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Research article

Integrated single-cell and spatial transcriptomic analysis reveals YBX1 drives immune regulation in GBM progression

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ABSTRACT

The RNA modification 5-methylcytosine (m5C) is widespread across various RNA types, significantly impacting RNA stability and translational efficiency. Accumulating evidence highlights its significant role within the tumorigenesis and progression of multiple malignancies. Nevertheless, the specific process through m5C is implicated in Glioblastoma (GBM) remains unclear. We conducted acomprehensive analysis of m5C expression distribution in single-cell GBM data. Our findings revealed elevated m5C scores in GBM single-cell data compared to the normal group. Additionally, multiple tumors exhibited significantly higher m5C scores than the normal group. Moreover, there was a positive correlation observed between the m5C score and inflammation score. m5C regulatory factor YBX1 exhibited a heightened expression in GBM, correlating closely with metastatic tendencies and an unfavorable prognosis across various cancer types. YBX1 has different biological functions in myeloid cells 1 and myeloid cells 2. YBX1 may act as immunosuppressive regulator by inhibiting the NF-κB pathway and inflammatory response in myeloid cells 1. YBX1 is essential for immune infiltrates, which creates a highly immunosuppressive tumor microenvironment by TNF signaling pathway in myeloid cells 2. YBX1+ neoplastic cells promote cell proliferation by NF-κB pathway. APOE mediates the interaction of YBX1+ myeloid cells and neoplastic cells by NF-κB.

1. Introduction

Glioblastoma (GBM) stands out as the most prevalent and aggressive primary brain tumor in adults, with an annual incidence of approximately 3.23 cases per 100,000 population [1]. Recent statistical analyses from the Central Brain Tumor Registry of the United States (CBTRUS) reveal a median survival of approximately 8 months, irrespective of treatment. Notably, one-year, five-year, and ten-year survival rates are reported at 42.8 %, 7.2 %, and 4.7 %, respectively [2,3]. GBM is prone to recurrence, the fundamental reason is that GBM cells show diffuse infiltrative growth, the boundary of the tumor tissue is not clear. GBM belongs to malignant neuroepithelial tumors that invade the surrounding brain parenchyma in vivo mainly in the form of mesenchymal motility [4].

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Epithelial-mesenchymal transition (EMT) is the basis of GBM cell invasion [5,6]. The triggers of EMT are complex and appear to be associated with stimuli such as inflammation, ROS, hypoxia, metabolic changes, tumor microenvironment and radiotherapy [7]. However, the signaling pathways and effector molecular mechanisms that sense these stimuli and drive EMT in GBM remain unelucidated.

The critically intricate relationship is underscored by the intricate interplay between cancer cells and the tumor microenvironment (TME) [8]. TME is regarded as a pivotal factor significantly driving tumor progression [9]. Playing a crucial role in governing the proliferation, metastasis, and treatment response of tumor cells, TME serves as the supportive "soil" for tumor growth [10]. Immune cells are a major component of TME [11]. The utilization of single-cell techniques is progressively enhancing our ability to describe the phenotypic and functional adaptability of the TME within GBM [12]. Analyzing individual cells within GBM is transforming our understanding of the phenotype and functional adaptability of the TME [13]. Additional research is needed to delve into the influence of m5C modifications on the diversity within the TME and the control of immune cell infiltration in GBM.

Presently, over 170 modifications have been identified on RNAs, with methylations constituting two-thirds of the total modifications and being ubiquitously present on nearly all RNAs [14,15]. The escalating interest in the involvement of RNA modifications in cancer is noteworthy, with ongoing comprehensive research specifically focusing on m5C RNA methylation in cancer [16]. The incorporation of m5C is facilitated by intricate processes overseen by 'writers,' involving the methyltransferase complex. Additionally, 'erasers' are responsible for removing m5C through demethylase activity, while 'readers,' constituted by RNA-binding proteins, are entrusted with recognizing m5C [17,18]. In this study, we scrutinized 15 key modulators, encompassing 11 writers (NOP2, NSUN2, NSUN3, NSUN4, NSUN5, NSUN6, NSUN7, DNMT1, TRDMT1, DNMT3A, and DNMT3B), three readers (YTHDF2, ALYREF, and YBX1), and one eraser (TET2) [19]. It is worth studying further the genetic alterations and dysfunctions of m5C regulatory factors on a variety of cancer therapeutic targets.

Within this study, a thorough profiling of the expression of 15 m5C modulators was conducted, revealing discernible m5C modification profiles in GBM. Interestingly, we found that m5C modulators are closely related to myeloid cells-related immune activation. Additionally, $YBX1^{+}$ myeloid cells were the largest proportion of myeloid cells. YBX1 activate myeloid cells by NF-κB pathway. Moreover, $YBX1^{+}$ neoplastic cells promote cell proliferation by NF-κB pathway. Finally, we identified APOE mediated the interaction of $YBX1^{+}$ myeloid cells and neoplastic cells by NF-κB pathway. These investigations aim to unravel the intricate regulatory mechanisms governed by m5C modulators, furnishing a theoretical foundation for the development of strategies targeting and immunotherapy against YBX1.

2. Materials and methods

This study's overall workflow diagram are illustrated in Fig. 1.

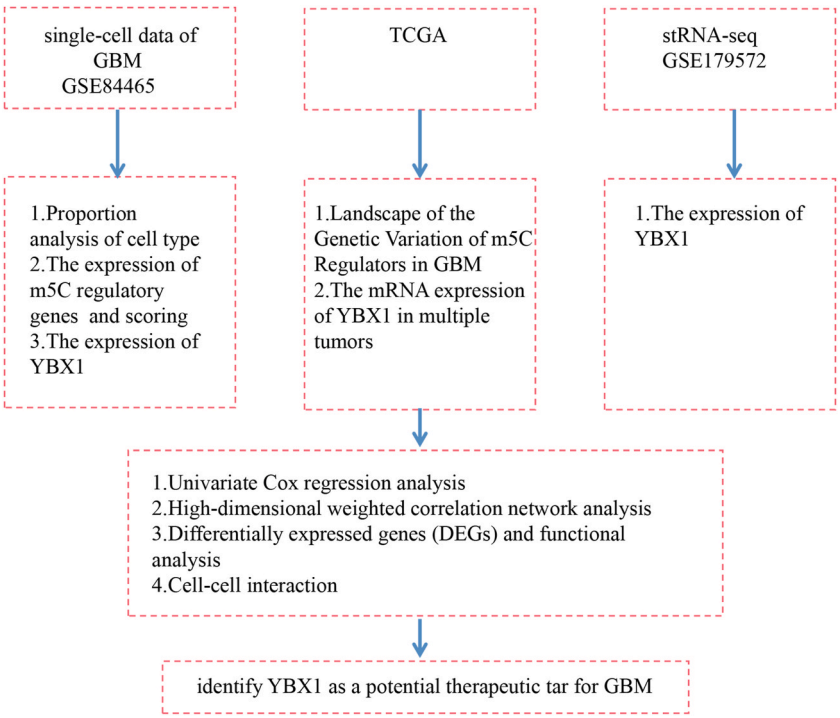

Fig. 1. Graphical summary of the study protocol.

