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Supplemental Information

Modular Architecture of the STING C-Terminal Tail Allows Interferon and NF-κB Signaling Adaptation

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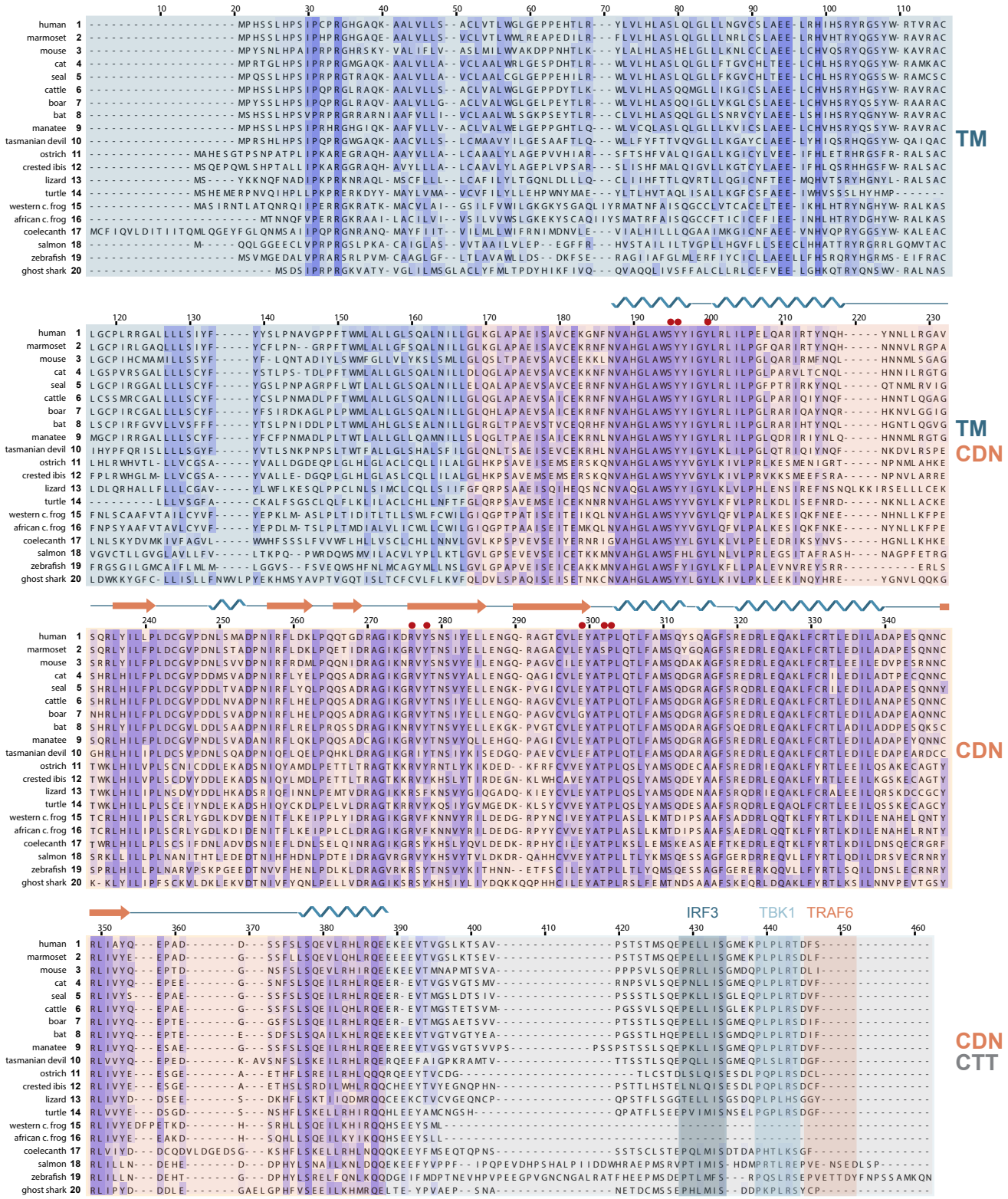


Figure S1. STING species alignment, Related to Figure 1

Alignment of representative vertebrate STING proteins selected for this study. Darker shadings indicate higher physiochemical conservation according to BLOSUM62 score using Jalview (Waterhouse et al., 2009). The STING protein regions are highlighted according to the transmembrane domain (TM, blue), cyclic dinucleotide binding domain (CDN, orange), and C-terminal tail (CTT, gray). Known secondary structure elements are shown on top for human STING. 2′3′cGAMP binding residues are marked with red dots and the three different CTT signaling motifs are depicted as IRF3 (dark blue), TBK1 (light blue) and TRAF6 (orange) binding sites.

