

Targetable genetic alterations of *TCF4* (E2-2) drive immunoglobulin expression in the activated B-cell subtype of diffuse large B-cell lymphoma

Running title: Targeting TCF4 (E2-2) in ABC-like DLBCL

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ABSTRACT

The activated B-cell (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) is characterized by the chronic activation of signaling initiated by immunoglobulin- μ (IgM). By analyzing DNA copy profiles of 1,000 DLBCLs, we identified gains of 18q21.2 as the most frequent genetic alteration in ABC-like DLBCL. We show that these alterations target the *TCF4* (E2-2) transcription factor, and that over-expression of *TCF4* leads to its occupancy on immunoglobulin gene enhancers and increased expression of IgM at the transcript and protein level. The *TCF4* gene is one of the top BRD4-regulated genes in DLBCL. Using a BET proteolysis-targeting chimera (PROTAC) we show that TCF4 and IgM expression can be extinguished, and ABC-like DLBCL cells can be killed *in vitro* and *in vivo*. This highlights a novel genetic mechanism for promoting immunoglobulin signaling in ABC-like DLBCL and provides a functional rationale for the use of BET inhibitors in this disease.

Key words: DLBCL, TCF4, IgM

1 INTRODUCTION

2 Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoma and is curable in
3 ~60% of patients using a combination chemo-immunotherapy regimen, R-CHOP^{1,2}. However,
4 those that are refractory to, or relapse following, first-line therapy have a dismal outcome³.
5 Chimeric antigen receptor (CAR)-T cells are likely to change the landscape of outcomes in
6 relapsed/refractory patients, but a large number of patients are not eligible for CAR-T therapy and
7 ~50% of those that received CAR-T progress within 12 months⁴. Novel rationally-targeted
8 therapeutic strategies are therefore needed for DLBCL.

9
10 The clinical heterogeneity of DLBCL is underpinned by molecular heterogeneity, with the major
11 distinction being between the germinal center B-cell (GCB)-like and activated B-cell (ABC)-like
12 'cell of origin' (COO) subtypes that were identified by gene expression profiling⁵. The GCB-like
13 subtype shows transcriptional similarities to normal germinal center B-cells, whereas the ABC-
14 like subtype shows transcriptional similarities to CD40-activated B-cells or plasmablasts. Patients
15 with ABC-like DLBCL have significantly worse overall survival compared to patients with GCB-
16 like DLBCL, when treated with the standard-of-care combination chemotherapy (CHOP) plus
17 rituximab (R-CHOP) regimen⁶. The ABC-like DLBCL subtype expresses immunoglobulin μ (IgM)⁷
18 in >90% of cases, which forms the B-cell receptor (BCR) signaling complex in association with
19 CD79A and CD79B and drives chronically active BCR signaling. Several genetic alterations have
20 been shown to promote this signaling, including mutations of the *CD79A*, *CD79B*, *CARD11*, and
21 *MYD88* genes⁸⁻¹¹. However, these mutations only account for approximately two thirds of ABC-
22 like DLBCL cases¹², suggesting that other significant genetic drivers remain to be defined.

23
24 A common mechanism for tumorigenesis is the gain or loss of DNA encoding oncogenes or tumor
25 suppressor genes, respectively. These copy number alterations (CNAs) perturb a higher fraction
26 of the cancer genome than somatic nucleotide variants (SNVs) and small insertion/deletions
27 (InDels) and are critically important to cancer etiology¹³. Here, we have integrated multiple
28 datasets, including DNA copy number profiles of 1,000 DLBCLs, and identified DNA copy number
29 gain of the E2 transcription factor *TCF4* as the most frequent genetic alteration in ABC-like
30 DLBCL. We show that *TCF4* is capable of driving IgM expression and is amenable to therapeutic
31 targeting through BET inhibition. These data therefore highlight a novel genetic basis for ABC-
32 like DLBCL with potential implications for future clinical studies.

33

34

35 RESULTS

36 DNA copy number gains of chromosome 18 are the most frequent genetic alteration in DLBCL.

37 In order to identify significant CNAs in DLBCL, we interrogated the genomic profiles of 1,000
38 DLBCLs using the GISTIC2 algorithm¹⁴. These included high-resolution SNP microarrays from
39 860 previously published cases, in addition to next generation sequencing (NGS)-derived profiles
40 from our own cohort of 140 cases (Table S1-2). Our analysis revealed 20 significant DNA copy
41 number gains and 21 significant DNA copy number losses (false discovery rate [FDR] <0.1; Fig.
42 1A and Table S3). Using a subset of 448 cases for which COO subtype data was available, we
43 identified 9 DNA copy number alterations that were significantly more frequent in ABC-like DLBCL
44 and 11 that were significantly more frequent in GCB-DLBCL (Fisher Q-value<0.1; Fig. 1B and S1;
45 Table S4). The most frequent genetic alteration in ABC-like DLBCL was gain of 18q21.2, which
46 was observed in 44% of tumors. In line with the association with ABC-like DLBCL, 18q21.2 gains
47 were associated with significantly reduced overall survival in both CHOP and R-CHOP treated
48 patients (Fig 1C-D). Using 199 tumors with matched COO subtype, DNA copy number data and
49 mutation status for 40 genes, we observed that the frequency of 18q21 gains (23.1% of all tumors;
50 40.7% of ABC-like tumors) was higher than other ABC-like DLBCL-associated somatic mutations
51 including *MYD88* mutation (16.6% of all tumors; 33.3% of ABC-like tumors), *CD79B* mutation
52 (7.5% of all tumors; 18.5% of ABC-like tumors) and other ABC-associated genes (Fig 1E, S2 and
53 Table S5). Because multiple genetic alterations are associated with ABC-like DLBCL, we
54 employed the REVEALER algorithm¹⁵ to identify the set of genetic alterations that best explained
55 the ABC-like DLBCL signature. Using a set of 87 DNA copy number alterations and recurrently
56 mutated genes as the feature set and *MYD88* mutations as the seed feature, REVEALER
57 identified an additional 4 genetic alterations including 18q21.2 gain as those best associating with
58 the ABC-like signature (Fig. 1F). Gains of 18q21 are therefore the most frequent genetic feature
59 of ABC-like DLBCL and are predicted to contribute to this molecular phenotype.

60

61 The TCF4 (E2-2) transcription factor is the target of 18q21 gains in ABC-like DLBCL

62 Gains of 18q have been previously attributed to the *BCL2* oncogene^{16,17}. However, our analysis
63 of this large cohort provided the resolution to identify two significant peaks of DNA copy gain on
64 chromosome 18; 18q21.2 (16 genes, $Q=4.8 \times 10^{-14}$) and 18q22.1 (70 genes, $Q=1.1 \times 10^{-7}$; Table
65 S3). We further integrated GEP data from 249 tumors to identify the likely targets of these lesions
66 by testing for the increase in expression of genes within the most significant peaks of DNA copy
67 gain. This highlighted *TCF4* and *BCL2* as likely targets of the 18q21.2 and 18q22.1 gains,
68 respectively (Fig. 2A; Table S6). Notably, most 18q copy number alterations incorporated both of

69 these genes (Fig 2A-C). Only 7.3% or 1.0% of ABC-like DLBCLs have copy number alterations
70 targeting *TCF4* or *BCL2* alone, respectively (Fig. 2B-C). In addition, we observed that *TCF4* was
71 more highly expressed in ABC-like DLBCL compared to GCB-like DLBCL generally, but was
72 further increased by DNA copy gain (Fig 2D). In line with this, ABC-like DLBCL cell lines
73 expressed TCF4 protein irrespective of DNA copy number, but these levels were significantly
74 increased by DNA copy gain (Fig 2E).

