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### The missing piece

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CHAPTER 4

## Long noncoding RNAs as a novel component of the Myc transcriptional network

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## Abstract

Myc is a well-known transcription factor with important roles in cell cycle, apoptosis, and cellular transformation. Long noncoding RNAs (lncRNAs) have recently emerged as an important class of regulatory RNAs. Here, we show that lncRNAs are a main component of the Myc-regulated transcriptional program using the P493-6 tetracycline-repressible MYC model. We demonstrate that both Myc-induced mRNAs and lncRNAs are significantly enriched for Myc binding sites. In contrast to Myc-repressed mRNAs, Myc-repressed lncRNAs are significantly enriched for Myc binding sites. Subcellular localization analysis revealed that compared to mRNAs, lncRNAs more often have a specific subcellular localization with a markedly higher percentage of nuclear enrichment within the Myc-repressed lncRNA set. Parallel analysis of differentially expressed lncRNAs and mRNAs identified 105 juxtaposed lncRNA-mRNA pairs, indicative for regulation in *cis*. To support the potential relevance of the Myc-regulated lncRNAs in cellular transformation, we analyzed their expression in primary Myc-high and Myc-low B-cell lymphomas. In total, 54% of the lncRNAs differentially expressed between the lymphoma subsets were identified as Myc-regulated in P493-6 cells. This study is the first to show that lncRNAs are an important factor within the Myc-regulated transcriptional program and indicates a marked difference between Myc-repressed lncRNAs and mRNAs.

# 1 Introduction

Myc is frequently overexpressed both in solid tumors and B-cell lymphoma. Overexpression of Myc has significant effects on cell growth and proliferation, causes a general de-differentiation, and induces malignant transformation<sup>1</sup>. Myc targets a large number of protein-coding genes as well as microRNAs, of which several have been shown to contribute to the oncogenic effects of Myc<sup>2</sup>. To what extent Myc regulates transcription of long noncoding RNAs (lncRNAs) is a largely unexplored area of research.

lncRNAs are defined as RNA transcripts of >200 nucleotides in length that lack protein coding potential. Classification of lncRNAs is mainly based on their location and orientation with respect to protein coding genes, e.g., intronic, intergenic, or antisense. Many studies have described crucial roles for lncRNAs in a multitude of cellular processes<sup>3,4</sup>. In these processes, lncRNAs can influence protein coding genes in multiple ways, e.g., by functioning as protein scaffolds<sup>5,6</sup>, interacting with epigenetic complexes<sup>7,8</sup>, regulating transcription in *cis* or *trans*<sup>8-10</sup>, or sequestering microRNAs<sup>11,12</sup>. In contrast, lncRNA loci have been shown to be regulated by the same epigenetic and transcriptional mechanisms as protein coding loci<sup>13-16</sup>. Together, this suggests a complex regulatory network.

Deregulated lncRNA expression patterns have been implicated in cancer cell biology and multiple lncRNAs functioning as oncogenes and tumor repressor genes have been identified (reviewed in<sup>17</sup>). The altered lncRNA expression patterns observed in cancer can be caused by various mechanisms, including DNA amplification, chromosomal translocations, chromatin modifications, and altered transcription factor activity such as that of Myc.

To broaden our understanding of Myc biology, we identified Myc-regulated lncRNAs in an *in vitro* B-cell lymphoma model and primary cases of B-cell lymphoma. We found that Myc strongly influences the lncRNA expression profile of the *in vitro* model and also of primary B-cell lymphoma samples. In addition, we studied several of the characteristics of the Myc-regulated lncRNAs including the presence of Myc binding sites, subcellular localization, and putative co-regulation of adjacent protein coding genes.

## 2 Materials and Methods

### 2.1 Cell lines, cell culture, and tetracycline treatment

Burkitt lymphoma (BL) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) (ST486) and the German Collection of Microorganisms and



Cell Cultures (DSMZ, Braunschweig, Germany) (DG75). P493-6 B cells were a kind gift of Prof. D. Eick (Helmholtz Center, Munich, Germany). Cell lines were cultured at 37°C under an atmosphere containing 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM ultraglutamine, and 20% ST486 or 10% P493-6 and DG75 fetal calf serum (Cambrex Biosciences, Walkersville, MD, USA). For repression of Myc in P493-6, 0.1 µg/ml of tetracycline hydrochloride (University Medical Center Groningen [UMCG] Pharmacy, Groningen, The Netherlands) was added to the culture medium for 72 hours. For Myc re-induction, the cells were spun down (200 g, 5 minutes) and resuspended in fresh, tetracycline-free, complete medium for the indicated time.

## 2.2 Patient material

Frozen tumor samples of 13 BL and 9 CLL patients were collected from the Academisch Medisch Centrum Amsterdam and UMCG tissue banks. Each individual diagnosis was reviewed by an experienced hematopathologist according to the World Health Organization classification<sup>18</sup>. All BL cases carry a Myc translocation and are Epstein-Barr virus negative, CD20+, CD10+ and BCL2-. All CLL cases are lymph node derived, CD20+, CD5+ and cyclin D1- and have variable zeta-chain-associated protein kinase 70 (ZAP-70) expression. The procedures were performed according to the guidelines of the medical ethics board of the UMCG.

## 2.3 RNA isolation from cell lines, tissue samples, and nuclear and cytoplasmic fractions

For the RNA isolation from patient material, 10 to 20 10 µm sections were cut for each tumor sample (depending on the size of the tissue block). Tissue samples and cell lines were subjected to standard Trizol RNA isolation (Life Technologies, Carlsbad, CA, USA). RNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), RNA integrity was assessed by 1% agarose electrophoresis. Nuclear and cytoplasmic fractions were separated from P493-6 MycOFF cells by adding 200 µL lysis buffer (140 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH8.0, 1 mM DTT, 0.5% Nonidet P-40) to pellets of ~8 million cells, followed by 5 minutes incubation on ice and centrifugation for 3 minutes at 4°C and 100 g<sup>19,20</sup>. The supernatant was collected as the cytoplasmic fraction. The pellet containing the nuclei was washed twice with lysis buffer. Qiazol (1 ml; Qiagen, Germantown, MD, USA) was added to the ~200 µL cytoplasmic fraction, to the nuclear pellet, and to the total cell pellet. RNA was isolated using Phase Lock Gel Heavy (5 Prime, Hilden, Germany) and the RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

## 2.4 Quantitative RT-PCR

cDNA was synthesized using random primers, dNTP mix, and the Superscript II Reverse Transcriptase Kit (Life Technologies Europe BV, Bleiswijk, The Netherlands) according to

the manufacturer's instructions. An input of 500 ng RNA was used per sample in a total reaction volume of 20  $\mu$ L. For detection of Myc, carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), transcription factor A, mitochondrial (TFAM) and phosphoglycerate kinase 1 (PGK1) transcripts, TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's instructions. All other transcripts were assessed using SYBR Green mix (Applied Biosystems) in a quantitative PCR reaction volume of 10  $\mu$ L with 300 nM primers. Triplicate quantitative PCR reactions were performed with 1 ng of cDNA on a LightCycler 480 system (Roche, Penzberg, Germany). Primer sequences and gene expression assays used in this study are listed in TABLE S1.