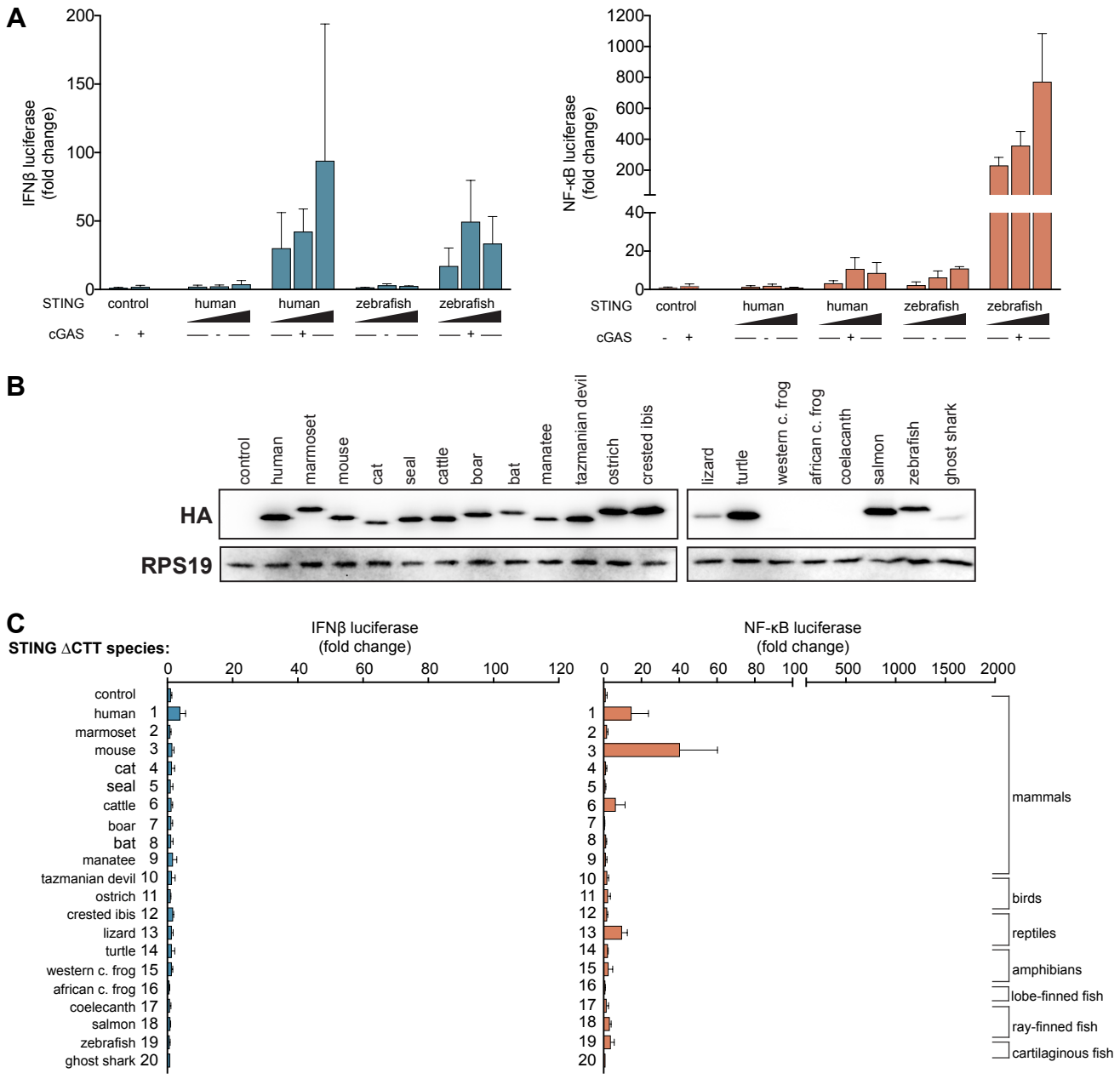


Figure S2. The STING CTT is required for signaling, Related to Figure 1

(A) Stimulation assays using the cellular reporter assay as in (Figure 1A). Increasing concentrations of cGAS plasmid were co-transfected with either human or zebrafish STING in HEK293T cells. The zebrafish STING-mediated increase in NF-κB signaling is cGAS-dependent. (B) Western blot analysis of expression levels of transfected HA-STING alleles in HEK293T cell reporter assays compared to RPS19 loading control. Data are representative of two independent experiments. (C) Cellular reporter assay as in (Figure 1A) including STING ΔCTT species alleles from 1- human (*homo sapiens*), 2- marmoset (*Callithrix jacchus*), 3- mouse (*Mus musculus*), 4- cat (*Felis catus*), 5- seal (*Leptonychotes weddellii*), 6- cattle (*Bos taurus*), 7- boar (*Sus scrofa*), 8- bat (*Rousettus aegyptiacus*), 9- manatee (*Trichechus manatus latirostris*), 10- tasmanian devil (*Sarcophilus harrisii*), 11- ostrich (*Struthio camelus australis*), 12- crested ibis (*Nipponia nippon*), 13- lizard (*Anolis carolinensis*), 14- turtle (*Chelonia mydas*), 15- western clawed frog (*Xenopus tropicalis*), 16- african clawed frog (*Xenopus laevis*), 17- coelacanth (*Latimeria chalumnae*), 18- salmon (*Salmo salar*), 19- zebrafish (*Danio rerio*), 20- ghost shark (*Callorhinchus milii*). Cellular reporter assay data are representative of at least three independent experiments.

