SUPPLEMENTAL EXPERIMENTAL PROCEDURES

RNA Isolation and RT-PCR - Total RNA was isolated from $5x10^3$-$1x10^4$ trypsinized cells grown at low density by using Quick-RNA™ MicroPrep kit (Zymo Research) according to the manufacturer's instructions. RT-PCR reactions were performed following standard procedures using the Titan One Tube RT-PCR kit (Roche).

Internalization Assays - For β1 integrin internalization experiments, HT1080 cells were seeded on glass coverslips 12h prior to use in complete media. Coverslips were rinsed with ice-cold PBS before addition of a 1/40 dilution of 9EG7 anti-β1 integrin rat monoclonal antibody at 4°C for 45min. Coverslips were rinsed and either fixed or submerged in complete media at 37°C for 30min. Internalization was stopped by transferring the cells to ice and washing with cold PBS. Antibody at the cell surface was removed by washing with ice-cold 0.2N Acetic acid containing 500mM NaCl pH=2.0 for 45sec. Cells were fixed and processed for immunofluorescence with an anti-rat IgG antibody conjugated to Cy3. Z-stack images of 8-10 random fields of about 50 cells per field were captured using the same exposure times. For image analysis, stacks were converted to single composite images. Background fluorescence was removed using the ImageJ's subtract background feature with a rolling ball radius of about 3 microns. Fluorescence intensity of each image was measured and divided by the number of cells. The internalized fraction was then determined using average intensity after and before internalization.
For EGF-TMR internalization experiments, cells were seeded on glass coverslips and starved for 12h. Cells were then rinsed with ice-cold PBS and labeled with 0.5μg/mL EGF-TMR at 4°C for 45min. Next, coverslips were transferred to 37°C DMEM and for 8min to allow internalization before rinsing with cold PBS and removing residual surface-bound ligand as described above. Fixation, microscopy and image processing was performed as described above. Then the average fluorescence intensity of transfected cells was measured and multiplied by the cell area to obtain total fluorescence units for individual cells.

FRET Biosensor Imaging of Rac1 Activation - NIH3T3 cells were mock siRNA-treated or depleted of epsins then transfected with Rac1 Raichu probes (1) created and kindly provided by Dr. Matsuda (Riken institute, Japan). Cells were trypsinized and allowed to recover in suspension for 1h before plating on coverslips coated with 10μg/mL fibronectin for 3h. Cells were fixed and migrating cells were imaged for FRET activity by Ziess Axiovision 4 FRET analyzer software. FRET intensity was determined by using the Acceptor Ratio method.

SUPPLEMENTAL REFERENCE

1. Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M. (2002) *Mol. Cell. Biol.* **22**, 6582-91

SUPPLEMENTAL TABLES

Supplemental Table 1: siRNAs used in this study	
SMARTpool (Dharmacon)	**Specificity**
L-064822	*Mus musculus* Epsin1
L-043345	*Mus musculus* Epsin2
M-046603	*Mus musculus* RalBP1
L-004724	*Homo sapiens* Epsin1
L-004725	*Homo sapiens* Epsin2
M-009266	*Homo sapiens* RalBP1

