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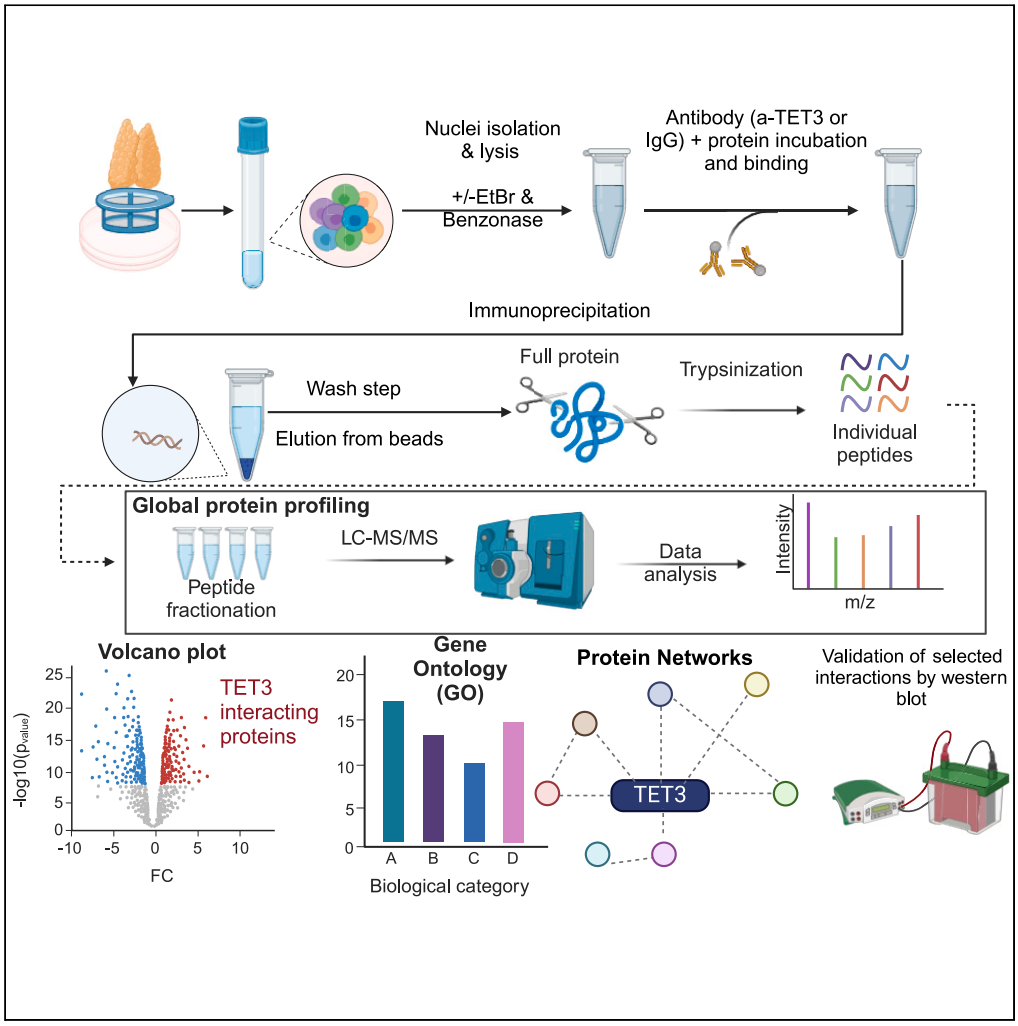

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Highlights

We identify TET3 interacting partners in developing thymocytes

TET3 participates in complexes to regulate polymerase elongation and splicing

TET3 interacts with proteins involved in DNA repair and replication

TET3 participates in networks orchestrated by CTCF, BCL11b, and GATA3

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Deciphering the TET3 interactome in primary thymic developing T cells

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SUMMARY

Ten-eleven translocation (TET) proteins are DNA dioxygenases that mediate active DNA demethylation. TET3 is the most highly expressed TET protein in thymic developing T cells. TET3, either independently or in cooperation with TET1 or TET2, has been implicated in T cell lineage specification by regulating DNA demethylation. However, TET-deficient mice exhibit complex phenotypes, suggesting that TET3 exerts multifaceted roles, potentially by interacting with other proteins. We performed liquid chromatography with tandem mass spectrometry in primary developing T cells to identify TET3 interacting partners in endogenous, *in vivo* conditions. We discover TET3 interacting partners. Our data establish that TET3 participates in a plethora of fundamental biological processes, such as transcriptional regulation, RNA polymerase elongation, splicing, DNA repair, and DNA replication. This resource brings in the spotlight emerging functions of TET3 and sets the stage for systematic studies to dissect the precise mechanistic contributions of TET3 in shaping T cell biology.

INTRODUCTION

Ten-eleven translocation (TET) proteins are a family of three dioxygenases that through their enzymatic activity can catalyze the oxidization of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)[1] as well as downstream oxidized cytosines (oxi-mCs), namely 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).[2,3] Thus, TET proteins can regulate DNA demethylation and ultimately gene expression. In addition, the oxi-mCs can be preferentially recognized by readers and act as distinct, stable epigenetic marks[4,5] that can mediate specific recruitment of transcription factors or proteins involved in DNA repair across the genome.[6–10]

TET proteins have distinct expression patterns across development. Specifically, TET1 is most highly expressed in embryonic stem cells (ESCs) and its expression is progressively diminished as cells differentiate.[11,12] TET2 is expressed across distinct cell types whereas TET3 follows an opposite expression pattern compared to TET1 and is more abundant in differentiated cells such as immune cells and neural cells.[11,13] Interestingly, TET1 and TET3 share a CXXC DNA binding domain at their N-terminal domain whereas TET2 does not have a CXXC DNA binding domain.[14] However, this CXXC DNA binding domain does not confer specificity and thus cannot explain the focal DNA demethylation that we and others have discovered when studying TET2/3-deficient cells such as T cells,[15] including regulatory T cells (Tregs),[16] B cells,[17,18] and hematopoietic stem cells.[19] Thus, it is hypothesized that TET proteins are recruited at specific loci across the genome via interacting partners to oxidize 5mC. We have previously demonstrated that 5hmC is dynamically distributed across the genome of distinct T cell subsets during the process of T cell lineage specification.[20] An additional finding was that 5hmC decorates active enhancers and coincides with histone H3K27Ac[20] during thymic development. H3K27Ac is a bona fide mark of active enhancers.[21,22]

T cell development is a tightly regulated process that takes place in a dedicated organ, the thymus, and aims to ensure that only T cells with optimal response against antigens will exit to peripheral organs where they will execute their immune surveillance functions. During T cell development in the thymus, double-negative (DN) subsets that do not express CD4 or CD8 in their surface will differentiate and proliferate to give rise to double-positive (DP) T cells that express CD4 and CD8 on their surface. Approximately 85% of thymocytes are DP T cells. Most DP cells do not recognize antigens and are eliminated by a process known as death by neglect.[23] DP cells that recognize and react too strongly upon antigen encounter are eliminated by the process of negative selection.[24] Only the DP cells that exhibit optimal antigen recognition will survive and differentiate to give rise to mature T cells by positive selection.[24,25] Specifically, DP cells that recognize peptide antigens presented by major histocompatibility complex (MHC) class I will become selected toward the CD8 lineage that is governed by the

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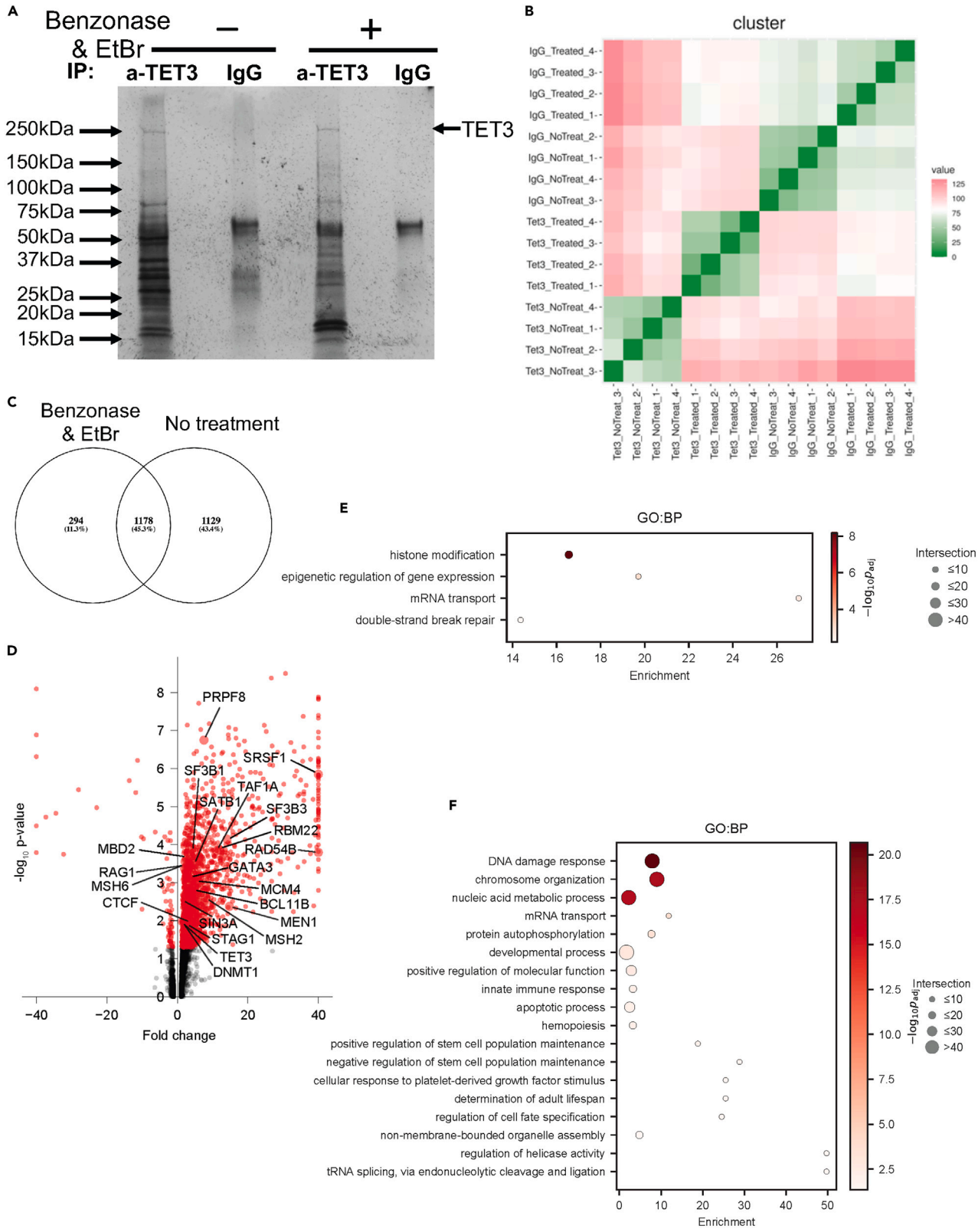

A
Benzonase & EtBr
–
+
IP:
a-TET3
IgG
a-TET3
IgG
250kDa
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
TET3
B
cluster
IgG_Treated_4
IgG_Treated_3
IgG_Treated_2
IgG_Treated_1
IgG_NoTreat_2
IgG_NoTreat_1
IgG_NoTreat_4
IgG_NoTreat_3
Tet3_Treated_4
Tet3_Treated_3
Tet3_Treated_2
Tet3_Treated_1
Tet3_NoTreat_4
Tet3_NoTreat_1
Tet3_NoTreat_2
Tet3_NoTreat_3
value
125
100
75
50
25
0
C
Benzonase & EtBr
No treatment
294
(11.3%)
1178
(45.3%)
1129
(43.4%)
D
-log10 p-value
Fold change
PRPF8
SRSF1
SF3B1
TAF1A
SATB1
SF3B3
RBM22
RAD54B
MBD2
GATA3
RAG1
MSH6
MCM4
CTCF
BCL11B
MEN1
SIN3A
STAG1
MSH2
TET3
DNMT1
E
GO:BP
histone modification
epigenetic regulation of gene expression
mRNA transport
double-strand break repair
Enrichment
−log10padj
Intersection
≤10
≤20
≤30
>40
F
GO:BP
DNA damage response
chromosome organization
nucleic acid metabolic process
mRNA transport
protein autophosphorylation
developmental process
positive regulation of molecular function
innate immune response
apoptotic process
hemopoiesis
positive regulation of stem cell population maintenance
negative regulation of stem cell population maintenance
cellular response to platelet-derived growth factor stimulus
determination of adult lifespan
regulation of cell fate specification
non-membrane-bounded organelle assembly
regulation of helicase activity
tRNA splicing, via endonucleolytic cleavage and ligation
Enrichment
−log10padj
Intersection
≤10
≤20
≤30
>40

Figure 1. Identifying TET3 interacting partners in murine primary thymocytes by immunoprecipitation followed by LC-MS/MS

(A) Single-cell suspensions from wild-type murine thymi were prepared. Nuclei were isolated and were treated with EtBr and benzonase or left untreated. Immunoprecipitation with anti-TET3 or IgG of the nuclei extracts was performed and the extracts were run on a gel that was submitted to silver staining. Note that in the presence of benzonase and EtBr, there is less intensity of the staining compared to the untreated samples, indicating less immunoprecipitated proteins.

