RNA m^{6}A modification enzymes shape innate responses to DNA by regulating interferon β

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List of Supplemental Material

SUPPLEMENTAL MATERIALS & METHODS

Immunoblotting and antibodies. Total cellular protein was collected by lysis in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.7M β-mercaptoethanol) followed by boiling for 3 min. Lysates were fractionated by SDS-PAGE and and analyzed by immunoblotting using the following antibodies: anti-ALKBH5 (*Sigma SAB1407587*), anti-FTO (*Abcam ab124892*), mouse anti-m^6A monoclonal (*Synaptic Systems 202111*), rabbit anti-m6A polyclonal (*Synaptic Systems 202003),* anti-METTL14 (*Sigma HPA038002*), anti-METTL3 (*Proteintech 15073-1-AP*), anti-WTAP (*Proteintech 60188-1-Ig*), anti-YTHDF1 (*Proteintech 17479-1-AP*), anti-YTHDF2 (*Proteintech 24744-1-AP*), anti-YTHDF3 (*Proteintech 25537-1-AP*), anti-YTHDC1 (*Abcam ab122340*), anti-STAT1 (*Cell Signaling 9172*), anti-STAT1 Y701 (*Cell Signaling 7649*), anti-MDA5 (*Proteintech 21775-1-AP*), anti-pTBK1 S172 (*Cell Signaling 5483*), anti-TBK1 (*Cell Signaling 3504*), anti-pIRF3 S396 (*Cell Signaling 4947*), anti-IRF3 (*Proteintech 11312-1-AP*), anti-IkBα (*Proteintech 10268-1-AP*), anti-4E-BP1 (*Bethyl Laboratories A300-501A*), anti-actin (*Cell Signaling 3700*), anti-actin (*Cell Signaling 3700)*, anti-GAPDH (Cell Signaling 2118); anti-pp28 (*Virusys CA004-100*), anti-UL44 (*Virusys CA006*), and anti-IE1/IE2 (*Millipore MAB8*10). Primary antibodies were detected using either anti-mouse IgG HRP (*GE Healthcare NA931V*) or anti-rabbit IgG HRP (*GE Healthcare NA934V*) secondary antibodies and visualized by chemiluminescent detection.

RNA interference. siRNAs (20 nM) were transfected using RNAimax (*Invitrogen 13778075*) according to the manufacturer's instructions. METTL3-specific siRNA [5'-CUGCAAGUAUGUUCACUAUGA-3', *Liu et al., 2014*] was synthesized by Sigma.

The following siRNAs were purchased from Sigma: METTL14 (*SASI_Hs01_00179440*), ALKBH5 (*SASI_Hs01_00013942*), FTO (*SASI_Hs02_00314786*), YTHDF1 (*SASI_Hs01_00233688*), YTHDF2 (*SASI_Hs01_00133214*), YTHDF3 (*SASI_Hs01_ 00202277*), STING (*SASI_Hs02_00371843*); AllStars negative-control siRNA was purchased from Qiagen.

Real-time PCR. Total RNA was extracted using TRIzol (*Invitrogen*) according to manufacturer's instructions.cDNA was prepared using qScript XLT cDNA SuperMix (*Quantabio 84358*). Quantitative real-time PCR (qRT-PCR) was performed using SsoAdvanced Universal SYBR Green Supermix (*Bio-Rad 172-5274*) in a Bio-Rad C1000 Touch Thermal Cycler with the following primers:

mRNA	Forward	Reverse
IFNB1	5'-GAAAGAAGATTTCACCAGGG-3'	5'-CCTTCAGGTAATGCAGAATC-3'
GAPDH	5'-TCTTTTGCGTCGCCAGCCGA-3'	5′-ACCAGGCGCCCAATACGACC-3′
DICER1	5'-AAATGGGAAATGTGATCCAG-3'	5'-AGTATACCTGTCTAAGACCAC-3'
METTL3	5'-CGGGTAGATGAAATTATTTGGG-3'	5'-GATTTCCTTTGACACCAACC-3'
METTL14	5'-ACTAGAAATGCAACAGGATG-3'	5'-GATTTAAGCTCTGTGTTCCC-3'
ALKBH5	5'-CGGCGAAGGCTACACTTACG-3'	5'-CCACCAGCTTTTGGATCACCA-3'
YTHDF1	5'-CCAGAGAACAAAAGGACAAG-3'	5'-TTTGACTGTCCAGTAAGGTAG-3'
YTHDF2	5'-CCAAGAGGAAGAAGAAAGTG-3'	5'-AGTCCTAATTCTCTTGAAGGTC-3'
YTHDF3	5'-ATCAGAGTAACAGCTATCCAC-3'	5'-CCCAGGTTGACTAAATACAC-3'
YTHDC1	5'-AAGAGAGCTAGAGGCATATC-3'	5'-ATGCTTCTTTTCTGAACCTG-3'
PPIA	5'-CCCACCGTGTTCTTCGACAT-3'	5'-TCTTTGGGACCTTGTCTGCAA-3'

Type I interferon (IFN) Bioassay. Type I IFN was quantified using the reporter cell line HEK-Blue™ IFN-α/β (*InvivoGen, hkb-ifnab*) according to the manufacturer's protocol. Briefly, HEK-Blue IFN- α/β cells in 180μL media were incubated with 20 μL NHDF cell culture supernatant at 37° C and 5% CO_2 for 24 hours. Secreted alkaline phosphatase (SEAP) activity was detected by incubating 20 μL HEK-Blue IFN- α/β supernatant with 180 μL QUANTI-Blue™ (InvivoGen, rep-qb1) alkaline phosphatase substrate at 37°C and 5% CO_2 for 15 min. SEAP activity was quantified by measuring the optical density at 640nm in a SpectraMax M3 plate reader and converted to IFN units using a standard curve generated by quantifying SEAP activity in the supernatant of HEK-Blue IFN- α/β cells incubated with recombinant IFNβ protein (*PBL Assay Science, 11415-1*)

Indirect Immunofluorescence. NHDFs grown on coverslips were fixed with 4% formaldehyde for 15 min, permeabilized with 0.5% TritonX-100 in PBS for 10 min and then blocked with 4% FBS. Immunostaining was performed using the appropriate primary and secondary antibodies. DNA was stained with 4',6'-diamidino-2-phenylindole (DAPI). Images were captured using a Leica DM5000 microscope equipped with Leica Imaging Software.

