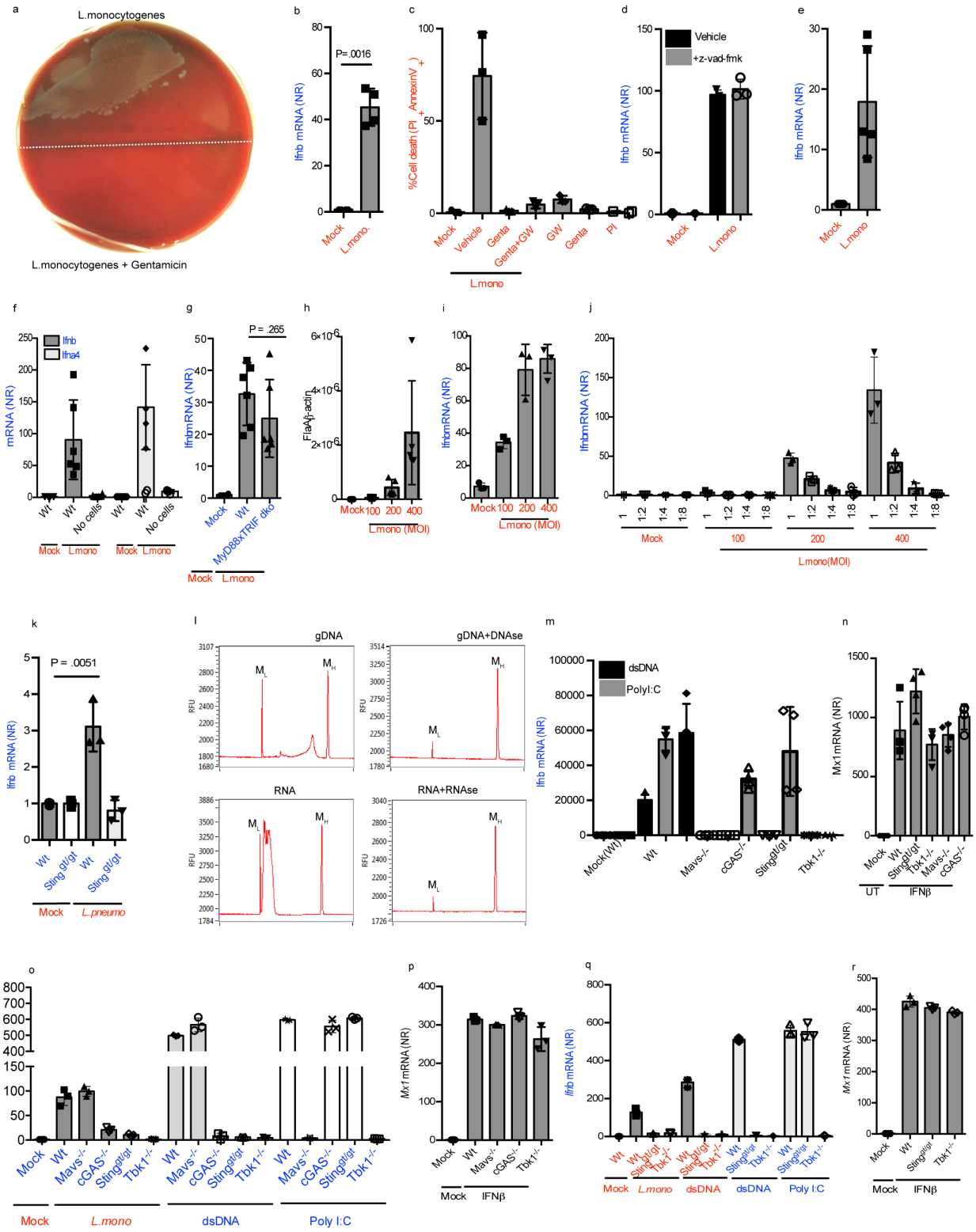
Intracellular bacteria engage a STING-TBK1-MVB12b pathway to enable paracrine cGAS-STING signaling

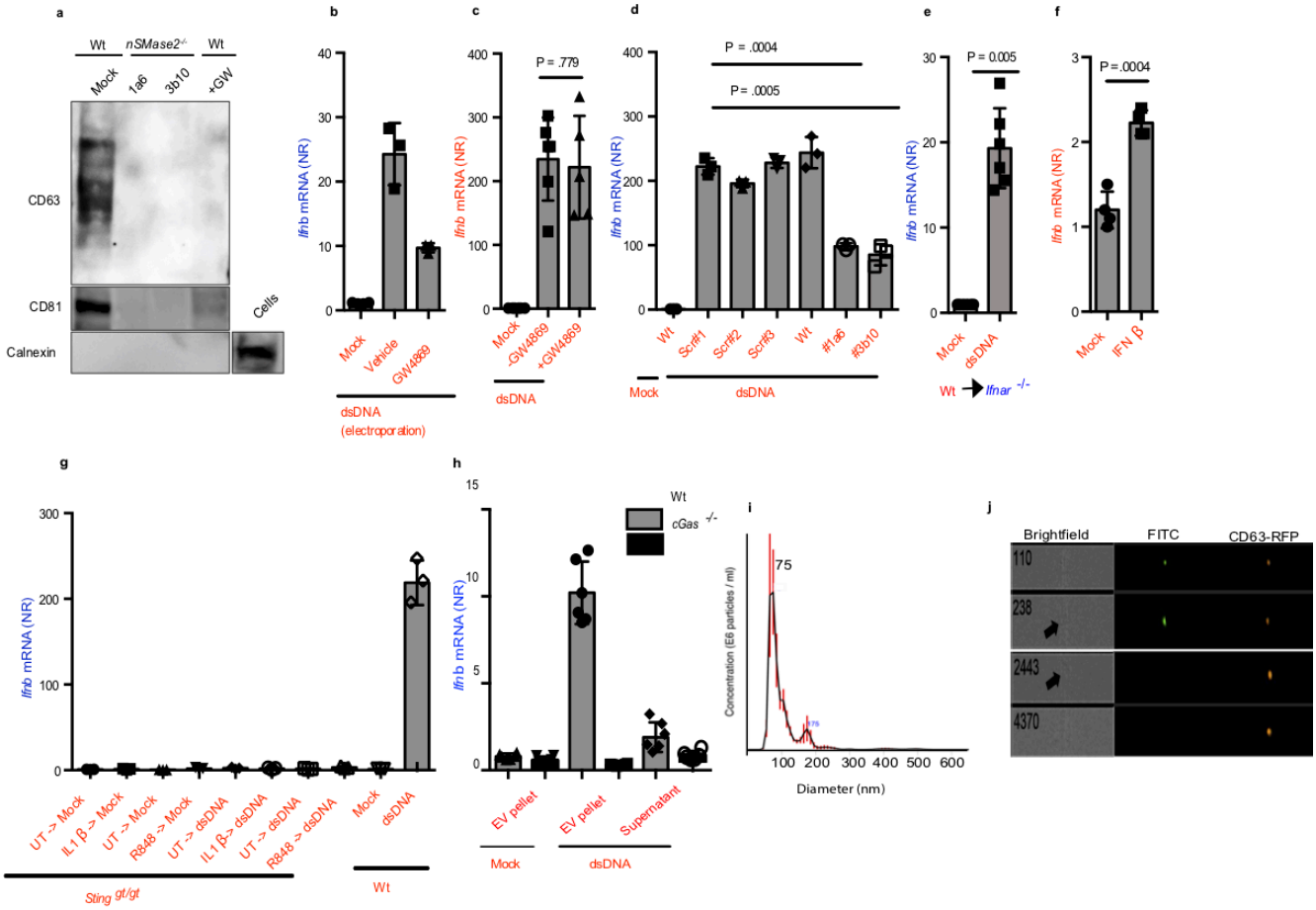
Supplemental Figure 1



For legend, see next page.

