ARTICLE

N-terminally truncated FOXP1 protein expression and alternate internal FOXP1 promoter usage in normal and malignant B cells

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ABSTRACT

Strong FOXP1 protein expression is a poor risk factor in diffuse large B-cell lymphoma and has been linked to an activated B-cell-like subtype, which preferentially expresses short FOXP1 ($FOXP1_S$) proteins. However, both short isoform generation and function are incompletely understood. Here we prove by mass spectrometry and N-terminal antibody staining that $FOXP1_S$ proteins in activated B-cell-like diffuse large B-cell lymphoma are N-terminally truncated. Furthermore, a rare strongly FOXP1-expressing population of normal germinal center B cells lacking the N-terminus of the regular long protein ($FOXP1_L$) was identified. Exon-targeted silencing and transcript analyses identified three alternate 5′ non-coding exons [*FOXP1-Ex6b(s), FOXP1-Ex7b* and *FOXP1-Ex7c*], downstream of at least two predicted promoters, giving rise to $FOXP1_S$ proteins. These were differentially controlled by B-cell activation and methylation, conserved in murine lymphoma cells, and significantly correlated with $FOXP1_S$ protein expression in primary diffuse large B-cell lymphoma samples. Alternatively spliced isoforms lacking exon 9 (e.g. isoform 3) did not encode $FOXP1_S$, and an alternate long human FOXP1 protein ($FOXP1_{AL}$) likely generated from a *FOXP1-Ex6b(L)* transcript was detected. The ratio of $FOXP1_L$:$FOXP1_S$ isoforms correlated with differential expression of plasmacytic differentiation markers in U-2932 subpopulations, and altering this ratio was sufficient to modulate CD19 expression in diffuse large B-cell lymphoma cell lines. Thus, the activity of multiple alternate *FOXP1* promoters to produce multiple protein isoforms is likely to regulate B-cell maturation.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease entity originating from germinal center (GC) or post-GC B cells such as plasmablasts.[1-4] The majority of DLBCL can be classified according to cell-of-origin gene expression profile, as either germinal center (GC-DLBCL) or activated B-cell (ABC-DLBCL) subtype.[5-9] While addition of rituximab to CHOP chemotherapy has improved DLBCL patients' survival significantly,[10] new therapies are needed for non-responding or relapsed patients (reviewed by Sehn and Gascoyne).[11]

Novel molecularly-targeted therapies are being sought particularly for the poorer prognosis ABC-DLBCL subtype following identification of key biological pathways contributing to disease pathogenesis, such as NF-κB pathway mutations and activation,[12-15] B-cell receptor (BCR) signaling,[16] MALT1 activity,[17] and *BLIMP1* muta-

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tions.[18] Maintenance of BCR signaling and prevention of plasma cell maturation to disrupt normal maturation/differentiation pathways is a common paradigm.

High FOXP1 expression correlates with the ABC-DLBCL subtype[4] and poor clinical outcome in both the pre- and post-rituximab eras.[19-22] *FOXP1* amplification and trisomy have been described in ABC-DLBCL,[23] and translocations involving the *immunoglobulin heavy chain (IGH)* locus[24] drive expression of a long ~75kDa FOXP1 protein ($FOXP1_L$) that may contribute to GC-DLBCL tumor growth by potentiating Wnt/β-catenin signaling.[25] Also, we have described abundant expression of short ~65kDa activation-induced FOXP1 proteins ($FOXP1_S$) in ABC-DLBCL.[26] Oncogenic activity of N-terminally truncated FOXP1 has been proposed following its truncation by an oncogenic virus[27] and non-IGH translocations targeting the *FOXP1* coding region in lymphoma.[24,28,29]

Studies manipulating Foxp1 expression have established biological roles in early B-cell development[30,31] and in mature B cells.[32] Direct FOXP1 target genes, including *PAX5, PRDM1*, and *POU2F1*, support a functional role in the GC reaction.[32] Recent studies have provided insight into FOXP1 function in DLBCL cells, indicating contributions to proliferation,[33] inhibition of apoptosis to compliment NF-κB-dependent regulation of proliferation,[34] and Wnt signaling.[25] However, further understanding of FOXP1 isoform expression and functionality is required to pursue emerging evidence indicating that $FOXP1_L$ and $FOXP1_S$ proteins have distinct functions.[22,31]

Here we demonstrate that $FOXP1_S$ proteins in DLBCL are encoded by transcripts with alternate 5′ non-coding exons, not internal exon skipping.[26] We identify a novel alternate long FOXP1 protein (designated $FOXP1_{AL}$) and a GC B-cell population lacking the $FOXP1_L$ N-terminus. Isoform-biased FOXP1 depletion altering $FOXP1_L$:$FOXP1_S$ stoichiometry regulates expression of CD19 in DLBCL cell lines, providing further evidence for isoforms having distinct functional roles in B-cell biology.

Methods

Cell culture

Diffuse large B-cell lymphoma and myeloma lines were sourced and cultured as described previously.[26] COS-1, 293T and NIH-3T3 (ATCC) were cultured in DMEM containing 10% fetal bovine serum (Life Technologies, Paisley, UK), and murine B-cell lines 5TGM1-GFP (a gift of Claire Edwards, Oxford, UK) and A20 (ATCC) in RPMI containing 10% serum, 100 μM non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol.

Primary human samples

Diffuse large B-cell lymphoma patient samples were collected with informed consent in accordance with the Declaration of Helsinki, and the study was performed under local ethics committee approval from the Leeds West Regional Ethics Committee, Leeds, UK. RNA was isolated from DLBCL samples obtained prior to therapy as described.[26] Primary B cells from blood buffy-coat preparations (National Blood Service, Bristol, UK) were activated with 50 μg/mL anti-IgM or 1:20,000 SAC plus 5 ng/mL recombinant human IL-2 (Sigma, St Louis, USA). Reactive tonsils were obtained with informed consent from John Radcliffe Hospital, Oxford, UK, and studies were conducted under ethical approval from NRES Committee South Central – Oxford B (C02.162).

Gene expression analyses

Random-primed cDNA was prepared from total RNA according to the manufacturer's protocols (Life Technologies), and real-time PCR analyses performed by Chromo4 (BioRad, Herts., UK) using Express qPCR supermix or SYBR GreenER supermix (Life Technologies), and primers/probes as detailed in *Online Supplementary Table S1*. Standard RT-PCR to discriminate *FOXP1-6b(L/S)* transcripts used forward Ex6b(L)#1, Ex6b(L)#2, Ex6b(S), or control forward primers Ex6 or Ex8, all paired with reverse primer Ex10 (*Online Supplementary Table S1*). RNAseq analysis was performed on NCBI Gene Expression Omnibus (GEO) lymphoma dataset GSE50721.[35]

Immunoblot and immunoprecipitation

Whole cell extracts were prepared in RIPA, or nuclear and cytoplasmic extracts by commercial reagent (Affymetrix, High Wycombe, UK). Immunoprecipitations from 50 μg nuclear lysate were performed in IP buffer (1% Triton, 150 mM NaCl, 10 mM Tris, 1 mM EDTA) and complexes purified using μMACS Protein G microbeads (Miltenyi Biotec, Surrey, UK). Primary antibody details are provided in *Online Supplementary Table S1*.

Mass spectrometry

JC12 immunoprecipitates were subjected to parallel SDS-PAGE analyses for immunoblotting and silver staining. Stained bands corresponding to ~65kDa and ~75kDa FOXP1 proteins were excised, subjected to reduction, alkylation and trypsin digestion, and resulting peptides identified by mass spectrometry as previously described[36] with formic acid modified to 0.1% in the gradient.