Supplemental Table 2: Plasmid used in this study		
Constructions	**Description**	**Source**
GST	pGEX-4T-1	GE Healthcare
GST-RalBP1	*Homo sapiens* RalBP1 in pGEX-4T-1	This study
His_6-epsin1 ENTH domain	*Rattus norvegicus* $Epsin1^{1-168}$ in pET28a	This study
His_6-epsin1 $ENTH^{YTQV}$ domain	*Rattus norvegicus* $Epsin1^{1-168\ Y101R,\ T105D,\ Q110A\ and\ V112A}$ in pET28a	This study
GFP	pEGFP	Clontech
GFP-epsin1 isoform 1	*Rattus norvegicus* Epsin1 in pEGFP	H. McMahon (MRC Cambridge)
GFP-epsin2a	*Homo sapiens* Epsin2a in pEGFP	P. De Camilli (Yale University)
GFP-epsin3	*Homo sapiens* Epsin3 in pEGFP	B. Pilcher (Bioform Medical), W. Parks (Washington University, Seattle)
Myc_6-RalBP1	*Homo sapiens* RalBP1 in pRK5-myc	This study
Myc_6-$RalBP1^{209-655}$	*Homo sapiens* $RalBP1^{209-655}$ in pRK5-myc	This study
Myc_6-$RalBP1^{1-209,\ 383-655}$ □ □(ΔGAP□)	*Homo sapiens* $RalBP1^{1-209,\ 383-655}$ in pRK5-myc	This study
Myc_6-$RalBP1^{1-383}$	*Homo sapiens* $RalBP1^{1-383}$ in pRK5-myc	This study
GFP-epsin1 ENTH	*Rattus norvegicus* $Epsin1^{1-168}$ in pEGFP	This study
GFP-epsin1 ΔENTH	*Rattus norvegicus* $Epsin1^{166-550}$ in pEGFP	This study
GFP-epsin1 $ENTH^{YTQV}$	*Rattus norvegicus* $Epsin1^{1-168,\ Y101R,\ T105D,\ Q110A\ and\ V112A}$ in pEGFP	This study
HA-RalBP1	*Rattus norvegicus* HA-tagged RalBP1 in pCDNA3	This study (cDNA kindly provided by Dr. A. Quaroni, Cornell University)
HA-$RalBP1^{209-655}$	*Rattus norvegicus* HA-tagged $RalBP1^{209-655}$ in pCDNA3	This study
HA-Arf6	*Homo sapiens* HA-tagged Arf6 in pSRa	P. Chavrier (Institut Curie)
HA-$Arf6^{TA}$	*Homo sapiens* HA-tagged $Arf6^{T157A}$ in pSRa	This study
Myc-ARNO	*Homo sapiens* Myc-ARNO in pCB7	J. Casanova (University of Virginia)
GST- CRIB	PAK1B CRIB domain in pGEX	J. Collard (Netherlands Cancer Institute)
GST- VHS+GAT domain	$GGA3^{1-316}$ in pGEX	J. Bonifacino (NIH)

Supplemental Table 3: Antibodies used in this study		
Antibody	**Characteristics**	**Source**
H-130	Rabbit Polyclonal anti-Epsin1	Santa Cruz
C-16	Goat Polyclonal anti- Epsin2	Santa Cruz
C-19	Goat Polyclonal anti- RalBP1	Santa Cruz
G1544	Rabbit Polyclonal anti- GFP	Sigma
7G9	Mouse Monoclonal anti-GFP	Abmart
HA.11	Mouse Monoclonal anti- HA	Covance
9E10	Mouse Monoclonal anti- Myc	DBS
6xHIS	Mouse Monoclonal anti- His_6	Clontech
ARC03	Mouse Monoclonal anti- Rac1	Cytoskeleton
9EG7	Rat Monoclonal anti-β1 Integrin	BD Biosciences
DM1A	Mouse monoclonal anti- αTubulin	Abcam

LEGEND TO SUPPLEMENTAL FIGURES

Suppl. Fig. 1: Examples of knock-down and transfection of RalBP1 and epsins. **A**. The presence of epsin transcripts in total RNA from the HT1080, MDA-MB-231 and NIH3T3 cell lines and from mouse pancreatic cancer models was tested by RT-PCR. Oligos designed to amplify different portions (ranging from 0.71-1kb) of the mouse and human transcripts were used. **B.** Cells were treated with the indicated siRNAs against single or combination of human (Hs) epsin paralogs. Samples were analyzed by immunoblot with anti-epsin specific antibodies. **C**. Mouse NIH3T3 cells treated or not with siRNA towards *Mus musculus* (Mm) RalBP1; transfected with (siRNA-resistant, myc-tagged) HsRalBP1 full length or ΔNT, as indicated, were analyzed by immunoblot with an anti-RalBP1 antibody. **D.** Lysates from cells expressing the indicated constructs were analyzed by immunoblotting with an anti-GFP, anti-myc and anti-Tubulin antibodies.

