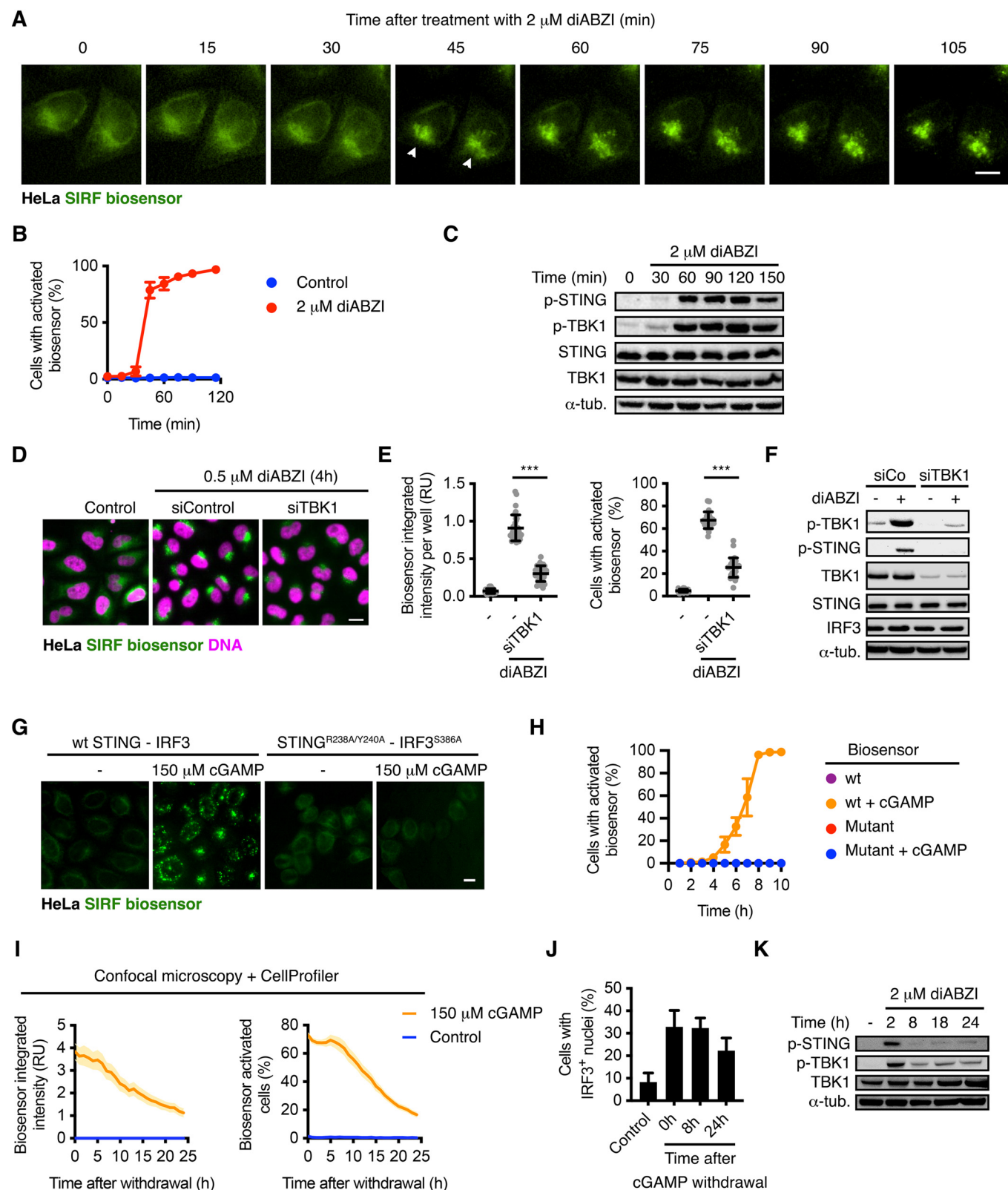


Expanded View Figures

Figure EV1. STING signalling analyses in HEK293T, HFF1 and HeLa cells.

