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Identification of novel Myc target genes with a potential role in lymphomagenesis

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ABSTRACT

The c-Myc transcription factor regulates a wide set of genes involved in processes such as proliferation, differentiation and apoptosis. Therefore, altered expression of Myc leads to deregulation of a large number of target genes and, as a consequence, to tumorigenesis. For understanding Myc-induced transformation, identification of these target genes is essential. In this study, we searched for Myc target genes involved in lymphomagenesis using different mouse T and B cell lymphoma cell lines transformed by a conditional Myc-allele. Target genes obtained by microarray experiments were further subjected to a kinetic analysis of mRNA expression upon Myc inactivation/reactivation, bioinformatic examination of Myc binding sites and chromatin immunoprecipitation. This approach allowed us to define targets whose activation is a direct consequence of Myc binding. Among the 38 novel Myc targets, we identified several genes implicated in the tumor development. These genes are not only relevant for mouse lymphomas because we observed their upregulation in human lymphomas as well. Our findings further the understanding of Myc-induced lymphomagenesis and help toward developing more efficient antitumor strategies.

INTRODUCTION

The c-Myc (Myc) proto-oncogene belongs to a family of related proteins that also include N-Myc, L-Myc, B-Myc and s-Myc (1); however, only c-Myc, N-Myc and L-Myc possess neoplastic potential (2,3). Under physiological conditions, Myc is broadly expressed during embryogenesis, as well as in adult tissues with high proliferative capacity. A null mutation in the Myc gene causes lethality at day 10.5 of gestation, which suggests that, it is critical for early development (4). Generally, Myc expression correlates closely with the proliferation status of a cell: in quiescent cells, Myc is almost undetectable whereas, upon mitogen stimulation, mRNA and protein levels are rapidly induced (3). Cell cycle, differentiation, apoptosis, metabolism, cell adhesion, and hematopoietic homeostasis are key processes that are regulated by Myc (5–9). Altered expression of Myc was found in a wide variety of human and animal tumors including breast carcinomas, colon carcinomas, cervical carcinomas, small cell lung carcinomas, osteosarcomas, glioblastomas, myeloid leukemias, and lymphomas (10).

The Myc proto-oncogene encodes a transcription factor with a C-terminal basic-helix–loop–helix/leucine zipper (bHLHZ) domain that promotes DNA and protein–protein interactions (11–13). For its function, Myc needs to dimerize with a related bHLH protein named Max. Myc–Max heterodimers are capable of binding specific DNA sequences, called the E-boxes (14,15). Dimerization with Max and binding to the E-box are essential for Myc to promote gene expression. Max homodimers compete with Myc–Max heterodimers for binding to DNA target sites (16). Mutations in the N-terminal Myc transactivation domain lead to abrogation of Myc activity (17–19). In addition to activation of a large set of genes, Myc has also been shown to repress some genes. The mechanism of Myc repression of target genes is not well understood. Possibly, this repression occurs via interference with transcription factors that is required for gene activation (20,21). In addition, Myc is recruited to core promoters through protein–protein interactions with TFII-I, YY-1, Sp-1 and Miz-1(22). Finally, interactions of Myc with Smad-2 and -3 and NF-Y proteins may contribute to repression of individual promoters (22).

Different approaches toward identification of the Myc target genes have been reported over the last few years (23–30). In essence, they are based on identification of genes that are differentially expressed, as a result of enforced Myc expression or on analysis of regulatory elements of genes that contain Myc–Max consensus binding sites. Since Myc plays such a critical role in a wide variety of physiological and pathophysiological processes, different model systems have been used to investigate target genes. Only a portion of the described genes overlapped in the separate studies. In addition, a huge number of genes that are not dependent on Myc change its expression as a consequence of Myc-induced cell growth and proliferation, differentiation or apoptosis, thus leading to the isolation of indirect targets.

We recently established a transgenic mouse model in which Myc is conditionally expressed in lymphoid cells using the tetracycline-controlled system of gene regulation (31). In these

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mice, the tetracycline-transactivator (tTA) is under the control of the intronic Ig-μ heavy chain enhancer (μE) and a minimal promoter, and is specifically expressed in lymphocytes. This transactivator then activates the transcription of a second transgene cassette consisting of a Myc proto-oncogene and a luciferase reporter gene. In the presence of doxycycline, the expression of both Myc and luciferase are aborted. Double transgenic mice (tTA/Myc mice) develop lymphomas that regress upon doxycycline administration (31).

Different cell lines were established from lymphoma-bearing mice. Further characterization by fluorescence activated cell sorting (FACS) showed expression of cell surface markers that were characteristic for T or B cells. In addition, fluorescence *in situ* hybridization (FISH) analyses revealed that T cell lines could be divided in two subgroups: one containing two copies of chromosome 15 and one with a chromosome 15 trisomy. Doxycycline treatment aborts expression of transgenic Myc mRNA in lymphoma cell lines within 2–8 h. As a consequence, 24–48 h later, the cells change their morphology, stop proliferation and begin to show early signs of apoptosis (31).

Using this system, we aimed at identifying genes commonly expressed in different subtypes of Myc-induced lymphomas. In a step-by-step analysis under stringent conditions, by the use of microarray hybridization, kinetic experiments, bioinformatic analysis and chromatin immunoprecipitation, we were able to identify several novel direct Myc targets with a potential role in lymphomagenesis.

MATERIALS AND METHODS

Cells and culture conditions

The cell lines used in this study were derived from mouse T or B cell lymphomas bearing a conditional human c-Myc allele (31). Two B cell lines, 5522 B and 5532 B and four T cell lines, 4502 T, 1415 T, 4347 T and 7755 T, were used in the Gene Chip experiment. FISH analysis had revealed that T cell lines 4347 T and 7755 T have a trisomy of chromosome 15. An additional set of two B (5542 B and 5527 B) and four T cell lines (4702 T, 377 T, 1749 T and 4491 T) were used to confirm results obtained by microarray hybridization. All the cell lines were established and characterized as described previously (31). The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum(FCS), penicillin/streptomycin, non-essential amino acids, glutamine and beta-mercaptoethanol (all from PAN Biotech, Germany).

To suppress the expression of Myc oncogene, the cells were treated with 2 μg/ml of doxycycline (ICN Biomedicals Inc., OH) for 12 h. For kinetic experiments, the cells were pre-treated with doxycycline for 12 h, extensively washed, and further incubated in cell culture medium without doxycycline for the indicated time intervals.

Human primary B cells were isolated from buffy coats of three healthy donors using B Cell Isolation Kit II (Miltenyi Biotec, Germany) according to the manufacturer's instruction.

