SUPPLEMENTARY INFORMATION for

Aminoacyl-tRNA Synthetase Inhibition Activates a Novel Pathway that Branches from the Canonical Amino Acid Response in Mammalian Cells

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Other supplementary materials for this manuscript include the following:

Detailed Methods
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Datasets S1 to S3
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Supplementary Information Text

DETAILED METHODS

Primary human synovial fibroblasts. Briefly, human synovial tissues from patients with RA were obtained by after synoviectomy or joint replacement surgery performed as part of indicated clinical care at Brigham and Women's Hospital. Synovial fibroblasts were released from synovial tissue by mincing followed by collagenase digestion (1 mg/ml, collagenase type IV, Worthington Biochemicals) were purified by serial passage as previously described (1), and were used in experiments between passages 6 and 10. Primary synoviocytes were cultured at 37°C under an atmosphere containing 10% CO2 in DMEM supplemented with 10% FBS, 2mM L-Glutamine, 1mM Sodium Pyruvate (Lonza), 1X MEM Non-Essential Amino Acid (Lonza), 1X MEM Amino Acid (Hyclone), 55μM 2-Mercaptoethanol (Gibco), 50μg/mL Gentamycin (Amresco) and 1X Penicillin/Streptomycin/Amphotericin B (Lonza). For gene expression analysis, 80% sub-confluent cells were left in serum reduced media (0.2% FBS) for 24-48 hours, treated with HF (300 nM) and/or proline (2mM) for 16 hours and treated with TNFα (10ng/ml) for 4 hours.

Immortalized K4 synoviocytes were a kind gift from Dr Evelyn Murphy (University College Dublin). Cells were cultured at 5% CO2 at 37°C in DMEM supplemented with 10% FBS, L-glutamine, sodium pyruvated, and antibiotics. For TNFα-stimulated experiments, cells were shifted into K4 cell media with 0.2% FBS instead of 10%FBS 24 hours before the HF treatment.

Human lung fibroblasts (LL29, AnHa), were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured DMEM + 10%FBS. Cells were put into DMEM with reduced (0.2%) FBS 24 hours before treatment with 200nM HF-/+ 2mM proline for 6 hours, and then TGFß for 16 hours. Cells were used before 12th passage.

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Human Vascular Endothelial Cells (HUVEC, Lonza EGM (CC-2517)) were cultured in EGM-2 (CC-3162, Lonza). For experiments, cells were serum starved in 0.2% FBS for 24hr, treated with 200nM HF and 2mM proline for 16hr, and then treated 10ng/mL TNFα for 4hr.
Normal human dermal fibroblasts (NHDF) were purchased from Lonza (CC-2511) and according to the manufacturer's instructions. For later passages, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 2mM L-glutamine (Life Technologies) and 1X of penicillin and streptomycin (Lonza). For gene expression experiments, NHDF cells were plated on collagen type 1 plates (BD biosciences), serum-starved in serum-reduced media (0.2 % FBS) for 24 hours, treated with HF and/or proline (2mM; Sigma-Aldrich) and/or TGFß (10ng/ml; R&D Systems) for 48 hours and re-treated for additional 48 hours. NHDF cells were used between passage 4 and 12.

***GCN2$^{-/-}$* FLS isolation and culture**. Primary cell cultures were obtained from wild type and general control nonderepressible 2 GCN2$^{-/-}$ (*eif2ak4$^{-/-}$*) mice purchased from Jackson Laboratories (Bar Harbor, Maine) (stock no. 008240). Primary murine synoviocytes isolated as previously described (1). Murine synoviocytes were cultured in the same media used for human primary synoviocytes. For treatment, murine synoviocytes were cultured in serum-reduced media (2%) for 72 hours , as further lowering FBS concentration reduced cell viability.

Primary CD4^{+} T cells from wild type (C57Bl/6J; stock no. 000664) or Gcn2$^{-/-}$ (*eif2ak4$^{-/-}$*) mice were isolated, treated +/- HF +/- L-proline, and cultured as previously described (2). Briefly, "naïve" CD4+CD25- T cells were magnetically isolated from single cell splenocyte suspensions using an EasySep mouse CD4+ T cell enrichment kit (Stem Cell Technologies, Cat. #: 19752). A biotinylated anti-mouse CD25 antibody (clone PC61.5, from eBioscience) was added at 1 µg/mL to the biotinylated antibody cocktail prior to incubation with streptavidin beads. These cells were activated with plate-coated anti-CD3 (Clone: 145-2C11, 0.3 µg/mL) and anti-CD28 (Clone: 37.51, 0.5 µg/mL) antibodies (both from Bio X Cell), and treated with additives (HF, L-proline) and/or Th17 polarizing cytokines (TGFß + IL-6) at the time of activation. For Th17 polarization, activated T cells were cultured with recombinant human TGFß (0.3 µg/mL; cat #: 240-B) and recombinant mouse IL-6 (3 µg/mL; cat. #: 406-ML) (both from R&D systems). Activated T cells were removed from antibody-coated wells after 48 hours; non-polarized cells (no cytokines) were expanded in media containing 10 µg/mL recombinant human IL-2 (eBioscience, cat. #: BMS334). TGFß and IL-6 were re-added to Th17-polarized cell cultures at 48 hours, and these cells were expanded in the absence of IL-2. Cells were expanded until day 4, and cytokine expression was determined by intracellular staining as previously described (2) following 3-4 hr stimulation with phorbol 12-myristate 13-acetate (PMA, 10 nM,, cat. #: P8139) and ionomycin (1 µM, cat. #: I3909) in the presence of brefeldin A (10 µg/mL, cat. #: B7651) (all from Sigma-Aldrich). For some experiments, CD4+CD25- T cells activated in Th17-polarizing conditions were collected at different time points for analysis of protein or mRNA expression. CCR6+ memory (CD3+CD4+CD25-CD62LloCD44hi) T cells were FACS-sorted from single cell splenocyte suspensions as previously described (2). These cells were activated in 96-well round-bottom tissue culture plates (Corning) with anti-CD3/anti-CD28-coated beads (Invitrogen; 3 beads:1 cell) plus 10 µg/mL recombinant human IL-2. Recombinant human IL-23 (20 ng/ml; R&D Systems, cat. #: 1290), as well as HF and/or L-proline were added as indicated. These cells were stimulated for 3-4 hr with

PMA and ionomycin in the presence of brefeldin A after 48 hours for analysis of intracellular cytokine expression.

Antibodies and Q-RT-PCR probes

Antibodies against total GCN2 (Cat. #3302), total eif2α (Cat#5324) and pS51 eif2α (Cat #3398) were purchased from Cell Signaling Technologies (Danvers, MA). Antibody against ATF4 (10835-1-AP) was from ProteinTech. Antibody against cytoplasmic actin was from Sigma (Clone AC-40). Anti-GCN1L1 was from Novus (NB100-97851). HF and HFol were prepared as described previously (3). Amino acids L-proline, L-threonine and the threonyl-tRNA synthetase inhibitor borrelidin were purchased from Sigma-Aldrich (Cat #5607, #T8625 and #B1936 respectively). The selective inhibitor of c-jun N-terminal kinase (JNK) SP600125 and the mTOR inhibitor Torin1 were purchased from Sigma-Aldrich (Cat. #S5567) and Tocris (Cat. #4247) respectively.

