MEF2B mutations lead to deregulated expression of the oncogene BCL6 in diffuse large B cell lymphoma

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MEF2B encodes a transcriptional activator and is mutated in ~11% of diffuse large B cell lymphomas (DLBCLs) and ~12% of follicular lymphomas (FLs). Here we found that MEF2B directly activated the transcription of the proto-oncogene BCL6 in normal germinal-center (GC) B cells and was required for DLBCL proliferation. Mutation of MEF2B resulted in enhanced transcriptional activity of MEF2B either through disruption of its interaction with the corepressor CABIN1 or by rendering it insensitive to inhibitory signaling events mediated by phosphorylation and sumoylation. Consequently, the transcriptional activity of Bcl-6 was deregulated in DLBCLs with MEF2B mutations. Thus, somatic mutations of MEF2B may contribute to lymphomagenesis by deregulating BCL6 expression, and MEF2B may represent an alternative target for blocking Bcl-6 activity in DLBCLs.

Diffuse large B cell lymphoma (DLBCL) is the most common form of human non-Hodgkin’s lymphoma in adults; it accounts for approximately 40% of diagnoses and also arises from the transformation of follicular lymphoma (FL)1. Gene-expression profiling studies have identified the heterogeneity of this germinal center (GC)-related malignancy by distinguishing three phenotypic subtypes—GC B cell–like (GCB) DLBCL, activated B cell–like (ABC) DLBCL and primary mediastinal B cell lymphoma—with a small subset of cases that remain unclassified. Those subtypes differ in their genotype, phenotype and, notably, clinical features, including different response to the currently adopted immunochemotherapy-based regimen3. Although a subset of patients with DLBCL can be cured, a substantial fraction of them (~40%) die of the disease3, which indicates the need to develop more specific targeted therapies.

Technological advances, including whole-genome sequencing of DNA and RNA and genomewide copy-number analysis, have provided a comprehensive view of the genomic landscape of GCB DLBCLs and ABC DLBCLs, which has allowed new insights in the genetic lesions associated with the pathogenesis of this malignancy4–7. Such approaches have identified several recurrent lesions that are present in both subtypes of DLBCL, including those involving the functions of chromatin acetylation and methylation, alterations that deregulate the GC master regulator Bcl-6 and those that lead to escape from the immune system4–5,8–10. In addition, such studies have confirmed or newly identified genetic lesions ‘preferentially’ associated with GCB DLBCLs, including chromosomal translocations involving MYC (which encodes the proto-oncoprotein c-Myc) and BCL2 (which encodes the antiapoptotic protein Bcl-2), and mutational activation of EZH2 (which encodes a chromatin-remodeling factor), as well as lesions ‘preferentially’ associated with ABC DLBCL, including those that lead to activation of the transcription complex NF-kB, translocations involving BCL6 and mutational inactivation of PRDM1 (which encodes Blimp-1, the master regulator of plasma–cell differentiation)10–16.

Among the genetic alterations recurrently found in DLBCL and FL but that remain of unclear functional relevance are mutations affecting MEF2B1–7. MEF2B is a member of the MEF2 (‘myocyte enhancer-binding factor 2’) family of transcription factors (MEF2A, MEF2B, MEF2C and MEF2D), which are characterized by high homology in their MADS (‘MCM1 agamous deficiens SRF’) box and an adjacent MEF2 domain17. Together those two conserved domains in the amino (N)-terminal half of MEF2B direct DNA binding, homodimerization of MEF2 polypeptides and interaction with specific transcriptional cofactors. The highly divergent carboxy (C)-terminal half of MEF2 proteins has been suggested to modulate their transcriptional activity17,18. The spectrum of targets activated by MEF2 transcription factors in different cell types is dependent on association with specific corepressors and coactivators in response to multiple signaling pathways17. In particular, MEF2B functions as a transcriptional activator by binding to specific AT-rich DNA sequences originally identified in the control regions of muscle-specific and growth factor–related genes18,19. Its activity is regulated by the alternative binding of either the corepressor CABIN1 or class II histone deacetylases to its N terminus, depending on the specific cellular context20,21. MEF2B can express at least two protein isoforms (A and B) with distinct C-terminal domains. In addition, several transcripts, some of which are tissue specific, are generated via alternative splicing. In lymphocytes,
MEF2D is involved in T cell antigen receptor–mediated apoptosis and the response to calcium signaling in thymocytes\textsuperscript{1,2}, whereas MEF2C is required for the formation of the GC\textsuperscript{23,24}. In the present study, we identify functional consequences of genetic alterations that affect MEF2B in DLBCLs and FLs and reveal a new role for MEF2B as a master regulator of the GC gene BCL6.

RESULTS

MEF2B mutations in DLBCL and FL

To further investigate the mutations that affect MEF2B in DLBCL and FL, we extended published analyses\textsuperscript{4} to include a total of 134 DLBCL samples (111 cases of primary DLBCL and 23 cell lines), as well as 35 cases of primary FL (Fig. 1). Using genomic PCR amplification and Sanger sequencing of the coding region of MEF2B, we identified 11 sequence variants, distributed in 10 of 134 cases of DLBCL and 1 of 35 cases of FL (Supplementary Table 1). We confirmed the somatic origin of the mutations by analysis of paired normal DNA, available in 3 cases from either our own panel or other reported data sets\textsuperscript{5,6}. We verified expression of the mutated MEF2B alleles in cases of primary DLBCL and confirmed the heterozygous nature of the mutations in all mutated DLBCL cell lines. With the exception of a frameshift deletion, all MEF2B mutations affected the two known isoforms (A and B) of MEF2B, both of which are expressed in B cells (Supplementary Table 1 and Supplementary Fig. 1).

Including the mutations reported in other studies\textsuperscript{5–7}, most observed variants were missense mutations (69%; 27 of 39), while eight frameshift and four nonsense mutations accounted for the remainder (Fig. 1a). Most missense mutations (85%) were clustered in the region encoding the N-terminal conserved MADS box and MEF2 functional domains (Fig. 1a), which suggested that they may affect the transcriptional function of MEF2B. In this region, six amino acid changes of confirmed somatic origin (K4E, K5N, Y69H, E77K, N81Y and D83V) recurrently affected the same codons in distinct cases of DLBCL and FL (Fig. 1a).

While 81% (73 of 90) of all reported cases with MEF2B mutation had alterations affecting the N-terminal region of MEF2B, a second group of cases (19%; 17 of 90) carried MEF2B mutations that affected the C-terminal half of the protein and included a mixture of frameshift, missense and nonsense mutations and no recurrent events (Fig. 1a). The nonsense mutations and the majority of frameshift mutations were predicted to generate truncated proteins through deletion of different lengths of the C terminus. Two of those alleles (encoding the substitutions R171X and Y201X) expressed MEF2B proteins with truncation of the C terminus (~19 kilodaltons (kDa) and ~22 kDa, respectively), whereas the frameshift mutations (G242fs, P256fs and L269fs), when expressed as mRNA encoding isoform A, were predicted to generate a full-length protein similar to wild-type isoform B, except for a unique stretch of inserted amino acids (Supplementary Fig. 1). Collectively, these alleles encoded proteins with truncation of the C terminus or that mimicked the wild-type MEF2B isoform B, which has a distinct C terminus (Supplementary Fig. 1). In conclusion, MEF2B was mutated in 7.5% of the cases of DLBCL in our panel, including both GCB DLBCL (7 of 66; 10.6%) and ABC DLBCL (3 of 68; 4.4%), and in 3% of FL cases (1 of 35) (Fig. 1b).

