

SUPPLEMENTARY MATERIALS

ESCRT-dependent STING degradation inhibits steady-state and cGAMP-induced signalling

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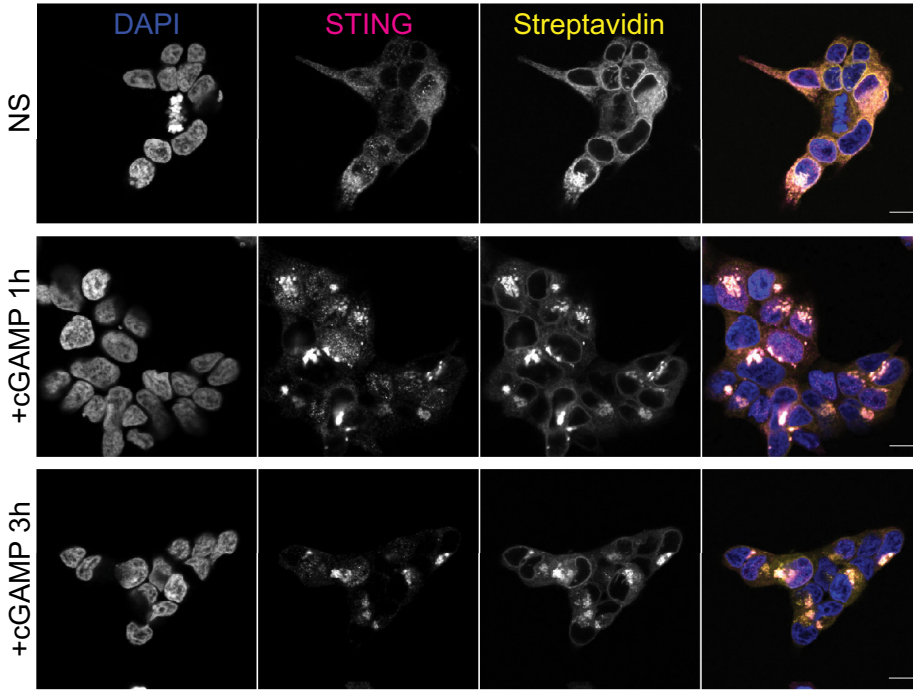
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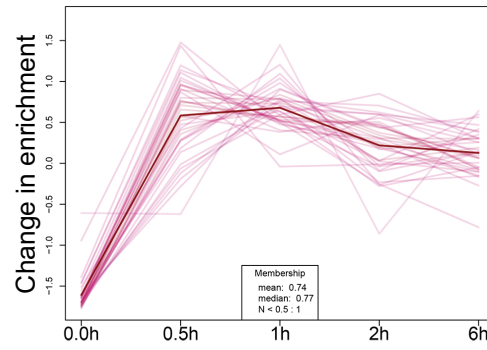
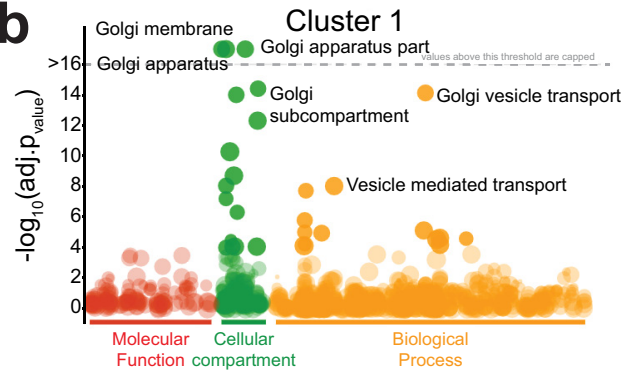
*Correspondence: nhacohen@mgh.harvard.edu (N.H.)

Figure S1

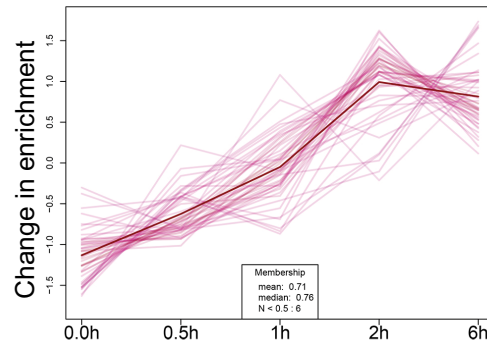
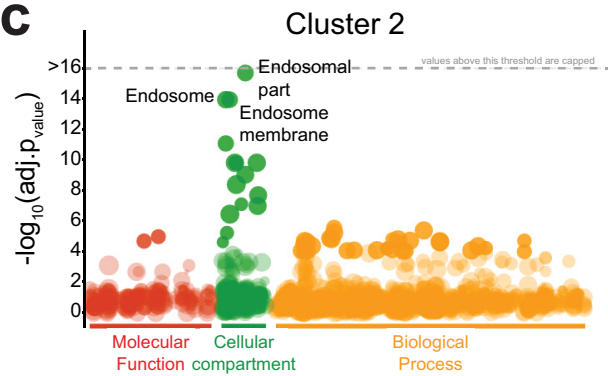
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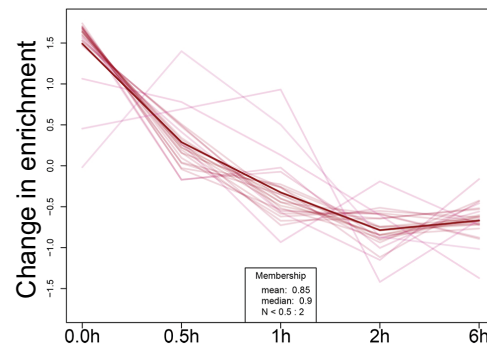
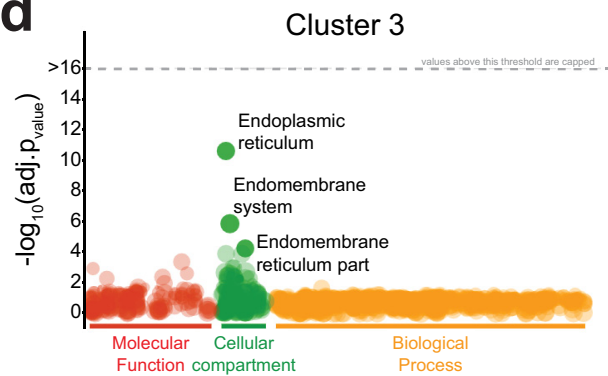
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c



d



e

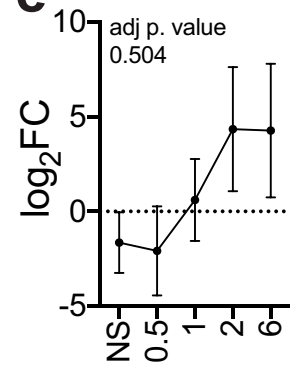


Figure S1. Related to Figure 1. a) Immunofluorescence of DAPI (blue) STING-TurboID (magenta) and Streptavidin-Cy5 (yellow) at the indicated time-points in 293T STING-TurboID cells post cGAMP stimulation or non-stimulated (NS). One representative field of $n \geq 3$ fields. Scale bar is 10 μ m. **b)** Cluster 1 from cluster analysis. Molecular Function, Cellular compartment and Biological process GO terms enrichment (left) and plot of change in enrichment of statistically significant proteins at the indicated time-points. **c)** Same as in b) for Cluster 2. **d)** Same as in b) for Cluster 3. **e)** log₂FC of p62 at the indicated time-points. Line represents average and error bars SD.