75
76 The *TCF4* gene encodes an E2 family transcription factor, E2-2. Mutations of another E2
77 transcription factor, *TCF3*, and its negative regulators *ID2* and *ID3* are frequent in Burkitt's
78 lymphoma (BL) and promote immunoglobulin signaling^{18,19}. We therefore interrogated the
79 mutation status of these genes and *TCF4* copy gains in our cohort of 140 DLBCLs and a prior
80 cohort of 108 BLs that were sequenced and analyzed with the same approach²⁰. We did not
81 observe recurrent mutations of *TCF4* or *ID2* in this BL cohort, and mutations of *TCF3* and *ID3*
82 were infrequent in DLBCL (data not shown). However, in BL, gains of *TCF4* were present at the
83 same frequency as *TCF3* mutations (18%). Furthermore, *TCF4* gains were significantly mutually-
84 exclusive from *TCF3* and *ID3* mutations (Fisher P=0.019; Fig. 2F), suggesting that *TCF4* gains
85 may serve a similar function as *TCF3/ID3* mutations in promoting immunoglobulin signaling.
86 These data therefore show that the *TCF4* gene is highly expressed in ABC-like DLBCL, with
87 expression further promoted by frequent 18q21.2 DNA copy gains, and implicates *TCF4* in
88 immunoglobulin signaling.

89

90 *TCF4* regulates *IgM* and *MYC* expression in ABC-like DLBCL

91 To identify potential target genes of TCF4, we performed differential gene expression analysis of
92 primary DLBCL tumors with TCF4 DNA copy gain (n=51) compared to those without (n=59). This
93 analysis was limited to ABC-like tumors so as to eliminate the confounding effect of genes that
94 differ in expression between COO subtypes. A total of 355 genes (472 probe-sets) and 87 genes
95 (107 probe-sets) were found to be expressed at significantly higher or lower levels in tumors with
96 *TCF4* gain, respectively (Q<0.1, fold-change≥1.2; Fig 3A; Table S7). We performed CHIP-seq of
97 ABC-like DLBCL cell lines, SUDHL2 and TMD8, with tetracycline-inducible Myc-DDK-tagged
98 *TCF4* in order to define whether these genes were direct transcriptional targets of TCF4 (Fig 3A).
99 Importantly, *TCF4* was expressed at a level comparable to that in the U2932 cell line with TCF4
100 copy gain (Fig. S3). Using the intersection of significant peaks from both cell lines, we identified
101 TCF4 binding proximal to 180/355 genes with increased expression and 46/87 genes with
102 decreased expression in tumors with TCF4 copy gain (Fig. 3A-B; Table S8). These peaks showed

103 a highly significant enrichment of motifs containing E-box consensus sequences (CANNTG; Fig.
104 S4), and many of the same regions are also bound by TCF4 in plasmacytoid dendritic cell
105 neoplasms²¹ (Fig. S4), providing strong evidence that we detected on-target binding. Among the
106 most significant ChIP-seq peaks were those within the immunoglobulin heavy chain locus (Fig.
107 3B), in line with the significantly higher expression of *IGHM* in ABC-like DLBCL tumors with *TCF4*
108 copy gain (Fig. 3C). This included peaks immediately upstream and downstream of the *IGHM* and
109 *IGHD* genes, respectively, in regions with corresponding H3K27Ac in normal CD20+ B-cells that
110 indicates they are *bona fide* enhancers (Fig. 3D). Tetracycline-inducible expression of *TCF4* in
111 ABC-like DLBCL cell lines led to a marked increase in *IGHM* at the transcript (Fig. 3E) and protein
112 level (Fig. 3F). In comparison, *BCL2* expression was not induced by *TCF4* over-expression and
113 *MYC* induction was restricted to the two cell lines that lacked *MYC* translocation (SUDHL2 and
114 TMD8; Fig. S5). These data show that IgM is a direct target of *TCF4* and can be induced by its
115 over-expression in ABC-like DLBCL.

116

117 TCF4 can be targeted by the BET proteolysis-targeting chimera (PROTAC), ARV771.

118 The *TCF4* gene is one of the most highly BRD4-loaded genes in DLBCL, including in ABC-like
119 DLBCL cell lines with *TCF4* copy gain (Fig. S6). We therefore evaluated small molecule BET
120 inhibitors and a BET protein degrader, ARV771, as a potential avenue for reducing *TCF4*
121 expression in ABC-like DLBCL cell lines with high-copy number of *TCF4*. The small molecule BET
122 inhibitors, JQ1 and OTX015, resulted in an up-regulation of BRD4 that was not observed with
123 ARV771 due to its role as a sub-stoichiometric BRD4 degrader (Fig 4A and S7). This was
124 associated with a greater efficacy of ARV771 in reducing the BRD4 target genes, *MYC* and *TCF4*
125 (Fig 4A), and the ability of ARV771 to induce apoptosis of these cell lines (Fig 4B). However, as
126 *MYC* is also a target of *TCF4*, the down-regulation of *MYC* is likely partially mediated through
127 *TCF4*.

128

129 Reductions of *TCF4* by ARV771 treatment were accompanied by reduced expression of the *TCF4*
130 target genes. This included significant reductions of IgM at the transcript and protein level (Fig.
131 4C-D and Table S9). ARV771 treatment led to significant down-regulation of the set of genes that
132 were identified as being increased in association with *TCF4* DNA copy number gain in primary
133 tumors (Fig. 4E). The promising *in vitro* activity of ARV771 led us to test whether this compound
134 would be efficacious *in vivo*. In xenografts of the U2932 (Fig. 4F-I) and RIVA (Fig. 4J-M) cell lines
135 that express high levels of *TCF4*, we observed that ARV771 was able to significantly reduce tumor
136 growth. At the end of treatment, tumors were significantly smaller in ARV771-treated mice and

137 this led to a significant prolongation of survival in these mice (Log Rank P-value < 0.05). Together
138 these data demonstrate a clear functional rationale for BET inhibition in ABC-like DLBCL, and
139 show that ARV771 is effective at eliminating TCF4 and its target genes and treating ABC-like
140 DLBCL *in vivo*.

