Core regions in immunoglobulin heavy chain enhancers essential for survival of non-Hodgkin lymphoma cells are identified by a CRISPR interference screen

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Received: November 16, 2023.
Accepted: June 19, 2024.
Early view: June 27, 2024.

https://doi.org/10.3324/haematol.2023.284672

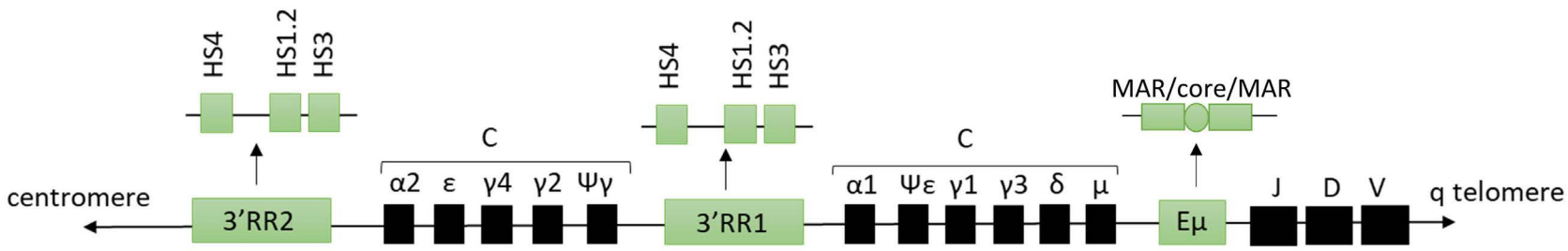
Abstract

Chromosomal translocations in non-Hodgkin lymphoma (NHL) result in activation of oncogenes by placing them under the regulation of immunoglobulin heavy chain (*IGH*) super-enhancers. Aberrant expression of translocated oncogenes induced by enhancer activity can contribute to lymphomagenesis. The role of the *IGH* enhancers in normal B-cell development is well established, but knowledge regarding the precise mechanisms of their involvement in control of the translocated oncogenes is limited. The goal of this project was to define the critical regions in the *IGH* regulatory elements and identify enhancer RNA (eRNA). We designed a single guide RNA library densely covering the *IGH* enhancers and performed tiling CRISPR interference screens in three NHL cell lines. This revealed three regions crucial for NHL cell growth. With chromatin-enriched RNA sequencing we showed transcription from the core enhancer regions and subsequently validated expression of the eRNA in a panel of NHL cell lines and tissue samples. Inhibition of the essential *IGH* enhancer regions decreased expression of eRNA and translocated oncogenes in several NHL cell lines. The observed expression and growth patterns were consistent with the breakpoints in the *IGH* locus. Moreover, targeting the Eμ enhancer resulted in loss of B-cell receptor expression. In a Burkitt lymphoma cell line, MYC overexpression partially rescued the phenotype induced by *IGH* enhancer inhibition. Our results indicated the most critical regions in the *IGH* enhancers and provided new insights into the current understanding of the role of *IGH* enhancers in B-cell NHL. As such, this study forms a basis for development of potential therapeutic approaches.

Introduction

Non-Hodgkin lymphomas (NHL) account for 3% of all cancer cases worldwide.[1,2] They arise from B cells at various stages of maturation, which is a multistep process involving several rearrangements occurring at the immunoglobulin heavy chain (*IGH*) locus. Obligatory intermediates during rearrangement of the *IGH* locus are DNA double-strand breaks. These breaks can result in illegitimate recombination and various chromosomal translocations including the t(8;14)(q24;q32) *MYC/IGH* in Burkitt lymphoma (BL) and t(14;18)(q32;q21) *IGH/BCL2* in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma.[3] As a result, the translocated oncogene is placed under the control of *IGH* enhancers, which leads to its overexpression. MYC is a transcription factor involved in many processes such as proliferation, apoptosis, and DNA-damage response.[4] BCL2 suppresses cell death by preventing the activation of caspases which contributes to treatment resistance and poor prognosis.[5] Interestingly, oncogenic translocation itself may not be sufficient to drive lymphomagenesis,[6] yet can contribute to instability that can in turn lead to accumulation of other mutations and malignant transformation.[7] The activity of the *IGH* locus is governed by enhancers: Eμ (the intronic enhancer) and 3' regulatory regions (3'RR1 and 3'RR2) (Figure 1A).[8] Among others, a feature of active enhancers is expression of enhancer RNA (eRNA). This class of non-coding RNA was regarded as a by-product of an active transcription machinery, but increasing evidence

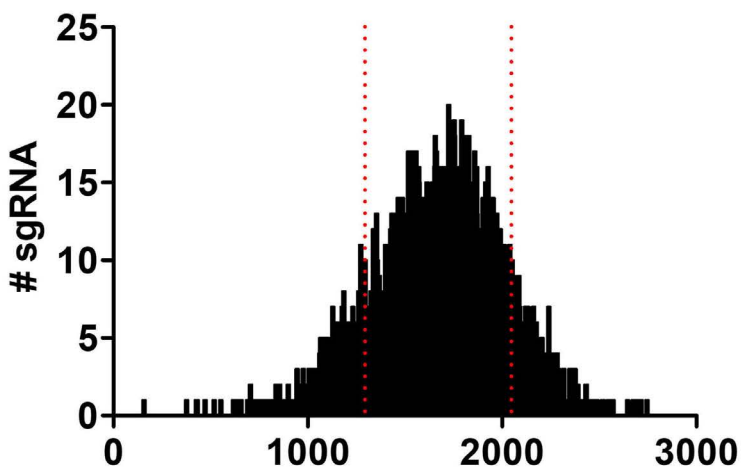
A



B

Region	Localization (hg19)	Number of sgRNA	Mean distance	Median distance	Min distance	Max distance
Eμ	chr14:106322800-106331124	2,219	8.1	4	1	302
3'RR1	chr14:106145100-106176250	3,784	10.7	4	1	1,850
3'RR2	chr14:106024800-106050011	3,322	10.6	4	1	1,285

C



MiSeq analysis	
% sgRNA with perfect match	88.6
% undetected sgRNA	0.0
Skew ratio	1.58