(A) PiggyBAC construct containing the SIRF biosensor. (B) Western blots of wt and SIRF biosensor HeLa cells treated as indicated. Note that the samples of HeLa biosensor cells correspond to the same experiment shown in Fig. 1C. (C) qRT-PCR analyses of wt and SIRF biosensor HeLa cells treated as indicated and represent as mean $\pm$ SD of 7-8 biological replicates. (D, E) Immunofluorescence analyses of wt HeLa cells treated as indicated. (F) Live cell confocal imaging analyses of HEK293T and HFF-1 cGAMP-biosensor cells treated with 2 μ M diABZI. Data in the mean $\pm$ SEM of relative fluorescence (compared to $t = 0$) of 20 randomly selected cells per condition. (G) IFN β -Luc reporter assays in HEK293T cells transfected with empty plasmid or wt STING, or HEK293T cGAMP-biosensor cells, and treated with the indicated concentrations of the STING agonist diABZI, and represent as mean $\pm$ SD of 4 biological replicates. Note that HEK293T do not express endogenous cGAS or STING as shown in (H). (H) Western blots of wt HEK293T and HeLa cells. Scale bars = 10 μ m. Source data are available online for this figure.





◀ **Figure EV2. S1RF biosensor activation dynamics and requirements.**

(A, B) Live cell confocal imaging analyses of HeLa S1RF biosensor cells in the presence or absence of 2 μ M diABZI. In (A), arrows indicate the initial clustering of the biosensor. In (B), activation of the biosensor was scored as clustering of the GFP signal in 1 or more puncta, and it is represented as mean $\pm$ SEM of $N = 3$ independent time-lapses with >200 cells per condition. (C) Western blots of wt HeLa cells treated as in (A, B). (D, E) Immunofluorescence analyses of HeLa S1RF biosensor cells transfected with the indicated siRNAs and treated as indicated for 4 h. Note that cells were imaged in a 384x high content microscope prepared for screening and analysed using CellProfiler. Data represents mean $\pm$ SD of $N = 32$ different samples per condition, each analysed for % of active cells or integrated intensity (RU) using CellProfiler. In (E), P -values from one-way ANOVA between the indicated groups with $N = 32$ biological replicates are indicated as *** $P < 0.0001$ in both cases. (F) Western blots of HeLa cells treated as in (D, E). siCo, unrelated control siRNA siLRP6. (G, H) Live cell confocal imaging analyses of S1RF biosensor (wt) and STING(R238A/Y240A)-P2A-IRF3(S386A) (mutant) HeLa cells in the presence or absence of 150 μ M cGAMP. In (G) activation of the biosensor was scored as clustering of the GFP signal in 1 or more puncta, and it is represented as mean $\pm$ SEM of $N = 3$ independent time-lapses with >200 cells per condition. (I) CellProfiler analyses of HeLa S1RF biosensor H2B-mCherry cells after live cell confocal imaging following cGAMP withdrawal (20-hour pre-treatment) or untreated. Data represent mean $\pm$ SEM of 8 different parallel time-lapses per condition each automatically analysed and with >200 cells. (J) Immunofluorescence analyses of wt HeLa cells after cGAMP withdrawal (20-hour pre-treatment) represented as mean $\pm$ SD of $N = 3$ biological replicates with >100 cells per condition (K) Western blots of wt HeLa cells treated as indicated. Scale bars = 10 μ m. Source data are available online for this figure.