Analysis of m^{6}A RNA sequencing. Following demultiplexing, sequence reads were deduplicated using BBtools (bbduk.sh) after which sequencing adapters were removed and low quality ends trimmed using TrimGalore (https://github.com/FelixKrueger/TrimGalore) [trim_galore --length 25 infile --clip_R1 5]. Sequence reads were subsequently mapped to hg19 using bowtie2 (*Langmead &*

Salzberg, 2012) and parsed using SAMTools (*Li et al., 20019*) to yield sorted, indexed BAM files. m^6A-peak regions were subsequently identified using exomePeak (*Meng et al., 2014*) and visualized using IGV (*Robinson et al., 2011*) and GVIZ (*Hahne & Ivanek, 2016*).

SUPPLEMENTARY REFERENCES

Hahne F, Ivanek R. 2016. Visualizing Genomic Data Using Gviz and Bioconductor. *Methods Mol Biol*. **1418**: 335-351.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* **9**: 357-359.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R; 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. **25**: 2078-2079.

Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, Jia G, Yu M, Lu Z, Deng X, Dai Q, Chen W, He C. (2014). A METTL3–METTL14 complex mediates mammalian nuclear RNA *N*6-adenosine methylation. *Nat. Chem. Biol.* **10:** 93-95.

Meng J, Cui X, Rao MK, Chen Y, Huang Y. 2013. Exome-based analysis for RNA epigenome sequencing data. *Bioinformatics*. **29**:1565-1567.

Meng J, Lu Z, Liu H, Zhang L, Zhang S, Chen Y, Rao MK, Huang Y. 2014. A protocol for RNA methylation differential analysis with MeRIP-Seq data and exomePeak R/Bioconductor package. *Methods*. **69**: 274-281.

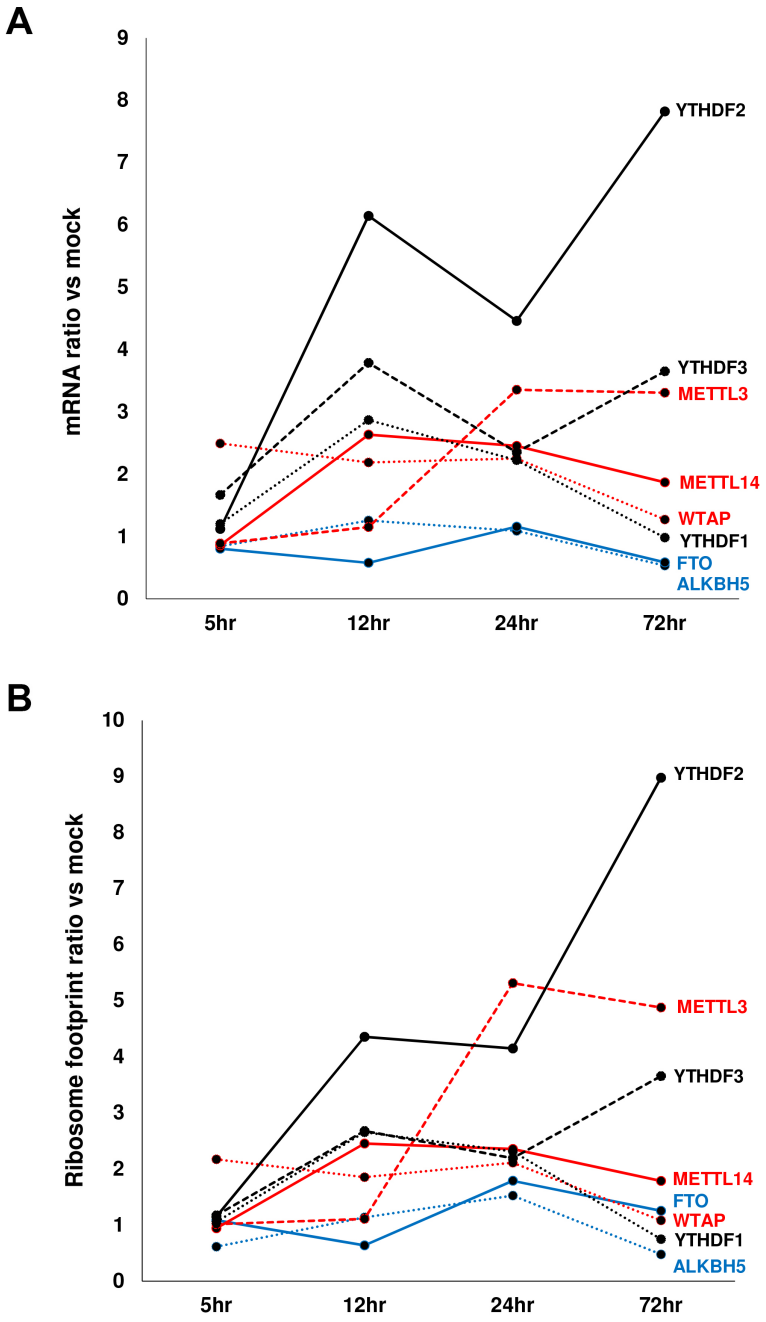
Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. 2011. Integrative genomics viewer. *Nat Biotechnol.* **29**: 24-26.

Supplementary Table S1: m^6A peak regions (ExomePeak output) identified in buffer-treated and dsDNA-treated samples.

Supplementary Table S2: Genes commonly regulated by METTL14 and ALKBH5 depletion.

Supplementary Table S3: Sequencing metrics and sequence read archive (SRA) accessions.

Supplementary Table S4: m^6A sequencing metrics and sequence read archive (SRA) accessions.



<u>Figure S1</u>. mRNA abundance and ribosome footprints of m^{6}A readers, writers, and erasers. (A) Abundance of selected mRNAs, relative to mock treatment, as profiled during a 72 hr HCMV infection. (B) Ribosome footprint profiling of the same mRNAs. All data were extracted from Tirosh et al., 2015, Supplementary Table S1.