Figure S2

Gene ontology

Reactome

direction
of enrichment

● down
● up

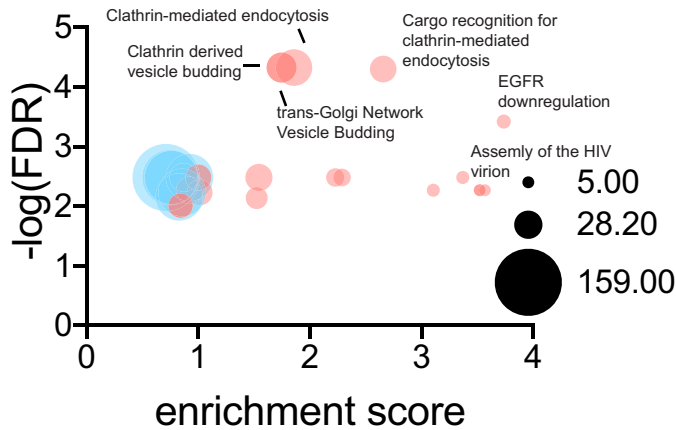
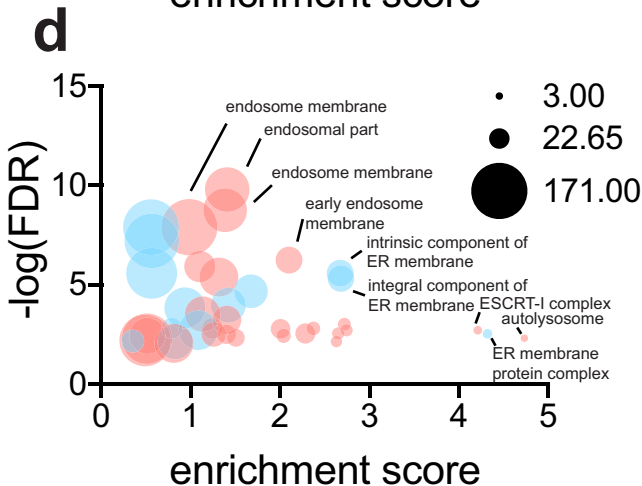
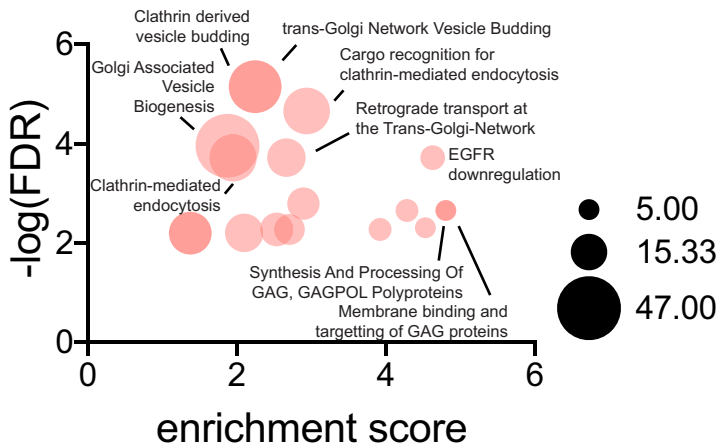
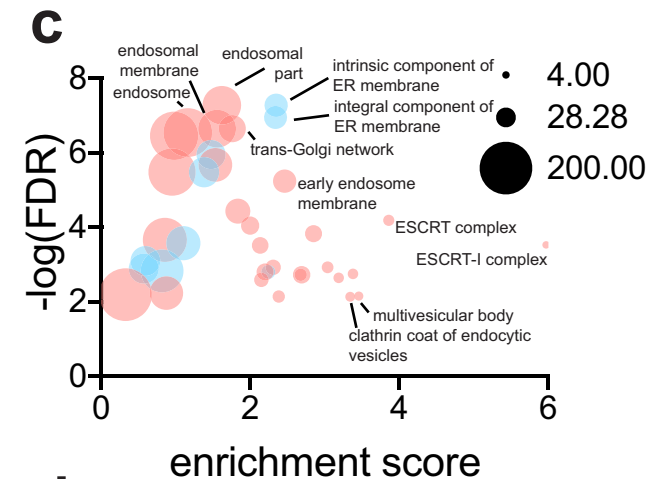
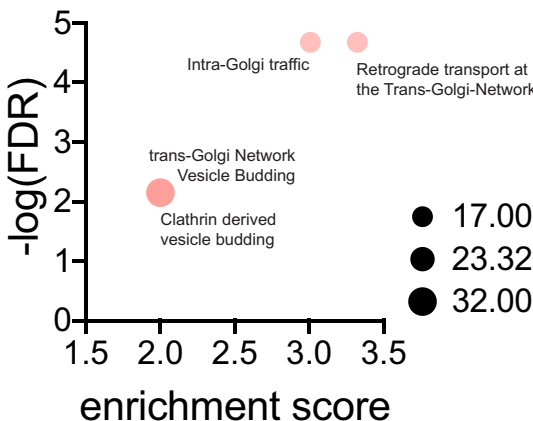
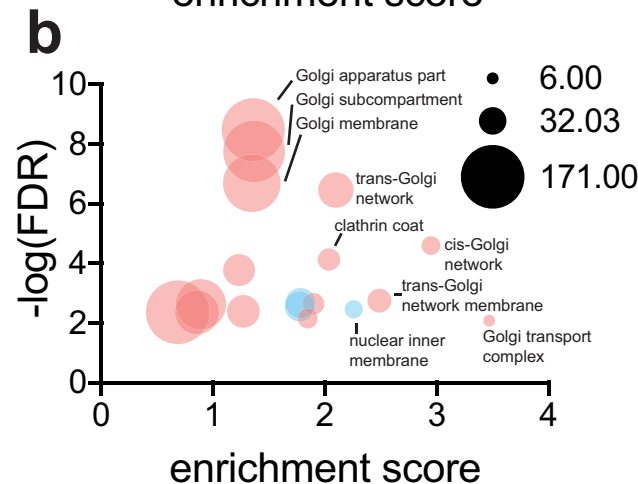
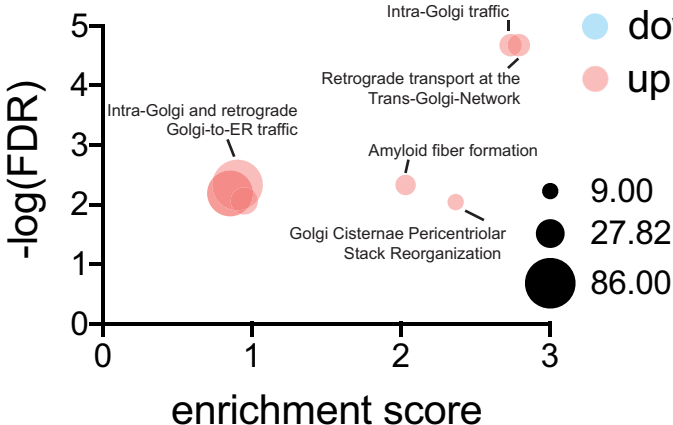
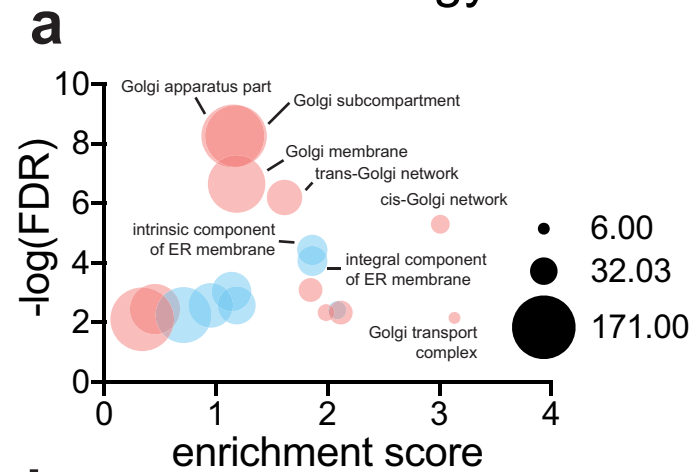
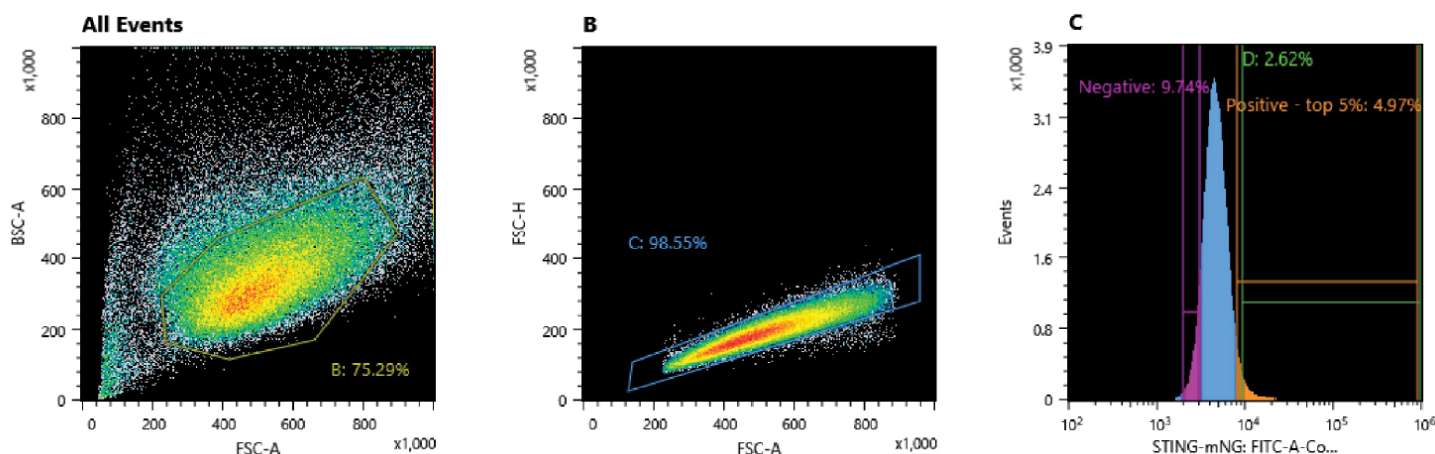


Figure S2. Related to Figure 1. Gene Ontology (GO) (left panels) and Reactome (right panels) enrichments calculated through STRING for the full TurboID dataset at **a)** 30 minutes, **b)** 1 hour, **c)** 2 hours and **d)** 6 hours post cGAMP stimulation. Terms that are positively enriched are in red, terms that are negatively enriched are in blue. Size of bubbles represent the number of genes mapped in each category. FDR: False Discovery Rate.