## 2.5 Western blot analysis

Cell lysates were prepared, separated on polyacrylamide gels, and transferred onto nitrocellulose membranes using standard protocols. All antibodies were diluted in 5% milk in Tris-buffered saline + Tween-20. Myc protein levels were determined by Western blot analysis using anti-c-Myc (rabbit mAb, N-term; 1:5000; Epitomics, Burlingame, CA, USA) and anti- $\beta$ -actin (mouse mAb; 1:5000; Abcam Inc, Cambridge, MA, USA) as an internal loading control. As secondary antibodies, polyclonal horseradish peroxidase-conjugated goat anti-rabbit Ig (1:2000) and rabbit anti-mouse Ig (1:1000; both from Dako, Glostrup, Denmark) were used. For detection, the membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. ChemiDoc MP scanner and Image Lab 4.0.1 Software (both from Bio-Rad, Veenendaal, The Netherlands) were used for visualization and quantification of protein bands, respectively.

## 2.6 Fluorescence-activated cell sorting analysis

Cell cycle distributions of P493-6 cells were analyzed using propidium iodide staining. In brief, cells were washed 3 times with PBS supplemented with 0.1% bovine serum albumin. Hypotonic propidium iodide staining solution (0.1% sodium citrate, 0.3% Triton X-100, 0.01% propidium iodide, 0.002% ribonuclease A in demineralized water) was added to the cell pellet, resuspended, and left on ice for ~15 minutes before measurement with fluorescence-activated cell sorting (FACS) Calibur Flow Cytometer and Cell Quest software (BD Biosciences, San Jose, CA, USA). Forward scatter of live cells in complete medium were measured to determine cell size. Data were analyzed by FlowJo Flow Cytometry Analysis Software, v7.6 (Tree Star, Ashland, OR, USA).

## 2.7 Microarray study

LncRNA expression was investigated using a custom-designed microarray that contained 31,456 lncRNA and 27,186 mRNA probes. A total of 28,533 of the lncRNA probes were custom designed using eArray software (Agilent Technologies, Santa Clara, CA, USA)



covering 97.3% of a published catalog of lncRNAs (8195 lncRNA loci, 2305 transcripts of uncertain coding potential loci, and 9 additional known lncRNA loci)<sup>21</sup> with an average of 3 probes per locus. The remainder of the lncRNA probes as well as all mRNA probes were derived from AMADID no. 028004 (Agilent Technologies). All of the following procedures were done according to the manufacturer's instructions. 50 to 100ng total RNA was spiked with the RNA Spike-in kit and labeled using the LowInput QuickAmp Labeling kit and the Cyanine 3 CTP Dye Pack (all Agilent Technologies). For the subcellular enrichment experiments, the dual-color LowInput QuickAmp Labeling Kit with cyanine 3 and 5 CTP Dye Packs were used. The total fraction was labeled with cyanine 3 and the nuclear and cytoplasmic fraction with cyanine 5. After labeling, cRNA samples were purified using the RNeasy Mini Kit (Qiagen), quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and hybridized on the custom array using the Gene Expression Hybridization Kit (Agilent Technologies). Arrays were scanned with the Agilent DNA Microarray Scanner and analyzed with Agilent Feature Extraction software v10.7.3.1. Resulting raw data were analyzed with GeneSpring GX 12.5 software (Agilent Technologies) using quantile normalization without baseline transformation. Probes used for further analyses were flagged as present by the feature extracting software in at least 1 of the 4 conditions (or 1 in 2 conditions for the BL and CLL samples) as well as consistently expressed in the 10th to 100th percentile. Using these conditions, a total of 15,355 mRNA and 9,559 lncRNA probes were consistently expressed above background in P493-6 and 13,163 mRNA and 6,517 lncRNA probes in BL and CLL samples. Statistical significant changes in expression upon Myc induction in P493-6 cells were determined by 1-way ANOVA using Benjamini-Hochberg multiple testing correction and Tukey's honestly significant difference post hoc test. Of these, all probes with a  $\geq 2$ -fold change in expression were selected for the final list. Significant expression changes in BL vs. CLL were determined by moderated Student's t test, Benjamini-Hochberg multiple testing correction, and a  $\geq 2$ -fold change in expression. Heat maps were generated with Genesis software v1.7.6<sup>22</sup> (Institute for Genomics and Bioinformatics Graz, Graz, Austria) and Pearson correlation as the distance metric. Array data used for this publication have been deposited in the Gene Expression Omnibus (GSE59480).

## 2.8 Gene set enrichment analysis and gene ontology analysis

Gene set enrichment analysis (GSEA) was performed using the Molecular Signatures Database (<http://www.broad.mit.edu/gsea>)<sup>23</sup>. Gene ontology (GO) analysis was performed using the DAVID ease Gene Functional Classification Tool<sup>24, 25</sup>. Myc-regulated mRNAs differentially expressed in P493-6 cells in the MycON or MycOFF state were assessed for functional gene cluster enrichment on the background of all genes expressed.

## 2.9 Myc binding site analysis

For the P493-6 Myc chromatin immunoprecipitation (ChIP) analysis, raw data were retrieved<sup>26</sup> for the 24 h MycON time point, aligned to the genome using Bowtie 2<sup>27</sup>,

and analyzed using MACS software v1.4.2 with default settings<sup>28</sup>. This resulted in the identification of 2398 binding sites. This data set and a second publically available data set<sup>29</sup> were used to determine the distances between the center of the Myc binding sites and the location of the nearest differentially expressed mRNA or lncRNA probes. The percentage of Myc-induced or -repressed mRNA and lncRNA transcripts with a Myc binding site in close proximity (5, 10, or 20kb) was calculated. The control group comprised the percentage of Myc binding sites within 5, 10, or 20kb of all mRNA and lncRNA probes present on the array. Significant enrichment of Myc binding sites compared to the control was calculated by the chi-square test.

## 2.10 Myc inhibition using short hairpin RNAs in BL cell lines

Short hairpin RNAs (shRNAs) targeting MYC were cloned in the pGreenpuro lentivector (SBI, Mountain View, CA, USA) using 5' BamHI and 3' EcoRI sites. Scrambled control vector was purchased (SBI). Virus was generated with a third-generation lentiviral system in 293T cells using CaPO4 transfection. Virus was collected 2 days after transfection, filtered, and directly used or stored at  $-80^{\circ}\text{C}$ . ST486 and DG75 cells were infected overnight, washed, and cultured. Four days after infection, the infection percentage was determined by FACS, and cells were collected for Western blot analysis and quantitative RT-PCR (qRT-PCR) 8 days after infection. Depending on the infection percentage, cells were collected without sorting (ST486, all samples >90% green fluorescent protein [GFP] positive) or GFP sorted (DG75, all samples >98% GFP positive). For each shRNA infection, 3 biologic replicates were generated and used for the lncRNA quantification by qRT-PCR (for ST486 shRNA2, only 2 biologic replicates were available). ShRNA sequences used are listed in TABLE S1.

