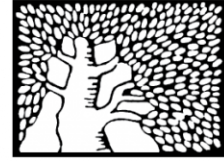


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1 **m⁶A modification controls the innate immune response to infection by**
2 **targeting type I interferons**

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16
17 **ABSTRACT**

18 N⁶-methyladenosine (m⁶A) is the most common mRNA modification. Recent studies revealed
19 that depletion of m⁶A machinery leads to alterations in the propagation of diverse viruses.
20 These effects were proposed to be mediated through dysregulated methylation of viral RNA.
21 Here we show that following viral infection or stimulation of cells with an inactivated virus,
22 the deletion of m⁶A ‘writer’, METTL3, or ‘reader’, YTHDF2, led to an increase in the
23 induction of interferon-stimulated genes. Consequently, propagation of different viruses was
24 suppressed in an interferon signaling dependent manner. Significantly, the mRNA of *IFNB*,
25 the main cytokine that drives type I interferon response, was m⁶A-modified, and was
26 stabilized upon repression of METTL3 or YTHDF2. Furthermore, we show that m⁶A-
27 mediated regulation of interferon genes was conserved in mouse. Altogether, our findings
28 uncover the role of m⁶A as negative regulator of interferon response, by dictating the fast
29 turnover of interferon mRNAs and consequently facilitating viral propagation.

30 INTRODUCTION

31 Methylation at the N⁶ position of adenosine (m⁶A) is the most abundant internal mRNA
32 modification, which is present in over 25% of human transcripts and typically enriched near stop
33 codons and terminal exons¹⁻³. It has been linked to various stages along the post-transcriptional
34 trajectory of mRNA, and in particular to promoting mRNA decay³⁻⁸. Deposition of m⁶A
35 occurs co-transcriptionally through a large protein complex ('m⁶A writers'), comprising the
36 catalytic subunit METTL3 and co-factors such as METTL14 and WTAP⁸⁻¹⁰. The modification is
37 then functionally 'interpreted' through the binding of m⁶A 'reader' proteins, which multiple of
38 them have been identified. Among m⁶A 'readers', the cytoplasmic YTH-domain family 1
39 (YTHDF1), YTHDF2 and YTHDF3 proteins have been shown to directly bind and recognize
40 m⁶A through their C-terminal YTH domain. These proteins are thought to mediate a myriad of
41 cellular processes including mRNA decay⁸ and it has been recently proposed that m⁶A and its
42 YTHDF 'readers' play a central role in shaping the cellular 'identity' by regulating a
43 synchronized processing of groups of transcripts¹¹. Finally, two potential demethylase 'erasers'
44 (ALKBH5 and FTO) were suggested to remove m⁶A modification from mRNAs¹²⁻¹⁴.

45 Functionally, m⁶A has been shown to impact fundamental cellular processes in diverse
46 organisms, including meiosis¹⁵, circadian clock¹⁶, DNA damage repair¹⁷, differentiation
47 of embryonic stem cells¹⁸, sex determination and neuronal functions¹⁹. More
48 recent *in vivo* studies conducted in mice uncovered deficits in differentiation^{20,21} and in immune
49 homeostasis^{22,23} in mice deficient in m⁶A machinery proteins. These studies have established
50 critical roles for m⁶A-dependent mRNA decay in regulating cellular machineries¹⁰.

51 The presence of m⁶A on transcripts of diverse viruses has long been known (reviewed in
52 ²⁴). The identification of the m⁶A machinery components stimulated new research into the roles
53 of m⁶A modification in viral RNA processing. Recent studies demonstrated that m⁶A 'writers'
54 and 'readers' play important roles in modulating the life cycle of numerous RNA and DNA
55 viruses²⁵⁻³⁴. Although in most of these studies the mechanistic basis of m⁶A effects on viral
56 propagation remained unclear, in all studies viral mRNAs were shown to be m⁶A-modified and
57 m⁶A effects were suggested to occur by direct m⁶A-mediated regulation of viral RNA
58 processing.

59 Here we reveal that upon depletion of the m⁶A 'writer' METTL3, viral infection
60 resulted in a modular and highly-specific induction of hundreds of interferon-stimulated genes

61 (ISGs), which constitute one of the first lines of antiviral defense. Consistent with these
62 observations, we show that drug-induced blocking of interferon (IFN) signaling restored viral
63 proliferation in METTL3- or YTHDF2-depleted cells. Importantly, this modular ISG
64 induction was also seen after stimulation of cells with a UV-inactivated virus, illustrating that
65 this effect was not driven by viral mechanisms. We demonstrate that the mRNA of IFN- β , the
66 central cytokine that drives the type I IFN response, was modified by m⁶A and was
67 significantly stabilized upon depletion of METTL3 or YTHDF2. Furthermore, m⁶A
68 methylation of *IFNB1* was conserved in murine cells and *Ifna* mRNA was also modified by
69 m⁶A. Finally, by constructing gene-deficient mice, we show that mice lacking the m⁶A
70 ‘reader’ protein YTHDF3 exhibit enhanced *Ifna* and *Ifnb* induction upon viral infection.
71 Altogether, our findings highlight the central role of m⁶A as negative regulator of type I IFN
72 response, by dictating the fast turnover of *IFNA* and *IFNB* mRNAs.

73

74 RESULTS

75 The m⁶A machinery is required for human cytomegalovirus propagation

76 The herpesvirus, human cytomegalovirus (HCMV), replicates in the nucleus relying on
77 cellular machinery for viral gene transcription and processing. We hypothesized that m⁶A is
78 likely to be involved in HCMV propagation. Supporting this hypothesis, we observed that m⁶A
79 ‘writers’ and ‘readers’ were both transcriptionally and translationally induced along HCMV
80 infection³⁵ (Supplementary Fig. 1a). We confirmed those findings in primary human foreskin
81 fibroblasts, in which the m⁶A ‘writer’ proteins METTL3 and METTL14 and ‘reader’ proteins
82 YTHDF2 and YTHDC1 were upregulated by HCMV infection at the protein level (Fig. 1a).
83 This induction of the m⁶A machinery prompted us to examine how depletion of m⁶A ‘writers’,
84 ‘readers’ and ‘erasers’ impacts HCMV propagation. Using CRISPR/Cas9 and sgRNAs
85 targeting the m⁶A ‘writer’ proteins METTL3, METTL14 and WTAP, the m⁶A ‘reader’
86 proteins YTHDF1, YTHDF2, and YTHDF3 or the putative m⁶A demethylases FTO and
87 ALKBH5, we generated fibroblasts in which these proteins were depleted. Since we used
88 primary fibroblasts, we did not isolate single cell clones but instead we confirmed the
89 efficient depletion of the targeted proteins in a mixed population (Supplementary Fig. 1b,c).
90 These cells were infected with an HCMV strain containing an SV40 promoter-driven
91 expression of green fluorescent protein (GFP)³⁶, which allows for fluorescence-based
92 monitoring of infection. Supernatants were collected and used to infect fresh wild-type
93 fibroblasts and the percentage of GFP positive cells was measured, as proxy for viral titers, by
94 flow cytometry (Fig. 1b,c) and microscopy (Supplementary Fig. 1d). Notably, we observed
95 strong reduction in viral titers when viruses were propagated in cells depleted of m⁶A
96 ‘writers’ or ‘readers’ (Fig. 1b) and elevation in viral titers when ALKBH5-depleted cells were
97 used (Fig. 1c). These effects were not due to differences in the cells viability before or after
98 HCMV infection (Supplementary Table 1). Furthermore, the efficiency of initial infection was
99 comparable in wild-type and depleted cells, as we did not observe any differences in the
100 abundance of the major immediate early viral protein (IE1-pp72) and the virally encoded GFP
101 at 24 hours post infection (hpi) (Fig. 1d and Supplementary Fig. 1e). Significant reduction in
102 viral protein expression was observed at 48 hpi, in cells depleted of METTL3 compared to
103 control cells, illustrating that the block in viral propagation occurred at relatively late stages
104 of HCMV infection (Fig. 1e and Supplementary Fig. 1f).

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m⁶A-mediated inhibition of HCMV growth is driven by enhanced type I IFN response

To date studies have suggested that the effect of m⁶A ‘writers’ and ‘readers’ on viral propagation are mediated by methylation and dysregulation of viral transcripts²⁵⁻³⁴. To assess whether HCMV transcripts were m⁶A-modified, we performed genome-wide m⁶A methylation profiling in HCMV-infected cells. Using relatively conservative thresholds, we identified 21 viral transcripts that contain enriched m⁶A peaks that were specific to wild-type but not to METTL3-depleted cells (Supplementary Table 2). To investigate the effects of m⁶A modification on viral gene expression, we conducted RNA-seq on METTL3-depleted and control cells 28 hpi with HCMV. This relatively early time point was chosen to allow capturing direct effects of m⁶A modification. Although we observed subtle but significant reduction in the overall viral gene expression in METTL3-depleted cells (Fig. 2a), we did not detect significant changes in the expression of viral transcripts that were found to be m⁶A-modified (Fig. 2b). In contrast, when we examined differences in cellular gene expression we discovered a modular and specific induction of interferon-stimulated genes (ISGs) upon METTL3 depletion (Fig. 2c). These results suggested that the inhibition in viral growth might not stem from m⁶A-mediated regulation of viral gene expression but rather from m⁶A-mediated regulation of the type I IFN response.

To confirm that the observed inhibition in viral growth in the absence of m⁶A is indeed due to more potent IFN response, we tested whether inhibition of IFN signaling affects HCMV propagation in cells depleted of m⁶A machinery proteins. To this end we used Ruxolitinib, a potent and selective Janus kinase (JAK) 1 and 2 inhibitor³⁷ that blocks the signaling downstream of the type I IFN receptors. In agreement with our expression measurements, HCMV propagation in cells depleted of either METTL3 or YTHDF2 were rescued by Ruxolitinib treatment, whereas propagation in control cells was impacted to a reduced extent (Fig. 2d). We further confirmed that Ruxolitinib treatment abolished the differences in ISG expression between METTL3-depleted and control cells (Supplementary Fig. 2a). The rescue in viral growth when IFN signaling is blocked illustrates that the main mechanism underlying HCMV inhibition in cells depleted of the m⁶A pathway proteins involves an enhanced IFN response.

136 **The elevated ISG expression in METTL3-depleted cells is independent of viral gene**
137 **expression**

138 We considered three possibilities for how depletion of m⁶A ‘writers’ or ‘readers’
139 results in an enhanced IFN response. Since it was suggested that m⁶A modification may
140 diminish recognition of viral RNAs by cellular immune sensors such as Toll-like receptor 3
141 (TLR3) and RIG-I^{38,39}, we first considered the possibility that the absence of m⁶A residues on
142 viral transcripts is sensed as “non-self” by host sensors, triggering stronger innate immune
143 response. To test this possibility, we infected METTL3-depleted and control cells with a UV-
144 inactivated virus (from which no viral genes can be transcribed), and conducted RNA-seq at
145 22 hpi. Although after UV inactivation no viral transcripts were expressed, we still observed
146 significant increased induction of ISG expression in METTL3-depleted cells (Fig. 3a,b),
147 demonstrating that the elevation in ISG expression was independent of viral RNA expression.
148 Furthermore, when METTL3-depleted and control cells were infected with HCMV for 5 h, we
149 observed high but similar ISG expression in control and METTL3-depleted cells (Fig. 3a,c),
150 indicating that the differences in ISG expression occurs only at later stages of infection and
151 are therefore probably not related to differences in host recognition which takes place at the
152 first hours post infection. These results support a direct effect of m⁶A modification on the IFN
153 pathway.

154
155 **ISGs are not directly regulated by m⁶A modification**

156 Since m⁶A was shown to promote destabilization of transcripts and was suggested to
157 act on groups of co-regulated transcripts¹¹, we next considered a second possibility, namely
158 that ISG mRNA stability is directly regulated by m⁶A, resulting in greater abundance of ISG
159 mRNAs in cells depleted of m⁶A ‘writers’ or ‘readers’. Mapping cellular transcripts that were
160 m⁶A-modified in HCMV-infected cells (Supplementary Table 3) revealed that ISGs were not
161 enriched in m⁶A peaks (Fig. 3d). Furthermore, we measured RNA decay in METTL3-depleted
162 and control cells infected with HCMV and found no differences in the decay rates of ISGs
163 (Fig. 3e,f and Supplementary Fig. 2b-e). These results led us to conclude that the increased
164 ISG expression in cells lacking m⁶A is probably related to their enhanced transcription and
165 not to changes in their decay rates.

