

## Supporting Supplemental Information

**Title:** The molecular ontogeny of follicular lymphoma: gene mutations succeeding the *BCL2* translocation define common precursor cells

**Authors:** Sarah Haebe<sup>1,2\*</sup>, William Keay<sup>1,2\*</sup>, Stefan Alig<sup>1,2</sup>, Anne-Wiebe Mohr<sup>3</sup>, Larissa K. Martin<sup>3</sup>, Michael Heide<sup>1,2</sup>, Ramona Secci<sup>4,5</sup>, Stefan Krebs<sup>6</sup>, Helmut Blum<sup>6</sup>, Andreas Moosmann<sup>2,7</sup>, Abner Louissaint Jr.<sup>8</sup>, David M. Weinstock<sup>8</sup>, Silvia Thoene<sup>4,5,9,10</sup>, Michael von Bergwelt-Baildon<sup>1,2,9,10</sup>, Jürgen Ruland<sup>4,5,9,10</sup>, Deepak Bararia<sup>1,2,9,10</sup>, Oliver Weigert<sup>1,2,9,10</sup>

\*These authors contributed equally.

### Affiliations:

<sup>1</sup>Laboratory for Experimental Leukemia and Lymphoma Research (ELLF), Hospital of the Ludwig-Maximilians-University (LMU), Munich, Germany. <sup>2</sup>Department of Medicine III, Hospital of the Ludwig-Maximilians-University (LMU), Munich, Germany. <sup>3</sup>Helmholtz Center Munich, German Research Center for Environmental Health, Research Unit Gene Vectors, Munich, Germany. <sup>4</sup>Institute of Clinical Chemistry and Pathobiochemistry, Technical University of Munich, School of Medicine, Munich, Germany. <sup>5</sup>Center for Translational Cancer Research (TranslaTUM), Munich, Germany. <sup>6</sup>Laboratory for Functional Genome Analysis, Gene Center, Ludwig-Maximilians-University (LMU) of Munich, Munich, Germany. <sup>7</sup>DZIF Research Group Host Control of Viral Latency and Reactivation, DZIF - German Center for Infection Research, Munich, Germany. <sup>8</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, USA. <sup>9</sup>German Cancer Consortium (DKTK), Munich, Germany. <sup>10</sup>German Cancer Research Center (DKFZ), Heidelberg, Germany.

**Corresponding author:** Oliver Weigert, MD, [oliver.weigert@med.uni-muenchen.de](mailto:oliver.weigert@med.uni-muenchen.de)

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## **Supplemental Methods**

### **Patient information**

Three patients with follicular lymphoma (FL) with available diagnostic biopsies and autologous leukapheresis products, collected at the time of first remission, were included in this study. All patients had received frontline R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; FL1 and FL2) or CHOP (FL3) within the GLSG2000 trial (1). An additional leukapheresis product from a patient with multiple myeloma was included as a control. Written informed consent including approval of molecular analyses was obtained from all patients (LMU #056/00 and #445/13).

### **DNA isolation**

Genomic DNA from formalin-fixed paraffin-embedded (FFPE) tumor biopsies was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen). Genomic DNA from viable / sorted cells was isolated using QIAamp DNA Blood Mini Kit (Qiagen).

### **Hybrid-capture DNA target sequencing**

Genomic DNA from FFPE tumor biopsies was used for customized hybrid-capture target enrichment (SureSelect, Agilent) and Illumina sequencing, as previously described (2). Non-tumor DNA was sequenced to exclude germline polymorphisms and platform-specific artifacts. Single nucleotide variants and short insertions/deletions (indels) were called with MuTect v1.1.4 and SomaticIndelDetector (GATK), respectively. We called nonsynonymous variants (missense, frameshift, nonsense, start-codon, and splice site mutations as well as in-frame insertions and deletions) with a variant allele frequency of  $\geq 5\%$ .

