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EGR1 addiction in diffuse large B cell lymphoma

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Abstract

Early growth response gene (EGR1) is a transcription factor known to be a downstream effector of B cell receptor signaling and Janus kinase 1 (JAK1) signaling in diffuse large B cell lymphoma (DLBCL). While EGR1 is characterized as a tumor suppressor in leukemia and multiple myeloma, the role of EGR1 in lymphoma is unknown. Here we demonstrate that EGR1 is a potential oncogene that promotes cell proliferation in DLBCL. Immunohistochemical analysis revealed that EGR1 expression is elevated in DLBCL compared with normal lymphoid tissues and the level of EGR1 expression is higher in activated B-cell-like subtype (ABC) than germinal center B-cell-like subtype (GCB). EGR1 expression is required for the survival and proliferation of DLBCL cells. Genomic analyses demonstrated that EGR1 upregulates expression of MYC and E2F pathway genes through the CBP/p300/H3K27ac/BRD4 axis while repressing expression of the type I interferon pathway genes by interaction with the co-repressor NAB2. Genetic and pharmacological inhibition of EGR1 synergizes with the BRD4 inhibitor JQ1 or the type I interferon inducer lenalidomide in growth inhibition of ABC DLBCL both in cell cultures and

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Authorship

L.R. conceived and designed the study, and supervised research. T. A.W. and C.M.C. established the xenograft models, supervised research and revised the manuscript. S.K., L.L., N.M.H., F.Z., P.D.B., A.D., S.Z., D.T.Y., A.H., Y.L., Y.L., A. R., A.K., S. B., and M.C. performed research. S.K., L.L., N.M.H., F.Z. and L.R. analyzed data. S.K., L.L. N.M.H. and L.R. drafted and revised the manuscript.

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xenograft mouse models. Therefore, targeting oncogenic EGR1 signaling represents a potential new targeted therapeutic strategy in DLBCL, especially for the more aggressive ABC DLBCL.

Introduction

Diffuse large B cell lymphoma (DLBCL) represents 30% to 40% of newly diagnosed lymphomas, and comprises two main molecular subtypes: activated B cell-like (ABC) and germinal center B cell-like (GCB), based on gene expression profiling (1). Genetic lesions that alter the structure and/or the expression pattern of oncogenes and tumor suppressor genes underlie the molecular pathogenesis of DLBCL (2,3). For example, inactivating mutations in the acetyltransferase genes CREBBP/EP300 are present in one-third DLBCL samples (4). These mutations only affect one allele, consistent with a haploinsufficient tumor suppressor function (4). Chromosomal translocations of the oncogene MYC to the immunoglobin heavy or light chain loci detected in 10 to 15% GCB DLBCL lead to constitutive expression of MYC (5) and are associated with a poor prognosis in DLBCL (6). Chronic activation of the B cell receptor (BCR) signaling pathway in ABC DLBCL results from genetic events targeting BCR signaling regulators, including CD79B, CARD11, BCL10 and MALT1 (3). Activating mutations in MYD88, a gene encoding an adapter protein mediating Toll-like receptor signaling, are present in ~40% of ABC DLBCL, thus activating NFκB and JAK/STAT signaling (7).

ABC DLBCL is more aggressive with a current cure rate of only ~40% (8). The BCR signaling pathway is known for its critical role in the pathogenesis of ABC DLBCL (9,10). IL6- and IL10-mediated JAK1/STAT3 activation regulates gene expression, which promotes the survival and proliferation of ABC DLBCL cells (11–18). Our recent study has revealed that early growth response gene 1 (EGR1), a transcription factor known to be a downstream effector of BCR signaling (19), is also a downstream epigenetic target of JAK1 signaling through histone modifications in ABC DLBCL (13). While EGR1 has been characterized as a tumor suppressor in leukemia and multiple myeloma (20–24), the role of EGR1 in human lymphoma remains unknown.

EGR1 binds to GC rich DNA sequences via 3 zinc-finger motifs in its carboxyl terminus and regulates gene transcription through recruitment of activating (e.g. CBP/p300) or repressing factors (e.g. NAB2) (25–30). Here, we found that EGR1 expression is elevated in DLBCL patient samples with higher levels of EGR1 expression in ABC DLBCL. EGR1 expression is required for the survival and proliferation of ABC DLBCL. Genomic analysis demonstrated a large overlap of EGR1 genomic occupancy with that of BRD4, a highly expressed bromo and extraterminal (BET) protein in DLBCL; EGR1 upregulates expression of MYC, MYC target genes and E2F target genes through the CBP/p300/H3K27ac/BRD4 axis while repressing transcription of the lethal type I interferon pathway genes by interacting with NAB2.

Materials and Methods

(See Supplementary Materials and Methods for detail.)

Cell Lines and Culture.

ABC-DLBCL cell lines (TMD8, OCI-Ly10, SUDHL2, U2932, HBL1,OCI-Ly3 and RIVA), GCB-DLBCL cell lines (OCI-Ly19, OCI-Ly8, BJAB, HT, OCI-Ly1, SUDHL7, TOLEDO and OCI-Ly7), and mantle cell lymphoma cell lines (JEKO-1, Granta519, UPN-1, Rec-1, Z138, Maver-1 and Mino) were engineered to express the bacterial tetracycline repressor as described previously (31). All cultures were routinely tested for mycoplasma contamination. All lymphoma cell lines were purchased from the American Type Culture Collection (ATCC) and used for previous studies (31,32) and authenticated by gene expression profiling.

Immunohistochemical staining and quantification.

To study EGR1 expression in DLBCL, tissue microarrays were constructed from archival cases of *de novo* DLBCLs diagnosed at Indiana University Health Pathology Laboratory during 2000–2014, with approval from the Indiana University IRB (Protocol#1407470378). A tissue microarray (TMA) of mantle cell lymphoma cases was obtained from our previous study (33), with approval from the University of Wisconsin-Madison Institutional Review Boards (UW protocol M-2008–1011 and MCRF protocol SHA 10109). The study was approved by the Institutional Review Board of Indiana University. The study was performed in accordance with the Declaration of Helsinki Ethical Principles of Medical Research.

Protein expression was scored using InformTM advanced image analysis software. Scores of duplicated cores were averaged for each case.

Naive B cell isolation.

Naive human B cells were isolated from peripheral blood mononuclear Cells (PBMCs) or human tonsils using naive B cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. All tissues were collected with institutional review board (IRB) approval (Protocol #2013–1570, #2015–1483) by the University of Wisconsin-Madison.

Cell viability assay.

Cell viability was measured with an automatic cell counter according to the manufacture’s instruction. Cells were harvested and suspended, and mixed with equal volume of 0.4% trypan blue in PBS. Ten μl of the cell suspension was loaded onto TC20 system (Bio-Rad) counting slides, and the number of viable cells was quantified on a TC20 automated cell counter (Bio-Rad).

Xenografts.

Male and female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) breeder pairs were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred under specific pathogen-free conditions in sterile ventilated racks in the animal care facility at the University of Wisconsin-Madison and the National Cancer Institute. The study was approved by the Animal Care and Use Committee of the University of Wisconsin-Madison and the National Cancer Institute, NIH.

shRNA survival assay.

shRNA toxicity was assayed as described (25). Briefly, the pRSMX-PG vector that co-expresses shRNA and GFP was transduced into lymphoma cell lines. Within 2 weeks after retroviral transduction, 20 ng/ml doxycycline was added to induce shRNA expression. The fraction of GFP-positive and shRNA-expressing cells was monitored over various time points by flow cytometry using MAQS Quant (miltenyi biotec). The GFP-positive fractions in each shRNA were divided by each day 0 data and then compared with the fraction of control shRNA. The reduction of the GFP-positive and shRNA-expressing fraction at later time points indicates shRNA toxicity.

Cell cycle assay.

