MINCR is a MYC-induced lncRNA able to modulate MYC's transcriptional network in Burkitt lymphoma cells

Gero Doose, Andrea Haake, Stephan H. Bernhart, Cristina López, Sujitha Duggimpudi, Franziska Wojciech, Anke K. Bergmann, Arndt Borkhardt, Birgit Burkhardt, Alexander Claviez, Lora Dimitrova, Siegfried Haas, Jessica I. Hoell, Michael Hummel, Dennis Karsch, Wolfram Klapper, Karsten Kleo, Helene Kretzmer, Markus Kreuz, Ralf Küppers, Chris Lawerenz, Dido Lenze, Markus Loeffler, Luisa Mantovani-Löffler, Peter Möller, German Ott, Julia Richter, Marius Rohde, Philip Rosenstiel, Andreas Rosenwald, Markus Schilhabel, Markus Schneider, Ingrid Scholz, Stephan Stilgenbauer, Hendrik G. Stunnenberg, Monika Szczepanowski, Lorenz Trümper, Marc A. Weniger, Steve Hoffmann, Reiner Siebert, Ingram Iaccarino, Matthias Schlesner

Angaben zur Veröffentlichung / Publication details:

Doose, Gero, Andrea Haake, Stephan H. Bernhart, Cristina López, Sujitha Duggimpudi, Franziska Wojciech, Anke K. Bergmann, et al. 2015. "MINCR is a MYC-induced lncRNA able to modulate MYC's transcriptional network in Burkitt lymphoma cells." *Proceedings of the National Academy of Sciences* 112 (38): E5261–70. https://doi.org/10.1073/pnas.1505753112.

MINCR is a MYC-induced lncRNA able to modulate MYC's transcriptional network in Burkitt lymphoma cells

Gero Doose[a], Andrea Haake[b], Stephan H. Bernhart[a], Cristina López[b], Sujitha Duggimpudi[c], Franziska Wojciech[b], Anke K. Bergmann[b,d], Arndt Borkhardt[c], Birgit Burkhardt[e,f], Alexander Claviez[d], Lora Dimitrova[g], Siegfried Haas[h], Jessica I. Hoell[c], Michael Hummel[g], Dennis Karsch[i], Wolfram Klapper[j], Karsten Kleo[g], Helene Kretzmer[a], Markus Kreuz[k], Ralf Küppers[l], Chris Lawerenz[m], Dido Lenze[g], Markus Loeffler[k], Luisa Mantovani-Löffler[n], Peter Möller[o], German Ott[p], Julia Richter[b], Marius Rohde[f], Philip Rosenstiel[q], Andreas Rosenwald[r], Markus Schilhabel[q], Markus Schneider[l], Ingrid Scholz[m], Stephan Stilgenbauer[s], Hendrik G. Stunnenberg[t], Monika Szczepanowski[j], Lorenz Trümper[u], Marc A. Weniger[l], ICGC MMML-Seq Consortium[1], Steve Hoffmann[a], Reiner Siebert[b], and Ingram Iaccarino[b,v,2]

[a]Transcriptome Bioinformatics, Leipzig Research Center for Civilization Diseases, University of Leipzig, D-04107 Leipzig, Germany; [b]Institute of Human Genetics, University Hospital Schleswig-Holstein, Christian Albrechts University, D-24105 Kiel, Germany; [c]Department of Pediatric Oncology, Hematology and Clinical Immunology, University Children's Hospital, Heinrich Heine University, Medical Faculty, D-40225 Düsseldorf, Germany; [d]Department of Pediatrics, University Hospital Schleswig-Holstein, D-24105 Kiel, Germany; [e]Department of Pediatric Hematology and Oncology, University Hospital Münster, D-48149 Munster, Germany; [f]Department of Pediatric Hematology and Oncology, University Hospital Giessen, D-35392 Giessen, Germany; [g]Institute of Pathology, Charité University Medicine Berlin, D-12200 Berlin, Germany; [h]Friedrich-Ebert Hospital Neumünster, Clinics for Hematology, Oncology and Nephrology, D-24534 Neumünster, Germany; [i]Department of Internal Medicine II: Hematology and Oncology, University Medical Centre, D-24105 Kiel, Germany; [j]Hematopathology Section, University Hospital Schleswig-Holstein, Christian Albrechts University, D-24105 Kiel, Germany; [k]Institute for Medical Informatics Statistics and Epidemiology, University of Leipzig, D-04107 Leipzig, Germany; [l]Institute of Cell Biology (Cancer Research), University of Duisburg-Essen, D-45122 Essen, Germany; [m]Division Theoretical Bioinformatics, German Cancer Research Center, D-69120 Heidelberg, Germany; [n]Hospital of Internal Medicine II, Hematology and Oncology, St. Georg Hospital Leipzig, D-04129 Leipzig, Germany; [o]Institute of Pathology, Ulm University, D-89070 Ulm, Germany; [p]Department of Clinical Pathology, Robert Bosch Hospital and Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, D-70376 Stuttgart, Germany; [q]Institute of Clinical Molecular Biology, Christian Albrechts University, D-24105 Kiel, Germany; [r]Institute of Pathology, University of Würzburg and Comprehensive Cancer Center Mainfranken, D-97080 Würzburg, Germany; [s]Department of Internal Medicine III, University of Ulm, D-89081 Ulm, Germany; [t]Department of Molecular Biology, Radboud Institute for Molecular Life Sciences, Radboud University, 6525 Nijmegen, The Netherlands; [u]Department of Hematology and Oncology, Georg August University of Göttingen, D-37075 Göttingen, Germany; and [v]Institute of Genetics and Biophysics "A.Buzzati-Traverso," Consiglio Nazionale delle Ricerche, I-80131 Naples, Italy

Despite the established role of the transcription factor MYC in cancer, little is known about the impact of a new class of transcriptional regulators, the long noncoding RNAs (lncRNAs), on MYC ability to influence the cellular transcriptome. Here, we have intersected RNA-sequencing data from two MYC-inducible cell lines and a cohort of 91 B-cell lymphomas with or without genetic variants resulting in MYC overexpression. We identified 13 lncRNAs differentially expressed in *IG-MYC*-positive Burkitt lymphoma and regulated in the same direction by MYC in the model cell lines. Among them, we focused on a lncRNA that we named MYC-induced long noncoding RNA (MINCR), showing a strong correlation with MYC expression in MYC-positive lymphomas. To understand its cellular role, we performed RNAi and found that MINCR knockdown is associated with an impairment in cell cycle progression. Differential gene expression analysis after RNAi showed a significant enrichment of cell cycle genes among the genes down-regulated after MINCR knockdown. Interestingly, these genes are enriched in MYC binding sites in their promoters, suggesting that MINCR acts as a modulator of the MYC transcriptional program. Accordingly, MINCR knockdown was associated with a reduction in MYC binding to the promoters of selected cell cycle genes. Finally, we show that down-regulation of Aurora kinases A and B and chromatin licensing and DNA replication factor 1 may explain the reduction in cellular proliferation observed on MINCR knockdown. We, therefore, suggest that MINCR is a newly identified player in the MYC transcriptional network able to control the expression of cell cycle genes.

MYC is a transcription factor belonging to the basic helix–loop–helix zipper family that was originally identified in Burkitt lymphoma (BL) because of a chromosomal translocation that juxtaposes the MYC oncogene with one of three immunoglobulin (Ig) loci (1–3). In BL, the deregulation of the oncogenic transcription factor MYC is considered to be the major driving force in lymphoma development (4, 5). MYC overexpression is not restricted to BL and has been found to be a common feature

Significance

Gains of the MYC gene are the most common imbalances in cancer and are associated with poor prognosis, particularly in B-cell lymphoma. Recent advances in DNA sequencing have revealed the existence of thousands of long noncoding RNAs (lncRNAs) with unknown functional relevance. We have here identified a MYC-regulated lncRNA that we named MYC-induced long noncoding RNA (MINCR) that has a strong correlation with MYC expression in cancer. We show that MINCR is functional and controls cell cycle progression by influencing the expression of MYC-regulated cell cycle genes. MINCR is, therefore, a novel player in MYC's transcriptional network, with the potential to open new therapeutic windows in the fight against malignant lymphoma and, possibly, all cancers that rely on MYC expression.

Author contributions: G.D., S.H., R.S., and I.I. designed research; C. López, S.D., F.W., L.D., K.K., M. Schneider, M. Szczepanowski, M.A.W., and I.I. performed experiments; A.B., B.B., A.C., S. Haas, M.H., D.K., W.K., R.K., D.L., L.M.-L., P.M., G.O., M.R., A.R., S.S., L.T., and I.M.-S.C. contributed new reagents/analytic tools; G.D., A.H., S.H.B., J.I.H., H.K., M.K., C. Lawerenz, M.L., P.R., A.R., M. Schilhabel, I.S., S. Hoffmann, and I.I. analyzed data; R.S. and I.I. wrote the paper; A.H., J.R., and R.S. coordinated the IGCG MMML-Seq Project; A.K.B. and H.G.S. provided data in the context of the BLUEPRINT project.

The authors declare no conflict of interest.

Data deposition: The sequence data reported in this paper has been deposited in the European Genome-Phenome Archive, www.ebi.ac.uk/ega/ (study no. EGAS00001001199).

[1]A complete list of the ICGC MMML-Seq (International Cancer Genome Consortium Molecular Mechanisms in Malignant Lymphoma by Sequencing) can be found in the *Supporting Information*.

[2]To whom correspondence should be addressed. Email: iiaccarino@medgen.uni-kiel.de.

of many tumors of different origins (6). Amplification of the genomic region containing MYC was actually found to be the most frequent somatic copy number alteration in a panel of 3,131 cancer specimens belonging to 26 histological types (7).