# 3 Results

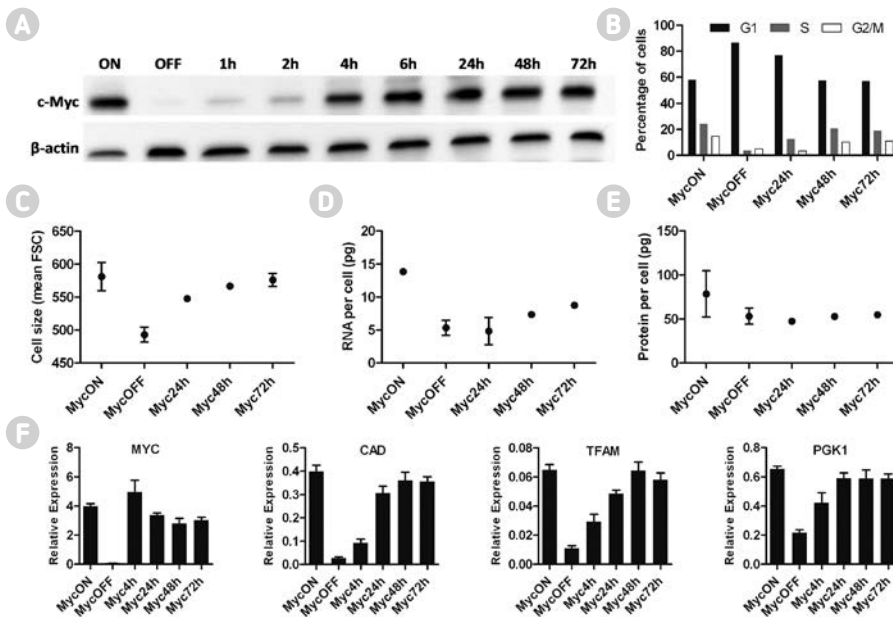
## 3.1 Identification of Myc-regulated lncRNAs

To investigate the effects of Myc on lncRNA expression, we used the widely applied P493-6 B cell line that carries a conditional, tetracycline-repressible MYC allele<sup>30</sup>. Tetracycline treatment (72 h) strongly decreased MYC mRNA and Myc protein levels, which returned to baseline within 4 and 6 hours after tetracycline removal, respectively (FIGURE 1A, F). Consistent with previous findings<sup>30</sup>, phenotypic changes upon inhibition of Myc included cell cycle arrest in G1 phase as well as a decrease in cell size and protein and RNA content per cell (FIGURE 1B–E). On the basis of the expression pattern of several known Myc-regulated mRNAs<sup>31</sup>, we selected 2 independent biologic replicates at the 4 and 24 hours time points after Myc induction and the steady-state MycOFF and MycON samples for lncRNA profiling (FIGURE 1F). Expression profiling was performed using a

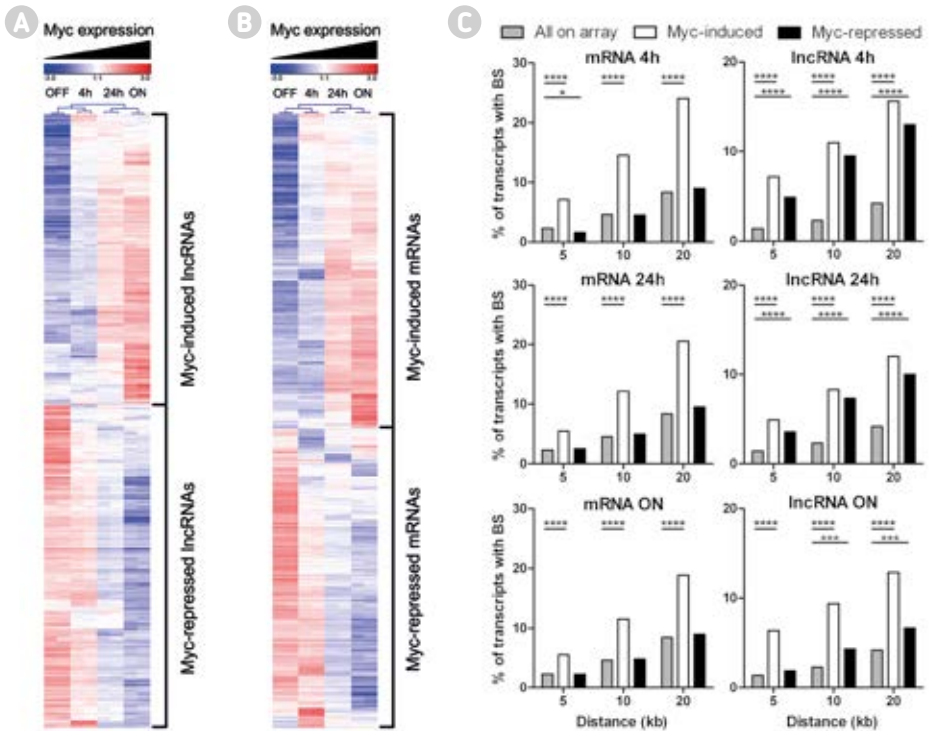




custom designed microarray that covers >10,000 intergenic lncRNAs<sup>21</sup>, ~1,300 known lncRNAs, and all known protein coding genes. In concordance with the literature<sup>32</sup>, we observed a lower median expression level of lncRNAs compared to that of mRNAs (data not shown). We identified 6,555 mRNA and 2,014 lncRNA probes (1,244 lncRNA loci) with a significant differential expression between MycOFF and any of the 3 MycON time points (1-way ANOVA, false discovery rate [FDR] <0.05, fold change  $\geq 2$ ). GSEA and GO analysis of the Myc-regulated mRNAs revealed a significant enrichment of a previously identified Myc-responsive protein-coding gene set<sup>33</sup> and Myc-related gene ontologies<sup>1</sup> (FIGURE S1A; data not shown). This confirms that our samples indeed had the expected Myc-dependent gene expression profile. Unsupervised hierarchical clustering of the 2,014 differentially expressed lncRNA probes (47% Myc-induced and 53% Myc-repressed) revealed a pairwise clustering of MycOFF with Myc at 4 hours and of Myc at 24 hours with MycON samples (FIGURE 2A). An early Myc response (4 hours) was observed for 30% of the lncRNA probes, whereas 56% showed a Myc-induced change in expression within 24 hours. Similar Myc response patterns were observed for mRNAs (FIGURE 2B).



**FIGURE 1** Effects of Myc in P493-6 cells. **(A)** Myc protein levels are strongly increased upon release from tetracycline-mediated repression (MycOFF; 72 hours tetracycline; 0.1  $\mu\text{g}/\text{ml}$ ). A representative Western blot is shown. **(B)** Downstream effects of Myc repression include cell cycle arrest in G1 phase and a decrease in cell size **(C)**, RNA **(D)**, and protein content per cell **(E)**. Two independent experiments were performed. Within each independent experiment, samples were treated and analyzed in duplicate; mean  $\pm$  sd. Cell cycle distributions of P493-6 cells were analyzed using propidium iodide staining; Forward scatter of live cells in complete medium was measured to determine cell size. **(F)** qRT-PCR analysis for Myc and 3 known Myc-induced target genes revealed that expression of Myc target genes is maximal within 24 hours after Myc induction. Relative expression on the y axis is depicted as  $2^{-\Delta\text{Ct}} \times 10^6$ . 18S was used for normalization.



**FIGURE 2** LncRNAs are a main component of the Myc network. **(A)** Unsupervised hierarchical clustering of the 2,014 significantly differentially expressed lncRNA probes (FDR <0.05, fold change  $\geq 2$ ) in P493-6 samples (4 time points, each consisting of 2 independent biologic replicates). The heat map shows that MycOFF samples cluster with Myc at 4 hours, separate from the Myc at 24 h and the MycON samples. **(B)** Same analysis as in (A), but for the 6,555 differentially expressed mRNA probes. **(C)** Myc binding sites are enriched in Myc-induced mRNAs and in Myc-induced and -repressed lncRNAs, but not in Myc-repressed mRNAs. The percentage of Myc-induced or -repressed mRNA and lncRNA transcripts with a Myc binding site in close proximity (5, 10, or 20 kb) was calculated using previously published data of P493-6 cells. A similar result was obtained with a Myc binding site set identified in 5 BL cell lines (data not shown). Distances refer to the center of the binding site and location of the probe. As a control, the percentage of Myc binding sites within 5, 10, or 20 kb of all mRNA and lncRNA probes present on the array was calculated. Significant enrichment of Myc binding sites compared to the control is calculated by the chi-square test; significance is indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .

As a validation, we performed qRT-PCR and confirmed the Myc-induced up-regulation for 10 out of 10 successfully designed primer sets (FIGURE S1B). These results demonstrate that lncRNAs are an important component of the Myc-regulated transcriptional program.