Cell cycle was analyzed with the APC BrdU Kit (BD Pharmingen, San Diego, CA, USA), following manufacturer's instructions. Cells were incubated with BrdU for 16 h. The cells were washed in the staining buffer, fixed/permeabilized with the Cytofix/Cytoperm buffer or Cytofix/Cytoperm buffer plus and washed with the Perm/Wash buffer. After permeabilization, cells were treated with DNase for 1 h at 37°C, and then stained with APC-conjugated anti-BrdU antibody and 7-AAD (25 μg/ml). The stained samples were analyzed using MACS Quant (Miltenyi Bioteq).

RNA-seq.

Total RNA was extracted using RNeasy plus mini kit (Qiagen) according to the manufacturer's protocol. RNA-seq libraries were prepared by using the Illumina TruSeq stranded mRNA LT sample preparation kit (Illumina). Gene Set Enrichment Analysis (GSEA) was performed by GSEA software (V2.0) (http://software.broadinstitute.org/gsea/index.jsp). RNAseq data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession number: GSE123291.

ChIP-seq.

ChIP experiments were performed as previously described (18). ChIP-seq data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession number: GSE123291.

Apoptosis assay.

Cell apoptosis was measured using APC-Annexin V and propidium iodide (PI) staining kit (BD Pharmingen# 556547) following the manufacture's protocols. Samples were evaluated by MAQS Quant (miltenyi biotec) and data were analyzed by FlowJo software.

RT-qPCR.

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacture's protocol, and quantified with Nanodrop lite spectrophotometer (Thermo Scientific). Samples were run at 2 min at 95°C, followed by 40 cycles at 95°C for 5s and 60°C for 30s. Data were normalized to β-Actin or GAPDH mRNA expression.

Results

EGR1 overexpression in DLBCL.

Our recent genomic analysis demonstrated a non-canonical gene regulation mechanism of JAK1 by which JAK1 catalyzes phosphorylation of H3Y41, which leads to transcription of ~3000 genes including EGR1 in ABC DLBCL cells (13). We verified EGR1 as a JAK1 target using either the JAK1 inhibitor AZD1480 (Supplementary Fig. S1A) or JAK1 shRNA (Supplementary Fig. S1B). EGR1 expression is also regulated by BCR signaling since knockdown of the key early BCR pathway gene BTK reduces EGR1 expression in ABC DLBCL cells (Supplementary Fig. S1B). Consistent with previous studies (19), BCR stimulation with anti-IgM antibody induces EGR1 expression in normal tonsillar B cells (Supplementary Fig. S1C).

Next, we performed immunohistochemical analysis of two tumor tissue microarrays for EGR1 expression. We found that EGR1 level is elevated in DLBCL as compared to normal human tonsils, lymph nodes (LN) and mantle cell lymphomas (MCL), and its level is higher in ABC DLBCL (non-GCB) than GCB DLBCL (Fig. 1A,1B). Higher levels of EGR1 expression were also observed in ABC DLBCL cell lines compared with those in GCB DLBCL cell lines with the exception of OCI-Ly3 in ABC DLBCL cell lines and SUDHL7 in GCB DLBCL cell lines (Fig. 1C), and those in MCL cell lines with the exception of the Mino cell line (Supplementary Fig. S1D). These results suggest that the constitutively activated JAK1 and BCR signaling sustain EGR1 expression in ABC DLBCL cells.

EGR1 expression is required for the survival and proliferation of DLBCL cells.

To test the functional specificity of EGR1, we used RNA interference technology. In this assay, the different small hairpin RNA (shRNA) sequences were placed into the retroviral vector containing the GFP marker gene, and shRNA-expressing cells were analyzed by flow cytometry. Cells from cultures were collected every 3 days for up to 12 days, and then the percentage of shRNA-expressing cells was calculated relative to day 0 (Supplementary Fig. S2A). If any shRNA is toxic for cancer cells, we would observe reduced GFP-positive cells over time. We were able to identify two shRNAs that reduced EGR1 expression by 50% to 70%. During 12 days of expression, the EGR1 shRNA exhibited a time-dependent toxicity in all ABC DLBCL cell lines except the Riva cell line, while only one of 5 GCB DLBCL cell lines and 3 of 7 MCL cell lines were sensitive to the EGR1 shRNA (Fig. 2A, Supplementary Fig. S2B, S2D). The toxicity is EGR1 shRNA specific since doxycycline addition and control shRNA expression did not change the percentage of GFP-positive cells during 12 days of culture (Supplementary Fig. S2A).

A recent study demonstrates that the RIVA cell line has low levels of IL6 receptor expression and lacks STAT3 activation, a downstream of IL6 receptor signaling (34). This suggests that EGR1 expression in this cell line is independent of JAK-STAT signaling, a survival pathway in ABC DLBCL, which may explain why knockdown of EGR1 did not affect cell viability. Cell cycle analysis demonstrated that EGR1 shRNA-mediated reduction of viable cells is due to decreased cell proliferation rather than triggering cell apoptosis, since EGR1 knockdown reduces G1 to S phase entry but does not change subG1 population

(Fig. 2B). The result was confirmed by apoptosis assay with annexin V and propidium iodide staining (Supplementary Fig. S2C). Therefore, EGR1 expression is required for the fitness of DLBCL cells, especially for ABC DLBCL cells, suggesting that EGR1 is a potential oncogene whose expression drives cell proliferation.

Identification of EGR1 target genes in ABC DLBCL.

To identify EGR1 target genes, we used two representative ABC DLBCL cell lines, OCI-Ly10 and TMD8, both of which have high levels of EGR1 expression (Fig. 1C). We performed EGR1 ChIP-seq analysis to determine genome-wide gene occupancy by EGR1 using EGR1 shRNA expressing OCI-Ly10 cells or input TMD8 cells as a ChIP control. We identified a total of 12,387 EGR1 binding sites (peaks) in OCI-Ly10 cells and 9,601 in TMD8 cells compared with the control sample (Fig. 3A, Supplementary Table S1), based on a peak calling analysis used for our recent study (17). We then applied the MEME motif enrichment analysis to test specificity of these EGR1 binding sites and found a similar distribution pattern of EGR1 motifs between the two cell lines (Fig. 3B, 3C, Supplementary Fig. S3A). Based on the genomic loci of these peaks, we performed peak annotation analysis (17,18) and identified 7,390 potential EGR1 target genes in TMD8 cells and 8,262 in OCI-Ly10 cells (Fig. 3D, Supplementary Table S1). Among these target genes, 4,818 genes are shared between the two cell lines (Fig. 3D, Supplementary Table S1). The overlapping genes are considered common target genes in ABC DLBCL.

Next, we performed the whole transcriptome analysis by RNA-seq in the OCI-Ly10 cell line using EGR1 shRNA expressing cells as a control. The result demonstrated that 32% (2,684/8,262) of EGR1 target genes changed their expression when EGR1 was knocked down by two different shRNAs and the number of downregulated genes was less than that of upregulated genes (Fig. 3E). This finding suggests that EGR1 functions as a transcriptional activator but predominantly as a transcriptional repressor in ABC DLBCL cells. A similar result was obtained in TMD8 and SUDHL2 cells (Supplementary Table S1).

EGR1-mediated transcriptional activation through the CBP/p300/H3K27ac/BRD4 axis.

We performed gene set enrichment analysis (GSEA) of RNA-seq data and found that MYC and E2F pathway genes were on the list of downregulated genes by EGR1 shRNAs in all three ABC DLBCL cell lines, OCI-Ly10, TMD8 and SUDHL2 (Fig. 4A, Supplementary Fig. S3B, Supplementary Fig. S4A–D, Supplementary Fig. S5A–C). Since the cellular function of the two pathways is closely related each other (35,36), we focused on the MYC pathway for further investigation. Interestingly, although EGR1 knockdown by shRNAs reduces MYC expression (Fig. 4B), there is no significant enrichment of EGR1 ChIP peaks on the MYC locus (Supplementary Table S1). This result suggests that EGR1 indirectly regulates MYC expression. Previous studies have demonstrated that EGR1 can bind the promoter of CREBBP/EP300 (encoding CBP/p300) and activates their expression (25,26). Consistently, our immunoblot analysis revealed that knockdown of EGR1 reduced expression of CBP and p300 in TMD8 and OCI-Ly10 cells (Supplementary Fig. S5D). EGR1 binding to EP300 regulatory regions (5’UTR and enhancer) was confirmed by EGR1 ChIP PCR analysis (Supplementary Fig. S6A). CBP/p300 catalyze H3K27 acetylation (H3K27ac), an active histone mark that recruits BRD4, a highly expressed bromo and

extraterminal (BET) protein in DLBCL known to induce transcription of MYC and MYC pathway genes, among others (27,35,37,38). Next, we analyzed published BRD4 ChIP-seq data in ABC DLBCL cells (37) for common EGR1 genomic binding sites and, indeed, 67% (2484/3694) of EGR1 peaks overlap those of BRD4 (Fig. 4C, Supplementary Table S1), as shown on the loci of representative MYC target genes (Fig. 4D, Supplementary Fig. S5B, Supplementary Table S1). Reduced expression of these MYC target genes in EGR1 shRNA expressing cells was confirmed by quantitative real-time PCR analysis (Supplementary Fig. S5C).