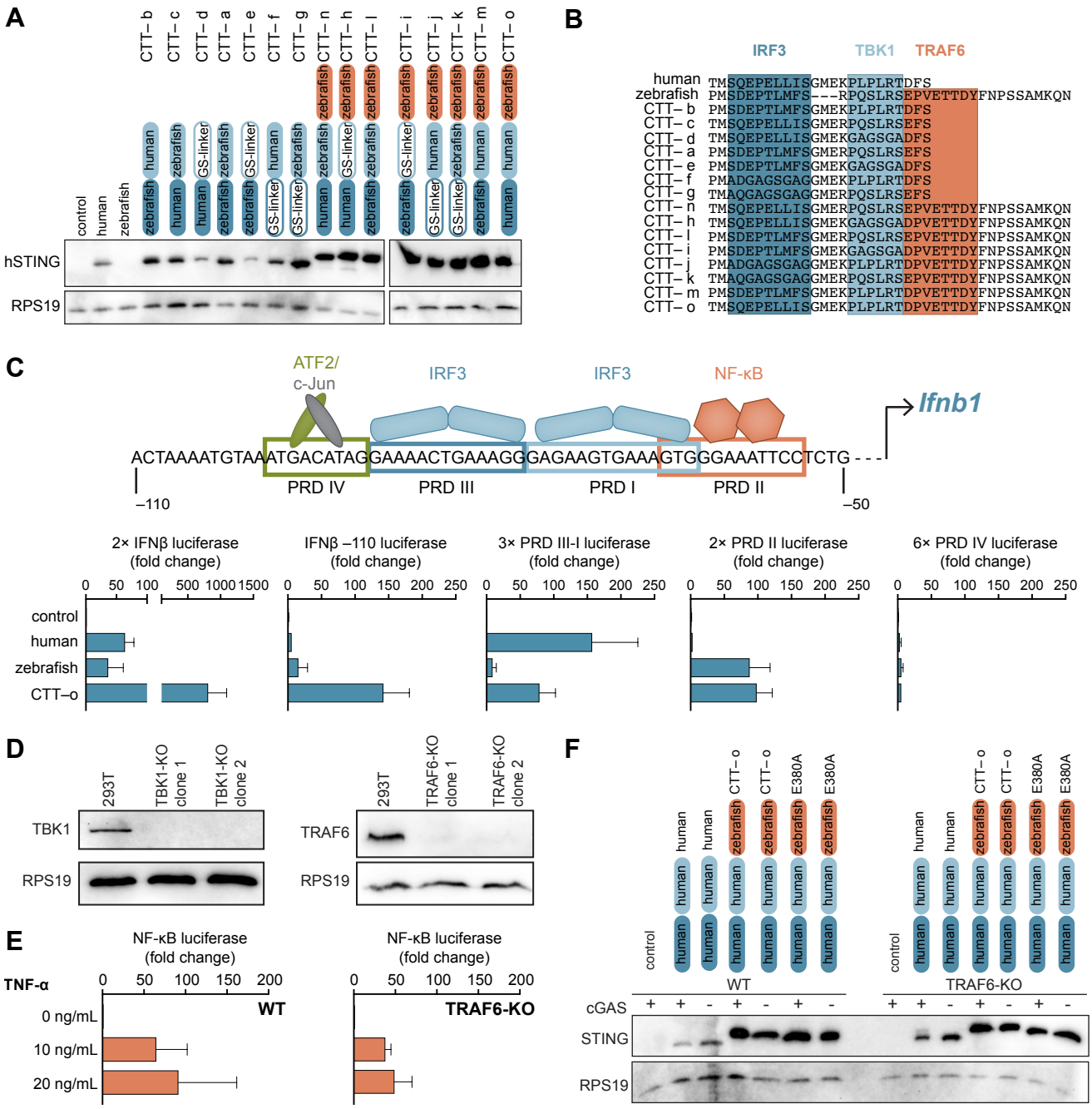
