

ORIGINAL ARTICLE

Burkitt's lymphoma-associated c-Myc mutations converge on a dramatically altered target gene response and implicate Nol5a/Nop56 in oncogenesis

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Burkitt's lymphomas (BLs) acquire consistent point mutations in a conserved domain of Myc, Myc Box I. We report that the enhanced transforming activity of BL-associated Myc mutants can be uncoupled from loss of phosphorylation and increased protein stability. Furthermore, two different BL-associated Myc mutations induced similar gene expression profiles independently of T58 phosphorylation, and these profiles are dramatically different from MycWT. Nol5a/Nop56, which is required for ribosomal RNA methylation, was identified as a gene hyperactivated by the BL-associated Myc mutants. We show that Nol5a is necessary for Myc-induced cell transformation, enhances MycWT-induced cell transformation and increases the size of MycWT-induced tumors. Thus, Nol5a expands the link between Myc-induced regulation of nucleolar target genes, which are rate limiting for cell transformation and tumor growth.

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INTRODUCTION

Burkitt's lymphoma (BL) is an aggressive mature B-cell lymphoma,¹ in which the Myc proto-oncogene is translocated to the immunoglobulin loci.² In addition to the consistent chromosomal translocations, a majority of BLs have mutations in the coding region of Myc, which cluster in a conserved region known as Myc Box I (MBI), including E39D, A44V, P57S, T58A and T58I.^{3–9} The repeated identification of these mutations in tumors suggests that they are selected for during Myc-driven tumorigenesis. The majority of investigations into the activity of the BL Myc mutants have focused on MycT58A.^{10–12} Myc is phosphorylated on T58, and this phosphorylation is reported to be required for Myc degradation.¹³ MycT58A has been found to have an increased half-life and expression level, an increased transforming activity and/or an impairment in Myc-induced apoptosis.^{10,14–18} Studies in systems in which Myc does not induce apoptosis still show increases in transformation by MycT58A-expressing cells,^{10,14,15} suggesting that changes in apoptosis are not solely responsible for changes in Myc oncogenic activity.

Myc likely drives cell proliferation through its broad influence on gene expression, which controls a number of essential functions; metabolism, protein synthesis, mRNA cap methylation and promoting the activity of RNA pol I and III.^{19–25} The question arises whether the transformed phenotype induced by Myc overexpression is the result of a general increase in all Myc functions or due to altered regulation of specific genes. In cancers of several origins, it has been demonstrated that deregulation of specific Myc targets genes has an integral role in promoting the

transformed phenotype.^{19,20} Studies on the effect of the T58A mutation on target gene regulation vary widely, but some specific examples of genes and proteins differentially expressed in response to MycWT and MycT58A expression have been found.^{17,26,27} To date, there has been scant investigation of the more common BL-associated Myc mutants, MycE39D and MycT58I.^{10,28} In this study, we applied global gene expression analysis to examine how the BL-associated MBI mutants modulate Myc function and to identify the target genes that these mutants regulate to enhance Myc-induced transforming activity. We find that different mutations converge on remarkably similar and substantial changes in Myc-responsive genes compared with MycWT and that Nol5a/Nop56, a gene involved in ribosome biogenesis, may be a critical gene involved in Myc-mediated oncogenic transformation.

RESULTS

BL-associated Myc mutations increase the oncogenic potential of Myc, independent of T58 phosphorylation

To investigate the role of BL-associated Myc mutants in Myc-mediated tumorigenesis, we created a panel of mutants corresponding to the most common mutations; MycE39D, MycT58I and MycT58A.¹⁰ Upon stable overexpression of these mutants, we observed variable levels of Myc expression (Figure 1a). The mutants MycE39D and MycT58I expressed similarly to Myc wild type, whereas MycT58A showed a substantial increase (2.6-fold) in expression compared with Myc wild type.