Induction of MEF2B expression in GC B cells

We investigated the expression pattern of MEF2B in normal mature B cell subpopulations isolated from human tonsils\textsuperscript{25}. MEF2B mRNA, but not mRNA encoding other members of the MEF2 family, had abundant expression in GC B cells, with low expression in naive and memory B cells (Fig. 2a). The expression pattern of MEF2B in these B cell subpopulations was similar to that of the proto-oncogene BCL6 (Fig. 2a,b), which encodes a transcriptional repressor (Bcl-6) that is selectively expressed in GC B cells of the mature B cell lineage and is required for GC formation and whose deregulated expression is linked to lymphomagenesis\textsuperscript{26,27}. We detected transcripts corresponding to the A and B isoforms of MEF2B in GC B cells (data not shown), and expression of MEF2B protein was high in GC B cells, while it was absent in naive B cells and follicular mantle zones (Fig. 2c,d). We further confirmed the association of MEF2B expression with the fate of GC B cells in secondary lymphoid tissues of mice challenged with T cell–dependent antigens (Fig. 2e), in which expression of MEF2B protein appeared in activated B cells soon after immunization (day 2) and slightly preceded that of Bcl-6, a known marker of GC commitment. MEF2B was coexpressed with Bcl-6 throughout the GC reaction, as confirmed by immunofluorescence staining (Fig. 2e and Supplementary Fig. 2a,b). These observations indicated that MEF2B, like Bcl-6, is a bona fide GC B cell marker.

MEF2B directly regulates BCL6 transcription

The coexpression of MEF2B and Bcl-6 in GC B cells prompted us to investigate the relationship between these two transcription factors.
The GC reaction, assessed by flow cytometry above. M, mantle zone. Scale bars, 20 µm. (e) Kinetics of the expression of MEF2B and Bcl-6 protein on days 0–5 (left margin) during the GC reaction, assayed by flow cytometry (left) and immunofluorescence analysis (right). Outlined areas (left) indicate GC-committed (PNA<sup>hi</sup>CD95<sup>hi</sup>) B cells (far left) and immunofluorescence staining of MEF2B (green) and the B cell–specific surface antigen CD20 (red) in a human tonsil section (top); below, enlargement of area outlined above. M, mantle zone. Scale bars, 20 µm. (f) Representative of one experiment (a, b; mean and s.d. of three technical replicates in b), two independent experiments (c), four independent experiments (d) or two independent experiments with one mouse per time point (e).

Analysis of the 'human B cell interactome' (the entire set of molecular interactions in human B cells)<sup>28</sup> showed that MEF2B was specifically connected to Bcl-6 and the transcription cofactor ZMYND11, which showed positive (Bcl-6) and negative (ZMYND11) transcriptional relationships with MEF2B<sup>29</sup> (Fig. 3a). Accordingly, MEF2B expression had a strong positive correlation with Bcl-6 expression in a variety of B cell phenotypes (Fig. 3b). Moreover, MEF2B seemed to be a main node in the Bcl-6 regulatory network predicted by the ARACNe algorithm, a reverse-engineering approach applied to a large data set of normal and malignant B cells<sup>30</sup>.

While MEF2B was not included in the list of direct transcriptional targets bound by Bcl-6<sup>21</sup> in vivo<sup>31</sup>, we identified several AT-rich DNA sequences resembling canonical MEF2-binding motifs in a ~1.5-kilobase region of the BCL6 promoter proximal to the transcription start site (Supplementary Fig. 2c), which suggested that MEF2B might bind to the BCL6 promoter region. In support of that hypothesis, chromatin immunoprecipitation (ChIP) analysis of tonsillar GC B cells and two DLBCL cell lines (U2932 and SUDHL4) revealed binding of MEF2B to a region of the BCL6 promoter approximately 1 kilobase upstream of the BCL6 transcription start site (Fig. 3c and Supplementary Fig. 2c,d). To determine the transcriptional outcome of the binding of MEF2B to the BCL6 promoter, we analyzed the response to MEF2B of a luciferase reporter driven by a native BCL6 promoter region (positions −1593 to −672). Cotransfection of that reporter with increasing amounts of a plasmid expressing wild-type MEF2B in HEK293T human embryonic kidney cells led to a dose-dependent increase in the reporter activity (up to fivefold; Fig. 3d). While both MEF2B isoforms were able to transactivate the BCL6 reporter construct, isoform B exhibited higher transcriptional activity than did isoform A (Fig. 3d), both in HEK293T cells and in DLBCL cells (Supplementary Fig. 3). Deletion of the DNA-binding and dimerization domains of MEF2B or selective mutation of a potential MEF2B consensus site in the promoter region of BCL6 abrogated the ability of MEF2B to transactivate the BCL6 promoter (Fig. 3d and Supplementary Fig. 3), which confirmed the specificity of the observed transcriptional effects.

To demonstrate the relevance of the MEF2B-dependent regulation of Bcl-6 expression in vivo, we silenced MEF2B in two DLBCL cell lines (U2932 and SUDHL4) through the use of two different short hairpin RNAs (shRNAs) that target both isoforms of MEF2B. Consistent with the results of the luciferase reporter assays, knockdown of MEF2B led to downregulation of Bcl-6 expression, while knockdown of Bcl-6 did not affect the expression of MEF2B protein or MEF2B mRNA (Fig. 3e and Supplementary Fig. 4). Accordingly, silencing of MEF2B expression in DLBCL cell lines (LY8 and VAL) carrying BCL6 translocations that remove the MEF2B-binding region of the BCL6 promoter did not affect the expression of Bcl-6 protein (Fig. 3f), in contrast to results obtained with DLBCL cell lines with wild-type BCL6 alleles (U2932 and SUDHL4; Fig. 3e). Finally, knockdown of MEF2B in a B cell line (P3HR1) led to increased expression of Bcl-6 target genes<sup>32</sup> (Supplementary Fig. 4). Together these data demonstrated that BCL6 is a direct transcriptional target of MEF2B in normal and malignant GC B cells.

**DLBCL proliferation requires MEF2B**

To investigate whether, like Bcl-6, MEF2B is required for DLBCL growth, we infected the cell lines SUDHL4 (carrying a MEF2B mutation encoding D83V) and U2932 (carrying wild-type MEF2B) with an inducible lentiviral vector system in which transcription of a cassette encoding the red (orange) fluorescent protein turboFP<sup>2</sup> (rFP) and shRNA is dependent on the addition of doxycycline<sup>32</sup> (Fig. 4a). In SUDHL4 cells, two different shRNAs targeting MEF2B caused a progressive depletion of rFP<sup>2</sup> cells, in direct correlation with their ability to decrease the abundance of MEF2B mRNA and MEF2B protein (Fig. 4a,b and Supplementary Fig. 4). That growth defect was associated with a substantial decrease in the expression of BCL6.
mRNA and Bcl-6 protein, and it did not occur in cells treated with shRNA targeting renilla luciferase, used here as a control (Fig. 4a,b and Supplementary Fig. 4). As expected, knockdown of Bcl-6 resulted in defective cell growth, with no effect on MEF2B expression (Fig. 4a,b). Analysis of cell-cycle distribution by flow cytometry revealed that tRFP+ SUDHL4 cells expressing either MEF2B- or Bcl-6-specific shRNA were mainly arrested at the G1 phase of the cell cycle (Fig. 4c and Supplementary Fig. 4), whereas we observed no increase in markers of early apoptosis (annexin V) or cell death (7-AAD; data not shown). We obtained similar results for U2932 cells (data not shown). Enforced expression of Bcl-6 alone was not sufficient to ‘rescue’ the proliferative defects of B cells after knockdown of MEF2B (Supplementary Fig. 5). Therefore, GC-derived lymphoma cells were dependent on MEF2B for their growth and proliferation, and this effect was related only in part to the ability of MEF2B to control Bcl-6 expression.