The correlation between the changes in gene expression and the phenotypic changes induced by MYC has been the focus of several reports. Although activation of genes involved in protein biosynthesis, energy metabolism, and cell cycle regulation by MYC is consistent with its ability to drive cell growth and proliferation, the search for "the oncogenic" MYC target genes has been relatively frustrating. This inconsistency is partly due to the fact that MYC is able to modulate the transcription of up to 15% of all protein coding genes (8, 9). Another layer of complexity is added by the finding that MYC can interact with several proteins able to induce chromatin modifications associated with active transcription, like TRRAP (10), GCN5 (11), and TIP60 (12). MYC was also found bound to components of the P-TEFb complex and to influence general transcriptional pause release (13). Recent findings suggest that MYC may behave as an amplifier of a cell/tissue-specific transcriptional program (14, 15). Nevertheless, this effect cannot be observed universally and has been suggested to be an indirect effect of the regulation by MYC of specific sets of genes (16, 17).

Next generation sequencing experiments have revealed that up to 70% of the human genome is actively transcribed from one or both strands (18), with only 2% of the genome being dedicated to coding genes (19, 20). The functional relevance of this large amount of noncoding transcripts remains mostly unknown, but the number of reports showing effects on cellular physiology and aberrant expression in several diseases is quickly increasing (21–25).

The largest portion of the mammalian noncoding transcriptome is made of a class of heterogeneous RNAs: the long noncoding RNAs (lncRNAs) (26). The GENCODE v7 catalog of human lncRNAs estimates a total number of 14,880 lncRNAs (27), but with the rapid increase in RNA sequencing (RNA-seq) data, the number of lncRNAs may soon overcome that of coding genes. lncRNAs have been associated with diverse functions, including X-chromosome inactivation (28, 29), differentiation (30), modulation of p53 activity (21), and formation of subnuclear domains (31, 32). Several lncRNAs were found to recruit RNA binding proteins and affect histone modifications, with immediate ramifications on gene regulation (33, 34). lncRNAs have also been shown to be differentially expressed in specific cancer types, where they can have a key role in the control of cellular proliferation (22, 23). Evidence for a strong requirement of lncRNA expression for normal mammalian development and physiology has also come from functional studies in knockout mouse models (24).

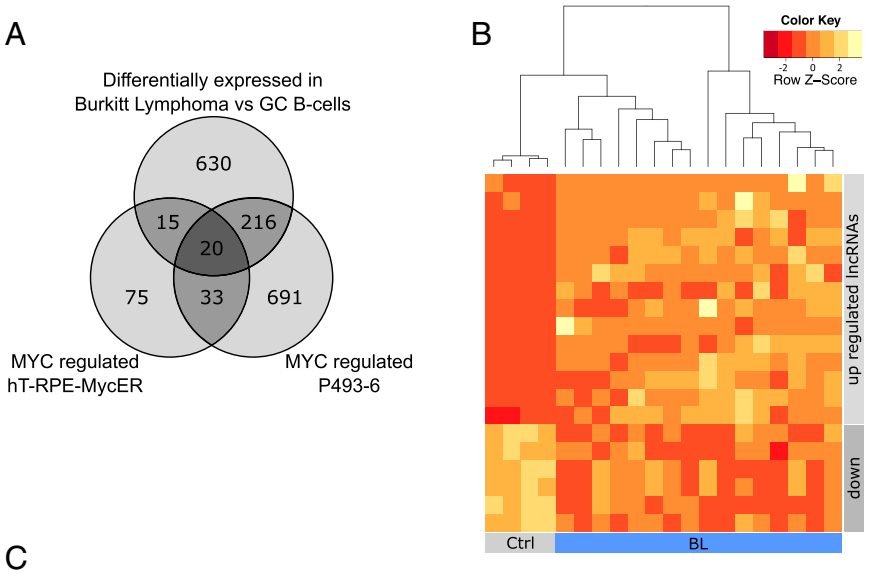
Much work has been done on the analysis of specific patterns of transcription associated to different subtypes of B-cell lymphomas (35–37). Theses analyses typically addressed the expression level of annotated coding genes by means of microarray technology. In this study, we used RNA-seq to focus on gene expression changes associated to lncRNAs, with the aim of identifying MYC-regulated lncRNAs potentially involved in lymphoma development. To do this, we analyzed RNA-seq data of samples from the major subtypes of mature B-cell lymphomas, namely BL, diffuse large B-cell lymphoma (DLBCL), and follicular lymphoma (FL), compared with data from normal germinal center (GC) B cells. These data were intersected with data coming from cell lines expressing MYC in an inducible manner to identify MYC-regulated lncRNAs. This approach led to the identification of a group of lncRNAs differentially expressed in BL relative to normal GC B cells and regulated by MYC in the same direction in two cell model systems. We focused our analysis on an up-regulated transcript showing the highest positive correlation with MYC expression, which we named MYC-induced long noncoding RNA (MINCR). By using RNAi, we show that MINCR knockdown is associated with reduced cellular proliferation in three different cell types. In line with this finding, RNA-seq of cells knocked down for MINCR expression showed a significant reduction in the expression of genes functionally important for cell cycle progression.

Results

Identification of BL-Specific MYC-Regulated lncRNAs. With the aim of identifying MYC-regulated lncRNAs that could play a driving role in lymphoma development, we set up a bioinformatic pipeline to screen for lncRNAs differentially regulated in MYC-inducible model cell lines and a set of BL samples. In particular, we used RNA-seq data obtained from (*i*) hT-RPE-MycER cells, an immortalized retinal pigment epithelial cell line expressing the MycER fusion protein (a cell line shown to be a relevant tool for the study of several aspects of MYC activity in oncogenic transformation) (38, 39); (*ii*) P493-6 cells, an immortalized B-lymphoblastic cell line carrying a tetracyclin-inducible MYC construct that was shown to be a good model to study MYC-induced lymphomas (40, 41); and (*iii*) 91 GC B cell-derived lymphoma samples subjected to RNA-seq in the framework of the International Cancer Genome Consortium project on malignant lymphoma [ICGC MMML-Seq (International Cancer Genome Consortium Molecular Mechanisms in Malignant Lymphoma by Sequencing)], including 16 BLs, 35 DLBCLs, 40 FLs, and 4 controls (normal GC B-cell samples) (Table S1).

We identified lncRNAs that are differentially regulated on MYC activation in either of two model cell systems. This analysis revealed 960 and 143 lncRNAs significantly differentially expressed by activation of MYC in the P493-6 cells and the hT-RPE-MycER cells, respectively. Parallel analysis of 16 BL samples of the ICGC MMML-Seq Cohort compared with the normal GC B-cell samples revealed 514 and 367 lncRNAs significantly up- and down-regulated in BL, respectively. Finally, to identify lncRNAs that are both MYC-regulated and -deregulated in BL, we intersected the data coming from the two model cell lines with those obtained from the lymphomas. This approach led to the identification of 20 lncRNAs that were regulated in the same direction by MYC in the cell model systems and primary BL (Fig. 1*A*). A manual inspection of the identified lncRNAs revealed that 7 of these 20 could be considered false positives, corresponding to misannotated 3′UTRs of coding genes or pseudogenes. A list of the remaining 13 bona fide BL-specific and MYC-regulated lncRNAs is presented in Fig. 1*C*, and their sequence-based expression values in BL and normal GC B cells are shown in Fig. 1*B*.

The finding that the identified lncRNAs are regulated by MYC in two different MYC-inducible model cell systems suggested that MYC could be either directly or indirectly involved in transcriptional regulation of those RNAs. To address this point, we analyzed 13 lncRNAs at the genomic and transcriptomic levels to find out if (*i*) the promoters of these transcripts are bound by MYC in ChIP experiments, (*ii*) the promoters of these transcripts are characterized by other chromatin marks associated with active transcription, and (*iii*) there is a positive correlation between MYC expression and the expression of 13 lncRNAs. To answer the first question, we analyzed available data from MYC ChIP-sequencing (ChIP-seq) experiments performed with P493-6 cells under MYC low or MYC high expression (16) as well as MYC ChIP-seq experiments performed in BL cell lines (42). We found that 7 of 13 lncRNAs were bound by MYC under MYC high conditions in the region around the transcriptional start site (TSS) (Fig. 1*C*). Furthermore, five of them were also bound by MYC in at least one of the BL cell lines analyzed in ref. 42. These data suggest that some of the identified lncRNAs may be direct MYC target transcripts. Next, we asked if the 13 lncRNAs could be identified as actively transcribed regions by looking at chromatin marks and the DNA methylation status of their promoters. We took advantage of data generated by the BLUEPRINT Project on B-cell malignancies using the cell lines



C

Identity		MYC correlation		Expression		MYC ChIP			Chromatin			Methylation			
Gene ID	Genome location	Spearman's rank	p-value	mean RPKM Ctrl	mean RPKM BL	L	H	BL	GM	BL	NBL	Ctrl	BL	non BL	DHS
ENSG00000253716	Chr8:144362331-144363860	0.647	0.008	2.055	5.304	84	862	v							
ENSG00000259187	Chr15:45117747-45124231	0.412	n.s.	2.394	5.923	-	-	.							
ENSG00000271576	Chr1:8454201-84543614	0.382	n.s.	0.132	1.214	-	1033	v							
TCONS_l2_00001839	Chr1:229394028-229406775	-0.362	n.s.	0.095	0.305	-	-	.							
ENSG00000261490	Chr4:10069713-10074643	-0.332	n.s.	1.728	0.727	-	-	.							
ENSG00000214719	Chr17:28903482-28999807	-0.329	n.s.	0.505	0.290	-	501	.							
ENSG00000226380	Chr7:130561495-130598069	0.294	n.s.	0.134	0.051	-	1106	v							
ENSG00000250899	Chr12:3150603-3154116	-0.253	n.s.	0.022	0.341	-	-	.							
ENSG00000271270	Chr3:129612768-129627960	0.238	n.s.	0.031	0.262	-	380	v							
ENSG00000175701	Chr2:110969106-111002997	0.206	n.s.	0.094	0.246	-	575	v							
ENSG00000213971	Chr19:23442708-23457053	-0.147	n.s.	0.703	0.421	-	219	.							
ENSG00000257925	Chr12:47631517-47672804	-0.135	n.s.	1.197	0.189	-	-	.							
ENSG00000244306	Chr14:19854098-19925348	-0.050	n.s.	0.041	0.130	-	-	.							

