Nuclear FOXO1 promotes lymphomagenesis
in germinal center B cells

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Supplementary information

Supplementary methods

Cell culture conditions

Human BL cell lines were cultured in RPMI medium 1640 supplemented with 15% fetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin. For PI3K pathway inhibition cell lines were treated with 200nM wortmannin (Sigma) 1 hour prior analysis. De-acetylation or de-glycosylation was prevented by Trichostatin A (5μM) or PUGNAc (100μM) treatment 2 or 1 hrs prior analysis. Mouse BL cell lines were cultured in DMEM medium with 15% FCS, 1% L-glutamine, 1% non-essential amino acids, 1% HEPES, 1% sodium pyruvate and 0.1% beta-mercaptoethanol. If applicable, puromycin (Sigma) was used in a final concentration of 3 μg/ml for selection of transfected mouse lymphoma cells.

FOXO1 sequencing

Genomic DNA and RNA was extracted from human and mouse samples (using AllPrep DNA/RNA Kit by QIAgen) and Exon 1 and 2 of FOXO1 were amplified using the following primers:

Human Ex1 Fwd: 5'-CCCTCTTGGCTCTCCTGCGGCTGG-3'

Human Ex1 Rev: 5'- GTAGGGCACGCTCTTGACCATCCACTCG -3'

Human Ex2 Fwd: 5'-ATGGAAATCTGTAGCACACACTCTTA-3'

Human Ex2 Rev: 5'- AACACATTGTCAAAGTTAAAATCCAAT-3'

Mouse Ex1 Fwd: 5'-GTCCTAGGCACGAACTCGGAGGCTC-3'

Mouse Ex1 Rev: 5'- GTTGCTGTCGCCCTTATCCTTGAAGTAGG-3'

Mouse Ex2 Fwd: 5'- CGAGCTGTTAGGCTGCTCAGAATGG-3'

Mouse Ex2 Rev: 5'- TTAGCCTGACACCCAGCTGTGTGTTGTAG-3'

cDNA synthesis from total RNA was performed according to manufacturer's instructions (Thermoscript RT-PCR system from Invitrogen) and the coding sequence of mouse and human *FOXO1* was amplified using

Human Fwd: 5'-ATGGCCGAGGCGCCTCAGGTGGTGGAGA-3',

Human Rev: 5'- CCCTCAGCCTGACACCCAGCTATGTGTC-3'

Mouse Fwd: 5'- ATGGCCGAAGCGCCCCAGGTGGTGGAGAC-3'

Mouse Rev: 5'- TTAGCCTGACACCCAGCTGTGTGTTGTAG-3'.

For detection of CRISPR/Cas9 mutagenesis Exon1 primer sets were used to amplify a DNA region including the gRNA/PAM sequence and the T24 position. PCR products were subcloned (Zero blunt cloning kit, Invitrogen) and after transformation of bacteria single colonies were analyzed for the cloned insert.

Electroporation and retroviral infection

Mouse *Foxo1* gRNAs were cloned in vectors as previously described [1] and 5 μg of DNA were electroporated in 1–2 × 10^6 lymphoma cells using a Nucleofector 2b device (Lonza). Reporter+ cells were sorted at 48hrs after electroporation. For retroviral infections, the coding sequence of *Foxo1* was cloned into the MSCV-IRES-GFP vector (Addgene, plasmid# 20672) where GFP was replaced by BFP. Forty-eight hours after plasmid transfection in PLATE packaging cells retrovirus containing supernatant was collected. Mouse lymphoma cells were transduced by spin infection in the presence of Polybrene 8μg/ml (Sigma).

Lentiviral infection

Human *FOXO1* gRNA was cloned into pKLV-U6gRNA(BbsI)-PGKpuro2ABFP vector (Addgene, plasmid# 50946). Lentiviral particles containing pKLV-U6gRNA(BbsI)-PGKpuro2ABFP or FUCas9Cherry (Addgene, plasmid# 70182) were produced at the Centre International de Recherche en Infectiologie, École Normale Supérieure de Lyon (Lyon, France). Human lymphoma cells were transduced similarly to mouse cells.