Given that H3K27ac is a substrate of CBP and p300, our immunoblot assay confirmed a reduction in H3K27ac expression in TMD8 and OCI-Ly10 cells after knockdown of EGR1 (Fig. 4E). As expected, occupancy of H3K27ac and BRD4 on the loci of these MYC target genes but not the negative control DNLZ was reduced in EGR1 shRNA expressing cells (Fig. 4F, 4G, Supplementary Fig. S5E, S5F). These results suggest that EGR1 regulates expression of MYC target genes through the CBP/p300/H3K27ac/BRD4 axis. Notably, addition of the BRD4 inhibitor JQ1 enhanced the toxicity of EGR1 shRNAs (Fig. 4H).

No specific EGR1 inhibitor is currently available but the selective sp1 inhibitor mithramycin can prevent EGR1 from binding to GC rich DNA sequences for gene transcription (39–42). Mithramycin has been used for the treatment of leukemia patients (43–45). Our ChIP-seq data indicate that EGR1 binds to its own promoter region and therefore regulates self-expression (Supplementary Fig. S5G). EGR1 binding to its own promoter regions was confirmed by EGR1 ChIP PCR analysis (Supplementary Fig. S6B). We tested whether EGR1 self-regulation is affected by the drug by immunoblot analysis. Indeed, we observed reduced expression levels of EGR1 and MYC in two ABC DLBCL cell lines when treated with 10 nM mithramycin (Supplementary Fig. S7A, S7B). Mithramycin treatment reduced EGR1 binding to its own promoter region and the EP300 enhancer region based on our ChIP qPCR analysis (Supplementary Fig. S7C), suggesting that mithramycin inhibits EGR1 transcriptional activity.

To test the anti-tumor effect of mithramycin, we performed ABC DLBCL xenografts in immunocompromised NOD scid gamma (NSG) mice. Based on recent xenograft studies in solid cancers (46,47), we treated the OCI-Ly10 engrafted NSG mice with 1 mg/kg mithramycin or PBS control 3 times a week for 3 weeks. As shown in Supplementary Figure S7D, mithramycin treatment completely inhibited tumor growth and significantly extended survival of the mice. To examine whether mithramycin can enhance JQ1 anti-tumor effects, we used a suboptimal dose of mithramycin at 0.5 mg/kg and 50mg/kg JQ1 based on a published DLBCL study (48). The result demonstrated that both JQ1 and mithramycin treatment inhibited OCI-Ly10 tumor growth in the NSG mice as compared with the vehicle group (Fig. 4I). More importantly, a combination of the two drugs produced greater tumor growth inhibition than the single drug alone (Fig. 4I). Taken together, these findings suggest that co-targeting EGR1 and BRD4 is a potential therapeutic strategy in ABC DLBCL.

EGR1 represses expression of the type I interferon pathway genes by interaction with NAB2.

Our GSEA of RNA-seq data demonstrated that a set of the type I interferon signaling pathway genes is at the very top of the list of upregulated genes by EGR1 shRNAs, which is only one shared by three ABC DLBCL cell lines (Fig. 5A, 5B, Supplementary Fig. S3B). ChIP-seq data demonstrated EGR1 binding to the transcription start site (TSS) of IRF7 and STAT1, two key transcription factors in the pathway (Fig. 5C). Repression of IRF7 and STAT1 expression was confirmed by immunoblot assay since EGR1 knockdown increased the protein level of IRF7 and STAT1 (Fig. 5D). In ABC DLBCL cells, IRF7 induces production of the type I interferon, which can be repressed by IRF4/SPIB (49). Our recent work revealed that expression of IRF7 and the type I interferon downstream effectors including STAT1 are also inhibited by STAT3 (18). Since activation of the type I interferon signaling is lethal, ABC DLBCL cells maintain their fitness through multiple mechanisms to suppress this pathway.

EGR1 is known to interact with NAB2, a corepressor that is required for transcriptional repression by EGR1 in other cell types (28,30). We examined whether the type I interferon signaling is repressed through this mechanism in DLBCL cells. We found that NAB2 knockout by sgRNA indeed increased expression of 5 representative genes including IRF7 and STAT1 in the type I interferon signaling pathway (Supplementary Fig. S8A). Immunoblot analysis revealed NAB2 sgRNA increased STAT1 phosphorylation after 30 min stimulation with the type I interferon IFN-α (Fig. 5E) in TMD8 and OCI-Ly10 cells. The level of total STAT1 was also elevated after 4 h stimulation with IFN-α (Fig. 5E). We observed a similar pattern using NAB2 shRNA (Fig. 5F). Altogether, these results suggest that interaction of EGR1 with NAB2 provides an additional layer of repression to the lethal type I interferon signaling in ABC DLBCL.

Co-targeting EGR1 and the type I interferon pathway in ABC DLBCL.

To test whether co-targeting EGR1 and type I interferon signaling synergistically inhibits growth of ABC DLBCL cells, we conducted *in vitro* cell culture analysis and xenografts. We used lenalidomide, an active agent in ABC DLBCL that induces type I interferon response by downregulation of IRF4 and SPIB, which otherwise inhibit IRF7 expression (49,50). We performed *in vitro* survival assay in TMD8 and OCI-Ly10 cells when EGR1 shRNA expression was induced in the presence of lenalidomide. After 9 days of culture, EGR1 shRNA reduced cell viability and the number of the viable shRNA expressing cells was further reduced in the presence of lenalidomide compared with the DMSO control (Fig. 6A).

The synergistic effect was validated by TMD8 ABC DLBCL xenografts in the NSG mice. EGR1 shRNA expression and lenalidomide caused complete tumor growth inhibition during the period of treatment but EGR1 shRNA expression or lenalidomide treatment alone only achieved partial inhibition (Fig. 6B). As expected, knockdown of EGR1 augmented lenalidomide-mediated expression of IRF7, STAT1 and the type I interferon IFNβ (Fig. 6C, 6D). Similarly, mithramycin treatment enhanced lenalidomide-mediated cell apoptosis (Supplementary Fig. S8B) and expression of IRF7 and IFNβ (Supplementary Fig. S8C).

Therefore, these results provide a mechanistic rationale for therapeutic co-targeting of EGR1 and the type I interferon pathway in ABC DLBCL.

Discussion

EGR1 is a zinc finger transcription factor that can be upregulated by a variety of external stimuli. EGR1 functionally regulates cell activation, proliferation, differentiation, and apoptosis (51–54). EGR1 is dysregulated in solid cancers, and functions as either an oncogene or a tumor suppressor, depending on the stage and type of cancers (22,55–60). EGR1 deletion together with other genes in chromosome 5 is a recurrent genetic alteration in myelodysplastic syndromes and acute myeloid leukemia (20). Loss of EGR1 function has been shown to promote leukemiagenesis in murine models (20,21). In multiple myeloma, EGR1 is a direct target of the transcription factor JUN and low levels of EGR1 and JUN expression are associated with an inferior clinical outcome (23). Knockdown of EGR1 enhances resistance of multiple myeloma cells to bortezomid, a proteasome inhibitor used as a front-line treatment for newly diagnosed multiple myeloma (23). Missence mutations of EGR1 have been detected in multiple myeloma (24). Altogether, these findings suggest that EGR1 functions as a tumor suppressor in leukemia and multiple myeloma.

