





METTL3-dependent m⁶A modification programs T follicular helper cell differentiation

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T follicular helper (T_{FH}) cells are specialized effector CD4⁺ T cells critical to humoral immunity. Whether post-transcriptional regulation has a function in T_{FH} cells is unknown. Here, we show conditional deletion of METTL3 (a methyltransferase catalyzing mRNA N⁶-methyladenosine (m⁶A) modification) in CD4⁺ T cells impairs T_{FH} differentiation and germinal center responses in a cell-intrinsic manner in mice. METTL3 is necessary for expression of important T_{FH} signature genes, including *Tcf7*, *Bcl6*, *Icos* and *Cxcr5* and these effects depend on intact methyltransferase activity. m⁶A-miCLIP-seq shows the 3' UTR of *Tcf7* mRNA is subjected to METTL3-dependent m⁶A modification. Loss of METTL3 or mutation of the *Tcf7* 3' UTR m⁶A site results in accelerated decay of *Tcf7* transcripts. Importantly, ectopic expression of TCF-1 (encoded by *Tcf7*) rectifies T_{FH} defects owing to METTL3 deficiency. Our findings indicate that METTL3 stabilizes *Tcf7* transcripts via m⁶A modification to ensure activation of a T_{FH} transcriptional program, indicating a pivotal function of post-transcriptional regulation in promoting T_{FH} cell differentiation.

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The production of high-affinity antibodies and generation of memory cells are critical for establishing protective immunity^{1,2}. During acute viral infection, T follicular helper (T_{FH}) cells provide help to cognate antigen-presenting B cells to facilitate the formation of germinal centers (GCs) and the development of long-lived plasma cells and memory B cells³. After priming by dendritic cells (DCs) in the T-cell zone, CD4⁺ T cells upregulate the expression of chemokine receptor CXCR5, the costimulatory receptor ICOS and the transcriptional repressor B-cell lymphoma 6 (Bcl-6), which together coordinate early fate commitment of T_{FH} cells and their migration to the follicles. After interacting with cognate antigen-presenting B cells, these activated CD4⁺ T cells mature into T_{FH} cells and further differentiate into GC T_{FH} cells, providing help to GC B cells for humoral immunity^{4,5}.

T_{FH} differentiation is a complicated biological process⁶, which is tightly controlled by multiple transcription factors. As a master regulator of T_{FH} differentiation, Bcl-6 represses the T_H1, T_H2, and T_H17 lineage-specific transcription factors to promote the differentiation of activated CD4⁺ T cells into T_{FH} cells^{7–9}. On the contrary, Blimp1 (encoded by *Prdm1*) negatively modulates Bcl-6 transcription by binding to the *Bcl6* promoter and acting as a repressor, thus preventing T_{FH} differentiation and promoting the formation of non-T_{FH} effector helper T cells^{9,10}. Accumulative studies have demonstrated that TCF-1 (encoded by *Tcf7*) plays key roles in T_{FH} differentiation by directly acting upstream of the Bcl-6–Blimp1 axis^{11–13}. Although the regulation of these transcription factors has been intensely investigated at the transcriptional level, it remains unknown if post transcriptional mechanisms are involved in the balanced expression of these key factors during T_{FH} differentiation.

N⁶-methyladenosine (m⁶A) is the most prevalent modification on eukaryote mRNAs, catalyzed by m⁶A methyltransferases¹⁴. Methyltransferase like 3 protein (METTL3, encoded by *Mettl3*) is the core catalytic subunit of m⁶A methyltransferases, and METTL14 is an allosteric activator of METTL3^{15,16}. The recruitment of m⁶A-binding proteins is actively involved in almost every stage of mRNA metabolism, from processing in the nucleus to translation and decay in the cytoplasm, adding another layer of regulatory mechanisms in gene expression¹⁷. Early studies showed that m⁶A-tagged transcripts had shorter half-life¹⁸, and binding of YTHDF2 to m⁶A promoted m⁶A-modified mRNA decay^{18,19}. Recently, it was discovered that a group of novel m⁶A-binding proteins, IGF2BPs, stabilized methylated transcripts by guarding them against degradation^{20,21}. The distinct roles of YTHDF2 and IGF2BPs suggest that the impact of m⁶A modification on mRNA stability is highly dependent on cell context.

Several studies have uncovered critical roles of m⁶A modification in immune regulation. It has been documented that m⁶A methylation is essential for normal hematopoiesis and leukemia development^{22–24}. m⁶A methylation regulates T-cell homeostasis, as well as suppressive functions of Treg cells by targeting *Socs* genes^{25,26}. In the context of viral infection, m⁶A represses type I interferon production in an innate antiviral state^{27,28}. Despite these profound effects of m⁶A modification on immunoregulation, its role in T_{FH} differentiation has not been determined.

In this study, by conditional targeting the *Mettl3* gene in T cells, we demonstrate that METTL3-mediated m⁶A modification is critical for T_{FH} cell differentiation. Mechanistically, METTL3 deficiency impairs the stability of m⁶A-modified *Tcf7* mRNA, resulting in compromised activation of T_{FH} transcriptional program.

Results

METTL3 controls T_{FH} differentiation and GC reactions. To investigate the role of METTL3 in T_{FH} differentiation, *Mettl3*-

floxed mice were crossed with *Cd4*-Cre mice to generate conditional deletion of METTL3 in T cells (*Mettl3^{fl/fl}Cd4*-Cre mice), and the deletion efficiency of METTL3 in splenic CD4⁺ T cells was validated by quantitative RT-PCR (Supplementary Fig. 1a). We then infected *Mettl3^{fl/fl}Cd4*-Cre mice and their wild-type control littermates (Ctrl) with LCMV-Armstrong strain. The frequency and numbers of CD44⁺CXCR5⁺ T_{FH} cells were significantly diminished in *Mettl3^{fl/fl}Cd4*-Cre mice compared with those in their control littermates on day 8 post viral infection (Fig. 1a, b). In contrast, METTL3-deficient CD4⁺ T cells were dramatically skewed toward the CD44⁺CXCR5⁻ T_H1 proportion, albeit the numbers of T_H1 cells were also decreased with the ablation of METTL3 (Fig. 1a, b). It is worth mentioning that ablation of METTL3 resulted in more severe defects in T_{FH} cells (21.4-fold change) than T_H1 cells (1.9-fold change) upon acute viral infection (Fig. 1b). Given T-bet is expressed at a relatively high level on T_H1 lineage and directs its commitment²⁹, we also analyzed T-bet expression on both T_H1 and T_{FH} cells. We found T_H1 cells expressed a much higher level of T-bet than T_{FH} cells, and both T_{FH} and T_H1 cells downregulated T-bet expression in the absence of METTL3 (Supplementary Fig. 1b). Moreover, *Mettl3^{fl/fl}Cd4*-Cre mice exhibited remarkably lower percentages and absolute cell numbers of GC T_{FH} cells (identified as PD-1^{hi}CXCR5⁺, ICOS^{hi}CXCR5⁺, or Bcl-6^{hi}CXCR5⁺) than those of their control littermates (Fig. 1c, d). Accordingly, the expression levels of CXCR5, PD-1, ICOS, and Bcl-6 were dramatically lower on METTL3-deficient T_{FH} cells than on wild-type cells (Supplementary Fig. 1c, d). These results indicated that METTL3 deficiency severely impairs T_{FH} differentiation.

Given the main function of T_{FH} cells is to provide cognate B-cell help, which is a fundamental aspect of humoral immunity and generation of immunological memory¹. We next examined whether METTL3 deficiency in CD4⁺ T cells affects GC formation during viral infection. A robust reduction in the proportions and numbers of GL-7⁺Fas⁺ GC B cells (Fig. 1e, f) and PNA⁺Fas⁺ GC B cells (Supplementary Fig. 1e, f) were observed in *Mettl3^{fl/fl}Cd4*-Cre mice, compared with those in their wild-type counterparts. Furthermore, the frequency and cell numbers of IgD^{lo}CD138⁺ plasma cells were also much lower in *Mettl3^{fl/fl}Cd4*-Cre mice than those in wild-type mice (Fig. 1e, f). The immunohistochemical analysis further confirmed substantially reduced PNA⁺ GCs within B-cell follicles in spleens from *Mettl3^{fl/fl}Cd4*-Cre mice (Fig. 1g). To assess the consequences of defective T_{FH} and GC responses in *Mettl3^{fl/fl}Cd4*-Cre mice, we measured the LCMV-specific serum concentration of immunoglobulin G (IgG). The LCMV-specific IgG concentration was significantly lower in *Mettl3^{fl/fl}Cd4*-Cre mice than that in their wild-type control littermates on day 8 and day 56 post viral infection (Fig. 1h). Collectively, these results suggested that METTL3 is required for T_{FH} differentiation and GC responses.

To further validate these findings, we immunized *Mettl3^{fl/fl}Cd4*-Cre mice and their wild-type control littermates by intraperitoneal administration of keyhole limpet hemocyanin (KLH) emulsified in Complete Freund's Adjuvant (CFA). On day 8 post immunization, *Mettl3^{fl/fl}Cd4*-Cre mice exhibited impaired development of T_{FH} cells and GC T_{FH} cells, but not T_H1 cells (Supplementary Fig. 2a, b). Meanwhile, the percentages and cell numbers of GC B cells and plasma cells (Supplementary Fig. 2c, d) were also impaired in *Mettl3^{fl/fl}Cd4*-Cre mice compared with their wild-type counterparts. These data jointly indicated METTL3 promotes T_{FH} differentiation upon different antigen stimulation.

Meanwhile, the differentiation of other T helper lineages was also examined by using KLH immunization model. We found that both GATA3⁺ and IL-4-producing cells, as well as Foxp3⁺ cells were not altered in *Mettl3^{fl/fl}Cd4*-Cre mice (Supplementary

