EXTENDED EXPERIMENTAL PROCEDURES

RNA, cDNA synthesis, and quantitative RT-PCR

RNA from mammalian cell lines was extracted using Trizol reagent (Invitrogen) or RNeasy Mini Kit (Qiagen). RNA was treated with RQ1 RNase-free DNase (Promega). RNA was reverse transcribed with Superscript III (Invitrogen). Mouse *ifnB* was quantified relative to mouse *rps17* as described previously (*26*). Human *ifnB* was quantified relative to human *S9* as described previously (*27*).

Protein purifications

WspR construct (pQE-WspR*) was a generous gift from Steve Lory (Harvard). WspR purification and c-di-GMP synthesis reactions were carried out as previously described (*28*). Overexpression strains and plasmids for DncV and mutant DncV were provided by J. Mekalanos. DncV protein was overexpressed and purified as previously described (*22*). Briefly, DncV protein production was induced in mid-log phase for 3h at 37° C with 1mM IPTG. Cells were lysed and DncV protein was purified under denaturing conditions. Cleared lysate was incubated with Ni-NTA and eluted in Urea Elution buffer (2M Urea, 10mM Tris pH=8.0, 150mM NaCl, 250mM Imidazole). Eluted protein was dialyzed to 25mM Tris-Cl, pH=7.5, 300mM NaCl, 5mM $Mg(OAc)_2$, 10% glycerol, 2mM DTT. H6SUMO-mcGAS was expressed in Rosetta(DE3) pLysS cells by overnight induction with 0.5mM IPTG at 18° C. Cells were lysed into 50mM Tris-Cl, pH=8, 300 mM NaCl, 20mM Imidazole, 5mM BME and 0.2mM PMSF by French Press. Cleared lysate was incubated with Ni-NTA and bound protein was eluted with 20mM Tris-Cl, pH=7.4, 150mM NaCl, 300mM Imidazole. Eluant was dialyzed to 20mM Tris-

Cl, pH=7.4, 150mM NaCl, 5mM β-mercaptoethanol with 10% glycerol. Protein was flash frozen and stored at -80°C.

Nuclease Digests

Nuclease P1from *Penicillium citrinum* and Snake venom phosphodiesterase I from *Crotalus adamanteus* were purchased from Sigma. Reactions from *in vitro* cyclic-di-nucleotide synthesis labeled with α^{32}P-GTP were diluted 1:5 in either P1 buffer (40mM Tris-Cl, pH=6, 2mM $ZnCl_2$) or SVPD buffer (40mM Tris-Cl, pH=8, 10mM $MgCl_2$) followed by digestion with 2.5mU of nuclease P1 or SVPD, respectively. Digestions were incubated for 45 minutes at 37° C and nucleotide products were resolved by TLC.

EXTENDED DISCUSSION OF THE NMR DATA

In the ^{1}H-^{31}P HMBC spectrum shown in Figure 3C, the phosphorous nucleus, P-11, is correlated to the 2′ ribose proton (H-12) of guanosine as well as to the 5′ ribose methylene protons (H-10) and the 4′ ribose proton (H-9) of adenosine. The other phosphorous nucleus (P-22) is correlated to the 3′ ribose proton (H-8) of adenosine as well as to the 5′ ribose methylene protons (H-21) and 4′ ribose proton (H-20) of guanosine. Thus, the regiochemistry of the phosphodiester linkages was determined to be cyclic[G(2′-5′)pA(3′-5′)p].

In order to assign the above peaks, it was critical to accurately identify the ribose spin systems corresponding to guanosine and adenosine, respectively. The protons corresponding to the adenine nucleobase (H-2, H-5) and guanine nucleobase (H-17) were assigned based upon reference spectra for the individual nucleobases, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC NMR (Figure S4A and S4B). The ^{1}H-^{1}H NOESY experiment showed through-space interactions

between the adenine proton H-5 and the 3' ribose proton (H-8) as well as between the guanine proton H-17 and the 1' ribose proton (H-18) (Figure S4D). The remaining protons in the corresponding ribose spin systems were identified by ^{1}H-^{1}H COSY (Figure S4C), and multiplicity edited ^{1}H-^{13}C HSQC (Figure S4A and S4B), which distinguished the 5' methylene protons in particular (H-10 and H-21).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Sequence alignment of hSTING variants, related to Figure 1. hSTING was cloned from THP-1 cells compared to the reference STING allele (NCBI NP_938023.1).