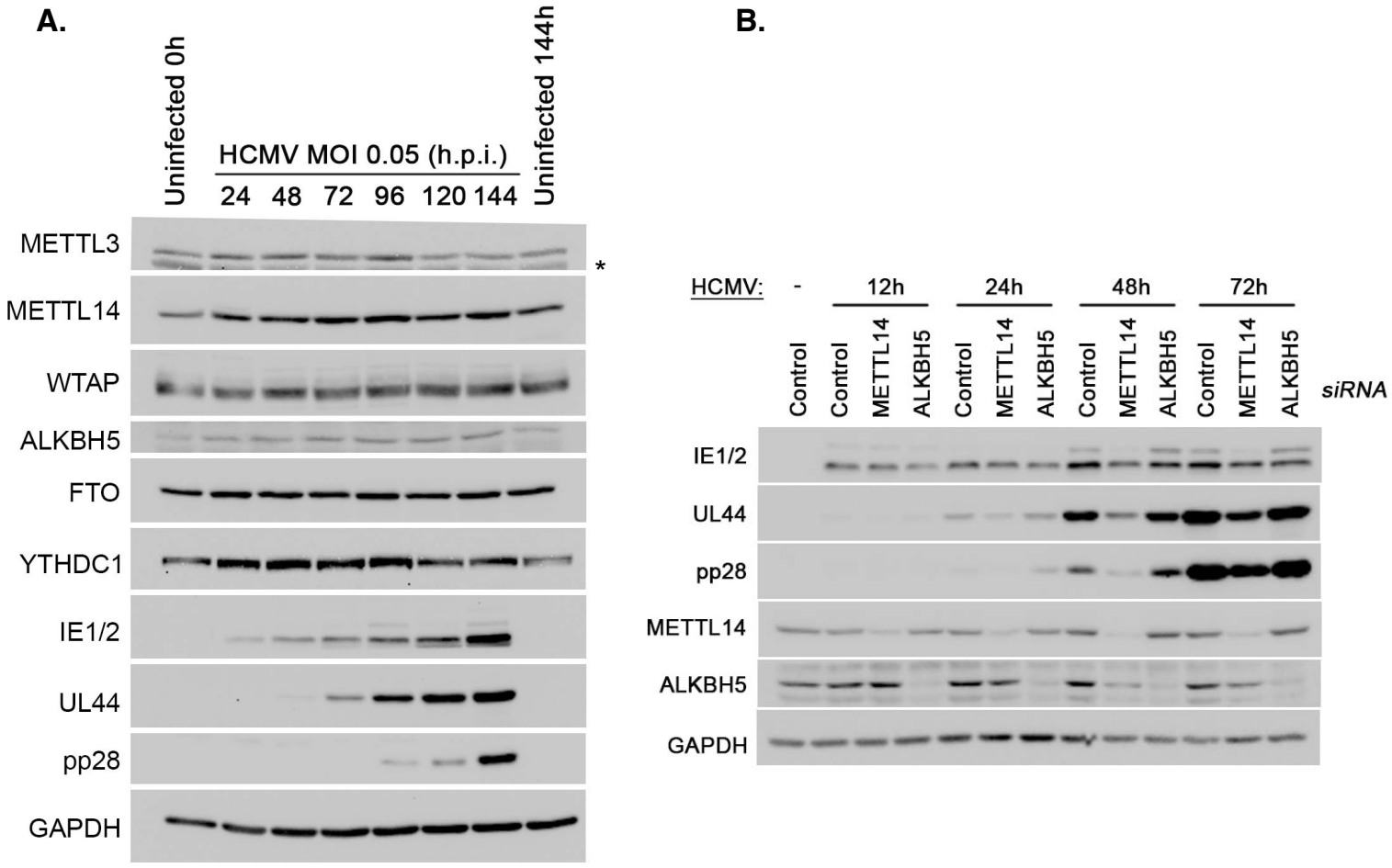
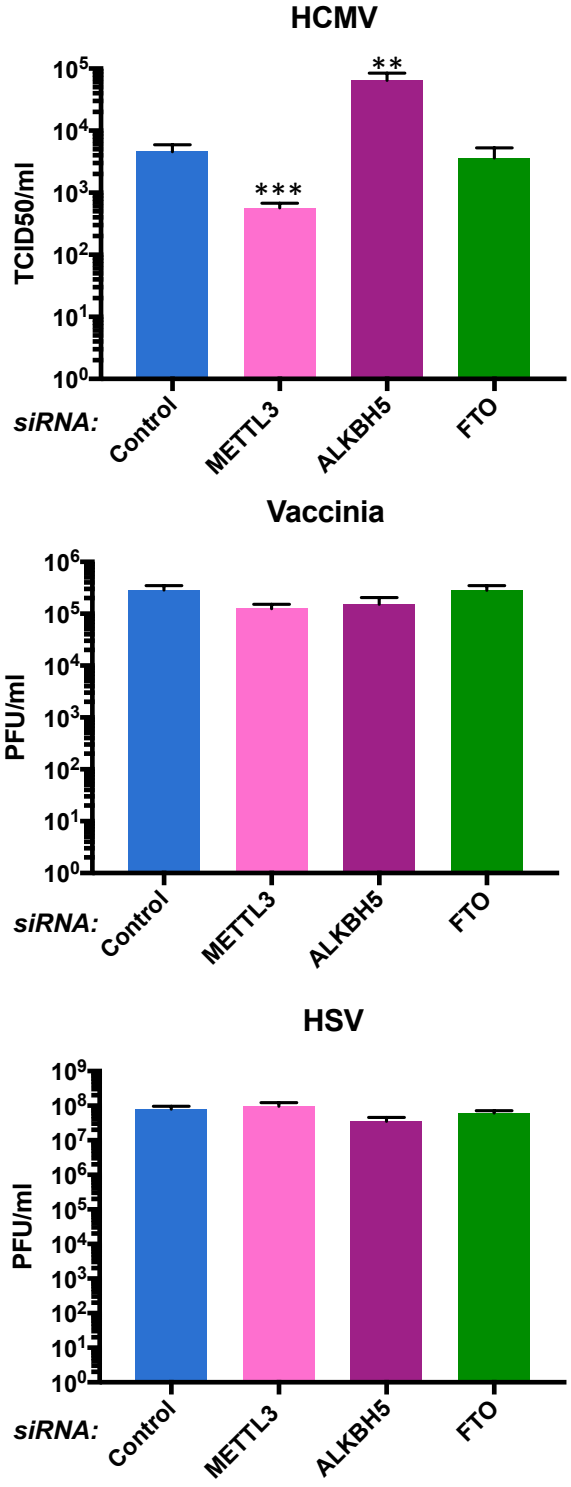


Figure S2. Cellular m^{6}A machinery responds to HCMV infection and regulates virus gene expression. A) NHDFs were mock-infected (uninfected) or infected with HCMV (MOI=0.05). At the indicated hours post-infection (hpi), total protein was collected, fractionated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. GAPDH represents a host antigen whose levels remain unchanged during infection. B) NHDFs were treated with control, non-silencing siRNA, siRNA specific for the m^{6}A methyltransferase METTL14 subunit, or siRNA specific for the ALKBH5 demethylase. After 72h, cultures were infected with HCMV (MOI=3). At the indicated h post-infection, total protein was isolated, fractionated by SDS-PAGE and analyzed by immunoblotting with the indicated antisera.