**N-terminal substitutions enhance MEF2B transcriptional activity**

We then investigated the consequences of all DLBCL- and FL-associated MEF2B mutations detected in our panel (Supplementary Table 1), as well as two additional somatic missense mutations (encoding Y69H and N81Y), which are highly recurrent in DLBCL and FL, for their effects on the MEF2B–Bcl-6 transcriptional axis. Analysis of the transcriptional response of the BCL6 promoter to those mutants, through the use of a luciferase reporter assay, showed that a subset of mutations encoding substitutions located in the N-terminal half of MEF2B, including the most recurrent D83V, resulted in increased transcriptional activity (Fig. 5a and Supplementary Fig. 3). The enhanced transcriptional activity of these mutants suggested a probable dominant effect, as all these MEF2B mutations are present in heterozygous form in cases of primary DLBCL. We confirmed that hypothesis by showing that cotransfection of equimolar amounts of wild-type MEF2B and mutant MEF2B with the D83V substitution, a combination that mimics primary DLBCL, caused an increase in transactivation of the BCL6 promoter similar to that achieved with the D83V MEF2B mutant alone (Supplementary Fig. 6).

To confirm the physiological relevance of the findings reported above, we investigated the Bcl-6 transcriptional signature31, as a proxy of Bcl-6 activity, in the gene-expression profiles of cases of primary DLBCL ‘stratified’ by the presence or absence of MEF2B mutations. By gene-set enrichment analysis33, we observed that tumors with mutated MEF2B alleles encoding mutant MEF2B with enhanced transcriptional activity showed significant downregulation of Bcl-6 target genes31 (Fig. 5b). This result suggested that MEF2B mutants with enhanced transcriptional activity at the BCL6 promoter resulted in increased Bcl-6 biological activity; thus, these may contribute to the deregulation of the proto-oncogene BCL6 in DLBCL and FL.

![Figure 3](image_url) **Figure 3** BCL6 is a direct transcriptional target of MEF2B in GC B cells. (a) Connections between transcription factors identified as master regulators of the GC28, as predicted by the ARACNe algorithm30, including MEF2B (yellow arrow), Bcl-6 (red arrow) and ZMYND11 (green arrow); red and green lines indicate positive and negative correlation, respectively. (b) Ranked correlation between MEF2B expression and BCL6 expression in normal B cells (red, GC n samples per phenotype = 16); dark blue, naive (n = 5); and green, memory (n = 5) and tumor cells (yellow, chronic lymphocytic leukemia (n = 26); dark pink, mantle cell lymphoma (n = 8); light blue, FL (n = 6); light pink, Burkitt lymphoma (n = 11); and gray, DLBCL (n = 51)). Spearman’s rank correlation coefficient (R2) = 0.7. (c) The BCL6 promoter (top), including the MEF2B-binding region (A) and control region (B), with positions numbered relative to the transcription start site (TSS), and ChIP–quantitative PCR analysis (bottom) of the binding of MEF2B to those regions in GC B cells and U2932 cells; results are presented relative to those obtained with immunoglobulin G (IgG; control), set as 1. (d) Luciferase activity (top) of HEK293T cells transfected to express a luciferase reporter driven by a native BCL6 promoter (positions −1593 to −672 (encompassing the MEF2B-bound region); BCL6-luc) together with increasing amounts (wedge) of hemagglutinin (HA)-tagged wild-type MEF2B (HA-MEF2B WT) or with a HA-tagged transcription-defective mutant with deletion of the DNA-binding and dimerization domains (HA-MEF2B ΔMADSMEF2); results are presented relative to those of cells transfected to express the luciferase reporter alone (–), set as 1. Below, immunoblot analysis of lysates of the cells above. (e) Immunoblot analysis of MEF2B, Bcl-6 and actin in U2932 and SUDHL4 cells transduced with lentiviral vector expressing control shRNA (Ctrl.sh) or either of two different MEF2B- or Bcl-6-targeting shRNAs (.sh1 or .sh2). (f) BCL6 locus, with upward arrows indicating translocation (Tx) breakpoints in the LY8 and VAL DLBCL cell lines (top), and immunoblot analysis of MEF2B, Bcl-6 and actin in LY8 and VAL cells transduced with lentiviral vector expressing control or MEF2B-targeting shRNA (below). Data are representative of three independent experiments (c); mean and s.d. of three technical replicates, two experiments with two technical replicates each (d); mean and s.d. of four samples) or two independent experiments (e,f).
N-terminal mutants escape CABIN1 corepressor activity

To identify the mechanisms by which lymphoma-associated MEF2B mutants affect its transcriptional activity, we used publicly available structural data of the N terminus domain of human MEF2B in complex with DNA and with CABIN1, its known corepressor20 (Fig. 6a). We used PyMOL molecular graphics software to study potential structural alterations due to mutant residues and predicted that several MEF2B missense mutations (resulting in the L54P, Y69H, E77K, S78R and D83V substitutions) could impair the ability of MEF2B to bind CABIN1 (Fig. 6a and Supplementary Table 1). Additionally, we found that the L38I, L54P and N81Y substitutions could alter the homodimerization of MEF2B, whereas the K4E substitution could affect DNA binding (Fig. 6a and Supplementary Table 1).

To test those predictions, we used coimmunoprecipitation assays to investigate the ability of wild-type and mutant MEF2B to interact with CABIN1 in HEK293T cells transfected to express those two proteins. Six substitutions (L54P, Y69H, E77K, S78R, N81Y and D83V) abrogated the binding of MEF2B to CABIN1, whereas the remaining mutations acted similarly to wild-type MEF2B (Fig. 6b).

Accordingly, mutant proteins with the L54P, Y69H, E77K, S78R, N81Y or D83V substitution that did not bind CABIN1 in communoprecipitation assays (Fig. 6b,c) or recruit CABIN1 to the BCL6 promoter (Fig. 6d) also escaped the corepression effects of CABIN1 in promoter reporter assays (Fig. 6e). Reciprocally, an experimentally generated mutation that resulted in the L2172A substitution in CABIN1, which abrogates binding to MEF2B20, blocked that corepressive effect (Supplementary Fig. 7); this confirmed the specificity of the MEF2B-CABIN1 interaction. We obtained analogous results for the same mutations in the context of MEF2B isoform A (Supplementary Fig. 3). An exception to the observations reported above was the L38I mutant, which did not respond to CABIN1 despite its ability to physically interact with this transcriptional corepressor (Fig. 6b,e). Although the substitution L38I and two other missense mutations (resulting in the L54P and N81Y substitutions) were predicted to affect the dimerization of MEF2B monomers, given the crystal structural data of the N terminus domain of human MEF2B in complex with DNA and with CABIN1, its known corepressor20 (Fig. 6a) we used PyMOL molecular graphics software to study potential structural alterations due to mutant residues and predicted that several MEF2B missense mutations (resulting in the L54P, Y69H, E77K, S78R and D83V substitutions) could impair the ability of MEF2B to bind CABIN1 (Fig. 6a and Supplementary Table 1). Additionally, we found that the L38I, L54P and N81Y substitutions could alter the homodimerization of MEF2B, whereas the K4E substitution could affect DNA binding (Fig. 6a and Supplementary Table 1).