Supplementary Figure S2. R232 of human STING is required for responsiveness to c-di-GMP, but not for binding of c-di-GMP; related to Figure 2. (**A**) 293T cells were transfected with the indicated alleles of mouse (m)STING or human (h)STING and were then stimulated with c-di-GMP (cdG). STING activity was detected by the induction of a co-transfected IFN-luciferase reporter construct and expressed as fold-induction over luciferase activity of unstimulated cells. (**B**) Lysates of transfected 293T cells were UV crosslinked in the presence of α^{32}P-c-di-GMP, resolved by SDS-PAGE, and then analyzed by autoradiography. Lysates were also western blotted for STING and ACTIN as expression controls in parallel.

Supplementary Figure S3. G230A and H232R are both required for optimal responsiveness to c-di-nucleotides but are not required for binding to c-di-nucleotides; related to Figure 3. (**A, B**) 293T cells were transfected with the indicated alleles of mouse (m)STING or human (h)STING and were then stimulated with c-di-GMP (cdG). STING activity was detected by the induction of a co-transfected IFN-luciferase reporter construct.

Supplementary Figure S4. Additional NMR analysis of the cGAS product; related to Figure 4. All data acquisition was performed in D_2O and at 50°C. (**A, B**) Multiplicity-edited ^{1}H-^{13}C HSQC experiment in a 900 MHz field. Positive phased signals corresponding to methine and methyl protons are shown in green, negative phased signals corresponding to methylene protons are shown in blue. (**C**) ^{1}H-^{1}H COSY experiment in a 600 MHz field. (**D**) ^{1}H-^{1}H NOESY experiment in a 900 MHz field.