T7EI assay

CRISPR/Cas9 targeting efficiency was assessed by the T7EI endonuclease assay (NEB) according to manufacturer' s protocol and indel calculation was performed as previously published [2]. Primer sequences for amplification of mouse *Foxo1*: Fwd: 5'-GTCCTAGGCACGAACTCGGAGGCTC-3',   Rev: 5'-GTTGCTGTCGCCCTTATCCTTGAAGTAGG-3'.

Quantitative RT-PCR

Total RNA from human BL cells was extracted using the AllPrep DNA/RNA Kit (QIAgen) and cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen). For quantitative RT-PCR we used Power SYBR Green and the StepOnePlus system (Applied Biosystems) for analysis. Samples were assayed in triplicate and messenger abundance was normalized to that of *ACTB.* Primer

sequences: *FOXO1*_Fwd: 5'-CGAGTGGATGGTCAAGAGCG-3', *FOXO1*_Rev: 5'-CAGTTCCTTCATTCTGCACACG-3', *ACTB*_Fwd: 5'-GAGCACAGAGCCTCGCCTTT-3', *ACTB*_Rev: 5'- TAGCAACGTACATGGCTGGG-3'

Flow cytometry

pAKT levels were determined by intracellular FACS analysis using α-pAKT (S473) and pAKT (S473) blocking peptide (Cell Signaling) following manufacturer's instructions. Flow cytometry and cell sorting was performed in BD LSR Fortessa and BD FACS Aria flow cytometers. Data were analyzed using FlowJo software (Tree star).

Viability and cell cycle assay

Viabiliy and proliferation were determined using the BrdU Flow Kit (BD Pharmingen). Briefly, cells were incubated with BrdU for 1 hrs prior to harvesting. After BrdU labeling cells were fixed, permeabilized and stained according to the manufacturer's instructions. Lastly, DNA was stained with 7-AAD and its content determined by flow cytometer. Diploid cells characterized by a DNA content less than 2X were considered dead (R1 gate in Figure 4B). Living cells (DNA content ≥2X) were gated (R2) and subsequently analyzed for BrdU incorporation.

Pre-processing of gene expression data

The Robust Multi-array Average (RMA) function was used to normalize the data during import of the raw Affymetrix data (CEL files) of a total of 12 samples into Partek Genomics Suite (PGS). The background value was defined as the median of the entire normalized dataset using all probes. Gene probes were only kept for further analysis if their mean expression values were higher than 100 in at least one condition within the whole dataset. Next, multiple probes for one gene locus were filtered allowing only one probe with the highest overall mean expression. These pre-processing steps resulted in 20,000 unique genes to be present in at least one group or condition. These genes were retained for further analyses.

Identification of differentially expressed genes and hierarchical clustering

Differentially expressed (DE) genes were defined in PGS by analysis of variance (ANOVA) models by setting certain fold change (FC) thresholds and anadjusted P-value cutoffs to determine differences between the compared groups The respective cutoffs used for a particular comparison are described in the result section. The most variable genes were determined in ANOVA models to compare the variations

throughout groups by t-test, that is, genes with most significant P-values. Hierarchical clustering using the Euclidean distance on both genes and samples was performed.

Gene Ontology (GO) enrichment analysis and GO network visualization

To link the data to prior knowledge, GO enrichment analysis was applied by using the Cytoscape plug-in BiNGO (v2.44)[3]. To include only significant results, the FDR q-value threshold was set to 0.05 as default. The Cytoscape plugins Enrichment Map (v1.1)[4] and Word Cloud[5] were used to visualize the GO networks.

Gene set enrichment analysis (GSEA) on single samples

To study relative pathway activity on single samples, we have used the combined z-score method[6]. Since the used GSEA relies on a parametric assumption, the combined z-score method transforms the expression values into z-scores and uses those as input for computing an enrichment score for each gene set and individual sample. GSEA on single samples was performed on 147 genes being up-regulated and 170 genes down-regulated in Sander_DZ using the R package "GSVA"[6] by setting the method parameter to "zscore".

Statistical analyses

Data were analyzed by Wilcoxon-Mann-Whitney test or by the standard one-way of variance (ANOVA) test and a p value ≤ 0.05 was considered significant.