2.1. Data collection and processing

The publicly available single-cell data for GBM (accession number GSE84465) were acquired from the University of California at Santa Cruz (UCSC) Cell Browser. Single-cell RNA sequencing (RNA-seq) was performed on 3589 cells in a cohort of four GBM patients. Cells were obtained from the tumor core as well as surrounding peripheral tissue. tumor core was group as tumor and surrounding peripheral tissue group as nomal. Moreover, the bulk RNA-seq expression dataset, somatic mutations and copy number variants (CNV) data for GBM, including 168 glioblastoma and 5 normal brain tissues, as well as the corresponding phenotype dataset for multiple tumors from The Cancer Genome Atlas (TCGA) [20]. Visualisation was performed using R packages "maftools" and "Rcircos." Immunohistochemical images depicting human normal tissues and tumor tissues were sourced from the Human Protein Atlas (HPA, https://www.proteinatlas.org/). Adherence to the published guidelines of TCGA and UCSC ensured ethical compliance, obviating the need for explicit ethical approval and informed consent from patients. Additionally, we retrieved spatial transcriptome RNA sequencing (stRNA-seq) data for GBM using the 10× Visium platform from GSE179572.

2.2. Prognosis analysis

M5C feature scores for GBM patients within the TCGA cohort were computed using Gene Set Variation Analysis (GSVA). We used Kaplan-Meier analysis in conjunction with a log-rank test to assess the relationship between YBX1 expression levels and clinical outcomes, particularly overall survival (OS), in diverse cancer types present in the TCGA pan-cancer dataset. Prognostic parameters, such as WHO grade and IDH status were analyzed using glioma patient data from the TCGA in the clinical meaning module of the Xiantao platform (https://www.xiantao.love/).

2.3. Immune infiltration analysis

The TISIDB database (http://cis.hku.hk/TISIDB/index.php) is an integrated repository portal for tumour-immune system interactions based on TCGA datasets.

2.4. Differentially expressed genes (DEGs) and functional analysis

A comprehensive set of 15 m5C RNA methylation modulators was curated from published literature (refer to Supplementary Table S1) [21]. DEGs analysis was executed using the "FindMarkers" function within the Seurat packages, employing default parameters. Adjusted $p < 0.05$ and $|\log2FC| > 0.25$ were used to define DEGs' significance. Subsequent gene set functional analyses were carried out utilizing the R packages 'clusterProfiler' [22] and 'GSVA' [23]. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for GSVA analyses. Hallmark gene sets were obtained from R package 'msigdbr'. The microenvironment in GBM exhibited a pronounced level of inflammation. To assess the inflammatory state in both GBM and normal groups, a functional gene set scoring method was employed, evaluating inflammatory signaling with gene signatures sourced from MSigDB.

2.5. Protein-protein interaction (PPI) network analysis

Protein-protein interaction information of DEGs was acquired from Search Tool for the Retrieval of Interacting Genes (database (http://www.stringdb.org/).

2.6. Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) [24] was executed in the GBM cohort to gain deeper insights into the molecular mechanisms underlying the DEGs. Gene sets meeting criteria of a false discovery rate (q-val) less than 0.25 and a normalized (NOM) p-value less than 0.05 were deemed significant. A positive normalized enrichment score (NES) indicated a positive association of the gene with the pathway, while a negative NES suggested a negative association.

2.7. HdWGCNA analysis

High dimensional weighted gene co-expression network analysis (hdWGCNA) was employed to construct a scale-free network at the single-cell level, utilizing the R package 'hdWGCNA'. The scale-free topology model fit threshold was set at > 0.85, and the optimal soft threshold of 5 was chosen to maximize connectivity.

2.8. Cell-cell interaction

The NicheNetr package was utilized to investigate signaling pathways, aiming to identify instances where intracellular communication might contribute to variances in gene expression among different disease stages and healthy controls [25]. We combined the expression of YBX1 and immune cell infiltration to analyze its impact on tumor cells. Consequently, we constructed a recognized cell interaction network among myeloid cell YBX1-positive cells, myeloid cell YBX1-negative cells, and tumor cells. We chose myeloid cells as sender cells due to their high correlation with lymphocyte activation, while tumor cells were selected as receiver cells because of

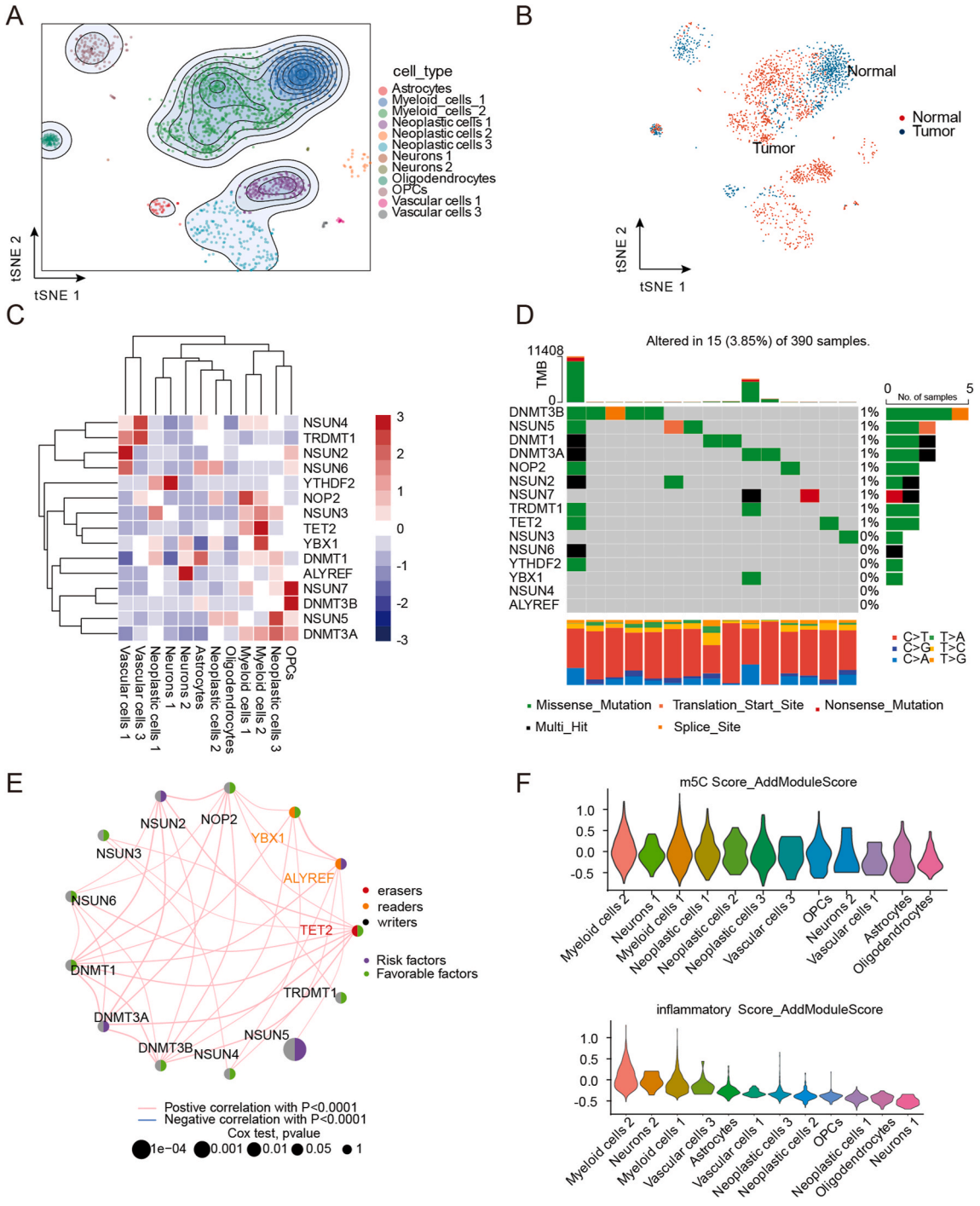

Fig. 2. Landscape of m5C methylation modulators in normal and GBM tissues. A: tSNE plot depicting the major cell types identified by single-cell sequencing. B: tSNE plot depicting the major cell types identified by healthy controls and tumor tissues. C: Heatmap showing the expression level of 15 m5C modulators in GBM. D: 15 of 390 samples have genetic alterations of 15 m5c modulators. E: Interaction between m5c modulators. Red dots represent erasers, orange dots represent readers, and gray dots represent writers. Pink lines represent positive correlation between m5c modulators, and blue lines represent negative correlation between m5c modulators. The size of each circle represents the prognostic effect of each adjustment factor and is scaled by P value. Purple indicates risk factors, and green indicates favourable factors. F: we utilized AddModuleScore to calculate the m5c score and inflammatory gene set score. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

their sustained increase in GBM compared to healthy control groups.

