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Supplementary Materials for

Lysyl-tRNA synthetase produces diadenosine tetraphosphate to curb STING-dependent inflammation

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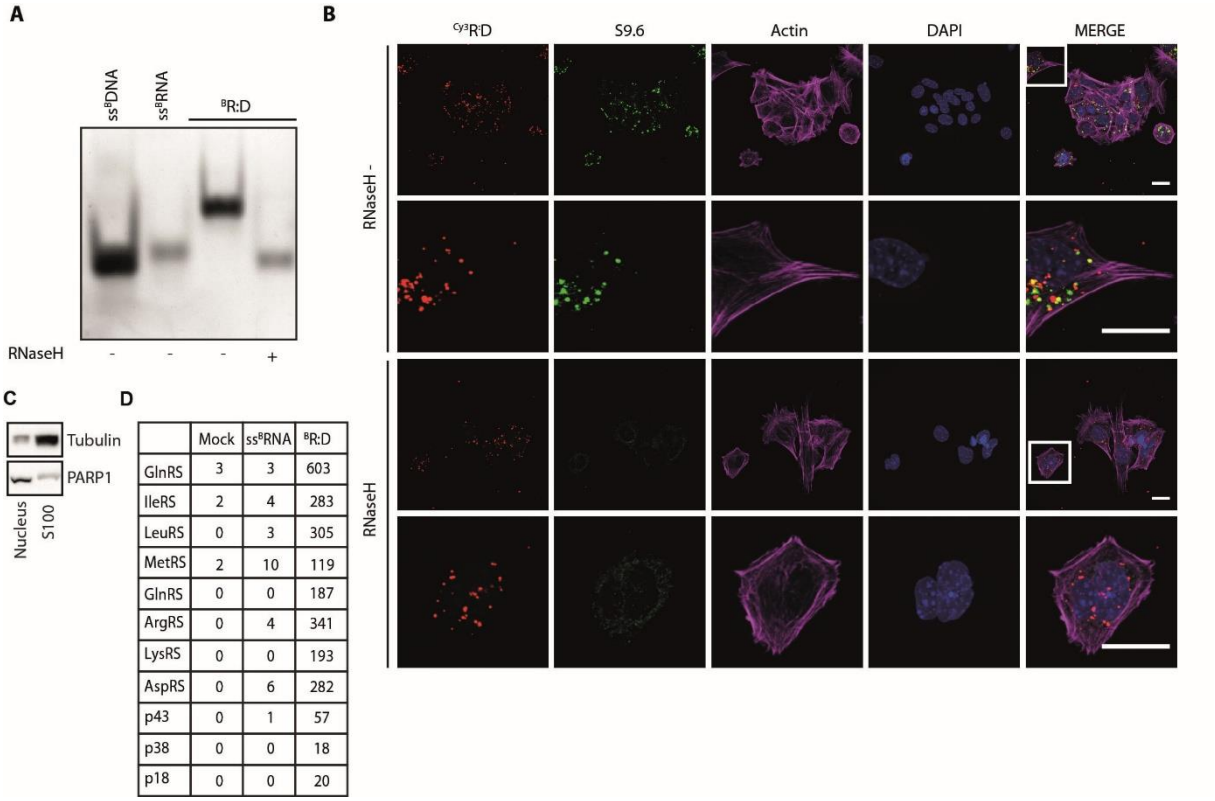
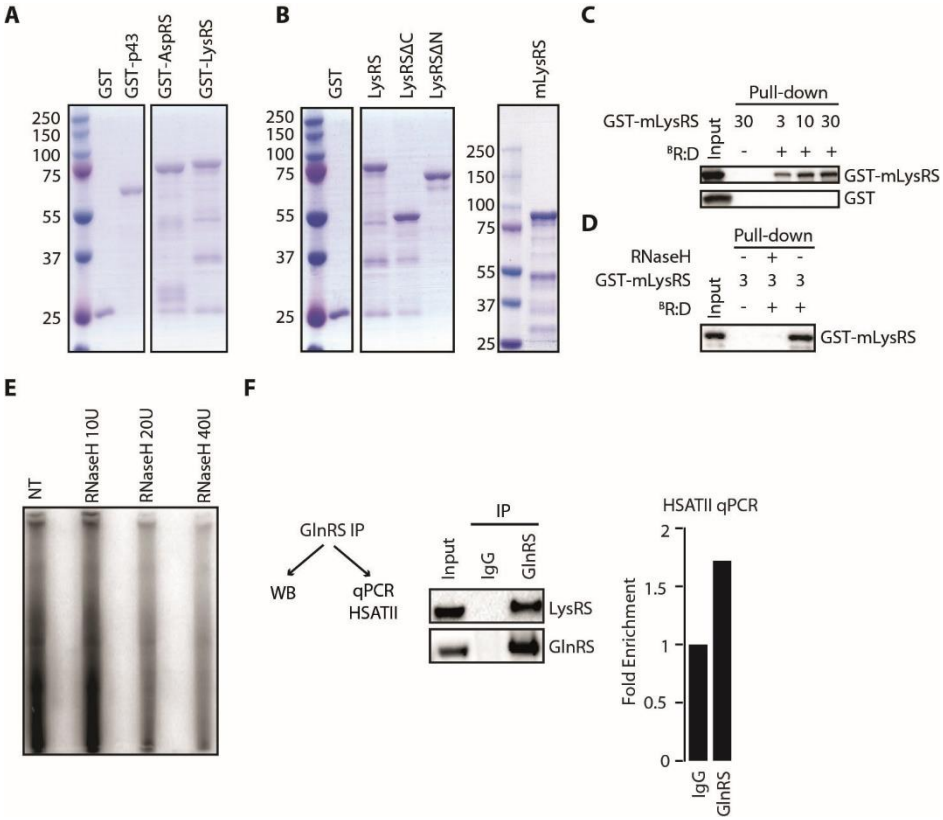
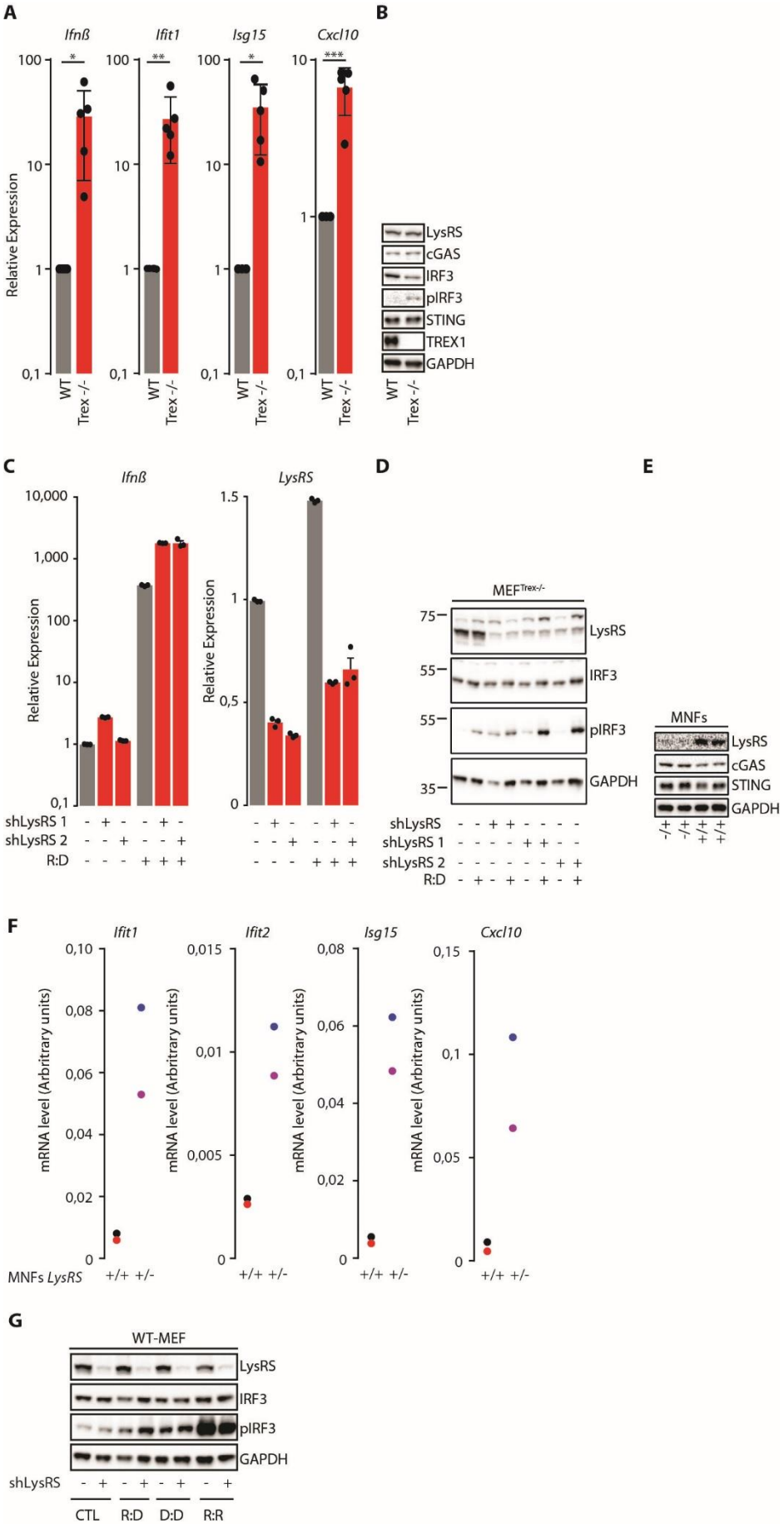