(B) Two-dimensional enhanced distance matrix visualization (cluster) was created based on trim-mean normalized protein expression values using Euclidian distance of similarity. The color level is proportional to the value of the dissimilarity between observations: pure green corresponds to zero distance and thus indicates the highest similarity and pure pink corresponds to the highest value of Euclidean distance computed. Objects belonging to the same cluster are displayed in consecutive order. Four biological replicates/condition were evaluated. Four clusters were identified and each of the clusters had four samples.

(C) Venn diagram depicts the interacting partners that were identified as significantly bound to TET3 over the IgG control in EtBr and benzonase-treated samples versus untreated samples. 1,472 proteins were identified to interact with TET3 in the treated samples and 2,307 were identified to interact with TET3 in the untreated samples. 294 proteins were exclusively identified in the treated samples whereas 1,178 proteins were common among the treated and non-treated samples.

(D) Volcano plot depicts the proteins that were identified in the LC-MS/MS analysis in the a-TET3 and IgG samples that were treated with EtBr and benzonase. In red are proteins that were significantly ($p < 0.05$) enriched or depleted in the anti-TET3 sample. Fold change values are clipped at −40 and 40. Selected proteins are highlighted.

(E) Gene ontology (GO) of biological processes (BP) of the 294 proteins that were exclusively identified to interact with TET3 in the presence of benzonase and EtBr. Only significant categories are visualized.

(F) GO of the BP of the 1178 proteins that were identified to interact with TET3 in the presence or absence of benzonase and EtBr. Only significant categories are visualized.

transcription factor Runt domain transcription factor 3 (RUNX3).[26,27] DP cells that recognize peptides presented by MHC class II molecules will be selected toward the CD4 lineage.[26] The transcription factor GATA binding factor 3 (GATA3) is required for CD4 lineage commitment,[28] whereas T helper-inducing POZ-Kruppel factor (ThPOK) is instrumental for the maturation of the lineage. CD4 single-positive (SP) cells in the thymus that react strongly to antigens upregulate the transcription factor forkhead box P3 (FOXP3) and differentiate to the Treg lineage,[29–31] with critical immunosuppressive functions. Finally, those DP T cells that recognize lipid antigens presented by the MHC class I molecule CD1d give rise to a subset known as invariant natural killer T (iNKT) cells.[32]

Notably, *in vivo* studies aiming to decipher the biological implications of TET-regulated gene expression in the context of thymic development revealed redundant roles among all 3 TET proteins. Specifically, concomitant deletion of both TET2 and TET3 was critical for stable expression of the Treg lineage specifying factor FOXP3 and for maintenance of Treg stability.[33] TET2 and TET3 exert instrumental roles in shaping iNKT cell lineage specification and regulating their proliferation.[15] In addition, TET1 and TET3 are important for regulating DNA demethylation of an enhancer that regulates stability of CD4 expression both *in vitro* and *in vivo*.[34,35] Moreover, TET2 and TET3 fine-tune the expression of the transcription factor ThPOK both in CD4 SP and in iNKT cells, by regulating DNA demethylation of the locus and binding of the transcription factor GATA3.[36] While in the aforementioned T cell subsets at least 2 TET proteins must be deleted to observe impactful phenotypes, we have previously reported that deficiency of TET3 alone is sufficient to impact iNKT cell lineage specification in the thymus and promote skewing toward iNKT cells that express RORγt and secrete IL-17, known as NKT17 cells.[15,37,38]

In the present study, we focus on TET3, the most highly expressed of the TET proteins in thymic murine T cell subsets[11,36] and yet the least studied among the TET proteins. We seek to discover the interacting partners that mediate recruitment of TET3 across the genome in developing thymic T cells and to decipher the biological implications of these interactions. We anticipate that this endeavor will reveal functions of TET3 and will enhance our understanding of TET3-mediated regulation of gene expression.

To this end, we performed immunoprecipitation (IP) experiments using nuclear extracts from total thymocytes, followed by quantitative mass spectrometry. Our goal was to identify protein partners of TET3 in a system where TET3 plays important roles and yet its interactome remains elusive. In this resource, we report proteins that interact with TET3 *in vivo*, in primary murine developing thymocytes. We perform bioinformatic analysis to identify key biological functions where TET3 may be involved. Our data reveal that TET3 participates in numerous fundamental biological processes, such as transcriptional regulation, RNA polymerase elongation, splicing, DNA repair, and DNA replication. Next, we discover protein networks that control the identified functions. For selected interactions, we perform further validation by IP followed by western blot. We revisit published work in the field, and we identify links between phenotypes observed in TET-deficient immune cells and TET3, assigning emerging functions of TET3 under the lens of our identified interactions. Finally, we identified shared interacting partners of TET proteins across different cell types and systems establishing broad implications of TET proteins in regulating gene expression in a multifaceted manner.

RESULTS

Experimental strategy to identify TET3 interacting partners in primary developing T cells

To identify TET3 interacting partners in developing primary T cells, we isolated nuclear extracts and we performed IP with anti-TET3 antibody that has been generated against the conserved catalytic domain located at the C-terminal domain of TET proteins or IgG (negative control) (Figure 1A). We have previously validated that this TET3 antibody recognizes specifically TET3.[36] In addition, in the present study, in our enriched TET3 interactors, we did not identify TET2 or TET1 indicating that indeed our antibody does not cross-react with other members of the TET family that share the conserved catalytic domain (Table S1). We used IgG as negative control since germline deletion of TET3 results in

perinatal lethality.[14,39,40] To study the function of TET3 *in vivo*, in developing T cells in the thymus, we have generated mice where the deletion of TET3 starts specifically at the DP stage by crossing $Tet3^{flx/flx}$ mice[40,41] with CD4cre mice[42] as previously described.[15] However, in thymocytes isolated from the conditional Tet3 knockout (KO) mice, we detected some remaining expression of TET3 both at RNA and protein level, most likely due to the lack of Cre recombinase expression at the DN stage.[36] Thus, for our study, we opted to use IgG as negative control as it has been previously described for immunoprecipitation experiments followed by mass spectrometry.[43–46] We chose to investigate TET3 interactors in primary developing T cells that are abundant in the thymus, due to the requirement of large amounts of total protein in order to achieve efficient pull-down of endogenous TET3. Specifically, 600 μg of nuclear proteins were used per each IP sample. Before proceeding with the mass spectrometry experiment, we performed silver staining assay to evaluate the optimal concentration of antibody for our assay (Figure S1). We tested two different concentrations: 3.5 and 5 μg/IP. Our results indicated enhanced enrichment when using 5 μg and we chose to proceed with 5 μg of antibody/IP (Figure S1). In addition, the silver staining experiment indicated that the IP with anti-TET3 was resulting in high enrichment of proteins compared to the IgG control (Figure S1). TET3 has a CXXC DNA binding domain. However, we have observed only focal gain of DNA methylation upon TET deletion in T cells.[15] In addition, mapping of 5hmC in distinct T cell subsets revealed targeted deposition of 5hmC.[20] Thus, when we embarked on the pursuit of identifying the TET3 interactome in developing T cells in the thymus, we had to consider all plausible scenarios: (a) TET3 could directly bind to the DNA via its CXXC DNA binding domain without the requirement of physical interaction with other proteins, (b) based on the focal pattern of DNA demethylation, TET3 could participate in protein complexes that finely tune TET3 recruitment at specific loci across the genome, and (c) TET3 could be part of protein complexes that are in the same genomic area with other proteins/protein complexes with which TET3 does not interact. In order to distinguish interactions, meaning proteins that can physically associate with TET3, either directly or by participating in protein complexes, from proteins that are recruited in the same vicinity across the genome without interacting with TET3, we lysed nuclei in the presence of 10 μg/mL ethidium bromide (EtBr) that inhibits indirect interactions, which occur due to binding to the DNA,[47–49] and 250 U/mL benzonase as previously described.[50–52] Benzonase digests DNA and RNA, so interactions that are identified in the presence of benzonase are protein-protein interactions that occur independently of the presence of nucleic acids (DNA or RNA).[50,53] We performed the IP step in the absence or presence of EtBr and benzonase (Figure 1A). Indeed, the comparison of samples treated with EtBr and benzonase versus untreated samples by performing silver staining revealed that the treatment was reducing the number of identified proteins (Figures 1A and S2). We proceeded with liquid chromatography with tandem mass spectrometry (LC-MS/MS). 4 biological replicates per condition were evaluated across the following conditions: (a) TET3 IP untreated, (b) IgG IP untreated, (c) TET3 IP treated, and (d) IgG IP treated (where "treated" indicates treatment with EtBr and benzonase). Our dissimilarity analysis confirmed that the biological replicates for each condition were similar with each other (Figure 1B). We identified 2,307 significantly enriched proteins in the untreated samples, when comparing untreated nuclear extracts immunoprecipitated using anti-TET3 against untreated nuclear extracts immunoprecipitated using IgG (negative control). When the nuclear extracts were treated with EtBr and benzonase, the identified TET3-interacting proteins were 1,472 in the samples that were immunoprecipitated with anti-TET3 compared to the samples immunoprecipitated with IgG (Figures 1C; Table S1).

Comparison of the differentially enriched proteins in the treated versus untreated samples revealed 1,129 unique proteins exclusively in the untreated samples. 1,178 proteins overlapped whereas 294 proteins were differentially enriched specifically in the samples that were treated with benzonase and EtBr (Figures 1C; Table S1). This comparison further confirmed our conclusion that treatment with EtBr and benzonase renders more stringent the immunoprecipitation conditions and most likely the identified proteins interact with TET3, meaning they are in direct contact or they form together complexes, whereas the 1,129 proteins that are unique to the untreated samples may reflect proteins that bind to similar genomic regions as TET3 (Figures 1C; Table S1). Volcano plot analysis of the 1,472 proteins that were differentially enriched in the treated samples further confirms the efficiency of the anti-TET3 IP as demonstrated by the enrichment of proteins in the anti-TET3 samples compared to the IgG control (Figure 1D).

As indicated in the volcano plot, numerous proteins that exert significant biological functions in T cells were identified as interacting partners of TET3, including proteins involved in chromatin architecture such as special AT-rich sequence-binding protein 1 (SATB1),[54–56] B cell lymphoma/leukemia 11B (BCL11B),[57] and CCCTC-binding factor (CTCF)[58–60] (Figures 1D; Table S1). Among the interacting partners of TET3, we were able to identify OGT (Table S1), an established interacting partner of all TET proteins[61,62] (Table S2). An additional well-studied partner of TET proteins that appeared among our data was SIN3A[43,62,63] (Figure 1D; Tables S1 and S2). In addition, we identified paraspeckle component 1 (PSC1) (Table S1) that has been previously reported to interact with TET2 in ESCs[52] and in MCF7 cells[45] (Table S2). PSC1 has been suggested to recruit TET2 to regulate expression of retroviral elements by mediating their suppression.[52] In support of our proteomic data, we have previously identified upregulation of long terminal repeat retrotransposons as well as long interspersed elements in *Tet2/3* double-knockout (DKO) iNKT cells.[64]

We also identified the transcription factor GATA3 (Figures 1D; Table S1) as well as proteins involved in DNA repair and DNA recombination,[65–68] such as mini-chromosome maintenance complex 4 (MCM4), RAD54B, and RAG1 (Figures 1D; Table S1), and splicing, such as SF3B1, SF3B3, and SRSF1[69] (Figure 1D; Table S1).

