAGAA-3' [2],

RalB: 5'-GGTGGTTCTCGACGGAGAA-3' [3],

Sec5 #1 : 5'-CGGCAGAAUGGAUGUCUGC-3' [4],

Sec5 #2 : 5'-GGUCGGAAAGACAAGGCAGAU-3' [4].

Tissue Culture.

Mouse embryonic fibroblasts from $Cav1^{-/-}$ and $Cav1^{+/+}$ littermate mice (provided by Dr. Richard Anderson, University of Texas Health Sciences Center, Dallas TX) were cultured in high glucose DMEM medium with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen, Carlsbad, CA). MiaPaCa2 cells were

obtained from ATCC and cultured in RPMI 1640 with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen, Carlsbad, CA).

For transfections, 10^6 cells were electroporated using the GenePulser Xcell (Bio-Rad Laboratories, Hercules, CA) with 40μg of DNA (10μg DNA plasmid + 30μg of salmon sperm DNA). Cells were incubated for 6h with 5mM sodium butyrate to promote protein expression. If required, cells were serum starved 12 h after electroporation in medium with 0.2% fetal bovine serum. Cells analyzed ~24 h after electroporation generally showed 90-95% transfection efficiency. For knockdown, cells were transfected first with 80μM siRNA or control oligos using the RNAiMax transfection reagent (Invitrogen) followed the next day by a second transfection with 80 μM siRNA. Cells were used 48hours after the second transfection. For reconstitutions, cells were electroporated 24hrs after second siRNA transfection with the rescue vectors (30ug plasmid +10ug salmon sperm DNA), allowed to recover for 24hours and then used. For experiments, cells were detached with 1x trypsin, which was stopped with soybean trypsin inhibitor. Cells were then washed and held in suspension with 2% methylcellulose in high glucose DMEM before replating on fibronectin-coated coverslips. For proaerolysin labeling, cells were detached with Accutase (Innovative Cell Technologies Inc.). Fibronectin for coating coverslips was used at 2μg/ml or 10μg/ml as indicated in figure legends.

Stable knockdown of RalA and RalB were done in human pancreatic MiaPaCa2 cells using retroviruses derived from pSuper-Retro-Puro plasmids encoding shRNA against RalA, RalB, or a scramble sequence as described earlier [5, 6]

Analysis of surface bound proaerolysin.

Detached cells in suspension (0 min, 90min) or re-adherent cells were chilled on ice for 15min then incubated with 0.1μg/ml of proaerolysin (Protox Biotech) for 30min. Cells were washed with cold PBS and $2x10^5$ cells were lysed in 100μl of SDS sample buffer. Cell-equivalent amounts of lysate were resolved by SDS PAGE, transferred to PVDF and blocked with 5% nonfat dry milk in TBS+0.5%

Tween-20 (TBS-T). Blots were incubated with anti-aerolysin antibody (0.5μl/ml) followed by anti-mouse antibody conjugated to horse radish peroxidase and developed using the ECL detection system (Amersham). Blots were stripped using Re-blot Plus stripping solution (Chemicon International) and re-probed with monoclonal anti-beta tubulin antibody as a loading control. Proaerolysin band intensities were normalized to tubulin. For knockdowns, blots were probed for RalA (antibody 1μg/ml), RalB (antibody 1μg/ml) and Arf6 (antibody 1μg/ml). If required, blots were developed with Immobilon Western HRP Luminal Reagent (Millipore) to boost signal. Bands were quantified by densitometry using the Image J software.

Analysis of total GM1 and GPI linked protein levels.

Cells were incubated with 1ug/ml proaerolysin or 10μg/ml of cholera toxin B subunit (CTxB) for 2hours. Cells were washed, lysed and cell equivalent dot blotted on nitrocellulose (for detecting bound CTxB) or resolved by western blot and transferred to PVDF membrane (for detecting bound proaerolysin). Blots were incubated with anti-CtxB antibody or anti-aerolysisn antibody followed by anti-goat and anti-mouse HRP. Expression of RalA79L was detected with anti-HA antibody and anti-mouse HRP. Blots were developed with ECL (Amersham) immunodetection reagents

Cell labeling with CTxB.