Supplementary references

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Supplementary figure legends

Supplementary Figure 1. FOXO1 is abundantly expressed in BL

(A) Quantitative RT-PCR analysis of *FOXO1* expression in human BL and HL cell lines. Results were normalized to the expression of the control gene *ACTB*. Each symbol represents an individual cell line; horizontal lines indicate the mean. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Wilcoxon-Mann-Whitney test)

(B) Western blot analysis for FOXO1 expression in human BL and HL cell lines. ACTB served as loading control. Data are representative of two experiments.

(C) Immunofluorescence analysis of FOXO1 (red) in human BL cell lines. Cell nuclei were counterstained with DAPI (blue).

(D) Western blot analysis of FOXO1 expression in subcellular fractions of human BL cells. The purity of the cytoplasmic and nuclear fraction was determined by Actin (ACTB) and Histone H3 (H3) antibodies, respectively. (C) indicates the cytoplasmic fraction; (N) the nuclear fraction; (W) whole cell lysate; (KO) FOXO1 knockout cells. Data are representative of at least two experiments.

(E) Western blot analysis of phospho-AKT (pAKT(S473)), AKT, phospho-FOXO1 (pFOXO1(T24)) and FOXO1 expression in human BL cell lines. Cells were treated either with the PI3K inhibitor wortmannin (+) or DMSO (-) 1hr prior to protein extraction. ACTB served as loading control. Data are representative of two experiments.

Supplementary Figure 2. FOXO1 is recurrently mutated in BL and the gene locus is accessible for genome editing

(A) Summary tables and electropherograms showing non-synonymous mutations in mouse and human *FOXO1* as detected by Sanger sequencing. Mutations interfering with T24 phosphorylation of FOXO1 were marked in red. Genomic DNA of lymphoma cells was used for the analysis.

(B) Analysis of *Foxo1* gRNA targeting efficiency in NIH3T3 cells (wt[+]/wt[+]). Top: gRNAs used in this assay; CRISPR/Cas9 target sequences were given in blue (protospacer) and red (protospacer adjacent motif, PAM). Bottom: *Foxo1* T7EI assay in NIH3T3 after transfection of Cas9 and individual gRNA expression plasmids. Arrows indicate the expected positions of DNA bands cleaved by mismatch-sensitive T7EI.

(C) Sequence alignments generated in isogenic mouse and human BL cell line clones in which the mt *FOXO1* allele was ablated by CRISPR/Cas9 (wt$^+$/mt$^-$) as determined by Sanger sequencing.

(D) Western blot analysis (left) of phospho-AKT (pAKT(S473)), AKT, phospho-FOXO1 (pFOXO1(T24)) and FOXO1 in isogenic mouse BL cell line clones after CRISPR/Cas9 guided editing of the *Foxo1* locus. Per genotype 3 (or 2) individual cell line clones were analyzed in the presented experiment. ACTB served as loading control. right: sequence alignments generated in isogenic cell line clones in which the wt *Foxo1* allele was ablated by CRISPR/Cas9 (wt$^-$/mt$^+$) as determined by Sanger sequencing.

Supplementary Figure 3. CRISPR/Cas9 introduces deletions and insertions in the mt and wt *FOXO1* locus of BL and HL

(A) Sequence alignments generated in mouse BL cells after CRISPR/Cas9 guided editing of the F*oxo*1 locus. Two cell lines were characterized by a heterozygous T24 mutation (mouse BL#19 and #82) whereas the others expressed exclusively wt FOXO1 (mouse BL#81 and #88).

(B)   Sequence alignments generated in human BL cells after CRISPR/Cas9 guided editing of the F*oxo*1 locus. Namalwa and BL2 carried a mt FOXO1 allele whereas CA46 was characterized by wt FOXO1.

(C) *FOXO1* sequence analysis after CRISPR/Cas9 editing in human HL cell lines (L1236, HDLM2) at indicated time points after FACS-based sorting. CRISPR edited sequences were analyzed and the proportion of "out of frame" and "in frame" sequences was depicted in the pie charts. The total number of sequences analyzed was given in the center of the chart.

Supplementary Figure 4. Mouse BL cells loose their FOXO1 expression after genome editing

(A) Sequence alignments generated in isogenic mouse BL cell line clones after CRISPR/Cas9 guided FOXO1 knockout (KO). Two of the parental cell lines were characterized by a heterozygous T24 mutation (mouse BL#19 and #82) whereas the others expressed exclusively wt FOXO1 (mouse BL#81 and #88).