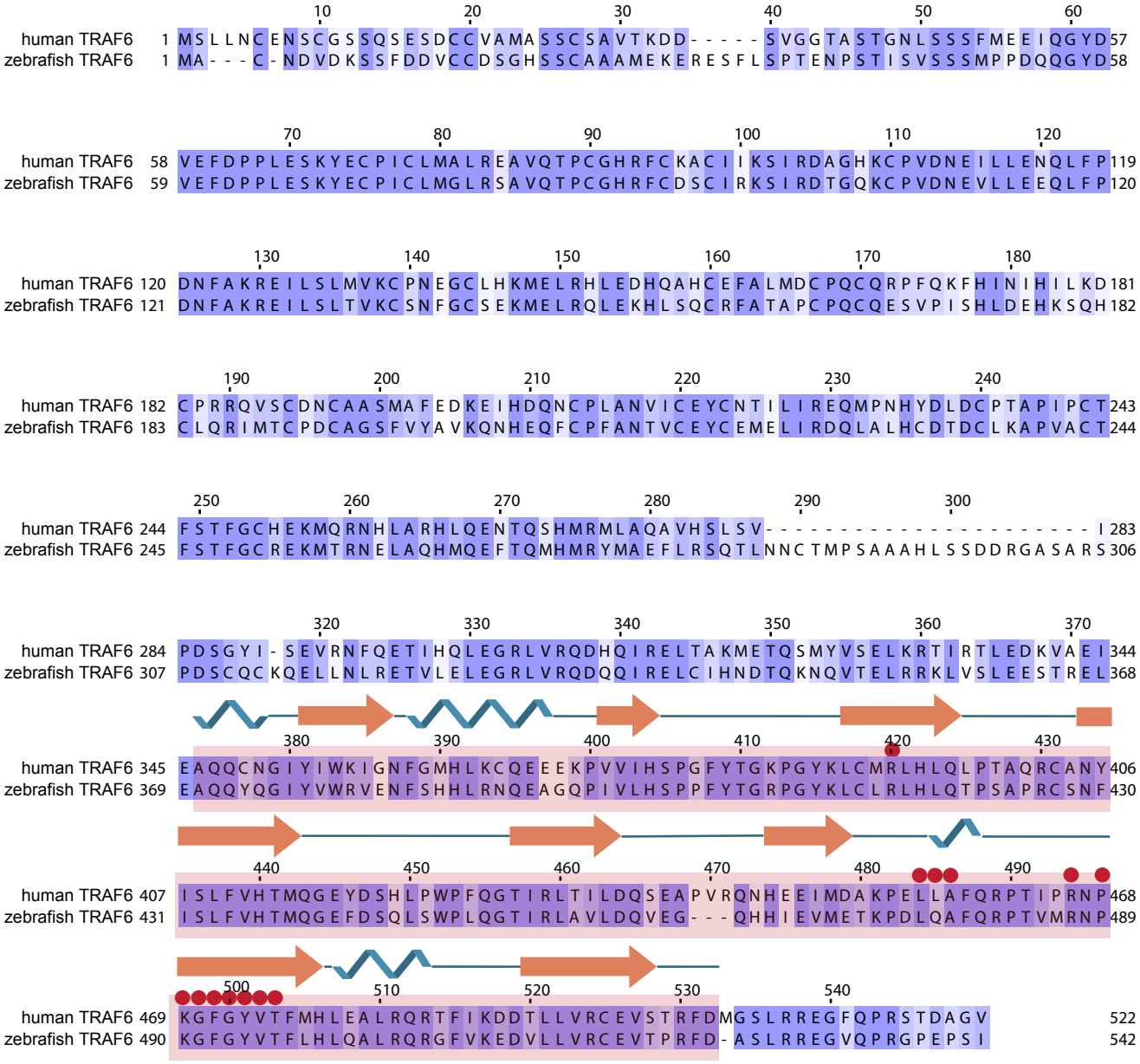