### 3.2 Myc enhances lncRNA levels rather than causing de novo induction

Two studies argued that in general Myc acts as an amplifier of already expressed protein-coding genes rather than inducing de novo transcription<sup>26,34</sup>. In line with these

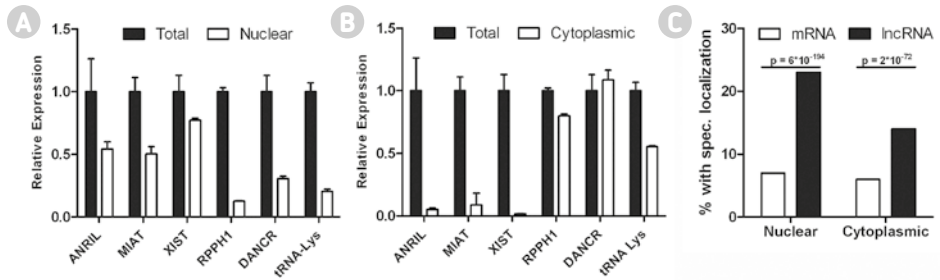
findings, we observed that most (94%) of the Myc-induced protein-coding genes were already expressed in the MycOFF state, albeit at lower levels. A similar pattern was observed for Myc-induced lncRNAs, with 92% of the Myc-induced transcripts already being expressed in the MycOFF samples. The few putative de novo-induced lncRNAs and mRNAs showed expression levels that were close to the detection limit of the array in the MycON samples. These data demonstrate that in general, Myc-induced mRNAs and lncRNAs are already expressed when Myc levels are low.

### 3.3 Myc-regulated lncRNA loci are enriched for Myc binding sites

To confirm a direct Myc regulation of the differentially expressed lncRNAs and mRNAs, we assessed the presence of Myc binding sites using 2 previously generated Myc ChIP data sets<sup>26, 29</sup>. The distances between the Myc binding sites and the differentially expressed mRNAs and lncRNAs at the different time points were determined. As a control, the distances between the Myc binding sites and all lncRNAs or mRNAs on the array were calculated. Myc-induced mRNA transcripts showed consistent, highly significant enrichment for binding sites compared to control. In contrast, Myc-repressed mRNAs did not show any enrichment for Myc binding sites identified in P493-6 (FIGURE 2C) or only a minor enrichment for binding sites identified in BL cell lines (data not shown). For Myc-induced lncRNAs, a significant enrichment of Myc binding sites similar to that of Myc-induced mRNAs was observed. Interestingly, the Myc-repressed lncRNA set also showed a significant enrichment for Myc binding sites, especially in the 4 and 24 hours samples. Thus, Myc-induced lncRNAs and mRNAs as well as early Myc-repressed lncRNAs are enriched for Myc binding sites, whereas Myc-repressed mRNAs are not.

### 3.4 lncRNAs more often have a specific subcellular localization

The subcellular localization of lncRNAs may give a first indication of their putative function. For instance, a nuclear localization has been reported for myocardial infarction associated transcript, involved in splicing, and for ANRIL and X-inactive specific transcript, involved in epigenetic transcriptional control<sup>35</sup>. To study the subcellular localization of Myc-regulated lncRNAs, we analyzed cytoplasmic and nuclear fractions of P493-6 cells. As a control for the isolation of the fractions, 6 transcripts with a known subcellular localization were analyzed and showed the expected enrichment and depletion in the respective fractions. (FIGURE 3A AND B). lncRNAs significantly more often showed a specific subcellular localization compared to mRNAs (37% vs. 13%, FIGURE 3C). Of the lncRNAs with a specific subcellular localization, >60% showed enrichment in the nuclear fraction. Within the Myc-regulated lncRNAs, a strong prevalence for nuclear enrichment could be observed for Myc-repressed lncRNAs. For Myc-regulated mRNAs, a similar trend was observed, although less pronounced (data not shown).



**FIGURE 3** LncRNAs more often show a specific subcellular localization. To validate the isolation of nuclear (A) and cytoplasmic fractions (B), the enrichment of 3 nuclear (ANRIL, MIAT, XIST) and 3 cytoplasmic (RPPH1, DANCR, tRNA-Lys) RNAs was analyzed by qRT-PCR.  $\Delta\Delta Ct$  values are shown with total fractions set to 1; mean  $\pm$  se. 18S and U3 were used for normalization of cytoplasmic and nuclear fractions, respectively. (C) Percentages of all mRNAs and lncRNAs expressed in P493-6 with a specific subcellular localization as determined by microarray. Significance was calculated by chi-square test.

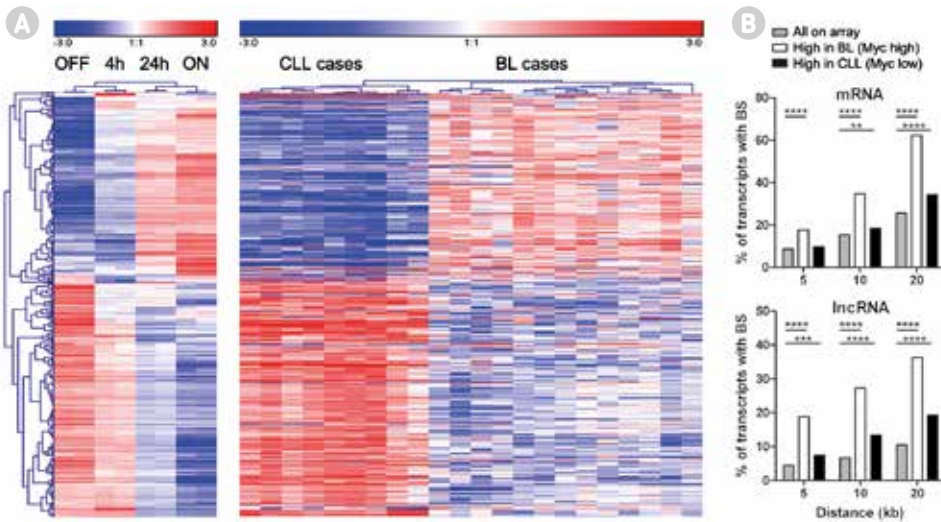
### 3.5 Expression correlation of lncRNAs and mRNAs in close vicinity

Because multiple lncRNAs have been shown to positively or negatively influence gene expression of neighboring genes in *cis* (reviewed in<sup>36</sup>), we explored putative lncRNA-mediated *cis* regulation. We defined putative *cis* regulation as juxtaposed pairs of Myc-regulated lncRNAs and Myc-regulated mRNAs within a probe-to-probe distance of 20kb. Using these settings, we identified 105 *cis*-regulatory lncRNA candidates (TABLE S2), with a sense orientation for 38, a tail to tail for 36, a head to head for 18, an antisense for 12, and an intronic localization for one lncRNA-mRNA pair. The direction of expression was concordantly up- and downregulated for 41 and 32 pairs, respectively. For 32 (30%) of the 105 pairs, we observed an inverse correlation. Inverse correlations were more often observed within the lncRNA sets with antisense and tail-to-tail orientations (respectively, 42% and 36%) and less within the sets with sense and head-to-head orientations (respectively, 23% and 22%).

