SUPPLEMENTARY APPENDIX

Early lesions of follicular lymphoma: a genetic perspective

Emilie Mamessier,[1] Joo Y. Song,[2] Franziska C. Eberle,[2] Svetlana Pack,[2] Charlotte Drevet,[1] Bruno Chetaille,[3] Ziedulla Abdullaev,[2] José Adelaïde,[4] Daniel Birnbaum,[4] Max Chaffanet,[4] Stefania Pittaluga,[2] Sandrine Roulland,[1] Andreas Chott,[5] Elaine S. Jaffe,[2]* and Bertrand Nadel[1]*

[1]CIML, Genomic Instability and Human Hemopathies, Marseille, France; [2]National Institutes of Health, Laboratory of Pathology, Bethesda, USA; [3]IPC, Laboratory of Pathology, Marseille, France; [4]IPC, Molecular Oncology, Marseille, France; and [5]Wilhelminenspital, Institute of Pathology and Microbiology, Vienna, Austria

Current affiliation of FCE: Department of Dermatology, Eberhard Karls University, Tübingen, Germany

***ESJ and BN contributed equally to this work.**

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Correspondence: elainejaffe@nih.gov or nadel@ciml.univ-mrs.fr

Supplemental Material and Methods
Online Supplemental Revised tables 1-6 uploaded as Excel files

Samples

Most cases were selected from FFPE archived specimens submitted to the Hematopathology Section at the National Cancer Institute (NCI) for consultation, from 1999 through 2011. Seven cases of FLIS and 5 cases of PFL (all Grade 1-2), with suitable material were identified based on previously published criteria (12). Patients with FLIS and PFL had no other evidence of disease during the period of follow-up; 3 of 7 FLIS (#1, #2, and #4) were included in a previously published series (12). Five cases of DFL were obtained from the archival files of the Institute of Pathology, Medical University Vienna, Austria, collected from January 1997 to April 2008, as previously reported by Schmatz *et al* (11). The principal cohort of FLIS, PFL and DFL was compared to 5 cases of FL Grade 1-2 and 5 cases of FL Grade 3A included as controls of the most frequent alterations occurring in low grade and presumptively more advanced FL. Finally, reactive follicular hyperplasia sections (RFH, n=2) and laser micro-dissected lymphoid cells from uninvolved areas of one FLIS (FLIS background) were also hybridized and used as reference for normal (non-neoplastic) cells (**Supplemental Table 1**). Only cases with confirmed t(14; 18) were included in the study. The Institutional Review Boards of the National Cancer Institute and the Medical University of Vienna approved the study.

FISH validation at the 1p36, 7p and 12q loci, and FISH analysis of the BCL2 gene rearrangements on paraffin-embedded tissue sections

Deletion in the 1p36, amplifications in the 7p and 12q loci, and *BCL2* gene rearrangement status were assessed using Fluorescence *In Situ* Hybridization (FISH). For BCL2 interphase FISH analysis, BCL2 Split Signal FISH DNA Probe, (DAKO, Denmark, Code Y5407) was used. FISH for 1p36, 7p12, 12q 13.3-14.1 chromosomal regions was performed using Vysis probes Vysis LSI 1p36 / LSI 1q25 Dual-Color Probe covering MEGF6 and TP73 genes for Chr. 1p36 region, Vysis LSI EGFR SpectrumOrange/ CEP 7 SpectrumGreen Probe (Abbott Molecular, Chicago, IL) for Chr 7p12, and custom-made probe for Chr. 12q13.3-14.1 region using RP11-571M6 BAC clone (that includes CDK4 locus and 11 more genes) labeled with Red-5-ROX dUTP combined with the control centromeric probe for chromosomes 12 labeled with Green-5-Fluorescein (Empire Genomics, Buffalo, NY).

FISH assays were performed on 5 micron Formalin-Fixed-Paraffin-Embedded (FFPE) tumor sections using laboratory standardized protocol with slight modifications (17). In brief, 5 µm thick sections of FFPE tissue blocks were de-paraffinized, and rehydrated. Antigen retrieval was performed with IHC-Tek Epitope Retrieval Solution (IHC World, Woodstock, MD) with steaming for 25 min. After cooling, slides were subjected to 50 µkg/ml pepsin treatment at 37°C, rinsed in PBS solution followed by dehydration in ethanol series. Co-denaturation of the probe and target DNA at 73°C in HYBrite (Abbott Molecular, Chicago, IL) for 5 min was followed by overnight hybridization at 37°C. The next day, slides were washed at 72°C in 0.4X SSC/0.3% Tween-20 for 2 min and then in 2X SSC/0.1% Tween-20 at room temperature for 1 min. The slides were counterstained, mounted with DAPI/Antifade (Vector Laboratories, Burlingame, CA) and analyzed on the BioView Duet-3 fluorescent scanning station using 63X-oil objective and DAPI/FITC/Rhodamine single-band pass filters (Semrock, Rochester, NY). At least 100 tumor cell nuclei were scored for each specimen. The cutoff >10% was applied for the cells with the split Red and Green fluorescence signals to be scored as positive for *BCL2* gene rearrangement. For 1p deletion, ratio of 1p-Red/1q-Green signals <0.8 was scored as positive for a 1p36 deletion. For 7p and 12q copy number gain presence of >3 signals in more than 40% of cells, as well as ratio Red/Green signal>1.3 was scored as copy number gain, while ratio >2 was indicative of focal amplification.