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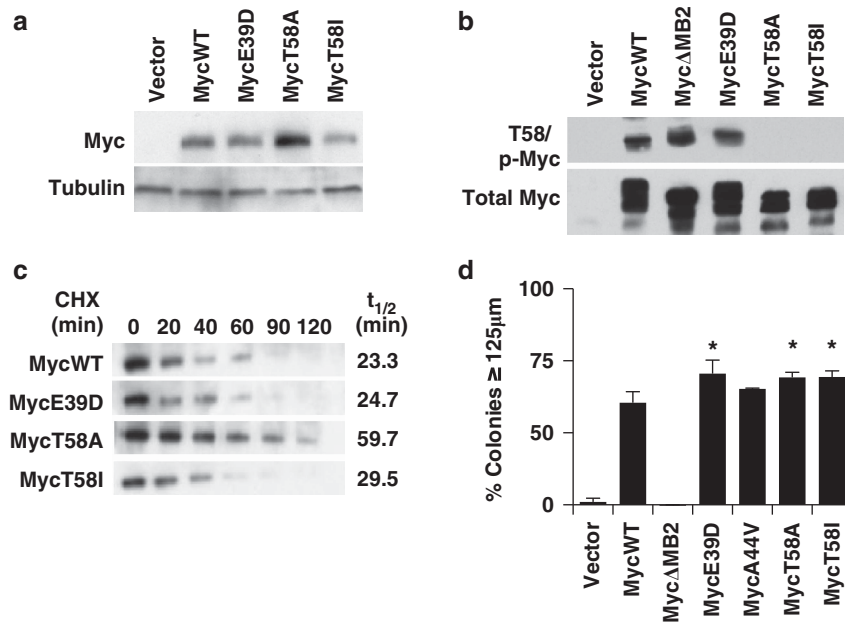


Figure 1. Burkitt's Lymphoma (BL) Myc mutants enhance Myc-induced cell transformation independently of loss of T58 phosphorylation and increased expression. **(a)** Rat1a fibroblasts were engineered to express MycWT, the indicated BL Myc mutants or vector control. Cell extracts were analyzed by western blot using anti-Myc or anti-tubulin antibodies. **(b)** MycWT and the BL-associated Myc mutants were transiently expressed in 293 cells. Cell extracts were analyzed by parallel western blots using anti-phospho-Myc (p58/p62) or anti-Myc (phospho-independent). **(c)** The half-life of MycWT and BL-associated mutants was determined by engineering *myc*^{-/-} fibroblast to express the indicated proteins, and cells were treated with 50 $\mu\text{g/ml}$ cycloheximide (CHX) for up to 120 min. Cell extracts were analyzed by western blot using anti-Myc antibodies. **(d)** Log phase Rat1a fibroblasts expressing MycWT or BL-associated Myc mutants were used in a soft agar transformation assay. The chart depicts the diameter of colonies from each cell lines after 1 week of growth. Error bars indicate the standard deviation (s.d.) for three biological replicates using two independently engineered cell lines. Significance was determined using Student's *t*-test; **P* \leq 0.05.

To determine whether the increase in expression was due to changes in protein stability, we treated the cells with cycloheximide and observed protein levels at varying times (Figure 1b). The half-life of MycE39D was equivalent to that of MycWT (23 \pm 6.2 min), similar to previously reported half-lives for Myc, whereas MycT58A had an increased half-life of more than twice that of MycWT (59.7 \pm 10.4 min). This result is consistent with previous studies of MycT58A half-life.²⁹ Surprisingly, we did not observe any appreciable increase in MycT58I stability compared with that of MycWT, consistent with the equivalent steady-state level. We observed that MycWT and MycE39D retained T58 phosphorylation, whereas both the MycT58A and MycT58I mutation where no longer phosphorylated in both HEK293 and *myc*^{-/-} fibroblasts (Figure 1c and data not shown). One explanation for the differential stability of MycT58A versus MycT58I could be previously observed differences in S62 phosphorylation,¹⁰ which contribute to changes in stability.¹³