### 3.6 Myc regulated lncRNAs in primary B-cell lymphoma

Next, we determined to what extent the Myc-regulated lncRNAs are deregulated in primary Myc associated B-cell lymphoma. To this end, we compared the lncRNA expression profiles of BL with those of chronic lymphocytic leukemia (CLL) samples, which are characterized by high and low Myc levels, respectively (median normalized intensity values for Myc 13,036 and 926, respectively). A total of 974 lncRNA probes were differentially expressed between BL and CLL cases (moderated t test, FDR < 0.05, fold change  $\geq 2$ ), with 319 being upregulated and 655 downregulated. Of these 974 lncRNA probes, 498 (54%) were identified as Myc-regulated lncRNAs in the P493-6 model (45 were not expressed in P493-6). More than 93% of the 498 lncRNAs showed the expected expression pattern, i.e., high levels of the Myc-induced lncRNAs in

BL compared to CLL and *vice versa* (FIGURE 4A). Similar to the lncRNAs differentially expressed in the P493-6 model, we also observed significant enrichment of Myc binding sites for both lncRNAs with increased and decreased expression levels in BL compared to CLL (FIGURE 4B). For mRNAs, a significant Myc binding site enrichment was observed for the genes with high expression levels in BL compared to CLL. For mRNAs with low levels in BL, a less pronounced Myc binding site enrichment was observed, in line with the P493-6 results. To confirm a Myc-dependent regulation in BL, we analyzed the expression of the validated Myc-induced lncRNAs in BL cell lines ST486 and DG75 treated with 2 different shRNA constructs against Myc. This revealed for 5 out of 9 (ST486) and 7 out of 7 (DG75) expressed lncRNAs the expected pattern of decreased levels upon Myc knockdown (FIG. 51C TO F).



**FIGURE 4 Myc-regulated lncRNAs show consistent expression patterns in primary B-cell lymphoma. (A)** Heat maps of the 498 overlapping lncRNAs that are Myc regulated in P493-6 cells and differentially expressed between BL (high Myc) and CLL (low Myc) cases. At left is the unsupervised hierarchical clustering of this lncRNA set in P493-6 samples; at right is the clustering with the same order of lncRNAs for primary BL and CLL cases. BL cases cluster separately from CLL cases. Most probes showed the expected increased levels in BL for Myc-induced lncRNAs and *vice versa* for the Myc-repressed lncRNAs. **(B)** Myc binding site proximity analysis for all mRNAs (3,204) and lncRNAs (974) differentially expressed between primary cases of BL and CLL using published Myc ChIP-seq data obtained from 5 BL cell lines. Myc-induced mRNAs and Myc-induced and -repressed lncRNAs are consistently enriched for Myc binding sites. Myc-repressed mRNAs show a less pronounced enrichment for Myc binding sites that is significant with lncRNA probe-Myc binding site distances within 10 or 20 kb. Significant enrichment of Myc binding sites compared to the control is calculated by the chi-square test; significance is indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .

## 4 Discussion

This study shows that lncRNAs, next to mRNAs and microRNAs, are an important component of the Myc-regulated gene expression network. In total, we identified 1,244 lncRNA loci that were regulated by Myc in the P493-6 model. So far, only 3 lncRNAs have been described to be induced by Myc, i.e., CCAT1-L, H19, and HOTAIR<sup>37,38</sup> [reviewed in<sup>39</sup>]. CCAT1-L and HOTAIR are not expressed in the cell types investigated in this study. H19 is not expressed in P493-6 cells, but it shows a higher expression in BL cases compared CLL cases, in accordance with an up regulation by Myc. Furthermore, our data are in line with a recent study showing that a substantial part of the deregulated lncRNA expression in murine dicer knockout embryonic stem cells can be attributed to deregulated Myc expression<sup>40</sup>. Next to lncRNAs that are regulated by c-Myc (which we referred to throughout as Myc), a few lncRNAs have also been described to be controlled by the structurally related n-Myc protein. Three transcribed ultraconserved regions (T-UCRs) were induced, and long intergenic noncoding (linc) 00467 was shown to be repressed by n-Myc<sup>41,42</sup>. No probes were available on our array for the T-UCRs. Linc00467 was approximately 3-fold down-regulated in P493-6 upon Myc induction but showed no clear difference in expression between BL and CLL cases. Thus, this is the first study to report a comprehensive overview of the lncRNAs regulated by Myc.

One of the remarkable findings in this study is the enrichment of Myc binding sites for Myc-repressed lncRNAs, indicating direct regulation by Myc. The lack of binding site enrichment for Myc-repressed mRNAs suggests that these transcripts are in general not directly targeted by Myc. To what extent Myc can repress the expression of mRNAs is currently under debate. Two studies proposed that Myc acts as an amplifier of practically all expressed genes without obvious specificity<sup>26, 34</sup>. However, 2 recent studies argued for a model in which Myc can directly induce and repress specific genes<sup>43,44</sup>. The globally increased RNA production was proposed to be an indirect effect due to the regulation of genes involved in RNA synthesis. In these 2 recent studies, the investigators did not discriminate between protein-coding and noncoding genes, which possibly explains why no noticeable difference in Myc binding was observed between induced and repressed target genes. Another factor that may explain this dissimilarity is the differences in the amount of Myc binding sites defined during Myc overexpression in the recent studies<sup>43, 44</sup> (30,000 and 45,645 sites) compared to the 2 data sets<sup>26,29</sup> we used (2,398 and 7,054 sites). Because Myc first occupies high-affinity target genes before binding to lower-affinity targets<sup>1, 44</sup>, it is likely that our smaller sets are more enriched for high-affinity genes. Possible enrichment of Myc-repressed lncRNAs and/or depletion of Myc-repressed mRNAs within the high-affinity Myc target gene set compared to the low-affinity target gene set could explain the observed differences in direct Myc binding between Myc-repressed mRNAs and lncRNAs. Thus, although the reason for the marked difference between Myc-repressed mRNAs and lncRNAs in our study is yet unclear, it suggests a crucial role for both Myc-induced and Myc-repressed lncRNAs.



Expression analysis of nuclear, cytoplasmic, and total fractions revealed that lncRNAs, compared to mRNAs, more often have a specific subcellular localization. In line with other studies<sup>21,32,45</sup>, a high number of lncRNAs were enriched in the nucleus. It has been reported that nuclear lncRNAs, similar to nuclear mRNAs, are significantly less stable than cytoplasmic transcripts or transcripts without specific localization<sup>45</sup>. Moreover, stable mRNAs are frequently involved in housekeeping or metabolic functions, while unstable mRNA species are involved in gene regulatory functions that require a fast response to external or internal stimuli and thus a rapid turnover<sup>45, 46</sup>. The frequent nuclear localization, combined with the observed Myc binding site enrichment of Myc-repressed lncRNAs, suggests that Myc directly down-regulates lncRNAs that regulate dynamic nuclear processes.

The parallel analysis of lncRNA and mRNA expression in P493-6 upon Myc induction identified >100 differentially expressed mRNAs that are directly adjacent to differentially expressed lncRNAs. Modulation of lncRNA expression at the endogenous locus in order to determine effects on the neighboring gene or genes should indicate whether these lncRNAs can indeed regulate gene expression in *cis*. The fact that we observed an inverse correlation in expression upon Myc induction between the lncRNA and mRNA for 30% of the pairs indicates that at least for this group, the changes in expression are not simply due to an open or closed local chromatin structure and supports putative regulation in *cis*.

To support the relevance of the identified lncRNAs in P493-6, we studied lncRNA expression in primary lymphomas characterized by high and low Myc levels. More than half of the lncRNAs differentially expressed in lymphoma were also identified as Myc-regulated in P493-6, indicating the potential relevance of these lncRNAs. We selected CLL as an example of a Myc-low lymphoma; although not tested, we expect similar results when compared to other Myc-low lymphoma subtypes, such as follicular or mantle cell lymphoma. The relevance of Myc in establishing this Myc-dependent profile in BL was further confirmed by analysis of lncRNA expression levels on shRNA-based inhibition of Myc in BL cell lines. At present, the diagnostic utility of the identified lncRNAs is uncertain. However, it might be of diagnostic value in diffuse large B-cell lymphoma. Genes that are Myc-responsive have been used to determine the Myc activity, and this was shown to be an independent negative prognostic factor in diffuse large B-cell lymphoma<sup>47</sup>.

