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Supplemental Information

The SNARE VAMP7 Regulates Exocytic Trafficking of Interleukin-12 in Dendritic Cells

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Supplemental experimental procedures

Mice

The animals used in this study were as follows: six to eight weeks old C57BL/6 mice were purchased from Harlan. OVA-specific, MHC class I and MHC class II restricted, TCR transgenic OT-I and OT-II mice were purchased from the Jackson Immuno Research Laboratories. Male VAMP7-deficient mice (Danglot et al., 2012) on a C57BL/6 background were compared with their male wild-type littermates. Mice were bred and maintained in sterile isolators. Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community [EEC] Council Directive 86/609; OJL 358; December 12, 1987). Protocols were approved by the Italian Ministry of Health.

Cell culture

Dendritic cells were obtained from crude BM cells cultured in 24-well plates (2×10^6 cells/well) in complete IMDM (Gibco BRL) supplemented with 30% GM-CSF supernatants or 50 ng/ml Flt3L (R&D Systems). DCs were used for experiments between day 7 and 8, when expression of CD11c was higher than 80%. GM-derived cells were used for overexpression of fluorescently tagged proteins. For silencing, functional analysis and confocal microscopy conventional DCs from FLT3 derived cultures were isolated by negative selection using $B220^+$ microbeads (Miltenyi Biotec). For isolation of DCs from spleens, cell suspensions were obtained by digestion with Collagenase D (1.6 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) for 30 min at 37°C. Splenic DCs were enriched from total spleen cells by positive selection using $CD11c^+$ microbeads (Miltenyi Biotec). BM from I-A^b GFP mice (Boes et al., 2002), were a kind gift of Ana-Maria Lennon Dumenil, (Insitut Curie, Paris, France).

OT-I and OT-II cells were isolated from total lymph nodes suspension by negative or positive selection respectively using CD8 or CD4 purification kit (Miltenyi Biotec). Effector CD4+ T cells used to visualize IL12R clustering were generated by stimulating naïve CD4+T cells for 7 days with OVA class-II pulsed DCs (1:10 ratio) in the presence of recombinant IL-2. Human dendritic cells were generated as follows: PBMCs were isolated from the blood of healthy donors using the Ficoll gradient. From this population, monocytes were positively selected via CD14 beads from Miltenyi and then cultured in Mo-DC Differentiation Medium (Miltenyi) for seven days to generate immature Mo-DC.

TLR agonists

CpG-B (1826) oligonucleotide was bought from InVivoGen, San Diego, CA, LPS (*Escherichia coli* O55:B5) from Enzo Life Sciences, Inc. Human Mo-DC cells were stimulated with a cocktail of poli:IC (10ug/ml) and LPS (1ug/ml) for 5 hrs (In VivoGen).

Molecular cloning

SNAREs sequences cloned into an EGFP plasmid (Syntaxin6 and VAMP3 in -C2 vector with the GFP at the N-terminal, VAMP7 in -N1 vector with the GFP at the C-terminal) were a kind gift of JL Stow (University of Maryland, Brisbane). For the in vitro transcription compatibility, constructs were further subcloned into pcDNA3.1+ vectors bringing a T7 promoter at N-terminus of the protein sequences. Then plasmids were linearized cutting a site at the C-terminus in the non-coding region. P35-GFP and p35-SV5 were obtained by subcloning a recombinant p35 murine sequence (pORF5-mp35 purchased from Invivogen) in a pcDNA3.1+ together with a GFP and a SV5 sequence respectively at the C-terminal. A nucleotidic linker was kept between p35 and GFP sequences. RFP-VAMP7 vector was provided by T.Galli.

Silencing of VAMP7

Non-targeting siRNA and ON-TARGET plus siRNA targeting VAMP7 was purchased from Thermo Scientific (Dharmacon RNAi Technology), and used as control siRNA. ON-TARGET plus siRNA targeting VAMP7 were purchased by Thermo Scientific (Dharmacon RNAi Technology) and used as specific VAMP7 siRNA. BM-DCs were collected at day 5 and transfected with 2μM siRNA using the Amaxa Nucleofector according to the manufacturer's instructions. The transfected cDCs were collected and seeded into 24-well plates containing complete IMDM plus Flt3L. Cells were collected 48 h after transfection for sequent experiments.

RNA isolation, RT-PCR and qRT-PCR

Total RNA was isolated by TRI Reagent Isolation Kit (Sigma-Aldrich), following manufacturer's instructions. DNA was removed from isolated RNA fraction by a treatment with RNAse-free DNAse I (Fermentas Inc, Massachusetts, USA). Total RNA was retro-transcribed to cDNA by Moloney murine leukaemia RT (M-MLV-RT) in the presence of random hexamers (IDT). qRT-PCR was based on SYBR Green Master Mix (applied Biosystems) technology.