	Mock	ssBRNA	BR:D
GlnRS	3	3	603
IleRS	2	4	283
LeuRS	0	3	305
MetRS	2	10	119
GlnRS	0	0	187
ArgRS	0	4	341
LysRS	0	0	193
AspRS	0	6	282
p43	0	1	57
p38	0	0	18
p18	0	0	20

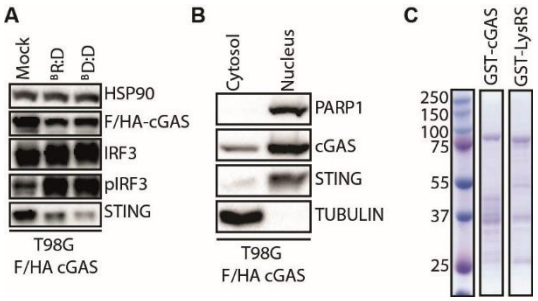
Supplementary figure 1. The Multi-tRNA Synthetase Complex interacts with RNA:DNA hybrids. (**A**) Biotinylated ssDNA (ss^{B}DNA), biotinylated ssRNA (ss^{B}RNA) and biotinylated ^{B}RNA:DNA hybrids (^{B}R:D) were digested or not with RNAseH prior to migration on a non-denaturing 10% acrylamide gel and staining with SYBR Gold. (**B**) Immunofluorescence analysis of WT-MEF transfected with Cyanin3-labelled RNA:DNA hybrids (^{Cy3}R:D) for 6 hours. Coverslips were treated or not with RNaseH prior to immunostaining with the S9.6 antibody. Nuclei and ACTIN were stained with DAPI and with the ActinGreen 488 ReadyProbes reagent, respectively. (**C**) Cytosolic and nuclear fractions from HeLa-S3, used in figure 1C, were analyzed by Western blot (WB) using the indicated antibodies. (**D**) Total peptide count of proteins of the MSC complex identified by Mass Spectrometry analysis of eluates from the *in vitro* pull-down performed using synthetic nucleic acids (ss^{B}RNA and ^{B}R:D) and HeLa-S3 cytosolic extracts as described in figure 1C.