Figure S1

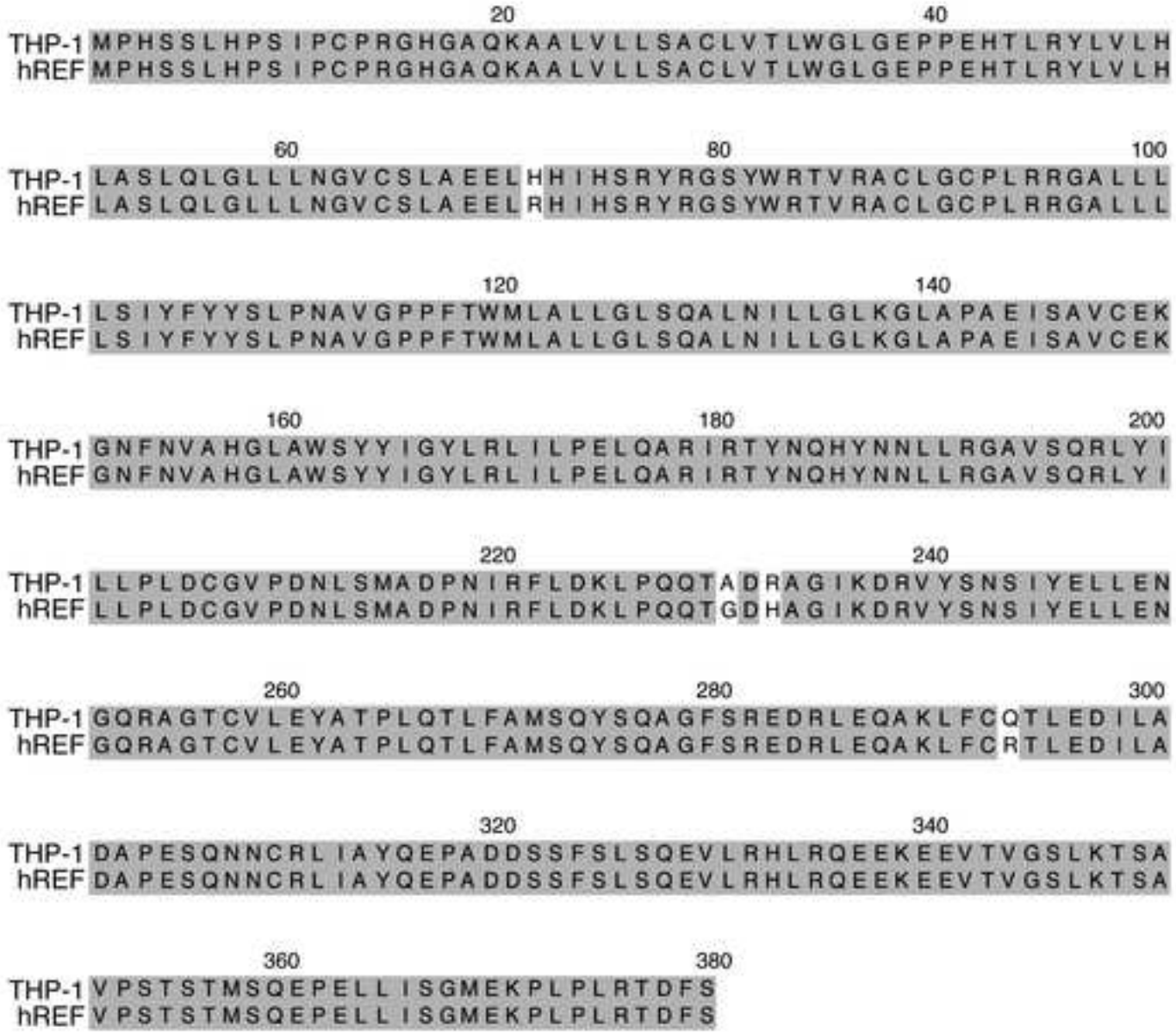
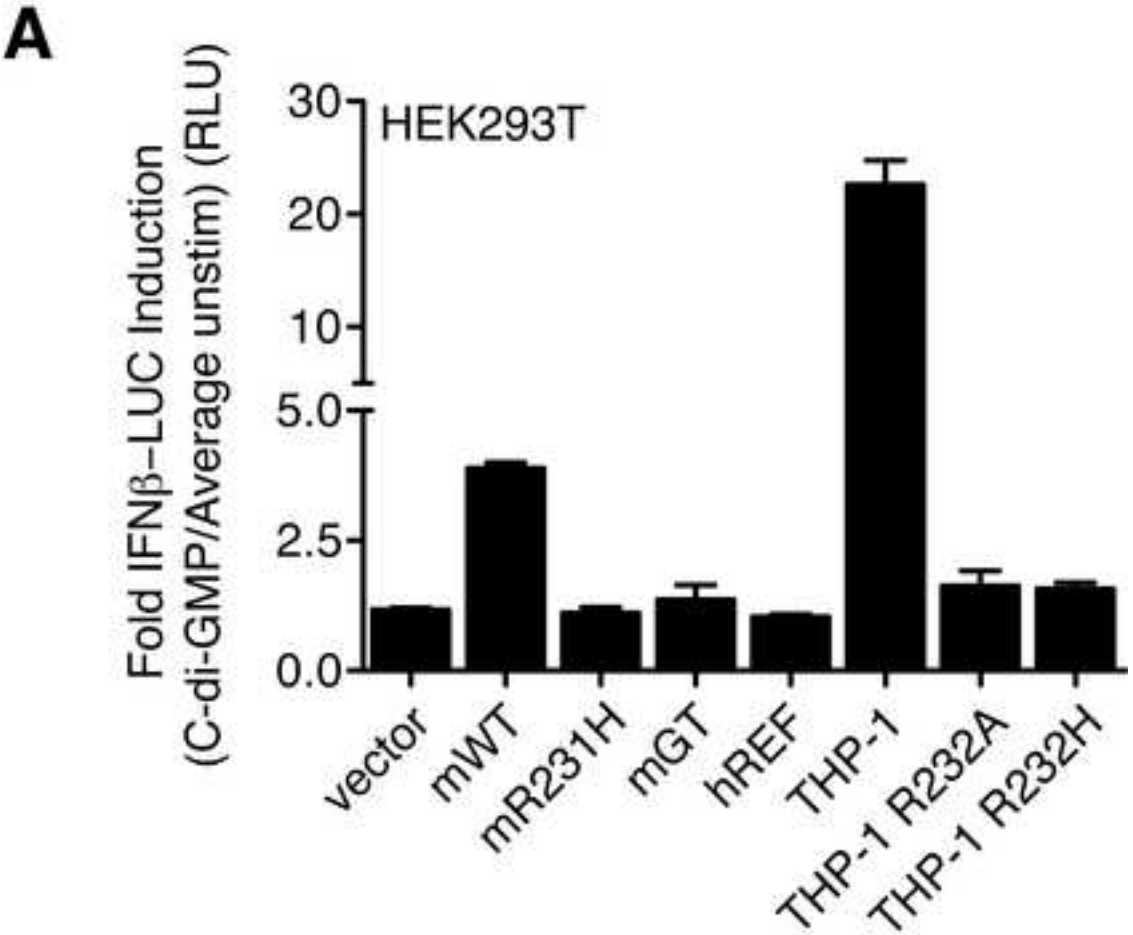