Heterochromatin (Het) | Transcribed region (TR) | Hypomethylation at TSS | Not detected
Active promoter | Either TR or Het | DNAse I hypersensitive site at TSS

Fig. 1. Identification of BL-specific, MYC-regulated lncRNAs. (*A*) Venn diagram showing the intersection of BL-specific lncRNAs (those differentially expressed in BL compared with normal GC B cells) and lncRNAs regulated by MYC in the same direction in hT-RPE-MycER cells and P493-6 cells. (*B*) Heat map showing an unsupervised hierarchical clustering of the expression intensities in the BL samples of 13 of 20 lncRNAs found at the intersection between three datasets compared as in *A*. The heat map clearly separates the control (Ctrl) and BL samples. Yellow indicates high expression, and red indicates low expression. (*C*) List of 13 lncRNAs identified as differentially expressed in BL samples relative to controls (normal GC B cells) and regulated by MYC in the same direction in the two MYC-inducible cell lines P493-6 and hT-RPE-MycER. For each lncRNA, the figure shows the Ensembl gene identification, the chromosomal location, the Spearman's rank correlation with MYC expression level (MYC correlation) and the significance of this correlation (*P* value; n.s., not significant), the mean expression (defined as reads per kilobase of transcript per million mapped reads, RPKM) in the four Ctrl samples (mean RPKM Ctrl), the mean expression in 16 BL samples (mean RPKM BL), the presence of MYC binding regions around the lncRNA's TSS (MYC ChIP) in P493-6 cells expressing low (L) or high (H) MYC according to ref. 16 and at least one BL cell line according to ref. 42. For the MYC-bound region in P493-6 cells, the peak intensity values are also shown. For each lncRNA the table also shows information on the chromatin status and the level of promoter accessibility in terms of DNA methylation and presence of DNase I hypersensitive sites (DHS) at the TSS according to data from ENCODE (43) and the BLUEPRINT Consortium. Analysis of TSS methylation was performed using whole-genome bisulfite sequencing data of normal GC B cells (Ctrl), BL samples (BL), and DLBCL and FL samples (non-BL). In the chromatin states analysis, the following abbreviations were used: GM, B lymphoblastoid cell line GM17828; BL, BL cell lines BL-2 and DG-75; NBL, non-BL cell lines SU-DHL-5 and KARPAS-422.

BL-2, DG-75, SU-DHL-5, and KARPAS-422 as well as data on a B-lymphoblastoid cell line (GM12878) from the Encyclopedia of DNA Elements (ENCODE) Project (43) and whole-genome bisulfite sequencing data from the IGCG MMML-Seq Project. The data presented in Fig. 1*C* show that at least 10 of 13 lncRNAs were defined as either transcribed regions or active promoters based on the analysis of several chromatin marks. Furthermore, most of them were characterized by hypomethylation of the region around the TSS and the presence of a DNase I hypersensitive site, suggesting that they can be considered active transcriptional units, at least in the B-cell lineage. Finally, as additional evidence of a direct regulation by MYC, we compared the expression level of the identified lncRNAs with MYC expression in 16 BL samples that are part of the ICGC MMML-Seq Cohort. In Fig. 1*C*, we ranked these lncRNAs according to their Spearman's rank correlation with MYC expression level and the significance of this correlation (*P* value). The correlation analysis shows that, although 6 of 10 lncRNAs identified had a positive correlation with MYC expression, only for 1 of them (ENSG00000253716) did the correlation have a significant *P* value (Fig. 1*C*).

Characterization of the lncRNA MINCR. Among 13 lncRNAs shown in Fig. 1*C*, ENSG00000253716 seemed to be the best candidate as the MYC-regulated transcript that could play a role in the development of MYC-positive B-cell lymphomas. ENSG00000253716 had the highest and most significant positive correlation with MYC expression levels and showed the presence of MYC binding regions around its TSS in BL cells and P493-6 cells, with increasing peak intensity in MYC high conditions in P493-6 cells. Finally, ENSG00000253716 had all of the marks associated with

open chromatin and active transcription in normal and malignant B cells, and its MYC inducibility could be validated by quantitative RT-PCR (qRT-PCR) in both hT-RPE-MycER and P493-6 cell lines (Fig. 2*B*). We also found that the expression of ENSG00000253716 was reduced after MYC knockdown in BL cell lines (Fig. S1*A*). Based on these observations, we decided to concentrate additional experiments on this transcript, which we named MINCR.

The MINCR gene is located on chromosome 8q24.3 and intergenic to the two coding genes GLI4 and ZNF696, with distances of 3 and 9.5 kb, respectively. Using BLAST, we found MINCR to be conserved throughout primates, with the exception of Galago (bushbaby), but not in other vertebrates (alignment shown in Fig. S2). MINCR also has a RefSeq entry defined as uncharacterized LOC100507316. ENCODE annotates at least six different isoforms transcribed from the MINCR gene locus, with a long isoform (MINCR_L) composed of three exons and all others containing two exons. A schematic depiction of MINCR intron–exon structure is shown in Fig. 2*A*. Analysis of sequence reads mapping of RNA-seq data from both MYC-inducible cell lines and lymphoma samples fits well with the junctions of the annotated transcripts and additionally, suggests a certain degree of intron retention (Fig. 2*A*). Our analysis also shows that the number of reads spanning exons 1 and 2 is much higher than those spanning exons 2 and 3, arguing that the expression of the short isoform (MINCR_S) may be two times higher than the expression of MINCR_L. A qRT-PCR analysis performed using isoform-specific sets of primers confirmed that, indeed, the most abundant isoforms are MINCR_S and MINCR_L, with MINCR_S being more expressed than MINCR_L (Fig. 2*C*). Finally, both MINCR_S and MINCR_L are induced after MYC activation in hT-RPE-MycER cells (Fig. S1*B*).

MINCR_L and MINCR_S are 696 and 379 nt long, respectively. To define MINCR coding potential, we analyzed the coding potential calculator (CPC) score for both isoforms. Fig. S3*A* shows that the CPC scores for MINCR_L and MINCR_S are −1.146 and −1.148, respectively, and they are in a range similar to that observed for the experimentally validated lncRNA XIST (−0.95). On the contrary, the CPC scores for the coding genes MYC and GAPDH are 6.77 and 12.4, respectively. MINCR can be, therefore, considered a lncRNA. Because lncRNAs were shown to be preferentially enriched in nuclear fractions (27) and because their subcellular localization can be indicative of their mechanism of action, we sought to define the intracellular localization of the MINCR_L and MINCR_S transcripts by using RNA fractionation followed by quantitative PCR. The analysis was performed in the BL cell line BL-2. As shown in Fig. S3*B*, similar to the nuclear lncRNA MALAT1, both MINCR transcripts L and S were preferentially enriched in the nuclear RNA fraction. We conclude that transcription of the MINCR gene produces two main transcripts, MINCR_L and MINCR_S, with very poor coding potential and preferential nuclear localization.

Although MINCR has been identified based on its differential expression in BL relative to normal GC B cells and found to be induced by MYC in an immortalized B cell line, it is clearly expressed and also, MYC-regulated in the retinal pigment epithelial

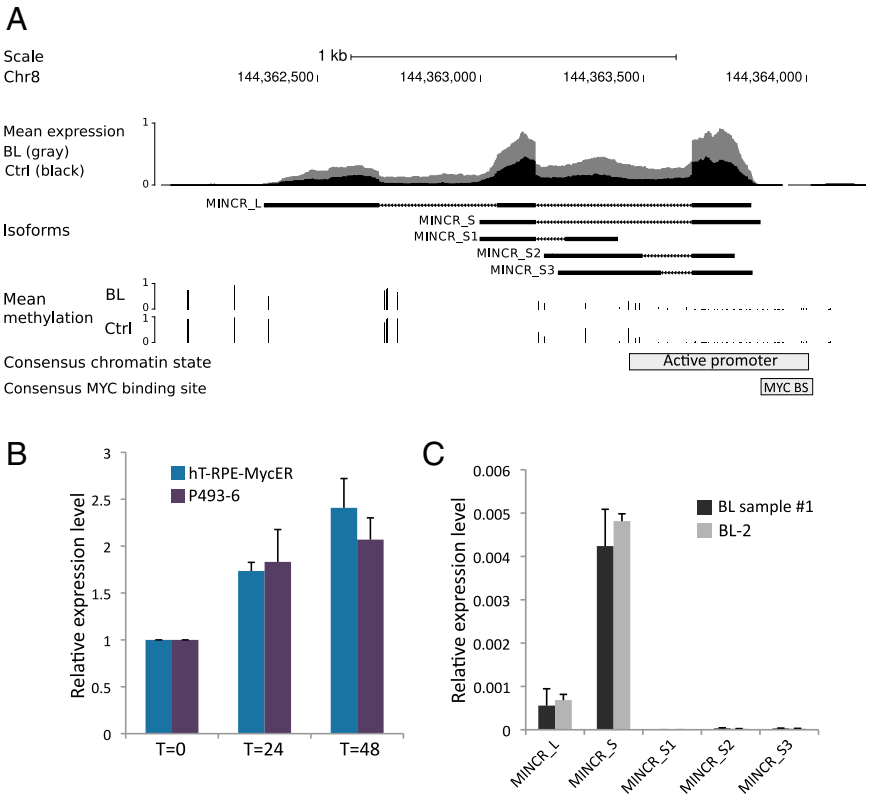


Fig. 2. Characterization of MINCR, a MYC-induced, BL-specific lncRNA. (*A*) Schematic representation of MINCR genomic locus on chromosome 8 (Chr8) with the mean expression profiles in BL (gray; $n = 16$) and the control samples (normal GC B cells; black; $n = 4$). The predicted isoforms based on the ENCODE annotations and the mean methylation of BL and control (Ctrl) are also shown as well as the intersection of chromatin states and MYC-bound regions (MYC BS) between the following cell lines: GM12878, K562, HeLa-S3, H1-hESC, HepG2, HUVEC and K562, HeLa-S3, HepG2, MCF-7 and NB4. (*B*) qRT-PCR analysis of MINCR expression in hT-RPE-MycER and P493-6 cells after MYC activation for 24 and 48 h. The expression of MINCR was calculated using GAPDH and RPL31 to normalize for cDNA content. $T = 0$ refers to cells treated with vehicle for hT-RPE-MycER cells and cells grown for 72 h in medium containing tetracyclin for P493-6 cells. (*C*) qRT-PCR analysis of MINCR isoforms expression in RNA derived from a BL sample of the ICGC MMML-Seq Cohort and the BL cell line BL-2.