2.9. Statistical analysis

Two-group differences were assessed using the Wilcoxon rank-sum test, and correlations were determined using the Spearman rank test. Survival analysis utilized Kaplan-Meier analysis with the log-rank test. Statistical analyses were performed using R (version 4.2.2), and significance was denoted by p-values: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

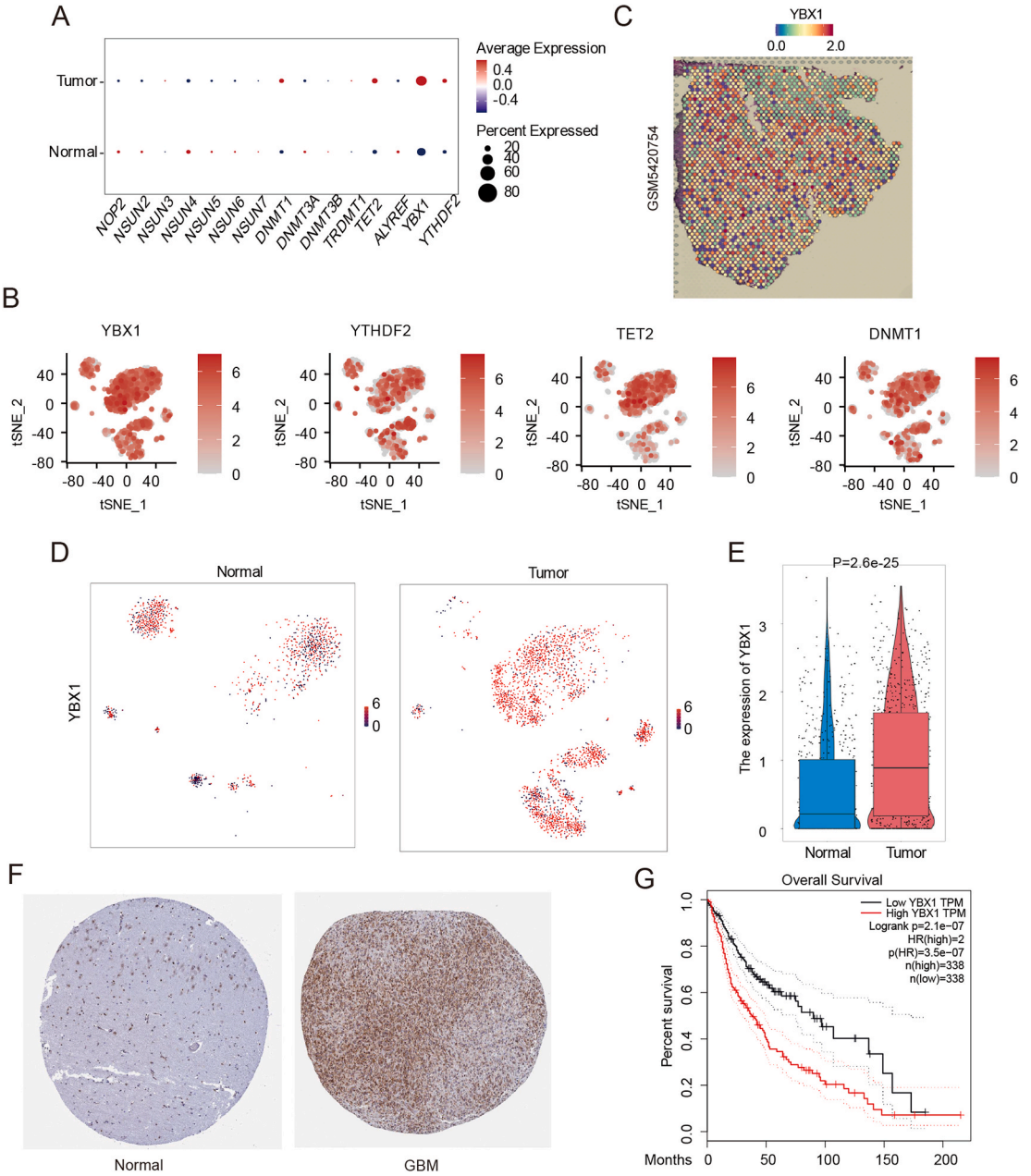

Fig. 3. YBX1+ myeloid cells exhibited the most largest proportion in myeloid cells A: 15 m5C modulators genes used to depicting cell types. Dot size represents % of cells of that cluster expressing the given gene, whereas colour indicates the expression level of that cluster. B: tSNE visualisation the expression of genes of YBX1,YTHDF2,TET2 and DNMT1 in normal and GBM. C: The expression of YBX1 in spatial transcriptome sequencing. D: tSNE visualisation the expression of genes of YBX1 in single-cell sequencing. E: Relationships between the expression of genes of YBX1 in normal and GBM. F: The IHC images of YBX1 in normal and tumor tissues extracted from the HPA. G: The Effects of YBX1 expression on OS in glioma. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. M5C modulators are closely associated with myeloid cells-related immune activation

We download the matrix, cell meta-annotations and dimensionality reduction coordinates from the UCSC database. After initial quality control, we obtained a total of 3588 cells in a cohort of four patients, 1189 from peripheral and 2399 from GBM tumor tissues. Major cell types were annotated as astrocytes, myeloid cells 1, myeloid cells 2, neoplastic cells 1, neoplastic cells 2, neoplastic cells 3, neurons 1, neurons 2, oligodendrocytes, OPCs, vascular cells 1, and vascular cells 3, based on established marker genes for specific cell types (Fig. 2A). To compare the expression profiles between cells from tumor and normal tissues, we visualized transcriptome of each sample cell by t-SNE analysis (Fig. 2B). In the tumor group, the number of myeloid cells 1 was reduced and neoplastic cells was increased compared to the normal group (Fig. S1A, Supplementary Table S2). 15 m5C regulatory genes and their expression profiles in GBM are illustrated in Fig. 2C. Alterations in copy numbers and somatic mutations in m5C regulatory genes in GBM were investigated, revealing a variation frequency of 3.85 % (15 of 390 samples), as depicted in Fig. 2D. The DNMT3B gene exhibited the highest mutation rate at 1 %, as illustrated in Fig. 2D. Additionally, ALYREF, DNMT1, NOP2, TRDMT1, and NSUN6 displayed higher frequencies of CNV amplification, while YTHDF2 and NSUN3 had increased probabilities of CNV deletions, as shown in Fig. S1B. Fig. S1C illustrates the chromosomal positions displaying CNV in m5C modulators.

Univariate Cox regression analysis highlighted the predictive significance of the 15 m5C modulators in patients with GBM (refer to Supplementary Table S3). Furthermore, the m5C modulators network highlighted the prognostic relevance of interactions among m5C modulators in patients with GBM, as shown in Fig. 2E. To further evaluate the 15 m5C regulatory genes and immune microenvironment around the GBM and normal groups, we used two scoring algorithms (AddModuleScore and AUCell) to gain a functional gene set scoring by using gene signatures from MSigDB.