D

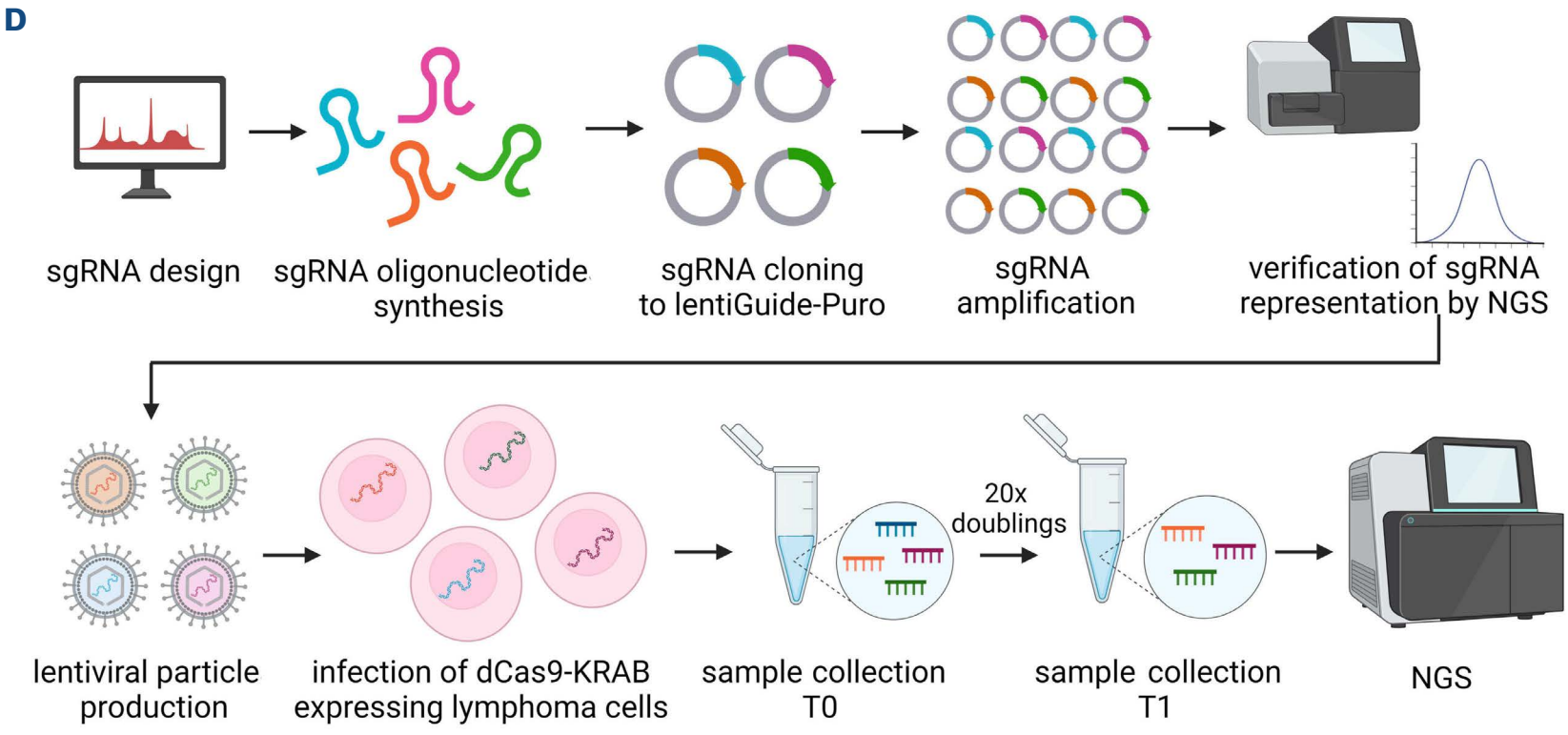


Figure 1. Design and generation of a tiling CRISPR interference library targeting *IGH* enhancers. (A) Scheme of the human immunoglobulin heavy chain (*IGH*) locus. Green boxes represent the *IGH* enhancers: two 3' regulatory regions (3'RR), which are composed of smaller enhancers (HS), and the intronic enhancer Eμ, composed of the core and two matrix attachment regions (MAR). Black boxes represent immunoglobulin genes: C – constant, J – joining, D – diverse, V – variable. (B) Summary of the single guide (sg)RNA distribution in the CRISPR-eIGH library. Note that since 3'RR1 and 3'RR2 are highly similar, 2,344 sgRNA are common for both regions. (C) sgRNA abundance in the CRISPR-eIGH library obtained with next-generation sequencing (NGS). Red dotted lines indicate the 10th (left) and 90th (right) percentiles. (D) Overview of the CRISPR interference screen experiment.

shows that eRNA can be functional.[9] Active transcription of 3'RR in activated B cells was first demonstrated by Peron *et al.*[10] Recently, the eRNA ARIEL was found to be a driver of oncogenesis in T-cell acute lymphoblastic leukemia.[11] In BL, the eRNA AL928768.3, expressed from 3'RR1, was shown to downregulate MYC expression upon knockdown.[12] Nevertheless, knowledge regarding the role of eRNA in NHL remains limited.

The function of *IGH* enhancers has been extensively studied in normal B cells. These studies showed that Eμ is important for earlier stages of B-cell development, mainly VDJ recombination,[13] while 3'RR takes over the locus control at later stages, namely somatic hypermutation and class-switch recombination (CSR).[14] Several mouse models demonstrated the involvement of *IGH* enhancers in the regulation of oncogene expression and lymphomagenesis, but still more research is necessary to understand these processes in malignant human B cells, where the *IGH* enhancer organization differs from that in mice.[15] As *IGH* enhancers encompass a region of approximately 64 kb in total, it is important to pinpoint the core regions crucial for lymphoma cells.

The CRISPR/Cas9 system can be applied to study tissue-specific *cis*-regulatory elements of enhancers.[16] Here, we employed a tiling CRISPR/dCas9-KRAB screen to pinpoint the critical regions within Eμ and 3'RR in BL and DLBCL cell lines. RNA sequencing of chromatin-enriched RNA revealed transcriptional activity of *IGH* enhancers, which we also confirmed in primary patients' samples. Further validation in several NHL cell lines revealed various patterns of dependency on *IGH* enhancers reflecting the differences in breakpoint location. The underlying molecular mechanisms involved disturbed expression of translocated oncogenes and in some cases also B-cell receptor (BCR) presentation.

Methods

Cell lines

The BL cell lines, BL41, CA46, DG75 (from DSMZ, Braunschweig, Germany), ST486 (from ATCC, LGC Standards, Lomianki, Poland), BL2, BL60, JI, LY19 (gifts from Prof. Reiner Siebert, Ulm University, Germany), and the DLBCL cell lines, SUDHL4, WSU-DLCL2 (from DSMZ) and P493-6[17] (a gift from Prof. D. Eick, Helmholtz Center, Munich, Germany) were grown in RPMI 1640 (Lonza, Basel, Switzerland) and the HEK293T cell line (from DSMZ) was grown in Dulbecco modified Eagle medium (Lonza), supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin (Biowest, Nuaillé, France) and 10-20% fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, USA), under standard conditions (37°C, 5% CO_2) in a humidified incubator. Cells were regularly tested for *Mycoplasma* contamination by polymerase chain reaction.

Patients' samples

Enhancer RNA expression was validated in patient-derived NHL samples: BL (8 cases, all *MYC* translocation positive) and DLBCL (14 cases: 6 germinal center B-cell [GCB], 7 activated B-cell [ABC] and 1 unclassified) as well as tonsil tissues obtained during tonsillectomy as controls (6 cases). Tissues were obtained from the pathology files of the University Medical Center Groningen tissue bank. They were used in accordance with the Declaration of Helsinki and the protocol was approved by the Medical Ethical Review board of the University Medical Center Groningen (RR#201800554).

Design and generation of the CRISPR-eIGH library

IGH enhancer regions were defined by the H3K27ac plus 5 kb flanking sequences (hg19): Eμ (chr14:106317800-106336124), 3'RR1 (chr14:106140100-106181250) and 3'RR2 (chr14:106019800-106055011). A tiling library of single guide RNA (sgRNA) covering those regions was designed and cloned into the lentiGuide-Puro vector.[18]

CRISPR screen

Cells ($27.5x10^6$) stably expressing dCas9-KRAB were transduced in duplicate with the CRISPR-eIGH library, with the previously established amount of virus. After 4 days of selection with puromycin (T0), $8x10^6$ cells were collected for DNA isolation. Remaining cells were further cultured for 20 population doublings. At each passage, the number of cells corresponding to 1,000x coverage of the library, that is $8x10^6$, were cultured in RPMI medium with 1 μg/mL (DG75) or 0.3 μg/mL (other cell lines) puromycin and collected at the final timepoint (T1).