cell line hT-RPE-MycER, suggesting that MINCR does not have a B cell-restricted expression pattern. Accordingly, analysis of MINCR expression in the Human Body Map lincRNAs Database shows that MINCR is ubiquitously expressed, with the highest expression values in brain, prostate, and testis (Fig. S3*C*) (44). A similar conclusion can be drawn by analysis of the genome segmentations based on the ENCODE data. This analysis is presented in Fig. 2*C* and shows that the intersection of the chromatin states around the TSS region of MINCR is typical of an active promoter region in the cell lines GM12878, K562, HeLa-S3, H1-hESC, HepG2, and HUVEC. Interestingly, in most of these cell lines (K562, HeLa-S3, HepG2, MCF-7, and NB4), MYC ChIP-seq data showed the presence of a uniform peak in the TSS region of MINCR, suggesting that MYC may regulate MINCR expression not only in B cells.

Analysis of MINCR Expression in Cancer. Translocations involving the MYC oncogene are a typical feature of BL, but they are also known to occur in subsets of DLBCL and FL (45). A total of seven DLBCL and three FL samples in the ICGC MMML-Seq Cohort contained a MYC break as detected by FISH (Table S1). For subsequent analyses, we, therefore, divided the non-BL lymphomas (DLBCL and FL) in MYC break-negative non-BLs (non-BLs) and MYC break-positive non-BLs (non–BL-MYCs). As shown in Fig. S3*D*, the non–BL-MYC lymphomas show a clearly higher MYC expression level relative to the non-BL samples negative for the MYC break (*P* value = 4.011e-05). We, therefore, analyzed the expression of MINCR in the entire ICGC MMML-Seq Cohort divided as described above. As shown in Fig. 3*A*, MINCR expression was found to be higher in the non–BL-MYC lymphomas relative to the non-BL without the MYC break. We, therefore, extended the correlation analysis between MYC and MINCR expression levels to all of the MYC break-positive lymphomas of the cohort and found that there is a highly significant correlation (P value $< 10^{-3}$) in the comparison of the expression values of MINCR and MYC in all MYC-positive lymphomas (16 BL cases + 10 non–BL-MYC cases) (Fig. 3*B*). The finding that MINCR expression correlates with MYC expression in all lymphomas examined (not only in BL) led us speculate that, because of the ubiquitous expression of MINCR, this correlation may also exist in other cancer types. To test this hypothesis, we searched in the total ICGC data for datasets where MINCR expression was detected. We found 356 datasets representing four groups of neoplasms (endocrine pancreatic neoplasm, pancreatic ductal adenocarcinoma, chronic lymphocytic leukemia, and ovarian cancer) where both MYC and MINCR were expressed. Interestingly, when we compared the MYC and MINCR expression intensities, we found a significant correlation in the pancreatic ductal adenocarcinomas group (Fig. 3*C*). These data suggest that the expression of the lncRNA MINCR strongly correlates with MYC expression in not only BL but more in general, GC-derived MYC-positive lymphomas and other cancer types, like pancreatic ductal adenocarcinomas, that are known to rely on MYC expression (46, 47).

MINCR Regulates Cell Cycle Progression by Controlling the Expression of Cell Cycle Genes. Changes in gene expression driven by oncogenes could be either essential for the development of the cancer phenotype and/or continuously required for cancer maintenance. In the second case, cancer cells become addicted to the expression of the gene of interest, and they will eventually cease growing or even die after the expression of the gene is reduced to basal level. We, therefore, decided to use RNAi to knockdown the expression of MINCR and measure a possible change in cell proliferation/viability. MINCR knockdown was performed in the MYC-inducible cell line hT-RPE-MycER using with two independent siRNAs (knockdown efficiency of the siRNAs used is shown in Fig. S4*A*). We also took advantage of the easy inducibility of the MYC protein in this cell line to analyze the effect of MINCR knockdown in cells with or without MYC activation. As shown in Fig. 4*A*, transfection of siRNAs directed against MINCR resulted in a time-dependent decrease in cellular proliferation independent of the MYC activation status. A more modest but reproducible effect of MINCR knockdown was also observed in the P493-6 cell line and the BL cell line BL-2 using a cell viability assay (Fig. 4 *B* and *C*). The weaker effect of the knockdown on cell viability in BL-2 cells is consistent with the lower MINCR silencing achieved in these cells relative to hT-RPE-MycER cells (Fig. S4*B*). To understand if the reduction in cell proliferation observed was because of a defect in cell cycle progression caused by MINCR knockdown, we analyzed the DNA content of hT-RPE-MycER cells with or without MYC activation 48 h after the transfection of control or MINCR-targeting siRNAs. As shown in Fig. 4*D*, MINCR knockdown resulted in a significant reduction in the percentage of cells having a DNA content corresponding to cells in S phase and G2/M phase. Concomitantly, an increase in the percentage of cells in G0/G1 phase was also observed. Finally, to investigate if apoptosis could also contribute to the reduction of cell growth observed in Fig. 4*A*, we measured the intracellular levels of caspase 3/7 activity after MYC activation in knocked down cells. Interestingly, although very few dead cells could be detected by visual inspection, we found that, in cells transfected with either of the siRNAs directed against MINCR, there was a significant increase in caspase 3/7 activity after MYC activation (Fig. S4*C*), suggesting that, at later time points, apoptosis could also be affecting cellular viability on MINCR knockdown, specifically when MYC is switched on.

Given that MINCR knockdown had a reproducible effect on cellular viability and cell cycle progression, we decided to investigate gene expression changes induced by MINCR knockdown in hT-RPE-MycER cells, the cell line where the effect was most pronounced. We performed RNA-seq of hT-RPE-MycER cells 48 h after transfection with the two siRNAs directed against MINCR. The analysis was performed using two biological replicas. Furthermore, to exclude possible off-target effects on gene expression associated with single siRNAs, we focused only on genes regulated in the same direction with both siRNAs in the two replicates. As shown in Fig. 5*A*, MINCR knockdown in hT-RPE-MycER cells resulted in the up-regulation of 568 genes and the down-regulation of 784 genes.

lncRNAs have been described to be able to regulate gene expression of neighboring genes (48, 49). We, therefore, asked if the expression of the most proximal genes upstream and downstream from MINCR were changed after MINCR knockdown. Given that no significant change was observed, we conclude that MINCR is not a *cis*-regulator of neighboring genes. Next, we looked at the groups of genes differentially up-regulated or down-regulated after MINCR knockdown to see if there was any enrichment in particular biological processes. Interestingly, we found a strongly significant enrichment of the biological process category "cell cycle" in the analysis of the genes down-regulated after MINCR knockdown (Fig. 5*B*). A heat map with the expression values of the down-regulated cell cycle genes in all conditions examined is shown in Fig. 5*C*, and a list of the same genes is shown in Table S2. To this list, we also added a few genes belonging to the same functional category that failed to pass the stringent significance criteria applied to the analysis but were still clearly down-regulated on MINCR knockdown. A selection of these genes chosen for their degree of down-regulation in MINCR knockdown cells and their relevance within lymphoma cell physiology was successfully validated by qRT-PCR (Fig. 5*D*).

We also analyzed the changes in gene expression observed in hT-RPE-MycER cells on knockdown of MINCR expression and treatment with 4-hydroxytamoxifen (4-OHT) to activate MYC. On MYC activation, the number of genes significantly differentially expressed was much lower than in the absence of MYC

