Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP

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Supplementary Material

Supplementary Figure 1: Stable overexpression of cGAS in HEK cells induces activation of HEK STING cells *in trans*. a, HEK cells and HEK STING cells were co-cultured with increasing amounts of HEK cGAS* cells (ratios ranging from 1:0.25 to 1:0.0156 HEK/HEK STING:HEK cGAS*). Co-cultures were transfected with pIFN-β-GLuc and after 20 h transactivation of the reporter construct was assessed. Mean and s.e.m. (biological duplicates) of one representative experiment out of two independent experiments are depicted. **b**, HEK STING cells were co-cultured with HEK cells or HEK cGAS* cells (ratios HEK STING:HEK/HEK cGAS* = 1:0.25) for 4 h and phosphorylation of IRF3 was determined in the cellular lysates by immunoblotting. **c**, Kinetics of IRF3 phosphorylation of HEK STING and HEK cGAS* co-cultures (ratio HEK STING:HEK cGAS* = 1:0.25) are depicted. CMA served as a control stimulus. Representative experiments of two independent experiments are shown (**b**, **c**).

Supplementary Figure 2: DNA-triggered cGAS activation induces IFN-β expression in adjacent cells via STING. a, **b**, HEK cells and HEK STING cells were co-cultured with increasing amounts of HEK $cGAS^{low}$ cells (**a**) or primary MEFs (**b**) as depicted in Fig. 2c, d (ratio of HEK/HEK $cGAS^{low}$/MEFs was titrated ranging from 1:0.125 to 1:0.0156). Co-cultures were transfected with pIFN-β-GLuc and after 20 h transactivation of the reporter construct was assessed. Mean and s.e.m. of six experiments (**a**) or eight experiments (**b**) are depicted (*$P < 0.05$, **$P < 0.01$). **c**, Schematic view of the experimental set-up is shown: primary MEFs were silenced for cGAS expression using two independent siRNAs targeting cGAS or a control siRNA. Forty-eight hours later MEFs were co-cultured with HEK STING cells and then transfected with pIFN-β-GLuc and after an additional period of twenty hours transactivation of the reporter construct was assessed. **d**, cGAS expression in MEFs treated as in **c** was analysed by qPCR (data normalized to control siRNA condition). Mean values and s.e.m. of two independent experiments are depicted. **e**, Mean values and s.e.m. of duplicate measurements of one representative experiment, in which the ratio of HEK STING cells over MEFs was titrated ranging from 1:0.5 to 1:0.0625 is depicted. **f**, Mean values and s.e.m. (data normalized to control siRNA condition) of three independent experiments are depicted (HEK STING/MEF ratio is 1:0.5) (**$P < 0.01$).

Supplementary Figure 3: Overexpression of a RIG-I-stimulatory RNA molecule cannot confer activation of bystander cells. a, HEK cells were transfected with empty vector (Cont.), cGAS–GFP (cGAS) or a construct encoding a RIG-I-stimulatory shRNA molecule (shRNA). Twenty hours after transfection cells were collected, washed and added onto HEK cells or HEK STING cells expressing pIFN-β-GLuc. After 20 h of co-culture luciferase activity was measured. **b**, HEK STING cells were transfected as in **a** together with pIFN-β-GLuc and luciferase activity was measured 20 h after transfection. Mean and s.e.m. (biological duplicates) of one representative experiment out of two independent experiments are shown.

Supplementary Figure 4: cGAS-dependent bystander cell activation requires direct cell-to-cell contact. a, **d**, Schematic view of the experimental set-up is depicted. HEK STING cells or LL171 cells were left untreated (i) or co-cultured with HEK cells (ii) or HEK cGAS* cells in the presence (iii) or absence (iv) of a trans-well system. **b**, **e**, After 4 h of co-culture, phosphorylation of IRF3 was determined in cellular lysates via immunoblotting. **c**, After 14 h, relative induction of *IFNB* and *CXCL10* in HEK STING cells was analysed via qPCR. In addition, HEK STING cells were transfected with pIFN-β-GLuc 20 h before donor cells were added. After 18 h luciferase activity in HEK STING cells was assessed. **f**, Relative induction of *Ifnb* in LL171 cells was determined via qPCR after 4 h. Furthermore, transactivation of an endogenous ISRE-reporter construct was assessed in LL171 cells after 14 h. **g**, LL171 cells were transfected with siRNA targeting STING or a control siRNA. Forty-eight hours after siRNA transfection relative expression of STING was determined by qPCR. Mean and s.e.m. of duplicate measurements of two independent experiments is shown. **h**, LL171 cells from **g** were co-cultured with HEK cGAS* cells and after 6 h phosphorylation of IRF3 was determined by immunoblotting. Mean and s.e.m. (biological duplicates) of one representative experiment out of two independent experiments are shown (**c**, **f**) or one representative experiment out of two independent experiments is shown (**b**, **e**, **h**).