As the E39D and T58I mutations do not increase protein stability, we wanted to determine if these mutations were still able to enhance Myc-mediated transformation (Figure 1d). Although we found no change in the proliferation rates (Supplementary Figure 1), all three mutants (MycE39D, MycT58A and MycT58I) enhanced anchorage-independent growth compared with MycWT or the transformation-defective Myc Δ MB2 mutant. We observed a 35–40% increase in Myc-mediated transformation on average. Thus, Myc-induced transformation is enhanced with BL-associated Myc mutants, independent of protein stability and T58 phosphorylation status. A previous study failed to show similar increases in these Myc mutant alleles.¹⁰ However, these studies used an oncogene cooperation assay to determine oncogenic potential, which may lack the sensitivity to observe these more

subtle qualitative difference from the more robust quantitative difference between c-Myc overexpression.

MycE39D and MycT58I regulate target gene expression similarly but significantly different from MycWT

We investigated whether the enhanced transforming activity of the BL-associated Myc mutants could be correlated with differential regulation of target genes. To avoid contributions of differential protein stability, we transduced MycE39D, MycT58I and Myc Δ MB2 (a non-transforming Myc mutant) into *myc*^{-/-} fibroblasts.³⁰ Myc protein expression in this polyclonal cell system is relatively low, and MycE39D and MycT58I were expressed equivalently to MycWT and at physiological levels similar to parental *myc*^{+/+} fibroblasts (Supplementary Figure 2a). Proliferation rates and cell morphology were also similar to parental fibroblasts. RNA was harvested from two independent log-phase pools for each Myc variant or vector control and analyzed for gene expression by whole-genome microarray. A graphic comparison of gene expression profiles can be visualized by hierarchical clustering of the complete data set (Figure 2a). After filtering and normalizing the data, the number of genes upregulated and downregulated by 1.5-, 1.75- and 2-fold in response to each Myc protein was compared with vector control (Supplementary Figure 2b). Consistent with microarray data from this and other cell systems, expression of MycWT was found to regulate a large number of genes in a MB2-dependent manner, and there were generally equivalent numbers of genes upregulated and downregulated.

A key question was whether MycWT and the BL-associated mutants regulated the same or different genes. We carried out a 'Significance Analysis of Microarrays' or SAM,³¹ which analyzes the