A
CTT– b
CTT– c
CTT– d
CTT– a
CTT– e
CTT– f
CTT– g
CTT– n
CTT– h
CTT– l
CTT– i
CTT– j
CTT– k
CTT– m
CTT– o
control
human
zebrafish
hSTING
RPS19
B
IRF3
TBK1
TRAF6
human
zebrafish
CTT– b
CTT– c
CTT– d
CTT– a
CTT– e
CTT– f
CTT– g
CTT– n
CTT– h
CTT– l
CTT– i
CTT– j
CTT– k
CTT– m
CTT– o
C
ATF2/
c-Jun
IRF3
IRF3
NF-κB
Ifnb1
ACTAAAATGTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCTG
PRD IV
PRD III
PRD I
PRD II
–110
–50
2× IFNβ luciferase (fold change)
IFNβ –110 luciferase (fold change)
3× PRD III-I luciferase (fold change)
2× PRD II luciferase (fold change)
6× PRD IV luciferase (fold change)
control
human
zebrafish
CTT–o
D
293T
TBK1-KO clone 1
TBK1-KO clone 2
TBK1
RPS19
293T
TRAF6-KO clone 1
TRAF6-KO clone 2
TRAF6
RPS19
E
NF-κB luciferase (fold change)
TNF-α
0 ng/mL
10 ng/mL
20 ng/mL
WT
TRAF6-KO
F
control
human
zebrafish
CTT– o
E380A
WT
TRAF6-KO
cGAS
STING
RPS19

Figure S3. Expression levels and CTT sequences of human–zebrafish STING chimera constructs, Related to Figures 2 and 3

(A) Western blot analysis of expression levels of transfected STING C-terminal tail (CTT) chimeras (constructs CTT–a through CTT–o) in HEK293T cells reporter assays compared to RPS19 loading controls. Data are representative of two independent experiments. (B) Aligned CTT sequences for human–zebrafish STING chimeras (constructs CTT–a through CTT–o) with highlighted interferon β (dark / light blue) and NF-κB (orange) signaling motifs. (C) Schematics of the –110 to –50 nucleotide region of the human IFNβ enhancer depicting the four positive regulatory domains (PRDI–IV) and the associated transcription factors. Luciferase reporter assay measuring activation of individual IFNβ enhancer elements by human, zebrafish and chimera CTT–o STING. Luciferase activity was measured for 2× IFNβ promoter and 1× IFNβ promoter (IFNβ–110) compared to individual enhancer elements: PRD III-I (3× copies, IRF3 binding site), PRD II (2× copies, NF-κB binding site) and PRD IV (6× copies, ATF-2–c-Jun binding site). (D) Western Blot validation of TBK1 and TRAF6 expression in the respective CRISPR/Cas9-mediated knockout cell line clones. (E) Luciferase reporter assay measuring NF-κB signaling in wild-type HEK293T and TRAF6-KO cells after stimulation with TNF-α as indicated. Cellular reporter assay data are representative of at least three independent experiments. (F) Western blot analysis of expression levels of transfected STING C-terminal tail (CTT) chimeras (human, CCT–o and CTT–o E380A) +/- cGAS in HEK293T and TRAF6-KO cells compared to RPS19 loading controls.