For gene expression studies, RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen), and retro-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Transcriptional analysis was performed using the Universal Probe Library System (UPL; Roche) on a LightCycler 480 Real-Time PCR machine. The following primers and probes were used:

- *Human ACTA2* (alpha-SMA): probe 21. FW primer: gcactgccttggtgtgtg; RW primer: tcccattcccaccatcac
- *Human COL1A1*: probe 21. FW primer: ccctctggagcctctggt; RW primer: gagtccatctttgccaggag
- *Human CXCL2:* probe 69. FW primer: cccatggttaagaaaatcatcg; RW primer: cttcaggaacagccaccaat
- *Human CXCL9:* probe 56. FW primer: ttgaatcactgctcacactgc; RW primer: gacgttcgggtgggatct
- *Human CXCL10:* probe 34. FW primer: gaaagcagttagcaaggaaaggt; RW primer: gacatatactccatgtagggaagtga
- *Human MMP1:* probe 7. FW primer: gctaacctttgatgctataactacga; RW primer: tttgtgcgcatgtagaatctg
- *Human MMP3:* probe 46. FW primer: ctccaaccgtgaggaaaatc; RW primer: catggaatttctcttctcatcaaa
- *Human MMP13:* probe 73. FW primer: ccagtctccgaggagaaaca; RW primer: aaaaacagctccgcatcaac
- *Human MMP14:* probe 15. FW primer: tggtctcggaccatgtctc; RW primer: aggtagccatattgctgtagcc
- *Human PGK1 (control for primary cells):* probe 56. FW primer: ggaagcgggtcgttatgag; RW primer: attgtccaagcagaatttgatg
- *Human GAPDH (control for Immortalized cells):* probe 60. FW primer: agccacatcgctcagacac; RW primer: gcccaatacgaccaaatcc
- *Human TRB3: probe16. FW primer: tccagatcgtgcaactgct. RW primer: cttcctggacggggtaca*
- Human ACTA2: probe 21. FW primer: gcactgccttggtgtgtg. RW primer: tcccattcccaccatcac
- *Mouse ACTA2* (□-SMA): probe 56. FW primer: caaccgggagaaaatgacc; RW primer: cagttgtacgtccagaggcata
- *Mouse COL1A1:* probe 15. FW primer: catgttcagctttgtggacct; RW primer: gcagctgacttcagggatgt.

- *Mouse CXCL9:* probe 1. FW primer: cttttcctcttgggcatcat; RW primer: gcatcgtgcattccttatca.
- *Mouse CXCL10:* probe 56. FW primer: aatgaaagcgtttagccaaaaa; RW primer: aggggagtgatggagagagg.
- *Mouse MMP1a:* probe 69. FW primer: tgacaccacttacgttccaaa; RW primer: tcaaatgggttgttgtcacc
- *Mouse MMP13:* probe 89. FW primer: gccagaacttcccaaccat; FW primer: tcagagcccagaattttctcc
- *Mouse TBP1 (control):* probe 107. primer FW: ggcggtttggctaggttt; primer RW: gggttatcttcacacaccatga

Probe and primers were designed using the ProbeFinder software (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000). Relative gene expression (fold change) levels were obtained using the ΔΔCt method and normalizing to the control genes.

Stable knock-down of GCN1 and GCN2 in K4 synoviocytes and human RA synoviocytes

Lentiviral-transduction was performed using the following pLKO.1 lentiviral constructs encoding shRNA with the following sequences:
GCN1 shRNA #698 Original Clone ID TRCN0000154964 Target gene specific sequence GCATAGACTCACTGGCAACAA
GCN1 shRNA #699 Original Clone ID TRCN0000154822 Target gene specific sequence GCCGTGCTGTATTTCTCTGAA
GCN2 shRNA #2 Original Clone ID TRCN0000078652 Target gene specific sequence GCCTAACTGGTGAAGAAGTAT
GCN2 shRNA #3 Original Clone ID TRCN0000078651 Target gene specific sequence CCAAAGGTCTATCAAATGAAA
Scrambled shRNA (Addgene plasmid #1864).

Lentiviruses were generated in 293T cells by Effectene (Qiagen) mediated co-transfection of the pLKO.1 plasmid, the psPAX2 packaging vector and the pMD2.G, VSV-G envelope expressing plasmid in a 2:1.5:1 ratio. 16 hours after transfection, the transfected 293T cells rinsed with PBS, and then they grew in the growth media for K4 synoviocytes or RA synoviocytes. At 48 hours post transfection, the lentiviral supernatant was harvested, spun at 2000xg for 5 minutes at 4℃, filtered with 0.45μM syringe filter. K4 synoviocytes or RA synoviocytes were treated with the lentiviral suprnatant with 5μg/mL polybrene. 6 hours after infection, the transduced cells rinsed with PBS, and then they grew in the fresh growth media. Next day, the lentiviral infection process was repeated. The transduced cells were selected with 2 μg/mL puromycin for 48~72 hours. After selection, cells grew in growth media with 0.25 ug/mL puromycin.

Transduced K4 synoviocytes or RA synoviocytes were left in serum-reduced media (0.2% FBS) for 24 hours, treated with HF (100nM or 200nM, respectively) for 16 hours and with TNFα (10ng/ml) for additional 6 hours. Western blot and gene expression analyses were performed as previously described. Results are representative of three independent experiments.

RNA-seq. For both primary RA-FLS and K4 RNAseq (supplementary Files S1 and S2 respectively), RNA quantity and quality was checked using a Bioanalyzer at the

Biopolymers Facility, Harvard Medical School. Poly-A enriched libraries were prepared with the TruSeq Stranded mRNA Library Prep Kit, sequenced using a single-end approach with 100 bp reads at a total sequencing depth of 30 million reads on an Illumina 4000 instrument, and mapped to the genome by the JP Sulzberger Columbia Genome Center. For RA-FLS RNAseq (S1), all samples were processed using an RNA-seq pipeline implemented in the bcbio-nextgen project (https://bcbio-nextgen.readthedocs.org/en/latest/). Raw reads were examined for quality issues using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to ensure library generation and sequencing are suitable for further analysis. Read were aligned aligned to UCSC build 19 of the human genome (hg19), augmented with transcript information from Ensembl release GRCh37 using STAR (4). Adapter sequences, other contaminant sequences such as polyA tails and low quality sequences were trimmed by STAR's soft-trimming feature. Alignments were checked for evenness of coverage, rRNA content, genomic context of alignments (for example, alignments in known transcripts and introns), complexity and other quality checks using a combination of FastQC, (5). http://doi.org/10.1093/bioinformatics/bts503], MultiQC (https://github.com/ewels/MultiQC) and custom tools. Counts of reads aligning to known genes were generated by (6). Differential expression at the gene level was called with limma-voom (7). For K4 RNAseq (File S2), Pairwise DEseq2 analysis of differentially expressed genes was performed on the Galaxy web platform. Normalized gene expression was analyzed using the GenePattern software suite (http://genepattern.broadinstitute.org), and visualized via the MultiPlot module. For analysis, only annotated genes displaying real normalized expression values (> 0) in all replicate samples were used (*n* = 12,973).