Transcription profiling public dataset

Public Affymetrix U133+2 data sets from purified Resting, Naïve, GC, memory and FL B cells were retrieved from the public GEO datasets GSE12195 (http://www.ncbi.nlm.nih.gov/gds). We used Robust Multichip Average (RMA) with the non-parametric quantile algorithm as normalization parameter. RMA was applied to the raw data, and quantile normalization and Loess' correction were done in R using Bioconductor and associated packages. The probesets corresponding to the gene of interest were retrieved from the normalized data sets and the corresponding log values were linearized for graphical representation. Ontology and pathway analyses were submitted to Ingenuity and DAVID's database for annotation, visualization, and integrated discovery.

Sanger Sequencing

DNA from FLIS (n = 7), DFL (n=4), PF (n=3)L, FL (n = 6) and RFH (n=5) samples was used to analyze all exons of TNFRSF14 genes, exons 25-30 of the CREBBP gene, and Exon 15 of the EZH2 gene by PCR amplification. Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). Mutations were sequenced twice from different PCR reactions to verify the aberration. Primers pairs are listed below:

TNFRSF14 Exon 1: TGAGCTAGGTCTGGGTTGACCCGAGA/
CTCTGCTGGAGTTCATCCTG
TNFRSF14 Exon 2: CCCAGGGCTTCATCACAC / CCCAAGTGCAGTCCAGGTAG
TNFRSF14 Exon 3: CACTGTGGCCATGGAGAGAG / GGCCATTTGAGTCCCCTTAG
TNFRSF14 Exon 4: TCTCTGTCCTGGAGCAGTTC / CTCCCAGGACCTTCCTGCAA
TNFRSF14 Exon 5: TGCTGGAGAAGACGGTCAT / GGTCTAGAAGCTCACAGACA
TNFRSF14 Exon 6: GCTTTCTTCTTTTCACACATATGATTAG/
CTGGGACCTGTCTTCACTG
TNFRSF14 Exon 7: TGAGCTACCGTGGCTGTACT / GTGTATTGCAGGTGATGTAG
TNFRSF14 Exon 8: GAAAACAGGAGCCGAATTC / TGGGTTGGCTGCAGTGTG
CREBBP Exon 25: GGACACTTAAGAGCCCTGGTC/ CATTCACAGAGGTGCAGTTCC
CREBBP Exon 26: CACCTGGAAAGAGGAGCTTTG/ CAGGGTGTTGTTTGTTGCTTG
CREBBP Exon 27: CTCCAACTGTGCTGCTCTCAG/ TCCTGGCTTTAGTCCTTGCTC
CREBBP Exon 28: AGGACCTAACAGTCGACACGC/ CACACATGCATGGGACTCTG
CREBBP Exon 29: ACTTCCCTCCCACCACAGAC/GTGACCTACTTTGGCCTGAGC
CREBBP Exon 30: CAGCCACCATCAGGTACAGAC/CTCAGCCACCTGCCTATTCTG
EZH2_Exon 15: TCTCAGCAGCTTTCACGTTG/ CAGGTTATCAGTGCCTTACCTCTCC

Statistics

Groups were compared using non parametric unpaired Mann & Whitney t-test. Only p-values inferior to 0.05 were considered as significant. * : $p < 0.05$