Stably adherent cells were chilled on ice for 15min then incubated with 10μg/ml of CTxB-Alexa 594 or 488 in PBS for 15 min. Cells were detached, held in suspension and replated on FN-coated coverslips for the indicated times prior to fixation in 3.5% paraformaldehyde. Alternatively, surface GM1 was detected by incubating cells on ice with 10μg/ml CTxB-Alexa594 in PBS for 15min then rinsed in cold PBS and fixed in 3.5% paraformaldehyde. Labeled cells were permeabilised in PBS containing 3% BSA and 0.05% Triton X100 for 15 min, then blocked with 3% BSA in PBS for 1 h and stained for other antigens. Cells expressing HA-RalA 79L were stained with anti-HA antibody, followed by anti-

mouse Alexa 488 antibody (Molecular Probes). Cells were mounted in with Fluoromount-G (Southern Biotech), observed using a Zeiss LSM 510 laser confocal microscope with a 40x or 63x objective and analysed using either the Zeiss LSM Image Browser or the Image J software (NIH). For quantitation of surface labeling, confocal images were recorded being careful to avoid pixel saturation. Using Image J software (NIH), thresholds were set to map the entire cell. The tracing tool was then used to select edges of the thresholded areas. Total intensity within the thresholded areas was determined and compared between samples as required.

Ral activity assay.

Stably adherent cells were detached, held in suspension 2h and replated on 10 μg/ml FN. Cells were lysed on ice in buffer containing 50mM Tris pH 7.5, 200mM NaCl, 10mM $MgCl_2$, 1% NP-40 and protease and phosphatase inhibitors (Calbiochem). Lysates were centrifuged for 5min at 20,000xg and incubated with 35 μg Sec5-RBD pre-coupled to GST-Sepharose beads (Amersham) for 45 min at 4°C. Beads were washed 3X in lysis buffer, eluted with SDS sample buffer and resolved by SDS PAGE. RalA and RalB were detected by Western blotting with anti RalA and RalB antibody and ECL or Immobilon detection system.

Measuring cell spreading.

Images of adherent cells were analyzed using Image J software. Thresholds were set to map the entire cell, the tracing tool was used to select the edge of each cell. The total area within the mapped edge for each cell was determined.

Statistical significance.

Comparison between data points were done using the Student's t test (Sigmaplot Stastical Analysis Software).

Fluorescent Image Colocalization Analysis.

Images of cells photographed using the Laser Confocal Microscope at both wavelengths were analysed using Image J software (NIH), and Pearson's coefficient determined using the colocalization threshold plugin.

INVENTORY OF SUPPLEMENTAL INFORMATION.

Figure S1.

Confirms the expression of the Ral Binding Domains of Sec5 and RLIP tested in Figure1 and their effect on surface GM1 endocytosis.

Figure S2.

Reveals that RalA knockdown studied in Figure 2 regulates the time course of cell spreading and membrane microdomain exocytosis, but not their endocytosis through caveolae.

Figure S3.

Shows that adhesion does not regulate RalB GTP loading. It also confirms the expression of active RalA mutant constructs used to test their role as part of the exocyst complex in membrane microdomain trafficking.

Figure S4.

Shows that loss of Sec5 does not affect Cav1-/- MEFs and that loss of RalA does not affect microdomain endocytosis.

Supplemental Figure Legends.

Supplementary Figure 1. **(A)** Untransfected WT MEFs (Control) or cells expressing the Sec5 RaI binding domain (Sec5-RBD), or the RLIP76 RaI binding domain (RLIP-RBD) were serum starved overnight. Left panels, cells were surface labeled with CTxB-Alexa 594 immediately after detachment 0min; Middle panels, these cells were held in suspension for 90min; Right panels, cells were suspended for 90 min then surface labeled. Cells with very low surface staining are marked by asterisks. **(B)** MEFs expressing Myc-tagged Sec5-RBD or RLIP-RBD were stained for Myc to demonstrate equivalent transfection efficiency and levels.