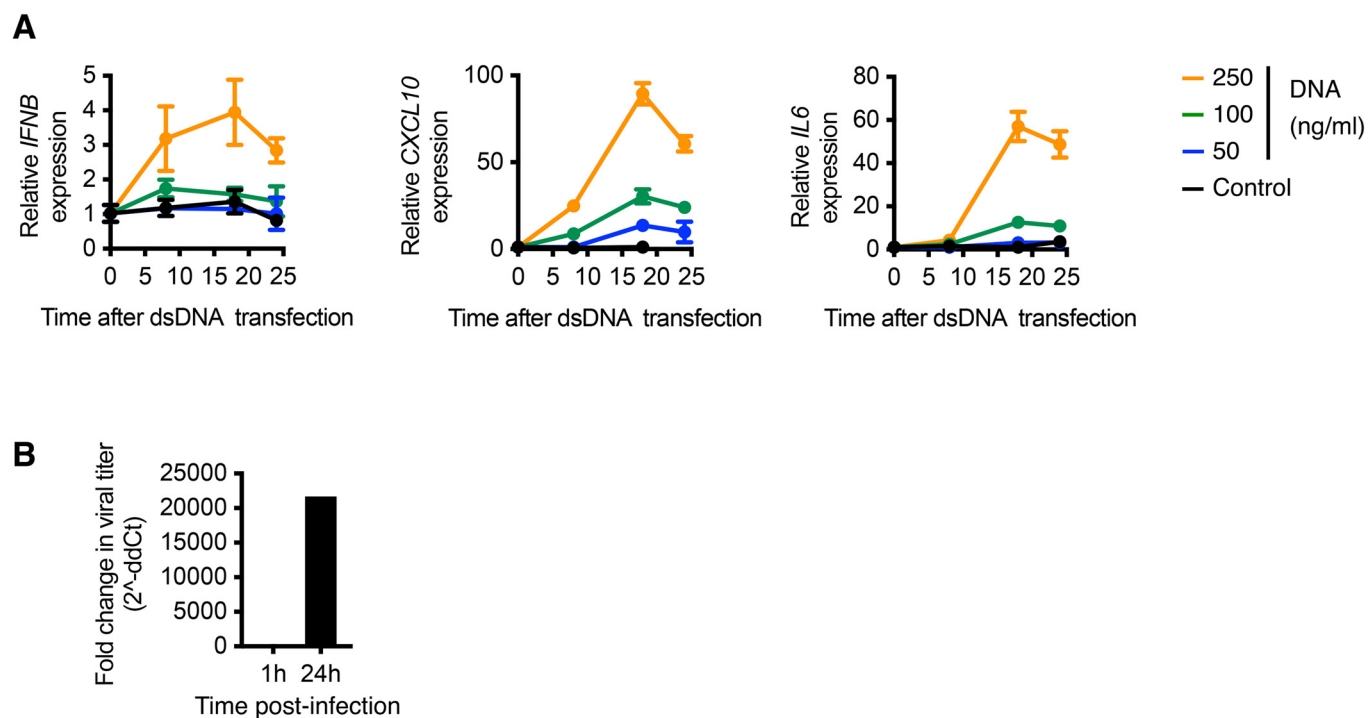
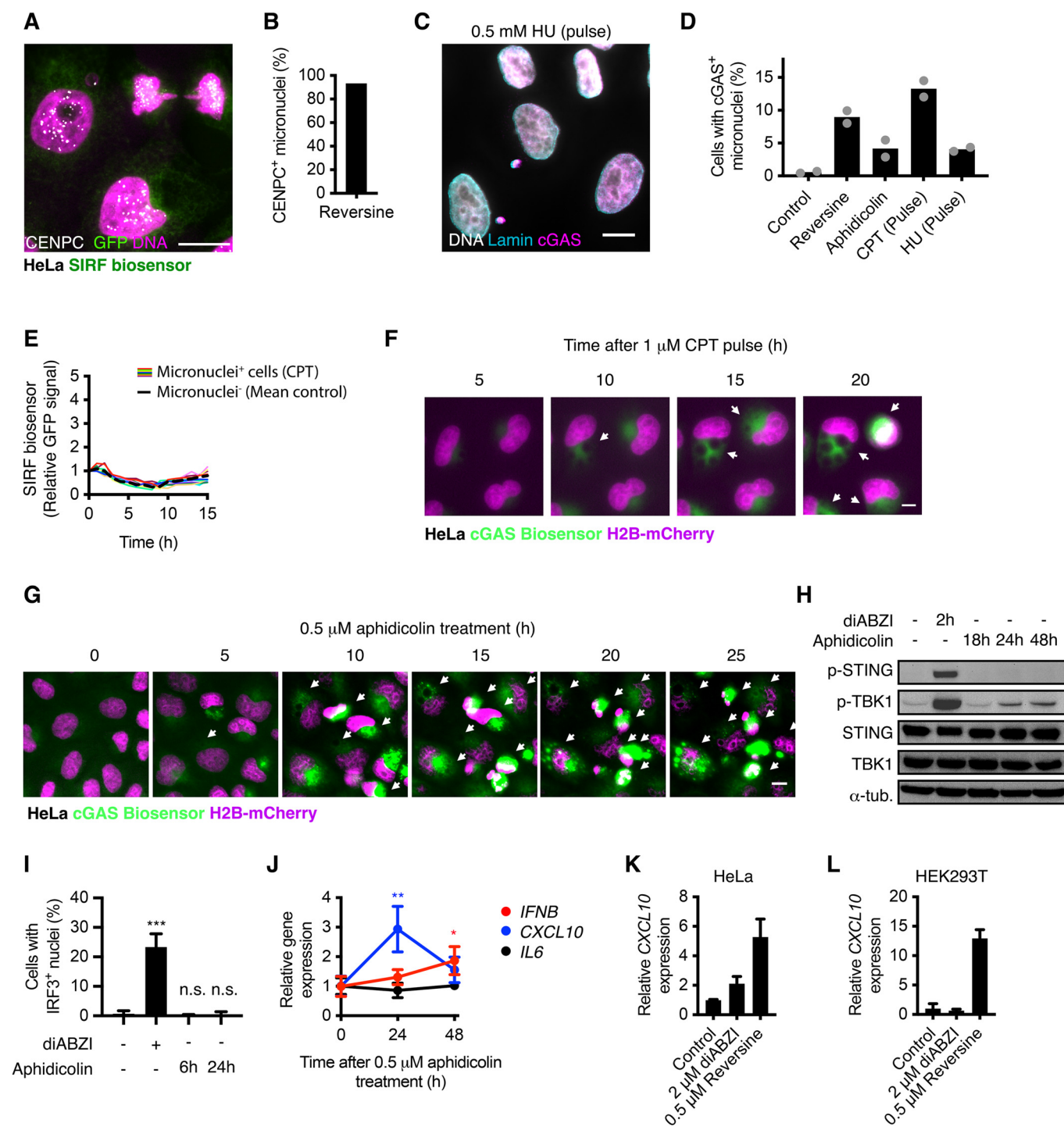


