INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Functional dissection of the immunoglobulin heavy chain enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas

mgr Marta Elżbieta Kasprzyk

Doctoral dissertation was conducted
at the Department of Molecular Pathology
under the supervision
of dr hab. n. med. Agnieszka Dzikiewicz-Krawczyk, prof. IHG

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I would like to thank

my supervisor prof. Agnieszka Dzikiewicz-Krawczyk for all the support, ideas and opportunities given during the implementation of this project, without which the preparation of my doctoral dissertation would not be possible,

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Weronika Sura for fruitful cooperation, help, understanding and constant flow of chocolate,

our collaborators – prof. Anke van den Berg, Joost Kluiver, Annika Seitz and Jeroen Guikema for their help, inspiring ideas and advices

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my students for their enthusiasm and allowing me to grow as a potential future supervisor,

and finally - my family for their understanding and patience, in times when I was not there, for keeping me sane and supporting me on a daily basis

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ABBREVIATIONS

3'RR – 3' regulatory region

ABC – activated B cell

AID – activation-induced cytidine deaminase

BCL2 – B-cell leukemia/lymphoma 2 apoptosis regulator

BCR – B-cell receptor

BL – Burkitt lymphoma

Cas9 – CRISPR associated protein 9

CRISPR - clustered regularly interspaced short palindromic repeats

CSR – class switch recombination

DLBCL – diffuse large B-cell lymphoma

DSB – double-strand break

Eμ – enhancer Eμ

eRNA – enhancer RNA

FBS – fetal bovine serum

GCB – germinal center B cell

GFP – green fluorescent protein

HS – hypersensitive site

IgG – immunoglobulin isotype G

IGH – immunoglobulin heavy chain

IgM – immunoglobulin isotype M

KRAB – Krüppel associated box

MBR – major breakpoint

MYC – proto-oncogene, bHLH transcription factor

NHEJ – non-homologous end joining

NHL – non-Hodgkin lymphoma

PBS – phosphate buffered saline

RAG – recombination-activating gene

RSS – recombination signal sequences

sgRNA – single guide RNA

SHM – somatic hypermutation

TdT – terminal deoxynucleotidyl transferase

TF – transcription factor

VDJ – variable, diverse, joining gene segments

STRESZCZENIE

Chłoniaki nieziarnicze (ang. *non-Hodgkin lymphoma*; NHL) to wysoce heterogenna grupa nowotworów krwi. Szybka diagnoza i zastosowanie odpowiedniego leczenia może zapewnić 5-letnie przeżycie nawet do 90%, ale różni się ono znacznie w zależności od typu NHL i stopnia zaawansowania choroby. Złożony proces rozwoju i różnicowania sprawia, że limfocyty B są szczególnie podatne na aberracje genetyczne. Locus genu łańcucha ciężkiego przeciwciał (ang. *immunogobulin heavy chain,* IGH) podlega rekombinacji VDJ, hipermutacji somatycznej i rekombinacji prowadzącej do przełączania klas immunoglobulin, które są niezbędne do powstania szerokiego spektrum przeciwciał o wysokim powinowactwie. Ekspresja z locus *IGH* jest kontrolowana przez jego sekwencje wzmacniające: Eμ oraz regiony regulatorowe 3'RR1 i 3'RR2. Przebudowa locus *IGH* obejmuje pęknięcia podwójnej nici DNA, które stwarzają zagrożenie powstania nieprawidłowych rearanżacji. Rzeczywiście, cechą charakterystyczną NHL są translokacje, w wyniku których onkogeny przenoszone są w pobliże sekwencji wzmacniających transkrypcję (enhancerów) *IGH*. Chłoniak rozlany z dużych komórek B (ang. *diffuse large B-cell lymphoma*; DLBCL) jest najczęściej rozpoznawanym podtypem NHL i często charakteryzuje się obecnością t(14;18)(q32;q21) IGH/BCL2 zestawiając regulator apoptozy – BCL2 – z sekwencjami wzmacniającymi ekspresję*IGH*. Chłoniak Burkitta (ang. *Burkitt lymphoma*; BL) należy do szybko proliferujących NHL. Jest to rzadszy podtyp, w większości przypadków powiązany z t(8;14)(q24.1;q32) MYC/IGH. Podobnie jak w przypadku DLBCL, w BL MYC jest zestawiany z sekwencjami wzmacniającymi *IGH*, co prowadzi do jego zwiększonej ekspresji. MYC jest czynnikiem transkrypcyjnym zaangażowanym w kontrolę kilku procesów komórkowych, takich jak wzrost, proliferacja i apoptoza. Deregulacja onkogenu przez sekwencje wzmacniające *IGH* jest wczesnym zdarzeniem patogennym, które nakierowuje komórkę B na drogę transformacji nowotworowej. Przeżycie i proliferacja komórek chłoniaków często zależy od ekspresji translokowanego onkogenu. Pomimo szerokiej wiedzy na temat roli sekwencji wzmacniających transkrypcję *IGH* w normalnych komórkach B, nasze obecne rozumienie ich funkcjonowania w chłoniakach B-komórkowych jest ograniczone.

Badania podjęte w tej rozprawie doktorskiej poszerzają wiedzę na temat roli sekwencji wzmacniających ekspresję *IGH* w NHL. W artykule przeglądowym **Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer** (Kasprzyk ME, Sura W, Dzikiewicz-Krawczyk A, 2021) opisałam, w jaki sposób sekwencje wzmacniające transkrypcję mogą być zaangażowane w powstawanie nowotworów z komórek B. Szczególnie skupiłam się na enhancerach genu *IGH*, przedstawiając

podsumowanie aktualnych doniesień literaturowych na temat ich roli w normalnym rozwoju komórek B i w komórkach chłoniaków. Na podstawie dostępnej wiedzy opisałam interakcje pomiędzy Eμ i 3'RR oraz wymieniłam modele mysie wykorzystane w badaniach. W artykule oryginalnym **CRISPRi screen identifies core regions in IGH enhancers essential for non-Hodgkin lymphoma cells survival** (Kasprzyk ME i in., w przygotowaniu) potwierdziłam wyniki uzyskane za pomocą naszego badania przesiewowego CRISPR/Cas9 ukierunkowanego na sekwencje wzmacniające transkrypcję genu *IGH* w BL i DLBCL. Badanie przesiewowe CRISPRi pozwoliło na identyfikację precyzyjnych regionów w Eμ i 3'RR, które są niezbędne do przeżycia komórek NHL. Wykazałam, że inhibicja tych istotnych regionów w sekwencjach wzmacniających *IGH* obniża proliferację komórek chłoniaka i zmniejsza ekspresję translokowanych onkogenów. W BL udało mi się częściowo odwrócić obserwowany fenotyp przez nadekspresję MYC. Ponadto wykazałam, że zablokowanie regionu istotnego dla Eμ prowadzi do utraty receptora komórek B (BCR) na powierzchni komórek, który jest istotny dla funkcjonowania i przeżywalności limfocytów B. Przeprowadziłam również sekwencjonowanie RNA (RNA-Seq) pochodzącego z frakcji chromatynowej i potwierdziłam aktywną transkrypcję z sekwencji wzmacniających *IGH* i z ich istotnych regionów. Potwierdziłam ekspresję enhancerowych RNA (eRNA) z istotnych regionów sekwencji wzmacniających *IGH* w szerokim panelu chłoniaków z komórek B, a także w próbkach pochodzących od pacjentów. W drugim artykule oryginalnym **7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity** (Kasprzyk ME i in., 2021) przetestowałam związek 30666, zaproponowany niedawno jako specyficzny inhibitor sekwencji wzmacniających genu *IGH*. Moje badania wykazały, że związek 30666 rzeczywiście zmienia aktywność enhancerów genu *IGH*, na co wskazuje obniżona ekspresja translokowanych onkogenów, zróżnicowana ekspresja eRNA i globalne zmiany poziomu modyfikacji histonów specyficznych dla enhancerów. Chociaż związek 30666 negatywnie wpływał na przeżywalność komórek, wykazałam, że efekt ten nie był ograniczony tylko do chłoniaków B-komórkowych.

ABSTRACT

Non-Hodgkin lymphoma (NHL) is a highly heterogeneous group of blood malignancies. Fast diagnosis and application of appropriate treatment can ensure the 5-year survival rate up to 90%, but it differs strongly depending on the NHL type and disease stage. The complex process of development and differentiation makes B cells especially prone to genetic aberrations. Immunoglobulin heavy chain locus (IGH) undergoes VDJ recombination, somatic hypermutation and class switch recombination, which are all necessary to establish a wide range of high-affinity antibodies. The expression from *IGH* locus is controlled by its enhancers: Eμ and 3' regulatory regions 3'RR1 and 3'RR2. IGH locus remodeling involves DNA-double strand breaks, which pose a threat of illegitimate rearrangements. Indeed, recurrent translocations placing oncogenes under the regulation of *IGH* enhancers are a hallmark of NHL. Diffuse large B-cell lymphoma (DLBCL) is the most frequently diagnosed subtype of NHL and is often characterized by t(14;18)(q32;q21) IGH/BCL2 juxtaposing an apoptosis regulator - BCL2 - with *IGH* enhancers. Burkitt Lymphoma (BL) belongs to the fast growing NHLs. It is more rare and associated with the t(8;14)(q24.1;q32) MYC/IGH. Similarly to DLBCL, in BL MYC is juxtaposed with *IGH* enhancers which leads to its increased expression. MYC is a transcription factor, involved in the control of several cellular processes, such as growth, proliferation and apoptosis. Oncogene deregulation by *IGH* enhancers is an early pathogenic event setting a B-cell on a path towards malignancy. Survival and proliferation of lymphoma cells often depends on the expression of the translocated oncogene. Despite wide knowledge about roles of *IGH* enhancers in normal B-cells, our current understanding of their functioning in malignant B-cells is limited.

The research undertaken in this doctoral dissertation broaden the knowledge regarding *IGH* enhancers is NHL. In the review article **Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer** (Kasprzyk ME, Sura W, Dzikiewicz-Krawczyk A, 2021) I explored how enhancers can contribute to the formation of malignant B-cells. I especially focused on *IGH* enhancers, providing the summary of the current literature reports of their roles in normal B-cell development and in lymphoma cells. Based on the available knowledge, I described the interactions between Eμ and 3'RRs and listed mouse models used in studies. In the original article **CRISPRi screen identifies core regions in IGH enhancers essential for non-Hodgkin lymphoma cells survival** (Kasprzyk ME et al., in preparation) I validated the results obtained by our CRISPR/dCas9 screen targeting I*GH* enhancers in BL and DLBCL. CRISPRi screen allowed for identification of precise regions within Eμ and 3'RRs that are necessary for survival of NHL cells. I showed that inhibition of those essential regions in IGH enhancers lowers lymphoma

cells proliferation and downregulated the expression of translocated oncogenes. In BL, I was able to rescue the observed phenotype by MYC overexpression. Moreover, I showed that blocking of the Eμ significant region leads to B-cell receptor (BCR) loss on the cell surface, which is necessary for proper functioning and survival of B-lymphocytes. I also performed chromatin-enriched RNA-Seq and confirmed ongoing transcription in *IGH* enhancers and their core regions. I validated enhancer RNAs (eRNAs) expression from *IGH* enhancers essential regions in a wide panel of B-cell lymphomas as well as patient derived samples. In the second original article **7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity** (Kasprzyk ME et al., 2021) I tested the compound 30666 proposed recently as a specific *IGH* enhancers inhibitor. My research showed that the compound 30666 indeed alters the *IGH* enhancers activity as indicated by lowered expression of translocated oncogenes, differential expression of eRNAs and global changes of enhancer-specific histone modifications. Although compound 30666 negatively affected cell survival, I demonstrated that the effect was not limited to B-cell lymphomas.

LIST OF ARTICLES INCLUDED IN THE DISSERTATION

This dissertation is based on two original articles and one review article listed below:

1. **Review article**

Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards cancer;
Marta E Kasprzyk, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk,

MDPI Cancers 2021, 13(13), 3270, published: 29th June 2021
doi: 10.3390/cancers13133270.
Impact factor upon publishing: 6.639
5-year impact factor: 6.886
MEiN points: 140

2. **Original article**

CRISPRi screen identifies core regions in *IGH* enhancers essential for non-Hodgkin lymphoma cells survival;
Marta E Kasprzyk*, Weronika Sura*, Marta Podralska, Marta Kazimierska, Wojciech Łosiewski, Annika Seitz, Tomasz Woźniak, Jeroen E. J. Guikema, Joost Kluiver, Anke van den Berg, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk

Article not yet published.

3. **Original article**

7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity;
Marta E Kasprzyk*, Wojciech Łosiewski*, Marta Podralska, Marta Kazimierska, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk

European Journal of Pharmacology, Vol 910:174505, published 14th September 2021
doi: 10.1016/j.ejphar.2021.174505
Impact factor upon publishing: 4.432
5-year impact factor: 4.721
MEiN points: 100

*authors contributed equally

Total impact factor: 11.071
Total 5-year Impact Factor: 11.607
Total MEiN points: 240

LIST OF CONFERENCES

Table below summarizes all posters and lectures, connected to this doctoral dissertation, which I had presented and co-authored.

9th May 2023	Next Level Project - H2020 – Staff Exchange 2023, Poznań, Poland **oral presentation:** Functional dissection of IGH enhancers and eRNA in B-cell lymphomas. *Marta E Kasprzyk*, Weronika Sura*, Marta Podralska, Marta Kazimierska, Wojciech Łosiewski, Annika Seitz, Tomasz Woźniak, Jeroen E. J. Guikema, Joost Kluiver, Anke van den Berg, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk*
6-7th October 2022	4th Interdisciplinary FNP Conference, Warsaw, Poland **poster:** Functional dissection of IGH enhancers and eRNA in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
26-30th June 2022	VI Kongres Genetyki 2022, Cracow, Poland **poster:** Functional dissection of IGH enhancers and eRNA in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk*,
12-17th June 2022	4th International Conference on the Long and the Short of Non-Coding RNAs, Rhodes, Greece **poster and oral presentation:** Functional dissection of IGH enhancers and eRNA in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
9th June 2022	The European Hematology Association (EHA) - EHA2022 Congress, Vienna, Austria **invited oral presentation:** Functional dissection of IGH enhancers and eRNA in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
28th March - 1st April 2022	Staff exchange week, Next Level Project - H2020 – Twinning, on-line **oral presentation:** Functional dissection of IGH regulatory regions in B-cell non-Hodgkin lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk*

19-20th November 2021	XXV Gliwice Scientific Meetings, on-line **poster selected for oral presentation:** Functional dissection of IGH enhancers and eRNA in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
21-23rd July 2021	MDPI - Noncoding RNA World: From Mechanism to Therapy, on-line **poster:** Functional dissection of IGH enhancers and eRNA in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
21-23rd May 2021	The 9th Intercollegiate Biotechnology Symposium SYMBIOZA, on-line **poster co-authorship:** Effect of IGH enhancers inhibition with compound 30666 on B-cell lymphoma survival and oncogene expression. *Wojciech Łosiewski*, Marta Kasprzyk*, Weronika Sura, Marta Podralska, Agnieszka Dzikiewicz-Krawczyk*
17-20th May 2021	EMBL – Chromatin and Epigenetics, on-line **poster:** Functional dissection of IGH enhancers in B-cell lymphoma. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
10-14th May 2021	Keystone Symposia, Non-Coding RNAs: Biology and Applications, online **poster co-authorship:** Functional dissection of IGH enhancers in B-cell lymphoma. *Weronika Sura*, Marta Kasprzyk*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
25-26th February 2021	International Young Scientists Conference on Molecular and Cell Biology, online by International Institute of Molecular and Cell Biology, on-line **poster:** Understanding the mechanism of oncogene expression regulation by IGH enhancers. *Marta Kasprzyk* Weronika Sura*, Wojciech Łosiewski, Marta Podralska, Marta Kazimierska, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk,*
20-21st November 2020	XXIV Gliwice Scientific Meetings, on-line **poster co-authorship:** CRISPRi tiling screen identifies regions in IGH enhancers crucial for Burkitt Lymphoma cell growth. *Weronika Sura*, Marta Kasprzyk*, Marta Kazimierska, Marta Podralska, Wojciech Łosiewski, Tomasz Woźniak, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk*, **poster co-authorship:** Effect of IGH enhancers inhibition with compound 30666 on B-cell lymphoma survival and oncogene expression. *Wojciech Łosiewski*, Marta Kasprzyk*, Marta Podralska, Agnieszka Dzikiewicz-Krawczyk*,

11-12th April 2019	3rd Interdisciplinary FNP Conference, Warsaw, Poland **poster:** Functional dissection of IGH regulatory regions in B-cell non-Hodgkin lymphoma. *Marta Kasprzyk, Agnieszka Dzikiewicz-Krawczyk*

*authors contributed equally

LIST OF AWARDS

During execution of this doctoral dissertation I was granted awards listed below:

- Helice 2022 - Best poster award at the conference: VI Kongres Genetyki 2022 - June 2022
- FEBS Travel Award - to attend the 4th International Conference on the Long and the Short of Non-Coding RNAs - June 2022
- stipend for the best phd student granted by the Director of Institute of Human Genetics - June 2022
- stipend for the best phd student granted by the Director of Institute of Human Genetics - October 2021
- stipend for the best phd student granted by the Director of Institute of Human Genetics - October 2020

DOCTORAL CANDIDATE'S PROFILE

Education

Doctoral Studies – Institute of Human Genetics, Polish Academy of Sciences (PAS) December 2018-2023
Poznań, Poland
Department of Molecular Pathology, Independent Research Group of Non-coding Parts of the Genome

Project: Functional dissection of IGH regulatory regions in B-cell non-Hodgkin lymphoma.
First Team FNP POIR.-04.04.00-00-5EC2/18-00
Supervisor: Agnieszka Dzikiewicz-Krawczyk, MD-PhD, Assoc. Prof.

Doctoral Studies – Institute of Bioorganic Chemistry, Polish Academy of Sciences August 2017 -December 2018
Poznań, Poland
Department of RNA Biochemistry

Project: Influence of non-coding RNA on renal cell development and carcinogenesis.
OPUS NCN 2016/21/B/NZ2/03468
Supervisor: Jan Wrzesiński, PhD, Prof.

Master of Science Degree in Biotechnology - Faculty of Biology, Adam Mickiewicz University (AMU) 2013-2016
Poznań, Poland
Polish Academy of Sciences, Institute of Bioorganic Chemistry, Department of Molecular Genetics,

Thesis: Influence of stress conditions on ribosome – tRF interactome in *Saccharomyces cerevisiae.*
SONATA NCN: 2014/13/D/NZ1/00061
Supervisor: Kamilla Bąkowska-Żywicka, PhD,

Bachelor's Degree in Biotechnology - Faculty of Biology, Adam Mickiewicz University 2010-2013
Poznań, Poland
Department of Molecular and Cellular Biology

Thesis: The organ-specific expression of plant S1/P1-like endonucleases.
Supervisor: Krzysztof Leśniewicz, PhD, Assoc. Prof.

Experience

Institute of Human Genetics, PAS, Department of Molecular Pathology, Independent Research Group of Non-coding Parts of the Genome Poznań, Poland **Position:** Doctoral Student **Assistance on the projects:** SONATA NCN 2016/23/D/NZ1/01611 Identification of functional MYC binding sites and target genes essential for tumor cell growth based on CRISPR / Cas9 screening OPUS NCN 2017/27/B/NZ1/00877 Identification and functional characterization of long non-coding RNAs involved in ATM-dependent DNA damage response	January 2019 - 2023
University Medical Center Groningen, Department of Pathology and Medical Biology Groningen, Netherlands **Position:** Research Trainee - International scholarship exchange of doctoral students and academic staff **Main project:** PROM NAWA 10 PPI/PRO/2019/1/00014/U/00001 Validation of eRNA expression in B-cell non-Hodgkin lymphomas in primary patient samples – granted to Marta Kasprzyk, MSc.	August 2021
International Institute of Molecular and Cell Biology, Laboratory of Zebrafish Developmental Genomics Warsaw, Poland **Position:** Research Technician **Main project:** POLONEZ NCN 2015/19/P/NZ2/03655 Deciphering the role of RNA editing in zebrafish development. **Assistance on:** SONATA NCN 2016/21/D/NZ2/03843 Elucidating the gene regulatory network of zebrafish heart development using genomics.	February 2017 - July 2017
Oklahoma Medical Research Foundation, Cell Cycle & Cancer Biology Research Program, Dean Dawson Lab Oklahoma City, Oklahoma, USA **Position:** Research Scholar – Visiting Research Graduate Traineeship Program **Project**: Revealing the Rec8 functional map by site directed mutagenesis.	July 2015 - June 2016
International Institute of Molecular and Cell Biology, Laboratory of Mitochondrial Biogenesis Warsaw, Poland Poland **Position:** Intern “Grasz o staż” - “Play for your training”	February 2015
Aarhus University - Department of Molecular Biology and Genetics Flakkebjerg, Slagelse, Denmark **Position:** ERASMUS intern	July 2014 -September 2014

Institute of Bioorganic Chemistry, PAS - Department of RNA Biology Poznań, Poland **Position:** Intern **Main project:** Influence of stress conditions on ribosome – tRF interactome in *Saccharomyces cerevisiae.* **Assistance on:** Revealing an unknown function of tRNA-derived small RNAs in *Saccharomyces cerevisiae.*	August 2013 -September 2013
Institute of Plant Genetics, PAS - Department of Plant Resistance Poznań, Poland **Position:** Intern	July 2012
Institute of Experimental Biology Faculty of Biology, AMU, Department of Microbiology Poznań, Poland **Position**: Intern	October 2011 - April 2012

Other activities

- Science popularization as part of Gen-i-już organization at the Institute of Human Genetics (October 2021-2022)
- Supervision over trainees and master student (2019-2022)

Other articles

Non-coding RNAs: Mechanisms of action (2023); Marta Elżbieta Kasprzyk, Marta Kazimierska, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk, Marta Podralska;
Elsevier. Book: Navigating Non-coding RNA. From Biogenesis to Therapeutic Application. Chapter 3:89-139. June 2023, ISBN: 9780323904063

CRISPR/Cas9 screen for genome-wide interrogation of essential MYC-bound E-boxes in cancer cells (2023); Marta Kazimierska, Marta Podralska, Magdalena Żurawek, Tomasz Woźniak, Marta Kasprzyk, Weronika Sura, Wojciech Łosiewski, Iwona Ziółkowska-Suchanek, Joost Kluiver, Anke van den Berg, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk;
Molecular Oncology, *accepted for publication in May 2023*, IF: 7.449

TransCRISPR–sgRNA design tool for CRISPR/Cas9 experiments targeting specific sequence motifs (2023);
Tomasz Woźniak, Weronika Sura, Marta Kazimierska, Marta Elżbieta Kasprzyk, Marta Podralska, Agnieszka Dzikiewicz-Krawczyk;
Nucleic Acid Research, gkad355; IF: 19.160

Adar-mediated A-to-I editing is required for establishment of embryonic body axes in zebrafish (2022);
Katarzyna Niescierowicz, Leszek Pryszcz, Cristina Navarrete, Eugeniusz Tralle, Agata Sulej, Karim Abu Nahia, Marta Elżbieta Kasprzyk, Katarzyna Misztal, Abhishek Pateria, Adrianna Pakuła, Matthias Bochtler & Cecilia Winata;
Nature Communications 13, 5520 (2022), 1-14; IF: 17.694

Human Long Noncoding RNA Interactome: Detection, Characterization and Function (2020);
Marek Kazimierczyk, Marta K Kasprowicz, Marta E Kasprzyk, Jan Wrzesinski;
International Journal of Molecular Sciences, 2020 Feb 4;21(3):1027; IF: 5.923

The widespread occurrence of tRNA-derived fragments in Saccharomyces cerevisiae (2016);
Kamilla Bąkowska-Żywicka, Anna M. Mleczko, Marta Kasprzyk, Marek Żywicki, Tomasz Twardowski;
FEBS Open Bio. 2016 Oct 21;6(12):1186-1200; IF: 2.517

tRNA-derived short RNAs bind to Saccharomyces cerevisiae ribosomes in a stress-dependent manner and inhibit protein synthesis in vitro (2016);
Kamilla Bąkowska-Żywicka, Marta Kasprzyk, Tomasz Twardowski;
FEMS Yeast Research 2016 Sep;16(6); IF: 3.332

RNA processing – unusual mechanism of generation of novel classes of noncoding RNAs from functional RNAs (2014);
Marta Kasprzyk, Anna M. Mleczko, Kamilla Bąkowska-Żywicka,
Postępy Biochemii 60(3): 295-304; IF: 0.535

*equal contribution

INTRODUCTION

Non-Hodgkin Lymphomas (NHLs) represent one of the most common blood malignancies and include a heterogenous group of cancers, originating from B cells, T cells and NK cells[1,2]. Although some NHL types are common in children, the usual onset of the disease occurs over the 55 year of age. Worldwide NHL incidence is 5.1 per 100,000 cases and accounts for 2.5 deaths per 100,000[3]. Several factors were identified to underlie the NHL pathogenesis. Among them are infections with certain viruses such as: Epstein-Barr virus (EBV)[4], Hepatitis C virus[5], Human T-cell leukemia virus type 1[6], Human herpesvirus[7], Human Immunodeficiency Virus[8] but also bacterial infection with *Helicobacter pylori*[9], environmental factors[10], chronic inflammation[11] and genetic factors such as chromosomal translocations[12].

In this doctoral dissertation I focused on B-cell derived, aggressive NHLs: Burkitt Lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL).

Burkitt Lymphoma (BL)

Burkitt Lymphoma is a rapidly proliferating NHL originating from mature germinal center B-cells. Historically, BL was divided into three subtypes: endemic – known also as African variant, occurring mainly in children and often connected with EBV infection, with a characteristic jaw swelling due to affected lymph nodes; sporadic – known as non-African or non-endemic, EBV infection is less frequently diagnosed, mostly affects the abdominal region; and immunodeficiency-associated – due to human immunodeficiency virus (HIV) infection, congenital or acquired immunodeficiency (e.g. as a result of bone barrow transplantation)[13,14]. This type of BL classification was already criticized in 1999[15]. Current knowledge shows that the BL classification based solely on EBV-infection allows for distinction of two biologically different groups with their own molecular characteristics. Indeed, the latest 5th edition of the World Health Organization Classification of Haematolymphoid Tumours[16] divides BL into EBV-positive and EBV-negative, regardless on their geographic location. Whole-genome and transcriptome data confirm that both types have different mutational landscapes[17]. Relative to EBV(-) BL, EBV(+) BL are characterized by higher expression of activation-induced cytidine deaminase (AICDA), fewer driver mutations, especially in genes connected with apoptosis and increased aberrant somatic hypermutation[17].

Common feature of all BLs is the chromosomal translocation placing the proto-oncogene MYC under the control of immunoglobulin enhancers[18,19]. Rearrangements involve either the heavy-chain (IGH), t(8;14)(q24;q32), presented on Figure 1 or light-chain immunoglobulin (IGL) loci, t(2;8)(p12;q24.1) IGK/MYC or t(8;22)(q24.1;q11.2) IGL/MYC.

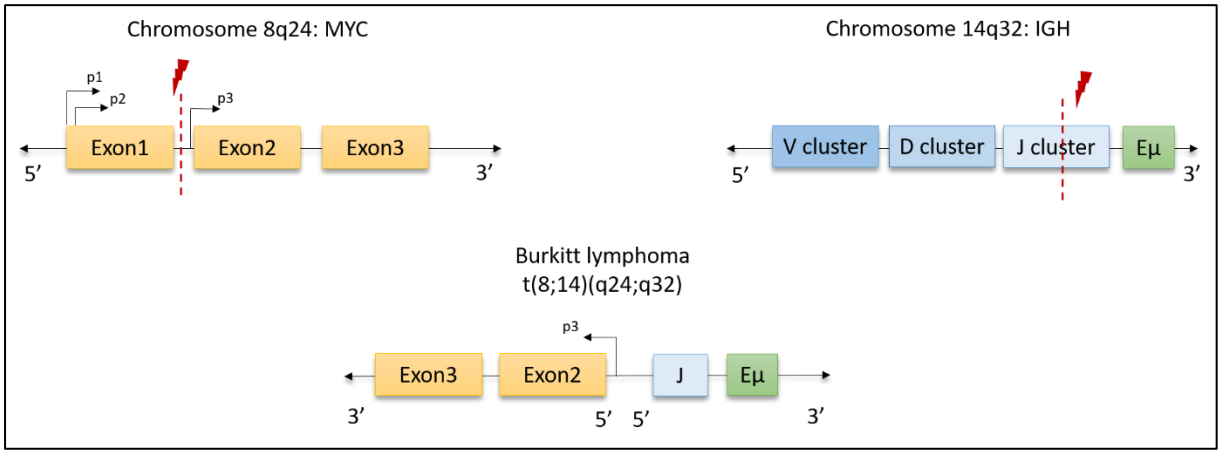

Figure 1. Scheme of MYC/IGH translocation in Burkitt lymphoma. p – promoter. Based on Atlas of Haematological Cytology (http://www.leukemia-cell.org/atlas).

Burkitt lymphoma patients experience night sweats, fever, weight loss, fatigue, nausea and abdomen pains[20]. Current treatment mainly involves chemotherapy. In pediatric patients cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP) are applied in combination with surgical resection. Stage III pediatric patients are additionally treated with high-dose methotrexate. Number of chemotherapy rounds depends on the disease stage. In adult BL patients other treatment regime is applied: R-hyper-CVAD or CODOX-M/IVACA with or without rituximab or dose-adjusted EPOCH with rituximab (summarized in Graham BS and Lynch DT, 2022[20]). Immunotherapy with rituximab (anti-CD20) is recommended for use alongside other treatments.

Since in BL MYC is deregulated, its targeting sounds promising. Unfortunately, this BHLH oncogenic transcription factor is involved in many cellular processes, such as cell cycle, proliferation and apoptosis. Due to that as well as its nuclear localization and disordered structure, MYC targeting remains challenging. Therapies for direct or indirect inhibition of the MYC oncogene are currently under investigation[21]. A promising compound Omomyc (OMO-103) prevents MYC from binding to its target E-boxes and therefore prevents activation of target genes[22]. This molecule specifically disrupts MYC interactions.

Diffuse Large B-cell Lymphoma (DLBCL)

Diffuse Large B-cell Lymphoma is the most frequently diagnosed subtype of NHL worldwide[23]. DLBCL can be found in children, but its occurrence increases exponentially with age. After diagnosis, the 5-year survival rate can reach up to 60%. DLBCL can arise from germinal center* B cells as well as activated B cells. DLBCLs are heterogenous, chromosomal translocations involving heavy and light immunoglobulin chain loci and oncogenes such as MYC, BCL2 or BCL6 are frequently observed [24]. Double and triple hit lymphomas with several concurrent translocations are also common. In this doctoral dissertation I focused on t(14;18)(q32;q21) IGH/BCL2 presented on Figure 2[25]. As a result of the chromosomal translocation, BCL2 – key negative regulator of apoptosis is upregulated which in turn favors the survival of lymphoma cells.

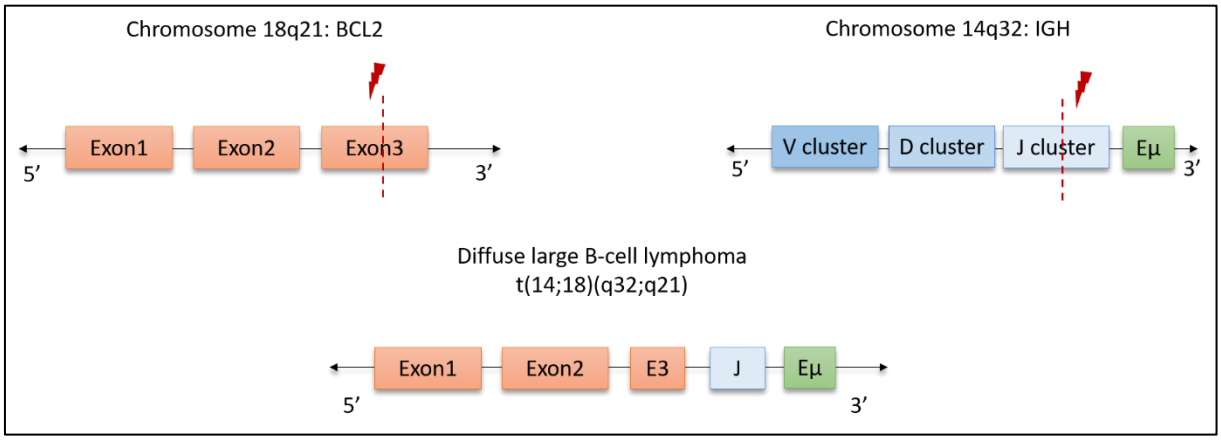

Figure 2. Scheme of IGH/BCL2 translocation in diffuse large B-cell lymphoma. Based on Atlas of Haematological Cytology (http://www.leukemia-cell.org/atlas).

DLBCL patients report swollen, quickly enlarging lymph nodes, abdominal pains, skin lumps, fever, night sweats, weight loss, fatigue[26]. Chemotherapy treatment is similar to BL, rituximab along with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) or more aggressive - rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin and prednisone (R-ACVBP). Choice of the appropriate treatment depends on the lymphoma stage, molecular subtype and whether the disease is aggressive or indolent.

BCL2 overexpression in DLBCL is connected with treatment resistance and poor prognosis. Venetoclax (ABT-199[27], GDC-0199) is the first specific BCL2 inhibitor approved for treatment of acute

* Germinal center – a structure (follicle) inside secondary lymphoid organs such as lymph nodes, in which mature B-cells undergo activation, differentiation and proliferation

myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) (reviewed in Kapoor I et al., 2020[28]). ABT-199 kills cancer cells in caspase dependent manner[27].

IGH and its enhancers

The immunoglobulin heavy chain (IGH) locus localized in human on chromosome 14, encodes a large polypeptide subunit, which is a part of an antibody (Figure 3). During B cell development in the bone marrow, *IGH* undergoes extensive remodeling. In VDJ recombination, first diverse (D) and joining (J) segments are combined forming D-J and then they are joined together with a variable (V) segment allowing formation of a primary immunoglobulin (Ig)s repertoire. V, D and J exons are flanked by recombination signal sequences (RSSs). Recombination activating gene (RAG) proteins 1 and 2 introduce a cut between RSS and the coding sequences of the two rearranging gene segments, therefore initiating VDJ recombination. The obtained ends undergo non-homologous ends joining (NHEJ), also releasing the DNA in between. Upon joining, nucleotides may be removed from the ends and moreover, terminal nucleotidyltransferase (TdT) can add non-germline nucleotides (N sequences), which can as well contribute to the diversity of the variable region.

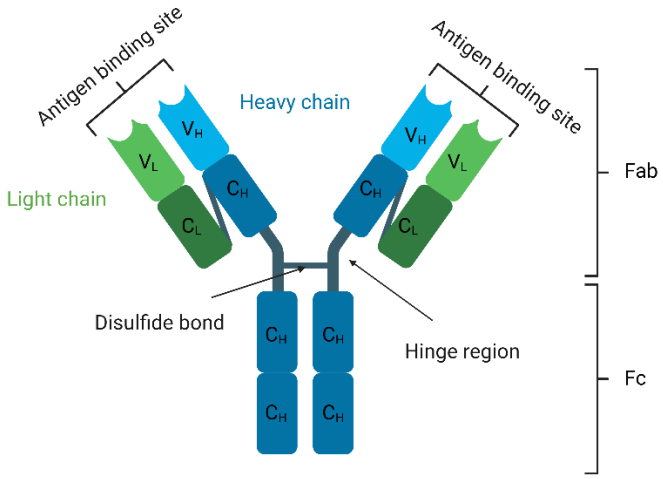

Figure 3. Scheme of an antibody. H – immunoglobulin heavy chain; L – immunoglobulin light chain; V – variable region; C – constant region; Fac - fragment antigen-binding region; Fc - fragment crystallizable region, this part interacts with Fc receptors on cell surface. Created in BioRender.

Two other important processes within the *IGH* locus include somatic hypermutation (SHM) and class switch recombination (CSR). The former introduces point mutations to the rearranged VDJ segment, allowing further diversification of antibodies. Activation-induced cytidine deaminase (AID) is a major player

in this process, deaminating cytosine in the DNA to uracil. Upon this change guanine-cytosine pair can no longer be established. To repair the resulting mismatch high-fidelity base excision repair enzymes come into action. Uracil-DNA glycosylase removes the uracil from the DNA, followed by DNA polymerases filling the created gap and creating the mutation. Those alterations tend to be accumulated in the complementarity determining regions (CDRs) of the V genes, which will be in direct contact with the antigen.

In naïve mature B cells immunoglobulins of the IgM and IgD classes are produced. However, they are of a low affinity. Upon antigen encounter IgM and IgD expressing B-cells proliferate and can be further specialized by CSR. This process allows switching the antibody class to IgG, IgA or IgE, each of a different functional characteristics. In CSR the *IGH* constant region (C) is changed, while the rearranged and hypermutated V(D)J region is not further altered. This allows an increase in antibody affinity. AID also plays a role in CSR, since uracil residues can be converted to DNA-double strand breaks (DSBs). The CSR complex includes also other components such as base excision repair enzymes, mismatch repair proteins, error-prone DNA polymerases and NHEJ proteins.

The *IGH* expression is controlled by the super-enhancer region composed of the intronic Eμ enhancer, localized between J and Cμ, and 3' regulatory region (3'RR) (reviewed in Kasprzyk ME, Sura W, Dzikiewicz-Krawczyk A, 2021[29]). Eμ plays an important role at the early stages of B cell development, namely VDJ recombination[30]. This intronic enhancer controls local chromatin accessibility, promoting histone acetylation in the *IGH* locus prior to recombination[31]. Eμ is also important for allelic exclusion, ensuring that the *IGH* expression will occur only from one allele[32]. 3'RR, often referred to as the locus control region takes control at the later developmental stages[33]. In contrast to mice, humans have two 3'RRs (3'RR1 and 2). They are highly similar in sequence and each is composed of a cluster of several DNase I hypersensitive sites. Upon deletion of 3'RR SHM and CSR are severely affected[34,35].

Mechanism of chromosomal translocation

Chromosomal translocation is an event in which two chromosomes are incorrectly joined together after a break repair[36]. RAG proteins recognize RSS sequences flanking VDJ segments, but those sites are also present outside the *IGH* locus[37]. The RSS is composed of the heptamer sequence (5'-CACAGTG-3') and a nonamer sequence (5'-ACAAAAACC-3'), which are separated by 12-23 non-conserved base pairs. Aberrant VDJ recombination, involving cutting of cryptic RSS and joining with *IGH* contributes to the chromosomal translocation[38]. Chromosomal breakages happen in consistent regions. In case of t(14;18)

IGH/BCL2, the major breakpoint (MBR) in *BCL2* locus occurs within specific approximately 150 bp region.[39] Mistakes can occur also during CSR. Activity of AID tends to be restricted to the Ig loci, but the enzyme can also target actively transcribed genes, including oncogenes like BCL6 or MYC[40]. As shown by Roix JJ et al., spatial proximity of translocation-prone oncogenes is another factor contributing to chromosomal aberrations involving *IGH*[41].

Interestingly, a number of B cells carrying *IGH* translocation can be found also in healthy individuals[42,43]. This suggests that the translocation alone is not sufficient to turn a B cell malignant. The altered activity of translocated oncogenes by *IGH* enhancers and accumulation of other mutations over time play a major role in lymphomagenesis[44].

B-cell receptor (BCR)

BCR is composed of a heterodimer Ig-α/Ig-β (CD79a/b) transducing signal to the cell interior and a membrane-bound immunoglobulin (IgD, IgM, IgA, IgG, or IgE) (Figure 3). Binding of an antigen by BCR, activates a signaling cascade in B cell leading to its proliferation and further differentiation into an antibody secreting plasma cell or a memory B cell.

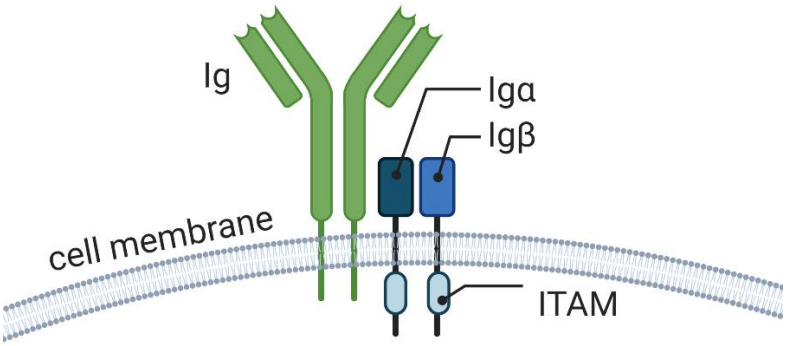

Figure 3. Scheme of B cell receptor. Ig – membrane-bound immunoglobulin; Igα, Igβ – signal transducing heterodimer; ITAM – immunoreceptor tyrosine-based activation motif. Created in BioRender.