141

142 **DISCUSSION**

143 The ABC-like subtype is one of two major molecular subtypes of DLBCL that is recognized by the
144 WHO classification²². These tumors are driven by chronic active B-cell receptor signaling that
145 emanates from autoreactive IgM that is localized to the cell surface and intracellular lysosomes^{8,23-}
146 ²⁵. Mutations in *CD79B* and *MYD88* deregulate this signaling through the reduction of LYN-
147 mediated negative feedback and by activation of IRAK signaling, respectively^{8,10}. However, recent
148 murine studies have shown that *MYD88* mutation alone drove a phenotype that was reminiscent
149 of peripheral tolerance, and this was only relieved by the combination of *MYD88* and *CD79B*
150 mutations together, or by increased expression of surface IgM²⁶. The ABC-like phenotype is
151 therefore the result of cumulative epistatic genetic alterations, rather than a single dominant driver
152 mutation. In further support of this notion, recent genomic studies have defined co-associated
153 sets of genetic alterations that co-segregate with unique genetic subsets of ABC-like DLBCL²⁷.
154 The “cluster 5” subset of ABC-like DLBCL included frequent *MYD88* and *CD79B* mutations, but
155 the most frequent genetic alteration in this subtype was DNA copy number gain of 18q²⁷.

156

157 We identified the *TCF4* (aka E2-2) gene as the most significant target of 18q DNA copy number
158 gains in DLBCL. The *TCF4* gene (aka E2-2) is closely related to *TCF3* (aka E2A), with both
159 encoding helix-loop-helix transcription factors that form dimers and recognize E-box consensus
160 sequences (CANNTG)²⁸. Murine conditional knock-out studies showed that TCF3 and TCF4 are
161 critical regulators of germinal center B-cell and plasma cell development, in part due to their role
162 in activating immunoglobulin heavy- and light-chain enhancer elements^{29,30}. The ID2 and ID3
163 proteins bind to and inhibit the activity of TCF3 and TCF4 by preventing their dimerization and
164 DNA binding²⁸. The *TCF3* and *ID3* genes are recurrently mutated in another form of B-cell
165 lymphoma, Burkitt Lymphoma, with the mutations residing in the interface between *TCF3* and *ID3*
166 and preventing their interaction^{18,19}. Mutations in *ID3* are approximately twice as frequent as
167 mutations of *TCF3*, and presumably also reduce the interaction between ID3 and TCF4
168 considering the high degree of homology between these two proteins. We observed that TCF4
169 DNA copy number gains are also frequent in Burkitt lymphoma, and that they mutually exclude
170 *TCF3* and *ID3* mutations, providing further evidence for the importance of *TCF3/TCF4*

171 deregulation in this disease. In contrast to Burkitt lymphoma, *TCF3* and *ID3* mutations are rare in
172 ABC-like DLBCL, but *TCF4* DNA copy number gains are present at more than twice the
173 frequency. In line with the murine studies, we observed a marked up-regulation of IgM transcript
174 expression in primary tumors with *TCF4* DNA copy number gain. We also identified binding sites
175 for TCF4 in the immunoglobulin heavy chain locus and showed that induced expression of TCF4
176 was sufficient for increasing the expression of IgM at the transcript and protein level. Together,
177 this provides strong evidence for a functional role of TCF4 in promoting IgM expression in ABC-
178 like DLBCL. This is particularly important in this disease, because >90% of ABC-like DLBCL cases
179 express IgM and the disease etiology centers on pathogenic signaling downstream of this
180 receptor^{7,8}. Notably, *TCF4* was more highly expressed in ABC-like DLBCL compared to GCB-like
181 DLBCL generally, even in cases without DNA copy number gain of the locus. This suggests that
182 this axis may be active in all ABC-like DLBCLs, and further enhanced in the ~40% that harbor
183 18q DNA copy number gains. This is akin to the role of EZH2 in GCB-like DLBCL, which promotes
184 the survival and proliferation of all germinal center B-cells but has enhanced activity in the context
185 of hypomorphic somatic mutations^{31,32}. We therefore hypothesize that *TCF4* may participate in a
186 critical functional axis of immunoglobulin regulation in all ABC-like DLBCL.

187
188 Proteins in the BET family, including BRD4, are attractive therapeutic targets in cancer due to
189 their role in the transcriptional activation of oncogenes such as *MYC*^{33,34}. In DLBCL, BRD4 targets
190 include key transcription factors such as *BCL6*, *PAX5* and *IRF4*³⁵. We have highlighted *TCF4* as
191 another prominent target of BRD4 in DLBCL, as has been previously described in plasmacytoid
192 dendritic cell neoplasms²¹. Due to the difficulty in directly drugging transcription factors, BET
193 inhibition therefore represents a logical avenue for reducing TCF4 expression in ABC-like DLBCL.
194 Notably, cell line studies have shown that the small molecule BET inhibitor OTX015 induces
195 apoptosis in ABC-like DLBCL cell lines, as compared to a predominantly cytostatic effect in GCB-
196 like DLBCL cell lines³⁶. However, small molecule inhibitors have also been shown to result in the
197 up-regulation of BRD4 expression³⁷. We therefore evaluated a novel BET protein PROTAC, ARV-
198 771, which combines a BET-targeting warhead from OTX015 with a moiety that recruits the VHL
199 ubiquitin ligase³⁷. Because the PROTAC is not degraded, this results in sub-stoichiometric
200 proteolysis of BET proteins, including BRD4. We found that ARV-771 was able to inhibit the
201 expression of TCF4 at 10-fold lower concentrations than small molecule BET inhibitors. The
202 inhibition of TCF4 expression in ABC-like DLBCL cell lines with high TCF4 copy number was
203 accompanied by the coordinate down-regulation of genes that were highly expressed in primary
204 tumors with TCF4 DNA copy gain, suggesting that a subset of the broad transcriptional changes

205 associated with BRD4 degradation were the consequence of reduced TCF4. This was also
206 associated with the induction of apoptosis at low nanomolar doses of ARV-771, significant
207 reduction of tumor growth *in vivo*, and a significant prolongation in the life of tumor-bearing
208 animals. Together, this highlights *TCF4* DNA copy gains as a functional rationale for BET
209 inhibition in ABC-like DLBCL and shows that the BET PROTAC ARV-771 has significant activity
210 in this context. The over-expression of *BCL2* has been described as a resistance mechanism for
211 BET inhibitors³⁸. We observed that the majority of 18q DNA copy number gains in DLBCL
212 encompass both the *TCF4* and the *BCL2* gene, and we therefore posit that the promising activity
213 of BET inhibitors in ABC-like DLBCL may be further enhanced by combination with a *BCL2*
214 inhibitor such as Venetoclax. In support of this, BET inhibitors have been shown to act
215 synergistically with Venetoclax in myeloid leukemia³⁸ and in another form of B-cell lymphoma,
216 mantle cell lymphoma³⁹. Combination of BET and *BCL2* inhibition therefore represents an
217 attractive therapeutic avenue for future investigation in ABC-like DLBCL.

218

219 In conclusions, we have identified DNA copy number gains of *TCF4* as the most frequent genetic
220 alteration in ABC-like DLBCL. Increased expression of *TCF4* leads to its occupancy on IgM
221 enhancer elements and increased expression of IgM at the transcript and protein level. We have
222 shown that BET-targeting PROTACs efficiently reduce the expression of *TCF4* and its target
223 genes, induce apoptosis in ABC-like DLBCL cells, and prolong the life of mice bearing ABC-like
224 DLBCL tumors. This study therefore highlights the BRD4-regulated *TCF4* and IgM axis as a
225 functional rationale for the use of BET inhibitors in ABC-like DLBCL.

