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## The role of non-coding RNAs in B-cell lymphoma

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# CHAPTER 2

## **Identification of MYC-regulated long non-coding RNAs in Burkitt lymphoma**

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*Manuscript in preparation*

### ABSTRACT

The transcription factor MYC plays an important role in the development and progression of B-cell lymphoma. Burkitt lymphoma (BL) is a well-known example of a MYC-driven malignancy with high expression of MYC due to a chromosomal translocation involving MYC and one of the immunoglobulin enhancer regions. Long non-coding RNAs (lncRNAs) have been identified as regulatory RNAs with important roles in several types of cancer. We previously identified MYC-regulated lncRNAs in P493-6 cells, which is a B-cell line with a tetracycline-repressible MYC allele. Here, we identified MYC-regulated lncRNAs in ST486 BL cells upon shRNA-induced MYC knockdown using a custom designed microarray. This revealed 387 significantly deregulated lncRNA probes corresponding to 319 lncRNA loci and 1,153 mRNA probes corresponding to 1,053 protein-coding genes. Comparison with our previous study of the P493-6 MYC model revealed that 71% of the lncRNA and 81% of the mRNA probes identified in ST486 were also differentially expressed in the P493-6 model. Moreover, 54% of lncRNA and 65% of mRNA probes were also significantly differentially expressed between BL and CLL patient cases. Differential expression of four selected MYC-regulated lncRNAs were confirmed by RT-qPCR. Re-analysis of published MYC chromatin-immunoprecipitation data of BL cell lines indicated a significant enrichment of MYC binding sites in the MYC-induced and MYC-repressed lncRNAs. Similar to our previous study on P493-6 cells, only the MYC-induced, but not the MYC-repressed mRNA transcripts were significantly enriched for MYC binding sites. Knockdown of one of the MYC-induced lncRNAs, i.e. lncRNA KTN1-AS1, resulted in a mild (DG75) and strong (CA46 and ST486) impaired growth of BL cell lines. Thus, we identified 319 MYC-regulated lncRNA loci in BL. The observed oncogenic role of the MYC-induced lncRNA KTN1-AS1 in BL cell lines suggests that this lncRNA may play a role in MYC-driven B-cell lymphoma.

## INTRODUCTION

Burkitt lymphoma (BL) is an extremely aggressive B-cell lymphoma subtype with a doubling time of 24-48 hours <sup>[1]</sup>. The tumor cells are derived from B cells that are at the germinal center (GC) stage of maturation. There are three clinical variants recognized: endemic, sporadic and immunodeficiency-related BL <sup>[2]</sup>. Endemic BL has been linked to malaria, is frequently associated with EBV and mainly occurs in the African equatorial belt. The sporadic variant is found throughout the rest of the world and immunodeficiency-related BL mostly occurs in HIV infected individuals.

Chromosomal translocations affecting the *MYC* gene region are the hallmark of BL. This translocation results in juxtaposition of the *MYC* gene to the immunoglobulin heavy or light gene enhancer elements <sup>[3]</sup>. This translocation results in increased *MYC* expression levels. Enhanced *MYC* levels results in a deregulated cell cycle control and inhibition of both differentiation and apoptosis <sup>[4]</sup>.

Over the last decade lncRNAs have been shown to be critical players in many cellular functions. A myriad of functions have been demonstrated for lncRNAs <sup>[5]</sup>, although the function for the vast majority of them remains unknown. One of the well-studied modes of action of lncRNAs is how they modulate gene expression in cis and in trans <sup>[6]</sup>. Several studies have shown interplay between *MYC* and specific lncRNAs (reviewed in <sup>[7]</sup>). For example, *MYC* induces histone acetylation of the H19 promoter, resulting in higher expression levels of lncRNA H19. H19 in turn influences cell growth by controlling *IGF2* expression in cis <sup>[8]</sup>. The *MYC*-induced lncRNA MINCR was shown to regulate expression of cell cycle genes <sup>[9]</sup>. We previously identified a large set of *MYC* regulated lncRNAs using the P493-6 cell line <sup>[10]</sup>. This cell line contains a tetracycline-repressible *MYC* allele that was engineered in transformed B cells derived from PBMCs <sup>[11, 12]</sup>. Here we studied to what extent *MYC* regulates lncRNA expression levels in BL cells and compared the results to our previously obtained data. In addition we showed an oncogenic effect of one of the *MYC*-induced lncRNAs, i.e. *KTN1-AS1*, in BL.

## **MATERIALS AND METHODS**

### ***Cell culture***

BL cell lines were purchased from DSMZ (DG75 and CA46) and ATCC (ST486) and cultured in RPMI-1640 medium (Cambrex Biosciences, Walkersville, USA) supplemented with 2mM ultra glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 20% (ST486) or 10% (DG75 and CA46) Fetal Calf Serum (all Cambrex Biosciences, Walkersville, MD, USA) at 37°C under an atmosphere containing 5% CO<sub>2</sub>.