There are no recurrent EGR1 mutations in DLBCL based on recent studies (61–63). This study together with our previous work demonstrates that EGR1 expression is driven by active BCR and JAK1 signaling (13,64). As a transcription factor and an effector in these two hallmark oncogenic pathways in ABC DLBCL, EGR1 regulates gene expression to promote the survival and proliferation of cancer cells. EGR1 is both a transcriptional activator and a transcriptional repressor. EGR1mediates transcriptional activation by a canonical transcriptional mechanism since about a third of EGR1 binding sites are located in the promoter regions. In addition, EGR1 induces gene expression including MYC and E2F pathway genes through the CBP/p300/H3K27ac/BRD4 axis. EGR1 can directly activate expression of CREBBP (encoding CBP) and EP300 (encoding p300) (25,26). CBP and p300 are histone acetyltransferases and function as a transcriptional coactivator for a large number of DNA-binding transcription factors (65,66). Genomic deletions and somatic mutations of these two genes are present in ~39% DLBCL but usually affect one allele (4). Consistent with previous studies (25,26), EGR1 is required for expression of CBP and p300 in DLBCL, which catalyze H3K27 acetylation (H3K27ac). This active histone mark then recruits BRD4, whose expression promotes the survival and proliferation of DLBCL cells by many mechanisms including activating MYC and E2F pathway genes (27,35,37,38).

MYC chromosomal translocation is frequently associated with BCL2 or, to a lesser extent, BCL6 translocation (5). These so-called “double-hit” or “triple-hit” lymphomas are included in the 2016 WHO classification in the new category of high-grade B cell lymphoma (67). In DLBCL, the arrangement of MYC, BCL2 or BCL6 is more common in the GCB subtype (5). Here we reveal a new epigenetic mechanism by which EGR1, a downstream effector of BCR and JAK1 signaling, regulates MYC expression through the CBP/p300/H3K27ac/BRD4 axis in ABC DLBCL. We analyzed RNA-seq data in 150 ABC DLBCL and 297 GCB DLBCL samples from a recent clinical study (68) and demonstrated that the expression of MYC and its target genes is higher in ABC DLBCL than in GCB DLBCL (Complementary

Fig. S9). Interestingly, there is no correlation between EGR1 and MYC expression either in these patient samples (Complementary Fig. S10A) or in DLBCL cell lines based on our immunoblot analysis (Complementary Fig. S10B, S10C). This may be explained by many other mechanisms underlying MYC overexpression in DLBCL, in addition to EGR1 transcriptional regulation. For example, we recently demonstrated that the protein arginine methyltransferase PRMT5 upregulates MYC expression by forming a positive-feedback loop with BCR/PI3K/AKT signaling in DLBCL (69).

EGR1 represses expression of the type I interferon pathway genes through interaction with NAB2 in DLBCL cells. This is consistent with previous studies on EGR-mediated transcriptional repression in other cell types (28,30). Our recent study and other work have demonstrated that the genes in the type I interferon pathway are transcriptionally repressed by IRF4/SPIB (49) as well as by STAT3 (18). EGR1 provides an additional mechanism to prevent this lethal pathway from activation in DLBCL cells. Although a set of the type I interferon pathway genes enriched after knockdown of EGR1 is only one shared by three cell lines, some other gene signatures are shown in individual cell lines, such as TNFα, inflammatory response and apoptosis (Complementary Fig. S3B).

Our study supports a working model demonstrating a dual function of EGR1 in gene regulation in DLBCL (Fig. 7). As a downstream effector of both BCR and JAK1 signaling, EGR1 represents a unique molecular target in ABC DLBCL. Here we provide a mechanistic rationale for co-targeting of EGR1 and BRD4 or the type I interferon signaling in ABC DLBCL. Targeting BCR signaling with ibrutinib, a specific inhibitor for BTK, has emerged as a promising therapy for ABC DLBCL (70,71). However, drug resistance is significant and affects the long-term survival of the patients (70,71). In our pilot study, we found increased EGR1 expression in ibrutinib resistant ABC DLBCL cells. Our future directions include to investigate the role of EGR1 overexpression in ibrutinib resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Implications

The study characterizes EGR1 as a potential oncogene that promotes cell proliferation and defines EGR1 as a new molecular target in diffuse large B cell lymphoma, the most common non-Hodgkin lymphoma.

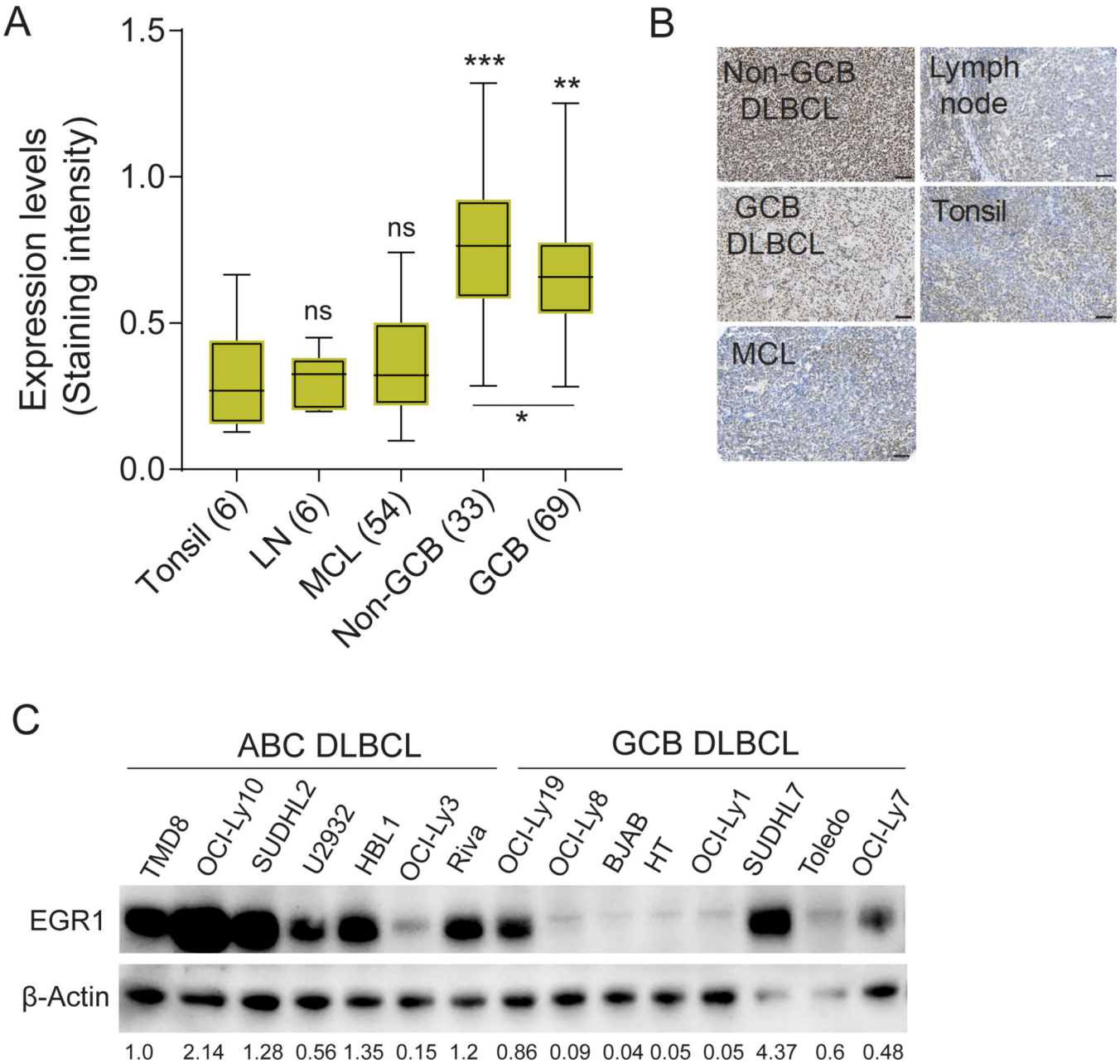

Figure 1. EGR1 expression is elevated in DLBCL cell lines and patient samples.