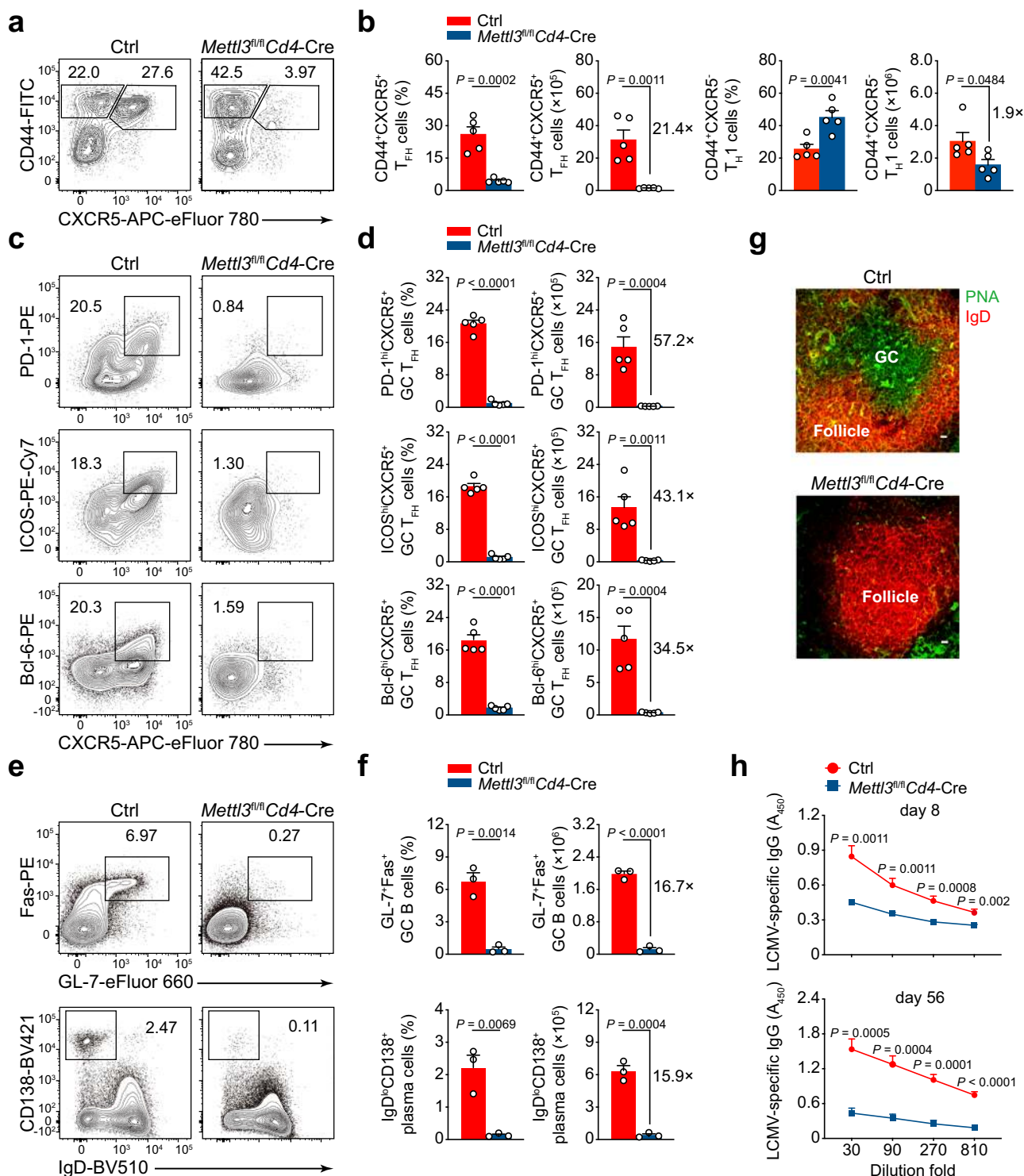


Fig. 1 Conditional ablation of METTL3 impairs T_{FH} differentiation and GC responses. **a, b** Flow cytometry analysis of CD44⁺CXCR5⁺ T_{FH} cells and CD44⁺CXCR5⁺ T_{H1} cells, gated on splenic CD4⁺ T cells from Ctrl and *Mettl3^{fl/fl}Cd4-Cre* mice on day 8 post infection (8 dpi). Summary of the frequency and cell numbers of indicated cell subsets are shown in **b** ($n = 5$ per group). **c, d** Flow cytometry analysis of PD-1^{hi}CXCR5⁺ GC T_{FH} cells (top panel), ICOS^{hi}CXCR5⁺ GC T_{FH} cells (middle panel), and Bcl-6^{hi}CXCR5⁺ GC T_{FH} cells (bottom panel), gated on splenic CD44^{hi}CD62L^{lo}CD4⁺ T cells from Ctrl and *Mettl3^{fl/fl}Cd4-Cre* mice on 8 dpi. Summary of the frequency and cell numbers of indicated cell subsets are shown in **d** ($n = 5$ per group). **e, f** Flow cytometry analysis of splenic GL-7⁺Fas⁺ GC B cells (top panel) and IgD^{lo}CD138⁺ plasma cells (bottom panel) on 8 dpi. Summary of the frequency and cell numbers of GC B cells and plasma cells are shown in **f** ($n = 3$ per group). **g** Immunofluorescent staining of spleens from Ctrl and *Mettl3^{fl/fl}Cd4-Cre* on 8 dpi. Green: PNA; Red: IgD; scale bar: 10 μ m. **h** Analysis of LCMV-specific IgG concentration in serum on 8 dpi (top) and on 56 dpi (bottom) by ELISA (day 8: $n = 12$ for Ctrl group, $n = 10$ for *Mettl3^{fl/fl}Cd4-Cre* group; day 56: $n = 5$ per group). Data are representative of at least three independent experiments. Error bars indicate standard error of the mean. P value was calculated by unpaired two-tailed Student's t test.

Fig. 2e–h), indicating T_{H2} and Treg cell differentiation are not affected in the absence of METTL3 upon KLH immunization. Interestingly, both $ROR\gamma^+$ and IL-17a-producing cells were significantly decreased in METTL3-deficient mice (Supplementary Fig. 2e–h), revealing METTL3 is essential for T_{H17} cell differentiation in vivo upon protein immunization.

METTL3 intrinsically regulates T_{FH} differentiation. We next focused on whether METTL3 regulates T_{FH} differentiation in a cell-intrinsic manner using both bone marrow chimeric and adoptive transfer mice models. We first generated bone marrow chimeric mice by reconstituting lethally irradiated wild-type recipient mice ($CD45.1^+$) with a mixture donor of bone marrow cells from *Mettl3^{fl/fl}Cd4-Cre* mice ($CD45.2^+$) and wild-type ($CD45.1^+$) competitor mice (Supplementary Fig. 3a). On day 8 post LCMV-Armstrong infection, *Mettl3^{fl/fl}Cd4-Cre* mice-derived $CD4^+$ T cells had much lower cell numbers of $CD44^+CXCR5^+$ T_{FH} cells than those of competitor bone marrow-derived $CD4^+$ T cells (Supplementary Fig. 3b, c). Correspondingly, $CD4^+$ T cells originated in *Mettl3^{fl/fl}Cd4-Cre* mice also exhibited a reduction of $PD-1^{hi}CXCR5^+$ GC T_{FH} cells (Supplementary Fig. 3b, c). The expression levels of CXCR5, PD-1, ICOS, and Bcl-6 were significantly lower on METTL3-deficient T_{FH} cells than those on competitor cells (Supplementary Fig. 3d).

To further rule out the effect of external factors on T_{FH} differentiation, we adoptively transferred $CD45.1^+$ SMARTA $CD4^+$ T cells into $CD45.2^+$ *Mettl3^{fl/fl}Cd4-Cre* or their wild-type control littermates (Fig. 2a). On day 8 post viral infection, we observed the percentages and numbers of $CD44^+CXCR5^+$ T_{FH} cells derived from $CD45.1^+$ SMARTA $CD4^+$ T cells were similar in the *Mettl3^{fl/fl}Cd4-Cre* mice and their wild-type counterparts (Fig. 2b, c), indicating the microenvironment in the *Mettl3^{fl/fl}Cd4-Cre* mice did not impair T_{FH} differentiation. In contrast, METTL3-deficient $CD4^+$ T cells displayed compromised T_{FH} differentiation compared with that of congenic wild-type $CD4^+$ T cells (Fig. 2b, c). Correspondingly, METTL3-deficient $CD4^+$ T cells also exhibited defects in their ability to differentiate into $CD44^+CXCR5^-$ T_{H1} cells (Fig. 2b, c). Besides, transferred $CD45.1^+$ SMARTA cells also profoundly promoted the GC B and plasma cells differentiation (Fig. 2d, e), GC formation (Fig. 2f), and excessive production of LCMV-specific IgG antibody (Fig. 2g) in *Mettl3^{fl/fl}Cd4-Cre* recipients. Together, our data demonstrated METTL3 is intrinsically required for T_{FH} cell differentiation and functions as a key role in GCs formation.

METTL3 is essential for the early initiation of T_{FH} cells. During acute viral infection, effector $CD4^+$ T cells' commitment to the T_{FH} lineage or T_{H1} lineage emerges before the initiation of GCs¹². To investigate whether METTL3 is required for early T_{FH} specification in vivo, naive Ctrl or *Mettl3^{fl/fl}Cd4-Cre* SMARTA $CD4^+$ T cells were labeled with cell-trace violet (CTV) and adoptively transferred into congenic recipient mice, followed by LCMV-Armstrong infection (Supplementary Fig. 4a). On day 3 post viral infection, SMARTA $CD4^+$ T cells of both genotypes showed similar upregulation of T-cell activation markers CD69 and CD44, and downregulation of CD62L (Supplementary Fig. 4b, c). In vitro cell culture analysis also revealed that *Mettl3^{fl/fl}Cd4-Cre* SMARTA $CD4^+$ T cells displayed no obvious defects in T-cell activation (Supplementary Fig. 4d). Ctrl SMARTA $CD4^+$ T cells exhibited vigorously proliferation post viral infection, whereas METTL3-deficient cells displayed a delayed proliferation (Fig. 3a). Lineage commitment to T_{FH} lineage has occurred on day 3 post viral infection, as detected by $Bcl-6^+CXCR5^+$ cells³⁰. Consistently, wild-type SMARTA cells developed robust numbers of $Bcl-6^+CXCR5^+$ T_{FH} cells, whereas METTL3-deficient cells

exhibited much lower percentages and cell numbers of $Bcl-6^+CXCR5^+$ T_{FH} cells (Fig. 3b). Tracking the cell division showed *Mettl3^{fl/fl}Cd4-Cre* SMARTA cells were predominantly in third and fourth divisions, while Ctrl SMARTA cells had advanced to fifth and sixth divisions (Fig. 3c, d), reflecting the impaired proliferation of activated $CD4^+$ T cells in the absence of METTL3. Consistently, *Mettl3^{fl/fl}Cd4-Cre* SMARTA cells exhibited a reduction of proportions and cell numbers of $Bcl-6^+CXCR5^+$ T_{FH} cells in indicated cell divisions compared with that of Ctrl SMARTA cells (Fig. 3e, f). Interestingly, as a critical regulator for early T_{FH} differentiation³¹, TCF-1 expression in $CXCR5^+$ cells was also dramatically reduced due to ablation of METTL3 (Fig. 3g, h). Moreover, we also observed compromised $CD25^-CXCR5^+$ T_{FH} cells in *Mettl3^{fl/fl}Cd4-Cre* SMARTA cells by using the combination of CXCR5 and CD25 to identify T_{FH} cells in activated $CD4^+$ T cells (Supplementary Fig. 4e, f), and apoptosis of T_{FH} cells was elevated in the absence of METTL3 (Supplementary Fig. 4g, h). We further assessed the expression levels of genes that are associated with T_{FH} cells, including *Tcf7*, *Cxcr5*, *Bcl6*, *Pdcd1*, and *Icos*. The expression levels of these genes were substantially decreased in *Mettl3^{fl/fl}Cd4-Cre* SMARTA T_{FH} cells compared with those of Ctrl cells (Fig. 3i). Correspondingly, the expressions of *Prdm1* and *Id2*, both well-known for promoting T_{H1} differentiation^{9,32}, were much lower in Ctrl T_{FH} cells than that of *Mettl3^{fl/fl}Cd4-Cre* SMARTA cells (Fig. 3i). These results thus demonstrated that METTL3 is indispensable for early T_{FH} commitment during acute viral infection.

METTL3 orchestrates the transcriptional profiles of T_{FH} cells.

We next investigated how METTL3 deficiency affects the transcriptional profiles of T_{FH} cells. Considering $CD4^+$ T cells generally differentiate into T_{H1} cells or T_{FH} cells upon acute viral infection³³ and METTL3 deficiency affects CXCR5 expression, we hence used CD44 and SLAM, which is expressed at low level on T_{FH} cells⁹, to identify T_{H1} and T_{FH} cells in activated $CD4^+$ T cells. $CD44^+SLAM^{hi}$ T_{H1} cells and $CD44^+SLAM^{lo}$ T_{FH} cells were sorted from *Mettl3^{fl/fl}Cd4-Cre* mice and their Ctrl littermates on day 8 post viral infection and subjected to RNA-seq analysis. Compared with those in wild-type cells, 515 upregulated genes and 252 downregulated genes in METTL3-deficient T_{FH} cells (Fig. 4a) and 763 upregulated genes and 332 downregulated genes (Supplementary Fig. 5a) in METTL3-deficient T_{H1} cells were identified, respectively (≥ 2 -fold expression change, adjusted P value < 0.01). In particular, the upregulated genes in T_{FH} cells were significantly enriched in the T-cell differentiation and defense response to virus; the downregulated genes in T_{FH} cells were apparently enriched in T-cell proliferation and differentiation (Fig. 4b). Then we selected a T_{FH} cell and a GC T_{FH} cell signature gene set¹¹ for gene set enrichment analysis (GSEA). Both the T_{FH} lineage gene set and the GC T_{FH} -associated gene set were enriched in wild-type T_{FH} cells, but not in METTL3-deficient T_{FH} cells (Fig. 4c). When applying GSEA to exhibit the signature genes of T_{H1} (ref. 12), T_{H2} (ref. 34), T_{H17} (ref. 35), and Treg³⁴ cells, we found that all of which were enriched in METTL3-deficient T_{FH} cells (Supplementary Fig. 5b). Similarly, we also observed T_{FH} , T_{H2} , T_{H17} , and Treg-related gene sets were positively enriched in METTL3-deficient T_{H1} cells (Supplementary Fig. 5c). These analyses indicated that loss of METTL3 leads to disordered gene profiles of both T_{FH} and T_{H1} transcription program.