<u>Figure S3.</u> Interfering with the host METTL3 m^{6}A methylase and ALKBH5 demethylase selectively impacts HCMV, but not HSV1 or Vaccinia productive growth. NHDFs were treated with control, non-silencing siRNA, siRNA specific for the m^{6}A methyltransferase METTL3 subunits, or siRNA specific for the ALKBH5 or FTO demethylase. After 72 h, cultures were infected with HCMV (MOI=0.05), HSV-1 (MOI=5x10^{-4}), or Vaccinia virus (MOI=5x10^{-4}). Supernatants were harvested after 7 d for HCMV, 72 h for HSV-1 and 48 h for Vaccinia virus and virus titer (TCID/50) determined using NHDFs for HCMV or by plaque assay in Vero cells for HSV-1 and Vaccinia. The error bars indicate SEM. **, $P \leq 0.01$; ***, $P \leq 0.001$, by Student's *t* test.

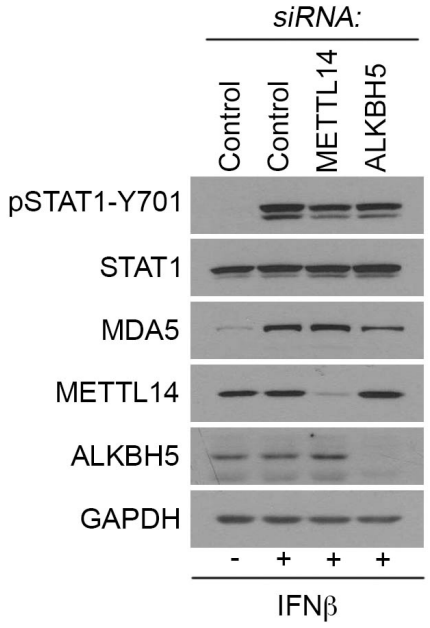
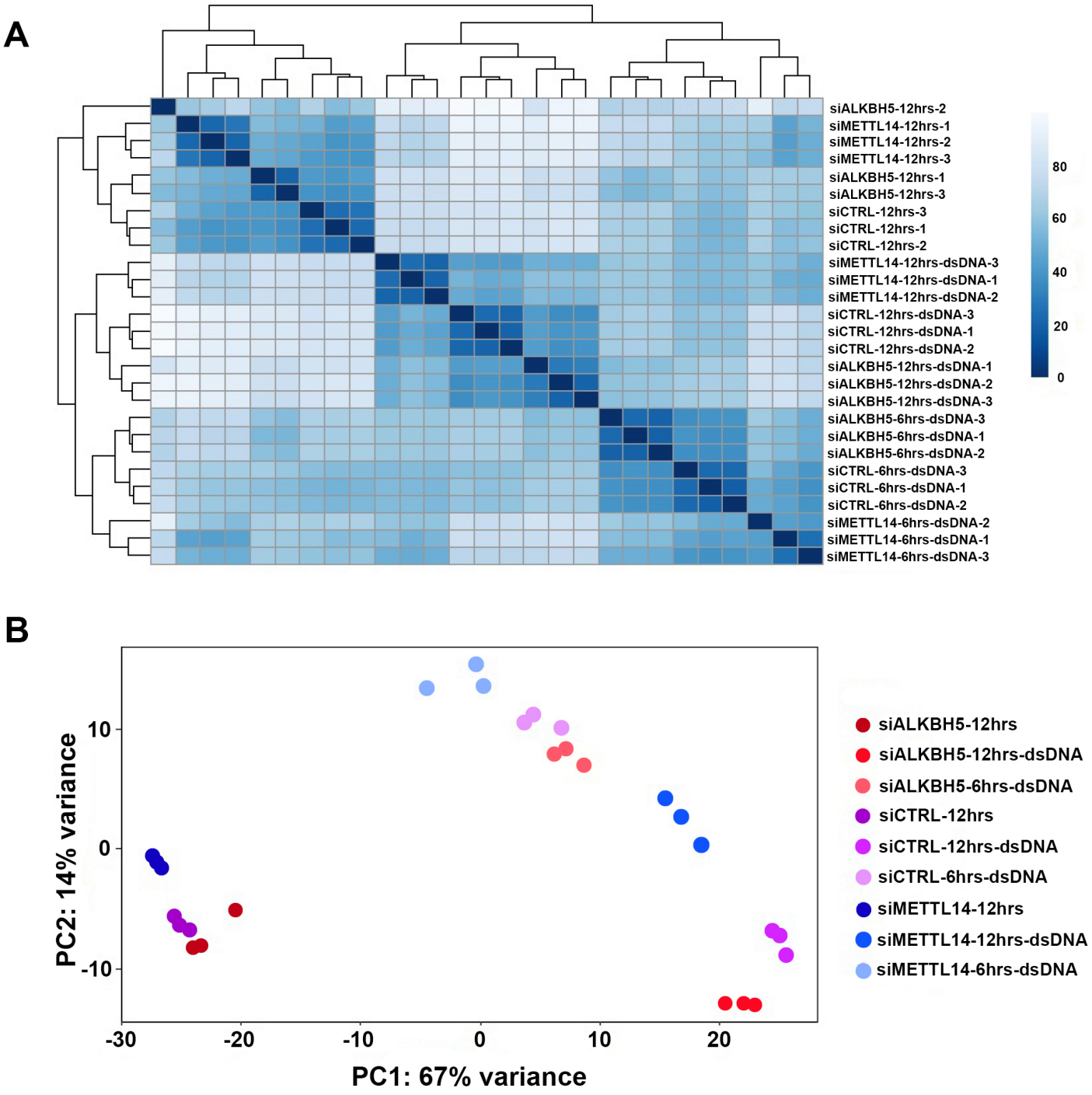


Figure S4. METTL14 or ALKBH5-depletion does not detectably impact STAT1 phosphorylation in response to IFNβ. NHDFs transfected with control, non-silencing siRNA or siRNA specific for METTL14 or ALKBH5 were untreated or treated with 100 U IFNb. After 4h, total protein was collected, fractionated by SDS-PAGE and analyzed by immunoblotting using the indicated anti-sera.



<u>Figure S5</u>. Assessment of sequencing quality. a, heatmap of sample-to-sample distances comparing all twenty-seven samples sequenced in this study. Sample siALKBH5-12hrs was excluded from further analyses due to aberrant clustering. **b,** principal component analysis (PCA) plot showing the same dataset organized according the principal components 1 and 2.