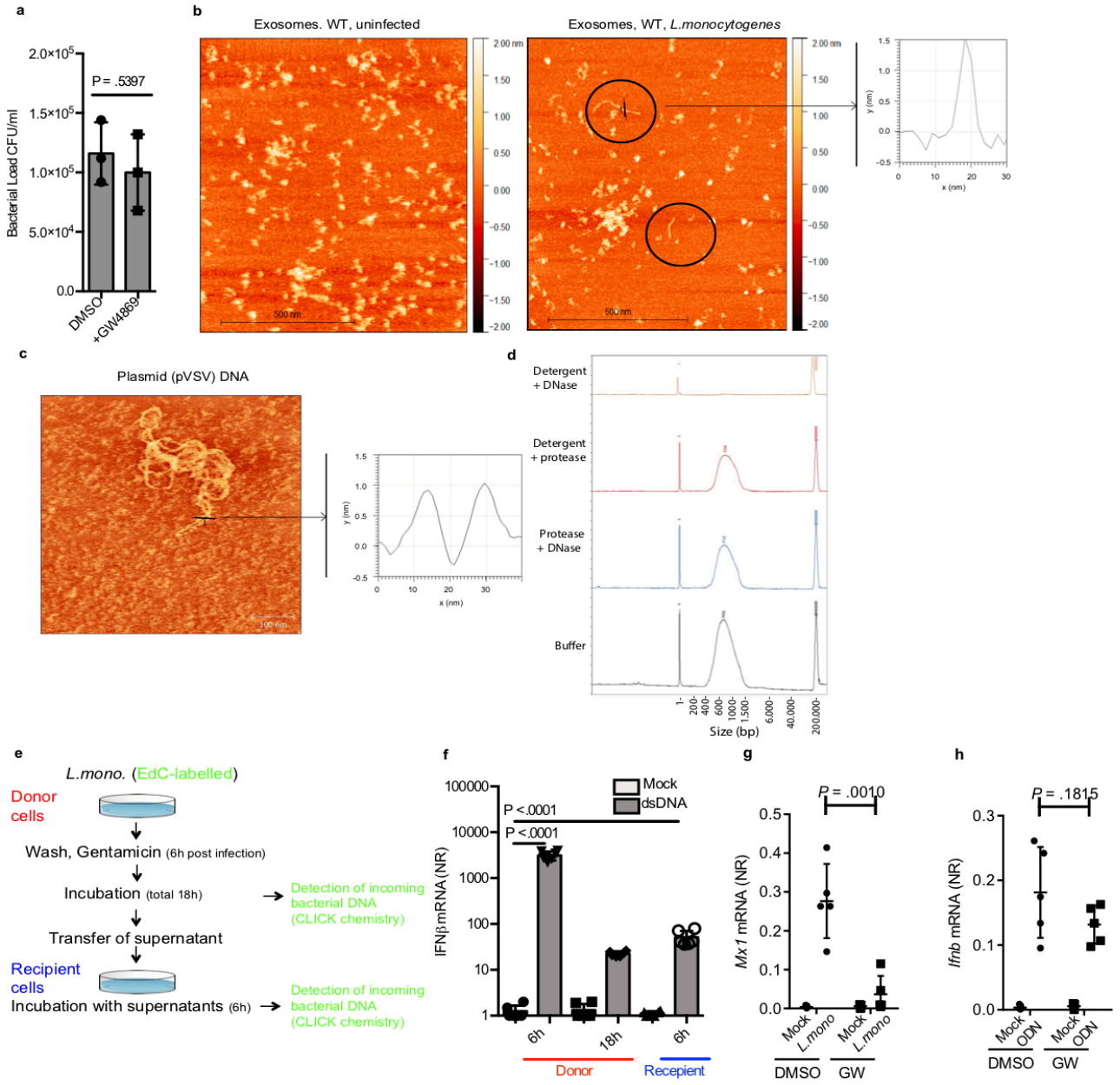
Supplementary Figure 1. Supernatants from cells infected with intracellular bacteria contain IFN-inducing potential. (**a**) Killing of extracellular bacteria with gentamicin. 50ul of cell supernatant of gentamicin-treated Listeria-infected cells were plated onto a blood agar plate, and this was compared to supernatants from infected cells not treated with gentamicin. (**b**) Induction of *Ifnb* in recipient MEFs stimulated for 6 h with supernatants from *L.monocytogenes*-infected Wt donor cells treated with chloramphenicol (50ug/ml) six hours post infection (n=4). (**c**) Cell death in MEFs infected with *L. monocytogenes* for 18 h in the presence or absence of gentamicin (50ug/ml) and GW4869 (10ug/ml) added 6 h post infection. The cells were stained with annexinV and PI (n=3). (**d**) Induction of *Ifnb* in recipient MEFs stimulated for 6 h with supernatants from *L.monocytogenes*-infected Wt donor cells treated with z-VAD-fmk (10 μM). The donor cells were treated with gentamicin (50ug/ml) six hours post infection (n=3). (**e**) Induction of *Ifnb* in recipient MEFs stimulated for 6 h with supernatants from *L.monocytogenes*-infected Wt donor cells treated with gentamicin (50ug/ml) 1 h post infection (n=5). (**f**) *Ifnb* and *Ifna4* induction in Wt recipient MEFs stimulated with supernatants from infected plates with or without cells (n=6). (**g**) Induction of *Ifnb* in Wt and *Myd88*$^{-/-}$*Trif*$^{-/-}$ recipient MEFs stimulated with supernatant from *L.monocytogenes*-infected Wt donor cells (n=6). (**h-j**) Wt MEFs were infected with *L. monocytogenes* at the indicated doses (gentamicin was added after 6 h). Total RNA and culture medium was isolated 6 h and 18 h post infection, respectively. (**h-i**) Levels of *FlaA* and *Ifnb* mRNA in donor cells (n=4;n=3). (**j**) *Ifnb* mRNA levels in recipient cells after incubation with different dilutions of culture medium from donor cells infected with different doses of *L. monocytogenes* (n=3). (**k**) Induction of *Ifnb* in Wt and *Sting*$^{gt/gt}$ recipient MEFs stimulated with supernatant from donor cells infected for 18 h with *L.pneumophila* (n=3). (**l**) Evaluation of the action of the DNase and RNase. M_L and M_H, low and high molecular marker, respectively. (**m-n**) Wt, *Sting*$^{gt/gt}$, *cGas, Mavs*$^{-/-}$, and *Tbk1*$^{-/-}$ MEFs were transfected with dsDNA (2 μg/ml) or polyI:C (0.2 μg/ml) or stimulated with IFNβ (25 U/ml) as indicated. Total RNA was isolated 6 h later and analyzed by RT-qPCR (n=3;n=4). (**o**) The experiments was performed as described in the figure legend to Figure 3a, and in addition a set of recipient cells were transfected with dsDNA or Poly I:C (2 μg/ml) for 6h. The data shown with black bars represent the data shown in Figure 3a, while the data represented with grey bars represent positive and negative controls (n=3). (**p**) Wt, *Sting*$^{gt/gt}$, *cGas, Mavs*$^{-/-}$, and *Tbk1*$^{-/-}$ MEFs treated with murine IFNbeta(25U) for 6hours were measured for Mx1 mRNA by RT-qPCR (n=3). (**q**) The experiments were performed as described in the figure legend to Figure 5a and 5b, and in addition a set of recipient cells were transfected with dsDNA or Poly I:C (2 μg/ml) for 6h (n=3). (**r**) Wt, *Sting*$^{gt/gt}$ and *Tbk1*$^{-/-}$ MEFs treated with murine IFNβ (25U) for 6hours were measured for Mx1 mRNA by RT-qPCR. The data shown to the right with black bars represent the data shown in Figure 5a and 5b, while the data represented with grey bars represent positive and negative controls. The presented data are representative of at least 3 independent experiments. The *Ifnb* and *Mx1* mRNA levels were normalized to *bactin* mRNA levels and shown as relative levels compared to mock (n=3). Data are shown as mean ± SD. P values were calculated using 2-tailed unpaired students t-test.