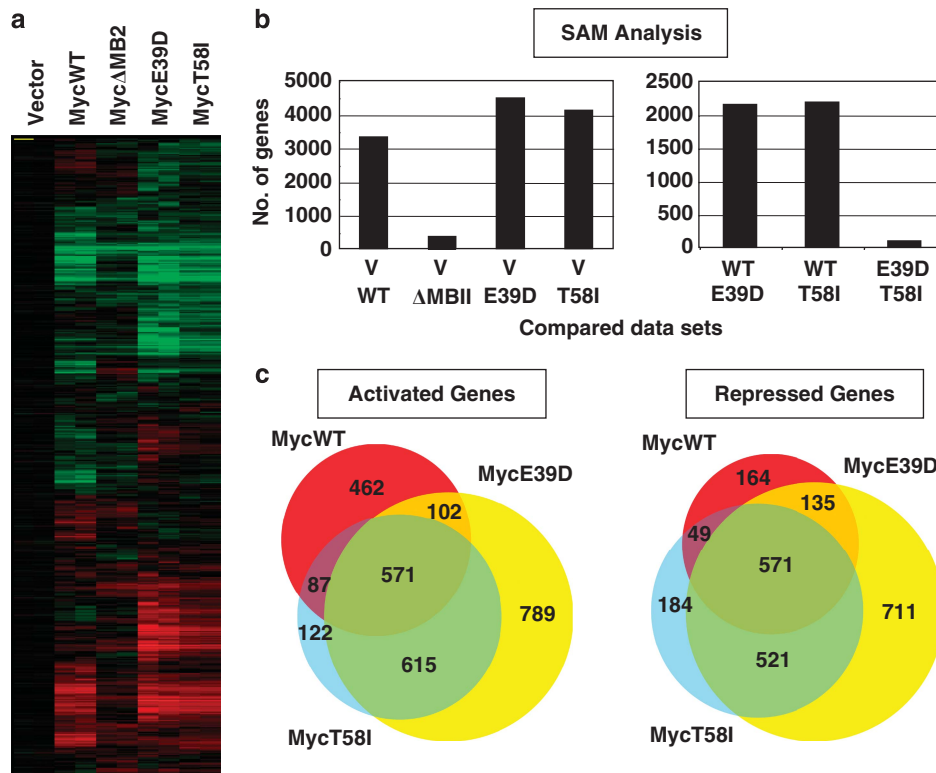


Figure 2. The BL-associated mutants, MycE39D and MycT58I, have a highly similar set of target genes, which is significantly different from MycWT target genes. **(a)** *myc*^{-/-} fibroblasts were engineered to express MycWT, a transactivation-defective mutant MycΔMB2, or the indicated BL-associated Myc mutants. RNA was extracted from two independent polyclonal cell populations expressing the indicated Myc protein or vector control. Expression microarrays were performed using Agilent 4 × 44K Whole Rat genome microarrays. The figure shows the clustered heat map of all genes with signals 1.5-fold above background and normalized to vector control. **(b)** Significance analysis of microarray (SAM) analysis was performed to compare the number of genes that were differentially regulated between the cell lines expressing the Myc proteins (WT, ΔMBII, E39D, T58I) and vector control (V) (left panel), and between the cell lines expressing the different Myc proteins (right panel). **(c)** Venn diagram representing target gene overlap of MycWT and BL-associated mutants for genes upregulated or downregulated 1.5-fold over vector.

differences in gene expression profiles between groups of cell lines (Figure 2b). At a false discovery rate of 1% in a two-class analysis, SAM determined that there were 3326 genes differentially expressed between MycWT-expressing and vector control *myc*^{-/-} cells. Similarly, 4494 genes were differentially expressed between MycE39D-expressing and vector control cells, and 4116 genes were differentially expressed between MycT58I-expressing and vector control cells. The most significant question is whether MycWT and the BL-associated Myc mutants activate the same or different genes. We made the unexpected finding that a large number of genes are differentially regulated by MycWT and the Myc mutants. Using the same 1% false discovery rate, there were 2150 genes differentially regulated between cells expressing MycWT and MycE39D, and 2186 genes between cells expressing MycWT and MycT58I (Supplementary Table 1). Most strikingly, there was little difference between the gene sets expressed in MycE39D and MycT58I. SAM determined that only 100 genes were expressed at a significantly different level between these BL-associated Myc mutants. As MycE39D is phosphorylated at both T58/S62, whereas MycT58I is not, this substantial shift in Myc-responsive genes is independent of phosphorylation status. Thus, two independent mutations skew the Myc response in a remarkably similar way from the MycWT profile, correlating with an enhanced oncogenic potential for both mutants.

We next examined the overlap of genes upregulated and downregulated 1.5-fold or more over vector in MycWT and BL-associated mutant gene sets (Figure 2c, Supplementary Tables 2 and 3). Of the 1222 genes activated by MycWT, 462 (38%) were activated by MycWT alone, whereas a nearly equivalent number (615; 50%) were jointly activated by the BL-

associated mutants alone. Similarly, 164 of 919 (18%) genes were repressed by MycWT alone, whereas 521 (56%) were jointly repressed by BL-associated mutants alone. We noted that the total number of genes activated and repressed by MycE39D was greater (approximately 50%) than Myc T58I and MycWT. However, 1186 of 1345 (89%) were upregulated and 1092 of 1329 (82%) were downregulated similarly between MycT58I and MycE39D. We performed a gene ontology (GO) term analysis on these subsets of genes (MycWT only, BL-associated mutant only and commonly regulated) to determine whether any particular pathway changes could account for the observed increase in tumorigenesis. Although there were slight changes in gene numbers in a variety of pathways, we did not observe any significant changes in important pathways for the BL-associated mutants.