166

167 ***IFNB* mRNA is m⁶A-modified and is more stable in METTL3- and YTHDF2-depleted**
168 **cells**

169 We thus considered a third possibility, namely that the induction of ISGs upon
170 METTL3 depletion was a consequence of stabilization of a common signaling component
171 upstream of them, mediated by the absence of m⁶A. The IFN response is initiated by
172 recognition of pathogen-associated molecular patterns (PAMPs) by cellular sensors. These
173 sensors trigger signaling cascades resulting in phosphorylation of the transcription factors
174 IRF3 and IRF7 that leads to transcription and secretion of type I IFNs, namely IFN- α and
175 IFN- β . Subsequently, type I IFNs bind to the interferon receptor and activate the Janus kinase
176 (JAK) - signal transducer and activator of transcription (STAT) pathway, leading to
177 transcription of hundreds of ISGs⁴⁰. Consistent with our hypothesis, at 24 hpi STAT1
178 phosphorylation was increased in METTL3-depleted cells compared to control cells (Fig. 4a
179 and Supplementary Fig. 2f). Conversely, we did not observe substantial differences in the
180 amount of IRF3 and IRF7 phosphorylation (Fig. 4a), indicating that the enhanced expression
181 of ISGs is mostly independent of differences in PAMP recognition by cellular sensors. The
182 absence of differences in IRF3 and IRF7 phosphorylation, as compared to differences
183 observed in STAT1 phosphorylation, pointed to the possibility that differential ISG
184 expression was related to differences in the abundance of type I IFNs which are induced by
185 IRFs and signal via JAK-STAT pathway. Since the main type I IFN that is expressed by
186 human non-immune cells is IFN- β , we examined whether *IFNB* mRNA is modified by m⁶A.
187 Genome-wide mapping of m⁶A methylation at 6 hpi, when *IFNB* mRNA is still highly
188 expressed, revealed that *IFNB* mRNA exhibited prominent m⁶A peaks in the vicinity of its
189 stop codon (Fig. 4b). The m⁶A signal was specific as it was reduced when METTL3 was
190 depleted (Supplementary Fig. 3a). In agreement with the equivalent efficiencies of the initial
191 infection and the similar expression of ISGs we observed at early time points post infection,
192 no differences in *IFNB* transcript abundance was detected at 5 hpi and 8 hpi in cells depleted
193 of METTL3 or YTHDF2 in comparison to control cells (Fig. 4c and Supplementary Fig. 3b).
194 However, when infection progressed and *IFNB* mRNA began to decline in control cells, *IFNB*
195 transcript abundance was significantly higher in METTL3- and YTHDF2-depleted cells (Fig.
196 4c and Supplementary Fig. 3b). The differences in IFN- β abundance were further validated by
197 ELISA, demonstrating that IFN- β protein concentrations were higher at 24 hpi in METTL3-

198 and YTHDF2-depleted cells (Fig. 4d and Supplementary Fig. 3c). Since we observed no
199 significant differences in *IFNB* and ISG mRNA abundance early in infection when these
200 genes were induced, and since m⁶A methylation was demonstrated to reduce RNA
201 stability^{3,4,7,18}, we hypothesized that m⁶A may directly regulate *IFNB* mRNA stability. To test
202 this possibility, we performed an RNA decay assay and found that *IFNB* mRNA stability was
203 increased in cells depleted of METTL3 and YTHDF2 as compared to control cells, whereas
204 *USP42* mRNA that served as control transcript showed no differences in stability (Fig. 4e and
205 Supplementary Fig. 3d).

206 To directly test the role of the methylated adenosines we identified by m⁶A-
207 immunoprecipitation in the regulation of *IFNB* stability, we ectopically expressed either a
208 wild-type *IFNB* or *IFNB* in which the three putative m⁶A-modified adenosines were mutated
209 to guanosines (Supplementary Fig. 3e). Consistent with a role of these adenosines in the
210 regulation of *IFNB* mRNA stability, although both constructs were expressed under the same
211 promoter, the abundance of the mutant *IFNB* transcripts was two-fold higher than the wild-
212 type *IFNB* (Fig. 4f). We further measured the stability of these transcripts and found that
213 mutant *IFNB* mRNA was significantly more stable than the wild-type *IFNB* transcripts (Fig.
214 4g), indicating that these three adenosines are indeed important for regulating *IFNB* mRNA
215 stability. Taken together, these results demonstrate that following infection, loss of m⁶A
216 modification leads to increased stability of *IFNB* mRNA and sustained IFN-β production, thus
217 facilitating a stronger antiviral response that blocks HCMV propagation.

218 We next tested if *IFNA*, the second cytokine that participate in type I IFN response, is
219 also regulated by m⁶A. Since *IFNA* is mainly expressed by immune cells we used
220 differentiated monocytic cell line, THP1. Depletion of METTL3 in THP1 cells
221 (Supplementary Fig. 3f,g) resulted in increased expression of both *IFNA* and *IFNB* following
222 HCMV infection, compared to control cells (Fig. 4h). These results illustrate that *IFNA*
223 expression is also likely regulated by m⁶A machinery.

224

225 **Depletion of m⁶A machinery led to elevated type I IFN response upon infection with** 226 **diverse viruses**

227 Since type I IFN response and ISG expression block the propagation of diverse
228 viruses⁴¹, we next examined whether the mechanism identified here, of m⁶A-mediated

229 destabilization of *IFNB*, could serve as a mechanism affecting the propagation of additional
230 viruses. Indeed, we found that depletion of METTL3 or YTHDF2 in Influenza A Virus (IAV)-
231 , Adenovirus- and Vesicular Stomatitis Virus (VSV)-infected cells was accompanied by
232 increased *IFNB* and *ISG15* expression (Fig. 5a-d). We further observed that depletion of
233 METTL3 inhibits IAV and Adenovirus gene expression (Fig. 5e), the former in agreement
234 with previous findings²⁵. Importantly, inhibition of IFN signaling by Ruxolitinib treatment
235 partially rescued IAV and Adenovirus gene expression in METTL3-depleted cells (Fig. 5e),
236 demonstrating that at least part of the inhibition in viral gene expression stems from enhanced
237 IFN response in m⁶A-depleted cells.

238

239 **Type I IFN regulation by m⁶A methylation is conserved in mouse**

240 Finally, we tested whether regulation of *IFNB* by m⁶A methylation is also conserved in
241 mouse. We re-analyzed m⁶A maps obtained in a time-course following stimulation of mouse
242 dendritic cells with lipopolysaccharide⁴². We found that the murine *Ifnb* mRNA was also
243 modified by m⁶A in vicinity to its stop codon (Fig. 6a). Importantly, out of the 14
244 *Ifna* isoforms, we detected expression of *Ifna9* and *Ifna14*, both of which were m⁶A-modified
245 in vicinity to their stop codon (Fig. 6b and Supplementary Fig. 4a). Using CRISPR/Cas9 we
246 generated mouse embryonic fibroblasts (MEFs) that were depleted of METTL3 or the m⁶A
247 ‘reader’ proteins YTHDF 1, 2 and 3 (Supplementary Fig. 4b,c). In agreement with our
248 findings in human cells, infection of MEFs lacking METTL3 or YTHDF1-3 with murine
249 CMV (MCMV) resulted in enhanced *Ifnb* and ISG expression (Fig. 6c,d). The differences in
250 IFN-β abundance were further validated by ELISA, confirming that IFN-β protein
251 concentrations were higher at 24 hpi in METTL3-depleted MEFs (Fig. 6e). Since non-immune
252 murine cells express both IFN-α and IFN-β, we also tested the expression of *Ifna* and found
253 that depletion of METTL3 or YTHDF1-3 resulted in enhanced *Ifna* expression (Fig. 6f). We
254 next performed an RNA decay assay demonstrating that *Ifna* and *Ifnb* mRNA stability is
255 increased in MEFs depleted of METTL3 compared to control cells, whereas a control mRNA,
256 *Usp42* showed no differences in stability (Fig. 6g). These results illustrate that in both human
257 and mouse cells, inhibition of m⁶A machinery is accompanied by increased abundance of type
258 I IFNs and ISGs after infection.

259 To test whether type I IFN regulation by m⁶A also plays a role for the IFN response *in*
260 *vivo*, we constructed a *Ythdf3*-deficient mouse (Supplementary Fig. 5a). *Ythdf3* deletion was
261 validated by sequencing (Supplementary Fig. 5a) and immunoblot analysis of MEFs from
262 *Ythdf3*^{-/-} embryos (Supplementary Fig. 5b). We infected wild-type and *Ythdf3*^{-/-} mice with
263 MCMV and at 48 hpi we examined *Ifnb* and *Ifna* expression. In agreement with our *in vitro*
264 findings, we observed significant increase in *Ifnb* and *Ifna* expression in *Ythdf3*^{-/-} mice
265 compared to wild-type controls (Fig. 6h,i), implicating the potential role of m⁶A methylation
266 in regulating type I IFN abundance *in vivo*.

267

268 DISCUSSION

269 Immunity to viral infection is characterized by the production of type I IFNs, which
270 induce autocrine and paracrine antiviral resistance state. In resemblance to most cytokines, the
271 type I IFN response is fine-tuned by opposing augmenting and suppressive signals; these
272 signals are responsible to induce a rapid and effective antiviral response while restraining the
273 magnitude and length of the response to avoid attendant toxicity. Several regulatory
274 mechanisms that suppress type I IFN-mediated response have been characterized, including
275 downregulation of cell surface IFN α / β receptor⁴³, induction of negative regulators (such as
276 suppressor of cytokine signaling (SOCS) proteins and ubiquitin carboxyl-terminal hydrolase
277 18 (USP18))^{44,45}, and the induction of miRNAs⁴⁶.

278 Here we have revealed an additional, evolutionarily conserved strategy, to regulate
279 type I IFN response, whereby m⁶A targets *IFNB* mRNA, enhancing its destabilization and
280 providing a novel mechanism for restricting the duration of the antiviral response. In murine
281 cells, which express both IFN- β and IFN- α , we showed that *Ifna* is also m⁶A-modified next to
282 its stop codon and that depletion of the m⁶A machinery in both mouse and human cells leads
283 to elevation in *IFNA* abundance. These results strongly suggest that the regulatory mechanism
284 we identified is conserved between *IFNB* and *IFNA*.

285 We demonstrate that upon viral infection, depletion of m⁶A ‘writer’, METTL3 or the
286 cytoplasmic m⁶A ‘reader’, YTHDF2, lead to elevated levels of type I IFNs and consequently
287 to a stronger induction of ISGs. Several observations support our conclusion that this effect is
288 directly mediated by m⁶A modification of IFN transcripts that regulates their decay rates.

289 First, we demonstrated that depletion of both METTL3 or YTHDF2 leads to specific elevation
290 in the stability of *IFNB* transcript. Second, by ectopically expressing *IFNB*, we show that the
291 m⁶A-modified adenosines located in the proximity of *IFNB* stop codon are important for
292 regulating *IFNB* mRNA stability. Finally, by both RNA-seq and real-time PCR, we observed
293 no significant differences in *IFNB* and ISG mRNA abundance early in infection, when these
294 genes are induced, but significant differences are seen at later time points post infection when
295 IFN levels start to decline. This kinetics further supports the notion that the differences in IFN
296 and ISG levels are mediated by differences in IFN decay rates. However, these observations
297 do not preclude the possibility that other mechanisms, beyond elevation in IFN mRNA
298 stability, may contribute to the stronger elevated IFN response upon depletion of m⁶A
299 machinery.

300 Since type I IFN affects the propagation of most viruses, our results suggest a potential
301 unifying model for interpreting some of the diverse viral phenotypes that have been
302 previously observed upon depletion of m⁶A machinery. It is likely that for different viruses,
303 the contribution of the mechanism we identified to the phenotypes observed upon depletion of
304 m⁶A machinery may vary. This variation will depend on the levels of type I IFN induction,
305 the sensitivity of a given virus to type I IFN and the contribution of direct effects of m⁶A
306 modification on viral mRNA processing.

307 Interestingly, it has been demonstrated that pathogens exploit some of the cellular IFN
308 negative regulatory mechanisms to escape immune responses⁴⁷. The strong induction of m⁶A
309 machinery following HCMV infection implies that HCMV may be using this mechanism as
310 an additional way to efficiently shut-off and escape the type I IFN response.

311 A recent study demonstrated that the RNA helicase DDX46 inhibits antiviral innate
312 responses by erasing m⁶A from several transcripts encoding for signaling molecules involved
313 in the activation of type I IFN (through specific recruitment of m⁶A demethylase, ALKBH5).
314 This demethylation was suggested to enforce these transcripts retention in the nucleus and
315 therefore to inhibit IFN production⁴⁸. Our results imply that depletion of m⁶A modification
316 leads to prolonged expression of IFN- β and to elevation in ISG expression. Future work will
317 have to delineate how these two, seemingly opposing mechanisms, act together.