Supplemental Figure 2



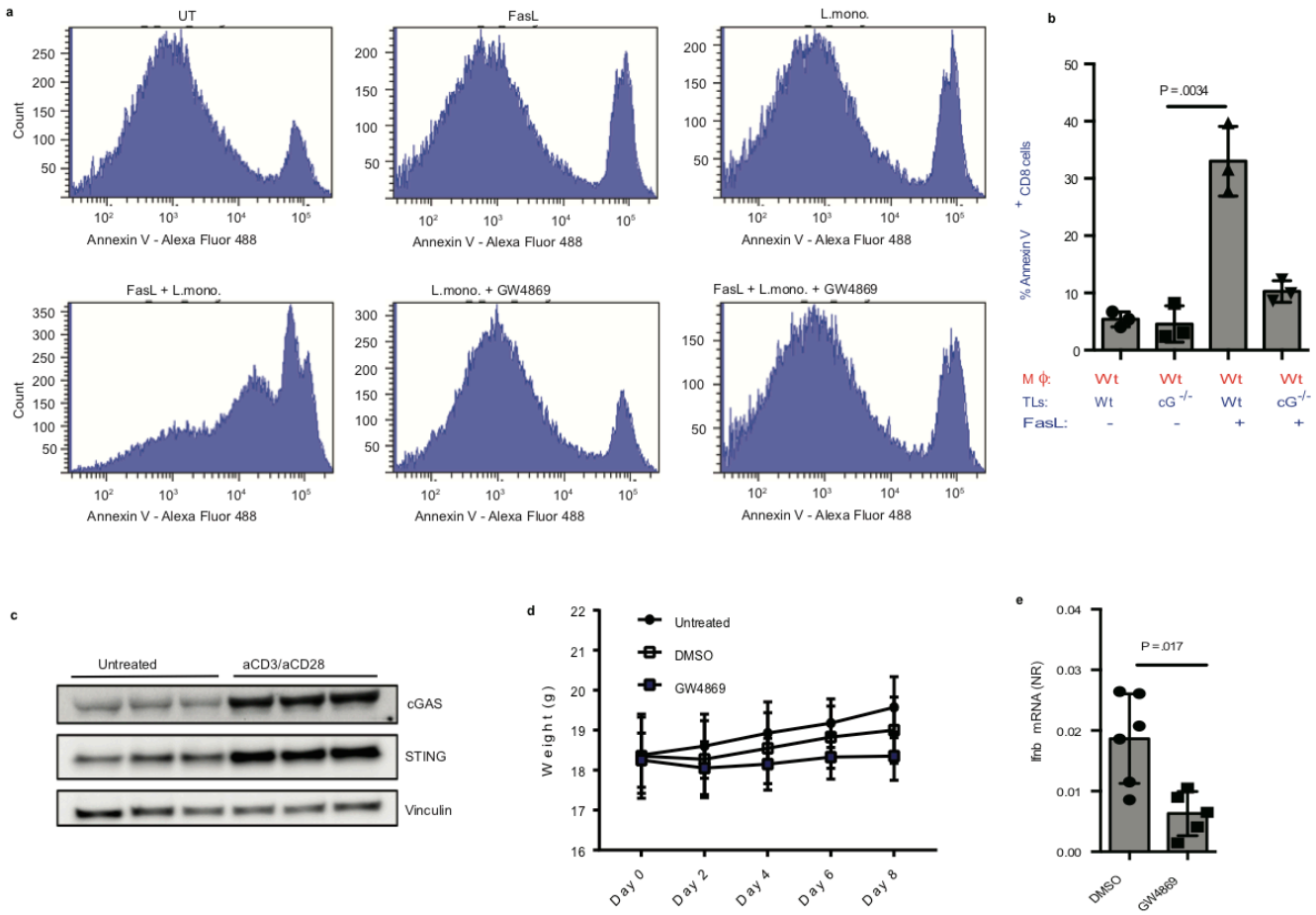
Supplementary Figure 2. Effect of exosome inhibition and type I IFN signaling on stimulation of bystander cells. (**a**) Supernatants from Wt (+/- GW4869, 10µM) and two clones of nSMase2-/- MEFs were subjected to EV isolation, and analyzed by Immunoblotting for exosomal markers CD81 and CD63, and the ER marker calnexin. A whole cell lysate was included as positive control for the Calnexin immunoblot. (**b**) *Ifnb* induction in BMMs stimulated for 6 h with supernatants isolated from MEFs (6x10^6) 18 h after electroporation with dsDNA (60µg) in the presence or absence of GW4869 (10µM) (n=3). (**c**) MEFs treated with or without GW4869 (10µM) were transfected with 1µg/ml dsDNA and mRNA was isolated after 6 hours and *Ifnb* was measured by real-time qPCR (n=5). (**d**) *Ifnb* mRNA levels in recipient Wt MEFs treated with supernatants from DNA-transfected (i) Wt MEFs, (ii) three monoclonal control clones with the same non-targeting sgRNA) or (iii) the two *nSmase2*-deficient clonal MEFs lines used in Figure 2 (n=3). (**e**) Induction of *Ifnb* in *Ifnar*$^{-/-}$ MEFs stimulated with supernatants from dsDNA-transfected Wt MEFs (n=6). (**f**) Induction of *Ifnb* mRNA in Wt MEFs stimulated for 6 h with IFNβ (25 U/ml) (n=3). (**g**) Induction of *Ifnb* in Wt MEFs treated for 6 h with supernatants from Sting$^{gt/gt}$ cells treated with IL1b (25 ng/ml) or R848 (1 µg/ml) as indicated (n=3). (**h**) Induction of *Ifnb* mRNA in Wt and cGas$^{-/-}$ MEFs MEFs stimulated with EVs from dsDNA- or mock transfected Wt MEFs, and with the remaining supernatant from the EV isolation procedure (n=6). (**i**) The supernatant from the EVs isolation procedure was subjected to Nanoparticle Tracking Analysis for evaluation of size distribution. The Red error bars indicate one standard error of the mean (+/-), while the black curve represents the mean of three independent measurements. (**j**) ImageStream analysis of EVs isolated from FITC-DNA-transfected CD63-RFP MEFs. Black arrows indicate EVs visibly identifiable by brightfield microscopy. The presented data are representative of at least 2 independent experiments. The *Ifnb* mRNA levels were normalized to *bactin* mRNA and shown as relative levels compared to mock. Data are shown as mean ± SD. P values were calculated using 2-tailed unpaired students t-test.

Supplemental Figure 3



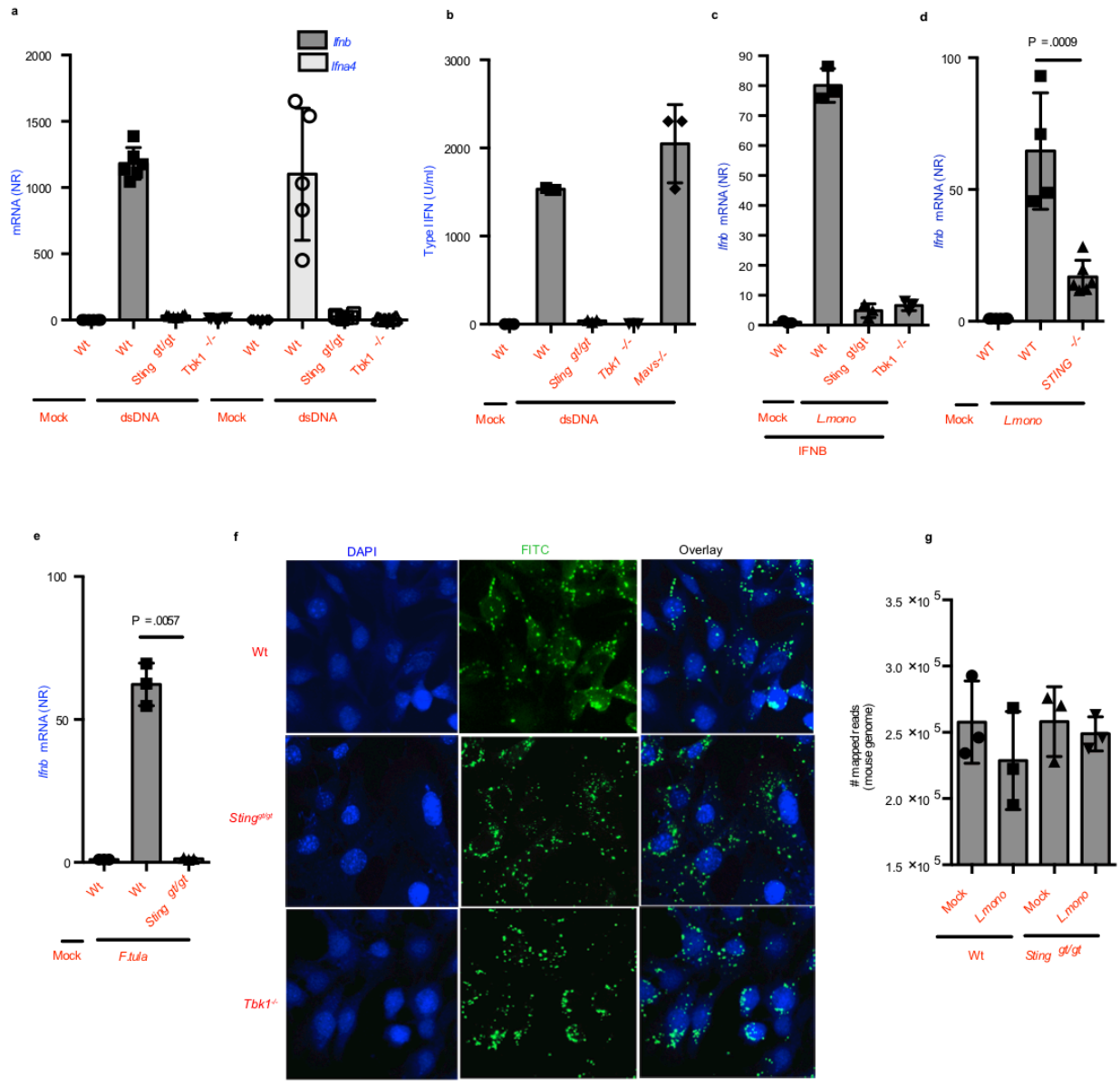
Supplementary Figure 3. Delivery of Listeria DNA to bystander cells. (**a**) Quantification of intracellular *L.monocytogenes* 18 h after infection of MEF at MOI 200 following treatment with gentamicin in the presence or absence of GW4869 (10 μM) (n=3). (**b**) Representative AFM pictures of DNA extractions from exosomes isolated from uninfected and *L.monocytogenes* (MOI 200) infected Wt MEFs. Circles are shown around extended structures with a width and height similar to DNA. Scale bar 500nm. The boxed part of the image is magnified in the image to the right for measurement of the height of exosomal DNA (~1.5 nm) (n=3). (**c**) Plasmid DNA was used as a positive control. Height of plasmid DNA was measured to be approximately 1nm. Scale bar 500nm (n=2). (**d**) Fragment analyzer electrophoresis of DNA extracted from DNAse-treated EVs from supernatants of MEFs infected with *L.monocytogenes*. Prior to electrophoresis, the material was treated with detergent (NP-40), Proteinase K and DNase as indicated. Markers, 1 bp (left) and 200,000 bp (right) (n=3). (**e**) MEFs were infected with EdC-labelled *L. monocytogeneses* (MOI 100), and treated as illustrated to the left. The localization of DNA from the bacteria used for infection was detected in the infected cells and in MEFs receiving supernatants from the infected cells by CLICK chemistry. Please note that this method does not detect DNA produced *de novo* in the infected cells. (**f**) Relative contribution of exosome-mediated *Ifnb* induction in DNA-transfected donor Wt MEFs 6h and 18h post transfection, and in recipient Wt MEFs 6h post transfer. (n=6). (**g, h**) *Mx1* and *Ifnb* mRNA levels in spleens of mice left untreated or infected with *L. monocytogenes* ($1x10^6$cfu) for 24 h or treated with ODN1826 (10 μg) for 6 h in the presence of GW4869 as indicated (GW, 0.125 μg per gram bodyweight) (n=5). The presented data are representative of at least 2 independent experiments. The *Ifnb* mRNA levels were normalized to *bactin* mRNA and shown as relative levels compared to mock. Data are shown as mean ± SD. P values were calculated using 2-tailed unpaired students t-test.