In the organism, B cells are tested for the functional BCR. Allelic exclusion mechanism plays an important role in ensuring that each B cell will express only one type of BCR (reviewed in Vettermann C. and Schlissel MS., 2011[45]). While the recombination of D and J segments in *IGH* occurs on both alleles, joining with a V segment occurs only on one allele, termed a productive allele. An attempt to use the other allele will happen only if the first try was unsuccessful (unproductive). In pro-B cells rearranged VDJ heavy chain is tested for functionality by coupling with the surrogate light chain, forming a pre-B cell receptor

(pre-BCR). Next, the rearrangement of the immunoglobulin light chain occurs. First kappa light chain undergoes remodeling. If it fails, another attempt will be made with the lambda light chain. B cells that fail to express functional heavy and light immunoglobulin chains undergo apoptosis. BCR signaling can be divided into tonic, chronic active and priming[46]. Tonic BCR signaling means constitutive, low-level signaling, independent of an antigen binding. It is important for the survival of resting B cells and is mediated by the PI3K/AKT/FOXO/mTOR pathways[47,48]. Priming BCR signaling is antigen-driven and necessary for the initiation of B cell activation and differentiation into antibody-secreting cells (recently reviewed in Kwak K., Akkaya M. and Susan K. Pierce S.K., 2019[49]). Another antigen-induced signaling is the chronic active BCR signaling, which is found necessary for the survival of in the activated B-cell-like (ABC) lymphomas[50–52]. This type of BCR signaling is characterized by constitutive NF-κB pathway activity[50].

In translocated *IGH* locus, one allele is unproductive, but the other can produce functional BCR. Deregulated BCR signaling due to genetic aberrations contributes to the survival and proliferation of B-cell NHLs[53]. Several therapeutics such as ibrutinib, idelalisib, dasatinib, fostamatinib, everolimus inhibiting downstream effectors of BCR proved efficient in chemo-resistant cases[54–56].

Enhancer RNAs

Enhancer RNAs (eRNAs) are a class of non-coding RNAs (ncRNAs) transcribed from active enhancer regions (Figure 4A)[57]. Initially considered as a transcriptional noise, nowadays are more often being proved functional. This ncRNAs are usually unstable and expressed at low levels and are subjected to RNA exosome degradation as a part of the regulation of their long-distance genomic interactions (Figure 4B)[58]. Enhancer RNAs can facilitate long-range promoter-enhancer interactions[59], allowing to achieve the proper spatial conformation (Figure 4C). They can serve as baits in recruitment of different proteins, for example cohesins[59] or transcription factors (TFs) (Figure 4D)[60]. Enhancer transcription, but not the transcript was found essential in deposition of mono- and dimethylantion of lysine 4 on histone 3 at *de novo* enhancers upon TLR4 signaling in macrophages[61]. In contrast, in oestrogen-dependent transcriptional activation the eRNA transcript itself was proven functional[59]. So far the knowledge regarding *IGH* eRNAs in NHL is very limited.

IGH enhancers as therapeutic targets

Several studies have attempted to shed light on the Eμ and 3'RR enhancers involvement in the control of translocated oncogenes. Developed mouse models clearly indicated that the deregulation of oncogene expression by *IGH* enhancers is the driving force of B cell malignant transformation (reviewed recently in more details in Kasprzyk ME et al. 2021[29]). Insertion of 3'RR DNase I hypersensitive sites in the

murine Bcl2 locus resulted in follicular lymphoma development[62]. Mimicking the t(3;14)(q27;q32) translocation in mice with the use of *Bcl6* transgene lead to development of lymphoma in small fraction

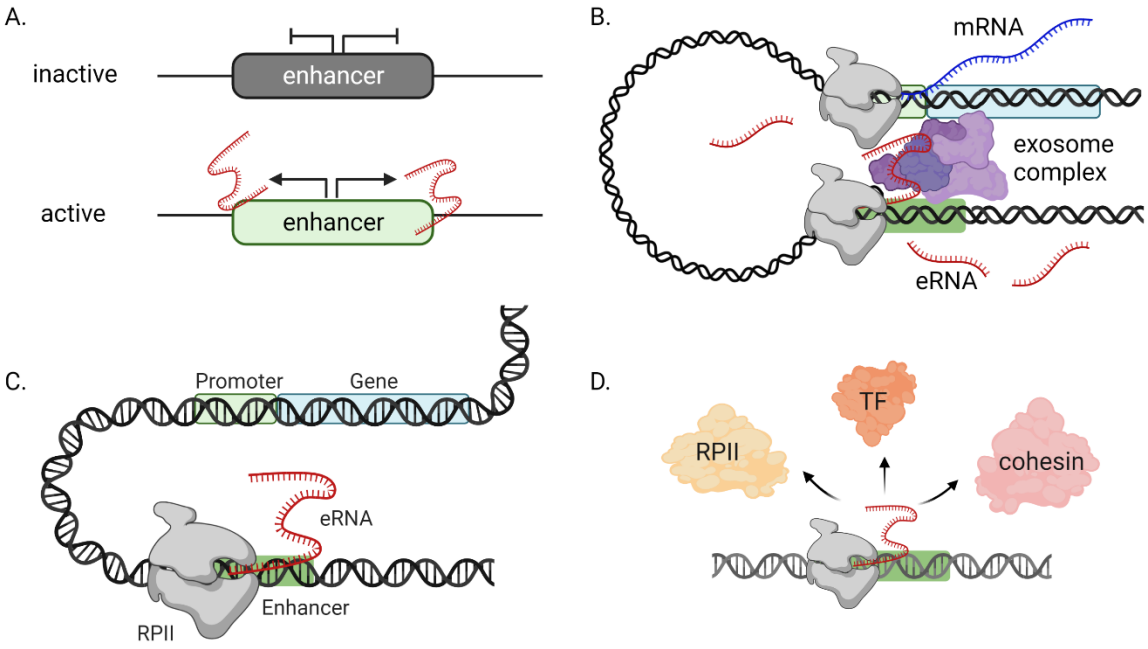

Figure 4. Enhnacer RNAs (eRNAs). A. eRNAs are transcribed from active enhancers. B. Exosome-mediated degradation of eRNAs. C. eRNAs can facilitate enhancer-promoter looping. D. eRNA can serve as baits for different proteins. Created in BioRender.

of animals, but the incidence was increased upon administration of carcinogen N-ethyl-N-nitrosourea[63]. Mouse model with *Myc* knock-in into the *IGH* locus imitates human Burkitt lymphoma[64].

Since the common feature of B-cell NHLs are recurrent chromosomal translocations, juxtaposing the oncogenes with the *IGH* enhancers, targeting their activity instead of the singular oncogenes could be a more universal approach in potential therapy. Eμ and 3'RRs are also important for normal B cells, therefore tackling their functionality could also negatively affect the healthy cells. However, B cell depletion therapies proven to be successful in the clinic[65]. So far only a few compounds were shown to alter *IGH* enhancers activity (discussed in more details in Kasprzyk ME et al. 2021[66]), but suggesting that they can be druggable. Understanding the gene expression regulation by Eμ and 3'RRs in malignant B cells is of a key importance.

SCIENTIFIC AIM

The role of *IGH* enhancers in B cells development and maturation is rather well established, but their involvement in lymphomagenesis and control of translocated oncogenes remains elusive. The main aim of this dissertation was the functional analysis of the *IGH* gene regulatory regions and enhancer RNA (eRNAs) in B-cell non-Hodgkin's lymphomas.

Detailed aims:

1. Providing an overview of the current state of knowledge about the involvement of enhancers in B-cell malignancies.
2. Identification of transcriptionally active regions in *IGH* gene enhancers and determination of the role of *IGH* enhancers and eRNAs in B-cell lymphomas.
3. Testing the potential of inhibition of *IGH* enhancers using small molecules.

1st ARTICLE

Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer

Marta E Kasprzyk, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk,
MDPI Cancers 2021, 13(13), 3270, published: 29th June 2021

Content:

Blood cancers can originate from B-cells at different developmental stages, giving rise to a heterogenous group of lymphomas (Hodgkin and non-Hodgkin) as well as leukemias. Several mechanisms of setting B-cells on the path towards malignant transformation have been described so far. Among them altered epigenetic regulation can be distinguished. Indeed, enhancers – regulatory DNA elements that control gene expression - play an important role in driving carcinogenesis in B-cells. The proper functioning of B-cell-specific enhancers ensures the correctness of the multistage development and differentiation of those cells and maintains their important role in the organism defense system – antibodies production. Enhancer function in B-cells may be profoundly disrupted by germline variants, somatic mutations, hijacking by viruses e.g. Epstein–Barr virus or Kaposi's sarcoma-associated herpesvirus, deregulated transcription factors activity or chromosomal translocations, juxtaposing enhancers with oncogenes. Understanding those mechanisms provides an opportunity for novel therapies development. This review summarized current knowledge regarding roles of IGH (Eμ and 3'RRs), lGK and IGL enhancers in normal and malignant B-cells. We provided overview of the mechanism of chromosomal translocations and prepared detailed figures visualizing breakpoints within immunoglobulin heavy and light chain genomic regions and frequently translocated oncogenes. Moreover, listed in this work are mouse models of *IGH* translocations. We also point out importance of enhancer variants and mutations in B-cell neoplasms and sum up recent reports of exploitation of B-cell enhancers by altered activity of transcription factors (such as PAX5 and RUNX1) and lymphoma-associated viruses.

Conclusions:

In the recent years, there is an increasing body of evidence, showing the important role on the non-coding genome in the cancer onset. In this review article, we summarized the current knowledge regarding B-cell enhancers involvement in malignant transformation. Those non-coding regions can be exploited through

several mechanisms. Notwithstanding, the cell-type specificity of enhancers makes them attractive therapy objectives. Further research is required to asses precise sites/regions that can be efficiently targeted.

MEK contribution:

In this work I was responsible for: literature review, writing, figures and tables preparation for chapters:

- 1. Introduction
- 2.1.2. The 3' Regulatory Region
- 2.1.3. Interplay between Eμ and 3'RR Enhancers
- 2.3. Role of IGH Enhancers in Regulating Oncogene Expression and Malignant Development.

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1st ARTICLE – full text

cancers

MDPI

Review

Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer

Marta Elżbieta Kasprzyk, Weronika Sura and Agnieszka Dzikiewicz-Krawczyk *

Institute of Human Genetics, Polish Academy of Sciences, 60-479 Poznań, Poland; marta.kasprzyk@igcz.poznan.pl (M.E.K.); weronika.sura@igcz.poznan.pl (W.S.)
* Correspondence: agnieszka.dzikiewicz-krawczyk@igcz.poznan.pl; Tel.: +48-61-657-9219

Simple Summary: B-cell malignancies are a heterogenous group of lymphomas and leukemias and are the 6th most common cancer-related cause of death. Apart from several oncogenes and tumor suppressors involved in their pathogenesis, recently the role of non-coding, regulatory sequences has been implied. Enhancers are DNA elements controlling gene expression to ensure proper cell development and function. However, the activity of enhancers can be redirected, setting cells on the path towards cancer. In this review, we discuss different mechanisms through which enhancers are exploited in malignant B cells. We also highlight the potential of therapeutic targeting of enhancers as a direction for future investigation.

Abstract: B-cell lymphomas and leukemias derive from B cells at various stages of maturation and are the 6th most common cancer-related cause of death. While the role of several oncogenes and tumor suppressors in the pathogenesis of B-cell neoplasms was established, recent research indicated the involvement of non-coding, regulatory sequences. Enhancers are DNA elements controlling gene expression in a cell type- and developmental stage-specific manner. They ensure proper differentiation and maturation of B cells, resulting in production of high affinity antibodies. However, the activity of enhancers can be redirected, setting B cells on the path towards cancer. In this review we discuss different mechanisms through which enhancers are exploited in malignant B cells, from the well-studied translocations juxtaposing oncogenes to immunoglobulin loci, through enhancer dysregulation by sequence variants and mutations, to enhancer hijacking by viruses. We also highlight the potential of therapeutic targeting of enhancers as a direction for future investigation.

Keywords: B-cell lymphoma; B-cell leukemia; enhancer; IGH; IGK; IGL; EBV

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1. Introduction

B-cell malignancies are a diverse group of blood cancers which include several types of leukemias and lymphomas: Hodgkin's lymphoma and non-Hodgkin lymphomas [1–3]. They originate from B cells at different developmental stages [4]. Among all cancers, lymphoid malignancies are reported to be the 6th cause of death in the United States [5]. Several factors have been implicated in the pathogenesis of B-cell neoplasms, from genetic mutations, altered miRNA and lncRNA expression to epigenetic changes [4,6–10].

Enhancers are regulatory DNA elements with a pivotal role in shaping cell type-specific transcriptional programs in response to intra- and extracellular signals [11]. They contain sequences recognized by transcription factors and serve as platforms for assembly of an enhanceosome [12]—a multi-protein complex, able to recruit chromatin remodelers and RNA polymerase at the promoter region of target gene, and eventually lead to its expression. Characteristic features of active enhancers include DNase I hypersensitivity indicating open chromatin, presence of binding sites for multiple transcription factors, binding of transcription co-activators and presence of specific chromatin signature marks, such as high histone H3 lysine 4 monomethylation (H3K4me1) but low trimethylation (H3K4me3),

and high histone H3 lysine 27 acetylation (H3K27ac) [13]. Enhancers are essential for proper development and functioning of organisms, while their dysregulation might lead to disease, including cancer [14,15].

B-cell neoplasms are a classical example of the enhancer involvement in malignant transformation. The first described eukaryotic enhancer was an intronic Eμ enhancer in the immunoglobulin heavy chain locus [16]. Up to date, several B-cell specific enhancers have been described. Their activity allows for a proper B-lymphocyte differentiation and fulfilling their main function: secretion of high-affinity antibodies [17–19]. However, the activity of enhancers can be redirected, setting B cells on the path towards cancer. In this review we discuss different mechanisms through which B-cell enhancers are exploited in malignant cells. In Sections 2 and 3, we describe the well-studied translocations juxtaposing oncogenes to immunoglobulin heavy or light chain loci. We also discuss mechanisms leading to Ig translocations and the role of Ig enhancers in regulating oncogene expression and malignant development. In Section 4, we present how enhancer dysregulation by germline variants and somatic mutations contributes to development of B-cell neoplasms. Exploitation of enhancers by deregulated transcription factors is described in Section 5. Finally, in Section 6 we focus on enhancer hijacking by certain viruses, showing how B-cell enhancers can be repurposed for viral replication and lymphomagenesis. We also highlight the potential of therapeutic targeting of enhancers as a direction for further investigation.

2. Immunoglobulin Heavy Chain Enhancers in B-Cell Malignancies

2.1. Structure and Function of IGH Enhancers in Normal B Cells

The *IGH* locus contains several variable (V), diversity (D), joining (J) and constant (C) segments which undergo sequential rearrangements in the course of B-cell maturation to produce the large polypeptide subunit of all classes of immunoglobulins. In the early stage of B-cell development, V(D)J recombination initiated by RAG1 and RAG2 endonucleases brings together one of the different V, D and J gene segments of the *IGH* variable region. Assembly of the recombined VDJ with the Cμ or Cδ constant region results in expression of IgM or IgD molecules, respectively [20]. In mature B cells, antigen-dependent activation triggers somatic hypermutation (SHM) during the germinal center reaction. This leads to further diversification of the variable region of *IGH* and allows selection of B cells with high affinity B-cell receptor. Similarly, upon antigen encounter, class switch recombination (CSR) brings the fused VDJ gene segment in proximity to one of the Cγ, Cε or Cα constant region exons, switching from the expression of IgM/IgD to IgG, IgE or IgA, respectively. SHM and CSR depend on the activation-induced cytidine deaminase (AID) [21,22]. The *IGH* locus contains two enhancers that govern its activity: Eμ and 3′ regulatory region (3′RR).

2.1.1. Intronic Eμ Enhancer

The Eμ enhancer (also known as the intronic enhancer) was the first eukaryotic enhancer described; it was proven to have strong promoter-, distance- and orientation-independent activity in *cis*, specific to B cells [16,23–25]. Eμ resides in the intron between the J_H region and Cμ exons (upstream to the switch recombination region). It consists of a 220 bp core enhancer element containing sites recognized by multiple transcription factors, flanked by two 310-350 bp matrix attachment regions (MARs) (Figure 1). Control elements within the core enhancer include C/EBP, E1, E5, E2, μA (bound by Ets-1), E3 (bound by TFE3, TFEB, and USF), μB (bound by PU.1), E4, and an octamer sequence (bound by Oct1 and Oct2 together with the specific coactivator OCA-B). Additionally, E2, E4 and E5 are positively regulated by E2A, E2-2, and HEB, in contrast to the negative regulation of E4 and E5 by ZEB (summarized in [26,27]). MARs comprise sites of attachment to the nuclear matrix and contain binding sites for Bright in B cells [28,29], otherwise bound by a negative regulator, Cux/CDP, in non-B cells [30]. Although a limited region containing μA, E3, and μB is sufficient to activate transcription in B cells [31,32], the whole core enhancer element and MARs are postulated to function as locus control region (LCR) [28] and are necessary for efficient transcription of the IGH μ transcript (from V_H promoter) [33–35].

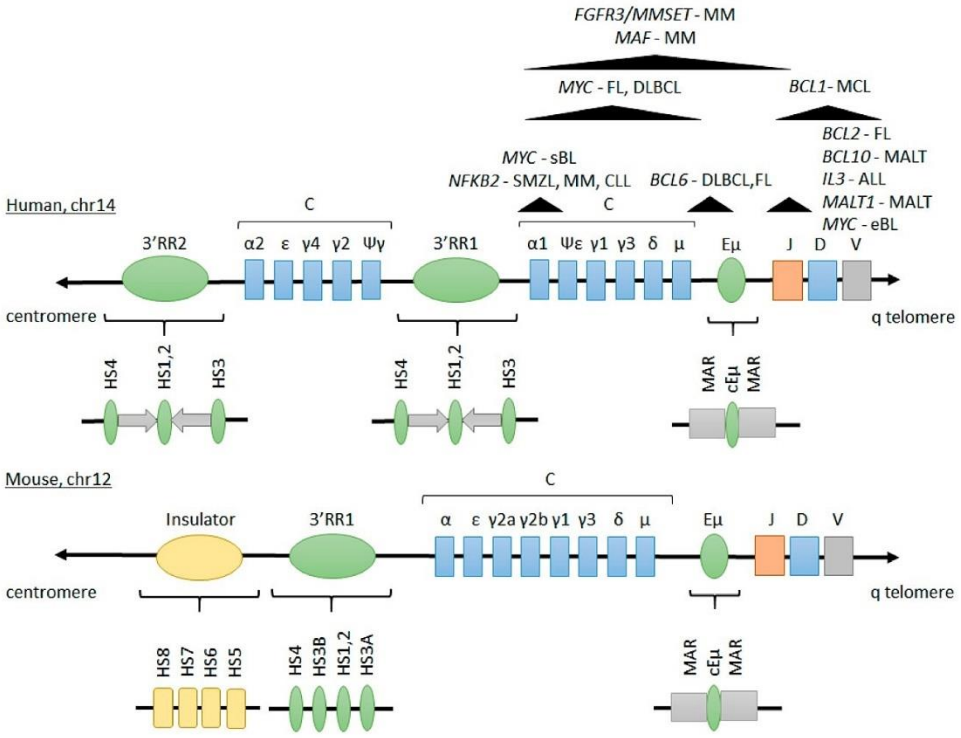

Figure 1. *IGH* locus organization in human and mice. Black triangles mark regions of breakpoints involved in translocations in malignant cells. C—constant region; J—joining; D—diversity; V—variable; HS—DNase hypersensitive site; MAR—matrix attachment region; 3′RR—3′ regulatory region; ALL, acute lymphoblastic leukemia; eBL, endemic Burkitt lymphoma; sBL, sporadic Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MM, multiple myeloma; SMZL, splenic marginal zone B-cell lymphoma.

The intronic enhancer is active throughout B cell development, although especially important in the early stages [27]. It is necessary for the V(D)J recombination—in the absence of its core element, D-J and V-DJ rearrangements are severely impaired [36–39]. Eμ control of this process is connected to transcription. Prior to D-J recombination, transcription of the Iμ transcript initiates from the Eμ enhancer [40]. At the same time Eμ-dependent D_H intergenic antisense transcription starts from the enhancer [41]. The intronic enhancer also promotes histone acetylation in the *IGH* locus before recombination, increasing its accessibility [42]. Moreover, Eμ seems to be responsible for the proper timing of V(D)J recombination, as it initiates the process in pro–B cells but not in pre–B cells [43]. Prior to recombination, the *IGH* locus undergoes radial repositioning and two levels of chromosomal compaction involving formation of multi-looped domains; these processes are also dependent on Eμ [44]. Crucial for the topological alterations are three transcription factors: PAX5, YY1 and CTCF, and the interaction between Eμ and intergenic control region 1 (IGCR1) (reviewed in [45]). Moreover, the Eμ/IGCR1 loop limits RAG1/2 tracking in the first step of V(D)J recombination from the J_H-related recombination centre (RC) to a domain containing D_H and J_H gene segments, so the recombination occurs only between D_H and J_H segments (no V_H gene segments) [46]. After V(D)J recombination, Eμ is involved in the checkpoint for allelic exclusion at the pre-B cell to immature B cell transition [47].

The enhancer ensures sufficient Ig μ chain expression required for proper signaling in this process [48].

Studies of the role of Eμ in SHM and CSR initially led to contrary conclusions (reviewed, e.g., in [49]), but it was likely due to the fact that in the absence of Eμ, V_H assembly is severely disturbed, which results in the arrest of B cell development. Analysis of mice devoid of Eμ enhancer, but carrying fully assembled V_H gene showed that Eμ contributes to both SHM and CSR, yet is not essential for them [50].

2.1.2. The 3′ Regulatory Region

The 3′RR lies downstream of the Cα gene segment and differs between human and mouse [51–54]. In humans and other Hominoidea (chimpanzee, gorilla, gibbon) 3′RR is duplicated and each region is composed of a 5′→3′ satellite repeat, containing 20 bp tandem repeats, and 3 enhancers: hs3, hs1.2 and hs4. Mouse and rat single 3′RR consist of a 5′→3′ satellite repeat and 4 enhancers: hs3a, hs1.2, hs3b, hs4 as well as 4 insulators: hs5, hs6, hs7, hs8 (Figure 1). In the 3′RR organization, proximal (containing enhancers hs3, hs1.2) and distal (containing hs4) elements are distinguished [53]. Phylogenetic analysis by D'Addabbo et al. showed high sequence similarity of both 3′RRs; 3′RR2 in human being evolutionary older than 3′RR1 [52]. Primate 3′RRs are characterized by the presence of locally repetitive elements with short tandem repeats, similar to switch sequences found in *IGH* locus. On the contrary, in rodents those short tandem repeats are organized in families and are interspaced through the 3′RR palindrome [55]. Hs1.2 is the center of the "quazi-palindrome" flanked by 3 kb inverted sequences, which are conserved in mammals, but not in evolutionarily distant species [52,56–58]. The orientation of human hs1.2 enhancers within 3′RR1 and 3′RR2 is also inverted. In mice, hs3a and hs3b enhancers, which are inverted copies of each other, are also part of the palindrome [52]. Preserving the palindromic organization is of key importance for some of 3′RR-controlled functions. Its deconstruction leads to decreased V_H germline transcription, AID recruitment and SHM, while IGH transcription and CSR remain relatively unaffected [52,56–59]. In humans four allelic variants of hs1.2 have been identified for 3′RR1 and two for 3′RR2 [60,61]. A polymorphism of hs1.2 enhancer is involved in immunological diseases, among others: herpetiform dermatitis, coeliac disease, rheumatic arthritis, diabetes or IgA deficiency [52,62–64].

3′RR is often referred to as the master regulator of the *IGH* locus [54,61]. Indeed, it has been implicated in control of majority of recombination events happening at this location [65]. Studies in 3′RR deficient mice revealed that V(D)J is not affected in pre-B cells, supporting the reports that 3′RR activity is obligatory for later developmental stages of B cells [53,65,66]. However, it is speculated that 3′RR might take part in allelic exclusion. 3′RR-mediated inhibition of the *IGH* variable region has been reported, resulting in suppression of V_H-DJ_H recombination. When V(D)J is completed, this effect is abolished [67]. 3′RR is indispensable for SHM and CSR. In B cells from mice lacking 3′RR, heavy chain cannot undergo SHM, while SHM in light chain is not affected [68–70]. 3′RR is controlling *IGH* accessibility for AID to enable SHM [58,70]. In order to study 3′RR function in CSR, several knock-out models have been applied [53,70–74]. It has been demonstrated that knocking-out the whole 3′RR significantly impairs CSR, but deletions of single enhancers from 3′RR leave CSR relatively unaffected [58,75]. Combined removal of hs3b and hs4, on the other hand, decreased IGH expression and CSR [71,72,76]. Another study suggested 3′RR involvement in CSR only at its early stages [77]. Interestingly, CSR to IgD was reported as independent of 3′RR regulation [78,79]. Recently, a long non-coding RNA CSR^{IgA} has been identified to interact with hs4 and play an important role in IgA CSR [80].

2.1.3. Interplay between Eμ and 3′RR Enhancers

Although *IGH* enhancers show developmental-dependent manner of activation, they do not act as solitary units. Complex spatial interactions between enhancers themselves, other components of the *IGH* locus and transcription factors were observed [44,54,81,82]. Eμ and 3′RR are separated by ~200 kb and this distance and their spatial relation (3′RR down-

stream of Eμ) are important for their synergy [83,84]. 3C experiments detected chromatin loop formation between 3′RR and *IGH* variable region [85]. The hs1.2 enhancer emerged as an important player in this interaction. Upon its substitution with Neo^R, loop formation and IGH transcription were abolished, while Eμ proved dispensable for this interaction. Moreover, hs1.2 quadruplex formation was speculated to regulate transcription factor binding [86]. During CSR, chromatin looping occurs between 3′ RR and Eμ, enabling isotype-specific S-S synapsis formation and possibly reducing the threat of unwanted chromosomal translocations [87,88]. Recent profiling of epigenetic marks and enhancer RNAs (eRNAs) transcription during CSR revealed that in later stages of B cell development, Eμ is actually placed under the 3′RR control. Despite the experimentally confirmed physical association of both enhancers during CSR, Eμ might be dispensable. Its deletion did not affect germline transcription, nor 3′RR epigenetic marks and eRNA expression, while on the other hand deletion of 3′RR reduced transcription rate around Eμ and decreased its H3K9ac [89]. These results further support 3′RR enhancer as the master regulator of the IGH locus.

2.2. *IGH* Translocations in B-Cell Malignancies

The V(D)J recombination and CSR machineries generate several DNA double strand breaks as obligate intermediates, whereas SHM may result in nonmandatory DSBs. These lesions pose a danger of illegitimate recombination outside of the *IGH* locus. The resulting translocations may lead to activation of oncogenes placed under the control of *IGH* enhancers, which is regarded as an early oncogenic hit driving lymphomagenesis. Indeed, several recurrent translocations involving *IGH* have been described in B-cell malignancies. Interestingly, *IGH* translocations occur as well in healthy B-cell populations, which implies that alone they are insufficient to invoke oncogenesis [90–93]. Likely, genomic instability caused by translocated oncogene deregulation leads to accumulation of other mutations [94]. This is also supported by the in vivo experiments where oncogene overexpression results in malignancy only in a favourable genetic background [95,96].

2.2.1. Mechanisms of *IGH* Translocations

Occurrence of translocations between *IGH* and oncogenes has been mainly attributed to the off-target activity of two key players involved in *IGH* rearrangements: recombination activating gene (RAG) 1 and 2 proteins and activation-induced cytidine deaminase (AID). RAG1 and RAG2 initiate V(D)J recombination in pro-B cells. These lymphocyte-specific endonucleases recognise recombination signal sequences (RSS) of the rearranging segments and cut them exactly between a pair of RSSs and coding sequences. Then, the ends may be additionally modified and finally are ligated by the enzymes of the non-homologous end joining pathway (NHEJ) [97]. However, cryptic RSSs are present throughout the genome and can be processed by RAG [98]. The off-target activity of RAG is determined by various factors, e.g., histone marks, CpG islands or chromatin architecture [46,99,100].

SHM and CSR are completely dependent on AID [101,102] which transforms deoxycytidine into deoxyuridine at the specified sections of Ig loci, inducing error-prone DNA repair. AID displays preference to deaminate cytosine within the WRC motif (where W = A/T, R = A/G), both in vitro [103] and in vivo [104,105], resulting in certain hotspots, influenced additionally by genomic context [106]. Importantly, switch regions contain a double-WRC motif AGCT, in which two adjacent deaminated cytidines lead to double strand breaks in CSR [105,107]. The *IGH* 3′RR enhancer interacting with the Eμ enhancer and appropriate germline transcription promoters of switch regions, brings them together to enable DNA recombination between the S regions [88]. Due to strict regulation, AID activity is mostly restricted to the Ig loci. However, the enzyme also targets a group of actively transcribed genes, including proto-oncogenes like *BCL6*, *MYC*, *CD79A*, *CD79B*, *CD95*, *PIM1*, *MYC*, *RHOH*, *PAX5* [97]. Both hypermutations of those genes [108–111] as well as their translocations (resulting mostly from erroneous CSR) occur in tumours and in a certain subset of normal B cells [112].

Table 1. Translocations involving immunoglobulin heavy chain locus in B-cell malignancies.

Genes Involved	Translocation	Disease	Consequences	References
BCL2	t(14;18)(q32;q2)	90% FL 15–30% DLBCL	Delayed apoptosis and accumulation of aberrant cells	[132–135]
BCL3	t(14;19)(q32;q13)	CLL, NHL	Modulation of the NF-kB pathway	[136–139]
BCL6	t(3;14)(q27;q32)	30% DLBCL 4-14% FL	Increased cell proliferation, block of terminal differentiation	[140–147]
BCL10	t(1;14)(p21;q32)	5% MALT	Activation of the NF-kB pathway (translocation involves a mutant BCL10 which lost pro-apoptotic functions)	[148–150]
CCND1 (BCL1)	t(11;14)(q13;q32)	95% MCL 15–20% MM B-PLL, PCL, SLVL	Accelerated passage through the G1 phase	[151–156]
CEBPA	t(14;19)(q32;q13)	ALL	Deregulated cellular proliferation and differentiation	[157–160]
CEBPB	t(14;20)(q32;q13)			
CEBPD	t(8;14)(q11;q32)			
CEBPE	t(14;14)(q11;q32)			
CEBPG	t(14;19)(q32;q13)			
FGFR3/MMSET	t(4;14)(p16;q32)	10% MM	Increased cell proliferation and survival	[161–166]
FOXP1	t(3;14)(p14;q32)	10% MALT DLBCL	Enhanced tumor cell survival	[167–169]
IL3	t(5;14)(q31;q32)	ALL	Increased cell proliferation and survival	[170,171]
MAF	t(14;16)(q32;q23)	MM	Increased cell proliferation	[172–175]
MALT1	t(14;18)(q32;q21)	15–20% MALT	Activation of the NF-kB pathway	[167,176,177]
MYC	t(8;14)(q24;q32)	70% BL ALL DLBCL	Increased cell proliferation	[135,178–183]
NFKB2	t(10;14)(q24;q32)	SMZL MM, CLL	Constitutional activation of the non-canonical NF-kB pathway	[184–186]
PAX5	t(9;14)(p13;q32)	50% LPL	Dysregulation of PAX5 target genes	[187,188]

ALL, acute lymphoblastic leukemia; BL, Burkitt lymphoma; B-PLL, B-prolymphocytic leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PCL, plasma cell leukemia; SLVL, splenic lymphoma with villous lymphocytes; SMZL, splenic marginal zone B-cell lymphoma.

2.3. Role of IGH Enhancers in Regulating Oncogene Expression and Malignant Development

Our knowledge of the precise roles of particular Ig heavy chain enhancers in different steps of B-cell maturation is rather well established. Occurrence of *IGH* translocations in B-cell malignancies prompted studies on the role of *IGH* enhancers in lymphoma. Since Eμ and 3′RR are important regulators of the *IGH* locus activity throughout the B-cell lifetime, the intuitive questions to ask are: if and how can they be implicated in expression of translocated oncogenes? Mouse models of chromosomal translocations, juxtaposing oncogenes with Eμ and/or 3′RR allowed to build our current understanding of their engagement in B-cell malignancies [66,189–191]. Three main study approaches can be distinguished: (1) regulation by Eμ; (2) regulation by 3′RR and (3) regulation by

both Eμ and 3′RR, the most resembling endogenous conditions. When choosing the mice model, main window of activity of each enhancer should also be kept in mind. Lymphomas developed in mice with an oncogene under regulation by Eμ only represent immature B-cell stage, while stimulation by 3′RR-only results in mature B-cell malignancies [189,192]. Animal models are important not only because they allow to understand the mechanisms driving oncogene expression and malignant transformation, but also provide an in vivo system for testing therapeutic approaches [193]. Therefore, mimicking the translocations is of key importance. It has been observed though, that even if the translocation is present, the development of lymphoma can be variable [193]. This indicates that other factors, besides translocation itself, play a role in lymphomagenesis. Up to date, several mouse models with *IGH* translocations have been established (Table 2).

Table 2. Mouse models—*IGH*.

Gene	Translocation	Enhacer Involved	Model Name	Disease	References
BCL2	t(14;18)(q32;q21)	3′RR	Igh-3′E-bcl2	FL	[194]
BCL6	t(3;14)(q27;q32)	Eμ	Eμ-tTA-BCL6	DLBCL, TL	[195]
BCL10	t(1;14)(p22;q32)	Eμ	Eμ-BCL10	MZL	[96]
CCND1	t(11;14)(q13;q32)	Eμ	Eμ-CCND1	no *	[196,197]
		3′RR	CCND1-3′RR	no	
MAF	t(14;16)(q32;q23)	Eμ	Eμ-c-MAF	MM	[198]
MYC	t(8;14)(q24;q32)	Eμ	Eμ-myc iMycEμ	BL	[189–191,199–205]
		Eμ + 3′RR	iMycCα		
		3′RR	IgH-3′E-myc, minimal 3′RR, iMycCμ		

* malignant transformation occurred when crossed with Eμ-myc mice; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MM, multiple myeloma; MZ, marginal zone lymphoma; TL, T-cell lymphoma.

Eμ-myc mice have been so far the most widely used model [199], reviewed recently in more details by Ferrad et al. [189]. It employs a construct in which Eμ enhancer is placed 5′ to exon 1 of *c-Myc*. Arising lymphomas represent mainly immature B-cell stages. Another knock-in model, iMycEμ, imitates endemic Burkitt lymphoma with *MYC-IGH* translocation t(8;14) in humans/t(12;15) in mice [200,201,206]. Here, *c-Myc* is under the regulation of both Eμ and 3′RR. iMycEμ helped to reveal an aberrant regulatory network involving PI3K, NF-κB and STAT3, important for Myc expression and tumor development, although the involvement of enhancers is not discussed in this work [201].

In contrast to Eμ, 3′RR contains several enhancers. Which of them are of key importance for translocated oncogene expression? Kovalchuk et al. showed that hs3a and hs1,2 enhancers are important drivers of Myc overexpression in mouse plasmacytomas, while hs3b and hs4 are dispensable [204]. Another study indicated that 3′RR is not obligatory for translocated c-Myc expression in pro-B cell lymphomas, but essential in peripheral B-cell lymphomas [205].

Several knock-in models placing *c-Myc* under control of 3′RR enhancers have been developed. Those include: IgH-3′E-myc knock-in mice, iMycCα, iMycCμ and the use of "minimal 3′RR" (also reviewed in [189,190]). The first approach utilizes introduction of murine 3′RR DNase I hypersensitive sites into the endogenous *c-Myc* locus [202]. Even though other *IGH* regulators were not involved, transgene insertion resulted in elevated c-Myc expression and led to Burkitt lymphoma-like malignancy. Although this model clearly demonstrated the ability of 3′RR to deregulate oncogene expression, it does not resemble the native organization of translocation in BL, where exons 2–3 of *c-Myc* are inserted into the *IGH* locus. To further validate the involvement of 3′RR in oncogene deregulation, the minimal 3′ locus control region (LCR) transgene was developed, consisting of

c-Myc with its P1 and P2 promoters fused with a fragment containing only the core 3′RR sequences: hs3a, hs1.2, hs3b and hs4 [203]. Authors reported increased c-Myc levels and appearance of BL-like cells at 34 weeks of age in animals bearing the transgene.

Recent study by Ghazzaui et al. revealed that 5′ and 3′ *IGH* enhancers cooperate in the induction of B-cell lymphomas [191]. Authors compared three commonly used, previously mentioned, mice models: iEμMyc, iMycCα and iMycCμ. They highlighted the elevated rate of lymphomagenesis and Ki67 index in animals with both Eμ and 3′RR enhancers present and the oncogene placed upstream of Eu (iEμMyc). This model resembles most closely BL cases. iEμMyc mice are characterized by shorter life expectancy and higher c-myc expression levels than other two models. Surprisingly, in iMycCμ, where Eμ is knocked-out, a specific group of B-cell lymphoma cells was reported—a CD19-negative population. The reason of this remains an open question. In iMycCα mice the oncogene is placed among Cα exons, and the Eμ enhancer remains intact [207]. In both iMycCμ and iMycCα mice elevated Myc expression was confirmed and they developed lymphoma, although the onset was delayed compared to the iEμMyc animals.

Apart from Myc, mouse models have been also developed for other oncogenes involved in IGH translocations. In Igh-3′E-bcl2 mice, which aimed to mimic human lymphoma with t(14;18)(q32;q21), 3′RR enhancers were inserted 3′ of *Bcl2* and led to increased mRNA and protein levels [194]. Moreover, *Bcl2* promoter change from P1 to P2 occurred, similarly to native follicular lymphoma cases. Chromosome conformation capture experiments revealed interaction of 3′RR with *Bcl2* locus in Igh-3′E-bcl2 mice, however the exact hs site involved in this interaction was not discussed. Similar interactions were observed in t(14;18) human cell lines. In addition, chromatin immunoprecipitation in human SU-DHL-4 cells revealed OCT-2 and BOB-1 binding to hs1.2 and hs4 enhacers [208]. Interestingly, OCT-1, OCT-2 and BOB-1 were found at promoter 2 of *BCL2*, even though this region does not contain their binding sites.

In another study CCND1-3′RR mice, mimicking human t(11;14)(q13;q32), were created to investigate mantle cell-like and myeloma-like phenotype [196]. Surprisingly, juxtaposition of cyclin D1 with 3′RR was not itself sufficient for malignant transformation. Eμ-cyclin D1 mouse model obtained similar results, but when crossed with Eμ-myc mice, lymphoma occurrence was rapid [197]. This further supports the observation, that other factors besides single translocation are required to drive carcinogenesis.

Eμ c-Maf TG mouse model was developed to study human t(14;16)(q32;q23) found in multiple myeloma [198]. Elevated levels of c-Maf mRNA and protein were confirmed in those transgenic animals, as well as 28% incidence of lymphoma. Transgenic animal models of other chromosomal translocations found in human lymphomas include also: Eμ-BCL10 mice to mimic t(1;14)(p22;q32) [96] or tet-o-BCL6 crossed with Eμ-tTA to study t(3;14)(q27;q32) [195], but those in vivo studies were more focused on investigation of molecular and physiological effects of aberrant oncogene expression than on pinpointing *IGH* enhancers function in malignant transformation.

Despite an important progress in elucidating the involvement of *IGH* enhancers in oncogene expression and lymphomagenesis achieved with the use of transgenic mice, the precise mechanisms still remain to be determined. It should also be kept in mind that besides clear homology between human and murine *IGH* loci, there are a few differences in their organization. Human 3′RR is duplicated, it contains only one hs3 enhancer and lacks hs5-8 insulators. Those differences may limit direct translation of findings from mouse models to humans.