Identifying biological functions for the TET3 interactome

To identify the biological significance of these interactions in an unbiased approach, we performed gene ontology (GO) analysis (Figures 1E and 1F; Figure S3). First, we focused on the 294 proteins that were uniquely differentially enriched in the most stringent conditions of immunoprecipitation, in the presence of benzonase and EtBr. Our GO analysis revealed that the top enriched biological process (BP) was histone modification (Figure 1E). Another BP that was significantly enriched was epigenetic regulation of gene expression consistent with the bona

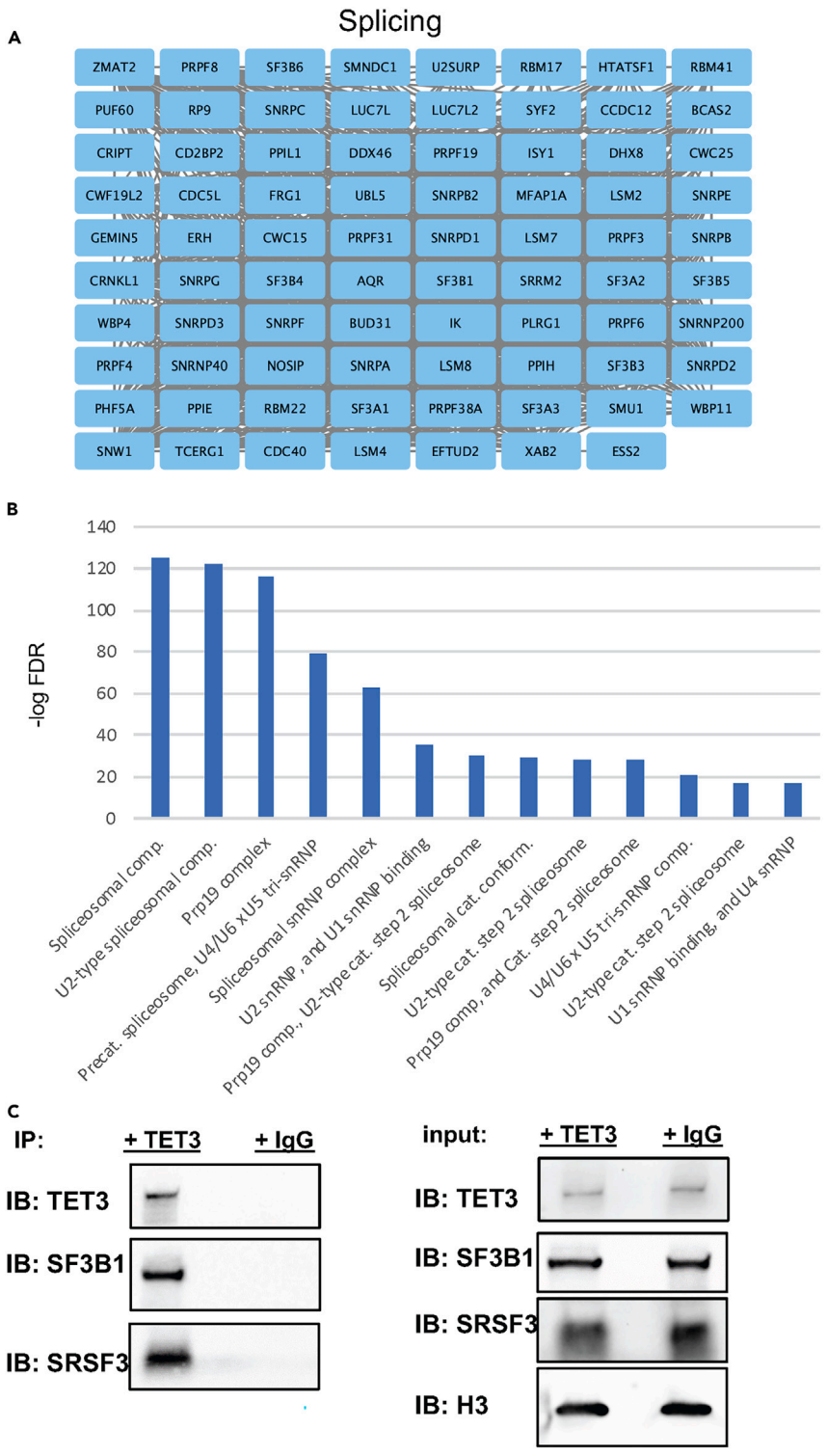


Figure 2. TET3 interacting partners involved in RNA splicing regulation

(A) Functional cluster analysis of TET3 interacting partners using STRING software reveals a network of 79 interacting proteins involved in RNA splicing.

(B) STRING analysis of the proteins of this cluster indicates the major biological functions that these proteins regulate. Note that spliceosomal complex is the top function and U2-type spliceosomal complex comes second.

Figure 2. *Continued*

(C) Immunoprecipitation (IP, *left*) of endogenous TET3 (+TET3) or IgG (+IgG) (negative control) from nuclear extracts of thymocytes in the presence of EtBr and benzonase followed by immunoblotting for TET3, SF3B1, and SRSF3. TET3 was specifically precipitated in the presence of anti-TET3, but not in the negative control. Note the presence of specific bands for SF3B1 and SRSF3 exclusively in the IP sample in the presence of TET3. Total nuclear extracts, treated with EtBr and benzonase, were used as input (*right*). Note that TET3, SF3B1, and SRSF3 are detected by western blot in both samples (the one used for TET3 IP and the one used for the IgG). Histone 3 (H3) was used as control to assess amount of total protein. One out of two representative experiments is shown.

fide role of TET3 in mediating DNA demethylation across various cell types, including T cells (Figure 1E). Intriguingly, mRNA transport and double-strand break repair were among the top BPs that were identified (Figure 1E).

GO analysis focusing on cellular component (CC) identifies categories such as chromosome, nucleoplasm, male-specific lethal complex, as well as CDC73/PAF1 complex that is involved in transcriptional elongation (Figure S3A). Importantly, this finding is consistent with our previous discovery regarding the enrichment of 5hmC with H3K36me3 and elongating polymerase II (Pol II) during thymic T cell differentiation.[20] GO analysis focusing on molecular function indicates that the identified interacting partners are involved in protein binding and DNA binding confirming our hypothesis that TET3 is recruited at specific loci in T cells by interacting with factors that can bind to DNA[6,70] (Figure S3B). Moreover, the unbiased GO demonstrated that additional categories were related to transcription/transcriptional elongation (RNA Pol II C-terminal domain phosphorylation, CDC73/PAF1 complex) and epigenetic regulation of gene expression.

Next, we asked what were the biological procedures that were regulated by 1,178 TET3-interacting proteins, which were identified as common hits both in the presence/absence of EtBr and benzonase (Figures 1F, S3C, and S3D). The top BP category was DNA damage response establishing further the implication of TET3 in DNA repair processes in thymic T cells (Figure 1F). In addition, chromosome organization also emerged (Figure 1F). Importantly, TET1-deficient oocytes show aneuploidies[71] and stem cells with inducible deletion of all three TET proteins exhibit aneuploidies.[72] Moreover, TET2 has been reported to interact with proteins that regulate DNA repair and chromosome segregation mainly in ESCs[43] and MCF7 cells[45] (Table S2). Thus, our findings raise the possibility that TET3 is involved in proper chromosome segregation and overall maintenance of genome stability in T cells. In agreement with the fact that our experiments were performed in developing thymocytes, other categories were developmental process, hemopoiesis, and regulation of cell fate specification (Figure 1F). Intriguingly, splicing emerged as a significant category (Figure 1F). To further support the implication of TET3 interacting partners in regulation of splicing, the CC analysis revealed spliceosomal complex among the most significant categories (Figure S3C). Notably, we have previously identified 5hmC enrichment in intron-exon junctions during T cell development.[20] In addition, TET2 has been shown to interact with proteins involved in splicing in ESCs[43,52] as well as in MCF7 cells[45] (Table S2). Thus, we hypothesize that TET3 by interacting with splicing factors can deposit 5hmC and other oxi-mCs to impact splicing in developing T cells in the thymus.

Finally, we examined the 1,129 proteins that were identified only when the samples were not treated with benzonase and EtBr. These proteins most likely indicate proteins that are recruited across the genome in proximity with TET3. Our analysis revealed top categories such as nucleus, chromosome organization, and catalytic activity. For the rest of the study, we focus on the analysis of the 1,472 proteins that were identified in the presence of EtBr and benzonase.

Next, we sought to identify functional networks defined by the discovered interacting partners of TET3 under treatment with EtBr and benzonase. We used 1,472 proteins: 294 that were identified exclusively in the treated samples as well as 1,178 common hits identified in both treated and untreated samples (Figure 1C). To this end, we leveraged the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software which aims to integrate both known and predicted associations between proteins[73] in order to discover functional clusters formed by TET3 and its interacting partners in primary developing T cells in the thymus.

We then focused on specific clusters of interest and we took a closer look in the proteins that interact with TET3. For specific proteins of interest in each cluster, we examined their enrichment across the treated samples versus the treated negative controls that were analyzed by mass spectrometry. Finally, we further validated these interactions by co-immunoprecipitation in the presence of EtBr and benzonase followed by western blot.

TET3 interacting partners are involved in RNA splicing

Among the largest clusters that emerged from the STRING analysis was a cluster of 79 proteins involved in RNA splicing (Figure 2A) as revealed by STRING network cluster analysis (Figure 2B). For instance, key members of the SF3B complex, such as SF3B1, SF3B3, SF3B4, and SF3B6, were among the TET3 interacting partners that were identified in the treated samples with EtBr and benzonase (Figure 2A). The SF3B complex is a component of the U2 small nuclear ribonucleoprotein (snRNP).[74,75] In addition, among the TET3 interacting partners were components of the PRP19 complex[76] such as PRPF3, PRPF4, PRPF6, PRPF8, PRPF19, PRPF31, and PRPF38a (Figures 2A and 2B). Moreover, snRNPs such as SNRPD1, SNRP70, SNRPC, SNRPF, SNRPD2, SNRPE, SNRPD3, SNRPA, SNRPB2, SNRP40, SNRNP200, and SNRPB were also among the interacting partners of TET3 that were identified under the stringent conditions of the IP, in the presence of EtBr and benzonase (Figures 2A and 2B). Collectively, the proteins of this cluster participate in the formation of the spliceosomal complex, a large RNA-protein molecular complex involved in splicing of pre-mRNA[69,75] (Figure 2B). Next, we focused on two of the interacting partners in this category, SF3B1 and SRSF3, which based on the mass spectrometry analysis were significantly enriched in all 4 biological replicates that were immunoprecipitated with anti-TET3 over the IgG control (Table S1). We further validated that SF3B1 and SRSF3 can interact with TET3 by performing IP in the presence of EtBr and benzonase followed by western blot (Figures 2C and S4). Notably, TET2 has been identified to interact with SF3B1[43] and SF3B2[52] in ESCs and MCF7 cells,[45] as well as numerous snRNPs, members of the PRP19 complex, subunits of the splicing factor

U2AF, and subunits of the SF3B complex and the SRSF complex (summarized in Table S2). Thus, our data provide a strong foundation for involvement of TET3 in splicing in developing thymic T cells, potentially in collaboration with TET2.

TET3 interactome regulates RNA polymerase elongation and transcription

Another cluster that emerged from the STRING analysis comprised 55 proteins that were involved in transcription, such as in transcriptional initiation and elongation (Figures 3A and 3B). Specifically, we identified interaction of TET3 with subunits of the RNA Pol II, the polymerase that synthesizes mRNAs, such as POLR2B, POLR2K, POLR2E, POLR2F, POLR2D, POLR2A, POLR2M, and POLR2H (Figures 3A and 3B). POLR2B has been identified as a TET3 interacting partner in HEK293 cells[62] (Table S2). TET2 has been reported to interact with POLR2A, POLR2B in ESCs[52] (Table S2). Notably, among the discovered proteins that interact with TET3 in primary thymocytes were components of the mediator complex, including MED11, MED12, MED17, MED20, MED25, MED27, and MED31 (Figures 3A and 3B). The mediator complex interacts with various transcription factors and with the RNA Pol II pre-initiation complex.[77–79] Another critical regulator of gene expression, the bromodomain protein 4 (BRD4), was also found to interact with TET3 (Figure 3A). BRD4 recruits the active form of transcription elongation factor PTEF-b.[80] Moreover, BRD4 can impact transcription by acting as an atypical kinase that can phosphorylate serine 2 of the RNA polymerase II carboxy-terminal domain.[81] In addition, BRD4 through its histone acetyltransferase activity mediates nucleosome eviction.[82]

Importantly, polymerase-associated factor (PAF1) complex that interacts with RNA Pol II and plays a role in transcription elongation was also among the proteins associated with TET3 in samples treated with EtBr and benzonase. We further validated this identified interaction by performing co-immunoprecipitation followed by western blot (Figure 3C). Our results confirm that PAF1 specifically interacts with TET3 in primary thymocytes (Figures 3C and S4).

TET3 interacting partners are involved in DNA replication and repair

Among the most significant biological functions to be regulated by TET3 interacting partners in primary T cells were DNA replication and DNA repair (Figures 4A–4D; Table S1; Figure S5). Our analysis revealed a network of 30 proteins that were interacting with TET3 and were involved in processes like DNA binding, DNA replication, and DNA repair (Figures 4A and 4B). For instance, X-ray repair cross complementing 1 (XRCC1) scaffold protein with critical role in DNA base excision repair[83] was among the interacting partners (Figure 4A) as well as XRCC6 (Figure 4A). Specifically, XRCC1 is recruited by PARP1 and/or PARP2 in single-strand breaks (SSBs) and then XRCC1 recruits proteins such as DNA polymerase b and DNA polymerase III that are required for repairing the breaks.[84,85] Previously, TET2 was reported to interact with PARP1 in ESCs[43] and MCF7 cells[45] (Table S2). Functionally, TET2 and PARP1 have been implicated in epigenetic remodeling during the reprogramming of MEFs to inducible pluripotent stem cells.[86] In addition, TET3 interacts with MutS homolog 2 (MSH2) and MSH6 proteins that exert instrumental roles in DNA mismatch repair (Figures 4A–4D; Table S1).[87] Other interesting interacting partners involved in DNA repair are BRCA2, RAD21, LIG1, TRP53, and NHEJ1.[88] Our mass spectrometry data revealed strong enrichment for MSH2 in all 4 replicates of the IP using anti-TET3 over the IgG control (Table S1). MSH2 has been found to interact with TET2 in ESCs[43](Table S2).