(B) Western blot analysis for FOXO1 expression in mouse BL cell line clones (parental clones and KO clones as shown in (A)). Three individual cell line clones (for most conditions) were analyzed in the presented experiment. ACTB served as loading control.

Supplementary Figure 5. Detrimental effect of P110* or mt FOXO1 ablation in mouse BL#19 cells

(A) Schematic depiction of CRISPR/Cas9 targeting at P110* transgene introduced in Rosa26 locus and percentage of BFP expressing cells over time. After infection of mouse BL#19 cells with constructs encoding individual gRNAs targeting wt Rosa 26, transgenic MYC and P110* the proportion of transgene expressing cells was monitored by FACS at indicated time points. Results represent a single experiment.

(B) Sequence alignments generated in mouse BL#19 cells after CRISPR/Cas9 guided editing of the P110* transgene at indicated time points after FACS-based sorting. Arrows mark the in-frame edited sequences.

(C) Hierarchical clustering of 1.000 most variable transcripts across all samples.

(D) Boxplots summarizing the enrichment scores for FOXO1 upregulated (Sander_DZ_up) or downregulated (Sander_DZ_down) DZ genes in each sample. The samples are grouped acoording to their genotypes after *Foxo1* locus editing.

Supplementary Figure 6. Mutant *Foxo1* can be successfully corrected by CRISPR/Cas9 technology

(A) Sequence alignments generated in isogenic mouse BL cell line clones after CRISPR/Cas9 guided repair of the mt *Foxo1* locus.

(B) Western blot analysis of phospho-AKT (pAKT(S473)), AKT, phospho-FOXO1 (pFOXO1(T24)) and FOXO1 in repaired cell line clones. Four individual cell line clones were analyzed in the presented experiment. The parental cell line carrying the heterozygous T24 mutation served as control (CT). ACTB served as loading control.

Supplementary Figure 7. Overexpression of nuclear FOXO1 induces lymphoma cell growth in mouse BL cells

(A) Western blot analysis of phospho-AKT (pAKT(S473)), AKT, phospho-FOXO1 (pFOXO1(T24)) and FOXO1 in FACS-sorted FOXO1 knockout BL cells after transduction with an empty vector (BFP) or constructs (as described in Figure 7D) encoding wt or mt FOXO1. ACTB served as loading control. Data are representative of two experiments.

(B) Representative FACS analysis of BFP expression in mouse BL cells at day 2 and day 10 after retroviral transduction.

(C) Western blot analysis of FOXO1 expression after immunoprecipitation with α-Acetylated-Lysine antibody in mouse BL cells (as described in Figure 7F). Cells

were either inhibitor treated (+) or DMSO (-) prior to analysis. The membrane was incubated with α-Histone H3 antibody to demonstrate protein precipitation. ACTB served as loading control. Data are representative of two experiments.

(D) Western blot analysis of O-glycosylation after immunoprecipitation with α-FOXO1 antibody in mouse BL cells (as described in Figure 7F). Cells were either inhibitor treated (+) or DMSO (-) prior to analysis. The membrane was incubated with α-FOXO1 antibody to demonstrate protein precipitation. ACTB served as loading control. Data are representative of two experiments.
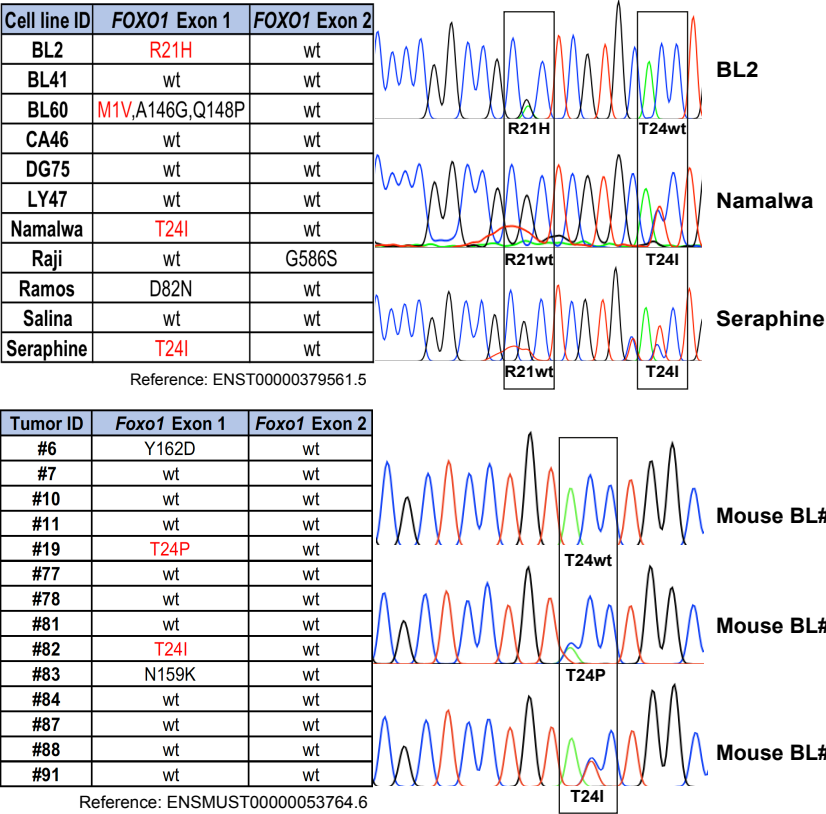
Supplementary Figure 1