Chromatin-enriched RNA-sequencing and data analysis

RNA from the chromatin fraction was sent for RNA-sequencing stranded library preparation, pair-end sequencing and bioinformatic data analysis to Novogene (Beijing, China). Sequencing was performed on an Illumina X-Ten platform. Quality control of raw data in FASTQ format was performed; clean data were obtained by removing reads containing adapter and poly-N sequences and reads with low quality. Approximately 77-$92x10^6$ clean reads were obtained per sample. All downstream analyses were based on clean data with high quality. Reads were mapped to the human reference genome version GRCh37.87 using HISAT 2 software. Reads mapping to the *IGH* enhancer regions (see Design of the CRISPR-eIGH library) were selected for further study. Using Galaxy,[19] BAM files provided by Novogene were sliced by genomic region chr14:106019800-106337000 and filtered by second mate to retrieve information about the strand from which the transcript was derived. Next, using Integrative Genome Browser,[20] BedGraph files were generated and visualized in the UCSC Genome Browser (*http://genome.ucsc.edu*).[21]

Results

Generation of the CRISPR-eIGH library targeting *IGH* enhancers

Target regions of the human Eμ, 3'RR1 and 3'RR2 were defined by the H3K27ac histone mark in B cells (GM12878 cell line from ENCODE) (*Online Supplementary Figure S1*) and a total of 18,732 sgRNA were designed. Due to homology within the 3'RR1 and 3'RR2 regions, several sgRNA were present multiple times in the design and sgRNA targeting up to two sites within the regions of interest were allowed (12,062 sgRNA remained). Further testing for off-targets elsewhere in the genome excluded 5,080 sgRNA. The final library included 6,982 sgRNA that cover the Eμ and 3'RR with an average distance between the sgRNA of 8 bp for the Eμ and 11 bp for 3'RR (Figure 1B); 2,344 sgRNA were common for 3'RR1 and 3'RR2. Including the negative and positive control sgRNA, the final library consisted of 7,971 sgRNA (*Online Supplementary Table S1*).

Next-generation sequencing-based quality control of the CRISPR-eIGH library confirmed its completeness and integrity (Figure 1C). Of all guides, 88.6% matched perfectly and there were no undetected guides. The skew ratio of top 10% to bottom 10% was 1.58. This confirms good quality of the generated CRISPR-eIGH library.

The CRISPR interference screen identifies elements of *IGH* enhancers essential for lymphoma cell growth

To identify essential elements of the *IGH* enhancers, we performed a CRISPR interference screen (Figure 1D). First, we generated lymphoma cell lines (BL41, DG75, SUDHL4) stably expressing the catalytically inactive Cas9 fused with the repressive KRAB domain (dCas9-KRAB) (*Online Supplementary Figure S2A*). These cells were thereafter transduced in duplicate with lentivirus carrying the CRISPR-eIGH library. We achieved transduction efficiency between 21.8% and 30.4% and coverage of 750x-1,045x (*Online Supplementary Figure S2B*). Cells were collected at T0 (after puromycin selection) and at T1 (after 20 population doublings) and the abundance of sgRNA constructs was assessed using next-generation sequencing. In each cell line we identified a few hundred sgRNA constructs showing a consistent ≥2-fold depletion in both screen replicates (Figure 2A, *Online Supplementary Table S2*). Non-targeting sgRNA did not show any major effects, while several sgRNA targeting CD79a and CD79b were depleted as expected.

Using a sliding window approach we identified three essential regions, hereafter called peaks, marked by sgRNA highly depleted over time (*Online Supplementary Table S3*): one Eμ-peak and two peaks in each of the 3'RR: 3'RR-peak1 and 3'RR-peak2 (Figure 2B). The profoundness of depletion varied between cell lines. For the Eμ-peak the strongest effect was observed in SUDHL4, with $\log_2$ fold change (FC) values reaching -1.4, followed by DG75 ($\log_2$ FC -0.8), while in BL41 it did not exceed -0.4. In both 3'RR peaks we observed the strongest effect in DG75 ($\log_2$ FC ~-2), while BL41 and SUDHL4 showed less depletion ($\log_2$ FC ~-1). The significant peaks within 3'RR overlapped with known DNase I hypersensitive sites (HS) HS4 and HS1.2.

We selected two sgRNA per peak to validate the effect on cell growth in a green fluorescence protein (GFP) competition assay in a larger set of BL and DLBCL cell lines. In line with the results of the screen, we observed progressive depletion of BL and DLBCL cells transduced with sgRNA constructs targeting enhancer-essential regions compared to non-targeting controls (Figure 2C, D). The dependency of B cells on *IGH* enhancers varied between cell lines, which is possibly linked to differences in the location of the breakpoints in *IGH* (Figure 2E). In DG75,[22] SUDHL4 and WSU-DLCL2[23] the breakpoint occurs in the V/D/J region, hence the Eμ is involved in the translocation. Accordingly, the strongest effect on survival in SUDHL4 and WSU-DLCL2 was observed upon inhibition of the Eμ-peak, while DG75 was very susceptible to blocking of all identified essential *IGH* regions. BL41 and CA46 have a breakpoint within the constant region Cα1[22] resulting in Eμ exclusion from the *IGH* locus on the translocated allele, and the strongest effect on survival in those cells was observed upon blocking of 3'RR-peak1, followed by 3'RR-peak2 and Eμ. The ST486 cell line with a breakpoint within the switch region μ[24] proved to be the most resistant to blocking of core *IGH* enhancer regions.

Altogether, our CRISPR interference screens identified specific regions in the *IGH* enhancers, essential for survival of B-cell lymphoma cells.

Chromatin-enriched RNA-sequencing reveals enhancer RNA transcripts from *IGH* enhancers

To identify transcription of eRNA from the *IGH* locus, we performed cellular fractionation combined with chromatin-enriched RNA-sequencing in BL41, DG75, and SUDHL4 lymphoma cells and P493-6 along with HEK239T as controls (no *IGH* translocation). This method allows for relatively fast, reproducible and cost-effective enrichment of eRNA (*Online Supplementary Figure S3A*). Proper fractionation was confirmed by RNA agarose gel electrophoresis (*Online Supplementary Figure S3B*), and by using appropriate markers on RNA and protein levels (*Online Supplementary Figure S3C, D*).

Focusing on transcripts mapping to the *IGH* enhancers, we observed bidirectional transcription from the Eμ and 3'RR (Figure 3A, *Online Supplementary Figures S4* and *S5*) in B-lineage cell lines, but not in HEK293T. The significant peaks identified in the CRISPR interference screen were also transcriptionally active. In the Eμ, transcription from the minus strand was 10-fold higher compared to that from the plus strand, with the highest read counts for BL cell lines. In 3'RR1, transcription rates were comparable from