Moreover, TET3 was found to interact with the (MCM2, which is a highly conserved DNA helicase and is critical for DNA replication[89,90] (Figures 4A–4D; Table S1). Our data indicate that TET3 can interact with MCM3, MCM4, MCM5, MCM6, and MCM9 (Figures 4A–4D; Table S1). MCM3 has been previously identified to interact with TET3 in HEK293T cells.[62] MCM3 and MCM7 have been also shown to interact with TET2 in ESCs[43] and in MCF7 cells[45](Table S2). The MCM proteins are critical factors for the initiation of DNA replication. Among them, six proteins, MCM2–7 proteins, form a hexameric complex, which is a component of the prereplication complex that assembles at replication origins during early G1 phase. MCM2–7 proteins may additionally play a role in the elongation of DNA replication.[89] Recently, it was shown that MCM2 can also promote stem cell differentiation in an H3-H4-mediated manner.[91]

For selected candidates MSH2, MCM2, and XRCC1, we further confirmed their interaction with TET3 by performing co-immunoprecipitation of thymic nuclei with anti-TET3 or IgG, in the presence of EtBr and benzonase (Figures 4E; Figure S5). Our experiments further establish that TET3 can interact with the aforementioned proteins. Collectively, our findings in this study and the mentioned data in the literature suggest that TET3 can exert roles in DNA replication and repair in developing thymocytes.

TET3 participates in CTCF and cohesin complex network in developing T cells

CTCF was significantly enriched as an interacting partner of TET3 in all 4 biological replicates of co-immunoprecipitation followed by MS/MS that were treated with EtBr and benzonase compared to the treated IgG control (Figure 5A; Table S1) as well as in the non-treated samples (Table S1). CTCF is an architectural protein that is sensitive to the presence of DNA methylation. It has been previously shown that CTCF binding is impacted upon gain of methylation in *Tet1/2* DKO ESCs.[92] In addition, CTCF has been shown to regulate splicing by preferentially binding to 5hmC while its high affinity for 5caC enables its binding at genomic loci that would not be recruited otherwise.[93–95] Notably, CTCF has been shown to interact with TET1 under endogenous conditions and TET2 upon overexpression in adipocytes.[96] In addition, CTCF has been reported to interact with a neural isoform of TET3 that lacks CXXC domain in retinal explants upon overexpression[97] (Table S2). Here, our data suggest that in developing thymic T cells, interaction of CTCF with TET3 can impact CTCF-mediated functions that depend on the presence of oxi-mCs across the genome, including chromatin and DNA binding, and potentially exerting an impact on RNA polymerase pausing[95] (Figure 5B). Specifically, our STRING analysis revealed a protein network formed by CTCF and other TET3 interacting partners identified in this study (Figure 5A). Among the proteins that participate in this network was the cohesin subunit STAG1 that works together with CTCF to shape three-dimensional genomic conformation, such as enhancer-promoter loops.[98,99] Structural maintenance of chromosomes 3 (SMC3) and

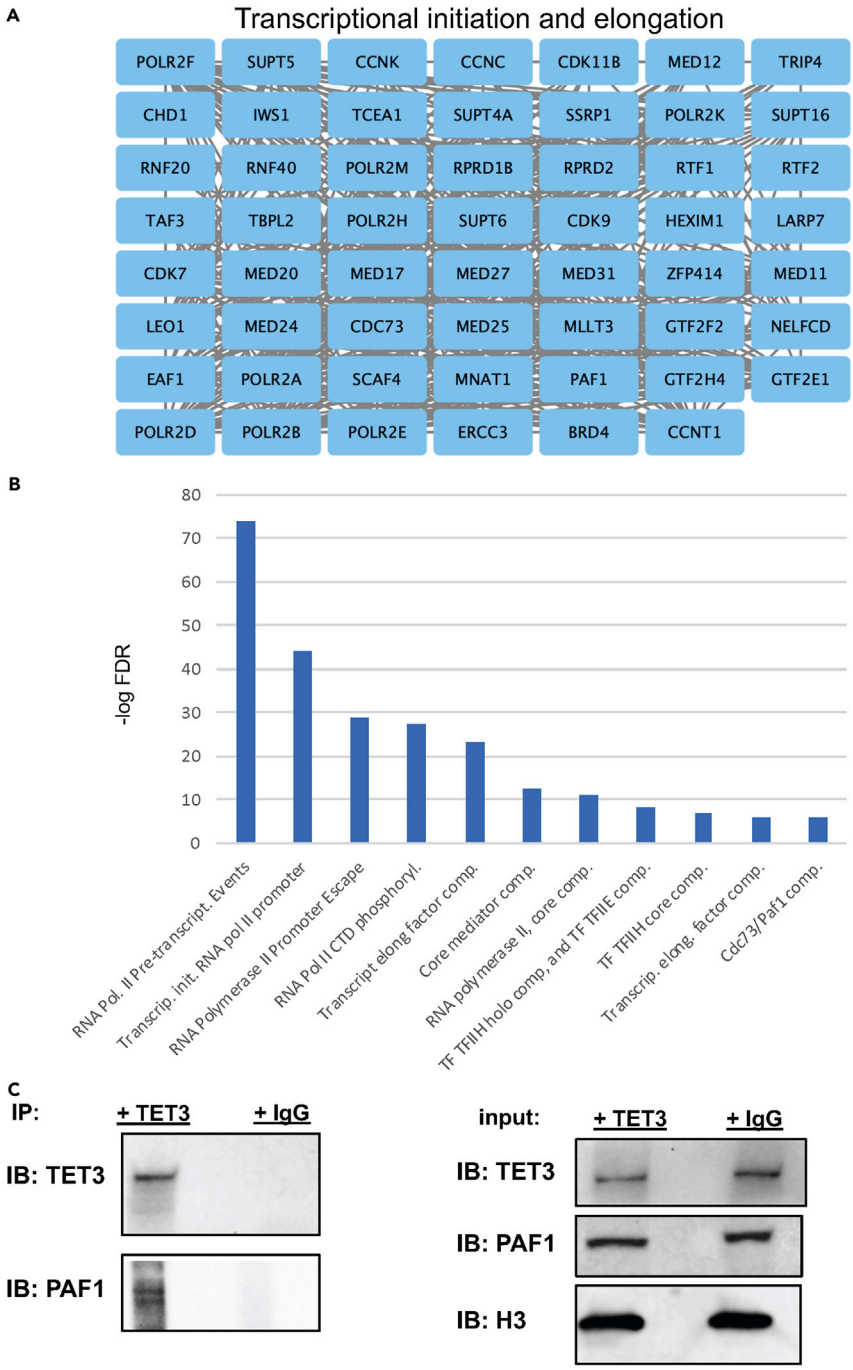


Figure 3. TET3 interacts with RNA Pol II and proteins involved in transcriptional initiation and elongation

(A) STRING analysis indicates that TET3 interacts with subunits of RNA Pol II as well as additional proteins involved in transcriptional initiation and elongation such as the mediator complex and BRD4.

(B) STRING analysis of the proteins identified in A reveals the major biological functions regulated by this cluster. The RNA Pol II pre-transcription events category is the most significant category, while transcription initiation RNA Pol II promoter follows.

(C) IP (*left*) of endogenous TET3 (+TET3) or IgG (+IgG) (negative control) from nuclear extracts of thymocytes in the presence of EtBr and benzonase followed by immunoblotting for TET3 and PAF1. Note that TET3 and PAF1 are specifically precipitated in the +TET3 condition but not in the negative control. Total nuclear extracts were used as input (*right*). Note that TET3 and PAF1 are detected by western blot in both samples. Histone 3 (H3) was used as loading control. One out of two representative experiments is shown.

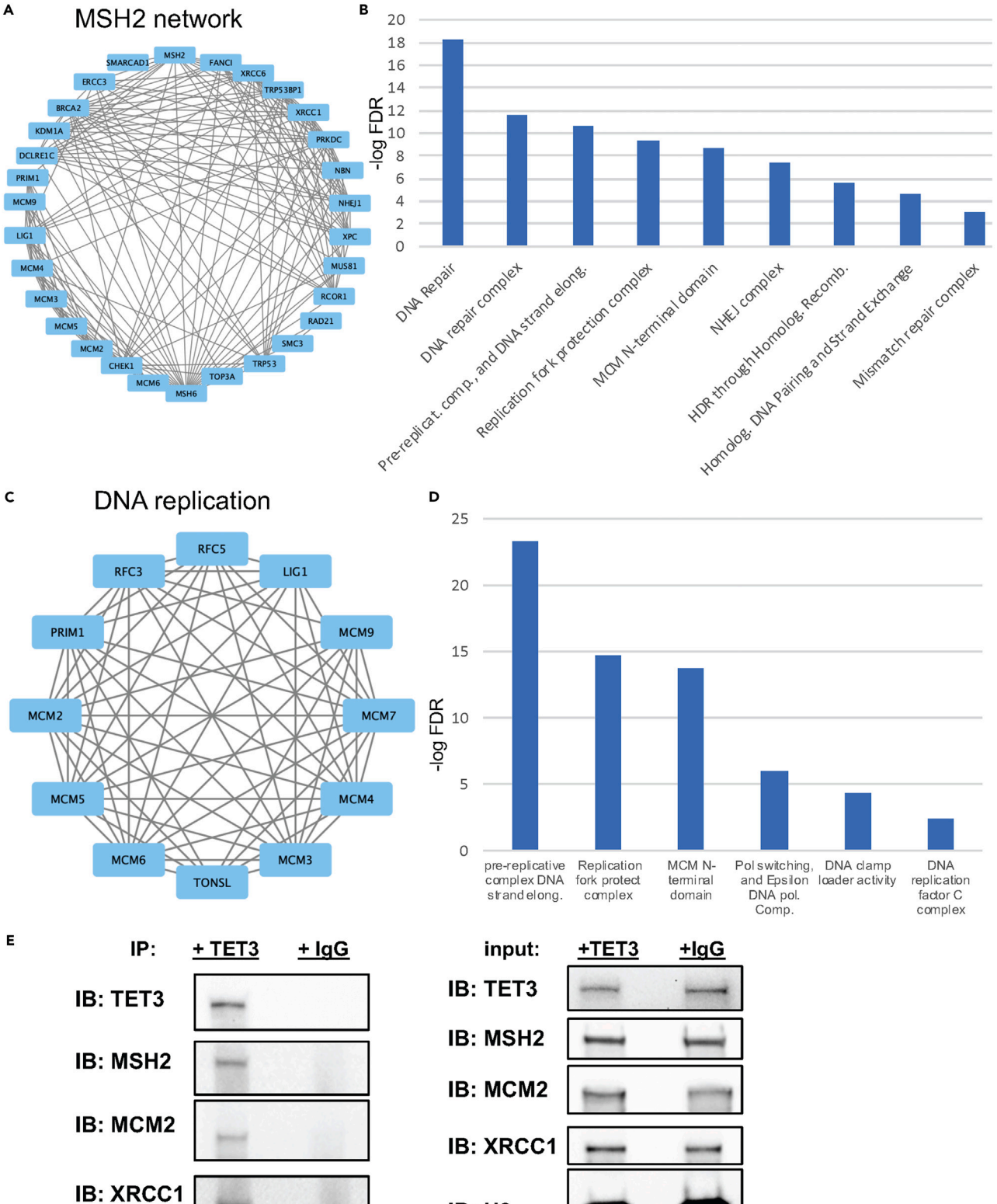

A
MSH2 network
SMARCAD1
MSH2
FANCI
XRCC6
ERCC3
TRP53BP1
BRCA2
XRCC1
KDM1A
PRKDC
DCLRE1C
NBN
PRIM1
NHEJ1
MCM9
XPC
LIG1
MUS81
MCM4
RCOR1
MCM3
RAD21
MCM5
SMC3
MCM2
TRP53
CHEK1
TOP3A
MCM6
MSH6
B
-log FDR
20
18
16
14
12
10
8
6
4
2
0
DNA Repair
DNA repair complex
Pre-replicat. comp., and DNA strand elong.
Replication fork protection complex
MCM N-terminal domain
NHEJ complex
HDR through Homolog. Recomb.
Homolog. DNA Pairing and Strand Exchange
Mismatch repair complex
C
DNA replication
RFC5
RFC3
LIG1
PRIM1
MCM9
MCM2
MCM7
MCM5
MCM4
MCM6
MCM3
TONSL
D
-log FDR
25
20
15
10
5
0
pre-replicative complex DNA strand elong.
Replication fork protect complex
MCM N-terminal domain
Pol switching, and Epsilon DNA pol. Comp.
DNA clamp loader activity
DNA replication factor C complex
E
IP:
+ TET3
+ IgG
IB: TET3
IB: MSH2
IB: MCM2
IB: XRCC1
input:
+TET3
+IgG
IB: TET3
IB: MSH2
IB: MCM2
IB: XRCC1
IB: H3

Figure 4. TET3 interacting partners with roles in DNA replication and DNA repair

(A) STRING analysis of TET3 interacting partners reveals a cluster with proteins involved in DNA binding, DNA helicase activity, and DNA repair.