Figure EV3. Innate immune response to foreign dsDNA and dsRNA in HeLa cells.

(A) qRT-PCR analyses of wt HeLa cells treated as indicated and represented as mean $\pm$ SD of $N = 3$ biological replicates. (B) qRT-PCR analyses of influenza A viral RNA in infected HeLa SIRF biosensor cells, as indicated, and represented as mean of a representative experiment from $N = 3$ independent experiments. Source data are available online for this figure.



◀ **Figure EV4. Characterisation of Micronuclei and replicative stress in HeLa cells.**

(A, B) Immunofluorescence experiments in HeLa SIRC biosensor cells treated with 0.5 μ M reversine for 18 h and stained as indicated. (C, D) Immunofluorescence analyses of HeLa cells treated with 2 μ M diABZI for 4 h, with 0.5 μ M reversine or 50 nM aphidicolin for 18 h, or pulsed for 4 h with 0.5 μ M CPT or 0.5 mM HU followed by 18 h release. CPT, Topoisomerase inhibitor Camptothecin; HU, Hydroxyurea. In (D), data is mean of $N = 2$ independent experiments each with >200 cells per condition. (E, F) Live cell imaging analyses of HeLa SIRC biosensor H2B-mCherry cells pre-treated with a pulse of 0.5 μ M CPT for 4 h and released for 18 h. In (E), single cells with micronuclei at the beginning of the recording were monitored for 15 h and are compared to the mean of control cells (dashed line). In (F), cells showing clear signs of autophagy and cell death are indicated with arrows. (G) Live cell imaging analyses of HeLa SIRC biosensor H2B-mCherry cells treated with 0.5 μ M aphidicolin (DNA polymerase inhibitor). Cells showing clear signs of autophagy and cell death are indicated with arrows. (H) Western blots of wt HeLa cells treated as indicated. (I) Immunofluorescence analyses of wt HeLa cells treated as indicated. Data represents mean $\pm$ SD of $N = 4$ biological replicates with P -values from one-way ANOVA between from the indicated groups are indicated as *** $P < 0.0001$, or not significant (n.s.) from left to right as $P = 0.983$, $P = 0.999$. (J, K) qRT-PCR analyses of wt HeLa cells treated as indicated. Data represents mean $\pm$ SD of $N = 3$ biological replicates. P -values from one-way ANOVA compared to control for each gene are indicated as ** $P = 0.0087$ for *CXCL10* and * $P = 0.049$ for *IFNB*. In (K), cells were treated with diABZI for 4 h or with reversine for 24 h. (L) qRT-PCR analyses of wt HEK29T cells treated with diABZI for 4 h or with reversine for 48 h. Data represents mean $\pm$ SD of $N = 3$ biological replicates. Scale bars = 10 μ m. Source data are available online for this figure.