Immunohistochemistry

After formalin fixation, paraffin embedding and sectioning, slides were dewaxed and antigen retrieved by microwaving in 50 mM Tris, 2 mM EDTA pH9.0. Immunostaining with primary antibodies (*Online Supplementary Table S1*) was followed by secondary antibody and detection (Envision-Dako, Ely, UK).

Flow cytometry

Cells were labeled in PBS containing 0.5% bovine serum albumin, 2 mM EDTA with primary antibodies (*Online Supplementary Table S1*) and/or isotype controls, and/or secondary antibody (streptavidin APC, eBioscience) and data obtained by FACSCalibur (Becton Dickinson, San Jose, USA).

Transfection

For overexpression, cells were harvested 48 h after transfection with pcDNA4-HisMax expression vectors encoding human FOXP1-4 proteins using Lipofectamine (Life Technologies). For knockdown, cells were electroporated in the presence of 1 μM Stealth siRNA duplexes (Life Technologies) (*Online Supplementary Table S1*) using Amaxa Nucleofector (Lonza, Slough, UK) generally using Solution L, program X-001.

Results

Short FOXP1 proteins ($FOXP1_S$) in ABC-DLBCL lack the N-terminus

We used mass spectrometry to characterize $FOXP1_S$ and $FOXP1_L$ proteins immunoprecipitated from GC- and ABC-DLBCL cell lines (Figure 1A and B). Peptides translated from most exons encoding the $FOXP1_L$ protein were identified, while peptides from the N-terminal coding exons *(Ex), Ex6* and *Ex7*, were absent from $FOXP1_S$ pro-

teins in ABC-DLBCL cell lines RIVA and OCI-Ly3 (Figure 1B). This is consistent with FOXP1$_S$ proteins deriving from transcripts where translation initiates in *Ex8* (e.g. isoform 9)[26] but inconsistent with internal deletion of *Ex8* and/or *Ex9* and/or *Ex10* identified in FOXP1 isoforms 3, 5 and 8, which retain *Ex6* and *Ex7*.[26]

To confirm N-terminal truncation of FOXP1 proteins in DLBCL, we validated a commercially available FOXP1 polyclonal antibody against an *Ex7*-encoded peptide. This antibody detected recombinant FOXP1$_L$ protein (*Ex6-Ex21*), but not FOXP1$_S$ protein (*Ex8-Ex21*), using both immunohistochemistry and Western blotting. However, there was cross-reactivity with both the related FOXP2 protein and an unknown, FOXP1 siRNA resistant, ~70kDa cytoplasmic protein in DLBCL cell lines (*Online Supplementary Figure S1A-D*). Thus, this reagent was used to study only nuclear expression in cell lines and tissues with known FOXP2 status. In DLBCL nuclear extracts, this N-terminal antibody recognized only FOXP1$_L$, while our anti-C-terminal JC12 antibody detected both FOXP1$_S$ and FOXP1$_L$ proteins (Figure 1C). Thus FOXP1$_S$ proteins in multiple ABC-DLBCL cell lines lack the N-terminal epitopes encoded by *Ex7*.[26] The N-terminal antibody was ineffective at routinely distinguishing ABC- *versus* GCB-DLBCL cell lines by immunohistochemistry (*Online Supplementary Figure S2A*), reflecting FOXP1$_L$ co-expression, non-specific cytoplasmic staining, and FOXP2 expression in RIVA. Interestingly, immuno-precipitation of FOXP1$_L$ using the N-terminal antibody co-immunoprecipitated FOXP1$_S$, providing evidence for a physical FOXP1$_L$-FOXP1$_S$ interaction in ABC-DLBCL (*Online Supplementary Figure S1E*).

A rare subset of germinal center B cells lack the FOXP1$_L$ N-terminus

The ABC-DLBCL cell-of-origin is proposed to be a plasmablastic B cell poised to exit the GC.[1-4] Tonsillar B-cell follicles (FOXP2-negative) consistently exhibited comparable intensity and pattern of mantle zone staining when immunolabeled with N- and C-terminal FOXP1 antibodies (Figure 1D). However, rare GCs in some tonsils contained a small subpopulation of strongly FOXP1$^+$CD20$^+$ B cells (Figure 1D; inset) that were not effectively labeled using the N-terminal antibody. Thus, a small population of GC B cells may share the abundant FOXP1$_S$ protein expression observed in ABC-DLBCL. However, we cannot exclude an alternate FOXP1$_L$ protein (FOXP1$_{AL}$), which we have identified in ABC-DLBCL.

Transcripts encoding FOXP1$_S$ proteins in ABC-DLBCL have variable 5' non-coding exons and share coding exons 3' from Ex8

N-terminally truncated FOXP1$_S$ proteins in ABC-DLBCL might derive from post-translational cleavage of the normal FOXP1$_L$ protein or alternate promoter usage. To distinguish these possibilities, we performed exon-targeted siRNA across the *FOXP1* locus (Figure 2A), thus identifying transcripts producing FOXP1 proteins in ABC-DLBCL