318 The cytoplasmic m⁶A ‘readers’, YTHDF1-3, were suggested to mediate different
319 functions. YTHDF2 was shown to regulate instability of m⁶A-containing mRNAs⁴, whereas
320 YTHDF1 was suggested to promote translation⁵ and YTHDF3 has been proposed to serve as a
321 co-factor to potentiate the effects of both YTHDF1 and 2^{49,50}. In similarity to other studies
322 that examined the effects of YTHDF ‘readers’ on viruses^{26,28,29,32}, depletion of YTHDF
323 ‘readers’ in our experiments presented comparable effects on CMV propagation and resulted
324 in an increase of *Ifnb* mRNA abundance. These results support the recent view that the
325 YTHDF proteins may, under certain circumstances, promote similar functions⁸.

326 Our results also demonstrate how rapid m⁶A-mediated turnover of a specific mRNA
327 can affect critical responses to external stimuli and help to maintain homeostasis. In this
328 sense, our study provides a rationalization of how relatively subtle destabilization of an
329 mRNA, caused by m⁶A, can lead to strong phenotypes. While it is probable that for the
330 majority of genes, subtle destabilization is unlikely to play a major regulatory role, in the
331 context of tightly regulated cytokines whose expression is regulated by several feedback
332 loops, such regulation can result in profound effect on cell physiology.

333 In summary, we uncover a significant and central role for m⁶A modification in
334 regulating innate immune homeostasis. Our findings suggest that development of m⁶A-
335 modulating agents may lead to novel therapeutic approaches to a range of infectious and
336 potentially also inflammatory diseases.

337 **ACCESSION CODES**

338 All RNA-seq data sets generated in this manuscript have been deposited in the GEO under
339 accession number GSE114019. Full images of immunoblots presented in this study have been
340 deposited to Mendeley Data and are available at <https://dx.doi.org/10.17632/3zb63b6ssj.1>.

341

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348

349 **AUTHOR CONTRIBUTIONS**

350 R.W., E.G., L.L., J.H.H., S.S. and N.S.-G. conceived experiments and interpreted data. L.L.,
351 S.G. and J.H.H. generated and characterized the gene-deficient mice. R.W., E.G., M.S., S.G.,
352 C.S., A.N., J.T.-S., N.F. and M.M. executed experiments and analysis. V.T.K.L.-T. and M.T.
353 provided critical reagents and advice. R.W., E.G. and N.S.-G. wrote the manuscript with
354 contribution from all other authors.

355

356 **COMPETING INTERESTS**

357 The authors declare no competing interests.

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470 444–447 (2017).
- 471
- 472

473 **Figure Legends**

474 Figure 1:

475 m⁶A ‘writers’ and ‘readers’ are upregulated during HCMV infection and required for viral
476 growth

477 (a) Immunoblot analysis of m⁶A machinery proteins along HCMV infection of human foreskin
478 fibroblasts. Actin was used as a loading control. (b and c) Viral supernatant was collected from
479 cells depleted of m⁶A machinery proteins and control cells and transferred to recipient wild-type
480 fibroblasts. 48 h later, the recipient cells were analyzed by flow cytometry. The values present
481 the ratio of the percentage of GFP positive cells relative to the control, indicating viral titers (n =
482 2, cell culture replicates). Dots, measurements; bars, mean. *P*-value by two-sided student’s t-test.
483 (d) Immunoblot analysis of HCMV Immediate-early (IE1-pp72) protein (upper panel) and
484 fluorescent microscopy of GFP signal (lower panel), at 24 hpi in m⁶A machinery depleted cells
485 and control cells. GAPDH was used as a loading control. (e) Immunoblot analysis of HCMV
486 immediate early (IE1-pp72), early (UL44) and late (pp28) proteins, in METTL3-depleted and
487 control cells, at 24, 48 and 72 hpi. GAPDH was used as a loading control. (a,d,e) Gel images
488 were cropped to present only relevant proteins. Data are representative of two (a,d,e) or three
489 (b,c) independent experiments.

490

491 Figure 2:

492 Inhibition of HCMV growth in m⁶A-deficient cells is driven by enhanced type I interferon
493 response

494 (a) Percentage of viral reads out of total uniquely aligned reads in METTL3-depleted and control
495 cells, as measured by RNA-seq at 28 hpi (n = 2). Dots, measurements; bars, mean. *P*-value by
496 likelihood ratio test. (b) Viral gene expression in METTL3-depleted versus control cells, as
497 measured in (a). Putative m⁶A-modified viral transcripts are marked in blue. (c) Volcano plot
498 showing changes in cellular transcripts levels in METTL3-depleted cells versus control cells at
499 28 hpi, as measured in (a). The log₂ fold change between METTL3-depleted and control cells
500 and -log₁₀ of the FDR are represented in the x and y axis, respectively. ISGs are marked in red.
501 *P*-value by hypergeometric test. (d) Viral supernatant was collected from METTL3- and
502 YTHDF2-depleted and control cells, treated or untreated with Ruxolitinib and transferred to
503 recipient wild-type fibroblasts. 48 h later, the recipient cells were analyzed by flow cytometry.

504 The values present the ratio of percentage of GFP positive cells relative to control (n = 2, cell
505 culture replicates). Dots, measurements; bars, mean. *P*-value by two-factor ANOVA test. Data
506 are representative of three independent experiments.

507

508 Figure 3:

509 ISG enhanced expression in METTL3-depleted cells is independent of viral gene expression
510 (a) ISG relative expression, as measured by RNA-seq, in METTL3-depleted cells versus control
511 cells at 22 hpi, 22 h after infection with UV-inactivated virus (22uv) and at 5 hpi. Expression
512 levels of each transcript were normalized to a scale of 0 to 1. ISGs showing significant difference
513 (FDR < 0.01) between control and METTL3-depleted cells at 22 hpi are presented. (b and c)
514 Cumulative distribution of cellular transcript expression in METTL3-depleted cells versus
515 control cells at 22 h after infection with UV-inactivated virus (b), or at 5 hpi with an active virus
516 (c), as measured in (a). *P*-value by two-sided student's t-test. (d) Quantification of putative m⁶A
517 methylation sites on ISGs compared to all other transcripts, measured by peaks identified by
518 RNA-seq of m⁶A immuno-precipitated samples (n = 3). (e) METTL3-depleted and control cells
519 were treated with Actinomycin D at 22 hpi and harvested for RNA-seq at 0, 2 and 4 h post
520 treatment. The decay ratio of ISGs compared to all other transcripts is presented (n = 2 for each
521 time point). (d,e) Thick line, median; box boundaries, 25% and 75% percentiles; whiskers, 1.5-
522 fold interquartile range. (f) mRNA decay of *OASL* in METTL3-depleted compared to control
523 cells, as measured in (e). Values represent the mean of RNA-seq replicates and error bars show
524 SD.

525

526 Figure 4:

527 *IFNB* mRNA is m⁶A-modified and is more stable in METTL3-depleted cells
528 (a) Immunoblot analysis of total and phosphorylated forms of STAT1, IRF3 and IRF7 in
529 METTL3-depleted and control cells at 24 hpi. GAPDH was used as a loading control. Gel image
530 was cropped to present only relevant proteins. (b) RNA-seq of input RNA and m⁶A immuno-
531 precipitated RNA from 6 hpi in replicates are presented for the *IFNB* transcript. m⁶A motif
532 sequences which correspond to IP-enriched region are marked in red. (c) *IFNB* mRNA and (d)
533 protein levels in METTL3-depleted and control cells at indicated time points post infection,
534 measured by qRT-PCR and ELISA, respectively. *18S* ribosomal RNA was used as a normalizing

535 gene in qRT-PCR. Dots, measurements; bars, mean of three technical (c) and cell culture (d)
536 replicates; *P*-value by two-sided student's t-test. (e) Nascent RNA was labeled for 2 h with 5-
537 Ethynyluridine (EU). EU was washed out and RNA was extracted at the indicated time points.
538 The relative remaining EU-labeled mRNA abundance, normalized to *I8S* ribosomal RNA, was
539 analyzed by qRT-PCR for *IFNB* and *USP42* that was used as control. Values represent the mean
540 of three technical replicates and error bars show SD. *P*-value by two-sided student's t-test. (f and
541 g) Wild-type (WT) and mutant (MUT) *IFNB* mRNA (in which three putative m⁶A-modified
542 adenosines were mutated to guanosines) were ectopically expressed in fibroblasts. At 24 hpi,
543 *IFNB* mRNA levels were measured by qRT-PCR (f), or cells were treated with Actinomycin D
544 and harvested at 0, 0.5, 1 and 4 h post treatment (g). Blasticidin-resistance gene, which was
545 expressed from the same construct, was used as a normalizing gene. *P*-value by two-sided
546 student's t-test. (f) Dots, measurements; bars, mean of three technical replicates. (g) Values
547 represent the mean of three technical replicates and error bars show SD. (h) mRNA levels of
548 *IFNA* and *IFNB* in HCMV-infected differentiated THP1 cells were measured by qRT-PCR at 24
549 hpi. *I8S* ribosomal RNA was used as a normalizing gene. Dots, measurements; bars, mean of
550 three technical replicates. *P*-value by two-sided student's t-test. Data are representative of two
551 (a,d) or three (c,e-h) independent experiments.

552

553 Figure 5:

554 Depletion of m⁶A machinery proteins leads to elevation in *IFNB* levels upon infection with
555 diverse viruses

556 (a-d) mRNA levels of *IFNB* and *ISG15* in Influenza A-, Adenovirus- and VSV-infected
557 METTL3-depleted (a-b) and YTHDF2-depleted (c-d) compared to control cells, were measured
558 by qRT-PCR at 24 hpi. *I8S* ribosomal RNA was used as a normalizing gene. (e) qRT-PCR
559 analysis of Influenza *M2* and Adenovirus *L3* expression in METTL3-depleted cells and control
560 cells (48 hpi), treated or untreated with Ruxolitinib. *I8S* ribosomal RNA was used as a
561 normalizing gene. (a-e) Dots, measurements; bars, mean of three technical replicates; *P*-value by
562 two-sided student's t-test (a-d) and by two-factor ANOVA test (e). Data (a-e) are representative
563 of three independent experiments.

564

565 Figure 6:

566 Type I IFN m⁶A methylation and destabilization are conserved in mouse
567 (a and b) RNA-seq of input RNA and m⁶A immuno-precipitated RNA from mouse dendritic
568 cells treated with lipopolysaccharide (LPS) for 3 and 6 h is presented for *Ifnb* (a) and *Ifna9* (b).
569 m⁶A motif sequences which correspond to IP-enriched region are marked in red. (c and d) MEFs
570 depleted of m⁶A ‘readers’ and ‘writer’ proteins were infected with MCMV and harvested at 24
571 hpi. mRNA levels of mouse *Ifnb* (c) and *Isg15* (d) were measured by qRT-PCR. *Gapdh* was used
572 as a normalizing gene. (e) IFN-β protein levels in METTL3-depleted and control MEFs were
573 measured by ELISA. (f) MEFs depleted of m⁶A ‘readers’ and ‘writer’ proteins were infected
574 with MCMV and harvested at 12 hpi. mRNA levels of mouse *Ifna* were measured by qRT-PCR.
575 *Gapdh* was used as a normalizing gene. (c-f) Dots, measurements; bars, mean of three technical
576 (c,d,f) or cell culture (e) replicates; *P*-value by two-sided student’s t-test. (g) Nascent RNA was
577 labeled for 2 h with 5-Ethynyluridine (EU). EU was washed out and RNA was extracted at the
578 indicated time points. The relative remaining EU-labeled mRNA abundance, normalized to *18S*
579 ribosomal RNA, was analyzed by qRT-PCR for *Ifnb*, *Ifna* and *Usp42* (control). Values represent
580 the mean of three technical replicates and error bars show SD. *P*-value by two-sided student’s t-
581 test. (h and i) *Ythdf3*^{+/+} (n=12) or *Ythdf3*^{-/-} (n=13) mice were infected with MCMV. *Ifnb* (h) and
582 *Ifna* (i) mRNA levels in spleen were measured by qRT-PCR at 48 hpi. *18S* ribosomal RNA was
583 used as a normalizing gene. Thick line, median; box boundaries, 25% and 75% percentiles;
584 whiskers, 1.5-fold interquartile range. *P*-value by two-sided student’s t-test. Data are
585 representative of three (c,d,f,g) or two (e) independent experiments.

586

587 **Tables**

588 **Supplementary Table 1**

589 Measurements of cells viability in control cells and cells depleted of m⁶A machinery proteins,
590 before and 96 h post infection with HCMV.