### **Flow cytometry sorting**

Leukapheresis-derived mononuclear cells (LMNCs) were separated by Ficoll-Paque gradient density centrifugation (GE Healthcare) and magnetic cell separation (EasySep Human progenitor cell enrichment, Stem Cell Technologies) and sorted by flow cytometry (BD Aria II, **Fig 1C**). A fixable stain was used to discriminate live from dead cells within each sample. Pre-gated live

lineage negative (lin-) haematopoietic cells (CD45<sup>+</sup>/CD34<sup>+</sup>/CD38<sup>-</sup>) were sorted into haematopoietic stem cells (HSC; CD45<sup>+</sup>/CD34<sup>+</sup>/CD38<sup>-</sup>/CD90<sup>+</sup>/CD45RA<sup>-</sup>), multipotent progenitor cells (MPP; CD45<sup>+</sup>/CD34<sup>+</sup>/CD38<sup>-</sup>/CD90<sup>-</sup>/CD45RA<sup>-</sup>), and multilymphoid progenitor cells (MLP; CD45<sup>+</sup>/CD34<sup>+</sup>/CD38<sup>-</sup>/CD90<sup>-low</sup>/CD45RA<sup>+</sup>), as previously described (3). T cells (CD45<sup>+</sup>/CD3<sup>+</sup>) were sorted from lineage positive cells.

Antibodies for flow cytometry:

Marker	Fluorochrome	Vendor / Clone
CD45	PE	BD H130
CD45RA	FITC	BD H100
CD34	APC-Cy7	Thermo Fisher 581
CD38	PE-Cy7	Biolegend HB-7
CD90	Qdot 605	Biolegend OX-7
CD10	APC	Thermo Fisher eBioCB-CALLA (CB-CALLA)
CD19	FITC	BD HIB19
CD3	APC	BD HIT3a
CD15	PE-Cy7	Biolegend W6D3
DAPI		Thermo Fisher Scientific

### Highly-sensitive mutation detection by amplicon deep sequencing

We performed highly-sensitive mutation detection in HSPC fractions by amplicon-deep sequencing as previously described (4). We analyzed the sequencing data (**Table S1**) by calculating the mutation rate for each candidate gene in each fraction. The mutation rate was defined as the ratio of all mutant reads to all non-reference reads. Amplicons with < 10,000 total reads were excluded from further analysis to ensure sufficient sensitivity. To correct for background signals, we calculated mutation rate ratios by dividing the mutation rate in each fraction by the mutation rate in control samples, *i.e.* in LMNC-derived T cells and in a peripheral blood sample from a healthy individual (normal control), respectively. Spiked-in tumor DNA from

FL3 initial (1:1,000) served as positive control. We used the Fisher's exact test and calculated Bonferroni-adjusted p-values to determine statistical significance.

### **Colony forming assay**

For methylcellulose colony forming units (CFUs), an aliquot of LMNCs was thawed in RPMI 1640 medium (Thermo Fisher Scientific) and washed twice in Hanks' balanced salt solution (Sigma-Aldrich) containing 2% fetal bovine serum. Following incubation with DNase 1 (Stem Cell Technologies) and magnetic cell separation (EasySep Human CD34 Positive Selection Kit, EasySep Human Progenitor Cell Enrichment Kit, Stem Cell Technologies), CD34<sup>+</sup> and lin<sup>-</sup> cells (20,000-100,000 per plate) were separately plated onto cytokine-supplemented methylcellulose-medium (MethoCult H4035 Optimum Without EPO, Stem Cell Technologies) and cultured for 14 days at 37°C with 5% CO<sub>2</sub>. In this assay, HSPCs with self-renewal capacity give rise to CFUs, which consist of at least 20-40 genetically identical cells. We used MethoCult™ H4035 Optimum Without EPO, although it is formulated to support primarily growth of granulocyte-macrophage progenitor cells (CFU-GM, CFU-G and CFU-M), as CFU assays for pre-B lymphoid progenitor are only available for murine cells. LMNCs from a patient with multiple myeloma were used as control. Plates were then either analysed individually or pooled (1-4 plates per pool, **Table S2**). In total, 5 pools of CD34<sup>+</sup> and 5 pools of lin<sup>-</sup> hematopoietic stem and progenitor cells (HSPC)-derived CFUs were tested for each patient.