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Figure 4 MEF2B is required for cell-cycle progression and proliferation in DLBCL. (a) Time course of the abundance of tRFP+ SUDHL4 cells infected with inducible lentivector expressing control shRNA (Ctrl) or either of two shRNAs (a) or (b) targeting MEF2B or Bcl-6 (key), then cultured for 18 d together with uninfected cells (ratio, 1:1) in the presence of doxycycline (for induction of shRNA expression); results are presented relative to those of cells at time 0, set as 100%. (b) Immunoblot analysis of MEF2B, Bcl-6 and actin in tRFP+ and tRFP− SUDHL4 cells at day 2 of coculture as in a. (c) Cell-cycle profiles of tRFP+ (+) and tRFP− (−) cell populations at day 2 of coculture as in a; numbers above bars indicate frequency of cells in G1, S or G2-M, detected through the use of the thymidine analog BrdU and staining with 7-AAD (specific phase gates used for quantitation, Supplementary Fig. 4d,e). Data are representative of two independent experiments with one cell pool per shRNA.

Figure 5 N-terminal substitutions of MEF2B enhance BCL6 transcription and correlate with increased biological activity of Bcl-6 in cases of primary DLBCL. (a) Luciferase activity in HEK293T cells transfected to express a luciferase reporter driven by the BCL6 promoter (as in Fig. 3d) together with expression vectors with alleles encoding wild-type or mutant MEF2B (horizontal axis); dashed line, activity of wild-type MEF2B; red bars, MEF2B mutants with significantly more transcriptional activity than wild-type MEF2B. * P < 0.05 (one-way analysis of variance paired with Tukey’s multiple-comparison test). (b) Gene-set enrichment analysis of the Bcl-6 core transcriptional signature31 in cases of primary DLBCL with wild-type MEF2B (WT; n = 8 samples) or mutant MEF2B (L54P, E77K or S78R) with increased transcriptional activity at the BCL6 promoter (Mut; n = 3 samples). P < 0.00001; false-discovery rate, <0.00001; normalized enrichment score, 2.05. Data are representative of three independent experiments with two technical replicates in each (a; error bars, s.d.).
structure (Fig. 6a), we did not observe altered homodimerization in coimmunoprecipitation assays, which suggested that these three mutations may instead result in stabilization of the MEF2B dimer (Supplementary Fig. 8). Together these results indicated that a subset of lymphoma-associated MEF2B mutations resulted in abrogation of the binding of MEF2B to CABIN1 and response to its corepressive activity and, consequently, deregulated MEF2B transcriptional activity. Given both the confirmation from biochemical analyses and the structural predictions, this mechanism accounts for 56% of all cases of DLBCL and FL with MEF2B mutations reported thus far (n = 47 of 90 and n = 3 of 90, respectively)\(^1\)–\(^6\).  

### C-terminal mutants escape inactivation by the kinase PKA

The predominance of nonsense and frameshift mutations leading to truncated MEF2B proteins suggested that those mutations may have been selected for the elimination of a C-terminal regulatory domain (Fig. 1a). That observation, together with the finding that protein kinase A (PKA) phosphorylates the C terminus of MEF2B and abrogates its transactivation activity\(^34\), prompted us to explore whether MEF2B is similarly regulated by PKA, a serine-threonine protein kinase whose activity is dependent on cytoplasmic concentrations of cyclic AMP\(^35\). To investigate whether MEF2B is phosphorylated by PKA in vivo, we metabolically labeled proteins in SUDHL4 DLBCL cells with \(^32\)P in the presence or absence of forskolin (an activator of adenylyl cyclase\(^36\) and PKA) and immunoprecipitated MEF2B from those cells (Fig. 7a). The basal content of phosphorylated endogenous MEF2B was substantially enhanced after the addition of forskolin and was completely blocked in the presence of H89, an established small-molecule inhibitor of PKA\(^37\) (Fig. 7a), which indicated the specific involvement of this kinase. We also observed additional bands indicative of phosphorylated high-molecular-weight proteins in the immunoprecipitates (Fig. 7a), which might correlate with other covalent modifications of MEF2B (such as sumoylation; discussed below). In contrast, mutant MEF2B proteins with the R171X or Y201X substitution (the largest truncated MEF2B proteins) were not phosphorylated in response to treatment with forskolin (Fig. 7b), which suggested that MEF2B, and more specifically its C terminus, was indeed targeted by PKA-dependent phosphorylation.

To assess the functional consequence of phosphorylation by PKA, we examined its effect on the ability of MEF2B to regulate BCL6 transcription in transient luciferase reporter assays. Similar to published reports of negative regulation of MEF2D by PKA\(^34\), the transcriptional activity of MEF2B was substantially decreased in the presence of forskolin (Fig. 7c) or exogenous PKA (Fig. 7d) but not in the presence of a ‘kinase-dead’ PKA mutant (K72H)\(^38\). Notably, mutant MEF2B with the R171X or Y201X substitution escaped the negative regulation by PKA (Fig. 7c,d), consistent with the absence of forskolin-induced phosphorylation of those mutants (Fig. 7b). Thus, MEF2B with DLBCL-associated C-terminal truncations disrupted the negative regulatory effects of PKA-dependent phosphorylation.

### Frameshift and nonsense mutations abrogate MEF2B sumoylation

In considering the effect of the three frameshift mutations (G242fs, P256fs and L269fs) affecting the most C-terminal portion of the MEF2B protein, we noted that they were predicted to cause the switch from isoform A to isoform B (Supplementary Fig. 1). The latter isoform lacks a highly conserved phosphorylation-dependent sumoylation motif (PDSM)\(^39\) found in all members of the MEF2 family (Fig. 8a). That domain would also be lost in the products of all alleles carrying premature truncating mutations. The PDSM is a bipartite motif composed of a SUMO consensus site (ψKxKxK, where ‘ψ’ is a hydrophobic amino acid and ‘x’ is any amino acid) and a proline-directed phosphorylation site separated from the SUMO site by two amino acids (ψKxExxSP) (Fig. 8a). The negative charge conferred by phosphorylation of the serine residue in the PDSM

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**Figure 6** MEF2B proteins with N-terminal substitutions fail to bind CABIN1 and escape its corepressor activity. (a) Crystal structure of MEF2B (blue) in complex with DNA (orange) and CABIN1 (green)\(^20\); yellow (stick model), residues that are mutant in DLBCL or FL. (b) Immunoassay of HEK293T cells cotransfected with expression vectors encoding wild-type or mutant HA-tagged MEF2B and Myc-tagged CABIN1 (residues 2037–2220), followed by immunoprecipitation (IP) with anti-HA and immunoblot analysis (IB) of the immunoprecipitates (top) or samples without immunoprecipitation (Input; bottom). Expt 1 and 2 (bottom) indicate two separate experiments. (c) Immunoblot analysis of nuclear extracts of GC B cells, U2932 cells and SUDHL4 cells before immunoprecipitation (Input; 10% of total sample) and after coimmunoprecipitation of proteins with anti-MEF2B (IP) or anti-IgG (IP: IgG); numbers between blots indicate quantification of CABIN1 by densitometry. (d) ChIP–quantitative PCR analysis of the binding of MEF2B or CABIN1 to the BCL6 promoter in U2932 and SUDHL4 cells (presented as in Fig. 3c). (e) Luciferase activity of HEK293T cells transfected to express a luciferase reporter driven by the native BCL6 promoter together with vector encoding HA-tagged wild-type or mutant MEF2B (horizontal axis) in the presence of Myc-tagged CABIN1 (residues 2037–2220), presented relative to results obtained in the absence of CABIN1, set as 1 (dashed line); shaded background indicates basal reporter activity in the absence of MEF2B (red bars as in Fig. 5a). Data are representative of three independent experiments (b), two independent experiments (c), one experiment (d); mean and s.d. of three technical replicates or two experiments (e); mean and s.d. of two biological replicates.
facilitates sumoylation of the lysine residue through recruitment of the E2 conjugating enzyme Ubc9 (ref. 40).