591 **Supplementary Table 2**

592 Dataset of 21 putative m⁶A sites in HCMV transcripts.

593 **Supplementary Table 3**

594 Dataset of 7,093 putative m⁶A sites in human transcripts, obtained following infection with
595 HCMV.

596

597 **Methods**

598 **Cells and viruses**

599 Human foreskin fibroblasts (HFF) (ATCC CRL-1634) and Mouse embryonic fibroblasts (MEF)
600 were maintained in Dulbecco Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 2
601 mM L-glutamine, and 100 units/ml penicillin and streptomycin (Beit-Haemek). THP1 cells
602 (ATCC TIB-202) were maintained in RPMI-1640 Medium with 10% fetal bovine serum, 2 mM
603 L-glutamine, 100 units/ml penicillin and streptomycin, 10 mM HEPES pH7.46, 1 mM sodium
604 pyruvate, 1500 mg/L sodium bicarbonate (Beit-Haemek) and 0.05 mM 2-mercaptoethanol. The
605 bacterial artificial chromosome (BAC) derived strain TB40E expressing an SV40-GFP reporter
606 protein (TB40E-GFP) was previously described³⁶. Virus was propagated by electroporation of
607 infectious BAC DNA into HFF cells using the Amaxa P2 4D-Nucleofector kit (Lonza) according
608 to the manufacturer's instructions. The human influenza virus A/Puerto Rico/8/34 H1N1 used in
609 this study was generated as previously described⁵¹. The MCMV used was of strain Smith-GFP,
610 which was previously described⁵². Adenovirus of serotype 4 was collected from patients, as
611 previously described⁵³. VSV was from ATCC (VR-1238). *In vitro* infection with Influenza virus
612 was done at multiplicity of infection (MOI) of 0.5. All other *In vitro* infections were done at
613 MOI of 5.

614

615 **Immunoblot analysis**

616 Cells were lysed using RIPA buffer. Lysates were centrifuged at 20,000 × g for 10 min at 4 °C.
617 Samples were then separated by 4–12% polyacrylamide Bis-tris gel electrophoresis (Invitrogen),
618 blotted onto nitrocellulose membranes and immunoblotted with primary antibodies: anti-IE1/IE2
619 (clone CH160, Abcam, ab53495); anti-UL44 (Virusys, CA006); anti-pp28 (Eastcoast, CA004);
620 anti-GAPDH (Cell Signaling Technology, 2118S); anti-ACTIN (Sigma Aldrich, A4700); anti-
621 METTL3 (Proteintech, 15073-1-AP); anti-METTL14 (Novus Biologicals, NBP1-81392); anti-
622 YTHDF1 (Proteintech, 17479-1-AP); anti-YTHDF2 (Aviva Systems Biology, ARP67917); anti-
623 YTHDF3 (Santa Cruz Biotechnology, SC-87503); anti-YTHDC1 (Abcam, ab122340); anti-
624 STAT1 (Cell Signaling Technology, 14994); anti-Phosphorylated-STAT1 (Cell Signaling
625 Technology, 9167); anti-IRF3 (Cell Signaling Technology, 4302); anti-Phosphorylated-IRF3
626 (Cell Signaling Technology, 4947); anti-IRF7 (Abcam, ab109255); anti-Phosphorylated-IRF7
627 (Cell Signaling Technology, 5184); anti-HSP90 (Epitomics, 1492-1). Secondary antibodies used

628 were Goat anti-rabbit, Goat anti-mouse or Goat anti-rat (IRDye 800CW or IRDye 680RD, Li-
629 Cor). Reactive bands were detected by Odyssey CLx infrared imaging system (Li-Cor).

630

631 **RT-PCR**

632 Total RNA was extracted using Tri-Reagent (Sigma) according to manufacturer's protocol. RNA
633 was then treated with DNaseI, using PerfeCTa DNaseI kit (Quantabio) and cDNA was prepared
634 using qScript cDNA Synthesis Kit (Quantabio).

635 Real-time PCR was performed using the SYBR Green PCR master-mix (ABI) on a QuantStudio
636 12K Flex Real-Time PCR System (life technologies) with the following primers (forward,
637 reverse):

638 Human IFNB; (5'-ACTGCAACCTTTTCGAAGCCT-3', 5'-AGCCTCCCATTCAATTGCCA-3')

639 Human *IFNA*; (5'-ATTTCTGCTCTGACAACCTC-3', 5'-CTGAATGACTTGGAAGCCTG-3')

640 Human *ISG15*; (5'-TTTGCCAGTACAGGAGCTTG-3', 5'-TTCAGCTCTGACACCGACAT-3')

641 Human *GAPDH*; (5'-TGGTATCGTGGAAGGACTCA-3', 5'-

642 CCAGTAGAGGCAGGGATGAT-3')

643 Human *18S*; (5'-GTAACCCGTTGAACCCCAT-3', 5'-CCATCCAATCGGTAGTAGCG-3')

644 Human *USP42*; (5'-ATGGCCAGGGTGATTGAAAAC-3', 5'-

645 CACCACGCAGATTGGAACAG-3')

646 Blasticidin resistance gene; (5'-AACGGCTACAATCAACAGCA-3', 5'-

647 CGATCGCGACGATAACAAGTC-3')

648 Mouse *Ifnb*; (5'-AACCTCACCTACAGGGCGGACTTCA-3', 5'-

649 TCCCACGTCAATCTTTCCTCTTGCTTT-3')

650 Mouse *Ifna*; (5'-GGACTTTGGATTCCCGCAGGAGAAG-3', 5'-

651 GCTGCATCAGACAGCCTTGCAGGTC-3')

652 Mouse *Isg15*; (5'-TCTGACTGTGAGAGCAAGCAG-3', 5'-ACCTTTAGGTCCCAGGCCATT-

653 3')

654 Mouse *Gapdh*; (5'-TCAAGCTCATTTCTGGTATGACA-3', 5'-

655 TAGGGCCTCTCTTGCTCAGT-3')

656 Mouse *Usp42*; (5'-TCTTCCTGGAAAGGTGACGC-3', 5'-CTTTGGAGAGCTTCCCCCTG-3')

657 Influenza *M2*; (5'-CGAGGTGAAACGCCTATCA-3', 5'-GAAGGCCCTCCTTTTCAGTCC-3')

658 Adenovirus *L3*; (5'-GGCACGGGACTCCGCGCAAGGAC-3', 5'-

659 CTTAAGCCCGCTCCAGAGAC-3')

660

661 **Plasmids and sgRNAs**

662 All gene silencing was done using CRISPR/Cas9 system, with lentiCRISPR v2 plasmid
663 (Addgene#52961)⁵⁴. The following sgRNAs were cloned downstream of U6 promoter:

664 Human & mouse *METTL3*: 5'-GGACACGTGGAGCTCTATCC-3'

665 Human *METTL14*: 5'-GCCGTAACCTTCTGCCGCTCC-3'

666 Human *WTAP*: 5'-GCGGGAGGAGCTACCATTACT-3'

667 Human *YTHDF1*: 5'-GAATGGACGGCGGGTAATAGC-3'

668 Human *YTHDF2*: 5'-GATGGAGGGACTGTAGTAACT-3'

669 Human *YTHDF3*: 5'-GCTAAGCGAATATGCCGTAAT-3'

670 Human *FTO*: 5'-GCACTTCATCTTGTCCGTTGT-3'

671 Human *ALKBH5*: 5'-GCCTCATAGTCGCTGCGCTCG-3'

672 Human *IGSF8* (Control): 5'-GCGGCAGCAGCGTGGGCCTGA-3'

673 Mouse *Igsf8* (Control): 5'-GGAGCGAACTCAGCGGCGTG-3'

674 Mouse *Ythdf1*: 5'-GAAGCATGTTCGGCCACCAGCG-3'

675 Mouse *Ythdf2*: 5'-GTGAGGATCCGAGAGCCATGT-3'

676 Mouse *Ythdf3*: 5'-GATATATGGATCTGACATTGG-3'

677

678 Lentiviruses were generated by co-transfection of lentiCRISPR v2 constructs and 2nd generation
679 packaging plasmids (psPAX2, Addgene#12260 & pMD2.G, Addgene#12259), using jetPEI
680 DNA transfection reagent (Polyplus transfection) into HEK293T cells, according to
681 manufacturer's instructions. 48 h post transfection, supernatants were collected and filtered
682 through 0.45µm PVDF filter (Millex). To induce gene silencing in fibroblasts, cells were
683 transduced with lentivirus expressing sgRNA and were puromycin-selected (1.75µg/mL) for 4–5
684 days. The depletion of target proteins was confirmed by immunoblot analysis and experiments
685 were done within 6 days after transduction of the lentivirus.

686

687 **Cells treatments and ELISA**

688 Cells were resuspended in PBS and propidium iodide was added at concentration of 1µg/mL for
689 1 min prior to analysis in flow cytometer. Ruxolitinib (InvivoGen) was used at final

690 concentration of 4 μ M and added to growth media 1 h post infection (hpi). For ELISA,
691 supernatant from METTL3- or YTHDF2-depleted and control cells was collected at 24 hpi and
692 analyzed by Human IFN Beta ELISA Kit or Mouse Ifn Beta ELISA Kit (pbl Assay Science).

693

694 **RNA decay assays**

695 Actinomycin D (Sigma Aldrich) was used at final concentration of 5 μ M. At each time point, two
696 independent wells of METTL3-depleted cells and two independent wells of control cells were
697 harvested. To calculate RNA decay rate, first we normalized the number of reads for each
698 transcript to follow the global degradation rate in each time point, based on median degradation
699 level in mammalian cells. Decay rate for each transcript was calculated as the slope of linear
700 regression of the log of the normalized number of reads as function of time.

701 5-Ethynyluridine (EU) labeling and pull-down was done using the Click-It Nascent RNA
702 Capture Kit (Invitrogen).

703

704 **Generation of plasmids**

705 The plasmids containing wild-type and mutant *IFNB* were derived from the pLex_TRC206
706 plasmid⁵⁵. eGFP sequence was replaced, using XmaI and BamHI sites, with *IFNB*, with or
707 without mutations of three putative m⁶A-modified adenosines.

708

709 **Next-generation sequencing and data analysis**

710 Raw sequences obtained from NextSeq500 (Illumina) were first trimmed at their 3' end,
711 removing the polyA tail. Alignment was performed using Bowtie (allowing up to 2 mismatches)
712 and reads were aligned to concatenation of the human (hg19) and the viral genomes (NCBI
713 EF999921.1). Reads aligned to ribosomal RNA were removed. Reads that were not aligned to
714 the genome were then aligned to the transcriptome.

715

716 **Statistics**

717 Differential gene expression was calculated using edgeR with default parameters⁵⁶.

718 ISGs were defined as genes which were ≥ 4 -fold induced, following a 5 h treatment with type I
719 interferons³⁵. ISG enrichment was calculated using hypergeometric test. *P*-value for reduction in

720 viral gene expression in METTL3-depleted cells was calculated using likelihood ratio test, with
721 logistic regression of viral reads.

722 Differences in Ruxolitinib effect between control and METTL3-depleted cells or control and
723 YTHDF2-depleted cells were calculated by two-factor ANOVA. For Fig. 2d, Degrees of
724 freedom = 4, F = 1010.5 (METTL3), F = 498.7 (YTHDF2). For Fig. 5e, Degrees of freedom = 8,
725 F = 1602.3 (IAV), F = 14.9 (Adenovirus).

726

727 **Detection of putative m⁶A sites**

728 m⁶A pull-down and preparation of RNA-seq libraries were done as previously described^{15,42}.
729 Two sets of m⁶A-seq experiments were performed at 72 hpi and at 6 hpi. Both experiments
730 included 3 replicates of wild-type samples and 3 replicates of METTL3-depleted samples. In
731 both experiments, both the immuno-precipitated fraction (IP) and non-immuno-precipitated
732 samples (Input) were sequenced. m⁶A-seq data was aligned separately to the human and HCMV
733 genomes using STAR aligner⁵⁷. We then applied a previously published approach to identify
734 putative m⁶A sites⁴², and to assign each putative site with a Peak Over Median (POM) score,
735 capturing the fold change of enrichment in the peak region over the median coverage of the gene
736 harboring it, and Peak Over Input (POI) score, capturing the fold change of enrichment over the
737 corresponding region in the Input (non-enriched) control experiment. To identify sites specific to
738 wild-type, but absent upon METTL3-depleted samples, we performed two separate T tests: The
739 first assessed, for each putative peak, whether the distribution of POM scores in wild-type
740 samples differed significant ($P < 0.05$) from METTL3-depleted samples; The second assessed
741 the same for the POI scores. A site was deemed as significant if (1) both its mean POM (across
742 the triplicates) and mean POI scores in the wild-type samples exceeded the corresponding value
743 in the METTL3-depleted samples, and (2) if at least one of the two above calculated P values
744 was significant.

745

746 **Generation of *Ythdf3*^{-/-} mice and infection with MCMV**

747 T7 promoter was added to the Cas9 coding region by PCR amplification of px330 plasmid
748 (Addgene#42230)⁵⁸, using T7-Cas9 F and Cas9 R primers. The T7-Cas9 PCR product was gel
749 purified and used as the template for *in vitro* transcription (IVT) using mMESSAGING
750 mMACHINE T7 ULTRA kit (Thermo Fisher Scientific). In order to generate *Ythdf3* sgRNA,

751 gRNA primers, g*Ythdf3* F+R, were cloned into px330 plasmid. T7 promoter was added to the
752 gRNA template by PCR amplification of px330, using T7-sg*Ythdf3* F and sg*Ythdf3* R. The T7-
753 sgRNA PCR product was gel purified and used as the template for IVT using MEGA shortscript
754 T7 kit (Thermo Fisher Scientific). Both the Cas9 mRNA and the sgRNAs were purified using the
755 MEGA clear kit (Thermo Fisher Scientific).