### ***MYC and KTN1-AS1 short hairpin RNA-based knockdown***

Two shRNAs targeting exon 3 of the MYC gene and two shRNAs targeting exon 3 of the KTN1-AS1 variant #2 (Figure 5A), were designed using the Invivogen siRNA wizard (Table 1). Sense and anti-sense oligo's were ordered from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Cloning of shRNAs, generation of viral particles and infection of cells has been performed as described previously <sup>[10]</sup>. The percentage of infected cells was assessed by flow cytometry based on expression of GFP, which is co-expressed from the viral constructs. Depending on the infection percentage, cells were harvested directly (ST486, all samples having >90% GFP+ cells) or after sorting based on GFP expression (DG75 and CA46 all samples having >98% GFP+ cells after sorting). The efficiency of the shRNAs were tested in BL cells harvested 8 days post-infection by western blot and RT-qPCR.

### ***GFP competition assay and statistics***

For the GFP competition assay, 3 biological replicates were performed per shRNA per cell line for MYC and 2 replicates for KTN1-AS1. The first GFP measurement was performed at day 4 (range 30%-60%) and this percentage was set to 1. The culture was followed for a period of 3 weeks and GFP was measured tri-weekly. The decrease in percentages of GFP positive cells in the BL cell lines infected by a MYC or KTN1-AS1 shRNA over time was compared with that of a non-targeting control shRNA using a mixed model with time and the interaction of time and

shRNA type as fixed effects and measurement repeat within shRNAs as random effects in SPSS (22.0.0.0 version, IBM, Armonk, New York, USA). In case of a non-linear relation between time and decrease of GFP positive cell percentages (determined by visual inspection of the graph) quadratic terms for time itself and for time interacting with shRNA were added to the model. No (fixed and random) intercept was included in the model, as the percentages at time 0 were set to 100% and percentages–100% were analyzed. The interaction term is the parameter of interest, as this identifies whether the decrease over time differs between the MYC / KTN-AS1 shRNAs and the non-targeting control shRNA. P values <0.05 were considered statistically significant.

### ***RNA isolation***

RNA was isolated as described earlier <sup>[10]</sup> using Qiagen miRNeasy Mini Kit (Life Technologies, Carlsbad, CA, USA). The RNA concentration was measured with a Nano Drop<sup>TM</sup> 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and RNA integrity was assessed by analysis of the 28S/18S bands on a 1% agarose gel.

### ***Quantitative RT-PCR***

First strand cDNA synthesis was performed with 500ng RNA in a reaction volume of 20 $\mu$ L containing 300 ng/ $\mu$ L random hexamer primers, 0.5mM dNTP mix, 5 $\times$  first-strand buffer, 0.1M DTT, 40 units/ $\mu$ L RNaseOUT<sup>TM</sup> Recombinant Ribonuclease Inhibitor and 200 units superscript II reverse transcriptase according to the manufacturer's instructions (Life Technologies Europe BV, The Netherlands).

The quantitative PCR reactions were performed in triplicate on 1ng cDNA in a volume of 10 $\mu$ L containing 1x SYBR Green Master Mix (Applied Biosystems) and 3 $\mu$ M forward and reverse primers on a Lightcycler 480 system (Roche, Penzberg, Germany). 18S ribosomal RNA served as an endogenous control for normalization. Primer sequences are listed in Table 1.

### *Western blotting*

Cells were washed with cold PBS and lysed in Lysis Buffer (#9803, Cell Signaling Technology, Danvers, MA, United States). Lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and supernatant was collected. Protein concentration was determined using Pierce BCA Protein Assay following the manufacturer's instructions (Thermo scientific). Samples were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes using standard procedures. Membranes were blocked in 5% milk and incubated with rabbit monoclonal anti-human MYC antibody (#1472-1, Epitomics, Burlingame, USA) diluted 5,000x and mouse monoclonal anti-human  $\beta$ -actin antibody (mAbcam 8226) diluted 20,000x in 5% milk supplemented with Tris-buffered saline and 0.1% Tween-20 at 4°C overnight. Polyclonal horseradish peroxidase-conjugated goat anti-rabbit Ig (2,000x) and rabbit anti-mouse Ig (1,000x; both from Dako, Glostrup, Denmark) were used as secondary and third incubation steps. Membranes were incubated with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions to visualize the signal on a ChemiDoc MP scanner (Biorad, Veenendaal, The Netherlands). Image Lab 4.0.1 Software (Bio-Rad) was used for quantification of protein bands.

### *Microarray analysis*

Gene and lncRNA expression analysis was performed by custom microarray analysis as described previously<sup>[10]</sup>. Arrays were scanned with Agilent DNA Microarray Scanner and analyzed with Agilent Feature Extraction software version 12.0. Resulting raw data were analyzed in Genespring GX version 13.1.1 (Agilent Technologies) using quantile normalization without baseline transformation. Probes consistently flagged as present (feature extraction software) and expressed in the 10th to 100th percentile in 1 of 2 conditions (control vs. knockdown) were used for further analysis. To determine differentially expressed transcripts we

applied a moderated T test with Benjamin Hochberg multiple testing correction and a fold change threshold of at least 1.5. To determine the overlap with our previously published data we re-analyzed the MYC on vs. MYC off data of the P493-6 cell line <sup>[10]</sup> using identical settings for the set of genes significantly differentially expressed in ST486 cells upon MYC knock down. Using a similar approach, we also re-analyzed our previously published expression data of MYC-high BL as compared to MYC-low chronic lymphocytic leukemia (CLL) cases <sup>[10]</sup>. Gene set enrichment analysis (GSEA) was performed using the Molecular Signatures Database (<http://www.broad.mit.edu/gsea>) using curated gene sets (C2) and oncogenic signatures sets (C6).