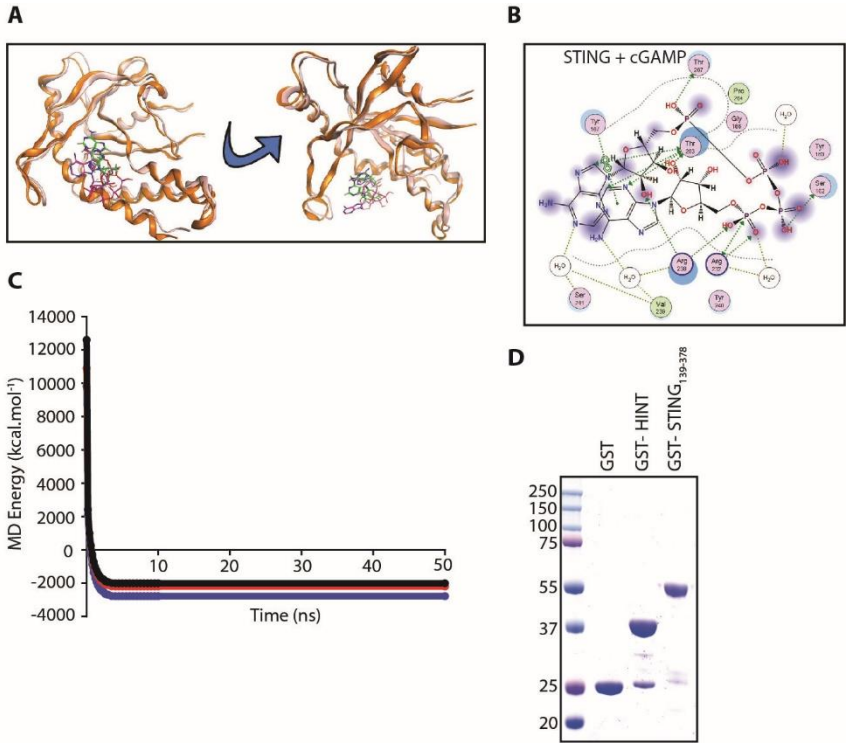
Supplementary figure 2. The Lyslyl t-RNA synthetase interacts directly with RNA:DNA hybrids. (**A**) Coomassie staining of recombinant proteins used in figure 2A-B. (**B**) Coomassie staining of recombinant proteins used in figure 2C-D. (**C**) Three, 10 and 30 pmols of recombinant mouse LysRS (mLysRS) protein or GST were incubated with 20 pmols of ^{B}R:D prior to pull-down on streptavidin affinity beads. Input and eluates were analyzed by WB with anti-GST antibody. (**D**) Three pmols of recombinant mLysRS were incubated with 20 pmols of ^{B}R:D prior to pull-down on streptavidin affinity beads. Where indicated, nucleic acids were treated with RNaseH prior to pull-down. Input and eluates were analyzed by WB using anti-GST antibody. (**E**) Nucleic acids extracted from HeLa cells were treated with 10, 20 or 40 Units of RNaseH prior to radiolabeling with γP32, migration on non-denaturing acrylamide gel and autoradiography. (**F**) Left panel shows the experimental scheme. Whole cell extracts from colorectal cancer cells (Colo205) were used to immunoprecipitate GlnRS. Eluates were either immunoblotted using the indicated antibodies (central panel) or DNA extracted and HSATII-derived DNA analyzed by quantitative PCR (right panel); (n=2). All blots show representative experiments.