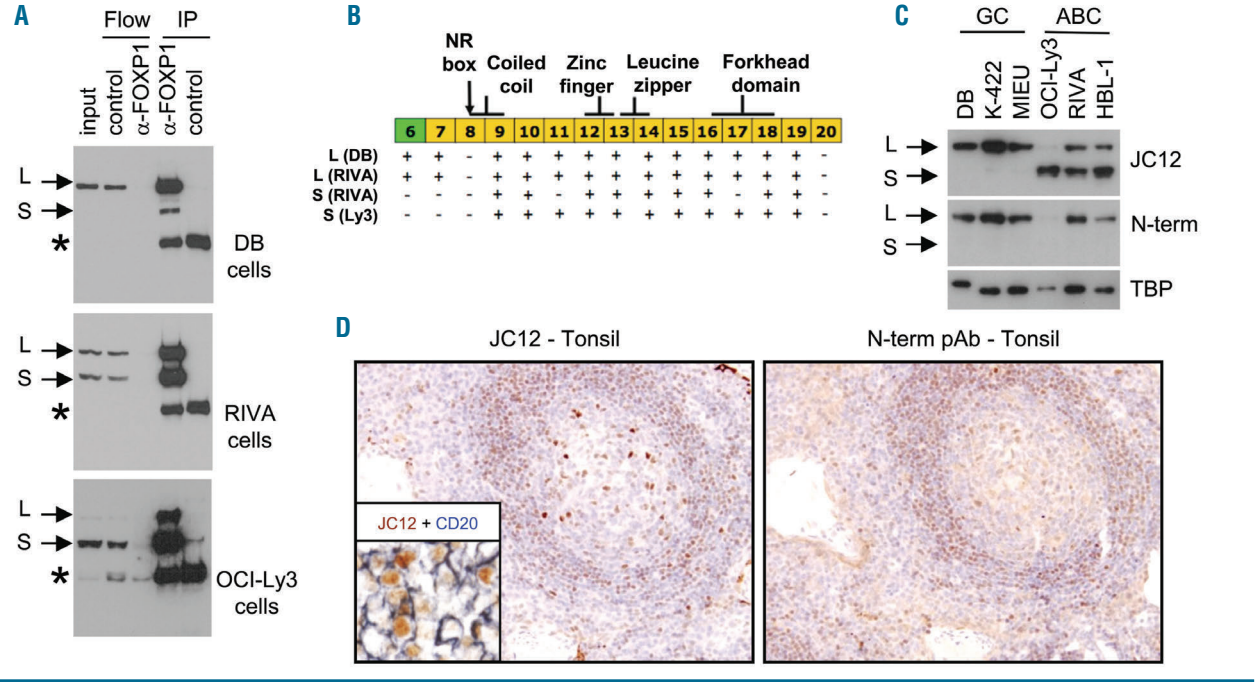


Figure 1. Short FOXP1 proteins (FOXP1$_S$) in ABC-diffuse large B-cell lymphoma (ABC-DLBCL) and rare germinal center B cells lack the FOXP1 N-terminus. (A) JC12 anti-FOXP1 immunoblot of immunoprecipitates from three DLBCL cell lines after JC12 (α-FOXP1) or isotype (control) immunoprecipitation. 'Input' represents 5%; 'flow' represents flow-through to show efficient precipitation; both FOXP1$_L$ (L) and FOXP1$_S$ (S) isoforms are indicated together with antibody heavy chain (asterisk); long and short FOXP1 proteins as indicated left were excised from silver-stained SDS-PAGE gels run in parallel to those in (A) and mass spectrometry performed. (B) Schematic to show presence (+) or absence (–) of FOXP1 peptides from each coding exon. (C) Representative FOXP1 immunoblot analysis of DLBCL cell line nuclear extracts using JC12 or FOXP1 N-terminal-specific antibodies. α-TBP blotting controlled for loading and transfer; (D) FOXP1 immunohistochemistry of serial sections through a secondary follicle (with strongly JC12-positive germinal center cells) from reactive human tonsil using both JC12 and N-terminal antibodies. Note similar mantle zone but less germinal center immunoreactivity with the N-term pAb. Inset shows CD20 surface expression on the majority of strongly JC12-positive germinal center cells by dual color immunohistochemistry for FOXP1 (JC12, brown nuclei) and CD20 (blue membranous).

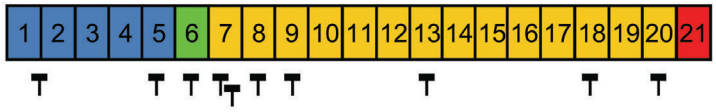
cell lines (RIVA and OCI-Ly3, and as a control the GC-DLBCL cell line DB) (Figure 2). *FOXP1* coding exon targeting generally reduced $FOXP1_L$ levels, although this was sometimes difficult to detect in OCI-Ly3 due to low $FOXP1_L$ expression (Figure 2B). Consistent with siRNA targeting of the 5′ coding region being inefficient for some genes, *Ex1-2* siRNA did not work at all, and *Ex5* and *Ex6* siRNAs targeted poorly. In contrast, targeting of *FOXP1 Ex8* onwards silenced FOXP1 protein expression effectively, confirming coding function of the 3′ exons and the absence of $FOXP1_S$ coding transcripts with internal *Ex8-9* deletions. *Ex5* and *Ex6* targeting had no effect on $FOXP1_S$ expression, suggesting that $FOXP1_S$ proteins were not post-translationally processed from $FOXP1_L$.

Interestingly, two independent siRNAs targeting *Ex7* that effectively silenced $FOXP1_L$ also partially depleted $FOXP1_S$ in both ABC-DLBCL cell lines (Figure 2B and C). As no *Ex7*-encoded peptides were identified in $FOXP1_S$ by mass spectrometry, a proportion of $FOXP1_S$-coding transcripts may contain a non-coding *Ex7*. Indeed one such transcript, with transcription starting in the 3′ end of alternative *Ex6b(S)* is described (Figure 3). Thus $FOXP1_S$-coding transcripts in ABC-DLBCL share common 3′ exons (from exon 8 onwards), have variable 5′ non-coding exons, and are not encoded by previously reported splice variants[26] lacking exons 8, 9 and/or 10.

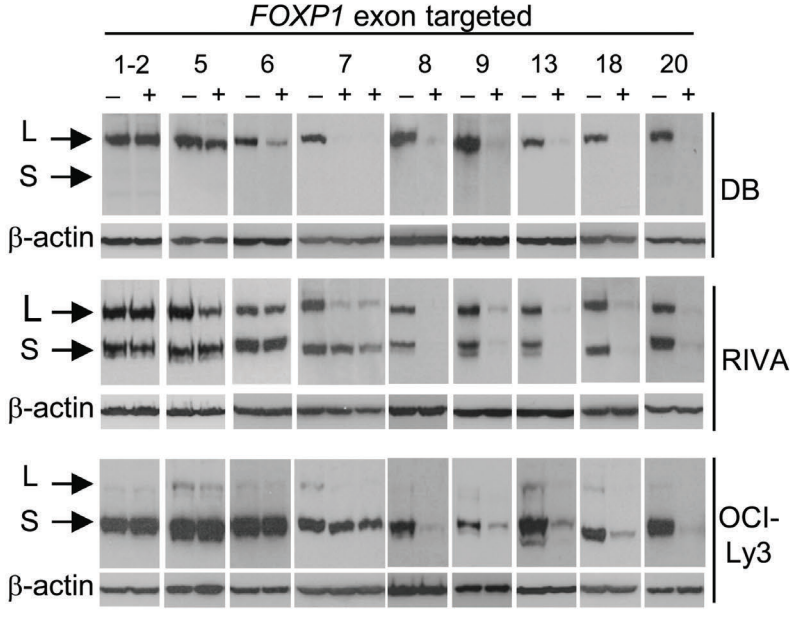
DLBCL cell lines expressing $FOXP1_S$ protein transcribe multiple 5′ alternate exon-containing FOXP1 *mRNA species*

To explore the relationship between FOXP1 proteins and transcripts, panels of GC- and ABC-DLBCL lines were ranked by increasing $FOXP1_S$:$FOXP1_L$ protein expression ratio (*Online Supplementary Figure S2B*). Based on our data,

A



B



C

Cell type	FOXP1 protein	Ex 1-2	Ex 5	Ex 6	Ex 7	Ex 8	Ex 9	Ex 13	Ex 18	Ex 20
DB	long	n/a	+/–	+/–	+	+	+	+	+	+
RIVA	long	n/a	+/–	+/–	+	+	+	+	+	+
OCI-Ly3	long	n/a	+/–	+/–	+	+	+	+	+	+
RIVA	short	n/a	–	–	+/–	+	+	+	+	+
OCI-Ly3	short	n/a	–	–	+/–	+	+	+	+	+

Figure 2. Transcripts encoding $FOXP1_S$ proteins in activated B-cell like-diffuse large B-cell lymphoma (ABC-DLBCL) share coding exons from Ex8 onwards with $FOXP1_L$. (A) Schematic illustration of human *FOXP1* exons to show location of siRNA target sequences. (B) Immunoblot analysis of whole cell extracts from DLBCL cells harvested 48 h after transfection with *FOXP1*-targeting (+) or matched control (–) siRNA duplexes, representative of two or more experiments. (C) Schematic summary of immunoblotting data in (B). + indicates successful siRNA targeting; - indicates the FOXP1 isoform was unaffected; +/- indicates suboptimal/partial targeting.

additional database searching, and published FOXP1 RNA-sequencing data from primary DLBCL biopsies,[24] we assembled a list of *FOXP1* transcripts with the potential to encode $FOXP1_L$ and $FOXP1_S$ proteins in ABC-DLBCL (Figure 3A). There appear to be two transcriptional start sites within *Ex6b*, with the 5′ longer *Ex6b* transcript (*Ex6b(L)*) predicted to encode a long FOXP1 protein with an alternate N-terminus ($FOXP1_{AL}$), while the shorter (*Ex6b(S)*) initiates translation from *Ex8*, as do transcripts containing other alternate 5′ non-coding exons *Ex7b* and *Ex7c*.