Figure S3

a



b

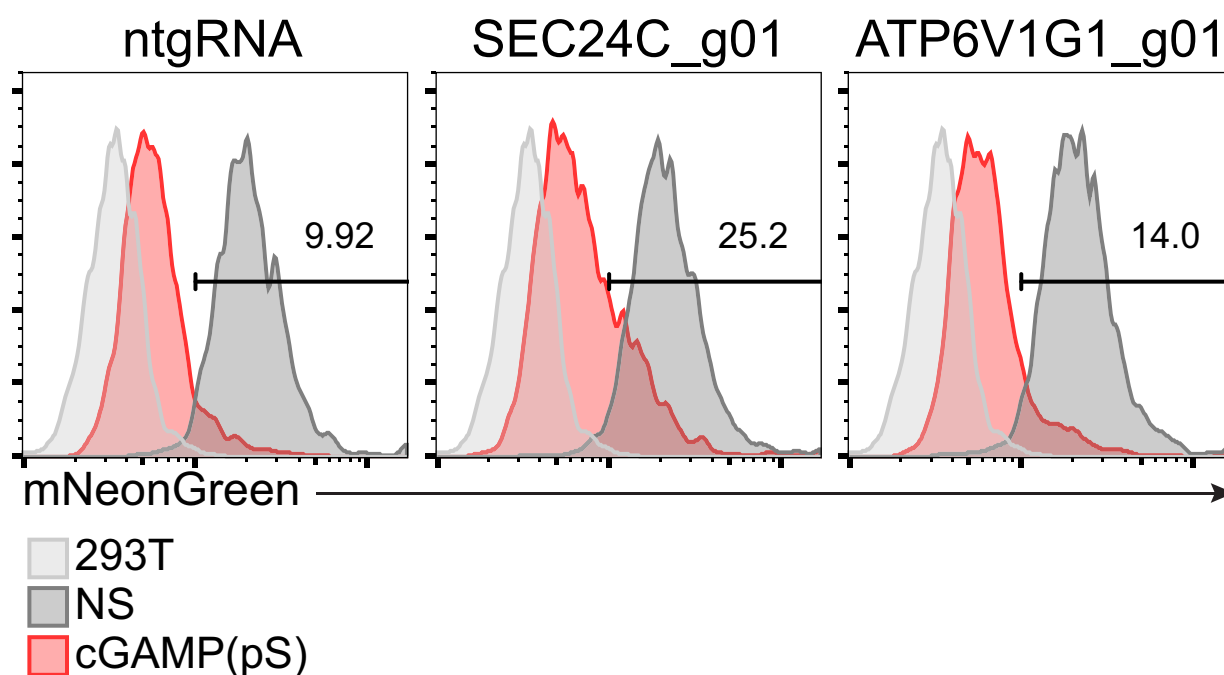


Figure S3. Related to Figure 2. **a)** Gating strategy for the genome-wide CRISPR screen. Gate-D in Panel C not used. **b)** mNeonGreen levels in reporter spCas9 expressing 293T STING-mNeonGreen cell lines transduced with the indicated sgRNAs before (dark gray – NS) or after (red) stimulation with 1 μ g/ml 2'3'-cGAMP(pS)₂ for 24 hours. Line represents gating strategy, and numbers represent %STING-mNeonGreen positive cells post stimulation. 293T (light gray) are shown as a reference for mNeonGreen negative cells. One representative plot of n=2 independent experiments with n=2 technical replicates per experiment.

Figure S4

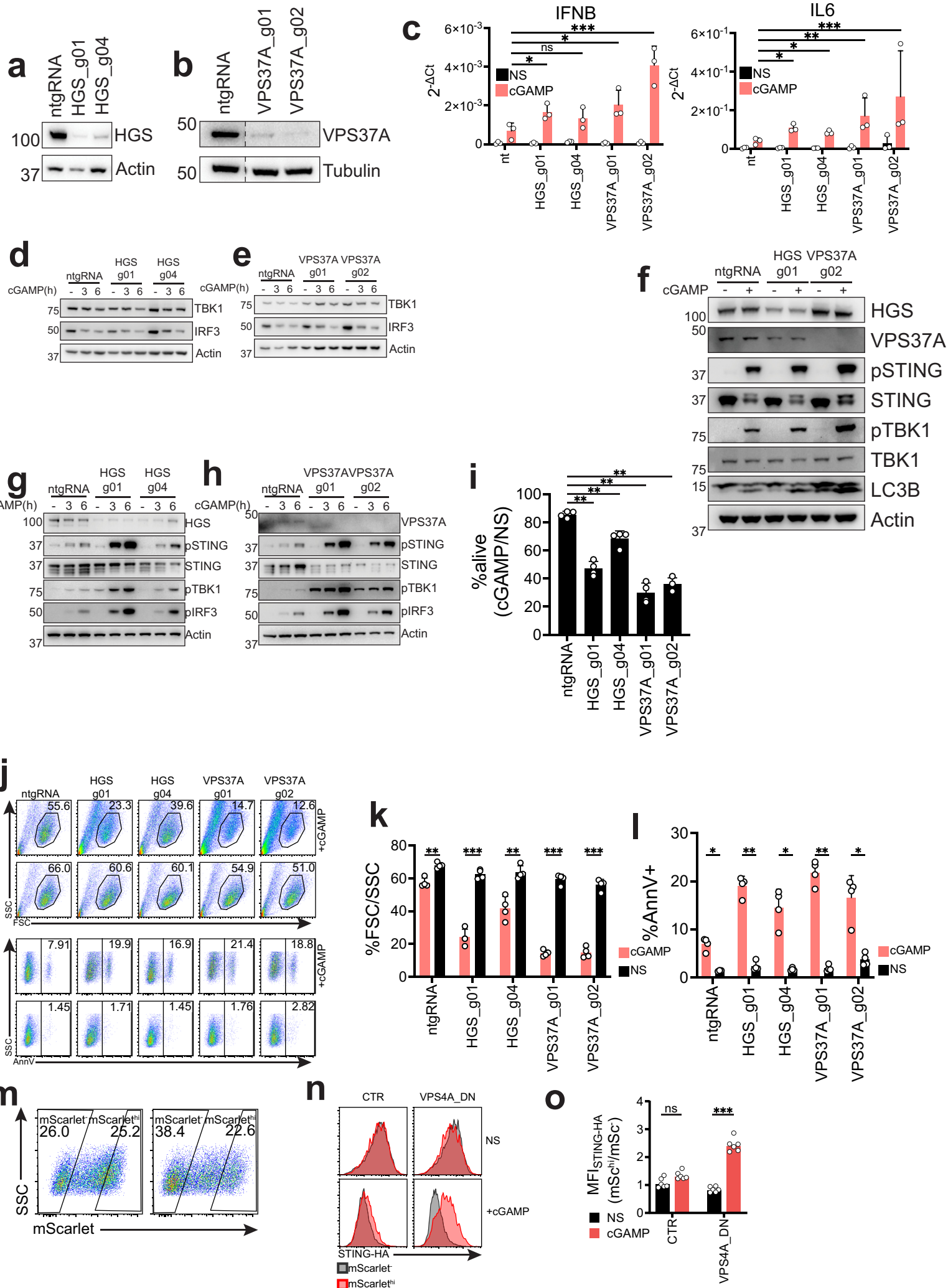


Figure S4. Related to Figure 3. **a)** HGS KO efficiency relative to Fig. 3b, 3c. **b)** VPS37A KO efficiency relative to Fig. 3b, 3c. **c)** $2^{-\Delta Ct}$ values related to Fig. 3j. n=3 independent experiments. One-way ANOVA on log-transformed data with Dunnet multiple comparison test. **d)** Immunoblot of the indicated proteins in BJ1 KO for HGS, relative to 3h. **e)** Same as in d) for VPS37A, relative to Fig. 3i. **f)** Immunoblot of the indicated proteins in primary non-hTERT immortalized BJ fibroblasts stimulated with 0.5µg/ml cGAMP (in perm buffer) for 6h. One representative experiment of n=3 experiments. **g)** Immunoblot of the indicated proteins in U937 KO for HGS stimulated with 20µg/ml cGAMP stimulation (in medium) for 6 hours. One representative blot of n=3 independent experiments. **h)** Same as in g), for VPS37A. **i)** U937 cells were treated with 20µg/ml cGAMP (in medium) for 24 hours and alive cells quantified with Cell Titer Glo. Alive cells shown as percentage ratio RLU in cGAMP treated samples over non-stimulated samples. n=4 independent experiments. One-Way ANOVA with Dunnet multiple comparison test. **j)** FSC/SSC and Annexin V for U937 treated as in i). One representative experiment of n=4 independent experiments. **k)** Percentage of cells in FSC/SSC gate for cells treated as in j). n=4 independent experiments. One-Way ANOVA with Holm-Sidak multiple comparison test. **l)** Percentage Annexin V positive cells in FSC/SSC gate for cells treated as in j). n=4 independent experiments. One-Way ANOVA with Holm-Sidak multiple comparison test. **m)** Expression of mScarlet-VPS4ADN (VPS4A E228Q) after transfection in 293T stably expressing STING-HA and gating strategy for mScarlet⁺ and mScarlet^{hi} cells. **n)** STING-HA levels in cells as in n) in mScarlet⁺ (dark grey) and mScarlet^{hi} (red) populations that were either non-stimulated (NS) or treated with 2µg/ml cGAMP (in perm buffer) for 6 hours. One representative plot of n=3 independent experiments with n=2 technical replicates per experiment. **o)** MFI of STING-HA signals shown as a ratio of MFI of the mScarlet^{hi} population over the MFI of the mScarlet⁺ population. n=3 independent experiments with n=2 technical replicates per experiment. Each dot represents an individual replicate. One-way ANOVA with Dunnet multiple comparisons test.

In all panels, bar plots show mean and error bars standard deviation. Marker unit for Westen blots is KDa. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 ns=not significant.