The findings revealed elevated m5C scores in GBM(Fig. 2F, Fig. S1D). Myeloid cells 2 exhibited the highest m5c score among all cell types, while both myeloid cells 1 and 2 displayed elevated inflammatory scores. We surmised that myeloid cells-related immune activation in GBM and an increase in inflammatory infiltration, may be closely related.

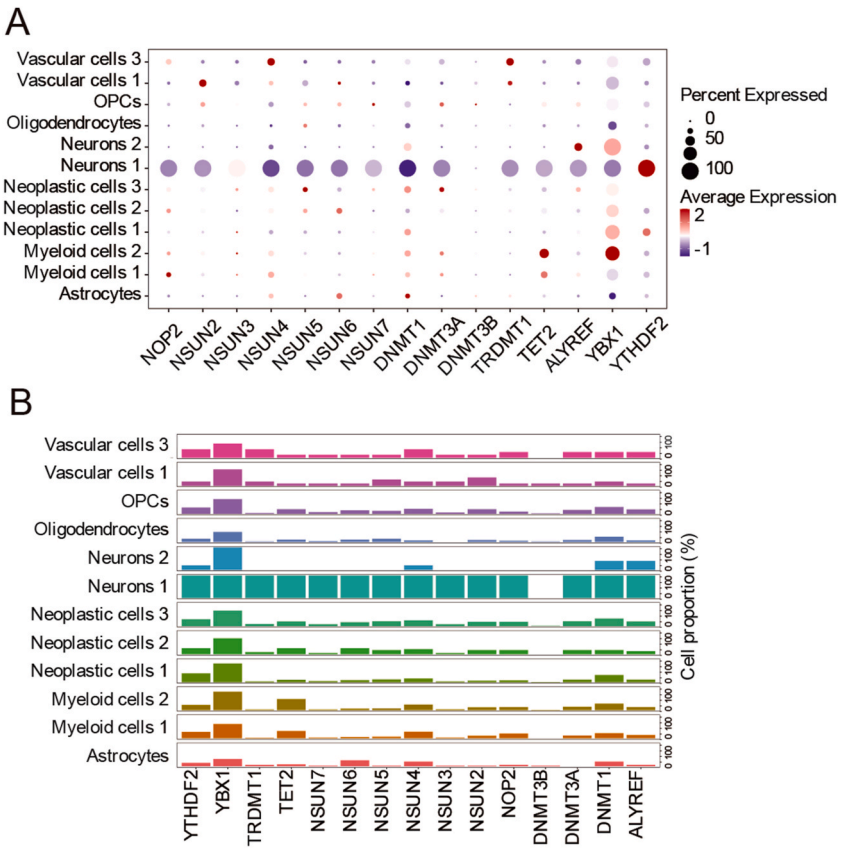

Fig. 4. 15 m5C modulators genes were used to depicting in cell type. A: 15 m5C modulators genes used to depicting in cell type. Dot size represents % of cells of that cluster expressing the given gene, whereas colour indicates the expression level of that cluster. B: Bar plot depicting the % of 15 m5C modulators genes in each cell type. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

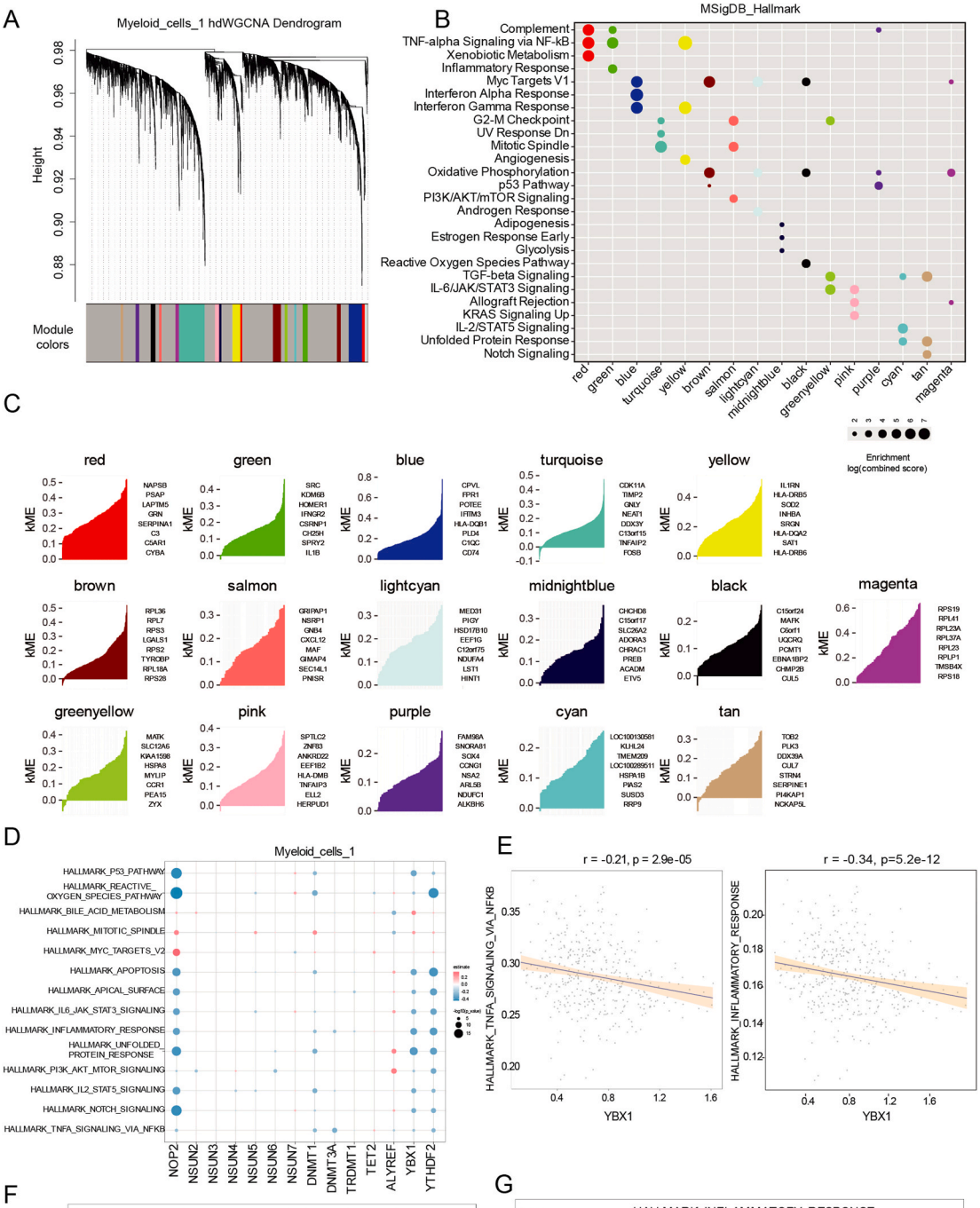

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Fig. 5. Identification of gene co-expression modules among myeloid cell 1. A: Weighed gene co-expression network analysis was constructed among myeloid cell 1. B: Dot plot of the KEGG functional enrich analysis of the module. C: The first 8 eigengenes of each module, ranked by eigengene-based connectivity (kME). D: Bubble diagram demonstrating the relevance of the m5c gene to the pathway in myeloid cells 1. E: Scatterplot demonstrating the correlation between YBX1 and the pathway in myeloid cells 1. F: Lollipop chart of correlations between YBX1 and genes in TNF-alpha signaling via NF-κB in myeloid cells 1. G: Lollipop chart of correlations between YBX1 and genes in inflammatory response pathway in myeloid cells 1.