Real-time PCR analysis of common 3′ exons (*Ex20-21*) demonstrated increased *FOXP1* expression in most ABC-DLBCL cell lines, while expression of 5′ *Ex6-7* was variable (Figure 3B). Alternate exons *Ex7b* and *Ex7c* were preferentially transcribed in ABC-DLBCL cell lines, while *Ex6b* (encoding $FOXP1_L$ or $FOXP1_S$ proteins) was only slightly more abundant in ABC-DLBCL lines (Figure 3C). RT-PCR analysis demonstrated that, in contrast to *Ex6b(S), Ex6b(L)* was abundant only in the ABC-DLBCL cell line HBL-1 (*Online Supplementary Figure S3A*). Interestingly, reduced recognition of long FOXP1 by the N-terminal antibody in HBL-1 (Figure 1C) is consistent with expression of an additional long FOXP1 protein containing an alternate N-terminus, $FOXP1_{AL}$. Transfection confirmed that *Ex6b(L)* encodes only the $FOXP1_{AL}$ protein (*Online Supplementary Figure S1F*).

Increased expression of alternate 5′ *Ex6b* and *Ex7b* tran-

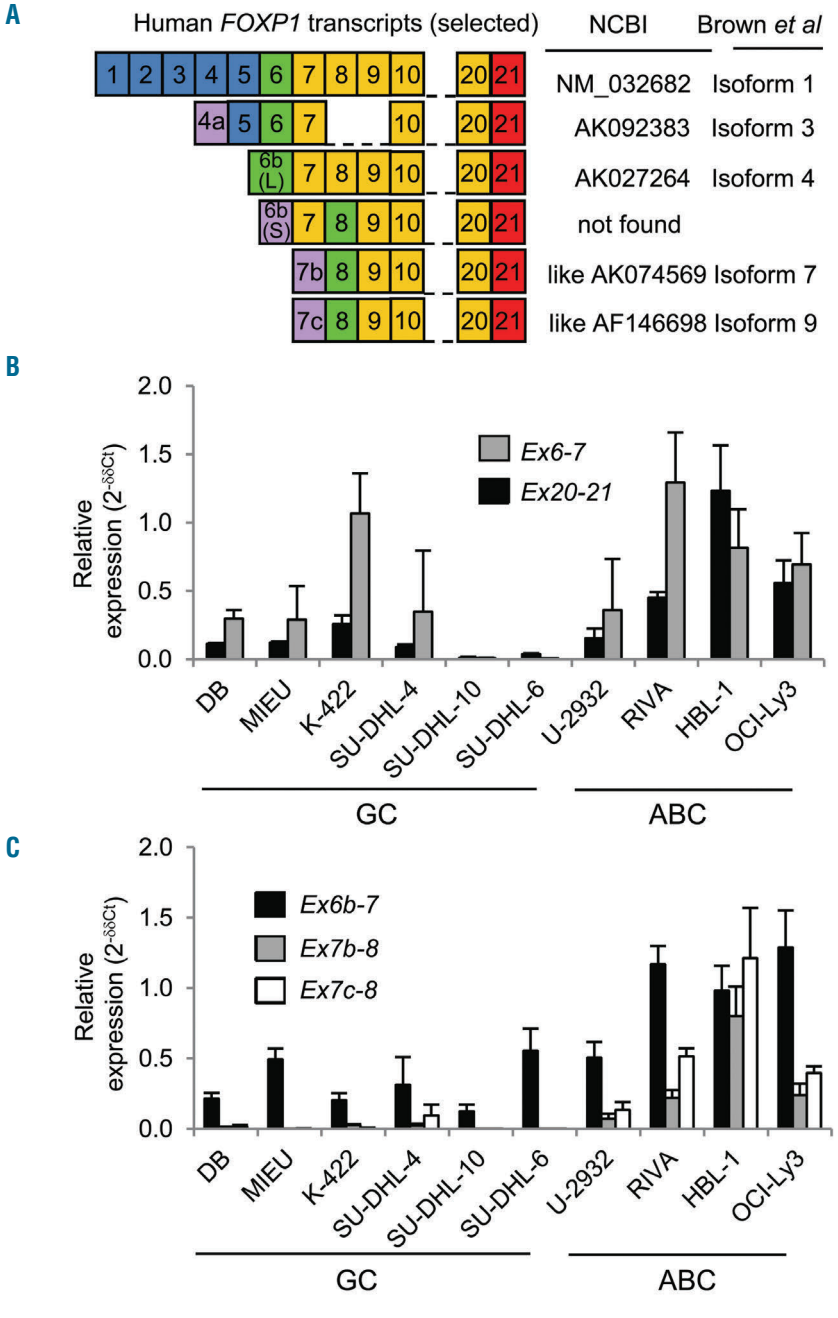


Figure 3. Diffuse large B-cell lymphoma (DLBCL) cells expressing $FOXP1_S$ protein transcribe multiple 5′ exon-containing *FOXP1* mRNA species. (A) Schematic illustration of human *FOXP1* transcripts containing alternative 5′ exons (purple), non-coding exons (light blue), coding exons (yellow), exons containing initiating methionine (green), and termination codons (red). Note *6b(L)* exon is an alternative exon colored green not purple due to presence of an initiating methionine. (B and C) Real-time PCR analyses of human *FOXP1* transcript expression in DLBCL cell lines ordered as in *Online Supplementary Figure S2B* (according to $FOXP1_S$ to $FOXP1_L$ protein ratio); n=3±SD.

scripts in ABC-DLBCL *versus* GC-DLBCL lines was also detectable by RNA-sequencing (Figure 4 and *Online Supplementary Table S2*), although only the *Ex6b(L)* increase was significant. *Ex7c* was not detected by this method, reflecting its relatively low abundance. In contrast to previously reported RT-PCR data,[26] *Ex4a* was not reliably increased in ABC-DLBCL (Figure 4 and *Online Supplementary Figure S3B*). Thus, no single alternate transcript was specifically associated with increased $FOXP1_S$ expression, and almost all ABC-DLBCL cell lines expressed multiple alternate transcripts.

FOXP1 transcripts with alternate exons 6 and 7 correlate with $FOXP1_S$ protein expression in primary human DLBCL

The expression of *FOXP1* total transcripts and those with alternate 5′ exons was further validated in a panel of primary DLBCL cases with known $FOXP1_L$ and $FOXP1_S$ protein expression, determined previously by immunoblotting.[26] Expression of *FOXP1* transcripts containing *Ex6b* or *Ex7c* was significantly increased in tumors where $FOXP1_S$ protein levels were equivalent to or exceeded $FOXP1_L$, while there was no relationship with total *FOXP1* transcripts (Figure 5A). Expression of *Ex6b, Ex7b, Ex7c* and total *FOXP1* was significantly higher in non-GC-DLBCL (Figure 5B). Combining expression profiles of all three $FOXP1_S$-encoding transcripts did not improve the accuracy of predicting GC or non-GC status (*data not shown*); some non-GC-DLBCL exhibited low expression of them all. Expression of *Ex7b* and *Ex7c* transcripts was significantly related to the intensity of tumoral nuclear FOXP1 protein positivity determined by immunohistochemistry (Figure 5C). Thus, despite lower relative abundance in DLBCL cell lines, only *Ex7c*-containing transcript levels correlate significantly with both FOXP1 protein expression levels, DLBCL cell-of-origin subtype, and predominant $FOXP1_S$ protein expression in primary tumors. *Ex4a* expression was not significantly related to FOXP1 protein or GC/non-GC status in primary DLBCL (*Online Supplementary Figure S3C*).