Supplementary Figure 2. **(A)** WT MEFs transfected with control, RalA or RalB siRNA were suspended for 90min and replated on FN (2μg/ml) for the indicated times. Cells were fixed, stained with phalloidin-Alexa 488 and images taken. Graph: spread area of cells/100; values are mean ± SE. (>25 cells were analysed per time point per experiment, 2 independent experiments gave similar results). ** =$p<0.001$ to * =$p<0.01$. **(B)** Expression of WT Cav1 (WT) and Y14Cav1 (Y14F) in RalA knockdown (RalAi) WTMEFs. Control (CON) cells were untransfected. **(C)** Cells were held in suspension for 90min then replated on FN (2μg/ml) for 30min. Cells were fixed and stained with phalloidin-Alexa 488 and for Cav1. Control untransfected cells are marked by asterisks. Graph: spread surface area/100; values are mean ± SE. (>31 cells were analysed, 2 independent experiments gave similar results). ** =$p<0.001$. **(D)** WT MEFs transfected with control, RalA or RalB siRNA were surface labeled with CTxB-Alexa 488, suspended for 90min, fixed and images taken. **(E)** Mouse RalA siRNA oligo (mRalA siRNA) (residues 1-19) compared to human RalA (hRalA*) with one endogenous mismatch(←) and two additional introduced silent mismatches (*).

Supplementary Figure 3. **(A)** WT MEFs that were stably adherent (SA), suspended for 90min (SUS), or replated on FN (10 μg/ml) for 30min (FN30') were

lysed and active RalB pulled down on GST-Sec5 RBD beads. Bound RalB and respective whole cell lysates (WCL) were Western blotted for RalB (WB: RalB). Graph: Active RalB bands were quantified and normalized to WCL. Values are means ± SE, n=5. **(B)** Expression of RalA79L was confirmed by immunostaining for HA (IF:anti HA). **(C)** Cells were labeled to steady state (2h 37°) and total aerolysin and CTxB detected by Western blotting (WB: Aero), anti-CtxB (WB: CTXB). β1 integrin antibody served as a loading control (WB : β1 int.). RalA 79L was detected with anti-HA antibody (WB:HA). **(D)** Control and RalA79L expressing WT MEFs surface labeled with CTxB-Alexa594 were suspended for 0min (0' susp) or 90min (90' susp), or suspended then replated on FN (2μg/ml) for 20min. The fraction of fluorescence in the perinuclear region was determined as described in Methods. **(E)** Expression of endogenous RalA (untransfected), HA-RalA79L, and untagged RalA 49E and 49N were assessed with anti-RalA (WB : RalA). Tubulin (WB :tubulin) was used as a loading control.

Supplementary Figure 4. **(A)** Cav1$^{-/-}$ MEFs transfected with control, Sec5 siRNA oligo #1 (sec5 KD-1) or #2 (Sec5 KD-2) were suspended for 90min and replated on FN (2 μg/ml) for 20min. Cells were fixed and stained with Phalloidin-Alexa 488. Graph: cells' spread area/100; values are mean ± SE (n=65 cells), representative of three independent experiments. **(B)** Re-adherent cells were incubated on ice with purified proaerolysin for 30min. Bound proaerolysin was detected in cell lysates by Western blotting (WB:Aero). Blots were also probed for RalA and RalB as controls (WB). Values represent aerolysin normalized to RalB relative to control. **(C)** RalA79L-WT MEFs transfected with Sec5-2 oligo (Sec5 KD) or left untransfected (CON) were serum starved overnight, detached and held in suspension for 90min. Cells were then surface labeled with CTxB-Alexa 594 (GM1-CTxB), fixed and images taken. Lysates from cells treated in parallel were analysed for Sec5 (WB : Sec5) and RalA79L (WB : HA). (Graph: Values are mean surface intensity ± SE, n=14, representative of two independent experiments. ** =p<0.001. **(D)** GFP-expressing WT MEFs were suspended for 90min, and replated on FN (2 μg/ml) for 20min. Cells were chilled, stained with

CTxB-Alexa 594 (Surface GM1), and fixed. **(E)**. WT MEFs left untransfected (CON) or transfected with siRNA for RalA or RalB (RalAi and RalBi) were surface labeled while adherent then detached and held in suspension for 90 min. Cells were then fixed and imaged. **(F)** Surface GM1 in MiaPaCa2 control, RalAi and RalBi cells were labeled with CTxB-Alexa 488 (GM1-CTxB), cells were detached and held in suspension for 90min, washed, fixed and images taken.

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