3. Immunoglobulin Light Chain Enhancers in B-Cell Malignancies

3.1. Structure and Function of IGK and IGL Enhancers in Normal B Cells

In a subset of B-cell malignancies, the immunoglobulin light chain loci—kappa and lambda—are involved in oncogenic translocations. The *IGK* locus contains three enhancers: the intronic enhancer (iEκ) located between the *IGK* joining and constant genes, and two enhancers localized 3′ of the *IGK* locus, the proximal (3′Eκ) and distal (Ed) enhancer

(Figure 3A). Functions of these enhancers have been studied in mouse models, and their genomic organization and sequence of their key elements is strongly conserved across mammals [209]. This suggests that mechanisms of *IGK* gene expression and rearrangements regulation by *IGK* enhancers are similar in human. During B cell development, *IGK* recombination is preceded by profound changes in chromatin structure organization and transcription factor occupancy within the *IGK* enhancers [210–213]. Moreover, iEκ is critically involved in maintaining the timing of *IGH* and *IGK* rearrangements: V(D)J recombination in *IGH* takes places in pro-B cells and only after it is stopped, recombination in *IGK* can be initiated in pre-B cells [43,214]. All three *IGK* enhancers interact with each other in active *IGK* loci to promote transcription and rearrangements [213,215–217], and their activity strongly depends on NF-κB binding to iEκ [215]. In human and mice expression of IGL and IGK is mutually exclusive. Rearrangements are initiated in the kappa locus and in case they are non-productive, the lambda locus is activated. Similar to *IGH* and *IGK* loci, rearrangements and expression of *IGL* genes are also regulated by enhancers [218,219]. There are marked differences between the murine and human *IGL* enhancers. While there are two enhancers in mice: $E\lambda_{2\text{-}4}$ downstream of Cλ4 and $E\lambda_{3\text{-}1}$ downstream of Cλ1 [220], human *IGL* locus contains one enhancer downstream of Cλ7 [221] (Figure 3B). Moreover, activity of the human but not mouse *IGL* enhancer strongly depends on NF-κB. At the same time, murine *IGL* enhancers are much weaker than human enhancers and this may be due to a mutated NF-κB binding site whose restoration increases activity of murine enhancers [222].

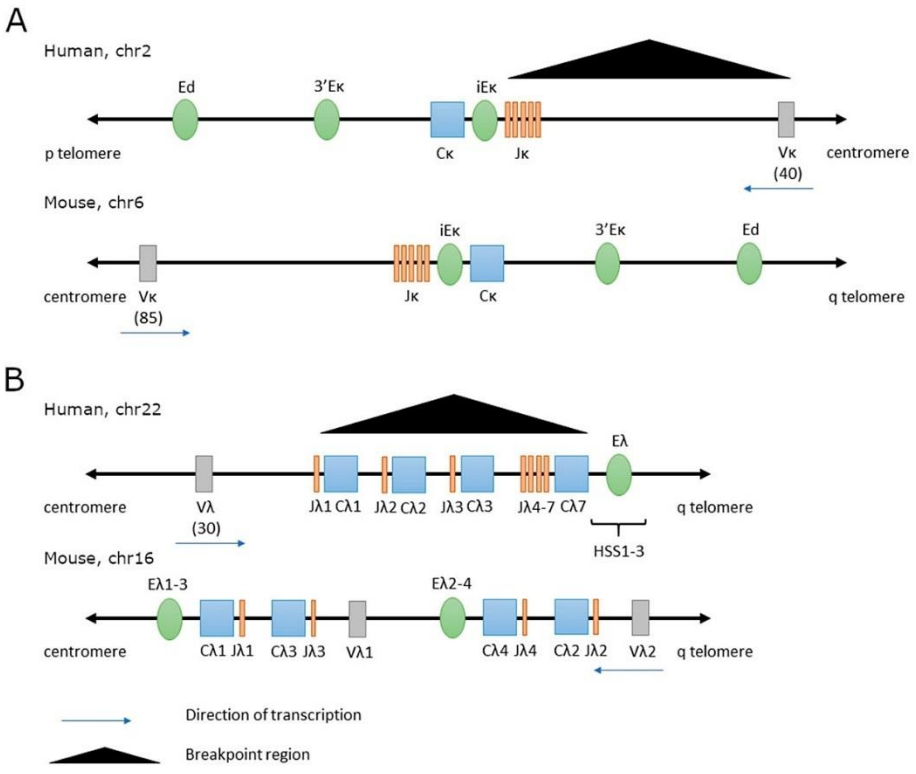

Figure 3. Organization of the human and murine *IGK* (**A**) and *IGL* (**B**) loci. Numbers below Vκ and Vλ indicate the number of variable gene segments. Blue arrows depict the direction of transcription. Black triangles mark regions of breakpoints involved in translocations in malignant cells.

3.2. IGK and IGL Translocations in B-Cell Malignancies

Given the crucial role of *IGK* and *IGL* enhancers in immunoglobulin light chain rearrangements and expression, it is not surprising that translocations juxtaposing light chain enhancers with oncogenes are found in B-cell malignancies, although less frequently than *IGH* translocations. Translocations of *MYC* to *IGL* [t(8;22)(q24.1;q11.2)] and *IGK* [t(2;8)(p11.2;q24.1)] have been described in several types of B-cell malignancies, such as BL, DLBCL, B-ALL and MM [223–227]. Unlike in the case of rearrangements with *IGH*, the breakpoint within *MYC* locus was localized up to 600 kb 3′ of *MYC* (Figure 2). As a result of the translocations, *MYC* was brought in the neighborhood of the *IGK* (up to 50 kb away) and *IGL* enhancers (100–300 kb away). Analysis of the chromatin organization in the BL cell line LY66 bearing the *IGK/MYC* translocation revealed that the physical distance between *MYC* and *IGK* was much shorter than expected for a linear distance [228]. This implies existence of a chromatin architecture allowing spatial interaction between *IGK* enhancers and *MYC*.

A comprehensive study of nearly 800 multiple myeloma patients revealed a wide repertoire of translocations, with 41% involving *IGH*, 10%—*IGL*, and 5%—*IGK*. *MYC* was juxtaposed to *IGH* and *IGL* with the same frequency, and was the most prevalent partner of *IGL* translocations (41%). *IGL* translocations were often accompanied by focal amplifications involving the *IGL* enhancer. Strikingly, patients with *IGL* translocations had worse outcome compared to patients with *IGH* and *IGK* translocations, despite similar levels of MYC expression. The authors propose that this phenomenon might be explained by high levels of IKZF1 bound to *IGL* and thus a weaker response to treatment with imide drugs targeting IKZF1 [229].

Rare variants of the *BCL2* translocation involving the *IGK* [t(2;18)(p11;q21)] or *IGL* [t(18;22)(q21;q11)] loci have been reported in follicular lymphoma (FL) [230–235] and chronic lymphocytic leukemia (CLL) [145,236,237]. Cases with these translocations were positive for BCL2 protein expression. Similarly to the variant *MYC* translocations, the breakpoint in *BCL2* was different from that involved in translocations with *IGH*, and was localized at the 5′ end of the *BCL2* gene (Figure 2).

CCND1 is commonly translocated to *IGH* in mantle cell lymphoma (MCL). Case studies also reported MCL patients with translocations involving *CCND1* or *CCND2* and *IGL* or *IGK* resulting in strong overexpression of cyclin D1 or D2 [238–243]. However, in a subset of cyclin D1-negative MCL cases the underlying molecular mechanism of the disease remained unclear. Recently, Martin-Garcia et al. investigated 56 cyclin D1-negative MCL cases using FISH, whole genome/exome sequencing and gene expression arrays. They found *CCND2* or *CCND3* rearrangements in 93% of the cases. Majority (70%) displayed conventional translocations with *IGL* or *IGK*. In a few cases the authors identified cryptic insertions of the *IGK* or *IGL* enhancers close to *CCND2* and *CCND3* genes which led to overexpression of those cyclins. Expression profiles and clinical outcome of cyclin $D1^-$ and cyclin $D1^+$ MCL cases was similar, indicating that the hijacking of *IGK/IGL* enhancers by *CCND2* and *CCND3* may be a molecular event involved in MCL pathogenesis [244].

Other, less frequent translocations found in B-cell lymphomas involved *IGK/IGL* and *BCL3*, *BCL6*, *BCL10* or *REL* or other regions with yet undefined partner genes [138,245–250] (Table 3).

Table 3. Translocations involving immunoglobulin light chain loci in B-cell malignancies.

Gene	IG Light Chain	Translocation	Disease	References
BCL2	lambda	t(18;22)(q21;q11)	CLL, FL	[145,230–237]
	kappa	t(2;18)(p11;q21)		
BCL3	lambda	t(19;22)(q13;q11)	FL, DLBCL	[138]
	kappa	t(2;19)(p12;q13)	HL, B-cell NHL	
BCL6	lambda	t(3;22)(q27;q11)	B-cell NHL	[247,250]
	kappa	t(2;3)(p11;q27)		

Table 3. *Cont.*

Gene	IG Light Chain	Translocation	Disease	References
BCL10	kappa	t(1; 2)(p22; p12)	MALT	[248,249]
CCND1	lambda	t(11;22)(q13;q11)	MCL	[238,240,242,243]
	kappa	t(2;11)(p11;q13)		
CCND2	lambda	t(12;22)(p13;q11)	MCL	[239,241,244]
	kappa	t(2;12)(p11;p13)		
CCND3	lambda	t(6;22)(p21;q11)	MCL	[244]
	kappa	t(2;6)(p11;p21)		
MYC	lambda	t(8;22)(q24;q11)	ALL, BL, DLBCL, MM	[223–227,229]
	kappa	t(2;8)(p11;q24)		
REL	lambda	t(2;22)(p16;q11)	HL	[245]

ALL, acute lymphoblastic leukemia; BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HL, Hodgkin lymphoma; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MM, multiple myeloma; NHL, non-Hodgkin lymphoma.

3.3. Role of IGK and IGL Enhancers in Regulating Oncogene Expression and Malignant Development

Increased expression of respective oncogenes in cell lines and patient samples bearing *IGK* or *IGL* translocations only indirectly indicates the role of immunoglobulin light chain enhancers in driving the expression of translocated genes. Overexpression of constructs mimicking the t(2;8) translocation identified the intronic and 3′ kappa enhancers together with the matrix attachment region (MAR) as the elements necessary and sufficient for high *MYC* transcription and change in *MYC* promoter usage from P2 (predominant in normal cells) to P1 (predominant for the translocated *MYC* allele) [251]. Since activity of iEκ critically depends on binding of NF-κB, and 3′Eκ—on SP1—their role in MYC activation was examined. Joint mutations of the respective binding sites completely abolished transcription from the P1 promoter. Similar effect was observed upon NF-κB depletion, while overexpression of both NF-κB subunit REL65 and SP1 synergistically promoted activity of P1 [252].

Further evidence for the role of *IGK/IGL* enhancers in tumorigenesis comes from mouse models. In parallel with the Eμ-Myc model where *Myc* is coupled with the Eμ *IGH* enhancer, mice mimicking the *IGK-MYC* translocation were generated. The Eκ-SV-Myc mice developed lymphomas, which confirms the role of the iEκ enhancer in lymphomagenesis. However, penetrance was lower and latency was higher compared to the Eμ-Myc mice [199]. Mice carrying the λ-Myc transgene under control of the *IGL* enhancer developed high penetrance lymphomas originating from lymph nodes; they presented the 'starry sky' appearance characteristic of BL [253]. This confirms the oncogenic potential of the translocated *IGL* enhancer. Compared to the Eμ-Myc model, λ-Myc mice developed lymphomas with more mature phenotype, closer reminiscent of the human BL. Another model of an *IGK/IGL*-driven malignancy is the mouse plasmacytoma (MPC). The disease is induced by pristine oil, alone or combined with Abelson virus, and is characterized by translocations of *Myc* with immunoglobulin loci. In majority of cases *IGH* is involved but translocations with *IGK* or *IGL* have also been reported [254–256]. The MPC model demonstrates that *IGK* and *IGL* are able to drive Myc expression which initiates the disease, although additional genetic lesions may be required for the full onset disease [257].

Altogether, this highlights the importance of immunoglobulin light chain enhancers as alternative drivers of B-cell malignancies, as well as the diagnostic and prognostic potential of detecting *IGK/L* translocations. However, more precise dissection of underlying mechanisms is still pending.

4. Enhancer Variants and Mutations in B-Cell Malignancies

Cancers are driven by accumulation of mutations. Moreover, inherited sequence variants can also influence susceptibility to malignant transformation. Whole genome sequencing (WGS) revealed a broad spectrum of recurrent, cancer-specific somatic mutations, while genome-wide association studies (GWAS) identified germline sequence variants associated with cancer risk. Recently, mutations and variants in the non-coding parts of the genome have attracted attention. Several risk loci and driver mutations in non-coding regions have been identified and shown to affect gene expression regulatory networks by e.g., interfering with transcription factor binding, shaping chromatin architecture or affecting miRNA binding to target genes [258,259]. Among them, variation in enhancers has been observed in B-cell malignancies and their functional consequences have been highlighted.

4.1. Somatic Mutations

A number of enhancers have emerged so far as mutational hot-spots in several B-cell malignancies (Table 4). WGS analysis of matched tumor-normal tissues from CLL patients revealed, in addition to mutations in protein-coding genes, several somatic mutations in non-coding regions. Among them, an intergenic region at chromosome 9p13 was densely mutated in 11% of cases. This region was enriched in transcription factor binding sites and chromatin marks for active enhancer specifically in B cells. 4C-seq revealed interaction with the *PAX5* locus. CRISPR-introduced specific point mutations in the enhancer or its deletion downregulated PAX5 expression by 40%, confirming the functional significance of mutations. However, the effect of mutations on chromatin architecture or TF binding was not investigated. Somatic mutations in the *PAX5* enhancer were also found by the authors in other types of B-cell lymphoma: DLBCL (29%), FL (23%), MCL (5%) [260]. An independent study focusing on somatic regulatory variants in DLBCL confirmed preferential mutation of the *PAX5* enhancer in 23% of the germinal center B-cell subtype of DLBCL [261]. The *PAX5* enhancer was also mutated in BL, especially in EBV-positive cases [262]. PAX5 is a transcription factor with an important role in B-cell commitment and development. Tight regulation of PAX5 levels is critical for normal B-cell lymphopoiesis but also to prevent tumor development. On one hand, *PAX5* is involved in translocations with *IGH*, which lead to PAX5 upregulation in aggressive B-cell lymphomas. On the other hand, PAX5 was shown to act as a haploinsufficient tumor suppressor in B-ALL [263,264]. So far, the effect of mutations in *PAX5* enhancer was studied only in CLL where the associated decrease in PAX5 expression suggests a tumor suppressor role of PAX5.

Table 4. Somatic mutations in enhancer regions identified in B-cell malignancies.

Gene	Disease	Effect on Gene Expression	Reference
BCL2	DLBCL	ND	[261,265]
BCL6	BL, DLBCL	ND	[261,262,265]
PAX5	BL, CLL, DLBCL, FL, MCL	Decreased	[260–262]
ST6GAL1	BL	ND	[262,265]
TPRG1	DLBCL	Increased	[266]

BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma.

Other mutation hot-spots in B-cell lymphoma were the enhancers of *BCL6*, *BCL2* and *ST6GAL1* [261,262,265]. A study focusing on mutations in transcription factor binding sites (TFBS), including the above-mentioned enhancers, in combination with RNA-seq data showed that in general mutations in TFBS are associated with altered gene expression. However, the direct effect of mutations in enhancers on their activity and expression of respective genes remains to be investigated. BCL6 and BCL2 are oncogenes with anti-apoptotic role, often mutated in B-cell malignancies and involved in translocations with immunoglobulin genes [267,268]. ST6GAL1 is involved in protein and lipid glycosylation,

its upregulation and oncogenic function was reported in several cancers [269]. Thus, mutations in *BCL2*, *BCL6* and *ST6GAL1* enhancers would be expected to augment their activity.

An alternative approach used data from Hi-Ci in naïve B cells to determine regions interacting with promoters as *cis*-regulatory elements (CREs), which were further sequenced in search for somatic mutations. This revealed 78 recurrently mutated CREs interacting with promoters of 72 genes in DLBCL, and 42 recurrently mutated CREs interacting with promoters of 37 genes in FL. As an example, a mutated CRE enriched in enhancer marks and interacting with the *TPRG1* promoter was further characterized. A mutation in the *TPRG1* enhancer was associated with higher TPRG1 expression in DLBCL. In addition, amplification of *TPRG1* gene was observed as an alternative mechanism of TPRG1 upregulation in DLBCL, implicating its significance in lymphoma. The function of TPRG1 is poorly characterized and requires further investigation [266].

Notably, several of those studies observed that enhancers were enriched in mutations affecting the C in the WRCY motif, which is a signature of AID-induced mutations [259–262,266]. This is in line with a previous report that AID off-targets at non-immunoglobulin loci are predominantly clustered in super-enhancer regions [115]. Characteristic features of enhancers targeted by AID mutations were active transcription of enhancer RNAs and engagement in long-range chromatin interactions. Analysis of BL and DLBCL tumors revealed that apart from the IG genes, main loci of AID mutations were active enhancers of genes with a known role in lymphoma: *BCL6*, *PAX5*, *ETS1*, *CIITA*, *CXCR4* [115]. This highlights AID as an important, and perhaps major, cause of somatic mutations in enhancers in B cells. A systematic analysis of enhancer mutations in B-cell malignancies could reveal other potential underlying mechanisms.

Although several mutations in enhancers were shown to affect expression of genes relevant for B-cell malignancies, significance of the mutations in tumorigenesis remains to be established. Targeted sequencing of 12 super-enhancers in B cells isolated from healthy individuals revealed ~9000 low frequency mutations in all samples. ~8000 of those were localized in the *BCL6* enhancer with a mutation frequency of 2.2×10^{-4}; other clusters mapped to the *PAX5* and *CD83* enhancers with a lower frequency ($6.9–9.7 \times 10^{-6}$). These mutations were specific for the memory B cells. Again, mutation pattern highlighted the role of AID [270]. A larger-scale study and follow-up of the individuals presenting mutations in enhancers would give insights into their prevalence and penetrance, but it is unlikely that they could lead to malignancy without additional genetic lesions. Similarly, oncogenic *IGH* translocations were observed in blood of up to 25% of healthy donors [90–93]. They persisted in the B-lymphocyte pool for years without any symptoms of B-cell malignancy, which indicates that additional events are required for lymphomagenesis.

4.2. Germline Sequence Variants

GWAS studies identified several risk loci for B-cell malignancies and some follow-up studies revealed that several of them harbor single nucleotide variants (SNVs) within enhancers and super-enhancers (Table 5). Two studies focused on enhancer variants within previously identified risk loci in CLL and identified several features indicating their functional importance [271,272]. Firstly, several enhancer SNPs were located in binding motifs for TFs such as SPI1, NFKB, PAX5, MEF2A, FOXI1, NFATC1 and TCF3, with a potential to disrupt or enhance their binding. Indeed, allelic imbalance was observed in ChIP experiments for several SNPs and TFs. Secondly, altered chromatin accessibility and levels of histone marks such as H3K27ac, H3K4me1 and H3K4me3 were observed for alternative alleles in those SNPs, and for some variant loci H3K27ac signals were significantly higher in CLL than in normal B cells. Thirdly, analysis of chromatin architecture revealed that the enhancers harboring risk SNPs interacted with several genes with established roles in B-cell development and malignancy, e.g., *MYC*, *BCL2*, *BCL6*, *IRF4*, *IRF8*, *BCL2L11*, *CDKNA*, *CDKNB*. Moreover, gene expression QTL analysis revealed risk loci with an effect on gene expression. These studies highlighted the potential role of enhancer variants in B-cell malignancies. It remains to be further investigated to what extent such SNPs can affect

chromatin interactions, TF binding and gene expression, and whether there is a direct link with development of B-cell malignancies.

Table 5. Germline variants in enhancer regions associated with B-cell malignancies.

Gene	SNP ID	Disease	Gene Expression	TF Binding	Reference
BMF	rs539846	CLL	Decreased	RELA (disrupted)	[273]
BMI1	rs11591377	ALL	ND	MYBL2, p300 (enhanced)	[274]
GATA3	rs3824662	ALL	Increased	NFIC (enhanced)	[275]
PIP4K2A	rs4748812	ALL	ND	RUNX1 (enhanced)	[274]

ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia.

Another study in CLL provided functional insights into a super-enhancer polymorphism at 15q15.1 risk locus. SNP rs539846 C > A is localized in a SE in the intron 3 of *BMF* gene, which encodes a pro-apoptotic member of the BCL2 family. The SNP alters a conserved RELA binding motif and was shown to disrupt RELA binding, reduce enhancer activity, and was associated with decreased BMF expression in primary CLL cases. BMF is a BCL2 antagonist, thus reduced BMF levels together with increased BCL2 expression observed in CLL may cooperate to attenuate apoptosis. Although no associations were found between the rs539846 genotype and prognosis or survival, this study revealed a mechanism underlying the 15q15.1 risk locus in CLL [273].

A follow up of two risk loci for childhood ALL identified previously in a GWAS revealed two SNPs located in enhancers of *BMI1* and *PIP4K2A*. rs11591377 lies in a region showing strong enhancer marks in hematopoietic cells and containing binding sites for multiple transcription factors. This enhancer interacted with the *BMI1* promoter in myeloid and B-cells but not T-cells. The risk G allele was predicted to enhance binding of MYBL2 and p300 transcription factors, which was demonstrated in K562 cells heterozygous for this SNP. Another SNP, rs4748812, was located in an enhancer region interacting with the *PIP4K2A* promoter in B cells. The rs4748812 risk allele T was predicted to create a RUNX1 binding site, but this was not proven experimentally [274].

A thorough functional investigation of a *GATA3* enhancer variant provided insights into B-ALL pathogenesis. rs3824662 located in a region with enhancer features in hematopoietic cells was associated with susceptibility to Ph-like ALL. The risk variant A allele increased activity of the enhancer in a reporter assay and was also associated with higher H3K4me1 mark and open chromatin in B cells. The enhancer formed a chromatin loop with the *GATA3* promoter. Accordingly, GATA3 expression was increased in primary leukemia samples with the risk allele and in a CRISPR-engineered LCL cell line with the A/A genotype, but no effect was observed on expression of other genes in the topologically associated domain. A binding site for the transcription factor NFIC was identified in the vicinity of the variant and ChIP confirmed stronger binding of NFIC to the A allele. Globally, the A allele induced binding of GATA3 to novel sites genome-wide and changes in the 3D genome organization and gene expression profile. An interesting observation was made that GATA3 binding motif was enriched near breakpoint regions in Ph-like ALL, which suggests that GATA3 may be involved in this translocation [275]. It would be interesting to investigate whether noncoding transcription at these loci may contribute to the rearrangements, as is the case for *IGH* translocations.

An integrative analysis of FAIRE-seq and histone marks ChIP-seq revealed distal regulatory elements (DREs) which differed in activity between follicular lymphoma samples and normal centrocytes. The variable DREs were enriched for SNPs and SNVs predicted to disrupt TF binding motifs. Three sequence variants, in BS for IKZF1, SP1 and TCF3, were further investigated. All three variants reduced binding of respective TFs and decreased enhancer activity. Analysis of gene expression in FL samples revealed that predicted target genes of these TFs were downregulated in FL samples with the sequence

variants. These included several genes which have been associated previously with B-cell malignancies (*HLA-DQA1*, *DUSP6*, *IRF8*) [276].

In summary, available data highlight the significance of somatic mutations and germline variants in enhancers as another mechanism of enhancer repurposing in B-cell malignancies. Functional studies revealed a profound impact of enhancer mutations and SNPs on chromatin architecture, TF binding and expression of genes involved in normal and pathological processes in B cells. Given the large number of non-coding mutations and variants observed in tumors and GWAS studies, more insights into the role of enhancer variants in B-cell malignancies are expected.

5. Exploiting Enhancers by Deregulated Transcription Factors

Enhancers are packed with transcription factors (TF) motif sequences. TF binding indicates active enhancer regions and is necessary for target genes activation [277]. In cancer cells, TF expression is often altered, which in consequence leads to aberrant binding at enhancers and ultimately changes expression of the controlled genes [278,279]. Here we describe a few examples of how deregulated TFs rewire enhancers' activity in B-cell neoplasms.

Sequential activation of the PAX5 transcription factor determines the B-cell commitment in early stages of lymphopoiesis. B-cell specific expression of PAX5 is controlled by several TFs (PU.1, IRF4, IRF8 and NF-κB) binding to an enhancer in intron 5 of *PAX5* [280]. Thus, deregulation of those TFs, which occurs in B-cell malignancies, affects expression of PAX5. Furthermore, PAX5 itself regulates expression of several target genes in B cells by rapidly recruiting chromatin modifying proteins to their promoters and enhancers. Presence of PAX5 on chromatin correlated with increased active chromatin marks in PAX5-induced genes, whereas an inverse pattern of histone modifications was observed in PAX5-repressed genes [281]. As demonstrated later, another B-cell specific transcription factor, EBF1, is required for the interaction of PAX5 with the MLL H3K4 methyltransferase complex and subsequent epigenetic modifications [282]. EBF1 and PAX5 have opposing roles in normal and malignant B cells with regards to the regulation of the *MYC* oncogene. Both EBF1 and PAX5 are bound to *MYC* enhancers in mouse pro-B cells as well as pro-B ALL NALM6 cells. While EBF1 promoted MYC expression, PAX5 negatively regulated MYC levels in normal B-cell progenitors [283]. Although it is not clear how this regulation looks in malignant cells, another report suggested that EBF1 and PAX5 prevent malignant transformation by limiting MYC levels [284].

Another transcription factor with a crucial role in hematopoiesis is RUNX1. Mutations and translocations involving *RUNX1* are frequent in hematologic malignancies [285]. In human pre-B leukemia cells RUNX1 together with FUBP1 bound to an intronic enhancer in the oncogene *c-KIT*. Overexpression of RUNX1 and FUBP1 upregulated c-KIT levels and enhanced cell proliferation, as well as decreased cell sensitivity to the c-KIT inhibitor and therapeutic drug imatinib mesylate [286]. RUNX1 also interacts with CBFA2T3 which enhances its transcriptional activity. They act in a self-activation loop, as RUNX1 binds its own promoter and the *CBFA2T3* enhancer located 2 kb upstream of the *CBFA2T3* promoter [287]. Since RUNX1 and CBFA2T3 are upregulated in ETV6-RUNX1 B cell precursor ALL (BCP-ALL) [288], it suggests that RUNX1 and CBFA2T3 may act as a driver loop in BCP-ALL. Indeed, use of a truncated CBFA2T3 protein significantly inhibited RUNX1 activity and reduced BCP-ALL cell proliferation [287].

The chimeric transcription factor TCF3-HLF, resulting from the t(17;19)(q22;p13) translocation, is associated with poor survival and resistance to therapy in B-ALL [289]. ChIP-Seq in leukemia cells revealed prevalent binding of TCF3-HLF to active enhancers, especially super-enhancers. Among them was a distal *MYC* SE possessing a HLF binding motif. CRISPR-mediated disruption of the HLF motif disturbed interactions between the SE and the *MYC* promoter, reduced MYC expression and decreased viability of HAL-01 cells. The activating effect of TCF3-HLF on enhancers was mediated by the recruitment of the p300 acetyltransferase and was thus vulnerable to an inhibitor of p300, A-485 [290].

MEF2B is a transcription factor often mutated in DLBCL and FL, which leads to its increased activity and upregulation of one of its target genes, *BCL6* [291]. ChIP-Seq revealed enrichment of MEF2B and the p300 acetyltransferase at *BCL6* super-enhancer. It was demonstrated that MEF2B directly activates BCL6 expression by increasing histone acetylation at its enhancer [292]. Similarly, activation of BCL2 is observed in MLL-rearranged leukemia patients [293]. The MLL-AF4 fusion protein resulting from the t(4;11)(q21;q23) translocation was shown to bind to the *BCL2* enhancer, consisting of two H3K27Ac clusters at the 3′ end of the gene. The authors demonstrated that MLL-AF4 regulates BCL2 expression by controlling H3K27Ac levels at its enhancer [294].

Global H3K27ac HiChIP analysis identified multiple interactions between enhancers and promoters in several primary effusion lymphoma (PEL) cell lines. In particular, super-enhancers of *MYC* and *IRF4* were critical for PEL cell growth. Transcription factors MEF2C and IRF4 bound to these SE and controlled expression of MYC and IRF4 by promoting H3K27ac. In addition, a global reduction in H3K27ac signals was observed upon CRISPR inactivation of the *IRF4* SE, which suggests that *IRF4* SE and IRF4 are master regulators of the enhancer landscape in PEL cells [295].

These studies demonstrate that physiological interactions between TFs and enhancers, essential for proper B-cell development and function, may become pathogenic upon dysregulation of TF levels.

6. Enhancer Hijacking by Lymphoma-Associated Viruses

Certain viruses have been implicated in B-cell malignancies, e.g., Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), human immunodeficiency virus (HIV), hepatitis C virus (HCV). Viruses rely on the host factors for their own replication and have mastered the ability to reprogram the host cell transcription and translation machinery as well as metabolism for their own purpose. One of the mechanisms exploited by viruses is hijacking host cell enhancers to change the epigenetic landscape and to promote a gene expression profile that creates a favorable environment for virus replication.

6.1. Epstein–Barr Virus

The best studied virus associated with B-cell lymphomas is Epstein–Barr virus (EBV). EBV is a human gamma-1 herpesvirus that shows tropism for B cells and is commonly present in the latent form in >90% of worldwide population. While majority of carriers are asymptomatic, in some cases infectious mononucleosis can develop. EBV has been also associated with B-cell malignancies: eBL, cHL and DLBCL. Endemic Burkitt lymphoma is a canonical example of EBV-linked malignancy. Virtually all cases of eBL are positive for EBV infection. Given the widespread persistence of EBV in the population, clearly EBV infection alone is not sufficient for lymphomagenesis. Compromised immune response, e.g., in case of malaria, AIDS or in post-transplantation patients releases EBV-infected cells from immune surveillance by T cells and increases risk of malignant transformation [296]. In vitro infection of B lymphocytes with EBV causes their immortalization and establishment of continuously proliferating lymphoblastoid cell lines (LCLs). A wide set of viral proteins is involved in B-cell immortalization but only a few are expressed later in the latent state, depending on the latency type (e.g., EBNA2, EBNA3 and EBNALP proteins). While the association of EBV with certain types of B-cell lymphomas is undisputable, still its precise role and mechanisms behind EBV-linked lymphomagenesis are not fully understood [297]. Recently, enhancer hijacking by EBV resulting in subsequent chromatin reorganization and transcriptional reprogramming has been highlighted in several studies.

Zhou et al. provided a global overview of EBV-controlled enhancers in a lymphoblastoid cell line GM12878. EBNA2-ChIP-seq identified 888 sites with very strong EBNA2 binding and high and broad H3K27ac signals, characteristic of super-enhancers (SEs). EBNA2 SEs were often localized near genes encoding essential B-cell TFs (e.g., MYC, MAX, RUNX3), and were often co-occupied by other B-cell TFs (e.g., ETS1, IRF4, SPI1, STAT5, PAX5). RBPJ, a TF which often mediates binding of EBNA proteins to DNA, was also found

in many of those sites. Apart from EBNA2, viral oncoproteins EBNA3A, EBNA3C and ENBALP are also involved in regulating gene expression in EBV-infected cells. Moreover, NF-κB is essential for LCLs survival. Thus, the authors searched for SEs with co-occupancy of all four oncogenic EBNAs and five NF-kB subunits. 187 such sites were identified and designated as EBV SEs. Genes associated with EBV SEs included *MYC*, *BCL2*, *RUNX3*, *IKZF3*, oncomiRs miR-155, miR-21 and let-71, and were involved in apoptosis, DNA damage repair and MAPK signaling. IGL enhancer was also occupied by EBNA [298].

Hijacking the *MYC* enhancer by EBV has been extensively studied. A region spanning 428-556 kb 5′ of *MYC* was strongly bound by EBNA2 an RBPJ and possessed features characteristic of active enhancers: high H3K4me1, H3K9ac, RNAPII and p300 signals. FISH assay with probes for the *MYC* promoter and distal enhancer confirmed their interaction. EBNA2 inactivation significantly diminished colocalized signals, indicating that the association of *MYC* enhancer and promoter depends on EBNA2 [299]. EBNA2-dependent loop formation between the *MYC* SE and promoter was confirmed later by chromosome conformation capture [300,301] and RNAPII ChIA-PET [302]. Importance of the *MYC* SE for EBV-infected cells was proved by reduced MYC expression and cell proliferation upon CRISPR/Cas9-mediated deletion of the SE [302]. Moreover, eRNAs transcribed from EBV SEs, including the *MYC* SE, were identified. Expression of *MYC* SE eRNAs was dependent on EBNA2, and their knockdown inhibited proliferation of LCLs, decreased MYC expression, and reduced H3K27ac signal and looping of *MYC* SE to promoter [300]. Altogether, EBV rearranges chromatin architecture in the *MYC* locus to promote its expression and proliferation of EBV-infected cells.

EBNA2 and EBNA3 proteins (3A and 3C) target common sites and genes. Majority of sites bound by EBNA2 and 3 carried histone marks characteristic for active enhancers: high H3K27ac and H3K4me1, while some were poised enhancers (H3K27ac−, H3K4me1+). However, Re-ChIP analysis revealed that EBNA2 and 3 do not bind simultaneously to the same sites, they are exclusive [303]. While EBNA2 is an activator of transcription, EBNA3 can act as both an activator and a repressor. Binding of EBNA2 and 3 to several enhancers was shown to affect genes crucial for B-cell survival, and in some instances the two EBNA proteins counteracted each other. Distant enhancers upstream and downstream of *BCL2L11* gene form loops with the *BCL2L11* promoter in EBV-negative cells, and these interactions are lost upon EBV infection. It has been shown that EBNA3A and 3C bind to those enhancers and disrupt looping with promoter by recruiting the PRC complex which deposits the silencing mark H3K27me3 across the *BCL2L11* promoter [301]. As a result, the pro-apoptotic BIM protein encoded by *BCL2L11* is repressed, which counteracts the MYC-induced apoptosis. Similar mechanism of EBNA3 and PRC-mediated disruption of chromatin interactions and repression of transcription was observed for the *CDKN2A/B* loci encoding the tumor suppressors p16INK4a, p15INK4b and p14 ARF [302].

Interplay between EBNA2 and 3 proteins affecting B-cell growth was revealed for RUNX transcription factors [304]. SE of *RUNX3* is bound by EBNA2, EBNA3A and EBNA3C which cooperatively promote RUNX3 expression in an RBPJ-dependent way. RUNX3 is required for proliferation of LCLs and was previously shown to negatively regulate expression of RUNX1 [305]. In EBV-positive BL cells, but not LCLs, *RUNX1* enhancer was also bound by EBNA2, which resulted in activation of RUNX1 expression. However, this effect was attenuated by EBNA3B and C which also bound *RUNX1* SE and repressed its expression [304]. Why EBNA2 activates RUNX1 in some EBV-positive cells and not in others requires further investigation. Possible role of MYC has been suggested as well.

An interesting link between EBV and somatic hypermutation in the immunoglobulins has been discovered by Kalchschmidt et al. They observed increased levels of AID mRNA and protein driven by EBNA3C. Furthermore, ChIP revealed EBNA3C occupancy at the SE of *AICDA* gene encoding AID. Again, binding of EBNA3C depended on the interaction with RBPJ. Increased levels of histone marks characteristic for enhancers, H3K4me3, H3K9ac, and H3K27ac, as well as recruitment of p300 to the *AICDA* SE was observed only in the presence of functional EBNA3C. Importantly, EBNA3C-induced AID was functional and

caused SHM in the V(D)J region of IGH [306]. In the light of the well-documented off-target AID activity in non-Ig genes which promotes translocations between Ig loci and oncogenes, this study provides a possible link between EBV and lymphomagenesis.

EBNA2 and 3 proteins have been also implicated in regulation of some oncogenic miRNAs. miR-221 and miR-222 are expressed from one pri-miR and they are often upregulated in several cancers, including DLBCL. In EBV-positive cells expression of mature and pri-miR-221/222 was regulated by EBNA3A and 3C. ChIP and chromosome conformation capture analyses revealed that this activation is mediated by EBNA3A and 3C binding to an enhancer upstream of miR-221/222 cluster, which leads to increased levels of active chromatin marks and looping between the enhancer and promoter. $P57^{KIP2}$, a negative regulator of cell proliferation, was validated as a target of miR-221/222. However, inhibition of miR-221/222 and subsequent upregulation of $P57^{KIP2}$ had only a mild effect on LCL cells proliferation, indicating that other targets of miR-221/222 may be relevant [307]. miR-155 is involved in normal hematopoiesis and overexpressed in B-cell lymphoma (HL, DLBCL). miR-155 was also upregulated in B cells upon EBV infection. EBNA2 was shown to promote expression of miR-155 two-way. First, directly by RBPJ-mediated binding to an enhancer upstream of the miR-155 host gene. Second, indirectly by RBPJ-mediated binding to an *IRF4* enhancer. IRF4 binds to the same miR-155 enhancer, thus additionally boosting miR-155 expression [308].

Taken together, these data indicate how hijacking cellular enhancers by EBV promotes B-cell proliferation and can contribute to lymphomagenesis (Figure 4). EBV upregulates MYC which boosts cell proliferation. At the same time, expression of the pro-apoptotic protein BIM is downregulated, counteracting the MYC-induced apoptosis. Increased activity of *MYC* enhancers can also promote translocations as it has been demonstrated that sites of active non-coding transcription are hotspots for AID-induced breakpoints [115,116]. In line with this, breakpoints in eBL are located in the 5′ distal region of *MYC*, in contrast to sporadic BL where they are mostly located within the *MYC* gene body. In addition, EBV also induces expression of AID, further promoting translocations. Since EBV-positive lymphomas do not express EBNA2 and 3 proteins, events described above are likely to contribute to development of lymphomas rather than maintaining established tumors.

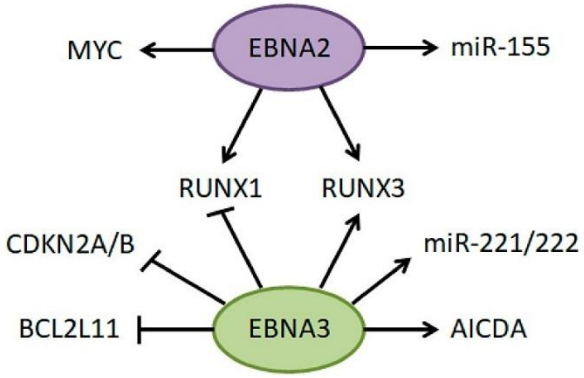

Figure 4. Enhancer hijacking by Epstein–Barr virus. Presented are interactions of Epstein–Barr Virus Nuclear Antigen 2 and 3 (EBNA2 and EBNA3) proteins with host gene enhancers. Arrows indicate activation of gene expression; bars represent inhibition.

6.2. Kaposi's Sarcoma-Associated Herpesvirus

Another virus involved in pathogenesis of B-cell malignancies is Kaposi's sarcoma-associated herpesvirus (KSHV) which causes primary effusion lymphoma (PEL). PEL is a rare, aggressive disease occurring in immunocompromised patients. 60–90% of PEL cases

are also positive for EBV [309]. In KSHV-infected cells the virus is maintained in a latent state with only a few viral genes expressed that sustain cell proliferation. Lytic state is activated in a subset of cells to allow virus replication. A master host transcription factor essential for PEL cells is IRF4 which binds to enhancers and drives expression of e.g., MYC and BATF3 [310]. Viral interferon regulatory factor 3 (vIRF3) was shown to associate with IRF4 and BATF at active enhancers to promote expression of several genes essential for PEL cells. Lack of either IRF4 or vIRF3 resulted in decreased enhancer activity. Over 60% of PEL essential genes were downregulated upon knockout of IRF4, BATF or vIRF3. Gene set enrichment analysis indicated MYC targets and cell cycle genes among genes regulated by IRF4 and vIRF3, which implies important function of KSHV in proliferation of PEL cells [311]. However, it is unclear how IRF4 and vIRF3 get hold of enhancers in PEL cells, e.g., whether vIRF3 and IRF4 shape chromatin architecture themselves or is their binding to enhancers facilitated by chromatin opening by other factors.

Another study performed a global analysis of epigenetic marks and nascent transcription in KSHV-positive PEL cells during virus latency and upon lytic reactivation. This revealed that during latency, super-enhancers for several oncogenes, including *MYC*, are activated by KSHV and repressed upon transition to the lytic state. GRO-seq confirmed that lytic reactivation resulted in a widespread shutdown of host gene transcription, including eRNAs. Further insights were gained into the regulation of MYC, which was previously shown to maintain KSHV latency and proliferation of PEL cells [312]. Strikingly, in PEL cells active enhancer marks and eRNA transcription were observed ~500 kb downstream of *MYC*, in contrast to EBV-infected cells where the active enhancer was located upstream of *MYC*. 4C experiments confirmed interaction of the downstream enhancer with *MYC* promoter in PEL cells, and CRISPRi targeting of the enhancer or eRNA inhibition reduced MYC expression and activated the lytic state. However, the role of viral proteins in the enhancer activation in latent state was not studied. Instead, it was shown that the host IRF4 activates the *MYC* enhancer during KSHV latency and upon viral reactivation the viral vIRF4 represses the cellular IRF4 leading to MYC repression [313].

