SUPPLEMENTARY APPENDIX

The small FOXP1 isoform predominantly expressed in activated B cell-like diffuse large B-cell lymphoma and full-length FOXP1 exert similar oncogenic and transcriptional activity in human B cells

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Supplementary Information

Supplementary Materials and Methods

Constructs

pcDNA3.1-FOXP1-myc-his encoding human FOXP1 was obtained from Daniel Simon (Harvard medical school, Boston, MA)[1]. The myc- and his-tag at the 3'end of the coding region were replaced by a stopcodon. FOXP1 was subsequently subcloned into LZRS-IRES-YFP via pcDNA4-TO to generate LZRS-FOXP1-IRES-YFP (referred to as FOXP1-IRES-YFP). A FOXP1-iso construct thet starts translation from the second coding ATG (in exon 6; M101), encoding a 100 AA N-terminally deleted FOXP1, was PCR-cloned and subcloned into LZRS. LZRS-BCL6-IRES-GFP was kindly provided by Dr. H. Spits[2].

B cell isolation

Buffy coats were obtained from Sanquin bloodbank (Amsterdam, the Netherlands). PBMCs were isolated by Ficoll separation. B cells were subsequently obtained by MACS separation with a memory B cell isolation kit (Miltenyi Biotech), or incidentally by FACSAria sorting of the $CD19^{+}CD27^{+}$ population using an APC-conjugated antibody against CD19 (BD Biosciences, San Jose, Ca) and a PE-conjugated antibody against CD27 (BD).

B cell cultures, cell lines, retroviral transductions and siRNA mediated knockdown

Isolated human B cells were cultured on CD40L-L cells (Arpin et al., 1995), IL-21(25 ng/ml, R&D systems, Abingdon, UK), and IL-2 (40U/ml, prospec, East Brunswick, NJ) in Iscove's modifies Dulbecco's medium (IMDM) containing 10% FCS, 100 units per ml of penicillin, and 100 µg per ml of glutamine (IMDM 10% FCS medium). For transduction, after culturing the cells for a minimum of 36 hours, cells were transferred to retronectin-coated plates and incubated with virus for 6-8 hours in IMDM 5% FCS medium. Thereafter, cells were cultured with CD40L-L cells, IL-21, and IL-2 in 10% FCS medium for 72 hours after which they were passaged and cultured either under the same conditions or without CD40L-L cells (for plasma cell differentiation experiments, or when specifically mentioned). For microarray analysis, after transduction cells were cultured without cytokines for three days.

DLBCL cell lines OCI-Ly1, OCI-Ly3, OCI-Ly7 and OCI-Ly10 were obtained as described[3]. U2932 was obtained from DSMZ and HBL1, TMD8, and RIVA were kindly provided by Dr. G. Lenz. OCI-Ly3 and OCI-Ly10 were cultured in IMDM 20% FCS medium, supplemented with 35 µM β-mercaptoethanol. OCI-Ly1, OCI-Ly7, and OCI-LY18 were cultured in IMDM 10% FCS medium. U2932 was cultured in RPMI 10%FCS medium, and HBL1, TMD8, and RIVA were cultured in RPMI 20% FCS medium For transductions, cell lines were transferred to retronectin-coated plates and incubated with virus for 24 hours. For siRNA-mediated knockdown, cells were transiently transfected using the Lonza nucleofection system. 5×10^{6} cells were resuspended in Nucleofector kit T (Lonza, Basel, Switzerland) mixed with 2.5 µg siRNA against FOXP1 or sigenome non-targeting siRNA pool #2 (Dharmacon, Thermo scientific, Waltham, MA) and pulsed with program G16.

Immunoblotting

Samples were applied on a 10% SDS-PAGE gel and blotted with rabbit polyclonal anti-FOXP1 ab16645 (Abcam) or #2005 (Cell Signaling), mouse-anti β-actin, or mouse-anti-β-tubulin antibodies (Sigma), followed by HRP-conjugated goat anti-rabbit or goat anti-mouse and developed by enhanced chemiluminescence (Amersham Pharmacia).

Quantitative RT-PCR

Total RNA was isolated using the RNeasy micro kit (Qiagen) or Trizol (Invitrogen), followed by isopropanol precipitation, and reverse-transcribed into cDNA with first strand buffer (Invitrogen), Moloney murine leukemia virus reverse transcriptase (Invitrogen), dNTPs, and Oligo (dT). For quantitative polymerase chain reaction (PCR), we used a Roche LightCycler 480 and mixed sensimix (Bioline) with cDNA and primer pairs for the indicated genes.