To determine whether MEF2B isoform A was sumoylated at the putative Lys319 sumoylation site in the MEF2B PDSM, we assessed whether MEF2B could be modified in vivo in the presence of the small ubiquitin-like modifier SUMO1 and Ubc9, in transient transfection assays. Wild-type isoform A of MEF2B was specifically sumoylated at Lys319, as replacement of that residue with arginine greatly impaired sumoylation; however, isoform B was not sumoylated, as it lacks the PDSM consensus sequence (Fig. 8b). The shift in the molecular mass of MEF2B by ~15 kDa suggested that MEF2B was modified by monosumoylation at this site (Fig. 8b). Notably, the serine residue in the PDSM of MEF2B isoform A (Fig. 8a) seems to be embedded in a potential PKA consensus site (RxS/T, where ‘S/T’ indicates ‘serine or threonine’). Replacement

Figure 7 MEF2B proteins with truncation at the C terminus escape negative regulation by PKA-dependent phosphorylation. (a) SDS-PAGE and autoradiography (top) of SUDHL4 cells metabolically labeled in vivo with 32P-orthophosphate and incubated in the presence (+) or absence (−) of forskolin (FSK) and H89, followed by immunoprecipitation (IP) of endogenous MEF2B; below, immunoblot analysis of MEF2B in the samples above. (b) Immunoassay of HEK293T cells transfected to express Flag-tagged MEF2B (wild-type WT or the R171X or Y201X mutant) and treated with forskolin and/or H89, followed by immunoprecipitation with anti-Flag and immunoblot analysis with antibody to phosphorylated serine (p-Ser) or anti-Flag. (c) Luciferase activity of HEK293T cells transfected to express a luciferase reporter driven by the native MADMEF promoter together with expression vector encoding HA-tagged MEF2B (wild-type or mutant), then treated overnight with various concentrations (key) of forskolin; results were normalized to renilla luciferase activity and are presented relative to those of cells treated with dimethyl sulfoxide (DMSO), set as 1. (d) Luciferase activity of HEK293T cells transfected to express a luciferase reporter and expression vector as in (c), plus expression vector encoding PKA (wild-type (+PKA) or a ‘kinase-dead’ mutant (+K72H)); results (normalized as in (c)) are presented relative to those of cells not transfected to express PKA (−PKA), set as 1. Data are representative of two independent experiments (a,b), two experiments (c), mean and s.d. of two biological replicates) or three independent experiments with two technical replicates in each (d; error bars, s.d.).

Figure 8 C-terminal substitutions of MEF2B abrogate negative regulation of MEF2B by phosphorylation-dependent sumoylation. (a) Sequence alignment of the conserved PDSM (ψKExxSP) in the C terminus of human MEF2A, MEF2C, MEF2D and MEF2B isoform A, composed of a sumoylation consensus site (gray box) and a proline-directed phosphorylation site (green box); red, Lys319 and Ser324 of the MEF2B PDSM. (b) In vivo sumoylation assay of HEK293T cells transfected with expression vector encoding Flag-tagged MEF2B isoform A (wild-type or mutant) or isoform B, with or without expression vectors encoding Ubc9 and hemagglutinin-tagged SUMO1, followed by immunoprecipitation with anti-Flag and immunoblot analysis with anti-HA or anti-Flag. MEF2B-SUMO1N (top), monosumoylated MEF2B; bottom, unmodified MEF2B. (c) Immunoassay of HEK293T cells transfected with expression vector encoding Flag-tagged MEF2B (wild-type or the S324A mutant) and incubated in the presence (+) or absence (−) of forskolin, followed by immunoprecipitation with anti-Flag and immunoblot analysis with antibody to phosphorylated serine (top) or anti-Flag (bottom). (d) Luciferase activity of HEK293T cells transfected to express a luciferase reporter driven by the native BCL6 promoter together with expression vectors encoding Ubc9 and SUMO1 (+ Ubc9-SUMO1) and MEF2B (wild-type MEF2B or mutant MEF2B with truncation of the C terminus (R171X and Y201X) or substitutions in the PDSM consensus site (K319R-S324A)); results are presented relative to those of cells not transfected to express Ubc9 and SUMO1 (− Ubc9-SUMO1), set as 1; dashed line indicates activity of cells transfected to express wild-type MEF2B. Data are representative of two independent experiments (b), two independent experiments (c) or two experiments (d; mean and s.d. of two biological replicates).
of that serine residue (Ser324) with alanine greatly impaired both the forskolin-dependent phosphorylation of MEF2B (Fig. 8c) and sumoylation of MEF2B (Fig. 8b), which suggested that sumoylation of MEF2B was dependent on PKA-mediated phosphorylation at that site in the PDSM consensus. Notably, mutant MEF2B with truncation at the C terminus via either the R171X or Y201X substitution was not sumoylated in the same assay (Fig. 8b), consistent with the loss of the sumoylation site at position 319. Given that this highly conserved sumoylation motif is not present in isoform B (Fig. 8a), sumoylation would also be lost in all frameshift mutations predicted to cause the switch from isoform A to B (Supplementary Fig. 1).

We then assessed whether that post-translational modification modulated the transcriptional activity of MEF2B, as is the case for other MEF2 proteins. Indeed, sumoylation of MEF2B in the presence of SUMO1 and Ubc9 substantially reduced the transcriptional activity of wild-type MEF2B (Fig. 8d), whereas substitution of both Lys319 and Ser324 in the PDSM consensus substantially relieved that negative effect on MEF2B activity (Fig. 8d). The presence of SUMO1 and Ubc9 did not reduce the transcriptional activity of the MEF2B mutants with the DLBCL-associated substitutions R171X and Y201X (Fig. 8d), consistent with both the loss of the PDSM consensus and the absence of sumoylation on these mutants in vivo (Fig. 8b).

Together these results showed that phosphorylation-dependent sumoylation negatively regulated the transcriptional activity of MEF2B in B cells. Thus, the majority of lymphoma-associated mutated alleles affecting the C terminus of MEF2B (73%; 11 of 15) escape such negative regulation. Overall, PKA- and sumoylation-mediated regulation of MEF2B is predicted to be lost in ~12% (11 of 90) of all reported cases of DLCBL and FL with mutation of MEF2B.

**DISCUSSION**

Our results suggest that MEF2B may actually be a hierarchically relevant transcription factor responsible for the initiation of a broad program of gene expression that substantially defines the GC B cell phenotype. Part of this broad MEF2B transcriptional program seems to rely on its ability to modulate the expression of Bcl-6, a key factor in GC biology that modulates the cell cycle, plasma-cell differentiation, responses to DNA damage and antiapoptotic molecules such as Bcl-2 (ref. 26). While our results suggest an apical role for MEF2B in ‘instructing’ the GC phenotype and indicate that Bcl-6 may represent one mediator of its function, additional analyses are needed to comprehensively define the role of this transcription factor in GC formation.

The critical role of MEF2B in the regulation of GC formation is consistent with its targeting by genetic alterations in DLBCL and FL, which represent malignant phenotypes of the GC. Overall, ~11% of cases of DLBCL and 12% of cases of FL tested so far carry mutations of MEF2B. Our results indicate that MEF2B mutations have important functional consequences via at least three distinct mechanisms, which differ by the nature and position of the mutations and lead to deregulated MEF2B activity.