Primers used for qRT-PCR were as follows: *IL-12 p35* (for. 5'-GGCATCCAGCAGCTCCTCTC-3', rev. 5'-ACCCTGGCCAAACTGAGGT G-3'); *IL-12 p40* (for. 5'-TGGTTTGCCATCGTTTTGCTG-3', rev. 5'-ACAGGTGAGGTTCA CTGTTTCT-3'); *IL-6* (for. 5′-GAGGATACCACTCCCAACAGA-3′, rev. 5′-AAGTGCATCATC GTTGTTCAT -3′); *Stx6* (for. 5'-GGAGAGGTACAGAAAGCAGTCA-3', rev. 5'-CCCTGAAG GAGCTCTGTCCAT-3'); *VAMP3* (for. 5'-TGCTGCCAAGTTGAAGAGAAAG-3', rev. 5'-TGA TCCCTATCGCCCACATC-3'); *VAMP7* (for. 5'-ACCTTCGCCCCTCAGTCAAT-3', rev. 5'-G GCAAGGATAGTGGTTCCCC-3'); *GAPDH (*for. 5'-AGAAGGTGGTGAAGCAGGCATC-3', rev. 5'-AGAAGGTGGAAGAGTGGGAGTTG-3').

Western blot

Cells were washed and lysed with NP-40 plus protease inhibitors. The lysates were centrifuged at 13,000*g* for 10 min at 4 °C. The supernatants were boiled for 10 min and separated by SDS-PAGE using 12% polyacrylamide gel. Antibodies used: rabbit anti-VAMP3 and mouse anti-VAMP7 (provided by A. Peden, University of Sheffield, Sheffield, UK), mouse anti-Syntaxin 6 (BD Transduction Laboratories), mouse anti-V5 (Invitrogen), mouse anti-γ–tubulin (Sigma), rabbit anti-phospho $IKK_{\alpha,\beta}$, rabbit anti-IKKα, rabbit anti-phospho p38, rabbit anti-p38 (Cell Signalling), mouse anti-phospho $ERK_{1,2}$, mouse anti-ERK (Cell Signalling), anti-GAPDH-HRP (Sigma), goat anti-rabbit-HRP (Molecular Probes) and goat α-mouse-HRP (Pierce).

Cytokine secretion

IL-12 (p70) and IL-12 (p40) were measured with Ready-Set-Go! ELISA kit purchased from eBioscience; IL-6 and TNF-α were quantified with ELISA MAX kit from Biolegend; IFN-γ was measured with ELISA antibodies from BD Pharmigen.

FACS antibodies

The following antibodies for FACS analysis were purchased from BioLegend: FITC- and PE-conjugated anti-CD11c, PE-Cy5- and PE-Cy7-conjugated anti-I-A^b, PE-Cy5-conjugated anti-CD86, anti-CD40 and anti-rat isotype, FITC-conjugated anti-B220, anti-CD3 and anti-Gr-1, APC-conjugated anti-CD8α. Stained cells were acquired with a FACSAria III flow cytometer and analyzed with FLOWJO software (version 4.5.4; Tree Star Inc.).

Synapse formation, confocal analysis and in vivo imaging

VAMP7 (mouse) and VAMP3 (rabbit) antibodies were generated in house by A. Peden (Gordon et al., 2010). In some staining a mouse anti VAMP7 antibody purchased by

Creative Diagnostic (clone 25G579) was used. Rabbit anti-Rab27a was a kind gift of M. Seabra (Imperial College London, UK). In two cases, due to incompatibility of antibodies-host species (in the co-labeling with Rab6 and Rab11 in Fig.1C), VAMP3 was detected by overexpressing a GFP-tagged version of the SNARE. The following commercial antibodies were used: mouse anti-Syntaxin 6 (BD Transduction Laboratories), goat anti-EEA1 (BD Pharmigen), mouse anti-CD63 (MBL), rabbit anti-Rab11, rabbit anti-Rab6 (Santa Cruz), rat anti-IL-12 p40/p70 (BD Pharmigen), rabbit anti-Rab7 (Cell Signaling Technology), Texas-red phalloidin (Molecular Probes), rabbit anti-SNAP23 (Covalab), mouse anti-V5 (Invitrogen), rabbit anti-GFP (Molecular Probes). p35 tagged with SV5 or GFP was used depending on the species of the antibodies to be used in colabeling. Human p35 was detected using goat anti p70 antibodies (R&D). All secondary Alexa-tagged antibodies were obtained from Molecular Probes. ProLong® Gold Antifade Mountant with DAPI (Molecular Probes) was used as mounting medium. In some time lapse movies T cells were stained with 0.5 μM Bodipy 630 (Molecular Probes). Imaging was performed at room temperature when analyzing fixed cells or at 37°C when performing time-lapse microscopy. Confocal images and time lapses were acquired with a LSM510 META Axiovert 200M reverse microscope with an Objective Plan-Apochromat 63x/1.4 Oil DIC and an Objective Plan-Apochromat 100x/1.4 Oil DIC (Carl Zeiss, Inc.). A camera AxioCam HRc and LSM 510 acquisition software (Carl Zeiss) were used. Video 2 was acquired with an Axio Observer.Z1 microscope with an Objective Plan-Apochromat 63x/1.4 Oil DIC and a camera AxioCam 506, using the ZEN lite software (Zeiss). Image Z-stack projection of slides, three-dimensional reconstruction and image analysis were performed using Volocity® 3D Image Analysis Software (Perkin Elmer) and ImageJ (National Institutes of Health). For the colocalization mask in Fig 2B and E the pixels containing both staining above the threshold were used to generate a white channel. The threshold for the