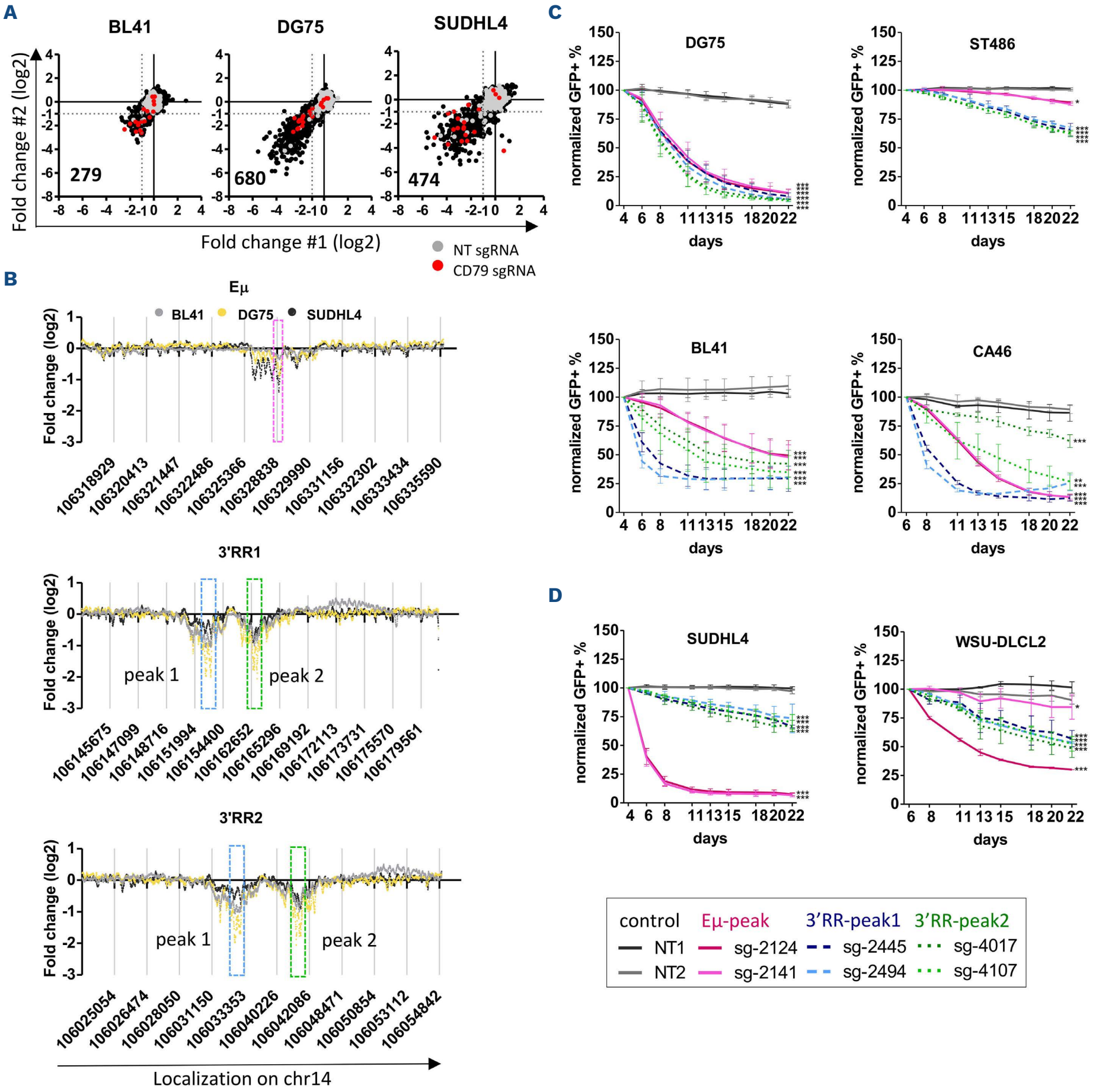


Figure 2. Tiling CRISPR interference screen of the *IGH* enhancers in B-cell non-Hodgkin lymphoma cells. (A) CRISPR interference screen results. Scatterplots represent changes of single guide (sg)RNA abundance relative to the initial pool in two screen replicates for each cell line. Numbers in the bottom left corner indicate the total number of sgRNA in the given cell line that were consistently >2-fold depleted from the initial pool. Black dots represent sgRNA targeting immunoglobulin heavy chain (*IGH*) en-

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hancers, gray dots represent non-targeting (NT) controls, and red dots represent positive controls targeting CD79. (B) Fold change values of 20 consecutive sgRNA calculated using the sliding window approach. Colored boxes mark regions identified as essential for cell survival. For Eμ: chr14:106328838-106329184, pink box, 3'RR1 and 3'RR2: chr14:106152156-106153203 and chr14:106032312-106033352, blue box – peak 1, chr14:106162681-106163347 and chr14:106041116-106041795, green box – peak 2. (C, D) Green fluorescent protein (GFP) growth competition assay. Assays performed with individual sgRNA over the course of 3 weeks in Burkitt lymphoma (C) and diffuse large B-cell lymphoma (D) cell lines. Average and standard deviation from three independent biological replicates are shown. *P≤0.05, **P≤0.01, ***P≤0.001, mixed model analysis. (E) Localization of breakpoints in the *IGH* locus for cell lines used in this study. Red: Burkitt lymphoma cell lines; purple: diffuse large B-cell lymphoma cell lines.

both strands. For the 3'RR2 more reads were mapped to the plus strand, especially for BL41 and SUDHL4.

Cellular localization of eRNA transcribed from core *IGH* enhancer regions was confirmed with real-time quantitative polymerase chain reaction analysis of subcellular fractions (Figure 3B). eRNA from the Eμ-peak and 3'RR-peak2 were enriched in the chromatin, while the 3'RR-peak1 eRNA was enriched in the cytoplasm.

IGH-eRNA expression in B-cell lymphoma cell lines and patient-derived samples

We verified eRNA expression from the essential *IGH* regions in a panel of B-cell lymphoma cell lines (Figure 3C). We observed statistically significant lower expression of all tested eRNA in Hodgkin lymphoma cell lines. There were no differences in expression levels between other groups, including ABC- and GCB-type DLBCL. In line with next-generation sequencing results, we observed higher expression from the Eμ-peak compared to the 3'RR-peaks.

We next confirmed eRNA expression in patient-derived formalin-fixed, paraffin-embedded samples (Figure 3D), including eight BL and 13 DLBCL, and six tonsils as control. We observed statistically significant higher expression of 3'RR in BL.

Downstream effects of targeting *IGH* enhancers on the expression of eRNA, translocated oncogenes and B-cell receptor

We next determined the effects of inhibition of significant *IGH* peaks on expression of eRNA, translocated oncogenes and BCR. Targeting *IGH* enhancer peaks with selected sgRNA significantly downregulated expression of eRNA from the respective regions (Figure 4A, *Online Supplementary Figure S6A*). In the BL cell lines BL41 and DG75, blocking each *IGH* peak led to a consistent, as much as 50% decrease of *MYC* transcript level. This was accompanied by reduced protein levels in DG75 (*Online Supplementary Figure S7*). In the BL cell line CA46 downregulation of *MYC* transcript was observed only upon blocking 3'RR, but not Eμ (*Online Supplementary Figure S6A*), while the MYC protein was consistently decreased in all samples (*Online Supplementary Figure S7*). ST486, which in the GFP assay appeared to be more resistant, did not exhibit downregulation of MYC, on either the RNA or protein level (*Online Supplementary Figures S6A* and *S7*). In DLBCL cell lines SUDHL4 and WSU-DLCL2, BCL2 expression was decreased on both RNA and protein levels in nearly all samples (Figure 4A, *Online Supplementary Figures S6A* and *S7*).