### ***Binding site analysis***

Previously published MYC ChIP datasets of 4 BL cell lines <sup>[13]</sup> were used to determine if the differentially expressed lncRNAs and genes have nearby MYC binding sites. We calculated the distance from the transcription start site (TSS) of each of the differentially expressed mRNAs and lncRNAs to the center of the reported MYC binding site using Galaxy (<https://usegalaxy.org>) <sup>[14]</sup> as follows: The genomic coordinates of binding sites and mRNA (RefSeq only) or lncRNA sets were uploaded in bed format. Operate on genomic intervals functions 'join' and 'fetch' closest non-overlapping feature were used to determine MYC binding sites that directly overlap a transcript or are adjacent to a transcript (up- and downstream), respectively. Lists were combined and TSS to MYC binding site distance was calculated per lncRNA and mRNA. Finally, the percentage of lncRNA and mRNA probes with a MYC binding site within 1, 2 or 5kb of their TSS was determined. Chi square test was used to determine significant differences. (\* =  $p < 0,05$ , \*\* =  $P < 0,01$ , \*\*\* =  $P < 0,001$ , \*\*\*\* =  $p < 0,0001$ ).



## RESULTS

### *Effect of MYC inhibition on BL cell growth*

Analysis of endogenous MYC levels in BL cell lines revealed the lowest level in ST486 cells, intermediate levels in DG75 and the highest levels in CA46 cells (Figure S1A). RT-qPCR analysis showed a similar expression pattern at the mRNA level (Figure S1B). Efficiencies of the MYC shRNA constructs determined by RT-qPCR on three BL cell lines harvested at day 8 after infection showed only a modest decrease (data not shown). At the protein level a decrease of 60-80% in MYC protein levels were observed compared to non-targeting control shRNA (Figure 1A).

To determine the consequence of MYC downregulation on BL cell growth we performed GFP competition assays. Cell growth was significantly impaired in all three BL cell lines with the strongest effects observed for MYC shRNA-2 (Figure 1B), consistent with the strongest decrease in MYC protein as observed by western blot. In ST486 a 50% reduction in GFP+ cells was observed about 9 days after infection with shRNA-2, and approximately 13 days after infection with shRNA-1. A 50% reduction of GFP+ cells in DG75 and CA46 was observed after ~13 days for shRNA-2 and between 18-22 days for shRNA-1. These experiments show that the MYC depletion was effective and also show that the BL cell lines are dependent on MYC expression.

### *LncRNA and mRNA expression analysis*

As the phenotypic effects of MYC depletion were strongest in ST486, we used this cell line to study lncRNA and mRNA expression changes upon MYC knockdown in BL. Two independent sets of samples were generated, including both shRNA constructs and a non-targeting control construct, harvested at day 8 after infection. As a validation for efficient MYC knockdown, RT-qPCR analysis were performed of three known MYC-induced genes, i.e. CAD, TFAM and PGK1<sup>[15]</sup>. This showed decreased expression levels upon MYC knockdown for all three genes, confirming efficient MYC knockdown (Figure 2A). In total, 7,192 lncRNA and 13,741

mRNA probes showed expression values above the background. Gene set enrichment analysis showed a strong enrichment for genes identified in a previous MYC-knockdown study<sup>[16]</sup> (FDR < 0.0001) and in a study using the P493-6 cell line<sup>[12, 17]</sup> (FDR = 0.0002) (Figure 2B). Together, these data confirm the robustness of our approach to efficiently downregulate MYC.

### ***Identification of MYC-regulated lncRNAs***

Upon knockdown *MYC* with shRNAs-1 and shRNA-2, 387 lncRNA and 1,153 mRNA probes were significantly differentially expressed compared to non-targeting control infected cells. These probes corresponded to 319 and 1,053 individual gene loci, respectively. LncRNAs were more often upregulated (65%, 250 probes) than downregulated (35%, 137 probes) upon *MYC* knockdown (Figure 3A). Analysis of the mRNAs showed a similar number of up- and downregulated transcripts (47%, 543 probes and 53%, 610 probes; Figure 3B). Clustering of the significantly differentially expressed lncRNA probes grouped one of the MYC knockdown replicates of shRNA-1 next to the control samples, while both replicates of shRNA-2 cluster separately and closely together with the other shRNA-1 replicate. For the mRNA probes, all shRNA samples form one cluster that is separate from the cluster of the control shRNA samples.

To support the MYC-dependency of the lncRNA transcripts differentially expressed upon MYC knockdown in BL cells, we analyzed their expression patterns in the MYC-on and MYC-off state of the P493-6 cell line in our previously published data set<sup>[10]</sup>. Out of 387 and 1,153 differentially expressed lncRNA and mRNA probes in ST486, 36 lncRNA and 50 mRNA probes were not expressed in P493-6 cells. The vast majority of the remaining probes, i.e. 249 (71%) lncRNAs and 898 (81%) mRNAs showed significant expression changes in the expected direction (Figure 3A).

The overlap with our previously published expression profiles of MYC-high BL and MYC-low chronic lymphocytic leukemia (CLL) cases<sup>[10]</sup> was 54% for the lncRNA and 65% for the mRNA probes (63 lncRNA and 49 mRNA probes were not expressed in BL vs. CLL). Overall, most of the probes showed expression changes in the expected direction (Figure 3B).