Figure S5

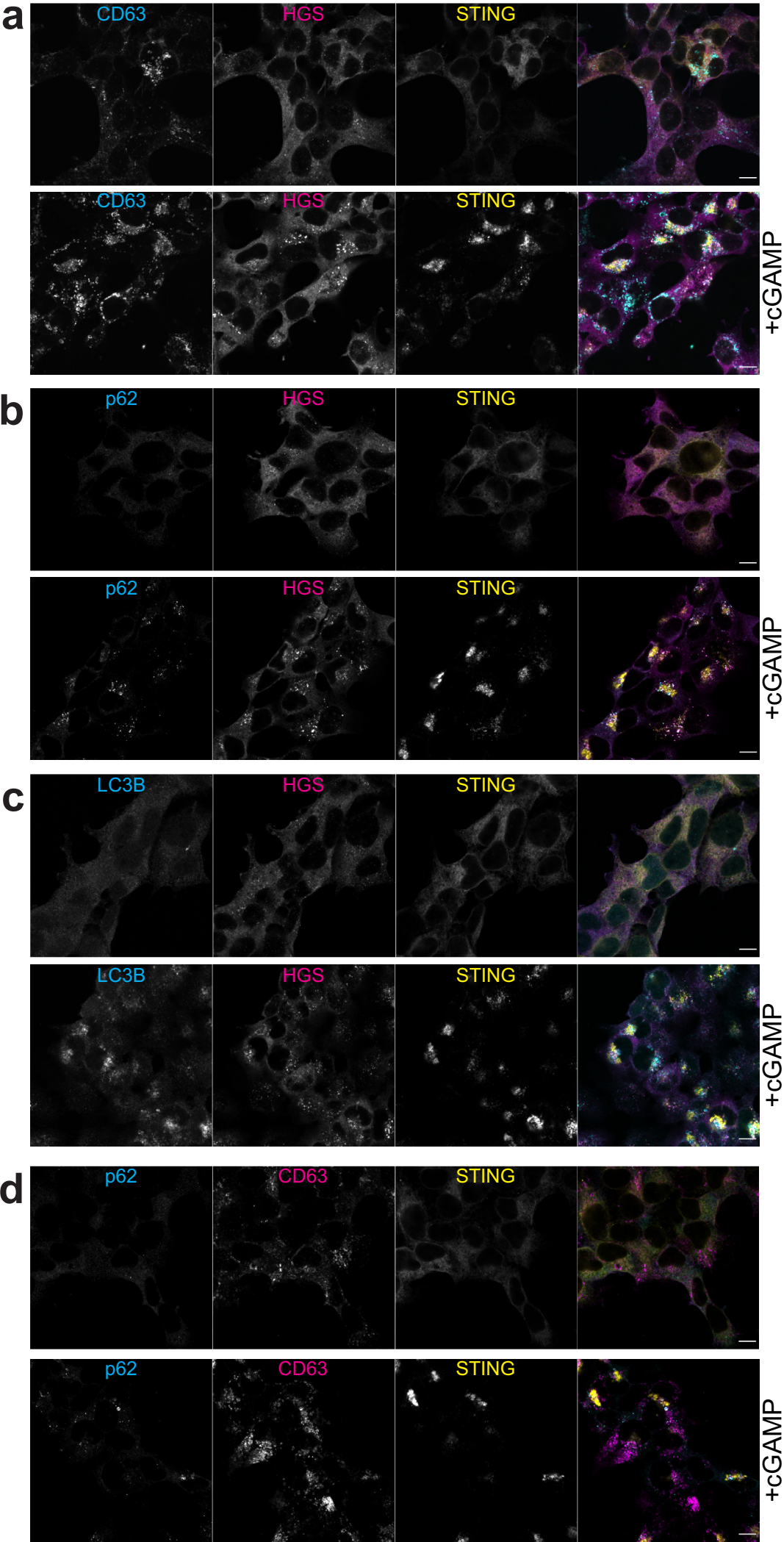


Figure S5. Related to Figure 4. Single color and merge of immunofluorescence of **a)** CD63 (cyan), HGS (magenta) and STING (yellow), **b)** p62 (cyan), HGS (magenta) and STING (yellow), **c)** LC3B (cyan), HGS (magenta) and STING (yellow), **d)** p62 (cyan), CD63 (magenta) and STING (yellow) in 293T stably expressing STING-HA non-stimulated or stimulated with cGAMP relative to Figure 4a-d. Scale bar is 10 μ m. One representative field of $n \geq 3$ independent fields in $n = 3$ independent experiments.

Figure S6

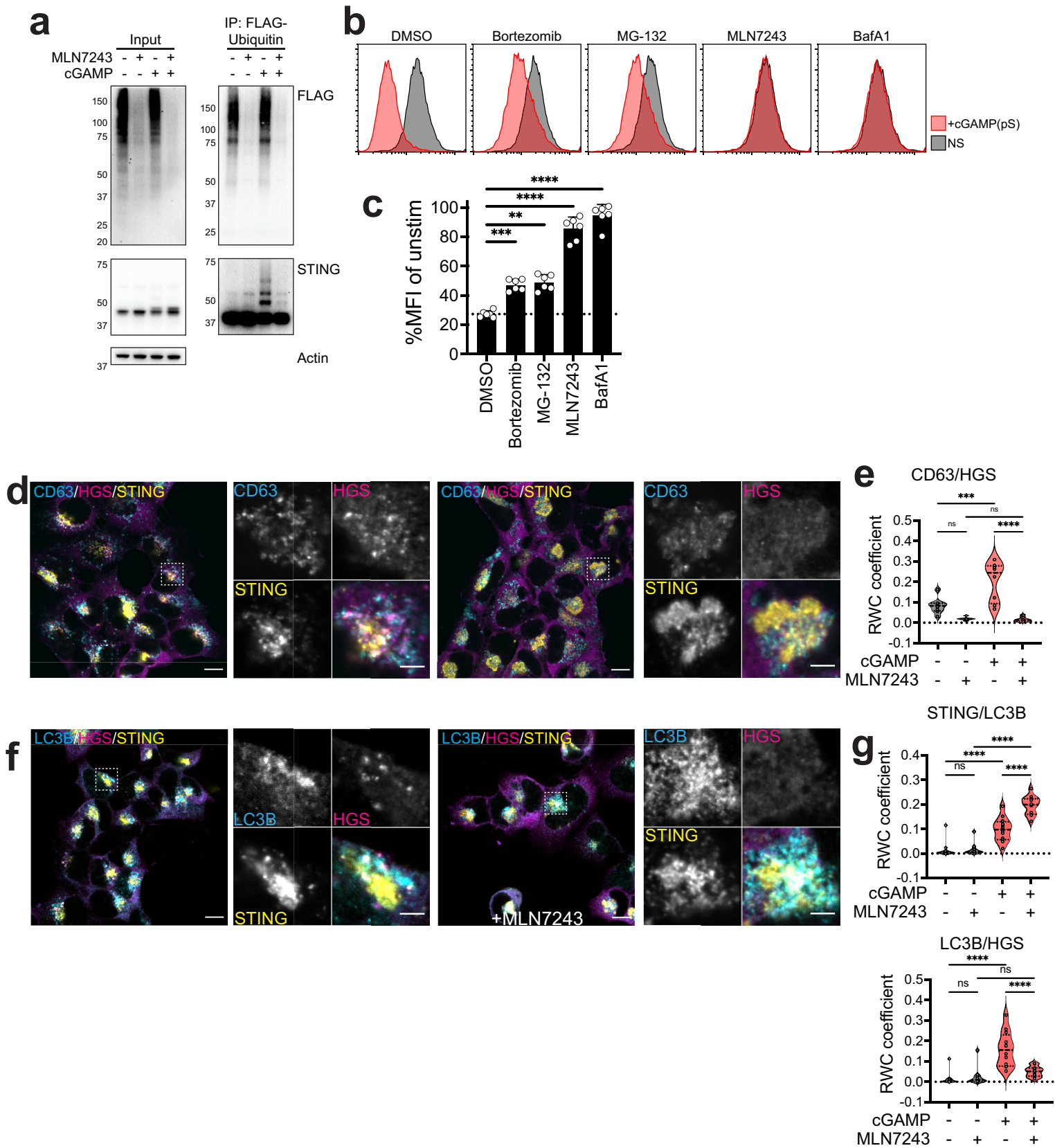


Figure S6. Related to Figure 5. **a)** Immunoblot of the indicated proteins in 293T stably expressing STING-HA and FLAG-ubiquitin in the input or after FLAG pulldown (IP: Flag-Ubiquitin) for cells stimulated with 2 μ g/ml cGAMP (in perm buffer) with 0.5 μ M MLN7243 for 2 hours. One representative blot of n=2 independent experiments. **b)** STING-mNG levels in 293T STING-mNG cells treated with 4 μ g/ml cGAMP(pS)2 (in medium) for 6 hours in presence of the indicated drugs. One representative experiment of n=3 independent experiments with n=2 technical replicates. **c)** Percentage MFI of STING-mNG signal expressed as ratio MFI of stimulated cells as in b) over MFI of unstimulated cells. n=3 independent experiments with n=2 technical replicates. Error bars represent SD. One-way ANOVA with Dunnet multiple comparisons test. **d)** Immunofluorescence of CD63 (cyan), HGS (magenta) and STING (yellow) in 293T stably STING-HA stimulated with 2 μ g/ml cGAMP (in perm buffer) and 0.5 μ M MLN7243 for 2 hours. Dashed boxes represent the cropped regions shown in the right panels. One representative field of n $\geq$ 5 fields in n=2 independent experiments. Scale bar is 10 μ m for wide-field and 3 μ m for enlargement. Control non-stimulated cells are in Fig. S7. **e)** RWC for CD63 and HGS in cells stimulated as in d). Each dot represents colocalization calculated in a field. n=2 independent experiments with n $\geq$ 5 fields. One way ANOVA with post-hoc Tukey test. **f)** Immunofluorescence of CD63 (cyan), HGS (magenta) and STING in cells treated as in d). Dashed boxes represent the cropped regions shown in the right panels. One representative field of n $\geq$ 5 fields in n=2 independent experiments. Scale bar is 10 μ m for wide-field and 3 μ m for enlargement. Control non-stimulated cells are in Fig. S7. **g)** RWC for LC3B and STING colocalization or LC3B and HGS colocalization in cells stimulated as in f). Each dot represents colocalization calculated in a field. n=2 independent experiments with n $\geq$ 5 fields. One way ANOVA with post-hoc Tukey test.

In all panels, bar plots show mean and error bars standard deviation. Marker unit for Westen blots is KDa. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 ns=not significant.