Supplemental Figure 4



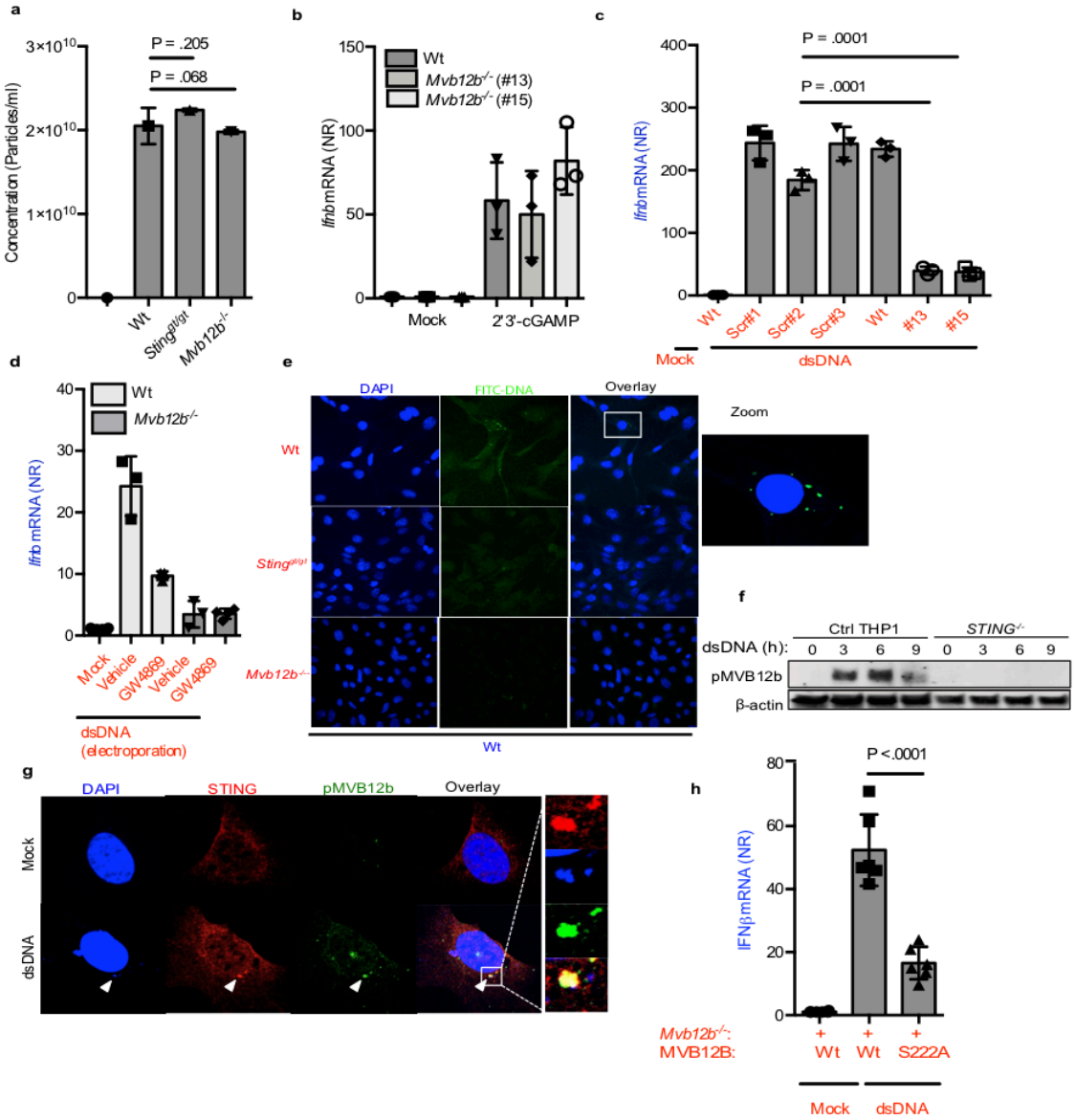
Supplementary Figure 4. EVs promote apoptosis in T cells during Listeria infection. (**a**) Representative flow cytometry plots for the groups in tested in Fig. 4b (n=2). (**b**) Apoptosis in Wt and *cGas*$^{-/-}$ (cG$^{-/-}$) Splenic T lymphocytes treated for 6 h with FasL (5 ng/ml) and supernatants from BMMs infected with *L. monocytogenes* (MOI 200) (n=3). (**c**) Immunoblot for cGAS, STING, and Vinculin in resting and αCD3/αCD28-activated splenic T cells. (**d**) Weight development in mice treated with GW4869 (0.03125 µg per gram bodyweight) (n=4). (**e**) *Ifnb* mRNA levels in spleens of mice infected with *L. monocytogenes* (1x10^{6}cfu) for 24 h in the presence of GW4869 (0.03125 mg per gram bodyweight) (n=5). The presented data are representative of at least 2 independent experiments. The *Ifnb* mRNA levels were normalized to *bactin* mRNA levels and shown as relative levels compared to mock. Data are shown as mean ± SD. P values were calculated using 2-tailed students t-test.

Supplemental Figure 5



Supplementary Figure 5. Sorting of DNA into exosomes is dependent on STING and TBK1. (**a-b**) Induction of *Ifnb* and *Ifna4* mRNA (n=5) (a) or type I IFN bioactivity (n=3) (b) in Wt MEFs stimulated with supernatants from dsDNA-transfected WT, *Sting*$^{gt/gt}$ or *Tbk1*$^{-/-}$ MEFs. (**c**) *Ifnb* mRNA levels in Wt MEFs treated with supernatants from Wt, *Sting*$^{gt/gt}$ or *Tbk1*$^{-/-}$ MEFs that had been treated with IFNb (25 U/ml) for 8 h followed by infection with i *L.monocytogenes* for 18 h (n=3). (**d**) Induction of *Ifnb* in BMDCs that received supernatant from DNA-transfected Wt and *STING*$^{-/-}$ THP1 cells (n=4). (**e**) *Ifnb* mRNA levels in Wt MEFs treated with supernatants from Wt or *Sting*$^{gt/gt}$ MEFs infected with *F.tularensis* (MOI 400) (n=3). (**f**) Transfection efficiencies of Wt, STING and TBK1 deficient MEFs using FITC-labelled DNA. Visualized by confocal microscopy (n=5). (**g**) Average number of mapped reads in EV DNA *versus* Mouse genome in EVs isolated from Mock- and *L.monocytogenes*-infeted Wt and *Sting*$^{gt/gt}$ MEFs (n=3). The presented data are representative of at least 2 independent experiments. The *Ifnb* mRNA levels were normalized to *bactin* mRNA and shown as relative levels compared to mock. Data are shown as mean ± SD. P values were calculated using 2-tailed unpaired students t-test.

Supplemental Figure 6



Supplementary Figure 6. Synthetic DNA activates a STING-TBK1-MVB12b pathway. (**a**) NTA analysis of EVs isolated from supernatants of *Wt, Sting* $^{gt/gt}$ and *Mvb12b*$^{-/-}$ MEFs (n=3). (**b**) Wt and *Mbv12b*$^{-/-}$ MEFs were stimulated with 2'3'-cGAMP for 6 h. Total RNA was isolated, and *Ifnb* mRNA levels were measured by RT-qPCR. Two clones of *Mbv12b*$^{-/-}$ MEFs were tested (#13 and #15) (n=3). (**c**) *Ifnb* mRNA levels in recipient Wt MEFs treated with supernatants from DNA-transfected (i) Wt MEFs, (ii) three monoclonal control clones with the same non-targeting sgRNA) or (iii) the two *Mvb12b*-deficient clonal MEFs lines used in Figure 6 (n=3). (**d**) Wt and *Mbv12b*$^{-/-}$ MEFs were electroporated with dsDNA (30μg) and incubated in the presence or absence of GW4869(10μM) and supernatants were used to stimulate Wt BMMs (n=3) (**e**) Wt MEFs treated for 2h with supernatants from Wt, *Sting*$^{gt/gt}$ or *Mvb12b*$^{-/-}$ MEFs stimulated with FITC-labelled DNA (1μg/ml, 18h) were subjected to confocal microscopy for visualization of FITC. Nuclei were stained with DAPI (n=5). (**f**) Control and *STING*$^{-/-}$ THP1 cells were treated with dsDNA (2μg/ml) for the indicated time period, and lysates were immunoblotted with anti-pMVB12b and anti-β-actin (n=3). (**g**) MEFs were stimulated with dsDNA (2μg/ml) for 3 h, fixed and stained for DNA (DAPI), STING, and pMVB12b. The cells were visualized by confocal microscopy. (**h**) *Mvb12b*$^{-/-}$ cells were reconstituted with Wt and S222A MVB12b, followed by stimulation with DNA (2μg/ml) for 18 h. Supernatants were transferred to Wt MEF recipient cells, and *Ifnb* mRNA levels in these cells 6 h post transfer was determined by RT-qPCR (n=5). The presented data are representative of at least 2 independent experiments. The *Ifnb* mRNA levels were normalized to *bactin* mRNA and shown as relative levels compared to mock. Data are shown as mean ± SD. P values were calculated using 2-tailed unpaired students t-test.