3.2. $YBX1^+$ myeloid cells exhibited the largest proportion in myeloid cells

Next, we want to know the percentage of 15 m5C regulatory genes in cell type by average expression. The findings indicated that four genes demonstrating the highest expression in GBM were YBX1, YTHDF2, TET2, and DNMT1(Fig. 3A). YBX1 expression was linked to the clinical features of glioma(Figure S1F, Figure S1G). WHO grade were associated with higher YBX1 expression. Moreover, IDH1 mutant cases have lower YBX1 expression. YBX1 expression is significantly correlated with infiltration levels of activated $CD4^+$ T cells, Effector memeory CD8 T cell, Type 1 T helper cell, Type 17 T helper cell, Type 2 T helper cell, activated B cells, Immature B cells and natural killer cell(Fig. S1H).

In Fig. 3B, their expression patterns in GBM samples are visualized at the single-cell level using tSNE. We acquired spatial transcriptome sequencing data from a GBM patient sourced from GSE179572. Notably, YBX1 exhibited high expression in GBM tissues (Fig. S1E, Figure S2A and Fig. 3C). Differential expression analysis and survival analysis showed YBX1 higher expression accompanies poor prognosis in multiple tumors from TCGA including GBM (Fig. 3D–G,Figs S2B–S2D). YBX1 is expressed in high a percentage of myeloid cells, neoplastic cells and neurons (Fig. 4A). Next, we analyzed the percentage of m5C regulatory genes in cell type (Fig. 4B–Supplementary Table S4), and found that YBX1 was expressed in an elevated percentage of myeloid cells 1 and 2 (63.5 % and 81.9 %, respectively), which correlates with unfavorable prognosis among GBM patients.

3.3. YBX1 activate myeloid cells by TNF-alpha signaling via NF-κB pathway

Tumor-associated myeloid cells play pivotal roles in regulating tumor progression and immune evasion, highlighting their potential as viable therapeutic targets [26,27]. To clarify the diversity in cancer and myeloid cells at the cellular and molecular levels within GBM, we conducted a comprehensive analysis specifically focusing on myeloid cells 1 and myeloid cells 2. To explore the intrinsic properties of myeloid cell 1, we conducted high-dimensional weighted correlation network analysis (hdWGCNA) to elucidate the gene expression distinctions among YBX1-positive and YBX1-negative cells. In myeloid cell 1, a soft power value of 6 was chosen for building the co-expression network, as depicted in Fig. S3A. The examination yielded 16 modules, derived from the scale-free network structure in myeloid cell 1. The dendrogram illustrating these modules is presented in Fig. 5A. The red module is primarily associated with complement, NF-κB and xenobiotic metabolism. The green module is primarily associated with inflammatory response (Fig. 5B). Module eigengenes (MEs) offer a comprehensive representation of gene expression patterns present in each module (Fig. 5C). In myeloid cells 1, we found that m5c modulators are primarily associated with the reactive oxygen species pathway (p53 pathway), inflammatory response, IL-6/JAK/STAT3 signaling, IL-2/STAT5 signaling, NF-κB, unfolded protein response, and bile acid metabolism (Fig. 5D). Among them, the expression of YBX1 shows a negative correlation with the NF-κB pathway and the inflammatory response pathway (Fig. 5E). In NF-κB pathway, YBX1 resulted in up-regulation of TNFAIP2, ZBTB10, FOSB, B4GALT5, KLF6 and down-regulation of BTG3, PLAUR, OLR1, IRS2, and PLEK (Fig. 5F). In the inflammatory response pathway, YBX1 led to up-regulation of KLF6, KCNMB2, PTAFR, ABCA1, CALCRL, and down-regulation of BST2, FPR1, PLAUR, OLR1, TNFRSF1B (Fig. 5G). As shown in Fig. 5F, YBX1 regulated myeloid cells 1 activation by increasing KLF6 expression and decreasing PLAUR and OLR1 expression to inhibit NF-κB pathway.

Moreover, we investigated intrinsic properties of myeloid cell 2. In myeloid cell 2, a soft power value of 8 was chosen to build co-expression network, as shown in Fig. S3B. Eighteen modules were obtained (Fig. 6A). The turquoise module is primarily associated with inflammatory response and NF-κB. The pink module is primarily associated with notch signaling, peroxisome, and TGF-beta signaling (Fig. 6B). MEs offer a comprehensive depiction of gene expression patterns within individual modules(Fig. 6C). In myeloid cells 2, we found that m5C modulators are primarily associated with epithelial mesenchymal transition, coagulation, glycolysis, apoptosis, inflammatory response, PI3K/AKT/mTOR signaling, TGF beta signaling, and NF-κB (Fig. 6D). YBX1 expression shows a negative correlation with NF-κB and a positive correlation with epithelial-mesenchymal transition(Fig. 6E). In epithelial mesenchymal transition pathway, YBX1 affected up-regulation of TIMP1, ANPEP, TGFBI, CD44, and VIM and down-regulation of HTRA1, RHOB, ID2, MMP2, and ITGAV (Fig. 6F). In NF-κB pathway, YBX1 promoted up-regulation of ATP2B1, CD44, CXCL3, SQSTM1, BTG1 and down-regulation of EGR3, DUSP1, NR4A1, RHOB, and OLR1 (Fig. 6G). YBX1 may induce myeloid cells 2 activation mediated NF-κB pathway molecules different from myelod cells 1.

3.4. $YBX1^+$ neoplastic cells promote cell proliferation by TNF-alpha signaling via NF-κB

Comparing gene expression between neoplastic cells and normal samples unveiled notable disparities in the expression levels of numerous genes. Altogether there were 2748 DEGs ($p < 0.05$). As shown in Fig. 7A the top upregulated gene in the neoplastic cells was TMSB10, whereas the top downregulated gene was ATP1A2. Furthermore, when comparing gene expression in YBX1-positive neoplastic cells to YBX1-negative neoplastic cells, a significant difference in the expression levels of multiple genes was observed.