We next validated expression changes of key T_{FH} genes and found that *Cxcr5*, *Bcl6*, *Pdcd1*, and *Icos* were significantly lower in METTL3-null T_{FH} cells than in wild-type cells (Fig. 4e). In addition, the expression of *Tcf7* was lower in METTL3-deficient T_{FH} cells than in wild-type T_{FH} cells (Fig. 4e). Among the genes with low expression, *Tcf7* was of interest because of its role in Tfh

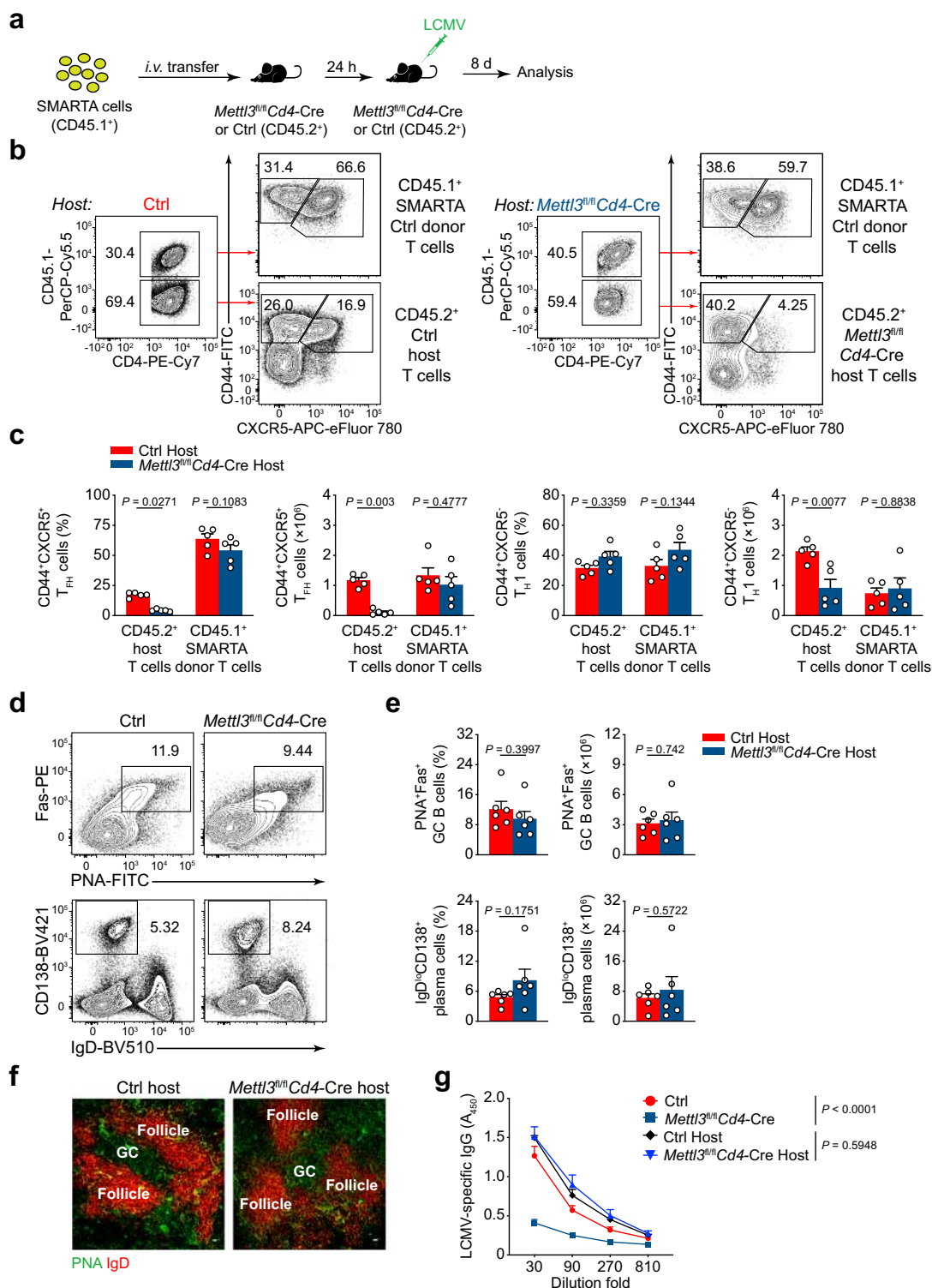


Fig. 2 METTL3 intrinsically controls T_H17 differentiation and GC responses. **a** Scheme of adoptive transfer model. 5×10^6 CD45.1⁺ SMARTA cells were adoptively transferred into CD45.2⁺ *Mettl3^{fl/fl}Cd4-Cre* mice or Ctrl host mice, followed by LCMV-Armstrong infection within 24 h. **b, c** Flow cytometry analysis of CD45.1⁺ SMARTA or CD45.2⁺ host T cell-derived CD44⁺CXCR5⁺ T_H17 and CD44⁺CXCR5⁻ T_H1 cells, gated on splenic CD4⁺ T cells from host mice on day 8 post viral infection. Frequency and numbers of CD44⁺CXCR5⁺ T_H17 cells and CD44⁺CXCR5⁻ T_H1 cells are shown in **c** ($n = 5$ per group). **d, e** Flow cytometry analysis of splenic PNA⁺Fas⁺ GC B cells (top panel) and IgD^{lo}CD138⁺ plasma cells (bottom panel) from host mice on 8 dpi. Summary of the frequency and cell numbers of GC B cells and plasma cells are shown in **e** ($n = 6$ per group). **f** Immunofluorescent staining of spleens from Ctrl or *Mettl3^{fl/fl}Cd4-Cre* host mice on 8 dpi. Green: PNA; Red: IgD; scale bar: 10 μ m. **g** Analysis of LCMV-specific IgG concentration in serum on 8 dpi by ELISA ($n = 5$ for Ctrl group, $n = 5$ for *Mettl3^{fl/fl}Cd4-Cre* group, $n = 6$ for Ctrl host group, $n = 6$ for *Mettl3^{fl/fl}Cd4-Cre* host group). Data are representative of at least three independent experiments. Error bars indicate standard error of the mean. P value was calculated by unpaired two-tailed Student's *t* test (**e**) or two-way ANOVA coupled with multiple comparisons (**c, g**).

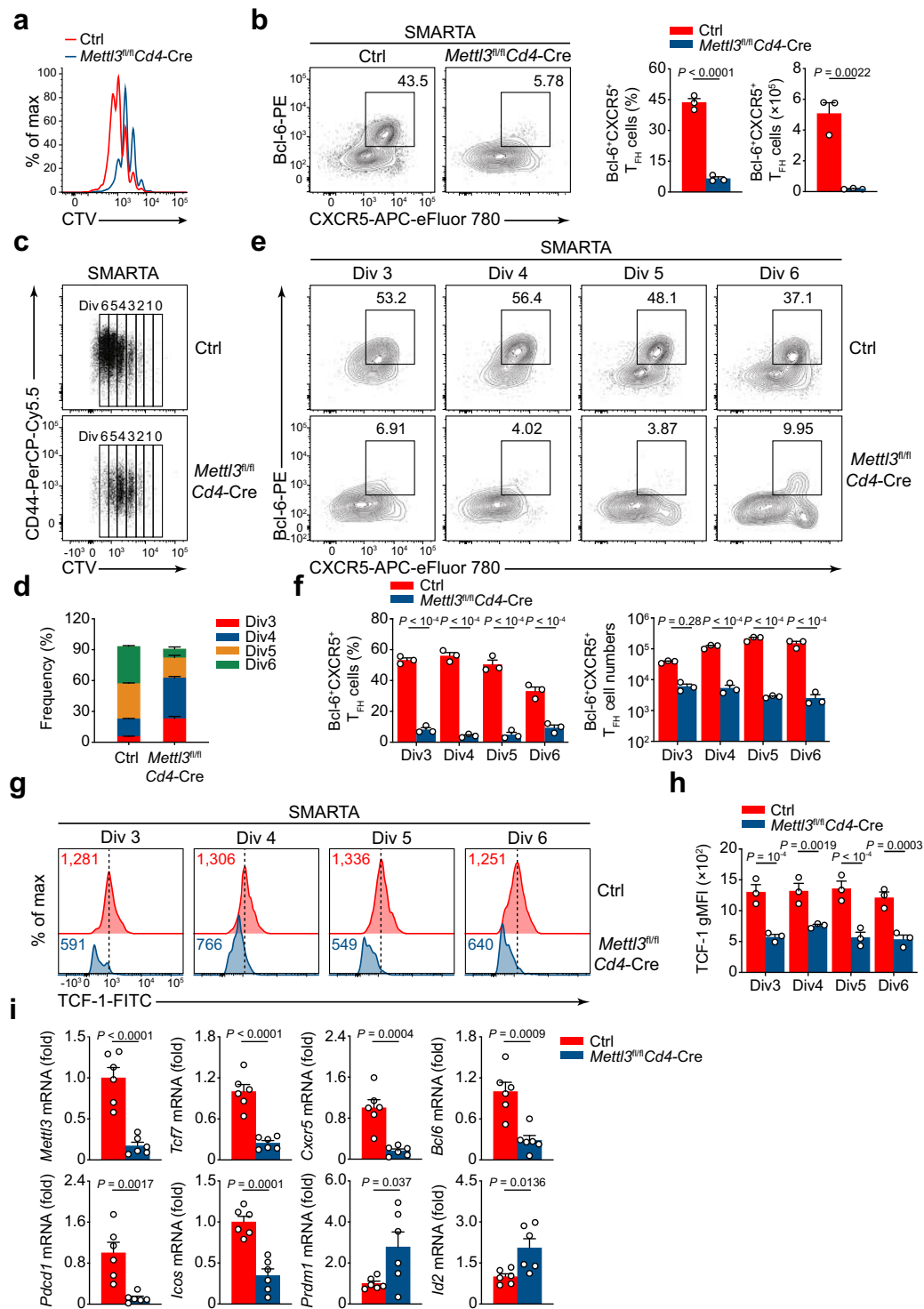


Fig. 3 METTL3 is indispensable for the initiation of T_{FH} development. **a** Flow cytometry analysis of cells from the wild-type recipient mice (CD45.1⁺) given adoptive transfer of naive CTV-labeled Ctrl or *Mettl3*^{fl/fl}*Cd4-Cre* SMARTA cells, followed by LCMV-Armstrong infection and analysis 3 days later as CTV dilution by the transferred cells. **b** Flow cytometry analysis of Bcl-6⁺CXCR5⁺ T_{FH} cells gated on SMARTA CD4⁺ T cells from recipient mice as in **a**. Summary of the frequency and cell numbers of T_{FH} cells are shown on the right (*n* = 3 per group). **c**, **d** Flow cytometry analysis of different cell divisions gated on SMARTA CD4⁺ T cells from recipient mice as in **a**. The frequency of third to sixth divisions is summarized in **d** (*n* = 3 per group). **e**, **f** Contour plots display Bcl-6⁺CXCR5⁺ T_{FH} cells in indicated divisions, among SMARTA cells from recipient mice as in **a**. Summary of the frequency and cell numbers of T_{FH} cells in indicated divisions are shown in **f** (*n* = 3 per group). **g**, **h** Flow cytometry analysis of the expression of TCF-1 in CXCR5⁺ cells in indicated divisions gated on SMARTA CD4⁺ T cells from recipient mice as in **a**. Quantification of geometric mean fluorescence intensity (gMFI) of TCF-1 in indicated divisions is shown in **h** (*n* = 3 per group). **i** Quantitative RT-PCR analysis of mRNA abundance of T_{FH} cell-related genes in CD25⁺CXCR5⁺ T_{FH} cells from recipient mice as in **a**, relative expression was normalized to Ctrl cells (*n* = 6 per group). Data are representative of two independent experiments. Error bars indicate standard error of the mean. P value was calculated by unpaired two-tailed Student's *t* test (**b**, **i**) or two-way ANOVA coupled with multiple comparisons (**f**, **h**).