Three sporadic European Burkitt lymphomas were included [for details see case number 1, 4 and 6; (32)]. Diagnosis was based on morphology, immunohistology and molecular cytogenetics. All three lymphomas had a diffuse growth pattern of cohesive small to medium sized cells and the typical starry sky pattern. The immunoprofile was: $CD20^+$ (3/3); $CD10^-$ (3/3); $CD38^+$ (2/3); $CD77^-$ (3/3); IgM^+ (3/3); MHC Class I^+ (3/3) and MHC Class II^+ (3/3); $Bcl2^-$ (2/3); $CD23^-$ (3/3). The proliferation index was assessed by Ki-67 staining and was above 90% in all lymphomas. EBV virus was not detected by Western blotting. FISH revealed a Myc rearrangement with a diagnostic signal constellation for the translocation t(8;14) in 2 lymphomas, whereas one lymphoma showed a signal pattern that indicates a breakpoint in the Myc locus with an elusive translocation partner.

Microarray analysis

About 2×10^7 cells were cultured in the presence or absence of doxycycline for 12 h, and RNA was analyzed by Affymetrix expression profiling using the Murine Array Gene Chip U74Av2.

Results obtained by scanning hybridized microarrays were further analyzed using Affymetrix Microarray Suite software Version 5.0.0.032 (Affymetrix Inc., CA) and NETAFFX Analysis Center Tools (Affymetrix Inc.). Quality control and normalization of results were performed using standard Affymetrix test genes and β-actin and GAPDH as housekeeping controls.

All raw data are made MIAME ('Minimal Information About a Microarray Experiment') compliant and submitted to ArrayExpress database (www.ebi.ac.uk/arrayexpress/) with an accession number E-MEXP-169.

Analysis of mRNA expression levels

Total RNA was isolated from cells using High Pure RNA Isolation Kit (Roche, Switzerland) and reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, CA) with random hexamer primers (Roche). The cDNAs were subjected to PCR using *Taq* Polymerase (Pharmacia, UK) and primers specific for indicated genes. As a housekeeping control we used primers specific for β-actin. All primer sequences are available upon request.

Proliferation assay

The cells were incubated for 12 h in the presence or absence of doxycycline. Afterwards, the cells were extensively washed, resuspended in RPMI and stained for 5 min with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, OR) at a final concentration of 2 μM. One aliquot of both treated and untreated cells was used for FACS analysis and the rest was replated in cell culture medium and analyzed after different time periods. Control samples of cells continuously treated with doxycycline were included. Reduction of CFSE fluorescence as a measure of proliferation was monitored at the indicated time points using FACSCalibur and CellQuest software (BD, CA).

Western blot

The cell lysates were prepared using a buffer containing 20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% Glycerol, 1 mM DTT and 1 mM PMSF. The proteins were separated on 12.5% SDS–polyacrylamide gel and blotted on Immobilion-P transfer membrane (Millipore, MA). Membrane blocking and incubation with antibodies involved standard procedures. Immunoblots were

probed with rabbit polyclonal c-Myc (N-262) and anti-RelA (both from Santa Cruz, CA). As a secondary antibody we used donkey anti-rabbit-HRP IgG (Jackson Immunoresearch, PA). Proteins were visualized using ECL detection kit (Amersham, UK).

Apoptosis assay

The apoptosis was assayed using the Annexin V-FITC Apoptosis Detection Kit obtained from Pharmingen. Annexin V and PI staining were performed according to the manufacturer's instructions.

Bioinformatic methods

Mouse genomic DNA sequences obtained from Celera Discovery System were searched for CACGTG motifs located within a distance of 2 kb on either side of the transcription start site. Preferentially, E-boxes situated in the first intron within CpG islands were chosen for further ChIP analysis.

Chromatin immunoprecipitation (ChIP) assay

About 2×10^7 cells were incubated for 12 h in the presence or absence of doxycycline, fixed in 1% formaldehyde, and chromatin was precipitated using a ChIP Assay Kit (Upstate, NY) following the instructions of the manufacturer. Rabbit polyclonal antibodies specific for c-Myc (N-262), Lyn, and PLCγ (all from Santa Cruz, CA), acetyl-histone H3, and acetyl-histone H4 (both from Upstate, NY) were used to precipitate chromatin. The primers used for amplification of genomic sequences containing Myc binding site for Ifrd2 were: 5′-ggg ggt gtt ccg tga ttc c-3′ and 5′-acg ctc ttt ctg gtg tgg ttt-3′; Pa2g4: 5′-cga aaa gga agc ggc cag ct-3′ and 5-ccc cac taa gaa gac ctg aag-3′; Picalm (phosphatidylinositol-binding clathrin assembly protein also annotated as clathrin assembly lymphoid-myeloid leukemia gene): 5′-cgg ctc gga tgc gtg ttc aa-3′ and 5′-ttg cct gcc gac tgc cta ac-3′; Clpp: 5′-gga cga gca gga agt agc ca-3′ and 5′-tgt gtc gta gat ggc cag gc-3′; Timm10: 5′-tct gct gag agc ctg gaa ct-3′ and 5′-cca agt acc cct caa aca agc-3′; Lap3: 5′-gca cag gtc cgg ctt tcc aa-3′ and 5′-cca gag acc tct cac tcc ca-3′.

RESULTS

Screening for Myc targets using microarray analysis

During the analysis of cell lines derived from tTA/Myc mice with advanced lymphomas (31), we observed that doxycycline treatment for 12 h, completely abolished expression of the transgenic Myc protein (Figure 1A). Yet cells still did not show obvious signs of apoptosis, i.e. Annexin V binding (Figure 1B). In addition, after such a 12 h treatment cell proliferation could be reactivated by washing out doxycycline (see below). Therefore, we decided to use these conditions to study the regulation of Myc target genes.

Three subtypes of lymphomas were used for microarray hybridization experiment: B cell lymphomas, T cell lymphomas that were diploid for chromosome 15 (T cell lymphoma 2× chromosome 15) and T cell lymphomas with trisomy of chromosome 15 (T cell lymphoma 3× chromosome 15). For each lymphoma subtype two cell lines were chosen, cultured to reach log phase of growth and then 2×10^7 cells were either