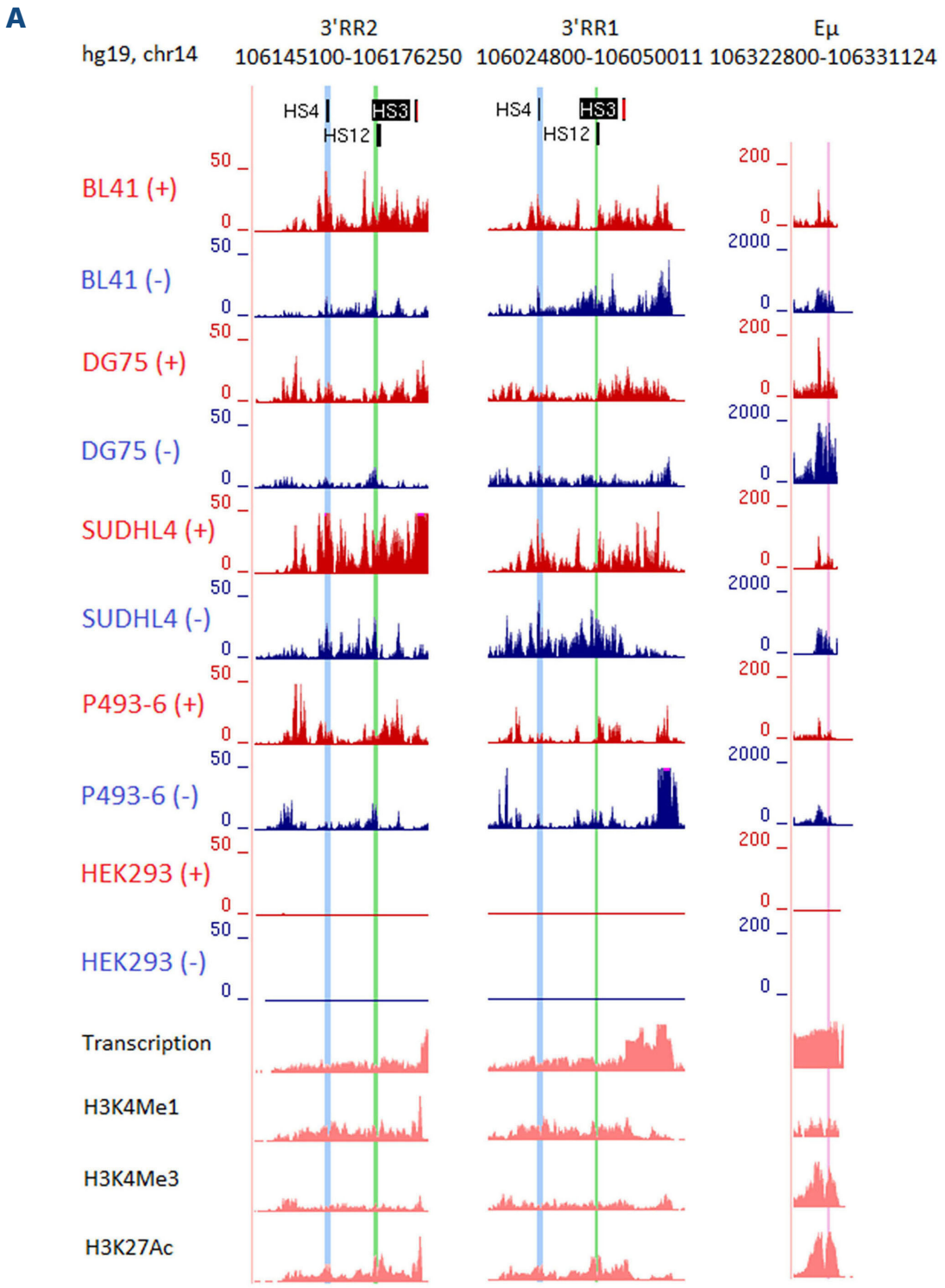
Usually, one *IGH* allele is involved in the translocation, while the other allele is productively rearranged and leads to production of secreted immunoglobulins and expression of the BCR on the cell surface (IgM or IgG).[25] Throughout their lifetime, B cells, including lymphoma cells, are constantly tested for proper BCR presentation on the cell surface[25] as their development and survival depend on it. Our CRISPR interference approach targets both the translocated and functional *IGH* alleles. Thus, we checked whether silencing *IGH* enhancers with dCas9-KRAB also leads to changes in BCR expression (*Online Supplementary Figure S8*). Targeting the Eμ-peak resulted in appearance of an IgM-negative population in BL cell lines BL41 (50% IgM$^-$), DG75 (up to 30% IgM$^-$) (Figure 4B, C), CA46 (70% IgM$^-$), but not in ST486 (*Online Supplementary Figure S6B, C*). A similar effect was observed in the IgG-expressing DLBCL cell line SUDHL4 with a BCR-negative population reaching 30-40% (Figure 4B, C). In contrast to the Eμ-peak, targeting the 3'RR-peaks led to only a slight reduction in BCR expression in SUDHL4 cells, but not in the other cell lines (Figure 4B, C, *Online Supplementary Figure S6B, C*).

IGH enhancers are active only in B cells. Therefore, no effect should be observed upon targeting them in non-B cells. In addition, based on the results of BCR expression analysis, we expected that our CRISPR interference approach would affect growth of B cells without *IGH* translocations only if the cells depended on BCR signaling and the Eμ enhancer was targeted. To test this, we assessed cell growth and BCR expression in: (i) BL cell lines with *MYC-IGL* t(8;22) translocation (BL2, BL60); (ii) BL cell lines with *MYC-IGK* t(2;8) translocation (JI, LY91); (iii) a B-cell cell line without *MYC* translocation (P493-6); and (iv) the embryonic kidney HEK293T cell line. We observed reduced cell growth in BL2, BL60 and P493-6 cells transduced with sgRNA targeting the Eμ peak. JI and LY91 cells did not respond to any construct, neither did HEK293T cells in which *IGH* enhancers are not active (*Online Supplementary Figure S9A-D*). In line with this, JI and LY91 cells are BCR-negative, they do not express either IgM or IgG (*data not shown*). BL2, BL60 and P493-6 cells express surface IgM and we observed 20-30% (BL2), 40-70% (BL60) and 82-85% (P493-6) decreases in IgM-positive cells upon targeting the Eμ peak (*Online Supplementary Figure S9E*). This confirmed that while the effect of silencing the Eμ enhancer may be attributed to

interfering with both the oncogene expression and BCR, reduced cell growth observed upon 3'RR targeting is not related to the BCR signaling.

MYC overexpression rescues cell proliferation in a Burkitt lymphoma cell line upon *IGH* enhancer inhibition

Since we observed a significant decrease in cell growth accompanied by MYC downregulation in DG75 upon blocking of *IGH* enhancers, we determined whether MYC overexpression could rescue the phenotype (Figure 5A). To this end we generated a DG75 cell line with doxycycline-inducible MYC expression (DG75-MYC-OE) (*Online Supplementary Figure S10A*). As a control, cells with empty vector (DG75-EV) were used. Induction of MYC expression was tested on both RNA and protein levels (*Online Supplementary Figure S10B, C*) with doxycycline doses of 0.1-0.5 μg/mL. Survival of DG75-MYC-OE cells upon induction of MYC expression was determined over the course of 3 weeks (*Online Supplementary Figure S10D*). We established that the use of 0.1 μg/mL is sufficient for MYC overexpression, while higher doses caused a strong decrease in cell survival. Next, DG75-MYC-OE and DG75-MYC-EV cells were transduced with the set of sgRNA targeting *IGH* enhancer peaks and non-targeting controls and their viability was tested. We observed a partial rescue of the effect exerted by inhibition of *IGH* enhancers in DG75-MYC-OE cells upon MYC induction (Figure 5B), but not in DG75-EV (Figure 5C). Although we could not fully rescue the observed effects on viability upon targeting *IGH* enhancers, our results suggest that the observed negative effect is at least in part caused by downregulation of MYC.



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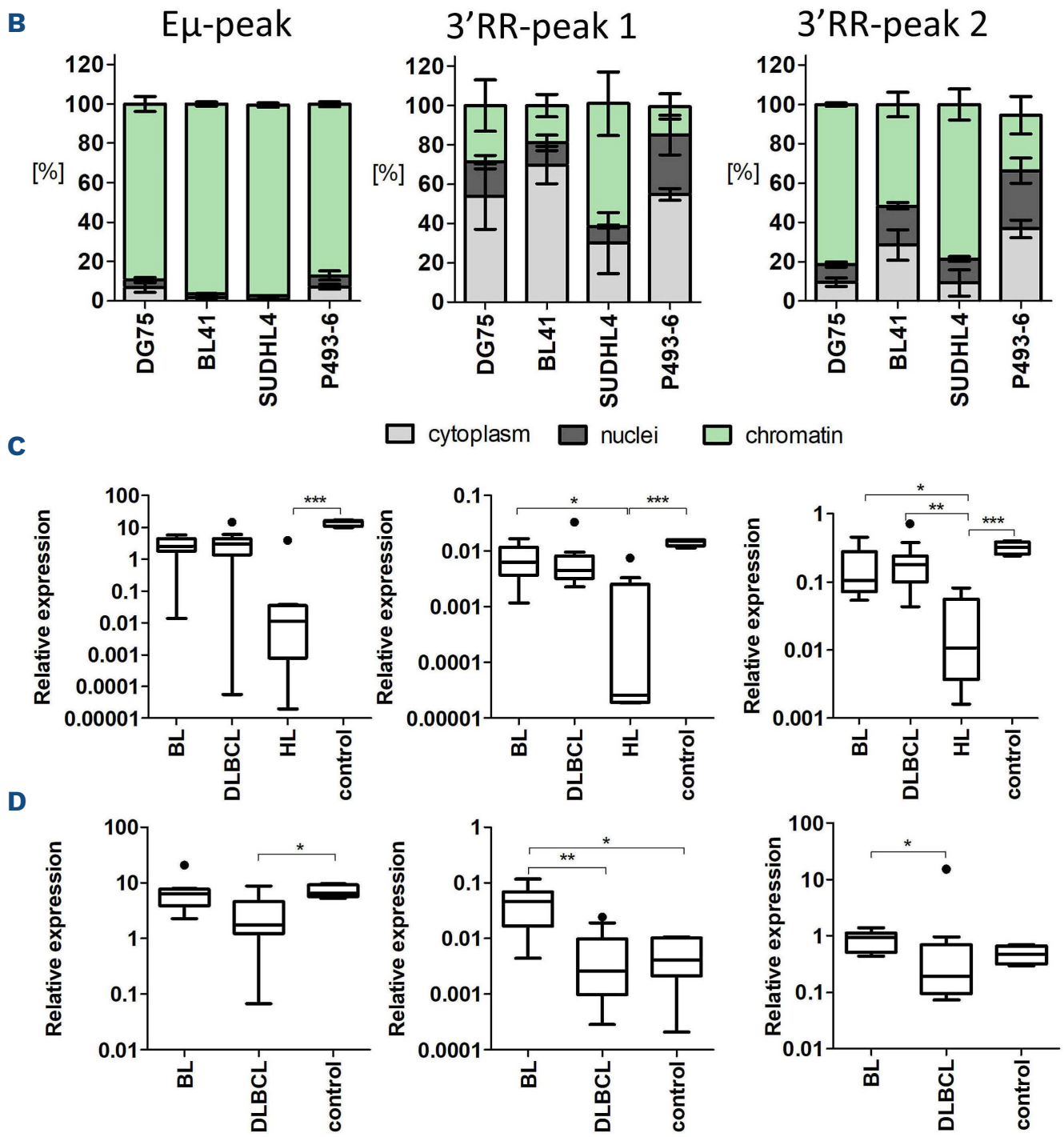