It is well known that inhibition of Myc in BL cell lines strongly impairs their growth<sup>48</sup> (data not shown). Because Myc regulates many mRNAs and microRNAs, it is intriguing that reversing the effect of Myc on the expression of a single Myc-regulated gene can have a strong negative effect on cell growth<sup>49,50</sup>. This strongly suggests that Myc has to orchestrate a wide range of genes in specific directions to be able to exert its effects on cell growth. Known functions of Myc relevant in oncogenesis include progression of cell cycle, blocking differentiation, and promoting transformation<sup>2</sup>. It may be expected

that several of the Myc-regulated lncRNAs identified in this study are essential in the above-mentioned processes.

In summary, this study shows that lncRNAs are a main component of the transcriptional program regulated by Myc. We identified >1,200 Myc-regulated lncRNAs, with 105 having putative *cis*-regulatory functions. In contrast to Myc-repressed mRNAs, Myc-repressed lncRNAs are potentially directly regulated by Myc and are often enriched in the nucleus. A large number of the Myc-regulated lncRNAs are differentially expressed in primary cases of B-cell lymphoma with high and low levels of Myc and are responsive to Myc knockdown in BL cell lines, confirming their relevance for Myc-associated B-cell lymphomas.





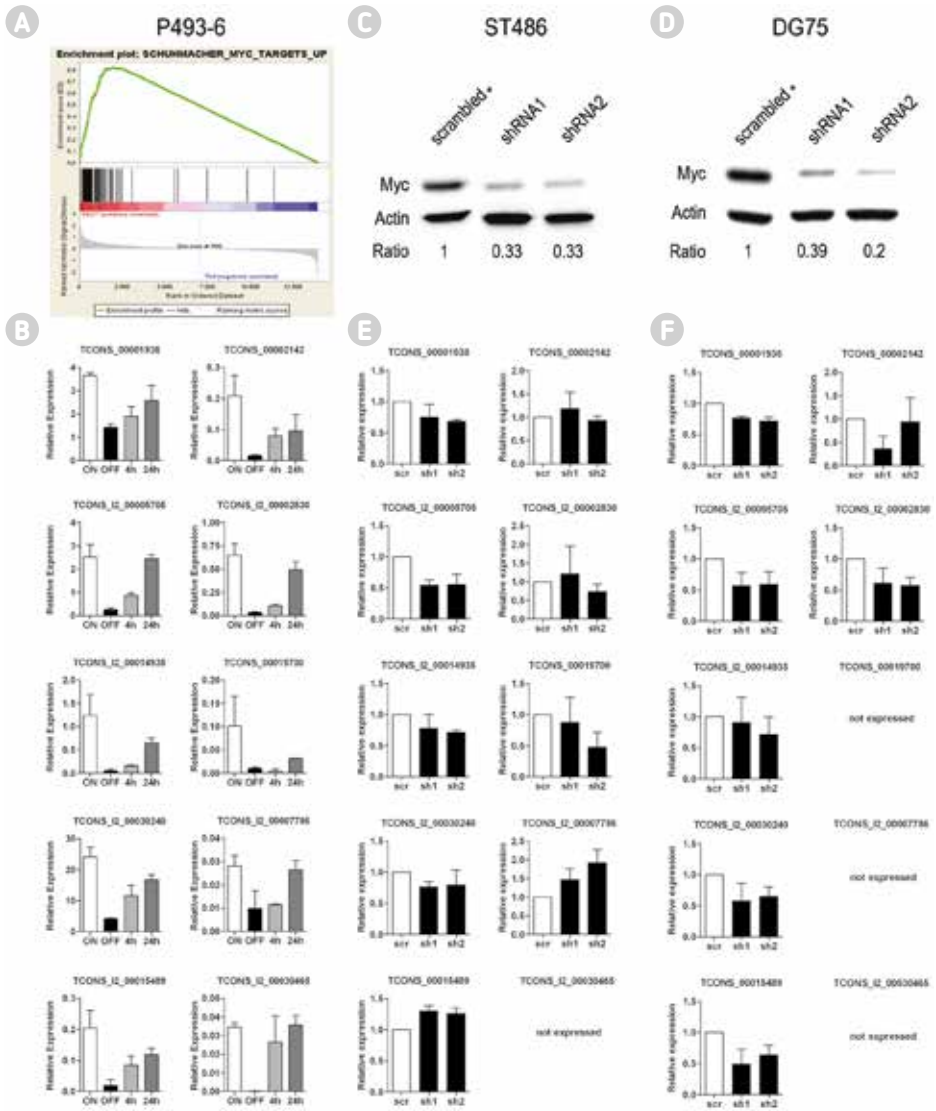
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**FIGURE S1 Validation of Myc-regulated lncRNAs by qRT-PCR. (A)** Gene set enrichment analysis shows strong enrichment for a previously identified Myc-induced target gene set in P493-6 cells with high Myc expression (FDR < 0.001; shown is MycOFF ["Tet"] against 4h, 24h and MycON ["REST"]). **(B)** Enhanced lncRNA levels were confirmed by qRT-PCR for 10 Myc-induced lncRNAs. For each time point the mean  $\pm$  error is shown. Relative expression on the y-axis is depicted as the  $2^{-\Delta Ct} \times 10^6$ . 18S served as endogenous control for normalization. **(C)** Representative Western blot for Myc on BL cell lines ST486 and DG75 **(D)** infected with 2 different shRNAs against Myc or a scrambled non-targeting sequence. \* indicates that this part was assembled next to another part of the same blot. **(E)** qRT-PCR results for the 10 validated Myc-induced lncRNAs in Myc shRNA infected ST486 and DG75 cells **(F)**. For ST486 1, and for DG75 3, lncRNAs were not expressed (Cp > 35). The shown lncRNA quantification is an average of 3 independent biological replicates (for shRNA2 infected ST486 only 2 replicates). 18S was used for normalization and  $2^{-\Delta Ct}$  was calculated and multiplied by  $10^6$ . For each independent biological replicate the scrambled control (SCR) was set to 1. Significance was calculated by t-test.

TABLE S1 Primer and shRNA sequences.