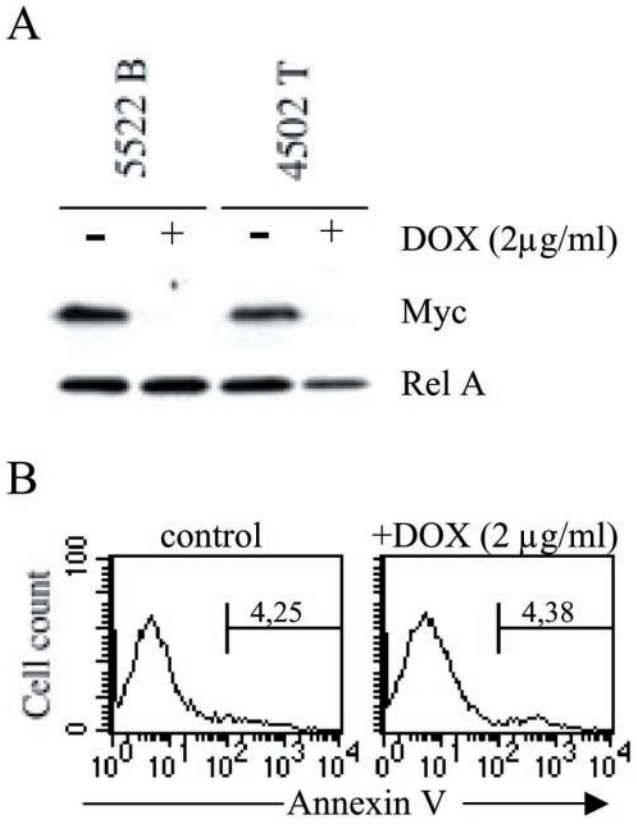

Figure 1. Expression of transgenic Myc is regulated by doxycycline treatment. Cell lines were incubated for 12 h in the presence or absence of doxycycline (2 μg/ml). Afterwards, cells were harvested, lysed in Dignam C buffer and 50 μg of the protein was analyzed by western blotting. After detection of Myc the membrane was stripped and reprobed with a rabbit polyclonal anti-RelA antibody as loading control. Representative examples of the transgenic Myc expression in B and T cell lines are shown (**A**). An aliquot of cells was used to test apoptosis by Annexin V staining (**B**).

treated or not treated with 2 μg/ml of doxycycline for 12 h to shut off Myc expression. Gene expression profiles of these two conditions were then compared by Affymetrix Murine Genome Array U74Av2 gene chip that contain about 12 000 sequences. All raw data are made MIAME ('Minimal Information About a Microarray Experiment') compliant and submitted to ArrayExpress database (www.ebi.ac.uk/arrayexpress/) with an accession number E-MEXP-169.

Our approach was to reduce the prevalence of non-specific genes and look for common events in Myc-induced lymphomagenesis by comparing the expression patterns of different lymphoma subtypes. As a first step, we compared mRNA expression of each of the six doxycycline-treated lines to the corresponding non-treated sample. Genes whose expression was increased by doxycycline treatment were considered as downregulated by Myc, whereas genes with decreased expression were considered as upregulated by Myc. We applied a threshold of a 2-fold change in expression levels between doxycycline treated and non-treated cells. Further, lists of upregulated and downregulated genes for each cell line from the same lymphoma subtypes were compared and only genes altered in both lines were considered as common for the certain lymphoma subtype (Figure 2). To obtain lists of

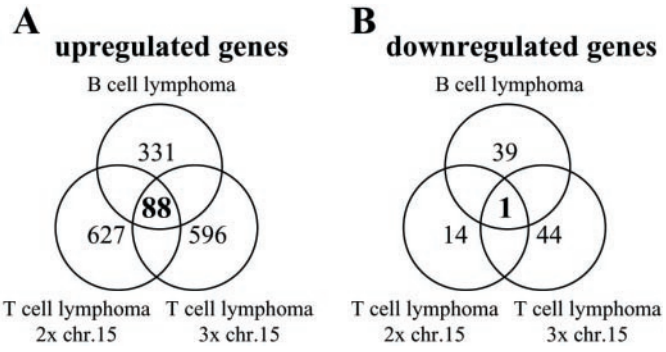

Figure 2. Myc regulates the expression of a large set of genes. Cell lines were divided into three subgroups: B cell lymphomas (cell lines 5522 B and 5532 B), T cell lymphomas with 2× chromosome 15 (4502 T and 1415 T) and T cell lymphomas with 3× chromosome 15 (4347 T and 7755 T). For the hybridization to Gene Chip Murine Genome Array U74Av2 cell lines were treated for 12 h with 2 μg/ml of doxycycline. Expression patterns for each treated line were compared with untreated control and genes that were up- and downregulated by Myc were further analyzed. Venn diagrams present numbers of genes altered in each of three subgroups of lymphomas. Numbers in each subgroup represents genes altered in both cell lines. (**A**) The 88 genes upregulated by Myc are common for all three subgroups of lymphomas. (**B**) Only one gene was found to be downregulated in all lymphoma subgroups.

genes that were commonly up- or downregulated in all analyzed lymphoma lines, lists of genes that were altered in each subtype were matched. Thus, as a final result, we obtained lists of genes up- or downregulated more than 2-fold in all six samples (ranging up to 27-fold of regulation).

The list of commonly upregulated genes contains 88 genes (Figure 2A and Table 1), whereas only one gene was consistently downregulated in all cell lines (Figure 2B and Table 1). Out of the 88 genes upregulated by Myc, 61 were described as genes with known protein function, whereas 27 were not annotated sequences (Table 1). The list of the upregulated genes that we obtained, showed substantial overlaps with the Myc target genes database published on the website (www.myc-cancergene.org) However, we observed the regulation of 37 additional genes that, to the best of our knowledge, had not been described previously as Myc targets (Table 1). To further analyze Myc target genes observed in this study, we first classified them according to their biological function (Table 1). As expected (1,33), the majority of genes regulated by Myc is involved in processes such as cell proliferation, differentiation, and signaling, or reflected increased requirements of highly proliferative cells for protein synthesis, modification, and metabolism.

Confirmation of Gene Chip data

To further confirm the results obtained by Gene Chip analysis, RT–PCR was performed using RNA isolated from cells that were identically treated as for the microarray hybridization. Several genes were chosen from the list of common upregulated Myc targets: Ifrd2, Pa2g4, Picalm, Timm10, Lap3, Sms and Gsto1. In agreement with the Gene Chip data, doxycycline treatment decreased mRNA levels for these genes in all tested lines (Figure 3A). In addition, expression of Itgam, the only gene commonly downregulated by Myc, was indeed increased in the presence of doxycycline (Figure 3A).

Myc target genes that were identified by Gene Chip analysis and additionally confirmed by RT–PCR were further evaluated using a supplementary set of two B and four T cell lines, as biological replicates, to analyze the pattern of expression of these genes in the presence or absence of Myc. In all cases, results obtained from additional cell lines corresponded to the results from Gene Chip analysis and RT–PCR obtained on the first set of six cell lines (Figure 3B). Therefore, the specific regulation of those target genes by Myc was not only true for the cell lines chosen for Gene Chip analysis, but also for the additional set of six other lymphomas.