Supplementary Figure 5: Carbenoxolone inhibits bystander effect in LL171 cells. a, **b**, LL171 cells were pre-treated with CBX (100 µM, 150 µM and 200 µM) 3 h before addition of HEK cells or HEK cGAS* cells. In addition, LL171 cells were stimulated with CMA (**a**) or recombinant IFN-α (250 U ml^{-1}). Phosphorylation of IRF3 (**a**) and luciferase activity of an endogenous ISRE-reporter construct (**b**) was determined in the cellular lysate 4 h and 14 h after stimulation, respectively. Mean and s.e.m. (biological duplicates) of one representative experiment out of two independent experiments are shown.

Supplementary Figure 6: Scrape loading assays reveal a direct transfer of cGAMP(2'-5') through gap junctions. a, **b**, HEK STING cells (STING in red) were either incubated with cGAMP(2'-5'), CMA or scratched in the presence of cGAMP(2'-5'). The latter condition was also performed in the presence of 150 µM CBX. STING activation was visualized 8 h later, whereas dashed lines follow the scratch margins and arrows highlight areas of STING complex assembly. Representative images of four independent experiments are shown (**a**) and STING-activated cells were quantified and depicted in a scatter plot (**b**). ***$P < 0.001$.

Supplementary Figure 7: Deep sequencing results of CX43/CX45-targeted HEK STING cells generated by CRISPR/Cas9-mediated genome editing and western blot analysis of HEK STING CX43/45DKO cells. a, **c**, For generating HEK STING CX43/45^{DKO} cells, a targeting strategy was devised based on hybrid gRNA sequences targeting Cas9 to the first coding

exons of the respective genes. The open reading frame of CX43 (**a**) and CX45 (**c**) are delineated in red. PAM, protospacer adjacent motif. **b**, **d**, Deep-sequencing-based allele calls of targeted HEK STING cell lines as well as control cell lines are presented. Mutations are indicated in red letters, whereas the numbers in brackets indicate the net frame shifts. **e**, HEK STING CX43/45^{WT} cells and HEK STING CX43/45^{DKO} cells were analysed for CX43 and CX45 expression via immunoblotting. Of note, HEK STING CX43/45^{DKO} cell line 2 harbours an in-frame deletion for CX43 (−12 bp) and for CX45 (−18 bp), which probably accounts for the faint signal observed in the immunoblot (asterisk). Data are representative of three independent experiments.

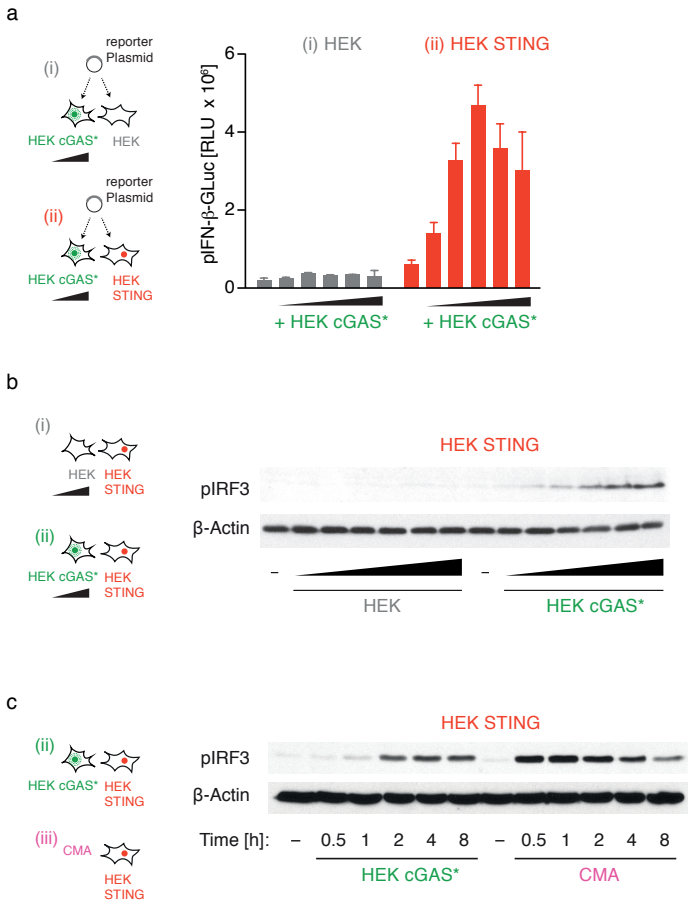
Supplementary Figure 8: Scrape loading of cGAMP(2'-5') into HEK STING CX43/45WT and CX43/45DKO cells and overexpression of distinct connexin members in HEK STING CX43/45DKO cells. a, Fluorescence images of HEK STING CX43/45^{WT} and CX43/45^{DKO} cells (STING in red) wounded and overlaid with cGAMP(2'-5'). Wounded cells without addition of cGAMP(2'-5') served as controls. Dashed line outlines the scratch margins. Representative images of n = 2 experiments are shown. **b**, Fluorescence images of HEK STING CX43/45^{DKO} co-cultured with HEK cGAS* and transfected with empty vector (pCI) and distinct members of human or murine connexins as indicated are depicted (pCI, CMA and mmCx45 as depicted in Fig. 4 are shown). Multimerization of STING was visualized 20 h after transfection. CMA stimulation for 8 h served as positive control. Representative images of n = 2 experiments are depicted.