Supplemental Figure 7

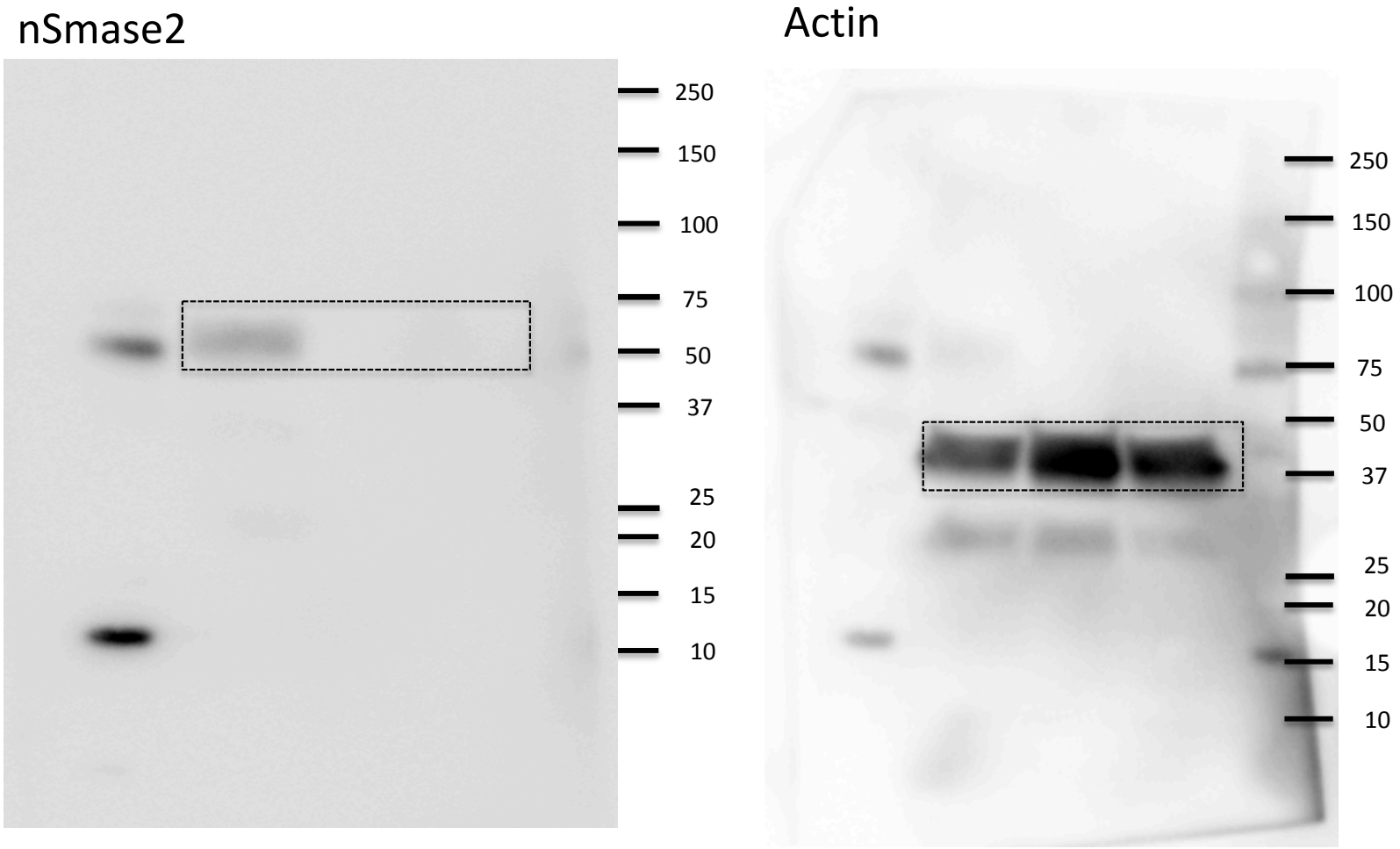


Figure2b

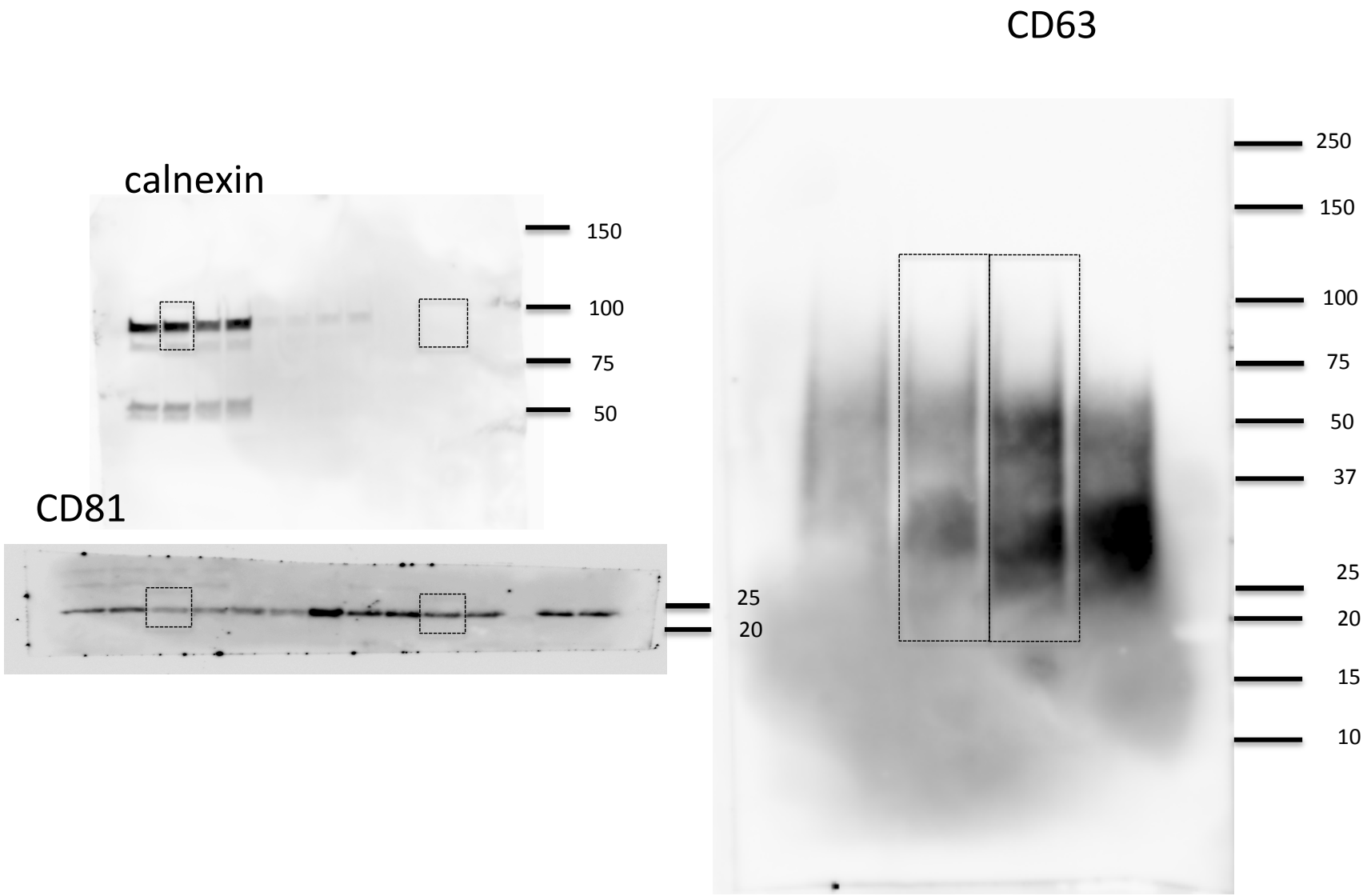

CD63
calnexin
150
100
75
50
CD81
25
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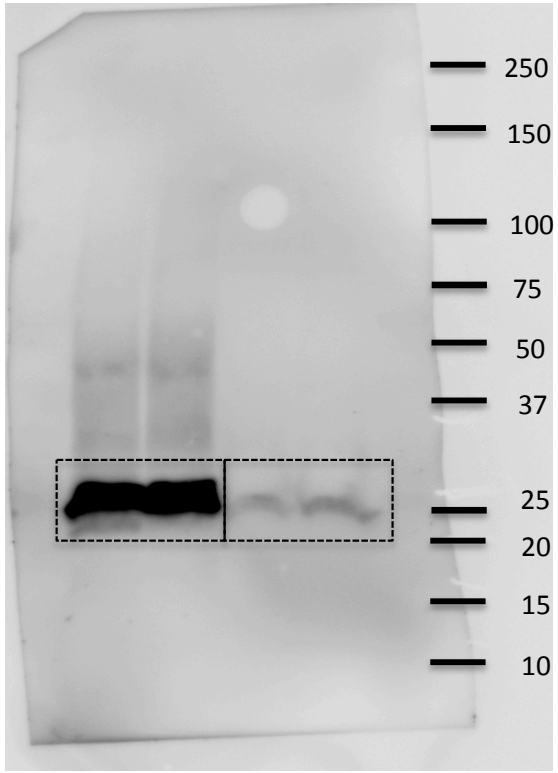
Figure 2g