(B) STRING functional analysis of proteins from network shown in A identifies as most significant function DNA repair, whereas DNA repair complex and pre-replication complex and DNA strand elongation follow.

(C) STRING analysis reveals cluster of proteins involved in DNA replication.

(D) Key functions of proteins forming the cluster in C. The most significant function is pre-replicative complex DNA strand elongation. Among the identified functions was polymerase switching and epsilon DNA polymerase complex.

(E) IP (*left*) of endogenous TET3 (+TET3) or IgG (+IgG) (negative control) from nuclear extracts of thymocytes in the presence of EtBr and benzonase followed by immunoblotting for TET3, MSH2, MCM2, and XRCC1. Note the presence of a specific band for each of the proteins exclusively in the +TET3 IP and not in the +IgG (negative control) IP. Total nuclear extracts, treated with EtBr and benzonase, were used as input (*right*). Note that TET3, MSH2, MCM2, and XRCC1 are detected by western blot in both samples. Histone 3 (H3) was used as loading control. One out of two representative experiments is shown.

RAD21 that are part of the cohesin complex were also among the TET3 interacting partners that appeared in the CTCF functional network (Figure 5A).[100–102] Interestingly, SMC3 was previously identified as a potential interacting partner of TET2 in ESCs[52](Table S2).

Additional proteins involved in this network were POLR2A, which is the largest subunit for RNA Pol II and POLR2F, which consists the sixth largest subunit of RNA Pol II (Figures 5A and 5B). Notably, CTCF has been reported to interact with the largest subunit of RNA Pol II.[103] Another protein that is part of this network is SIN3A (Figure 5A) which is part of the SIN3A co-repressor complex, which mediates histone deacetylation.[104] SIN3A was reported to interact with TET1 in ESCs[63] as well as with TET3 in overexpressed conditions in mouse embryonic fibroblasts[105] (Table S2). In this network of TET3 interacting partners that also interact with CTCF, the RNA helicase DDX5 was also included (Figures 5A; Table S1). DDX5 exerts critical roles in RNA processing, including splicing, transcript stability, mRNA export, and microRNA processing.[55,106] In addition, DDX5 is involved in R-loop disassembly and promotes DNA repair.[107] DDX5 has been previously identified as a TET2 interacting partner in ESCs[52] and in MCF7 cells[45] (Table S2). Finally, an interesting protein identified as a potential TET3 interacting partner in our study is MATRIN3 which is encoded by Matr3 (Table S1). MATRIN3 has been involved in organization and stabilization of chromatin and has been reported to interact with CTCF and cohesion.[108] Interestingly, it was identified previously as a candidate interacting partner of TET2 in ESCs[52] (Table S2). In summary, the aforementioned findings suggest that TET3 participates in protein complex with CTCF to regulate gene expression.

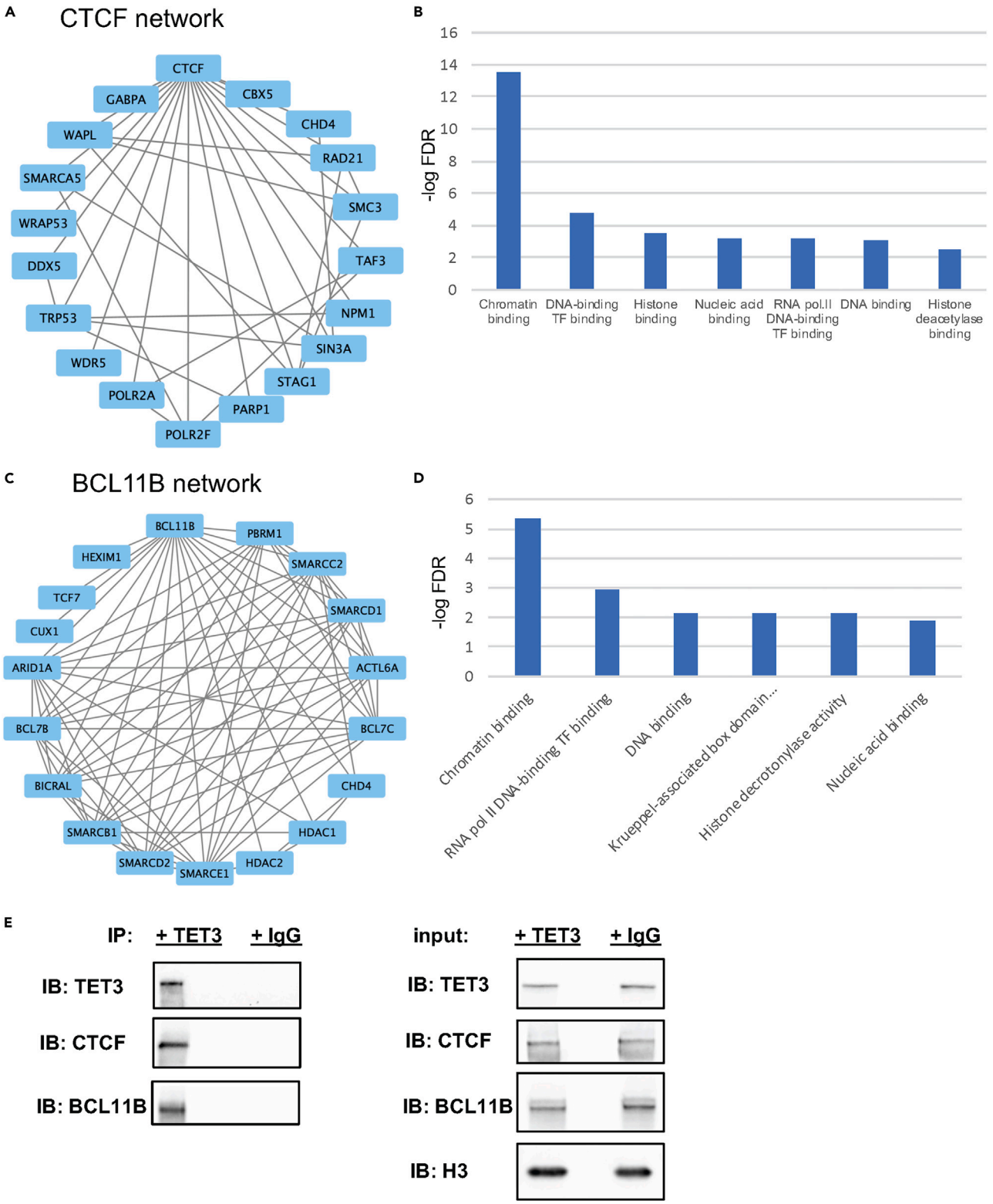
TET3 interacts with BCL11B and associated proteins in developing T cells

Among the enriched proteins in the TET3 immunoprecipitation followed by LC-MS/MS was BCL11B that plays critical roles in T cell development[109] (Figures 5C and 5D; Table S1). Similar to CTCF, BCL11B is also important in shaping 3D chromatin conformation.[57] CTCF was actually reported as an interacting partner of BCL11B in mass spectrometry experiments.[110] STRING analysis focused on BCL11B and other TET3 interacting partners revealed a network of proteins including switch/sucrose nonfermentable (SWI/SNF)-related matrix-associated, actin-dependent regulator of chromatin subfamily d member 1, SMARCD1, as well as SMARCD2, SMARCC2, SMARCE11, and SMARCB1, involved in chromatin remodeling[111] (Figure 5C). TET2 has been previously found to interact with members of the SWI/SNF complex, including SMARCB1, SMARCC2, SMARCD2, and SMARCE1 in MCF7 cells,[45] (Table S2). BCL11B has been shown by immunoprecipitation followed by mass spectrometry to interact with members of the SWI/SNF complex in T cells[110](Table S3). Also, BCL7B (Table S1; Figure 5C) and BCL11B sedimented with members of the SWI/SNF complex in sedimentation experiments,[112] and these interactions were further confirmed in co-immunoprecipitation assays.[112] AT-rich interaction domain 1A protein was also part of this network (Figure 5C) and is a subunit of the SWI/SNF complex.[111] Other interacting partners of TET3 that were included in the BCL11B functional network were the histone deacetylases (HDAC) 1 and HDAC2 as well as the chromodomain helicase DNA binding protein 4 (CHD4) that are subunits of the nucleosome remodeling and deacetylase (NuRD) complex[113] (Figure 5C) (Table S1). TET3 has been reported to interact with HDAC in overexpression experiments in HEK293 cells[62] while TET2 was shown to interact with HDAC2 in MCF7 cells[45](Tables S2 and S3). STRING analysis revealed that functions related to this group are chromatin binding, RNA Pol II DNA binding, and transcription factor binding (Figure 5C).

We further confirmed the interaction of TET3 with CTCF and BCL11B in murine thymocytes by isolating nuclei and performing immunoprecipitation, in the presence of EtBr and benzonase, with anti-TET3 or IgG followed by western blot using antibodies against CTCF and BCL11B (Figures 5E; Figure S6). Our data confirm that TET3 specifically interacts with these proteins. An additional validation of our findings stems from the fact that multiple of these interacting partners that form the CTCF network (Figure 5A) and the BCL11B network (Figure 5C) have been previously identified in a plethora of other studies (Table S3) as CTCF or BCL11B interactors, as mentioned earlier.

TET3 participates in network with transcription factors critical for T cell differentiation

Another interesting network that emerged from STRING analysis of TET3 interacting partners involved proteins that regulate CD4 versus CD8 lineage choice such as the transcription factor GATA3, which is critical for CD4 lineage commitment,[28] the RUNX1 and RUNX3 as well as the core binding factor CBFB that forms a complex with RUNX factors (Figures 6A and 6B; Table S1). RUNX1 has been previously reported to interact with TET2 in osteoclasts.[114] We used STRING software to interrogate the protein network that is formed by GATA3 and other identified TET3 interacting proteins in primary thymic T cells. RUNX1, RUNX3, and CBFB were identified to participate in this functional network that is critical for CD4 and CD8 lineage choice.[27] In addition, we identified STAT3 and STAT6 (Figures 6A; Table S1). STAT3 has been shown to interact with TET2 in dendritic cells[115] as well as in MCF7 cells[45](Table S2). It is worthwhile to emphasize that in MCF7 cells, STAT3 was identified as a common interactor of both TET2 and GATA3.[45]


A
CTCF network
CTCF
CBX5
GABPA
CHD4
WAPL
RAD21
SMARCA5
SMC3
WRAP53
TAF3
DDX5
NPM1
TRP53
SIN3A
WDR5
STAG1
POLR2A
PARP1
POLR2F
B
-log FDR
16
14
12
10
8
6
4
2
0
Chromatin binding
DNA-binding TF binding
Histone binding
Nucleic acid binding
RNA pol.II DNA-binding TF binding
DNA binding
Histone deacetylase binding
C
BCL11B network
BCL11B
PBRM1
HEXIM1
SMARCC2
TCF7
SMARCD1
CUX1
ARID1A
ACTL6A
BCL7B
BCL7C
BICRAL
CHD4
SMARCB1
HDAC1
SMARCD2
SMARCE1
HDAC2
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3
2
1
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Chromatin binding
RNA pol II DNA-binding TF binding
DNA binding
Krueppel-associated box domain...
Histone decrotonylase activity
Nucleic acid binding
E
IP:
+ TET3
+ IgG
IB: TET3
IB: CTCF
IB: BCL11B
input:
+ TET3
+ IgG
IB: TET3
IB: CTCF
IB: BCL11B
IB: H3

Figure 5. TET3 interacts with CTCF and BCL11B protein networks

(A) STRING analysis reveals CTCF network formed by TET3 interacting partners identified upon treatment with EtBr and benzonase.

(B) Significant functions performed by proteins in A are shown. The most significant function was chromatin binding followed by transcription factor (TF) DNA binding.

(C) STRING analysis reveals BCL11B network formed by TET3 interacting partners identified upon treatment with EtBr and benzonase.

(D) Significant functions performed by proteins in C are shown. The most significant function was chromatin binding followed by RNA Pol II DNA binding TF.