Supplementary Figure 9: MVA-infected MEFs activate HEK STING cells in trans in a gap-junction dependent fashion. a, Schematic view of the experimental set-up for **b**, **c**: MEFs were infected with MVA–GFP for 3 h, washed three times and then loaded onto HEK cells or HEK STING cells that were then incubated overnight. Subsequently, human IFN-β expression was analysed by qPCR. **b**, **c**, A representative experiment with a titration of MVA–GFP (1.6×10^6, 0.8×10^6 and 0.16×10^6 virus particles per ml) is depicted (**b**) and mean values and s.e.m. of three independent experiments at a concentration of 1.6×10^6 virus particles/ml are shown (**c**). **d**, Experiments were conducted as in **b**, now using HEK STING CX43/CX45^{WT} and HEK STING CX43/CX45^{DKO} cell lines as recipient cells. One representative experiment out of two independent experiments using 3.2×10^6 and 1.6×10^6 virus particles per ml is depicted in **d**.

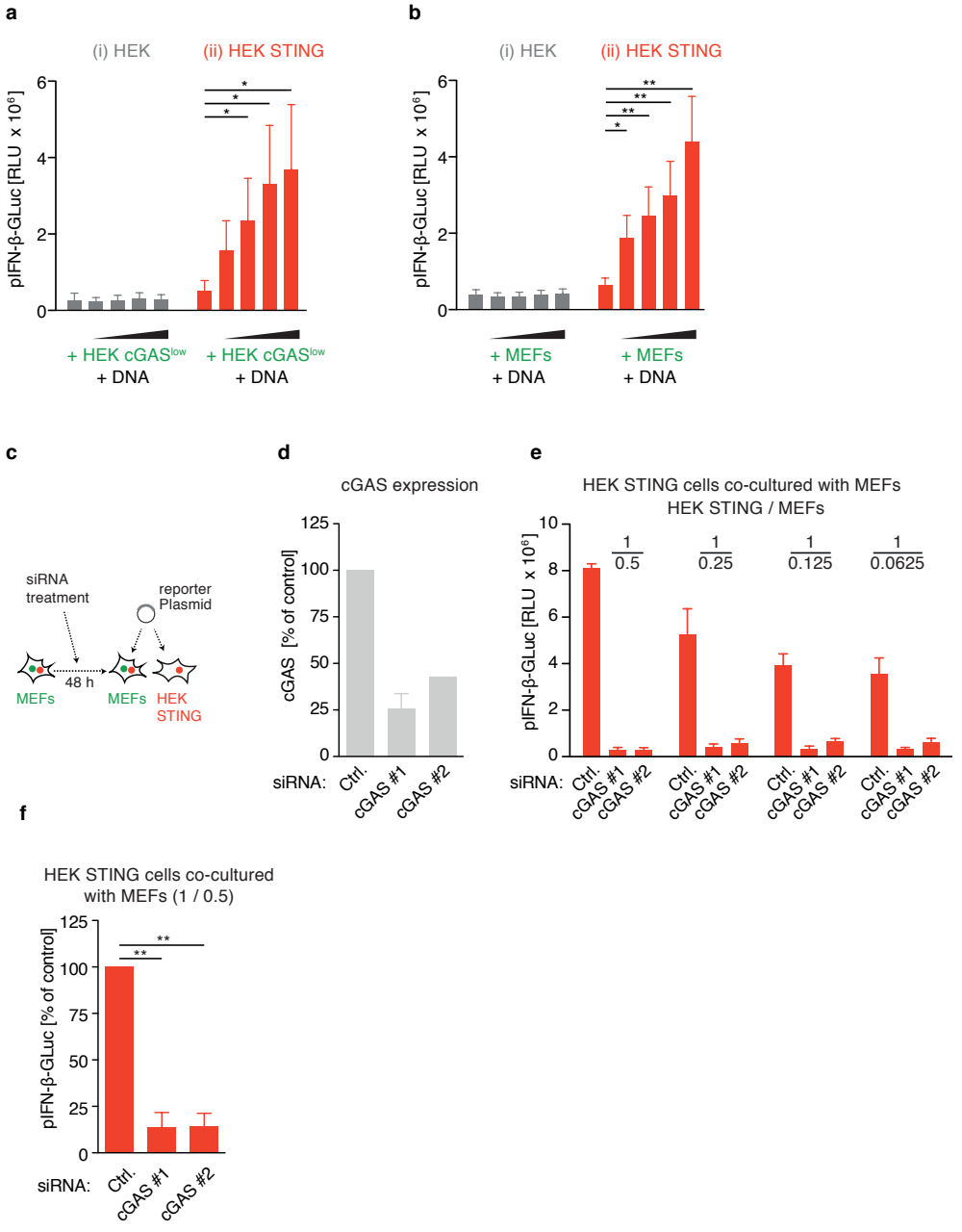
Supplementary Figure 10: Schematic model of the mechanism of gap-junction-mediated local immune collaboration. On infection with a DNA virus, a cell senses the presence of cytosolic viral DNA through the receptor cGAS, thus activating the synthesis of the second messenger cGAMP(2'-5'). cGAMP(2'-5') can pass through gap junctions into the cytosol of neighbouring cells, where it is detected by STING. The subsequent induction of an antiviral transcriptional program thus protects bystander cells from viral infection after the virus has successfully replicated in the cell initially infected.