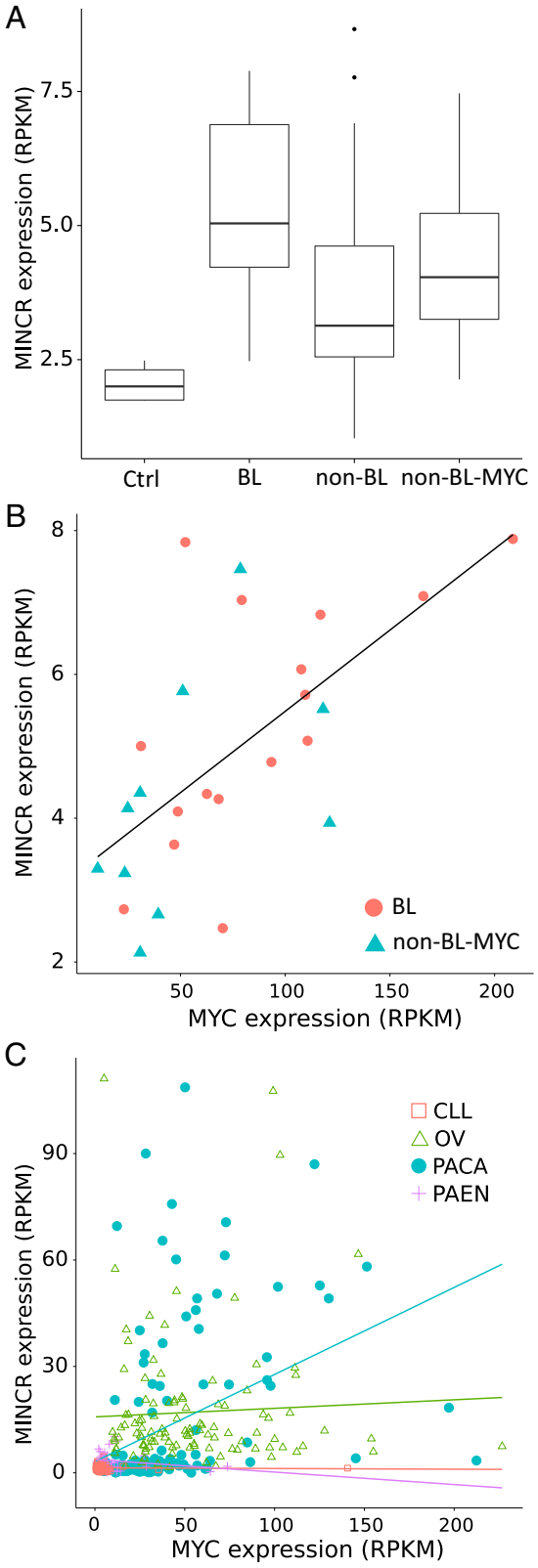


Fig. 3. Correlation between MINCR and MYC expression in cancer. (*A*) Boxplot showing the expression of MINCR in all samples of the ICGC MMML-Seq Cohort divided as control (Ctrl), normal GC B cells (n = 4), BL (n = 16), non-BL (DLBCLs and FLs negative for a MYC break; n = 66), and non–BL-MYC (DLBCLs and FLs positive for a MYC break; n = 10). Expression is defined in reads per kilobase of transcript per million mapped reads (RPKM). The significance of MINCR differential expression is characterized by the following P values: Ctrl vs. BL, P value = 1.618e-06; Ctrl vs. non-BL, P value = 0.0001043; Ctrl vs. non–BL-MYC, P value = 0.001919. (*B*) Correlation between the RPKM expression levels of MYC and MINCR in all lymphomas samples of the ICGC MMML-Seq Cohort carrying a MYC break; BL samples (n = 16) are indicated with red circles, and non–BL-MYC samples (n = 10) are indicated with blue triangles. The statistical dependence between the expressions of MYC and MINCR is characterized by a Spearman's correlation coefficient of 0.625 and a P value = 0.00083. (*C*) Correlation between the RPKM expression levels of MYC and MINCR in expression data of different cancer types from the ICGC data repository. CLL, chronic lymphocytic leukemia (correlation = –0.1247623; P value = 0.3221); OV, ovarian cancer (correlation = –0.0048745; P value = 0.9595); PACA, pancreatic ductal adenocarcinoma (correlation = 0.52926; P value = 4.88e-11); PAEN, pancreatic cancer endocrine neoplasm (correlation = –0.25568; P value = 0.1506).

activation. This finding could be due to the fact that many of the cell cycle genes identified are, indeed, weakly down-regulated on MYC activation in this cell system, a behavior not shared with all cell cycle genes (Fig. S5 *A* and *B* and Table S2). Nevertheless, our data clearly show that most of the cell cycle genes identified as down-regulated on MINCR knockdown in cells with MYC off are still down-regulated when MYC is activated but that they fail to pass the stringent significance criteria that we used for the definition of differentially expressed genes.

Overall, the data presented in Figs. 4 and 5 suggest that the reduction of proliferation observed in hT-RPE-MycER cells after MINCR knockdown depends on the down-regulation of a set of genes important for cell cycle progression. Next, we asked if these findings could be also extended to BL cell line BL-2. Initially, we found that only a few of the identified genes were also down-regulated in BL-2 cells (Fig. 6*A*). This observation might be explained by the lower degree of MINCR knockdown achieved in BL-2 cells relative to hT-RPE-MycER cells (Fig. S4*B*). Nevertheless, we found that the genes coding for Aurora kinase A (AURKA), AURKB, and chromatin licensing and DNA replication factor 1 (CDT1) were also reproducibly down-regulated in BL-2 cells after MINCR knockdown (Fig. 6*A*). Furthermore, these genes are among the top down-regulated on MINCR knockdown when MYC is activated (Table S2).

MINCR-Regulated Genes Are also MYC Targets. By closer inspection of the list of genes down-regulated on MINCR knockdown, we also noticed the presence of many known MYC target genes. To establish if there was a significant enrichment in MYC binding sites in the promoters of 125 cell cycle genes down-regulated on MINCR knockdown, we identified 242 promoter regions overlapping with our set of genes (each gene extended by 1,500 nt upstream). The promoter regions were defined based on the chromatin states of the GM12878 cell line (ENCODE 2012). Using this approach, we found 63 MYC binding sites (defined as in ref. 42) in these promoter regions. To check for significance, we analyzed the number of MYC-bound regions in a background set of cell cycle genes created by randomly selecting 125 of 1,324 genes belonging to the cell cycle functional category (Gene Ontology ID GO:0007049). Given that we found only 40 MYC binding sites in 259 promoter regions, we conclude that the 125 genes down-regulated on MINCR knockdown are significantly more enriched in MYC binding sites (P value = 0.004).

We, therefore, asked if the identified genes could differentiate between the lymphoma subtypes carrying and not carrying the MYC translocation. The analysis was done on eight genes found to be down-regulated on MINCR knockdown in BL-2 cells. Interestingly, the expression pattern of all of them clearly mirrored the MYC status of the lymphomas, with higher expression in BLs and non–BL-MYC lymphomas relative to the non-BLs without the break (Fig. 6*B*).

Given the observed enrichment of MYC binding sites in the promoters of the identified cell cycle genes, we asked if their

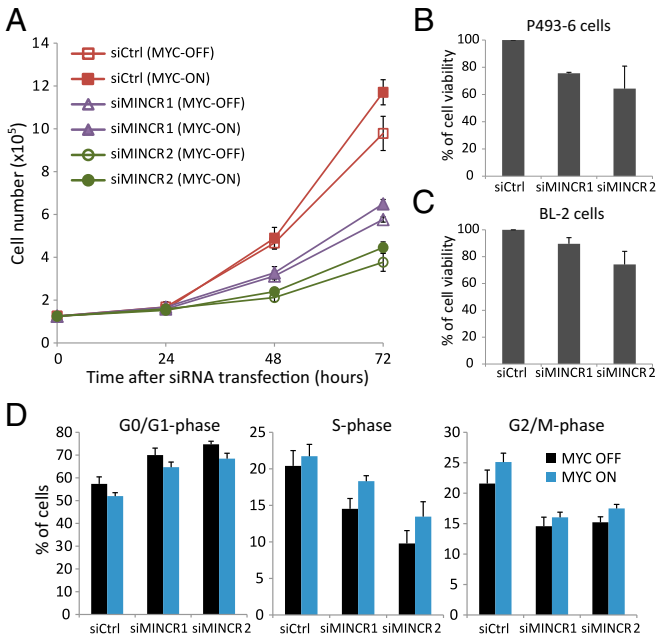


Fig. 4. MINCR expression is required for normal cellular viability. (*A*) Reduction of MINCR expression in hT-RPE-MycER cells using two independent siRNAs (siMINCR1 and siMINCR2) results in a significant decrease in cellular proliferation when MYC is either switched on by the addition of 4-hydroxytamoxifen (*P* value at 72 h < 0.01) or kept in an off status (*P* value at 72 h < 0.02). (*B* and *C*) Reduction of MINCR expression using two independent siRNAs (siMINCR1 and siMINCR2) results in decreased cellular viability of both (*B*) P493-6 cells grown without tetracycline (MYC high; *P* value < 0.02) and (*C*) the BL cell line BL-2 (*P* value < 0.01 with siMINCR2). Viability was measured using the Cell Titer Blue reagent (Promega). (*D*) Reduction of MINCR expression in hT-RPE-MycER cells using two independent siRNAs (siMINCR1 and siMINCR2) results in an increase of the percentage of cells in the G1 phase of the cell cycle and a decrease of the percentage of cells in the S and G2/M phases of the cell cycle (*P* values < 0.02). siCtrl, control siRNA.

reduced expression could be because of a reduced binding of MYC to their promoters. To answer this question, we performed ChIP using MYC and control IgG antibodies on hT-RPE-MycER cells transfected with two siRNAs directed against MINCR 24 h after MYC activation. Using quantitative PCR, we analyzed regions of the promoters of four cell cycle genes (AURKA, AURKB, CDK2, and NCAPD2) found to be bound by MYC in at least one of the ENCODE cell lines. The data presented in Fig. 6*C* show that at least three of four genes (AURKA, AURKB, and NCAPD2) are also significantly bound by MYC in hT-RPE-MycER cells. The result also shows that MYC binding is reduced in all promoter regions after knocking down of MINCR using siMINCR2, the siRNA that has the strongest effect on cellular proliferation (Fig. 4*A*), suggesting that MINCR silencing might reduce the ability of MYC to engage a transcriptionally active complex, at least for the genes tested.

The data presented suggest that MINCR is able to influence the transcription of a subset of MYC target genes at least in part by affecting the ability of MYC to bind their promoters. Given that not all MYC-regulated genes are affected by MINCR expression, we speculated that an additional specificity determinant might be required for transcriptional regulation by MINCR. We, therefore, analyzed the promoter regions of 125 cell cycle genes to search for motifs discriminating these genes from background cell cycle genes. This analysis showed a statistically significant enrichment of the two motifs shown in Fig. S5*C*. Interestingly, one of these motifs (motif 1) is almost identical to the DNA binding site of the homeobox protein family Sine Oculis Homeobox Homolog 1–6 (Six1–6).