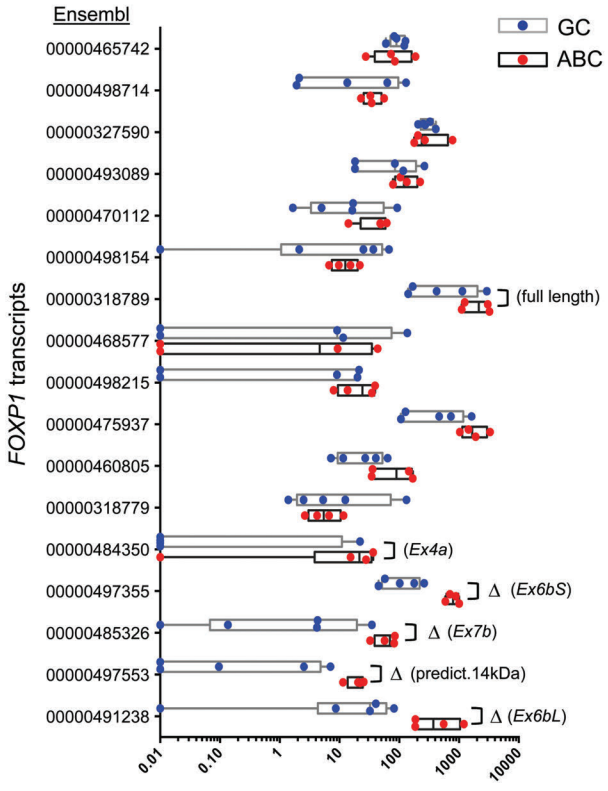


Figure 4. RNA-sequencing analysis detects increased *FOXP1* alternate 5′ promoter usage in ABC- *versus* germinal center (GC)-diffuse large B-cell lymphoma (DLBCL) cells. Analysis of *FOXP1*-annotated transcripts was performed in dataset GSE50721 that includes ABC-DLBCL cell lines OCI-LY3, OCI-LY10, SU-DHL2, U-2932, and GCB-DLBCL cell lines OCI-LY7, SU-DHL4, SU-DHL6, SU-DHL10, and OCI-LY19.[35] FASTQ files were downloaded, decrypted and aligned to hg19 genome using RSEM (bowtie2 mapping tool). Output gene and transcript count data were filtered to remove genes with 10 or fewer reads, then input into EdgeR (Bioconductor) and normalized to take into account the number of mapped reads and a normalization factor using a trimmed mean of M values (TMM). Output was log2 transformed normalized expression data. Expression of *FOXP1* transcripts containing *Ex6bS, Ex7b* or *Ex6bL* was higher in ABC cell lines (red data points, gray boxes) *versus* GCB-DLBCL cell lines (blue data points, black boxes), while *Exon7c* expression was not detected by this method. Differently-expressed transcripts are highlighted (triangle).

Malignant mature murine B cells express $Foxp1_S$ protein and N-terminally truncated Foxp1 transcripts partially conserved with mature human B cells

Cross-species conservation supports biological significance, and a murine $Foxp1_S$ isoform termed Foxp1D (initiating exon originally designated *mEx2b*,[37] revised to *mEx5b*)[38] has been described.[37] Multiple transcripts with alternate non-coding 5′ exons encoding murine $Foxp1_S$ protein in a murine B-cell lymphoma model, A20, that expresses both $Foxp1_L$ and $Foxp1_S$ proteins were investigated (*Online Supplementary Figure S4A*). Comparisons of the murine *Foxp1* genomic sequence with alternate 5′ exons in human *FOXP1* transcripts identified potential murine exons with homology to human *Ex6b* (*mEx4b*), *Ex7b* (*mEx5b*) and *Ex7c* (*mEx5c*) (*Online Supplementary Figure S4B and C*). However, the revised *mEx2b* alternate 5′ exon (predicted to encode $Foxp1_L$ or Foxp1A)[37] had only low similarity to human *Ex4a* sequences. Importantly, expression of $Foxp1_S$ -coding murine transcripts containing alternate 5′ exon *mEx4b* or *mEx5b* was detectable in the malignant mature B-cell lines A20 and 5TGM1 and associated with reduced expression of $Foxp1_L$ *Ex2-4* transcripts (*Online Supplementary Figure S4D*). Despite the significance of *Ex7c* in human DLBCL, transcripts containing its potential murine equivalent (*mEx5c*) could not be reliably detected. In summary, multiple transcripts with alternate 5′ exons contribute to $FOXP1_S$ protein expression in both murine and human lymphoma.

FOXP1 alternate 5′ exon usage is differentially induced by activation/maturation stimuli

The redundancy of multiple alternate 5′ exons giving rise to $FOXP1_S$ may enable several pathways (potentially different cell types or developmental stages) to fine-tune $FOXP1_S$ protein expression. Supporting this hypothesis, activation of primary human naïve B cells from 2 individuals with multiple stimuli, previously shown to induce $FOXP1_S$ protein,[26] increased expression of *Ex6b(L) Ex7b* and *Ex7c*, but had little effect on total *Ex6b* transcripts (Figure 6A). The latter indicates relatively high expression of *Ex6b(S) versus Ex6b(L)*, as in DLBCL cell lines (*Online Supplementary Figure S3A*). Activation of B-cell-derived cell lines induced *Ex7c* but not total *Ex6b* or reliably *Ex7b* expression, while *Ex6b* transcripts were induced only in the myeloid cell line HL-60, despite successful activation