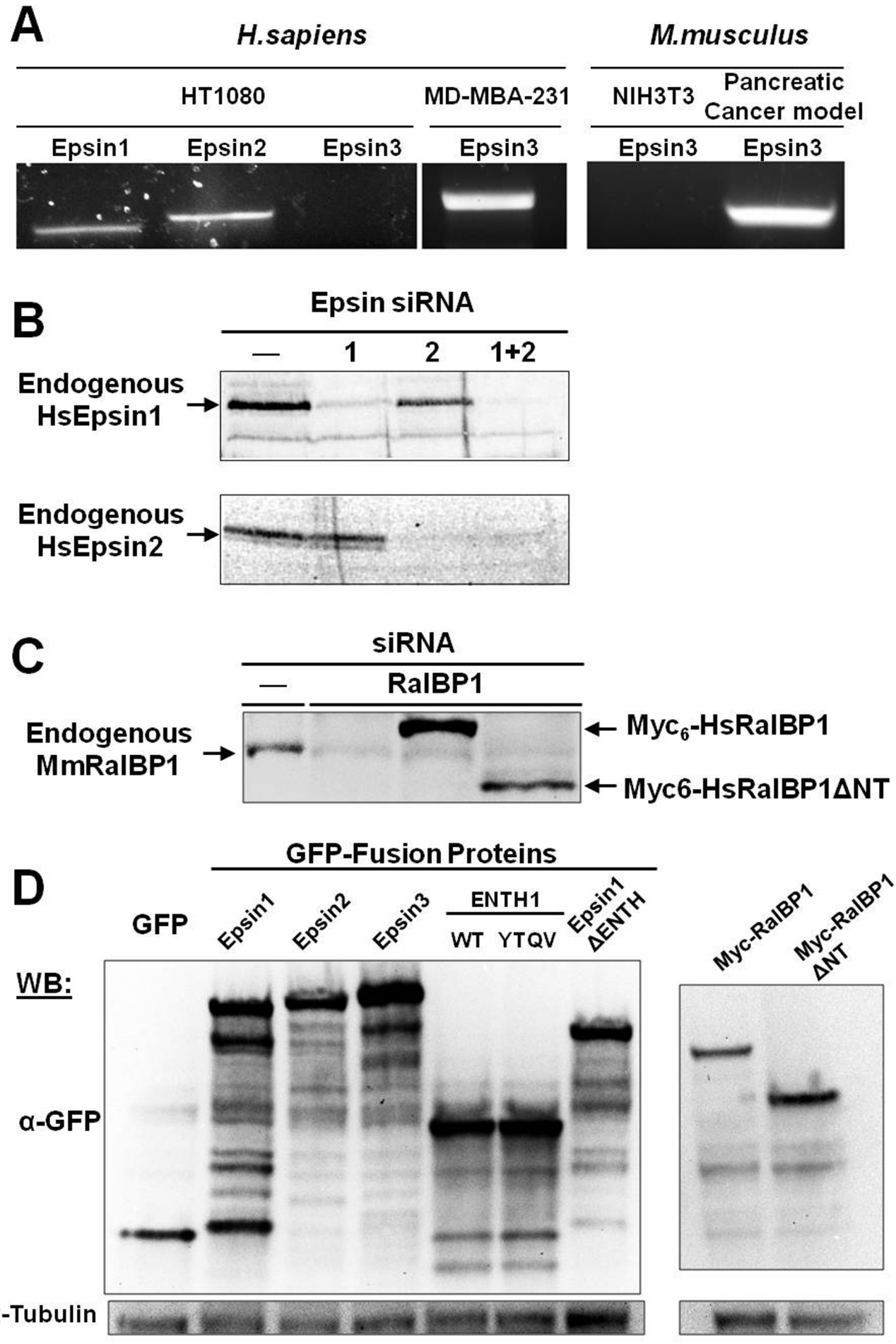
Suppl. Fig. 2: Effect of RalBP1 and epsin knock-down on Chemotaxis and Haptotaxis. A. RalBP1- and epsin-depleted cells were stimulated to migrate by addition of serum (Chemotaxis) in the transwell lower chamber (see Fig. 3A) or by coating the filter lower surface with fibronectin (Haptotaxis). Cells were analyzed for their ability to migrate as described in Materials and Methods and compared to controls. **B**. The effect of individual (RalBP1 or epsin1/2) or simultaneous knock-down on the ability of cell to migrate under haptotactic stimulation was performed as described in A. Statistical significance was determined by the t-test with Bonferroni correction (**: $p<0.05/2$).