756 CB6F1 (C57BL/6 × BALB/c) and ICR mice strains were used as embryo donors and foster
757 mothers, respectively. Superovulated CB6F1 mice (8-10 weeks old) were mated to CB6F1 stud
758 males, and fertilized embryos were collected from oviducts. Cas9 mRNAs and sgRNA (50 ng/μl)
759 was injected into the cytoplasm of fertilized eggs with well recognized pronuclei in M2 medium
760 (Sigma). The injected zygotes were cultured in KSOM with amino acids (Sigma) at 37 °C under
761 5% CO₂ in air until blastocyst stage by 3.5 days. Thereafter, 15–25 blastocysts were transferred
762 into uterus of pseudopregnant ICR females at 2.5 d post-coitum (dpc). Mutated animals were
763 screen for indels by sequencing with *Ythdf3* seq F+R primers. *Ythdf3*^{+/-} animal were backcrossed
764 with C57BL/6 mice for 2 generation before mating in order to generate *Ythdf3*^{-/-} mice. For *in*
765 *vivo* experiments mice (n = 25) were infected with 2 x 10⁵ pfu by intraperitoneal injection. One
766 *Ythdf3*^{+/+} mouse was mock-infected with PBS. At 48 hpi, they were euthanized and spleens were
767 harvested, homogenized and RNA was extracted. All animal studies were conducted according
768 to the guidelines and following approval of the Weizmann Institute of Science (IACUC approval
769 #33900217-2).

770 Primers (forward, reverse):

771 T7-Cas9; (5'-TAATACGACTCACTATAGGGAGAATGGACTATAAGGACCACGAC-3', 5'-
772 GCGAGCTCTAGGAATTCTTAC-3')

773 g*Ythdf3*; (5'-CACCGTTTGTCTGGCTACTTAAGTA-3', 5'-AAAC
774 TACTTAAGTAGCCAGACAAAC-3')

775 T7-sg*Ythdf3*; (5'-TTAATACGACTCACTATAGGTTTGTCTGGCTACTTAAGTA-3', 5'-
776 AAAAGCACCGACTCGGTGCC-3')

777 *Ythdf3* seq; (5'-CAAGGTTAGCCTGGGTTACAGAAGAAA-3', 5'-
778 CTAGTCATTATCCCATGAAAGTTTCCAGC-3')

779

780 **Reporting summary**

781 Further information on research design is available in the Life Sciences Reporting Summary,
782 which is linked to this article.

783

784 **DATA AVAILABILITY**

785 All RNA-seq data sets generated in this manuscript have been deposited in the GEO under
786 accession number GSE114019. Full images of immunoblots presented in this study have been
787 deposited to Mendeley Data and are available at <https://dx.doi.org/10.17632/3zb63b6ssj.1>. All
788 other data are available from the corresponding author upon reasonable request.

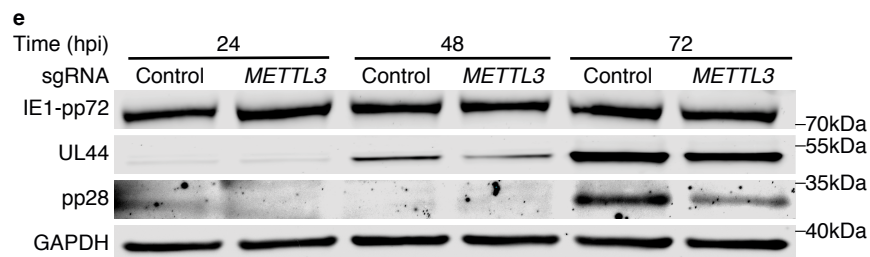
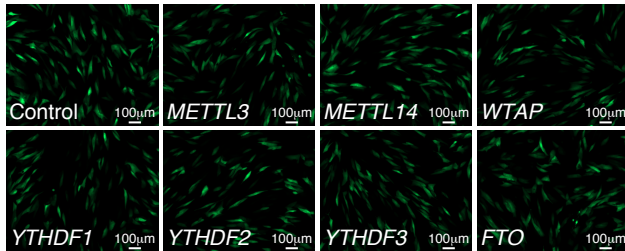
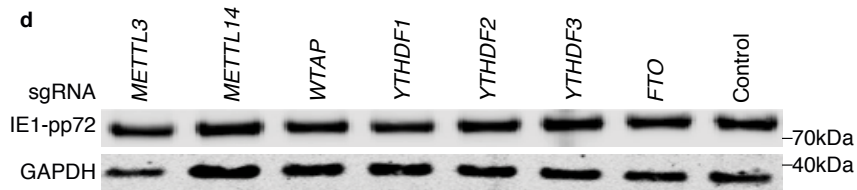
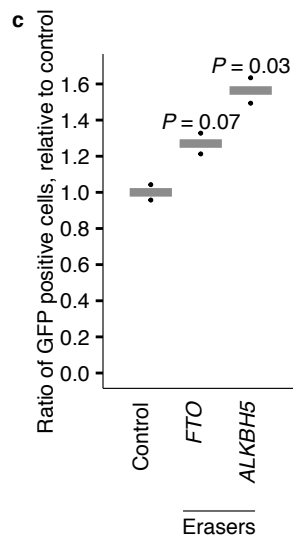
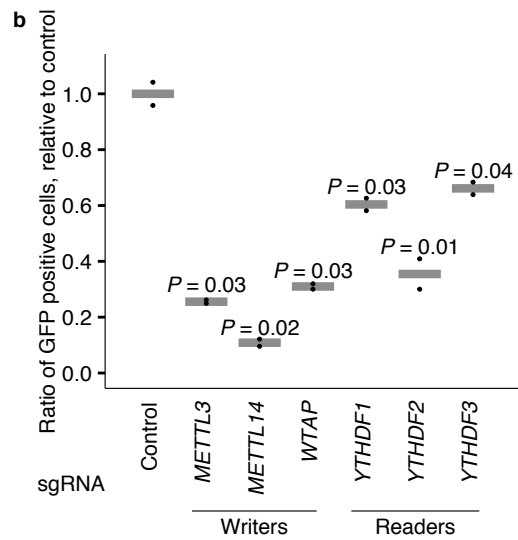
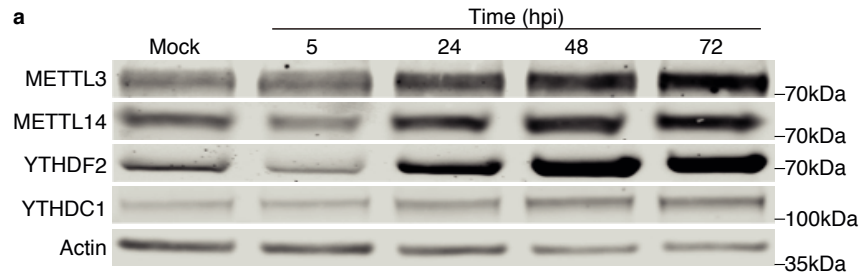
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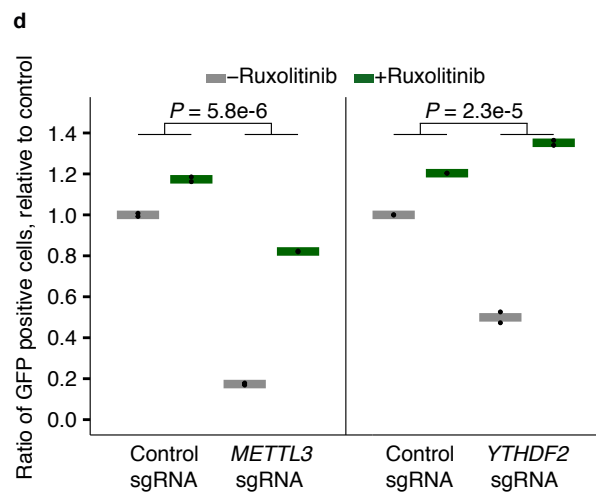
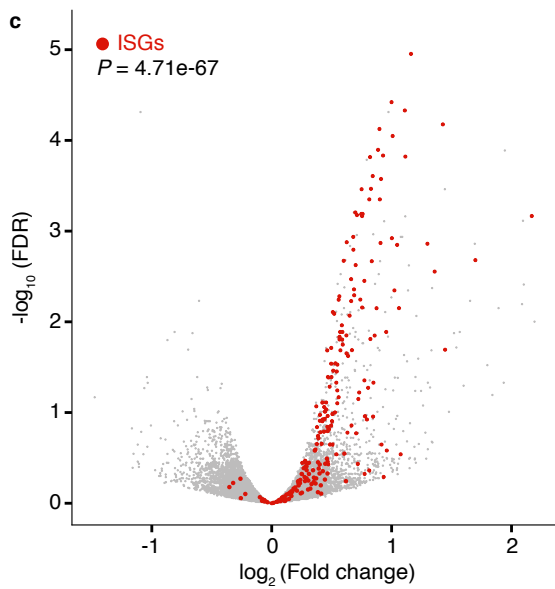
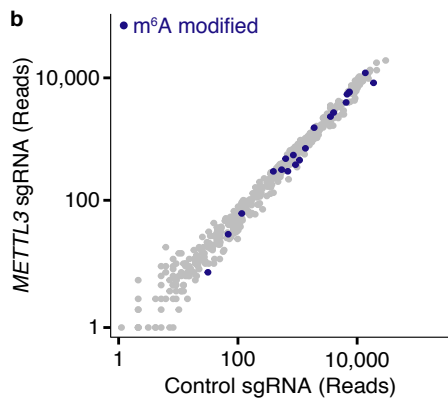
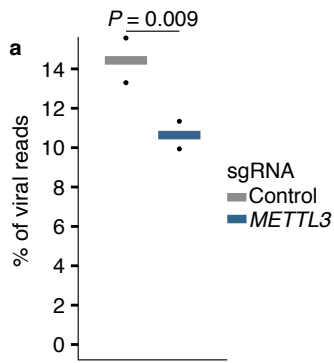
790 **REFERENCES**