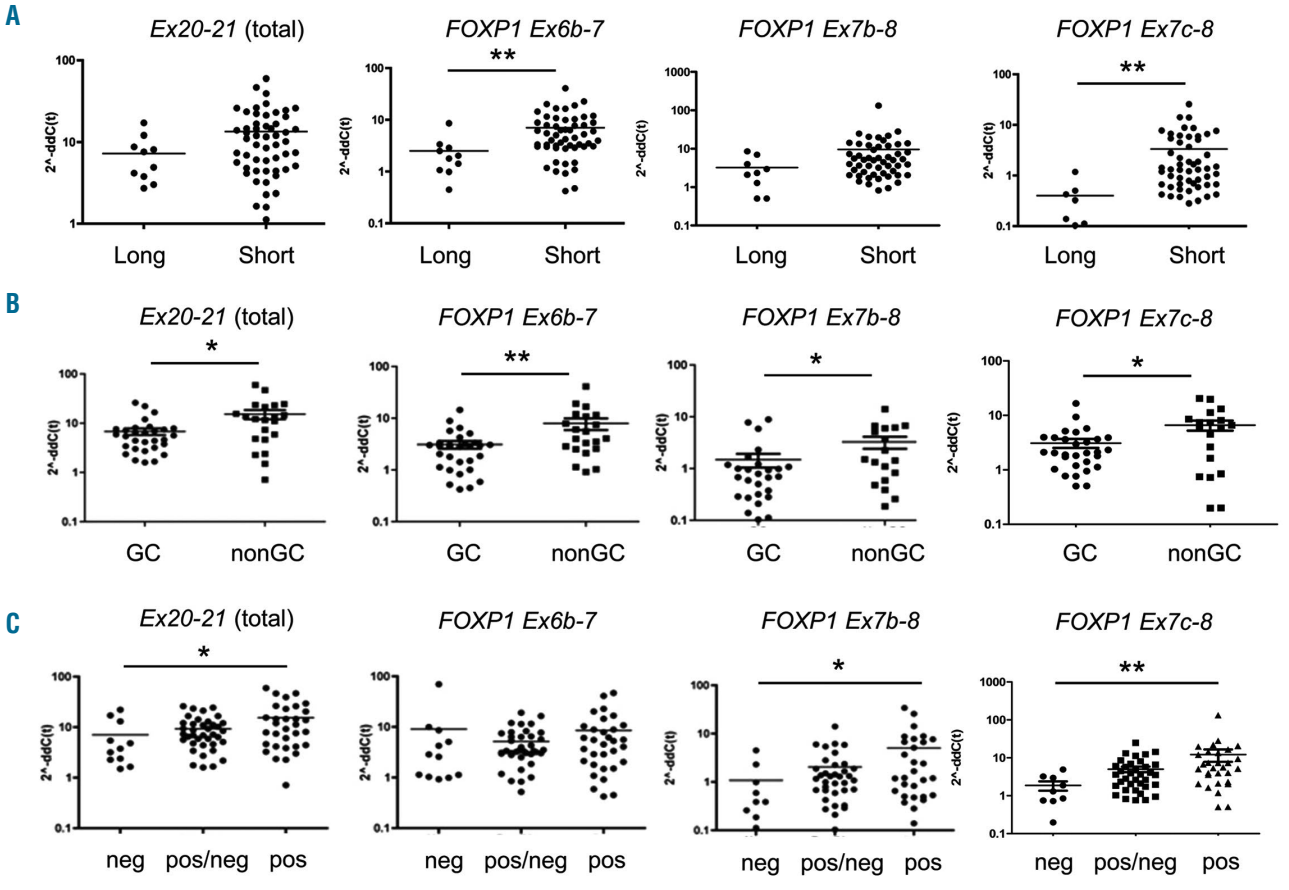


Figure 5. *FOXP1* transcripts with alternate exons *Ex6b* and *Ex7b/c* correlate with FOXP1$_S$ protein expression in primary human diffuse large B-cell lymphoma (DLBCL) samples. Real-time PCR analysis of multiple human *FOXP1* transcript expression in 79 primary DLBCL biopsies. Expression relative to RIVA cell line, normalized to 18S. Dots represent means of triplicate determinations *per* sample. Samples grouped according to (A) predominant FOXP1$_L$ (long: FOXP1$_L$ > FOXP1$_S$) or FOXP1$_S$ (short; FOXP1$_L$ ≤ FOXP1$_S$) protein expression as determined previously by western blot. (B) DLBCL subtype with germinal center (GC) or non-GC, as previously described,[26] or (C) intensity of tumoral FOXP1 positivity by immunohistochemistry, as previously described.[26] Horizontal bars within each scatterplot represent mean of each group. *P≤0.05, **P≤0.01.

of other myeloid lines (Figure 6B and *Online Supplementary Figure S5A and B*). Lack of *Ex6b(L)* transcripts (Figure 6B; inset) indicates this *Ex6b* to be FOXP1$_S$-coding. Thus, multiple conserved *FOXP1* transcripts with alternate 5′ exons are induced by activation/maturation events in a cell-type specific manner to produce FOXP1$_S$ protein. Treatment of several GC-DLBCL cell lines with 5-azacytidine increased *Ex7b* and *Ex7c* but not *Ex6b* expression (*Online Supplementary Figure S5C*), implicating methylation as another mechanism differentially regulating alternate *Foxp1* promoter activity between DLBCL subtypes.

FOXP1$_S$:FOXP1$_L$ ratio controls CD19 expression in human DLBCL

As GC- and ABC-DLBCL differ in their putative cell-of-origin, the relationship between FOXP1$_S$:FOXP1$_L$ ratio and expression of B-cell activation/maturation cell surface markers was investigated further across DLBCL cell lines. Expression of the B-cell marker CD20 was uniformly robust, and that of the plasma cell marker CD138 uniformly weak or absent (Figure 7A). In contrast, surface expression of CD19, CD45 (B220 isoform), and CD27 generally decreased as the FOXP1$_S$ to FOXP1$_L$ expression ratio increased (Figure 7A), with relatively few exceptions (e.g. CD19 in DB and CD45 in Karpas-422). Cell lines such as SU-DHL-4 and U-2932 exhibited both intermediate FOXP1$_S$:FOXP1$_L$ ratios and intermediate expression of the immaturity/memory markers CD19, B220 and/or CD27. Importantly, in these 'intermediate' DLBCL cell lines, transfection of siRNAs targeting *Ex7* to increase the FOXP1$_S$:FOXP1$_L$ ratio (Figure 2), but not targeting of total FOXP1, significantly reduced CD19 expression (Figure 7B and C).

The ABC-DLBCL cell line U-2932 has two subpopulations 'R1' and 'R2' (Figure 8A) both present as 'clones' in the original patient (CD20^{hi}CD38^{hi} and CD20^{lo}CD38^{lo}, respectively), which can be maintained stably and display both common and unique genetic aberrations.[39] Purified R1 and R2 populations exhibited clumped *versus* single-cellular growth habits, respectively (Figure 8B), and R1 showed higher FOXP1$_S$ protein expression and increased expression of FOXP1 *Ex6b, Ex7b* and *Ex7c* transcripts (Figure 8C and D). Furthermore, R1 has elevated expression of plasmablastic markers (*IRF4, XBP1*) and reduced expression of the FOXP1$_S$-repressed target gene *HIP1R*[22] (Figure 8D), again demonstrating a positive association between FOXP1$_S$ and B-cell maturity.

Discussion

Characterization of FOXP1 isoform complexity in both human and murine lymphoma cells is important in order to understand their roles in lymphoma biology and clinical relevance.

We have proven our original hypothesis[26] that $FOXP1_S$ proteins in ABC-DLBCL lack the N-terminus using both mass spectrometry and a commercially available polyclonal N-terminal antibody. An improved N-terminal antibody, lacking the non-FOXP1 cytoplasmic cross-reactivity and nuclear FOXP2 cross-reactivity seen with the current antibody, might routinely identify strongly $JC12^+$ (FOXP1 C-terminal epitope) DLBCL that lack the N-terminus. Despite its limitations, the current polyclonal antibody may be of interest within the research community, e.g. to validate expression of C-terminally truncated FOXP1 proteins, such as the FOXP1w isoform up-regulated by *SF3B1* mutation in poor prognosis B-cell chronic lymphocytic leukemia.

Western blotting data have demonstrated that normal lymphoid tissue and the microenvironment surrounding FOXP1-negative tumors express predominantly $FOXP1_L$ protein(s).[26] Thus, our identification of a rare GC B-cell population with high-level expression of FOXP1 protein(s) lacking *Ex7*-encoded epitopes is particularly interesting. Additional reagents (e.g. antibodies to novel epitopes in the N-terminus of $FOXP1_{AL}$) will help to definitively characterize this strongly $JC12^+$ population and examine whether $FOXP1_{AL}$ or $FOXP1_S$ isoforms predominate. Such studies may help to define a normal counterpart for ABC-DLBCL. FOXP1 exon-targeted siRNA and expression studies have identified at least three distinct FOXP1 proteins in DLBCL; $FOXP1_L$, $FOXP1_{AL}$ (long forms) and $FOXP1_S$, the latter two being primarily expressed in ABC-DLBCL. $FOXP1_{AL}$ detection in the HBL-1 ABC-DLBCL cell line is consistent with published transcript data describing potential expression of this isoform (FOXP1-011, ENST00000491238) in primary DLBCL.[24,26] Since all alternate 5′ exons were targeted inefficiently by siRNA in DLBCL cells, methods such as *FOXP1* locus editing could help to determine particular transcript contributions. Importantly, transcript conservation in murine lymphoma cells (e.g. human *Ex6b* and murine equivalent *mEx4b* being most abundant) should simplify $Foxp1_S$ functional studies. While no individual 5′ non-coding exon defines $FOXP1_S$ protein expression in primary DLBCL, *Ex7c* remains the best single transcript predictor and could be a useful addition to prognostic/diagnostic gene panels for DLBCL. Our data suggest that the $FOXP1_{S/L}$ ratio may also help define the stage of developmental block in DLBCL and improve stratification of DLBCL subgroups.