CD81



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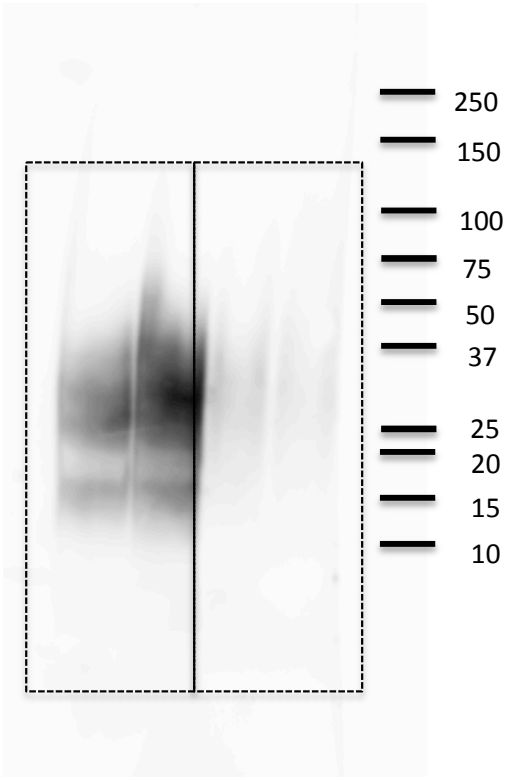


Calnexin

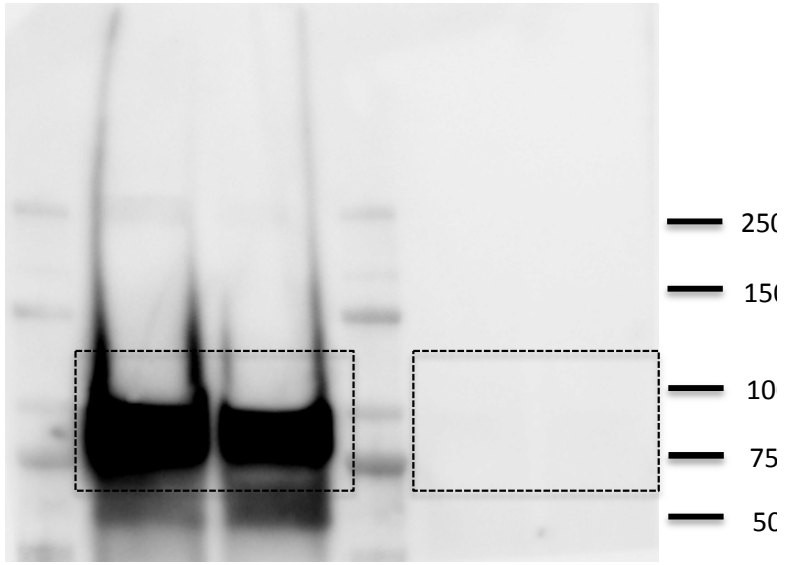
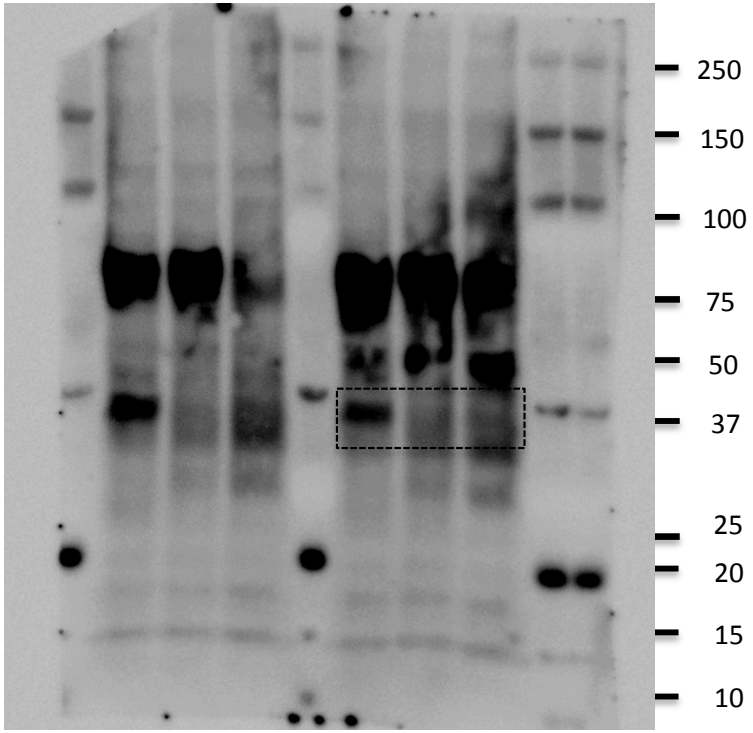