We also tested the mRNA expression levels of several genes, which, according to the approach we used, appeared to be specifically regulated by Myc in B or T cell lymphomas. We chose Amd1 and CD2 as examples for up- and downregulated genes specific for B cell lymphoma and Thop1 and CD28 as examples for T cell lymphoma specific genes. Amd1 was indeed downregulated and CD2 was upregulated in the presence of doxycycline in both B cell lines (Figure 3C). Similarly, the expression of Thop1 in all T cell lines was clearly reduced in the absence of Myc while CD28 expression was enhanced under the same conditions (Figure 3D).

Target gene expression follows the kinetics of Myc mRNA expression

Myc in combination with Max directly induces transcription of various genes (16). However, not only are direct targets activated by Myc expression but Myc regulated genes may also control the expression of a second wave of target genes. A prediction for direct target genes is that their expression should follow the kinetics of Myc expression. We treated cells with 2μg/ml of doxycycline for 12 h to suppress Myc expression and then later, an aliquot of cells was used to isolate RNA. The rest of the cells were intensively washed and replated in a medium without doxycycline. In order to follow the reexpression of Myc and potential reexpression of Myc target genes, cells were sampled at different time points (2,4,8 and 24 h) and RNA was isolated. We found that cells treated with doxycycline are capable of reestablishling the expression of Myc mRNA within 8 h of doxycycline withdrawal (Figure 4A).

To avoid the possibility that the reappearance of Myc after 8 h might reflect differences due to advanced proliferation of Myc-expressing cells, which perhaps did not respond to doxycycline treatment, we simultaneously followed cell proliferation, using staining with vital dye CFSE, immediately after doxycycline removal. As a control, cells that were not treated with doxycycline were used. No significant reduction of CFSE fluorescence was observed at the time point of 8 h in neither control nor doxycyline-treated cells (Figure 4B). This indicates that increased levels of Myc mRNA are indeed due to reactivation of transgenic construct and not just a consequence of overgrowth of cells that perhaps did not respond to doxycycline treatment. Interestingly, whereas all control cells divided at least once upon doxycycline withdrawal after 48 h, cells that were continuously treated did not proliferate at all and cells that were pretreated for 12 h showed a certain level of heterogeneity. Reexpression of transgenic Myc was sufficient to reactivate dormitory cells; however, a percentage of cells probably underwent a permanent arrest of growth and these cells did not proliferate upon doxycycline removal (Figure 4B).

Table 1. Myc regulates different sets of genes

Genes upregulated by Myc
Metabolism
Phosphoribosylglycinamide formyltransferase (*Gart*)[a]
Inosine 5′-phosphate dehydrogenase 2 (*Impdh2*)[a]
Phosphofructokinase, liver, B-type (*Pfk1*)
Expressed in non-metastatic cells 1, protein (*Nme1*)[a]
Hexokinase 2 (*Hk2*)[a]
ATPase, H+ transporting, V1 subunit F (*Atp6v1f*)
Phosphoserine aminotransferase 1 (*Psat1*)
Spermine synthase (*Sms*)
Serine hydroxymethyl transferase 1 (soluble) (*Shmt1*)[a]
Triosephosphate isomerase (*Tpi*)
Protein biosynthesis and modification
DnaJ (Hsp40) homolog, subfamily C, member 2 (*Dnajc2*)
Chaperonin subunit 6a (zeta) (*Cct6a*)[a]
FK506 binding protein 4 (*Fkbp4*)[a]
Importin 4 (*Ipo4*)
RRS1 ribosome biogenesis regulator homolog (*S.cerevisiae*) (*Rrs1*)[a]
Peptidylprolyl isomerase F (cyclophilin F) (*Ppif*)[a]
Nucleolin (*Ncl*)[a]
Cell cycling
Block of proliferation 1 (*Bop1*)[a]
CD40 ligand-activated specific transcript 3 (*Clast3-pending*)
Chromosome condensation 1 (*Chc1*)[a]
Pericentrin 2 (*Pcnt2*)
Peter pan homolog (*Drosophila*) (*Ppan*)
Cell differentiation
Interferon-related developmental regulator 2 (*Ifrd2*)[a]
Helicase, lymphoid specific (*Hells*)
DNA maintenance and repair
Checkpoint kinase 1 homolog (*S.pombe*) (*Chek1*)
Polymerase (*DNA directed), delta 2, regulatory subunit (Pold2)*[a]
Stress response
Stress-induced phosphoprotein 1 (*Stip1*)
Superoxide dismutase 2, mitochondrial (*Sod2*)
Glutathione S-transferase omega 1 (*Gsto1*)
Translation
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 7 (*Psmd7*)[a]
Nuclear proteins
Mki67 (FHA domain) interacting nucleolar phosphoprotein (*Mki67ip*)
Nucleolar and coiled-body phosphoprotein 1 (*Nolc1*)[a]
EBNA1 binding protein 2 (*Ebna1bp2*)[a]
Nucleosome assembly protein 1-like 1 (*Nap1l1*)[a]
Proteolysis and peptidolysis
Proliferation-associated 2G4 (*Pa2g4*)[a]
Insulin degrading enzyme (*Ide*)
Leucine aminopeptidase 3 (*Lap3*)
Caseinolytic protease, ATP-dependent, proteolytic subunit homolog (*E.coli*) (*Clpp*)
Mitochondrial proteins
Translocase of outer mitochondrial membrane 40 homolog (yeast) (*Tomm40*)
Translocase of inner mitochondrial membrane 10 homolog (yeast) (*Timm10*)
A kinase (PRKA) anchor protein 1 (*Akap1*)[a]
Transcription
Transcription elongation regulator 1 (CA150) (*Tcerg1*)
Transcription factor Dp 1 (*Tfdp1*)[a]
General control of amino acid synthesis-like 2 (yeast) (*Gcn512*)
Coactivator-associated arginine methyltransferase 1 (*Carm1-pending*)
Signaling
Thyroid hormone receptor interactor 13 (*Trip13*)[a]
RAN binding protein 1 (*Ranbp1*)[a]
Phosphatidylinositol binding clathrin assembly protein (*Picalm*)
Glucosidase 1 (*Gcs1*)[a]
Other
SET and MYND domain containing 5 (*Smyd5*)
Glutamate rich WD repeat protein GRWD (*Grwd-pending*)
G7e protein (*G7e-pending*)
Similar to KIAA0595 protein (*LOC226169*)
Expressed sequence AA408582 (*AA408582*)
Carnitine deficiency-associated gene expressed in ventricle 3 (*Cdv3*)
HIV-1 Rev binding protein 2 (*Hrb2*)
SET and MYND domain containing 2 (*Smyd2*)
NS1-associated protein 1-like (*Nsap 1l-pending*)
Fibroblast growth factor inducible 15 (*Fin15*)
Mus musculus goliath-related E3 ubiquitin ligase 4 (*Greul4*)
Myosin Va (*Myo5a*)
Not annotated sequences
RIKEN cDNA 2410012M04 gene (2410012M04Rik)
RIKEN cDNA 2410080P20 gene (2410080P20Rik)
RIKEN cDNA 6230425C22 gene (6230425C22Rik)
RIKEN cDNA C030006K11 gene (C030006K11Rik)
RIKEN cDNA 2810453H10 gene (2810453H10Rik)
RIKEN cDNA 2810026P18 gene (2810026P18Rik)
RIKEN cDNA 2700066J21 gene (2700066J21Rik)
RIKEN cDNA 5730507C05 gene (5730507C05Rik)
RIKEN cDNA 4930553M18 gene (4930553M18Rik)
RIKEN cDNA 2600001M11 gene (2600001M11Rik)
RIKEN cDNA 4833432B22 gene (4833432B22 Rik)
RIKEN cDNA 2810409H07 gene (2810409H07Rik)
RIKEN cDNA 2810470K21 gene (2810470K21Rik)
RIKEN cDNA 2310061O04 gene (2310061O04Rik)
RIKEN cDNA 4930553M18 gene (4930553M18Rik)
RIKEN cDNA 5730436H21 gene (5730436H21Rik)
RIKEN cDNA 2310061I04 gene (2310061I04Rik)
RIKEN cDNA 1110007M04 gene (1110007M04Rik)
RIKEN cDNA 2700079K05 gene (2700079K05Rik)
RIKEN cDNA 2610012O22 gene (2610012O22Rik)
RIKEN cDNA 2410004C24 gene (2410004C24Rik)
RIKEN cDNA 2410008G02 gene (2410008G02Rik)
RIKEN cDNA 2410008J01 gene (2410008J01Rik)
RIKEN cDNA 2410005K20 gene (2410005K20Rik)
RIKEN cDNA 0910001B06 gene (0910001B06Rik)
Target 100910 at MG-U74Av2
Target 98524_f_at MG-U74Av2
Genes downregulated by Myc
Integrin alpha M (Itgam)