Figure 3. Transcriptional activity of *IGH* enhancers. (A) Chromatin-enriched RNA-sequencing results for immunoglobulin heavy chain (*IGH*) enhancer regions accompanied by UCSC tracks from GM12878 B cells: transcription and histone marks: H3K4me1 and H3K27ac (characteristic for enhancer regions) and H3K4me3 (characteristic for promoters and active genes). Red indicates reads from the plus strand, blue indicates reads from the minus strand. Pink: Eμ peak; blue: 3'RR peak 1; green: 3'RR peak 2. (B) Cellular localization of eRNA transcripts determined by cellular fractionation. Average and standard deviation from two biological replicates are shown. (C) Validation of eRNA expression in a panel of B-cell lymphoma cell lines and control B cells: Burkitt lymphoma (BL) N=9; diffuse large B-cell lymphoma (DLBCL) N=12: activated B-cell subtype (ABC) N=5, germinal center B-cell subtype (GCB) N=7; Hodgkin lymphoma (HL) N=8; control (germinal center B cells) N=4. Expression normalized to TBP. Analysis of variance (ANOVA) Kruskall-Wallis with Dunn multiple comparison post-test was applied; **P*≤0.05, ***P*≤0.01; ****P*≤0.001. (D) Validation of eRNA expression in patient-derived formalin-fixed paraffin-embedded samples: BL N=8; DLBCL N=13: ABC N=7, GCB N=6; control (healthy donor tonsil) N=6. Expression normalized to TBP. ANOVA Kruskall-Wallis with Dunn multiple comparison post-test was applied. **P*≤0.05, ***P*≤0.01.

Discussion

In NHL, recurrent chromosomal translocations are known to bring oncogenes under the regulation of *IGH* enhancers – 5' intronic Eμ and 3' regulatory regions, 3'RR1 and 3'RR2. To date, targeting *IGH* enhancers as a therapeutic option remains elusive. Therefore, delineation of the core *IGH* enhancer regions, which control the expression of translocated oncogenes, as well as growth and survival of lymphoma cells is of a key importance. Our study is the first to define the exact regions crucial for survival of NHL. The use of a saturating CRISPR interference library allowed us to target and thoroughly screen the *IGH* enhancers. Interestingly, survival of lymphoma cells upon blocking *IGH* enhancer core regions varied between cell lines. Taking into account that chromosomal translocations can occur at various regions within the *IGH* locus, our data suggest that the observed patterns are connected to the breakpoint sites.

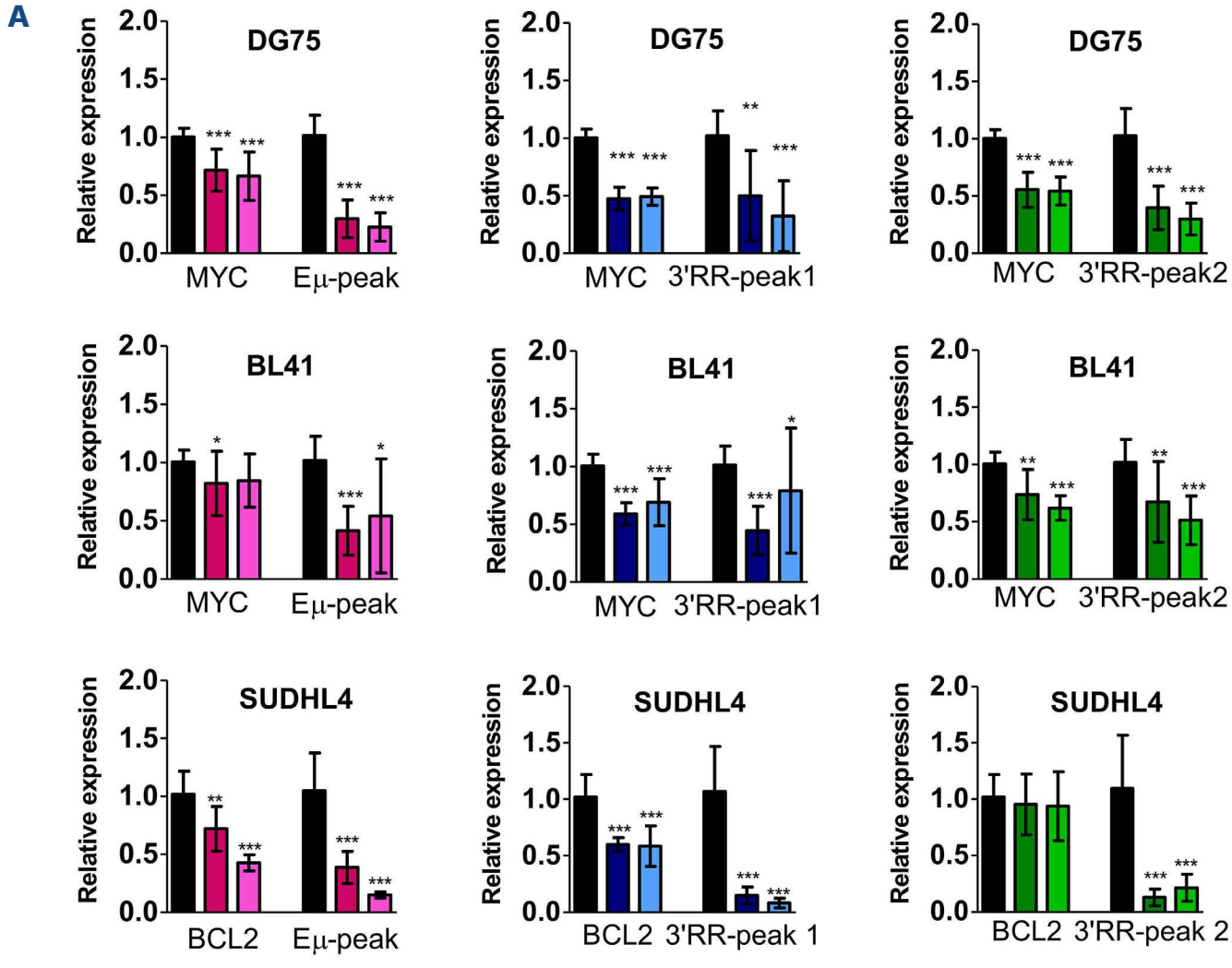

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Figure 4. Downstream effects of targeting *IGH* enhancers. (A) Expression of oncogenes involved in immunoglobulin heavy chain (*IGH*) translocation, *MYC* (DG75, BL41) or *BCL2* (SUDHL4) and expression of eRNA upon blocking *IGH* enhancer essential regions on a RNA level determined by real-time quantitative polymerase chain reaction. Mean and standard deviation of three independent biological replicates are shown. Expression normalized to HPRT. **P*≤0.05, ***P*≤0.01; ****P*≤0.001, Mann-Whitney test. (B) Immunostaining of B-cell receptor (BCR) on cell surface in DG75, BL41 (IgM) and SUDHL4 (IgG) cell lines. Representative histograms of overlaid data for non-targeting (NT) controls (gray) and single guide (sg)RNA targeting *IGH*-enhancer essential regions (pink, Eμ peak; blue, 3'RR peak 1; green, 3'RR peak 2). Arrows indicate samples from a separate staining in SUDHL4. (C) Average and standard deviation of percentage of BCR-positive cells (surface IgM or IgG) from two biological replicates. **P*≤0.05, ***P*≤0.01; ****P*≤0.001, *****P*≤0.0001, Student two-tailed *t* test.