(E) IP (*left*) of endogenous TET3 (+TET3) or IgG (+IgG) (negative control) from nuclear extracts of thymocytes in the presence of EtBr and benzonase followed by immunoblotting for TET3, CTCF, and BCL11B. Note the presence of a specific band for each of the proteins exclusively in the +TET3 IP and not in the +IgG (negative control) IP. Total nuclear extracts, treated with EtBr and benzonase, were used as input (*right*). Note that TET3, CTCF, and BCL11B are detected by western blot in both samples. Histone 3 (H3) was used as loading control. One out of two representative experiments is shown.

Among the transcription factors that we discovered to interact with TET3 was FOXP1, a factor that plays critical roles in T cell proliferation and memory formation as well as in the Treg lineage[116] (Table S1), and the transcription factors of the IKAROS family, AIOLOS and IKAROS[117] (Table S1). Interestingly, IKAROS and AIOLOS which are encoded by Ikzf1 and Ikzf3, respectively, were among the interacting partners of BCL11B in mass spectrometry experiments[110] (Table S3). FOXP1 has been reported to participate in complexes with HDAC1/2[118] which have been shown to interact with TET proteins (Tables S2 and S3). In addition, IKAROS has been identified as an interactive partner of FOXP1[110] (Table S3). Thus, cumulative data in the literature confirm the existence of the protein networks we identify in this study. We further validated the interaction of these factors with TET3 by IP experiments followed by immunoblot (Figures 6 and S7). It is worth to note that the antibody we used to detect FOXP1 was predicting molecular weight between 82 and 90 kDa. However, we detected for the IP a band with molecular weight around 97 kDa and for the input samples two bands with molecular weight around 97 and 72 kDa, respectively (Figures 6 and S7). This finding is consistent with previous observations that there are various FOXP1 isoforms.[119] In B cells, T cells, and organs where lymphocytes are abundant such as in spleen, FOXP1 isoform A has a molecular weight a bit higher than 95 kDa, whereas isoform C has molecular weight of 70 kDa,[119] consistent with our observations (Figures 6 and S7).

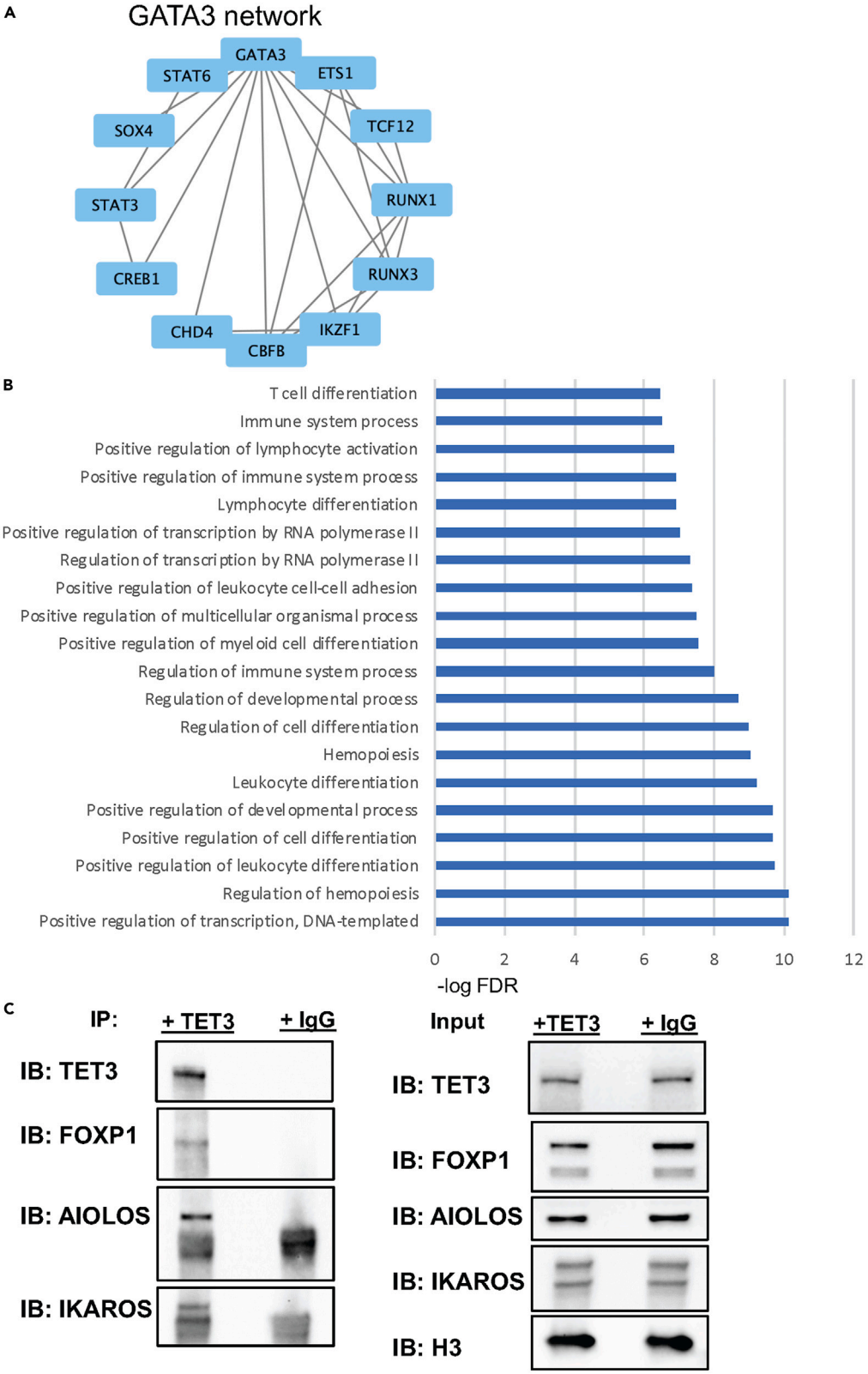
DISCUSSION

In this resource, we employ LC-MS/MS to decipher the TET3 interactome in murine primary developing T cells. Here, we discover protein networks where TET3 participates in endogenous conditions. Our data reveal that TET3 can form complexes with proteins involved in splicing (Figures 1 and 2). This finding agrees with our previous discovery that 5hmC is enriched intragenically, in exon/intron junctions in thymic T cell subsets.[20] An emerging question is how TET3 could regulate splicing. A plausible scenario is that TET3 and 5hmC by affecting the chromatin accessibility can impact the recruitment of the splicing machinery and promote the DNA binding of 5mC-sensitive factors.[120] Indeed, here we demonstrate that TET3 interacts with CTCF (Figure 5). CTCF is evicted in the presence of 5mC from the DNA and has been reported to be involved in co-transcriptional splicing.[69,95] Specifically, CTCF can bind 5hmC and its recruitment results in RNA polymerase pausing, allowing the inclusion of weaker exons during splicing.[95] However, presence of 5mC prohibits CTCF binding and as a result RNA Pol II can travel faster across the gene, resulting in exclusion of weaker exons.[95] An example of CTCF function in splicing regulation is the alternative splicing of CD45 in B cells and T cells.[93] Notably, 5mC oxidization in the case of CD45 was mainly regulated by TET1 and TET2, as demonstrated by deleting individual *Tet* gene expression, using short hairpin RNAs.[93] We attribute this to the different developmental and activation stage of the cells examined. Specifically, the authors assessed B cell lines that were in culture and human, activated CD4 T cells.[93] In these cells, TET2 was most highly expressed, while TET3 was less expressed. However, in developing, thymic T cells, TET3 is more highly expressed.[11,13,36] Collectively, our data suggest that TET3 can impact the recruitment of components of the spliceosome machinery to regulate splicing. An additional possibility is that TET3, by generating 5hmC and other oxi-mCs, can promote CTCF binding and accumulation of RNA Pol II, to allow the inclusion of weak exons. Additional studies in the literature implicate mainly TET2 in interactions with proteins involved in splicing regulation in ESCs[43,52] as well as MCF7 cells[45] (shown in Table S2). Thus, an emerging possibility is that TET proteins can regulate splicing across various cell types/developmental stages either individually or potentially in a collaborative manner. Future studies will shed light on the precise mechanisms of this regulation.

Another functional network formed by TET3 interacting partners is regulation of RNA Pol II and transcriptional elongation (Figure 3). We have previously discovered that 5hmC is highly enriched across the gene body of very highly expressed genes and positively correlates with marks of active transcription, such as H3K36me3, which is a histone mark that decorates actively transcribed genes, and elongating Pol II in DP T cells in the thymus.[20] Mechanistically, TET3 could interact with the elongating machinery to regulate the kinetics of transcription in developing T cells. Fueling this hypothesis, it has been recently shown in smooth muscle cells that TET3, through its catalytic activity, prohibits spurious transcription by preventing aberrant entry of RNA Pol II in the gene body of highly expressed genes.[121] Importantly, overexpression studies in HEK293 cells have shown that TET3 can interact with Pol II.[62]

In addition, our GO analysis revealed the unexpected category of mRNA transport (Figure 1). Further studies will reveal if TET3 may be involved in this process. Another plausible scenario is that in this category are proteins such as heterogeneous nuclear ribonucleoproteins such as hnRPNA2B1 that also exert roles in transcription that we have discussed previously.

Moreover, we discover significant interactions of TET3 with proteins involved in DNA repair (Figure 4). This is of fundamental importance in developing T cells where rearrangements of the alpha and beta chains of the T cell receptor are instrumental for the T cell function.[122] Notably, we have previously shown that *Tet2/3* DKO iNKT cells upon hyperproliferation can accumulate DNA double-strand breaks, as assessed by γH2Ax enrichment.[64] In addition, we demonstrated increased gene expression of DNA repair genes in the *Tet2/3* DKO iNKT cells,


A
GATA3 network
GATA3
STAT6
ETS1
SOX4
TCF12
STAT3
RUNX1
CREB1
RUNX3
CHD4
IKZF1
CBFB
B
T cell differentiation
Immune system process
Positive regulation of lymphocyte activation
Positive regulation of immune system process
Lymphocyte differentiation
Positive regulation of transcription by RNA polymerase II
Regulation of transcription by RNA polymerase II
Positive regulation of leukocyte cell-cell adhesion
Positive regulation of multicellular organismal process
Positive regulation of myeloid cell differentiation
Regulation of immune system process
Regulation of developmental process
Regulation of cell differentiation
Hemopoiesis
Leukocyte differentiation
Positive regulation of developmental process
Positive regulation of cell differentiation
Positive regulation of leukocyte differentiation
Regulation of hemopoiesis
Positive regulation of transcription, DNA-templated
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IB: TET3
IB: FOXP1
IB: AIOLOS
IB: IKAROS
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+ IgG
IB: TET3
IB: FOXP1
IB: AIOLOS
IB: IKAROS
IB: H3

Figure 6. TET3 participates in networks with important transcription factors for T cell development

(A) STRING analysis reveals a cluster of TET3 interacting partners including GATA3, RUNX1, RUNX3, CBFB, and IKAROS (indicated as IKZF1).

(B) STRING analysis indicates the major functions controlled by proteins participating in the protein network described in A. Positive regulation of transcription is the top category, while regulation of hemopoiesis and positive regulation of leukocyte differentiation categories follow. It is noteworthy that in this category, we detect regulation of transcription by RNA Pol II.

(C) IP (*left*) of endogenous TET3 (+TET3) or IgG (+IgG) (negative control) from nuclear extracts of thymocytes in the presence of EtBr and benzonase followed by immunoblotting for TET3, FOXP1, AIOLOS, and IKAROS. Note the presence of a specific band for each of the proteins exclusively in the +TET3 IP and not in the +IgG (negative control) IP. Total nuclear extracts, treated with EtBr and benzonase, were used as input (*right*). Note that TET3, FOXP1, AIOLOS, and IKAROS are detected by western blot in both samples. Histone 3 (H3) was used as loading control. One out of two representative experiments is shown.

which however fail to restore the breaks.[15] It has been shown in cell lines and ES cells that 5hmC is accumulated in DSBs and mediates recruitment of DNA repair proteins.[123] 5hmC has been reported to be enriched at sites of double-strand breaks and colocalizes with DNA repair proteins such as 53BP1 as well as histone γH2Ax.[123] In addition, 5caC and 5fC have been identified in mass spectrometry experiments to be preferentially recognized by DNA repair proteins, such as p53,[8] suggesting that they can play a role in promoting DNA repair. Moreover, TET1-deficient oocytes exhibit increased unresolved DNA breaks.[71] Here, our data suggest that TET3 may also be involved in DNA repair in total thymocytes by interacting with DNA repair proteins. Here, our analysis further implicates TET3 in interacting with DNA repair proteins in developing thymocytes. Moreover, TET2 has been shown to interact with proteins involved in DNA repair such as PARP1, XRCC6, DNAJA1, and MSH2[43,45] (Table S2). Given our data, the observed genomic instability upon loss of TET proteins in T cells can, at least in part, be attributed to defects in recruitment of the DNA repair machinery due to lack of oxi-mCs. We hypothesize that TET3 by oxidizing 5mC to oxi-mCs enhances recruitment of DNA repair proteins and can form complexes with some of these proteins to promote DNA repair. Collectively, the identified interactions of TET3 with DNA repair proteins provide a causal link between TET proteins, 5hmC, and genomic stability in primary T cells.