### **PCR and Sanger sequencing**

PCR primers were designed with Primer3 (<https://bioinfo.ut.ee/primer3-0.4.0/primer3/>). PCR (KOD Xtreme Hot Start DNA Polymerase, Novagen) for *BCL2/IGH* was performed using a two-step PCR approach utilizing specific primers for the *BCL2/IGH* major breakpoint region (MBR) and the *BCL2/IGH* minor cluster region (mcr). Standard curves for the MBR and mcr were generated using serial dilutions of tumor DNA from FL1 (MBR) and FL3 (mcr). Sanger sequencing was performed at GATC Services (Eurofins Genomics).

Primer sequences for *BCL2/IGH*:

Primer / Probe	Forward / Reverse	Remark
<b>Nested PCR</b>		
<i>BCL2/IGH</i> MBR	CAGCCTTGAAACATTGATGG / ACCTGAGGAGACGGTGACC TCTATGGTGGTTTGACCTTTAG / ACCAGGGTCCCTTGGCCCCA	First amplification Second amplification
<i>BCL2/IGH</i> mcr	CGTGCTGGTACCACTCCTG / ACCTGAGGAGACGGTGACC CCTGGCTTCCTCCCTCTG / ACCAGGGTCCCTTGGCCCCA	First amplification Second amplification

MBR, major breakpoint region; mcr, minor cluster region.

PCR protocol for *BCL2/IGH* major breakpoint region (MBR):

#### 1<sup>st</sup> PCR

	Temperature / Time (min)
Initial denaturation	94°C / 3
27 Cycles	94°C / 1
	55°C / 1
	72°C / 1
Final extension	72°C / 10

#### 2<sup>nd</sup> PCR

	Temperature / Time (min)
Initial denaturation	94°C / 3
30 Cycles	94°C / 1
	58°C / 1
	72°C / 1
Final extension	72°C / 10

PCR protocol for *BCL2/IGH* minor cluster region (mcr):

#### 1<sup>st</sup> PCR

	Temperature / Time (min)
1 Cycle	94°C / 3
	58°C / 1
	72°C / 1
30 Cycles	94°C / 1
	58°C / 1
	72°C / 30 sec
	72°C / 10

#### 2<sup>nd</sup> PCR

	Temperature / Time (min)
1 Cycle	94°C / 3
	60°C / 1
	72°C / 1
30 Cycles	94°C / 1
	60°C / 1
	72°C / 30 sec
	72°C / 10

### Real-time quantitative PCR (RT-qPCR)

RT-qPCR (TaqMan; Applied Biosystems) for *BCL2/IGH* MBR and mcr as well as *GAPDH* was performed on CD34<sup>+</sup>- and lin<sup>-</sup> HSPC-derived CFUs from FL1-FL3. 500 ng of CFUs were tested in duplicates for each biological replicate. Translocation-specific probes were utilized that hybridize to the *BCL2/IGH* MBR or mcr. Standard curves were generated using serial dilutions of spiked-in tumor DNA from OCI-Ly1 (MBR) or from the cloned *BCL2/IGH* from FL3 (mcr) and analysed according to published guidelines (5).

Primer sequences for *BCL2/IGH*:

Primer / Probe	Forward / Reverse
<b>RT-qPCR</b>	
<i>BCL2</i> MBR forward / JH consensus	CTATGGTGGTTTGACCTTTAGAG / CCTGAGGAGACGGTGACC
Probe for <i>BCL2/IGH</i> MBR	FAM-CTGTTTCAACACAGACCCACCCAGAG-TAMRA
<i>BCL2 mcr</i> forward/ JH consensus	CATTGAGTTATTTGTCTTTTGATTAACCTG / CCTGAGGAGACGGTGACC
Probe for <i>BCL2/IGH mcr</i>	FAM-AGAGCTCTTTGTATATTCAGGAAATTAGCACTTTGG- TAMRA
<i>GAPDH</i>	CAAAGCTGGTGTGGGAGG / CTCCTGGAAGATGGTGATGG
Probe <i>GAPDH</i>	JOE- CAAGCTTCCCGTTCTCAGCC-BHQ-1

MBR, major breakpoint region; *mcr*, minor cluster region.