[a]Genes previously described as a Myc targets.

It is not clear at present whether Myc expression was reactivated in this cell population.

The majority of analyzed genes showed the same expression pattern as Myc. After 12 h of doxycycline treatment, transcription of all genes that were positively regulated by Myc was decreased (Figure 4A). In contrast, transcription of Itgam, barely detectable in the control cells, significantly increased upon Myc inactivation (Figure 4A). Reappearance of Myc after 8 h led to an increase of mRNA levels of Pa2g4, Picalm, Timm10, Lap3, Gsto1 and Sod2, whereas transcription of Itgam decreased (Figure 4A). In contrast, the Myc effects on some target genes might be indirect. Umps was reexpressed after 24 h and mRNA level for Hells did not increase even during this time period (Figure 4A).

Myc binds to regulatory regions of direct target genes

An important mechanism by which Myc induces activation of direct target genes is by dimerization with Max and direct binding to the E-box (CANNTG) DNA sequence, mostly CACGTG (11,34,35). To identify direct targets of Myc, we used bioinformatics tools as a preselection method and continued further with experimental analysis of Myc binding to the CACGTG-containing sequence, using ChIP. We searched the mouse genomic DNA sequences of our putative target

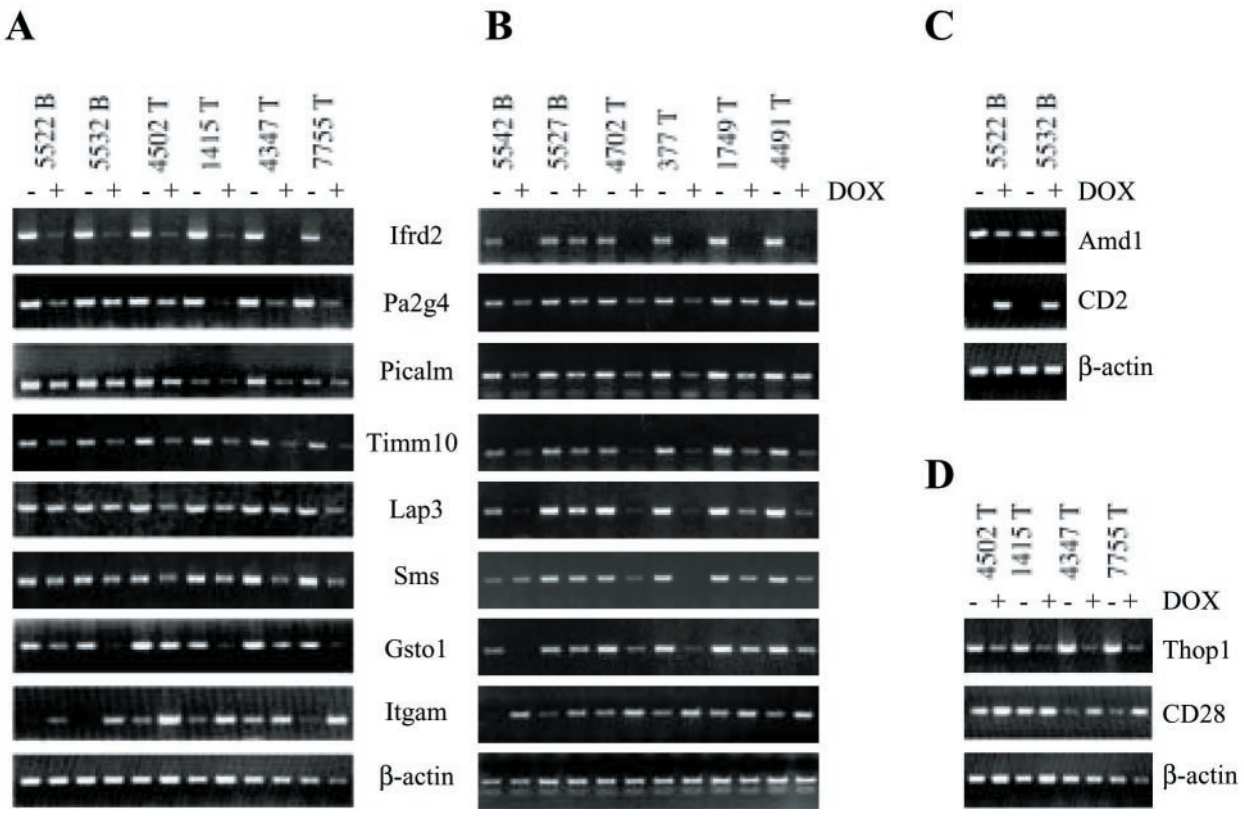

Figure 3. RT–PCR confirms the results obtained by Gene Chip analysis. An aliquot of cells that were prepared for microarray analysis was used to isolate total RNA and subsequently to perform the RT–PCR. Genes commonly upregulated by c-Myc in all analyzed cell lines (Ifrd2, Pa2g4, Picalm, Timm10, Lap3, Sms and Gsto1) showed a decreased expression upon doxycycline treatment (2 μg/ml for 12 h), while under the same conditions, expression of Itgam, a gene downregulated by Myc, is increased (**A**). Expression of the same genes was checked in an additional set of six lymphoma cell lines as biological replicates (**B**). Examples of up- and downregulated genes specific for B cell lymphoma (Amd1 and CD2) (**C**) and T cell lymphoma (Thop1 and CD28) (**D**) are presented. β-actin was used as a housekeeping gene.