Figure S7

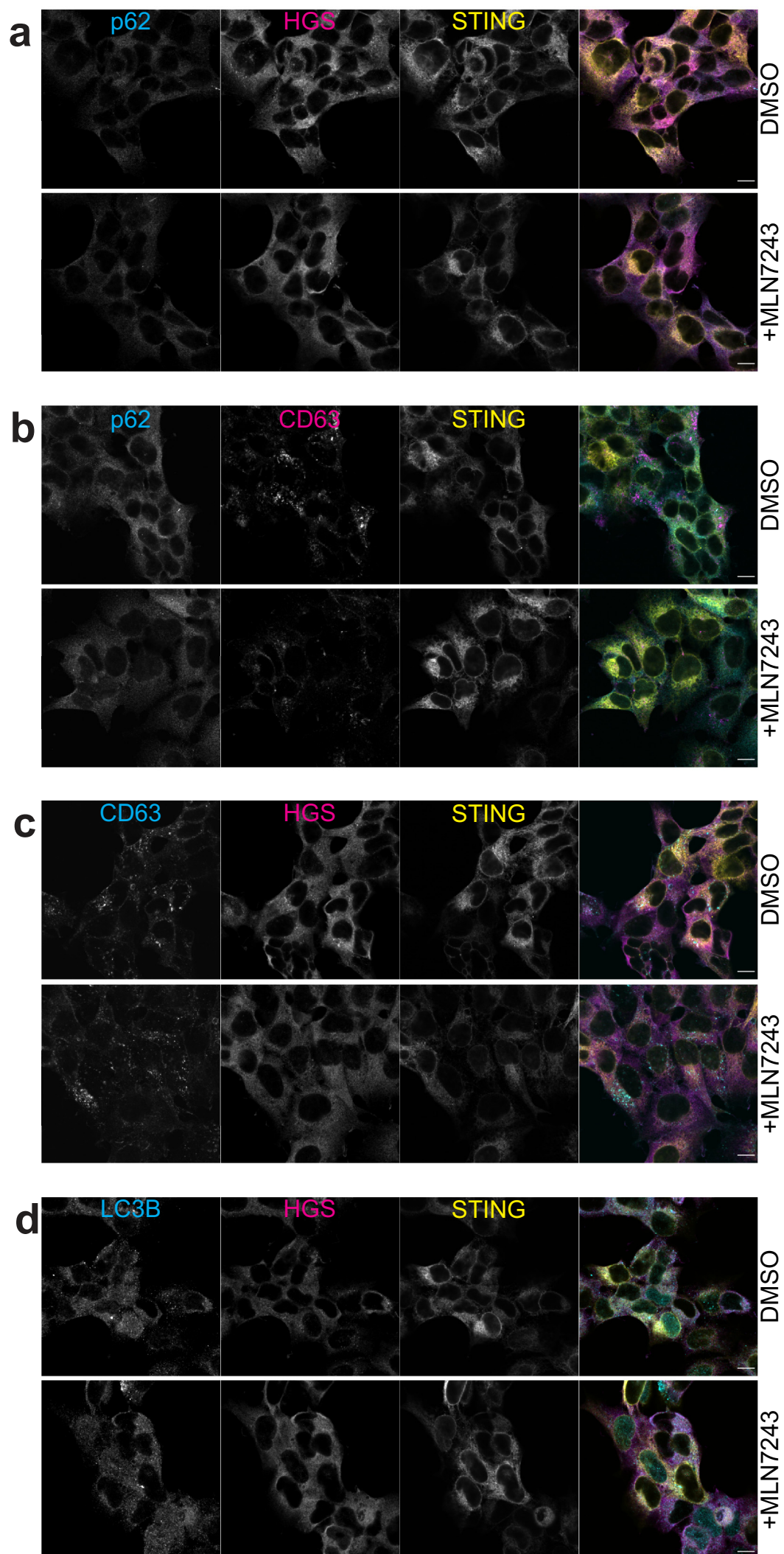


Figure S7. Related to Figure 5 and Figure S6. Immunofluorescence of **a)** p62 (cyan), HGS (magenta) and STING (yellow), **b)** p62 (cyan), CD63 (magenta) and STING (yellow), **c)** CD63 (cyan), HGS (magenta) and STING (yellow), **d)** LC3B (cyan), CD63 (magenta) and STING (yellow) in 293T stably expressing STING-HA treated (bottom panels) or non-treated (DMSO – top panels) with MLN7243.

Figure S8

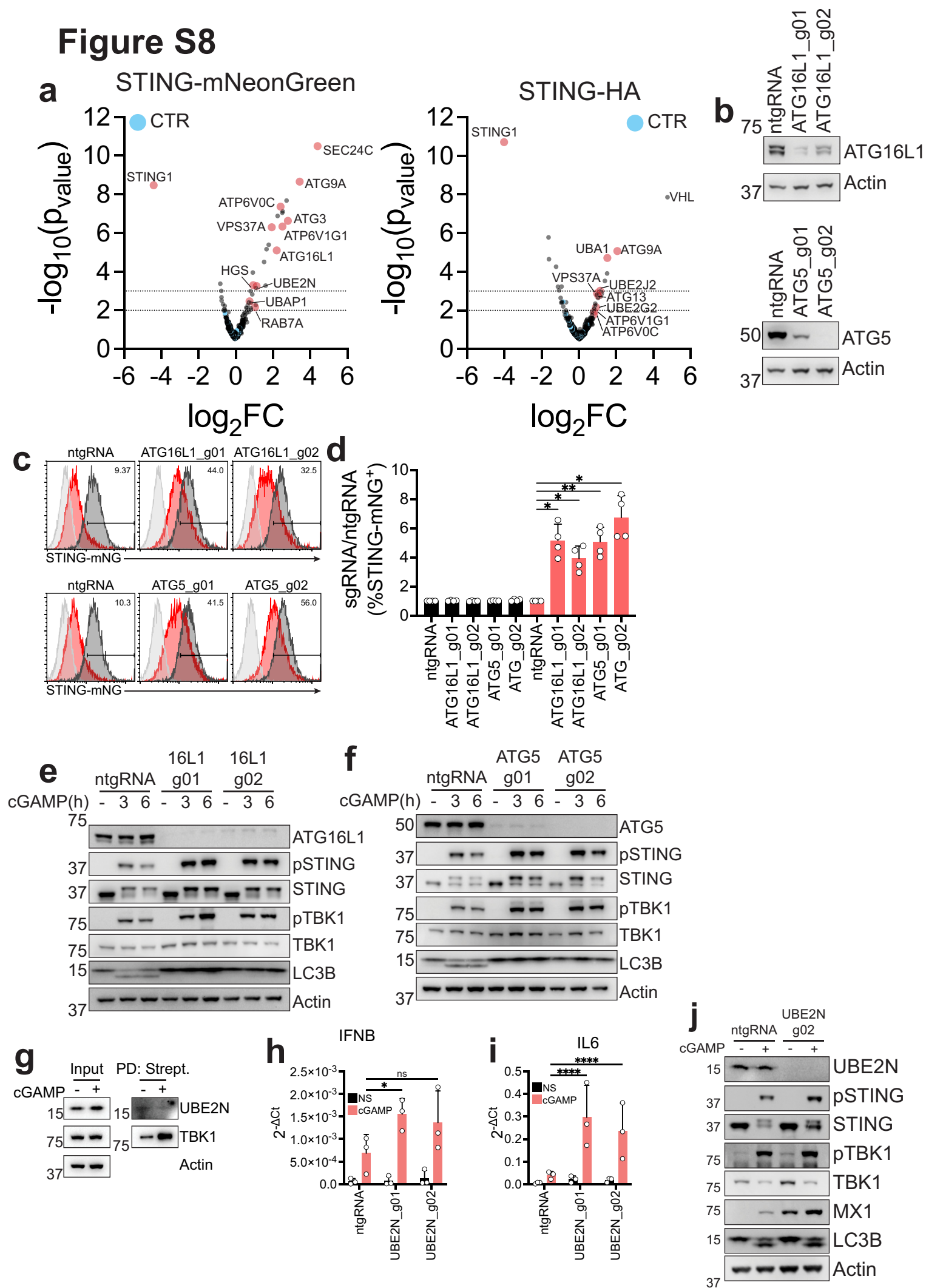


Figure S8. Related to Figure 6. **a)** Volcano-plots of $\log_2$ fold change ($\log_2FC$) vs $-\log_{10}(p_{value})$ after sequencing and analysis of the targeted screens in STING-mNeonGreen and STING-HA cell lines. VHL scoring highly in the STING-HA screen is due to the use of a hPGK promoter to drive STING-HA expression. KO of VHL stabilizes HIF1 α which drives transcription from the HRE elements present in the hPGK promoter. **b)** Immunoblot of the indicated proteins in 293T STING-mNG KO for the indicated genes. **c)** mNG levels in 293T STING-mNG cells lines same as in b) stimulated with 4 μ g/ml 2'3'-cGAMP(pS)2 (in medium) for 6 hours. One representative plot of n=2 independent experiments with n=2 technical replicates per experiment. **d)** Percentage of STING-mNG positive cells in cells stimulated as in c). n=2 independent experiments with n=2 technical replicates per experiment. Each dot represents an individual replicate. Error bars represent SD. One-way ANOVA with Dunnet multiple comparison test. **e)** Immunoblot of the indicated proteins in BJ1 fibroblasts KO for ATG16L1. One representative experiment of n=2 independent experiments. **f)** Immunoblot of the indicated proteins in BJ1 fibroblasts KO for ATG5. One representative experiment of n=2 independent experiments. Marker unit is KDa. **g)** Immunoblot of the indicated proteins in 293T STING-TurboID stimulated for 1 hour with 2 μ g/ml cGAMP (in perm buffer). **h)** $2^{-\Delta Ct}$ values related to Fig. 6f. **i)** and Fig. 6g. n=3 independent experiments. One-way ANOVA on log-transformed data with Dunnet multiple comparison test. **j)** Immunoblot of the indicated proteins in primary non-hTERT immortalized BJ fibroblasts stimulated with 0.5 μ g/ml cGAMP (in perm buffer) for 6h. One representative experiment of n=2 experiments. In all panels, bar plots show mean and error bars standard deviation. Marker unit for Westen blots is KDa. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 ns=not significant.