Supplementary Video 1: HEK cGAS* cells spread STING activating signals to bystander cells. HEK cGAS* cells loaded with calcein (in green) were added onto HEK STING cells (STING in red) and dye transfer and STING multimerization were continuously analyzed by confocal fluorescence microscopy for up to 210 min. Suppl. Video 1 shows the complete visual field.

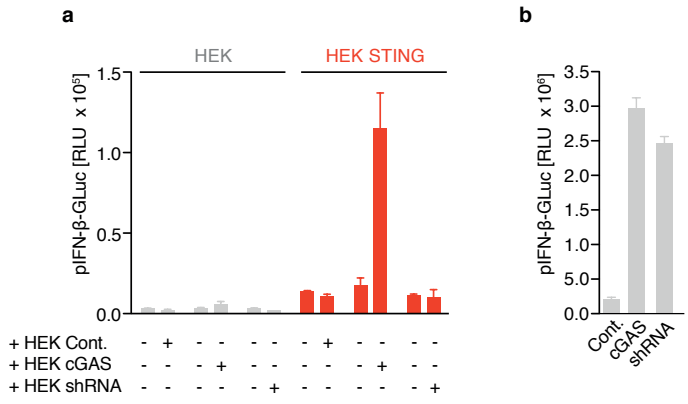
Supplementary Video 2: HEK cGAS* cells spread STING activating signals to bystander cells. HEK cGAS* cells loaded with calcein (in green) were added onto HEK STING cells (STING in red) and dye transfer and STING multimerization were continuously analyzed by confocal fluorescence microscopy for up to 210 min. Suppl. Video 2 highlights one particular region in higher resolution.