To confirm our microarray data, we selected four lncRNAs from the significantly differentially expressed lncRNAs upon MYC knockdown in ST486 cells for RT-qPCR validation. One was MYC-repressed, i.e. TUG1, and three were MYC-induced, i.e. KTN1-AS-1, TCONS\_00025860 and TCONS\_12\_00030240. The patterns observed in the array could be reproduced using RT-qPCR (Figure 3C).

### *MYC binding site analysis*

To assess the number of transcripts that are likely to be direct targets of MYC, we performed a MYC binding site analysis using publically available ChIP data. Seitz and colleagues<sup>[13]</sup> identified a total of 7,054 MYC binding sites in 5 BL cell lines (Raji, CA46, Blue1, BL41 and Ramos). We determined the percentage of differentially expressed transcripts with a MYC binding site within 1, 2 or 5kb of their transcription start site (TSS). As a control, we calculated the overall percentage of lncRNA and mRNA transcripts present on the array with a MYC binding site within a distance of 1, 2 and 5kb. Protein-coding genes more often had a MYC binding site in close proximity than lncRNAs (Figure 4). Both MYC-repressed and MYC-induced lncRNA transcripts were significantly enriched for MYC binding sites compared to the genome wide control. For the protein-coding genes only the MYC-induced transcripts were consistently significantly enriched for MYC binding sites. Only a slight enrichment in MYC binding sites was observed within MYC-repressed mRNAs when considering a 5kb distance between the TSS and the MYC binding site (Figure 4). These data indicate that MYC directly induces expression of protein-coding genes, while their repression in general seems to be an indirect effect. Conversely, our data show that MYC-induced and MYC-repressed lncRNAs are both direct targets of MYC, which is in line with our previous observations<sup>[10]</sup>.

### *KTN1-AS1 knockdown strongly impairs BL cell growth*

KTN1-AS1 is induced by MYC and has a MYC binding site ~900bp upstream of its TSS. This lncRNA has been identified as a MYC-regulated lncRNA in two earlier studies<sup>[10, 18]</sup>, but has not been functionally

characterized in BL. Four transcript variants have been described for KTN1-AS1 (Figure 5A). Based on our microarray data, variant #1 and/or #4 were differentially expressed, while the other variants were not expressed. RT-qPCR revealed that only variant #1 was expressed and followed the expected pattern upon MYC knockdown (Figure 3C). To determine the possible role of KTN1-AS1 on cell growth we knocked down KTN1-AS1 variant #1 using a shRNA approach. Both shRNAs resulted in knockdown of KTN1-AS1 with efficiencies ranging between 25-60% at the RNA level (Figure 5B). GFP competition assays revealed a modest (DG75) to strong (CA46 and ST486) growth disadvantage upon KTN1-AS1 knockdown (Figure 5C). The strength of the KTN1-AS1 KD phenotype in ST486 and CA46 was similar to the MYC KD phenotype (Figure 1B).

## DISCUSSION

In this study, we showed that a substantial number of lncRNAs are regulated by MYC in BL. We <sup>[10]</sup> and others <sup>[19]</sup> have previously shown that lncRNAs, next to mRNAs and miRNAs, are an important part of the MYC transcriptional network in BL cells. In this study we modulated endogenous MYC levels in BL cell lines to identify MYC-regulated lncRNAs, which was not done previously. This might better reflect the physiological situation than the P493-6 cell line model with a repressible MYC allele generated from EBV transformed peripheral B cells <sup>[11, 12]</sup>. We identified a markedly lower number of MYC-regulated transcripts in ST486 cells (387 lncRNA and 1,153 mRNA probes) as compared to those identified previously in the P493-6 model (2,014 lncRNA and 6,555 mRNA probes). This difference is likely to be caused by the less pronounced reduction in MYC protein levels in ST486 as compared to P493-6 cells (77% vs~95%, Figure 1A).

The overlap in MYC-regulated transcripts between ST486 and P493-6 was quite high with 71% of the lncRNA and 81% of the mRNA probes. The overlap with the comparison of BL vs. CLL patient cases was slightly less with an overlap of 54% of the lncRNA and 65% of mRNA probes (Figure

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2A/B). Tumor cells in BL have been shown to be supported by multiple micro-environmental factors<sup>[20, 21]</sup>. Admixture of other cells present in the microenvironment of BL patient cases, which is lacking in the ST486 and P493-6 cell lines may explain the higher overlap between ST486 and P493-6 compared to the overlap with BL vs. CLL patient cases.

We identified fewer lncRNAs with a MYC binding site in close proximity of the TSS as compared to the mRNAs (Figure 3A). The ChIP dataset for this analysis contains MYC binding sites that were identified in 5 BL cell lines, but this panel did not include ST486 cells. Therefore, it is possible that the difference is due to a higher percentage of lncRNAs unique to ST486 as compared to mRNAs. It would be interesting to determine whether a comparable percentage of MYC binding sites is observed for lncRNAs compared to mRNAs when MYC ChIP data of the ST486 cells would have been used.