T cell Microarray analysis. All procedures were performed as described in GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA, current version available at www.affymetrix.com) and Ambion® WT Expression Kit Protocol (Life Technologies, current version available at http://tools.invitrogen.com/content/sfs/manuals/cms_064619.pdf). Differentially expressed genes (≥ 2.5-fold) were analyzed using the Hieracrchical Clustering module in GenePattern (http://www.broadinstitute.org/cancer/software/genepattern). Microarray data are publicly available at Gene Expression Omnibus (GEO, Accession: GSE47478).

^{35}S Methionine Experiments

Primary human RA FLS were seeded in each well of a 6 well-plate, serum starved as described for TNFα treatment studies above, pre-treated with HF and labelled with 1 µCi/well S^{35}-labelled methionine in DMEM containing 0.2% FBS for 24 hours. Incorporation of S^{35}-methionine. Label incorporated into protein was isolated by total protein precipitation with trichloroacetic acid as described (8), and incorporated radioactivity determined as counts per minute using a liquid scintillation counter. Cell labelings were performed in triplicate. Background (unincorporated ^{35}S methionine) was measured in parallel trichloroacetic acid precipitations using an identical amount of ^{35}S methionine added to TCA/cell mixture after harvest.

Dataset S1. RNA-seq analysis of HF effects on TNFα responses. TNFα-inducible pro-inflammatory gene expression in primary RA- FLS, determined by RNA -seq. after 24 hr culture in media alone, TNFα, or TNFα plus HF. Data are mean of data from four separate biological samples.

Dataset S2.**RNA-Seq transcriptomics comparing HF and TNFα action in wild type and GCN2 depleted K4 FLS**. FLS were treated for 16 hrs with HF followed by 6 hours with TNFα (biological duplicates for each condition) and RNA harvested for RNAseq analysis.

Dataset S3. **HF effects on gene expression in wild type or GCN2-/- T cells under Th17 Differentiation conditions**. Primary mouse T cells were treated and RNA harvested at indicated times and analyzed by microarray.

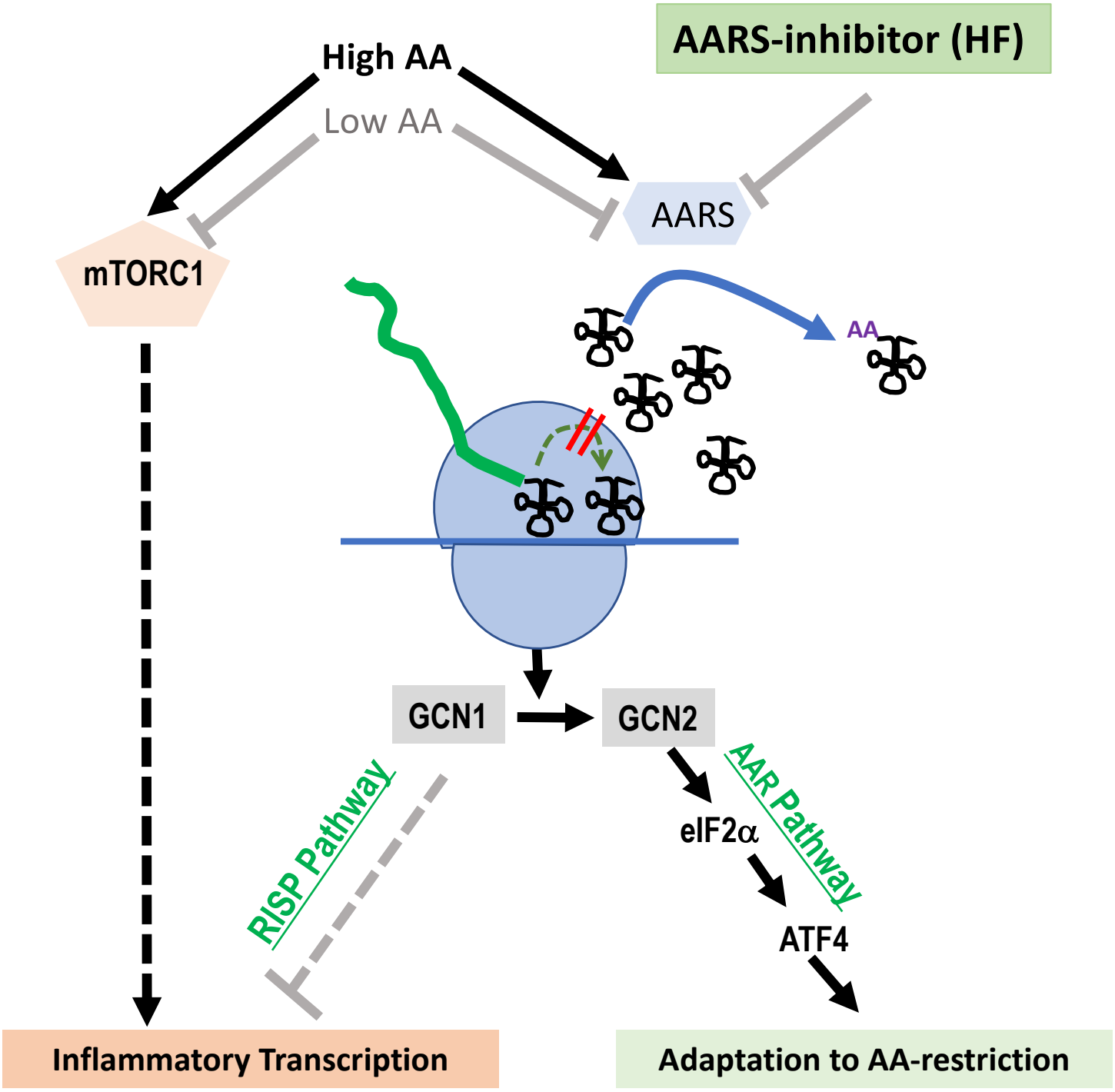

Fig. S1. (Related to Fig.5). Model of Ribosome-initiated Inflammatory Suppression Pathway (RISP) responsive to uncharged tRNA accumulation. AA-insufficiency or AARS-inhibition leads to accumulation of uncharged tRNAs, which, in turn, activate downstream signals to inhibit cytokine-induced inflammatory responses (dashed line indicates proposed new pathway, Ribosome-initiated Inflammatory Suppression Pathway) GCN1 is a shared component of both pathways, being required for GCN2 activation by uncharged tRNA.

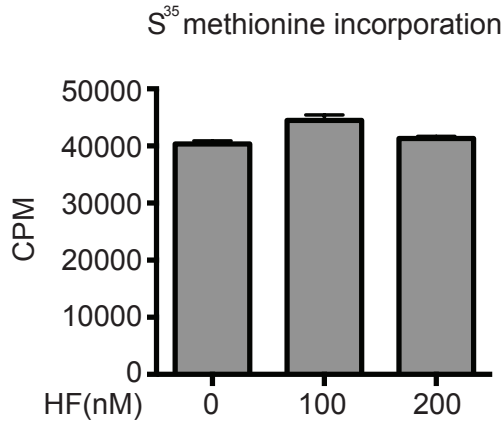

Fig.S2 (related to Fig.1). HF does not significantly inhibit general protein translation over 24 hours in quiescent cells. Human RA FLS were rendered quiescent by serum reduction as in Figure 1, and labelled with 35S-labelled methionine in DMEM+0.2% FBS for 24 hours in the presence of indicated concentrations of HF. TCA precipitable counts per minute were measured in triplicate.