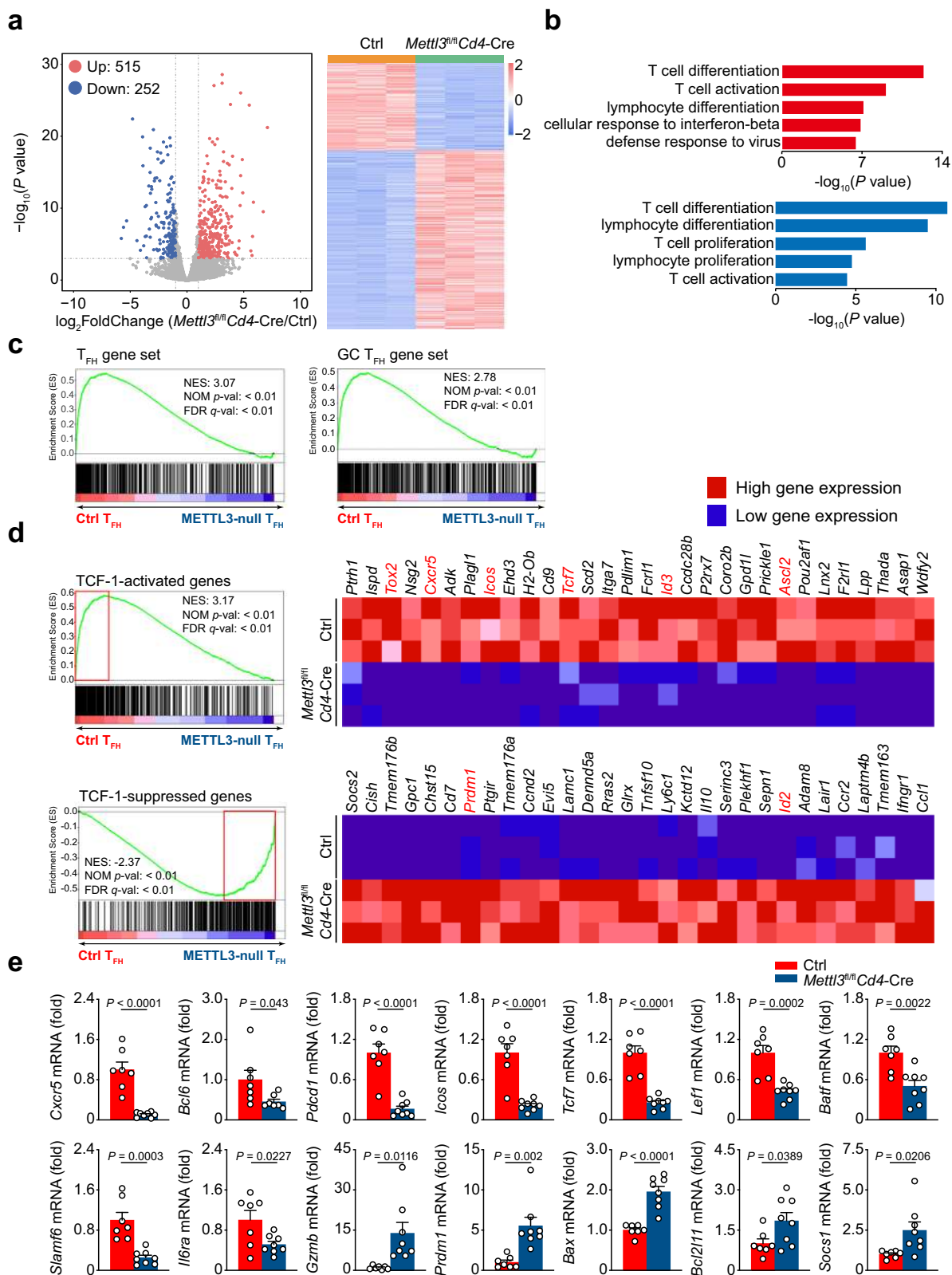
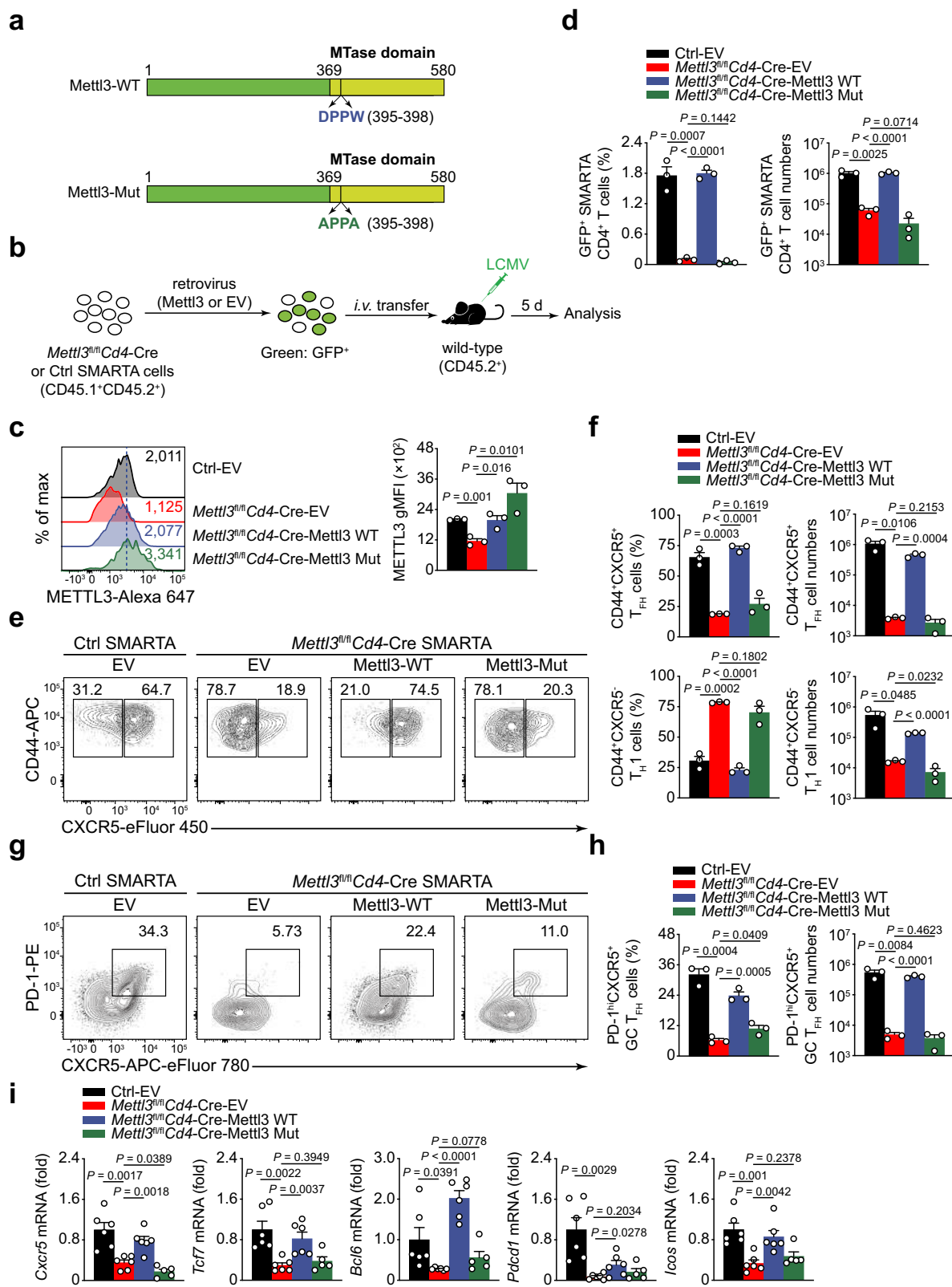


Fig. 4 Alteration of transcriptional profiles in METTL3-null *T_{FH}* cells. **a** Volcano map depicting genes upregulated (red) or downregulated (blue) 2-fold or more in *T_{FH}* cells on 8 *dpi*. Heatmap of differentially expressed genes is shown on the right. **b** Gene Ontology (GO) terms of the differentially expressed genes in *Mettl3^{fl/fl}Cd4-Cre* *T_{FH}* cells compared with Ctrl *T_{FH}* cells (Upregulated: top panel; Downregulated: bottom panel). **c** Gene set enrichment analysis (GSEA) of *T_{FH}* and GC *T_{FH}* gene set in *Mettl3^{fl/fl}Cd4-Cre* *T_{FH}* cells relative to expression in Ctrl *T_{FH}* cells as in **a**. **d** GSEA analysis of “TCF-1-activated genes in *T_{FH}* cells” and “TCF-1-suppressed genes in *T_{FH}* cells” (GSE65693) in *Mettl3^{fl/fl}Cd4-Cre* *T_{FH}* cells relative to expression in Ctrl *T_{FH}* cells as in **a**. Heatmaps representation of top 30 ranking genes in the leading edge are shown, respectively. **e** Quantitative RT-PCR analysis of selected interested genes in **a**, relative expression was normalized to that in Ctrl *T_{FH}* cells ($n = 7$ for Ctrl group, $n = 8$ for *Mettl3^{fl/fl}Cd4-Cre* group except *Bcl6* gene ($n = 7$ per group)). Data are from one experiment with triplicates (**a-d**) or pooled from two experiments (**e**). Error bars indicate standard error of the mean. *P* value was calculated by unpaired two-tailed Student’s *t* test (**e**).



cell differentiation, so we also performed GSEA analysis of a gene set containing TCF-1-activated genes in T_{HF} cells¹³. Interestingly, remarkable enrichment was exhibited in wild-type T_{HF} cells, whereas the TCF-1-suppressed gene set¹³ was notably enriched in METTL3-deficient cells, which suggested that METTL3 and TCF-1 share a common subset of target genes in

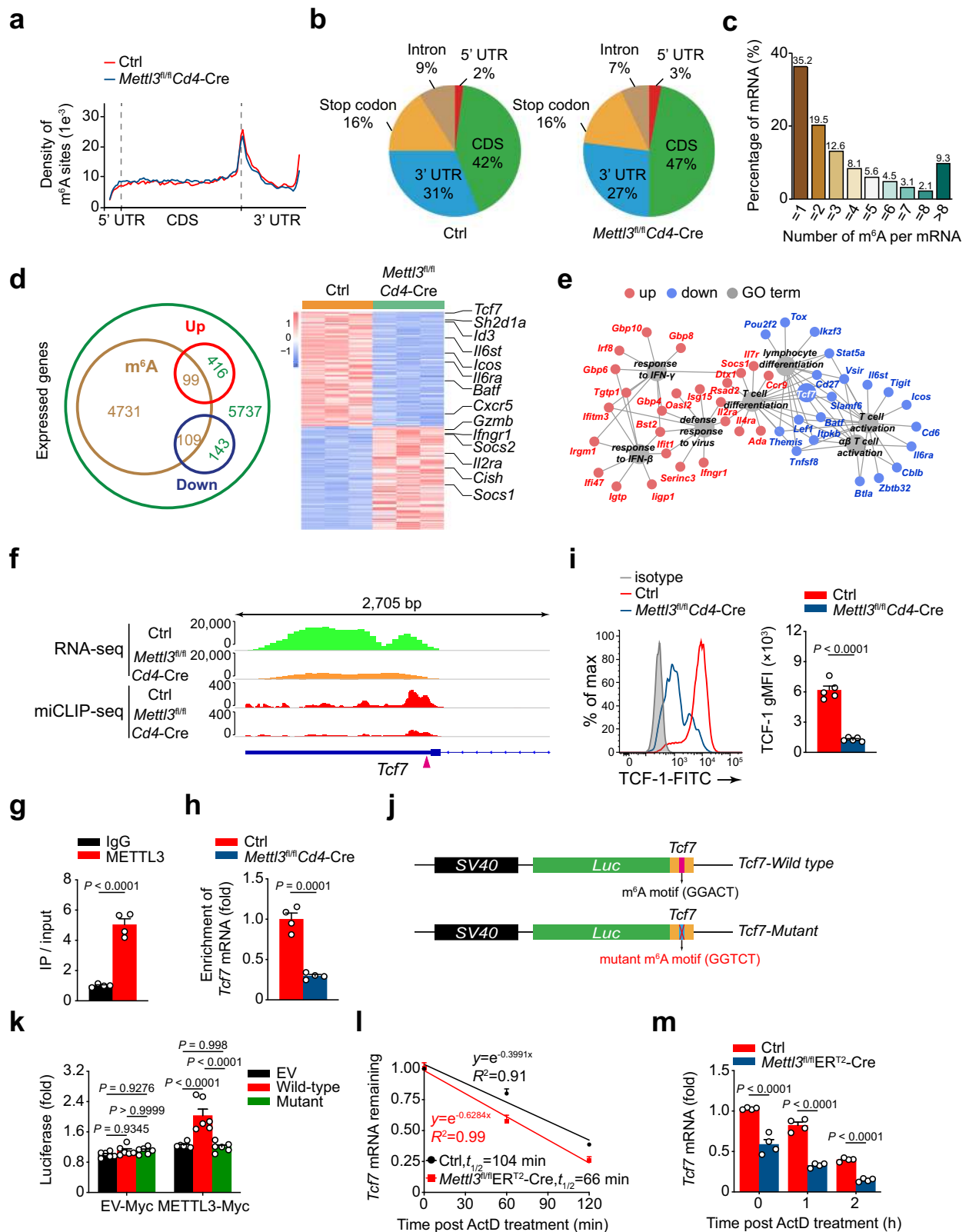
T_{HF} cells (Fig. 4d). Meanwhile, the expression of *Lef1* mRNA was also decreased in METTL3-deficient T_{HF} cells compared with wild-type cells (Fig. 4e). On the other hand, the expression of *Prdm1*, known as the antagonist of *Bcl6*, and *Gzmb* was higher in METTL3-null T_{HF} cells than that in wild-type T_{HF} cells (Fig. 4e). The expression levels of *Bcl2l11* and *Bax*, both known as