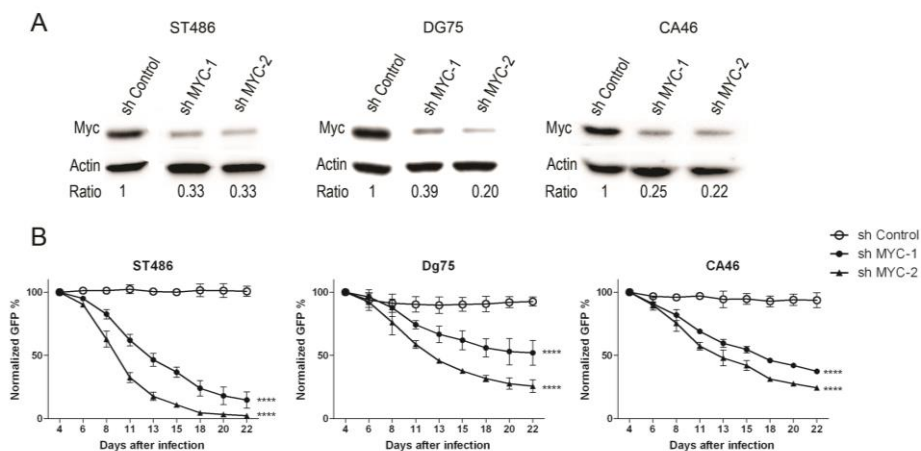
We identified two known lncRNAs as MYC-regulated genes in ST486 cells, i.e. taurine upregulated gene 1 (TUG1) and KTN1-AS1. TUG1 is repressed by MYC and has a MYC binding site ~560bp upstream its transcription start site. Knockdown of TUG1 was shown to increase proliferation of non-small cell lung cancer (NSCLC) *in vitro* and *in vivo*, and low TUG1 expression was identified as an independent predictor of poor overall survival in NSCLC patients<sup>[22]</sup>. Repression of TUG1 by MYC may thus be advantageous to the malignant properties of the cell. However, several other studies showed upregulation of TUG1 in different types of cancer, including gastric cancer<sup>[24]</sup>, urothelial carcinoma of the bladder<sup>[25, 26]</sup>, osteosarcoma<sup>[27]</sup>, hepatocellular carcinoma<sup>[28]</sup> and esophageal squamous cell carcinoma<sup>[29]</sup>. TUG1 has been shown to have an important role in cell cycle by binding to PRC2<sup>[24, 30]</sup> and this interaction was required for epigenetic repression of cyclin-dependent protein kinase inhibitors<sup>[24]</sup>.

KTN1-AS1, also known as MYCLO-3, has previously been identified as a MYC-regulated lncRNA in a wide range of cancer types including colorectal cancer<sup>[18]</sup>. KTN1-AS1 is overexpressed in colorectal and prostate cancer and was shown to act as an oncogene<sup>[18]</sup>. KTN1-AS1

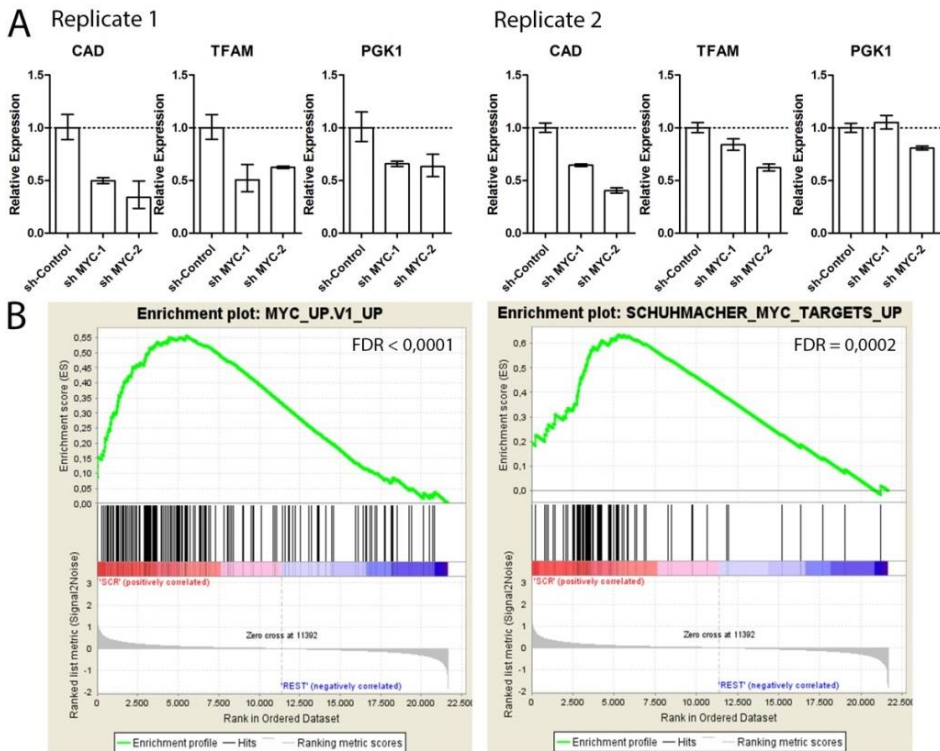
regulates known MYC target genes, such as *CDKN1A* and *CDKN2B*, through interactions with hnRNPK <sup>[18]</sup>. We showed that shRNA-induced depletion of KTN1-AS1 has a strong negative effect on BL cell growth, with an impact on cell growth that is quite similar to MYC knockdown itself.