Figure5e

Mvb12b



Actin

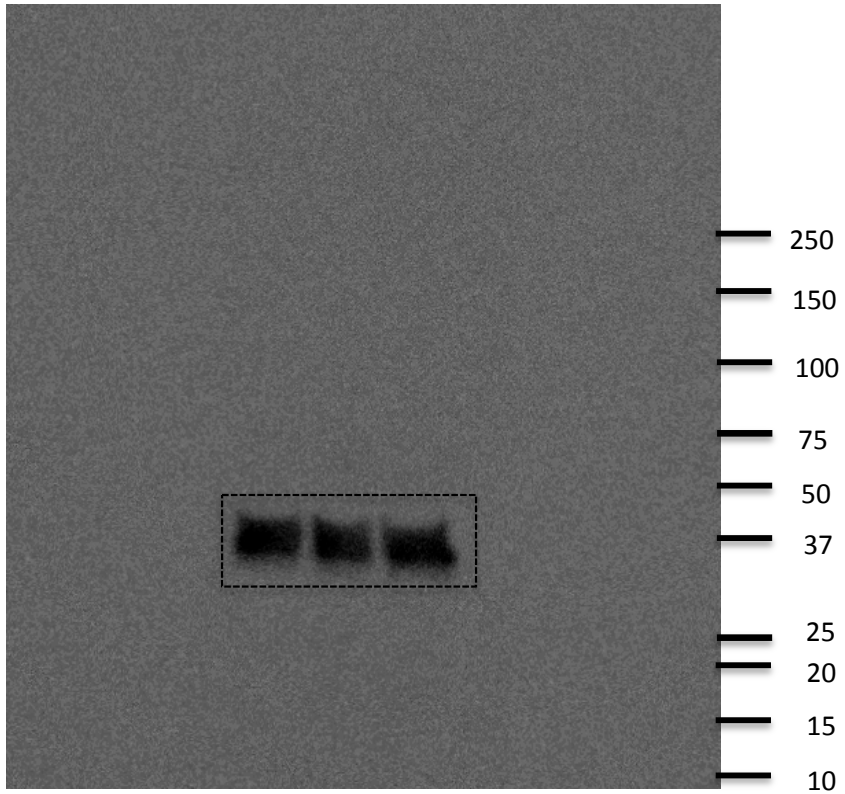


Figure6d

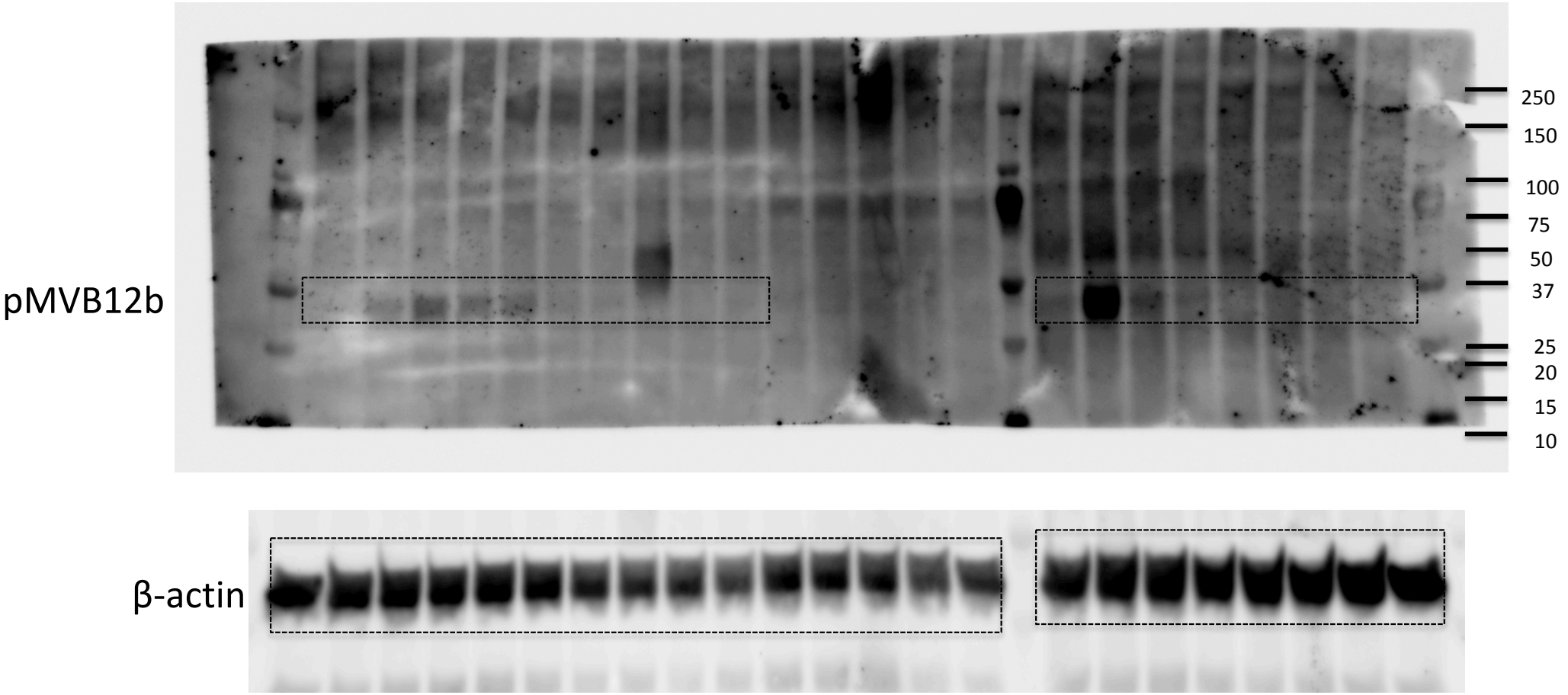
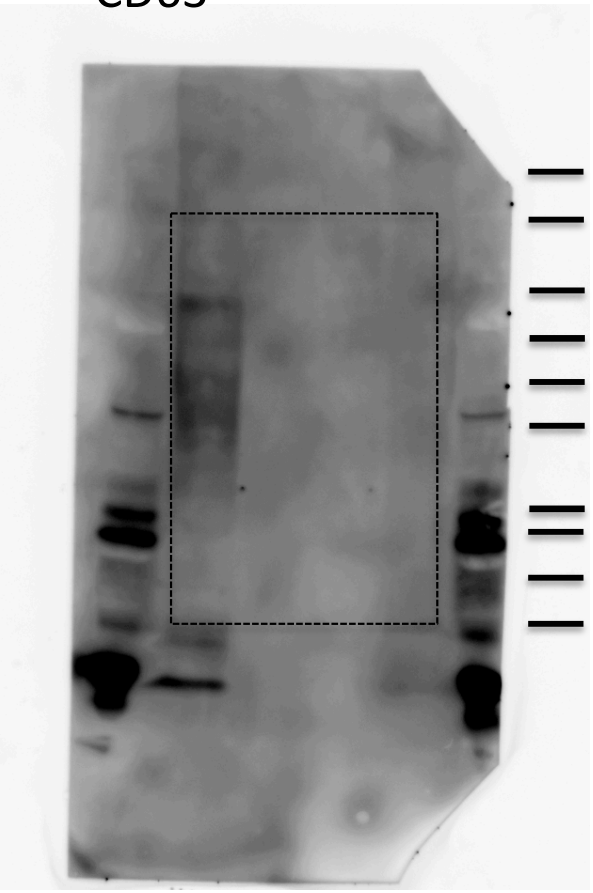
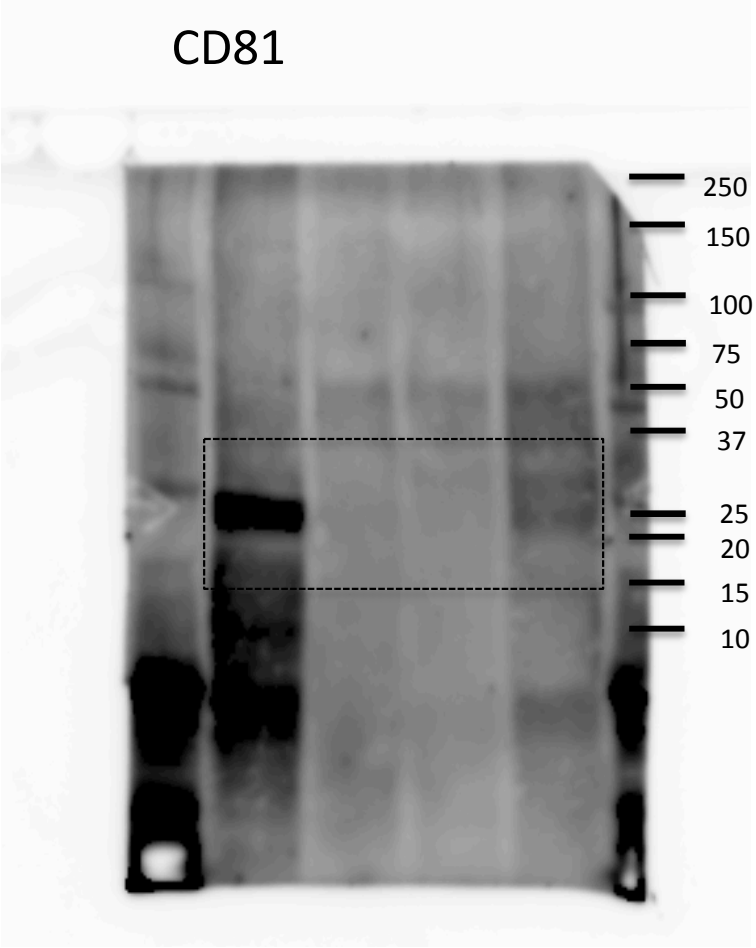