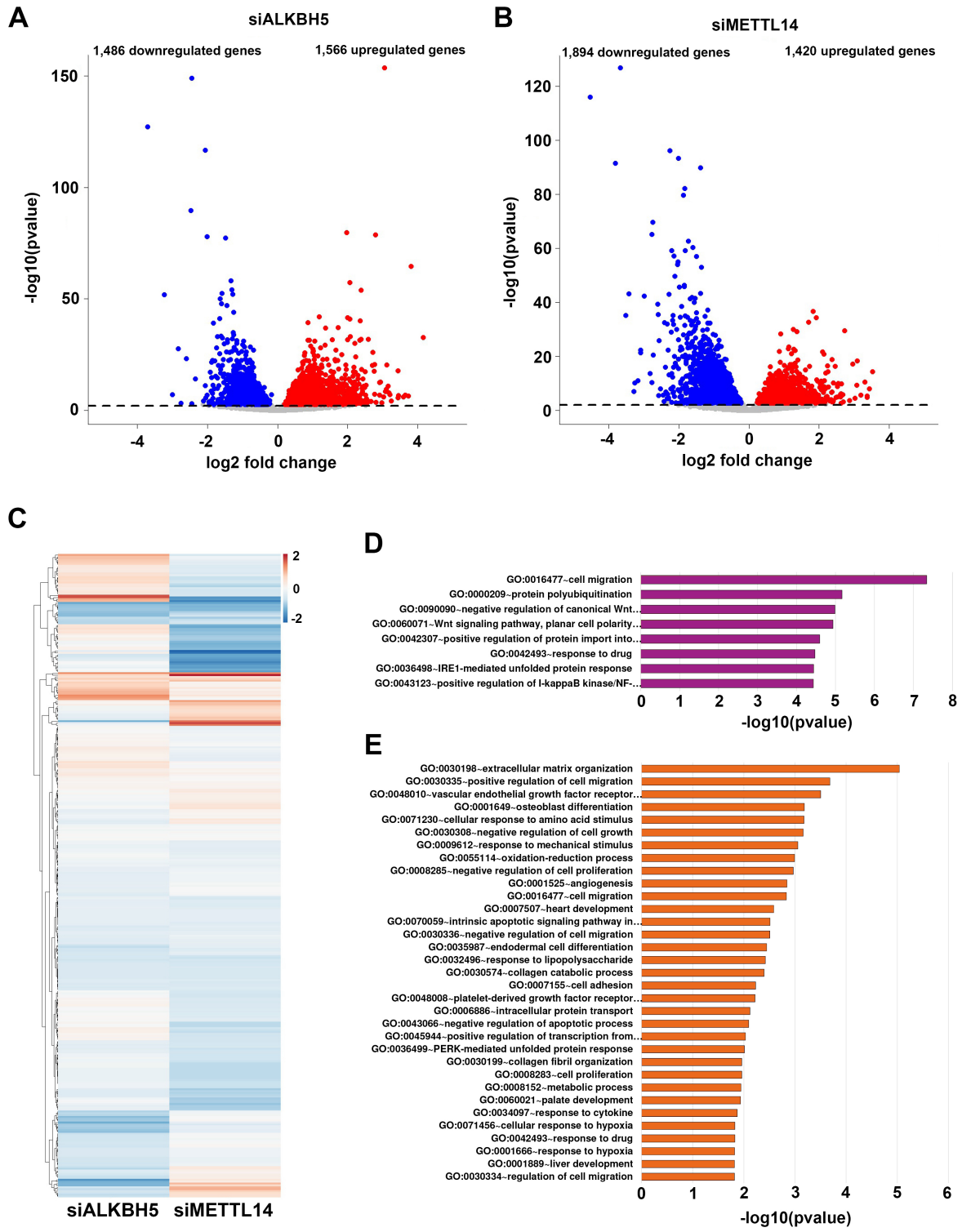


Figure S6. Control of genome-wide responses to dsDNA by m^{6}A demetyhylase ALKBH5 and m^{6}A methylase subunit METTL14.. Volcano plots show differentially expressed genes (adjusted p value < 0.01) identified from RNA-Seq of polyadenylated RNA, collected from cells transfected with (a) siALKBH5 (n=3 biological replicates), or (b) siMETTL14 (n=2 biological replicates), and stimulated with dsDNA for 6 h. Genes upregulated versus an non-silencing siRNA control (stimulated with dsDNA for 6 h, n=3 biological replicates) are shown in red, while downregulated genes are shown in blue. Non-regulated genes are shown in grey. (c) Heatmap depicting 349 interferon-stimulated genes (ISGs), colored according to log2fold change in expression versus the non-silencing siRNA control. (d-e) Pathway analyses (GO direct terms) of significantly differentially expressed genes from (a) and (b) were conducted using DAVID and filtered according to a Benjamini-Hochberg procedure (< 0.05).