A


human TRAF6
zebrafish TRAF6

B

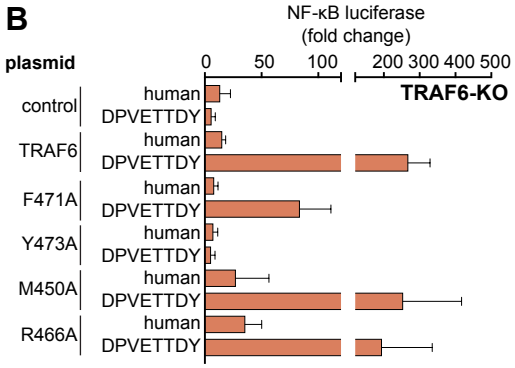

NF-κB luciferase
(fold change)
plasmid
0 50 100 200 300 400 500
TRAF6-KO
control
TRAF6
F471A
Y473A
M450A
R466A
human
DPVETTDY

Figure S4. Alignment of crystalized human and zebrafish TRAF6 TRAF C-domains, Related to Figure 4
(A) Alignment of human and zebrafish TRAF6 TRAF C-domains. Secondary structure elements are shown on top for the crystallized zebrafish homolog. Darker shadings indicate higher physiochemical conservation according to BLOSUM62 score. Residues involved in intermolecular interactions with the peptide ligand are marked in red. Crystallized zebrafish TRAF6 sequence is shaded in red. (B) Cell reporter assay as in Figure 3E monitoring STING-dependent signaling in the presence of TRAF6 mutants. Endogenous TRAF6 was inactivated (TRAF6-KO) and signaling was reconstituted with plasmids encoding TRAF6 with single amino acid mutations of the residues involved in interactions with the STING CTT. Cellular reporter assay data are representative of at least three independent experiments.