different signals was automatically generated by the software, considering the PSF of wavelenghts and optics used. For the colocalization of the 3 channels in Fig 2C, the colocalization mask was first created for channel 1 and 2 and then superimposed to channel 3. The mask (white channel/only) created was super-imposed on the merged images to highlight the colocalization points using ImageJ (Fiji Version 1.49q). Manders of A (p40 or p35) on SNAREs or endosomal markers and persons' coefficient were calculated using Volocity software (Perkin Elmer).

Electron microscopy

Sections were examined using a Tecnai Spirit electron microscope (FEI Company) equipped with an Olympus-SIS Quemesa digital camera.

Statistical analysis

All statistical analyses were performed using GraphPad Prism®. The error bars show the SEM between mean values of independent experiments (as in the functional analyses and SNARE polarization analyses) or between values of single cells analyzed (SNARE/p35 co-localization at the IS and p35 distance from the PM). P-values were obtained by student's, Wilcoxon paired non parametric or Mann-Whitney *t* tests.

Supplementary Figure Legends

Figure S1. Kinetic of IL-12 induction in bone marrow derived DCs (BM-DCs), related to Figure 1.

(A) DCs were stimulated with CpG-B or LPS. Data show the relative expression of mRNA for IL-12/p70 (p35) or IL-12/p40 (p40) at different times post-stimulation. Bars show mean±SEM of three independent experiments. ***:p<0,0001, Student's *t* test. *(B*) Kinetic of IL-12/p40 and p70 protein secretion upon CpG-B stimulation. Cell culture supernatants were harvested at the indicated time points and quantified by ELISA. Bars show the mean±SEM of two independent experiments.

Figure S2. Validation of p35-tagged constructs, related to Figure 2.

(A) p35-SV5 and mock-transfected DCs were stimulated or not with CpG-B for 7 hours. Cell lysates were immunoblotted with anti-SV5. g-tubulin was used as loading control. *(B)* DC overexpressing p35-SV5 were stimulated or not with CpG-B for 24 hours. Cell culture supernatants were immunoblotted with anti-SV5. *(C)* p35-SV5-transfected DCs were stimulated for 3 hours with CpG-B. Representative plane showing co-localization of SV5 (green) with the endogenous IL-12/p40 subunit (red). *(D,E)* p35-SV5- *(D)* and p35-GFP- *(E)* transfected DCs were stimulated with CpG-B for 8 hours. Cell culture supernatants were immunoprecipitated with anti-SV5 and immunoblotted with anti-p40. Mock-transfected DCs were used as negative control. *(F)* DCs were co-transfected with p35-SV5 and p35-GFP. Representative plane showing intracellular co-localization of the two proteins. (g). *(G)* Cells transfected with p35-SV5 were counter-labeled with antibodies against p40 and VAMP7. Single confocal planes showing single channels, color merges of channels 2 by 2 and a colocalization mask showing colocalization for the 3 channels are shown.

Figure S3. Phenotype of VAMP7 KO DCs, related to Figure 3.

A) BM-DCs lysates from WT and VAMP7KO mice were analyzed by immunoblotting for VAMP7 expression; γ-tubulin was used as loading control. (*B*) Representative dot plots of conventional DCs in the spleen. $B220^-/CD3^-/Gr\text{-}1^-$ splenocytes were labelled with antibodies against $CD11c^+$ and $I\text{-}A^b$ (left) or CD8a (right) *(C)* Frequency of total conventional DCs ($CD11c^+/I\text{-}Ab^{high}$, left panel) and CD8α positive and negative DCs (right panel) in the spleen of WT and VAMP7KO mice. Each data point represents one mouse. n.s., not significant, Student's t test.

Figure S4. TLR9 signaling and DCs maturation are not affected by VAMP7 deficiency, related to Figure 3.

(A) Immunoblot analysis of WT and VAMP7KO BM-DCs stimulated for 10, 30 or 60 min with CpG-B and probed with Abs against p-IKK, p-ERK and p-p38. GAPDH, total ERK and total p38 were used as loading controls. One representative experiment out of three with similar results is shown. *(B)* Expression of maturation markers in bone marrow (BM) and splenic DCs post CpG-B stimulation. WT, green histograms; VAMP7KO, blue histograms; dashed lines, isotype control. *(C)* Mean fluorescence intensities (MFI) for the indicated maturation marker. Data represent mean±SEM of four independent experiments. p values were determined by Student's t test. n.s., not significant. *(D)* mRNA expression of p35, p40 and IL-6 was measured by qPCR in BM-DCs. Data are mean±SEM of two independent experiments.

Figure S5. Selection of siRNA oligos for VAMP7 knockdown, related to Figure 3.