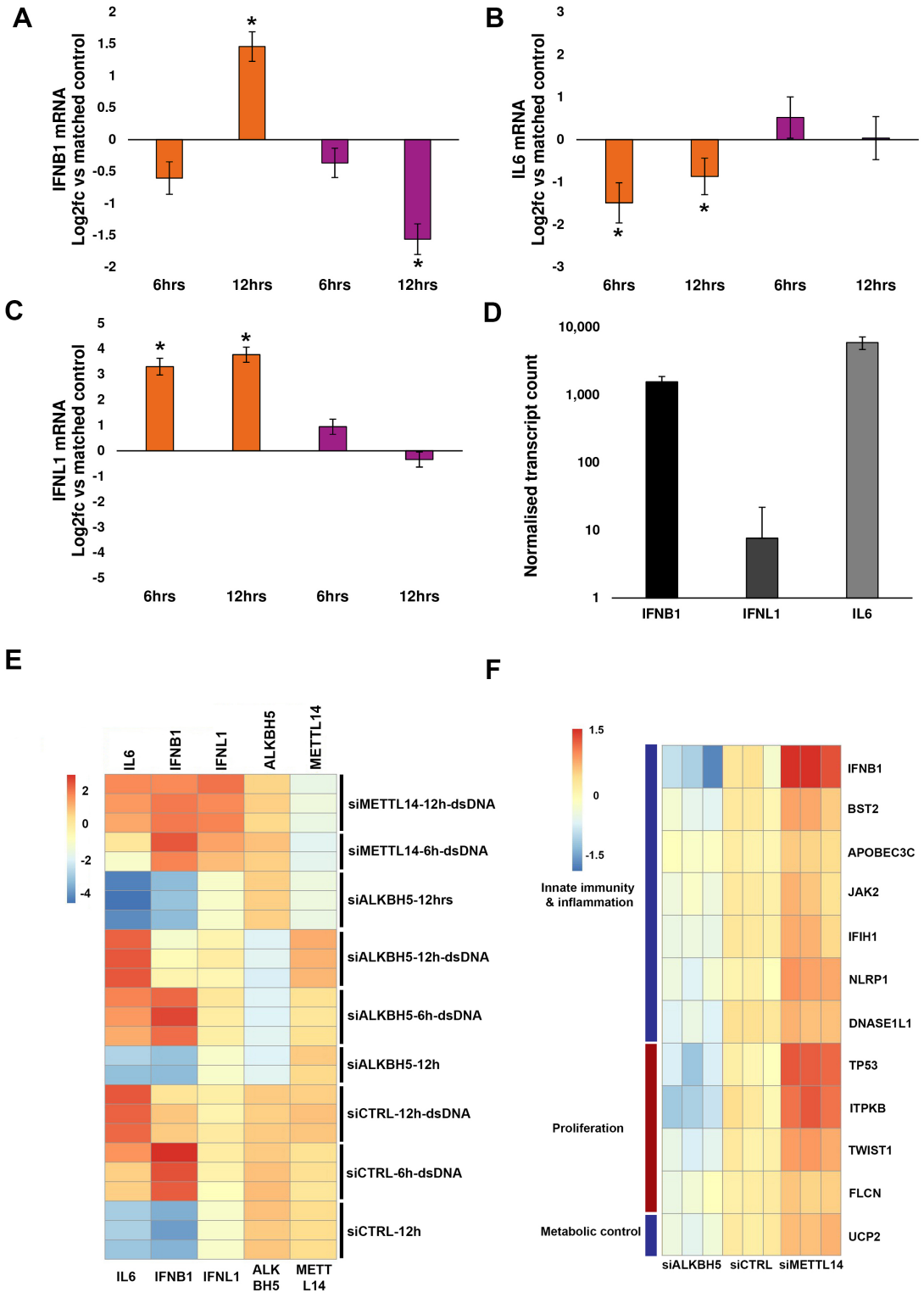
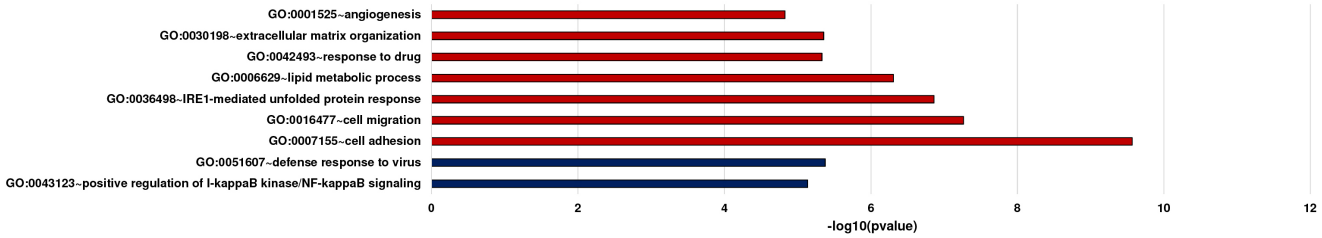
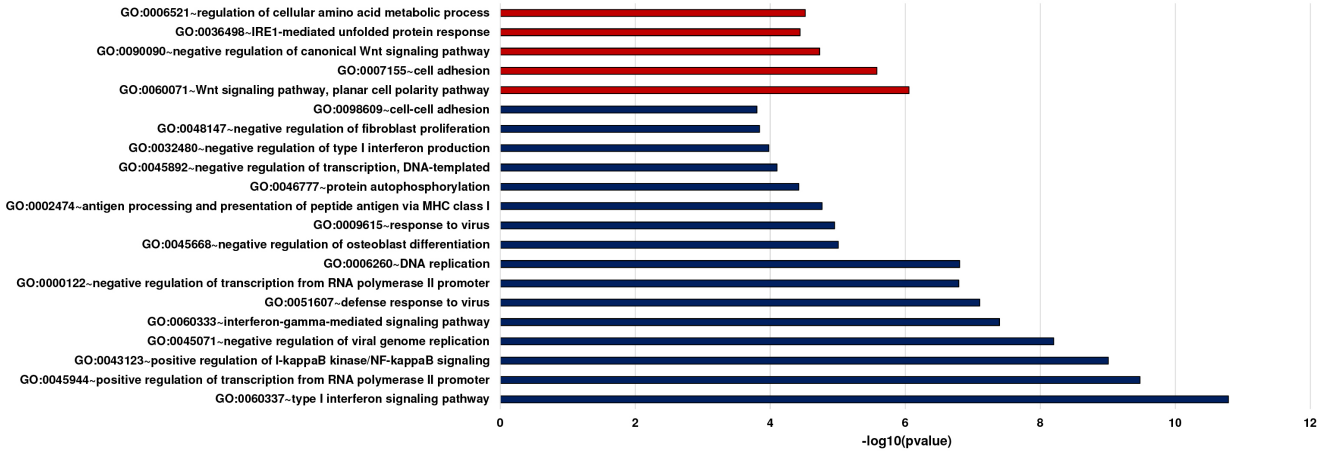


Figure S7. Profiling of regulated interferons and siRNA targets Following stimulation with dsDNA, increased cellular transcription of **A,** IFNB1, **B,** IL-6, and **C**, IFNL1 is variably modulated by siRNA-mediated suppression of METTL14 (orange) and ALKBH5 (purple) relative to siRNA controls at 6 and 12 hrs post-stimulation. Statistically significant differences log2 fold changes (adjusted p value < 0.05) are highlighted by asterisks (*). Error bars represent the log2 standard error. **D,** median normalized transcripts counts (mRNA abundance) for IFNB1, IFNL1, and IL6. Error bars indicated the standard error. **E**, heatmap showing relative expression of the three regulated interferons and two siRNA targets. **F**, heatmap showing relative expression of genes upregulated by METTL14-depletion and downregulated by ALKBH5-depletion.

A.



B.



<u>Figure S8.</u> Pathway analysis of differentially expressed genes in siALKBH5-treated cells A) after a 6 or 12 (B) hrs post dsDNA stimulation. Enriched pathways (GO direct terms) were identified via David (REF) using all significantly upregulated (red) or downregulated (blue) gene sets. Only pathways with a Benjamini-Hochberg score < 0.05 are shown.