Altogether, the data so far clearly highlight the hijacking of cellular enhancers by viruses as an important mechanism in B-cell lymphomagenesis. Given the limited repertoire of viral proteins, this is an efficient way to ensure proliferation of the host cells together with the virus and lytic reactivation to produce viral progeny. Genes controlled by the viruses for the sake of increased proliferation have often oncogenic properties and thus enhancer hijacking explains some aspects of the role of viruses in B-cell lymphomas.

7. Conclusions and Future Perspective

Cancer can be viewed as a disease of the genome caused by accumulation of acquired and hereditary alterations in the DNA. Recent advances clearly indicate that the non-coding, regulatory parts of the genome are critically involved in cancer pathogenesis. He we presented an overview of the role of enhancers in B-cell malignancies. Studies have demonstrated a variety of mechanisms through which enhancers controlling gene expression for proper B-cell development can be repurposed to direct the cell on a path toward malignant transformation. The emerging role of enhancers in the pathogenesis of B-cell malignancies marks a shift in cancer research: instead of paying attention to the ingredients that make up a malignant cell, focusing on the cook who determines their proportions.

Apart from broadening our understanding of B-cell malignancies and highlighting the role of non-coding sequences, this knowledge can also provide novel directions for therapeutic options. General enhancer inhibitors like BET-bromodomain protein inhibitor JQ1 or HDAC inhibitors have been investigated in different tumors [314,315]. Given the fundamental role of IGH enhancers in lymphomagenesis, they appear as attractive targets for therapeutic approaches [190,316]. Although disruption of *IGH* regulatory elements will likely affect normal B cells, transient impairment of humoral immune response is well-tolerated in humans as has been shown using the B-cell eradicating anti-CD20 antibody Rituximab that is commonly used for the treatment of B-cell lymphoma. So far, a limited num-

ber of compounds inhibiting the activity of *IGH* enhancers have been reported [317–319]. Further investigation of specific enhancers and mechanisms through which they are exploited by cancer cells can aid development of novel therapies. Cell-type specific activity of enhancers holds a promise for more precise targeting opportunities.

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Authors statements

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Marta Kasprzyk, MSc

Poznań, 8.05.2023

STATEMENT FOR DOCTORAL DISSERTATION

Regarding the procedure for awarding a doctoral degree, I hereby confirm that I have greatly contributed to the following publication which I am a co-author:

Marta Elżbieta Kasprzyk, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk

Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer

Cancers (Basel) Jun 29;13(13):3270; IF: 6.639

My contribution to the cited publication included: literature review, writing, figure and table preparation for chapters: 1. Introduction, 2.1.2. The 3' Regulatory Region, 2.1.3. Interplay between Eμ and 3'RR Enhancers, 2.3. Role of *IGH* Enhancers in Regulating Oncogene Expression and Malignant Development.

I would like to use the above publication in my doctoral dissertation entitled:
Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas.
This doctoral dissertation is prepared under the guidance of Agnieszka Dzikiewicz-Krawczyk, MD PhD.

With regards,

Kasprzyk Marta
.....
(doctoral candidate's signature)

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Weronika Sura, PhD

Poznań, 8.05.2023

STATEMENT FOR DOCTORAL DISSERTATION

Regarding the procedure for awarding a doctoral degree to Marta Kasprzyk, MSc, I hereby confirm that Marta Kasprzyk had greatly contributed to the following publication which I am a co-author:

Marta Elżbieta Kasprzyk, **Weronika Sura**, Agnieszka Dzikiewicz-Krawczyk

Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer

Cancers (Basel) Jun 29;13(13):3270; IF: 6.639

My contribution to the cited publication included literature review, writing, figure and table preparation for chapters: 2.1.1. Intronic Eμ Enhancer, 2.2.1. Mechanisms of IGH Translocations, 2.2.2. Recurrent IGH Translocations in B-Cell Lymphoma and Leukemia.

In addition, I agree for the use of the above publication in doctoral dissertation of Marta Kasprzyk entitled: *Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas*. This doctoral dissertation is prepared under the guidance of Agnieszka Dzikiewicz-Krawczyk, MD PhD.

With regards,

Weronika Sura

(co-author's signature)

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Agnieszka Dzikiewicz-Krawczyk, MD PhD

Poznań, 8.05.2023

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Marta Elżbieta Kasprzyk, Weronika Sura, **Agnieszka Dzikiewicz-Krawczyk**

Enhancing B-Cell Malignancies—On Repurposing Enhancer Activity towards Cancer

Cancers (Basel) Jun 29;13(13):3270; IF: 6.639

My contribution to the cited publication included literature review, writing, figure and table preparation for chapters: 3. Immunoglobulin Light Chain Enhancers in B-Cell Malignancies, 4. Enhancer Variants and Mutations in B-Cell Malignancies, 5. Exploiting Enhancers by Deregulated Transcription Factors, 6. Enhancer Hijacking by Lymphoma-Associated Viruses and 7. Conclusions and Future Perspective.

In addition, I agree for the use of the above publication in doctoral dissertation of Marta Kasprzyk entitled: *Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas*. This doctoral dissertation is prepared under my guidance.

With regards,

A. Dzikiewicz-Krawczyk

(supervisor's signature)

2nd ARTICLE

CRISPRi screen identifies core regions in *IGH* enhancers essential for non-Hodgkin lymphoma cells survival

Marta E Kasprzyk*, Weronika Sura*, Marta Podralska, Marta Kazimierska, Wojciech Łosiewski, Annika Seitz, Tomasz Woźniak, Jeroen E. J. Guikema, Joost Kluiver, Anke van den Berg, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk

Article pending submission.
*equal contribution

Backgroud:

Characteristic feature of B-cell non-Hodgkin lymphomas (NHL) are recurrent translocations juxtaposing an oncogene (e.g. MYC) with immunoglobulin heavy chain (IGH) enhancers: Eμ and 3'regulatory regions (3'RR1, 3'RR2). Survival and proliferation of many B-cell lymphomas depend on the expression of the translocated oncogene. The function of *IGH* enhancers in B-cell maturation is well established, while the precise mechanisms of their involvement in oncogene expression and lymphomagenesis are yet to be determined. Our goal was to identify functional elements in the *IGH* enhancers and enhancer RNAs (eRNAs) transcribed from them, which are essential for oncogene expression and B-cell lymphoma cell viability.

Results:

We designed and generated a tiling CRISPR-eIGH library targeting the human Eμ enhancer and 3'RRs (3'RR1 and 3'RR2). CRISPRi screen was performed in duplicate in 3 NHL cell lines with stable expression of dCas9-KRAB: BL41, DG75 (both Burkitt lymphoma, BL) and SUDHL4 (diffuse large B-cell lymphoma, DLBCL). We identified precise regions (300-1000 bp long), called further peaks: one in Eμ and two within each 3'RR that are necessary for B-cell lymphoma cell survival. Those results were validated for selected sgRNAs in a larger panel of lymphoma and control cells. Chromatin-enriched RNA-Seq confirmed bidirectional transcription of *IGH* enhancers and their essential regions. Those enhancer RNAs (eRNAs) were further validated in a panel of B-cell lymphomas as well as patient-derived samples. We established that eRNAs are expressed at low levels and are mainly localized in the chromatin fraction. Further investigation revealed that inhibition of *IGH* enhancer peaks in NHL leads to downregulation of translocated oncogenes expression on both RNA and protein level and decreased expression of eRNAs. The pattern of dependency on IGH enhancers differed between NHL cell lines. We also tested B-cell receptor (BCR) expression in cells

where *IGH* enhancers were blocked with CRISPRi. Our results revealed profound BCR loss mainly when Eμ enhancer peak was inhibited. Moreover, in BL (bearing IGH/MYC translocation) we were able to rescue the negative effect on cell viability observed upon blocking of core regions of IGH enhancers with MYC overexpression.

Conclusions:

This is the first study revealing core *IGH* enhancers regions, important for survival of human NHL cells. Differential dependence of B-cell lymphomas on *IGH* enhancers observed by us, may be contributed to their breakpoints pattern within the *IGH* locus – juxtaposing translocated oncogenes with the whole super-enhancer region or only a part of it. Blocking of Eμ enhancer-essential region leads to BCR loss in IGH-translocation positive B-cell lymphoma cells as well as IGH-translocation-negative cells. In agreement with previously published data, we suggest that targeting of 3'RRs can be a promising strategy in the fight against lymphoma. Inhibition of 3'RR's HS4 and HS1.2, led to downregulation of translocated oncogenes in majority of NHLs but did not affect BCR. Also, targeting of 3'RR core enhancer regions did not have negative impat on survival of IGH-translocation negative cells.

MEK contribution:

- experiments planning
- cell culture and handling,
- establishment of cell lines stably expressing dCas9-KRAB: DG75, WSU-DLCL2, OCI-LY19, HEK239T and establishment of DG75 cell line with doxycycline-induced MYC overexpression and empty vector control
- all cellular fractionations and preparation of samples for chromatin-enriched RNA-Seq
- eRNA detection and validation
- all Western Blots for cellular fractionations and oncogenes expression validation – MYC and BCL2
- RNA isolations, cDNA synthesis and qRT-PCRs for validation of CRISPR/dCas9-KRAB screens in cell lines: CA46, ST486, SUDHL4, WSU-DLCL2,
- all GFP-growth competition assays for cell lines: WSU-DLCL2, P493-6, HEK293T and replicates for CA46,
- immunostaining and flow cytometry analysis of BCR for cell lines: CA46, ST486, SUDHL4, P4936
- final data analysis and statistics for all qRT-PCRs, Western Blots, GFP-growth competition assays (without statistics) and BCR immunostainings, MYC resuce experiments
- preparation of tables, figures and original draft of the manuscript

2nd ARTICLE – full text

ORIGINAL ARTICLE

CRISPRi screen identifies core regions in IGH enhancers essential for non-Hodgkin lymphoma cells survival

Marta Elżbieta Kasprzyk[1*], Weronika Sura[1*], Marta Podralska[1], Marta Kazimierska[1], Annika Seitz[2], Wojciech Łosiewski[1], Tomasz Woźniak[1], Jeroen E. J. Guikema[3], Joost Kluiver[2], Anke van den Berg[2], Natalia Rozwadowska[1], Agnieszka Dzikiewicz-Krawczyk[1+]

[1] Institute of Human Genetics, Polish Academy of Sciences, Poznań, Poland
[2] Department of Pathology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[3] Department of Pathology, Amsterdam University Medical Centers, Amsterdam, The Netherlands
* Authors contributed equally
[+] Corresponding author. E-mail address: agnieszka.dzikiewicz-krawczyk@igcz.poznan.pl

Abstract

Chromosomal translocations in non-Hodgkin lymphoma (NHL) are known to bring oncogenes under the regulation of immunoglobulin heavy chain (IGH) super-enhancers, composed of the intronic Eμ enhancer and 3' regulatory regions (3'RR1, 3'RR2). Deregulation of translocated oncogenes by enhancer activity can contribute to lymphomagenesis. The role of *IGH* enhancers in normal B-cell development is well established, but the knowledge regarding precise mechanisms of their involvement in control of the translocated oncogenes is limited. The goal of this project was to identify the functional regions in *IGH* regulatory elements and enhancer RNAs (eRNA) essential for NHL cell growth. We designed sgRNA library densely covering the *IGH* enhancers and performed tiling CRISPR interference screens in three NHL cell lines. Our results revealed exact regions, one in the Eμ and two in each of the 3'RR enhancers, whose targeting profoundly inhibited NHL cells growth. With the use of chromatin-enriched RNA-Seq we confirmed transcription from those core enhancer regions and validated eRNAs expression in a large panel of NHL cell lines and in patient-derived samples. We showed in several NHL cell lines that inhibition of essential *IGH* enhancer regions decreased expression of eRNAs and translocated oncogenes in the fashion likely related to their breakpoints pattern within *IGH* locus. In addition, we reported loss of B-cell receptor (BCR) by targeting the Eμ enhancer but only slightly by inhibiting the 3'RRs essential regions. In Burkitt lymphoma, MYC overexpression partially rescued the phenotype of *IGH* enhancers inhibition. Our results provide new insights into the current understanding of *IGH* enhancers role in B-cell NHLs and indicate precise regions that may become a basis for development of potential therapeutic approaches.

Keywords: IGH, enhancer, lymphoma, BCR, MYC, BCL2

Introduction

Non-Hodgkin lymphomas (NHL) are the most common hematologic malignancy accounting 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: VDJ recombination, somatic hypermutation (SHM) and class switch recombination (CSR). Obligatory intermediates during the *IGH* locus rearrangements are DNA-double strand breaks. They create lesions, that can result in illegitimate recombination[3]. Indeed, several recurrent chromosomal translocations occur in NHL, e.g. t(8;14)(q24;q32) *MYC/IGH*[4] in Burkitt Lymphoma (BL) and t(14;18)(q32;q21) *IGH/BCL2*[5] in diffuse large B-cell lymphoma (DLBCL)[6] and follicular lymphoma (FL)[7]. 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, DNA-damage response[8–10]. It is a widely studied oncogene in hematological malignancies, and its deregulation may constitute a primary cause of lymphomagenesis or may occur as a secondary aberration. BCL2 suppresses cell death by preventing the activation of caspases[11,12]. Escape from apoptosis in lymphoma cells with BCL2 translocation contributes to treatment resistance and poor prognosis[13–15]. Interestingly, oncogenic translocation itself may not be sufficient to drive lymphomagenesis[16], yet can contribute to instability that can in turn lead to accumulation of other mutations and malignant transformation[17–19].

Usually one *IGH* allele is involved in the translocation, while the other is productively rearranged and leads to expression of the B-cell antigen receptor (BCR)[20]. BCR consists of a transmembrane immunoglobulin, which interacts with an antigen, and a signal-propagating domain Igα/Igβ (CD79A/CD79B). Throughout their lifetime B-cells, including lymphoma cells, are constantly tested for proper B-cell receptor (BCR) presentation on cell surface[20]. BCR signaling can be divided into (1) tonic, (2) chronic active, and (3) priming signaling necessary for the B cell differentiation into antibody-secreting cells (summarized recently in Liu W. et al. 2020[21]). The first one is antigen-independent and is necessary for B-cell development and survival. DLBCLs of germinal-center B-cell (GCB-DLBCL) subtype often rely on this type of signaling[22,23]. Chronic active BCR signaling is antigen-dependent and important for proliferation of DLBCLs derived from activated B-cells (ABC-DLBCL), FL and mantle cell lymphomas (MCL)[23]. While BL can rely on both tonic and active BCR signaling[24].

The activity of the *IGH* locus is governed by its enhancers: Eμ, often named an intronic enhancer, due to its location between the JH segment and the Cμ region, and 3'regulatory regions (3'RR1 and 3'RR2, Figure 1A)[25]. Enhancers organization differs between human and mice[26–28]. In human there are two copies of 3'RRs, composed of DNase I hypersensitive sites (HS): HS4, HS1.2, HS3, while in mice there is one 3'RR built of HS4, HS3b, HS1.2, HS3a. Active enhancers are characterized by open chromatin, supported by specific histone marks - high histone H3 lysine 4 monomethylation (H3K4me1)[29,30] but low trimethylation (H3K4me3)[31], and high histone H3 lysine 27 acetylation (H3K27ac)[32], binding of transcription factors and also - enhancer RNA (eRNA) expression. This class of non-coding RNAs was considered a by-product of an active transcription machinery, but increasing evidence shows that they can be functional (reviewed recently in Han Z. & Li W., 2022[33]). eRNAs can serve as scaffolds for recruitment of transcription factors, Mediator complex, RNA polymerase and are helping to achieve phase separation and proper enhancer-promoter looping. eRNAs can be transcribed bidirectionally and are characterized by rather low expression and stability. Recently, eRNA ARIEL was found to be a driver of oncogenesis in T-cell acute lymphoblastic leukemia[34]. In BL eRNA AL928768.3 was shown to downregulate MYC expression upon knock-down[35]. Nevertheless, knowledge regarding eRNAs roles in NHL remains limited.

Function of *IGH* enhancers has been extensively studied in normal B-cells. Studies showed that Eμ is important for earlier stages of B-cell development, mainly VDJ recombination[36], while 3'RR takes over the locus control at the later stages, namely SHM and CSR, and is referred to as the locus control region (LCR)[37]. Moreover, several mouse models demonstrated their involvement in oncogene expression regulation and lymphomagenesis, but still more research is necessary to understand regulation in malignant human B-cells (reviewed in Kasprzyk ME et al., 2021[18]). Lessons learned from animal models show that Eμ- and 3'RR-controlled oncogene expression usually leads in time to development of either immature or mature B-cell lymphomas, respectively. In lymphoma, chromosomal breakpoint regions within *IGH* locus differ, so juxtaposed oncogenes may be either under the regulation of the whole *IGH* super-enhancer region or only its part . Given the fact that *IGH* enhancers encompass 30-40 kb of DNA sequence, it is important to pinpoint the core regions crucial for lymphoma cells.

CRISPR/Cas9 system can be successfully applied in enhancer study[38] and help reveal functions of those tissue-specific *cis*-regulatory elements. Here, we employed tiling CRISPR/dCas9-KRAB screen to identify precise regions within Eμ and 3'RRs in BL and DLBCL cell lines that are necessary for their survival. We show that inhibition of core regions of *IGH* enhancers causes downregulation of translocated oncogenes and eRNA expression. Additionally, we report BCR loss upon blocking of the significant region of Eμ, but - in majority of cases - not 3'RRs. We confirm eRNAs expression from core *IGH* enhancer regions in both NHL cell lines and patient-derived samples and show that MYC overexpression can partially rescue proliferation of BL cells with blocked *IGH* enhancers-essential regions.

Materials and Methods

Cell lines

Several Non-Hodgkin Lymphoma cell lines bearing *IGH* translocations were used in this study. Four Burkitt Lymphoma cell lines, representing t(8;14) *MYC/IGH* translocation: BL41, CA46, DG75, (DSMZ, Braunschweig, Germany) and ST486 (ATCC, LGC Standards, Lomianki, Poland). Two Diffuse Large B-cell Lymphoma cell lines, representing t(14;18) *IGH/BCL2*: SUDHL4 and WSU-DLCL2 (DSMZ). We also used B-cells not bearing *IGH*-translocation: P493-6[39] (gift from prof. D. Eick, Helmholtz Center, Munich, Germany) and a lymphoblastoid cell line derived from a healthy donor (LCL) K1[40]. All B-lymphocyte cell lines were grown in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 2mM L-glutamine, 1% Penicillin-Streptomycin (Biowest, Nuaillé, France) and 10-20% fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, US). HEK293T (DSMZ) cells were cultured in DMEM (Lonza) supplemented as described above. Cell lines were cultured in standard conditions: 37°C and 5% CO_2 in humidified incubator. Cells were regularly tested for *Mycoplasma* contamination by PCR.

Patient samples

Enhancer RNA expression was validated in patient-derived NHL samples: BL (8 cases) and DLBCL (14 cases) as well as in germinal center B-cells, isolated from tonsils of healthy donors (6 cases). Tissues were used in accordance with the Declaration of Helsinki and the protocol was approved by the Medical Ethical Review board of the UMCG (RR#201800554).

Plasmids

Lenti-dCas9-KRAB-blast[41] (#89567) and lentiGuide-Puro[42] (#52963) were purchased from Addgene (Watertown, Massachusetts, USA) and used to establish NHL cell lines with stable expression of catalytically inactive Cas9 fused with KRAB domain and cloning of CRISPR-eIGH library, respectively. LentiGuide-Puro was also used for further validation of individual sgRNAs. pAW12.lentiguide.GFP[43] (#104374) purchased from Addgene was used in the GFP growth competition assays.

For MYC over-expression experiments, pCW57-MCS1-P2A-MCS2 (GFP)[44] (#80924, Addgene) with cloned MYC ORF was used to establish DG75-dCas9-KRAB-MYC OE cell line with inducible MYC expression (Supplementary Figure 7A). Cells transduced with empty vector were used as controls. pCW57-MCS1-P2A-MCS-MYC was a kind gift of Jeroen Guikema, PhD, University of Amsterdam, Netherlands.

For the production of lentiviral particles 2nd generation plasmids were used: envelope expressing plasmid pMD2.G (gift from Didier Trono, #12259, Addgene) and packaging plasmid psPAX2 (gift from Didier Trono, #12260, Addgene).

Design of the CRISPR-eIGH library

In this study we aimed to target the *IGH* enhancer regions as defined by the H3K27ac histone mark plus 5 kb flanking sequences: Eμ (hg19 chr14:106317800-106336124), 3'RR1 (hg19 chr14:106140100-106181250) and 3'RR2 (hg19 chr14:106019800-106055011). The sequences were searched for the presence of the PAM sequence (5'-NGG-3') on both strands and all possible sgRNAs were listed using a custom Python script. Since 3'RR1 and 3'RR2 are highly homologous, numerous sgRNAs could target both regions, and those were allowed. Off-target binding of sgRNAs was checked using the Cas-OT script[45]. Only sgRNAs with at least three mismatches to the potential off-targets or at least two mismatches including at least one in the seed region (nt 9-20) were retained. We also included in the library 900 non-targeting sgRNAs from the Brunello library as a negative control[46] and 10 sgRNAs targeting regions -50 bp to +100 bp relative to TSS of CD79a and CD79b as positive controls. The list of all sgRNA oligonucleotides is provided in Supplementary Table 1.

Cloning of the CRISPR-eIGH library

Designed sgRNAs were ordered from Twist Bioscience (San Francisco, CA, US) as oligonucleotides with the 20 nt sgRNA sequences flanked by sequences complementary to the lentiGuide-Puro vector TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG[sgRNA]GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGT. 2 ng of CRISPR-eIGH library was amplified with oligo-F and oligo-R primers (Supplementary Table 2) using NEBNextHiFidelity Master Mix (New England Biolabs, Ipswich, MA, US) in 20 x 25 μl PCR reactions. PCR program: 98°C 30 sec; (98°C 10 sec; 63°C 10 sec; 72°C 15 sec) x 6 cycles; 72°C 2 min. Pooled PCR reactions were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Purified PCR product was then run on agarose gel and extracted with QIAquick Gel Extraction Kit (Qiagen). LentiGuide-Puro was digested with BsmBI (New England Biolabs) and purified from agarose gel, same as oligo library. Purified sgRNA CRISPR-eIGH library was cloned into the lentiGuide-Puro vector using the circular polymerase extension cloning (CPEC) method as described previously[47]. Briefly, 20 CPEC reactions were

performed with 10:1 insert:vector molar ratio (17 ng amplified oligos, 100 ng vector) and NEB NextHiFidelity Master Mix. PCR program: 98°C 30 sec; (98°C 10 sec; 72°C 4,5 min) x 5 cycles; 72°C 5 min. All PCR reactions were pooled and purified by isopropanol precipitation. 300 ng of the purified CPEC product was used for transformation of electrocompetent Endura cells (Lucigen, Middleton, WI, USA) according to the manufacturer's protocol. Three electroporations were performed giving in total ~7.7 million colonies and resulting in ~710x coverage of the library. Bacteria were spread on 245x245 mm agar plates and grown for 13 h at 37°C. Colonies were scraped off the plates and plasmid DNA was isolated using Plasmid Plus Maxi Kit (Qiagen). Quality of the cloned CRISPR-eIGH library was verified by MiSeq next generation sequencing on Illumina platform (Laboratory of High-Throughput Technologies, Adam Mickiewicz University).

Cloning of individual sgRNA constructs

For cloning of individual sgRNAs into the lentiGuide-Puro and pAW12.lentiguide.GFP vectors, sense and antisense oligonucleotides containing overhangs compatible with BsmBI sticky ends (Supplementary Table 3) were synthesized by Genomed (Warsaw, Poland). They were resuspended in annealing buffer (10mM Tris-HCl pH 8, 1mM EDTA pH 8, 50mM NaCl) and annealed in a thermocycler under conditions: 95°C 5min, 95°C (-1°C/cycle) x 70 cycles. Annealed oligos were ligated into the BsmBI-digested lentiGuide-Puro and pAW12.lentiguide.GFP vectors at 1:5 vector:insert molar ratio with T4 DNA ligase (Invitrogen, Carlsbad, CA,US). 1 µl of ligation reaction was used for transformation of JM109 competent cells (Promega,Madison, WI, USA). Plasmid DNA from single colonies was isolated using Plasmid Plus Maxi Kit (Qiagen) and the sequences were confirmed by Sanger sequencing (Genomed).

Generation of lentiviral particles

HEK293T cells were seeded in 6-well plates one day prior to transfection. Next day, ~80% confluent cells were transfected with 2nd generation packaging plasmids psPAX (1.5 µg) and pMD2.G (1 µg), together with 2 µg of the transfer plasmid using Lipofectamine 2000 (Invitrogen) or calcium phosphate method (Invitrogen). One day after transfection medium was replaced with fresh DMEM supplemented with 10% FBS. 48 h and 72 h post-transfection lentiviral supernatants were filtered through 0.45 µm filter and stored at -80°C.

Establishing cell lines with stable expression of dCas9-KRAB

1-2 x 10^6 of optimally dividing cells were transduced with 2nd generation lentiviral particles carrying lenti-dCas9-KRAB-blast. Briefly, cells were seeded on a 12-well plate, cell line-optimized virus amount was added along with 4 µg/ml polybrene and placed in 37°C incubator. For some cell lines spinfection was performed at 33°C, 1000 x g, 2h. After 24 h, transduced cell lines were washed in PBS and seeded for blasticidin (bln; Gibco, Grand Island, New York, USA) selection as indicated in Table 1. Antibiotic selection lasted 6 days. Genomic insertion of dCas9-KRAB expressing cassette was confirmed on DNA level, followed by expression validation on RNA level by qRT-PCR and on protein level by Western Blot.

Table 1. Seeding densities and antibiotic concentrations used for cells selection after lentiviral transduction.

Cell line	Seeding density x10^6/ml	Puromycin concentration	Blasticidin concentration
BL41	0.25	1 µg/ml	2.5 µg/ml
CA46	0.15	2.3 µg/ml	5 µg/ml
DG75	0.17	3 µg/ml	20 µg/ml
ST486	0.25	0.3 µg/ml	8 µg/ml
SUDHL4	0.3	1 µg/ml	10 µg/ml
WSU-DLCL2	0.3	1 µg/ml	15 µg/ml
P493-6	0.25	1 µg/ml	20 µg/ml
HEK293T	0.2	NA	5 µg/ml

NA - not applicable

Determination of virus titer and cell transduction with CRISPR-eIGH library

For CRISPRi screening experiment, the lentivirus titer was determined in order to transduce ca. 30% cells, which guarantees that approximately 85% of them are infected by a single construct[48]. For this purpose, 2.5 x 10^6 cells per well were plated in 12-well plate and transduced with various amounts of lentivirus in the presence of 4 µg/ml polybrene. Cells were spinfected at 33°C, 1000g, for 2h and afterwards, additional 1 ml of medium was added. 24 h after transduction cells were washed and were plated out in duplicate with and without puromycin. Medium was changed after three days. After four days of selection cells were counted and the percentage of cells surviving puromycin treatment relative to cells without puromycin was calculated. Based on this, the amount of virus resulting in ~30% surviving cells was determined.

For the CRISPR screen, 27.5 x 10^6 cells were transduced in duplicate with CRISPR-eIGH library, with the previously established amount of virus. After four days of selection with puromycin (T0) 8 x 10^6 cells was collected for DNA isolation. Remaining cells were further cultured for 20 population doublings. At each passage, the amount of cells corresponding to 1,000x coverage of the library, that is 8 x 10^6, 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).

Preparation of libraries for next-generation sequencing of the plasmid pool or genomic DNA

sgRNA sequences from the CRISPR-eIGH library plasmids were amplified as described previously[49]. Briefly, PCR reactions were performed using High-Fidelity MasterMix 2x (New England Biolabs, #M0541L) and primers incorporating Illumina adaptors (Supplementary Table 4). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, # 28106), then analyzed by DNA electrophoresis and extracted from agarose gel with QIAquick Gel Extraction Kit (Qiagen, # 28706). Prior to NGS, the quality and quantity of library was determined using KAPA Library Quantification Kit (Roche, # 07960140001).

To analyze the sgRNA representation in the screening experiments, genomic DNA from cell lines was isolated using GENTRA Puregene Kit (Qiagen, #158722) and then sgRNA sequences were amplified as described above. DNA from 8 x 10^6 cells was amplified in 30 (BL41, DG75) or 40 (SUDHL4) individual 50 µl PCR reactions per sample with 2-2.5 µg DNA input.

NGS and data analysis

NGS was performed on Illumina X-Ten platform at BGI (Hong-Kong). Adaptor sequences were removed and reads were split for individual samples based on specific barcodes. Supplementary Table 5 summarizes the number of reads obtained for each sample. A Python script[50] was used for sgRNA enumeration. Only reads with no mismatches to sgRNA sequences were counted. Next, fold change (FC) between the beginning and end of screen was calculated for each sgRNA. FC values for both screen replicates were averaged. To identify regions whose targeting significantly affected lymphoma cell growth, we applied the sliding window approach[51]. Average FC values were calculated for 20 consecutive sgRNAs (Supplementary Table 6). Significance was calculated with t-test comparing FC values for 20 sgRNAs in each window with the negative control sgRNAs. We considered as significant windows which were at least 1.5-fold depleted (log2FC -0.585) and with FDR<0.001.

Cellular fractionation

20 x 10^6 cells (30 x 10^6 for SUDHL4) were used for cellular fractionation as described previously[52]. All steps were performed on ice. Buffers were supplemented with DTT (if noted), 1x EDTA-free protease inhibitors and 40U/ml RNaseOUT (Invitrogen) directly before use and kept on ice. Briefly, cells were washed in ice-cold PBS. Then cell pellet was resuspended in 500 µl Buffer W (300 mM sucrose, 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 2 mM $MgAc_2$ 0.5 mM DTT), followed by addition of 500 µl Buffer L (Buffer W supplemented with 6 mM $CaCl_2$, 0.2 % IGEPAL CA-630, 0.5 mM DTT). Lysates were centrifuged 1000 x g, 4°C, 10 min. Resulting supernatant was saved as cytoplasmic fraction, while nuclear pellet was processed further. Nuclei were washed in Buffer G (50 mM Tris-HCL pH 8.0, 25% glycerol, 5 mM $MgAc_2$, 0.1 mM EDTA, 5 mM DTT), then lysed with Buffer U (1 M urea, 20 mM HEPER pH 7.5, 7.5 mM $MgCl_2$, 0,1 mM EGTA, 300 mM NaCl, 1 mM DTT), vortexed and centrifuged 20 000 x g, 4°C, 10 min. Resulting supernatant was saved as nuclear fraction, while chromatin pellet was washed twice in Chromatin Wash Buffer (50 mM Tris-HCl pH 8.0 supplemented with 40 U/ml RNaseOUT). Chromatin pellet was then sonicated twice [5 sec on, 30 sec off] (Misonix 3000; Misonix, Farmingdale, NY, USA) on ice in 300 µl Buffer G for RNA isolation or in 500 µl RIPA buffer (Sigma, Saint Louis, MO, USA) supplemented with protease inhibitors for protein extraction. RNA was isolated from each fraction using Trizol method. Additionally, 10 µl 500 mM EDTA was added to chromatin fraction and heated 10 min 65°C with 600 rpm shaking on thermoblock. RNA and protein samples were stored in -80°C until analysis. To confirm successful separation of the fractions on RNA level, 2 marker genes were used per each fraction: RPPH1 and DANCER for cytoplasm, U3SNO and MIAT for nuclei and TBP_intron and KTN1_AS1_intron for chromatin (primers listed in Supplementary Table 2). To additionally confirm that the fractionation was successful, Western Blot was performed with fraction-specific antibodies (Supplementary Table 7), as described in Methods section **Western Blot**.

Chromatin-enriched RNA-Seq and data analysis

RNA from chromatin fraction of two biological replicates per each cell line was tested on 2% agarose gel for integrity. Prior to sequencing, RNA was treated with TURBO-DNase (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions, followed by purification using RNA Clean & Concentrator (Zymo Research, Irvine, CA, USA) with additional DNase treatment on the column. RNA-Seq stranded

library preparation, pair-end sequencing and bioinformatic data analysis was performed by Novogene (Beijing, China). EpicentreRibo-zeroTM rRNA Removal Kit (Epicentre, USA) and NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (NEB, USA) was used for library preparation. Sequencing was performed on Illumina X-Ten platform (Novogene). Quality control of raw data (raw reads) in FASTQ format was performed in Novogene. Clean data were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. Approximately 77-92 x 10^6 clean reads were obtained per sample. All downstream analyses were based on the 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[53] 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, BedGraph files were generated and visualized in UCSC Genome Browser.

RNA isolation, cDNA synthesis and RT-qPCR

RNA was isolated from 1-2 x 10^6 cells using RNA MiniPrep (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. DNase treatment was performed on the column. To obtain RNA from cellular fractions Trizol method was applied. Briefly, samples were resuspended in TRI Reagent (Sigma), chromatin fraction was additionaly heated as desribed in **Cellular fractionation.** Samples were then mixed with chloroform and centrifuged according to manufacturer's instructions. Next, RNA was precipitated with 96-99.9 % ethanol, 300 mM NaCl and 15 µg GlycoBlue™ Coprecipitant (Invitrogen) at -20^{o}C over-night. Next day, RNA pellet was washed in 75% ethanol and resuspended in UltraPure RNAse-free water. Chromatin fraction was additionally treated with TURBO-DNase (Invitrogen) according to manufacturer's instructions. Next, RNA from all fractions was purified and DNase treated on the column using RNA Clean and Concentrator (ZYMO Research). Reverse transcription was performed using 300-1,000 ng of total RNA or RNA fractions with QuantiTect Reverse transcription Kit (Qiagen) or SuperScript III and random primers (both Thermo Fisher Scientific).

qPCR analysis was performed using 5-15 ng cDNA with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) on CFX96 Touch qPCR System (Bio-Rad, Hercules, CA, US). Gene expression was normalized relative to the housekeeping gene. For eRNA expression validation in larger panel of B-cell cell lines and in FFPE patient samples qPCR was done with with 2X SYBR Green Master Mix (Applied Biosystems B.V., Bleiswijk, The Netherlands) on Light Cycler 480 (F. Hoffmann-La Roche, Basel, Switzerland). All primer sequences used in this study are available in Supplementary Table 2.

Validation of enhancer RNA expression

To validate eRNA expression, primers specific for transcriptionally active *IGH* regions, based on chromatin-enriched RNA-seq, were designed (Supplementary Table 2). eRNA expression was verified in a panel of B-cells and in patient-derived samples in collaboration with prof. Anke van den Berg group at University Medical Center Groningen, Netherlands. Cell panel consisted of 9 BL cell lines: EBV-negative - DG75, CA46, ST486, BL41, Ramos and EBV-positive - BL65, Namalwa, Jijoye, Raji; 12 DLBCL: derived from germinal center B-cells (GCB) - SUDHL16, SUDHL10, SUDHL6, SUDHL5, SUDHL4, SC1, WSU-DLCL2 and from activated

B-cells (ABC) - U2932, Ocily3, RI-1, Nu-dul1, SUDHL2; 8 Hodgkin lymphoma (HL) cell lines: L428, L1236, KMH2, DEV, L540, L591, SUPHD1, HDLM2 and 4 germinal center B-cell samples as controls. For DLBCL, 5 cell lines were of activated B-cell origin (ACB) and 7 of germinal center origin. Patient samples consisted of 8 BL cases, 14 DLBCL cases as well as 6 germinal center B-cell controls, isolated from tonsils of healthy donors (6 cases).

GFP competition assay

0.5-1 x 10^6 cells were transduced with individual sgRNA constructs in pAW12.lentiguide.GFP and cultured for 22 days. Cells were analyzed three times a week by flow cytometry using CytoFLEX S (Beckman Coulter, Indianapolis, Indiana, USA). The percentage of GFP-positive cells was calculated in reference to the start of the experiment (day 4 or day 6) set as 100%.

B-cell receptor (BCR) immunostaining

1 x 10^6 cells per sample were collected and washed with PBS, then with ice-cold staining buffer (2% FBS in PBS). Cells were resuspended in 95 µl of ice-cold staining buffer with addition of 5 µl FcR blocking reagent, human, (MiltenyiBiotec, Bergisch Gladbach, North Rhine-Westphalia, Germany, #130-059-901) and incubated 10 min in a fridge. Then, 20 µl of APC Mouse Anti-Human IgM (BD Biosciences, Franklin Lakes, New Jersey, USA, #551062) or 0.1 µg of Goat F(ab')2 Anti-Human IgG-AF647 (Southern Biotech, Birmingham, Alabama, USA, 2042-31) was added and cells were incubated 25 min on ice, protected from light. Next, cells were washed twice with 2 ml of staining buffer and finally resuspended in 100 µl of this buffer for flow cytometry analysis, performed on CytoFLEX S (Beckman Coulter).

MYC overexpression

1 x 10^6 DG75-dCas9-KRAB cells were transduced with 2^{nd} generation lentiviral particles carrying pCW57-MCS1-P2A-MCS2-MYC OE (GFP) or empty vector (EV). GFP-positive cells were sorted in PBS supplemented with 1% FBS and collected in culture medium supplemented with 50% FBS in 5 ml cytometric tubes. DG75-dCas9-KRAB-MYC OE cells were sorted on SH800S cell sorter (Sony Biotechnology, San Jose, California, USA) using 100 µM nozzle, normal mode. DG75-dCas9-KRAB-EV cells sorting was carried out using the BD FACS Aria™III (Becton Dickinson, Franklin Lakes, New Jersey, USA) cell sorter using 100 µM nozzle, 20 psi (0,138 MPa), 4-way sorting purity mode was selected for gaining highest purity level. After sorting cells were seeded at high density 1 x 10^6 cells/ml in complete RPMI medium and then split depending on cell fitness. MYC overexpression upon addition of doxycycline (Merck, Darmstadt, Germany) was evaluated on RNA and protein level. To assess cell survival upon MYC overexpression, DG75-MYC-OE cells were mixed 1:1 with DG75-dCas9 (no GFP, no ectopic MYC overexpression) and GFP growth competition assay was performed for 3 weeks. For the rescue experiment, 1 x 10^6 DG75-dCas9-KRAB-MYC OE and EV cells were transduced with 2^{nd} generation lentiviral particles carrying individual sgRNAs cloned into lentiGuidePuro, followed bypuromycin selection.

Cell viability assay

Cell viability was measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Briefly, at day 6 post-infection, 2000 cells per well were seeded on 96-well plates in triplicate. Doxycycline was added to the final concentration of 0.1 µg/ml. As control, wells without doxycycline were prepared. CellTiter-Glo reagent was mixed 1:2 in PBS and 100 µl per well was added. Luminescence was measured on GloMax-Multi Detection System (Promega) at 1h (baseline) and 72h after seeding cells. Cell viability was determined as the relative luminescence (RLU) calculated relative to the first measurement (1h). For each experiment three biological replicates were made. Change in cell growth upon doxycycline administration in cells infected with sgRNAs targeting *IGH* enhancers was calculated relative to the non-targeting controls.

Western Blot

5-20 x 10^6 cells were washed in PBS, then lysed in RIPA buffer (Sigma, Saint Louis, MO, USA), supplemented with protease inhibitors. Protein concentration was determined using Bicinchoninic Acid Kit (Sigma). 20 µg of total protein was mixed with the Laemmli 4X sample buffer (Sigma), heat-denatured and separated at 120V on the 8% (for Cas9 detection) or 12% polyacrylamide gel (PAA, acrylamide:bisacrylamide, 49:1) supplemented with 2,2,2-trichloroetanol (Merck, Kenilworth, NJ, USA) to allow for stain-free total protein detection. For cellular fractionation control, equal portion of each faction was loaded on 12 % PAA. Proteins were transferred onto PVDF (Bio-Rad, #1620177) or Low Fluorescence PVDF (Bio-Rad, #1620264) membrane for 1.5h 75V at room temperature with cooling, blocked for 1h in 5% milk in TBST and incubated with primary antibody at 4^{o}C over-night. Next membranes were washed in TBST, followed by TBS wash and incubated with secondary HRP-conjugated antibody for 1h at room temperature. Signal was detected by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad) with ChemiDoc™ Imaging Systems (Bio-Rad). Quantitative analysis was performed using Image Lab™ Software (Bio-Rad). For expression analysis bands were normalized using total protein method. Full list of antibodies used in this study can be found in Supplementary Table 7.