<b>18S</b>	F: 5'-CGGCTACCATCAAGGA-3' R: 5'-CCAATTACAGGGCTCGAAA-3'
<b>RPII</b>	F: 5'-CGTACGCACCAGTCCAAT-3' R: 5'-CAAGAGAGCCAAGTTCGGTAA-3'
<b>lRNA Lys</b>	F: 5'-CGGCTAGCTCAGTCGGTAGA-3' R: 5'-CCAACGTGGGGCTCGAAC-3'
<b>RPPH1</b>	F: 5'-AGCTTGGAACAGACTCACGG-3' R: 5'-AATGGGCGGAGGAGAGTAGT-3'
<b>DANCR</b>	F: 5'-CGTCTCTTACGTCTGCGGAA-3' R: 5'-TGGCTTGTGCCTGTAGTTGT-3'
<b>U3 SNORNA</b>	F: 5'-AACCCCGAGGAAGAGAGGTA-3' R: 5'-CACTCCCAATACGGAGAGA-3'
<b>ANRIL</b>	F: 5'-AAGCCGCTCCGCTCCTTCT-3' R: 5'-GCCGTGTCCAGATGTCGCGT-3'
<b>MIAT</b>	F: 5'-TGGAGGCATCTGTCCACCATGT-3' R: 5'-CCCTGTGATGCCGACGGGT-3'
<b>XIST</b>	F: 5'-GTCCTTTCTTTTGACCCAGAA-3' R: 5'-GAGCCTGGCACTTTTTTTTCC-3'
<b>TCONS_00001938</b>	F: 5'-CCAGGTTGCTCTGCTTAC-3' R: 5'-TCTATCTCACCACCGTAAAAC-3'
<b>TCONS_00002124</b>	F: 5'-TAGCCGTGAGATGCTACTGAC-3' R: 5'-CAAACAACCCAGCACTAGG-3'
<b>TCONS_00019700</b>	F: 5'-GGGAGAGTATATTAACAGGGCTTG-3' R: 5'-TTGACTTGGTCTGGCTTTC-3'
<b>TCONS_00002830</b>	F: 5'-TGGCCCTTCAAACCTGGAT-3' R: 5'-CAAGGACAGCAGCTGGTAGGT-3'
<b>TCONS_L2_00015489</b>	F: 5'-CATCTCTCAGCTGTGTTGA-3' R: 5'-CATTGGAGCCATACTGTTGAACC-3'
<b>TCONS_L2-00014935</b>	F: 5'-CGTCAAGCTCAGGTGATGG-3' R: 5'-AGCTTCTTGGGACGGAAGTG-3'
<b>TCONS_L2_00005705</b>	F: 5'-GTTGTTGATGCTGTAATTGCTGAA-3' R: 5'-GTTTTCCATCCTGTGCAATTC-3'
<b>TCONS_L2_00007786</b>	F: 5'-GGGGTTATTTGTCATTACAATTTGG-3' R: 5'-GTAAGGTAACCATTAAGCCTGC-3'
<b>TCONS_L2_00030240</b>	F: 5'-CACACTCAAGGAAACGCAA-3' R: 5'-GGGATGACTGACCTCCTTACC-3'
<b>C-MYC</b>	TAQMAN ASSAY: HS00153408_M1
<b>CAD</b>	TAQMAN ASSAY: HS00188977_M1
<b>PGK1</b>	TAQMAN ASSAY: HS00943178_G1
<b>TFAM</b>	TAQMAN ASSAY: HS01082775_M1
<b>MYC SH1</b>	S: 5'-GATCCGATGAGGAAGAAATCGATGTTCAAGAGACATCGATTTCT TCCTCATCTTTTG-3' AS: 5'-AATTCAAAAAGATGAGGAAGAAATCGATGCTCTTGAACATCGATT TCTTCTCATCG-3'
<b>MYC SH2</b>	S: 5'-GATCAACGACGAGAACAGTTGAAACATTCAAGAGATGTTTCAA CTGTTCTCGTCGTTTTTTTG-3' AS: 5'-AATTCAAAAACGACGAGAACAGTTGAAACATCTCTTGAATG TTTCAACTGTTCTCGTCGTTG-3'

TABLE S2 List of putative *cis*-acting lncRNAs.

Chr	lncRNA		mRNA	lncRNA to mRNA			
	Probe name	Transcript(s)		Gene Symbol	Distance (kb)	Orientation	Direction
1	CUST_4109_P1427622066	TCONS_I2_00001619	A_24_P942030*; A_33_P3409904	VAMP4	-17	sense	up/down
1	CUST_4136_P1427622066	TCONS_00002518	A_24_P28295	RABGAP1L	8	tail-to-tail	up/down
1	A_23_P37245	NR_037869.1/LOC653160	A_24_P370670	ZMYM6NB	-8	sense	down/down
1	CUST_983_P1427622066	TCONS_I2_00001960,TCONS_I2_00000051,-2,-3	A_23_P126908	TNFRSF14	13	sense	down/down
1	CUST_1765_P1427622066	TCONS_I2_00001143	A_23_P97021	AG03	7	tail-to-tail	down/down
1	CUST_4192_P1427622066	TCONS_00002525	A_33_P3314386	TOR1AIP1	7	tail-to-tail	down/down
1	CUST_4136_P1427622066	TCONS_00002518	A_24_P84428*; A_32_P114574; A_24_P45379	CACYPB	-7	head-to-head	up/up
1	CUST_983_P1427622068	TCONS_00001938	A_33_P3300395	AP1TD1	17	sense	up/up
1	<b>CUST_2653_P1427622068</b>	<b>TCONS_00002142</b>	<b>A_33_P3256685</b>	<b>TF2</b>	<b>2</b>	<b>sense</b>	<b>up/up</b>
1	CUST_2084_P1427622066	TCONS_I2_00001240	A_24_P276791	LRRCA2	14	tail-to-tail	up/up
1	A_33_P3326285	TCONS_00000336, NR_037605.1 / GAS5-AS1	A_23_P148984	DARS2	6	tail-to-tail	up/up
10	A_33_P3400152	NR_038444.1 / ENT1P1-AS1	A_33_P3218980*; A_33_P3218975	ENT1P1	-1	antisense	up/down
11	CUST_43006_P1427622066	TCONS_00019700	A_23_P36305	ATG16L2	-17	head-to-head	up/down
11	CUST_34393_P1427622068	TCONS_00019181	A_23_P172737*; A_33_P3423941	IFITM1	18	head-to-head	down/down
11	CUST_34393_P1427622068	TCONS_00019181	A_23_P87545	IFITM3	14	head-to-head	down/down
11	A_23_P127367	NR_046413.1 / POLD4	A_23_P138760	CLCF1	-13	sense	down/down
11	CUST_42918_P1427622066	TCONS_00019150	A_23_P115955	MRPL21	-19	tail-to-tail	down/up
11	CUST_35166_P1427622068	TCONS_00019374	A_23_P244444	DHCR7	14	head-to-head	up/up
11	CUST_34618_P1427622068	TCONS_00019861	A_33_P3356270	NCR3LG1	4	sense	up/up
11	A_24_P785293	NR_003098.1 / SNHG1	A_23_P36157	WDR74	18	sense	up/up
12	A_33_P3308585	NR_026947.1 / CIRL-AS1	A_23_P363968	CIRL	17	antisense	down/down
12	CUST_45502_P1427622066	TCONS_00021409	A_24_P124662	MAPKAP5	1	tail-to-tail	down/up
12	CUST_43854_P1427622066	TCONS_00020209,-10,-12,-13	A_23_P53363	XRCC68P1	-17	head-to-head	up/up
12	CUST_45606_P1427622066	TCONS_00021410,-11,-13,-14	A_23_P13604	PEBP1	-10	head-to-head	up/up
12	A_33_P3339336	TCONS_I2_00006623	A_23_P151059	FAM90A1	10	sense	up/up
14	CUST_40347_P1427622068	TCONS_00022531	A_23_P313632	FUT8	11	sense	down/down
14	A_32_P982475	NR_003138.3 / SNHG10	A_23_P65370	GLRX5	-11	head-to-head	up/up