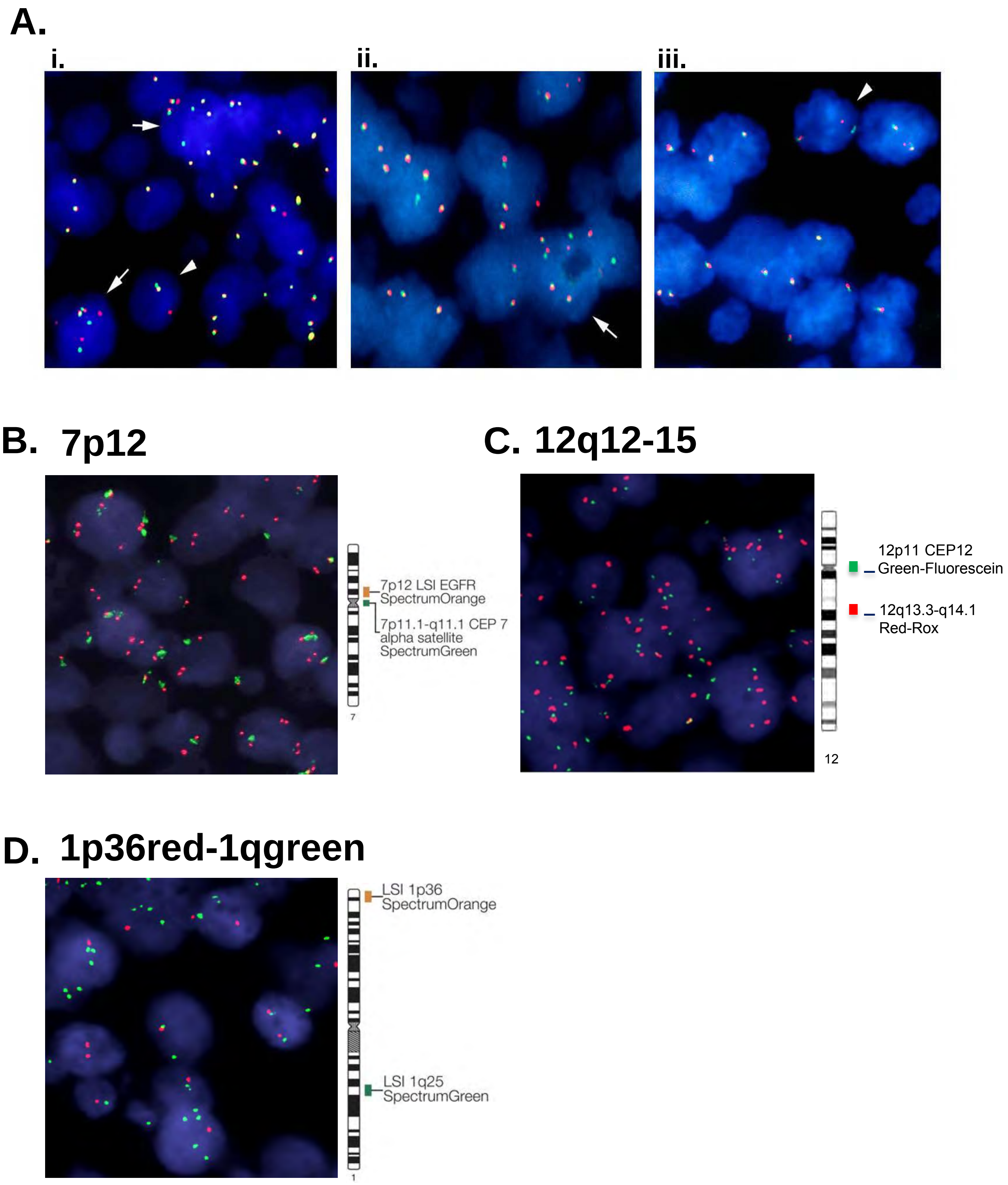


Figure S1. FISH validation for *BCL2* gene rearrangements in early FL entities with and without copy number variations.

Figure S2. FISH validation for *BCL2* gene rearrangements in early FL entities with and without copy number variations.

A. Dual-color Interphase FISH on FFPE tissue sections using *BCL2* split signal probe showing *BCL2* gene rearrangement that results in split red and green fluorescence signals. Cells with rearranged *BCL2* gene are marked by arrowheads. Cells having *BCL2* gene rearrangement and CNV are depicted by long arrows (**i, ii**) **i**. PFL #4 (left panel). Cell at lower left contains two fused signals, two red and two green split signals 2F/2R/2G, indicative of additional copies of wild –type and rearranged *BCL2.* **ii**. PFL #2 (center panel). One normal cell in center of the field contains two fused signals, 2F (normal diploid), whereas PFL cells contain 3-4 fused signals and one red, one green split signals 3-4F/1R/1G (arrow). **iii**. FLIS #4 (right panel). FLIS cells without CNV contain one normal fused signal, one red and one green split signals, 1F/1R/1G. Abbreviations: CNV, gain of *BCL2* copy number; F, fused; R, red; G, green.

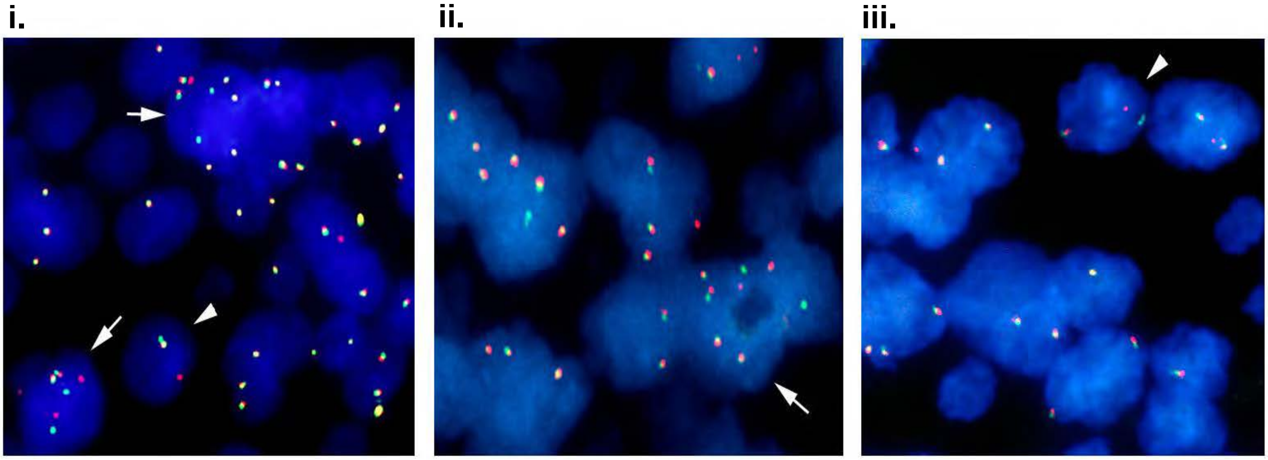
B-D.

Amplifications of the 7p14 region were confirmed on the PFL#2 sample, as well as on all available FL 1-2 (n = 1) and FL 3A (n = 4) samples displaying this alteration. A representative example of FISH result is shown in **B**.