AURKA, AURKB, and CDT1 Down-Regulation Recapitulates the Effect of MINCR Knockdown. Finally, we asked if at least some of the cell cycle genes found down-regulated on MINCR knockdown could be responsible for the phenotypic changes observed after MINCR silencing. We selected a set of cell cycle genes based on their degree of down-regulation in hT-RPE-MycER and BL-2 cells and their functional relevance. As shown in Fig. 7, among the chosen genes, knockdown of AURKA, AURKB, and CDT1 was responsible for a reproducible reduction in cellular viability in hT-RPE-MycER cells. Furthermore, the effect was even more pronounced after MYC activation. These data, therefore, suggest that down-regulation of cell cycle genes, like AURKA, AURKB, and CDT1, may be, at least in part, the cause of the reduced basal proliferation observed after MINCR knockdown.

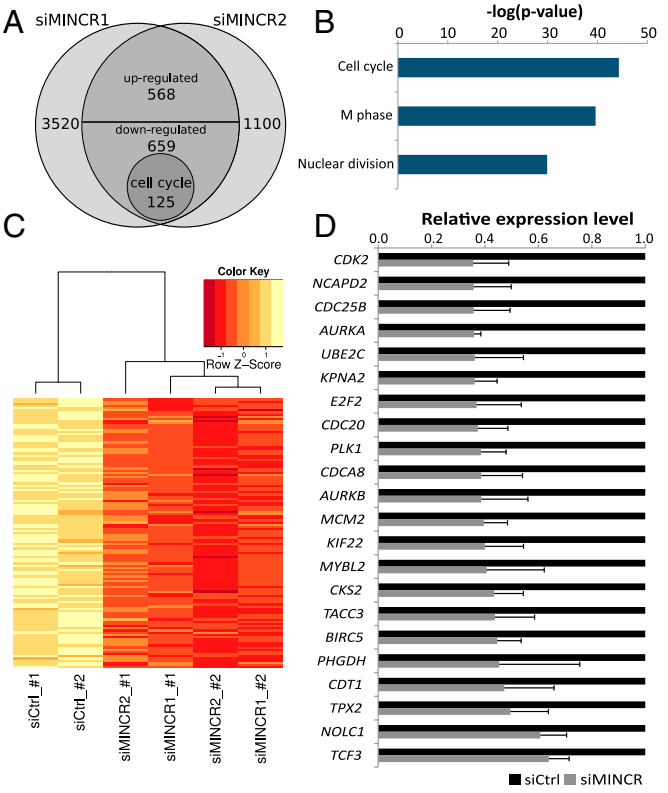


Fig. 5. Cells with reduced MINCR expression show a significant reduction in the expression of cell cycle genes. (*A*) Venn diagram showing the intersection between genes differentially regulated in hT-RPE-MycER cells after transfection of the two independent siRNAs against MINCR (siMINCR1 and siMINCR2). The diagram also shows the number of genes differentially up-regulated and down-regulated common to the two siRNA transfections and the number of cell cycle genes enriched among the down-regulated genes. (*B*) The cell cycle-related biological processes are significantly enriched among the genes down-regulated after MINCR RNAi. The graph shows the *P* values of the three most highly significant biological processes. (*C*) Heat map showing hierarchical clustering of the expression intensities of 125 cell cycle genes identified in hT-RPE-MycER cells transfected with two independent siRNAs against MINCR (siMINCR1 and siMINCR2) in two biological replicas (1 and 2). Yellow indicates high expression, and red indicates low expression. (*D*) qRT-PCR analysis of the expression of a selection of 125 identified cell cycle genes in hT-RPE-MycER cells after MINCR RNAi. The graph shows the mean of the change in expression relative to a control siRNA (siCtrl) observed after transfection of the two independent siRNAs against MINCR.

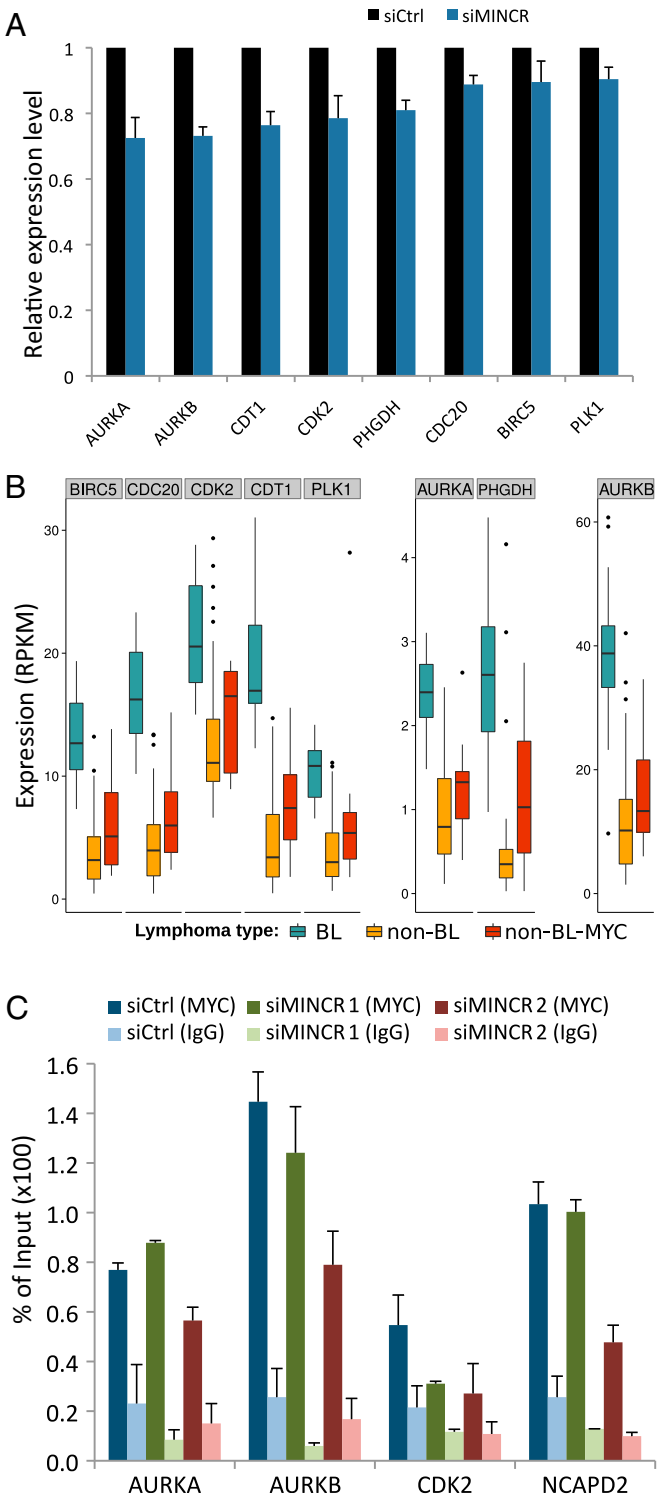


Fig. 6. Cell cycle genes down-regulated in BL-2 cells after MINCR knockdown. (*A*) qRT-PCR analysis of the expression of a selection of 125 identified cell cycle genes in BL-2 cells after MINCR RNAi. The graph shows the mean of the change in expression relative to a control siRNA (siCtrl) observed after transfection of two independent siRNAs against MINCR (P values > 0.05 only for BIRC5 and PLK1). (*B*) Expression of some of the identified cell cycle genes in B-cell lymphomas. Boxplots showing the expressions of eight cell cycle genes in all samples of the ICGC MMML-Seq Cohort divided as BL (n = 16), non-BL (DLBCLs and FLs MYC break-negative; n = 65), and non–BL-MYC (DLBCLs and FLs MYC break-positive; n = 10). Expression is defined in reads per lilobase of transcript per million mapped reads (RPKM). (*C*) ChIP–quantitative PCR analysis of hT-RPE-MycER cells after transfection of two independent siRNAs against MINCR (siMINCR1 and siMINCR2) using either control IgG or MYC-specific antibodies.

Discussion

Since the discovery that eukaryotic cells express thousands of long RNAs with no clear coding potential, one of the main challenges is to understand the biological functions associated to these novel transcripts. Several lncRNA have already been shown to be important players in the execution of physiological cellular and organismal homeostasis (24). Accordingly, altered expression of lncRNAs has been linked to several disease conditions, particularly cancer (22, 23). There are now several examples of lncRNAs that are able to influence the cellular transcriptional program, acting either at the transcriptional level by influencing the establishment of chromatin marks (34, 50) or the posttranscriptional level by controlling mRNA stability (51), compartimentalization (31), or translation (52). Given that the oncogenic transcription factor MYC is known to play a crucial role in the development of many neoplasms by controlling the expression of thousands of genes, we asked in this study if lncRNAs could also play a role in MYC-induced oncogenic transformation. We approached this aim by investigating the extent of changes that MYC is able to induce in lncRNA expression. We used two different cell line model systems carrying a MYC-inducible construct and through the analysis of BL, a cancer in which MYC is known to play a fundamental role. BL can be considered the best cancer model to study the role of MYC in oncogenic transformation for at least three reasons: it is invariably characterized by an IG-MYC translocation (resulting in the deregulated overexpression of MYC), MYC translocation is considered to be the primary oncogenic event in BL development, and it is usually characterized by a relatively simple karyotype, with secondary aberrations mostly representing singular events (53, 54). We, therefore, hypothesized that deregulated lncRNAs in BL are the result of MYC overexpression and could, therefore, play a major role in the development of the lymphoma. Using this approach, we identified more than 50 lncRNAs regulated by MYC in two different cell lines carrying two different MYC-inducible constructs and almost 1,000 lncRNAs differentially regulated in BL patient samples relative to normal GC B cells. Although several reports investigating the effect of MYC activation on lncRNA transcription were recently published (55–58), our study is the first, to our knowledge, to report an RNA-seq–based systematic analysis of MYC-regulated lncRNAs and at the same time, lncRNAs deregulated in GC-derived B-cell lymphomas.