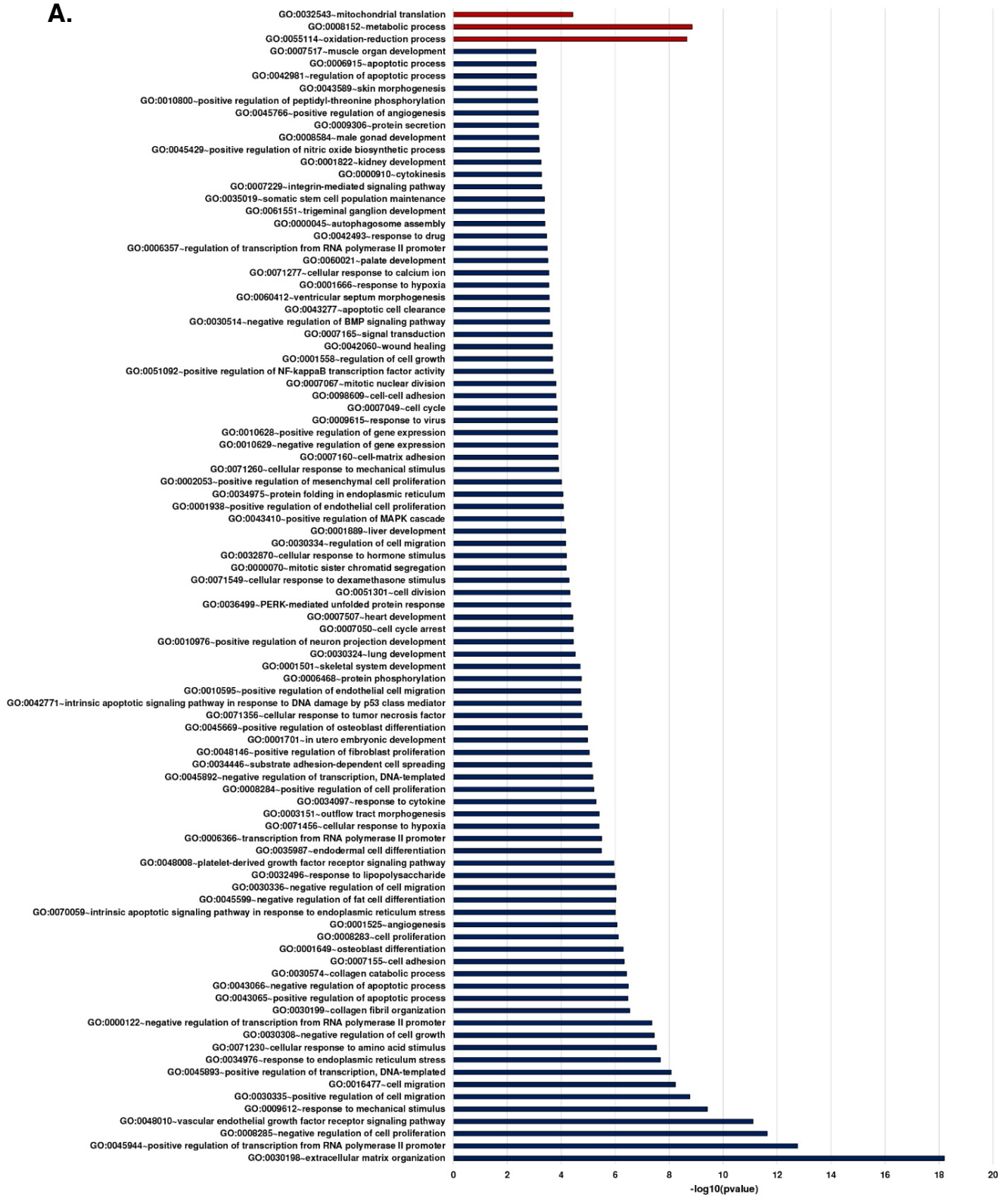
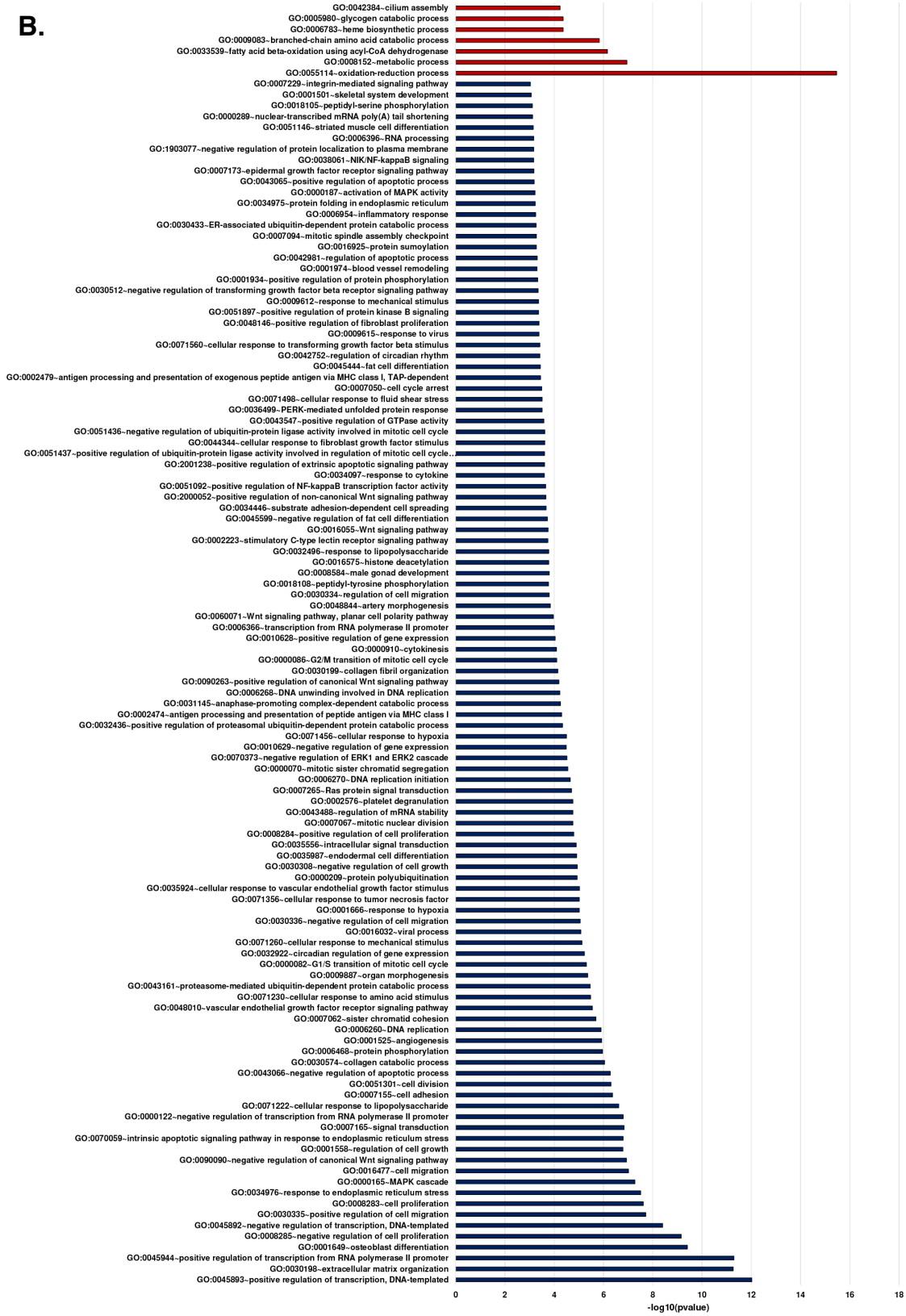
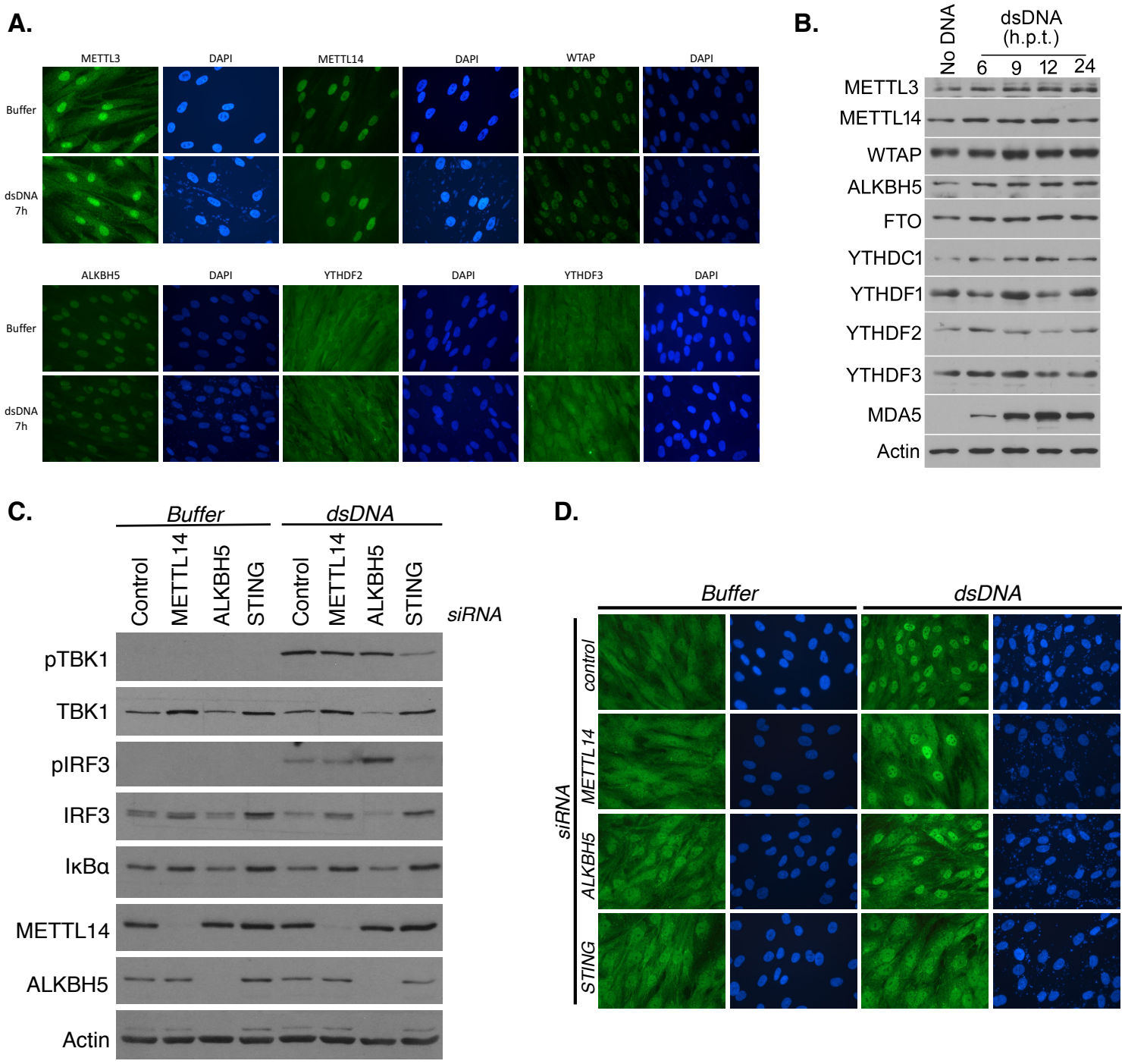