Fig. 5 METTL3 promotes T_{FH} differentiation in an m⁶A catalytic activity-dependent manner. **a** Graphic representation of the wild-type (Mettl3-WT) and catalytic domain dead (Mettl3-Mut; DPPW to APPA) METTL3 constructs. **b** Scheme of retrovirus transduction experiment. SMARTA cells were transduced with indicated structures by using a retrovirus transduction system. Then, the transduced cells were adoptively transferred into congenic CD45.2⁺ wild-type mice followed by LCMV-Armstrong infection, and analyzed on day 5 post viral infection. **c** Flow cytometry analysis of METTL3 gMFI in GFP⁺CD4⁺ SMARTA cells from recipient mice. Quantitation of METTL3 gMFI is shown on the right ($n = 3$ per group). **d** Summary of the frequency and cell numbers of retrovirus-transduced SMARTA CD4⁺ T cells in host mice ($n = 3$ per group). **e, f** Flow cytometry analysis of CD44⁺CXCR5⁺ T_{FH} populations and CD44⁺CXCR5⁻ T_{H1} subsets gated on SMARTA GFP⁺CD4⁺ T cells from host mice adoptively transferred with empty vector (EV), Mettl3-WT, or Mettl3-Mut retrovirus-introduced SMARTA cells. Summary of the frequency and cell numbers of T_{FH} cells and T_{H1} cells are shown in **f** ($n = 3$ per group). **g, h** Flow cytometry analysis of PD-1^{hi}CXCR5⁺ GC T_{FH} populations gated on SMARTA GFP⁺CD4⁺ T cells from host mice adoptively transferred with EV, Mettl3-WT, or Mettl3-Mut retrovirus-introduced SMARTA cells. Summary of the frequency and cell numbers of GC T_{FH} cells are shown in **h** ($n = 3$ per group). **i** Quantitative RT-PCR analysis of mRNA abundance of T_{FH} cell-related genes in CD44⁺CXCR5⁺ T_{FH} cells ectopically expressed with EV, Mettl3-WT, or Mettl3-Mut, relative expression was normalized to Ctrl cells transduced with EV retrovirus ($n = 6$ for Ctrl EV, *Mettl3^{fl/fl}Cd4-Cre-EV*, and *Mettl3^{fl/fl}Cd4-Cre-Mettl3 WT* group; $n = 5$ for *Mettl3^{fl/fl}Cd4-Cre-Mettl3 Mut* group). Data are representative of two independent experiments. Error bars indicate standard error of the mean. *P* value was calculated by one-way ANOVA, followed by unpaired two-tailed Student's *t* test for indicated pairwise comparisons.

pro-apoptotic regulators^{36,37}, were significantly elevated in METTL3-null T_{FH} cells compared with those in the wild-type T_{FH} cells (Fig. 4e), which corresponded to accelerated apoptosis in *Mettl3^{fl/fl}Cd4-Cre* T_{FH} cells (Supplementary Fig. 4g, h). In addition, the transcripts encoding other essential transcription factors, receptors and ligands for T_{FH} differentiation, including *Batf*, *Slamf6*, and *Il6ra*, were downregulated in *Mettl3^{fl/fl}Cd4-Cre* T_{FH} cells compared with those in wild-type cells (Fig. 4e). Besides, we observed elevated expression of *Socs1* mRNA, a known target of METTL3^{25,26}, in *Mettl3^{fl/fl}Cd4-Cre* T_{FH} cells (Fig. 4e). Collectively, these results suggested that METTL3 regulates T_{FH} differentiation program by activating T_{FH} program but suppressing T_{H1} lineage-associated genes.

METTL3 promotes T_{FH} differentiation in an m⁶A catalytic activity-dependent manner. m⁶A methylation is catalyzed by a multicomponent methyltransferase complex, while METTL3 functions as the predominant catalytic subunit^{15,16}. Next, we analyzed whether METTL3-mediated T_{FH} differentiation is dependent on its m⁶A methyltransferase activity. To achieve this goal, we generated wild-type METTL3 (Mettl3-WT) and catalytic domain mutant METTL3 (D395A and W398A; Mettl3-Mut) constructs¹⁵ (Fig. 5a). These METTL3 proteins were then expressed in *Mettl3^{fl/fl}Cd4-Cre* SMARTA CD4⁺ T cells with a retrovirus transduction system (Fig. 5b). On day 5 post infection, forced expression of Mettl3-WT and Mettl3-Mut both resulted in elevated METTL3 expression in *Mettl3^{fl/fl}Cd4-Cre* SMARTA cells compared with the empty-vector (EV) retrovirus (Fig. 5c). EV retrovirus-infected *Mettl3^{fl/fl}Cd4-Cre* SMARTA CD4⁺ T cells exhibited reduced expansion than EV retrovirus-infected Ctrl cells, which could be restored by overexpression of Mettl3-WT but not Mettl3-Mut (Fig. 5d). Moreover, EV retrovirus-infected *Mettl3^{fl/fl}Cd4-Cre* SMARTA CD4⁺ T cells remained defective in the generation of CD44⁺CXCR5⁺ T_{FH} cells, while Mettl3-WT but not Mettl3-Mut retrovirus promoted differentiation of *Mettl3^{fl/fl}Cd4-Cre* SMARTA CD4⁺ T cells into a T_{FH} fate (Fig. 5e). In addition, forced expression of Mettl3-WT could restore both the cell numbers of *Mettl3^{fl/fl}Cd4-Cre* T_{FH} cells and T_{H1} cells (Fig. 5f). Accordingly, ectopic expression of Mettl3-WT could largely rectify defective PD-1^{hi}CXCR5⁺ GC T_{FH} cells in METTL3-deficient cells (Fig. 5g, h). We also observed that the expression of T_{FH} cell-related genes, including *Cxcr5*, *Tcf7*, *Bcl6*, *Pdcd1*, and *Icos*, were restored in *Mettl3^{fl/fl}Cd4-Cre* cells with inducing Mettl3-WT expression (Fig. 5i). The data collectively indicated that the m⁶A-catalytic activity of METTL3 is necessary for T_{FH} differentiation.

m⁶A modifies *Tcf7* mRNA in 3' UTR to control its stability. To examine how m⁶A methylation modulates T_{FH} transcriptional program, we performed m⁶A-miCLIP-SMARTer-seq to map global m⁶A landscape in Ctrl or *Mettl3^{fl/fl}Cd4-Cre* SMARTA CD4⁺ T cells primed in vivo. Bioinformatic analysis revealed that m⁶A peaks were significantly abundant in 3' UTR and near the stop codon of mRNAs (Fig. 6a, b). Approximately 45.3% methylated mRNAs contained three or more peaks (Fig. 6c). Biological duplicates of m⁶A-miCLIP-SMARTer-seq yielded about 4939 transcripts (called 'm⁶A-modified transcripts' hereafter; Fig. 6d). By stratifying m⁶A-modified transcripts with differentially expressed genes, we found that 208 differentially expressed transcripts were potentially regulated by m⁶A methylation (Fig. 6d). GO term analysis showed these transcripts were enriched for functions related to defense response to the virus, T-cell activation and differentiation (Fig. 6e). Among these potential m⁶A methylated target genes, a set of T_{FH} cell-relevant genes were directly marked by m⁶A, such as *Cxcr5*, *Icos*, *Il6ra*, and *Il6st* (Supplementary Fig. 6a). Interestingly, the 3' UTR of *Tcf7* mRNA had highly enriched m⁶A peaks, whereas *Bcl6* and *Prdm1* mRNAs were not tagged by m⁶A (Fig. 6f and Supplementary Fig. 6a). Accordingly, RNA immunoprecipitation (RIP) assay suggested METTL3 directly binds to *Tcf7* mRNA (Fig. 6g). The m⁶A-RIP-qPCR results further indicated *Tcf7* mRNA was tagged by m⁶A methylation and the m⁶A levels on *Tcf7* mRNA were substantially decreased in METTL3-deficient cells (Fig. 6f, h). These data jointly indicated *Tcf7* is a bona fide m⁶A target. Accordingly, both the *Tcf7* mRNA and the TCF-1 protein were significantly decreased in METTL3-deficient T_{FH} cells compared with those in wild-type T_{FH} cells (Fig. 4e and Fig. 6i). To further validate METTL3 regulation of *Tcf7* expression in an m⁶A-dependent manner, we identified a high-confidence m⁶A site (GGA₁₀₁₁CT, a conserved m⁶A methylation motif) in the *Tcf7* 3' UTR region (Fig. 6f). We generated a minigene vector placing the *Tcf7* m⁶A site to the 3' end of luciferase reporter cDNA. Then the 'GGACT' consensus motif in the *Tcf7* m⁶A site was mutated into 'GGTCT' to abrogate m⁶A modification (Fig. 6j). Wild-type *Tcf7* 3' UTR exhibited increased luciferase activity compared to that of the empty vector (pGL4.23) in the presence of overexpressed METTL3; notably, mutation of *Tcf7* 3' UTR almost completely compromised the increase (Fig. 6k). These results revealed that METTL3 regulates *Tcf7* mRNA levels via m⁶A modifications in the 3' UTR region (m⁶A₁₀₁₁). Given m⁶A modification regulates gene expression by multiple approaches, including mRNA splicing, stability, and translation^{17,38}, alternative splicing assay was applied and exhibited no significant difference in the alternative splicing of *Tcf7* mRNA between Ctrl and METTL3-deficient T_{FH} cells (Supplementary Fig. 6b). We then performed RNA decay



assay via actinomycin D (ActD) treatment to detect the stability of *Tcf7* mRNA, and found that *Tcf7* mRNA exhibited substantially accelerated decline in METTL3-deficient cells compared with Ctrl cells at checkpoints after treatment (Fig. 6l, m). Taken together, these data suggested that METTL3 enhances *Tcf7* mRNA stability via catalyzing m⁶A methylation at its 3' UTR, to ensure TCF-1 expression in promoting T_{FH} differentiation.