(A) Cells were transfected with a siRNA targeted against VAMP7 (siVAMP7) or with an unrelated siRNA (CTRL). The level of protein expression was assessed by Western Blot 48 hrs post transfection. On the right, densitometry quantification of VAMP7 expression, one representative of 4 blots with similar results. *(B)* Control siRNA does not affect protein secretion. DCs transfected with mock or with not targeting siRNAs (#1-6) were stimulated for 6 hours with CpG-B and the levels of secreted IL-12/p70 were measured. SiRNA#1 was chosen for the experiments. *(C)* DCs transfected with non-targeting siRNA#1 (siIrrelevant) and a VAMP7-targeting (siVAMP7) siRNA were labelled after 48 hours and stained with with Annexin V and 7-AAD to assess viability. *(D)* Control (black line) and VAMP7-silenced (green line) DCs were stimulated or not (n.s.) with CpG-B for 15 hours and expression of maturation markers CD86, I-A^b and CD40 evaluated by FACS. One representative of three experiments is shown. *(E)* Cytokines gene induction in control and VAMP7 knock-down cells was determined by RT-PCR at 3 hours post stimulation. Mean values±SEM of four independent experiments, n.s. not significant, Student's *t* test.

Figure S6. Synaptic clustering of p35 in human DCs, related to Figure 4.

(A) Representative confocal planes showing the distribution of endogenous p35 in human Mo-DCs in synapse with $CD4^+$ T cells. Mo-DCs were loaded with 1 mg/ml of superantigens (SEB and TSST1) and mixed with CD3/CD28 activated $CD4^+$ T cells. After 30 min of

interaction, cells were fixed, labeled with antibodies against human p35, VAMP7 or Rab27 and analyzed by confocal microscopy. Insets show a 3x magnification of the synaptic regions. All bars shown in micrographs correspond to 5mM.

Legends to supplemental Movies

Movie S1. Related to Figure 5A.

Time-lapse confocal microscopy of VAMP7-RFP expressing DC during formation of conjugates with naïve T cells stained with membrane dye Bodiby630 (blu). The movie shows that cluster of VAMP7 polarize toward the interaction plane upon a T cell contact. Interestingly, VAMP7 transiently moved away from the first T cell to travel towards a second T cell contacting the same DCs, and repolarized again to the first contacted T cell within few min. Frames were acquired every 30s for 50 min.

Movie S2. Related to Figure 5D.

Epifluorescence imaging of DC transfected with p35-GFP in synapse with a $CD4^+$ T cell. The position of the T cell is indicated by white dotted lines. Imaging was started short after contact formation. The time-lapse series show bright fast moving dots of p35 in the cell periphery and a highly motile enrichment of vesicles underneath the synaptic membrane. Frames were taken every 400 ms for 2 min 45 sec.

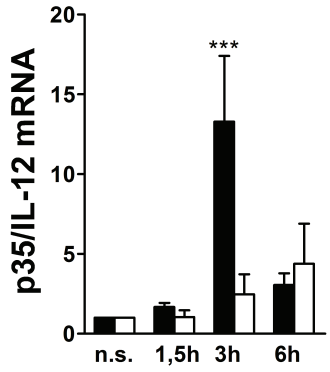
Movie S3. Related to Figure 5D.

High magnification of Movie S2 in the synaptic region shows $p35^+$tubules docking at the plasmamembrane.

Movie S4. Related to Figure 5E.

Time-lapse confocal microscopy of DCs co-transfected with p35-GFP and VAMP7-RFP, forming a conjugate with a T cell (blu). Frames were taken every 30s for 30 min. Video to Fig.5.

A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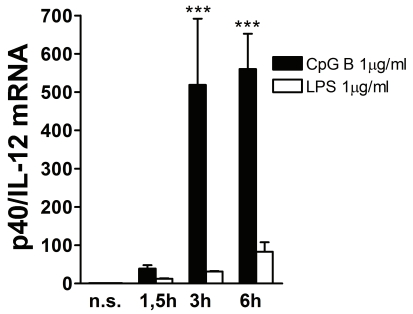
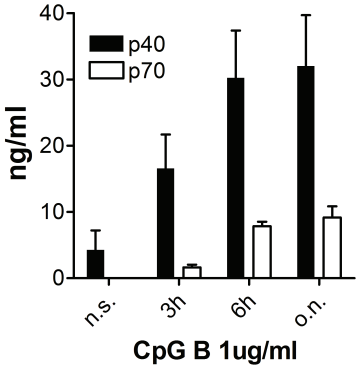
20
15
10
5
0
***
n.s.
1,5h
3h
6h


p40/IL-12 mRNA
700
600
500
400
300
200
100
0
***
***
n.s.
1,5h
3h
6h
CpG B 1μg/ml
LPS 1μg/ml