(A) Immunohistochemical analysis of EGR1 expression in GCB or non-GCB DLBCL and MCL tumor tissues, human tonsils and lymph nodes, analyzed with InformTM advanced image analysis software. Shown are box and whisker plots. Data were compared to the mean of tonsil samples and statistically analyzed by Dunnett's multiple comparison test (**$p<0.0021$, ***$p<0.0002$). Data between GCB and non-GCB DLBCL were analyzed by student's *t*-test (*$p<0.05$). (B) Representative images of EGR1 expression in a GCB DLBCL, a Non-GCB DLBCL, a MCL, a lymph node and a human tonsil. Scale bar, 50 μm. (C) Immunoblot analysis of EGR1 expression in the indicated DLBCL cell lines. β-Actin served as a loading control.

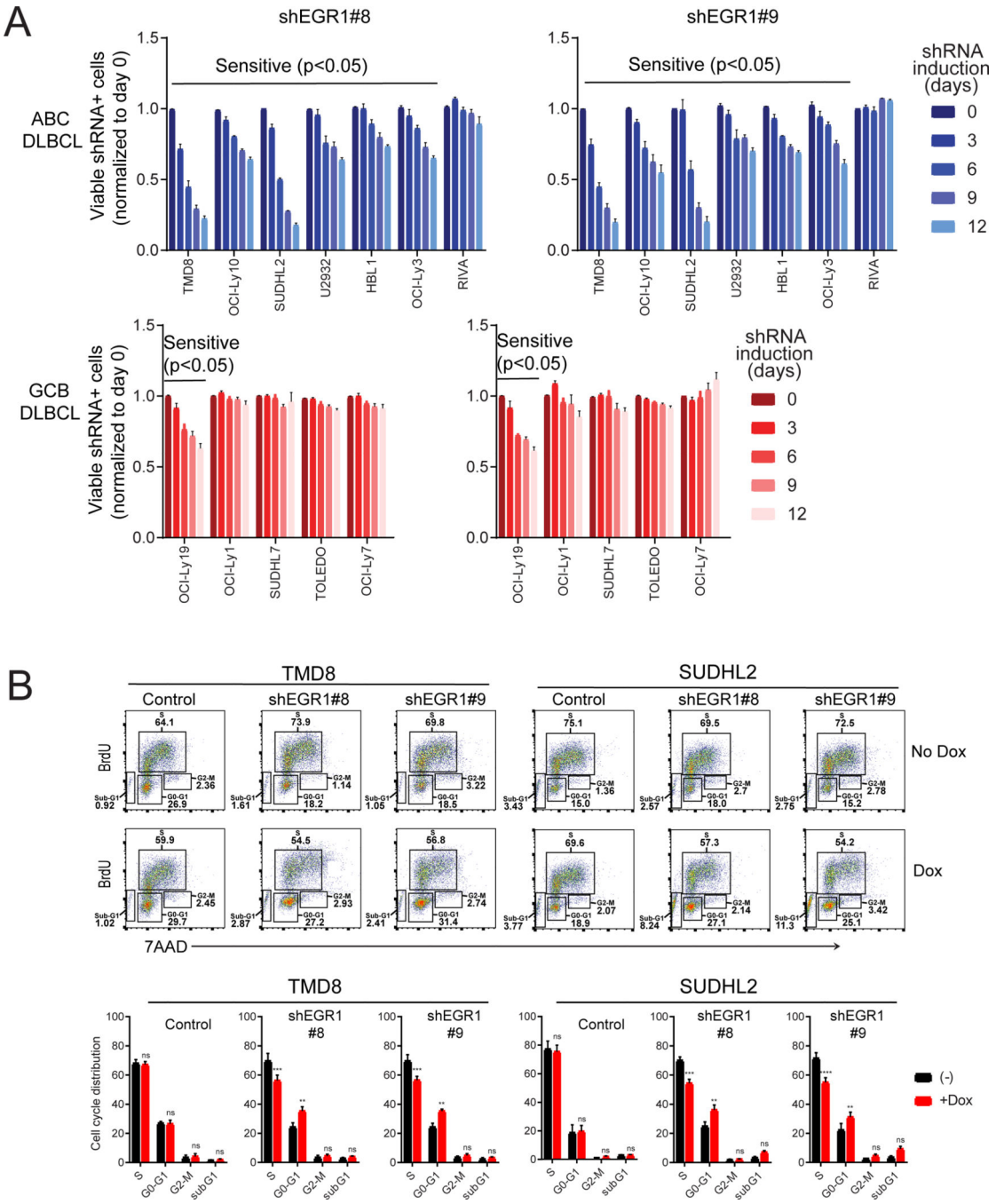

Figure 2. Sensitivity of DLBCL cell lines to EGR1 shRNAs.

(A) Flow cytometric analysis of cell viability of EGR1 shRNA expressing cells. The percentage of viable GFP+ shEGR1 expressing cells was normalized to that of the control shRNA for each time point. Error bars represent mean ± SEM of 3–10 replicates. The p value was calculated with Dunnett's multiple comparison test. (B) Flow cytometric analysis of cell cycle in TMD8 and SUDHL2 cells after 4 days of induction of shRNA expression. Error bars represent mean ± SD (Sidak's multiple comparisons test, **p<0.0021, ***p<0.0002, ****p<0.0002, N=3).