20
40
THP-1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH
hREF MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH
60
80
100
THP-1 LASLQLGLLLNGVCSLAEELHHIHSRYRGSYWRTVRACLGCPLRRGALLL
hREF LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL
120
140
THP-1 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK
hREF LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK
160
180
200
THP-1 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI
hREF GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI
220
240
THP-1 LLPLDCGVPDNLSMADPNIRFLDKLPQQTADRAGIKDRVYSNSIYELLEN
hREF LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN
260
280
300
THP-1 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCQTLEDILA
hREF GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA
320
340
THP-1 DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA
hREF DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA
360
380
THP-1 VPSTSTMSQEPELLISGMEKPLPLRTDFS
hREF VPSTSTMSQEPELLISGMEKPLPLRTDFS

Figure S2


A
HEK293T
Fold IFNβ–LUC Induction
(C-di-GMP/Average unstim) (RLU)
30
20
10
5.0
2.5
0.0
vector
mWT
mR231H
mGT
hREF
THP-1
THP-1 R232A
THP-1 R232H

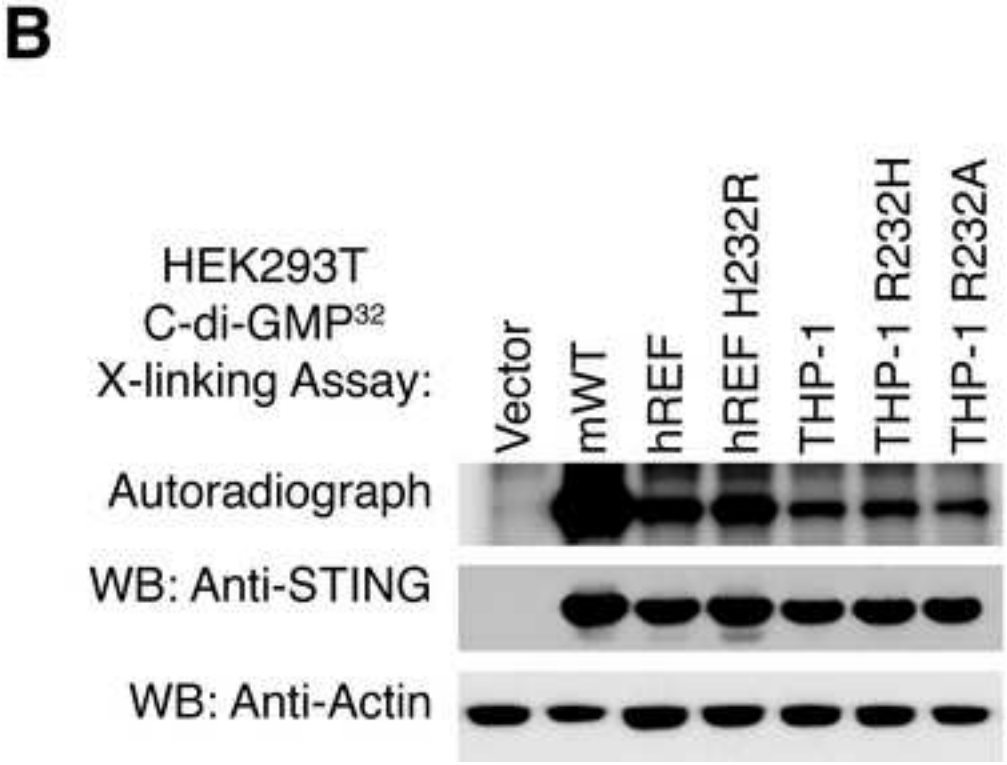

B
HEK293T
C-di-GMP[32]
X-linking Assay:
Vector
mWT
hREF
hREF H232R
THP-1
THP-1 R232H
THP-1 R232A
Autoradiograph
WB: Anti-STING
WB: Anti-Actin