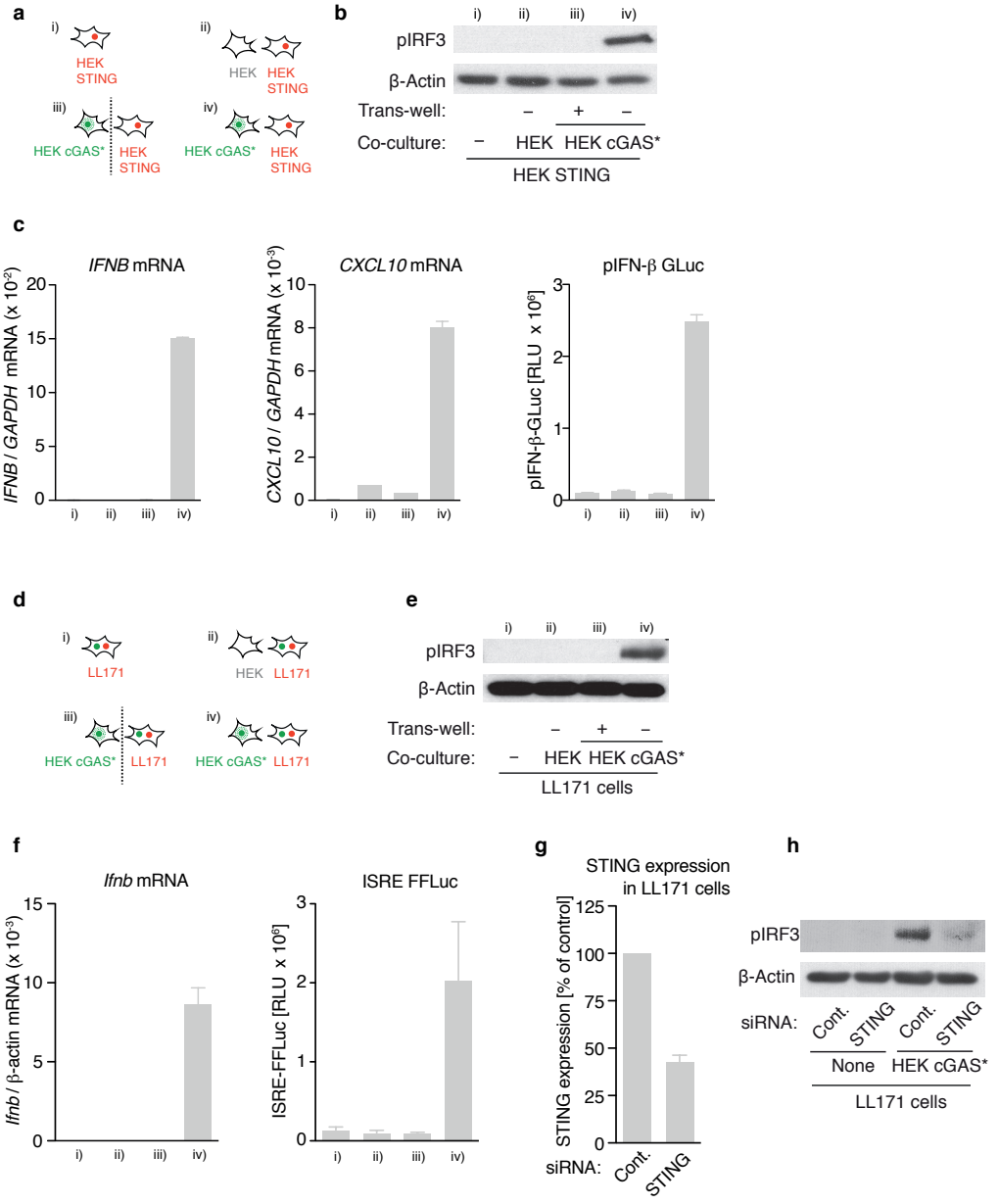
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