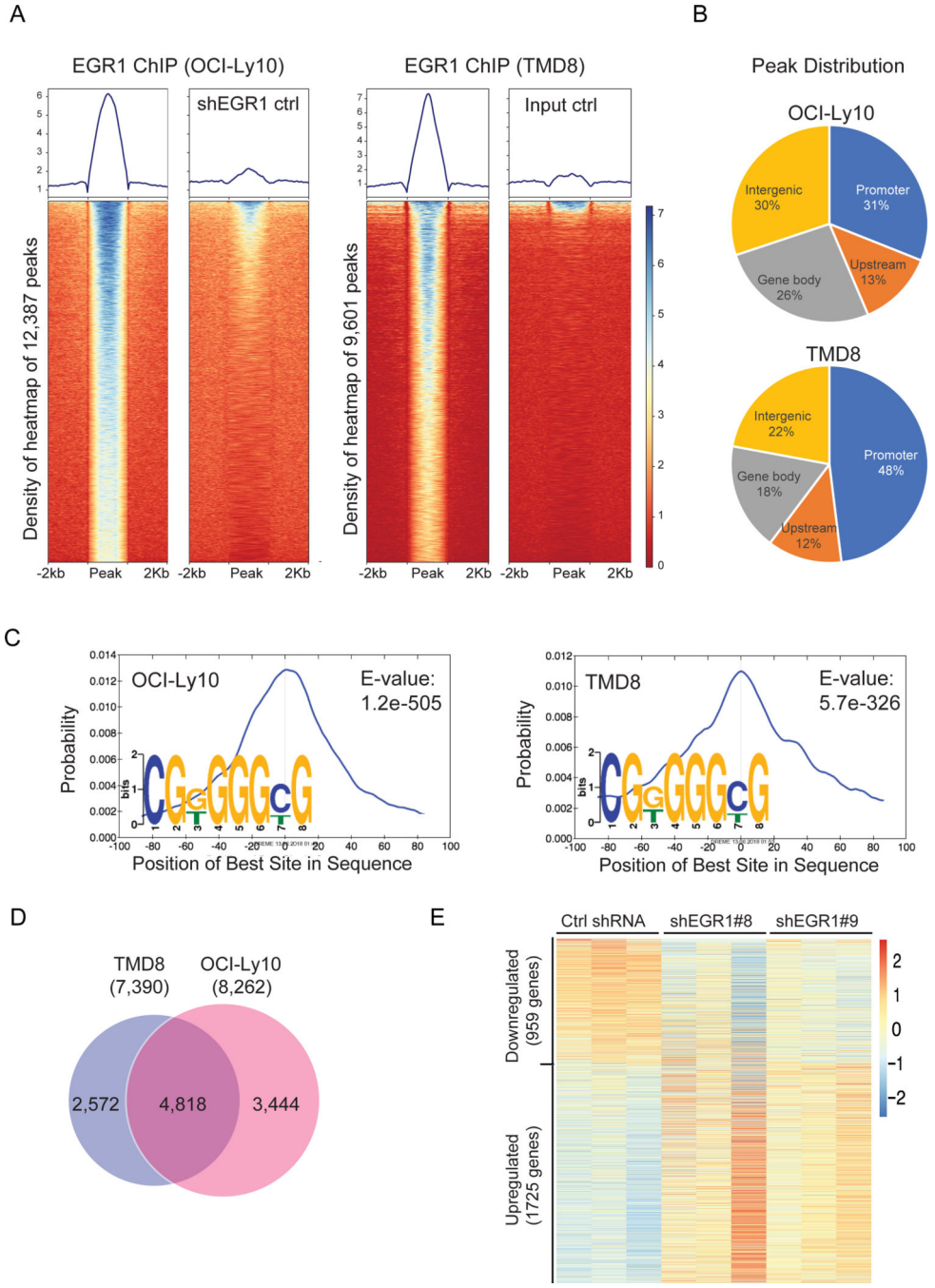

Figure 3. Genome-wide analysis reveals EGR1 transcriptional targets in ABC DLBCL.

(A) Heat maps of EGR1 ChIP-seq in OCI-Ly10 and TMD8 cells. shEGR1 expressing OCI-Ly10 cells or input TMD8 cells served as a control. EGR1 peak summits were centered within 2 kb of the flanking sequence on either side. Darker color indicates higher density of reads. EGR1 peaks were ranked by signal intensity at the peak center, the same order was used to display the control sample. (B) Distributions of EGR1peaks in the gene promoter (±1 kb to TSS), upstream enhancer (−15 kb to TSS), gene body and intergenic regions. (C) The CentriMo plots show the distribution of known EGR1 motif in the ChIP-seq peak

summit regions. De novo motif discovery from EGR1 ChIP-seq peaks show identical sequence logs to the known EGR1 consensus motif. (D) Venn diagram shows 4,818 EGR1 binding genes that are shared between TMD8 and OCI-Ly10 cells. (E) Heat maps show expression changes of EGR1 binding genes in OCI-Ly10 cells after 2 days of EGR1 knockdown by two shRNAs with 3 replicates for each sample.

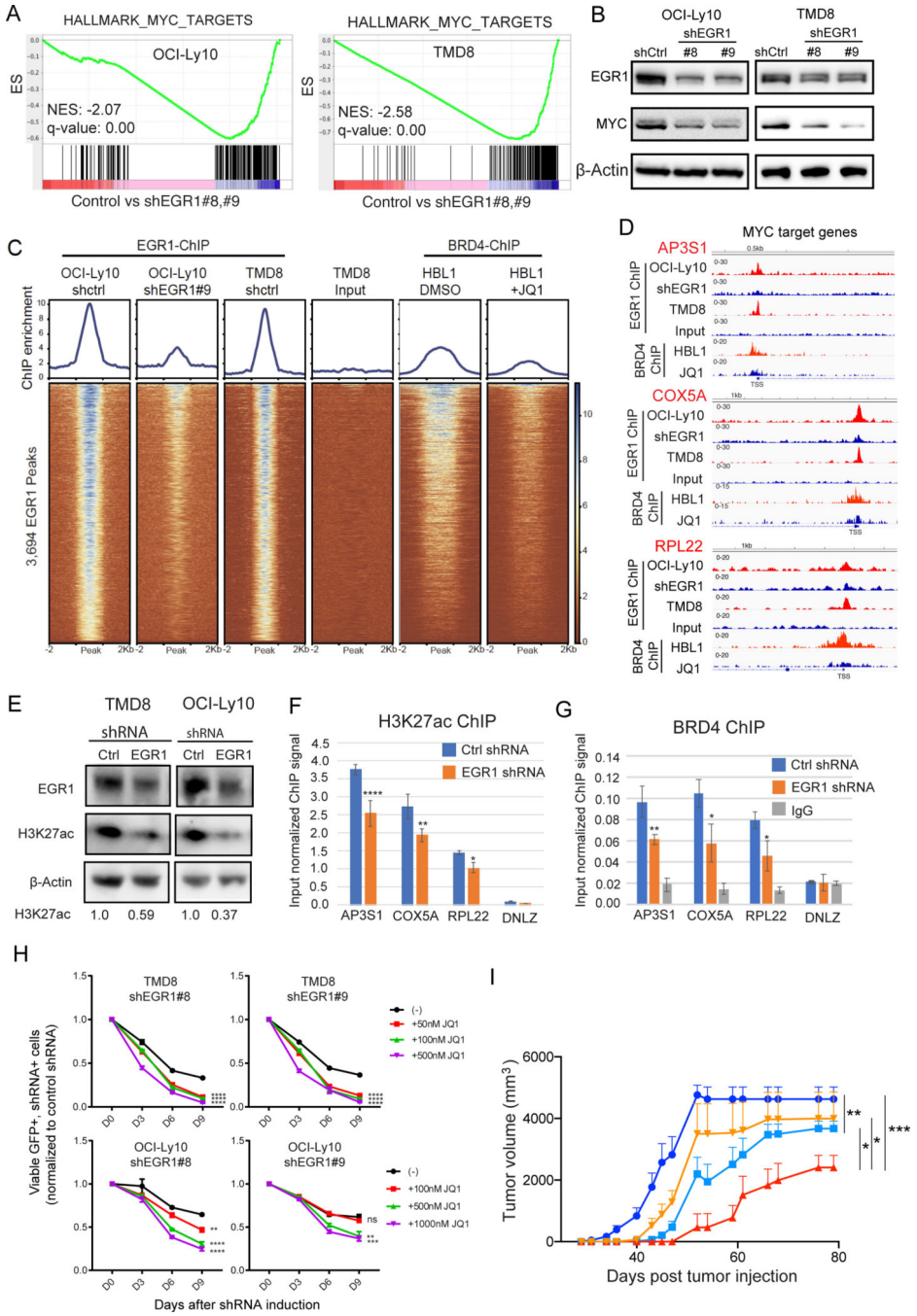

Figure 4. EGR1 regulates expression of MYC and MYC targets through the CBP/p300/ H3K27ac/BRD4 axis.

(A) Gene set enrichment analysis of MYC signature genes in OCI-Ly10 and TMD8 cells after EGR1 knockdown by 2 shRNAs. (B) Immunoblot analysis shows reduced MYC expression after EGR1 knockdown. β-Actin served as a loading control. (C) Heat maps show common 3,694 EGR1 peaks between TMD8 and OCI-Ly10 cells that overlap those of BRD4 ChIP-seq in HBL1 cells. BRD4 ChIP-seq data are from a previous study (37). (D) ChIP-seq histograms of three representative MYC target genes show EGR1 ChIP-seq in OCI-Ly10 and TMD8 cells and BRD4 ChIP-seq in HBL1 cells in red, and controls of

shEGR1 expressing OCI-Ly10 cells, TMD8 input sample and JQ1 treated HBL1 cells in blue. (E) Immunoblot analysis shows reduced H3K27ac expression in OCI-Ly10 and TMD8 cells after 2 days of shEGR1 induction. β-Actin served as a loading control. Data represent 3 independent experiments. (F) (G) ChIP analysis of H3K27ac (F) and BRD4 (G) on three MYC target genes in TMD8 cells. Quantitative PCR was performed using the primers targeting their promoter regions and negative control primers targeting the DNLZ promoter. The mean values of ChIP signals were normalized to the input DNA signal. ChIP signal from IgG control was negligible in F but shown in G. Error bars represent mean ± SD (student's *t*-test, *p<0.05; **p<0.01, ****p<0.0001, n = 4). (H) Flow cytometric analysis of EGR1shRNA expressing cells when treated with the indicated concentrations of JQ1 or DMSO control. The percentage of viable GFP+ shEGR1 expressing cells was normalized to that of the control shRNA for each time point. Error bars represent mean ± SEM (n=4). The p value was calculated with Dunnett's multiple comparison test (**p<0.0021, ***p<0.0002, ****p<0.0001). (I) ABC DLBCL xenografts. OCI-Ly10 cells were established as a subcutaneous tumor (average 100 mm^3) in the immunocompromised NSG mice, and then treated daily for 21 days with JQ1 in DMSO (i.p.) at a dose of 50 mg/kg or DMSO vehicle or mithramycin 0.5 mg/kg in PBS (i.p.) on Monday, Wednesday and Friday for 3 weeks until the endpoint (day 21) or with a combination of the two drugs. Error bars represent mean ± SEM (Two-way ANOVA, *p<0.05, ** p<0.01, ***p<0.001).