A

B

C

DAPI   FOXO1   MERGED

BL41

Raji

CA46

BL60

5 μm

D

E

Supplementary Figure 2

A

Cell line ID	*FOXO1* Exon 1	*FOXO1* Exon 2
BL2	R21H	wt
BL41	wt	wt
BL60	M1V,A146G,Q148P	wt
CA46	wt	wt
DG75	wt	wt
LY47	wt	wt
Namalwa	T24I	wt
Raji	wt	G586S
Ramos	D82N	wt
Salina	wt	wt
Seraphine	T24I	wt

Reference: ENST00000379561.5

Tumor ID	*Foxo1* Exon 1	*Foxo1* Exon 2
#6	Y162D	wt
#7	wt	wt
#10	wt	wt
#11	wt	wt
#19	T24P	wt
#77	wt	wt
#78	wt	wt
#81	wt	wt
#82	T24I	wt
#83	N159K	wt
#84	wt	wt
#87	wt	wt
#88	wt	wt
#91	wt	wt

Reference: ENSMUST00000053764.6

B

C

D

Supplementary Figure 3

A

B

C

Supplementary Figure 4

A

wt⁺/mt⁺

Mouse BL#19

KO#10
wt 5'-CTGGAGGAGAGTGAGGA----GCGCGGGCG-3'
mt 5'-CTGGAGGAGAGTGAGGA----GCGCGGGCG-3'

KO#14
wt 5'-CTGGAGCCTGCTGGACTTCCAGGGCCCCGA-3'
mt 5'-CTGGAGGAGAGTGAGGACTTCCGCGCGGGC-3'

KO#20
wt 5'---------------------------------3'
mt 5'-CTGGAGGAGAG------------------3'

Mouse BL#82

KO#1
wt 5'---------------------------------3'
mt 5'---------------------------------3'

KO#2
wt 5'-CT-----------------------------3'
mt 5'-CTGGAGG------------------------3'

wt⁺/wt⁺

Mouse BL#81

KO#4
wt 5'-CTGGAGGAGAGTGAGGACT-------------3'
wt 5'-CTGGAGGAGAGTGAGG---------------3'

KO#5
wt 5'-CTGGAGGAGAGTGAGGACT----GCGGGCG-3'
wt 5'-CTGGAGGAGAGTGAGG-------GCGGGCG-3'

KO#7
wt 5'-CTGGAGGAGAGTGAGGACT--GCGCGGGCG-3'
wt 5'-CTGGAGGAGAGTG----------CGGGCG-3'

Mouse BL#88

KO#5
wt 5'-CTGGAGGAGAGTGAGGACTT-------GCG-3'
wt 5'-CTGGAGGAGAGTGAGGA----GCGCGGGCG-3'

KO#6
wt 5'-CTGGAGGAGAGTGAGGAC----CGCGGGCG-3'
wt 5'-CTGGAGGAGAGTGAGGACTT--CGCGGGCG-3'

KO#7
wt 5'-CTGGA-----------------------3'
wt 5'--------------------------3'