In summary, we identified a set of MYC-regulated lncRNAs in BL and further delineated the regulatory mechanisms of MYC on lncRNA expression levels. We showed that the MYC-regulated lncRNA KTN-AS1 acts as an oncogene in BL, as depletion of KTN-AS1 resulted in growth disadvantage of BL cells. Our results suggest that lncRNAs regulated by MYC, such as KTN1-AS1, may play important roles in MYC-driven BL.

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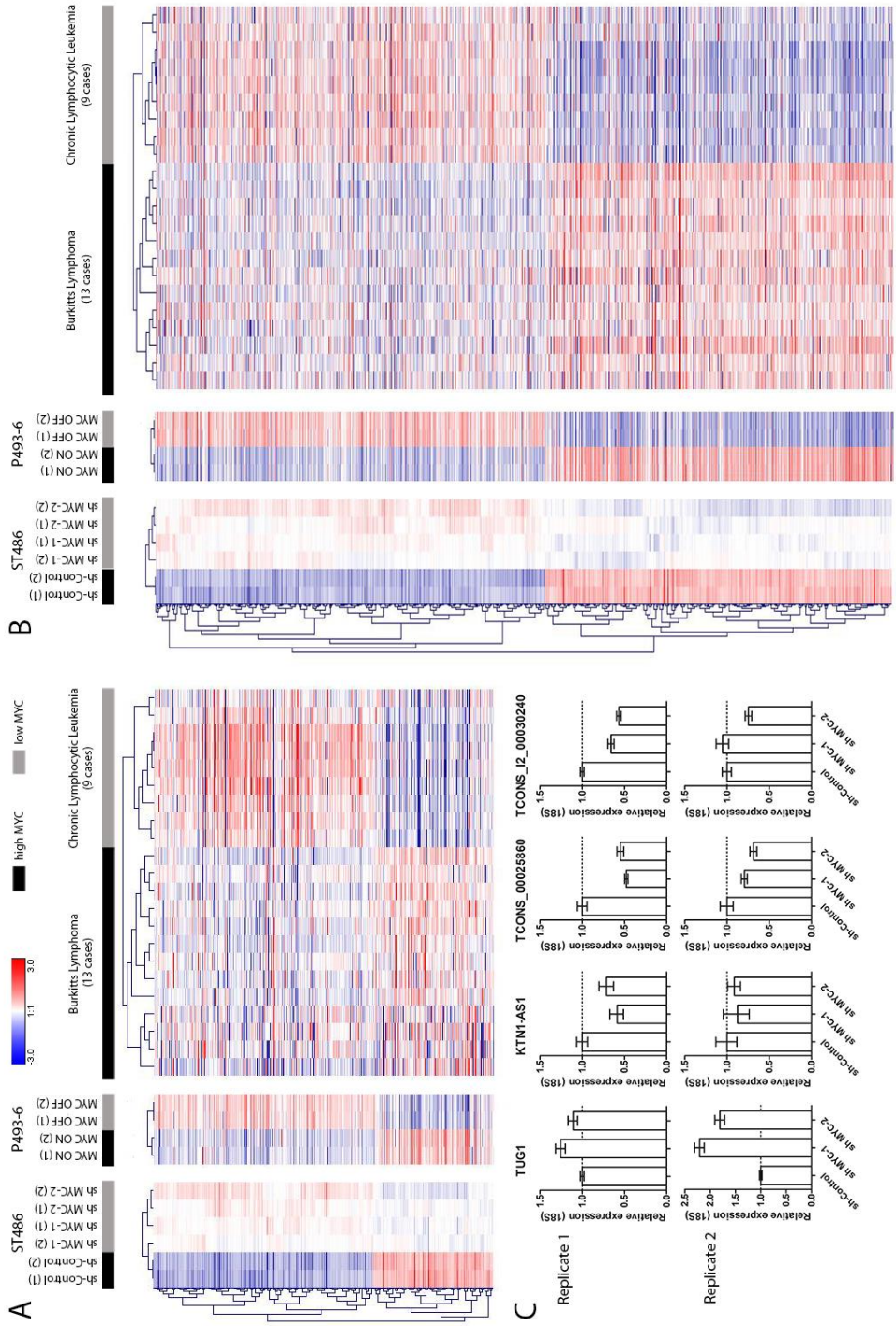
**Figure 1. Inhibition of MYC in BL results in a strongly reduced growth.** (A) Western blotting of BL cells treated with shRNAs against MYC revealed decreased MYC protein levels. Shown is a representative western blot of one out of three biological replicates. (B) GFP competition assays revealed a significant growth disadvantage upon MYC inhibition in all three BL cell lines. Shown are the average and standard deviation of three independent GFP competition assays per cell line. ShRNAs were stably expressed in cells using a viral vector which co-expresses GFP. The GFP percentage was measured triweekly for 22 days and GFP percentages were normalized to day 4. Asterisks indicate significant differences between each MYC shRNA and the non-targeting control shRNA. \*\*\*\* $P < 0.0001$ .