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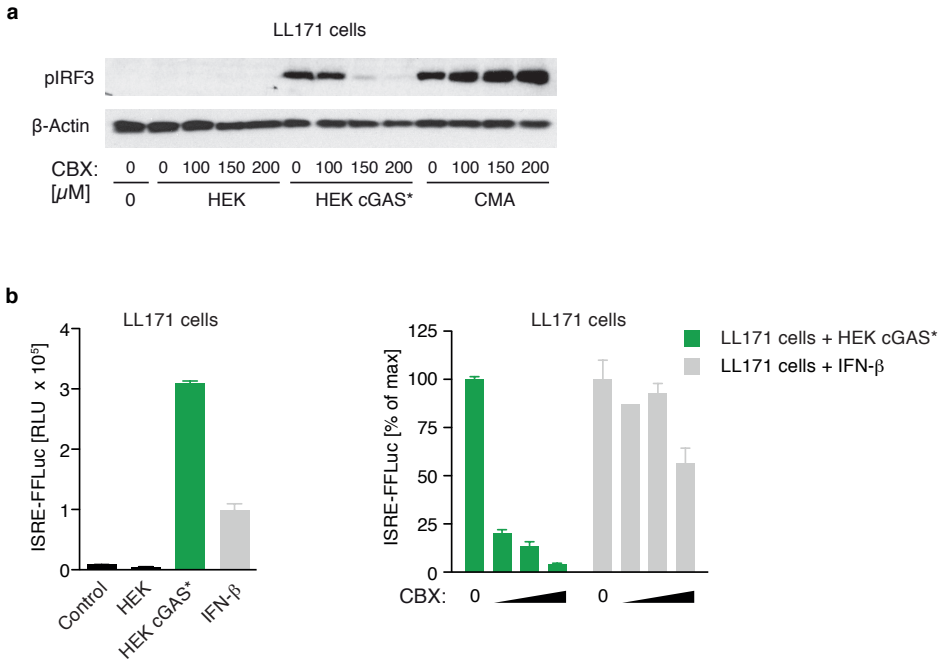
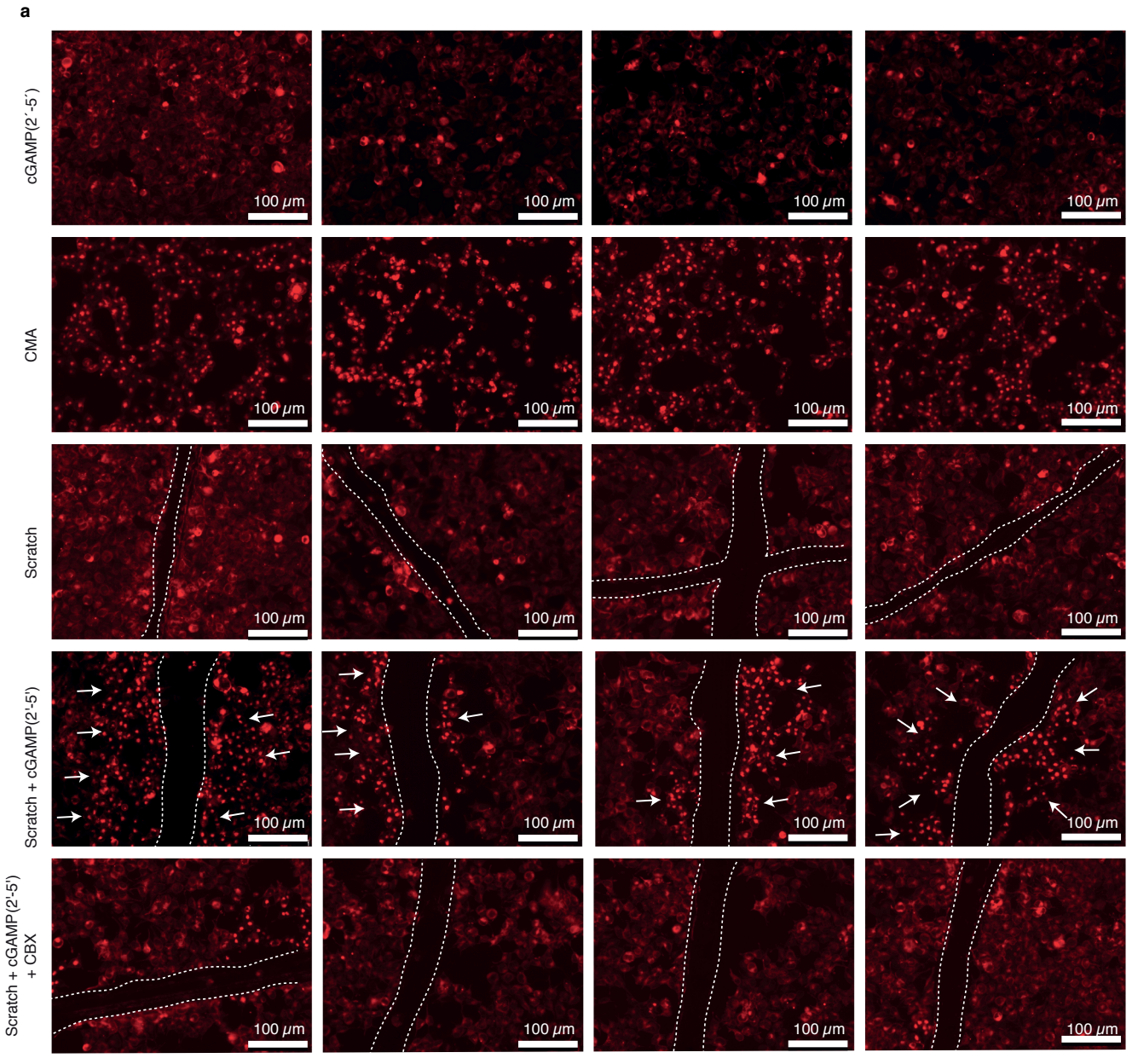