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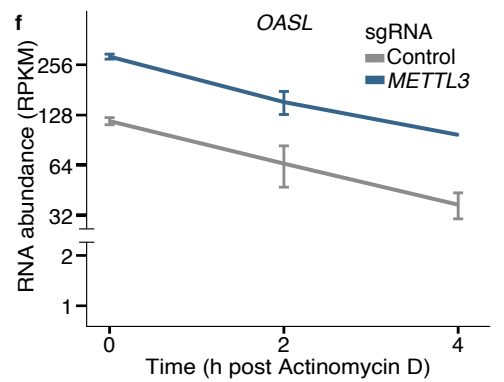
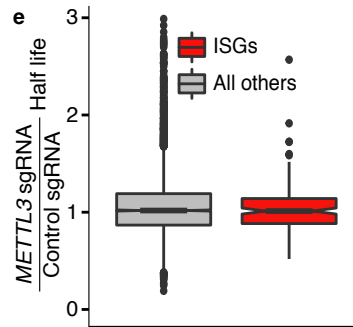
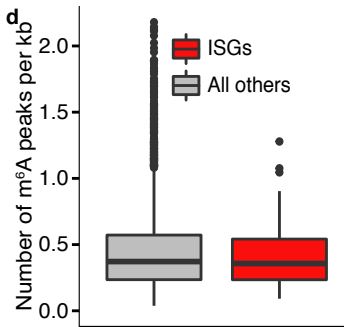
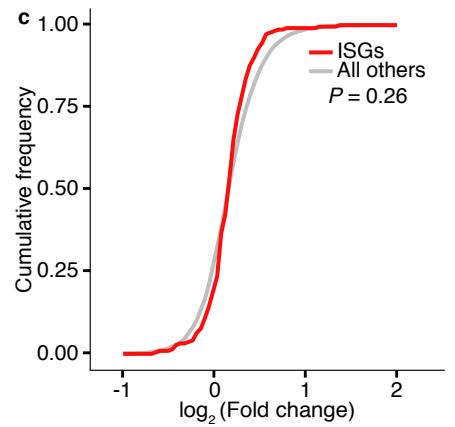
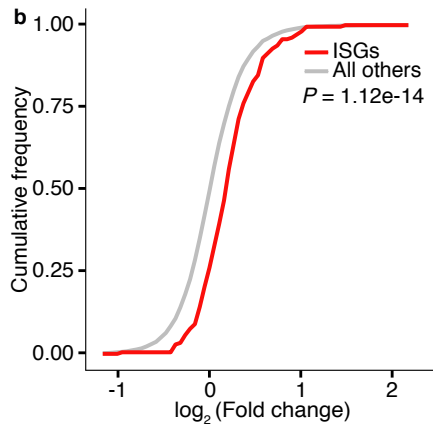
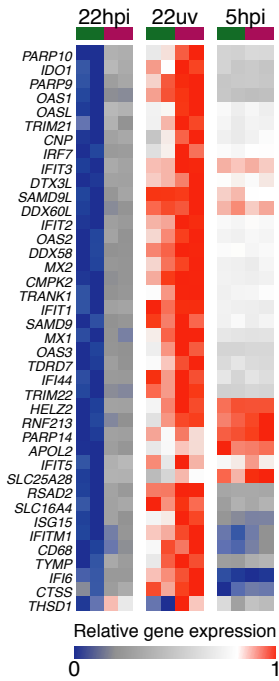
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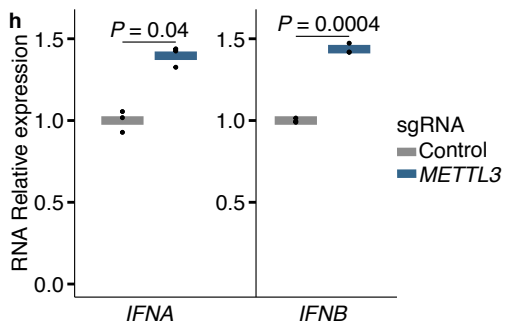
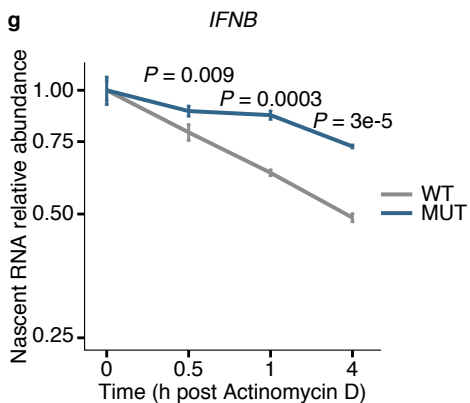
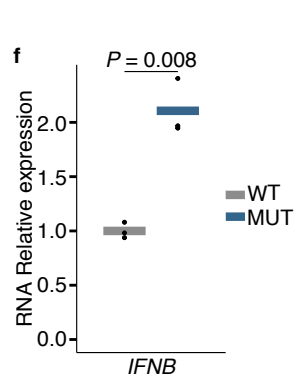
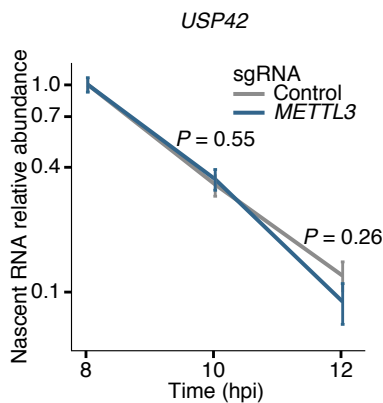
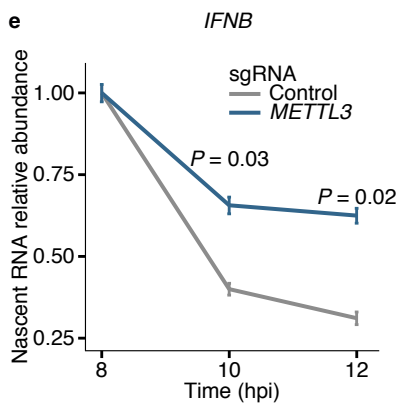
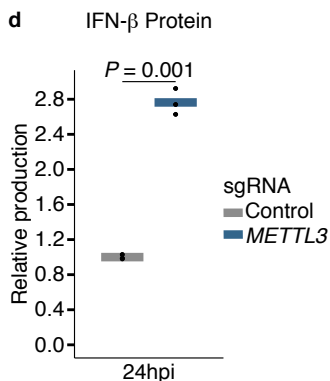
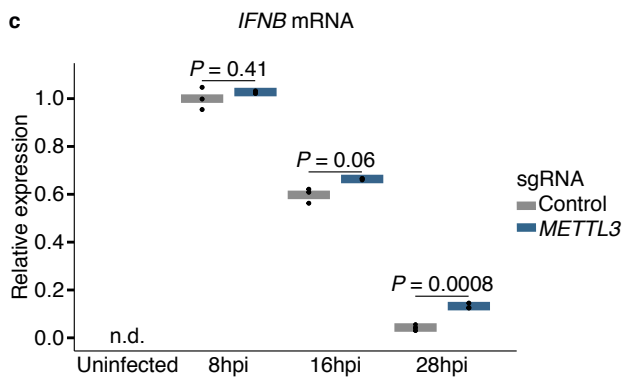
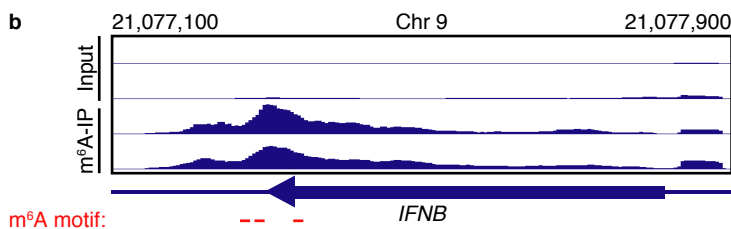
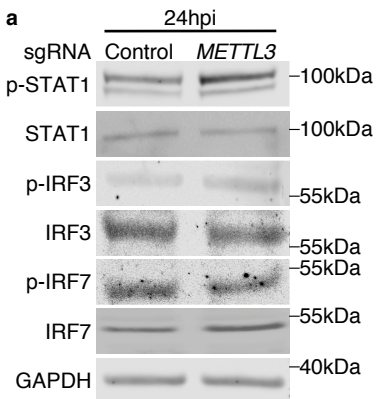
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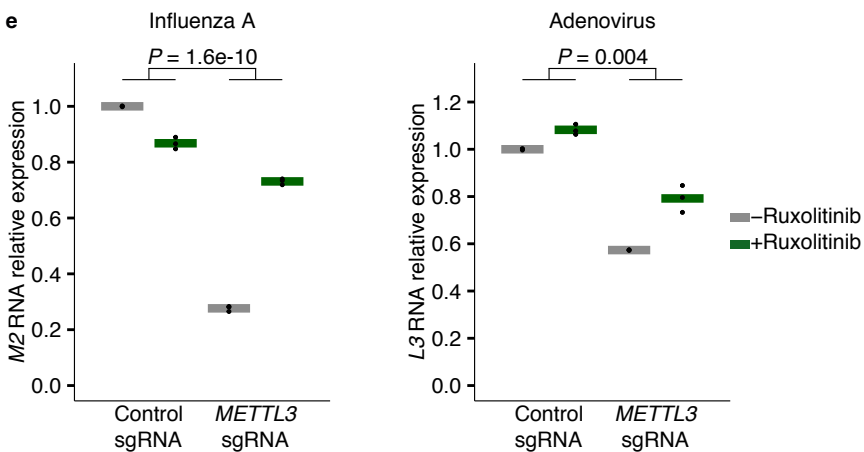
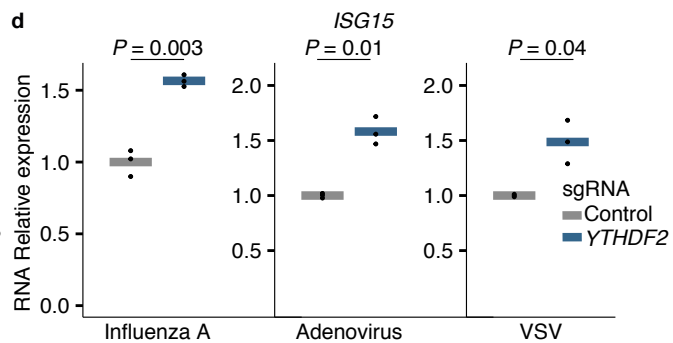
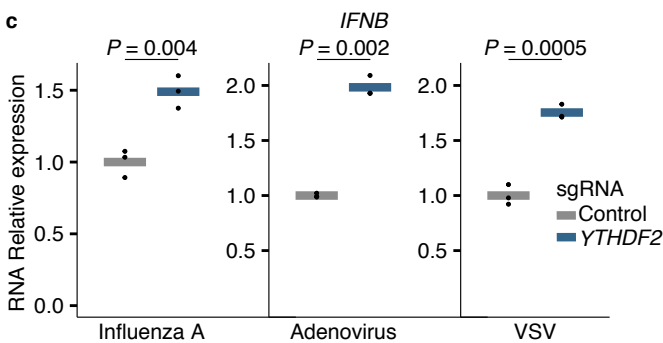
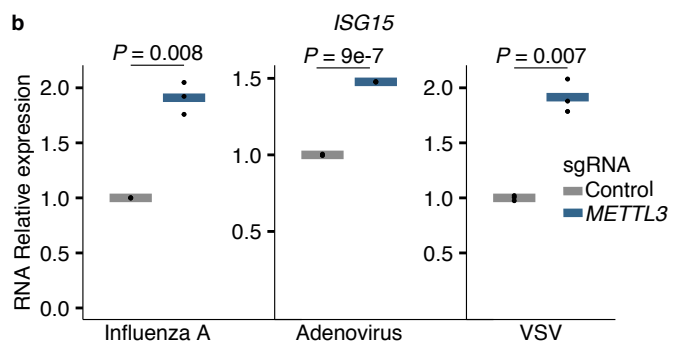
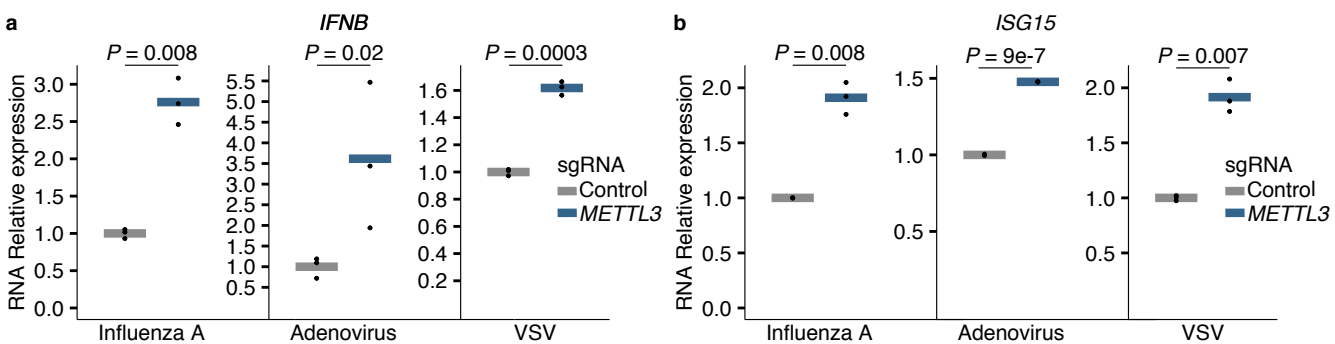