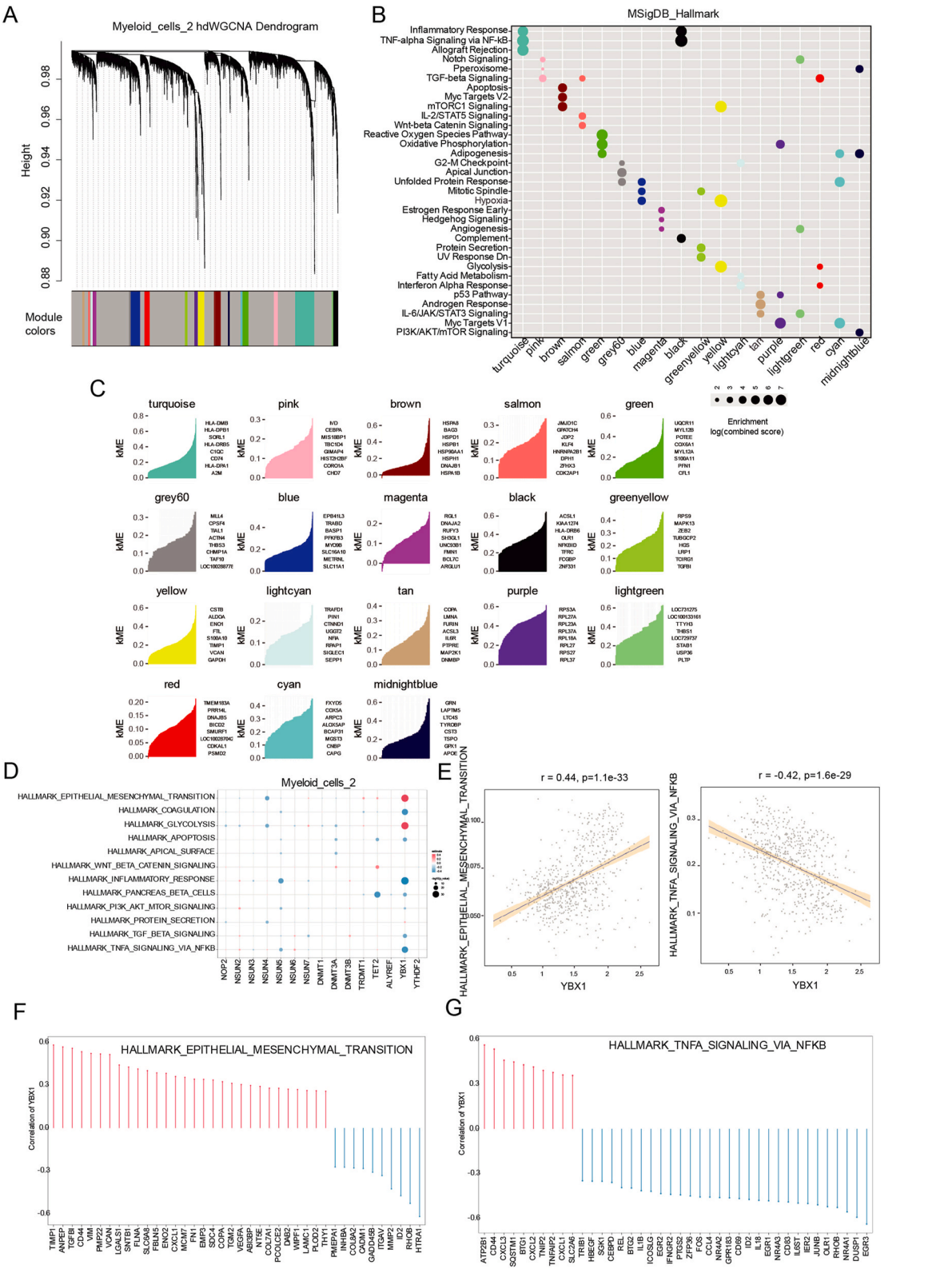

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Fig. 6. Identification of gene co-expression modules among myeloid cell 2. A: Weighed gene co-expression network analysis was constructed among myeloid cell 2. B: Dot plot of the KEGG functional enrich analysis of the module. C: The first 8 eigengenes of each module, ranked by eigengene-based connectivity (kME). D: Bubble diagram demonstrating the relevance of the m5c gene to the pathway in myeloid cells 2. E: Scatterplot demonstrating the correlation between YBX1 and the pathway in myeloid cells 2. F: Lollipop chart of correlations between YBX1 and genes in epithelial mesenchymal transition pathway in myeloid cells 2. G: Lollipop chart of correlations between YBX1 and genes in TNF-alpha signaling via NF-κB in myeloid cells 2.

Altogether there were 2123 DEGs($p < 0.05$). The volcano map shows that the top upregulated gene in the neoplastic cells YBX1 positive was ATP5A1, whereas the top downregulated gene was ID3, compared with the neoplastic cells YBX1 negative group in Fig. 7B. Moreover, comparing gene expression between YBX1-positive and YBX1-negative myeloid cells revealed significant differences in the expression levels of numerous genes. Altogether there were 710 DEGs ($p < 0.05$). The volcano map shows the top upregulated gene in the neoplastic cells YBX1 positive was S100A10, whereas the top downregulated gene was CCL3, compared with the neoplastic cells YBX1 negative group in Fig. 7C. By comparing the DEGs in the three cohorts, we identified 82 genes in Fig. 7D. Afterward, we conducted GO enrichment analysis to functionally annotate these 82 DEGs. Gene enrichment analysis displayed notable rises in cytoplasmic translation, regulation of fibroblast proliferation in neoplastic cells YBX1 positive in Fig. 7E. PPI information of DEGs was acquired from Search Tool for the Retrieval of Interacting Genes (database (http://www.stringdb.org/)). The results showed that YBX1 interacted with 24 proteins, suggesting its key role in GBM (Fig. 7F). The results of the GSEA analysis show that Hedgehog signaling, K-ras signaling DN, Apical junction signaling, NF-κB, and G2M checkpoint were activated. TGF beta signaling, MYOGENESIS signaling, and oxydative phosphorylation were suppressed as illustrated in Fig. 7G and H.

3.5. APOE mediated the interaction of YBX1$^+$ myeloid cells and neoplastic cells by TNF-alpha signaling via NF-κB

YBX1 activate myeloid cells by NF-κB pathway. Meanwhile, YBX1$^+$ neoplastic cells promote cell proliferation also by NF-κB pathway. To assess the influence of YBX1$^+$ myeloid cells on neoplastic cells, We employed the CellChat package for a ligand-receptor pairs interaction analysis, aiming to discern differences between YBX1-positive and YBX1-negative myeloid cells and neoplastic cells. We analyzed and compared their interactions as shown in Fig. 8A. NicheNet is a method that predicts ligand-target links between interacting cells by combining their expression data with prior knowledge on signaling and gene regulatory networks. NicheNet was employed to predict interactions between selected myeloid cells and neoplastic cells. Remarkably, within the top predicted ligands, YBX1-positive myeloid cells showed expression of APOE and NAMPT(Fig. 8B and. C). The target genes regulated by APOE are mainly IL1B, IL1A, and TNF (Fig. 8D). Ligand-receptor interactions indicate that APOE from myeloid cell YBX1 positive, regulates SDC3, LRP1, and SORL1 from neoplastic cells (Fig. 8E). These findings hinted that YBX1$^+$ myeloid cells interact with neoplastic cells by APOE and SDC3 or LRP1 or SORL1 ligand-receptor binding to promote GBM cells proliferation by NF-κB.

4. Discussion

RNA modification is an emerging gene regulatory mechanism, which involves m5C, N6-methyladenosine (m6A), and N1-methyladensoine (m1A) [28,29]. Among the more than 160 chemical modifications, m6A and m5C are the two most dominant and representative types of RNA post-transcriptional alterations [30,31]. M5C stands out as the most common epigenetic alteration observed on messenger RNA (mRNA) and long noncoding RNA in eukaryotes [32]. This modification relies on the activities of methyltransferases for addition, demethylases for removal, and binding proteins for recognition and functional regulation [16]. m5c-RNA methylation modification plays a variety of biological functions [33]. For example, it regulates mRNA transport, increases RNA stability, regulates protein translation, and maintains the normal structure of RNA [34]. Recent studies have demonstrated the involvement of m5C-RNA methylation modifications in regulating the development, invasion, and metastasis of various tumors [35]. Although RNA modifications have been implicated in disease pathogenesis and cancer tumorigenesis [36], the precise relationship between GBM and m5C remains elusive. Advancements in sequencing technologies, especially single-cell sequencing, have become a promising approach to unveil the role of m5C in the pathogenesis of GBM.

This study delineated aberrant expression patterns of m5C modification-related modulators in the GBM single-cell atlas. It has been reported that the m5C modification plays a role in mRNA stability, translation, splicing, and export [37]. The existing studies predominantly concentrate on cancer cells, with minimal exploration the functionality of immune cells within the GBM TME. In our study, we observed m5C regulatory factor contributes to GBM progression, exhibiting elevated expression scores across various tumors. CNV and somatic mutation of the m5c regulatory factor in GBM suggests that YBX1 is variation at a low frequency. However, YBX1 had an impact on the prognosis of stomach cancer (STAD), colon cancer (COAD), GBM, head and neck cancer (HNSC), liver cancer (LIHC), pancreatic adenocarcinoma (PAAD), rectal cancer (READ). YBX1 was highly expressed in COAD, ESCA, STAD, LIHC, HNSC, and GBM from TCGA; m5C score is highly expressed specifically in COAD, ESCA, STAD, READ, LIHC, HNSC and GBM.