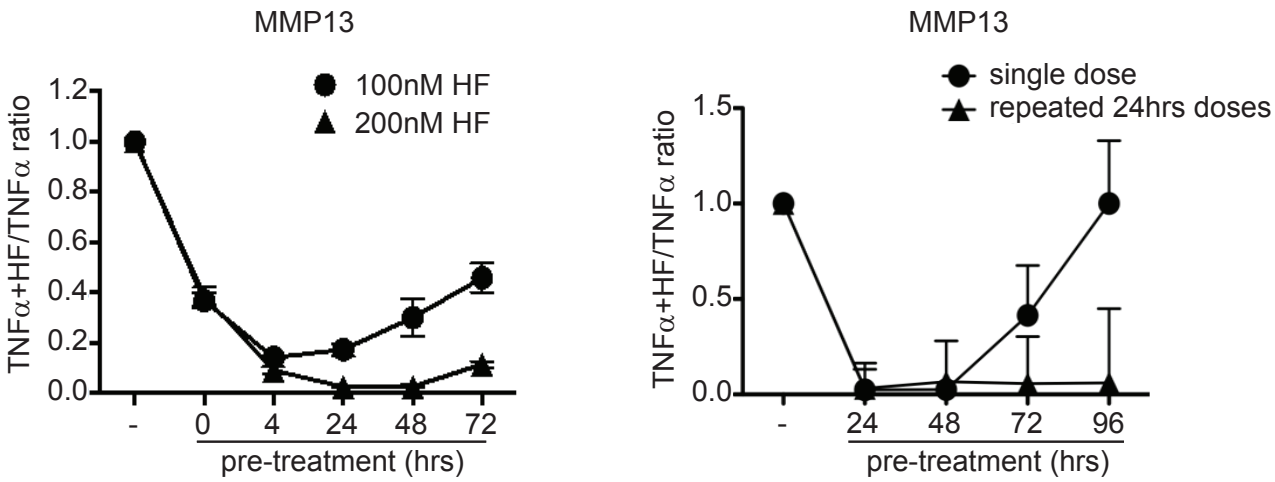

Figure S3 (related to Fig.1). HF induces a sustained change in cell responses. Human RA synoviocytes were serum starved as before, pre-treated with a single or multiple 24hrs doses of HF (200nM) at the time indicated and treated with TNFα (10ng/ml) for 6 hours. Results were normalized to control gene (PGK1) and reproduced in triplicate in 4 different cell lines from RA patients.

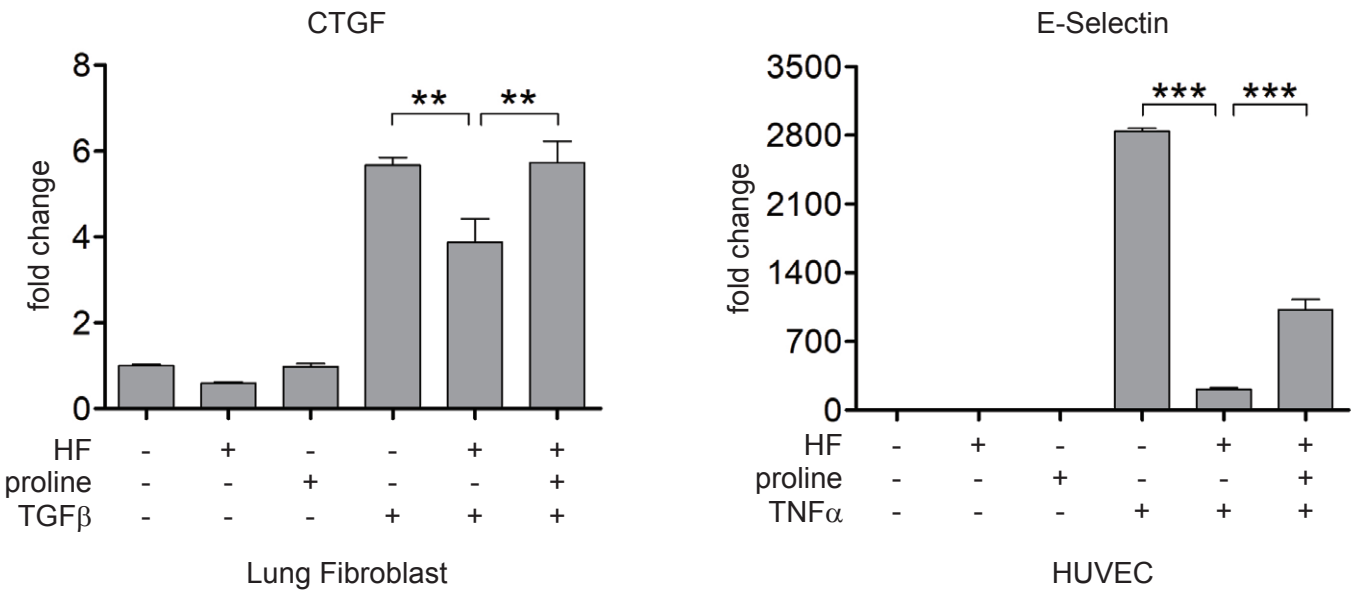

Fig.S4 (Related to Fig.1). HF affects multiple effectors of inflammation and remodeling in different primary cell types. Cells were treated and analyzed as described in legend to Fig.1A.