Statistical analysis

In eRNA expression validation, difference in expression between tested groups was evaluated with ANOVA Kruskall-Wallis with Dunn's Multiple Comparison Post-Test. For analysis of other qRT-PCR results Mann-Whitney test was applied. BCR staining was analyzed with Student's two-tailed t-test and cell viability change upon MYC overexpression with Student's paired one-tailed t-test. Those statistical analyses were calculated with GraphPad Prism version 5.0.0 (GraphPad Software Inc., San Diego, California, USA) with a P-value significance cut-off $P<0.05$. Statistics for GFP-growth competition assay was calculated as described previously[54] using SPSS software (IBM, Armonk, New York, USA). Mixed model analysis was used for comparison of GFP-positive cells decrease over time between controls and samples with blocked *IGH*-enhancers essential regions, time and the interaction of time and given sgRNA were treated as fixed effects and the measurement repeat for a given sgRNA as random effect.

Results

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

Target regions of the human Eμ enhancer, 3'RR1 and 3'RR2 were defined by the presence of the H3K27ac histone mark in B cells (Figure 1B). Restricted by the availability of the PAM sequence (NGG), in total 18,732 sgRNAs can target these regions. However, due to homology within 3'RR1 and 3'RR2, several sgRNAs were present multiple times in the design. Of these, we excluded those sgRNAs that targeted 3 or more sites within the regions of interest, resulting in a total of 12,062 sgRNAs. Further testing for off-targets elsewhere in the genome using the Cas-OT script excluded 5,080 sgRNAs. The final library contains 6,982 sgRNAs that cover the Eμ enhancer, 3'RR1 and 3'RR2 with average distances between sgRNAs of 8 bp for the Eμ enhancer and 10 bp for 3'RR1 and 3'RR2 (Figure 1C). 2,344 sgRNAs were common for 3'RR1 and 3'RR2. Including the negative and positive control sgRNAs, the final library size was 7971 sgRNAs (Supplementary Table 5).

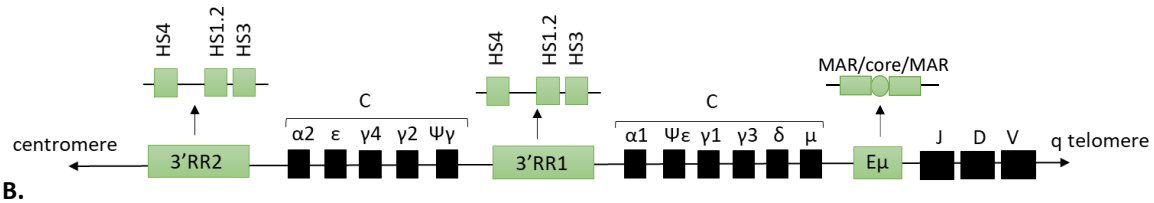
Quality check with MiSeq of the CRISPR-eIGH library cloned into the lentiGuide_puro vector confirmed its completeness and integrity (Figure 1D). 88.6% of guides matched perfectly and there were no undetected guides. Skew ratio of top 10% to bottom 10% was 1.58. This confirms good quality of the generated CRISPR-eIGH library.

CRISPRi screen identifies elements of *IGH* enhancers essential for lymphoma cell growth

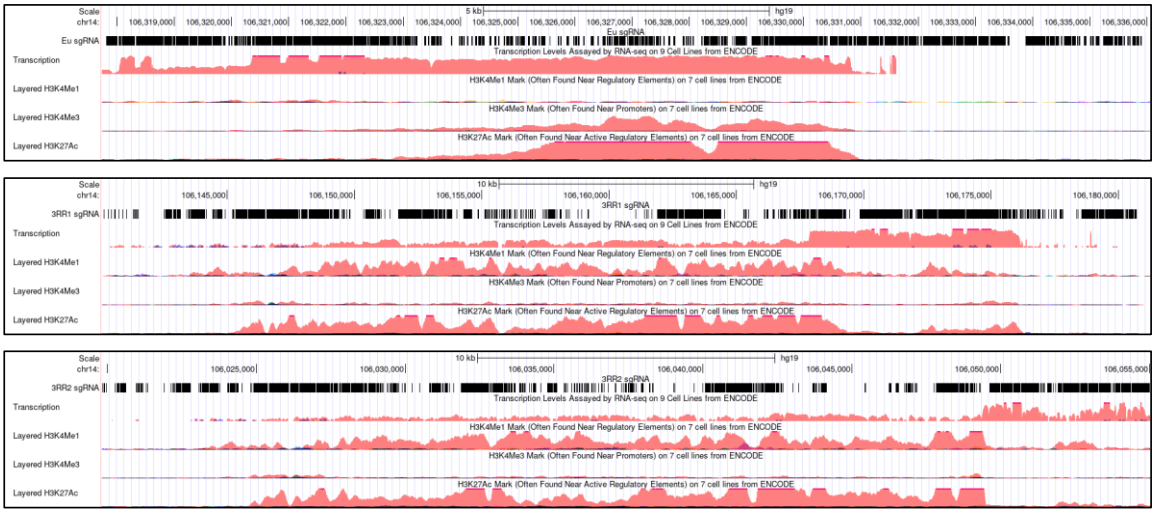
To identify essential elements of *IGH* enhancers, we performed CRISPR interference screen in which catalytically inactive Cas9 (dCas9) fused with the repressive KRAB domain silenced regions targeted by the CRISPR-eIGH library (Figure 1E). First, we generated lymphoma cell lines (BL41, DG75, SUDHL4) stably expressing dCas9-KRAB. Expression of dCas9-KRAB protein was monitored throughout the time of 4 weeks (Supplementary Figure 1A). These stable cell lines were thereafter transduced in duplicate with lentivirus carrying the CRISPR-eIGH library, aiming at 1000x coverage of the library and 30% transduction efficiency. The actual coverage ranged between 750 and 1,045x and the transduction efficiency – between 21.8% and 30.4% (Supplementary Figure 1B). After transduction, cells were collected immediately after puromycin selection (T0) and then after ca. 20 cell doublings (T1). The representation of sgRNA library in the cell populations was then assessed by next-generation sequencing.

In each cell line we identified a few hundreds of sgRNAs consistently ≥ 2-fold depleted in both screen replicates (Figure 2B). Nontargeting sgRNAs did not show any major effects, while several sgRNAs targeting CD79a and CD79b were also depleted. All read counts are available in Supplementary Table 5.

A. Human, *IGH* locus, chr14



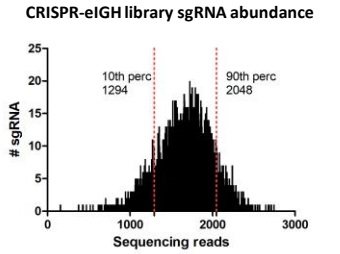
B.



C.

Region	Localization (hg19)	Number of sgRNAs	Mean distance	Median distance	Min distance	Max distance
Eμ	chr14:106322800-106331124	2219	8.1	4	1	302
3'RR1	chr14:106145100-106176250	3784	10.7	4	1	1850
3'RR2	chr14:106024800-106050011	3322	10.6	4	1	1285

D.



MiSeq analysis (~1,600x coverage)	
Percentage of guides that matched perfectly	88.6
Percentage of undetected guides	0.0
Skew ratio of top 10% to bottom 10%	1.58

E.

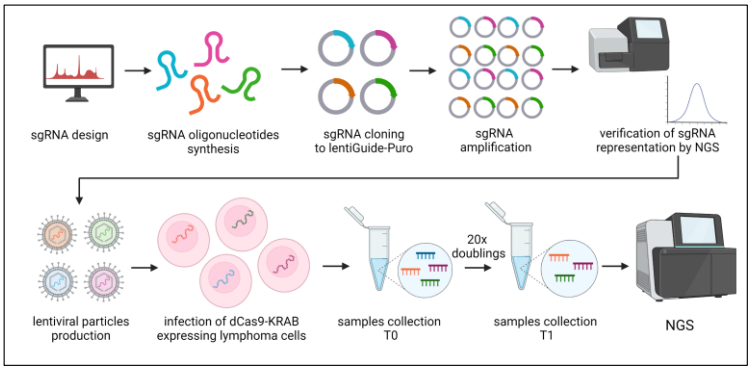


Figure 1. Design and generation of a tiling CRISPRi library targeting IGH enhancers. A. Scheme of the human *IGH* locus. Green - *IGH* enhancers: two 3' regulatory regions (3'RR), which are composed of smaller enhancers (HS) and the intronic enhancer Eμ, composed of the core and two matrix attachment regions (MAR); black - immunoglobulin genes: C- constant, J – joining, D – diverse, V – variable. **B.** Regions used for the design of the CRISPR-eIGH library. Black lines indicate localization of sgRNAs oligonucleotides. From the top: Eμ, 3'RR1 and 3'RR2. Peaks represent UCSC tracks : transcription, H3K4me1, H3K4me3, H3K27ac of GM12878 B-cells from ENCODE. **C.** Summary of the sgRNA distribution in the CRISPR-eIGH library. Note that since 3'RR1 and 3'RR2 are highly similar, 2344 sgRNAs are common for both regions. **D.** SgRNA abundance in the CRISPR-eIGH library obtained with Mi-Seq. **E.** Overview of the CRISPRi screen experiment.

To identify essential regions based on the results from multiple sgRNAs, we used a sliding window approach and calculated the mean fold change for the windows of 20 consecutive sgRNAs (Supplementary Table 6). This led to distinction of three essential regions, called hereafter peaks, marked by sgRNAs highly depleted in T1 comparing to T0 (one peak in Eμ enhancer – named Eμ-peak - and two peaks in 3'RR1 and 3'RR2 enhancers – named 3'RR-peak1 and 3'RR-peak2) (Figure 2C). These peaks were identified in all cell lines, although the profoundness of the depletion varied between them. For the Eμ-peak, encompassing region chr14:106328838-106329184, the strongest effect was observed in SUDHL4, with log2 FC values reaching -1.4, followed by DG75 (log2 FC -0.8), while in BL41 it did not exceed -0.4. In addition, in SUDHL4 we also observed profound depletion in a broader region, encompassing chr14: 106326696- 106329196. Two significant peaks were identified in the homologous regions of both 3'RR enhancers, encompassing chr14:106152156-106153203 and chr14: 106162681-106163347 in 3'RR1 and chr14:106032312-106033352 and chr14:106041116-106041795 in 3'RR2. In both 3'RR peaks we observed the strongest effect in DG75 (log2 FC ~-2), while BL41 and SUDHL4 showed less depletion (log2 FC ~-1). We noticed that the significant peaks within 3'RRs overlapped with DNase I hypersensitive sites (HS) HS4 and HS1.2 (Supplementary Figure 4).

We chose two sgRNAs per each peak identified in the CRISPRi screen and validated the effect on cell growth in a GFP competition assay in a larger set of BL and DLBCL cell lines. We also performed this experiment in P493-6 cell line (B cell without *IGH* translocation) and HEK239T (non-B cell). In line with the results of the screen, throughout the experiment, we observed progressive depletion of BL and DLBCL cells transduced with sgRNA constructs targeting enhancer-essential regions compared to non-targeting controls (Figure 2D-E). The dependency of B-cells on *IGH* enhancers varied between cell lines, which may be possibly linked to the various patterns of translocations (Figure 2A). In DG75[55], SUDHL4 and WSU-DLCL2[56] the breakpoint occurs in V/D/J region, hence the Eμ enhancer is involved in the translocation. Accordingly, the strongest effect on survival in those cell lines was observed upon inhibition of the Eμ peak. In SUDHL4 less than 10% of GFP(+) cells survived by the end of the experiment (similar for both sgRNAs) and 30% GFP(+) with sg-2124 in WSU-DLCL2 (sg-2141 had weaker effect). DG75 was very susceptible to blocking of all identified by us essential regions within *IGH* enhancers with only 20% of GFP-positive cells remaining at the end of experiment. While DLBCL cell lines SUDHL4 and WSU-DLCL2 were less affected by targeting of 3'RRs, reaching 60-70 % of GFP(+) cells after 3 weeks of culture. BL41 and CA46 cell lines have a breakpoint within the constant region Cα1[55] resulting in Eμ exclusion from the *IGH* locus on the translocated allele, and they exhibited a similar pattern of dependency on *IGH* enhancers. The strongest effect on survival in those cells was observed upon blocking of 3'RR peak 1 – only up to 30% GFP(+) cells left. BL41 and CA46 were also affected by inhibition of Eμ - 50% GFP(+) in BL41 and 15 % in CA46 and 3'RR peak 2 – 40% GFP(+) in BL41 and 30-60% (depending on sgRNA) in CA46. ST486 cell line with breakpoint at *IGH* within the switch region μ[57] proved to be the most resistant to blocking of core *IGH* enhancers regions, resulting in cell survival of up to 60% for 3'RRs and 90% for Eμ. Interestingly, in P493-6 targeting the Eμ peak also strongly decreased cell growth (Figure 2F). As expected, we observed no negative effect on cell survival in HEK239T (Figure 2G).

Altogether, our CRISPRi screens allowed to pinpoint specific regions in *IGH* enhancers, essential for survival of B-cell lymphoma cells.

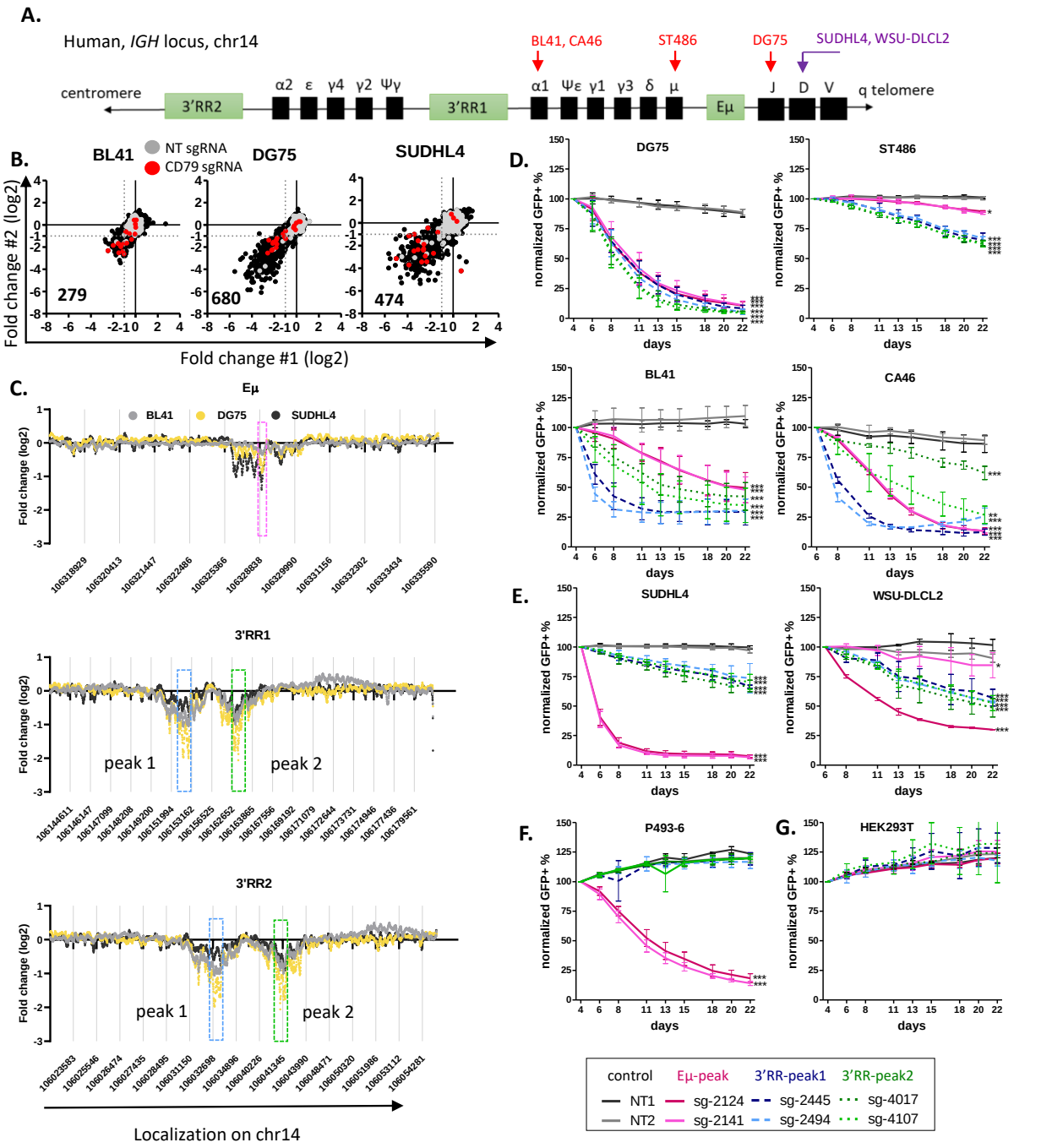


Figure 2. Tiling CRISPRi screen of the *IGH* enhancers in B-cell non-Hodgkin lymphoma cells. A. Localization of breakpoints in the *IGH* locus for cell lines used in this study. Red – BL cell lines, purple – DLBCL cell lines. **B.** CRISPRi screen results. Scatterplots represent change of sgRNAs abundance 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 – sgRNAs targeting *IGH* enhancers, grey – non-targeting controls, red – positive controls targeting CD79. **C.** 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: pink box, 3'RR1 and 3'RR2: blue box – peak 1, green box – peak 2. **D-G.** GFP growth competition assay. Assay performed with individual sgRNAs over the course of 3 weeks **D.** in BL and **E.** DLBCL cell lines, along with control cell lines **F.** P493-6,

B-cell cell line without *IGH* translocation and **G.** HEK239T. Average and standard deviation from 3 independent biological replicates is shown. *, P ≤0.05, **, P≤0.01; ***, P≤0.001, mixed model analysis.

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

Enhancer RNAs (eRNAs) are the class of non-coding RNAs transcribed from enhancer regions. They are rather elusive, unstable transcripts strongly associated with the chromatin. To identify eRNAs transcribed from the *IGH* locus, we performed cellular fractionation combined with chromatin-enriched RNA-Seq. Our preliminary data showed that this method allows for relatively fast, reproducible and cost-effective enrichment of eRNAs (Supplementary Figure 2A). Proper fractionation was confirmed by RNA agarose gel electrophoresis where we observed enrichment of small RNAs in the cytoplasmic fraction and the presence of additional precursor rRNA bands only in sonicated chromatin fraction (Supplementary Figure 2B). In addition, separation of fractions was confirmed with appropriate markers on RNA and protein levels (Supplementary Figure 2C-D). We prepared samples from two Burkitt lymphoma cell lines bearing *MYC/IGH* translocation t(8;14)(q24;q32) – BL41, DG75, diffuse large B-cell lymphoma cell line bearing *IGH/BCL2* translocation t(14;18)(q32;q21) – SUDHL4 and as controls we used cell lines without *IGH* translocation: B-cell line P493-6 and non-B-cell HEK293T.

During analysis of chromatin-enriched RNA-Seq we focused on transcripts mapping to the *IGH* enhancers. We observed bidirectional transcription from Eμ and 3'RRs (Figure 3A, Supplementary Figures 3-4) in B-lineage cell lines, but not in HEK293T, in which those enhancers are not active. Significant peaks identified in CRISPRi screen were also transcriptionally active. In Eμ transcription mainly occurs from the minus strand with the higher read counts for BL cell lines. In 3'RR expression was comparable from both strands, except for BL41 and SUDHL4 3'RR2 enhancer, where more reads were mapped to the plus strand.

Cellular localization of eRNAs transcribed from core *IGH* enhancers regions was then confirmed with RT-qPCR upon cellular fractionation (Figure 3D). eRNAs from the Eμ peak and 3'RR peak2 were enriched in the chromatin fraction, but surprisingly, we observed that 3'RR peak1 eRNA was enriched in the cytoplasmic fraction.

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

Based on the chromatin-enriched RNA-seq and CRISPRi screens results we designed qPCR primers to validate eRNAs expression within essential regions in *IGH* enhancers.

We first tested the eRNA expression in a panel of B-cell lymphomas (Figure 3B). We observed statistically significant lower expression of all tested eRNAs in Hodgkin lymphoma cell lines. There were no differences in expression level between other groups, including distinction for ABC and GCB lymphomas in DLBCLs. In line with NGS results, we observed higher expression from the Eμ enhancer-peak compared to 3'RR peaks.

To confirm that expression of eRNAs identified by us is not present only in *in vitro* cell cultures, we performed validation in patient-derived FFPE samples (Figure 3C). For this analysis, we used 8 BL FFPEs, 13 DLBCL FFPEs, of which 6 were GCB-derived and the rest ABC-derived, and 6 controls in the form of GCB from heathy donors tonsils. The expression of all eRNAs chosen for validation was confirmed in patient samples. We observed statistically significant higher expression of 3'RR in BL.

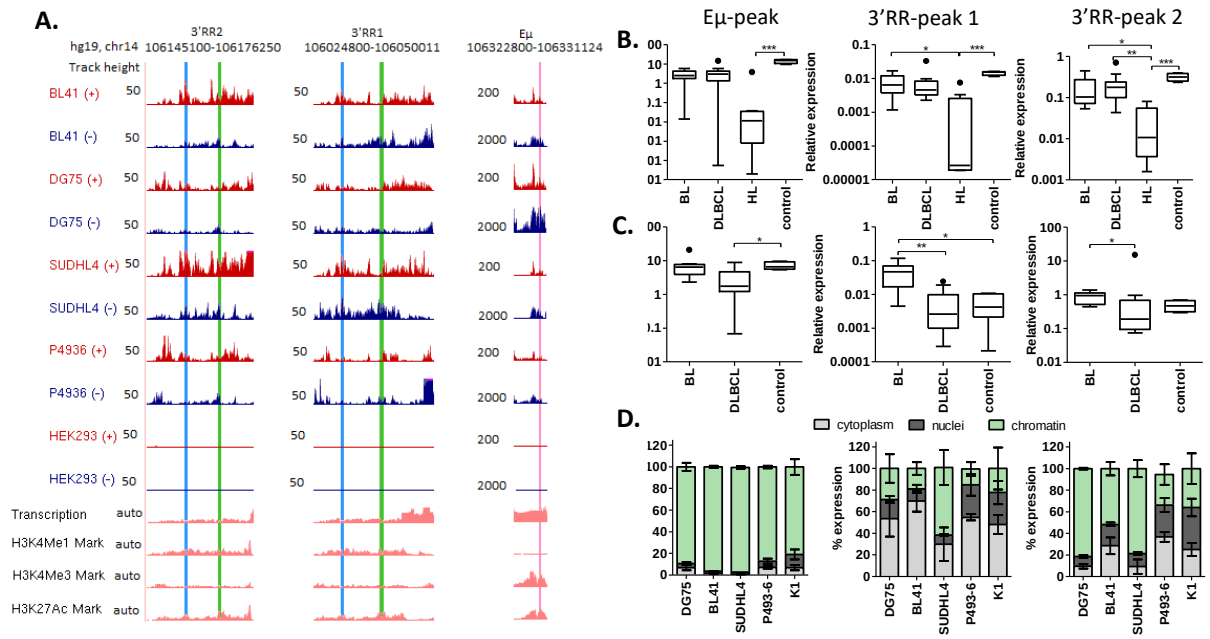


Figure 3. **Transcriptional activity of *IGH* enhancers. A.** Chromatin-enriched RNA-Seq results for *IGH* enhancer regions accompanied by UCSC tracks from GM12878 B-cells: transcription and histone marks: H3K4me1 and H3K27ac – characteristic for enhancer regions and H3K4me3 – characteristic for promoters and active genes. Red – indicates reads from the plus strand, blue – indicates reads from the minus strand. Pink - Eμ peak, blue – 3'RRs peak 1, green – 3'RRs peak 2. **B-C.** Validation of eRNA expression **B.** in a panel of B-cell lymphoma cell lines and control B cells: BL n=9, DLBCL n=12: ACB n=5, GCB n=7, HL n=8, control (germinal center B-cells) n=4. Expression normalized to TBP. ANOVA Kruskall-Wallis with Dunn's Multiple Comparison Post-Test was applied; *, P≤0.05; **, P≤0.01; ***, P≤0.001 **C.** in patient-derived FFPE 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's Multiple Comparison Post-Test was applied; *, P≤0.05; **, P≤0.01. **D.** Cellular localization of eRNA transcripts determined by cellular fractionation. Average and standard deviation from two biological replicates is shown.

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

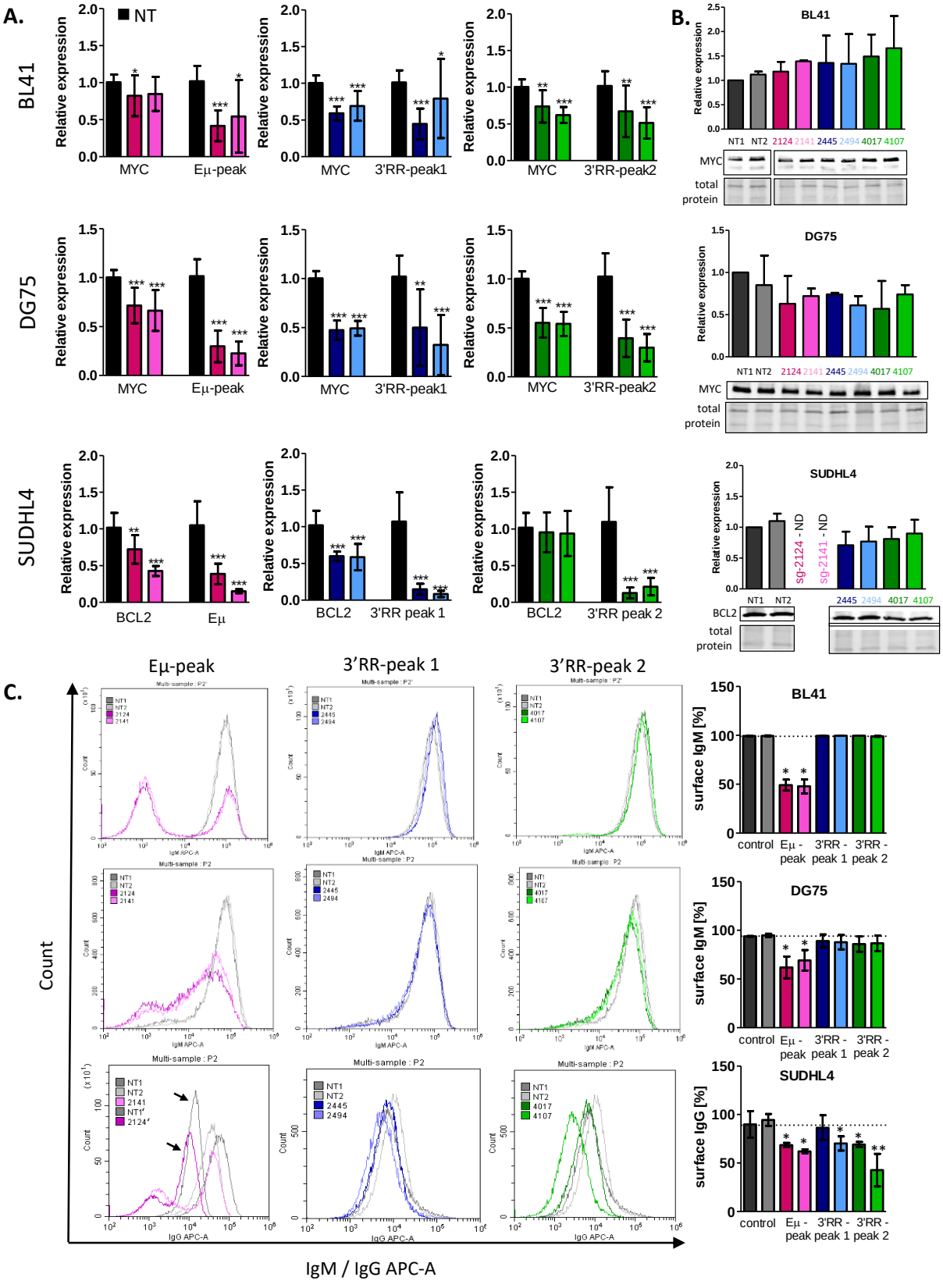
We hypothesized that inhibition of significant peaks in *IGH* enhancers should affect expression of eRNAs, translocated oncogenes and BCR. We observed that CRISPR/dCas9-KRAB targeting of *IGH* enhancers peaks with selected sgRNAs led to significant downregulation of their eRNAs expression (Figure 4 A-B, Supplementary Figure 6 A-B). BL cell lines carry the t(8;14) MYC/*IGH* translocation. In BL41 and DG75 blocking of all *IGH* peaks led to consistent, up to 50% decrease of MYC expression on RNA level, which was accompanied by reduced protein level only in DG75 (Figure 4A). In another tested BL cell line: CA46 (Supplementary Figure 6A-B), downregulation of the MYC transcript was observed only upon blocking of 3'RRs, but not Eμ. MYC protein however was consistently decreased in all samples in this cell line. ST486, which in GFP assay appeared to be more resistant to *IGH* enhancers peaks blocking, did not exhibit downregulation of MYC, neither on RNA nor protein level (Supplementary Figure 6A-B). As for DLBCL cell lines, bearing the t(14;18) *IGH*/*BCL2* translocations, BCL2 oncogene expression was decreased on both RNA and protein levels in nearly all samples. The exception was targeting of 3'RR peak 2 (sg-4017 and sg-4107) in SUDHL4, which did not affect BCL2 on RNA level and only slightly reduced the protein level. Also, in

SUDHL4, we were not able to obtain enough viable cells for protein isolation upon use of sg-2124 and sg-2141 (both targeting the Eμ-peak), therefore BCL2 protein expression could not be assessed there.

In the studied cell lines one *IGH* allele is involved in the translocation, while the other allele is functional and leads to production of secreted immunoglobulins and the presence of the B-cell antigen receptor (BCR) on the cell surface. BL41, DG75, CA46 and ST486 cells express surface IgM, and SUDHL4 – IgG, and their survival and proliferation depends on both the translocated oncogene as well as BCR signaling. The design of our CRISPR-eIGH library does not allow to directly distinguish those effect. Thus, we checked whether silencing *IGH* enhancers with dCas9-KRAB leads to the changes in BCR expression (Supplementary Figure 5). Indeed, targeting of the Eμ-peak resulted in appearance of an IgM-negative population in BL cell lines: BL41 (50% IgM(-)), DG75 (up to 30% IgM(-)) (Figure 4C), CA46 (70% IgM(-)), but not ST486 (Supplementary Figure 6C). Similar effect was observed in IgG-expressing DLBCL cell line SUDHL4 with BCR-negative population reaching 30-40% (Figure 4C). Since in GFP-growth competition assay we observed strong dependency on the Eμ enhancer in P493-6 cell line, which does not bear *IGH* translocation, we decided to test it for BCR (this cell line expresses IgM). Consistently with other cell lines, targeting of the Eμ-peak led to strong BCR loss, in over 80% of cells (Supplementary Figure 6D). On the other hand, targeting both 3'RR peaks led to slight reduction of BCR only in SUDHL4, but not other cell lines (Figure 4C and Supplementary Figure 6C).

Altogether, these results indicate that while the effect of silencing of Eμ enhancer may be mediated by interfering with both the oncogene expression and BCR, reduced cell growth observed upon 3'RR targeting may rather depend on the oncogene itself.

Figure 4. Downstream effects of targeting *IGH* enhancers. (next page) A. Expression of oncogenes involved in *IGH* translocation – MYC (BL41, DG75) or BCL2 (SUDHL4) and expression of eRNAs upon blocking of *IGH* enhancers essential regions on RNA level determined by qRT-PCR. Mean and SD of three independent biological replicates is shown. Expression normalized to HPRT. *, P ≤ 0.05; **, P ≤ 0.01, ***, P ≤ 0.001, Mann-Whitney test. **B.** Expression of oncogenes (MYC orBCL2) on protein level. Mean and SD from two independent biological replicates is shown. Protein bands were normalized to the total protein. ND – not determined. **C.** Immunostaining of B-cell receptor (BCR) on cell surface in BL41, DG75 (IgM) and SUDHL4 (IgG). Representative histograms of overlaid data for non-targeting controls (grey) and sgRNAs targeting *IGH*-enhancers essential regions (pink, Eμ peak; blue, 3'RR peak 1; green, 3'RR peak 2). Arrows indicate samples from a separate staining in SUDHL4. Column graphs show average percentage and SD of BCR-positive cells (surface IgM or IgG) from two biological replicates. *, P ≤ 0.05; **, P ≤ 0.01, Student's two-tailed t-test.


A.
BL41
DG75
SUDHL4
NT
Relative expression
MYC
Eμ-peak
3'RR-peak1
3'RR-peak2
BCL2
Eμ
BCL2
3'RR peak 1
BCL2
3'RR peak 2
B.
BL41
DG75
SUDHL4
NT1 NT2 2124 2141 2445 2494 4017 4107
MYC
total protein
sg-2124 - ND
sg-2141 - ND
BCL2
C.
Eμ-peak
3'RR-peak 1
3'RR-peak 2
Count
IgM / IgG APC-A
surface IgM [%]
surface IgG [%]
control
Eμ - peak
3'RR - peak 1
3'RR - peak 2

MYC overexpression rescues cell proliferation in Burkitt lymphoma cell line with blocked *IGH* enhancers-essential regions

Since we observed significant decrease in cell proliferation accompanied by MYC downregulation in DG75 cell line upon blocking of *IGH* enhancers, we asked whether MYC overexpression could rescue the phenotype (Figure 5A). To this end established the DG75 cell line with doxycycline-inducible MYC expression (DG75-MYC-OE). As control, cells with empty vector (DG75-EV) were used. Induction of MYC expression was tested on both RNA and protein level (Supplementary Figure 7B-C) with several doxycycline doses ranging from 0.1 to 0.5 µg/ml. Moreover, the survival of DG75-MYC-OE cells upon induction of MYC expression was verified over the course of 3 weeks (Supplementary Figure 7C). 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 EV cells were transduced as previously with the set of sgRNAs targeting *IGH* enhancers peaks and non-targeting controls. At day 6 post infection cells were seeded for CellTiter assay measuring cell viability with or without doxycycline. 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). This proved that the observed negative effect of targeting *IGH* enhancers is in part caused by downregulation of MYC.

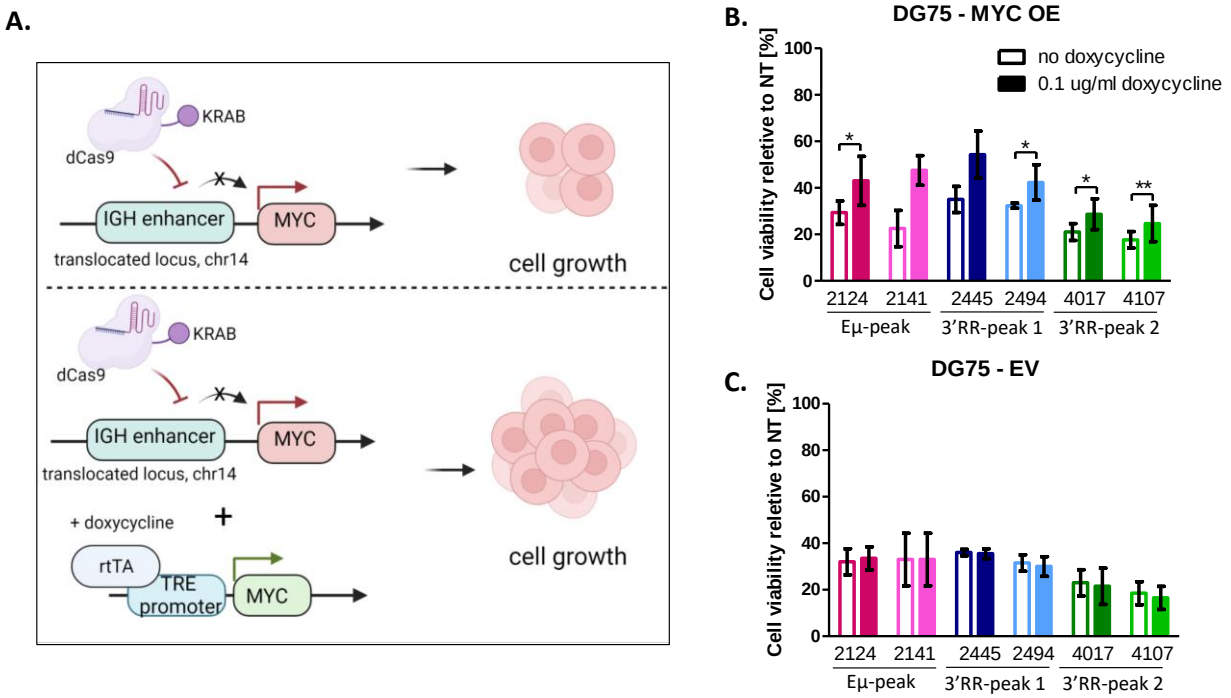


Figure 5 MYC overexpression rescues cell viability in BL cell line DG75 upon inhibition of *IGH* enhancer-essential regions. A. Overview of the MYC-rescue experiment. **B. and C.** Viability of **B.** DG75-MYC OE cell line and **C.** Control cell line DG75-EV transduced with sgRNAs targeting *IGH* enhancers and treated/untreated with doxycycline for induction of MYC expression. Average and SD from three independent biological replicates is shown. *, P ≤ 0.05; **, P ≤ 0.01, Student's t-test.

Discussion

Understanding of gene expression regulation in malignant cells is an important, yet challenging task. Cancer cells are characterized by many genomic and epigenomic abnormalities and what follows – gene expression changes. Moreover, those cells can accumulate additional mutations in their lifetime, making studying them even more complicated. In NHL recurrent chromosomal translocations[58] are known to bring oncogenes under the regulation of *IGH* enhancers – 5' intronic Eμ and 3'regulatory regions, 3'RR1 and 3'RR2[59,60]. Even though the translocation itself may not be sufficient to drive lymphomagenesis, deregulation of oncogene expression by enhancers becomes a driving force of malignant transformation[61–64]. Up to date, targeting of *IGH* enhancers remains elusive. Therefore, finding the core *IGH* enhancers 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 one so far, revealing exact regions – one in Eμ and two in each 3'RR - which are crucial for human NHL cells survival. The use of saturating CRISPRi library allowed us to target and screen throughout the whole *IGH* enhancers. Interestingly, we observed that survival of lymphoma cells upon blocking of *IGH* enhancers core regions varies between cell lines. Taking into account that chromosomal translocations can occur at various spots of the *IGH* locus, we suggest that the observed pattern may be connected to the breakpoint sites.

In DG75, SUDHL4 and WSU-DLCL2, both the Eμ enhancer and 3'RRs 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 a cooperation of *IGH* enhancers in driving expression of the translocated oncogene. Spatial interaction between Eμ and 3'RR occurs for example during *IGH* locus rearrangements[65–67]. Ghazzaui N. et al.[59] 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 for c-myc-KIEμ mice, where both Eμ and 3'RR enhancers were involved. They concluded that Eμ and 3'RR enhancers cooperate in driving translocated oncogenes expression and lymphomagenesis. We tested in our study whether inhibition of one core *IGH* enhancer region will affect transcription of others, but we did not observe any consistent pattern on eRNAs expression from 3'RR when blocking Eμ and *vice versa* (data not shown).

In other cell lines tested by us: BL41, CA46 and ST486 (all BL), the intronic Eμ is not involved in the translocation with MYC. In agreement with this, we did not observe MYC downregulation on transcriptomic level in CA46 and ST486 and only slight downregulation in BL41 upon targeting of Eμ enhancer essential region. However, blocking of 3'RRs core regions in BL41 and CA46 had a significant effect on MYC transcript levels as well as cell survival. In those two cell lines breakpoint site in *IGH* resides in constant region Cα1, in the proximity of 3'RR1. In NHLs despite differential translocations pattern, the 3'RR always remains in the *IGH* locus. This regulatory region was suggested previously to be a good potential target for therapy[68–71] and was found sufficient to deregulate oncogene expression[19,59,70,72]. Several factors were shown so far to affect transcriptional activity of 3'RR, proving that this enhancer may be druggable[73–78]. The 3'RRs span for 25-30 kb, therefore determination what sites to target is necessary. In mice, HS3a and HS1.2 were shown to be important for translocated MYC deregulation[79]. Our approach revealed HS4 (peak 1) and HS1.2 (peak 2) within 3'RRs as crucial for human B-cell lymphoma cells survival. Importantly, P493-6 cell line was not negatively affected by blocking of 3'RRs, in contrast to Eμ. This makes the identified by us 3'RR peaks

good candidates for potential therapeutic targeting. However, it still needs to be determined whether inhibition of HS4 and HS1.2 is toxic specifically for *IGH*-translocation positive lymphomas.