B


ng/ml
40
30
20
10
0
p40
p70
n.s.
3h
6h
o.n.
CpG B 1ug/ml

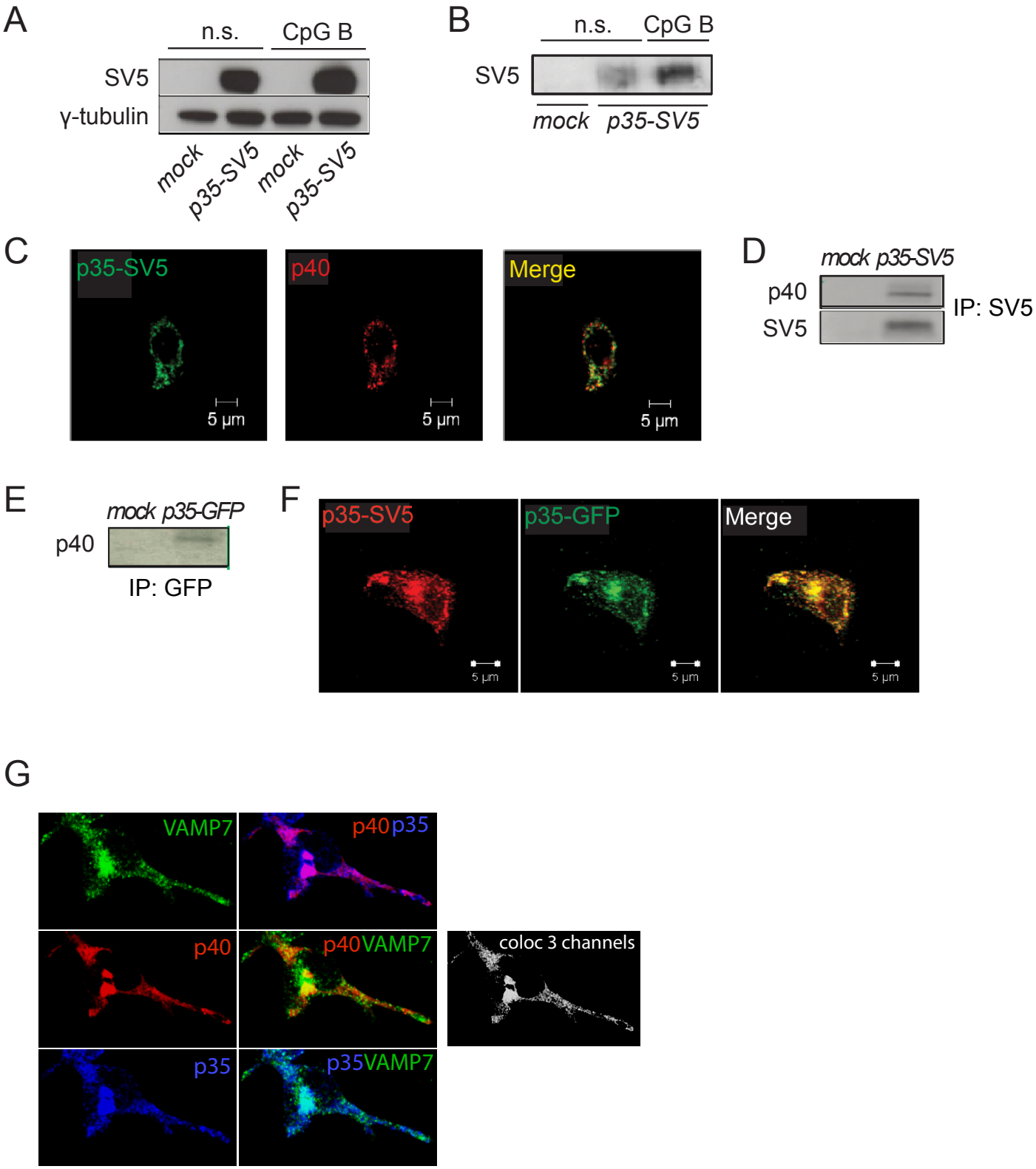
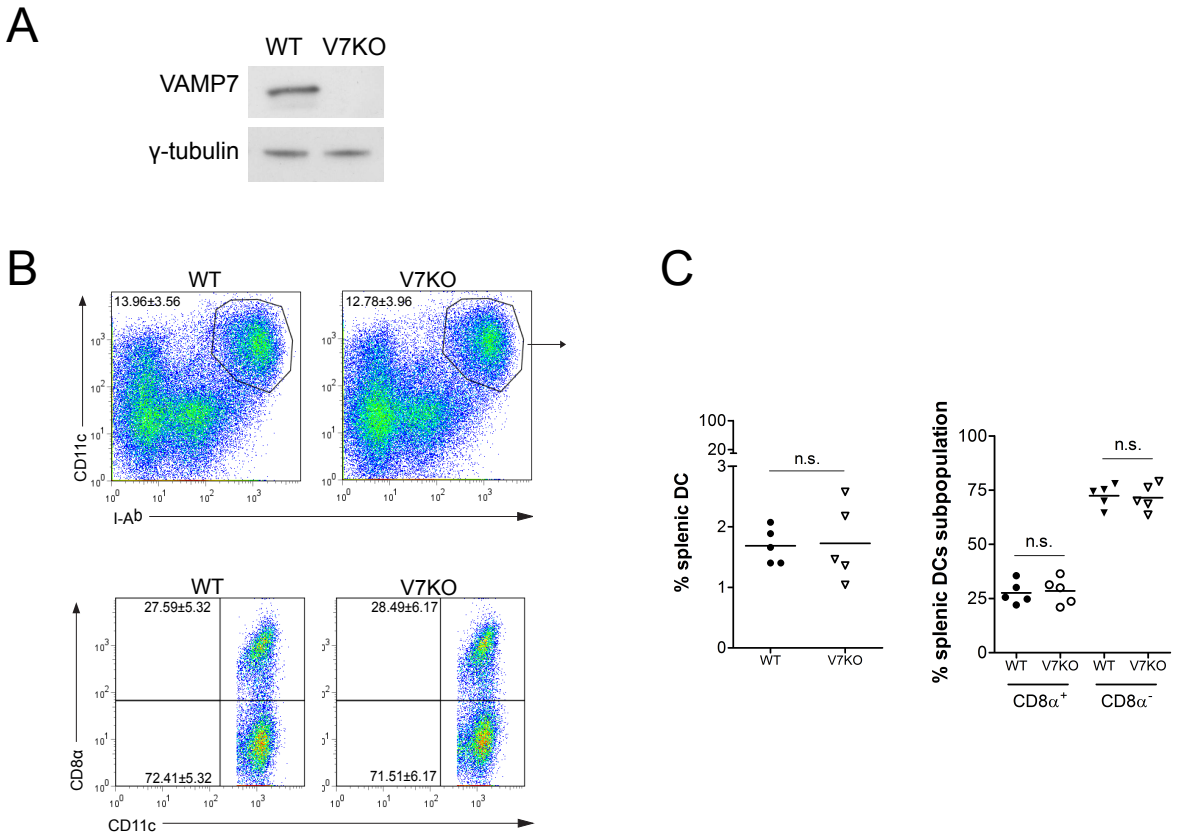