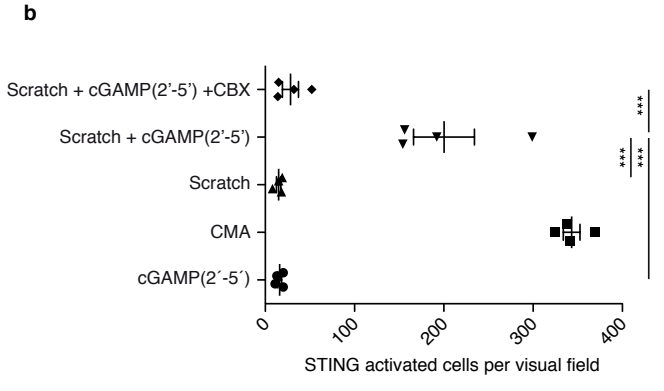
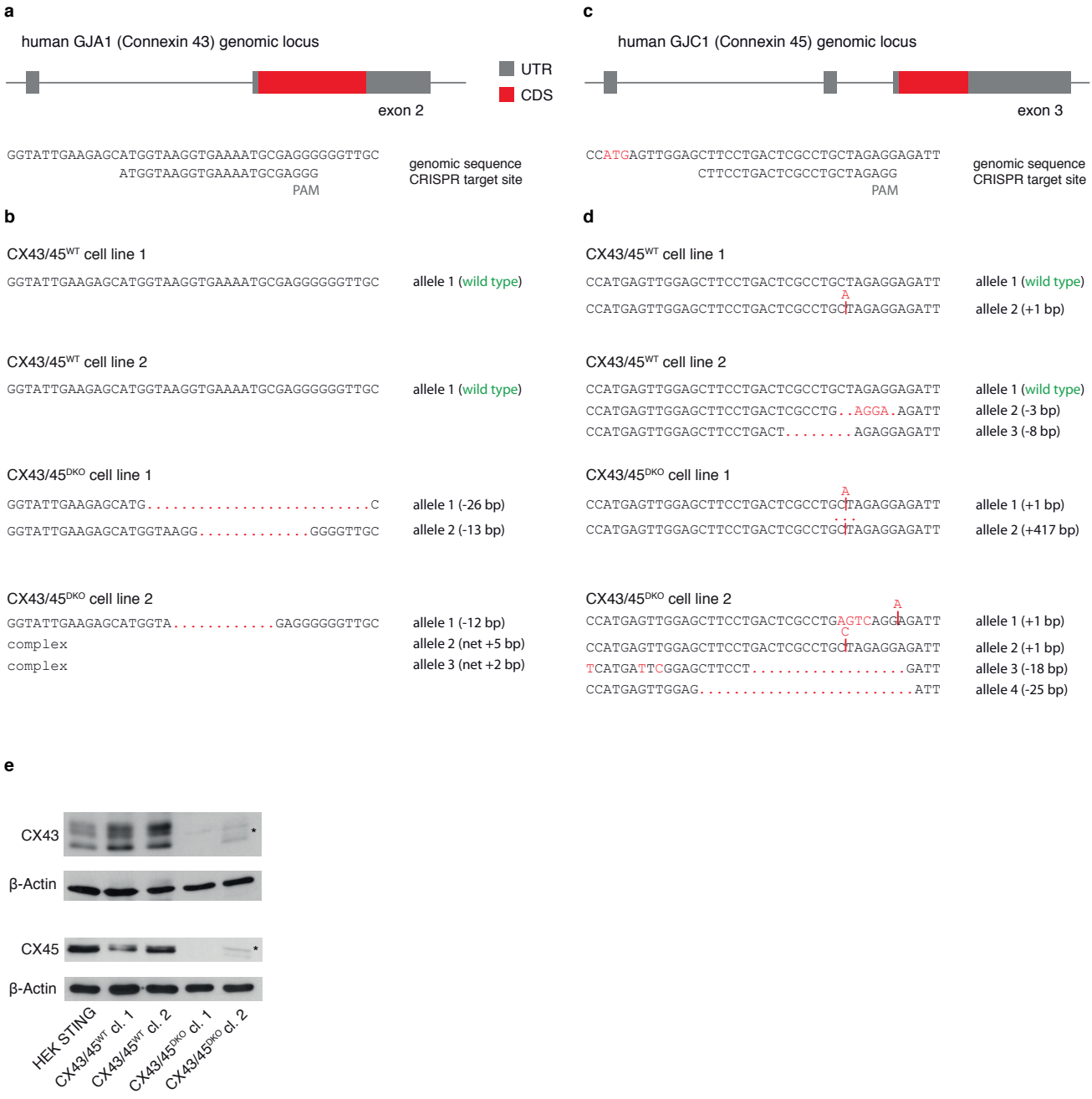