Forced expression of TCF-1 restores defective T_{FH} differentiation in METTL3-deficient mice. To determine the functional link between METTL3 and *Tcf7* transcript stability in T_{FH} differentiation, we next examined the impact of ectopic expression of TCF-1 in METTL3-deficient cells. Upon transducing in vivo primed SMARTA CD4⁺ T cells with TCF-1 (full-length CDS of P45 isoform without 3' untranslated region³⁹) retrovirus

Fig. 6 m^6A modifies *Tcf7* mRNA to control its stability. **a** Metagene profiles of m^6A site distribution along a normalized transcript containing three rescaled non-overlapping segments: 5' UTR, CDS, and 3' UTR in Ctrl and *Mettl3^{fl/fl}*Cd4-Cre SMARTA CD4⁺ T cells. **b** Pie chart showing the distribution of m^6A sites in five regions of Ctrl and *Mettl3^{fl/fl}*Cd4-Cre SMARTA CD4⁺ T cells. **c** Bar chart depicting percentage of mRNAs with different internal m^6A abundance. **d** Venn diagram showing the overlapping differentially expressed genes from RNA-seq and m^6A -modified transcripts from m^6A -miCLIP-SMARTer-seq. Heatmap of 208 differentially expressed genes with m^6A modification is shown on the right. **e** Representative GO terms of the biological process categories enriched in differentially expressed transcripts with m^6A peaks (Red: upregulated genes; blue: downregulated genes; gray: GO terms). **f** Integrative Genomics Viewer (IGV) tracks displaying RNA-seq (top panel) and m^6A -miCLIP-SMARTer-seq (bottom panel) reads distribution of *Tcf7* gene. The high-confidence m^6A site is marked as a triangle. **g** RIP-qPCR analysis showing enrichment of METTL3 on *Tcf7* mRNA in SMARTA CD4⁺ T cells. The *Tcf7* mRNA enrichment is presented as IP/input and normalized to IgG group ($n = 4$ per group). **h** m^6A -RIP-qPCR analysis of m^6A enrichment on *Tcf7* mRNA of Ctrl and *Mettl3^{fl/fl}*Cd4-Cre SMARTA CD4⁺ T cells ($n = 4$ per group). **i** Flow cytometry analysis of expression level of TCF-1 on T_{FH} cells on 8 dpi. Quantification of gMFI of TCF-1 is shown on the right ($n = 5$ per group). **j** Constructions of plasmids with wild-type or m^6A site mutant in *Tcf7* 3' UTR. **k** Luciferase reporter assay. Results were normalized to the luciferase activity of cells co-transfected with the pGL4.23 empty plasmid and EV-Myc plasmid ($n = 6$ per group). **l** RNA decay assay. The half-life of *Tcf7* mRNA was detected by quantitative RT-PCR. The remaining mRNAs were normalized to $t = 0$ ($n = 4$ per group). **m** Quantitative RT-PCR analysis of *Tcf7* mRNA abundance as in **l**, relative expression was normalized to $t = 0$ of Ctrl cells ($n = 4$ per group). Data are from one experiment with duplicate (**a–e**) or representative of at least three independent experiments (**i–h, k–m**). Error bars indicate standard error of the mean. P value was calculated by unpaired two-tailed Student's t test (**g–i**) or two-way ANOVA coupled with multiple comparisons (**k, m**).

(Fig. 7a), on day 8 post LCMV-Armstrong infection, we observed a significant increase in TCF-1 expression in METTL3-deficient SMARTA cells (Fig. 7b). We then analyzed T_{FH} populations from transduced SMARTA CD4⁺ T cells in recipient mice. Compared with Ctrl SMARTA CD4⁺ T cells infected with EV retrovirus, the EV-infected *Mettl3^{fl/fl}*Cd4-Cre SMARTA CD4⁺ T cells exhibited defects in CD44⁺CXCR5⁺ T_{FH} differentiation; whereas TCF-1 retrovirus could largely rectify the ability of *Mettl3^{fl/fl}*Cd4-Cre SMARTA CD4⁺ T cells to differentiate into T_{FH} cells (Fig. 7c). In addition, TCF-1 overexpression also elevated the cell numbers of *Mettl3^{fl/fl}*Cd4-Cre T_{FH} cells, but not T_{H1} cells (Fig. 7d). Consistently, PD-1^{hi}CXCR5⁺ GC T_{FH} cells could also be rescued with TCF-1 overexpression (Fig. 7e, f). Meanwhile, forced expression of TCF-1 could largely restore the expression levels of TCF-1, CXCR5, PD-1, ICOS, and Bcl-6 on *Mettl3^{fl/fl}*Cd4-Cre T_{FH} cells (Fig. 7g, h). These data collectively demonstrated that METTL3 stabilizes *Tcf7* mRNA expression to promote T_{FH} differentiation.

Discussion

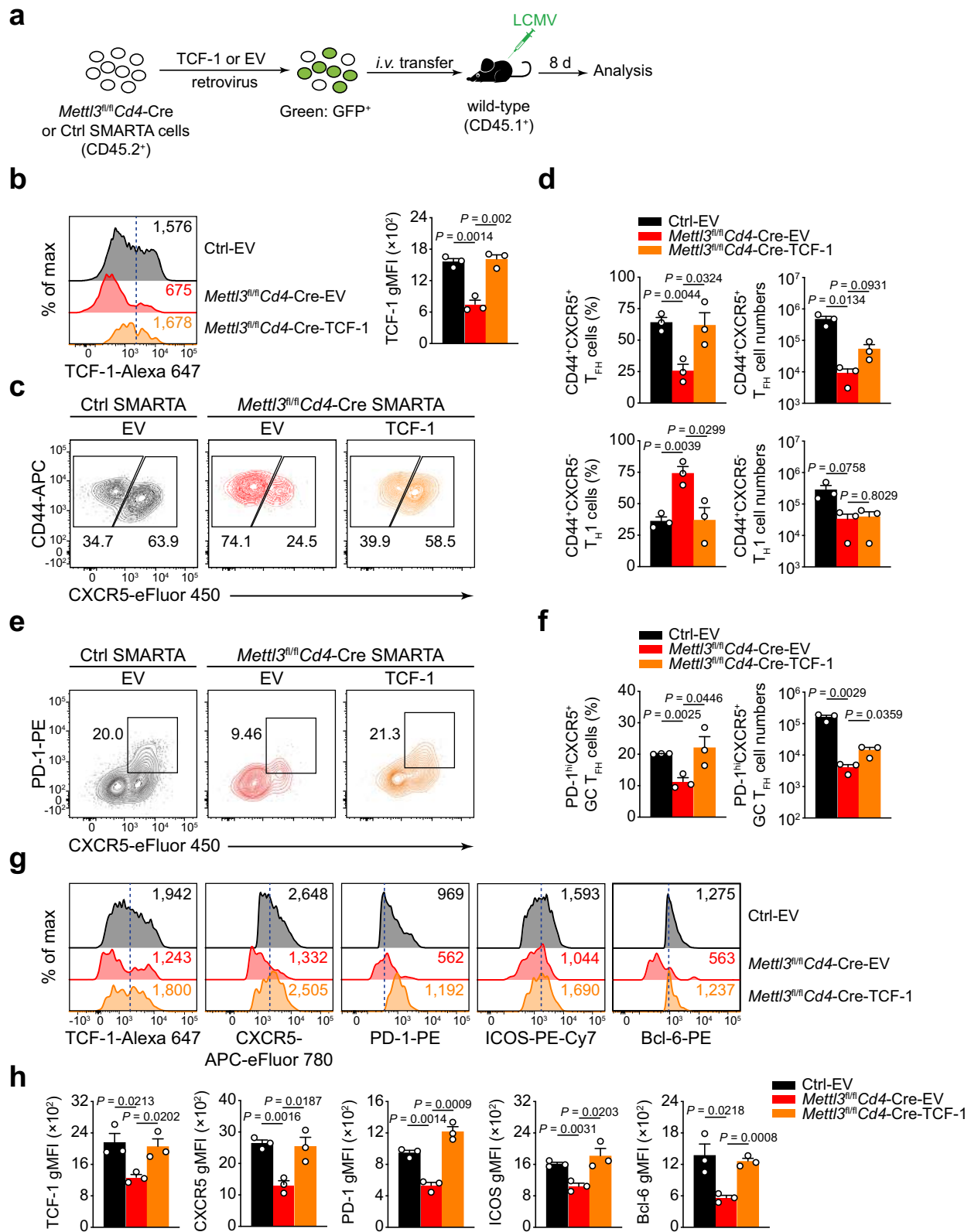
N^6 -methyladenosine (m^6A) accounts for the prevalent mRNA modifications and has recently emerged as a critical epitranscriptomic regulator to affect the translation and stability of the modified transcripts. Here, we showed that ablation of METTL3 leads to substantial defects in both T_{FH} and T_{H1} differentiation upon LCMV-Armstrong and KLH challenges. Based on our results, a more severe phenotype was exhibited in T_{FH} cells than that of T_{H1} cells from METTL3-deficient mice, which strongly supports the notion that the METTL3 acts as an intrinsic modulator of T_{FH} cells.

During acute viral infection, bifurcation of effector CD4⁺ T cells into T_{FH} cells or T_{H1} cells can be observed as early as the second to third division⁴⁰. A recent report demonstrated that METTL3-null CD4⁺ T cells remained naive state and exhibited defective proliferation when tested both in vivo and in vitro²⁵. Using an adoptive transfer model, we found that METTL3-deficient CD4⁺ T cells showed a delayed differentiation and a slower proliferation upon acute viral infection, although they were activated normally as shown by expression of the surface markers via both in vivo and in vitro assay. Meanwhile, at 72 h post infection, the activated CD4⁺ T cells were mostly in the fifth and sixth divisions, whereas METTL3-deficient CD4⁺ T cells were dominant in third and fourth divisions. These METTL3-deficient CD4⁺ T cells also showed defects in differentiation into T_{FH} cells and exhibited elevated apoptosis. Further, METTL3

deficiency also resulted in a decreased expression of T_{FH} -defining genes in these nascent T_{FH} cells. Strikingly, the abundances of *Tcf7* and *Bcl6* mRNAs, both essential for the early commitment of T_{FH} cells⁴¹, were profoundly decreased in early METTL3-null T_{FH} cells. Correspondingly, the protein levels of TCF-1 and Bcl-6 were also notably reduced. These findings revealed that METTL3 is required for early T_{FH} commitment, proliferation, and survival by maintaining the key T_{FH} gene expression.

As an RNA binding protein, METTL3 is the essential catalytic component of the conserved heterodimeric m^6A writer complex¹⁵. Does METTL3-mediated T_{FH} differentiation also directly depend on its m^6A catalytic activity? Our results showed that forced expression of METTL3 with a mutated catalytic domain failed to rectify the defects in T_{FH} differentiation of *Mettl3^{fl/fl}*Cd4-Cre CD4⁺ T cells, while wild-type METTL3 did. We thus concluded that METTL3 promotes T_{FH} generation in an m^6A catalytic activity-dependent manner. Giving m^6A methylation is essential for gene expression regulation⁴², we hence focused on the methylation status of those differentially expressed genes. The expression patterns of a series of T_{FH} -defining genes were altered at both early and mature stages, including *Tcf7*, *Cxcr5*, *Bcl6*, *Pdcd1*, and *Icos*. Through m^6A -miCLIP-SMARTer-seq, we found *Tcf7* mRNA was tagged by m^6A in the 3' UTR, whereas the other key T_{FH} cell regulator *Bcl6* mRNA was not modified. Further study indicated that METTL3-mediated m^6A modification regulates the stability of *Tcf7* mRNA, ultimately maintains TCF-1 level. Moreover, forced expression of TCF-1 could restore the T_{FH} differentiation in the absence of METTL3. These data collectively suggested that METTL3 directly modulates the *Tcf7* expression level in an m^6A -dependent manner to promote T_{FH} differentiation.

The functional outcome of m^6A modification is determined by an expanding list of m^6A reader proteins in a cell type and cellular context-dependent fashion^{14,43}. Although early evidence demonstrated that m^6A deposition destabilizes mRNAs, resulting in their faster decay, a recent study has reported that the insulin-like growth factor 2 mRNA binding proteins (IGF2BP1-3), as a class of distinct m^6A readers, promote m^6A -modified mRNA stability and translation²⁰. Similarly, a more recent study reported Prrc2a as a novel m^6A reader that regulates oligodendrocyte specification and myelination by stabilizing target mRNA⁴⁴. Our data also indicated m^6A modification in the 3' UTR of *Tcf7* enhances its mRNA stability to promote T_{FH} differentiation. However, the currently known IGF2BPs family readers are expressed at extremely low levels to detect in CD4⁺ T cells (Supplementary Fig. 8), implying the possibility of other unidentified proteins might be responsible



for deciphering the m⁶A-modified transcripts in T_{FH} cells. Therefore, it will be of great interest to identify new RNA-binding proteins virtually involved in T_{FH} cells via post-transcriptional networks in future studies.