The first and most frequent set of mutations affect the N-terminal domain of the protein and represent gain-of-function mutations that enhanced the transcriptional activity of MEF2B by preventing its binding to CABIN1 and by blocking corecruitment of CABIN1 to MEF2B-bound genomic regions. Notably, another member of the MEF2 family, MEF2C, is also targeted by mutations in ~2% of cases of DLBCL; these mutations affect exclusively the conserved N-terminal MADS box–MEF2 domain known to recruit transcriptional coactivators and corepressors. In fact, residue Tyr69, which is involved in the binding to CABIN1, is also mutant in MEF2C, which suggests a common theme in the selection of mutations of MEF2 family members during B cell lymphomagenesis. However, different from what has been described for the other members of the MEF2 family, we did not detect physical interaction between MEF2B and the coactivator EP300 or its related acetyltransferase molecule CREBBP in coimmunoprecipitation assays (data not shown). Finally, an additional ~26% (23 of 90) of cases with mutant MEF2B, which express MEF2B variants with alteration in the N terminus, probably do not involve the mechanism noted above. For these mutants, alternative mechanisms can be invoked on the basis of structural predictions and published reports; i.e., altered affinity for DNA (predicted for mutations encoding the G2E, K4E, K5E, I8V, R15G and K23V substitutions) or for interactions with basic helix-loop-helix proteins.

A second, less-frequent set of cases have mutations that affect the C terminus of the protein and encode MEF2B proteins still able to bind CABIN1 but with a distinct C terminus due to either truncating nonsense or frameshift mutations or, more rarely, to frameshift mutations that cause the switch from isoform A to the predominant production of isoform B proteins. Such mutations cause MEF2B to escape from PKA-mediated phosphorylation and sumoylation, which probably render MEF2B insensitive to upstream signals of a nature as yet unclear. PKA-mediated phosphorylation of MEF2B may be relevant, given the importance of PKA activity in GC physiology, in which it is required for activation of the cytidine deaminase AID and thus for somatic hypermutation of immunoglobulin-encoding genes and the affinity maturation of antibodies. Therefore, by a different mechanism (i.e., resistance to negative regulatory modifications), the second group of MEF2B mutants (with alterations at the C terminus) may also have deregulated transcriptional activity.

While transcriptional deregulation of BCL6 is probably not the only consequence of aberrant activity of MEF2B, it is clearly a relevant contributor to lymphomagenesis, as shown by the presence of chromosomal translocations that deregulate BCL6 expression in DLBCL and FL and by the demonstration that those events contribute to lymphomagenesis in transgenic mice. Mutational deregulation of MEF2B may represent another mechanism that leads to BCL6 deregulation, as an alternative to those mechanisms reported before, which include aberrant transcriptional regulation by promoter substitution caused by chromosomal translocations, mutations in the BCL6 promoter sequence, defective acetylation in cases with inactivating mutations of CREBBP or EP300 (ref. 8), or defective ubiquitin- and/or proteasome-mediated degradation due to inactivating mutations of the gene encoding the ubiquitin ligase FBXO11 (ref. 50). Accordingly, chromosomal rearrangements of BCL6 seem to be mutually exclusive in cases of DLBCL with activating mutations of MEF2B, which supports the idea that both types of lesions act in the same oncogenic pathway.

In conclusion, the functional importance of the large fraction of MEF2B mutations characterized so far suggests their selection for a role in lymphomagenesis. Their distribution in both GCB DLBCL and ABC DLBCL as well as in FL suggests a general role in transformation common to all subtypes. Finally, given the dependence of DLBCL cells on MEF2B for their normal growth and the current efforts to pharmacologically inactivate Bcl-6 (ref. 51) as a targeted strategy for the treatment of these malignancies, the results presented here suggest that MEF2B may represent a therapeutic target for the inhibition of Bcl-6 activity and possibly of a broader GC program to which mature B cell lymphomas may be ‘addicted’.

**METHODS**

Methods and any associated references are available in the online version of the paper.
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AUTHOR CONTRIBUTIONS
C.Y.Y. and R.D.-F. designed the study and wrote the manuscript; C.Y.Y. did experiments and analyzed data; D.D.-S. did and contributed to the design and execution of the experiments and data analysis; M.F. did coimmunoprecipitation assays; I.C.L. did structural analysis; S.H. contributed to the immunofluorescence staining; M.B. did bioinformatics analysis, supervised by A.C.; L.P. did and supervised genomics analysis; K.B. contributed to the original design of the study; D.D.-S., K.B., I.P. and I.C.L. edited the manuscript; and all authors read and approved of the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Primary samples and mutation analysis. High-molecular-weight genomic DNA from 111 samples from patients with newly diagnosed DLBCL and 35 samples from patients with FL were studied by protocols approved by the Institutional Review Board of Columbia University. This research involved coded information of archival biological specimen and is classified as Exempt Human Subject Research of existing pathologic specimens made anonymous and/or with identification information removed, under regulatory guideline 45 CFR 46.101(b)(4). The fraction of tumor cells, assessed by Southern blot analysis of the rearranged immunoglobulin heavy-chain locus and/or by histo-

logical analysis of frozen sections isolated before and after obtaining tissue for molecular studies, corresponded to >80% in most cases and to >50% in all cases. Detailed characterization of the DLBCL cohort (cell lines and biopsies of primary DLBCL), including classification by gene-expression profile analysis into the ABC or GCB subtype, has been reported4,13.

The MEF2B coding exons were targeted to DNA sequencing by the Sanger method on PCR products obtained from whole genome amplified DNA (oligonucleotides, Supplementary Table 2). Mutations were confirmed by PCR amplification and double-strand DNA sequencing of independent products obtained from genomic DNA. Somatic origin was verified by analysis of paired normal DNA, where available.

All mutations reported4–6 were verified for their absence in databases of reported single-nucleotide polymorphisms. Mutations resulting in the R114Q substitution were thus excluded here.

Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FCS, penicillin, streptomycin, and Lipofectamine LTX according to the manufacturer’s instructions (Invitrogen). BJAB cells were transiently transfected through the use of polyethylenimine (Polysciences) as described52. BJAB cells were transiently transfected through the use of Lipofectamine LTX according to the manufacturer’s instructions (Invitrogen). Equimolar amounts of pCMV-MEF2B vectors encoding wild-type or mutant MEF2B were cotransfected along with the JC16-luc reporter construct (pGL4.26 vector) and a renilla luciferase control reporter (pRL-SV40) in the presence or absence of expression vector pCMV-myc-CABIN1. Cells were harvested 48 h after transfection and the Dual Luciferase Reporter Assay was done according to the manufacturer’s instructions (Promega). Cells transfected with the BCL6-luc reporter construct (pNL1.1 vector) were harvested and processed for the Nano-Glo Luciferase Assay according to the manufacturer’s instructions (Promega).

Lentiviral transduction. Lentiviral vectors (pLKO.1) expressing MEF2B-specific shRNA (sh.1 (unique library identifiers of The RNAi Consortium in parentheses: TRCN0000032095), 5′-CCGGGGAATGAAACCCCTCCA AGAACCTGGAGCTTGGAGTGTGTTAGTCTCTTGGT-3′; or sh.2 (TRCN000013578), 5′-CCGGGGGCGTCTTCCCTGTCGGA TGCTGTCATAGTGAAGCCACAGATGTATGACACGCGGTATTGCACCTT-3′) were used for transduction of SUDHL4 and U2932 cells. Of note, we observed substantial variation of the shRNA sequences tested. Of note, we observed substantial variation of the shRNA sequences tested. SUDHL4 and U2932 cells were infected with the vectors in the absence or presence of expression vector pCMV-myc-CABIN1. Cells were harvested after 48 h for transfection and the Transcription Factor Reporter Assay was done according to the manufacturer’s instructions (Promega). Cells transfected with the BCL6-luc reporter construct (pNL1.1 vector) were harvested and processed for the Nano-Glo Luciferase Assay according to the manufacturer’s instructions (Promega).