A
n.s.
CpG B
SV5
γ-tubulin
mock
p35-SV5
mock
p35-SV5
B
n.s.
CpG B
SV5
mock
p35-SV5
C
p35-SV5
p40
Merge
5 µm
5 µm
5 µm
D
mock p35-SV5
p40
SV5
IP: SV5
E
mock p35-GFP
p40
IP: GFP
F
p35-SV5
p35-GFP
Merge
5 µm
5 µm
5 µm
G
VAMP7
p40 p35
p40
p40 VAMP7
coloc 3 channels
p35
p35 VAMP7

Figure S2


A
WT
V7KO
VAMP7
γ-tubulin
B
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V7KO
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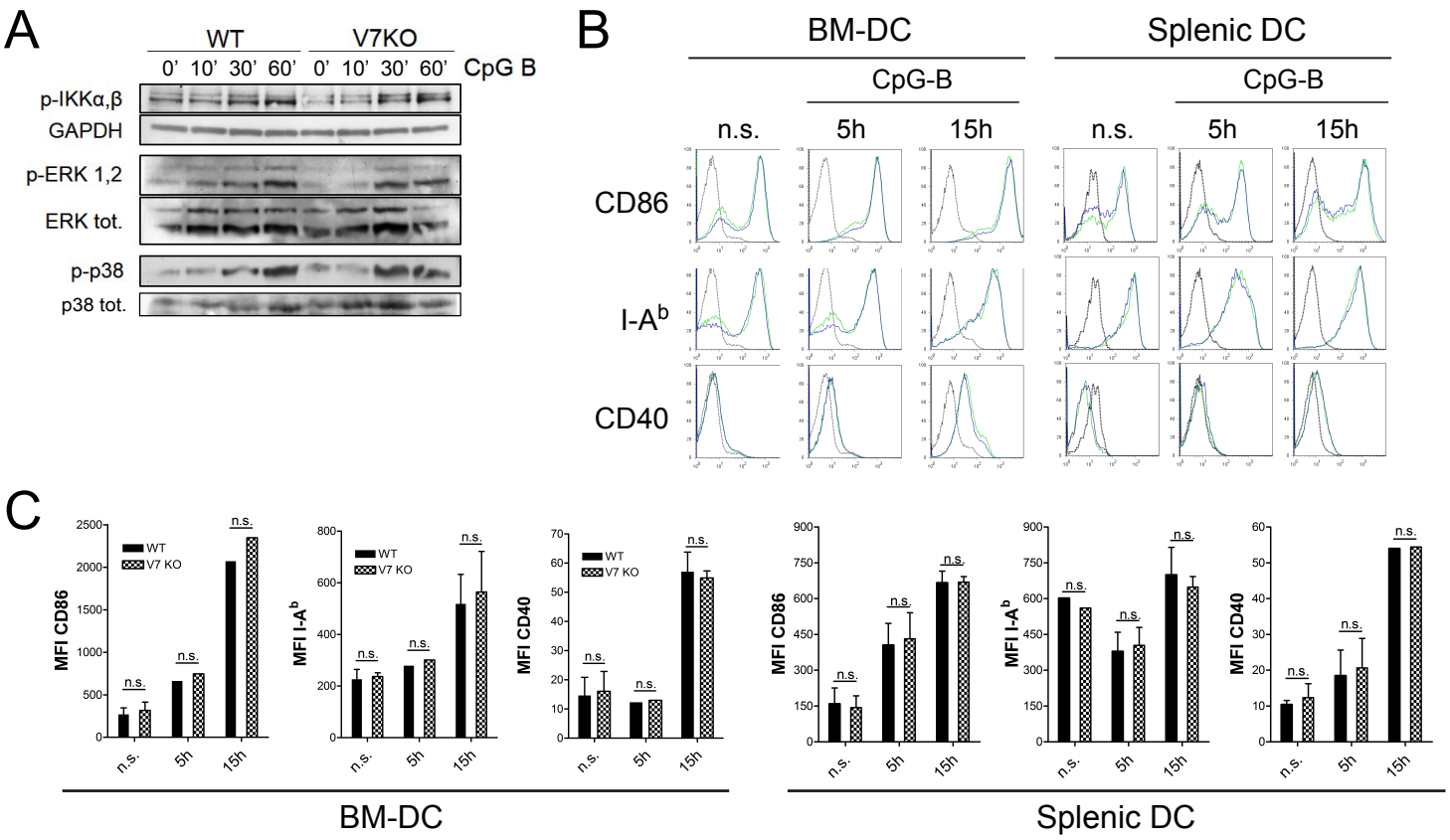
12.78±3.96
CD11c
I-Ab
WT
V7KO
27.59±5.32
28.49±6.17
72.41±5.32
71.51±6.17
CD8α
CD11c
C
% splenic DC
n.s.
WT
V7KO
% splenic DCs subpopulation
n.s.
n.s.
WT
V7KO
WT
V7KO
CD8α+
CD8α-