In METTL3-deficient cells, destabilization of *Tcf7* mRNA impacted T_{FH} cells at least in two major aspects. The first aspect is

that loss of TCF-1 expression results in massive apoptosis in T cell⁴⁵, which is coordinated with the decreased T_{FH} cell numbers in the absence of METTL3. The other point lies in that TCF-1 directly regulates *Bcl6* mRNA expression^{11–13}, and two of them thus collectively affect T_{FH} differentiation. This post-transcriptional regulation of *Tcf7* mRNA represents a distinct

Fig. 7 Enhanced TCF-1 expression rectifies defective T_{FH} differentiation in METTL3-null cell. **a** Scheme of TCF-1 rescue experiment. SMARTA cells were transduced with TCF-1 structure (full-length CDS of P45 isoform without 3' UTR region) by using a retrovirus transduction system. Then, the transduced cells were adoptively transferred into congenic CD45.1⁺ wild-type mice followed by LCMV-Armstrong infection, and analyzed on day 8 post viral infection. **b** Flow cytometry analysis of TCF-1 gMFI in SMARTA GFP⁺CD4⁺ cells from recipient mice on 8 dpi. Quantitation of TCF-1 gMFI is shown on the right ($n = 3$ per group). **c, d** Flow cytometry analysis of CD44⁺CXCR5⁺ T_{FH} populations and CD44⁺CXCR5⁻ T_{H1} subsets gated on SMARTA GFP⁺CD4⁺ T cells from different host mice adoptively transferred with empty vector (EV) or TCF-1 retrovirus-introduced SMARTA cells at 8 days post infection. Summary of the frequency and cell numbers of T_{FH} cells and T_{H1} cells are shown in **d** ($n = 3$ per group). **e, f** Flow cytometry analysis of PD-1^{hi}CXCR5⁺ GC T_{FH} populations gated on SMARTA GFP⁺CD4⁺ T cells from different host mice adoptively transferred with EV or TCF-1 retrovirus-introduced SMARTA cells. Summary of the frequency and cell numbers of T GC T_{FH} cells are shown in **f** ($n = 3$ per group). **g, h** Flow cytometry analysis of gMFIs of TCF-1, CXCR5, PD-1, ICOS, and Bcl-6 on CD44⁺CXCR5⁺ T_{FH} cells transduced with EV or TCF-1 retrovirus. Quantification of the gMFIs is shown in **h** ($n = 3$ per group). Data are representative of three independent experiments. Error bars indicate standard error of the mean. *P* value was calculated by one-way ANOVA, followed by unpaired two-tailed Student's *t* test for indicated pairwise comparisons.

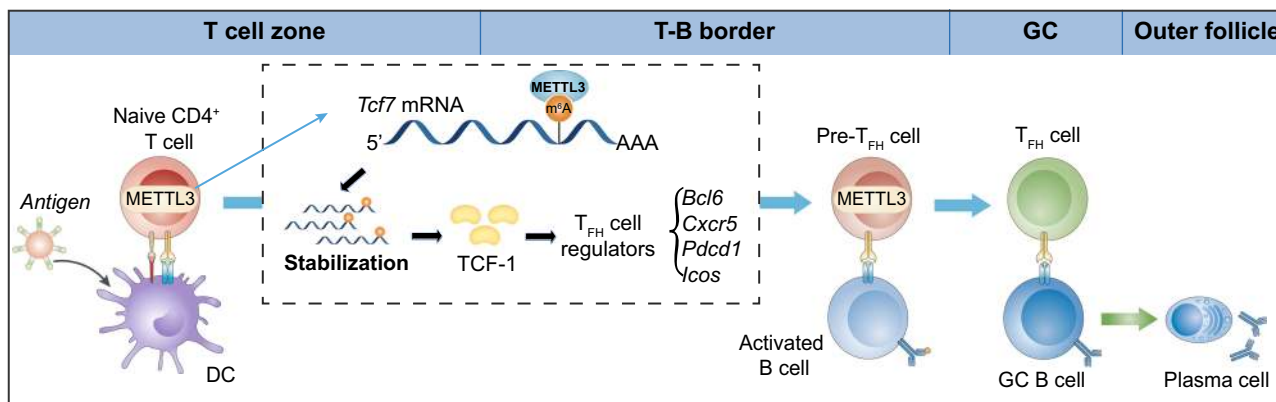


Fig. 8 Proposed model for m⁶A modification in promoting T_{FH} differentiation. During acute infection, METTL3-sufficient CD4⁺ T cells were activated. With m⁶A machinery, *Tcf7* mRNA was m⁶A modified and stabilized, allowing normal production of TCF-1 protein. TCF-1 in turn regulates expressions of T_{FH} cell regulators, which ultimately program T_{FH} commitment, proliferation, survival, and functional maturation.

mechanism that drives T_{FH} differentiation. It should be noted that other post-transcriptional regulators such as RNA-binding proteins and miRNAs also contribute to modulating T_{FH} differentiation program^{46–48}. Hence, exploring T_{FH} fate determination on the layer of post-transcriptional level might be a fruitful effort in future investigations.

A recent study referred that induced GAPDH protein by VHL deficiency reduced *Icos* expression through METTL3/METTL14-catalyzed m⁶A modification on *Icos* mRNA, implying that elevated m⁶A modification on *Icos* mRNA in VHL-deficient cells decreases *Icos* expression which is associated with attenuated T_{FH} differentiation⁴⁹. By analyzing high-throughput data, we also observed m⁶A modification in the 3' UTR of *Icos* mRNA, and the m⁶A level on *Icos* mRNA was decreased in the absence of METTL3. However, we found both the mRNA and protein level of ICOS were blunted in METTL3-deficient T_{FH} cells, indicating that loss of m⁶A modification impairs *Icos* expression. In addition, Zhu et al. reported that knockdown of METTL3 expression with short hairpin RNA (shRNA) in CD4⁺ T cells could promote T_{FH} differentiation⁴⁹, which differs in phenotypes from our genetic knockdown mice model. The varies may be contributed by the distinct experimental approaches and the divergent viewpoints from two studies also reflect the complex regulatory mechanism of m⁶A modification, which needs to be further disclosed.

In summary, our study uncovers a critical role of METTL3-dependent m⁶A methylation in directing T_{FH} lineage differentiation. Conditional ablation of m⁶A 'writer' METTL3 in CD4⁺ T cells intrinsically impaired the T_{FH} differentiation, proliferation, and survival. Consequently, the GC reactions were significantly compromised in METTL3-deficient mice in response to acute viral infection. Our data indicated that METTL3 directs

m⁶A modification in 3' UTR of *Tcf7* mRNA to stabilize the transcript and hence sustain TCF-1 protein expression (Fig. 8). Thus, m⁶A functions as an important modulator of the METTL3-TCF-1 axis to initiate and secure the differentiation of T_{FH} cells post-transcriptionally.

Methods

Mice. *Mettl3*^{fl/fl} mice were kindly provided by Drs. Qi Zhou and Wei Li (Institute of Zoology, Chinese Academy of Sciences). SMARTA mice⁵⁰ (expressing MHC II I-A^b-restricted TCR specific for LCMV glycoprotein amino acids 66–77) were generously provided by Dr. Rafi Ahmed (Emory University). *Cd4*-Cre, ER^{T2}-Cre, and C57BL/6J (CD45.2 and CD45.1) mice were purchased from the Jackson Laboratory. All mouse strains used in this study are on a fully C57BL/6J background. All mice were kept in group housing (3–5 mice per cage) in a specific pathogen-free facility with controlled environmental conditions of humidity (50 ± 10%), lighting (a 12-h light/dark cycle), and temperature (21 ± 1 °C) at China Agricultural University. All animal experiments were performed in accordance with the protocol of the Institutional Animal Care and Use Committee of China Agricultural University.

LCMV infection. LCMV-Armstrong strain was grown in BHK-21 cells and titers were determined as described before⁵¹. *Mettl3*^{fl/fl}*Cd4*-Cre mice and their wild-type littermates were intraperitoneally infected with 2 × 10⁵ plaque-forming units (pfu) LCMV-Armstrong strain. In adoptive transfer experiments, recipient mice were infected 1 day after cell transfer. For bone marrow chimeric mice, LCMV infection was performed after 8 weeks reconstitution.

Immunization. *Mettl3*^{fl/fl}*Cd4*-Cre mice and their wild-type littermates were intraperitoneally immunized with 100 µg of KLH (Sigma-Aldrich) emulsified in CFA (Sigma-Aldrich). Eight days later, splenic T cells were analyzed.

Flow cytometry and antibodies. Single-cell suspensions of spleens were used for flow cytometry analysis or cell sorting. Surface staining was performed in PBS containing 1% FBS. The antibodies and reagents used for flow cytometry staining are listed as: anti-CD19 (1D3; 1:100), anti-CD25 (PC61.5; 1:100), anti-CD4 (RM4-5; 1:100), anti-CD44 (IM7; 1:100), anti-CD45.1 (A20; 1:100), anti-CD45.2

(104; 1:100), anti-CD62L (MEL-14; 1:100), anti-CD69 (H1.2F3; 1:100), anti-CD8a (53-6.7; 1:100), anti-B220 (RA3-6B2; 1:100), anti-GITR (DTA-1; 1:100), anti-GL7 (GL7; 1:100), anti-PD-1 (J43; 1:100), anti-TCR Vα2 (B20.1; 1:100) (from Thermo Fisher Scientific); anti-CD138 (281-2; 1:100), anti-Fas (Jo2; 1:100) (from BD Biosciences); anti-SLAM (TC15-12F12.2; 1:100), anti-ICOS (C398.4A; 1:100) (from BioLegend), and peanut agglutinin (PNA; Cat. no. FL-1071; 1:500; Vector laboratories). CXCR5 staining was performed with a three steps staining protocol as described before⁵². Briefly, single-cell suspensions were first stained with purified anti-CXCR5 (2G8; 1:100; BD Biosciences) for 1 h, followed by biotin-conjugated goat anti-rat IgG (Cat. no. 111-066-144; 1:1,000; Jackson ImmunoResearch) for 30 min, and then by APC-eFluor 780-, or eFluor 450-labeled streptavidin (1:500; Thermo Fisher Scientific) at 4 °C for 30 min in PBS supplemented with 2% normal mouse serum (Cat. no. 015-000-120; Jackson ImmunoResearch), 2% FCS, and 0.5% BSA. For detection of cytokines, the splenocytes from KLH-immunized mice were cultured *in vitro* for 5 h in 2 μg/mL of PMA (Cat. no. P8139; Sigma-Aldrich), 2 μg/mL of Ionomycin (Cat. no. I0634; Sigma-Aldrich), GolgiStop (BD Biosciences), and GolgiPlug (BD Biosciences). Intracellular staining for cytokines was performed with monoclonal antibody against to IL-4 (11B11; 1:100; Thermo Fisher Scientific) and IL-17a (TC11-18H10; 1:100; BD Biosciences), using the Fixation/Permeabilization buffer kit (BD Biosciences). For intracellular staining of Bcl-6 (K112-91; 1:20; BD Biosciences), Foxp3 (FJK-16s; 1:100; Thermo Fisher Scientific), GATA3 (TWAJ; 1:100; Thermo Fisher Scientific), RORγt (AFKJS-9; 1:100; Thermo Fisher Scientific), TCF-1 (C63D9; 1:100; Cell Signaling Technology), and METTL3 (Cat. no. ab195352; 1:100; Abcam), Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) was used following the manufacturer's instructions. Active Caspase-3 was detected using the CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Cat. no. 88-7004-42; 1:200; Thermo Fisher Scientific). All data were collected on a FACVerse (BD Biosciences) with FACSuite software (v1.0.5) or an LSRFortessa (BD Biosciences) with FACSDiva software (v8.0.2) and were analyzed with FlowJo software (v10; Treestar). The gating strategies for flow cytometry data analysis are illustrated in Supplementary Fig. 7.

Adoptive transfer. To characterization of cell division at early T_{FH} differentiation, SMARTA CD4⁺ T cells were labeled with 5 μM of CTV (Invitrogen), and 2 × 10⁶ of labeled Vα2⁺ SMARTA CD4⁺ cells were transferred followed by intravenously infected with 2 × 10⁶ pfu of LCMV-Armstrong. To investigate the intrinsic effects with adoptive transfer model, 5 × 10⁶ wild-type SMARTA cells were transferred into Ctrl and *Mettl3*^{fl/fl}*Cd4*-Cre host mice, followed by 2 × 10⁵ pfu LCMV-Armstrong infection intraperitoneally.

Bone marrow chimeric mice. To generate bone marrow chimeric mice, lethally irradiated B6.SJL (CD45.1⁺) mice were transferred intravenously with a 1:1 mixture of 2.5 × 10⁶ *Mettl3*^{fl/fl}*Cd4*-Cre (CD45.2⁺) and 2.5 × 10⁶ B6.SJL (CD45.1⁺) bone marrow cells. After 8 weeks reconstitution, recipient mice were infected with LCMV-Armstrong strain.

ELISA. Analysis of the LCMV-specific antibody in serum was performed as previously described⁵³. Briefly, lysates of LCMV-infected BHK-21 cells were used as substrate and LCMV-specific antibody (IgG) was titrated in serial dilutions of serum using HPR-conjugated goat-anti mouse IgG antibodies (Cat. no. A90-131P-39; 1:5,000; Bethyl laboratories).