A
Ifnß
Ifit1
Isg15
Cxcl10
Relative Expression
WT
Trex -/-
B
LysRS
cGAS
IRF3
pIRF3
STING
TREX1
GAPDH
C
Ifnß
LysRS
shLysRS 1
shLysRS 2
R:D
D
MEF Trex-/-
shLysRS
E
MNFs
F
Ifit1
Ifit2
Isg15
Cxcl10
mRNA level (Arbitrary units)
MNFs LysRS
+/+ +/-
G
WT-MEF
CTL
R:D
D:D
R:R

Supplementary figure 3. The Lysyl t-RNA synthetase inhibits RNA:DNA hybrids-induced interferon production. (**A**) Mean (± SEM) mRNA expression levels of *Ifnβ* and ISGs (*Ifit1*, *Isg15* and *Cxcl10*) in MEF$^{Trex1-/-}$, as compared to WT-MEF (n=4). Unpaired t-test. *: p<0.05; **: p< 0.01; ***: p< 0.005. (**B**) Whole cell extracts from WT-MEF and MEF$^{Trex1-/-}$ were analyzed by WB using the indicated antibodies. (**C**) MEF$^{Trex1-/-}$ were transduced with a *LysRS*-targeting shRNA for 72 hours prior to transfection or not with R:D for 6 hours. *Ifnβ* and *LysRS* mRNA levels were analyzed by RT-qPCR and are expressed as mean relative expression (± SEM) as compared to cells transduced with a *Luciferase*-targeting shRNA. Representative experiment (n=3). (**D**) Whole cell extracts from cells in C were analyzed by WB using the indicated antibodies. (**E**) Whole cell extracts of WT Mouse Neonatal Fibroblast (MNFs) or MNF$^{LysRS+/-}$, derived from 2 independent 1 day-old mice, were analyzed by WB using the indicated antibodies. (**F**) *Ifit1*, *Ifit2*, *Isg15* and *Cxcl10* mRNA levels were analyzed in WT-MNF and MNF$^{LysR+/-}$. Different colors indicate different mice. (**G**) WB analysis of whole cell extracts from cells used in Figure 3M-O using the indicated antibodies. All blots show representative experiments.



Supplementary figure 4. LysRS delays the detection of RNA:DNA hybrids by cGAS. (**A**) Whole cell extracts of T98G$^{F/HA-cGAS}$, transfected or not with ^{B}R:D and ^{B}D:D for 6 hours were analyzed by WB using the indicated antibodies. (**B**) WB analysis of cytosolic and nuclear fraction of T98G expressing F/HA-tagged cGAS (T98G$^{F/HA-cGAS}$) used in figure 4A. (**C**) Coomassie staining of recombinant proteins used in figure 4C-F.



Supplementary figure 5. Diadenosine tetraphosphate intracts with STING. (**A**) Superposition of the 3D structures of cGAMP and Ap_4A compounds in the proximity of the active site of STING. Ap_4A is in blue and green, cGAMP is in red and pink. (**B**) Molecular interactions that stabilize cGAMP in the active site of STING. (**C**) Molecular dynamics trajectories. Black: STING, red: STING:Ap_4A, blue: STING:cGAMP. All molecular systems equilibrate fast, with the STING:cGAMP system espousing the most stable complex (lowest equilibrium energy). (**D**) Coomassie staining of recombinant proteins used in figure 5K-L.

Supplementary Table 1. STING:Ap_4A and STING:2'3'-cGAMP energies calculation. ALL: complex energy; INT: interaction energy; VDW: Van Der Waals. Calculated energies are expressed in $kcal.mol^{-1}$

		Energy	Strain	VDW	Electrostatic
Ap_4A	ALL	-2217.16	371.379	888.739	-4460.82
	INT	-270.969	N/A	-3.908	-267.062
2'3'-cGAMP	ALL	-2734.97	388.777	1831.373	-6757.68
	INT	-132.188	N/A	-10.376	-121.811