Figure S3


A
WT
V7KO
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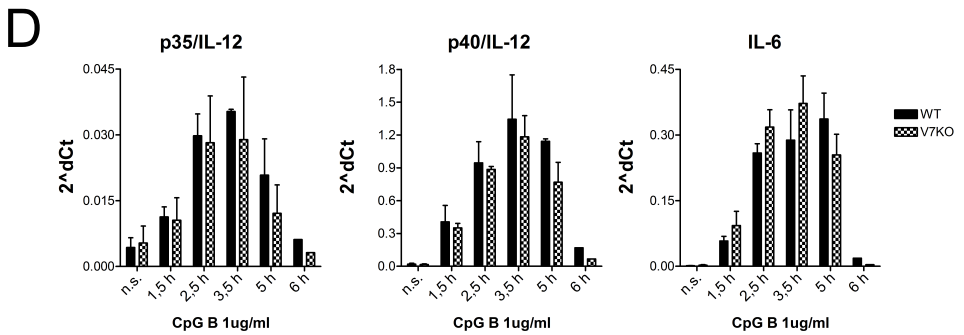
GAPDH
p-ERK 1,2
ERK tot.
p-p38
p38 tot.
B
BM-DC
Splenic DC
CpG-B
n.s.
5h
15h
CD86
I-Ab
CD40
C
MFI CD86
MFI I-Ab
MFI CD40
WT
V7 KO
n.s.
5h
15h
BM-DC
Splenic DC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p35/IL-12
p40/IL-12
IL-6
2^dCt
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V7KO
n.s.
1,5 h
2,5 h
3,5 h
5 h
6 h
CpG B 1ug/ml