PCR protocol for *BCL2/IGH* for MBR and *mcr*:

	Temperature / Time (min)
Initial denaturation	50°C / 2 95°C / 10
45 Cycles	95°C / 15 sec 59°C / 1

### Unique molecular identifier (UMI)-aided deep next generation sequencing

For each mutation, we assayed the equivalent of 10,000 cells (500 ng genomic DNA) in quintuplicate using a UMI-aided deep next sequencing approach. Briefly, a 1<sup>st</sup> PCR was used to amplify ( $\leq 5$  cycles; Phusion HF, NEB) the region of interest and to introduce a 15-mer UMI barcode, followed by a cleanup (exonuclease I treatment, NEB). A 2<sup>nd</sup> PCR (35 cycles) adds index primers to each amplicon (single index primers for Illumina (NEB)). PCR products were then run on a 2% agarose gel in 0.5% Tris Borate EDTA (TBE, Thermo Fisher Scientific) for 1h. The corresponding band for the successfully amplified and tagged region was excised, and the DNA extracted (QIAquick gel extraction Kit, Qiagen). Amplicon sequencing was performed on an Illumina Hi-Seq 2500 with 5 million paired-end 100bp reads per sample. Each amplicon was covered with a read depth of approximately 150,000 to 700,000 reads. Standard curves were generated using pooled results from serial dilutions of spiked-in tumor DNA from FL1 and FL3 for five different amplicon assays. After deduplication and consensus sequence calling, single nucleotide variants (SNV) and short insertions/deletions (indels) were called with MuTect v1.1.4 and SomaticIndelDetector (GATK), respectively.

Primer sequences for UMI-aided next generation sequencing:

Patient no.	Primer	Forward / Reverse
FL1	<i>KMT2D</i> K2548_fs	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNAGACCAGGCTGAGGGACA / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNAGGCAGTAGGGGAGCCTTC
	<i>KMT2D</i> T4787_fs	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNGGTACCCTAGGACACACCTTG / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNAAAAGGGCAAAGGAAGTGAGG
	<i>CREBBP</i> D1435V	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNACGTGGCCGGAAGAAATG / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNCCTGAGTTAAACATGTGCCTCCTT
	<i>SMARCA4</i> G883D	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNCGTTGGAAGTACATGATTGTGG / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNGCACCTGCGTCAGCTTG
	<i>ARID1A</i> C1968*	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNCCCCACAGTAAGGATGAGACC / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNCGAATGGTATTGGACACACAGA
	<i>EZH2</i> Y641S	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNCAGGTTATCAGTGCCTTACCTCTC / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNCTATTGCTGGCACCATCTGAC
	<i>TP53</i> T284P	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNGGTGAGGCTCCCTTTTCTT / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNGCTTTGAGGTGCGTGTTTGT
	<i>TNFAIP3</i> C607*	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNCTGCCTGTCTCAAGCTGCAC / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNTGTGCAAAGCCCTTGTTTTTC
	<i>PTEN</i> V85_ss	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNTTGTGCTGAAAGACATTATGACAC / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCAGTAAGATACAGTCTATCGGGTTT
FL2	<i>KMT2D</i> Q1377R	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNTTCCCATCTATCCTCTCACCAAA / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNCCGTGGTTCTCTTCTCCAACA
	<i>EP300</i> H1451Y	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNCAGGGCATATTTGGGCATGT / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCATTCTGCAGTCGCTTGG
	<i>STAT6</i> D419G	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNTCCCACAGGATAGTGGCTTTG / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNACCCCTGCTCACCCTCTT
FL3	<i>KMT2D</i> N5447_ss	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNTCAACGTAGCATCAATCACATGTTT / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNACAGCCACCACTGCCACTCT
	<i>CREBBP</i> P1476L	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNGTGTTTTGGCTTGGGTATTTT / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNGTGCGTCTGCAGGTATGTG
	<i>FAS</i> K274I	TCCCTACACGACGCTCTTCCGATCTNNNNNNNNAGGCTTTGTTTCAAAGAATGGTG / GTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCCATGAAGTTGATGCCAATTACG