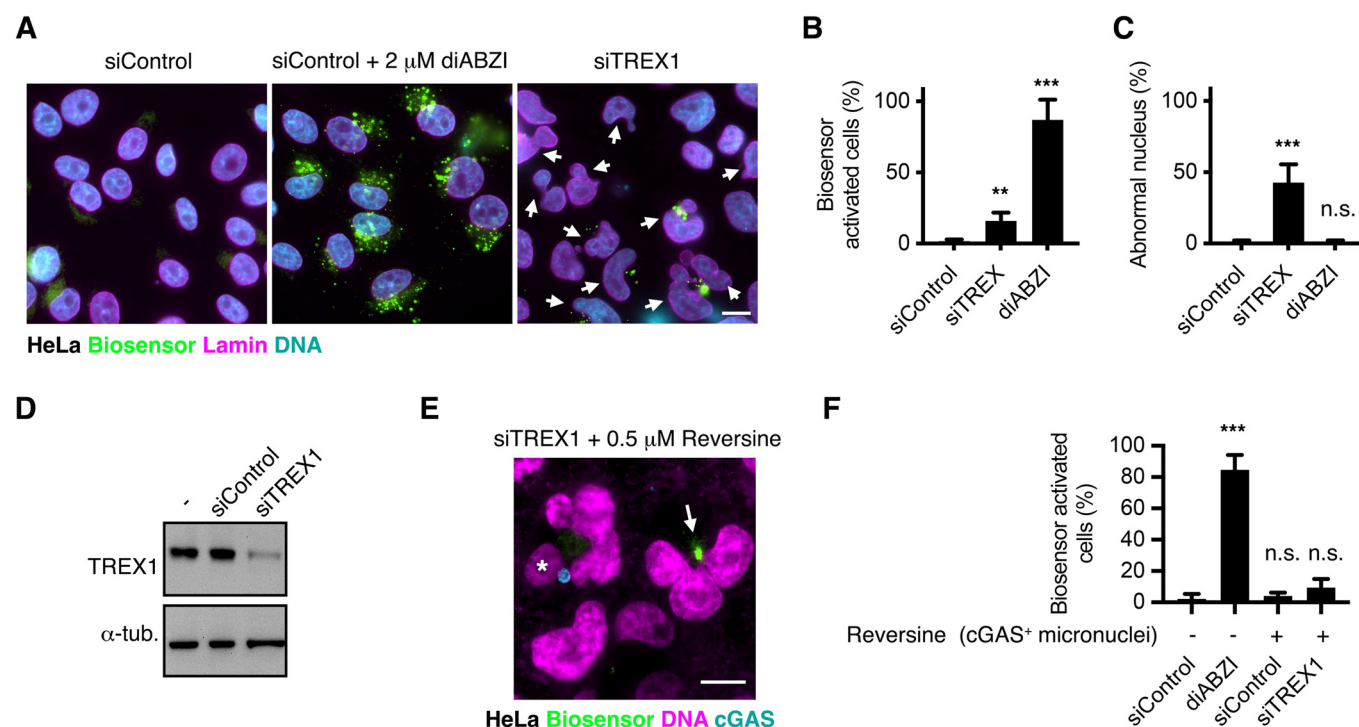


Figure EV5. SIRF biosensor activation upon TREX1 knock down.

(A–C) Immunofluorescence analyses of HeLa SIRF biosensor cells transfected with the indicated siRNAs for 72 h, or treated with 2 μ M diABZI for 4 h. In (C), abnormal nuclear shape was scored in cells showing similar morphology as those pointed in (A). Data represents mean $\pm$ SD of $N = 8$ –10 biological replicates with at least 25 cells per sample. P -values from one-way ANOVA between the indicated groups are indicated in (B) as $**P = 0.0035$, and $***P < 0.0001$, and in (C) as $***P < 0.0001$ and $n.s. = 0.9996$. (D) Western blots of HeLa cells transfected as in (A–F). (E, F) Immunofluorescence analyses of HeLa SIRF biosensor cells transfected with the indicated siRNAs for 72 h, or treated with either 2 μ M diABZI for 4 h or 0.5 μ M reversin for 18 h. Note that reversine treated siTREX1 cGAS⁺ micronuclei cells (F) show less percentage of activation compared to cells only transfected with siTREX1 (B) suggesting that active biosensor in siTREX1 concentrate in cells with nuclear shape abnormalities and/or division failure. In (F), Data represents mean $\pm$ SD of $N = 3$ independent experiments. P -values from one-way ANOVA compared to control are indicated as $***P < 0.0001$ and $n.s. = 0.94$ and 0.35 , respectively. In (A, E) arrows indicate active biosensor upon siTREX1, while an asterisk marks a cGAS⁺ micronucleus. Scale bars = 10 μ m. Source data are available online for this figure.