Figure S4

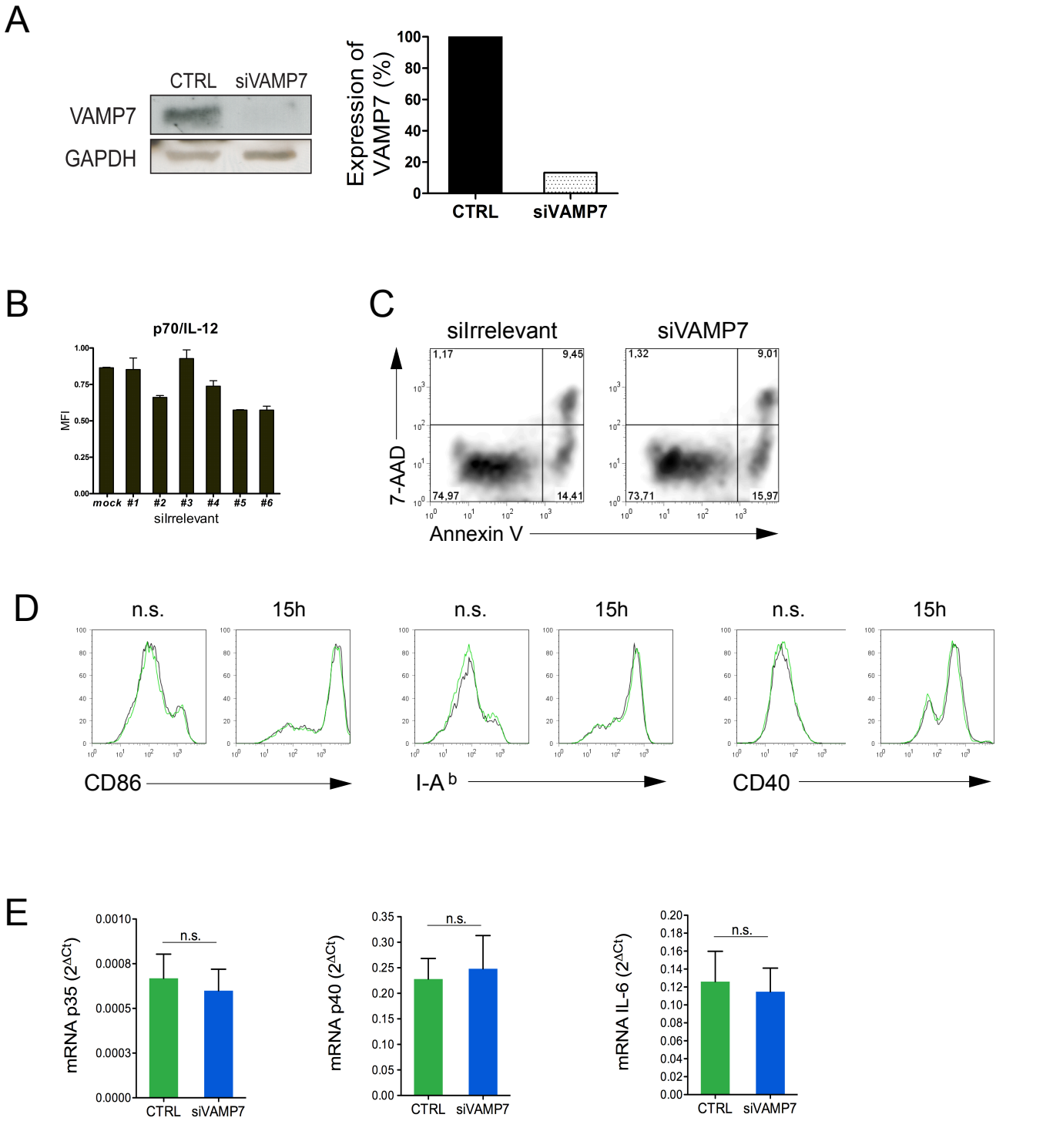
A
CTRL
siVAMP7
VAMP7
GAPDH
Expression of VAMP7 (%)
100
80
60
40
20
0
CTRL
siVAMP7
B
p70/IL-12
MFI
1.00
0.75
0.50
0.25
0.00
mock
#1
#2
#3
#4
#5
#6
silrrelevant
C
silrrelevant
siVAMP7
1,17
9,45
74,97
14,41
1,32
9,01
73,71
15,97
7-AAD
Annexin V
D
n.s.
15h
CD86
I-A b
CD40
E
mRNA p35 (2ΔCt)
0.0010
0.0008
0.0005
0.0003
0.0000
n.s.
CTRL
siVAMP7
mRNA p40 (2ΔCt)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
mRNA IL-6 (2ΔCt)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

Figure S5

A

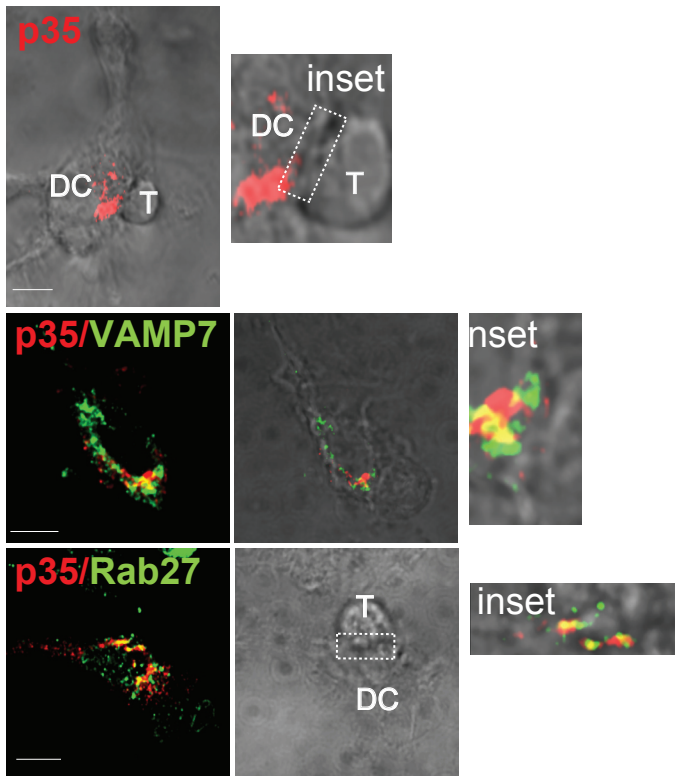

Figure S6