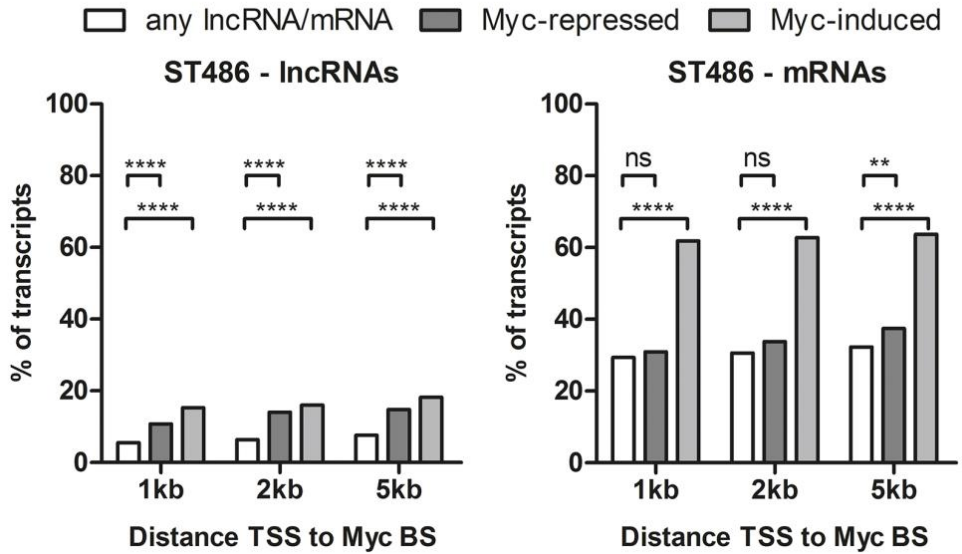
**Figure 2. Validation of MYC knockdown in ST486 cells.** (A) RT-qPCR analysis of three known MYC-induced genes shows their expected decrease in expression upon knockdown. The sh-Control is set to 1, shown are the means  $\pm$  error. (B) Gene Set Enrichment Analysis shows that two previously identified MYC-induced gene sets are enriched in sh-Control and depleted in the MYC knockdown cells (sh MYC-1 and sh MYC-2).



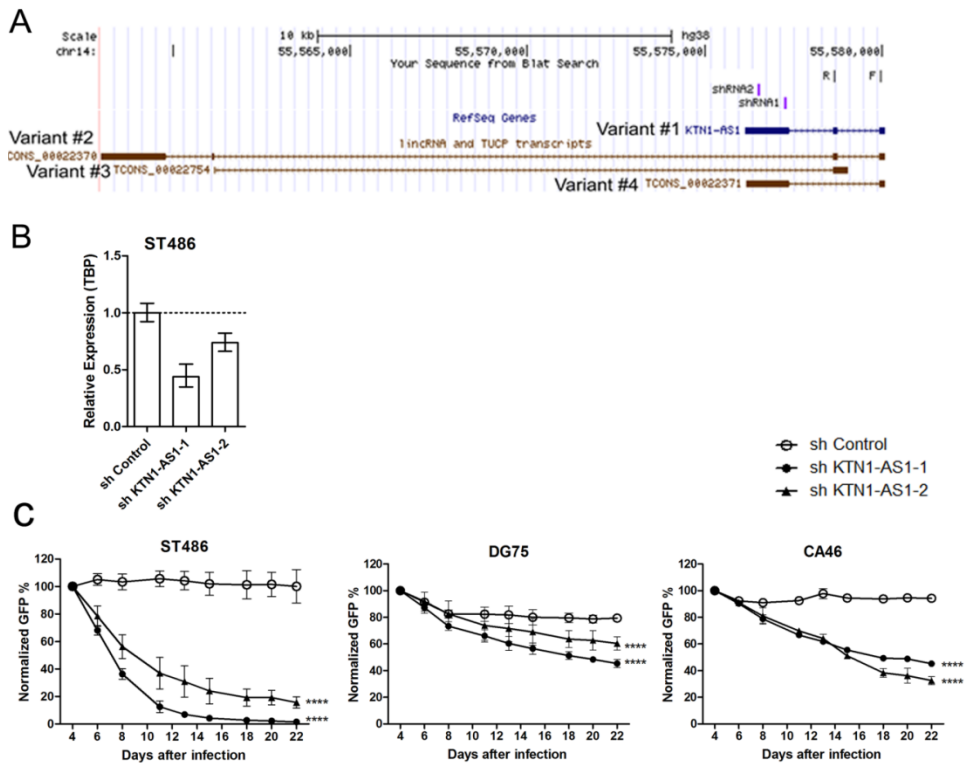
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**Figure 3. MYC-regulated lncRNAs in Burkitt lymphoma.** (A) Shown are heatmaps of the 387 lncRNA probes that were significantly differentially expressed between ST486 cells infected with sh-Control and sh-MYC-1 or sh-MYC-2. Expression of the same set of probes is shown in P493-6 cells in the presence and absence of MYC and in MYC-high BL cases vs. MYC-low CLL cases. (B) The same heatmaps as in (A) but now for the 1,153 differentially expressed mRNA probes. (C) Four MYC-regulated lncRNAs were validated by RT-qPCR and their relative expression in relation to 18S is shown for both replicates. TUG1 is MYC-repressed while the other three lncRNAs are MYC-induced. Observed expression patterns are in line with the array data. sh-Control is set to 1, mean  $\pm$  error.