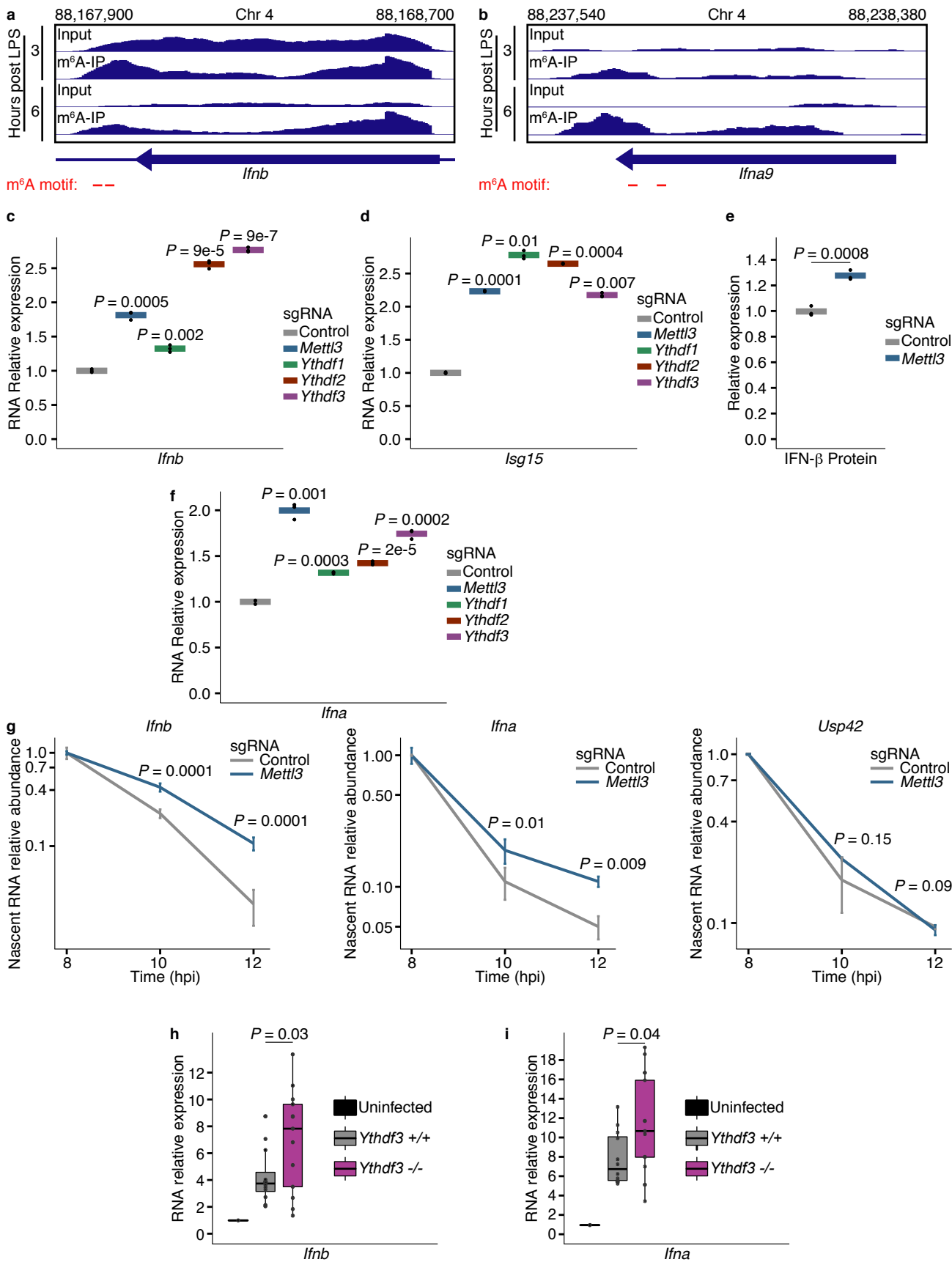


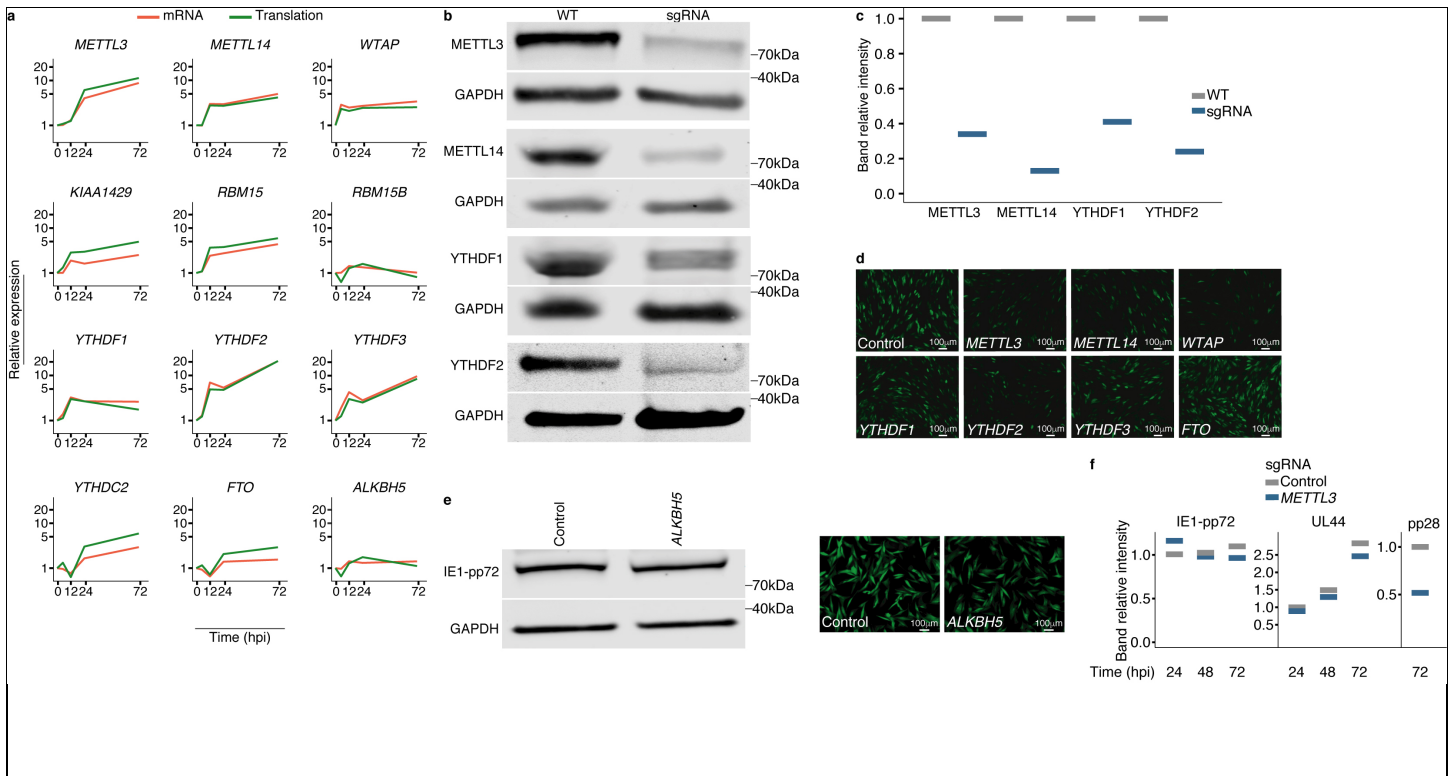
a ■ Control sgRNA ■ *METTL3* sgRNA







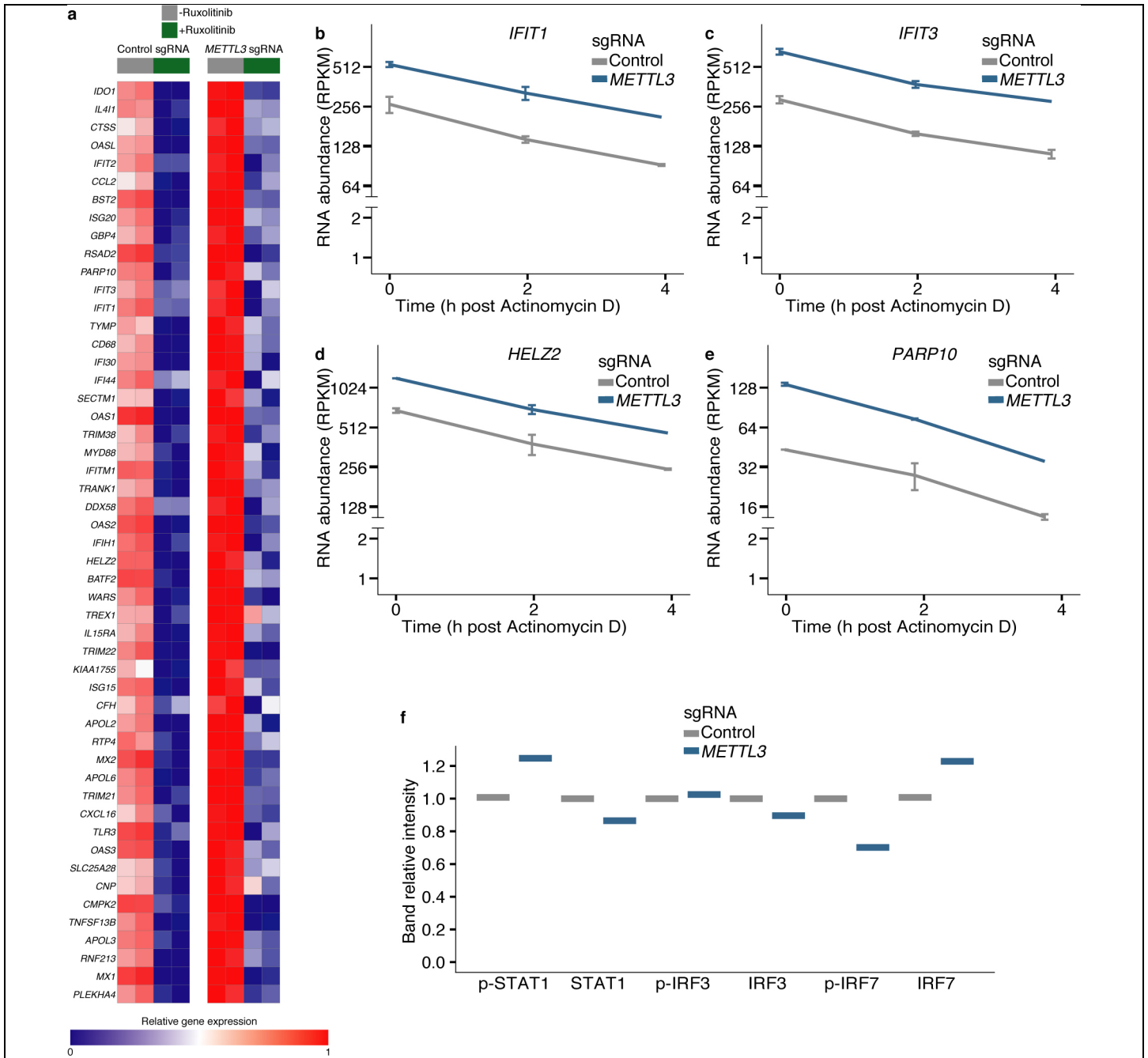




Supplementary Figure 1

m⁶A machinery is elevated along HCMV infection and is important for its propagation

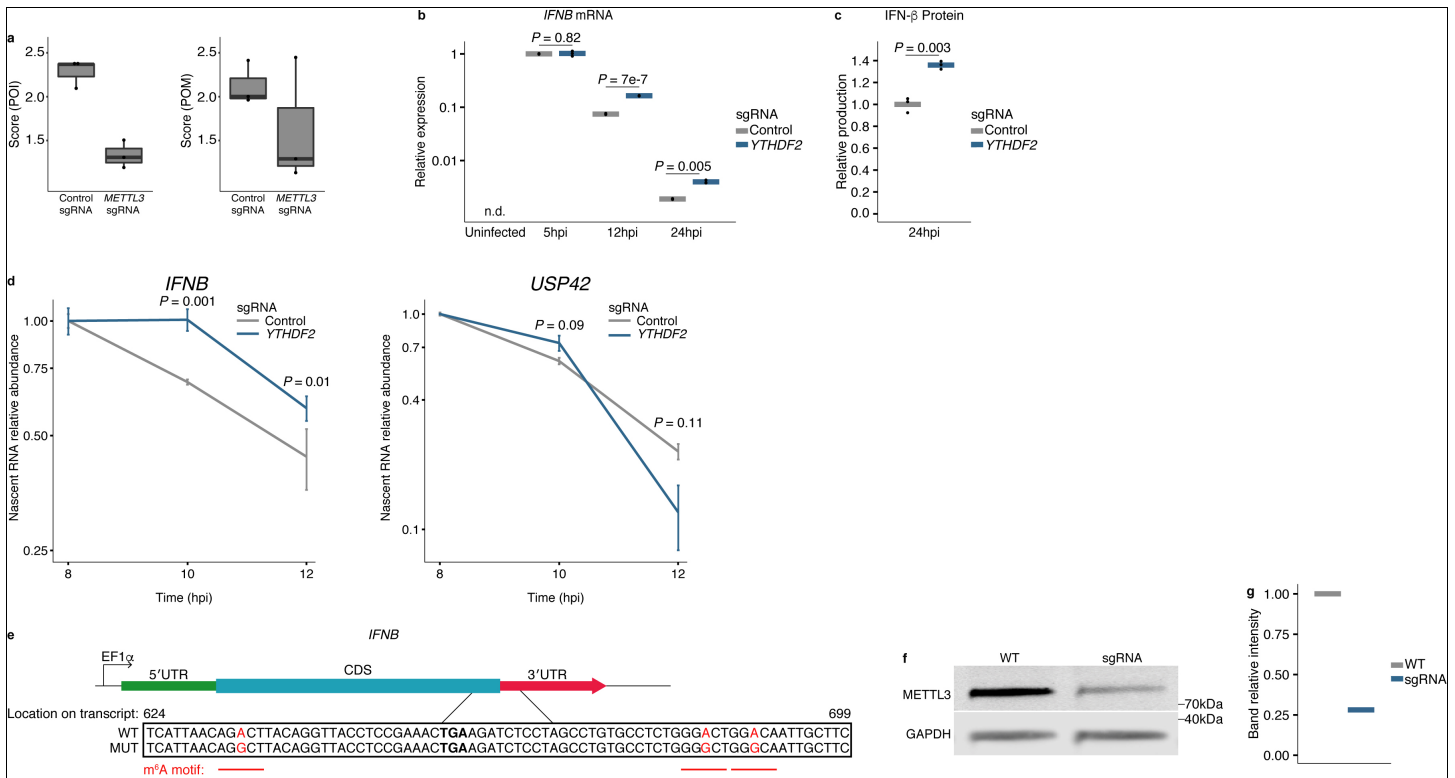
(a) mRNA and translation levels of genes encoding for m⁶A machinery along HCMV infection, as measured by RNA-seq (red) and ribosome profiling (green)³⁵. (b) Immunoblot analysis of m⁶A machinery proteins in cells expressing sgRNAs targeting control gene (WT) or the various m⁶A machinery genes (indicated on the left) in fibroblasts. GAPDH was used as a loading control. Gel image was cropped to present only relevant proteins. (c) Quantification of m⁶A machinery protein levels from the immunoblot analysis in (b) normalized to the levels of GAPDH. (d) Fluorescent microscopy of GFP signal in WT fibroblasts infected with supernatant from infected cells in which m⁶A machinery genes were depleted (indicated at the bottom). (e) Immunoblot analysis of HCMV Immediate-early protein (IE1-pp72) (left panel) and fluorescent microscopy of GFP signal (right panel), at 24 hpi in ALKBH5-depleted and control cells. GAPDH was used as a loading control. Gel image was cropped to present only relevant proteins. (f) Quantification of viral protein levels from the immunoblot analysis in Fig.1e normalized to the levels of GAPDH. Data are representative of three (d) or two (e) independent experiments.