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Chr	IncRNA		mRNA		IncRNA to mRNA		
	Probe name	Transcript(s)	Probe name	Gene Symbol	Distance (kb)	Orientation	Direction
15	CUST_42699_P1427622068	TCONS_00023239	A_23_P77228	CRTC3	20	sense	up/down
15	CUST_42499_P1427622068	TCONS_00023489	A_32_P99902	C15orf40	5	head-to-head	up/up
15	A_24_P655268	TCONS_00023383, -84, TCONS_00023186, TCONS_00023648, TCONS_00024099, OIP5-AS1	A_23_P379614	OIP5	-10	tail-to-tail	up/up
16	A_33_P3294372	NR_0364801 / VPS9D1-AS1	A_23_P129476	VPS9D1	11	antisense	up/down
16	CUST_43643_P1427622068	TCONS_I2_00009664	A_23_P256375	STX4	3	sense	down/down
16	CUST_43643_P1427622068	TCONS_I2_00010403, TCONS_I2_00009664	A_33_P3290487	ZNF668	-18	tail-to-tail	down/up
16	CUST_44622_P1427622068	TCONS_00024899, -900	A_23_P89030	C16orf95	15	head-to-head	up/up
16	A_32_P101699	RP11-505K9.1	A_23_P206324	HSDL1	-5	tail-to-tail	up/up
16	CUST_44622_P1427622068	TCONS_00024899, -900	A_23_P395566	FBXO31	-11	tail-to-tail	up/up
17	A_23_P363896	NR_0450291 / FAM211A-AS1	A_23_P207911	TRPV2	5	sense	up/down
17	A_23_P207319	NR_110324-1 / MAP3K14-AS1	A_24_P390668*, A_33_P3382303	FMNL1	17	sense	down/down
17	A_24_P857624	NR_0400711 / TNRC6C-AS1	A_23_P101013	TMC6	-8	tail-to-tail	down/down
17	CUST_45182_P1427622068	TCONS_I2_00011626	A_32_P452655	LGALS9C	16	tail-to-tail	down/down
17	CUST_54782_P1427622066	TCONS_I2_00011614, TCONS_I2_00011082, LINC00324	A_23_P130182	AURKB	14	sense	down/up
17	CUST_45655_P1427622068	TCONS_00025374, -5, -6, -7	A_33_P3315801	CDK12	20	sense	down/up
17	CUST_54652_P1427622066	TCONS_00025268	A_24_P333444	YWHAE	-14	tail-to-tail	down/up
17	CUST_46307_P1427622068	TCONS_00025488	A_33_P3358099	CD300E	-3	tail-to-tail	down/up
17	CUST_55852_P1427622066	TCONS_00025238	A_23_P152807	RSAD1	18	tail-to-tail	down/up
17	A_23_P363896	NR_0450291 / FAM211A-AS1	A_33_P258339	FAM211A	-1	antisense	up/up
17	A_33_P3258712	AC012146.7	A_23_P4294	ZNF232	8	antisense	up/up
17	A_33_P3269869	NR_028335.1 / LOC284-009	A_33_P3246733	MNT	19	sense	up/up
17	A_33_P3238543	TCONS_00025107, TCONS_00025525	A_23_P130194	PYCR1	-2	tail-to-tail	up/up
19	CUST_59469_P1427622066	TCONS_I2_00012778, -79, -80, TCONS_I2_00013348, TCONS_I2_00013382	A_33_P3243168	MZF1	19	antisense	up/down
19	CUST_58919_P1427622066	TCONS_I2_00013069, -72, -75, -76, -79, -80, -81, -82, -83, 84	A_24_P186342	ZFP14	-5	sense	up/down
19	CUST_58139_P1427622066	TCONS_00027673, TCONS_00027183, -84	A_24_P10657*, A_33_P3330549	SLC44A2	9	tail-to-tail	up/down
19	CUST_48715_P1427622068	TCONS_I2_00012703, -04	A_33_P3267799	LILRB4	4	sense	down/down
19	A_33_P3222753	TCONS_I2_00012668, TCONS_I2_00013339	A_33_P3256868	ZNF83	-13	tail-to-tail	down/down





Chr	IncRNA		mRNA	Gene Symbol		IncRNA to mRNA		Direction
	Probe name	Transcript(s)		Probe name	Gene Symbol	Distance (kb)	Orientation	
4	CUST_13927_P1427622068	TCONS_I2_00021578,-79	A_33_P3396607	UGDH	-17	tail-to-tail	up/up	
4	CUST_15077_P1427622068	TCONS_00007658, TCONS_00008904	A_23_P92552	PET112	-2	tail-to-tail	up/up	
6	A_24_P359067	LOC100130357	A_24_P943566	PHACTR1	4	antisense	down/down	
6	CUST_26643_P1427622066	TCONS_I2_00024520	A_23_P110961*; A_33_P3242873	MPC1	-17	tail-to-tail	down/up	
7	A_33_P3734384	RP4-647J21.1	A_24_P206343	MYO1G	-6	sense	down/down	
7	CUST_23585_P1427622068	TCONS_I2_00025931	A_32_P46191	ZNF727	6	sense	down/down	
7	CUST_24621_P1427622068	TCONS_00014220	A_23_P215132	WDR91	-15	tail-to-tail	down/down	
7	A_33_P3565787	PSMG3-AS1	A_24_P21044	PSMG3	19	antisense	down/up	
7	A_33_P3333480	LINC00035	A_23_P8558*; A_23_P362712	ABHD11	-2	tail-to-tail	down/up	
7	A_33_P3264042	TCONS_I2_00026188	A_23_P20022	HILPDA	11	sense	up/up	
7	CUST_22947_P1427622068	TCONS_I2_00025783	A_23_P93750	LSM5	-15	tail-to-tail	up/up	
8	CUST_31324_P1427622066	TCONS_00014599,-600,-601,-602, TCONS_00015212,-13	A_24_P201064	PPPR3B	20	head-to-head	up/down	
8	A_23_P149050	RP5-855D21.3	A_32_P187663	ZNF596	-12	intronic	up/down	
8	CUST_31578_P1427622066	TCONS_I2_00028127,-28	A_33_P3396951	BNIP3L	-13	head-to-head	down/down	
8	CUST_25688_P1427622068	TCONS_00015252	A_23_P157495	PPP3CC	5	sense	down/down	
8	CUST_31606_P1427622066	TCONS_00015261,-62	A_23_P134684*; A_33_P3276638	HMBOX1	13	sense	down/down	
8	CUST_37186_P1427622066	TCONS_00014558	A_23_P146187	RRS1	-4	head-to-head	down/up	
8	CUST_32946_P1427622066	TCONS_00015189,-90	A_23_P60101	ZNF696	-16	tail-to-tail	up/up	
9	CUST_35863_P1427622066	TCONS_00016695	A_23_P390148	RALGPS1	13	sense	up/down	
9	CUST_27271_P1427622068	TCONS_I2_00028588	A_23_P11744	WASH1	-1	tail-to-tail	up/down	
9	CUST_35880_P1427622066	TCONS_00016871,-73, TCONS_00015886	A_24_P355267	SLC25A25	9	tail-to-tail	up/down	
9	CUST_27671_P1427622068	TCONS_00015664	A_23_P123622	NPR2	-19	sense	down/down	
9	A_33_P3753757	RP11-401.2	A_23_P216610	SUSD1	-7	sense	down/down	
9	CUST_35880_P1427622066	TCONS_00016871,-73, TCONS_00015886	A_24_P106953	PTGES2	-6	sense	up/up	
9	CUST_29156_P1427622068	TCONS_00015749	A_33_P3260322*; A_33_P3659678	NR6A1	-1	tail-to-tail	up/up	
9	A_24_P352116	SNHG7	A_23_P431305	FAM69B	2	tail-to-tail	up/up	
X	CUST_36360_P1427622066	TCONS_I2_00030147,,48	A_23_P96641	PRPS2	4	sense	up/up	
Y	A_23_P60793	ASMTL-AS1	A_23_P159539	ASMTL	10	antisense	down/up	

\* Probe to probe distance in kb, positive distance means the IncRNA probe is upstream of the protein-coding gene, negative distance indicates the reverse. # The direction of the IncRNA is indicated first, the direction of the mRNA is indicated second. Direction is based on expression in Myc high (i.e. MYC-on, MYC-4h and MYC-24h) vs Myc low (i.e. MYC-off) samples in P493-6 cells.