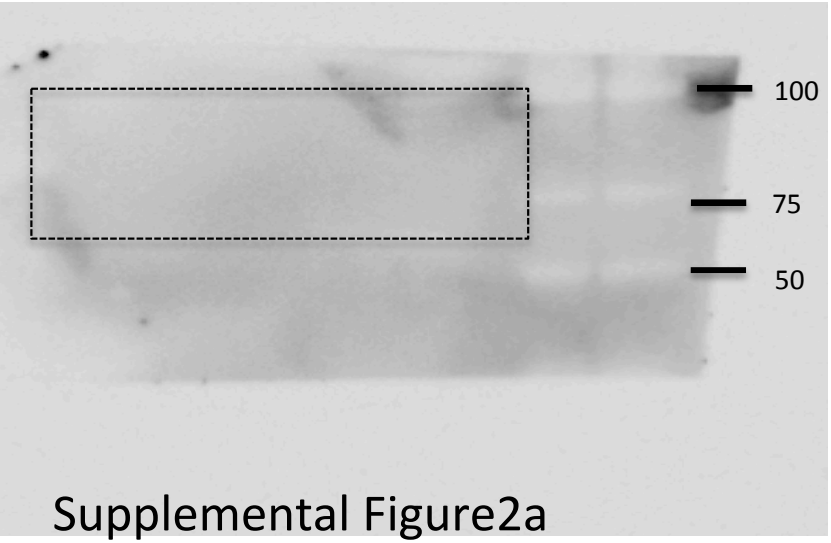
Figure6i

CD63



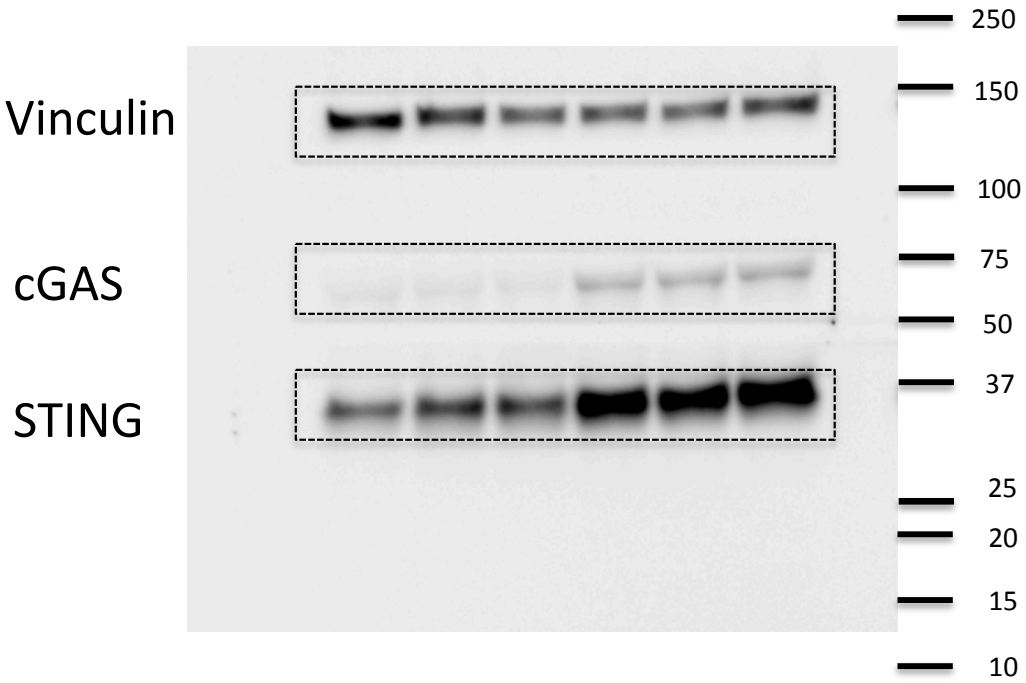
CD81



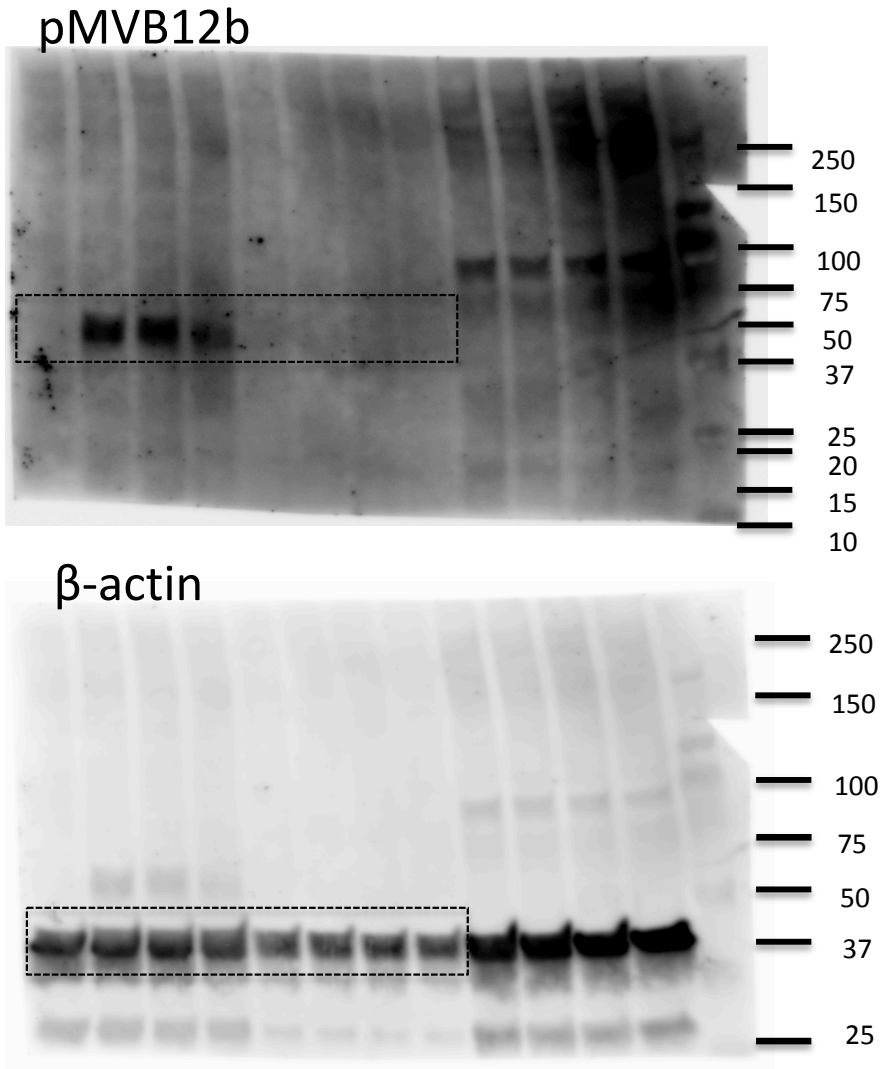


Calnexin

Supplemental Figure2a



Supplemental Figure4c



Supplemental Figure 6e

Supplementary Table 1: Distribution of mapped reads vs. Mouse genome

Mouse Chromosome	length	Wt Uninfected Sample 01	Wt Uninfected Sample 02	Wt Uninfected Sample 03	Wt *L.mono* Sample 04	Wt *L.mono* Sample 05	Wt *L.mono* Sample 06	*Sting*$^{gt/gt}$ Uninfected Sample 07	*Sting*$^{gt/gt}$ Uninfected Sample 08	*Sting*$^{gt/gt}$ Uninfected Sample 09	*Sting*$^{gt/gt}$ *L.mono* Sample10	*Sting*$^{gt/gt}$ *L.mono* Sample 11	*Sting*$^{gt/gt}$ *L.mono* Sample 12	Std.Dev.	Avg
1	195471971	6,84 %	6,91 %	6,80 %	6,76 %	7,27 %	7,12 %	6,98 %	7,17 %	7,16 %	7,19 %	7,17 %	7,20 %	0,18 %	7,05 %
2	182113224	11,15 %	10,98 %	11,14 %	11,46 %	9,44 %	10,22 %	10,90 %	9,32 %	9,49 %	8,96 %	8,92 %	9,16 %	0,97 %	10,10 %
3	160039680	5,22 %	5,34 %	5,18 %	5,08 %	5,57 %	5,46 %	5,53 %	5,56 %	5,64 %	5,61 %	5,49 %	5,63 %	0,19 %	5,44 %
4	156508116	5,59 %	5,63 %	5,64 %	5,60 %	5,80 %	5,75 %	5,54 %	5,78 %	5,79 %	5,77 %	5,81 %	5,73 %	0,09 %	5,70 %
5	151834684	5,55 %	5,51 %	5,64 %	5,61 %	5,72 %	5,61 %	5,38 %	5,52 %	5,47 %	5,66 %	5,77 %	5,60 %	0,11 %	5,59 %
6	149736546	5,48 %	5,52 %	5,41 %	5,40 %	5,75 %	5,67 %	5,57 %	5,70 %	5,74 %	5,71 %	5,66 %	5,67 %	0,13 %	5,61 %
7	145441459	5,36 %	5,24 %	5,38 %	5,42 %	5,47 %	5,39 %	5,00 %	5,32 %	5,22 %	5,32 %	5,41 %	5,27 %	0,12 %	5,32 %
8	129401213	4,73 %	4,71 %	4,78 %	4,77 %	4,88 %	4,82 %	4,64 %	4,81 %	4,77 %	4,83 %	4,89 %	4,82 %	0,07 %	4,79 %
9	124595110	7,34 %	7,18 %	7,37 %	7,71 %	6,28 %	6,72 %	7,11 %	6,25 %	6,29 %	6,03 %	6,03 %	6,13 %	0,60 %	6,70 %
10	130694993	4,57 %	4,62 %	4,61 %	4,37 %	4,56 %	4,53 %	4,59 %	4,66 %	4,65 %	4,81 %	4,77 %	4,72 %	0,12 %	4,62 %
11	122082543	4,93 %	4,81 %	5,15 %	4,83 %	4,73 %	4,73 %	4,59 %	4,84 %	4,75 %	4,99 %	5,21 %	4,89 %	0,18 %	4,87 %
12	120129022	4,49 %	4,51 %	4,43 %	4,53 %	4,60 %	4,60 %	4,48 %	4,53 %	4,54 %	4,47 %	4,48 %	4,50 %	0,05 %	4,51 %
13	120421639	4,24 %	4,32 %	4,29 %	4,13 %	4,33 %	4,29 %	4,33 %	4,45 %	4,42 %	4,53 %	4,54 %	4,47 %	0,12 %	4,36 %
14	124902244	4,55 %	4,57 %	4,50 %	4,59 %	4,64 %	4,66 %	4,53 %	4,57 %	4,57 %	4,44 %	4,37 %	4,49 %	0,08 %	4,54 %
15	104043685	3,68 %	3,69 %	3,74 %	3,67 %	3,82 %	3,73 %	3,63 %	3,77 %	3,75 %	3,83 %	3,87 %	3,81 %	0,07 %	3,75 %
16	98207768	3,38 %	3,41 %	3,34 %	3,32 %	3,56 %	3,48 %	3,46 %	3,53 %	3,53 %	3,55 %	3,50 %	3,51 %	0,08 %	3,46 %
17	94987271	3,62 %	3,56 %	3,68 %	3,65 %	3,67 %	3,58 %	3,47 %	3,60 %	3,57 %	3,67 %	3,78 %	3,61 %	0,08 %	3,62 %
18	90702639	3,17 %	3,20 %	3,14 %	3,12 %	3,32 %	3,25 %	3,23 %	3,31 %	3,28 %	3,33 %	3,31 %	3,33 %	0,08 %	3,25 %
19	61431566	2,26 %	2,23 %	2,28 %	2,20 %	2,24 %	2,19 %	2,24 %	2,30 %	2,24 %	2,42 %	2,45 %	2,35 %	0,08 %	2,28 %
X	171031299	3,76 %	3,98 %	3,42 %	3,69 %	4,26 %	4,11 %	4,78 %	4,97 %	5,11 %	4,87 %	4,58 %	5,09 %	0,59 %	4,39 %
Y	91744698	0,06 %	0,06 %	0,06 %	0,06 %	0,07 %	0,07 %	0,02 %	0,02 %	0,02 %	0,01 %	0,02 %	0,02 %	0,02 %	0,04 %
MT	16299	0,00 %	0,00 %	0,00 %	0,01 %	0,01 %	0,02 %	0,00 %	0,00 %	0,00 %	0,00 %	0,00 %	0,00 %	0,00 %	0,00 %