PCR Protocol for UMI-aided next generation sequencing:

#### 1<sup>st</sup> PCR

	Temperature / Time (sec)
Initial denaturation	98°C / 30
5 Cycles	98°C / 10
	56°C / 40
	72°C / 5
	72°C / 5 min

#### 2<sup>nd</sup> PCR

	Temperature / Time (sec)
35 Cycles	94°C / 10
	60°C / 40
	72°C / 15
Final extension	72°C / 5 min



### Digital droplet PCR (ddPCR)

ddPCR assays for somatic alterations in FL3 were performed on a QX200 ddPCR system with automatic droplet generation (Bio-Rad Laboratories). Reactions were carried out in ddPCR 96-well plates (Bio-Rad Laboratories) and were performed in duplicate in a reaction volume of 21 µl using the ddPCR Supermix for probes (no UTP, Bio-Rad Laboratories) and with a template input of 10 ng (tumor specimens, LMNC-sorted populations) or 100 ng DNA (PBMCs, *ex vivo* expanded B cells) per reaction. The primers and probes were synthesized by Integrated DNA Technologies. Positive controls (gBlocks), nuclease-free water and negative controls (genomic DNA either derived from FFPE lymph node samples or PBMCs of healthy subjects) were included in every run. Data were analysed using QuantaSoft software (Bio-Rad) according to the digital MIQE guidelines (6).

Primer and probes sequences for ddPCR:

Patient no.	Primer	Forward / Reverse	Probe WT 5' HEX / 3' IBFQ Probe MUT 5' FAM / 3' IBFQ
FL3	<i>KMT2D</i> Q4200_fs	CGCAGCTGTGGGTTTTTG / GTTGGAATCATGCCTACGGT	AGCTGTG+C+TCG+AAG / AGCTGTG+T+GCTCGA
	<i>KMT2D</i> N5447_ss	CGGTCAACGTAGCATCAATC / ACTCTTGTCCTTCTTCTGC	T+G+CCTTTCTA+GAA+TCG / T+CT+A+A+AATC+GA+GGC
	<i>CREBBP</i> P1476L	GGGTGGCAATGGAAGATGTA / GGTCCTGCAGGTATGTGAC	CA+CT+T+G+GAGGAC / CA+CT+T+A+GAGGACAGG
	<i>FAS</i> K274I	TGAAGCCAAAATAGATGAGATCAA / TCAATGTGTCATACGCTTCTTT	A+GAA+CAG+A+A+AGTT+CA / A+GAA+CAG+A+T+AGTT+CA

### Clinical course of patient 3

The 60-year-old woman was diagnosed with symptomatic advanced stage FL grade 1 in 2003. She received frontline chemotherapy with CHOP and autologous stem cell transplantation within the GLSG2000 trial (1). She relapsed in 2007 and was treated with R-DHAP (rituximab, dexamethasone, cytarabine and cisplatin), which resulted in a partial remission. Due to progressive disease during R-maintenance in 2008, the patient received two courses of R-FCM (rituximab, fludarabine, cyclophosphamide, and mitoxantrone). She achieved a partial remission and received a reduced-intensity conditioning regimen followed by transplantation of unmanipulated bone marrow from an HLA-matched unrelated male donor. Graft-versus-host

prophylaxis consisted of cyclosporine and mycophenolate mofetil. After a follow-up of more than 10 years, the patient is in ongoing clinical remission.