226

227 **MATERIALS AND METHODS**

228 DNA copy number data acquisition and processing

229 Publicly available data for single nucleotide polymorphism microarrays and array comparative
230 genome hybridization platforms with >200,000 markers were downloaded from the gene
231 expression omnibus^{16,17,40-46} (Table S1; www.ncbi.nlm.nih.gov/geo/). These included Affymetrix
232 250K and SNP 6.0 platforms, and the Agilent 244K platform. For Affymetrix microarrays, raw CEL
233 files were extracted and copy number predicted using the Affymetrix Copy Number Analysis for
234 Genechip (CNAG) tool, with reference to data from 100 Caucasian HapMap samples. Agilent data
235 was analyzed using BioConductor, as previously described¹⁷. Data for all arrays were represented
236 as Log₂ copy number change and segmented using the circular binary segmentation (CBS) tool
237 in GenePattern⁴⁷. Peaks of significant DNA copy number loss and gain were identified using

238 GISTIC2.0¹⁴. The thresholds utilized for DNA copy number gain and loss were 0.2 copies over a
239 region encompassing 100 markers.

240

241 Targeted next generation sequencing (NGS) and variant calling.

242 Genomic DNA for 140 fresh/frozen DLBCL tumors were obtained from the University of Nebraska
243 Medical Center (UNMC) lymphoma tissue bank (IRB 161-95-EP) and interrogated by targeted
244 sequencing of a panel of 380 genes, as previously described²⁰. In brief, 500-1,000ng of genomic
245 DNA was sheared using a Covaris S2 instrument and libraries prepared using KAPA Hyper Prep
246 kits (Roche) and Illumina TruSeq Adapters (Bioo Scientific) according to the manufacturer's
247 protocol. A maximum of 6 cycles of PCR was used for library preparation. Samples were 12-
248 plexed, subjected to hybrid capture with a 5.3Mbp Nimblegen SeqCap custom reagent (Roche),
249 and amplified by 8 cycles of PCR. Each pool was sequenced on a single lane of an Illumina HiSeq
250 2500 instrument in high-output mode using 100bp paired-end reads at the Hudson Alpha Institute
251 for Biotechnology Genome Sequencing Laboratory. Raw sequencing reads were aligned to the
252 human genome (hg19) using BWA-Mem⁴⁸, realigned around InDels using GATK⁴⁹, sorted and
253 deduplicated using Picard tools, and variants were called according to a consensus between
254 VarScan2⁵⁰ and GATK Unified Genotyper⁴⁹. This approach has been validated to have a
255 specificity of 92.9% and a sensitivity of 86.7%⁵¹. Average on-target rate for this dataset was 88%
256 and average depth of coverage 623X (min = 122X, max = 1396X). Raw FASTQ files for the
257 targeted NGS of previously published DLBCLs (n=119; European Nucleotide Archive Accession
258 ERP021212)⁵² and Burkitt's lymphomas²⁰ were also analyzed with the same pipeline, and the
259 results integrated. The DNA copy number of UNMC DLBCL and Burkitt lymphoma cohorts was
260 determined using CopyWriteR⁵³ with 200kB windows.

261

262 DLBCL Cell Lines

263 The SU-DHL-2 cell line was obtained from ATCC. The RIVA (aka RI-1), HBL1, TMD8, U2932 and
264 OCI-Ly10 cell lines were obtained from DSMZ. The DNA copy number profile of DLBCL cell lines
265 were derived from previously reported SNP6.0 data⁵⁴ or targeted next generation sequencing, as
266 described above. Cell of origin subtype was determined according to previous descriptions⁸.
267 U2932, RIVA, TMD8, HBL1, and SUDHL2 were maintained in RPMI-1640 media with 10% FBS
268 and 1% penicillin/streptomycin. OCI-LY1, OCI-LY7 and OCI-LY10 were maintained in IMDM
269 supplemented with 20% human serum and 1% penicillin/streptomycin. Cell lines were regularly
270 tested for mycoplasma, and identity confirmation by Short Tandem Repeat at core facility of MD

271 Anderson Cancer Center. Tetracycline-inducible expression of *TCF4* was performed in the TMD8,
272 HBL1, and SUDHL2 cell lines. Detailed methodology can be found in the supplementary methods.
273

274 ChIP-sequencing of TCF4

275 For inducible TCF4 expression, TMD8-TCF4, or SU-DHL-2-TCF4 cell lines were treated with
276 doxycycline (60ng/ml) for 24 hours. For chromatin immuno-precipitation, five million cells were
277 fixed with 1% formaldehyde for 10 min, quenched by addition of 125 mM Glycine for 5 min at RT,
278 washed with ice-cold PBS then resuspended and incubated in ice-cold ChIP buffer (10mM Tris-
279 HCl pH 8.0, 6.0 mM EDTA, 0.5% SDS and protease inhibitor) for 1hour. In the same time, 5µg of
280 antibodies (TCF4²¹) or control rabbit IgG (Cell Signaling; 2729) were allowed to bind to dynabeads
281 Protein-G (Invitrogen; 10003D) in binding buffer (0.2% BSA, 0.1% Tween-20 in PBS) for 2 hours.
282 Chromatin was sheared using Covaris M220. Sonicated lysates were diluted in dilution buffer
283 (10mM Tris-HCl pH8, 140 mM NaCl, 1mM EDTA pH 8, 0.5 mM EGTA, 1% Triton X-100, and 0.1%
284 Sodium Deoxycholate) and added to antibody bound Protein-G beads for immunoprecipitation
285 overnight at 4°C. Note; for ChIP normalization, spike in chromatin/antibody was added to
286 sonicated lysates (53083/61686; Active Motif). Next day, bead-bound complexes were washed 5
287 times with RIPA buffer (1% NP40, 0.1% SDS, and 0.5% Sodium Deoxycholate in PBS), 2 times
288 with LiCl buffer (10 mM Tris-HCl pH8, 250 mM LiCl, 0.5% NP40, 0.5% Sodium Deoxycholate, 1
289 mM EDTA), once with TE buffer pH 8.0 and finally resuspended in 50µl of TE buffer containing
290 20µg of proteinase K and RNase A (0.2 µg/µl). TE buffer, RNase A and proteinase K mixture was
291 also added in total chromatin samples in parallel as input reference. Reverse cross-linking was
292 performed at thermal cycler (4 hours 37°C, 4 hours 50°C, and overnight 65°C). DNA purification
293 was performed with SPRIselect beads (Beckman Coulter; B23317) and further processed for
294 library generation with KAPA HyperPrep kit (KK8502) according to the kit protocol.