BL cell line ST486 exhibited resistance to inhibition of *IGH* core enhancers regions. In GFP-growth competition assay performed with individual sgRNAs, this cell line showed survival at 70-90% and the expression of MYC was not affected. ST486 cell line bears several translocations involving MYC: typical BL reciprocal translocation MYC/IGH t(8;14)(q24;q32) but also complex t(8;14;18)(q24;q32;q23)[80]. 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 only from one site was not enough to observe a significant change in overall levels, nor 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. B-cells are continuously tested in the organism for functional BCR expression, their survival and proliferation depend on it[81–83]. In lymphoma cells, upon locus aberration, translocated allele does not produce BCR, which can still be expressed from the other functional *IGH* allele. In our approach, sgRNAs for *IGH* enhancer-essential regions can target both alleles. We observed partial BCR loss upon blocking of the Eμ essential region in nearly all tested lymphoma cell lines and also in P493-6 B cell line which does not harbor the *IGH* translocation. In contrast, targeting 3'RR essential peaks did not affect BCR expression, only a minor reduction was observed in SUDHL4. This is in line with the fact that the Eμ enhancer is especially important for proper VDJ during B-cell development (reviewed in Kasprzyk ME et al. 2021[18]) and Eμ deletion was shown to lower Igμ expression, resulting in lower BCR levels and weaker tonic signaling[84]. On the other hand, singular deletions of 3'RR enhancer components in mice were not sufficient to strongly affect BCR expression (reviewed in Pinaud e. et al, 2011[37]) and only combinatorial deletion of HS4 and HS3b downregulated BCR in B-cells[85,86]. 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[87]. This is similar to the effect on cell survival observed by us upon Eμ enhancer-essential region 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 case of the Eμ enhancer – also to the loss of BCR.

So far knowledge regarding eRNAs in NHL is very limited. Recently, a B-cell specific eRNA AL928768.3 was shown to regulate MYC expression in BL[35]. This eRNA resides within human 3'RR1 region. Authors modulated the expression of AL928768.3 by either siRNA-mediated knock-down or overexpression, and observed down- and upregulation of MYC, respectively. The effect on MYC was specific to cells bearing *MYC/IGH* translocation. Upon knock-down of eRNA AL928768.3 lower BL cells proliferation was also observed. Verification of this observation in wider panel of NHLs would be of interest. In our screen, inhibition of AL928768.3 region with CRISPR/dCas9 had no significant impact on cell survival overall; however a few individual sgRNAs were strongly depleted (Supplementary Table 6). Here, we confirmed transcription from core *IGH* enhancers regions and validated arising eRNAs in a panel of B-cell lymphomas, both NHL and HL, as well as NHL patient-derived samples. Blocking transcription of those regions with CRISPRi resulted in downregulation of eRNAs derived from them, which was accompanied by oncogene downregulation. Whether *IGH* eRNA from core regions are indeed involved in the regulation of those translocated oncogenes requires further study. For some eRNAs the very act of their transcription and not necessarily the transcript itself is important for carrying out their function[33,88]. On the other hand, enhancer RNAs may help achieve proper chromatin conformation and recruit transcriptional machinery to

target regions[89]. Transcription of 3'RR in mice was shown to recruit activation-induced deaminase (AID), which leads to *IGH* locus suicide recombination and BCR loss, potentially contributing to B-cell homeostasis[90]. eRNAs can also interact with other classes on non-coding RNAs. Long non-coding RNA CSR (lncRNA-CSR) interacts with 3'RR's HS4 eRNA, which promotes CSR[91]. So far we were not able to efficiently knock down *IGH* eRNAs with the use of either Gapmers or shRNAs, therefore the potential role of *IGH* eRNAs requires further investigation.

A more detailed analysis of the *IGH* enhancers core regions would be of interest. Enhancers are known to be packed with transcription factors biding sites. Elucidation if and how transcription factor binding is affected upon blocking essential enhancer sites will help understand expression regulation at those regions. In addition, Eμ and 3'RRs can form chromatin loops, and a closer look at the chromatin architecture in the *IGH* locus upon blocking the essential 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, HDAC inhibitors and aryl hydrocarbon receptor ligands have been shown to affect activity of the 3'RR and IGH transcription[73,76]. However, their effect on lymphomas driven by *IGH* translocations has not been evaluated. Recently, a small molecule reducing the activity of the Eμ enhancer has been reported, with an inhibitory effect on growth of *IGH* translocation positive multiple myeloma cells *in vitro* and *in vivo*[92]. However, our results showed that this compound is also toxic to other cell types[78].

In summary, we pinpointed regions within *IGH* enhancers Eμ and 3'RR crucial for survival of B-cell lymphomas. We showed that the observed negative effect on cell survival may be attributed to downregulation of translocated oncogenes and in 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.

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Authors contribution

MEK, WS: Investigation, Data analysis, Writing, Figure and table preparation; MP, MK, MŁ, AS: Investigation; TW: Data analysis; JEJG, JK, AvdB, NR: Project conceptualization, providing materials and samples; ADK: Project conceptualization, Supervision, Funding acquisition, Project administration, Data analysis, Writing. All authors read and approved the manuscript.

Declaration of competing interest

None of the authors have a conflict of interest.

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Supplementary materials

Supplementary Figures and Tables are attached after References section.

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2nd ARTICLE – Supplementary Files

Supplementary Materials

Supplementary Figures:

Supplementary Figure 1. Verification of dCas9 expression and performance of CRISPRi screens.

Supplementary Figure 2. Subcellular RNA fractionation**.**

Supplementary Figure 3. UCSC Genome Browser zoom in on *IGH* Eμ enhancer.

Supplementary Figure 4. UCSC Genome Browser zoom in on *IGH* 3'RR enhancers regions.

Supplementary Figure 5. Flow cytometry analysis of B-cell receptor (BCR) immunostaining**.**

Supplementary Figure 6. Downstream effects of targeting *IGH* enhancers.

Supplementary Figure 7. Establishment of MYC-overexpressing DG75 cells.

Supplementary Tables:

Supplementary Table 1. List of all CRISPR-eIGH library sgRNAs. *(attached only in the electronic version)*

Supplementary Table 2. List of primers used in this study.

Supplementary Table 3. List of sgRNA oligonucleotides used in CRISPRi screens validation.

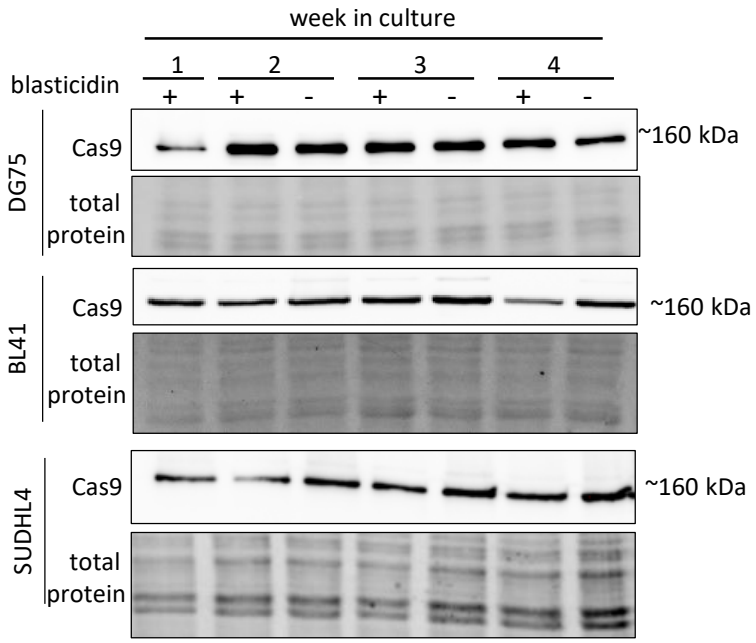
Supplementary Table 4. List of primers used in preparation of CRISPR-eIGH library for NGS.

Supplementary Table 5. CRISPRi screens read counts. *(attached only in the electronic version)*

Supplementary Table 6. CRISPRi screens fold change and sliding window analysis. *(attached only in the electronic version)*

Supplementary Table 7. List of antibodies used in this study.

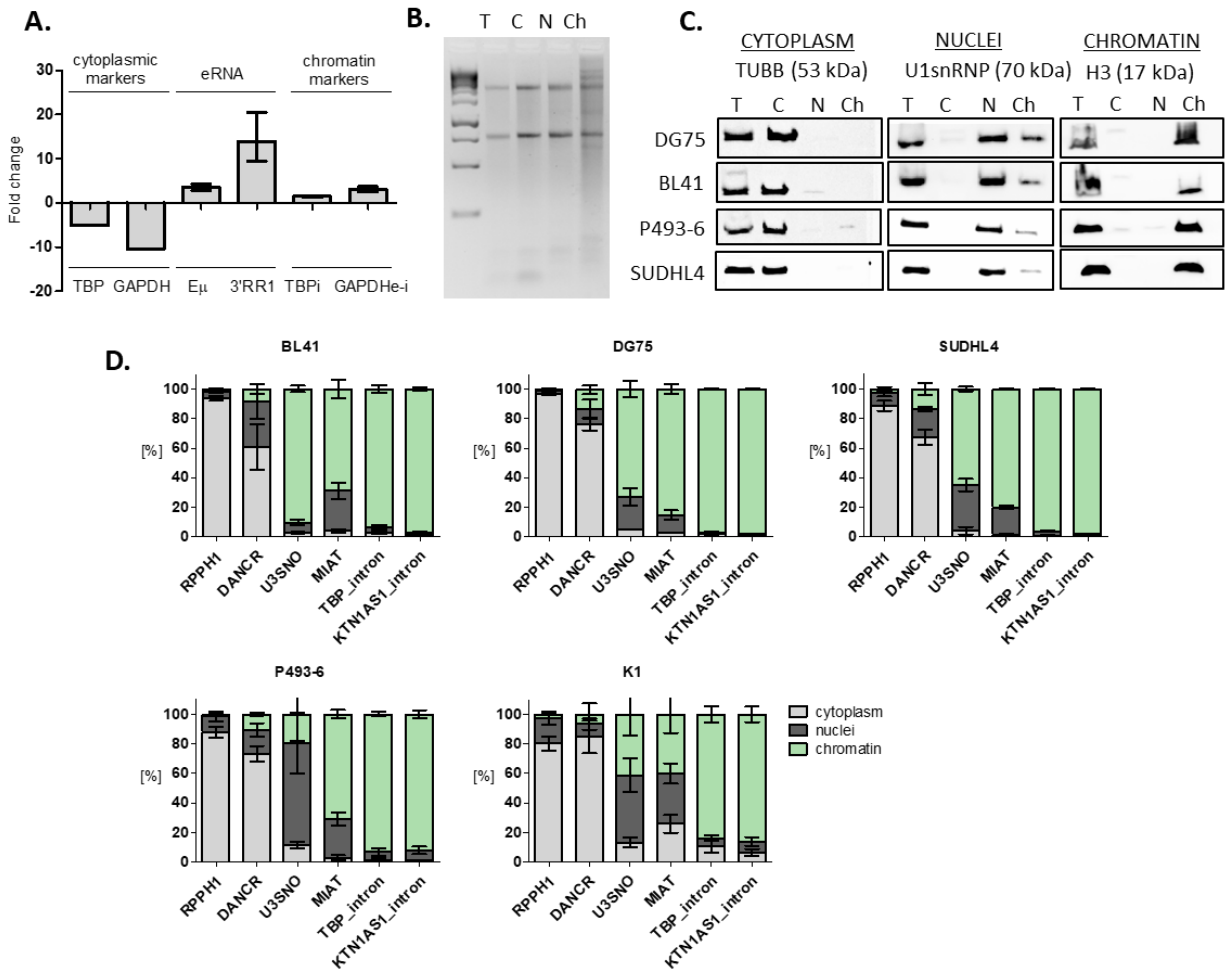
A.



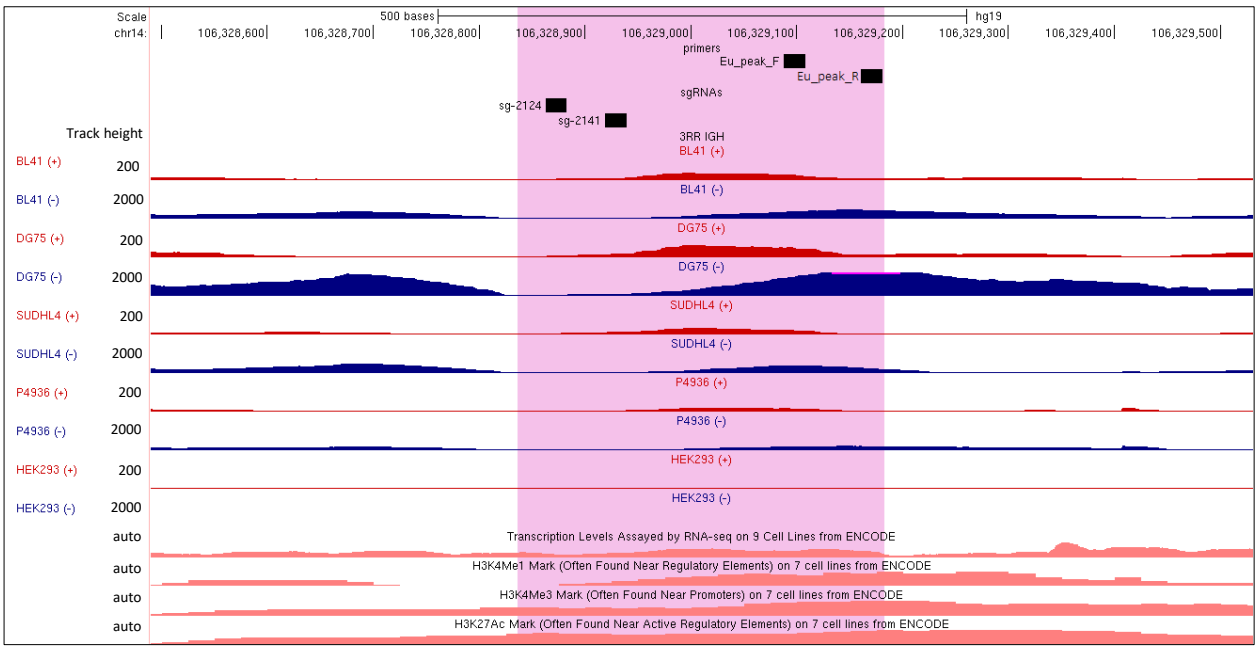
B.

Experiment	Percent of transduced cells	Number of transduced cells	CRISPR-IGH library coverage
BL41 screen #1	25.1 %	6.9 M	860x
BL41 screen #2	28.1 %	7.7 M	965x
DG75 screen #1	30.4 %	8.4 M	1045x
DG75 screen #2	21.8 %	6 M	750x
SUDHL4 screen #1	28.4 %	7.8 M	975x
SUDHL4 screen #2	22.6 %	6.2 M	775x

Supplementary Figure 1. **A.** Verification of dCas9 expression on protein level over time with or without addition of blasticidin in cell lines used in CRISPRi screens. **B.** Performance of CRISPRi screens for each replicate.

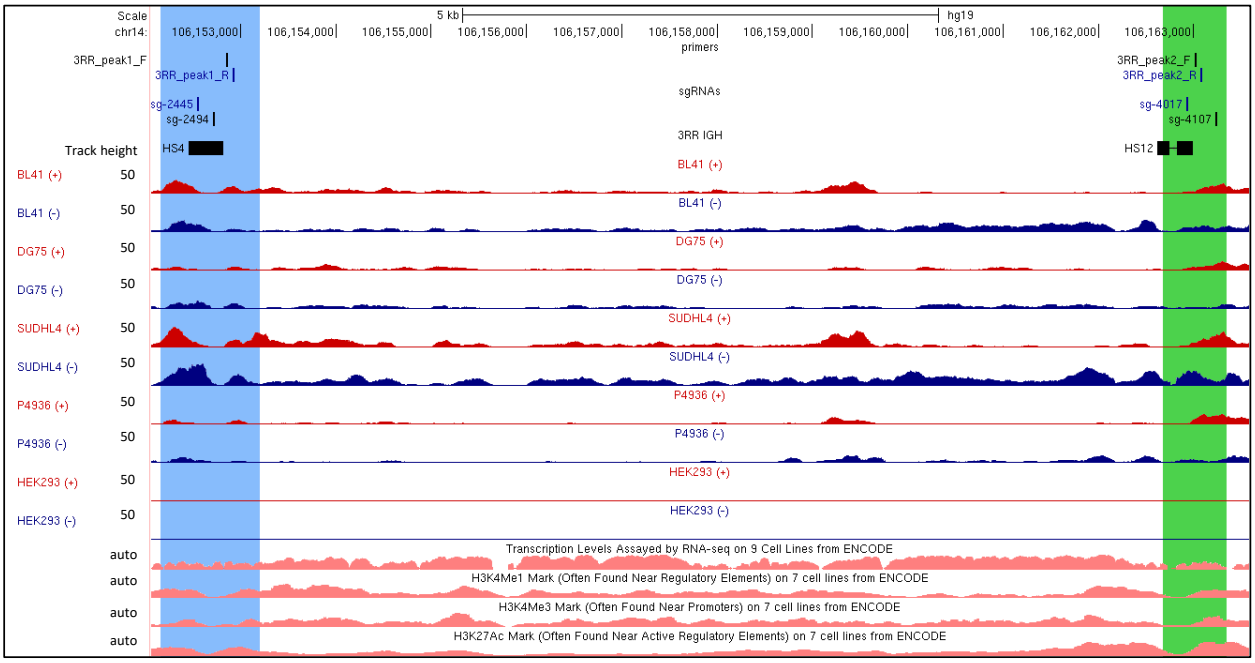


Supplementary Figure 2. **Subcellular RNA fractionation. A.** Abundance of transcripts in the chromatin fraction relative to total RNA. Cytoplasmic markers target spliced transcripts, while chromatin markers target unspliced transcripts. TBPi – intron, GAPDHe-i – transcript from exon-intron boundary. *IGH* enhancer RNA transcripts were predicted based on available transcription data from GM12878 cells (ENCODE). **B.** RNA from cellular fractionation on 2% agarose gel. Enrichment of small RNAs is visible in the cytoplasmic fraction. In chromatin fraction the pattern of observed ribosomal RNAs differs due to presence of rRNA precursors. T – total RNA, C – cytoplasmic fraction, N – nuclear fraction, Ch – chromatin fraction. **C.** Cellular fractionation control on protein level. T – total protein, C – cytoplasmic fraction, N – nuclear fraction, Ch – chromatin fraction. Fraction markers: cytoplasm – TUBB, nuclei – U1 snRNP 70, chromatin – H3. A representative blot is shown. **D.** Cellular fractionation control on RNA level. Fraction markers: cytoplasm – RPPH1, DANCR, nuclei – U3SNO, MIAT, chromatin – introns of TBP and KTN1_AS1. Average and SD from two independent biological replicates is shown.

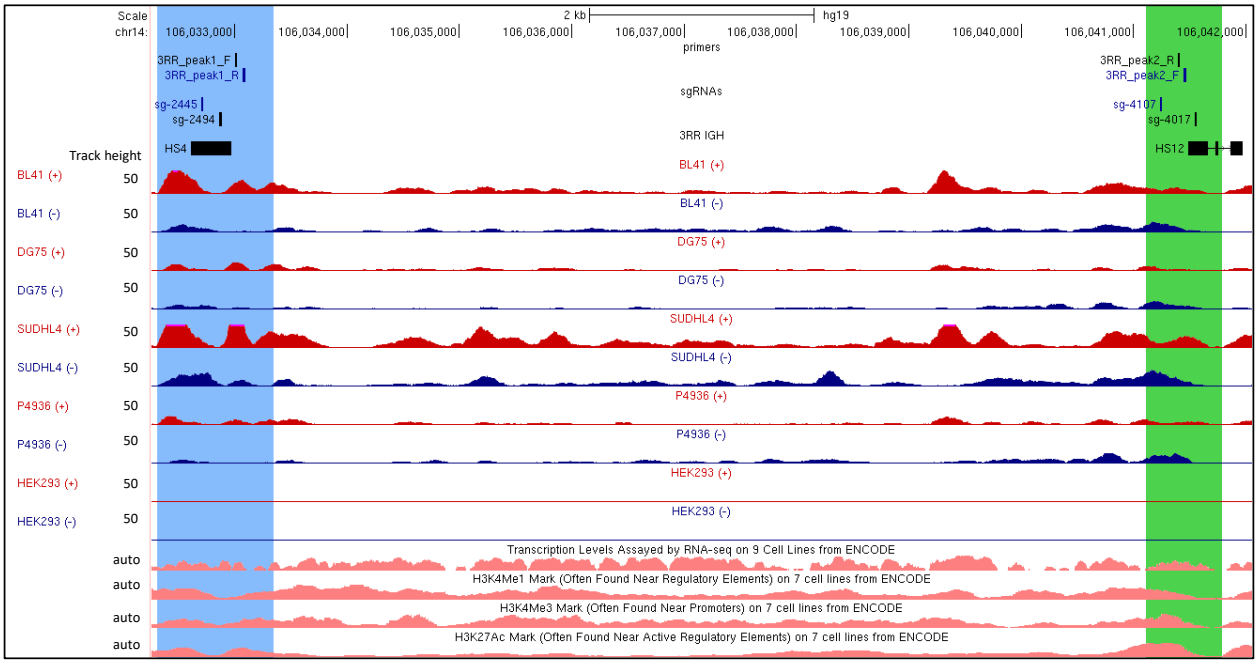


Supplementary Figure 3. **UCSC Genome Browser zoom in on *IGH* Eμ enhancer.** CRISPRi-identified enhancer-essential region is highlighted in pink. Localization of sgRNAs and primers used in CRISPRi screens and eRNA expression validation is marked as black boxes. Presented coverage are reads obtained from chromatin-enriched RNA-Seq. Red – reads from the plus strand, blue – reads from the minus strand obtained from chromatin-enriched RNA-Seq. Bottom tracks represent transcription and layered histone marks of GM12878 cells from ENCODE.

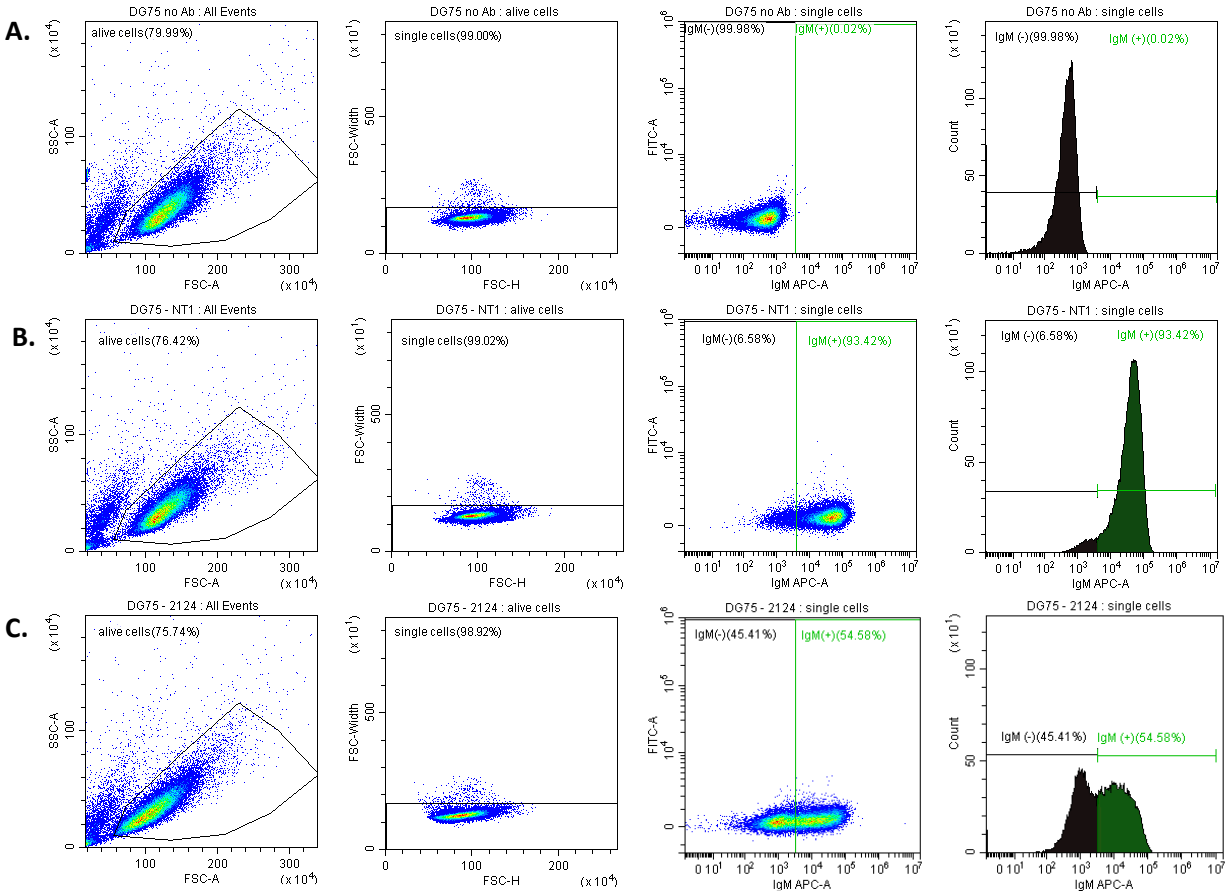
A.



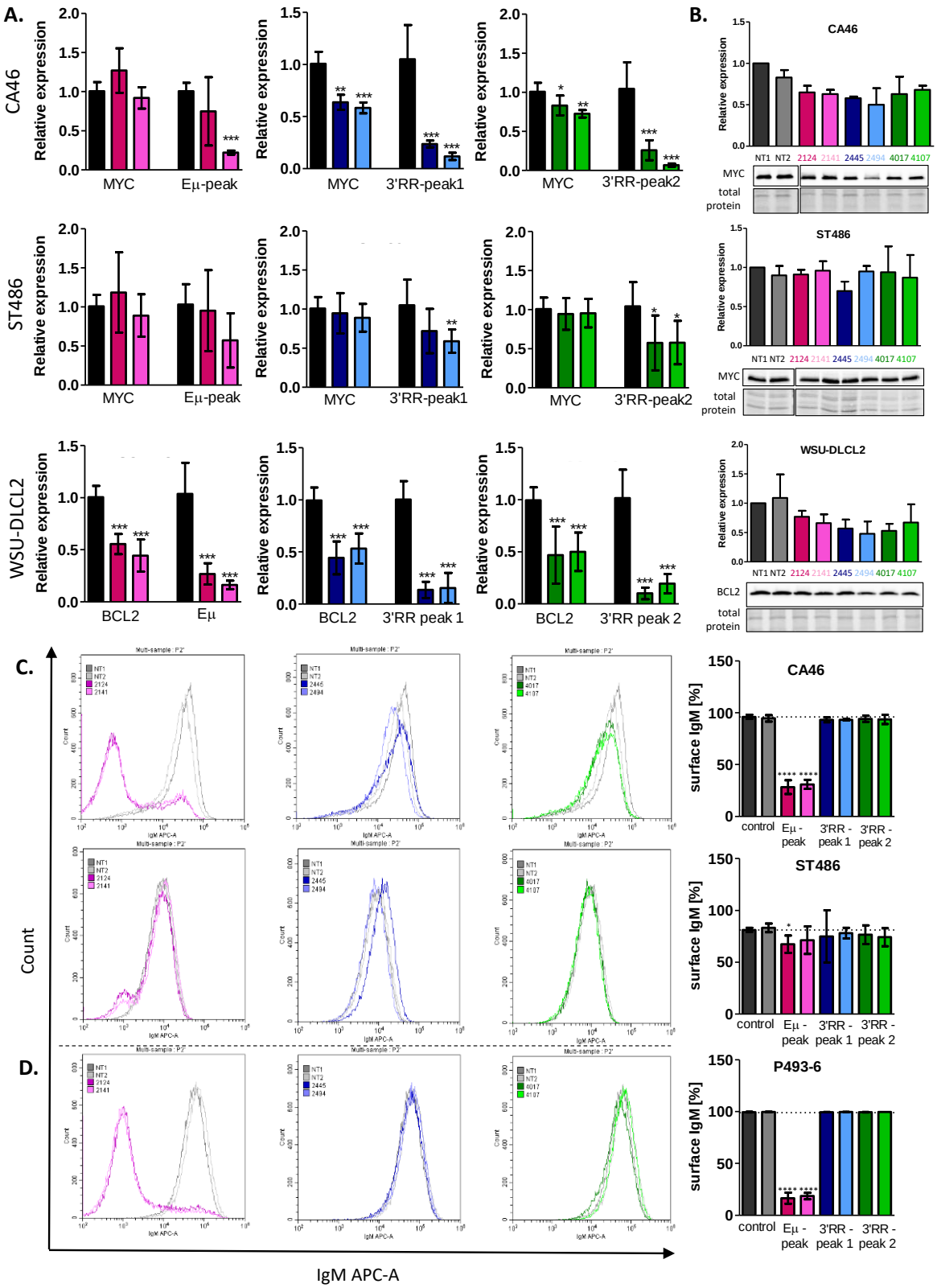
B.



Supplementary Figure 4. **UCSC Genome Browser zoom in on *IGH* 3'RR enhancers regions.** Presented coverage are reads obtained from chromatin-enriched RNA-Seq. Red – reads from the plus strand, blue – reads from the minus strand obtained from chromatin-enriched RNA-Seq. Localization of primers and sgRNAs used in CRISPRi screens and eRNA expression validation is marked as black boxes. **A.** 3'RR1 enhancer and enhancer-essential region 1 (peak 1) highlighted in blue and region 2 (peak 2) in green. **B.** 3'RR2 enhancer and enhancer-essential region 1 (peak 1) highlighted in blue and region 2 (peak 2) in green. Bottom tracks represent transcription and layered histone marks of GM12878 cells from ENCODE

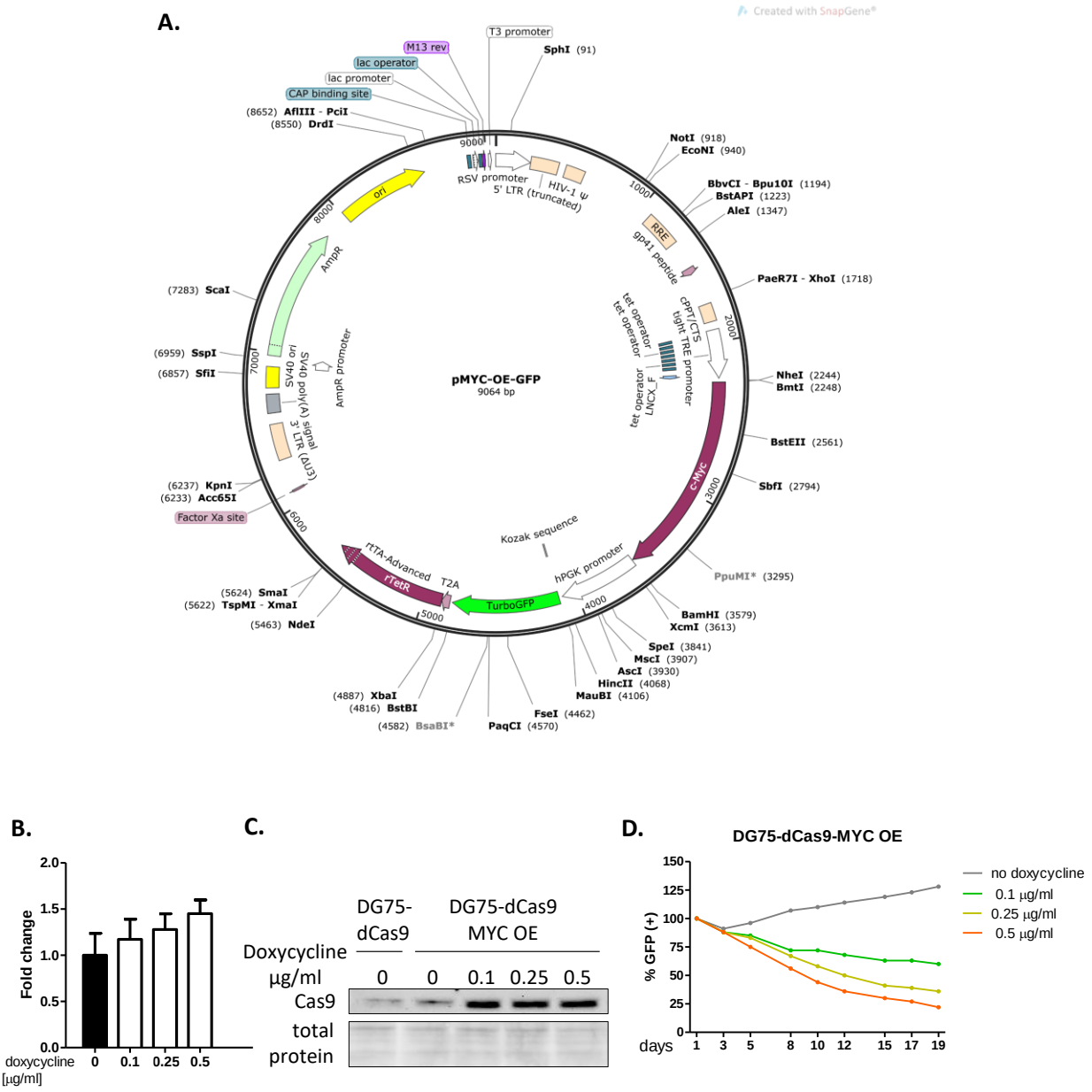


Supplementary Figure 5. Flow cytometry analysis of B-cell receptor (BCR) immunostaining. APC-based gating strategy for surface BCR analysis, from left: living cells selection, single cells selection, surface BCR positive (IgM or IgG) cells selection and histogram visualization. Examples are given for **A.** sample without anti-BCR antibody, **B.** control sample NT1, **C.** sample in which Eμ enhancer is targeted with sgRNA.



Supplementary Figure 6. Downstream effects of targeting IGH enhancers. A. Expression of oncogenes involved in *IGH* translocation – MYC (CA46, ST486) or BCL2 (WSU-DLCL2) and expression of eRNAs upon blocking of *IGH*

enhancers essential regions on RNA level determined by qRT-PCR. Mean and SD of three independent biological replicates is shown. Expression normalized to HPRT. *, P ≤ 0.05; **, P ≤ 0.01, ***, P ≤ 0.001, Mann-Whitney test. **B.** Expression of oncogenes (MYC/BCL2) on protein level. Mean and SD from two independent biological replicates is shown. Protein bands were normalized to the total protein. **C-D.** Immunostaining of B-cell receptor (BCR) on cell surface in **C.** CA46, ST486, **D.** P4936 (all IgM). Representative histograms of overlaid data for non-targeting controls (grey) and sgRNAs targeting *IGH*-enhancers essential regions (pink, Eμ peak; blue, 3'RR peak 1; green, 3'RR peak 2). Column graphs show average percentage and SD of BCR-positive cells (surface IgM or IgG) from two biological replicates. *, P ≤ 0.05; ****, P ≤ 0.0001, Student's two-tailed t-test.



Supplementary Figure 7. Establishment of MYC-overexpressing DG75 cells. A. Map of plasmid pMYC-OE-GFP used for establishment of DG75-MYC-OE cell line. **B-C.** Validation of doxycycline-induced MYC overexpression in DG75-MYC-OE cell line on **B.** RNA level, expression normalized to HPRT and **C.** on protein level using several doxycycline doses (0.1-0.5 μg/ml). **D.** DG75-MYC-OE cells (expressing GFP) survival upon doxycycline-induced MYC overexpression over 3 weeks of culture determined by GFP growth competition assay.

Supplementary Table 2. List of primers used in this study.

Name	Sequence - Fwd 5'-3’	Sequence - Rev 5'-3’
Gene expression analysis		
HPRT	GGCAGTATAATCCAAAGATGGTCAA	GTCTGGCTTATATCCAACACTTCGT
TBP	GCCCGAAACGCCGAATAT	CCGTGGTTCGTGGCTCTCT
GAPDH	GAGTCCACTGGCGTCTTCAC	"TGATGACCCTTTTGGCTCCC"
Eμ-peak	TCCTACAGACACCGCTCCTG	GGCTTGGGGAGCCACATTT
3'RR peak 1	TGACCCCCGATGAGTGTGAG	TGGATAACGCTCAGGACGGG
3'RR peak 2	GCCCAGAGATGCCGAAAACT	CTAGGGGCAAGCTGGTGAG
MYC	CACCAGCAGCGACTCTGA	ATCCAGACTCTGACCTTTTGC
BCL2	TGAACTGGGGGAGGATTGTG	CGTACAGTTCCACAAAGGCA
TBP_intron	TTTGTCTGAAGCCCTGATGTGT	CTGTGAAGAGAGCGCAGTGT
GAPDH_exon-intron	AATCCCATCACCATCTTCCAG	GAGCCACACCATCCTAGTTG
Fractionation controls		
TBP_intron	TTTGTCTGAAGCCCTGATGTGT	CTGTGAAGAGAGCGCAGTGT
KTN1_AS1_intron	TTGGCTGCTATTTACTACCCTCC	GCTGGGTGTGTTGCTAATCC
RPPH1	AGCTTGGAACAGACTCACGG	AATGGGCGGAGGAGAGTAGT
DANCR	CGTCTCTTACGTCTGCGGAA	TGGCTTGTGCCTGTAGTTGT
U3 snoRNA	AACCCCGAGGAAGAGAGGTA	CACTCCCCAATACGGAGAGA
MIAT	TGGAGGCATCTGTCCACCCATGT	CCCTGTGATGCCGACGGGGT
CRISPR-eIGH library amplification		
oligo-F/R	GTAACTTGAAAGTATTTCGATTTCTTGGCTTTA TATATCTTGTGGAAAGGACGAAACACC	ACTTTTTCAAGTTGATAACGGACTAGCCTTATT TTAACTTGCTATTTCTAGCTCTAAAAC

Supplementary Table 3. List of sgRNA oligonucleotides used in CRISPRi screens validation. Bold– overhangs for cloning.

Name	Target region	Sequence - Sense 5'-3'	Sequence - Antisens 5'-3'
sg-2124	Eμ peak	**CACCG**TCCCTAAGCCCCTGTCAGGA	**AAAC**TCCTGACAGGGGCTTAGGGA**C**
sg-2141	Eμ peak	**CACCG**CCCTGCTCTCATCAAGACCG	**AAAC**CGGTCTTGATGAGAGCAGGG**C**
sg-2445	3'RR_peak 1	**CACCG**TGGGGGGAAGGCTGGCACCC	**AAAC**GGGTGCCAGCCTTCCCCCCA**C**
sg-2494	3'RR_peak 1	**CACCG**GCTGCGGCCCGGTGCCCATG	**AAAC**CATGGGCACCGGGCCGCAGC**C**
sg-4017	3'RR_peak 2	**CACCG**TGACTCATTCTGGGCAGACT	**AAAC**AGTCTGCCCAGAATGAGTCA**C**
sg-4107	3'RR_peak 2	**CACCG**CCCGAGGCTAGGCTGTGGGA	**AAAC**TCCCACAGCCTAGCCTCGGG**C**
sg-NT1	non-targeting control	**CACCG**ACGGAGGCTAAGCGTCGCAA	**AAAC**TTGCGACGCTTAGCCTCCGT**C**
sg-NT2	non-targeting control	**CACCG**ATCGTTTCCGCTTAACGGCG	**AAAC**CGCCGTTAAGCGGAAACGAT**C**

Supplementary Table 4. List of primers used in preparation of CRISPR-eIGH library for NGS.

Name	Sequence 5'-3'
Fwd-1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTAAGTAGAGGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCATGCTTAGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATGCACATCTGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATTGCTCGACGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATAGCAATTCGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCGATAGTTGCTTGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCGATCCAGTTAGGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCGATTTGAGCCTGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGATCGATACACGATCGCTTTATATATCTTGTGGAAAGGACGAAACACC
Fwd-10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTACGATCGATGGTCCAGAGCTTTATATATCTTGTGGAAAGGACGAAACACC
Rev-1	CAAGCAGAAGACGGCATACGAGATTCGCCTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Rev-2	CAAGCAGAAGACGGCATACGAGATATAGCGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Rev-3	CAAGCAGAAGACGGCATACGAGATGAAGAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTTCAA
Rev-4	CAAGCAGAAGACGGCATACGAGATATTCTAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Rev-5	CAAGCAGAAGACGGCATACGAGATCGTTACCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Rev-6	CAAGCAGAAGACGGCATACGAGATGTCTGATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Rev-7	CAAGCAGAAGACGGCATACGAGATTTACGCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA
Rev-8	CAAGCAGAAGACGGCATACGAGATTTGAATAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGACTCGGTGCCACTTTTTCAA

Supplementary Table 7. List of antibodies used in this study. WB – Western Blot; IC – Immunostaining.