genes for CACGTG motifs located within 2 kb up- or downstream of the start site of transcription (Figure 5A) (24). The majority of the analyzed genes were indeed found to contain such E-boxes (Figure 5A). In most of the cases, potential Myc-binding sequences were located within the first intron or exon1 (Figure 5A). Interestingly though, some of the genes of physiological interest, Gsto1 and Sod2, did not contain CACGTG sequence within 2 kb upstream of the transcription start site nor within the gene sequence. However, more distant E-box elements cannot be excluded. Recent reports suggested CACGTG motifs, that were located in the first exon or intron and surrounded by CpG islands (correlating with an open, preacetylated state of chromatin), to be preferential Myc binding sites (24). Therefore, taking into account the position of E-boxes toward a transcription start site, the number of CACGTG motifs in this region and, the location within CpG islands, genes presented in Figure 5B were chosen as candidates for subsequent ChIP analysis.

Formaldehyde-cross linked chromatin was precipitated with antibodies specific for Myc, acetylated histone H3 or acetylated histone H4. Anti-Lyn or anti-PLCγ antibodies were used (irrelevant Ab) as isotype controls. PCR analysis with specific primers revealed binding of Myc at Ifrd2, Pa2g4, Picalm, Clpp, Timm10, Lap3, and ATP6v1f sequences (Figure 5B). PCR bands obtained in this experiment were indeed Myc specific since 12 h of doxycycline treatment, which completely abolish Myc expression (Figure 1), results in the disappearance of the signal (Figure 5B). Therefore, according to the kinetic and chromatin immunoprecipitation data, we concluded that regulation of these genes by Myc is direct. As expected (36,37), the analyzed Myc binding sequences represented open chromatin. We were able to precipitate the same sequences using an antibody specific for acetylated histone H3 both in untreated and doxycycline-treated cells (Figure 5B). Similar results were obtained using acetylated histone H4 antibody (data not shown). This implies that short-term abrogation of Myc expression, although enough to stop synthesis of mRNA for the particular gene, did not result in gross alterations in chromatin structure. This is consistent with the previous report that recruitment of Myc to a target promoter does not immediately influence the amount of acetylated histones at the promoter (38).

Myc target genes identified in mouse lymphomas are also regulated in human lymphomas

The mouse model for conditional Myc-induced lymphomagenesis allowed us to identify several new Myc target genes. The next obvious question was whether Myc might be involved in regulating the expression of these genes in

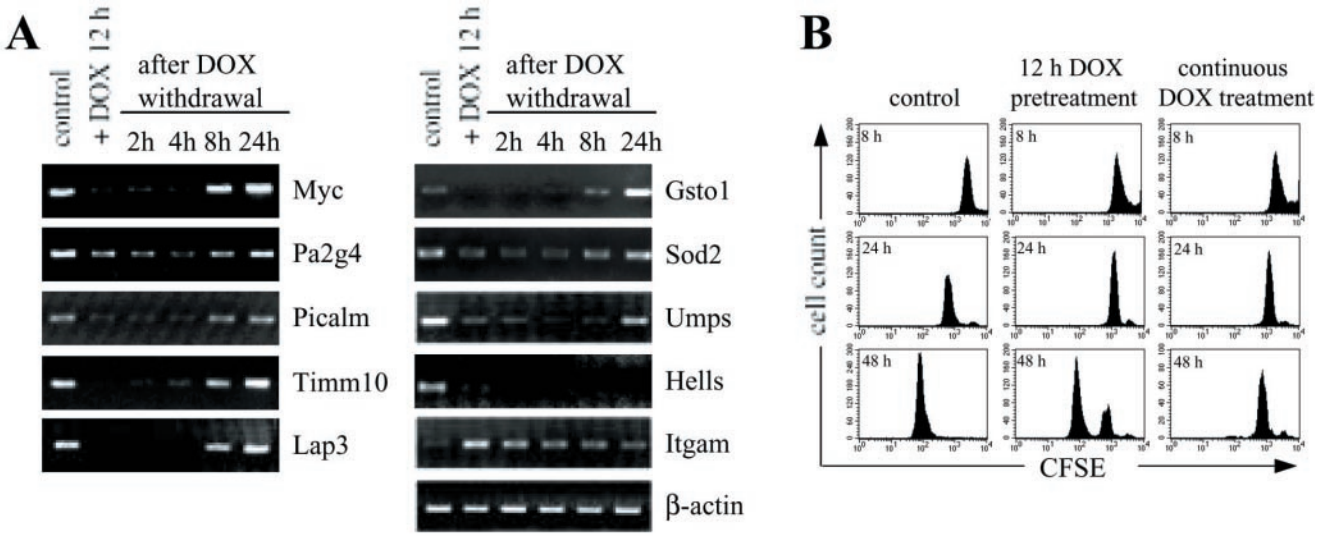

Figure 4. Myc target genes follow the kinetics of Myc expression. (**A**) Myc expression was blocked by treating lymphoma cells with 2 μg/ml of doxycycline for 12 h. Cells were than extensively washed and replated in normal medium. At indicated time points, an aliquot of cells was taken and analyzed for mRNA expression of Myc regulated genes. Pa2g4, Picalm, Timm10, Lap3, Gsto1 and Sod2 followed kinetic of Myc (expression after 8 h of Myc activation), while Umps and Hells showed later expression (24 h or later). β-actin was used as a control. Data presenting representative results are shown. (**B**) Cells were incubated for 12 h in the presence (panel '12 h DOX pretreatment') or absence ('control') of doxycycline. Subsequently, cells were extensively washed, resuspended in RPMI and stained for 5 min with 2 μM of vital dye CFSE. Cells were replated in cell culture medium and one aliquot of both treated and untreated cells was used for FACS analysis at indicated time points. In addition, sample of cells continuously that was treated with doxycycline was included. Reduction of CFSE fluorescence indicates proliferation of cells.