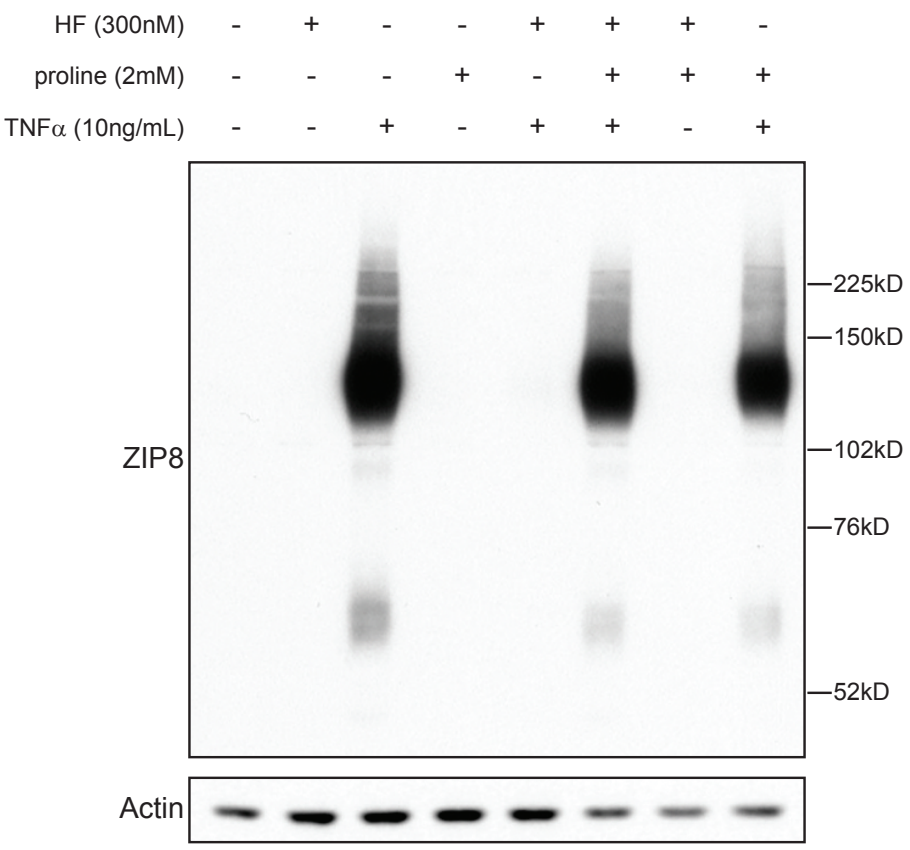

Fig. S5 (related to Fig.1). HF inhibits TNFα induction of ZIP8 protein. Human RA-FLS were pre-treated with 200 nM HF and/or 2 mM proline (Pro) for 16 hours, and treated with TNFα for 24 hours. Protein levels were assessed by Western Blot. Results are representative of three independent experiments.

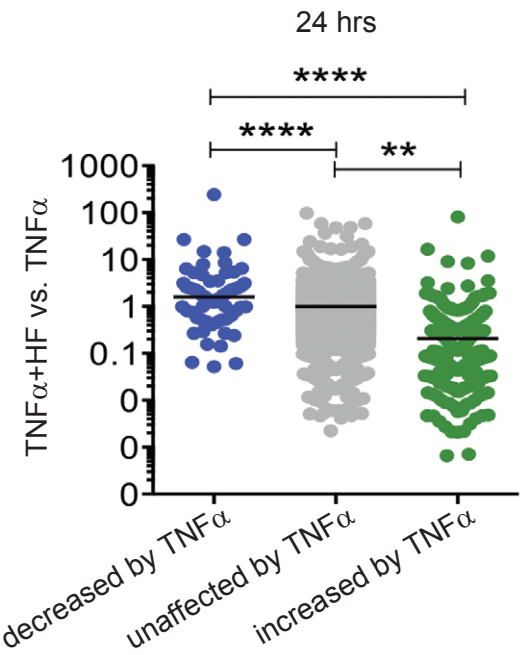

Fig. S6 (related to Fig.1B). HF effects on TNFα induced genes in RA-FLS. Opposing effects of TNFα and HF on FLS gene expression. Effects of HF treatment on the expression of genes that are strongly decreased (>10-fold, blue), not affected (within 10-fold, grey), or strong induced ((>10-fold, green) by TNFα stimulation after 24 hrs (compared to cells cultured in media alone). Data are presented as fold-change in TNFα + HF vs. TNFα only-treated cells. ** $P < .01$, **** $P < .0001$, One-way ANOVA with Tukey's correction for multiple comparisons

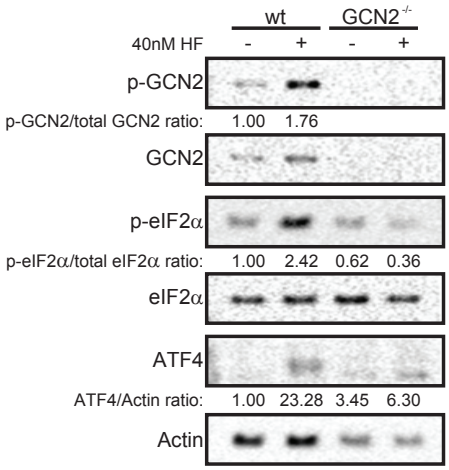

Fig. S7 (related to Fig.2). Mouse GCN2-/- FLS lack canonical GCN2 signaling. Mouse primary FLS from wild type (WT) and GCN2 knockout (GCN2-/-) were serum starved (2% FBS) for 72 hours, treated with 40 nM HF for 2 hours (for GCN2 and eIF2α) or 5 hours (for ATF4), and analyzed by western blotting for total GCN2, phosphorylated GCN2 (on Thr899), total eIF2α, phosphorylated eIF2α (on Ser51), and ATF4.

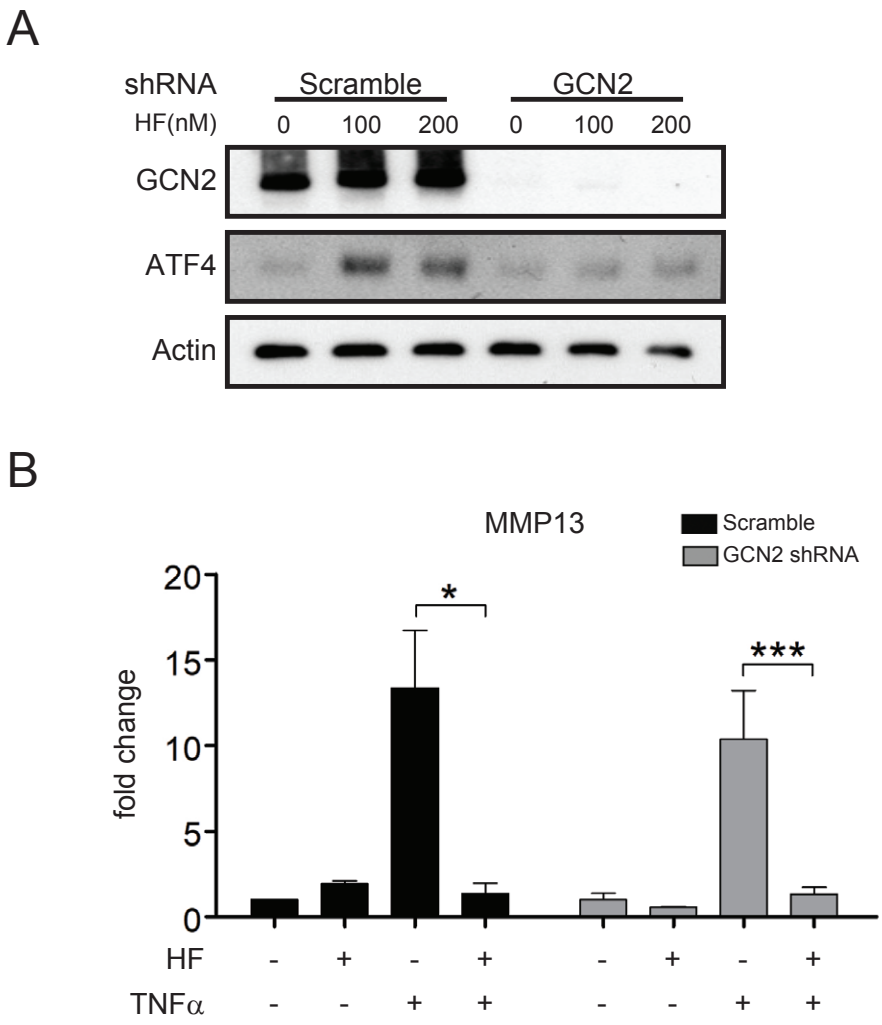

Fig. S8 (related to Fig.2). HF Inhibits TNFα induction of MMP13 in GCN2 depleted primary RA FLS. A) HF does not activate the AAR pathway in GCN2-knock down RA FLS. Human RA FLS were transfected with a lentiviral vector carrying shRNA against GCN2 or scrambled. Following antibiotic selection, GCN2-knock down FLS treated with HF (100-200nM) for 6 hours. Western blot analysis was performed as previously described. Results are representative of three independent experiments. B) GCN2 knockdown does not affect inhibition of TNFα induced MMP13 by HF. GCN2-knock down synoviocytes treated with HF (200 nM) for 16 hours, followed by TNFα for 6 hours. Transcript levels of target genes were quantified by qPCR and standardized to a housekeeping gene (PGK1) . Results are representative of three independent experiments.