Name	Host species	Company	Catalog number	Amount / dliution used	Purpose
Primary Antibodies					
Cellular Fractionation					
H3	Rabbit	Abcam	ab18521	1:1000	WB
Beta tubulin	Mouse	Abcam	ab131205	1:5000	WB
U1 snRNP 70	Mouse	Santa Cruz Biotechnology	sc-390899	1:500	WB
dCas9 expression validation					
Cas9	Mouse	Cell Signaling	#14697	1:1000	WB
Oncogene expression					
Bcl2	Rabbit	Abcam	ab32124	1:5000	WB; SUDHL4
Bcl2	Mouse	BD Biosciences	610538	1:1000	WB; WSU-DLCL2
Myc	Rabbit	Abcam	ab32072	1:10 000	WB
BCR immunostaining					
IgG	Goat	Sothern Biotech	2042-31	0.1 µg	IC
IgM	Mouse	BD Biosciences	551062	20 µl	IC
Secondary Antibodies					
Anti-Mouse	Goat	Santa Cruz Biotechnology	sc-2005	1:10 000	WB
Anti-Rabbit	Goat	Abcam	ab6721	1:5000	WB

Authors statements

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Marta Kasprzyk, MSc

Poznań, 24.05.2023

STATEMENT FOR DOCTORAL DISSERTATION

Regarding the procedure for awarding a doctoral degree, I hereby confirm that I have greatly contributed to the following publication which I am a co-author:

Marta Elżbieta Kasprzyk*, Weronika Sura*, Marta Podralska, Marta Kazimierska, Annika Seitz, Wojciech Łosiewski, Tomasz Woźniak, Jeroen E. J. Guikema, Joost Kluiver, Anke van den Berg, Natalia Rozwadowska, Agnieszka Dzikiewicz-Krawczyk
*co-first authors

CRISPRi screen identifies core regions in *IGH* enhancers essential for non-Hodgkin lymphoma cells survival.

Pending submission

My contribution to the cited publication included: cell culture and handling, establishment of cell lines stably expressing dCas9-KRAB: DG75, WSU-DLCL2, HEK239T and establishment of DG75 cell line with doxycycline-induced MYC overexpression and empty vector control, all cellular fractionations, preparation of samples for chromatin-enriched RNA-Seq, eRNA detection and validation, all Western Blots for cellular fractionations and oncogenes expression validation – Myc and Bcl2, RNA isolations, cDNA synthesis and qRT-PCRs for validation of CRISPR/dCas9-KRAB screens in cell lines: CA46, ST486, SUDHL4, WSU-DLCL2, OCI-LY19, all GFP-growth competition assays for cell lines: WSU-DLCL2, OCI-LY19, P493-6, HEK293T and replicates for CA46, immunostaining and flow cytometry analysis of BCR for cell lines: CA46, ST486, SUDHL4, P4936, final data analysis and statistics for all qRT-PCRs, Western Blots, GFP-growth competition assays and BCR immunostainings, MYC rescue experiments, preparation of tables, figures and original draft of the manuscript.

I would like to use the above publication in my doctoral dissertation entitled:
Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas.
This doctoral dissertation is prepared under the guidance of Agnieszka Dzikiewicz-Krawczyk, MD PhD.

With regards,

Kasprzyk Marta
.....
(doctoral candidate's signature)

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Weronika Sura, PhD

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With regards,

Weronika Sura

(co-author's signature)

Annika Seitz, BAS

Groningen, 24.05.2023

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With regards,

Annika Seitz
(co-author's signature)

umcg

AMC, Meibergdreef 9, Amsterdam
Department of Pathology
Jeroen Guikema, PhD.
L2-117
+31-20-5665708
j.e.guikema@amsterdamumc.nl

Date: 12 June 2023
Subject: Statement for Doctoral Dissertation Marta Kasprzyk, MSc.

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With regards,

Jeroen Guikema, PhD, Principal Investigator
Department of Pathology, Amsterdam University Medical Centers, Amsterdam, The Netherlands

Joost Kluiver, PhD

Groningen, 24.05.2023

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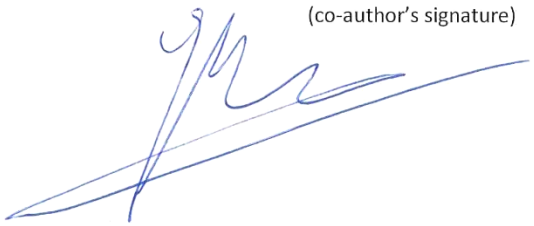
With regards,

Joost Kluiver
(co-author's signature)



umcg

P.O. Box 30 001, 9700 RB Groningen, The Netherlands

University Medical Center Groningen

Pathology & Medical Biology
Head of department Prof.dr. S.M. Willems

To
Whom it may concern

Enclosure(s)
Ref.

Date May 30, 2023
Re. Statement for doctoral dissertation

To whom it may concern,

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Kind regards,
Sincerely,

Anke van den Berg
Prof. Dr. A. van den Berg
Department of Pathology & Medical Biology
University Medical Center Groningen
The Netherlands
Phone: +31-50-3611476
Email: a.van.den.berg01@umcg.nl

Lymphoma Research Groningen

university of groningen

University Medical Center Groningen

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Agnieszka Dzikiewicz-Krawczyk, MD PhD

Poznań. 24.05.2023

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In addition, I agree for the use of the above publication in doctoral dissertation of Marta Kasprzyk entitled: *Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas*. This doctoral dissertation is prepared under my guidance.

With regards,

A. Dzikiewicz-Krawczyk

(supervisor's signature)

3rd ARTICLE

7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity

Marta E Kasprzyk*, Wojciech Łosiewski*, Marta Podralska, Marta Kazimierska, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk
European Journal of Pharmacology, Vol 910:174505, published 14th September 2021
*equal contribution

Background:

B-cell development and maturation involves multiple rearrangements at immunoglobulin heavy chain locus (IGH). DNA double-stranded breaks are obligatory intermediates in those process, making IGH locus a hot-spot for translocations. In non-Hodgkin lymphomas MYC/IGH t(8;14) or IGH/BCL2 t(14;18) are common and bring oncogenes under the regulation of IGH enhancers - Eμ and 3' regulatory regions. They in turn lead to oncogenes overexpression and can drive lymphomagenesis. 7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol, named hereafter compound 30666, was recently proposed as a potential inhibitor of Eμ enhancer activity, by an yet undetermined mechanism. In this article, we take the first steps in broadening the current understanding of compound 30666 mechanism of action.

Results:

We tested the effect of compound 30666 in two types of NHL, Burkitt lymphoma with t(8;14) *IGH/MYC* translocation and diffuse large B-cell lymphoma with t(14;18) *IGH/BCL2*. We report that both *IGH*-translocation positive NHL cells as well as *IGH*-translocation negative B cells and non-B cell controls treated with compound 30666 exhibited consistent growth inhibition. Moreover, during cell cycle analysis, we observed significant increase in the amount of cells in the sub-G1 phase, suggesting induction of apoptosis. We found that compound 30666 downregulated MYC levels in BL cell lines, but BCL2 levels in DLBCL were rather unaffected. This might be explained by differential expression regulation of the translocated oncogenes in those NHLs. Moreover, a global decrease of H3K27ac and an increase of H3K4me1 was observed upon 30666 treatment, which suggests switching enhancers to a poised or primed state. Active enhancers are characterized by the bi- or unidirectional transcription of non-coding RNAs named enhancer

RNAs (eRNAs). In this work, we report altered *IGH* eRNA expression upon 30666 treatment, suggesting that enhancer activity might indeed be out of balance. Initially, upon 30666 treatment, Eμ eRNA expression was significantly downregulated in majority of NHL cell lines and in control *IGH*-translocation negative P493-6 cells, but with time this effect was diminished. On the other hand, eRNA transcribed from *IGH* regulatory regions: 3'RR1 and 3'RR2 differed in their response to compound 30666. 3'RR1 eRNA showed a trend for upregulation, while 3'RR2 eRNA exhibited strong downregulation. So far, the information regarding Eμ and 3'RR eRNA expression in NHL is very limited, therefore our results showing that eRNA expression may be affected upon potential enhancer inhibitor treatment provide an important addition to the field.

Conclusions:

Our findings indicate that 30666 inhibitor affects enhancer activity but might not be as specific for *IGH* enhancers as previously reported and might affect cell viability by a more general mechanism.

MEK contribution:

- project organization and planning
- cell culture and handling
- 7-AAD cells staining for flow cytometry analysis of cell cycle
- total protein isolation and Western Blots
- data analysis of qRT-PCRs, Western Blots and cell cycle
- *in silico* analysis of compound 30666
- preparation of the original draft of the manuscript
- review and editing of the final version of the manuscript
- supervision over Wojciech Łosiewski, MSc

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7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity

Marta Elżbieta Kasprzyk [1], Wojciech Łosiewski [1], Marta Podralska, Marta Kazimierska, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk [*]

Institute of Human Genetics, Polish Academy of Sciences, Poznań, Poland

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ABSTRACT

B-cell non-Hodgkin lymphoma (NHL) is among the ten most common malignancies. Survival rates range from very poor to over 90% and highly depend on the stage and subtype. Characteristic features of NHL are recurrent translocations juxtaposing an oncogene (e.g. *MYC*, *BCL2*) to the enhancers in the immunoglobulin heavy chain (*IGH*) locus. Survival and proliferation of many B-cell lymphomas depend on the expression of the translocated oncogene. Thus, targeting *IGH* enhancers as an anti-lymphoma treatment seems a promising strategy. Recently, a small molecule - 7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) was identified to decrease activity of the Eμ enhancer and reduce the expression of translocated oncogenes in multiple myeloma and some NHL cell lines (Dolloff, 2019). Here, we aimed to test the effect of compound 30666 in Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) and shed light on its mechanism of action. We report that both *IGH*-translocation positive NHL cells as well as *IGH*-translocation negative B cells and non-B cell controls treated with compound 30666 exhibited consistent growth inhibition. A statistically significant increase in cell percentage in sub-G1 phase of cell cycle was observed, suggesting induction of apoptosis. Compound 30666 downregulated MYC levels in BL cell lines and altered *IGH* enhancer RNA expression. Moreover, a global decrease of H3K27ac and an increase of H3K4me1 was observed upon 30666 treatment, which suggests switching enhancers to a poised or primed state. Altogether, our findings indicate that 30666 inhibitor affects enhancer activity but might not be as specific for *IGH* enhancers as previously reported.

1. Introduction

During B-cell differentiation, immunoglobulin heavy chain locus (*IGH*) undergoes several types of rearrangements. V(D)J recombination, class switch recombination (CSR) and somatic hyper mutation (SHM) are all necessary for development of mature B cells capable of producing a wide range of antibodies and ensure memory-based response to antigens (Wang et al., 2020). Activity of *IGH* is regulated at each step of B-cell maturation by its enhancers: Eμ – intronic enhancer placed between 3′ J and Cμ segments (Pinaud et al., 2011), and two 3′ regulatory regions (3′RR1 and 3′RR2) located downstream of Cα segment (Ghazzaui et al., 2017). Eμ is mainly involved in early stages of *IGH* locus rearrangements – VDJ recombination (Perlot and Alt, 2008) while 3′RRs take more control over SHM and CSR (Pinaud et al., 2011). DNA double strand breaks (DSBs) are obligatory intermediates in those processes, making the *IGH* locus a hot-spot for translocations. Chromosomal rearrangements are often found in cancer cells (Hasty and Montagna, 2014). Several recurring translocations linking oncogenes with *IGH* enhancers are hallmarks of non-Hodgkin's lymphomas (NHL) (Vega and Medeiros, 2003). *IGH/MYC* t(8;14)(q24;q32) and *IGH/BCL2* t(14;18)(q32;q21) are often found in Burkitt lymphoma (BL) (Burmeister et al., 2013) and diffuse large B-cell lymphoma (DLBCL) (Zhang et al., 2011), respectively.

Survival and proliferation of B-cell lymphomas depend on expression of the translocated oncogenes, which makes them attractive therapeutic targets (Adams et al., 2019; Chen et al., 2018; Kapoor et al., 2020; Li et al., 2019). Despite relentless efforts, currently no MYC-targeting therapies are clinically available, and only a few agents targeting

* Corresponding author.
E-mail address: agnieszka.dzikiewicz-krawczyk@igcz.poznan.pl (A. Dzikiewicz-Krawczyk).
[1] Authors contributed equally.

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BCL2 are used in the clinic (Hafezi and Rahmani, 2021; Kapoor et al., 2020). Moreover, since those oncogenes also play important roles in normal cells, targeting them could have too broad effects. On the other hand, *IGH* enhancers are active only in B cells and could offer a more specific therapeutic approach for B-cell lymphomas. Indeed, targeting *IGH* enhancers has been pinpointed as a promising therapeutic strategy (Saintamand et al., 2015).

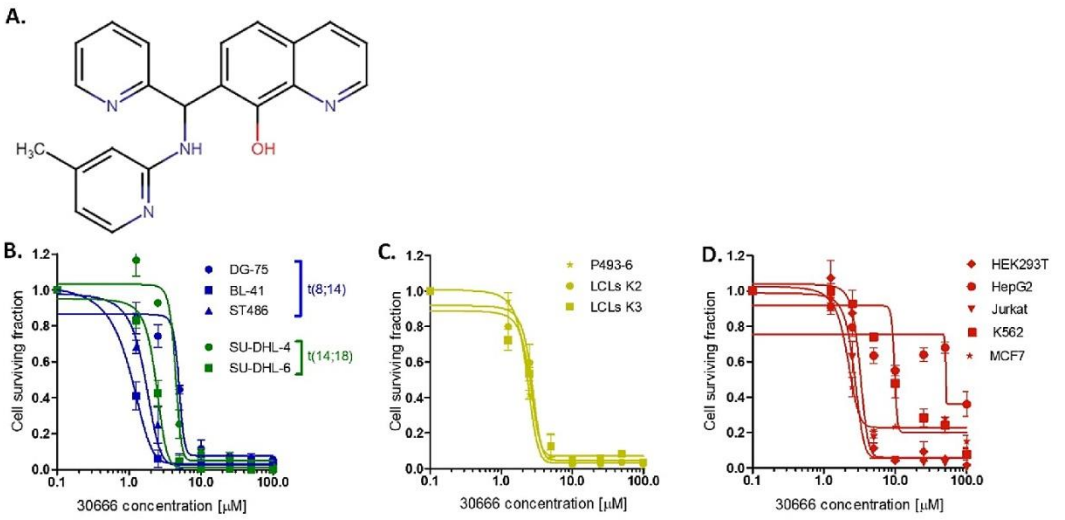
Recently, a small molecule - 7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (named by the author as compound 30666) was identified by N. G. Dolloff to decrease activity of the Eμ enhancer in a reporter assay (Dolloff, 2019). Reduced survival of multiple myeloma (MM) and some non-Hodgkin lymphoma cell lines bearing *IGH* translocations [t(+)] was observed upon treatment with 30666. However, some control *IGH* translocation-negative [t(−)] cell lines were also susceptible to compound 30666, and differences between *IGH* t(+) and t(−) samples were not statistically significant. 30666 treatment led to reduced expression of translocated oncogenes and decreased immunoglobulins secretion. Promising results were also obtained in *in vivo* tests on a plasmacytoma mouse model, where a trend for tumor growth suppression was observed.

Our aim was to broaden the understanding of 30666 mechanism and its specific anti-lymphoma properties. We tested its cytotoxicity in *IGH*-translocation positive lymphoma cell lines (BL and DLBCL) and *IGH*-translocation negative cells (B and non-B). We investigated the influence of 30666 inhibitor on expression of the translocated oncogenes: *MYC* and *BCL2* as well as expression of enhancer RNAs (eRNAs) arising from the Eμ and 3′RR enhancers. Next, we tested compound 30666 influence on cell cycle progression and global histone modifications characteristic for active enhancers: H3K4me1 and H3K27ac (Kang et al., 2021).

2. Methods

2.1. Compounds

7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol was purchased from Mcule (Budapest, Hungary), cat. no. MCULE-1536200389/STK506800. In line with the name used by Dolloff (2019), we further refer to it in the text as compound 30666. The purity of the compound, as stated by the supplier is >90% (Supplementary File 1). Compound 30666 was dissolved in DMSO at 100 mM and stored in aliquots at −80 °C. Structural formula of compound 30666 can be found in Fig. 1A.



E.

t(+)	EC50 [μM]	t(-)	EC50 [μM]	non B-cell	EC50 [μM]
BL-41	0.98	P493-6	2.31	HEK293T	3.23
DG-75	4.94	K2	2.71	HepG2	50.63
ST486	1.65	K3	2.57	Jurkat	2.7
SU-DHL-4	4.41			K562	9.8
SU-DHL-6	2.36			MCF7	2.14

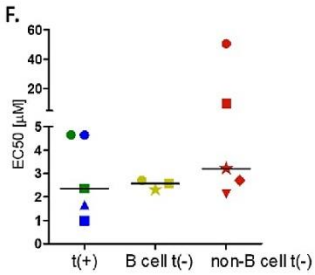


Fig. 1. Effect of compound 30666 on cell survival. A. Structural formula of compound 30666. **B-D.** Cell viability was measured after 48 h incubation with compound 30666 and surviving cell fractions were calculated relative to DMSO-treated cells. Average and SD from three independent experiments, each performed in triplicate are shown. **B.** Cell surviving fractions in *IGH* t(+) B-cell lymphoma cells BL (BL-41, DG-75, ST486) and DLBCL (SU-DHL-4, SU-DHL-6). **C.** Cell surviving fractions in *IGH* t(−) B cells (P493-6, LCLs K2, K3). **D.** Cell surviving fractions in non-B cell group. **E.** Summary of established EC50 doses of compound 30666 for each cell line, divided by the group type. **F.** EC50 values calculated based on A-C. Lines represent median values for each group. ANOVA Kruskall-Wallis test showed no significant differences between groups.

2.2. Cell lines

Several types of cell lines were used in the project. B-cell lymphoma cell lines included Burkitt lymphoma: with t(8;14) (DG-75, BL-41), and with t(8;14;18) (ST486); diffuse large B-cell lymphoma with t(14;18) (SU-DHL-4, SU-DHL-6). Non-lymphoma B-cells negative for *IGH* translocation included lymphoblastoid cell lines P493–6, K2 and K3. Cells of non-B cell origin included chronic myeloid leukemia (K562), hepatocellular carcinoma (HepG2), human embryonic kidney (HEK293T), breast adenocarcinoma (MCF7), leukemic T-cell lymphoblast (JURKAT). Cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland) or DMEM (Lonza) supplemented with 2 mM L-glutamine, 1% Penicillin-Streptomycin (Biowest, Nuaillé, France) and fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, US). Cell line source and media composition are listed in Table 1. Cell lines were cultured in standard conditions: 37 °C and 5% CO_2 in humidified incubator. Cells were regularly tested for mycoplasma contamination by PCR.

2.3. Cell survival

The effect of the compound 30666 on cell survival was measured with CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Cells were cultured with compound 30666 in concentrations: 100 μM, 50 μM, 25 μM, 10 μM, 5 μM, 2.5 μM and 1.25 μM or with the corresponding DMSO concentration. Luminescence was measured after 48 h on the Glo Max luminometer (Promega). EC50 values were calculated in GraphPad Prism 5. Experiments were performed in at least three independent biological replicates, each with three technical replicates.

2.4. qRT-PCR

Cell lines were cultured with 30666 at the EC50 concentration or with the corresponding DMSO dilution as control. Ca. 2 million cells were collected in two time points: 24 h and 48 h. RNA was isolated using Quick-RNA™ Miniprep or Microprep kits (Zymo Research, Irvine, CA, US). Reverse transcription was conducted with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) using 500 ng RNA for each reaction. qPCR was performed with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and primers listed in Table 2 on CFX96 Touch qPCR System (Bio-Rad, Hercules, CA, US). 5 ng of cDNA was used for each reaction. Gene expression was normalized relative to the *HPRT* gene. Experiments were performed at least in two independent biological replicates, each with three technical replicates.

Table 1
Cell lines used in this study and seeding cells concentrations for cell survival tests upon compound 30666 treatment.

Cell line	Medium	Source	Conc. for cell survival test [cells/well]
BL-41	RPMI + 10% FBS	DSMZ	10,000
DG-75	RPMI + 10% FBS	DSMZ	10,000
ST486	RPMI + 20% FBS	ATCC	20,000
SU-DHL-4	RPMI + 10% FBS	DSMZ	30,000
SU-DHL-6	RPMI + 20% FBS	DSMZ	30,000
P493-6	RPMI + 10% FBS	Gift from prof. D. Eick (Pajic et al., 2000) [Helmholtz Center, Munich, Germany]	30,000
LCL K2, K3	RPMI + 15% FBS	Patient derived (Dzikiewicz-Krawczyk et al., 2012)	40,000
JURKAT	RPMI + 1% sodium pyruvate (1 mmol/L) and 0.25% glucose (0.5 g/L) + 10% FBS	ECACC	40,000
K562	RPMI + 10% FBS	DSMZ	20,000
HEK293T	DMEM + 10% FBS	DSMZ	10,000
HepG2	DMEM + 10% FBS	DSMZ	13,000
MCF7	DMEM + 10% FBS + 1x NEAA	ECACC	6,500

RPMI, Roswell Park Memorial Institute; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; NEAA, Non-Essential Amino Acids; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany); ATCC, American Type Culture Collection (LGC Standards, Lomianki, Poland); ECACC, European Collection of Authenticated Cell Cultures (Salisbury UK).

2.5. Cell cycle analysis

For cell cycle analysis, cells were cultured for 24 h and 48 h in the presence of the compound 30666 at the previously established cell line-specific EC50 concentration. As control, cells were cultured in corresponding DMSO concentration. 7-Amino-Actinomycin D (7-AAD; BD Biosciences, Franklin Lakes, NJ, USA) was used for DNA content staining, according to manufacturer instructions. Briefly, 1×10^6 cells were washed in PBS and fixed in 70% cold EtOH. Cells were stored at −20 °C, until use. Before staining, cells were washed in PBS. 7-AAD staining was performed using 5 μl (0.25 μg) per sample, followed by 15 min incubation at room temperature. Samples were resuspended in 100 μl PBS for flow cytometry analysis using CytoFLEX (Beckman Coulter, Brea, CA, USA). Cell cycle analysis to establish cell percentage in G1, S and G2/M phase was performed using Watson algorithm in FlowJo (BD Biosciences). To establish the sub-G1 population, cells containing less than diploid (2n) DNA content were presented as the percentage of total single cells.

2.6. Western blot

For histone modification analysis, cells were cultured with DMSO or compound 30666 for 48 h. Next, 5×10^6 cells were lysed in RIPA buffer (Sigma, Saint Louis, MO, USA), supplemented with protease inhibitors, followed by two rounds of sonication on ice [5 s on, 30 s off] (Misonix 3000; Misonix, Farmingdale, NY, USA). Protein concentration was determined using Bicinchoninic Acid Kit (Sigma). Protein samples were mixed with the Laemmli 4X sample buffer (Sigma), heat-denatured and separated on the 12% polyacrylamide gel (acrylamide:bisacrylamide, 49:1) supplemented with 2,2,2-trichloroetanol (Merck, Kenilworth, NJ, USA) to allow for stain-free total protein detection. Proteins were transferred onto low-fluorescence PVDF membrane (Bio-Rad), blocked in 5% milk in TBST and incubated with primary antibodies against histone modifications H3K4me1 (Cell Signaling Technology, Danvers, MA, USA, #5326, 1:000), H3K27ac (ActiveMotif, Carlsbad, CA, USA, #39034, 1:1000) and total histone H3 (Abcam, Cambridge, MA, USA, ab18521-100, 1:1000). After incubation with secondary Ab-HRP (Abcam, ab6721, 1:5000) the signal was detected by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad) with ChemiDoc™ Imaging Systems (Bio-Rad). Quantitative analysis was performed using Image Lab™ Software (Bio-Rad). Bands were normalized using total protein method. Changes in histone H3 modifications were calculated in relation to total histone H3.

Table 2
qRT-PCR primers used in this study.

Gene	Forward	Reverse
HPRT	GGCAGTATAATCCAAAGATGGTCAA	GTCTGGCTTATATCCAACACTTCGT
MYC	CACCAGCAGCGACTCTGA	ATCCAGACTCTGACCTTTTGC
BCL2	CCAAGAATGCAAAGCACATCCA	CAACGGCACCTCTCGCC
Eμ	TCTCATCAAGACCGGGGCTA	TTATGAGGTGCGCTGTGTGT
3′RR1	CAGGGGTCATTGACTGGGTC	CCCTTGTTCCCGATTGCTGA
3′RR2	GGTGATGGGGTACAAGAGGC	TCTCGACTTAGCACTGGGGA

2.7. Statistical analysis

All statistics were calculated with GraphPad Prism version 5.0.0 with a *P*-value significance cut-off $P < 0.05$. EC50 values were compared between groups of cells using Kruskal–Wallis one-way analysis of variance. qRT PCR statistical analysis employed the Mann-Whitney test. Differences in cell cycle phases between cells treated with DMSO control and compound 30666 were calculated using chi-squared test. Statistical significance in the sub-G1 cell population was calculated with Student's unpaired *t*-test.

3. Results

3.1. Compound 30666 exhibits similar cytotoxicity in IGH translocation positive and negative cells

To test 7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol effect on survival of *IGH* t(+) BL (BL-41, DG-75, ST486), DLBCL (SU-DHL-4, SU-DHL-6) and *IGH* t(−) B cells (P493-6, LCLs K2, K3) and non-B cells (HepG2, Jurkat, K562, HEK293T, MCF7), the half maximal effective concentration (EC50) of the compound for each cell line was determined. We established, that similarly to N. G. Dolloff (2019) results, *IGH* t(+) cells exhibited growth inhibition at low micromolar concentration range (EC50 1–5 μM, Fig. 1B). However, *IGH* t(−) B cells showed similar sensitivity to 30666 (EC50 2.3–2.7 μM, Fig. 1C). There was more heterogeneity in the non-B cell group (Fig. 1D), with two outliers K562 (EC50 9.8 μM) and HepG2 (EC50 50.63 μM). All established EC50 doses are listed in Fig. 1E. Statistical analysis showed no significant differences between groups (Fig. 1F).

3.2. Compound 30666 induces cell death

We observed growth inhibition of NHL *IGH* t(+) cells as well as control cell lines upon compound 30666 treatment. To establish the reason of this effect, we checked the effect of compound 30666 on cell cycle progression. 7-aminoactinomycin D DNA (7-AAD) content staining, followed by flow cytometry analysis revealed rather slight changes in cell cycle. For BL cells DG-75, ST486 and control MCF7, an increase in S phase could be observed after 24 h culture in the presence of 30666, but this effect was abolished after 48 h (Fig. 2A). In DLBCL cells (SU-DHL-4, SU-DHL-6) after 24 h treatment with 30666 a decrease in G2/M phase was noticeable. Similarly to N. G. Dollof (Dolloff, 2019), we observed an increase in cell percentage in sub-G1 phase in B-cells treated with compound 30666 (Dolloff, 2019), but not in MCF7 (Fig. 2B). This effect was persistent in both time points, 24 h and 48 h. An increase in cell percentage in sub-G1 phase (Plesca et al., 2008) and lack of apparent changes in cell cycle phases suggests that compound 30666 induces apoptosis rather than affects proliferation.

3.3. MYC expression is downregulated in Burkitt lymphoma cell lines upon compound 30666 treatment

Oncogene translocation under the regulation of *IGH* enhancers is an early pathogenic event, setting the B-cell on the path towards malignancy. Survival of many NHL cells depends on the expression of misplaced oncogenes (Hachem and Gartenhaus, 2005). We tested MYC expression in BL cells with *IGH/MYC* translocation (DG-75, ST486) and BCL2 expression in DLBCL cells with *IGH/BCL2* translocation (SU-DHL-4, SU-DHL-6) upon compound 30666 treatment. B cells without *IGH*-oncogene translocation (P493-6) were used as a control. We established that MYC expression was downregulated in BL cell lines upon compound 30666 treatment (Fig. 3A). DG-75 showed persistent MYC downregulation in both tested time points, while in ST486 this effect was abolished after 48 h. Surprisingly BCL2 expression was generally not affected when treated with 30666 (Fig. 3B). Only SU-DHL-4 cells exhibited BCL2 downregulation after 24 h incubation, but after 48 h this effect was diminished. In P493-6 MYC expression was elevated after 24 h treatment with compound 30666, while BCL2 expression was consistently elevated in both tested timepoints, although the reason of this observation is not clear.

3.4. Altered expression of IGH eRNAs by compound 30666

Enhancer RNA expression is a feature of active enhancers (Arnold et al., 2020). Previous results in a reporter system showed that 30666 affects the transcriptional activity of the Eμ enhancer (Dolloff, 2019). Therefore, we decided to test the influence of compound 30666 on expression of endogenous eRNAs from Eμ and 3'regulatory regions: 3'RR1 and 3'RR2. We observed that indeed eRNA expression was altered upon 30666 treatment. Eμ eRNA expression was significantly downregulated after 24 h incubation in majority of NHL cell lines as well as *IGH* t(−)P493-6 cells. However, after 48 h this effect was diminished, with the exception of ST486 and SU-DHL-6 cells (Fig. 4A). 3'RR1 and 3'RR2 differed in their response to compound 30666. 3'RR1 eRNA showed a trend for upregulation in both time points (Fig. 4B), while 3'RR2 eRNA showed strong downregulation in all tested cell lines after

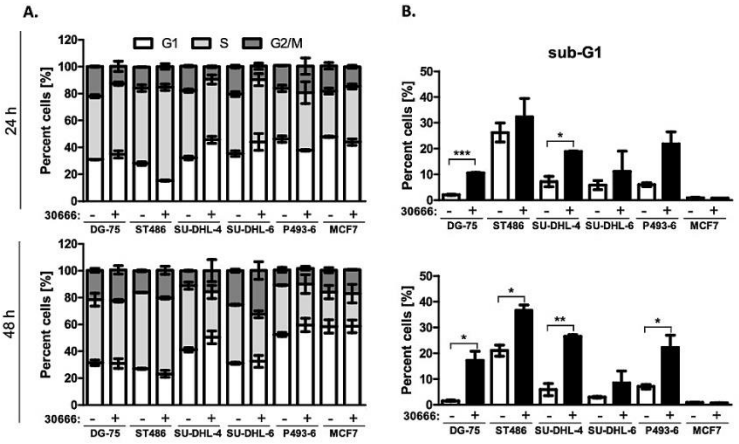


Fig. 2. Cell cycle analysis of NHL *IGH* t(+) cell lines (DG-75, ST486, SU-DHL-4, SU-DHL-6), *IGH* t(−) B-cells (P493–6) and non-B cell control (MCF7) upon treatment with compound 30666 for 24h and 48h. Cells were stained with 7-AAD to visualize DNA content and analyzed with flow cytometry **A.** Percent of cells in G1, S and G2/M phases. Data analyzed in FlowJo using Watson algorithm. There were no significant differences in cell cycle between cells treated with DMSO and compound 30666, chi-squared test. Detailed histograms can be found in Supplementary Fig. S1. **B.** Percent of cells in sub-G1 phase. Single cells exhibiting less than 2n DNA content were gated as sub-G1. Gating strategy to analyze cells in sub-G1 can be found in Supplementary Fig. S2. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001., Student's unpaired *t*-test. Mean and SD of two independent experiments are shown.

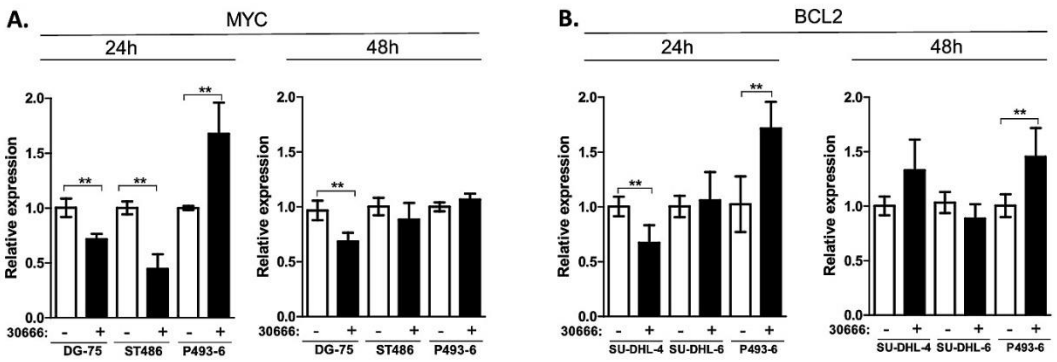


Fig. 3. Effect of compound 30666 on the expression of translocated oncogenes. Cells were cultured in the presence of compound 30666 for 24 h and 48 h. **A.** Effect on MYC expression in BL cell lines (DG-75, ST486) and control *IGH* t(−) P493-6. **B.** Effect on BCL2 expression in DLBCL (SU-DHL-4, SU-DHL-6) cell lines and control *IGH* t(−) P493-6. Mean and SD of two independent experiments, each performed in triplicate, are shown. Expression normalized to HPRT. **P ≤ 0.01, Mann-Whitney test.

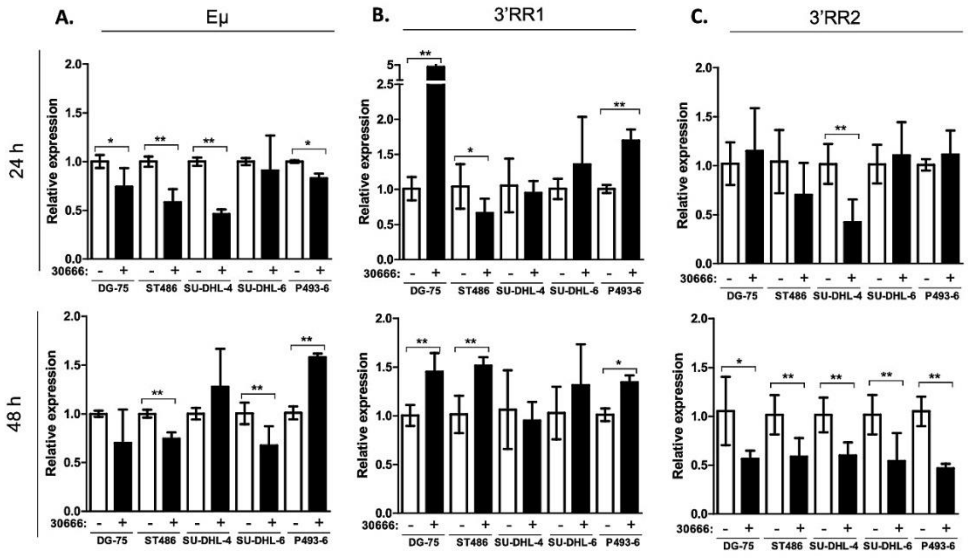


Fig. 4. Effect of the compound 30666 on the expression of *IGH* eRNAs. NHL cell lines and control *IGH* t(−) P493-6 cells were cultured in the presence of compound 30666 for 24 h and 48 h. **A.** Effect on Eμ eRNA expression. **B.** Effect on 3′RR1 eRNA expression. **C.** Effect on 3′RR2 eRNA expression. Mean and SD of two independent experiments, each performed in triplicate, are shown. Expression normalized to HPRT. *P ≤ 0.05, **P ≤ 0.01, Mann-Whitney test.

48 h (Fig. 4C).

3.5. Compound 30666 globally alters histone H3 epigenetic modifications

Since our results indicated that compound 30666 is neither specific for B cells with *IGH* translocations, nor consistently affects transcriptional activity of *IGH* eRNAs, we hypothesized that it may have a broader effect on enhancers. Histone H3 modifications H3K4me1 and H3K27ac define active enhancers (Calo and Wysocka, 2013). Thus, we investigated the effect of 30666 treatment on the presence of those chromatin marks in NHL cells and P493-6 and MCF7 as controls. We observed increased H3K4 mono-methylation in NHL cells (Fig. 5A), with mild effect on P493-6 and MCF7 cells. H3K27 acetylation was decreased in the majority of B cells with the strongest effect in DG-75 (Fig. 5A). Presence of H3K4me1 and lack of H3K27ac at enhancer regions is characteristic for primed and poised enhancers (Calo and Wysocka, 2013; Creyghton et al., 2010). Thus, our results might suggest that some enhancers are not transcriptionally active and are set in either primed or poised state upon 30666 treatment, but further study should be conducted to verify other H3 modifications at specific enhancers.

3.6. In silico prediction of compound 30666 target proteins

We reasoned that elucidation of compound 30666 protein interactors might help uncover its mechanism of action. SwissTargetPrediction offers a starting point (Daina et al., 2019). With the use of this web-based

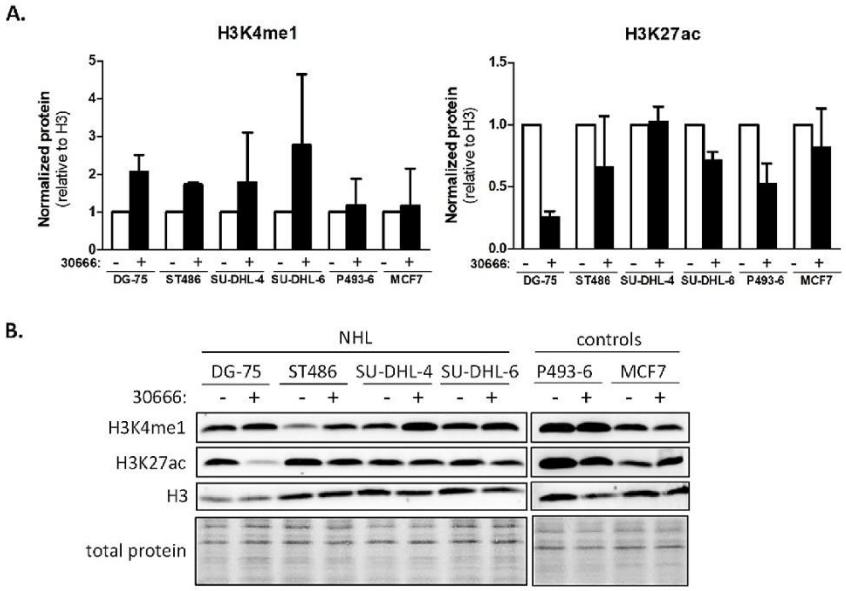


Fig. 5. Analysis of active enhancer-associated histone H3 modifications: H3K4me1 and H3K27ac. A. Changes in H3K4me1 and H3K27ac upon treatment with DMSO or compound 30666 for 48 h. Levels of H3K4me1 and H3K27ac are calculated in relation to total H3. Changes in samples treated with 30666 are presented in relation to DMSO control. Mean and SD of two independent experiments are shown. **B.** Representative Western blots used in calculations. Bands were normalized to total protein. Whole blots used to analyze changes in H3K4me1 and H3K27ac, along with H3 can be found in Supplementary Fig. S3.

tool, protein targets of small molecules can be predicted. For 7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol, potential targets in human are dominated by kinases (Fig. 6). Among other potential interactors, two epigenetic erasers appear - lysine demethylase 4 A (KDM4A) and NAD-dependent deacetylase sirtuin 1 (SIRT1). Both proteins have been reported as potential therapeutic targets in cancer treatment (Berry and Janknecht, 2013; Bur et al., 2016; Frazzi et al., 2014; Huang et al., 2019; Jha et al., 2018; Lee et al., 2020; Lin and Fang, 2013; Massett et al., 2021; Zhang et al., 2013; Wang and Cheng, 2013). Inhibition of KDM family was reported recently to increase H3K4me3 and was proposed as a promising target in germinal center-derived lymphomas (Heward et al., 2021). SIRT1 has several targets in hematological malignancies (Huang et al., 2019). In BCL6-dependent Burkitt lymphoma, treatment with cambinol inhibits SIRT1, as well as SIRT2 and leads to hyperacetylation of BCL6 and p53 and results in apoptosis (Heltweg et al., 2006).