Figure S3

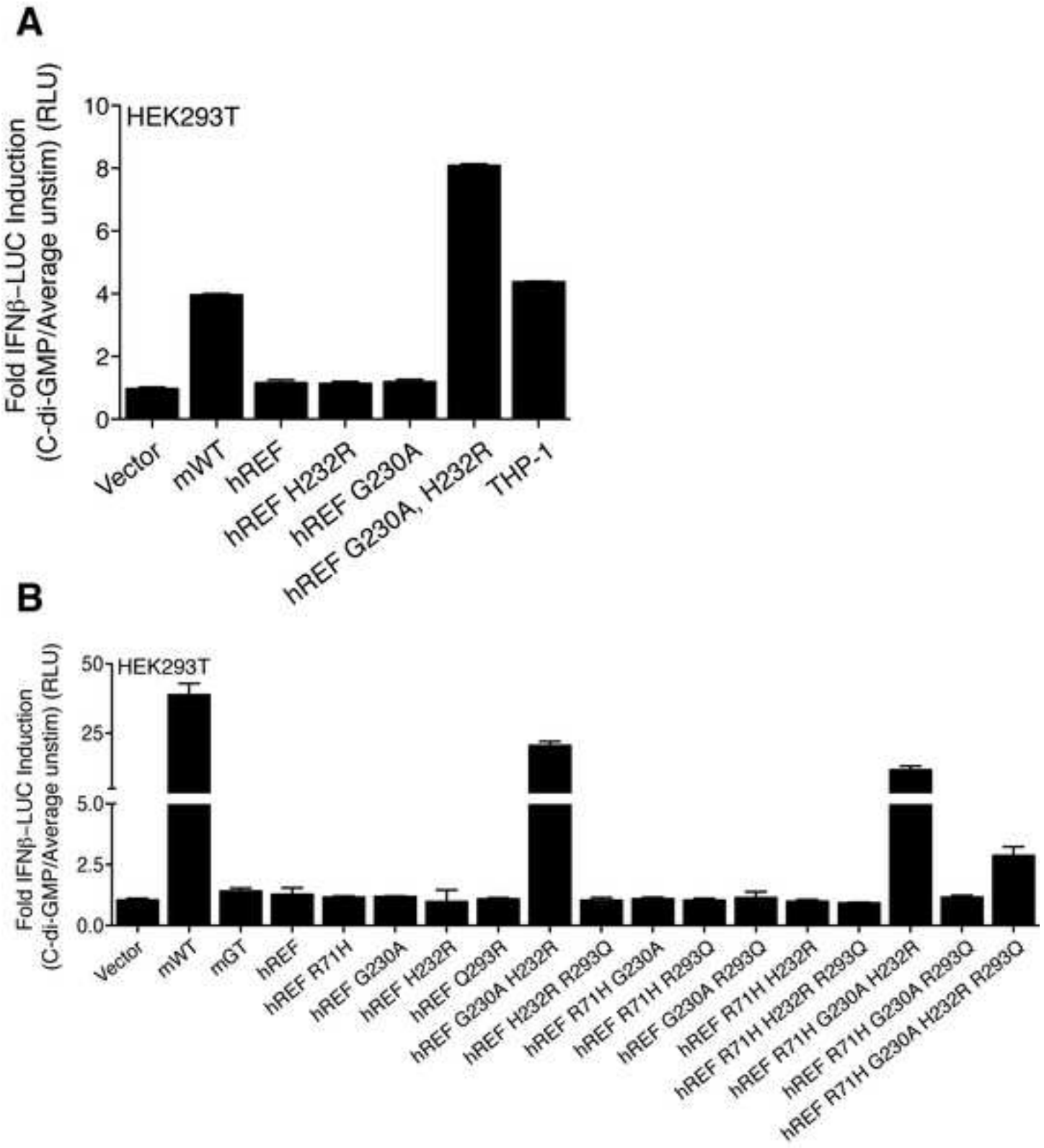

A
HEK293T
Fold IFNβ–LUC Induction
(C-di-GMP/Average unstim) (RLU)
10
8
6
4
2
0
Vector
mWT
hREF
hREF H232R
hREF G230A
hREF G230A, H232R
THP-1
B
HEK293T
Fold IFNβ–LUC Induction
(C-di-GMP/Average unstim) (RLU)
50
25
5.0
2.5
0.0
Vector
mWT
mGT
hREF
hREF R71H
hREF G230A
hREF H232R
hREF Q293R
hREF G230A H232R
hREF H232R R293Q
hREF R71H G230A
hREF R71H R293Q
hREF G230A R293Q
hREF R71H H232R
hREF R71H H232R R293Q
hREF R71H G230A H232R
hREF R71H G230A R293Q
hREF R71H G230A H232R R293Q

Figure S4