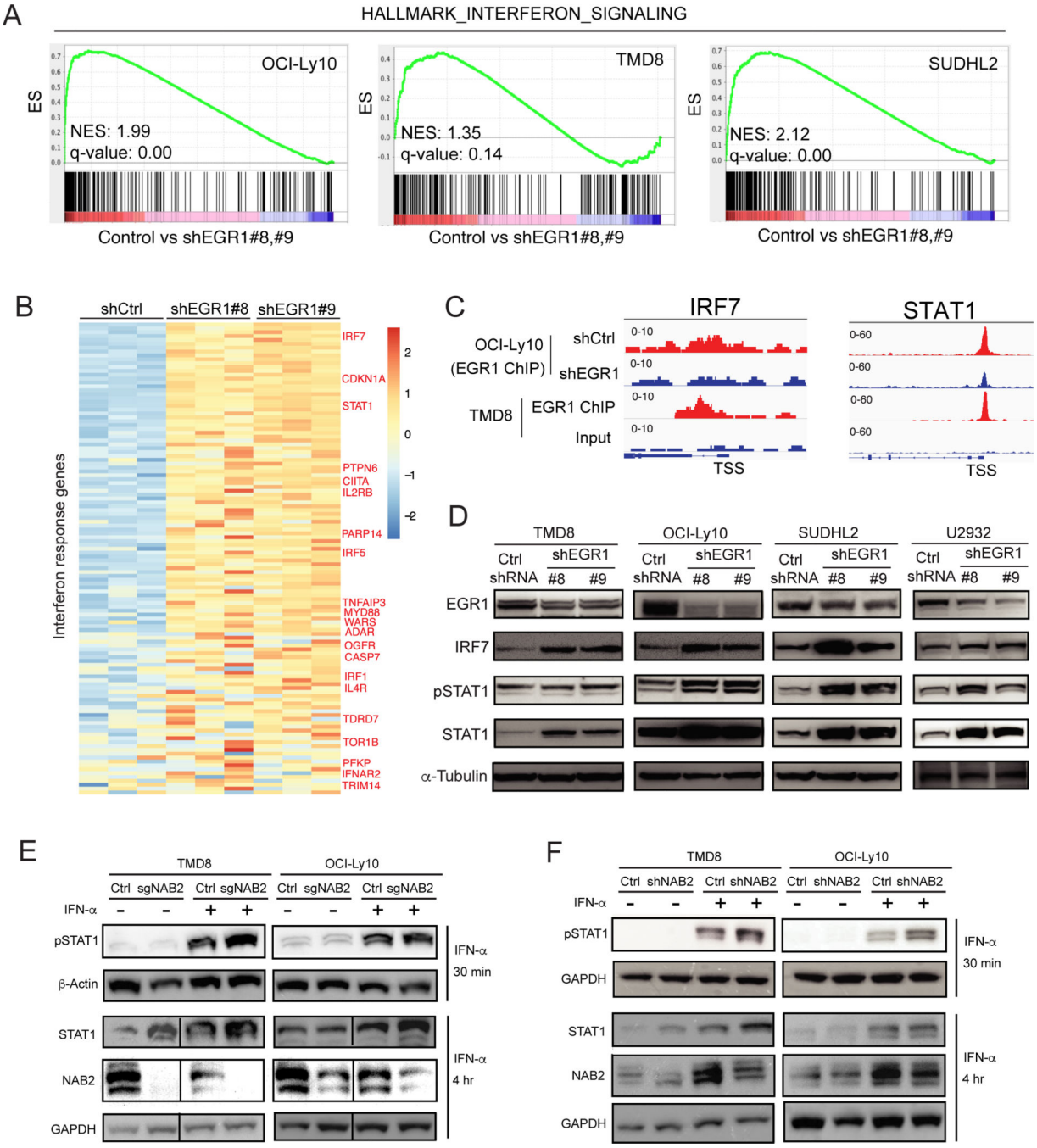

Figure 5. Repression of type I interferon signaling by EGR1 in ABC DLBCL.

(A) Gene set enrichment analysis of type I interferon signature genes in OCI-Ly10, TMD8 and SUDHL2 cells after 2 days of EGR1 knockdown by two shRNAs. (B) Heat maps show expression changes of the interferon response genes in OCI-Ly10 cells after 2 days of EGR1 knockdown by two shRNAs. EGR1 binding genes are indicated. Three replicates are shown for each sample. (C) EGR1 is recruited to regulatory regions of IRF7 and STAT1, as shown by read density tracks (OCI-Ly10 and TMD8 ChIP-seq samples in red, and control samples of shEGR1 expressing OCI-Ly10 cells and TMD8 input in blue). (D) Immunoblot analysis

of IRF7, pSTAT1 and STAT1 expression after 2 days of EGR1 knockdown in the indicated ABC DLBCL cell lines. α-Tubulin served as a loading control. Data represent 3 independent experiments. (E) Immunoblot analysis of pSTAT1 and STAT1 expression after 7 days of NAB2 knockout by sgRNA with or without stimulation by 10 ng/ml INF-α in the indicated ABC DLBCL cell lines. GAPDH served as a loading control. (F) Immunoblot analysis of expression of pSTAT1 and STAT1 after 2 days of NAB2 knockdown by shRNA with or without stimulation by 10 ng/ml INF-α in the indicated ABC DLBCL cell lines. β-Actin or GAPDH served as a loading control.