Suppl. Fig. 3: Knock-down of epsins or RalBP1 or overexpression of ENTH domain do not affect activated β1-integrin or EGF internalization. Mock and siRNA-treated HT1080 cells were labeled with 9EG7 anti-β1 integrin antibody at 4°C. Internalization was allowed for 30min at 37°C in complete media. Antibody bound to the cell surface was removed by acid wash to only show the internalized fraction or treated with PBS ("Int" and "Total", respectively). Scale bar = 50 microns**. B.** Internalized fraction of anti-β1 antibody was determined by dividing the fluorescence intensity of images showing internalized antibody and total surface antibody as described in Supplemental Materials and Methods. **C**. GFP or ENTH1-GFP-transfected HT1080 cells were allowed to internalize EGF-TMR for 8min. Surface EGF was removed and cells were fixed. Images show internal EGF accumulation in transfected cells (traced) and neighboring untransfected cells. **D**. The internalized EGF was quantified according to Supplemental Materials

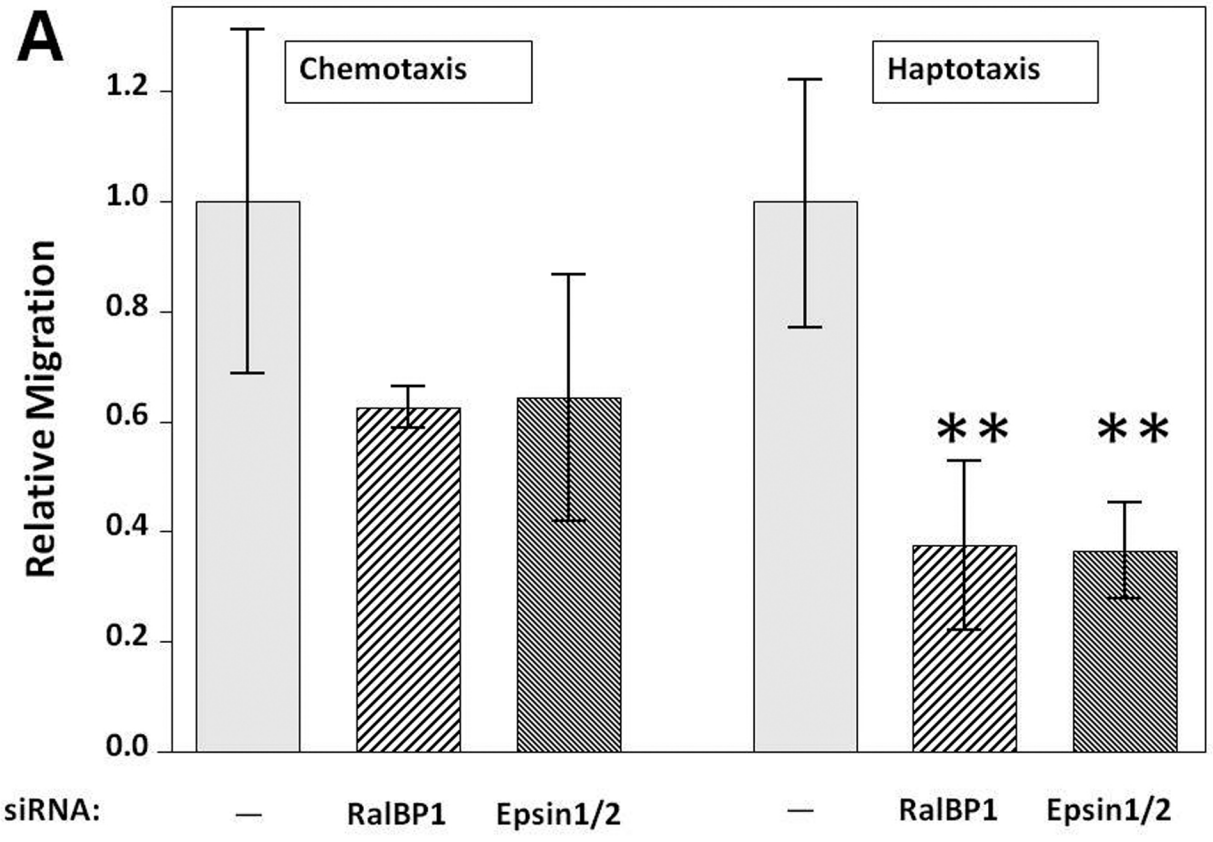
and Methods. Results show average and standard deviation of arbitrary fluorescence intensity values of transfected cells.