Cell lines. HEK293T cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FCS, 100 µg/ml penicillin and streptomycin. The DLBCL cell lines SUDHL4, U2932, SUDHL10, DB, LY8, VAL and Bjab were grown in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FCS, 100 µg/ml penicillin and streptomycin. All cell lines were tested negative for mycoplasma contamination.

ChiP. ChiP assays of purified CD77+ GC B cells or DLBCL cell lines (SUDHL4 and U2932) were described16. Crosslinked chromatin was fragmented by sonication with the Bioruptor (Diagenode) or, alternatively, with a Covaris S2200 to achieve a fragment size of 300–500 base pairs. Antiserum directed against MEF2B (ab3350; Abcam) or CABIN1 (ab3349; Abcam), or isotype-matched polyclonal IgG (Sigma-Aldrich) was used for ChiP, followed by quantitative PCR analysis with Absolute QPCR SYBR green mix (Thermo Scientific) and a 7300 Real Time PCR system (Applied Biosystems). Primers spanning the ~1.5-kilobase proximal promoter region of BCL6 (Supplementary Table 2) were used to define the peak of MEF2B binding in that region (Supplementary Fig. 2c). Primer pair selection was limited by the efficiency of amplification of the target site or region. For some assays (Figs 3c and 6d), the primer pair used was ‘Bpom_Tiling_F5’ and ‘Bpom_Tiling_R5’, which correspond to the main peak of binding of MEF2B in the BCL6 promoter (positions –1167 to –971 relative to the transcription start site). Change-in-cycling-threshold (ΔCT) values were calculated for DNA fragments immunoprecipitated with anti-MEF2B, anti-CABIN1 and control IgG relative to that of input DNA. The enrichment noted after immunoprecipitation with anti-MEF2B or anti-CABIN1 relative to that obtained with IgG was further normalized to the enrichment observed for the ACTB locus (encoding β-actin; negative control). Of note, we observed substantial variation between batches of MEF2B-specific antibody (ab3350; Abcam) in terms of immunoprecipitation efficiency.

Expression constructs, transient transfection and luciferase reporter assay. MEF2B and CABIN1 (residues 2037–2220) were amplified by PCR from human normal GC B-cell cDNA. GenBank accession numbers for the reference sequence are NM_001145785.1 (MEF2B isoform A), NM_005919 (MEF2B isoform B) and NM_001199281.1 (CABIN1). Those cDNAs were subcloned into pcMV vectors with an HA tag, Myc tag (Clontech) or Flag tag (Stratagene) for expression in mammalian cells. Misseense and nonsense mutant expression constructs of MEF2B were generated by site-directed mutagenesis with the mammalian expression vector encoding wild-type human MEF2B as a template. The construct with deletion of 31 base pairs (L269fs) was obtained by PCR amplification with the pCMV-MEF2B isoform A expression vector as a template; the final L269fs mutant construct was completed by ligation to the C-terminal fragment (Nael and Not1 sites) of isoform B. The construct with deletion of MADS-box–MEF2 domain (residues 8–91) was generated by PCR amplification with pCMV-MEF2B as a template. The CABIN1 coding sequence (residues 2037–2220) was amplified by PCR with cDNA from human GC B cells and was subcloned into the pCMV-Myc expression vector (Clontech). The CABIN1_L2172A mutant was generated by site-directed mutagenesis with a mammalian expression vector encoding wild-type CABIN1 (residues 2037–2220) as a template. Plasmid pCMV–HA PKACA was generated before44 and was used as a template to generate a ‘knase-dead’ PKA mutant (K72H) by site-directed mutagenesis. Plasmid pcDNA3-Ubc9 (2008; Addgene) was deposited by E. Yeh. Hemagglutinin-tagged SUMO1 was a gift from R. H. The luciferase reporter construct (BCL6-luc) was generated by cloning of a 921–base pair HindIII–HindIII fragment of the BCL6 promoter (positions –1593 to –672) digested from the published pLa/Sswt construct44 into pGL4.26 and pNL1.1 vectors (Promega). All final constructs were verified by digestion and confirmed by Sanger sequencing analysis.

HEK293T cells were transiently transfected through the use of polyethylenimine (Polysciences) as described52. BJAB cells were transiently transfected through the use of Lipofectamine LTX according to the manufacturer’s instructions (Invitrogen). Equimolar amounts of pCMV-MEF2B vectors encoding wild-type or mutant MEF2B were cotransfected along with the BCL6-luc reporter construct (pGL4.26 vector) and a renilla luciferase control reporter (pRL-SV40) in the presence or absence of expression vector pCMV-myc-CABIN1. Cells were harvested 48 h after transfection and the Dual Luciferase Reporter Assay was done according to the manufacturer’s instructions (Promega). Cells transfected with the BCL6-luc reporter construct (pNL1.1 vector) were harvested and processed for the Nano-Glo Luciferase Assay according to the manufacturer’s instructions (Promega).

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MEF2B-specific shRNA (a) (V2LHS 253202; GIPZ; mature antisense sequence, 5′-ACTCGTGTGACTCTTGAGC-3′) was used for transduction of the pLNDUCER11 inducible lentiviral vector12 (used in Fig. 4). SUDHL4 and U2932 cells were infected with viral supernatants (MEF2B- or BCL6-luc targeting shRNA or control shRNA targeting the gene encoding luciferin luciferase45). Cells were treated with doxycycline (1 µg/ml) for induction of the tRF–shRNA cassette. To obtain pure populations of tRF+ and tRF− cells for analysis of mRNA and protein, exponentially growing cultures of infected cells were sorted with a FACSaria II (HiCCC Flow Cytometry Facility). Cells with a mean fluorescence intensity for tRF of >102 were collected, pelleted and lysed for analysis.

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Coimmunoprecipitation. In transient assays and 48 h after transfection, HEK293T cells were lysed in the following immunoprecipitation buffer: 50 mM Tris, pH 7.05, 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.05% NP40, 1 mM NaF, 10 mM β-glycerophosphate, 0.5 mM PMSF and protease inhibitors. Lysates were incubated overnight at 4 °C with anti-HA affinity beads (Sigma). Beads were washed in the buffer noted above, and bound proteins were eluted in the presence of 0.25 mg/ml HA peptide (Sigma). Eluates were resolved by SDS-PAGE and analyzed by immunoblot.

Nuclear extracts were generated from SUDHL4 and U2932 cells as previously described55. Lysates were incubated overnight with the MEF2B antibody (Abcam) at 4 °C, and supplemented with Protein G beads (GE Healthcare) for 1 h at 4 °C. Beads were washed 5 times in wash buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 0.2% NP40, 10 mM beta-glycerophosphate, 0.5 mM PMSF, protease inhibitors); immunocomplexes were eluted in SDS-PAGE sample-loading buffer, and the eluates were resolved by SDS-PAGE for immunoblot analysis.

For detection of in vivo somoylation events (HA-tagged SUMO1), 25 mM N-ethylmaleimide (Sigma) was added during lysis and was maintained throughout the immunoprecipitation procedure.