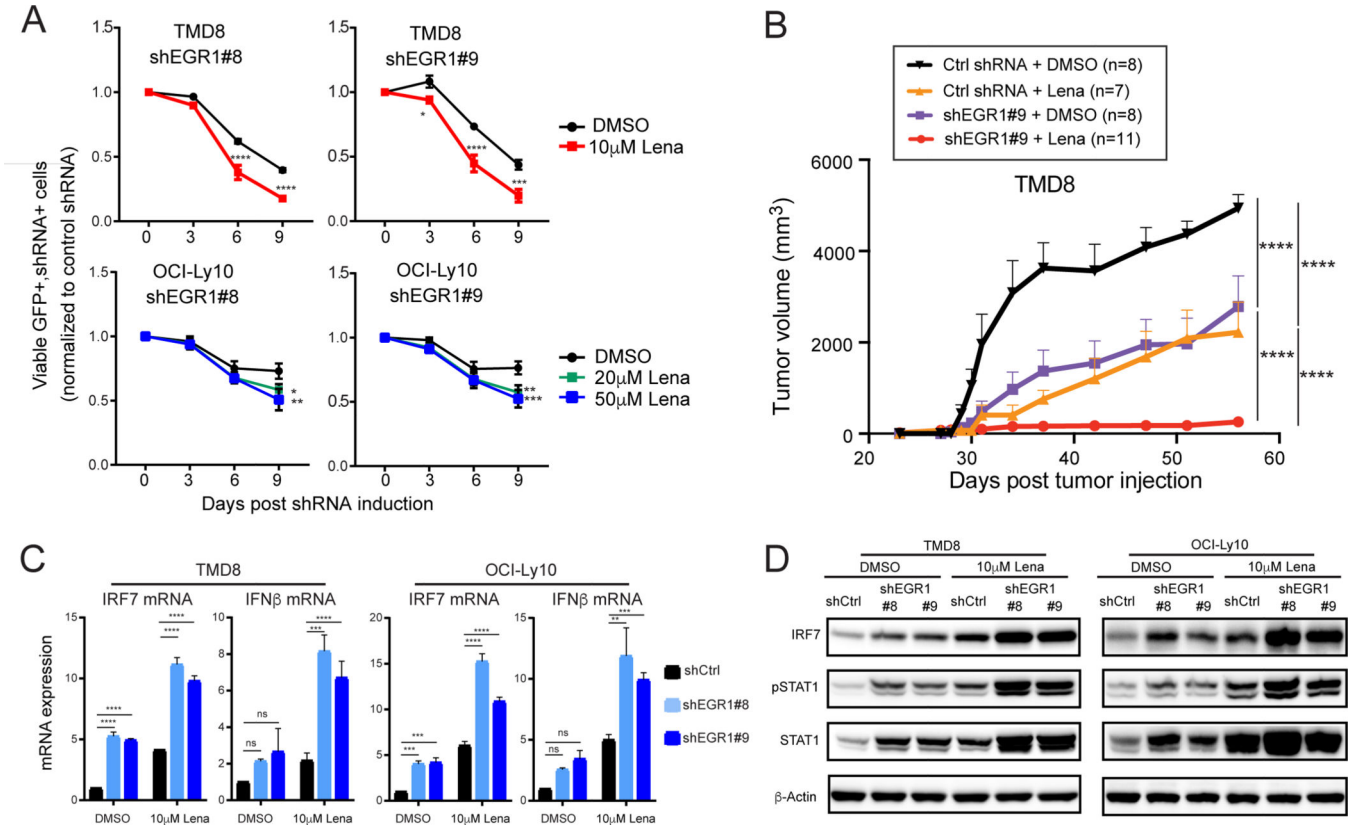

Figure 6. EGR1 inhibition enhances anti-tumor effects of lenalidomide in ABC DLBCL.

(A) Flow cytometric analysis of EGR1shRNA expressing cells when treated with the indicated concentrations of lenalidomide or DMSO control. The percentage of viable GFP+ shEGR1 expressing cells was normalized to that of the control shRNA for each time point. Error bars represent mean ± SEM of triplicates. The p value was calculated with Dunnett's multiple comparison test (*p<0.0332, **p<0.0021, ***p<0.0002). (B) ABC DLBCL xenografts. TMD8 cells were established as a subcutaneous tumor (average 100 mm^3) in the immunocompromised NSG mice, and then treated daily for 21 days with lenalidomide (i.p.) at a dose of 10 mg/kg/day or DMSO vehicle. shRNA expression was induced with 2 mg/ml doxycycline in drinking water. Error bars represent mean ± SEM (Two-way ANOVA, ****p<0.0001). (C) Quantitative PCR analysis of expression of IRF7 and IFNβ mRNA (normalized to β-actin mRNA levels) in TMD8 and OCI-Ly10 cells. Expression of EGR1 shRNAs was induced for 2 days in the cells with treatment of 10 μM lenalidomide or DMSO for 2 days. Error bars represent mean ± SEM of triplicates. The p value was calculated with student's *t*-test (*p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001). (D) Immunoblot analysis of IRF7, pSTAT1 and STAT1 expression in TMD8 and OCI-Ly10 cells. Expression of EGR1 shRNAs was induced for 4 days in the cells with treatment of 10 μM lenalidomide or DMSO for 4 days. β-Actin served as a loading control. Data represent 3 independent experiments.

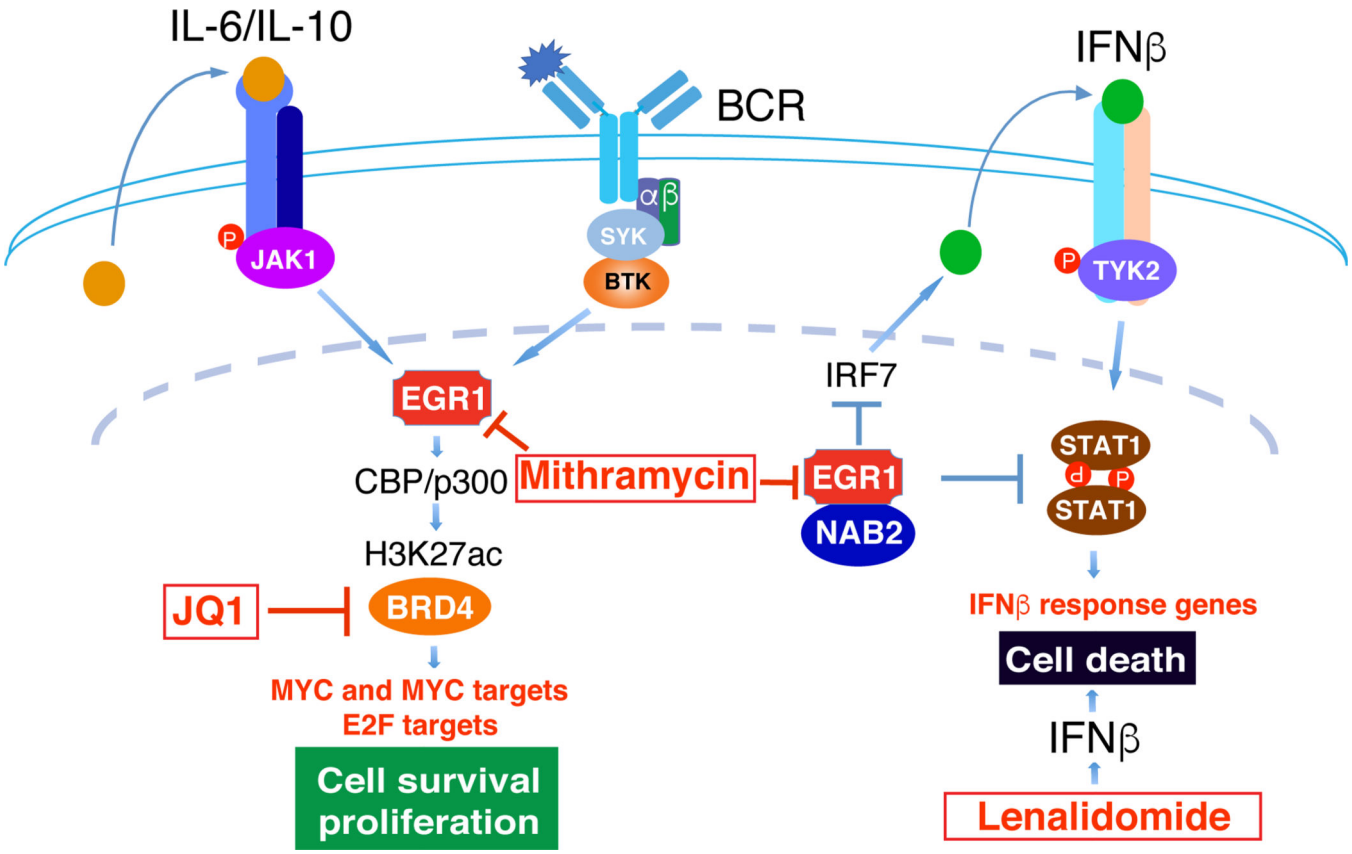

Figure 7. Schematic illustration of gene regulation by EGR1 in ABC DLBCL.

In ABC DLBCL, higher EGR1 expression is due to JAK1 through targeting the histone H3 and chronic activation of BCR signaling. Based on our EGR1 ChIP-seq and RNA-seq analyses, EGR1 upregulates gene expression in multiple oncogenic pathways such as MYC and E2F while damping the type I interferon pathway genes. In particular, EGR1 genomic occupancy largely overlaps that of BRD4, likely through inducing CBP/p300 expression and H3K27 acetylation. JQ1 is a BRD4 inhibitor and mithramycin is an EGR1 inhibitor. Lenalidomide is an inducer of the type I interferon signaling pathway.