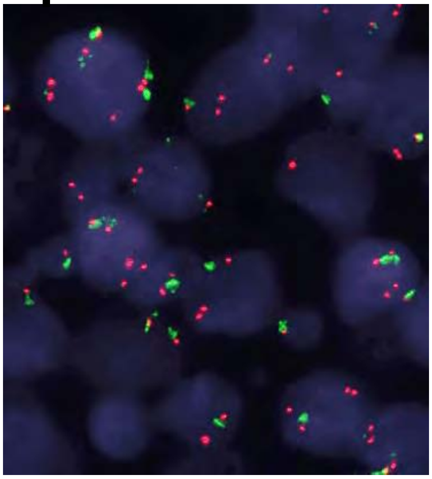
Amplifications of the 12q12-13 and 12q15 regions were confirmed on the PFL#1 sample, as well as on available FL 1-2 (n = 3) and FL 3A (n = 3) samples displaying this alteration. A representative example of FISH result is shown in **C**.

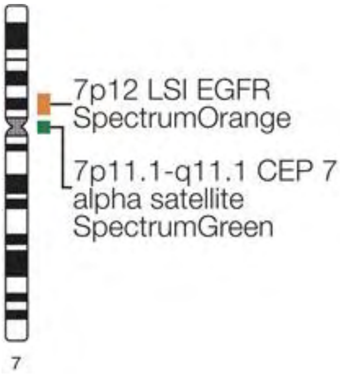
Losses of the 1p36 region were confirmed on the PFL#2 sample, as well as on available FL 3A samples displaying this alteration (n = 2) . A representative example of FISH result is shown in **D**.

A.

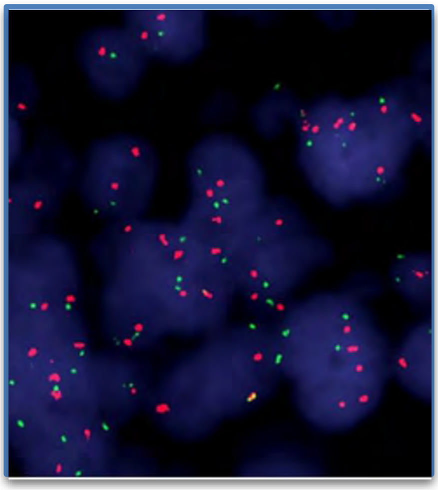


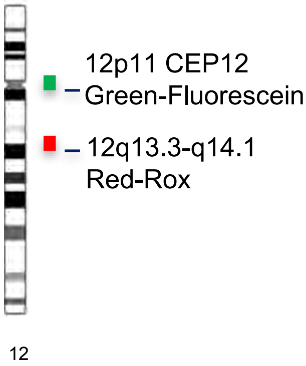
B. 7p14



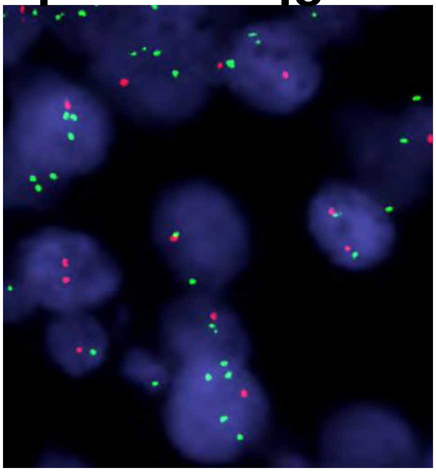


C. 12q12-15





D. 1p36red-1qgreen



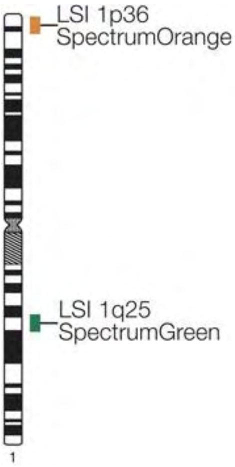


Figure S3. Mean number of gained and lost segments per sample in early-FL entities and FL samples.