Although FOXP1 proteins expressed in malignant and normal B cells are similar, the control of FOXP1 expression appears distinct. Genetic abnormalities truncating FOXP1 are infrequent,[24,28,29] and our data indicate the majority of N-terminally-deleted FOXP1 expression in lymphoma is generated by alternate internal 5′ promoter usage (*Ex6b* being particularly abundant). Thus, it will be important to understand the likely oncogenic mechanisms controlling these transcriptional events. Significantly, BLIMP1 has been shown to bind the *FOXP1* locus,[40] and thus *BLIMP1* mutation[18] may drive ABC-DLBCL lymphomagenesis at least in part by mis-regulating FOXP1 isoform expression.

Historically, many clinical studies have not evaluated heterogeneity of marker expression across areas of a tumor. However, studies of clonal evolution have highlighted the importance of heterogeneity in a variety of malignancies, particularly in developing treatment resistance. We previously noted in a lymphoma biopsy with *FOXP1* translocation that the localized area of tumor containing the translocation had stronger FOXP1 protein expression (*(PJ Brown et al, unpublished data, 2014*). Here we demonstrate that subpopulations from a single DLBCL patient (present in U-2932) exhibit distinct FOXP1 isoform patterns alongside different immunophenotypes and expression of transcription factors involved in B-cell differentiation. Greater understanding of FOXP1 isoform heterogeneity may help to identify therapy-resistant DLBCL clones, as elevated FOXP1 is linked to resistance in response to both CHOP-R and rituximab monotherapy in lymphoma.[19,21,22,41]

Abundant *Ex6bS* transcript expression in GC-DLBCL cell lines lacking $FOXP1_S$ protein expression suggests post-transcriptional regulatory mechanisms may also regulate $FOXP1_S$ protein expression in DLBCL. Alternate exon-specific miRNA activity, in addition to the previously report-

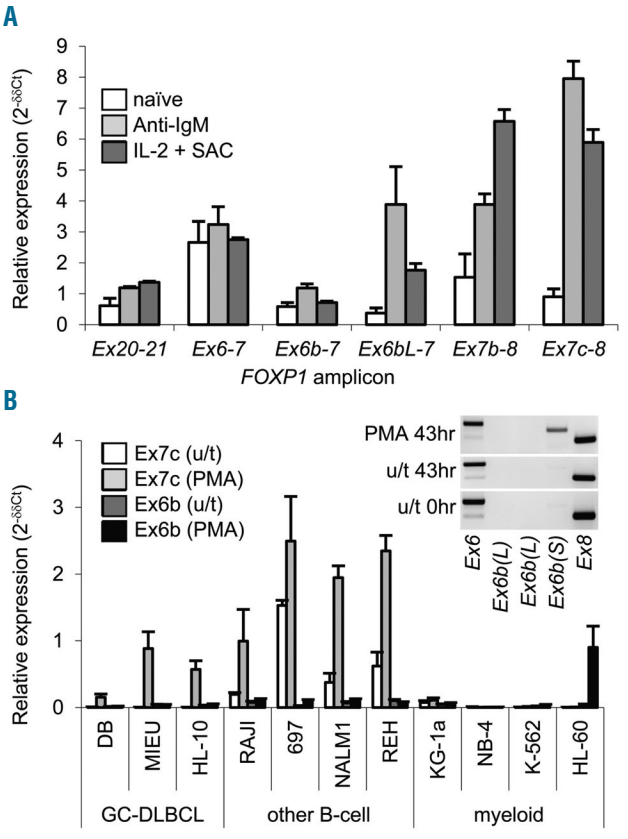


Figure 6. *FOXP1* alternate 5' exon usage is differentially induced by activation/maturation stimuli. (A) Real-time PCR analysis of multiple human *FOXP1* transcripts as indicated in primary naïve B cells purified from peripheral blood, cultured for 48 h without (Naïve) or with (anti-IgM or IL-2 + SAC) stimuli. Expression relative to the RIVA cell line, normalized to 18S. Mean of data from 2 individuals±S.E (B) Real-time PCR analysis of multiple human *FOXP1* transcripts as indicated in B lineage and myeloid cell lines cultured for 48 h with either carrier alone (u/t) or phorbol ester (PMA), expression relative to RIVA cell line, normalized to *18S*, representative of at least two experiments. (B) (Inset) Standard RT-PCR amplification to detect human *FOXP1* exon expression (top) in HL-60 cells treated with PMA identified *Ex6b(S)* but not *Ex6b(L)* transcripts.