B

Clone ID:	Mouse BL#19						Mouse BL#82			Mouse BL#88					Mouse BL#81					
	wt⁺/mt⁺			wt⁻/mt⁻			wt⁺/mt⁺	wt⁻/mt⁻		wt⁺/wt⁺		wt⁻/wt⁻			wt⁺/wt⁺			wt⁻/wt⁻		
	#2	#26	#42	#10	#14	#20	#1	#1	#2	#3	#5	#4	#5	#7	#2	#3	#8	#5	#6	#7
FOXO1																				
ACTB																				

Supplementary Figure 5

A

Rosa26 P110* allele

Mouse BL#19

- empty vector
- gRNA_Rosa26
- gRNA_MYC_1
- gRNA_MYC_2
- gRNA_P110*

B

Day 1 (bulk population)

wt sequence

5'-TACCTTGTT CCA ATCCTAGGTGGGAATG-3'

→ 5'-TACCTTGTT CCA - - -CTAGGTGGGAATG-3'
5'-TACCTTGTT CCA ATC-TAGGTGGGAATG-3'
5'-TACCTTGTT CCA AT- - - - - -GTGGGAATG-3'
→ 5'- - - - - - - - - - - - - - - -GTGGGAATG-3'
5'-TACCTT- - - - - - - - - - - - - GAATG-3'
→ 5'-TACCTTGTT CTA ATCCTAGGTGGGAATG-3'
→ 5'-TACCTTGTT CCA ATCGTAGGTGGGAATG-3'
5'-TACCTTGTT CCAATC-TAGGTGGGAATG-3'
5'-TACCTTGTT CCA ATC-TAGGTGGGAATG-3'
5'-TG- - - - - - - - - - GAATG-3'

Day 28 (single cell clones)

wt sequence

5'-TACCTTGTT CCA ATCCTAGGTGGGAATG-3'

→ clone #1 5'-TACCTTGTT CCA ATC- - -GGTGGGAATG-3'
→ clone #2 5'-TACCTTGTT CCA - - -CTAGGTGGGAATG-3'
→ clone #3 5'-TACCTTGTT CCA - - -CTAGGTGGGAATG-3'
→ clone #4 5'-TACCTTGTT CCA ATC- - -GGTGGGAATG-3'
→ clone #5 5'-TACCTTGTT CCA ATC- - -GGTGGGAATG-3'
→ clone #6 5'-TACCTTGTT CCA ATC- - -GGTGGGAATG-3'
→ clone #7 5'-TACCTTGTT CCA - - -CTAGGTGGGAATG-3'
→ clone #8 5'-TACCTTGTT CCA - - -CTAGGTGGGAATG-3'
→ clone #9 5'-TACCTTGTT C- - - - - -TAGGTGGGAATG-3'

C

Hierarchical Clustering

D

Sander_DZ_up

Sander_DZ_down

Supplementary Figure 6

A

Foxo1 wt allele

5′-CCCCGGCAGCGCTCCTGT**A**CCTGGCCGCTGCCCAGGCC-3′

Rep #2 CCCCGGCAGCGCTCCTGT**A**CCTGGCCGCTGCCCAGGCC
Rep #3 CCCCGGCAGCGCTCCTGT**A**CCTGGCCGCTGCCCAGGCC
Rep #5 CCCCGGCAGCGCTCCTGT**A**CCTGGCCGCTGCCCAGGCC
Rep #7 CCCCGGCAGCGCTCCTGT**A**CCTGGCCGCTGCCCAGGCC

Foxo1 mt allele

5′-CCCCGGCAGCGCTCCTGT**C**CCTGGCCGCTGCCCAGGCC-3′

Foxo1 repaired allele (wt^rep)

5′-CCCCGGCAGCGCTCCTGC**A**CCTGGCCGCTGCCCAGGCC-3′

Rep #2 CCCCGGCAGCGCTCCTGC**A**CCTGGCCGCTGCCCAGGCC
Rep #3 CCCCGGCAGCGCTCCTGC**A**CCTGGCCGCTGCCCAGGCC
Rep #5 CCCCGGCAGCGCTCCTGC**A**CCTGGCCGCTGCCCAGGCC
Rep #7 CCCCGGCAGCGCTCCTGC**A**CCTGGCCGCTGCCCAGGCC

B

Supplementary Figure 7

A

B

C

D