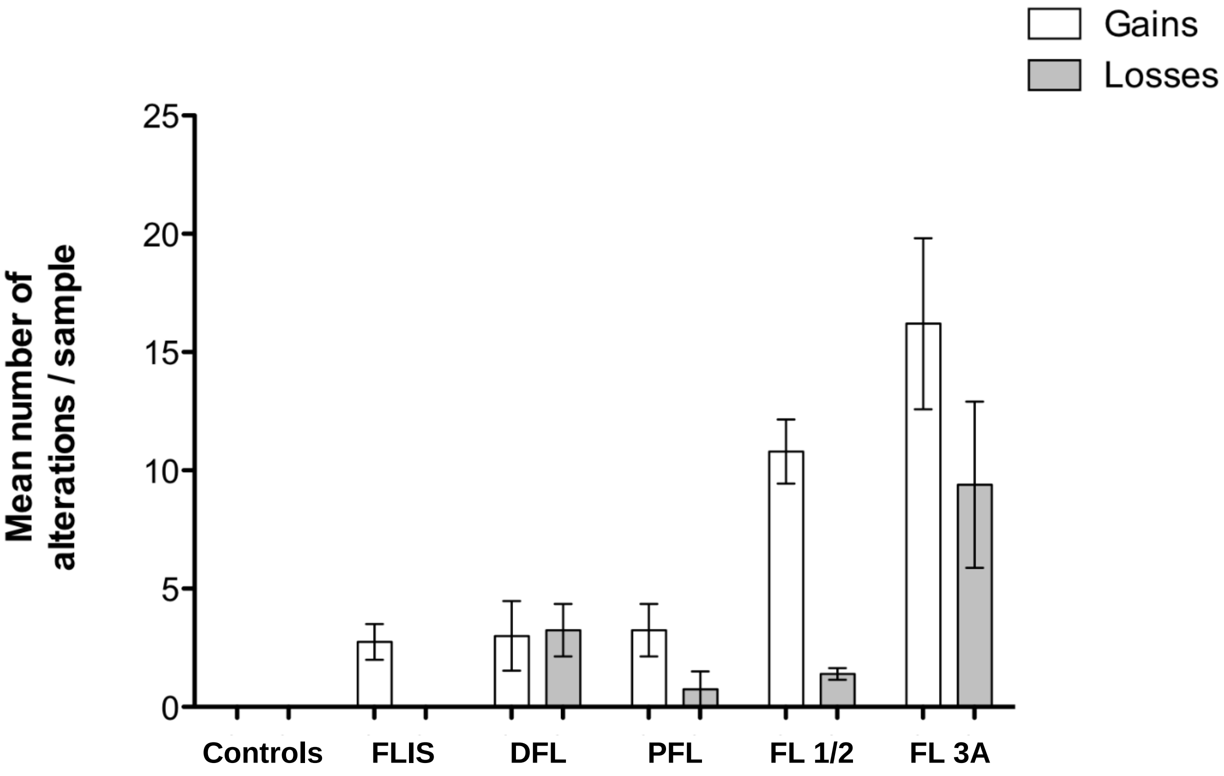


Figure S4. Selected examples of focal gains in microdissected FLIS samples (orange and pink) versus their backgrounds (grey). Some of these alterations involved GC-related genes (*AFF3, EBF1, BACH2, TOX*).