Supplementary Figure 5

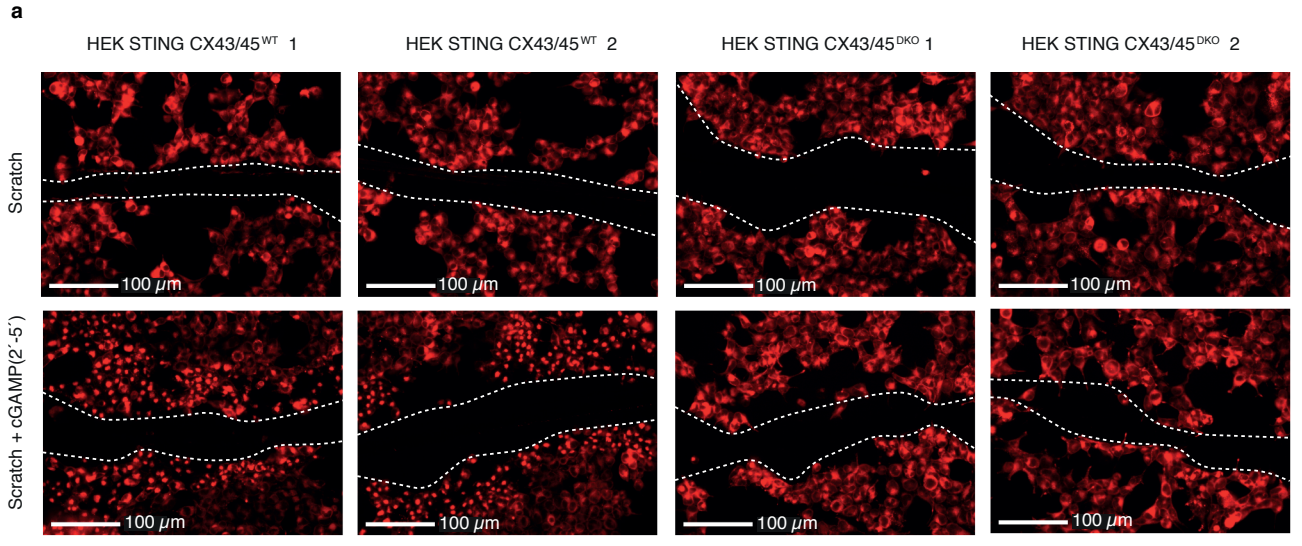


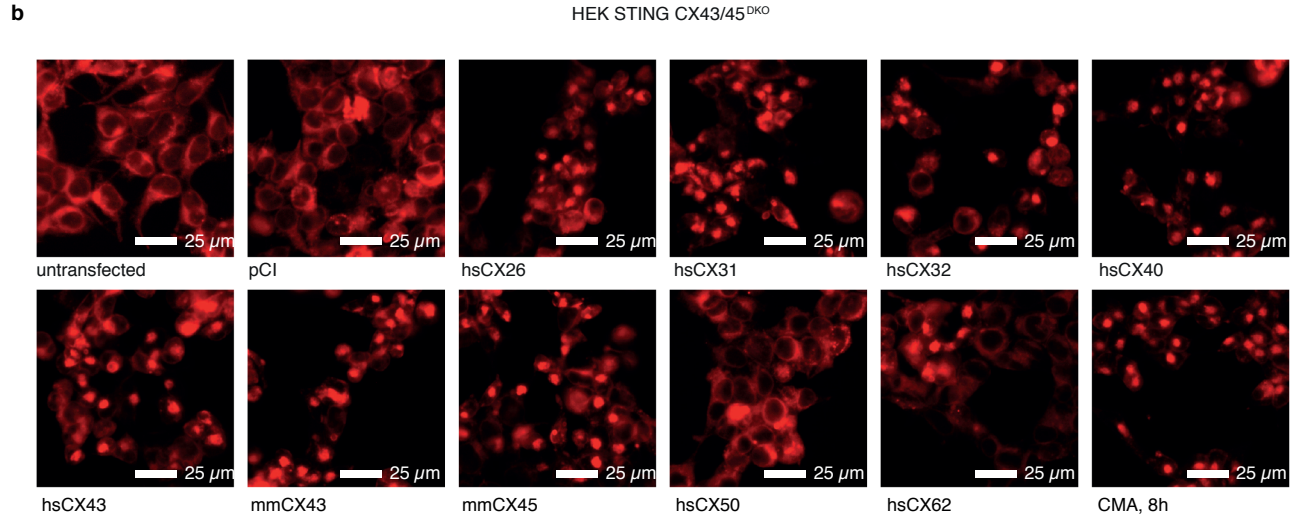


Supplementary Figure 6

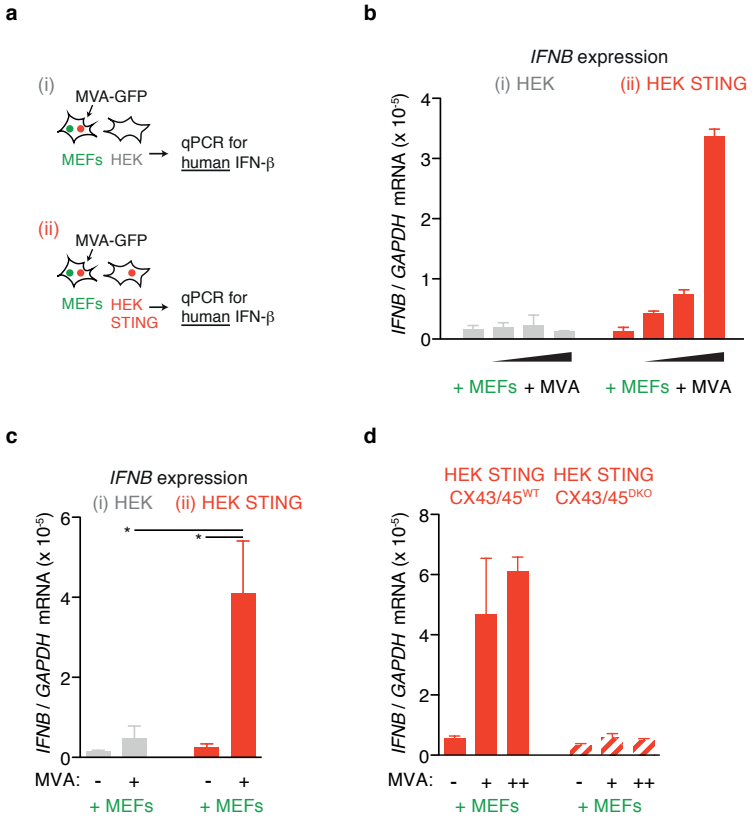


Supplementary Figure 7

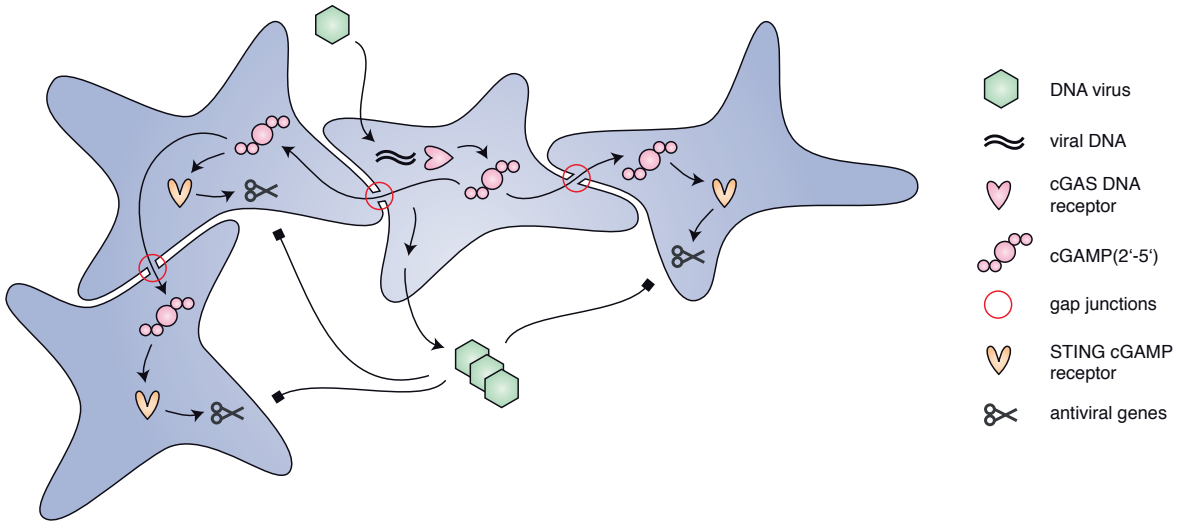




Supplementary Figure 8



Supplementary Figure 9



Supplementary Figure 10