The intersection of the cell line data and the data obtained from the sequencing of BL patient samples led us to the identification of the MINCR lncRNA. MINCR turned out to be the best candidate as a MYC-regulated BL-specific lncRNA, but it will certainly not remain the only lncRNA playing a role in BL development. To increase the chance to identify true MYC target transcripts, we decided to filter our RNA-seq data using two cell model systems carrying inducible MYC expression. Although this choice has probably shifted the focus toward lncRNAs with broader tissue-specific expression, the findings that MINCR shows a high correlation with MYC expression levels in MYC-positive lymphoma samples and that it is bound by MYC at the TSS region suggest that it was a successful strategy to identify MYC-regulated lncRNAs. Furthermore, the observation that MINCR has broad tissue specificity (Fig. S3*C*) suggests that MINCR could play an important role in not only MYC-positive lymphomas but also, several other cancers that rely on MYC overexpression. Accordingly, we identified a significant correlation between MINCR and MYC expression in RNA-seq data from pancreatic ductal adenocarcinomas (Fig. 3*C*). Given that amplification of the genomic region containing MYC is a very frequent event in cancer (7), we

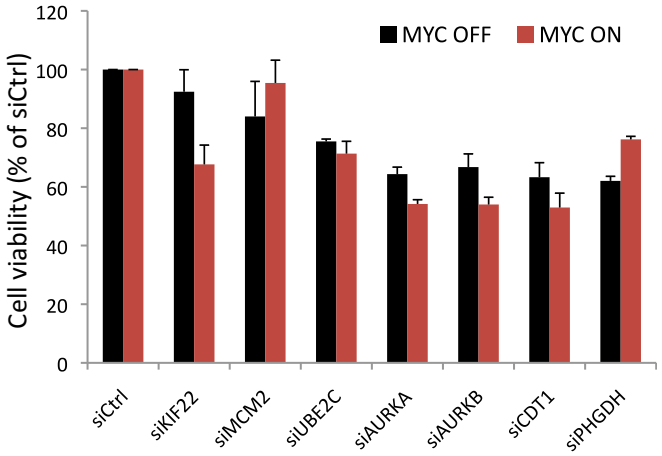


Fig. 7. Knockdown of AURKA, AURKB, and CDT1 recapitulates the effect of MINCR RNAi on hT-RPE-MycER cellular viability. hT-RPE-MycER cells were transfected with the indicated siRNAs directed against a set of cell cycle genes and then grown in the presence of 150 nM 4-hydroxytamoxifen (MYC on) or vehicle (MYC off). Viability was measured 48 h later using the Cell Titer Blue reagent (Promega). Cells transfected with siRNAs against AURKA, AURKB, and CDT1 had a significant reduction in cellular viability (*P* values with MYC on < 0.005). siCtrl, control siRNA.

can forecast a general role of MINCR in mediating the effect of MYC overexpression in cancer development.

Analysis of the transcriptional changes observed after MINCR knockdown suggested that the lncRNA MINCR is able to control the expression level of a set of genes involved in cell cycle initiation and progression. In particular, genes playing a role in initiation of genome replication and the assembly of the mitotic spindle are found strongly reduced after MINCR RNAi. This observation is in complete agreement with the data that we present in Fig. 4*D*, showing a strong perturbation of the cell cycle phases after MINCR knockdown. The other interesting observation was that those genes showed a significant enrichment of MYC-binding sites in their promoters. MINCR, therefore, seems to be involved in controlling a subset of MYC target genes. Interestingly, also, the MYC-regulated lncRNAs identified in colorectal cancer by Kim et al. (56) seem to share with MINCR the ability to modulate the expression of MYC target genes involved in cell cycle regulation. Although more experiments are needed to shed light on MINCR mechanism of action, we can speculate that at least two possible mechanisms may take place. One possibility is that MINCR directly influences MYC transcriptional activity. In this model, we can imagine MINCR binding either directly to MYC or to a MYC binding partner and influencing the ability of the MYC transactivation complex to bind promoters and affect transcription. The lncRNA PCGEM1, for instance, has recently been shown to bind MYC directly and enhance its ability to regulate the transcription of metabolic genes (59). In support of this model, we have observed a decrease in MYC binding to the promoters of a set of the identified cell cycle genes on MINCR knockdown (Fig. 6*C*). Alternatively, MYC has been shown to bind at least two of the core subunits of KMT2 methyltransferase complexes that target histone H3 lysine 4, a mark associated with open chromatin (60). Interestingly, one of these subunits, WDR5, has recently been shown to bind several lncRNAs through an RNA binding pocket essential for its function as a transcriptional activator (61).

In a similar scenario, MINCR could bind to a transcription factor that cooccupies the MYC binding regions in the promoters of the target genes, influencing in this way the ability of MYC to transactivate those genes. We have, indeed, identified two DNA motifs significantly enriched in the promoters of the cell cycle genes (Fig. S5*C*). One of these motifs is almost identical to the DNA binding site for the family of homeobox proteins Six1–6. Based on this finding, we can speculate that the transcriptional regulation of the identified cell cycle genes could depend on the interplay between MYC, MINCR, and one of the members of the Six family of transcription factors. Interestingly, the Six1 protein has recently been found to promote DNA replication and cell proliferation in cervical cancer by regulating the expression of multiple genes related to the initiation of DNA replication (62).

It is interesting to speculate that MINCR and lncRNAs in general, by conferring increased selectivity to a transcriptional factor like MYC that recognizes an extremely common DNA binding motif, may, in the end, constitute the missing link that could explain the choice between MYC as a general transcriptional amplifier and MYC as a selective gene regulator (16).

Whatever the mechanism involved, our data show that MINCR has a clear effect on the establishment of the expression level of the genes coding for AURKA and AURKB as well as CDT1. Additionally, we also show that down-regulation of these three genes may in the end explain the reduced proliferation observed after MINCR RNAi, because their knockdown results in reduced cellular viability (Fig. 7). Interestingly, AURKA and AURKB have already been shown to play a crucial role in MYC-induced oncogenic transformation, with AURKB showing a synthetic lethal interaction with MYC (63). Of note, the synthetic lethal effect of AURKB inhibition was identified in the same cellular system used in this study, the hT-RPE cells, albeit comparing vector-transfected cells with cells stably transfected with MYC. An oncogenic role in lymphoma development was also previously reported for CDT1 (64).

In conclusion, our findings suggest that the expression level of a group of genes involved in cell cycle initiation and progression may be regulated through the interplay between the transcription factor MYC and a newly identified lncRNA, which we named MINCR. The identification of the mechanism of MINCR action could in the future suggest novel therapeutic opportunities for the fight against not only malignant lymphoma but possibly, all cancers that rely on MYC expression.

Materials and Methods

Extended materials and methods are provided in *SI Materials and Methods*. The sequences of all primers and siRNAs/shRNAs used in this study are listed in Tables S3 and S4, respectively. The ICGC Malignant Lymphoma study was approved by the Institutional Review Board of the Medical Faculty of the University of Kiel (A150/10).

Experimental procedures for whole-genome, whole-genome bisulfite, and transcriptome sequencing of the ICGC MMML-Seq have been published previously (54) and are briefly repeated in *SI Materials and Methods*. Samples were sequenced using HiSeq 2500 (Illumina), and mapping was performed with the segemehl suite, version 0.1.7 (65, 66).

RNAi in BL-2 and P493-6 cells was performed using an Amaxa Nucleofector using a two-step transfection protocol, and viability was scored after growing cells for 24 h in 3% (vol/vol) FBS. hT-RPE-MycER cells were knocked down with HiPerFect (Qiagen) 5 or 24 h before activation of the MycER protein. Total RNAs were extracted with the RNeasy Mini Kit (Qiagen) and reverse-transcribed using the QuantiTect RT Kit (Qiagen). qRT-PCR was performed using the Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific). Cell cycle analysis was performed using propidium iodide 48 h after RNAi and 24 h after MYC activation. ChIP was performed according to protocols published by Lee et al. (67) 48 h after RNAi and 24 h after MYC activation.

ACKNOWLEDGMENTS. We thank Dirk Eick for the P493-6 cells and Pasquale Verde for critical reading of the manuscript. The members and technical staff of the ICGC MMML-Seq (International Cancer Genome Consortium Molecular Mechanisms in Malignant Lymphoma by Sequencing) and BLUEPRINT Consortium are acknowledged for providing samples and extensive datasets within that project. G. Richter is acknowledged for administrative coordination of the ICGC MMML-Seq Consortium. The ICGC MMML-Seq Project is funded by the Federal Ministry of Education and Research in Germany (BMBF) within Program for Medical Genome Research Grants 01KU1002A–01KU1002J. The BLUEPRINT Consortium is funded by the European Union's Seventh Framework Programme Grant 282510. The research on MYC-positive lymphomas is

supported by the BMBF in the framework of E:bio Network Molecular Mechanisms in Malignant Lymphoma with MYC-Deregulation (MMML-MYC-SYS) Grant 0316166. Support of research infrastructure by the Deutsche Forschungsgemeinschaft and the KinderKrebsInitiative Buchholz/Holm-Seppensen is acknowledged. C. López is a recipient of a postdoctoral fellowship from the Alexander von Humboldt Foundation.