Mass spectrometry

FOXP1 protein was immunoprecipitated from OCI-LY10 cells using anti-FOXP1 antibody (JC12) and analyzed by SDS-PAGE. Proteins were silver-stained using successive incubations in 0.02% sodium thiosulfate for 2 min, 0.2% silver nitrate for 20 min, and 0.05% formaldehyde. 6% sodium carbonate, and 0.0004% sodium thiosulphate for 5 min. Protein bands corresponding to FOXP1 full length and the smaller isoform were cut into small pieces and washed, dehydrated, reduced, alkylated and trypsin digested as described before.[4] Fragments were analyzed by nano-LC-ESI-ion trap MS/MS using an Ultimate LC system (Thermo Fischer), coupled to an HCTultra ion trap mass spectrometer (Bruker Daltonics) and by MALDI-ToF MS, using an Ultraflex II time-of-flight mass spectrometer (Bruker Daltonics). FOXP1 peptides identified by both methods (following Mascot database searches and manual inspection) in the FOXP1-full length and isoform protein bands, respectively, were combined to get the overall picture of exons covered by these peptides.

Flow cytometry

For eFluor 670 labeling, cells were washed in PBS and resuspended in 100 μl PBS. An equal volume of 2 μM eFluor 670 (eBioscience, San Diego, CA) was added, and cells were incubated at 37°C for 15 min, quenched in 100% FCS, and washed twice with medium containing 10% FCS. For cell cycle analysis, cells were fixed with ethanol, washed in PBS 0.5% BSA and incubated for 20 minutes with 50 μg/ml RNAse A and 50 μg/ml Propidium Iodide (invitrogen, Carlsbad, CA) in PBS 0.5% BSA. Propidium iodide, eFluor 670, GFP and/or YFP fluorescence were acquired on a FACSCanto (BD Bioscience, San Diego, CA) flowcytometer and analyzed using FlowJo software (TreeStar, Ashland, OR). For indetification f plasma cells, cells were stained with anti-human CD38 (BD), conjugated with APC and CD20 conjugated with PE and analyzed on a FACSCanto.

Caspase-Glo 3/7 assay

Activation of Casase 3 and/or Caspase 7 was measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI) according to the manufacturer's instructions. Luminescence was measured in a Glomax microplate-reading luminometer (Promega). Live cell numbers were determined by flow cytometry and luminescence values were normalized for numbers of live cells in each sample.

Enzyme-linked immunosorbent assay (ELISA)

Costar EIA 96-well plates (corning) were coated overnight at 4˚C with capture antibodies anti-human IgG (CLB-MH 16/l, Sanquin), IgM (UHB, southern biotech), or IgA (Sanquin). After blocking with 1% BSA for 1 hr , wells were incubated with serial dilutions of cell culture supernatants for 2 hours at room temperature, followed by incubation with HRP-conjugated mouse anti-human IgM, IgG, or IgA (Sanquin) and developed ad previously described (Bende et al., 1992). IgG isotype ELISA was performed using the Human IgG subclass profile ELISA kit. (Invitrogen) according to the manufacturer's instructions.

ELISPOT

IgG and IgM ELISPOTs were performed using IgG and IgM ELISpot kits (Mabtech) according to the manufacturer's instructions.

Microarray analysis

Microarray analysis was performed essentially as described[5;6]. Biotinylated complementary RNA was amplified with a double in vitro transcription, according to the Affymetrix small sample labeling protocol vII (Affymetrix). The biotinylated complementary RNA was fragmented and hybridized to the HG-U133 Plus 2.0 GeneChip oligonucleotide arrays according to the manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified and analyzed using the GCOS software (Affymetrix). The threshold for significant change was set to a P-value of ≦0.0025. Genes with 100% absent call were considered not to be biologically relevant and were removed. Data were analyzed and heatmaps were generated using R2: microarray analysis and visualization platform (http://r2.amc.nl).