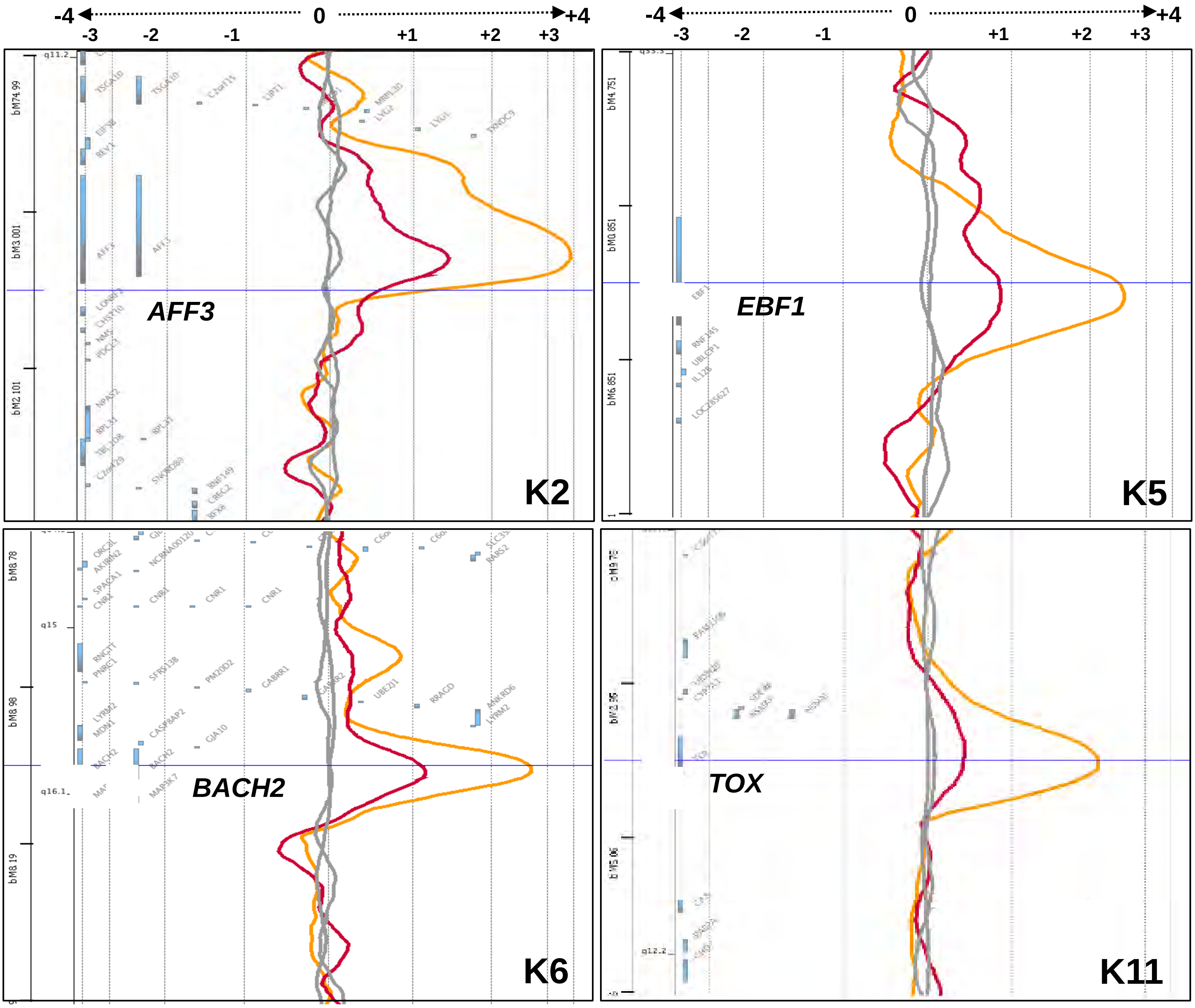


Figure S5. mRNA expression of candidates (identified by CGH) in overt FL samples. **A.** Genes found in altered regions from PFL, FLIS and FL samples. **B.** Oncogenes (previously involved in NHL lymphomagenesis) found in PFL. **C.** Oncogenes amplified in DFL samples.

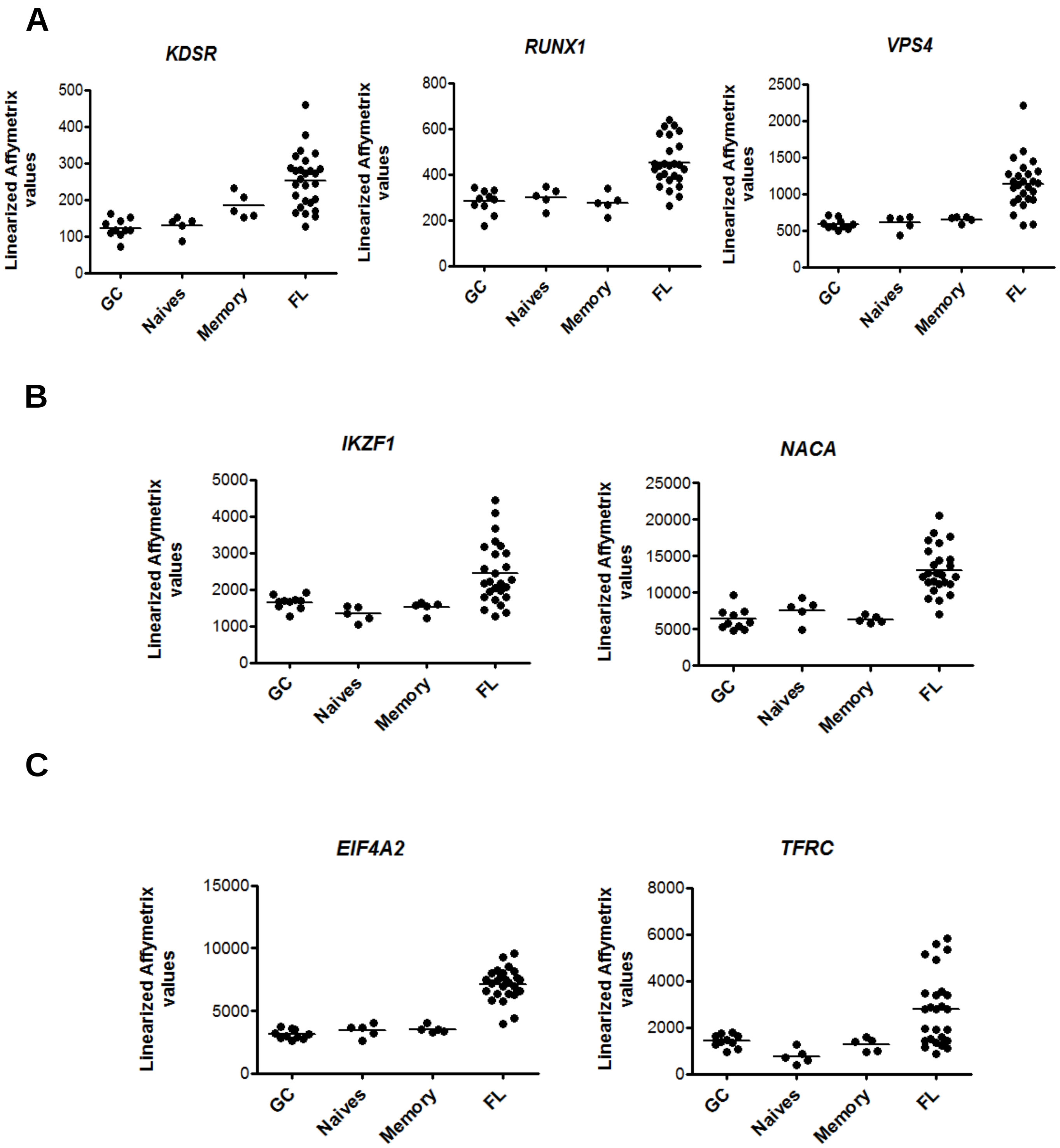


Figure S6. CGH profile of the chromosome 1, showing the loss at the 1p36 locus in PFL and DFL samples. In total, loss of the 1p36 locus are found in 17% of early-FL samples. PFL (**A**) and DFL (**B**) samples are in *colors* (Blue and purples shades respectively). Genes of interested covered in this region (TNFRSF14, TP73 and TNFRSF25) are indicated with black arrows