### ***Ex vivo* B cell expansion**

*Ex vivo* expansion of B cells from peripheral blood, collected in 2017, were generated by co-culture of PBMCs with murine fibroblastic L929 cells stably expressing human CD40 ligand (7) as previously described (8). Briefly, after seeding 96-well plates with irradiated (180 Gy) feeder cells, PBMCs were plated on top (2.5, 5, 10 or  $20 \times 10^4$  cells per well), in 200  $\mu$ l RPMI 1640 medium containing 10% fetal calf serum (PAN Biotech), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and sodium selenite (100 nM; Thermo Fisher Scientific), as well as recombinant human interleukin 4 (2 ng/ml, R&D Systems) and cyclosporin A (1  $\mu$ g/ml, Novartis). B cells were harvested and plated on top of freshly irradiated feeder cells every week. Cyclosporin A was omitted after 4 weeks.

## Supplemental Tables

**Supplemental Table 1. Summary of somatic mutations identified by hybrid-capture DNA target sequencing and subjected to highly-sensitive sequencing.**

**Supplemental Table 2. Summary of CFU-derived HSPC sequencing.**

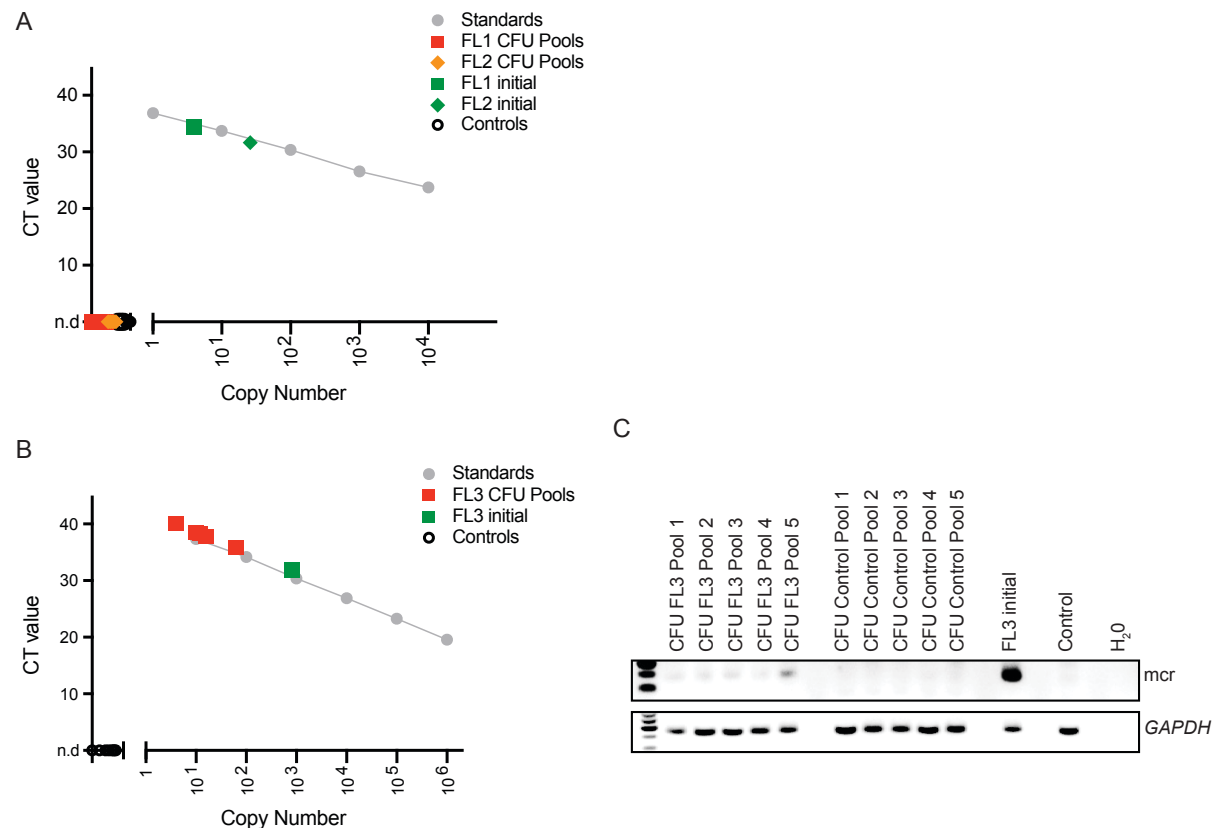
	CD34 <sup>+</sup> enriched CFUs						Lineage negative CFUs					
Patient no.	No. of pools (biological replicates)	No. of plates / pool	No. of CFUs / pool (median, min.-max.)	<i>BCL2/IGH</i> detection (by qPCR / nested PCR)	gene mutations (tested* / identified**)	UMI-aided sequencing	No. of pools (biological replicates)	No. of plates / pool	No. of CFUs / pool (median, min.-max.)	<i>BCL2/IGH</i> detection (by qPCR / nested PCR)	gene mutations (tested* / identified**)	UMI-aided sequencing
FL1	5	3	1,519 (1,420 -1,670)	negative (MBR)	9/12	n.d.	5	4	2,488 (2,049 -3,115)	negative (MBR)	9/12	n.d.
FL2	5	1	411 (344-445)	negative (MBR)	3/3	n.d.	5	2	1,297 (1,168 -1,403)	negative (MBR)	3/3	n.d.
FL3	5	n.a.	n.a.	positive (mcr)	n.a.	n.a.	5	2	644 (566 -779)	negative (mcr)	3/4	n.d.
Control	5	4	1,315 (1,259-1,411)	negative (MBR/mcr)	2/n.a.	n.d.	5	2	749 (618-774)	negative (MBR/mcr)	6/n.a.	n.d.