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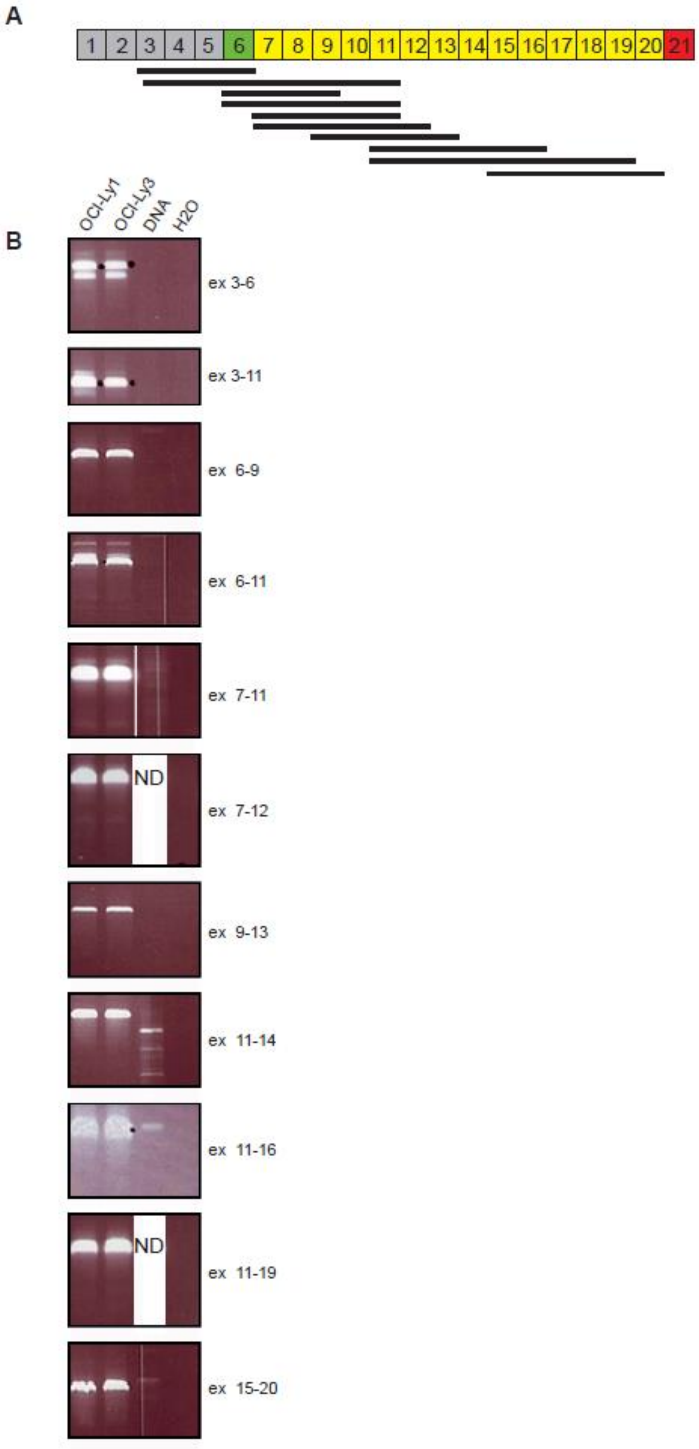
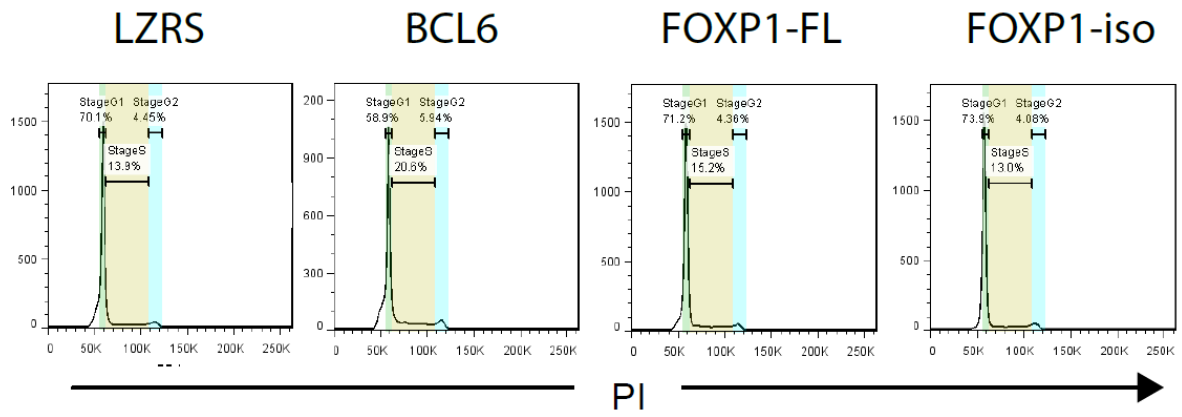
Supplementary figures



Supplementary Figure 1. Analysis of FOXP1 transcripts in DLBCL cell lines
(A,B) RT-PCR analysis of several regions of FOXP1, as schematically represented (A), in the DLBCL cell lines OCI-Ly1 and OCI-Ly3 (B). ND=not determined



Supplementary Figure 2. Cell cycle analysis of transduced B cells

Human primary B cells were transduced with ctrl-IRES-YFP, BCL6-IRES-GFP, FOXP1-FL-IRES-YFP, or FOXP1-iso-IRES-YFP and cultured on CD40L-L cells in the presence of IL-21 and IL-2. 12 days after transduction, cell cycle stage was determined by PI staining. The percentage of cells in each stage of the cell cycle was determined using the Watson Pragmatic model. Representative graphs of two independent experiments are shown.