Another emerging function of TET3 from our data analysis is its interaction with transcription factors and chromatin-modifying complexes. Specifically, we provide evidence that TET3 interacts with CTCF and additional proteins that participate in DNA looping (Figure 5), raising the possibility that TET3 can be involved in regulating the 3D chromatin conformation in T cells. Adding on this is our finding that TET3 can also physically interact with BCL11B (Figure 5), a protein with multifaceted roles in T cell differentiation, proliferation, and survival, which has also been recently implicated in regulating chromatin conformation during the differentiation of DN cells to DP cells.[57] While there have not been reports for this in T cells, it has been suggested that 5hmC may promote binding of the Yin Yang 1 factor (YY1) in mouse ESCs.[124] As YY1 mediates looping to bring in proximity promoter-enhancers and regulate gene expression, murine ESCs that lack all three TET proteins exhibit reduced YY1 binding and altered chromatin conformation compared to the wild-type ESCs.[124] Based on the focal DNA demethylation regulated by TET proteins in T cells,[15,16] we speculate that TET3 and 5hmC play a role to regulate chromatin conformation of genes that are involved in shaping lineage choice and forging cell identity. In-depth studies are required to systematically address the precise role and impact of TET3 and oxi-mCs in forming higher-order chromatin structures.

Importantly, we have previously identified downregulation of *Bcl11b* gene expression in the thymic T cell subpopulation known as iNKT stage 2 cells.[15] BCL11B suppresses NKT17 cell differentiation and promotes NKT2 and NKT1 differentiation.[125] A plausible scenario is that one of the mechanisms that result in NKT17[37] skewing of *Tet3* KO and to a larger degree *Tet2/3* DKO iNKT cells[15,38] is aberrant BCL11B function due to loss of interactions with TET3.

Moreover, BCL11B is known to further be part of chromatin remodeling complexes such as the SWI/SNF complex and the NuRD complex.[109] Importantly, in our study, we were able to identify as TET3 interacting partners various SMARC proteins that are subunits of the SWI/SNF complex as well as CHD4, HDAC1, and HDAC2 that are subunits of the NuRD complex (Figure 5). In addition, TET2 has been shown to interact with various SMARC proteins that we identify as TET3 interactors (Table S2). Specifically, TET2 was found to interact with Smarcb1, Smarcc2, Smarcd2, and Smarce1 in rapid immunoprecipitation mass spectrometry of endogenous protein experiments performed in MCF7 cells.[45] TET3 has been previously shown to interact with HDAC1 in overexpressed conditions in HEK293T cells.[105] Participation of TET3 in these complexes may be important to suppress gene expression.

Another critical factor that emerged in our study as TET3 interacting partner was SATB1 that exerts critical roles in shaping T cell biology.[54,56,126] Proteomic data in the thymus indicate that SATB1 interacts with proteins such as NONO, NUMA1, MYBBP1A, SIN3A, Polymerase 2, and CHD4 (Table S3), which we report as interacting partners of TET3 (Tables S1 and S3). Adding on, SATB1 is reported as a potential interacting factor of BCL11B[110] (Table S3). Collectively, these findings implicate TET3 in common protein networks with SATB1 and BCL11B and suggest that together they may play roles in regulating chromatin organization.

Despite the critical impact of TET proteins on T cell biology,[6,70] the cell-specific factors that mediate recruitment of TET proteins across the genome in developing T cells remain elusive. We identified as TET3 direct interactors the transcription factors GATA3 (Figures 6A; Table S1), FOXP1, as well as AIOLOS and IKAROS (Figures 6C; Table S1). We have previously demonstrated that GATA3 expression is reproducibly reduced in the absence of TET2 and TET3 in CD4 SP cells.[36] Moreover, we have shown that GATA3 cannot bind a regulatory element located in the gene body of *Zbtb7b*.[36] That site in *Tet2/3* DKO cells exhibited gain of methylation.[36] Adding on to this discovery, in the light of our data, we hypothesize that TET3 interacts with GATA3 to regulate expression of ThPOK in CD4 SP cells. We wish to note that we were not able to validate the interaction of GATA3 with TET3 by immunoblot. However, the enrichment in all 4 biological replicates immunoprecipitated with TET3 and subjected to LC-MS/MS was clear (Table S1). To add further confidence to our data, GATA3 and TET2 have been shown to

interact in MCF7 cells, and various proteins that we identify in this study as TET3 interactors were reported to be common interactors of GATA3 and TET2 in MCF7 cells.[45] We summarize some of these interactions that are relevant to our data in Table S2.

IKAROS and AIOLOS are two of the five members of the IKAROS family of transcription factors that play critical and multifunctional roles in lymphocyte development and differentiation.[117] FOXP1 is a transcription factor involved in regulating FOXP3 binding and function of Tregs.[127] FOXP1 acts as a pioneer transcription factor that binds genomic sites in both Tregs and non-Tregs that are occupied and regulated by FOXP3 in Tregs.[127] Importantly, loss of FOXP1 impairs Treg function and is associated with reduced CD25 expression and response to the cytokine IL-2.[127] Recently, it was shown that TET3-deficient Tregs express reduced levels of CD25 and show defects in response to IL-2.[128] Thus, FOXP1 may recruit TET3 to impact this process. In addition, *Tet2/3* DKO Tregs show impaired functions that are only partially explained by aberrant DNA demethylation of the intronic enhancer CNS2 that regulates the stability of FOXP3 expression.[33] Along these lines, it has been demonstrated that loss of FOXP1 results in unstable FOXP3 expression by binding the promoter and the intronic CNS2 enhancer of FOXP3. Importantly, FOXP1 has been involved in establishing quiescence of T cells,[116] and downregulation of FOXP1 in T cells has been reported in humans who suffer from lymphoproliferative disorders.[129] Thus, impaired interaction of FOXP1 with TET3 could be involved, at least in part, to the loss of quiescence and the hyperproliferation of *Tet2/3* DKO iNKT cells that we have previously described.[15]

Contemplating on the biological significance of our data, we note that while the interacting partners of TET3 are involved in fundamental procedures, such as transcription, genomic stability, splicing, and lineage choice, TET3-deficient thymic T cells do not exhibit noticeable aberrations in conventional T cell development, with the exception of the unconventional iNKT cell lineage specification.[15] Instead, TET3 must be concomitantly deleted either with TET1 to significantly affect demethylation of enhancers that are critical for later stages of CD4 function[34,35] or with TET2 to affect lineage choice, stability, proliferation, and genomic integrity in T cells.[15,33,36,64] Indeed, comparison of the discovered interacting partners of TET3 in thymocytes with existing datasets of TET1 and mainly TET2 interacting partners in other cell types indicates shared proteins involved in functions such as splicing, transcriptional elongation, and DNA repair (Table S2), suggesting cooperativity and functional redundancy of TET proteins. One could speculate that TET3 forms complexes with other TET proteins. However, our LC-MS/MS data did not identify either TET1 or TET2 among the TET3 interacting partners in the stringent or non-stringent conditions (Table S1). This could be attributed to technical limitations, the low expression levels of TET proteins, and/or to the fact that only after TET3 is deleted other TET proteins can participate in the identified complexes to compensate for TET3 loss. Another attractive scenario is that different TET proteins might play different roles in regulating these processes in T cells. For instance, it has been suggested that in ESCs, TET1 interacts with SALL4 to recruit the factor at genetic loci and then SALL4 recruits TET2 to mediate DNA demethylation.[130] Thus, we can envision that TET3, similarly to TET1, can be recruited to the DNA to regulate subsequent binding of other factors that can in turn bring in TET2. Future studies will reveal the precise kinetics of TET recruitment and most importantly the *in vivo* impact of the identified interactions.

Limitations of the study

In this study, we provide a resource of the TET3 interactome in primary developing T cells isolated from murine thymus. A limitation of the study is that we cannot distinguish which of the identified proteins directly interact with TET3 and which form protein complexes in which TET3 also participates. Moreover, in this study, we used total thymocytes that consist of distinct subsets. Most total thymocytes are DP cells that are positively selected to give rise to distinct subsets: CD4 SP cells that also comprise precursors of the Tregs (the known precursors are CD25+Foxp3- cells, as well as CD25-Foxp3+ cells,[131,132] iNKT cells, and CD8 SP cells. This approach may have resulted in lower representation of lineage specifying factors. However, we note that in the LC-MS/MS data, we do identify transcription factors such as GATA3, FOXP1, members of the IKAROS family (namely AIOLOS and IKAROS), and members of the RUNX family as specific interactors of TET3. We consciously opted to use total thymocytes as our goal was to identify proteins that interact with TET3 at endogenous level *in vivo*. The cell-specific subsets are much less frequent than the DP cells and TET3 is a lowly expressed protein. Thus, it would not be feasible to perform fluorescence-activated cell sorting to isolate sufficient number of the positively selected subsets. In addition, if we had attempted to enrich for specific subsets, the long processing time could affect the protein complexes. Single-cell proteomics would be an interesting approach to allow detection of specific interactions while we identify the precise identity of the cells that we assess. However, we should keep in mind that a single-cell approach may fail to identify lowly expressed proteins. Another technical limitation that we would like to mention is the lack of a germline TET3 knockout that would potentially have allowed us to use as additional negative control TET3-deficient cells. However, we emphasize that researchers should carefully evaluate the biological features of deficient cells since frequently deletion of critical proteins for gene expression can alter the identity of the cells, compromising thus their value as a proper control for identifying protein interactions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

D.T.: immunoprecipitation experiments and immunoblot, immunoblot data analysis, and relevant methods writing, T.H.: sample preparation for LC-MS/MS, data collection, data analysis, and LC-MS/MS report writing, G.W.: LC-MS/MS data analysis, T.Ä.: data analysis and data visualization, L.M.S.: data analysis and study design, E.J.S.: study design, data analysis, LC-MS/MS report writing, and scientific oversight, and A.T.: study design, data analysis, manuscript writing, funding acquisition, and study supervision. All the authors have critically read the manuscript, provided feedback, and agree with its content.

DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-AIOLOS (clone: D1C1E, dilution in 5% BSA 1:500 for IP and 1:1000 for input)	Cell Signaling Technology	Cat# 15103; RRID: AB_2744524
anti-BCL11B (clone: D6F1, dilution in 5% BSA, 1: 1500 for IP and 1:3000 for input)	Cell Signaling Technology	Cat# 12120; RRID: AB_2797823
anti-CTCF (clone: D31H2, dilution in 5% BSA, 1: 1500 for IP and 1:3000 for input)	Cell Signaling Technology	Cat# 3418; RRID: AB_2086791
anti-FOXP1 (clone: D35D10, dilution in 5% non-fat milk, 1:750 for IP and 1:1000 for input)	Cell Signaling Technology	Cat# 4402; RRID: AB_10545755
anti-IKAROS (clone: D6N9Y, dilution in 5% non-fat milk, 1: 1000 for IP, 1:3000 for input)	Cell Signaling Technology	Cat# 14859; RRID: AB_2744523
anti-MCM2 (clone: D7G11, dilution in 5% BSA, 1:1500 for IP, 1:3000 for input)	Cell Signaling Technology	Cat# 3619; RRID: AB_2142137
anti-MSH2 (clone: D24B5, dilution in 5% BSA, 1:1500 for IP and 1:3000 for input)	Cell Signaling Technology	Cat# 2017; RRID: AB_2235387
anti-PAF1 (clone: D9G9X, dilution in 5% non-fat milk, 1:250 for IP and 1:1000 for input)	Cell Signaling Technology	Cat# 12883; RRID: AB_2798052
anti-SF3B1 (clone: D7L5T, dilution in 5% BSA, 1:500 for IP,1:3000 for input)	Cell Signaling Technology	Cat# 14434; RRID: AB_2798479
anti-SRSF3 (clone: E9U9C, dilution in 5% BSA, 1:1500 for IP, 1: 3000 for input)	Cell Signaling Technology	Cat# 35073; RRID: AB_3065261
anti-TET3 (clone: E6J8A, dilution in 5% BSA, 1:1000 dilution for IP and input)	Cell Signaling Technology	Cat# 99980; RRID: AB_2928150
anti- XRCC1 (clone: E4A3V, dilution 5% in non-fat milk, 1:1000 for IP, 1:2000 for input)	Cell Signaling Technology	Cat# 76998; RRID: AB_2936252
anti-Histone H3 (dilution 1:10,000 in 5% non-fat milk)	Sigma Aldrich	Cat# H0164; RRID: AB_532248
anti-rabbit IgG antibody	Cell Signaling	Cat# 2729; RRID: AB_1031062
Veriblot for IP detection reagent HRP (dilution 1:500)	Abcam	Cat# 131366; RRID: AB_2892718
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (dilution 1:2000)	Cell Signaling Technology	Cat# 1721019; RRID: AB_11125143
Chemicals, peptides, and recombinant proteins		
Restore western blot stripping buffer	Thermo Scientific	Cat# PI21059
cOmplete, EDTA-free Protease Inhibitor Coctail	Roche	Cat# 11873580001
Benzonase nuclease	Sigma Aldrich	Cat# E8263
UltraPure Ethidium bromide	Thermo Scientific	Cat# 15585011
Precision Plus Protein All Blue Pre-stained Protein Standards (protein marker)	BIO-RAD	Cat# 161-0373
Color Pre-stained Protein Standard, Broad Range (10 -250kDa)	NEB	Cat# P7719S
PMSF	Sigma Aldrich	Cat# P7626
Critical commercial assays		
NE-PER nuclear and cytoplasmic extraction kit	Thermo Scientific	Cat# 78833
BCA protein assay kit	Thermo	Cat# 23235
Silver stain plus kit	Biorad	Cat# 1610449