1. Dalla-Favera R, et al. (1982) Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci USA* 79(24):7824–7827.
2. Hecht JL, Aster JC (2000) Molecular biology of Burkitt's lymphoma. *J Clin Oncol* 18(21): 3707–3721.
3. Molyneux EM, et al. (2012) Burkitt's lymphoma. *Lancet* 379(9822):1234–1244.
4. Felsher DW, Bishop JM (1999) Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol Cell* 4(2):199–207.
5. Boxer LM, Dang CV (2001) Translocations involving c-myc and c-myc function. *Oncogene* 20(40):5595–5610.
6. Nesbit CE, Tersak JM, Prochownik EV (1999) MYC oncogenes and human neoplastic disease. *Oncogene* 18(19):3004–3016.
7. Beroukhim R, et al. (2010) The landscape of somatic copy-number alteration across human cancers. *Nature* 463(7283):899–905.
8. Fernandez PC, et al. (2003) Genomic targets of the human c-Myc protein. *Genes Dev* 17(9):1115–1129.
9. Li Z, et al. (2003) A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci USA* 100(14):8164–8169.
10. McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* 94(3):363–374.
11. Zhang N, et al. (2014) MYC interacts with the human STAGA coactivator complex via multivalent contacts with the GCN5 and TRRAP subunits. *Biochim Biophys Acta* 1839(5):395–405.
12. Frank SR, et al. (2003) MYC recruits the TIP60 histone acetyltransferase complex to chromatin. *EMBO Rep* 4(6):575–580.
13. Rahl PB, et al. (2010) c-Myc regulates transcriptional pause release. *Cell* 141(3):432–445.
14. Lin CY, et al. (2012) Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 151(1):56–67.
15. Nie Z, et al. (2012) c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 151(1):68–79.
16. Sabò A, et al. (2014) Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. *Nature* 511(7510):488–492.
17. Walz S, et al. (2014) Activation and repression by oncogenic MYC shape tumour-specific gene expression profiles. *Nature* 511(7510):483–487.
18. Carninci P, et al.; FANTOM Consortium; RIKEN Genome Exploration Research Group and Genome Science Group (Genome Network Project Core Group) (2005) The transcriptional landscape of the mammalian genome. *Science* 309(5740):1559–1563.
19. Mattick JS, Makunin IV (2006) Non-coding RNA. *Hum Mol Genet* 15(Spec No 1):R17–R29.
20. Djebali S, et al. (2012) Landscape of transcription in human cells. *Nature* 489(7414): 101–108.
21. Huarte M, et al. (2010) A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell* 142(3):409–419.
22. Prensner JR, et al. (2011) Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nat Biotechnol* 29(8):742–749.
23. Flockhart RJ, et al. (2012) BRAFV600E remodels the melanocyte transcriptome and induces BANCR to regulate melanoma cell migration. *Genome Res* 22(6):1006–1014.
24. Sauvageau M, et al. (2013) Multiple knockout mouse models reveal lincRNAs are required for life and brain development. *eLife* 2:e01749.
25. Holdt LM, et al. (2013) Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. *PLoS Genet* 9(7):e1003588.
26. Esteller M (2011) Non-coding RNAs in human disease. *Nat Rev Genet* 12(12):861–874.
27. Derrien T, et al. (2012) The GENCODE v7 catalog of human long noncoding RNAs: Analysis of their gene structure, evolution, and expression. *Genome Res* 22(9):1775–1789.
28. Borsani G, et al. (1991) Characterization of a murine gene expressed from the inactive X chromosome. *Nature* 351(6324):325–329.
29. Lee JT, Lu N, Han Y (1999) Genetic analysis of the mouse X inactivation center defines an 80-kb multifunction domain. *Proc Natl Acad Sci USA* 96(7):3836–3841.
30. Kretz M, et al. (2012) Suppression of progenitor differentiation requires the long noncoding RNA ANCR. *Genes Dev* 26(4):338–343.
31. Yang L, et al. (2011) ncRNA- and Pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. *Cell* 147(4):773–788.
32. Bond CS, Fox AH (2009) Paraspeckles: Nuclear bodies built on long noncoding RNA. *J Cell Biol* 186(5):637–644.
33. Rinn JL, et al. (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129(7):1311–1323.
34. Tsai M-C, et al. (2010) Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329(5992):689–693.
35. Alizadeh AA, et al. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403(6769):503–511.
36. Hummel M, et al.; Molecular Mechanisms in Malignant Lymphomas Network Project of the Deutsche Krebshilfe (2006) A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N Engl J Med* 354(23):2419–2430.
37. Dave SS, et al.; Lymphoma/Leukemia Molecular Profiling Project (2006) Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* 354(23):2431–2442.
38. Alfano D, et al. (2010) Modulation of cellular migration and survival by c-Myc through the downregulation of urokinase (uPA) and uPA receptor. *Mol Cell Biol* 30(7):1838–1851.
39. Pocsfalvi G, et al. (2011) Analysis of secretome changes uncovers an autocrine/paracrine component in the modulation of cell proliferation and motility by c-Myc. *J Proteome Res* 10(12):5326–5337.
40. Schuhmacher M, et al. (1999) Control of cell growth by c-Myc in the absence of cell division. *Curr Biol* 9(21):1255–1258.
41. Yustein JT, et al. (2010) Induction of ectopic Myc target gene JAG2 augments hypoxic growth and tumorigenesis in a human B-cell model. *Proc Natl Acad Sci USA* 107(8): 3534–3539.
42. Seitz V, et al. (2011) Deep sequencing of MYC DNA-binding sites in Burkitt lymphoma. *PLoS One* 6(11):e26837.
43. Rosenbloom KR, et al. (2013) ENCODE data in the UCSC Genome Browser: Year 5 update. *Nucleic Acids Res* 41(Database issue):D56–D63.
44. Cabili MN, et al. (2011) Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev* 25(18):1915–1927.
45. Aukema SM, et al.; Molecular Mechanisms in Malignant Lymphomas Network Project (2014) Biological characterization of adult MYC-translocation-positive mature B-cell lymphomas other than molecular Burkitt lymphoma. *Haematologica* 99(4):726–735.
46. Stellas D, et al. (2014) Therapeutic effects of an anti-Myc drug on mouse pancreatic cancer. *J Natl Cancer Inst* 106(12):106.
47. Ischenko I, Petrenko O, Hayman MJ (2014) Analysis of the tumor-initiating and metastatic capacity of PDX1-positive cells from the adult pancreas. *Proc Natl Acad Sci USA* 111(9):3466–3471.
48. Lee JT, Jaenisch R (1997) The (epi)genetic control of mammalian X-chromosome inactivation. *Curr Opin Genet Dev* 7(2):274–280.
49. Trimarchi T, et al. (2014) Genome-wide mapping and characterization of Notch-regulated long noncoding RNAs in acute leukemia. *Cell* 158(3):593–606.
50. Gupta RA, et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 464(7291):1071–1076.
51. Liu X, Li D, Zhang W, Guo M, Zhan Q (2012) Long non-coding RNA gadd7 interacts with TDP-43 and regulates Cdk6 mRNA decay. *EMBO J* 31(23):4415–4427.
52. Carrieri C, et al. (2012) Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* 491(7424):454–457.
53. Salaverria I, et al.; Molecular Mechanisms in Malignant Lymphoma Network Project; Berlin-Frankfurt-Münster Non-Hodgkin Lymphoma Group (2014) A recurrent 11q aberration pattern characterizes a subset of MYC-negative high-grade B-cell lymphomas resembling Burkitt lymphoma. *Blood* 123(8):1187–1198.
54. Richter J, et al.; ICGC MMML-Seq Project (2012) Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. *Nat Genet* 44(12):1316–1320.
55. Hart JR, Roberts TC, Weinberg MS, Morris KV, Vogt PK (2014) MYC regulates the non-coding transcriptome. *Oncotarget* 5(24):12543–12554.
56. Kim T, et al. (2015) Role of MYC-regulated long noncoding RNAs in cell cycle regulation and tumorigenesis. *J Natl Cancer Inst* 107(4):107.
57. Kim T, et al. (May 11, 2015) MYC-repressed long noncoding RNAs antagonize MYC-induced cell proliferation and cell cycle progression. *Oncotarget*.
58. Winkle M, et al. (2015) Long noncoding RNAs as a novel component of the Myc transcriptional network. *FASEB J* 29(6):2338–2346.
59. Hung C-L, et al. (2014) A long noncoding RNA connects c-Myc to tumor metabolism. *Proc Natl Acad Sci USA* 111(52):18697–18702.
60. Ullius A, et al. (2014) The interaction of MYC with the trithorax protein ASH2L promotes gene transcription by regulating H3K27 modification. *Nucleic Acids Res* 42(11): 6901–6920.
61. Yang YW, et al. (2014) Essential role of lncRNA binding for WDR5 maintenance of active chromatin and embryonic stem cell pluripotency. *eLife* 3:e02046.
62. Liu D, et al. (2014) Sine oculis homeobox homolog 1 promotes DNA replication and cell proliferation in cervical cancer. *Int J Oncol* 45(3):1232–1240.
63. Yang D, et al. (2010) Therapeutic potential of a synthetic lethal interaction between the MYC proto-oncogene and inhibition of aurora-B kinase. *Proc Natl Acad Sci USA* 107(31):13836–13841.
64. Seo J, et al. (2005) Cdt1 transgenic mice develop lymphoblastic lymphoma in the absence of p53. *Oncogene* 24(55):8176–8186.
65. Hoffmann S, et al. (2009) Fast mapping of short sequences with mismatches, insertions and deletions using index structures. *PLOS Comput Biol* 5(9):e1000502.
66. Hoffmann S, et al. (2014) A multi-split mapping algorithm for circular RNA, splicing, trans-splicing and fusion detection. *Genome Biol* 15(2):R34.
67. Lee TI, Johnstone SE, Young RA (2006) Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc* 1(2):729–748.
68. Haralambieva E, Kleiverda K, Mason DY, Schuuring E, Kluin PM (2002) Detection of three common translocation breakpoints in non-Hodgkin's lymphomas by fluorescence in situ hybridization on routine paraffin-embedded tissue sections. *J Pathol* 198(2):163–170.
69. Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 17(1):10–12.
70. Breese MR, Liu Y (2013) NGSUtils: A software suite for analyzing and manipulating next-generation sequencing datasets. *Bioinformatics* 29(4):494–496.
71. Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biol* 11(10):R106.
72. Bailey TL, et al. (2009) MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res* 37(Web Server issue):W202–W208.