a



Figure S9. Related to Figure 7. a) Immunofluorescence of NBR1 (cyan), HGS (magenta) and STING (yellow) in 293T STING-HA cells stimulated with 2 μ g/ml cGAMP (in perm buffer) for 2h. Dashed boxes represent the cropped regions shown in the right panels. One representative field of $n \geq 5$ fields in $n = 2$ independent experiments. Scale bar is 10 μ m for wide-field and 3 μ m for enlargement. **b)** HA level in 293T cells expressing STING WT-HA or STING L374A-HA stimulated with 2 μ g/ml cGAMP (in perm buffer) for 6h. One experiment representative of $n = 3$ independent experiments with $n = 2$ technical replicates. **c)** MFI of cells as in b) shown as %MFI of cGAMP stimulated over non-stimulated (NS) for each mutant. $n = 3$ independent experiments with $n = 2$ technical replicates per experiment. Each dot represents an individual replicate. Two-tailed paired t-test. **d)** Alignment of STING in different species. Lysines are highlighted and red numbers refer to positions in human STING. **e)** HA levels in 293T stably expressing HA tagged STING WT or K289R. One representative plot of $n = 3$ technical replicates. **f)** MFI for cells as in e). Each dot represents one technical replicate. Two-tailed paired t-test. **g)** Immunoblot of the indicated proteins in 293T expressing STING 5KR-HA and FLAG-ubiquitin in the input or after FLAG pulldown (IP: Flag-Ubiquitin) stimulated with 2 μ g/ml cGAMP (in perm buffer) with 0.5 μ M MLN7243 for 2 hours. One representative blot of $n = 2$ independent experiments. Marker unit is KDa. **h)** Immunofluorescence of DAPI (blue), GM130 (magenta) and STING (yellow) in 293T stably expressing either STING WT or STING 5KR stimulated with 2 μ g/ml cGAMP (in perm buffer) for 2h. One representative field of $n = 3$ fields for $n = 2$ independent experiments. Scale bar is 20 μ m. **i)** HA levels in 293T expressing STING WT or STING 2KR (K338R/K370R) stimulated with 2 μ g/ml cGAMP (in perm buffer) for 6h. One representative experiment of $n = 3$ independent experiments with $n = 2$ technical replicates. **k)** MFI of cells as in i) shown as %MFI of cGAMP stimulated over non-stimulated (NS) for each mutant. $n = 3$ independent experiments with $n = 2$ technical replicates per experiment. Each dot represents an individual replicate. Two-tailed paired t-test. In all panels, bar plots show mean and error bars standard deviation. Marker unit for Westen blots is KDa. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ ns=not significant.

Figure S10

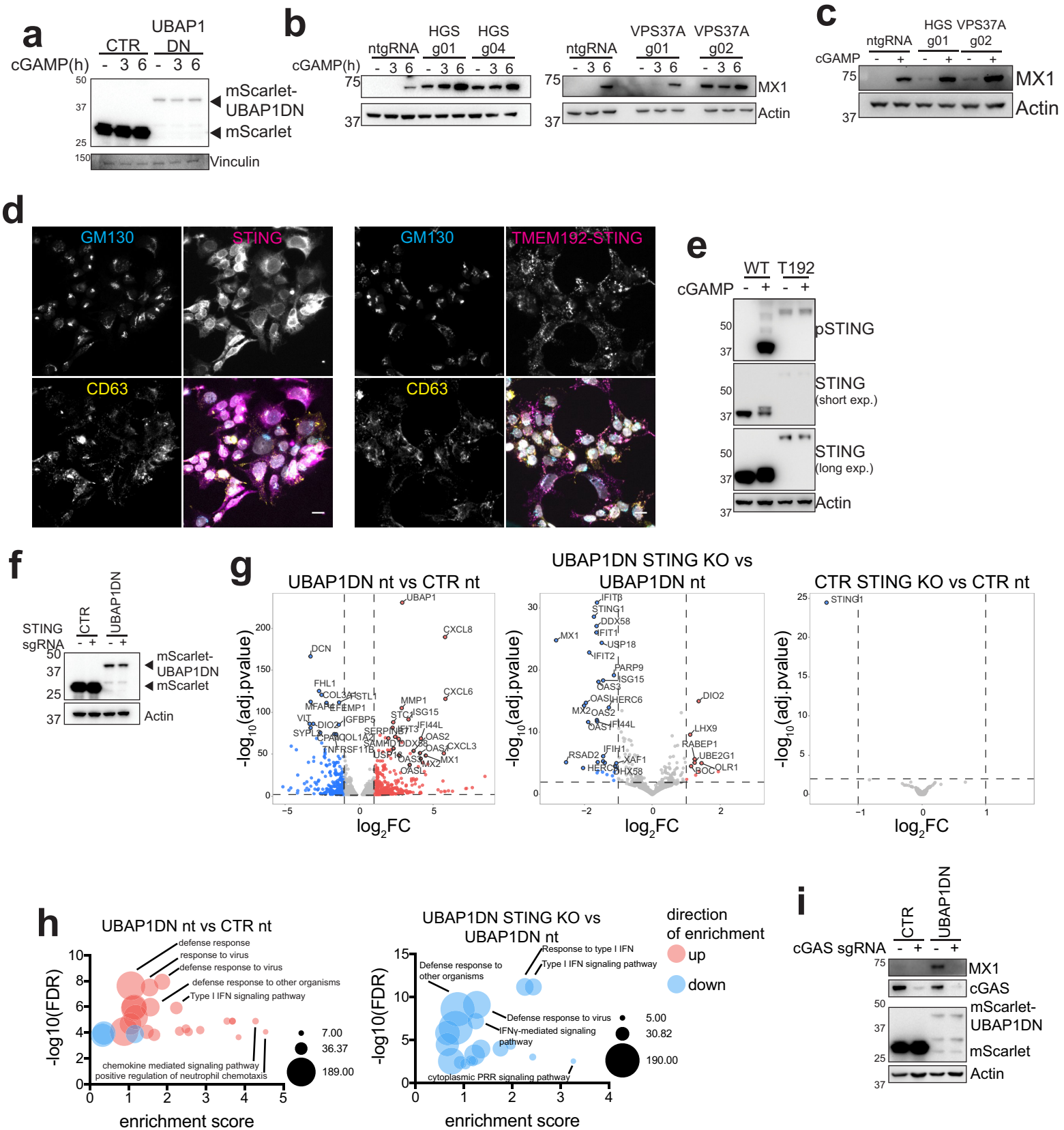
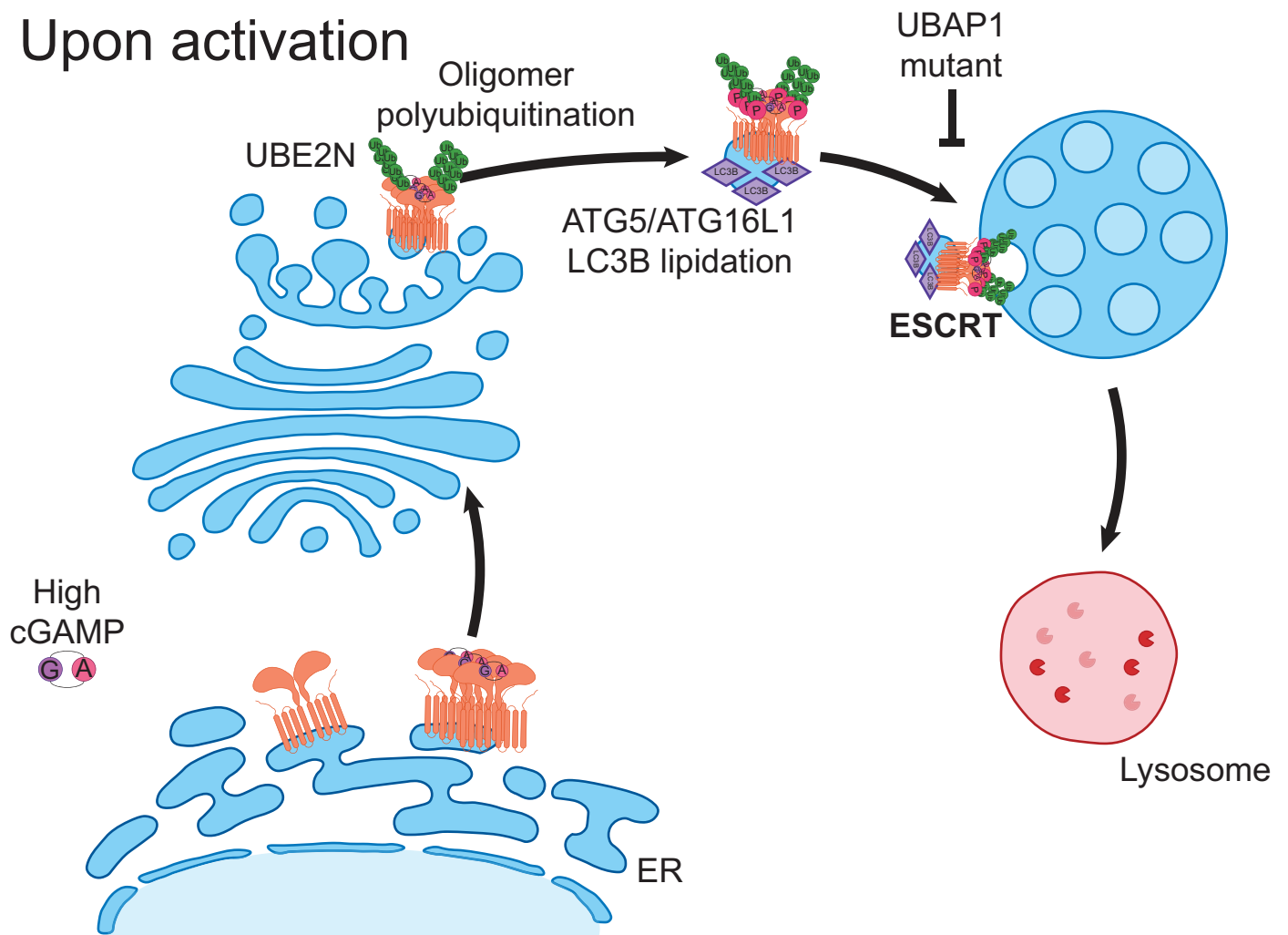


Figure S10. Related to Figure 8. **a)** Immunoblot of the indicated proteins. Related to Fig. 8f. Marker unit is KDa. **b)** Immunoblot of the indicated proteins in BJ1 fibroblasts stimulated with 0.5µg/ml cGAMP (in perm buffer) for the indicated times. One representative experiment of n=3 experiments. **c)** Immunoblot of the indicated proteins in primary non-hTERT immortalized BJ fibroblasts stimulated with 0.5µg/ml cGAMP (in perm buffer) for 6h. One representative experiment of n=2 experiments. **d)** Immunofluorescence of GM130 (cyan), HA (magenta) and CD63 (yellow) in 293T stably expressing STING-HA (left panel) or TMEM192-STING-HA (right panel). DAPI is grey in the merged image. Scale bar is 20µm. One field representative of n≥5 fields per construct of n=2 technical replicates. **e)** Immunoblot of the indicated proteins in 293T stably expressing WT STING-HA (WT) or TMEM192-STING-HA (T192) stimulated with 2µg/ml cGAMP (in perm buffer) for 2h. One experiment representative of n=2 independent experiments with n=2 technical replicates. **f)** Immunoblot of the indicated proteins. Related to Fig. 8k. Marker unit is KDa. **g)** Volcano-plots of differentially expressed genes (DEGs) for the indicated comparisons. Dashed lines to indicate significant DEGs are drawn at $\log_2FC \geq 1$ and $\log_2FC \leq -1$ and $-\log_{10}(\text{adjusted } p \text{ value}) \geq 2$. Significant downregulated genes are in blue, upregulated in red. nt: non-targeting sgRNA **h)** GO analysis of significantly enriched processes in the indicated conditions. Positively enriched terms are in red, negatively enriched in blue. Size of bubbles represents the number of mapped genes in each category. FDR: False Discovery Rate. **i)** Immunoblot of the indicated proteins in BJ1 expressing mScarlet (CTR) or mScarlet-UBAP1DN transduced with spCas9 and a control sgRNA or a cGAS targeting sgRNA. One experiment representative of n=3 independent experiments.