In DG75, SUDHL4 and WSU-DLCL2 both the Eμ enhancer and 3'RR are involved in the translocation. We observed consistent downregulation of the translocated oncogene (*MYC* or *BCL2*) upon blocking *IGH* enhancers, with a similar effect for each of the identified essential regions. This may indicate cooperation between *IGH* enhancers in driving expression of the translocated oncogene. Spatial interaction between Eμ and 3'RR occurs for example during *IGH* locus rearrangements.[26] Ghazzaui *et al.*[27] developed several mouse models of c-myc knock-in juxtaposed with *IGH* enhancers and demonstrated that the dynamics of lymphoma development and mice survival varied depending on the oncogene insertion site. The shortest lifespan was observed when both Eμ and 3'RR enhancers were involved. Ghazzaui *et al.* concluded that Eμ and 3'RR enhancers cooperate in driving translocated oncogene expression and lymphomagenesis. We tested in our study whether inhibition of one core *IGH* enhancer region affects transcription of others, but we did not observe any consistent effect on eRNA expression from 3'RR when blocking Eμ and *vice versa* (*data not shown*).

In BL cell lines BL41, CA46 and ST486 the intronic Eμ is not involved in the translocation with *MYC*. In agreement with this, we observed little to no MYC downregulation upon targeting the Eμ enhancer essential region. In contrast, blocking the 3'RR core regions in BL41 and CA46 had a significant effect on *MYC* transcript levels as well as cell survival. In NHL, despite differences in the location of the breakpoint in *IGH*, the 3'RR always remains in the *IGH* locus. This regulatory region was suggested previously to be a good target for therapy[28,29] and was found sufficient to deregulate oncogene expression.[7,27] So far, several factors have been shown to affect transcriptional activity of 3'RR, proving that this enhancer may be druggable.[30-32] The 3'RR span 25-30 kb each, so it is necessary to further pinpoint which sites to target. In mice, HS3a and HS1.2 were shown to be important for deregulation of the translocated *MYC*.[33] Our approach revealed HS4 (peak1) and HS1.2 (peak2) within 3'RR as crucial for the survival of human B-cell lymphoma cells. Importantly, the P493-6 cell line was not affected by blocking the 3'RR, in contrast to Eμ. This suggests that the 3'RR-peaks identified by us are attractive candidates for therapeutic targeting. However, it still needs to be determined whether inhibition of HS4 and HS1.2 is toxic specifically for *IGH*-translocation-positive lymphomas and not for normal B cells.

ST486 cells exhibited resistance to inhibition of *IGH* core enhancers regions. This cell line bears several translocations involving *MYC*: the typical BL reciprocal translocation *MYC/IGH* t(8;14)(q24;q32) but also complex t(8;14;18)(q24;q32;q23).[34] This leads to the presence of *MYC* at as many as four different locations: chromosome 8, der(8), der(14) and der(18). Probably downregulation of *MYC* expression from only one site was not enough to observe either a significant change in overall levels, or a more profound effect on cell proliferation.

We were able to partially rescue the phenotype of blocking *IGH* enhancers by MYC overexpression in a BL cell line. This suggests that other elements controlled by *IGH* enhancers may be involved. Most lymphoma cells retain expression of BCR from the non-translocated *IGH* allele. In our approach, sgRNA for *IGH* enhancer-essential regions can target both alleles. We observed partial BCR loss upon blocking the Eμ-peak in nearly all tested lymphoma cell lines with *IGH* translocations but also in BL cells with *IGH* translocations and in the P493-6 B cell line which does not harbor any *IG* translocation. In contrast, targeting 3'RR-peaks in general did not affect BCR expression. This is in line with previous studies showing a reduction of IgM- and IgG-expressing B cells in mice with deletion of the Eμ enhancer.[8,35-37] On the other hand, singular deletions of 3'RR enhancer components in mice were not sufficient[38–41] and only combinatorial deletion of HS4 and HS3b downregulated BCR in B cells.[42,43] However, other studies showed that deletion of HS3b and HS4 or even the whole 3'RR did not affect surface IgM; only IgG was reduced.[29,44-46] Interestingly, it has been shown that while BCR ablation *per se* does not negatively influence lymphoma growth, BCR-negative BL cells are outcompeted by their BCR-expressing counterparts.[47] This is similar to the effect on cell survival observed by us upon Eμ-peak inhibition in the GFP-growth competition assays. Taken together, our results indicate that the reduced cell growth observed upon inhibition of essential *IGH* enhancer regions can be attributed to downregulation of oncogene expression and in the case of the Eμ, also to the loss of BCR.

Current knowledge regarding the function of eRNA in NHL is very limited. Recently, a B-cell specific eRNA AL928768.3 was shown to regulate MYC expression in BL.[12] This eRNA resides within the human 3'RR1 region (hg19, chr14:106170301-10617093). Researchers modulated the expression of AL928768.3 by either small interfering (si)RNA-mediated