Figure S9. Pathway analysis of differentially expressed genes in siMETTL14-treated cells. A. Enriched pathways (GO direct terms) 6 hours post-DNA stimulation were identified via David (REF) using all significantly upregulated (red) or downregulated (blue) gene sets. Only pathways with a Benjamini-Hochberg score < 0.05 are shown. B. As in A except after 12 hours post-DNA stimulation



<u>Figure S9 - *cont'd.*</u> Pathway analysis of differentially expressed genes in siMETTL14-treated cells .



<u>Figure S10.</u> Response of m^{6}A machinery to dsDNA in uninfected cells. A) NHDFs exposed to buffer or dsDNA for 7h were fixed, permeablized and stained with DAPI. Subcellular distribution of host m^{6}A methylase subunits (*METTL3, METTL14, WTAP*), the demethylase ALKBH5, or m^{6}A readers (*YTHDF2, YTHDF3*) was visualized by indirect immunofluorescence using the indicated antibodies. B) Total protein isolated from NHDFs exposed to buffer or transfected with dsDNA for the indicated times (h.p.t.= h post-transfection) was fractionated by SDS-PAGE and analyzed by immunoblotting with the indicated antisera. Actin is a loading control; MDA5 is a control showing dsDNA-induced protein accumulation. C. NHDFs were treated with control, non-silencing siRNA, or siRNA specific for the indicted targets (*METTL14, ALKBH5, STING*). After 72 h, cultures were treated with buffer or dsDNA for 6 h. Total protein was harvested, fractionated by SDS-PAGE and analyzed by immunoblotting with the antibodies indicated on the left. Actin is a loading control. D) As in C except cultures were fixed, permeabilized, and stained with DAPI. Subcellular IRF3 distribution was determined by indirect immunofluorescence using an IRF3-specific antibody.