295
296 Sequencing reads were aligned to the human genome (hg19) using BWA-Mem⁴⁸, realigned
297 around InDels using GATK⁴⁹, sorted and deduplicated using Picard tools. Peaks were called in
298 TCF4 ChIP samples compared to their input control using EaSeq with global thresholding. Peaks
299 were annotated according to the transcription start site of the nearest RefSeq gene and filtered
300 based upon FDR (<0.1), log2ratio of TCF4 ChIP vs. isotype control (≥2.0), peaks that overlapped
301 between TMD8 and SUDHL2, and peaks corresponding to genes with differential expression
302 between ABC-like DLBCL tumors with or without TCF4 DNA copy number gain. Peaks within
303 2kbp of the transcription start site were defined as ‘promoter’ peaks, those outside of the promoter
304 region but within the coding region of the gene were defined as ‘intragenic’ peaks, and those

305 outside of these regions but within 50kbp of the transcription start site were defined as distant
306 'enhancer' peaks. For visualization, files were converted to wiggle format and viewed using the
307 Integrative Genomics Viewer⁵⁵. The wiggle file for H3K27Ac ChIP-seq for CD20+ B-cells was
308 downloaded directly from UCSC Genome Browser
309 (<https://genome.ucsc.edu/ENCODE/downloads.html>). Significantly over-represented DNA
310 sequence motifs (FDR<0.05) were identified in TCF4 ChIP-seq peaks compared to the reference
311 genome (hg19) using CisFinder⁵⁶ with the default settings. Motifs with 75% homology were
312 collapsed to motif clusters.

313

314 For ChIP-PCR, chromatin immuno-precipitation was performed with BRD4 antibody (Bethyl, Cat
315 No. A301-985A) following the protocol as described above for TCF4. Chromatin DNA was also
316 purified from the input samples. The purified DNA was used to perform quantitative PCR using
317 SYBR Green/ROX qPCR Master Mix (Applied Biosystem; 4309155). Percentage of input was
318 quantified from the adjusted input Ct values and further used to determine Δ Ct values for BRD4
319 or IgG ChIP. Primers used for ChIP-PCR have been listed in Table S10.

320

321 BET Inhibitors and Treatments

322 BRD4 inhibitors JQ1 and OTX015 were obtained from Selleck Chemicals. BRD4-PROTAC (ARV-
323 771) was provided by Arvinas, Inc. (New Haven, CT). U2932 and RIVA cell lines were treated
324 with indicated concentrations of BET-inhibitors (JQ1, OTX015) or BRD4-PROTAC (ARV771) for
325 24 hours before immunoblotting. For apoptosis analysis, U2932 and RIVA cell lines were seeded
326 at 2.5×10^5 cells/ml and treated with ARV771 at indicated concentration for 48 hours. Cells were
327 stained with Annexin V (Thermo Fisher; A35122)/To-PRO-3 and analyzed using flow cytometry
328 (BD LSRFortessa) and FlowJo software. For gene expression analysis, cell lines (U2932 and
329 RIVA) were un-treated or treated with ARV771 (50ng/ml) for 24 hours. Total RNA was extracted
330 using All prep DNA/RNA kit (Qiagen; 80204) and RNA integrity was assessed using an Agilent-
331 4200 TapeStation system. Libraries were generated using KAPA RNA HyperPrep kit with
332 RiboErase (KK8560) according to the manufacturer's instructions. Libraries were pooled and run
333 on a single lane of a HiSeq 4000 instrument at the MD Anderson Sequencing and Microarray
334 Core Facility. Fastq files were first aligned to the GRCh37 assembly with
335 GENCODE37lift37 annotations using STAR 2.6.0c, using a two-pass protocol with
336 alignment parameters from the ENCODE long RNA-seq pipeline. The transcript-
337 coordinate output files were then pre-processed with RSEM version 1.2.31's convert-

338 sam-for-rsem tool before quantifying with rsem-calculate-expression, assuming the data
339 is from an unstranded paired-end library. Tximport version 1.6.0 was then used under R
340 version 3.4.3 to read individual RSEM output files and aggregate to gene-level
341 expressions based on the gene-transcript relationships in GENCODE27lift37's Gene
342 symbol metadata. DESeq2 version 1.18.1 was used to identify differentially expressed
343 genes using a two-variable (Cell line and Treatment) analysis with default settings. Gene
344 set enrichment analysis⁵⁷ was performed using GenePattern and a list of all genes from
345 RNA-seq ranked by the fold-change in expression following ARV771 treatment. The gene
346 set consisted of all genes that showed significantly higher expression in ABC-like DLBCL
347 tumors with *TCF4* DNA copy number gain compared to those without, as shown in Figure
348 3.

349 Murine Xenograft Experiments

350 *Reagents and antibodies.* ARV-771 was kindly provided by Arvinas, Inc. (New Haven, CT) D-
351 Luciferin (potassium salt) was obtained from Gold Biotechnology, Inc. (St Louis, MO). BD Matrigel
352 Matrix High Concentration was obtained from BD Biosciences (Franklin Lakes, NJ) (Catalog
353 number 354248).

354
355 *Cell lines.* Luciferase-expressing RIVA and U2932 cells were created by transducing cells with
356 Luc-ZSGreen. pHIV-Luc-ZsGreen was a gift from Bryan Welm (Addgene plasmid # 39196). High
357 GFP-expressing cells were isolated by flow sorting for GFP expression in the M. D. Anderson
358 Flow Cytometry and Cellular Imaging Core Facility (FCCICF), a shared resource partially funded
359 by NCI Cancer Center Support Grant P30CA16672.

360
361 *In vivo studies.* All animal studies were performed under a protocol approved by the IACUC at
362 M.D. Anderson Cancer Center, an AAALAC-accredited institution. Five million RIVA or U2932
363 cells (mixed with Matrigel at a volume ratio of 1:1) were subcutaneously injected in the left flank
364 of male athymic nude mice (nu/nu) (n = 8 per group). Tumor volume was calculated by the
365 $\frac{1}{2}(\text{length} \times \text{width}^2)$ method. Treatment was initiated when the mean tumor volumes reached ~150
366 mm³. Mice were treated with vehicle (10% [1:1 solutol: ethanol] and 90% D5-water, s.c. daily x 5
367 days per week) or ARV-771 (30 mg/kg, s.c., daily x 5 per week). The RIVA mouse model was
368 treated for two weeks. Due to slower tumor growth, the U2932 mouse model was treated for three
369 weeks. For bioluminescent imaging, mice were IP-injected with 100 μ L of 75 mg/kg D-Luciferin

370 potassium salt (reconstituted in 1X PBS and sterile-filtered through a 0.2 um filter) incubated for
371 5 minutes, anesthetized with isoflurane and imaged once per week utilizing a Xenogen IVIS-200
372 imaging system (PerkinElmer) to monitor disease status and treatment efficacy. One mouse from
373 each cohort was euthanized after three weeks of treatment for biomarker analysis. Mice bearing
374 tumors greater than 1500 mm³ were removed from study and humanely euthanized (carbon
375 dioxide inhalation and cervical dislocation) according to the IACUC-approved protocol.
376 Veterinarians and veterinary staff assisting in determining when euthanasia was required were
377 blinded to the experimental conditions of the study. Tumor size was compared among cohorts by
378 unpaired t-test. The survival of the mice is represented by a Kaplan Meier plot. Differences in
379 survival were calculated by a Mantel-Cox log-rank test. P values less than 0.05 were considered
380 significant.