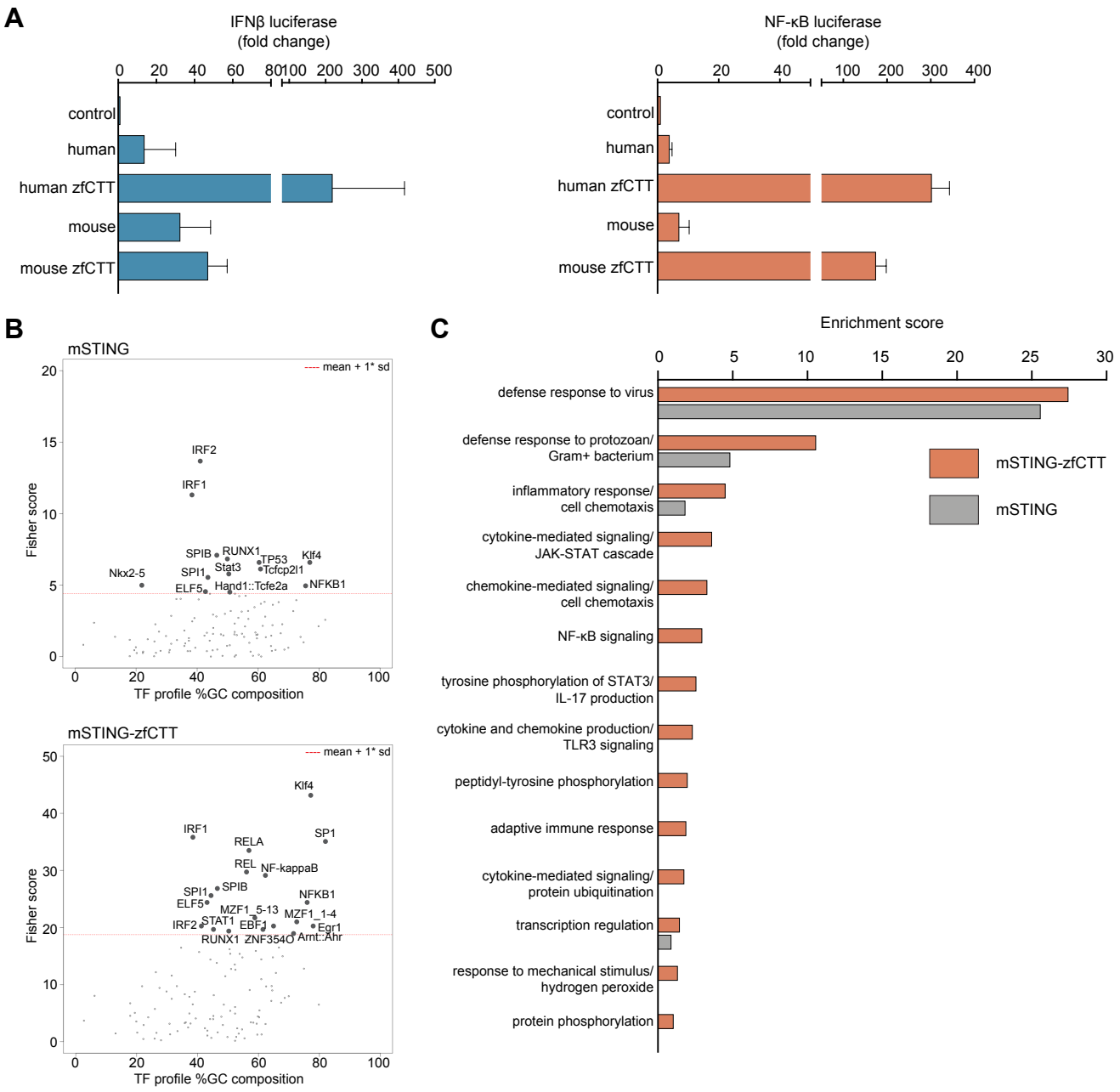


Figure S5. RNA sequencing analysis of STING-dependent signaling, Related to Figure 5

(A) Cellular reporter assay as in Figure 1A comparing the effect of addition of the zfCTT sequence on human STING or on mouse STING. (B) Visualization of transcription factors activated by mSTING or mSTING zfCTT. Differentially expressed genes for mSTING and mSTING zfCTT were analyzed using oPOSSUM for mouse single site analysis with default values and plotted as Fisher score vs %GC content. (C) Enrichment scores for biological processes activated by mSTING compared to mSTING zfCTT. Differentially expressed genes in mSTING or mSTING zfCTT were analyzed using GOTERM Gene Ontology analysis within DAVID.

	TRAF6–STING
Data Collection	
Resolution (Å)[a]	48.52–1.40 (1.42–1.40)
Wavelength (Å)	0.97918
Space group	P 1 21 1
Unit cell: a, b, c (Å)	46.62, 84.38, 53.72
Unit cell: α, β, γ (°)	90.0, 115.42, 90.0
Molecules per ASU	2
No. reflections: total	253469
No. reflections: unique	73125
Completeness (%)[a]	99.0 (94.2)
Multiplicity[a]	3.5 (3.2)
$I/\sigma I$[a]	4.8 (1.2)
CC(1/2)[1] (%)[a]	98.9 (49.5)
Rpim[2] (%)[a]	7.0 (58.4)
Refinement	
Resolution (Å)	48.52–1.40
Free reflections (%)	10
R-factor / R-free	18.1 / 19.9
Bond distance (RMS Å)	0.005
Bond angles (RMS °)	0.859
Structure/Stereochemistry	
No. atoms: protein	2501
No. atoms: peptide	134
No. atoms: ligands	25 (SO_4)
No. atoms: water	474
Average B-factor: protein	18.65
Average B-factor: peptide	23.34
Average B-factor: ligand	47.50
Average B-factor: water	33.49
Ramachandran plot: favored	98.09%
Ramachandran plot: allowed	1.91%
Ramachandran plot: outliers	0.00%
Rotamer outliers:	0.34%
MolProbity[3] score	1.20
Protein Data Bank ID	6MYD

[a] Highest resolution shell values in parenthesis
[1] (Karplus and Diederichs, 2012)
[2] (Weiss, 2001)
[3] (Chen et al., 2010)

Table S2. Crystallographic statistics, Related to Figure 4