In all panels, bar plots show mean and error bars standard deviation. Marker unit for Westen blots is KDa. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 ns=not significant.

Figure S11

Upon activation



Steady state

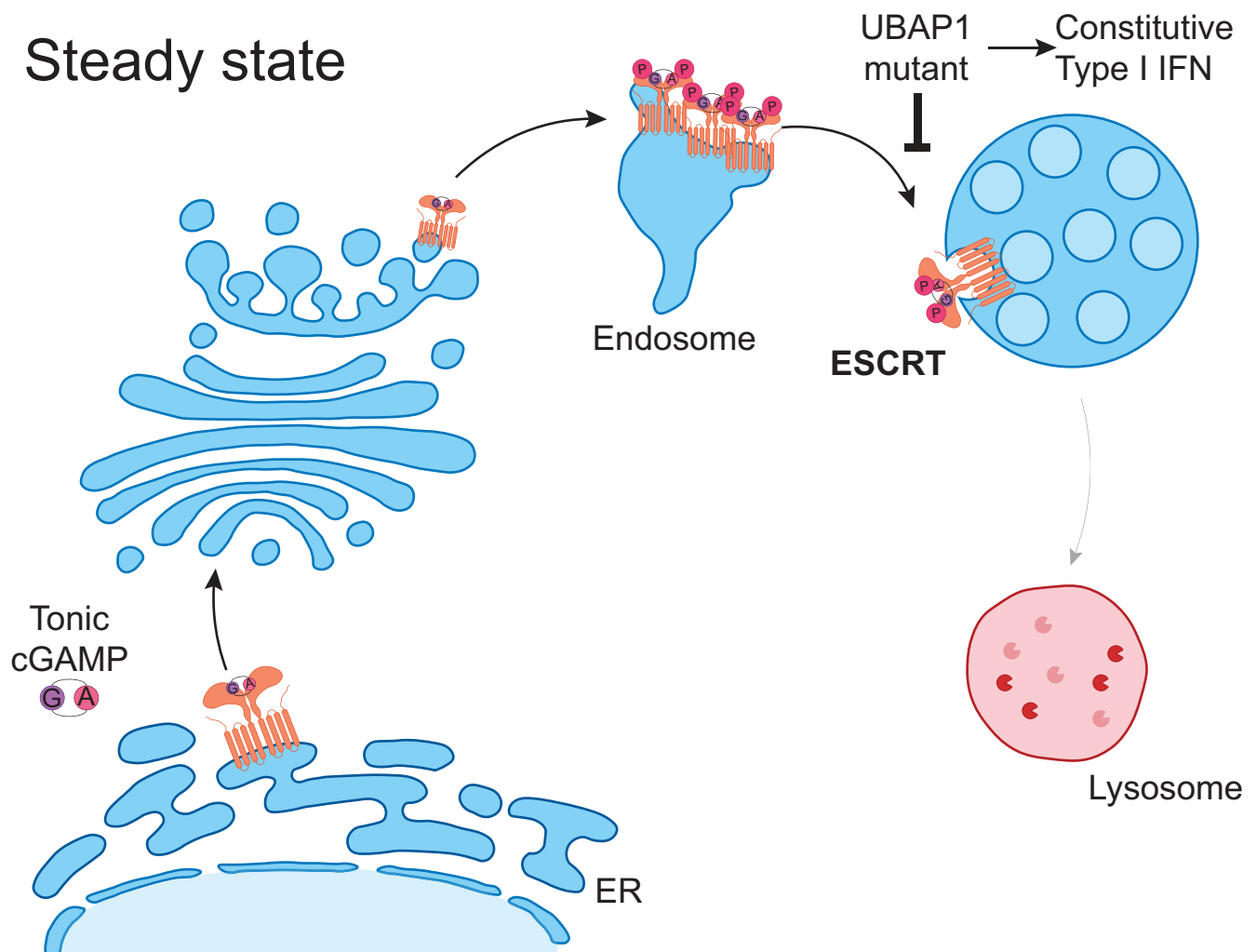


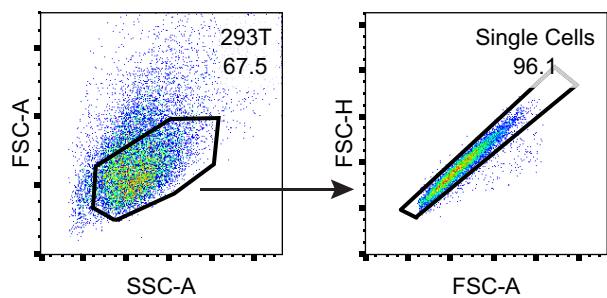
Figure S11. ESCRT-dependent STING degradation curtails steady-state and cGAMP-induced signaling

Top. Activated STING traffics from the ER to the Golgi and then to the endosome. High levels of intracellular cGAMP drive STING oligomerization which in turns lead to UBE2N dependent STING polyubiquitination and ATG5/ATG16L1 dependent LC3B lipidation of STING containing vesicles. Ubiquitination of STING drives its association with ESCRT at the late endosome. Association of ESCRT to STING creates an organizing center for fusion with the endolysosomal compartment leading to STING degradation. Pathogenic mutants of the ESCRT-I subunit UBAP1 block this process and lead to exacerbated STING responses.

Bottom. At steady state, cGAS primes tonic STING trafficking between the ER and the lysosomes through the endosomal compartment. ESCRT ensures removal of STING at steady state preventing spontaneous activation of the sensor. A UBAP1 mutant, or KO of HGS and VPS37A, blocking ESCRT function leads to accumulation of phosphorylated STING at the endosome consequently driving constitutive STING activation. Mutations in genes regulating post-Golgi STING trafficking could therefore lead to spontaneous activation of the sensor and underlie disease.

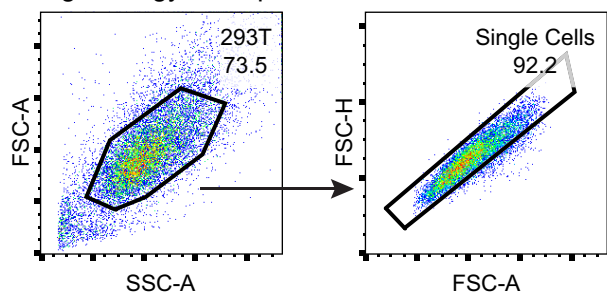
Figure S12

Gating strategy for experiments with STING-mNG cells



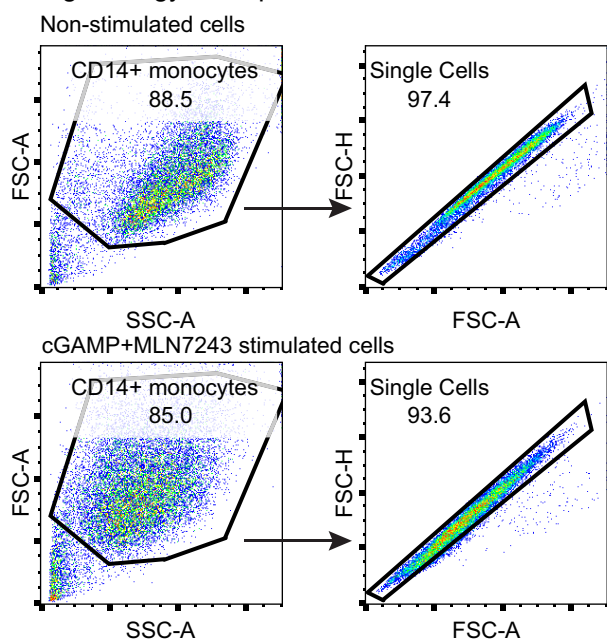
Related to Fig. 3b, 5a, 6c, 7a, S6b, S6c

Gating strategy for experiments with STING-HA cells



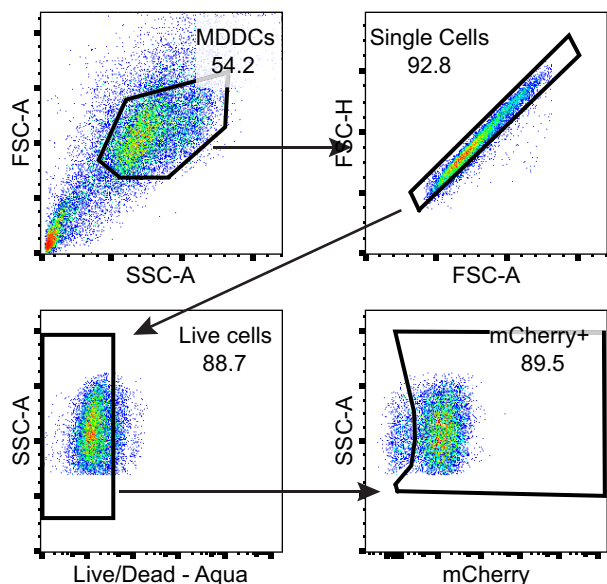
Related to Fig. 7a, 7d, 8c, S4m, S9b, S9e, S9i