CFU, colony forming units; HSPC, hematopoietic stem and progenitor cells; MBR, *BCL2/IGH* major breakpoint region; mcr, *BCL2/IGH* minor cluster region; n.a., not applicable; n.d., not detectable; UMI, unique molecular identifier; \*number of mutations tested; \*\* number mutations identified in the initial tumour.

## Supplemental Figures

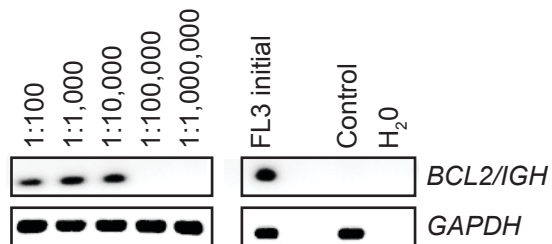
### Supplemental Figure 1. Detection of *BCL2/IGH* MBR and *mcr* in LMNC-derived CD34<sup>+</sup> CFUs.

**(A)** RT-qPCR for *BCL2/IGH* MBR. Standard curves were generated using serial dilutions of spiked-in DNA from OCI-Ly1 (grey). Genomic DNA from LMNC-derived CFUs from patient FL1 and FL2 was tested in replicates. Genomic DNA from PBMCs from a healthy donor (Controls) was used as negative controls, initial tumour DNA from FL1 and FL2 served as positive controls. **(B)** RT-qPCR for patient-specific *BCL2/IGH* *mcr*. Standard curves were generated using the *BCL2/IGH* translocation cloned from FL3 (grey). Genomic DNA from LMNC-derived CD34<sup>+</sup> CFUs (n=5) was tested in duplicates and results were plotted against the standard curve. Genomic DNA from PBMCs from a healthy donor (Controls) was used as negative controls, initial tumour DNA from FL3 served as positive control. **(C)** RT-qPCR products of the LMNC-derived CD34<sup>+</sup> CFUs. Genomic DNA from LMNC-derived CFUs from a patient with multiple myeloma (CFU Control Pools 1-5) and from PBMCs from a healthy donor (Control) were used as negative controls, FL3 initial served as a positive control. PCR for *GAPDH* was used as a loading control.



**Supplemental Figure 2. Sensitivity of nested PCR assay for BCL2/IGH mcr.**

Serial dilutions of spiked-in tumor DNA from FL3 initial for the patient-specific BCL2/IGH mcr. Genomic DNA from PBMCs from a healthy donor (Control) was used as negative control. PCR for GAPDH was used as a loading control.



## Supplemental References

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