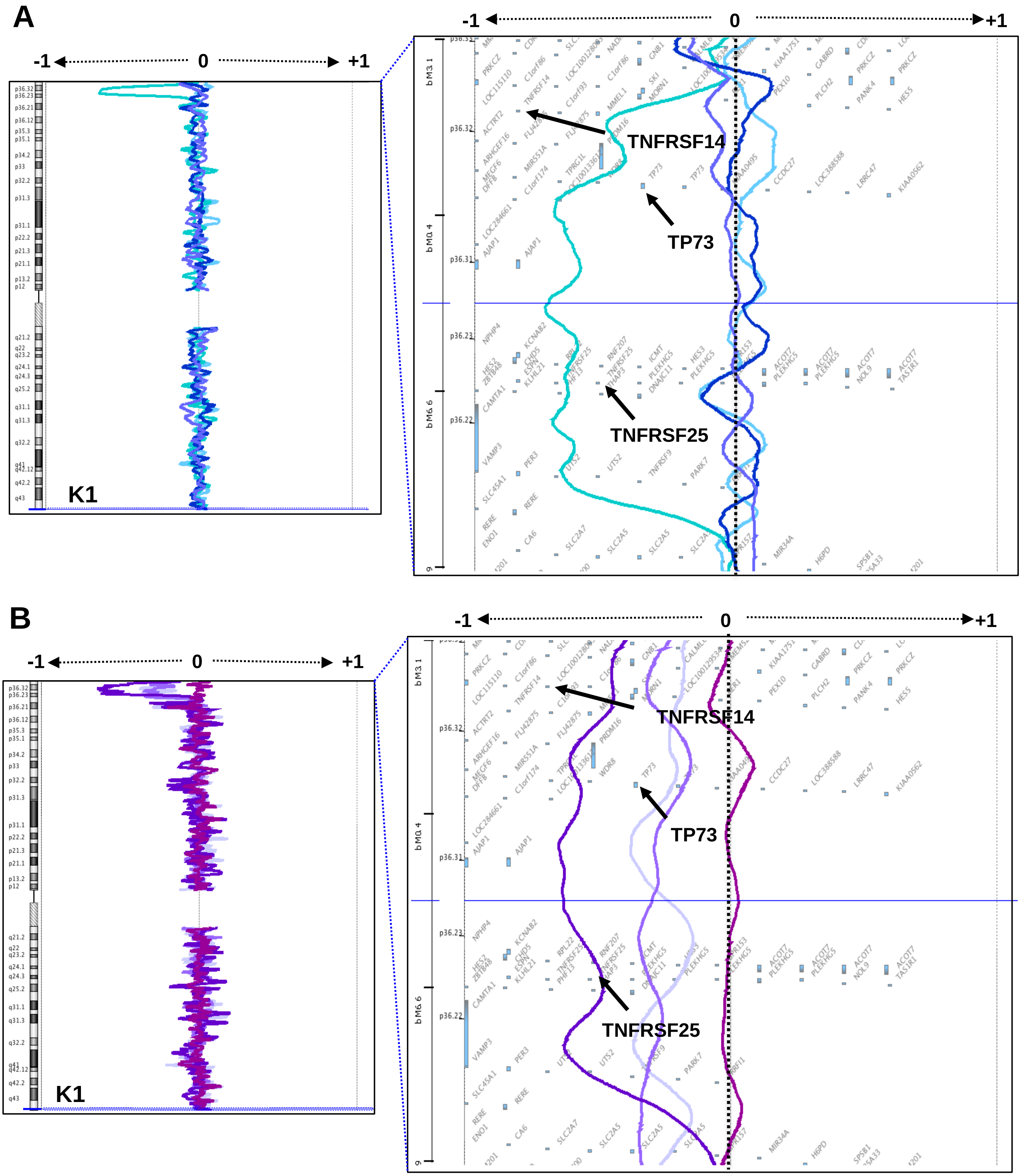


Figure S7.
Mean number of alterations between 100 and 700kb (gains and losses) in early-FL and FL