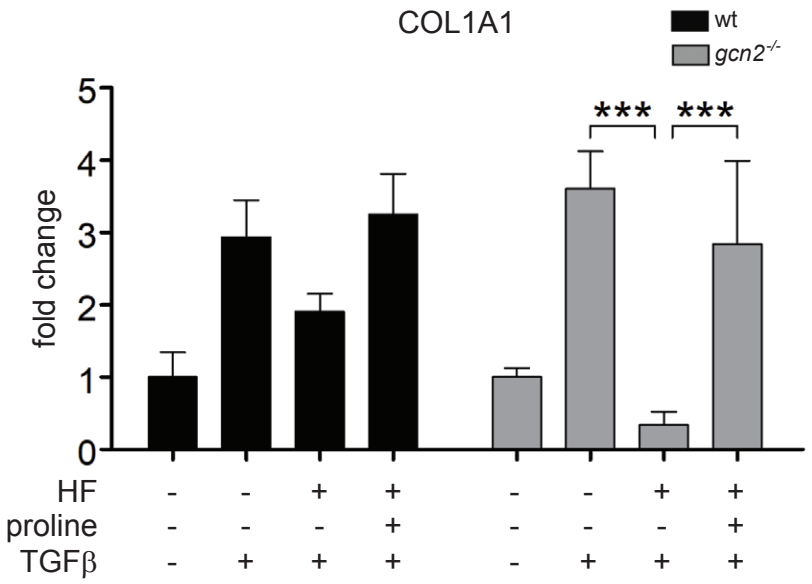

Fig. S9 (related to Fig.2). HF inhibits TGFß effects in GCN2 null cells. Mouse primary dermal fibroblasts from wild type (WT)and GCN2 knockout (GCN2$^{-/-}$) were treated with HF and/or TGFβ. Transcript levels of target genes were quantified by qPCR and normalized to a housekeeping gene control (GAPDH) . Results are representative of three independent experiments.

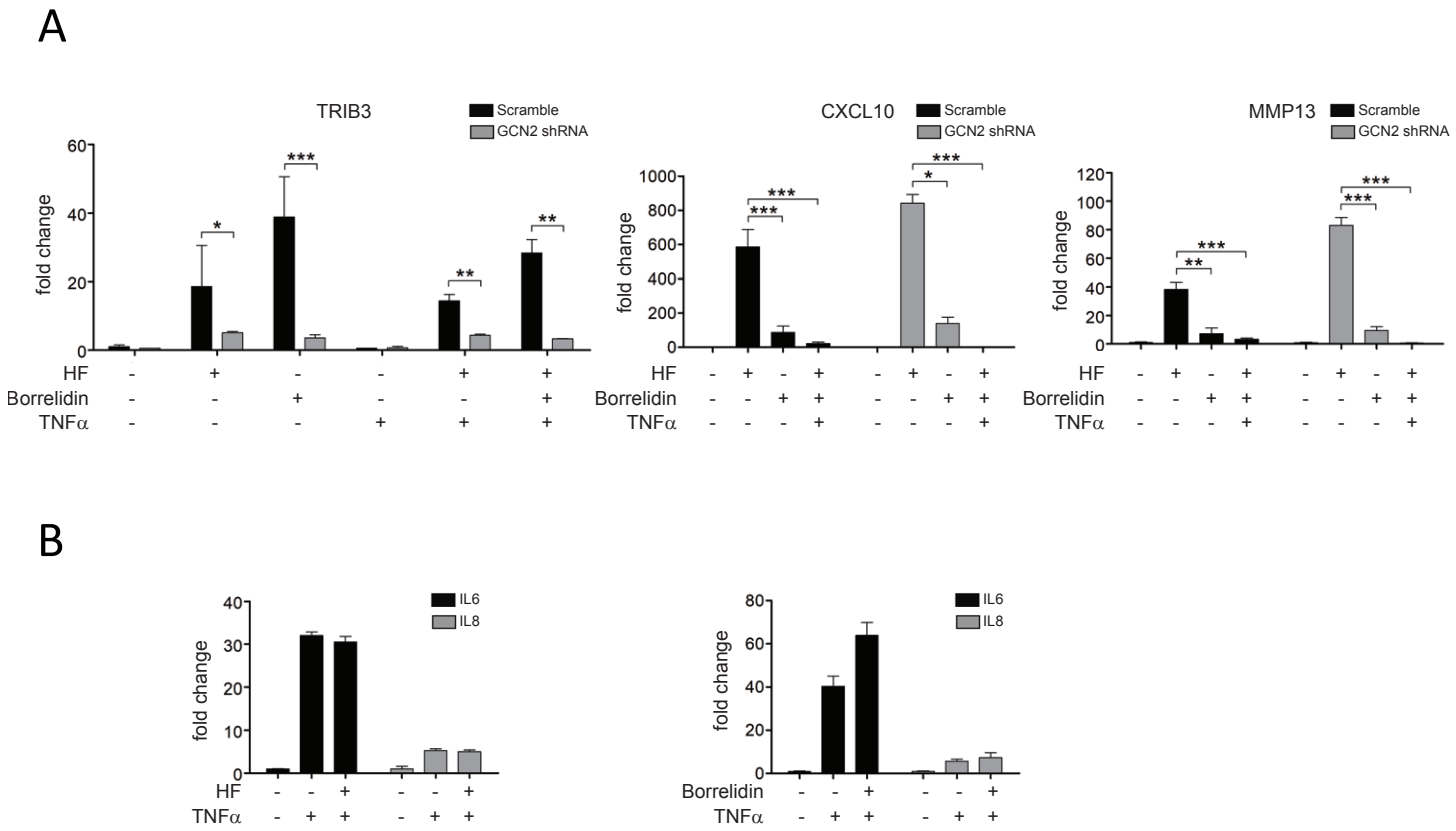

Fig. S10. Borrelidin blocks responses to TNFα in wild type and GCN2 depleted cells. (related to Fig.2). A) Borrelidin effects on TNFα responses are not reduced by GCN2 depletion. wt or shRNA GCN2 depleted K4 fibroblasts were pre-treated with 1000 nM Borrelidin or 200 nM HF for 16 hours, then treated with10 ng/mL TNFα for 6 hours and then analyzed by Q-RT-PCR for expression of a canonical AAR response (TRIB3) or TNFα responses (CXCL10, MMP13) B) Borrelidin, like HF does not inhibit IL6 or IL8 induction by TNFα. FLS were treated with 300 nM HF or 1μM of Borrelidin for 16 hours, treated with TNFα for 4 hours, and analyzed by qRT-PCR. The transcript levels were normalized to PGK1 levels. Results are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001..