Supplementary Figure 2

Differences in ISG expression between METTL3-depleted and control cells is abolished by Ruxolitinib and does not stem from changes in their stability

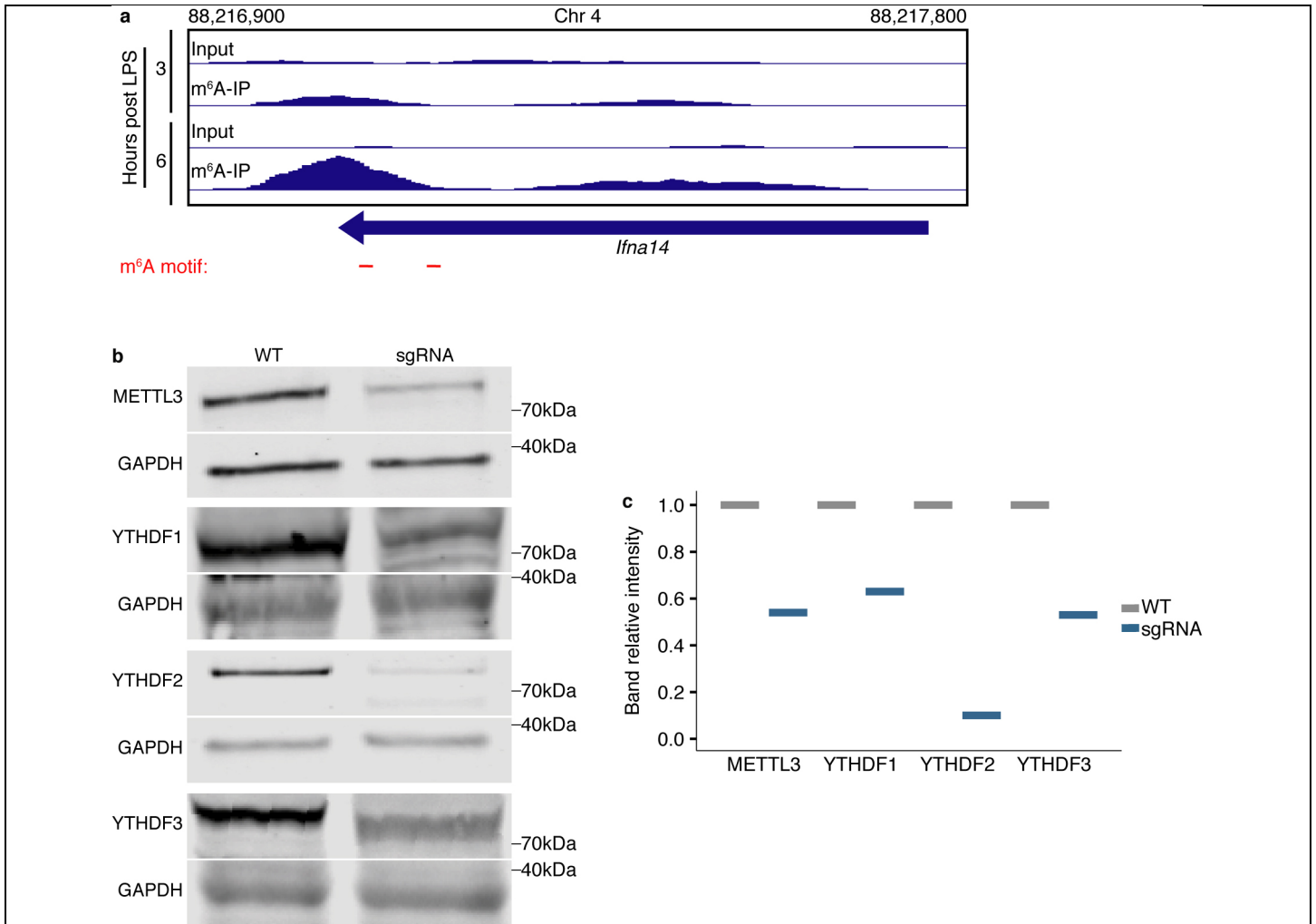
(a) ISG relative expression, as measured by RNA-seq, in METTL3-depleted cells versus control cells at 28 hpi, treated or untreated with Ruxolitinib. Expression levels of each transcript were normalized to a scale of 0-1. ISGs showing significant difference (FDR < 0.01) between control and METTL3-depleted cells are presented. (b-e) METTL3-depleted and control cells were treated with Actinomycin D at 22 hpi and harvested for RNA-seq at 0, 2 and 4 hours post treatment. The mRNA decay of several ISGs that showed enhanced expression in METTL3-depleted cells are presented (n = 2 for each time point). Values represent the mean of RNA-seq replicates and error bars show SD. (f) Quantification of protein levels from the immunoblot analysis in Fig.4a normalized to the levels of GAPDH.



Supplementary Figure 3

IFNB mRNA is m⁶A-modified and its levels are higher in YTHDF2-depleted cells compared to control cells

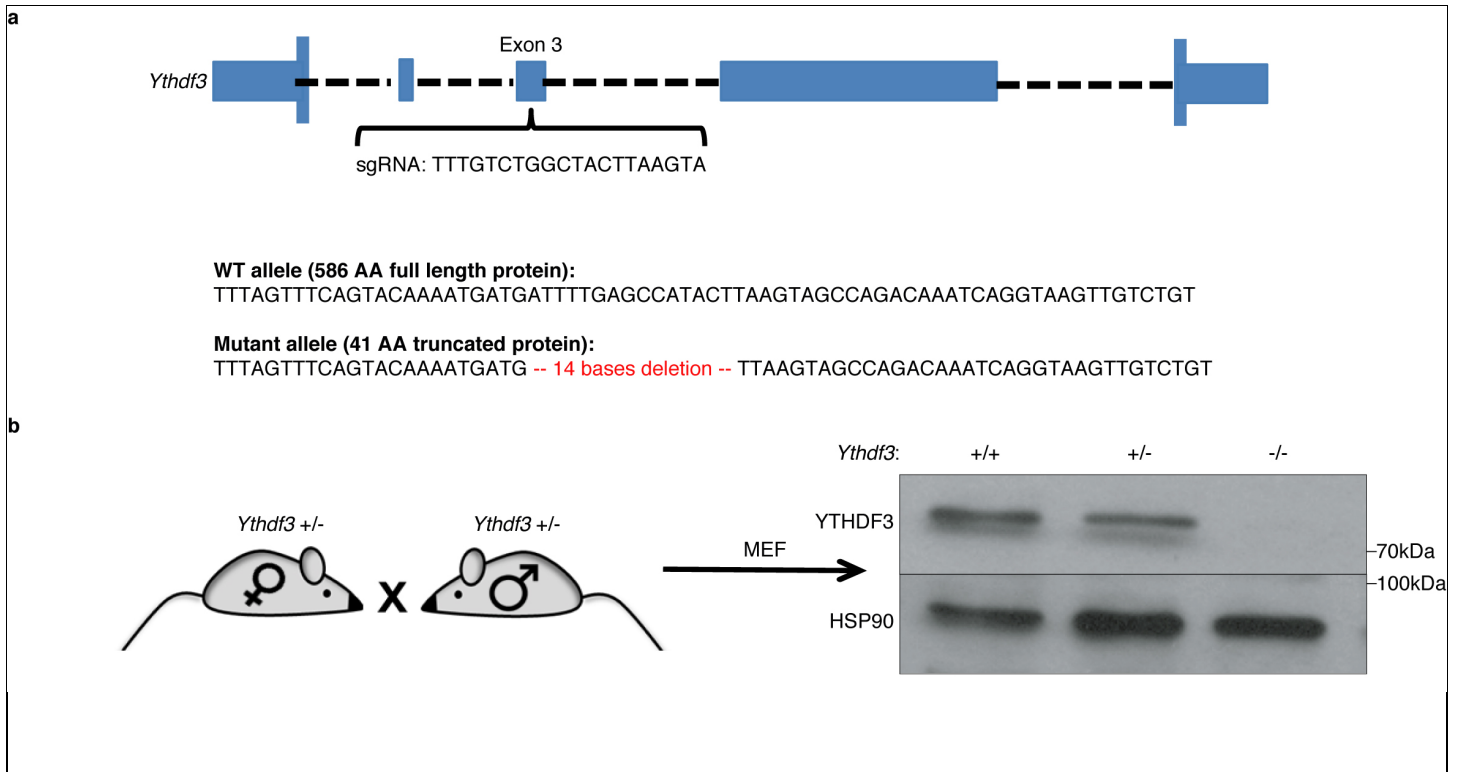
(a) Specificity of m⁶A signal on *IFNB* transcript in immuno-precipitated (IP) samples compared to input (POI, Peak Over Input) and to median coverage across the gene (POM, Peak Over Median), in METTL3-depleted (n = 3) and control cells (n = 3). Thick line, median; box boundaries, 25% and 75% percentiles; whiskers, 1.5-fold interquartile range. (b) *IFNB* mRNA and (c) protein levels in YTHDF2-depleted and control cells at indicated time points post infection, measured by qRT-PCR and ELISA, respectively. *18S* ribosomal RNA was used as a normalizing gene in qRT-PCR. Dots, measurements; bars, mean of three technical (c) and cell culture (d) replicates. *P*-value by two-sided student's *t*-test. (d) Nascent RNA was labeled for 2 h with 5-Ethynyluridine (EU). EU was washed out and RNA was extracted at the indicated time points. The relative remaining EU-labeled mRNA abundance, normalized to *GAPDH*, was analyzed by qRT-PCR for *IFNB* and *USP42* that was used as control. Values represent the mean of three technical replicates and error bars show SD. *P*-value by two-sided student's *t*-test. (e) *IFNB* gene (5'UTR, coding sequence and 3'UTR) was cloned into a plasmid in its wild-type (WT) version and in a mutant version (MUT), in which three putative m⁶A-modified adenosines were mutated to guanines (labeled in red). (f) Immunoblot analysis of METTL3 in THP1 cells expressing sgRNAs targeting control gene (WT) or METTL3. GAPDH was used as a loading control. Gel image was cropped to present only relevant proteins. (g) Quantification of METTL3 levels from the immunoblot analysis in (f) normalized to the levels of GAPDH. Data (b-d) are representative of two independent experiments.



Supplementary Figure 4

m⁶A-mediated IFN regulation is conserved in mouse

(a) RNA-seq of input RNA and m⁶A immuno-precipitated RNA from mouse dendritic cells treated with lipopolysaccharide (LPS) for 3 and 6 h is presented for *Ifna14*. (b) Immunoblot analysis of m⁶A machinery proteins in MEFs expressing sgRNAs targeting control gene (WT) or the various m⁶A machinery genes (indicated on the left). GAPDH was used as a loading control. Gel image was cropped to present only relevant proteins. (c) Quantification of m⁶A machinery protein levels from the immunoblot analysis in (b) normalized to the levels of GAPDH.



Supplementary Figure 5

Construction of *Ythdf3*^{-/-} mouse

(a) *Ythdf3*^{-/-} mice were generated via one-cell embryo CRISPR/Cas9 injection. sgRNA targeting *Ythdf3* exon3 was used. The mutated *Ythdf3* gene contains an out of frame 14bp deletion, which leads to the production of a stop codon. (b) Immunoblot analysis of YTHDF3 protein expression in MEFs extracted from *Ythdf3*^{+/+}, *Ythdf3*^{+/-} and *Ythdf3*^{-/-} embryos. HSP90 was used as a loading control. Gel image was cropped to present only relevant proteins.