Immunofluorescence staining. Tissue specimens submerged in OCT compound were quickly frozen in liquid nitrogen and cut into 10 μm thickness. Frozen tissue sections were then fixed in cold acetone for 30 min at -20 °C, blocked with 1% BSA and Fc-blocker (2.4G2; 1:100; BD Biosciences) in PBS. The tissue sections were then stained with biotinylated PNA (Cat. no. BA-0074; 1:20; Vector laboratories), followed by staining with BV510-labeled anti-CD4 (RM4-5; 1:100; BD Biosciences), APC-labeled anti-IgD (11-26c; 1:100; Thermo Fisher Scientific), and AF488-conjugated streptavidin (1:500; Invitrogen). Then the slides were washed at least three times with PBS. Coverslips were mounted on slides using an antifade kit (Beyotime Biotechnology) and then examined using an Andor Dragonfly confocal microscope. The images were processed with Imaris (v8.1; Bitplane) and Image J (v1.52 g; NIH).

Retroviral vectors, transduction, and cell transfer. *Tcf7* (full-length CDS region of P45 isoform without 3' UTR region³⁹) and *Mettl3* (wild-type and catalytic domain dead) coding sequences were amplified and cloned into the pMIG-R1 vector (MSCV-IRES-GFP). Retrovirus was packaged by transfection of HEK293T cells (Cat. no. CRL-3216; ATCC) with the retroviral vectors along with pCL^{eco} plasmid. SMARTA cells were activated *in vivo* by injection of 200 μg of LCMV GP61-80 (GLNGPDIYKGVYQKSFVEFD) peptide into *Mettl3*^{fl/fl}*Cd4*-Cre SMARTA mice or their littermate wild-type transgenic mice. After 16~18 h, activated SMARTA cells were isolated, purified, and 'spin-infected' for 120 min at 37 °C by centrifugation (1000 × g) with freshly harvested retroviral supernatants supplemented with 8 μg/mL of polybrene (Sigma-Aldrich), then cultured overnight in the presence of 20 ng/mL of IL-2 (Peprotech), and 250 nM of LCMV GP61-80. The spinofection was repeated the next day, and a total of 0.5~1 × 10⁶ retroviral infected SMARTA CD4⁺ T cells were then adoptively transferred into recipient

mice, followed by infection of the hosts with 2 × 10⁵ pfu LCMV-Armstrong within 24 h.

RNA-seq and data analysis. For isolation of T_{H1} cells and T_{FH} cells, splenocytes from *Mettl3*^{fl/fl}*Cd4*-Cre mice and their control littermates on day 8 post viral infection were subjected to depletion of cells positive for lineage markers by using biotin-conjugated antibodies (anti-B220 (RA3-6B2; 1:100), anti-CD8 (53-6.7; 1:100), anti-Gr.1 (RB6-8C5; 1:100), anti-CD11b (M1/70; 1:100), anti-CD11c (N418; 1:100), anti-TER119 (TER-119; 1:100), and anti-CD49b (DX5; 1:100); all from Thermo Fisher Scientific) coupled to Dynabeads M-280 Streptavidin (Invitrogen), followed by surface stained. CD44⁺SLAMF^{hi} T_{H1} cells and CD44⁺SLAMF^{lo} T_{FH} cells were sorted with a FACSAria II cell sorter (BD Biosciences) with FACSDiva software (v7.0) and subsequently lysed with TRIzol Reagent (Life technologies). Total RNAs were extracted and then subjected to Annoroad (Beijing, China) for library construction and RNA sequencing. The qualities of clean reads were assessed by FastQC (v0.11.5). Then the reads were mapped to mouse genome mm10 (version M17) using TopHat (v2.1.1). The read counts of all genes were estimated by HTseq (v0.6.1) and differentially expressed genes were identified by DESeq2 (v1.18.1). TPM, FPKM, and RPKM were calculated, and upregulated or downregulated genes in *Mettl3*^{fl/fl}*Cd4*-Cre T_{FH} or T_{H1} cells were identified by expression changes ≥ 2-fold and FDR < 0.01.

Quantitative RT-PCR. Total RNAs were extracted from sorted cells using RNeasy Mini Kit (Qiagen) followed by cDNA synthesis with FastQuant RT Kit (Tiangen). Quantitative RT-PCR was carried out with SuperReal PreMix Plus SYBR Green (Tiangen) on a CFX96 Connect™ Real-Time System (Bio-Rad). Results were processed by Microsoft Excel and then normalized to the expression of *Hprt1* transcripts. Fold differences in expression levels were calculated according to the 2^{-ΔΔCT} method. All primers used are listed in Supplementary Table 1.

Gene set enrichment analysis. GSEA was performed with GSEA desktop software (v4.1.0) from the Broad Institute. The T_{FH} gene set¹¹, GC T_{FH} gene set¹¹, T_{H1} gene set¹², T_{H2} gene set³⁴, T_{H17} gene set³⁵, and Treg gene set³⁴ have been described before. The 'TCF1-activated genes in T_{FH} cells' gene set contains 569 genes that are downregulated by ≥1.5-fold in *Tcf7*^{fl/fl}*Cd4*-Cre T_{FH} cells; The 'TCF1-suppressed genes in T_{FH} cells' gene set contains 513 genes that are upregulated by ≥1.5-fold in *Tcf7*^{fl/fl}*Cd4*-Cre T_{FH} cells (GSE65693).

Luciferase reporter assay. *Tcf7* 3' UTR segment (~200 nt) was amplified from a mouse double-positive (DP) thymocytes cDNA library by PCR and inserted into the pGL4.23 vector (Promega) by using XbaI and FseI restriction sites. The *Tcf7* 3' UTR mutation plasmids were generated from site-directed mutagenesis. All plasmids and mutations were verified by sequencing. HEK293T cells were seeded into 24-well plates in triplicate to allow 80% confluency in the next day. A total of 200 ng of reporter plasmids (Fluc) and 20 ng of Renilla luciferase (Rluc) control plasmids (pRL-TK) were co-transfected using Lipofectamine 2000 reagent (Invitrogen) under METTL3 overexpressing. Fluc and Rluc activities were measured 24 h later with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The relative luciferase activity was calculated by dividing Fluc by Rluc and normalized to pGL4.23 empty vector for each assay.

RNA decay assay. CD4⁺ T cells were purified from *Mettl3*^{fl/fl}ER^{T2}-Cre mice and their control wild-type mice as described above. 5 × 10⁵ purified CD4⁺ T cells in RPMI 1640 medium supplemented with 10% fetal bovine serum, 20 ng/mL of IL-2, 10 ng/mL of IL-7, and 5 μM of 4-Hydroxytamoxifen (Sigma-Aldrich) were seeded into 48-well plates. After 48 h, actinomycin D (MedChemExpress) was added to a final concentration of 5 μM, and cells were harvested at *t* = 0, 1, 2 h after actinomycin D treatment. Total RNAs were extracted and subjected to RT-qPCR analysis. Results were processed by Microsoft Excel and then normalized to the expression of *Gapdh* transcript. Fold differences in expression levels were calculated according to the 2^{-ΔΔCT} method. All primers used are listed in Supplementary Table 1.

m⁶A-miCLIP-SMARTer-seq and data processing. Total RNAs from CD4⁺ SMARTA cells sorted from LCMV GP61-80-primed *Mettl3*^{fl/fl}*Cd4*-Cre SMARTA or Ctrl SMARTA mice were extracted with TRIzol Reagent (Life Technologies). mRNAs were further isolated from total RNAs using Dynabeads mRNA purification kit (Ambion). The procedures of m⁶A-miCLIP-SMARTer-seq were according to the previously reported methods with some modifications⁵⁴. Briefly, 100 ng of mRNAs were fragmented to ~100 nt by using the fragmentation reagent (Life Technologies) and incubated with 5 μg of specific antibody against m⁶A (Abcam) in 500 μL of immunoprecipitation buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.05% NP-40) with gentle rotation at 4 °C for 2 h. The mixture was then transferred into a clear flat-bottom 96-well plate (Corning) on ice and irradiated three times with 0.15 J/cm² at 254 nm in a CL-1000 Ultraviolet Crosslinker (UVP). The irradiated mixture was then transferred to a new tube and incubated with 50 μL of pre-washed Dynabeads Protein A (Life Technologies) at 4 °C for 2 h. After extensive washing twice with high-salt wash buffer (50 mM Tris-HCl

(pH 7.4), 1 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS) and twice with immunoprecipitation buffer, the mixture on beads was subjected to dephosphorylation with T4 PNK (NEB) for 20 min at 37 °C. After extensive washing, the RNA was eluted from the beads by proteinase K (Sigma-Aldrich) digestion at 55 °C for 1 h, followed by phenol-chloroform extraction and ethanol precipitation. The purified RNA was subjected to library construction using a SMARTer smRNA-Seq Kit for Illumina (Clontech Laboratories) according to the manufacturer's instructions. Sequencing was carried out on an Illumina X-ten platform. m⁶A-miCLIP-SMARTer-seq data (paired-end) were analyzed as previously described⁵⁵. Adapter sequences at the 3' ends were removed first. R2 reads were then transformed to reverse complementary sequences and merged with R1 reads. PolyA tails from the library were trimmed by Cutadapt (v1.17) and fastq2collapse (v1.1.3) was used to remove duplicated reads; afterward, the barcodes of reads were removed. Low-quality bases were discarded, and only reads longer than 18 nt were retained. The remaining reads were mapped to the reference genome (mm10) using BWA (v0.7.17) with the parameter: -n 0.06. The mutation information was extracted and PCR duplicates according to BWA's results were removed separately by parseAlignment.pl (-map-qual 1 -min-len 18) and tag2collapse.pl (-EM 30 -seq-error-model alignment). CIMS.pl was used to calculate the coverage (n) of the mutation site and transition number (m). Mutation sites with parameters, $m \geq 3$, $k/m \geq 0.01$, and $k/m \leq 0.5$, were kept. The remaining sites within the RRACH motif sequence were considered as m⁶A.

m⁶A-RIP-qPCR. Purified mRNAs of CD4⁺ SMARTA cells sorted from *Mettl3^{fl/fl}* *Cd4-Cre* SMARTA or Ctrl SMARTA mice were prepared and fragmented into ~100 nt by RNA fragmentation reagents (Life Technologies). Immunoprecipitation was performed using anti-m⁶A antibody (Abcam) as described above. The enrichment of m⁶A was measured with quantitative RT-PCR. Primers for m⁶A-RIP-qPCR are listed in Supplementary Table 1.

RIP-qPCR. 5 × 10⁶ CD4⁺ SMARTA T cells were isolated and lysed with 1 mL cell lysis buffer (150 mM KCl, 10 mM HEPES (pH 7.6), 2 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1:100 proteinase inhibitor cocktail, and 0.4 U/μL RNasin) at 4 °C for 30 min. After centrifugation, the supernatant (10% of which was kept as input) was subjected to RNA immunoprecipitation with anti-METTL3 (Abcam) coupled with Dynabeads Protein A (Life Technologies). RNA was isolated from the beads and input samples for RT-qPCR. Primers for RIP-qPCR are listed in Supplementary Table 1.

Statistical analysis. Statistical analysis was performed with Prism 8.0 (GraphPad). An unpaired two-tailed Student's *t* test with a 95% confidence interval, one-way ANOVA, or two-way ANOVA analysis was used to calculate *P* values.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

RNA-seq and m⁶A-miCLIP-SMARTer-seq datasets have been deposited in Gene Expression Omnibus (GEO) under the accession number GSE129650. All data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions

Y. Yao and W.G. performed the overall experiments with Y. Yang and L.X. M.Y., Z.S., X.C., G.Y., Z.Q., Jingjing L., and T.Z. helped to carry out experiments and interpreted data. Juanjuan L. helped to breed B6.SJL mice. Y. Yang executed m⁶A-miCLIP-SMARTer-seq experiments. M.Y., Y.Z., and F.W. analyzed the RNA-seq and m⁶A-miCLIP-SMARTer-seq data. Y. Yao, W.G., and S.Y. were responsible for analysis of overall data. Y. Yao and S.Y. wrote the manuscript with the revision by all authors. S.Y., L.Y., and Y.-G.Y. conceived experiments and supervised the study.

Competing interests

The authors declare no competing interests.

Additional information

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