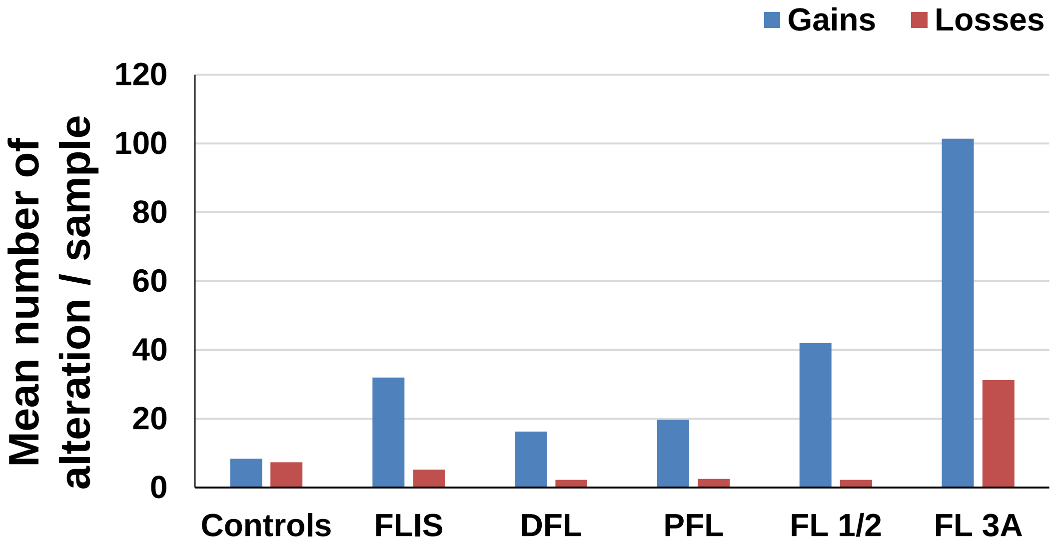
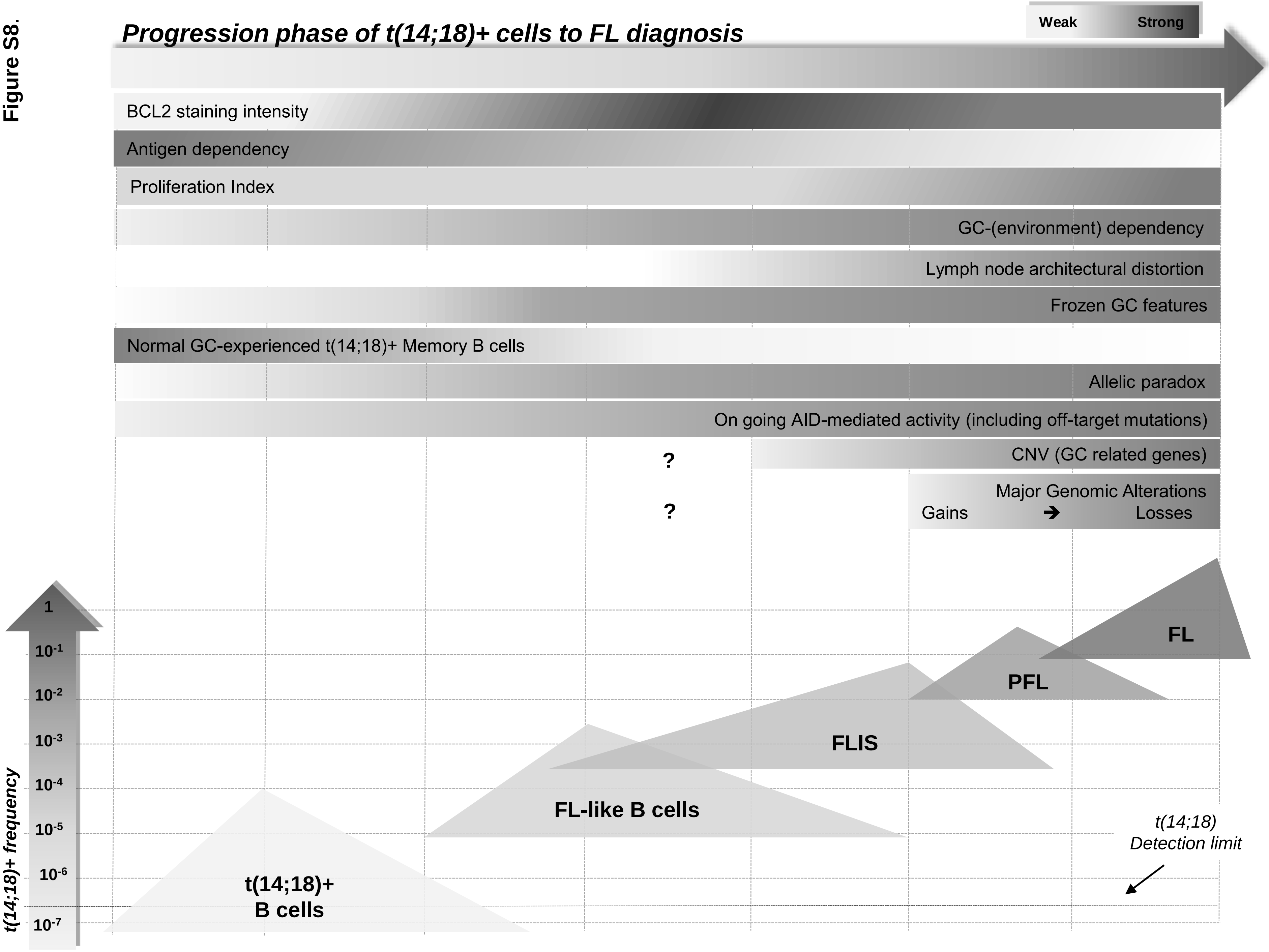


Figure S8.



Progression phase of t(14;18)+ cells to FL diagnosis
Weak
Strong
BCL2 staining intensity
Antigen dependency
Proliferation Index
GC-(environment) dependency
Lymph node architectural distortion
Frozen GC features
Normal GC-experienced t(14;18)+ Memory B cells
Allelic paradox
On going AID-mediated activity (including off-target mutations)
?
CNV (GC related genes)
?
Major Genomic Alterations
Gains ➔ Losses
t(14;18)+ frequency
1
10-1
10-2
10-3
10-4
10-5
10-6
10-7
t(14;18)+ B cells
FL-like B cells
FLIS
PFL
FL
t(14;18) Detection limit