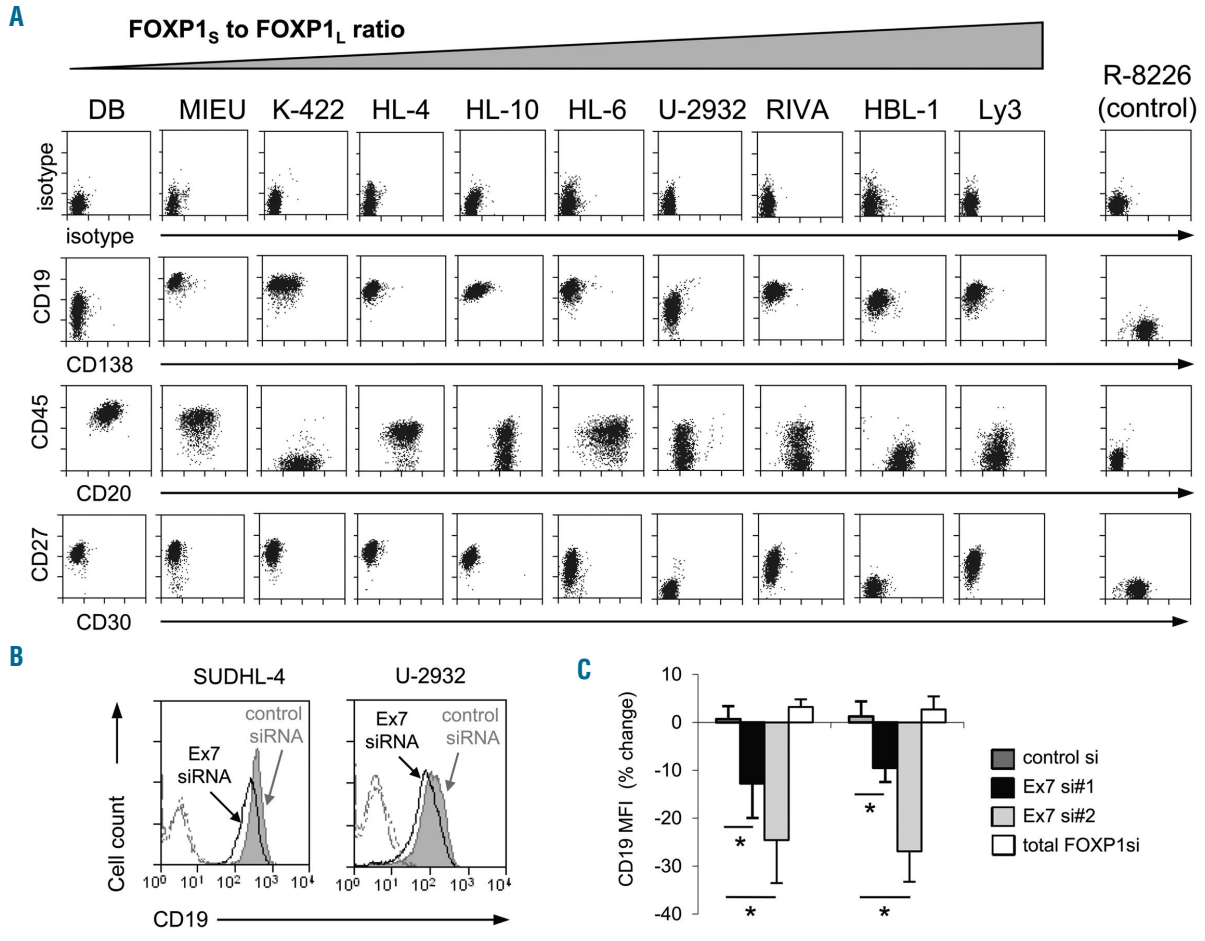


Figure 7. FOXP1$_S$ to FOXP1$_L$ expression ratio controls developmental status of human DLBCL cells. (A) Flow cytometric analysis of CD19, CD138, CD45, CD20, CD27 and CD30 surface expression on DLBCL cell lines with increasing FOXP1$_S$:FOXP1$_L$ protein ratio, as indicated. Note reasonably constant expression of CD20 and weak/absent CD138 and CD30 expression, while CD19, CD45 and CD27 show generally decreasing expression with increasing FOXP1$_S$ to FOXP1$_L$ ratio. (B) Representative flow cytometric analysis of CD19 surface expression on SUDHL-4 (GC-DLBCL) and U-2932 (ABC-DLBCL) cell lines 48 h after introduction of control siRNA or siRNA targeting *FOXP1* Exon 7 to increase the FOXP1$_S$ to FOXP1$_L$ ratio. (C) Mean fluorescence intensity±S.D. of surface CD19 determined by flow cytometric analysis of cells treated as in (B), both *FOXP1 Ex7* targeting siRNAs significantly reduced CD19 expression (P<0.05) [n=6 (SU-DHL-4) or n=3 (U-2932)] while *Ex13* targeting siRNA (total FOXP1si) did not; n=3 for both lines.

ed global miRNA targeting of FOXP1,[31,33] could be one regulatory mechanism, but there are many possibilities. Our finding that the first methionine codon in *hEx6b(L)* enabling translation of FOXP1$_{AL}$ is not conserved in mouse must be considered in studies of murine lymphoma, and may also prove significant outside of lymphoma and B-cell biology. Particularly the established neuronal roles of FOXP1,[42] emerging evidence for FOXP1 alterations in human behavioral disorders,[43] and the proposed contribution of related FOXP2 sequence evolution to language acquisition[44] indicate that neuronal studies of human-specific FOXP1$_{AL}$ may be warranted.

Total FOXP1 depletion studies indicate a contribution to cell viability and regulation of developmental target genes such as *PAX5* and *PRDM1* in lymphoma cells.[25,32,33,45] In addition, Dekker *et al.* have recently shown that total FOXP1 activates nearly all BCR-dependent genes; however, as FOXP1 silencing did not decrease BCR clustering, they concluded that it contributed to chronic B-cell receptor signaling (CABS) but only minimally to CABS-directed NF-κB activation.[46] Our findings, showing no decrease in CD19 expression upon total FOXP1 silencing, are consistent with these data. Interestingly, an siRNA that preferentially silenced FOXP1$_L$ was recently reported to be less effective at inducing cell death than total FOXP1 targeting in multiple DLBCL cell lines.[45] While the same study generally observed stronger regulation of gene expression on silencing all FOXP1 isoforms, there were some exceptions.[47] Our isoform-biased depletion studies presented here indicate that, in accordance with our previous findings (where the FOXP1 target gene *HIP1R* was preferentially regulated in ABC-DLBCL),[22] and complimentary to some common functions,[47] FOXP1$_L$ and FOXP1$_S$ have at least some distinct functional properties, including CD19 regulation. CD19 is a critical regulator of BCR signaling[48] *via* both BCR-dependent and independent mechanisms, thus FOXP1 isoforms (rather than total expression levels) may contribute to surface BCR activity. Distinct long and short FOXP1 functions are highly likely to derive from altered protein-protein interactions at the N-terminus. Given that induction of FOXP1$_S$ expression, for example

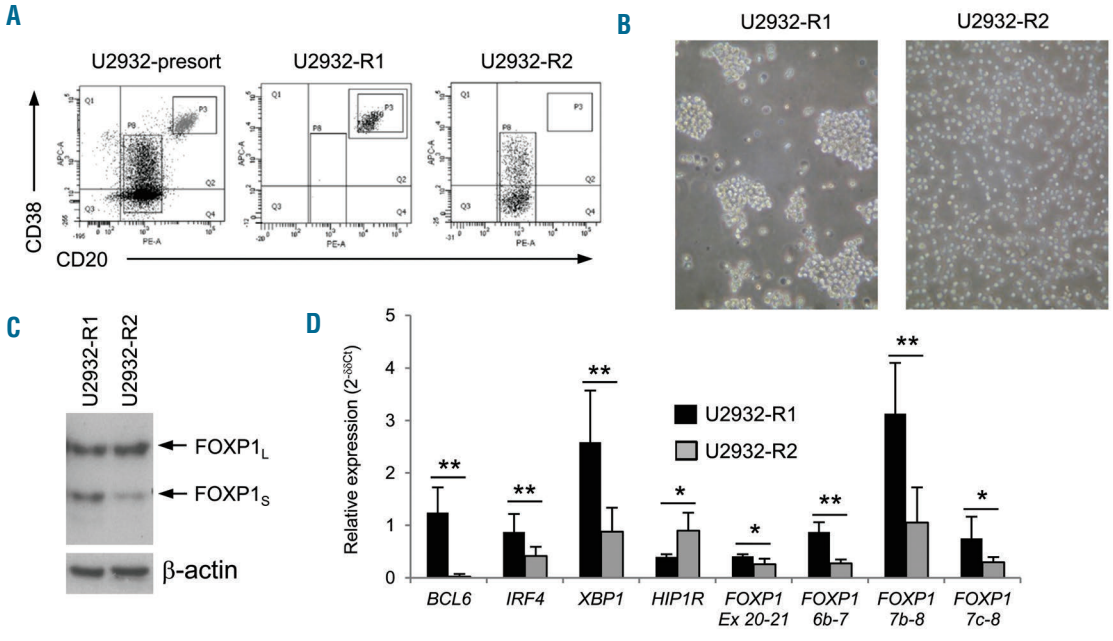


Figure 8. U-2932 DLBCL subpopulations display distinct FOXP1 expression patterns and maturation status. (A) Flow cytometric analysis of CD20 and CD38 expression in U-2932 parental (pre-sort sample) and subpopulations R1 and R2 sorted using gates as described previously.[39] Phenotypes were stable while samples were harvested over a 2-week period. (B) Representative phase-contrast light microscopy of sorted cultures to show clumping behavior of R1 on the left, as exhibited by multiple other ABC-DLBCL lines (*data not shown*). (C) Representative JC12 immunoblot analysis of FOXP1 in nuclear extracts. (C) Real-time PCR analysis as indicated, expression relative to RIVA cell line, normalized to 18S; n=5±S.D.

during B-cell activation, is associated with many other changes, the cellular environment is likely to play a crucial role in defining long and short FOXP1 functions.

Overall, we favor a model in which temporally- or spatially-distinct $FOXP1_L$ and $FOXP1_S$ expression have some conserved functions but mediate also distinct isoform-specific functions during mature B-cell development and in lymphoma pathogenesis. CD19 regulation may provide a potential mechanism for the reported FOXP1 regulation of BCR signaling,[46,49] and modulating FOXP1 could, therefore, have clinical benefit for therapies targeting CD19 in DLBCL, e.g. anti-CD19 chimeric antigen receptors.[50] Studying expression, regulation and function of FOXP1 isoforms in relation to developmental blocks in DLBCL may also lead to the identification of novel therapeutic differentiation strategies for this disease.

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