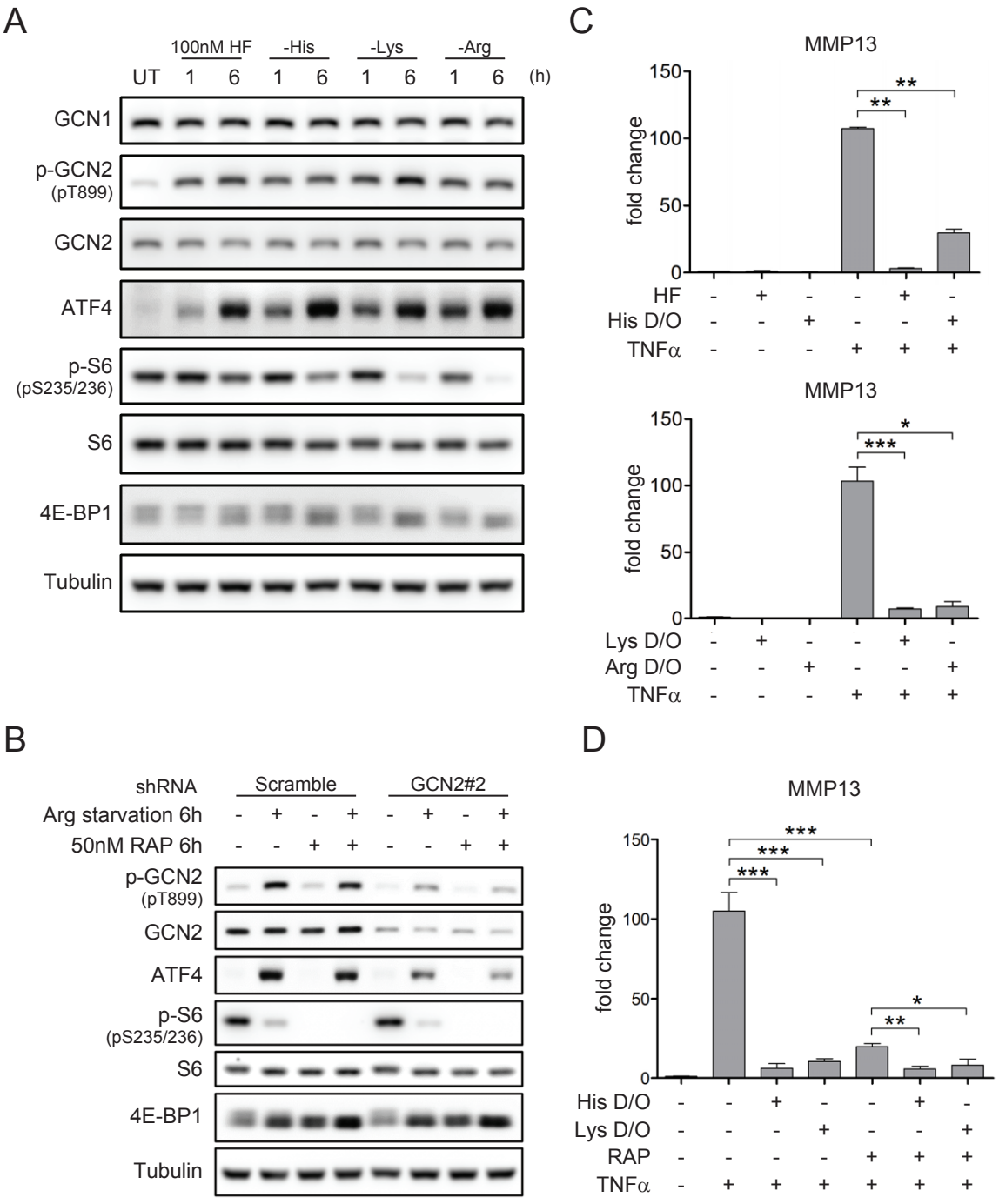

Fig. S11 (related to Fig.3). Effects of individual amino acid depletions on TNFα responses are insensitive to inhibition of GCN2 or mTORC1 signaling. A) Effects of HF treatment or incubation of cells in arginine (arg) or lysine (lys) dropout (D/O) DMEM on AAR pathway activity (p-GCN2, ATF4) and mTORC1 pathway activity (p-S6,4E-BP1). B) effects of GCN2 knockdown (GCN2KD) cells or rapamycin treatement on AAR pathway activity and mTORC1 pathway activity. C) Effect of single amino acid depletion on TNFα induction of MMP13 in wild type cells. D) Effect of histidine or lysine dropout (D/O) medium on TNFα induction of MMP13 in GCN2 knockdown (GCN2KD) cells in the presence or absence of rapamycin.