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pierce ECL Western blotting substrate	Thermo Scientific	Cat# 32209
Deposited data		
TET3 proteome	This study	PRIDE repository: identifier PXD045465
Experimental models: Organisms/strains		
C57/BL6J (male and female mice are used, age 4–6 weeks old)	The Jackson Laboratories (purchased during the past 4 years and used directly or bred in house at UNC)	Cat# 000664; RRID: IMSR_JAX:000664
Software and algorithms		
STRING	Szklarczyk et al.,[73]	https://string-db.org/
Venny	Oliveros et al.,[136]	https://bioinfogp.cnb.csic.es/tools/venny/index.html
GO	Raudvere et al.,[135]	https://biit.cs.ut.ee/gprofiler/gost
Proteome Discoverer 3.0	Thermo Scientific	N/A
NovoExpress	Agilent	N/A
Other		
Thermo Orbitrap Fusion Lumos high resolution accurate mass tandem mass spectrometer	Thermo Scientific	N/A
Synergy 5 microplate reader	Biotek	
Trans Blot Turbo transfer system	Biorad	Cat# 1704150
Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels	Biorad	Cat# 1658004
ChemiDoc MP imaging system	Biorad	N/A
Novocyte Cytometer	Agilent	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests regarding this resource study should be directed to and will be fulfilled by the lead contact Ageliki Tsagaratou (ageliki_tsagaratou@med.unc.edu).

Materials availability

This study did not generate any new reagents or materials.

Data and code availability

- All experimental data are available within this article and raw proteomic data are available via ProteomeXchange: PXD045465. Original western blot images are provided in the supplemental items.
- This study did not generate new code.
- Any additional information to reanalyze data in this study is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

Mice were housed under pathogen-free conditions at University of North Carolina (UNC) Genetic Medicine building, in a facility managed by the Division of Comparative Medicine at UNC Chapel Hill. All the procedures were approved by the UNC Institutional Animal Care and Use Committee and are described in our protocol 22–252. C57BL/6 (B6) (stock number: 000664) were obtained from Jackson Laboratories and subsequently were bred in our facility. Age-matched, 4–6 weeks old, male and female mice were assessed.

METHOD DETAILS

Isolation of primary murine thymic T cells

Mice were dissected and thymi were isolated and placed immediately in tubes containing ice-cold T cell medium as previously described.[133,134] The tubes were placed on ice until the tissue was processed. Each thymus was placed inside a 70 μm cell strainer (BD Falcon,

cat no: 352350) inside a 60 mm plate (BD Falcon) and lysed using the pestle of a 1 mL syringe without needle.[133,134] Cells were measured in a Novocyte cytometer (Acea Agilent) using the NovoExpress software (Agilent).

Isolation of nuclear extracts and immunoprecipitation (IP)

Proteins were isolated from nuclei of total thymocytes from C57BL/6 (B6) mice. For the extraction of the nuclear proteins, we used the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, cat no 78833) with some modifications. Specifically, the nuclei were isolated from total thymocytes as described in the NE-PER kit by utilizing the CERI and CERII buffers, supplemented with 1xProtease inhibitors (Roche, cat no: 11873580001) and 1 mM PMSF (Sigma Aldrich, cat no P7626). Subsequently, the nuclei were lysed in Lysis buffer (0.5 mM Tris-HCL pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 0.5% Triton and 5% glycerol) supplemented with 1xProtease inhibitors and 1 mM PMSF. In the experiments investigating the direct protein partners of TET3, 250 U/mL benzonase (Sigma Aldrich, cat no E8263) and 10 μg/mL ethidium bromide (EtBr) (Thermo Scientfic, cat no 15585011) are also added in the Lysis buffer. The cell lysates were incubated on a rotator for 40 min, at 4°C, followed by a centrifugation for 20 min, at 4°C. The supernatants, containing the nuclear proteins, were collected in pre-chilled 1.5 mL tubes and their quantity was determined using the BCA kit (Thermo Scientific, cat no 23235) in a Synergy 5 microplate reader (Biotek). For the IP, 600 μg of nuclear proteins diluted in 500 μL of Lysis buffer were incubated with 5 μg TET3 antibody (Cell Signaling Technology, cat no 99980) or with 5 μg anti-rabbit IgG antibody (Cell Signaling Technology, cat no 2729) that is used as a negative control. Again, in the experiments studying the direct protein-protein interactions, 250 U/mL benzonase and 10 μg/mL EtBr are added on each IP reaction. The samples are rotated for 3 h at 4°C, and then, are mixed with 20 μL pre-washed A/G Magnetic Beads (Thermo Scientific, cat no 88802) and are rotated for an additional 1 h at 4°C. Subsequently, the beads are washed three times with the Lysis buffer and two times with the Lysis buffer without Triton. Each wash includes a 3–5 min rotation at 4°C. After the final wash, the proteins are eluted from the beads by adding 40 μL 1× Elution buffer (25 mM Tris pH 6.8, 50 mM NaCl, 1% SDS, 10 mM DTT, 0.02% Bromophenol blue) and boiling them for 10 min with 500 rpm shaking. The input samples are diluted in 4× Elution buffer and boiled for 10 min.

SDS-PAGE electrophoresis, silver staining and immunoblotting

The immunoprecipitated proteins and 5% input of the nuclear extracts are separated by SDS-PAGE electrophoresis. Prior to electrophoresis, glycerol is added to the samples to a final concentration of 10% and the samples are boiled for 5 min. Subsequently, the samples are loaded on either 4–20%, or 12%, or 7.5% Mini-PROTEAN TGX Precast Protein Gels (Bio-rad) and subjected to SDS-PAGE electrophoresis. For the silver staining analysis, the gels were stained with the Silver Stain Plus (Bio-Rad, Cat. No. 161–0449) according to the manufacturer's instructions. The immunoblotting analysis was performed as previously described.[36] Briefly, after the preparation of electrophoresis, the proteins were transferred to PVDF membranes through the use of the Trans-Blot Turbo Transfer System (Biorad). The membranes were then blocked for 1 h, at room temperature (RT) with 5% non-fat milk (Biorad) diluted in TBS supplemented with 0.1% Tween (Sigma). For the protein-specific detection, the membranes were incubated with the primary antibodies described in key resources table, overnight (o/n) at 4°C and afterward were incubated with anti-HRP secondary antibodies for 1 h, RT. Specifically, for the membranes containing the IP samples, we used Veriblot for IP detection reagent (dilution 1:500, Abcam, cat no 131366) as a secondary antibody, while the membranes containing the input samples were incubated with an anti-rabbit secondary antibody (dilution 1:2000, Bio-Rad, cat no 1721019). The signals were visualized by utilizing the Pierce ECL Western blotting substrate (Thermo Scientific, cat no: 32209) and the ChemiDoc MP imaging system (Biorad). In some cases, to re-probe a membrane in order to detect another protein of interest, the membrane was stripped using the Restore western blot stripping buffer (Thermo scientific, cat no: PI21059), as instructed by the manufacturer.

Sample preparation for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

The Duke Proteomics and Metabolomics Core Facility (DPMCF) received 16 samples (4 of each TET3 IP, IgG IP, TET3 IP treated with EtBr and benzonase, and IgG IP treated with EtBr and benzonase) which were kept at −80°C, until processing. Samples were spiked with undigested bovine casein at a total of either 250 or 500 fmol as an internal quality control standard. Next, samples were supplemented with 5.9 μL of 20% SDS, reduced with 10 mM dithiolthreitol for 30 min at 80°C, alkylated with 20 mM iodoacetamide for 30 min at room temperature, then supplemented with a final concentration of 1.2% phosphoric acid and 383 μL of S-Trap (Protifi) binding buffer (90% MeOH/100 mM TEAB). Proteins were trapped on the S-Trap micro cartridge (Protify), digested using 20 ng/μL sequencing grade trypsin (Promega) for 1 h at 47°C, and eluted using 50 mM TEAB, followed by 0.2% FA, and lastly using 50% ACN/0.2% FA. All samples were then lyophilized to dryness. Samples were resolubilized using 12 μL of 1% TFA/2% ACN with 12.5 fmol/μL yeast ADH.

LC-MS/MS analysis

Quantitative LC-MS/MS was performed on 3 μL using an MClass UPLC system (Waters Corp) coupled to a Thermo Orbitrap Fusion Lumos high resolution accurate mass tandem mass spectrometer (Thermo) equipped with a FAIMSPro device via a nanoelectrospray ionization source. Briefly, the sample was first trapped on a Symmetry C18 20 mm × 180 μm trapping column (5 μL/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.8 μm Acquity HSS T3 C18 75 μm × 250 mm column (Waters Corp.) with a 90-min linear gradient of 5–30% acetonitrile with 0.1% formic acid at a flow rate of 400 nL/min (nL/min) with a column temperature of 55°C. Data collection on the Fusion Lumos mass spectrometer was performed for three difference compensation voltages (−40v, −60v, −80v). Within each CV, a data-dependent acquisition (DDA) mode of acquisition with an r = 120,000 (@ m/z 200) full MS scan from m/z 375–1500 with a target

AGC value of 4e5 ions was performed. MS/MS scans were acquired in the ion trap in Rapid mode with a target AGC value of 1e4 and max fill time of 35 ms. The total cycle time for each CV was 0.66s, with total cycle times of 2 s between like full MS scans. A 20s dynamic exclusion was employed to increase depth of coverage. The total analysis cycle time for each injection was approximately 2 h.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative data analysis

Following UPLC-MS/MS analyses, data were imported into Proteome Discoverer 3.0 (Thermo Scientific Inc.). In addition to quantitative signal extraction, the MS/MS data was searched against the SwissProt *M. musculus* database (downloaded in Nov 2019) and a common contaminant/spiked protein database (bovine albumin, bovine casein, yeast ADH, human keratin, etc.), and an equal number of reversed-sequence "decoys" for false discovery rate determination. Sequest with Infernys enabled (v 3.0, Thermo PD) was utilized to produce fragment ion spectra and to perform the database searches. Database search parameters included fixed modification on Cys (carbamidomethyl) and variable modification on Met (oxidation). Search tolerances were 2 ppm precursor and 0.8Da product ion with full trypsin enzyme rules. Peptide Validator and Protein FDR Validator nodes in Proteome Discoverer were used to annotate the data at a maximum 1% protein false discovery rate based on q-value calculations. Peptide homology was addressed by only using unique peptides for quantitation. Protein homology was addressed by grouping proteins that had the same set of peptides to account for their identification. A master protein within a group was assigned based on % coverage.

Statistical enrichment of interaction proteins

Relative fold-changes between various sample groups were performed based on the protein expression values and two-tailed heteroscedastic t-test on log2-transformed data were calculated. Those fold changes and *p*-values are presented for all proteins in Table S1. A protein was considered enriched over IgG control background if a fold-change of >1.5-fold and *p*-value of <0.05 were met.

ADDITIONAL RESOURCES

Gene ontology analysis

The Gene Ontology (GO) analysis was performed using the gProfiler: GOSt database (https://biit.cs.ut.ee/gprofiler/gost).[135]

Overlapping proteins

To identify overlapping proteins and design Venn diagrams we used Venny (https://bioinfogp.cnb.csic.es/tools/venny/index.html).[136]

Local STRING network clusters

We used the STRING software.[73] Specifically, STRING employs physical interactions as well as functional associations. For the purpose of our study, for the STRING analysis we used exclusively the proteins that were found to be interacting with TET3 in the treated samples and were statistically significant as indicated by a *p*-value lower than 0.05. To identify functional clusters formed by the proteins that we discovered to physically interact with TET3 we used Markov clustering. We used threshold of high confidence (0.70). Our analysis revealed 213 clusters. Some proteins participated in more than one clusters.