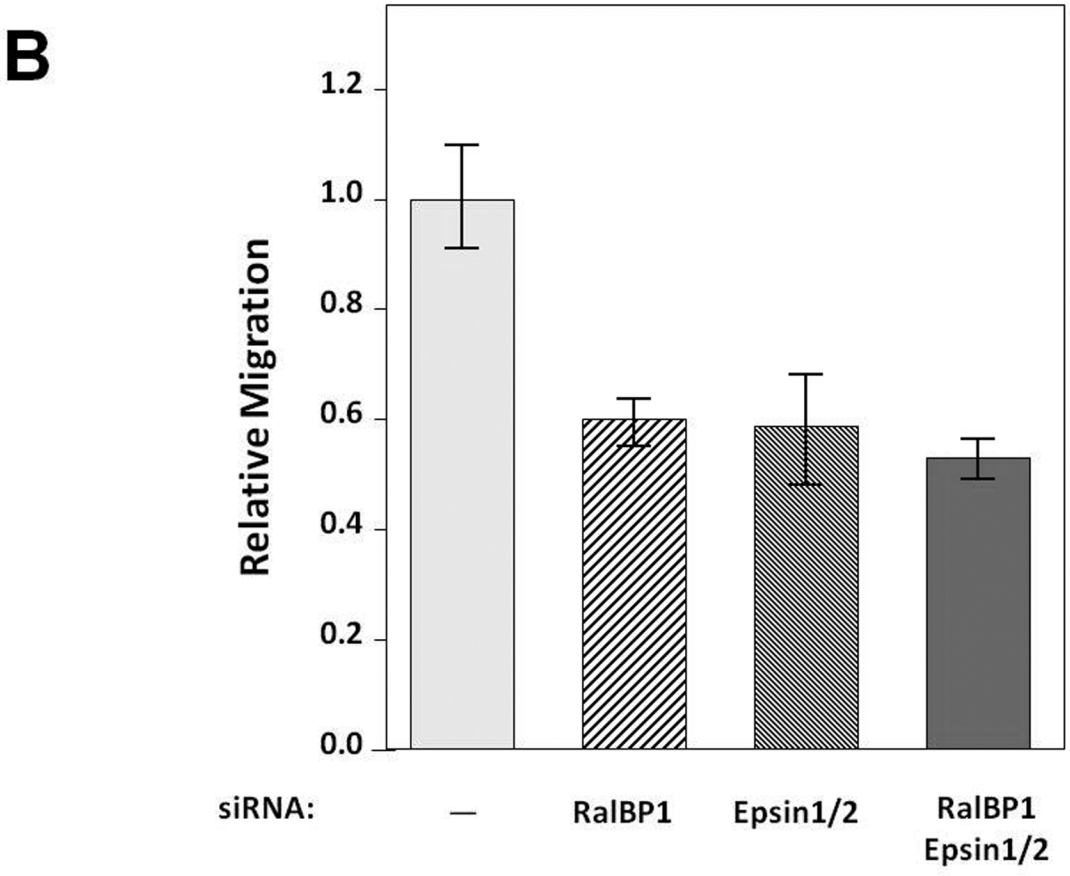
Suppl. Fig. 4: Epsin knock-down leads to Rac1 activation deficiencies. A. Scheme showing the structure of Raichu probes. The Fluorescence Resonance Energy Transfer (FRET) probe is made up of a Rac1 module fused to the PAK1 effector domain ([Rac1•GTP]-binding module), and YFP and CFP fused to the N- and C-termini of the molecule, respectively. Upon Rac1 activation the PAK1 domain binds Rac1 bringing the N- and C- terminal ends in close proximity, allowing CFP/YFP FRET. **B**. Cells were transfected with Rac1 Raichu probe. Migrating cells were imaged and FRET signals were analyzed by the Acceptor Ratio method, signal intensity was color coded for display.