Gating strategy for experiments with CD14+ monocytes



Related to Fig. 5k

Gating strategy for experiments with MDDCs



Related to Fig. 8h

Table S1. List of sgRNAs

sgRNA name	sgRNA sequence	Vector
ntgRNA	GTATTACTGATATTGGTGGG	CROPseq-guide-Puro or pXPR_BRD023
SEC24C_g01	GAAGATGACAGGAACAACCG	CROPseq-guide-Puro
SEC24C_g02	AAGAGCCCAGGGATAGCTCA	CROPseq-guide-Puro
ATP6V1G1_g01	TGAACAGTACCGCCTGCAGA	CROPseq-guide-Puro
ATP6V1G1_g02	TGCCACGGGATCCCAATGCC	CROPseq-guide-Puro
HGS_g01	CTTGGGGTACGAAGTGTACG	pXPR_BRD023
HGS_g04	CAGCCCCTCATAGTACACTG	pXPR_BRD023
VPS37A_g01	CATAAGGAGACATCCCACCTT	pXPR_BRD023
VPS37A_g02	GGTGGATAAACACTGATCAC	pXPR_BRD023
STING_g01	CATTACAACAACCTGCTACG	pXPR_BRD023
cGAS_g01	ATCCCTCCGTACGAGAATGG	pXPR_BRD023
UBE2N_g01	TGGTTCTGCCAGCAAACGCT	pXPR_BRD023
UBE2N_g02	CTGTTGCCTTCATAGATAAG	pXPR_BRD023
ATG16L1_g01	GCTGCAGAGACAGGCGTTCG	pXPR_BRD023
ATG16L1_g02	AAAAGCATGACGTACCAAAC	pXPR_BRD023
ATG5_g01	AAGAAGACATTAGTGAGATA	pXPR_BRD023
ATG5_g02	AAATGTACTGTGATGTTCCA	pXPR_BRD023

Table S2. List of antibodies

Target	Type	Application	Source	Reference	Dilution
STING (D2P2F)	Rbt monoclonal	WB/IF	CST	13647S	WB: 1:2000 IF: 1:200
STING	Ms monoclonal	WB	R&D	MAB7169	1:1000
pSTING (Ser366) (D7C3S)	Rbt monoclonal	WB	CST	19781S	1:1000
pSTING (Ser366) (D8K6H)	Rbt monoclonal	IF	CST	40818S	1:50
TBK1 (E8I3G)	Rbt monoclonal	WB	CST	38066S	1:1000
TBK1 (E9H5S)	Ms monoclonal	WB	CST	51872S	1:1000
pTBK1 (Ser172) (D52C2)	Rbt monoclonal	WB	CST	5483S	1:1000
IRF3 (D6I4C)	Rbt monoclonal	WB	CST	11904S	1:1000
pIRF3 (Ser386) (E7J8G)	Rbt monoclonal	WB	CST	37829S	1:1000
STAT1	Rbt monoclonal	WB	CST	9172S	1:2000
cGAS (D1D3G)	Rbt monoclonal	WB	CST	15102S	1:1000
pSTAT1 (Tyr 701) (58D6)	Rbt monoclonal	WB	CST	9167S	1:2000
MX1 (D3W7I)	Rbt monoclonal	WB	CST	37849S	1:1000
HRS (D7T5N)	Rbt monoclonal	WB/IF	CST	15087S	WB: 1:2000 IF: 1:200
LC3B	Rbt monoclonal	WB/IF	CST	2775S	WB: 1:1000 IF: 1:100
p62 (D5L7G)	Rbt monoclonal	IF	CST	88588S	1:200
Ubc13	Rbt monoclonal	WB	CST	4919	1:1000
CD63 (EPR22458-280)	Rbt monoclonal	IF	Abcam	ab252919	1:400
CD63 (MX-49.129.5)	Ms monoclonal	IF	Santa Cruz	sc-5275	1:400
VPS37A (G-3)	Ms monoclonal	WB	Santa Cruz	sc-376978	1:100
NBR1 (4BR)	Ms monoclonal	IF	Santa Cruz	sc-130380	1:200
UBAP1	Rbt polyclonal	WB	Proteintech	12385-1-AP	1:1000
RFP (6G6)	Ms monoclonal	WB	Chromotek	6g6-100	1:1000
ATG5 (D5F5U)	Rbt monoclonal	WB	CST	12994S	1:1000
ATG16L1 (D6D5)	Rbt monoclonal	WB	CST	8089S	1:1000
GM130 (35/GM130)	Ms monoclonal	IF	BD	610823	1:200

			Biosciences		
EEA1 (14/EEA1)	Ms monoclonal	IF	BD Biosciences	610457	1:200
β-actin (AC-15)	Ms monoclonal	WB	Abcam	ab6276	1:2000
α-Tubulin (DM1A)	Ms monoclonal	WB	Thermo Fisher	14-4502-82	1:1000
Vinculin	Rbt monoclonal	WB	CST	4650S	1:1000
HRP Anti-beta Actin antibody [AC-15]	Ms monoclonal	WB	Abcam	ab49900	1:20000
HRP-anti β-actin (mAbcam 8226)	Ms Monoclonal - HRP conjugate	WB	Abcam	ab20272	1:5000
HRP-Streptavidin	NA	WB	BioLegend	405210	1:1000
HA	Rat monoclonal	IF	Millipore Sigma	11867423001	1:200
DYKDDDDK Tag (D6W5B) - FLAG	Rabbit monoclonal	WB	CST	14793S	1:1000
Cy5-Streptavidin	NA	IF	Biolegend	405209	1:200
Alexa Fluor 647 anti-HA.11	Biolegend	FC	Biolegend	682404	1:200
Alexa 488 anti-mouse IgG (H+L)	Gt polyclonal	IF	Thermo Fisher	A-11029	1:200
Alexa 555 F(ab')₂ anti-Rabbit IgG	Gt polyclonal	IF	Thermo Fisher	A-21430	1:200
Alexa 647 anti-rat IgG (H+L)	Gt polyclonal	IF	Thermo Fisher	A-21247	1:200
Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)	Gt polyclonal	WB	Jackson ImmunoResearch	111-035-144	1:5000
Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L)	Gt polyclonal	WB	Jackson ImmunoResearch	111-035-146	1:5000

Rbt: rabbit; Ms: mouse; Gt: goat.

WB: western blot; IF: immunofluorescence; FC: flow cytometry.

Table S3. List of qPCR primers

Primer name	Primer sequence
IFNB1 FWD	CAGCATCTGCTGGTTGAAGA
IFNB1 RV	CATTACCTGAAGGCCAAGGA
IL6 FWD	CCCCTGACCCAACCACAAAT
IL6 RV	ATTTGCCGAAGAGCCCTCAG
GAPDH FWD	GTCTCCTCTGACTTCAACAGCG
GAPDH RV	ACCACCCTGTTGCTGTAGCCAA

Table S4. RNA sequencing reagents and conditions

RT mix 1		
Reagent	Reaction conc.	μL required
PEG 8000 (50% solution)	5%	3.2
Triton X-100 (10% solution)	0.10%	0.24
RNAse Inhibitor (40μ/μL)	0.5μ/μL	0.32
Smartseq3_OligodT30VN(100μM)	0.5μM	0.16
dNTPs (10mM/each)	0.5mM/each	1.6
mRNA template		μL for 15ng
Nuclease Free Water		Up to 24μL
Total μl		24

RT mix 2		
Reagent	Reaction conc.	μL required
Tris-HCl pH 8.5 (1M)	25mM	0.8
NaCl (1M)	30mM	0.96
MgCl ₂ (100mM)	2.5mM	0.8
GTP (100mM)	1mM	0.32
DTT (100mM)	8mM	2.56
RNAse Inhibitor (40u/μl)	0.5u/μl	0.4
TSO (100μM)	2μM	0.64
Maxima H-minus RT enzyme (200U/μl)	2u/μl	0.32
Nuclease Free Water		1.2
Total μl		8

First-strand synthesis		
Temperature	Time	Cycles
42 °C	90 min	1x
50 °C	2 min	10x
42 °C	2 min	
85 °C	5 min	1x

cDNA PCR mix		
Reagent	Reaction conc.	µl per. reaction
Kapa HiFi HotStart buffer (5X)	1X	10
dNTPs (10mM/each)	0.3mM/each	1.5
MgCl ₂ (100mM)	0.5mM	0.25
Fwd Primer (100µM) AAO270	0.5µM	0.25
Rev Primer (10µM) AAO271	0.1µM	0.5
First-strand synthesis reaction		20
Polymerase (1U/µl)	0.02U/µl	1
Nuclease Free Water		16.5
Total µl		30

cDNA amplification		
Temperature	Time	Cycles
98 °C	3 min	1x
98 °C	20 sec	25x
65°C	30 sec	
72 °C	6 min	
72 °C	5 min	1x
4 °C	Hold	

Tagmentation mix		
Reagent	Reaction conc.	µl per. reaction
Tagmentation buffer (4x) (Tris-HCl pH 7.5 - 40mM, MgCl ₂ - 20mM, DMF- 20%)	1X	2
Amplicon Tagmentation Mix (Tn5)		0.32
UltraPure water		1.68
Total		4

Final library amplification mix		
Reagent	Reaction conc.	µl per. reaction
Phusion HF buffer (5X)	1X	5.6
dNTPs (10mM/each)	0.2mM/each	0.6
Phusion HF (2U/µl)	0.01U/µl	0.16
Nextera Index Primers (0.5µM)	0.1µM	6
H2O		5.64
Tagmented cDNA		10
Total µl		28

Final library amplification		
Temperature	Time	Cycles
72 °C	3 min	1x
98 °C	3 min	1x
98 °C	10 sec	12x
55 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	1x
4 °C	Hold	