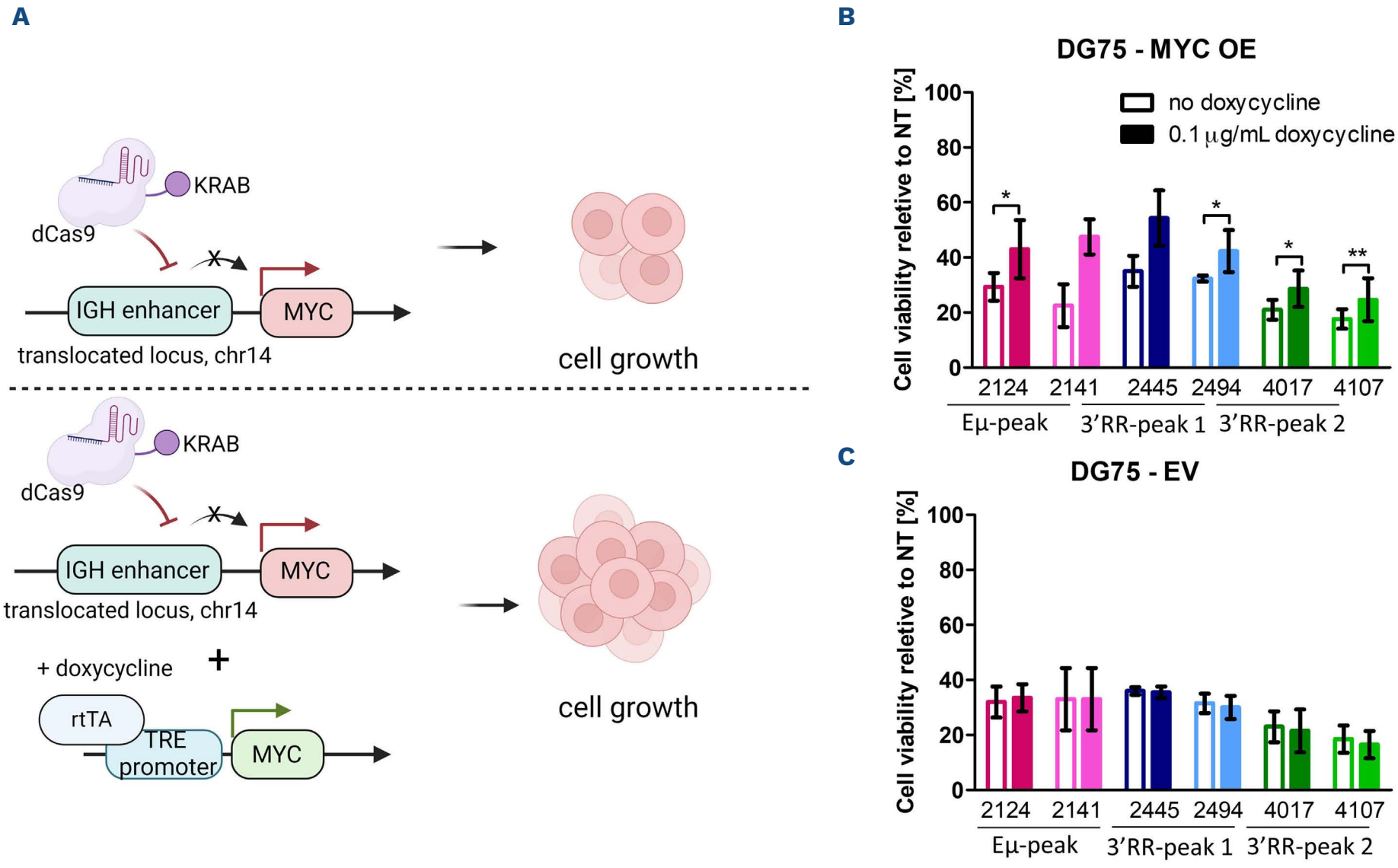

Figure 5. MYC overexpression rescues cell viability in the Burkitt lymphoma cell line DG75 upon inhibition of *IGH* enhancer-essential regions. (A) Overview of the MYC-rescue experiment. (B, C) Viability of the DG75-MYC overexpressing cell line (B) and a control cell line DG75-EV (C) transduced with single guide (sg)RNA targeting immunoglobulin heavy chain (*IGH*) enhancers and treated or not with doxycycline to induce MYC expression. Average and standard deviation from three independent biological replicates are shown. **P*≤0.05, ***P*≤0.01, Student *t* test.

knockdown or overexpression, and observed down- and upregulation of MYC, respectively. The effect on MYC was specific to cells bearing the *MYC/IGH* translocation. Knockdown of eRNA AL928768.3 resulted in a decrease in BL cell proliferation. Verification of this observation in a wider panel of NHL would be of interest. In our screen, inhibition of the AL928768.3 region with CRISPR/dCas9 did not, in general, have any significant impact on cell survival overall; however, a few individual sgRNA were strongly depleted, especially in the DG75 and SUDHL4 cell lines (*Online Supplementary Table S6*). Here, we confirmed transcription from core *IGH* enhancer regions and validated arising eRNA in a panel of B-cell lymphomas, both NHL and Hodgkin lymphomas, as well as NHL patient-derived samples. It was shown that the activity of *IGH* enhancers depends on transcription factors such as BOB.1 and PU.1.[48] Low expression of IGH eRNA in Hodgkin lymphomas is in line with absence of those transcription factors.[48,49] Levels of BOB.1 and PU.1 are high in BL and germinal center B cells but variable in DLBCL,[48,49] which may account for the observed differences.

Blocking transcription of the essential regions with CRISPR interference resulted in downregulation of eRNA derived from them, which was accompanied by oncogene and BCR downregulation. Whether *IGH* eRNA from core regions are indeed involved in the regulation of those translocated oncogenes and BCR requires further study. For some eRNA the very act of their transcription and not necessarily the transcript itself is important for carrying out their function.[9,50] On the other hand, eRNA may help the achievement of proper chromatin conformation and recruit transcriptional machinery to target regions.[51] Transcription of 3'RR in mice was shown to recruit activation-induced cytidine deaminase, which leads to *IGH* locus suicide recombination and BCR loss, potentially contributing to B-cell homeostasis.[10] eRNA can also interact with other classes of non-coding RNA. Long non-coding RNA CSR interacts with 3'RR HS4 eRNA, which promotes CSR.[52] Apart from these functions in chromatin, eRNA have also been reported to have a role in the cytoplasm.[53] We observed a predominant cytoplasmic localization of the eRNA from 3'RR-peak 1 which suggests that it may have an additional role beyond acting *in cis* in the *IGH* locus. So far, we have not been able to efficiently knockdown *IGH* eRNA with the use

of either Gapmers or short hairpin RNA and so the potential role of *IGH* eRNA requires further investigation.

A more detailed analysis of the *IGH* enhancer core regions would be of interest. Enhancers are known to be packed with transcription factor binding sites. Elucidation of if and how their binding is affected upon blocking essential enhancer sites will help to understand expression regulation at those regions. In addition, Eμ and 3'RR can form chromatin loops, so a closer look at the chromatin architecture in the *IGH* locus upon blocking of the core enhancer regions could provide insights into the mechanisms involved. Inhibition of the *IGH* enhancers allows for a precise, B-cell restricted, targeting of translocated oncogenes in B-cell lymphoma. This makes the core regions identified by us attractive targets for therapeutic approaches. So far, histone deacetyl inhibitors and aryl hydrocarbon receptor ligands have been shown to affect activity of the 3'RR and *IGH* transcription.[30,31] However, their effect on lymphomas driven by *IGH* translocations has not been evaluated. Recently, a small molecule reducing the activity of the Eμ has been reported, with an inhibitory effect on growth of *IGH* translocation-positive multiple myeloma cells *in vitro* and *in vivo*.[54] However, we showed that this compound is also toxic to other cell types.[32]

In summary, we have pinpointed regions within the *IGH* enhancers Eμ and 3'RR that are crucial for survival of B-cell lymphomas. We have shown that the observed negative effect on cell survival is likely to be attributed to down-regulation of translocated oncogenes and, in the case of Eμ inhibition, also to BCR loss. Our results set a frame for further studies to explore the therapeutic potential of inhibiting *IGH* enhancers in B-cell lymphoma.

Disclosures

No conflicts of interest to disclose.

Contributions

MEK and WS performed investigations, analyzed data, wrote the manuscript and prepared figures and tables. MP, MK, WŁ, and AS performed investigations. TW analyzed data. JEJG, AD, JK, AvdB, and NR conceived the project and provided materials and samples. ADK conceived and supervised the project, acquired funding, was responsible for project administration, analyzed data, and wrote the paper. All authors read and approved the manuscript.

Acknowledgments

The authors would like to thank Prof. Reiner Siebert from the Institute of Human Genetics, Ulm University for kindly providing the BL2, BL60, JI and LY91 cell lines; Dr. Natalia Derebecka from the Laboratory of High Throughput Technologies, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznań, Poland for performing MiSeq analysis and Dr. Wojciech Juzwa from the Department of Biotechnology and Food Microbiology, Faculty of Food Science and Nutrition, Poznań University of Life Sciences for cell sorting of the DG75-dCas9-EV cell line. Graphics for Figure 1D and Figure 5A were created with BioRender.

Funding

This research was carried out within the First Team program of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund (grant n. POIR.04.04.00-00-5EC2/18-00) and PROM program, The Polish National Agency for Academic Exchange (NAWA) n. 10 PPI/PRO/2019/1/00014/U/00001 co-financed by the European Union under the European Social Fund. AD-K, NR and AvdB were supported by the European Union's Horizon 2020 research and innovation program under grant agreement n. 952304.

Data-sharing statement

Raw and processed RNA-sequencing data have been deposited in Gene Expression Omnibus (GSE247393).

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