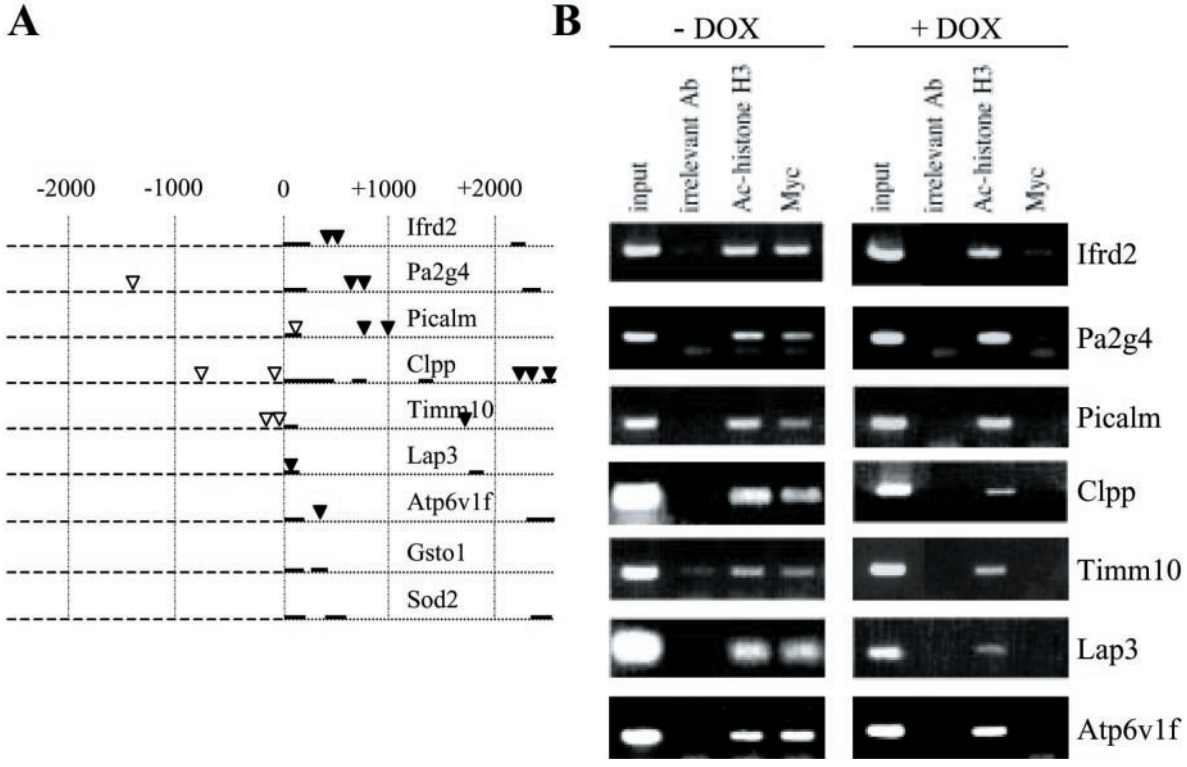

Figure 5. Myc binds to the consensus CACGTG sequences in target genes. (**A**) Mouse genomic DNA sequences for indicated genes, obtained from Celera Discovery System, were searched for CACGTG motifs located within a distance of 2 kb on either side of transcription start site. Triangles represent consensus binding sites, whereas full triangles symbolize consensus CACGTG sequences further analyzed by ChIP. Full lines represent exons, dark dashed lines introns and light dashed lines non-transcribed genomic sequence. (**B**) Cells treated or not treated with doxycycline, were cross-linked with formaldehyde and the chromatin was immunoprecipitated using anti-Myc or anti-AC histone H3 antibody. After reverse cross-linking, co-immunoprecipitated DNA was analyzed by PCR using primers specific for the regions containing CACGTG motifs into indicated Myc target genes. Lyn or PLCγ antibodies were used as a negative control (irrelevant antibody). Representative data obtained with the cell line 5522 B are shown.

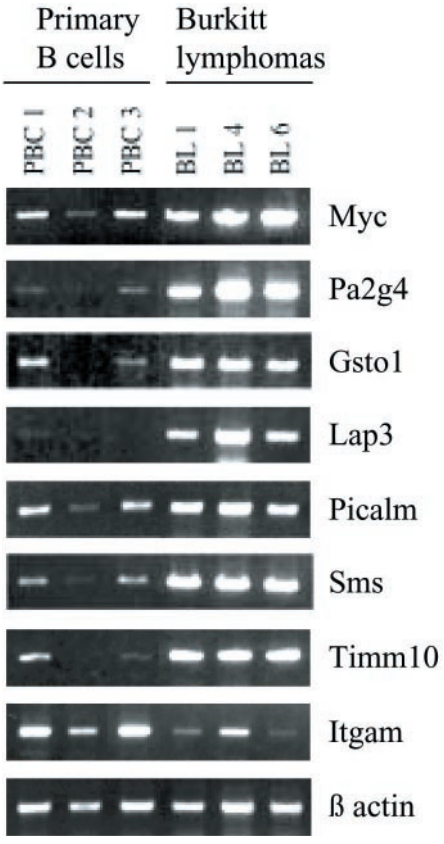

Figure 6. Identification of Myc target genes in human Burkitt's lymphoma. Total RNA was isolated from primary B cells of three healthy donors and three individual surgical samples of Burkitt's lymphoma. RT–PCR was performed to compare the expression level of Myc and its target genes. β-actin was used as a housekeeping gene.

human cells as well. Therefore, we compared the expression of several genes, which were identified as novel Myc targets in our mouse system, in human B cells. Primary B cells were isolated from peripheral blood of healthy donors and compared with primary cases of Burkitt lymphomas. In accordance with the main feature of Burkitt lymphoma, expression of Myc in lymphoma samples was significantly higher than in primary B cells (Figure 6). Importantly, the expression pattern of most tested Myc target genes correlated with the expression levels of Myc. The expression levels of Pa2g4, Gsto1, Lap3, Picalm, Sms, and Timm10 are significantly higher in Burkitt lymphoma samples when compared with primary B cells (Figure 6). Moreover, as in the mouse model, Burkitt lymphomas had lower level of mRNA for Itgam, which was the only target gene negatively regulated by Myc (Figure 6). Out of all tested target genes only Sod2 showed the same level of expression in Burkitt lymphomas and primary B cells (data not shown), indicating that the Myc-dependence differs between mouse and human lymphomas for this gene.

DISCUSSION

An analysis of molecular events leading to cancerogenesis is very important with respect to the attempts to create efficient antitumor therapies. The role of Myc as a transcription factor suggests that the initial steps in Myc-induced tumorigenesis probably involve the transcription of a certain set of genes critical in the transformation process. Previous reports on different experimental models revealed that Myc affects transcription of a large number of genes (23–30). The total number of these genes analyzed to date is more than 1000, as listed in the Myc target gene database (www.myccancergene.org). However, it is still an open question, as to which of the reported target genes triggered by ectopic Myc expression are indeed essential for tumorigenesis. In addition, not all of described genes found in this large list have been proven to be direct Myc targets.