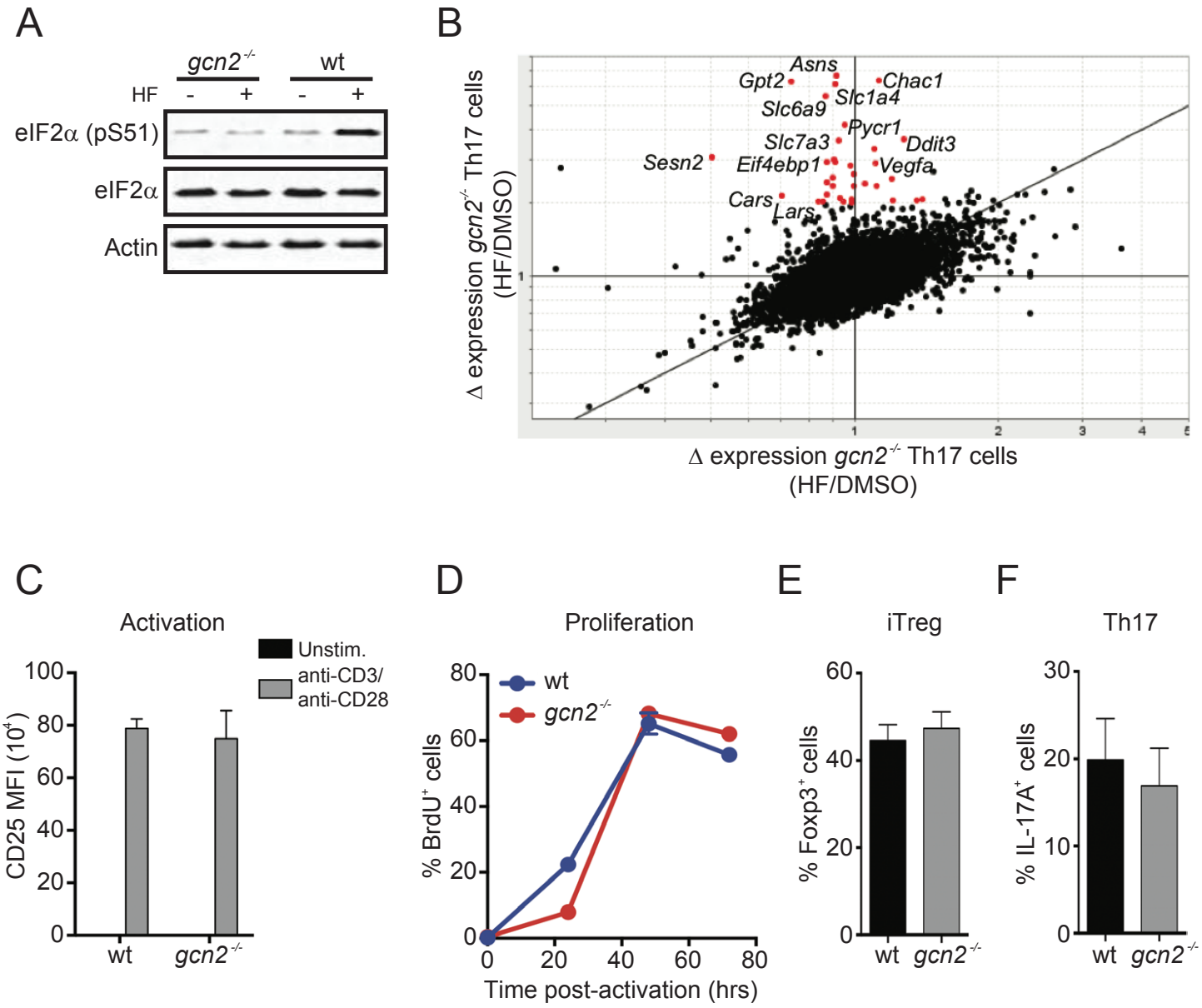

Fig. S12 (Related to Fig.4). HF responses and function of gcn2-deficient T cells. A) eIF2α phosphorylation (serine 51) in wild type or gcn2-deficient CD4+ T cells stimulated for 4hrs with anti-CD3/anti-CD28 antibodies +/- 10nM HF was determined by western blot. B) Differential gene expression, determined by microarray, in wild type or gcn2-deficient CD4+ T cells stimulated for 4hrs in Th17-polarizing cytokine conditions (anti-CD3/anti-CD28 plus TGFb and IL-6) in the absence or presence of 10nM HF. Data are presented as fold-change, fold-change plot, gene expression in HF vs. DMSO treated wild type (wt, y-axis) and gcn2-deficient (x-axis) T cells. Canonical gcn2-dependent amino acid starvation response genes are highlighted in red and labeled. C) CD25 expression (MFI, mean fluorescence intensity), determined by FACS analysis, in wild type or gcn2-deficient CD4+ T cells stimulated for 24hrs with anti-CD3/anti-CD28 antibodies. D) Anti-CD3/anti-CD28-induced proliferation in wild type or gcn2-deficient CD4+ T cells determined by BrdU incorporation and FACS analysis. Efficiency of induced T regulatory (iTreg) cell E) or Th17 F) differentiation in wild type or gcn2-deficient CD4+ T cells. iTreg and Th17 differentiation was assessed by FACS analysis of Foxp3+ or IL-17A+ cells, respectively. All data are representative of 2-3 independent experiments; microarray analysis incorporates data from biological duplicate samples. The number of genes affected by HF vs. DMSO treatment at each timepoint at least 2.5-fold are shown above the bar for each timepoint.

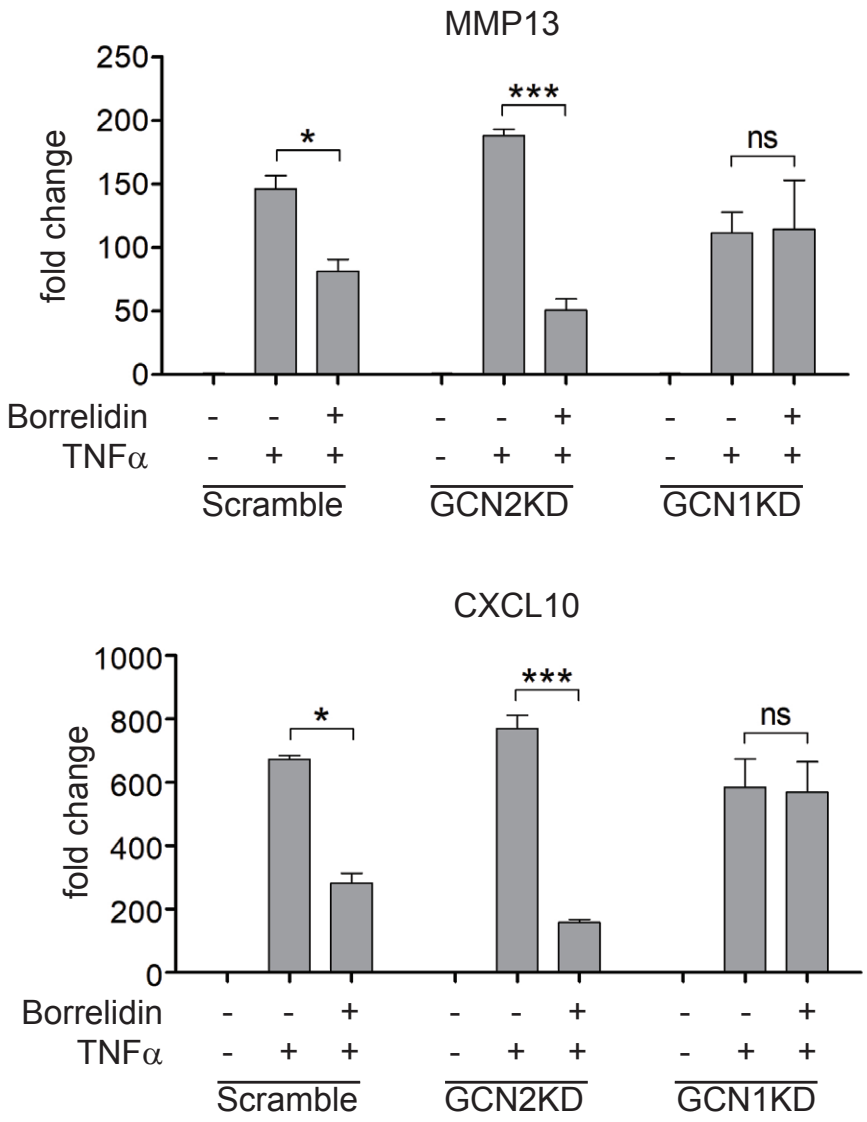

Fig S13 (related to Fig.5). Borrelidin effects on Inflammatory Responses are sensitive to GCN1 depletion and insensitive to GCN2 depletion. K4 wildtype, GCN2, GCN1 knockdown cells were serum starved in 0.2% FBS DMEM for 24h, followed by treatment with 750 nM Borrelidin for 16h and stimulated with 10 ng/ml TNFα for 6h. Relative mRNA levels of CXCL10 and MMP13 were measured by quantitative RT-PCR. Results are representative of three independent experiments (means ± SD, n=3). *p<0.05, **p<0.01, ***p<0.001.

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