Meanwhile, we found that YBX1 as m5C methyltransferase (writer) was significantly different between GBM samples and normal samples. In addition, we find that YBX1 is more often found in myeloid cell, more specifically in myeloid cell 2. YBX1 is a multifunctional DNA/RNA-binding protein involved in various cellular events such as DNA repair, transcription, and translational regulation. It plays a crucial role in cell proliferation, differentiation, and stress responses [38–40]. YBX1 showed sequence-specific binding to A/C-rich exon enhancers (ACE elements) in the human CD44 gene, crucial for in vivo splicing and spliceosome assembly. Wild-type YBX1 promoted exon v4 inclusion, reliant on ACE for YBX1 binding, indicating YBX1's role in vivo in recognizing exonic ACE

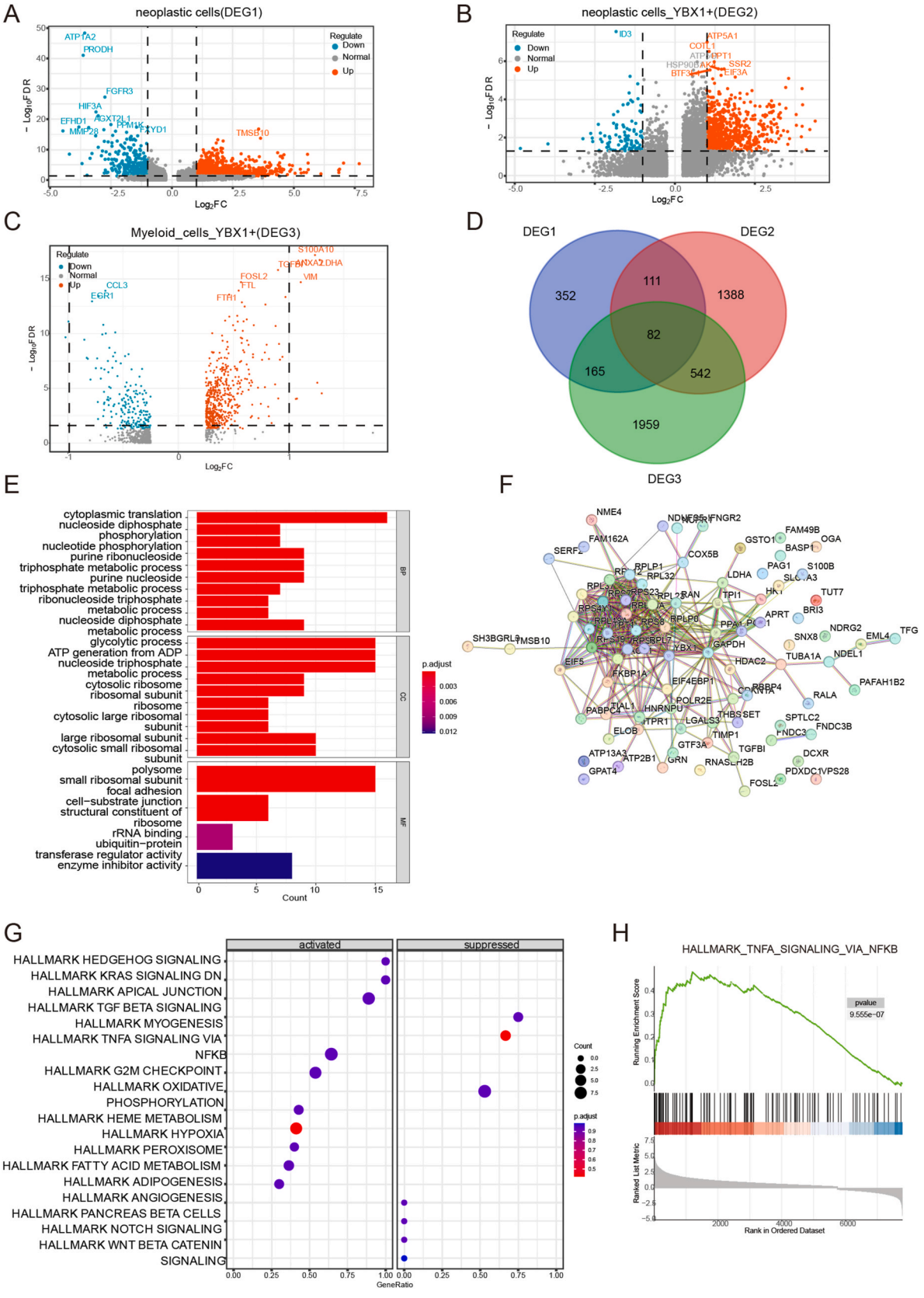

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Fig. 7. YBX1+ neoplastic cells promote cell proliferation by TNF-alpha signaling via NF-κB. A: Differential gene expression of neoplastic cells between GBM and normal. B: Differential gene expression of neoplastic cells YBX1 positive compared with neoplastic cells YBX1 negative. C: Differential gene expression of myelod cells YBX1 positive compared with myelod cells YBX1 negative. D: The venn plot of the differential gene. E: GO analyses of the 82 DEGs. F: PPI network for the 82 DEGs. G: GSEA reveals that Hedgehog signaling, K-ras signaling DN, Apical junction signaling, TNF-alpha signaling via NF-κB, G2M checkpoint were activated. TGF beta signaling, MYOGENESIS signaling, Oxidative phosphorylation were suppressed. H:TNF-alpha signaling via NF-κB in GSEA.

elements, The human YBX1 exhibits diverse DNA/RNA-binding roles, contributing to transcription, translation, and spliceosome-associated functions, particularly in alternative splicing [41,42]. YBX1 plays a crucial role in the function of epidermal progenitors, specifically in cycling keratinocytes, and its absence results in defects in skin architecture. Notably, YBX1 negatively regulates epidermal progenitor senescence by governing the translation of specific cytokine mRNAs, establishing its significance as a