In vivo metabolic labeling with 32P-orthophosphate. For radioactive labeling of the phosphorylated form of endogenous MEF2B in SUDHL4 cells, cell cultures were preincubated for 1 h in DMEM without phosphates and with 10% dialyzed FCS (Gibco/Life Technologies). 32P-orthophosphate (167 Ci/mmol) was subsequently added and cells were grown for 6 h at 37 °C in the presence of 10 mM HEPES, pH 7.0 (Gibco/Life Technologies), in a shielded incubator, with or without addition of 20 μM forskolin (Sigma) and/or 40 μM H89 (Cell Signaling). Cells were then washed in cold PBS and lysed in immunoprecipitation buffer supplemented with phosphatase inhibitors (sodium orthovanadate and sodium fluoride), and endogenous MEF2B was immunoprecipitated with anti-MEF2B (ab3350; Abcam). Immunoprecipitates were resolved by SDS-PAGE and the gel was dried and exposed to autoradiography film overnight at ~80 °C. A fraction of each immunoprecipitate was transferred to nitrocellulose membranes for immunoblot analysis.

Cell-cycle analysis. For cell-cycle analyses, cells were labeled by the addition of 10 mM BrdU (5-bromo-2-deoxyuridine) to the culture medium for 2 h before harvest. BrdU (active DNA synthesis) and 7-AAD (DNA content) were detected with the APC BrdU detection kit according to the manufacturer's instructions (BD Biosciences). Stained cells were analyzed with a FACSCalibur (BD Biosciences), with gating on IRFP® or IRF6® subpopulations. Final data analysis and plot rendering were done with FlowJo Software (version 9.5.2; TreeStar).

Structural analysis. PyMOL software was used to map and assess the molecular interactions of DLBCL-associated MEF2B mutants onto the crystal structures of MEF2B-CABIN1 and MEF2A-EP300. The coordinates used to generate the structural view were retrieved from the RCSB Protein Data Bank with the accession codes 1N6J (CABIN1-MEF2B-DNA ternary complex) and 3P57 (EP300-MEF2A-DNA ternary complex).

RNA extraction, cDNA synthesis and quantitative real-time PCR. Total RNA was extracted from DLBCL cell lines, naive B cells and GC B cells with the Trizol reagent according to the manufacturer's instructions (Invitrogen), then cDNA was synthesized with the SuperScript II First-Strand Synthesis System (Invitrogen). For the detection of normal and mutated MEF2B alleles in primary DLBCL, primers surrounding the mutation site were designed (Supplementary Table 2) and the amplified PCR products were analyzed by direct sequencing. Quantitative RT-PCR was done in triplicate with the appropriate primers (Supplementary Table 2) and ABsolute QPCR SYBR green mix (Thermo Scientific) in the 7300 Real Time PCR system (Applied Biosystems). GAPDH was used as negative control for quantitative RT-PCR.

Immunoblot analysis. For immunoblot analysis, nonspecific binding to membranes was blocked by 1 h of incubation in a solution of 0.2% Tween and 5% milk in PBS, then membranes were incubated with primary antibodies (Supplementary Table 3) diluted in a solution of 0.2% Tween and 3% BSA in PBS, overnight at 4 °C with constant rotation (except anti-β-actin, 1 h at 21 °C). ImageJ software was used for densitometry (Fig. 6 and Supplementary Fig. 7).

Immunofluorescence analysis of paraffin-embedded lymphoid tissues. Immunofluorescence analysis was done on sections of formalin-fixed, paraffin-embedded tissues (Dako) was used, and immunocomplexes were detected by tyramide-fluorochrome amplification (1:1,000 dilution; for 3 min; PerkinElmer). For biotin-conjugated primary and secondary antibodies (immunodetection of Pax5 on human tissues and of B220 in mouse tissues), streptavidin-fluorochrome was added as a final step.

Mouse immunization and tissue collection. C57BL/6 mice (The Jackson Laboratory) were housed in a dedicated pathogen-free environment. All experiments and procedures conformed to ethical principles and guidelines revised and approved by the Institutional Animal Care and Use Committee at Columbia University.

4-month-old mice were immunized by a single intraperitoneal injection of sheep red blood cells to trigger T cell-dependent immune responses and were killed at various times after immunization (2, 3, 4, 5 d; two mice per time point). Spleens were then isolated and divided in two fragments, which were processed for histology and flow cytometry as described56. Splenic mononuclear cells were isolated by stripping of the tissue through 40-μm cell strainers in a buffer of 0.5% BSA in PBS and lysing of the red blood cells as reported56. Mononuclear cell suspensions were stained for 20 min on ice with antigen-specific fluorochrome-conjugated antibodies (antibodies, Supplementary Table 3) and were analyzed with a BD LSR II (BD Biosciences); 200,000 events were collected per sample and were analyzed with FlowJo Software (version 9.5.2; TreeStar).

Bioinformatic analysis. To generate the ‘human B cell interactome’ (HBCI), we first generated a transcriptional network from a large compendium of 199 B cell gene expression profiles with an Affymetrix HG-U95Av2 GeneChip, which represented normal B cell types isolated from human tonsils and B cell tumors57. The transcriptional network was generated by the bootstrap version of the ARACNe algorithm57 with the following parameters: $P = 0.0000001$, dots per inch = 0 and 100 bootstraps. To generate the final HBCI, we used the Bayesian evidence integration algorithm to integrate transcriptional network with evidences from experimental assays, databases and literature mining, by filtering them in a context-specific criteria, as explained before28. The HBCI contains about 38,500 B cell–specific interactions, including both protein–protein and protein–DNA interactions.

To identify master regulators of the GC, we searched the HBCI with the MARina algorithm (master regulator inference algorithm)28. This algorithm first identifies the positive and negative regulon of each transcription factor by computing the Spearman correlation between the expression profile of the transcription factor with each of its targets from HBCI. Second, it computes the enrichment for the regulon of each transcription factor on the genes expressed differently by GC B cells (n = 10 samples) and naive B cells (n = 5 samples) by gene-set enrichment analysis33. We computed the enrichment for 308 transcription factors with at least 20 targets and ranked all transcription factors based on the DETOR (differentially expressed target odds ratio), defined as the ratio of the percentage of genes from the regulon before the leading edge to the total number of genes before the leading edge and the percentage of genes from the regulon after the leading edge to the total number of genes after the leading edge. This step identified 103 transcription factors as candidate master regulators with a P value of <0.01. Finally, shadow analysis identified...
and removed possible false-positive results. If the regulon of two transcription factors overlap significantly and only one of them is a master regulator, then the other may also seem to be a master regulator because of common target enrichment, and the shadow analysis identifies all such master regulators. After shadow analysis, we identified 65 master regulators, of which 22 were activated in GC B cells and 43 were inactivated in GC B cells.

Gene-set enrichment analysis software publicly available from the Broad Institute website was used as described for gene-set enrichment analysis, including \( P \) values, normalized enrichment score and false discovery rate. We used the default parameters, except we used 1,000 gene-set permutations, and the data set was collapsed to gene names (maximum of probes).

Expression heat maps were generated with the MultiExperiment Viewer (MeV v4.8), which is part of the TM4 Microarray Software Suite 59. GeneChIP HG-U133 Plus 2.0 (Affymetrix) expression data from normal B cell subsets available from Gene Expression Omnibus (GSE12195) were collapsed to gene names (maximum of probes) and then normalized to \( z \) values with the ‘standardize’ function of Excel before heat maps were built.

Circos software was used as a visualization tool to represent the connections between different GC master regulators.

Statistics. One-way analysis of variance paired with Tukey’s multiple-comparison tests were done with MATLAB (version R2010b). Two-tailed \( t \)-tests (equal variance) were used to determine statistical significance in luciferase reporter assays. Spearman’s rank correlation coefficient was used to determine the ranked correlation of the expression of MEF2B and BCL6 in normal and tumor B cells.

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