381

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386 771 for the studies.

387

388 **COMPETING INTERESTS**

389 The authors have no competing interests to declare.

390

391 **AVAILABILITY OF DATA**

392 The data produced in this study are available in the gene-expression omnibus
393 (www.ncbi.nlm.nih.gov/geo/), accession number GSE119241. The SNP and gene expression
394 microarray accessions for the previously published data are listed in Table S1. Raw next
395 generation sequencing data will be provided upon reasonable request to the corresponding author
396 and the completion of confidentiality non-disclosure and material transfer agreements.

397

398 **AUTHOR CONTRIBUTIONS**

399 NJ, KH, ST, WF, DK and OH performed experiments. NJ, KH, ST, KB and MRG analyzed data
400 and wrote the manuscript. MJM, AB, TH, QD, DM, CP, AG, SR, JI, FG, SSN, JW, RED and KB
401 analyzed or interpreted data. AA and CLL provided computational resources. EH, RK, KES, GJ,
402 RR, RDG, AR, JV, ML, and TG provided samples or data. MRG conceived and supervised the
403 study. All authors read and approved the manuscript.

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587 **FIGURE LEGENDS**

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589 **Figure 1: DNA copy number gains of 18q21.2 are the most frequent genetic alteration in**
590 **ABC-like DLBCL. A)** GISTIC analysis of DNA copy number profiles from 1,000 DLBCL tumors
591 identified 21 peaks of DNA copy loss (blue, left) and 20 peaks of DNA copy gain (red, right). The
592 green line indicates the significance threshold of q-value = 0.1. **B)** The GISTIC peaks from (A)
593 are shown with reference to their frequency in ABC-like (orange) compared to GCB-like (green)
594 cell of origin subtypes (*Q-value<0.1). DNA copy gains of 18q21.2 were the most frequent
595 alteration in ABC-like DLBCL cases. **C-D)** A Kaplan-Meier plot of overall survival for patients
596 treated with CHOP combination chemotherapy (C) or CHOP plus Rituximab (D) shows that the
597 presence of 18q21.2 gain is associated with poor outcome. **E)** The frequency of 18q21 gains is
598 shown relative to other somatic mutations that are significantly associated with the ABC-like
599 DLBCL subtype. This shows that gains of 18q21 are the most frequent genetic alteration in ABC-
600 like DLBCL. **F)** REVEALER analysis was performed to identify the set functionally-complementary
601 genetic features that likely contribute to the ABC-like DLBCL molecular phenotype. Mutations of
602 *MYD88* were used as the seed feature. Mutations of *IRF4*, *PIM1* and *CD79B*, and DNA copy
603 gains of 18q21 were selected as additional features that likely also contribute to the phenotype
604 (*Seed feature; IC, information coefficient; CIC, conditional information coefficient).

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620 **Figure 2: The TCF4 gene is the most significant target of 18q DNA copy number gains. A)**

621 A schematic of 18q DNA copy number gains is shown, with each line representing a single tumor
622 and deeper shades of red indicating higher DNA copy number. The GISTIC q-value is shown at
623 the top of the diagram and the two significant peaks are highlighted with arrows. The most
624 statistically significant peak harbors the *TCF4* gene, while the less significant peak harbors the
625 *BCL2* gene. However, it can be seen that in many cases the DNA copy number gains span both
626 the *TCF4* and *BCL2* regions. **B-C)** The frequency of tumors with DNA copy number gains that
627 include both the *TCF4* and *BCL2* genes (purple), the *TCF4* gene and not the *BCL2* gene (pink)
628 or the *BCL2* gene and not the *TCF4* gene (yellow) are shown for all tumors (B) and for the ABC-
629 like only (C). This shows that the majority of 18q DNA copy gains include both TCF4 and BCL2,
630 but *TCF4* is more frequently gained independently of *BCL2* than vice versa. **D)** The gene
631 expression level from microarrays are shown for GCB-like DLBCL (green) and ABC-like (orange)
632 DLBCL tumors that are diploid for 18q, and for ABC-like DLBCL tumors that harbor *TCF4* DNA
633 copy number gains (red). The expression of TCF4 is significantly higher in ABC-like DLBCL
634 compared to GCB-like DLBCL in diploid cases and further significantly increased by DNA copy
635 number gain. P-values are from students T-test. **E)** The protein level of TCF4 and BCL2 are shown
636 in ABC-like DLBCL cell lines, ordered according to DNA copy number of the *TCF4* locus. Two
637 GCB-like DLBCL cell lines are shown for reference. The ABC-like DLBCL cell lines express higher
638 levels of TCF4 than the GCB-like cell lines and there is a visible relationship between TCF4
639 protein abundance and DNA copy number that is less clear for BCL2. **F)** The frequency of *TCF4*
640 DNA copy gains, *TCF3* mutation and *ID3* mutation are shown for a cohort of 108 Burkitt lymphoma
641 tumors. Gains of the *TCF4* locus are present at the same frequency of *TCF3* mutations and are
642 significantly mutually exclusive from *TCF3* and *ID3* mutations.

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654 **Figure 3: TCF4 regulates IgM expression in ABC-like DLBCL. A)** Differential gene expression
655 analysis of 110 primary ABC-like DLBCL tumors with or without *TCF4* DNA copy number gain
656 identified a large set of genes with increased expression associated with *TCF4* gain. This included
657 the direct targets of 18q DNA copy number gains, *TCF4* and *BCL2*, and multiple genes with an
658 important role in the pathophysiology of DLBCL, such as *IRF4*, *MYC* and the immunoglobulin
659 heavy chain μ (*IGHM*), that are upregulated as a secondary effect of *TCF4* gain. ChIP-seq of
660 *TCF4* from SUDHL2 and TMD8 cell lines showed that the majority of these genes were marked
661 with *TCF4* binding in intragenic or distant enhancer elements, suggesting that their up-regulation
662 may be driven by transcriptional activation by *TCF4*. **B)** The significant *TCF4* ChIP-seq peaks
663 from SUDHL2 and TMD8 are shown, ordered from strongest (top) to weakest (bottom) signal ratio
664 compared to the input control. Significant peaks were detected for important genes such as *MYC*
665 and *IRF4*, but multiple *IGHM* peaks were amongst those with the highest *TCF4* binding. **C)** A
666 violin plot shows that primary DLBCL tumors with 18q21 gain express significantly higher
667 transcript levels of *IGHM*. **D)** Two of the *TCF4* peaks at the immunoglobulin heavy chain locus
668 are shown for *TCF4* ChIP (blue) compared to the equivalent input control (grey). A black box
669 indicates the significant peak. For reference, ENCODE data for H3K27 acetylation (H3K27Ac)
670 ChIP-seq in CD20+ B-cells is shown, which support the *TCF4* bound regions as bona fide
671 enhancer elements in B-cells. **E)** Tetracycline-induced expression of *TCF4* in ABC-like DLBCL
672 cell lines with low *TCF4* copy number resulted in a significant increase in *IGHM* transcript
673 compared to control cells. **F)** Tetracycline-induced expression of *TCF4* led to a marked increase
674 in IgM protein in ABC-like DLBCL cell lines with low *TCF4* copy number. An increase in *MYC* was
675 also observed in SUDHL2 and HBL1 cell line, but was not significant in TMD8. No change was
676 observed for *BCL2*. The quantification of triplicate experiments is shown in Figure S5.