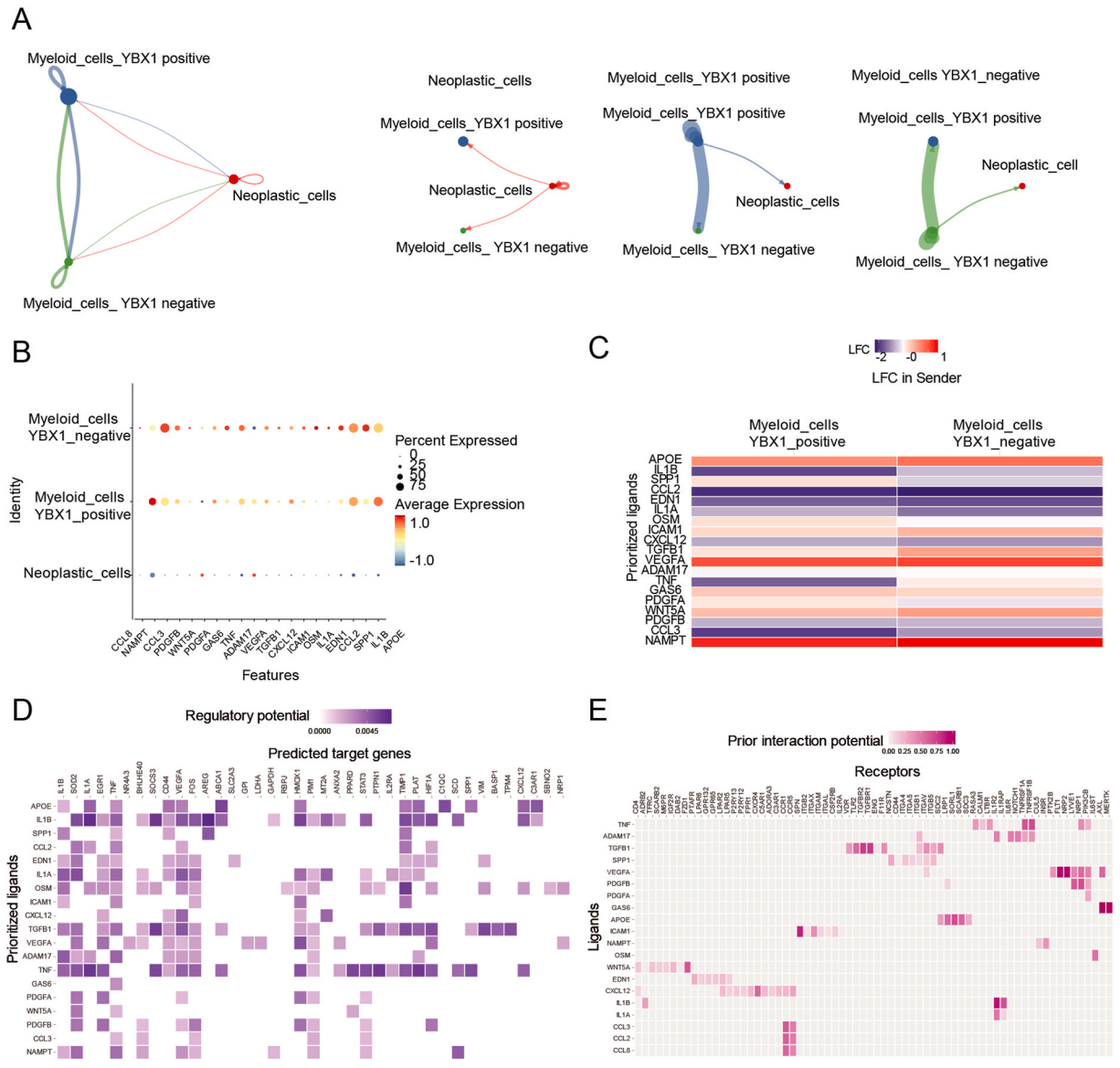

Fig. 8. APOE was potential regulatory targets of YBX1 in myeloid cell. A: Analysis of myelod cells YBX1 positive and myelod cells YBX1 negative. interacting with neoplastic cells in both GBM and normal groups. B: The dot plot shows the expression of top20 ligands in myeloid cell YBX1 positive cell, myeloid cell YBX1 negative cell. C: Heatmap demonstrates the differences expression of top20 ligands in myeloid cell YBX1 positive cell, myeloid cell YBX1 negative cell. D: Heatmap showed the predicted ligand activity and the regulatory potential of the ligand and their target genes expressed by myeloid cell YBX1 positive cell, where the matrix is colored according to the regulatory potential values. E: Ligand−receptor heatmap demonstrating strength of predicted interaction of ligands and receptors expressed by myeloid cell YBX1 positive cell.

posttranscriptional effector in maintaining epidermal homeostasis [43]. Recently, Dong et al. demonstrated DSCAM-AS1 interacted with YBX1, influencing its recruitment to FOXA1 and ERα promoters, suggesting its regulatory role in cancer progression [44]. Indeed, there are studies indicating that YBX1 integration with oncogenic PI3K/mTOR signaling is essential in regulating the fitness of malignant epithelial cells [45]. In T-cell acute lymphoblastic leukemia, YBX1 functions as an oncogenic factor [46]. Zhang et al. underscores the critical role of YBX1 in sustaining CML LSC survival, advocating for the potential targeting of YBX1 as a therapeutic strategy in CML treatment [47].

This study unveils the association of YBX1 with various immune cells. Notably, our findings suggest that the functionality of immune cells may be contingent upon YBX1 expression. Furthermore, enrichment analysis indicates that YBX1 may impact the progression of GBM through NF-κB pathway, inflammatory response, and unfolded protein response pathways in myeloid cell 1. However, YBX1 is negatively correlated with the inflammatory response, NF-κB. The expression of YBX1 is positively correlated with the epithelial mesenchymal transition, glycolysis. In epithelial mesenchymal transition pathway, YBX1 may be up-regulation of TIMP1, ANPEP, TGFBI, CD44, VIM in myeloid cell 2. YBX1 serves as a protective barrier against the transition from proliferation to invasion in mesenchymal-like epithelial cancer cells. Its absence amplifies partial EMT and encourages in vivo invasion within heterogeneous head and neck cancers. Moreover, YBX1 is thought to contribute to various DNA repair processes through a range of mechanisms [48,49]. We additionally uncovered the gene modules within GBM, identified signaling pathways mainly regulated by the red module complement, NF-κB, and xenobiotic metabolism. Signaling pathways mainly regulated by the green module cause inflammatory response. Signaling pathways mainly regulated by the blue module are associated with MYC targets V1, interferon alpha and interferon gamma responses. In myeloid cell 2, YBX1 is primarily associated with peptide antigen assembly with MHC protein complex in red module. YBX1 is primarily associated with phagolysosome assembly in pink module and with response to unfolded protein in brown module. YBX1 holds substantial influence in governing pathways associated with inflammatory response, NF-κB.

There exists widespread agreement regarding the extensive inter-tumor heterogeneity of the immune microenvironment in GBM. In the present study, we observed that YBX1 activate myeloid cells mainly by NF-κB pathway. Moreover, $YBX1^{+}$ neoplastic cells promote cell proliferation also by NF-κB pathway. APOE is one of the most significant survival predicting cell differentiation-related genes in human GBMs [50]. The gene APOE expression is upregulated in YBX1 positive myeloid cells. $YBX1^{+}$ myeloid cells have interaction with neoplastic cells by APOE and SDC3 or LRP1 or SORL1 ligand-receptor binding to promote GBM cells proliferation by NF-κB.

Due to the preliminary and exploratory nature of this study, it is important to acknowledge several limitations in our analyses. Firstly, the analysis was constrained by lacking of comprehensive analysis on specific GBM subtypes and broader patient cohorts: While the study conducted a comprehensive analysis of m5C expression distribution in single-cell GBM data, it did not address potential variations among GBM subtypes. Secondly, the analysis was constrained by a limited numbers of samples and cells, potentially affecting the generalizability of the findings. We also try to particular focus on YBX1 to demonstrate its heterogeneity across patients in this single-cell data. However, this single-cell data contains only 4 samples of GBM cases and cannot be compared. Thirdly, Despite the widespread recognition of RNA modification m5C and its significant impact on various malignancies, including tumorigenesis and progression, the exact mechanism by which m5C influences GBM remains unclear. We will focus on elucidating the specific mechanisms through which m5C influences GBM development and progression in the future. This could involve targeted experiments to investigate the functional consequences of m5C modifications on key signaling pathways implicated in GBM pathogenesis, such as proliferation, invasion, and immune evasion. Notwithstanding these constraints, employing scRNA-seq to delineate the intricate role of m5C in GBM represents a promising direction for further investigation. The future studies will also aim to address the expression variability of the YBX1 gene across patients.

5. Conclusions

In conclusion, this study suggests that the m5C modification pattern of YBX1 plays a variety of important roles in GBM. Analysis of YBX1 will deepen the understanding of TME in GBM and help develop better immunotherapy strategies.

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Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article(GSE84465,GSE179572).

Declaration of interest's statement

The authors declare no conflict of interest.

Ethics declarations

Informed consent was not required for this study because datasets presented in this study can be found in public databases.

CRediT authorship contribution statement

Yanshan Ge: Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Huiting Weng:** Project administration, Investigation, Formal analysis. **Yingnan Sun:** Supervision, Resources, Project administration, Conceptualization. **Minghua Wu:** Methodology, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29451.

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