Supplemental Fig. 1

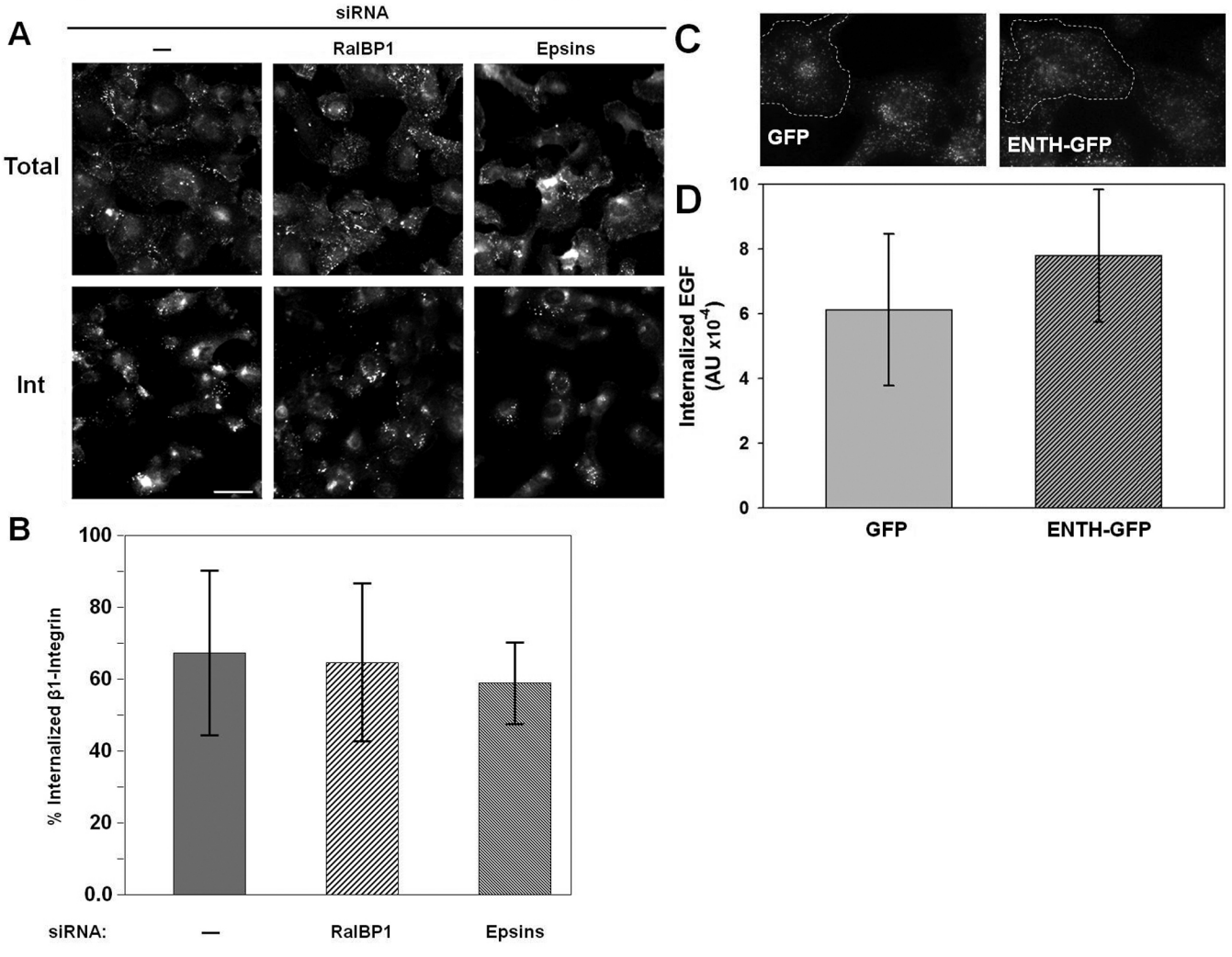


Supplemental Fig. 2





Supplemental Fig. 3



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Supplemental Fig.4