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687 **Figure 4: BET proteolysis-targeting chimeras (PROTAC) effectively inhibit TCF4 and show**
688 **efficacy in ABC-like DLBCL cell lines. A)** The treatment of ABC-like DLBCL cell lines with high
689 *TCF4* DNA copy number using small molecule BET inhibitors, JQ1 and OTX015, leads to an
690 accumulation of BRD4 but can reduce the BRD4-targets TCF4 and MYC. The BET PROTAC,
691 ARV771, effectively degrades BRD4 and leads to a more potent reduction of TCF4 and MYC at
692 10-fold lower doses than the small molecule inhibitors. **B)** Treatment of U2932 and RIVA cell lines
693 with 50nM of ARV771 for 48h led to the induction of apoptosis, as measured by TOPRO3 /
694 Annexin-V positive staining. **C)** The treatment of U2932 and RIVA cell lines with 50nM of ARV771
695 for 24h led to broad changes in transcript levels. This included the down-regulation of known
696 BRD4 target genes, *BCL6* and *PAX5*, as well as the down-regulation of *TCF4* and its target gene,
697 *IGHM*. **D)** The down-regulation of IgM following treatment with ARV771 is also observed at the
698 protein level. The quantification of triplicate experiments is shown in Figure S7. **E)** Gene set
699 enrichment analyses are overlaid for U2932 (green) and RIVA (blue) for the set of genes that
700 were more highly expressed in primary ABC-like tumors with TCF4 DNA copy number gain
701 compared to those tumors without DNA copy number gain, as shown in Figure 3A. Treatment
702 with ARV771 led to a significant and coordinate down-regulation of this TCF4-associated
703 signature in both the U2932 and RIVA cell lines. **F-I)** Murine xenografts of the U2932 cell line
704 were allowed to become established and then treated with 30mg/kg of ARV771 daily x 5 days per
705 week for 3 weeks. At the end of treatment the luminescence was significantly lower in ARV771-
706 treated mice compared to vehicle control (F, G) as a result of the significant reduction in tumor
707 growth (H). This led to significantly prolonged survival in ARV771-treated mice. **J-M)** Murine
708 xenografts of the RIVA cell line were allowed to become established and then treated with
709 30mg/kg of ARV771 daily x 5 days per week for 2 weeks. At the end of treatment the
710 luminescence was significantly lower in ARV771-treated mice compared to the vehicle control (J-
711 K), as a result of the significant reduction in tumor growth (L). Despite the short duration of
712 treatment, this led to a significant prolongation in survival of ARV771-treated mice (M).

713

Figure 1

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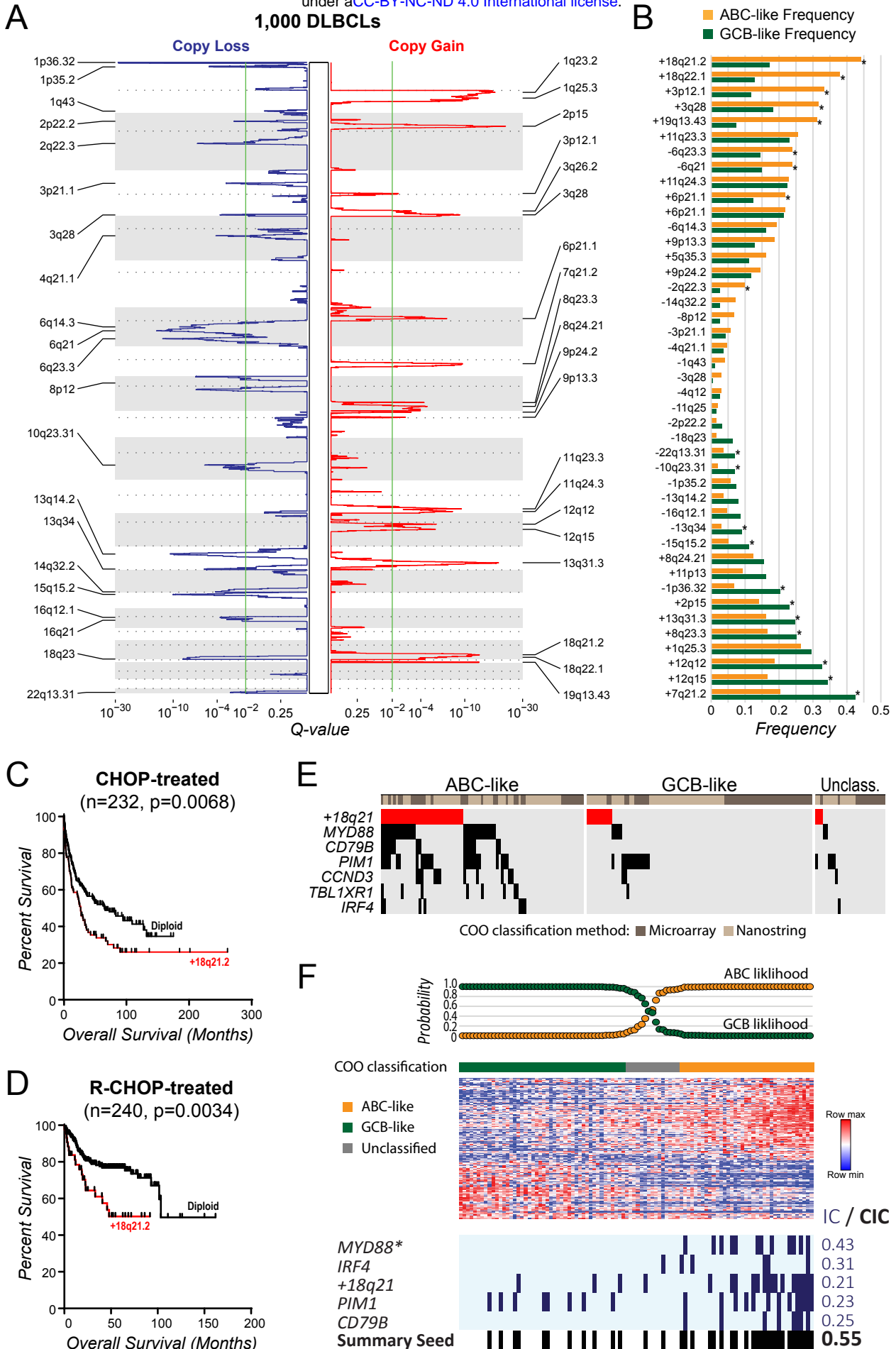