In this study, we identified Myc target genes in a system of conditional Myc-induced lymphomagenesis. Doxycycline treatment of cell lines used in our work specifically affects expression of Myc and Myc-dependent physiological processes (31). In our previous work, we observed that overexpression of Myc that was indirectly controlled by the μE-driven tTA led to development of both T and B lymphomas (31). In addition, it was possible to distinguish between two subgroups of T cell lymphomas according to the number of copies of chromosome 15 (31). Several recent papers also reported a link between Myc-induced tumors and an increased frequency of chromosome 15 trisomy in T cells (39–41). This study did not aim to identify potentially different expression patterns in T cell lymphomas that differed in their chromosome 15 content. For this more tumor samples need to be investigated. Nevertheless, we took advantage of this heterogeneity in lymphoma in attempts to identify potentially essential general players in Myc-induced lymphomagenesis and to reduce the prevalence of genes regulated as a side-effect of this process.

Microarray analysis revealed that abrogation of Myc expression by doxycycline affects the expression of a large number of genes. However, for further analysis we considered only genes that changed at least 2-fold in all six analyzed cell lines. Applying these stringent conditions, we reduced the chance for false positive results and ended up with a list of 88 (61 with known protein function and 27 with non-annotated sequences) upregulated genes and 1 downregulated gene. Expression of several genes identified genes in this manner was also confirmed by RT–PCR analyses of the same cell lines that were used for Gene Chip as well as an additional set of six cell lines. Genes were classified into functional groups according to their physiological role. The obtained pattern confirms that Myc regulates genes mainly involved in metabolism (10 genes), cell cycle control (5 genes), signaling (4 genes), protein biosynthesis and modification (7 genes).

In addition to 22 genes already reported as a Myc targets, our analysis revealed that Myc increases the expression of 37 novel genes with known protein function and decreases expression of only one such gene in all six tested lymphoma cell lines. We analyzed literature data for all novel Myc targets and tried to link them with the process of lymphomagenesis.

In all tested lymphomas, doxycycline treatment led to upregulation of Itgam (Mac-1) expression. It is known that Mac-1 is expressed on certain subsets of T and B cells; however, the precise function of Mac-1 in lymphocyte physiology is not fully clarified (42,43). Although we could presume that regulation of an adhesion molecule affects certain aspects of tumor physiology, i.e. ability to invade different tissues,

additional studies will be necessary to reveal the role of downregulation of Itgam expression in Myc-triggered lymphomagenesis.

We were able to identify a group of genes upregulated by Myc with roles in mitotic spindle checkpoint and genomic stability. This group includes Clast3 (CD40 ligand-activated specific transcript 3), pericentrin 2 and Chek1. Clast3 expression is controlled in a cell cycle-dependent manner and affects the regulation of G2-M transition (44). In addition, it interferes with the mitotic spindle checkpoint, and constitutive overexpression of Clast3 induces polyploidy and multinucleation (44). Pericentrin 2 is a calmodulin-binding protein and an integral component of pericentriolar material (45). Frameshift mutations of Chek1 were detected in different tumors (46–48). It was shown that Chek1 is critical for the S and G2 checkpoints and, via interactions with Cdc25, regulates the cell cycle progression (49,50). The well-known ability of Myc to induce genomic instability (51,52), therefore, could be at least partially mediated by altered expression of these genes.

It was interesting to find that Myc regulated the expression of Picalm. This gene fulfilled all criteria for a direct Myc target. Transcription of Picalm follows the kinetics of Myc expression and we could detect the binding of Myc protein at the E-box containing sequence in the first intron of this gene. A variety of leukemias and lymphomas have been associated with a translocation that fuses Picalm with the putative transcription factor gene AF10 (53–56). However, the specific role that overexpressed non-mutated Picalm plays for Myc-induced lymphomagenesis is not clear. Interestingly, mutations of Picalm appeared to be responsible for the defects in hematopoiesis and iron metabolism that were observed in fit-1 mutant mice (57). The significance of iron metabolism in tumor biology was already reported (58,59). There is also evidence that Myc activation disturbs iron homeostasis and it has been reported that iron regulates Myc expression (60,61). Elevated levels of Picalm could contribute to the mechanisms involved in the iron-dependent aspects of a Myc-induced lymphomagenesis.

The potential role of natural polyamines for the processes of tumorigenesis has been discussed for quite some time (reviewed in (62)). Indeed, one of the Myc target genes that was described first was ornithine decarboxylase (ODC), a critical enzyme in polyamine metabolism (63). We observed that Myc regulates the expression of another enzyme from this group, spermine synthase (Sms). Spermine is the most abundant polyamine in human tumors (64) and increases the resistance of tumor cells to therapy (65). During attempts to develop an antitumor strategy directed against polyamine production, it was observed that ODC inactivation alone did not alter the spermine concentration (65). It was suggested that blockade of the single enzyme triggers changes in polyamine metabolism and transport, which compensates for the defect (62). In addition to this, we presume that Myc could affect spermine concentration by direct induction of the spermine synthase transcription.

Several genes, known to be overexpressed during tumor development in our study, appeared to be upregulated by Myc. Gene Chip and ChIP analyses showed that Myc regulates expression of ATP6V1F, a vacuolar ATPase that is overexpressed in human prostate carcinomas (66). Next, we observed an elevated mRNA level for a lymphoid-specific helicase, Hells, a molecule shown to be altered in leukemia (67). However, the expression kinetic for Hells does not follow the Myc expression directly, and we could not identify E-boxes in the regulatory region of this gene. Therefore, activation of Hells by Myc is probably indirect.

A significant number of the upregulated genes have a role in different metabolic pathways (i.e. Lap3, Clpp, Psat1), mitochondrial function (Timm10, Tomm40) or signaling (Ipo4, Ide). Although, at least some of them (Lap3, Clpp, Timm10) are direct Myc targets, taking into account the high proliferative capacity of lymphoma cells, elevated expression of these genes could represent increased metabolic requirements of cells rather than being the cause of tumorigenic transformation.

We also detected upregulation of genes important for stress response, Stip1, Sod2 and Gsto1. In the case of these genes, we could not detect consensus Myc binding sites within the 2 kb sequences surrounding the transcription start. The increased expression of these genes might be a consequence of the exposure of cells to oxidative stress (68,69) and not directly induced by Myc overexpression. Alternatively, Myc binding sites might be present at more distinct sites relative to the sites of transcription.

Finally, we found that the results obtained in our mouse conditional model for lymphomagenesis are relevant for human neoplasia as well. The same pattern of regulation for several identified Myc target genes was also observed in Burkitt lymphoma samples. However, further work is necessary to highlight specific roles of the novel Myc targets and their importance for Myc-induced tumorigenesis in order to develop efficient antitumor strategy.

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