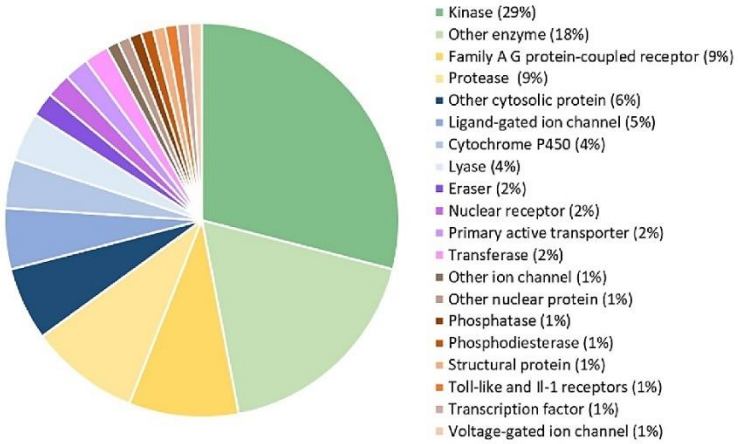


Fig. 6. SwissTargetScan prediction of potential protein targets of compound 30666. Graphical summary of all potential protein targets of compound 30666 in human. Full list of potential protein targets can be found in Supplementary Table 1.

4. Discussion

B-cell development is a multistep process, involving several rearrangements within immunoglobulin heavy chain locus (*IGH*), making it a hot spot for translocations. Juxtaposition of oncogenes with *IGH* enhancers: Eμ and 3'regulatory regions - 3′RR1 and 3′RR2 is an early pathogenic event leading to lymphomagenesis and is a common feature of NHL (Duan et al., 2008; Ghazzaui et al., 2020; Kasprzyk et al., 2021). *IGH* enhancers, besides playing important role in the control of B-cell maturation, can also drive expression of the translocated oncogene. Therefore, targeting them provides a promising anti-lymphoma strategy (Kasprzyk et al., 2021; Snyder et al., 2020; Sulentic et al., 2004; Wourms and Sulentic, 2015).

Lu et al. employed Trichostatin A (TSA), a histone deacetylases (HDACs) inhibitor, in the study on systemic lupus erythematosus (Lu et al., 2005). They found that HDAC1 binds to one of the 3′RR components: enhancer HS1.2. Inhibition of HDAC1 by TSA resulted in reduced *IGH* enhancers activity, which decreased the production of anti-dsDNA autoantibodies. *IGH* enhancer regions are packed with transcription factor binging sites(Calame and Sen, 2004; Hagman, 2015). Aryl hydrocarbon receptor (AhR) associates with HS4 within 3′RR (Sulentic et al., 2004). A recent study suggested that AhR is also involved in the activity of HS1.2 (Snyder et al., 2020). 2,3,7,8-tetrachlorodibenzo-p--dioxin (TCDD), an AhR ligand led to inhibition of 3′ *IGH* enhancer activity and decreased *IGH* transcription (Wourms and Sulentic, 2015).

7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) has been recently described (Dolloff, 2019) as a potential inhibitor of Eμ enhancer activity, by an yet undetermined mechanism. We decided to broaden the current knowledge regarding 30666. In our study we focused on two types of NHL, Burkitt lymphoma with t(8;14) *IGH/MYC* translocation and diffuse large B-cell lymphoma with t(14;18) *IGH/BCL2*. Similarly to the previous study, we observed that NHL *IGH* t(+) cells are susceptible to compound 30666 at low molar concentrations. However, in our hands, control cells not bearing *IGH* translocation were also affected to a comparable degree. This suggests that compound 30666 might not be as specific for *IGH*-translocation positive cells as previously reported.

To gain insight into a possible mechanism of action of the compound 30666, we performed several experiments. Similarly to N. G. Dolloff we observed only minor changes in cell cycle. There was a significant increase in the amount of cells in the sub-G1 phase. This suggests that compound 30666 induces apoptosis. Interestingly, this effect was seen only in B-cells.

The influence of compound 30666 on the expression of translocated oncogenes is ambiguous. While, in agreement with N. G. Dolloff results, we observed statistically significant downregulation of MYC in BL cell lines, BCL2 expression in DLCBL was rather not affected. It is unclear, whether compound 30666 affects *IGH* enhancers directly or indirectly. Non-homogenous effect on expression of different oncogenes might be connected to differential mechanisms of activation of particular oncogenes. Enhancer RNAs transcription provides another layer of gene expression regulation and is often described as a feature of active enhancers (Arnold et al., 2020; Sartorelli and Lauberth, 2020). eRNAs can facilitate enhancer-promoter looping, recruit and/or trap transcription factors as well as RNA polymerase II, control mRNA transcription and affect chromatin accessibility. We observed altered expression of Eμ and 3′RR eRNAs upon 30666 treatment, suggesting that enhancer activity might indeed be out of balance. Global changes in H3K4me1 and H3K27ac histone marks induced by compound 30666 further support this idea, however, also indicate a broader effect on enhancers, not specific for *IGH*. Increased H3K4me1 and decreased H3K27ac, suggest that compound 30666 sets enhancers into a primed or poised state (Creyghton et al., 2010). To further distinguish between those states, other histone modifications should be investigated. An increase of H3K4me1 in the poised state of enhancers is accompanied by H3K27me3 (Calo and Wysocka, 2013). ChIP-Seq analysis of several enhancer-associated chromatin marks would help to determine if Eμ and 3′RRs and potentially other enhancers are affected by 30666 treatment. Whether changes in histone H3 modifications are the primary mechanism of action of compound 30666 is not clear yet. With the help of SwissTargetScan, several potential protein targets of 7-[[(4-methyl-2--pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol were predicted, but their further experimental validation is necessary.

Taken together, our results suggest that compound 30666 might not be as specific for *IGH*-translocation positive cells as initially reported and might affect cell viability by a more general mechanism. Compound 30666 was previously tested in a mouse model of multiple myeloma and exhibited promising pharmacokinetics, as well as suppressed tumor growth (Dolloff, 2019), therefore further mechanistic study would be of interest.

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CRediT authorship contribution statement

Marta Elżbieta Kasprzyk: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Wojciech Łosiewski:** Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Marta Podralska:** Formal analysis, Investigation, Methodology. **Marta Kazimierska:** Investigation. **Weronika Sura:** Investigation. **Agnieszka Dzikiewicz-Krawczyk:** Conceptualization, Supervision, Funding acquisition, Project administration, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None of the authors have a conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174505.

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3rd ARTICLE – Supplementary Files

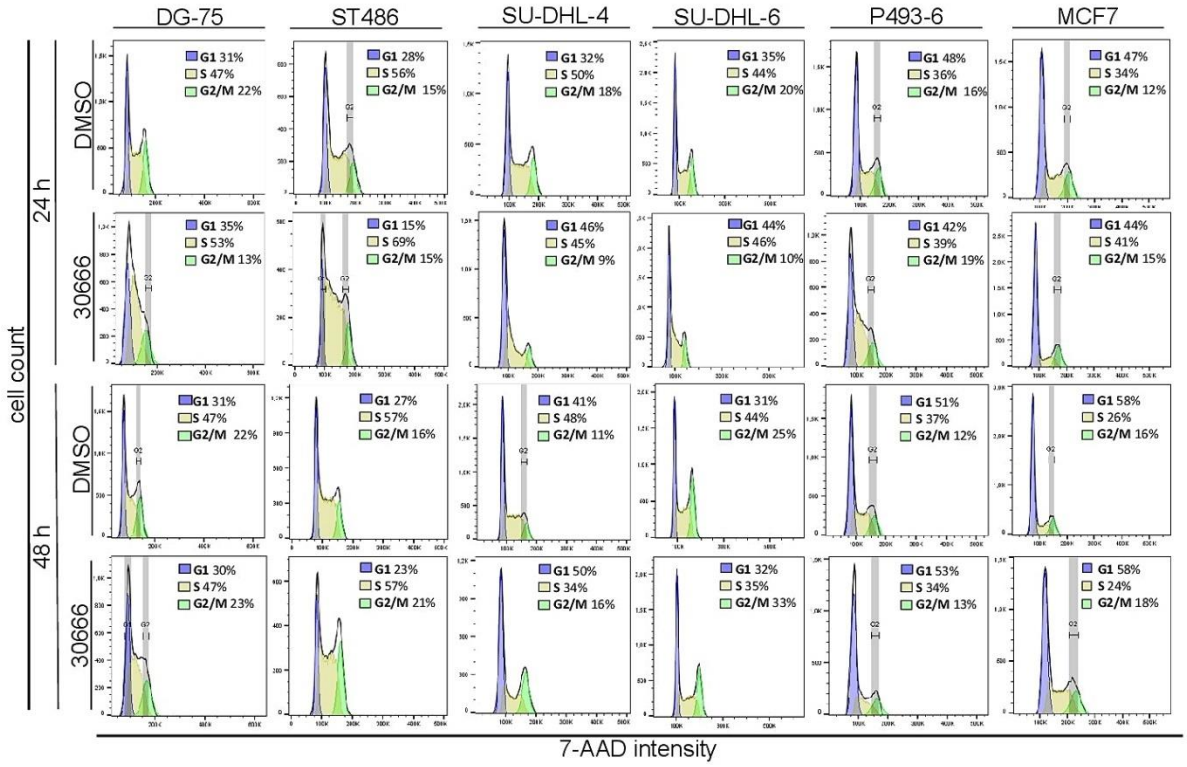

Figure S1. Flow cytometry analysis of the cell cycle distribution upon treatment with compound 30666. Cells were collected after 24h and 48h incubation with compound 30666 or DMSO, fixed with 70% EtOH and frozen. 7-AAD staining was done prior to flow cytometry analysis. Histograms show representative analysis using Watson algorithm in FlowJo. In majority of analyses cell cycle phases were automatically detected, but in some G1 and/or G2 phase had to be manually constrained (grey shade). Colors correspond to cell cycle phases: purple – G1 phase, yellow – S phase, green – G2/M phase. Y axis shows the cell count and X axis shows 7-AAD staining intensity.

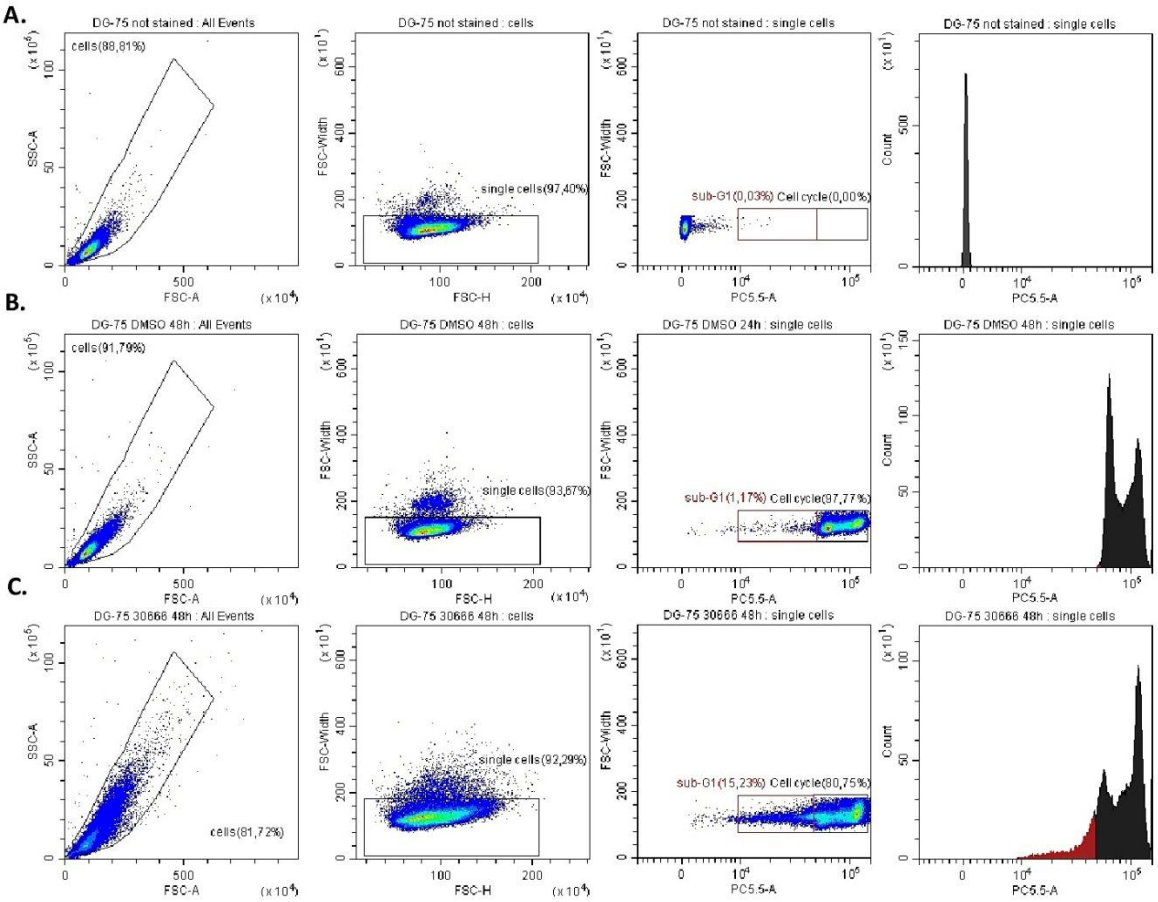

Figure S2. Gating strategy to analyze cells in sub-G1 phase. A. Unstained DG-75 cells. **B.** DG-75 cells cultured with DMSO for 48 h, stained with 7-AAD. **C.** DG-75 cells cultured with compound 30666 for 48 h, stained with 7-AAD. First dot plot in a row shows gating of cells. Second dot plot shows single cells. Third dot plot and histogram show gating of cells in sub-G1 – red gate and the cells analyzed by Watson algorithm – black gate.

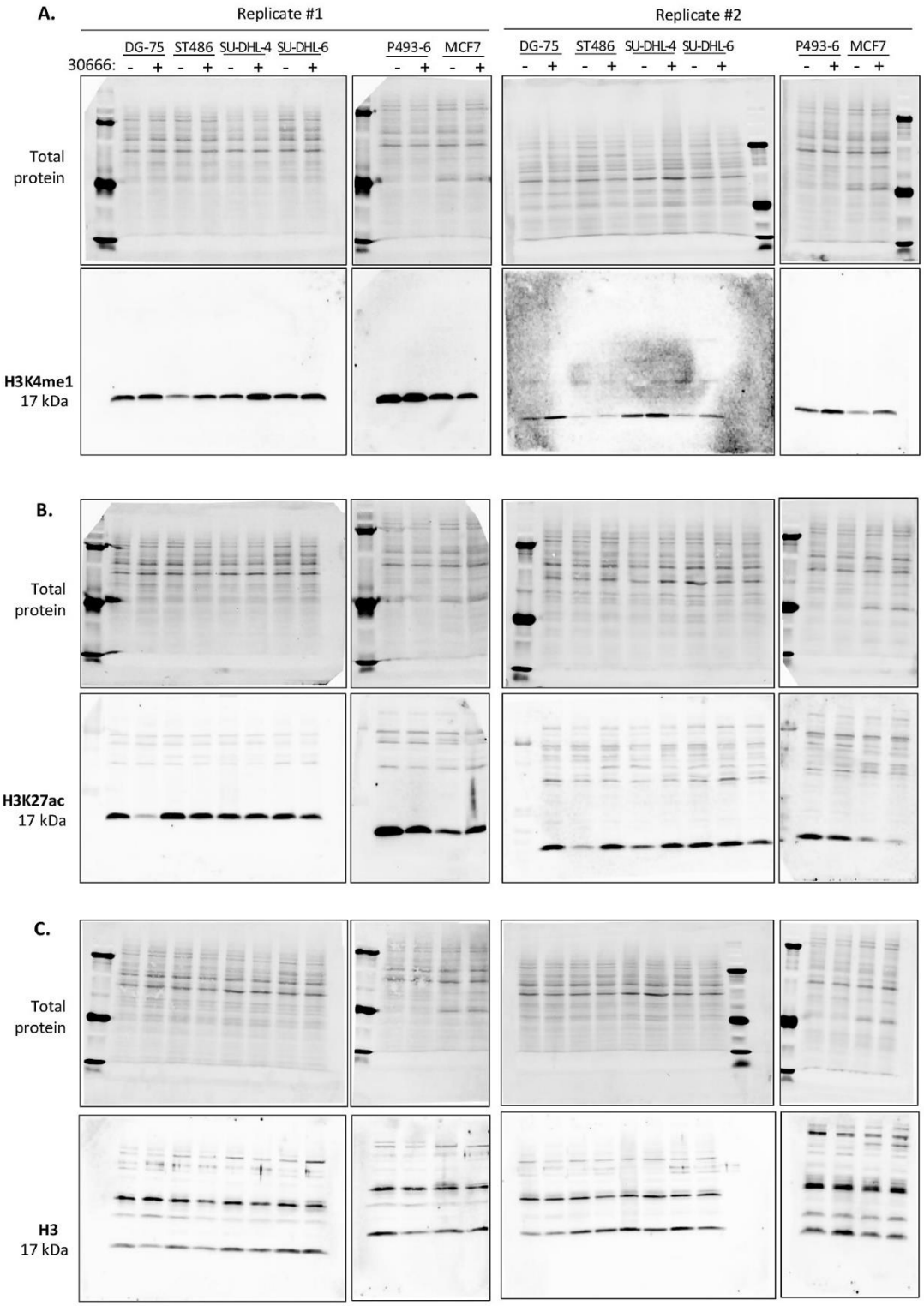

Figure S3. Western blot analysis of histone modifications associated with active chromatin. A. H3K4me1, **B.** H3K27ac, **C.** H3. Cells were treated with DMSO (-) or compound 30666 (+) for 48h. Pictures show activated membranes after transfer, which were used in total protein normalization and blots after incubation with antibodies, which were used in analysis. Two independent replicates for each used antibody are presented. Bands were normalized using total protein method in ImageLab (Bio-Rad). Levels of H3K4me1 and H3K27ac were calculated in relation to total H3.

Supplementary Table 1.

SwissTargetPrediction						
Target	**Common name**	**Uniprot ID**	**ChEMBL ID**	**Target Class**	**Probability**	**Known actives (3D/2D)**
Cytochrome P450 11B1	CYP11B1	P15538	CHEMBL1908	Cytochrome P450	0.11	25 / 0
Cytochrome P450 11B2	CYP11B2	P19099	CHEMBL2722	Cytochrome P450	0.11	30 / 0
Thromboxane-A synthase	TBXAS1	P24557	CHEMBL1835	Cytochrome P450	0.11	9 / 0
Cytochrome P450 19A1	CYP19A1	P11511	CHEMBL1978	Cytochrome P450	0.11	44 / 0
Macrophage migration inhibitory factor	MIF	P14174	CHEMBL2085	Enzyme	0.12	5 / 1
Quinone reductase 1	NQO1	P15559	CHEMBL3623	Enzyme	0.11	5 / 0
Quinone reductase 2	NQO2	P16083	CHEMBL3959	Enzyme	0.11	9 / 0
Steryl-sulfatase	STS	P08842	CHEMBL3559	Enzyme	0.11	11 / 0
Arachidonate 12-lipoxygenase	ALOX12	P18054	CHEMBL3687	Enzyme	0.11	14 / 0
L-lactate dehydrogenase A chain	LDHA	P00338	CHEMBL4835	Enzyme	0.11	8 / 0
1-acylglycerol-3-phosphate O-acyltransferase beta	AGPAT2	O15120	CHEMBL4772	Enzyme	0.11	19 / 0
Estradiol 17-beta-dehydrogenase 2	HSD17B2	P37059	CHEMBL2789	Enzyme	0.11	14 / 0
L-lactate dehydrogenase B chain	LDHB	P07195	CHEMBL4940	Enzyme	0.11	2 / 0
Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	O75874	CHEMBL2007625	Enzyme	0.11	81 / 0
Prostaglandin E synthase	PTGES	O14684	CHEMBL5658	Enzyme	0.11	20 / 0
Aldose reductase (by homology)	AKR1B1	P15121	CHEMBL1900	Enzyme	0.11	7 / 0
Serine/threonine-protein kinase/endoribonuclease IRE1	ERN1	O75460	CHEMBL1163101	Enzyme	0.11	5 / 0
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3	PFKFB3	Q16875	CHEMBL2331053	Enzyme	0.11	32 / 0
Tankyrase-2	TNKS2	Q9H2K2	CHEMBL6154	Enzyme	0.11	22 / 0
Tankyrase-1	TNKS	O95271	CHEMBL6164	Enzyme	0.11	16 / 0
Carnitine O-palmitoyltransferase 1, liver isoform	CPT1A	P50416	CHEMBL1293194	Enzyme	0.00	37 / 0

Fructose-1,6-bisphosphatase	FBP1	P09467	CHEMBL3975	Enzyme	0.00	12 / 0
Lysine-specific demethylase 4A	KDM4A	O75164	CHEMBL5896	Eraser	0.11	2 / 2
NAD-dependent deacetylase sirtuin 1	SIRT1	Q96EB6	CHEMBL4506	Eraser	0.11	5 / 0
Adenosine A2a receptor	ADORA2A	P29274	CHEMBL251	Family A G protein-coupled receptor	0.11	258 / 0
Cannabinoid receptor 1 (by homology)	CNR1	P21554	CHEMBL218	Family A G protein-coupled receptor	0.11	367 / 0
Adenosine A2b receptor	ADORA2B	P29275	CHEMBL255	Family A G protein-coupled receptor	0.11	58 / 0
Interleukin-8 receptor B	CXCR2	P25025	CHEMBL2434	Family A G protein-coupled receptor	0.11	51 / 0
Purinergic receptor P2Y1	P2RY1	P47900	CHEMBL4315	Family A G protein-coupled receptor	0.11	5 / 0
Cholecystokinin B receptor	CCKBR	P32239	CHEMBL298	Family A G protein-coupled receptor	0.11	212 / 0
Neurokinin 2 receptor	TACR2	P21452	CHEMBL2327	Family A G protein-coupled receptor	0.00	5 / 0
Calcium sensing receptor	CASR	P41180	CHEMBL1878	Family C G protein-coupled receptor	0.11	28 / 0
Metabotropic glutamate receptor 2	GRM2	Q14416	CHEMBL5137	Family C G protein-coupled receptor	0.00	11 / 0
G protein-coupled receptor kinase 6	GRK6	P43250	CHEMBL6144	Kinase	0.11	6 / 3
Serine/threonine-protein kinase 33	STK33	Q9BYT3	CHEMBL6005	Kinase	0.11	19 / 0

Cyclin-dependent kinase 5/CDK5 activator 1	CDK5R1 CDK5	Q15078 Q00535	CHEMBL1907600	Kinase	0.11	159 / 0
Glycogen synthase kinase-3 beta	GSK3B	P49841	CHEMBL262	Kinase	0.11	153 / 0
Serine/threonine-protein kinase Aurora-C	AURKC	Q9UQB9	CHEMBL3935	Kinase	0.11	9 / 0
Inhibitor of nuclear factor kappa B kinase beta subunit	IKBKB	O14920	CHEMBL1991	Kinase	0.11	37 / 0
Insulin receptor	INSR	P06213	CHEMBL1981	Kinase	0.11	13 / 0
Kinesin-1 heavy chain/ Tyrosine-protein kinase receptor RET	RET	P07949	CHEMBL2041	Kinase	0.11	56 / 0
Serine-protein kinase ATM	ATM	Q13315	CHEMBL3797	Kinase	0.11	3 / 0
Serine-protein kinase ATR	ATR	Q13535	CHEMBL5024	Kinase	0.11	14 / 0
Myosin light chain kinase, smooth muscle	MYLK	Q15746	CHEMBL2428	Kinase	0.11	7 / 0
Cyclin-dependent kinase 2/cyclin E1	CCNE1 CDK2	P24864 P24941	CHEMBL1907605	Kinase	0.11	41 / 0
ALK tyrosine kinase receptor	ALK	Q9UM73	CHEMBL4247	Kinase	0.11	57 / 0
Vascular endothelial growth factor receptor 3	FLT4	P35916	CHEMBL1955	Kinase	0.11	13 / 0
Tyrosine-protein kinase SRC	SRC	P12931	CHEMBL267	Kinase	0.11	132 / 0
Nerve growth factor receptor Trk-A	NTRK1	P04629	CHEMBL2815	Kinase	0.11	23 / 0
Serine/threonine-protein kinase 17B	STK17B	O94768	CHEMBL3980	Kinase	0.11	11 / 0
Serine/threonine-protein kinase mTOR	MTOR	P42345	CHEMBL2842	Kinase	0.11	87 / 0
Cyclin-dependent kinase 4	CDK4	P11802	CHEMBL331	Kinase	0.11	39 / 0
3-phosphoinositide dependent protein kinase-1	PDPK1	O15530	CHEMBL2534	Kinase	0.11	36 / 0
Dual-specificity tyrosine-phosphorylation regulated kinase 1A	DYRK1A	Q13627	CHEMBL2292	Kinase	0.11	66 / 0
Dual specificity tyrosine-phosphorylation-regulated kinase 1B	DYRK1B	Q9Y463	CHEMBL5543	Kinase	0.11	27 / 0
Tyrosine-protein kinase JAK3	JAK3	P52333	CHEMBL2148	Kinase	0.11	247 / 0

TGF-beta receptor type I	TGFBR1	P36897	CHEMBL4439	Kinase	0.11	122 / 0
Tyrosine-protein kinase Lyn	LYN	P07948	CHEMBL3905	Kinase	0.11	5 / 0
Tyrosine-protein kinase TXK	TXK	P42681	CHEMBL4367	Kinase	0.11	2 / 0
Fibroblast growth factor receptor 1	FGFR1	P11362	CHEMBL3650	Kinase	0.00	65 / 0
Serine/threonine-protein kinase RIPK2	RIPK2	O43353	CHEMBL5014	Kinase	0.00	8 / 0
Serine/threonine-protein kinase PLK4	PLK4	O00444	CHEMBL3788	Kinase	0.00	1 / 0
GABA-A receptor; alpha-1/beta-2/gamma-2	GABRA1 GABRB2 GABRG2	P14867 P47870 P18507	CHEMBL2095172	Ligand-gated ion channel	0.11	14 / 0
GABA-A receptor; alpha-3/beta-3/gamma-2	GABRB3 GABRA3 GABRG2	P28472 P34903 P18507	CHEMBL2094120	Ligand-gated ion channel	0.00	42 / 0
GABA-A receptor; alpha-1/beta-3/gamma-2	GABRB3 GABRG2 GABRA1	P28472 P18507 P14867	CHEMBL2094121	Ligand-gated ion channel	0.00	35 / 0
GABA-A receptor; alpha-5/beta-3/gamma-2	GABRB3 GABRG2 GABRA5	P28472 P18507 P31644	CHEMBL2094122	Ligand-gated ion channel	0.00	44 / 0
GABA-A receptor; alpha-2/beta-3/gamma-2	GABRA2 GABRB3 GABRG2	P47869 P28472 P18507	CHEMBL2094130	Ligand-gated ion channel	0.00	38 / 0
Carbonic anhydrase I	CA1	P00915	CHEMBL261	Lyase	0.11	193 / 0
Carbonic anhydrase IX	CA9	Q16790	CHEMBL3594	Lyase	0.11	142 / 0
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.11	220 / 0
Carbonic anhydrase XII	CA12	O43570	CHEMBL3242	Lyase	0.11	112 / 0
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0.11	20 / 7
Mineralocorticoid receptor	NR3C2	P08235	CHEMBL1994	Nuclear receptor	0.11	54 / 0
Cyclin-dependent kinase 1/cyclin B	CCNB3 CDK1 CCNB1 CCNB2	Q8WWL7 P06493 P14635 O95067	CHEMBL2094127	Other cytosolic protein	0.11	45 / 0

5-lipoxygenase activating protein	ALOX5AP	P20292	CHEMBL4550	Other cytosolic protein	0.11	19 / 0
Heat shock protein 75 kDa, mitochondrial	TRAP1	Q12931	CHEMBL1075132	Other cytosolic protein	0.11	4 / 0
Heat shock protein HSP 90-alpha	HSP90AA1	P07900	CHEMBL3880	Other cytosolic protein	0.11	41 / 0
Cyclin-dependent kinase 1/cyclin B1	CDK1 CCNB1	P06493 P14635	CHEMBL1907602	Other cytosolic protein	0.00	24 / 0
Cyclin-dependent kinase 7/ cyclin H	CDK7 CCNH	P50613 P51946	CHEMBL2111288	Other cytosolic protein	0.00	15 / 0
Apoptosis regulator Bcl-2	BCL2	P10415	CHEMBL4860	Other ion channel	0.11	4 / 0
p53-binding protein Mdm-2	MDM2	Q00987	CHEMBL5023	Other nuclear protein	0.13	25 / 19
Serine/threonine protein phosphatase PP1-alpha catalytic subunit	PPP1CA	P62136	CHEMBL2164	Phosphatase	0.68	6 / 4
Phosphodiesterase 4B	PDE4B	Q07343	CHEMBL275	Phosphodiesterase	0.11	35 / 0
ATP-binding cassette sub-family G member 2	ABCG2	Q9UNQ0	CHEMBL5393	Primary active transporter	0.11	12 / 0
P-glycoprotein 1	ABCB1	P08183	CHEMBL4302	Primary active transporter	0.00	12 / 0
Matrix metalloproteinase 9	MMP9	P14780	CHEMBL321	Protease	0.11	36 / 0
Matrix metalloproteinase 2	MMP2	P08253	CHEMBL333	Protease	0.11	42 / 0
ADAMTS4	ADAMTS4	O75173	CHEMBL2318	Protease	0.11	3 / 0
Carboxypeptidase B	CPB1	P15086	CHEMBL2552	Protease	0.11	2 / 0
Beta secretase 2	BACE2	Q9Y5Z0	CHEMBL2525	Protease	0.11	106 / 0
Beta-secretase 1	BACE1	P56817	CHEMBL4822	Protease	0.11	457 / 0
Prenyl protein specific protease	RCE1	Q9Y256	CHEMBL3411	Protease	0.11	5 / 10
Matrix metalloproteinase 1	MMP1	P03956	CHEMBL332	Protease	0.11	35 / 0
Cathepsin K	CTSK	P43235	CHEMBL268	Protease	0.00	113 / 0
Tubulin beta-1 chain	TUBB1	Q9H4B7	CHEMBL1915	Structural protein	0.00	4 / 0

Toll-like receptor (TLR7/TLR9)	TLR9	Q9NR96	CHEMBL5804	Toll-like and Il-1 receptors	0.00	14 / 0
Signal transducer and activator of transcription 6	STAT6	P42226	CHEMBL5401	Transcription factor	0.11	9 / 0
Thymidylate synthase	TYMS	P04818	CHEMBL1952	Transferase	0.11	44 / 0
2-acylglycerol O-acyltransferase 2	MOGAT2	Q3SYC2	CHEMBL2439944	Transferase	0.00	8 / 0
Calcium-activated potassium channel subunit alpha-1	KCNMA1	Q12791	CHEMBL4304	Voltage-gated ion channel	0.11	14 / 0

Material Safety Data Sheet

according to Regulation (EC) No 1907/2006 (REACH)

1. Identification of the substance and of the company

1.1.

Product identifier	Substance name
STK506800	7-{[(4-methylpyridin-2-yl)amino](pyridin-2-yl)methyl}quinolin-8-ol

CAS No.: N/A
Index No: N/A
EC No: N/A
REACH No: Registration number for this substance is not available. The substance is exempted from registration as the annual tonnage does not require a registration.
1.2. Relevant identified uses of the substance.
Laboratory chemicals, Research and Development use only.
1.3 Details of the supplier of the safety data sheet:
Name: **Vitas M Chemical Limited**,
Phone: +852 98659192
Address: 15/F, Radio City, 505 Hennessy Road, Causeway Bay, Hong Kong
E-mail: irina@vitasmlab.biz

2. Hazards identification

2.1. Classification of the substance according to Regulation (EC) No 1907/2006 (REACH)
Hazard description: Not classified.
Physical hazards Not classified
Health hazards Not classified
Environmental hazards Not classified

3. Composition/information on ingredients

Substance name	Mol Formula	Mol Weight	Purity	Product ID
7-{[(4-methylpyridin-2-yl)amino](pyridin-2-yl)methyl}quinolin-8-ol	C21H18N4O	342.4	>90%	STK506800

Percentage: individual state, not mixtures.
Classification: No components need to be disclosed according to the applicable regulations.

4. First aid measures

After inhalation: supply fresh air. If required, provide artificial respiration. Keep patient warm.
After skin contact: Immediately wash with water and soap and rinse thoroughly.
After eye contact: rinse opened eye for several minutes under running water. Then consult a doctor.
After swallowing: seek medical treatment.

5. Fire Fighting measures

Products are not flammable. Use fire fighting measures that suit the surrounding fire.
Protective equipment: Wear self-contained respirator.

6. Accidental release measures

Person-related safety precautions: Wear protective equipment. Ensure adequate ventilation.

Measures for environmental protection: Do not allow materials to be released to the environment without proper governmental permits.
Additional information: See section 11 for disposal information.

7. Handling and storage
Handling
Information for safe handling: Keep container tightly sealed. Store in cool, dry place in tightly closed containers. No special precautions are necessary if used correctly.
Information about protection against explosions and fires: The product is not flammable
Storage
Requirements for storage rooms and vessels: Keep vial tightly closed and upright. Store in a cool and well ventilated place.
Information about storage in one common storage facility:
Store away from oxidizing agents.
Do not store together with acids.

8. Exposure controls and personal protection
General protective and hygienic measures
The usual precautionary measures for handling chemicals should be followed. Keep away from foodstuff, beverages and food. Wash hands before breaks and at the end of work.
Breathing equipment: Use suitable respirator when high concentrations are present.
Protection of hands: Impervious gloves.
Body protection: Protective work clothing.

9. Physical and chemical properties
Form: odorless dry powder,
Boiling point: Not determined
Flash point: Not applicable
Flammability: Not determined.
Ignition temperature: Not applicable
Decomposition temperature: Not determined
Materials to be avoided: Acids, Oxidizing agents.
Dangerous reactions: React with strong acids. React with strong oxidizing agents.
Explosive properties: Not determined
Oxidizing properties: Not determined

10. Stability and reactivity
Chemical stability: stable under recommended storage conditions
Conditions to avoid: excess heat
Materials to avoid: strong oxidizing agents, strong acids, strong bases

11. Toxicological information
Toxicological properties are not fully investigated. No specific health hazards known.
Additional toxicological information: No classification data on carcinogenic properties of this material is available from the EPA, IARC, NTP, OSHA or ACGIH.

12. Ecological information.
General notes: Not regarded as dangerous for the environment. However, large or frequent spills may have hazardous effects on the environment.

13. Disposal considerations

Recommendation: Consult local or national regulations for proper disposal.

14. Transport information
Not a hazardous materials for transportation.
ICAO/IATA Class: None
Transport/Additional information: Not dangerous regarding EU Transport Directive (ADR).

15. Regulations
EU legislation Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18
December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).
Regulation (EC) No 1272/2008 - classification, labelling and packaging of substances and mixtures (CLP).

16. Other information:
Employers should use this information only as a supplement to other information gathered by them, and should make independent judgement of suitability of this information to ensure proper use and protect the health and safety of employees. This information is furnished without warranty, and any use of the product not in conformance with this Material Safety Data Sheet, or in combination with any other product or process, is the responsibility of the user.
For Research and Development use only. Not for drug, household and other uses. This is an experimental product whose properties are not fully evaluated yet. The information contained herein is based on the present state of our knowledge and therefore does not guarantee certain properties. Recipient of this product must take responsibility for observing existing law and regulations.

February 06, 2020

Authors statements

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Marta Kasprzyk, MSc

Poznań, 8.05.2023

STATEMENT FOR DOCTORAL DISSERTATION

Regarding the procedure for awarding a doctoral degree, I hereby confirm that I have greatly contributed to the following publication which I am a co-author:

Marta Elżbieta Kasprzyk*, Wojciech Łosiewski*, Marta Podralska, Marta Kazimierska, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk
*co-first authors

7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity

Eur J Pharmacol Sep 14;910:174505; IF: 4.432

My contribution to the cited publication included:

- project organisation and planning
- cell culture and handling
- 7-AAD cells staining for flow cytometry analysis of cell cycle
- total protein isolation and Western Blots
- data analysis of qRT-PCRs, Western Blots and cell cycle
- *in silico* analysis of compound 30666
- preparation of the original draft of the manuscript
- review and editing of the final version of the manuscript

I would like to use the above publication in my doctoral dissertation entitled:
Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas.
This doctoral dissertation is prepared under the guidance of Agnieszka Dzikiewicz-Krawczyk, MD PhD.

With regards,

Kasprzyk Marta
.....
(doctoral candidate's signature)

Wojciech Łosiewski, MSc

Poznań, 25.04.2023

STATEMENT FOR DOCTORAL DISSERTATION

Regarding the procedure for awarding a doctoral degree to Marta Kasprzyk, MSc, I hereby confirm that Marta Kasprzyk had greatly contributed to the following publication which I am a co-author:

Marta Elżbieta Kasprzyk*, **Wojciech Łosiewski***, Marta Podralska, Marta Kazimierska, Weronika Sura, Agnieszka Dzikiewicz-Krawczyk,
*co-first authors

7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity

Eur J Pharmacol Sep 14;910:174505; IF: 4.432

My contribution to the cited publication included cell culture and handling, preparation of Cell Titer Glo experiments, preparation of RNA isolation, cDNA and qRT-PCRs, 7-AAD cells staining for flow cytometry analysis of cell cycle, preparation of Western Blots, data analysis of Cell Titer Glo experiments and qRT-PCRs and preparation of the original draft of the manuscript.

In addition, I agree for the use of the above publication in doctoral dissertation of Marta Kasprzyk entitled: *Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas*. This doctoral dissertation is prepared under the guidance of Agnieszka Dzikiewicz-Krawczyk, MD PhD.

With regards,

Wojciech Łosiewski

(co-author's signature)

INSTITUTE
OF HUMAN GENETICS
POLISH ACADEMY OF SCIENCES

Strzeszyńska 32
60-479 Poznań, Poland

tel. +48/61/657 91 00
fax +48/61/823 32 35
e-mail: igcz@man.poznan.pl

www.igcz.poznan.pl

Agnieszka Dzikiewicz-Krawczyk, MD PhD

Poznań, 8.05.2023

STATEMENT FOR DOCTORAL DISSERTATION

Regarding the procedure for awarding a doctoral degree to Marta Kasprzyk, MSc, I hereby confirm that Marta Kasprzyk had greatly contributed to the following publication which I am a co-author:

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7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) inhibits enhancer activity and reduces B-cell lymphoma growth – A question of specificity

Eur J Pharmacol Sep 14;910:174505; IF: 4.432

My contribution to the cited publication included project conceptualization, funding acquisition, project administration, supervision of the project, review of the original draft of the manuscript, review and editing of the final version of the manuscript.

In addition, I agree for the use of the above publication in doctoral dissertation of Marta Kasprzyk entitled: *Functional dissection of IGH enhancers and enhancer RNAs in B-cell non-Hodgkin lymphomas*. This doctoral dissertation is prepared under my guidance.

With regards,

A. Dzikiewicz-Krawczyk

(supervisor's signature)

CONCLUSIONS

Research undertaken in the course of this doctoral dissertation have led to the following conclusions:

1. In B-cell lymphomas, enhancers can be exploited by malignant cells through several mechanisms and can contribute to the process of lymphomagenesis.

2. CRISPRi tiling screen indicated three exact regions in the immunoglobulin heavy chain (IGH) enhancers Eμ and 3'RRs (3'RR1 and 3'RR2), which are essential for Burkitt lymphoma (BL) and Diffuse Large B-cell lymphoma (DLBCL) cell growth.

3. Chromatin enriched RNA-Seq confirmed ongoing, bidirectional transcription within the IGH enhancers as well as their essential regions.

4. Different non-Hodgkin lymphoma cell lines display various patterns of dependency on *IGH* enhancers, possibly linked to their breakpoints sites within the *IGH* locus.

5. Blocking of the core regions of IGH enhancers with CRISPR/dCas9 leads to downregulation of translocated oncogenes expression.

6. Inhibition of Eμ enhancer-essential region leads to B-cell receptor (BCR) loss in B-cell lymphomas bearing *IGH* locus translocation as well as B-cell cell lines without IGH translocation.

7. Core regions of *IGH* enhancers appear as promising potential therapeutic targets.

8. 7-[[(4-methyl-2-pyridinyl)amino](2-pyridinyl)methyl]-8-quinolinol (compound 30666) tested in this dissertation, alters enhancer activity, although is not as specific for *IGH* enhancers as initially reported.

FUNDING

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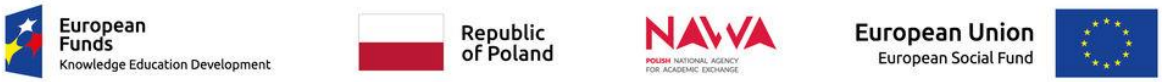
and by PROM program, The Polish National Agency for Academic Exchange (NAWA) no. 10 PPI/PRO/2019/1/00014/U/00001 granted to Marta Kasprzyk, MSc.



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