Figure 2

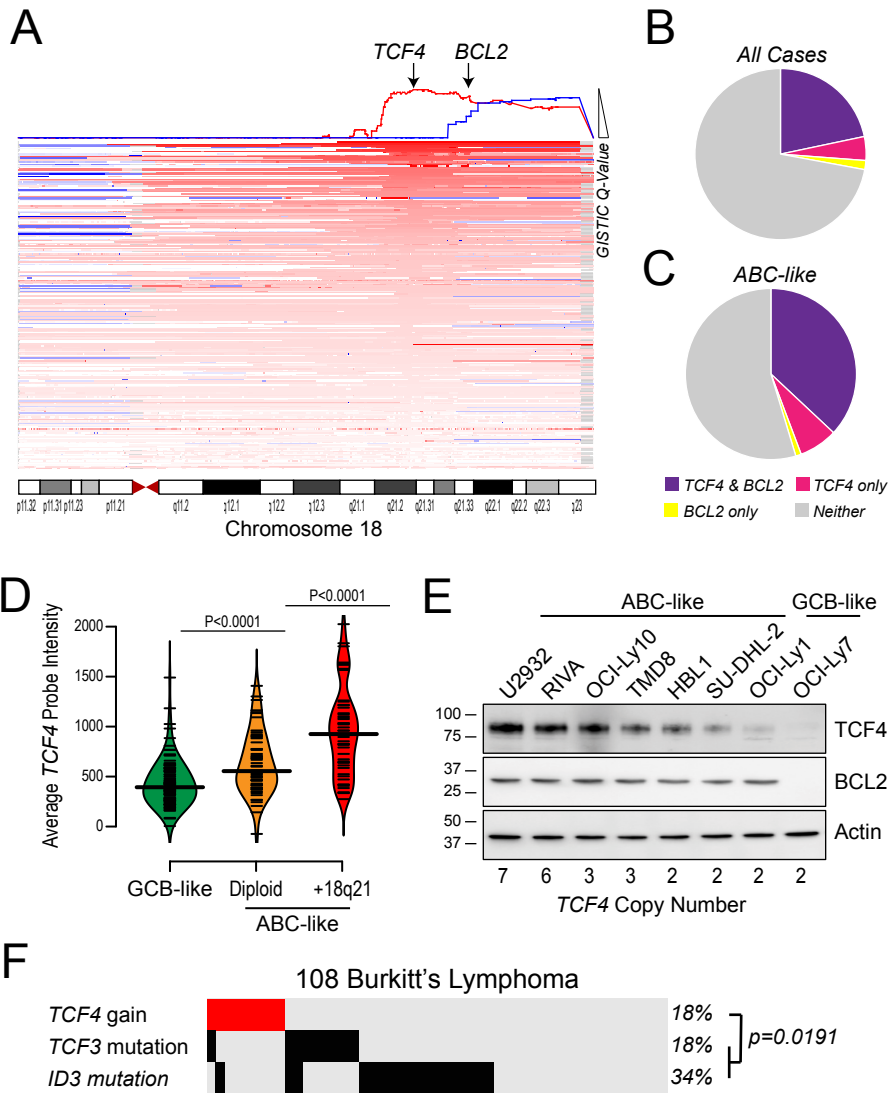


Figure 3

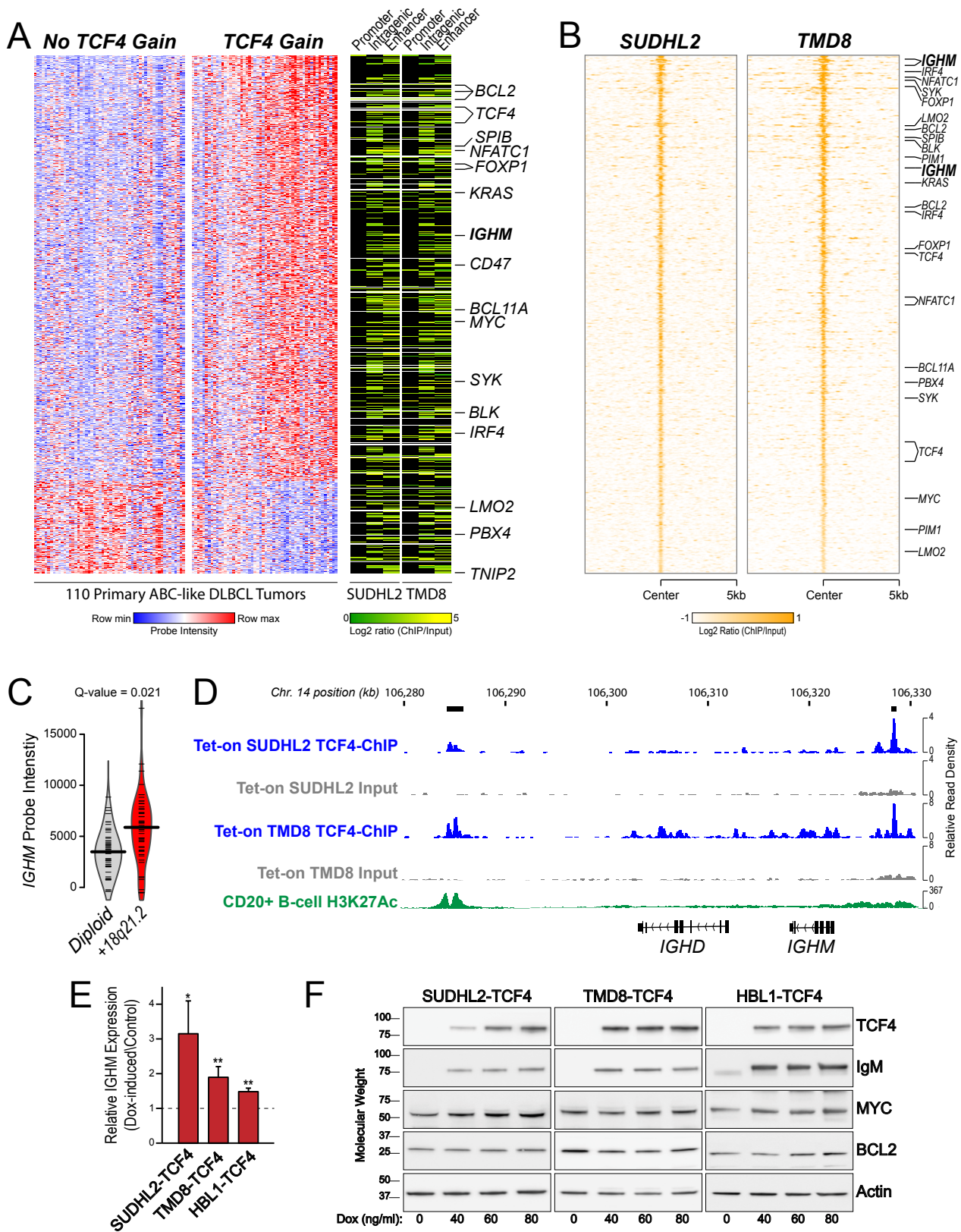


Figure 4