**Figure 4: MYC binding site enrichment of differentially expressed lncRNAs and mRNAs.** The percentage of transcripts with a MYC binding site (BS) within 1, 2 or 5kb of their transcription start site (TSS) are shown for lncRNAs and mRNAs differentially expressed in ST486. The percentage of all transcripts on the array with a BS within the same distance serves as control (white bars). Relative enrichment is calculated by Chi-square test. ns - non-significant; \*\* -  $p < 0.001$ ; \*\*\*\* -  $p < 0.00001$ .

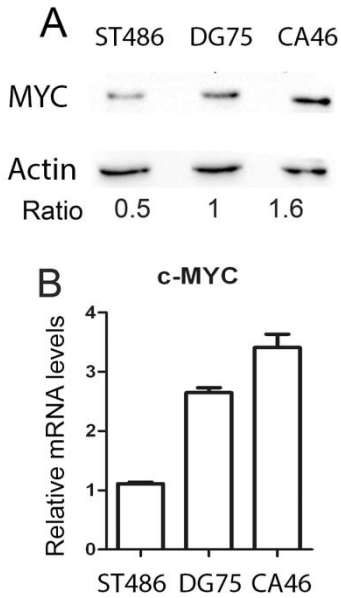


**Figure 5. Knockdown of KTN1-AS1 results in impaired cell growth in BL cell lines.** (A) UCSC genome browser view for KTN1-AS1. Forward (F), reverse (R) primers and location of shRNAs are indicated. Primers span exon 1 and exon 2 of transcript variants #1 and #2. Both shRNAs target exon 3 of KTN1-AS1 variants #1 and #4 (not expressed). (B) The efficiency of shRNA-mediated lncRNA knockdown is shown by RT-qPCR in ST486. sh-Control is set to 1, shown are the means  $\pm$  error. (C) GFP competition assay upon KTN1-AS1 knockdown in ST486, DG75 and CA46. ShRNAs were stably expressed in cells using a viral vector which co-expresses GFP. The GFP percentage was measured triweekly for 22 days and GFP percentages were normalized to day 4. Asterisks indicate significant differences between each shRNA and the sh-Control. \*\*\*\* $P < 0.0001$ .

**Table 1. ShRNA and Primer sequences (all 5' to 3')**

<b>sh Control</b>	Sense: TCGAGCAACAAGATGAAGAGCACCACAATTCAAGAGATTGGTGCTTTCATCTTGTGTTTTG Antisense: AATTCAAAAACAACAAAGATGAAGAGCACCACAATTCAAGAGATTGGTGCTTTCATCTTGTGTTG
<b>sh MYC-1</b>	Sense: GATCCGATGAGGAAGAAATCGATGTTCAAGAGACATCGAATTTCTCCTCACTTTTTG Antisense: AATTCAA AAAAGATGAGGAAGAAATCGATGCTCTTTGAA CATCGAATTTCTCCTCATCG
<b>sh MYC-2</b>	Sense: GATCCAACGACGAGAACAGTTGAAAATTCAAAGAGATGTTCAACTGTCTCGTCGTTTTTTG Antisense: AATTCAAAAAAGCAGCAGAACAGTTGAAACATCTTTGAAATGTTTTCAACTGTCTCGTCGTTTTG
<b>sh KTN1-ASI-1</b>	Sense: GATCCGAGATCTAGAAATCATTTAGTTCAAGAGACTAATGACTTCTAGATCTGCTTTTTG Antisense: AATTCAAAAAGCAGATCTAGAAAGTCAATTAGTCTCTTGAACATAAGACTTCTAGATCTGCG
<b>sh KTN1-ASI-2</b>	Sense: GATCCGAAAGTAGTAGAGAGGTAAATTCAGAGATTTACCTCTACCTACTTCTTTTTG Antisense: AATTCAAAAAAGAGTAGTAGAGAGGTAAATCTCTTTGAAATTTACCTCTACCTACTCTG
<b>MYC</b>	F: GCTCATTTTGAAGAGGACTTGTGG R: TTACGCCACAAGAGTCCGTAGCT
<b>CAD</b>	F: AACCTGTGGGCTAATGACTG R: ACACCCAAACACCACGTGAG
<b>PGK1</b>	F: CAGTGTGGTCTGTCAT R: GCTGGCTGGCTTTAAAC
<b>TFAM</b>	F: GTTCTCCGAAGCATGTGG R: AGATGAAAAACCACCTCGGTAAA
<b>TCONS_00030240</b>	F: CACACTCCAAGGAAACGCCAA R: GGGATGACTGACCTCCTCTACC
<b>TCONS_00025860</b>	F: CGCGATTTGCAGGATTCC R: CAAATGTGGCACITAAAAGCA
<b>TUG1</b>	F: GCAGCATGGACCACACCCCA R: ACAGGTCCAGTGACCTTCACG
<b>KTN1-ASI</b>	F: GTTCAGTAGCCCGCTCTC R: ACGTCAAGAGATAGCCAGT
<b>18s</b>	F: CGGTACCACATCCAAGGA R: CCAATTACAGGGCCTCGAAA

## SUPPLEMENTARY DATA



**Figure S1. Endogenous MYC levels in three BL cell lines.** (A) Western blotting revealed the lowest endogenous MYC expression level in ST486 cells, moderate levels in DG75 and the highest levels in CA46 cells. (B) MYC mRNA levels followed a pattern consistent with the western blot results.

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