It is apparent that broad groups of genes are differentially regulated by the Myc mutants versus MycWT, and gene expression sometimes reversed from activated to repressed (or *visa versa*) between MycWT and mutants. This demonstrates that, although all the Myc proteins regulate a subset of the same genes, there are large groups of genes that are regulated differently by the Myc mutants and MycWT. We also noted that the MycE39D responsive genes have generally more robust signals than MycT58I, consistent with the larger number of genes that pass each threshold.

Differential target gene regulation results in changes in gene expression and promoter histone modifications associated with Myc co-factor binding

We wanted to investigate how BL-associated Myc mutations could alter the Myc target gene expression profile. Myc promotes

transcription by binding to target genes and recruiting histone acetyltransferase complexes.^{12,32,33} We investigated Myc recruitment and histone acetylation on two genes, *HSP60* and *Nucleolin*, which are hyperactivated by the Myc mutants compared with MycWT. These were compared with two genes, *CAD* and *NME1*, which had reduced activation by the Myc mutants compared with MycWT. We used independent RNA samples to confirm differential expression by the Myc mutants compared with MycWT (Figure 3a). *CAD* and *NME1* were induced more by MycWT than the Myc mutants, whereas *HSP60* and *Nucleolin* were induced more by the Myc mutants. For all four genes, we used chromatin immunoprecipitation (ChIP) to investigate Myc recruitment to the E-box closest to the transcription initiation site (Figure 3b). We found that MycWT, MycE39D and MycT58I were all recruited equivalently to the E-boxes of all four Myc target genes. As a negative control, we found that the Myc proteins were not significantly recruited to the β -globin promoter (data not shown). We next investigated histone acetylation in response to MycWT and the Myc mutants at the same genes. We found Myc-dependent histone H4 acetylation at all four target genes (Figure 3c). In correlation with the increased *HSP60* and *Nucleolin* expression, we observed increased H4 acetylation at the *HSP60* and *NUCL* genes in response to expression of the Myc mutants compared with that induced by MycWT. Conversely, in correlation with the reduced *CAD* and *NME1* expression observed in response to MycE39D and MycT58I, we observed reduced histone

acetylation at the *CAD* and *NME1* genes compared with that induced by MycWT. Thus, the Myc mutations affect local chromatin modifications but do not affect promoter occupancy, consistent with their location in the Myc transactivation domain and not the DNA-binding domain.

Myc mutations differentially regulate *Nol5a*, a gene required for ribosome biogenesis

The BL-associated mutations result in increased Myc-induced cell transformation and a qualitative change in Myc responsive genes. We wanted to test the hypothesis that the increase in cell transformation was a result of specific changes in gene expression. Myc regulates all aspects of protein synthesis and ribosome biogenesis,^{34,35} and recent evidence suggests that ribosome biogenesis may be a primordial function of Myc, conserved throughout evolution in even the most primitive organisms that harbor Myc and Max orthologs.³⁶ Furthermore, mice that overexpress Myc but express only one functional copy of ribosomal protein L24 have significantly reduced translation rates and impaired Myc-mediated oncogenesis.³⁷ We therefore focused our studies on an essential ribosome biogenesis target gene that was differentially activated in Myc mutants and is used as a molecular diagnostic for BL.³⁸ *Nol5a* (nucleolar protein 5a/Nop56) encodes a component of an ribosomal RNA (rRNA) methylation complex that also regulates rRNA processing.^{39–41} We first established that *Nol5a* expression was also increased in

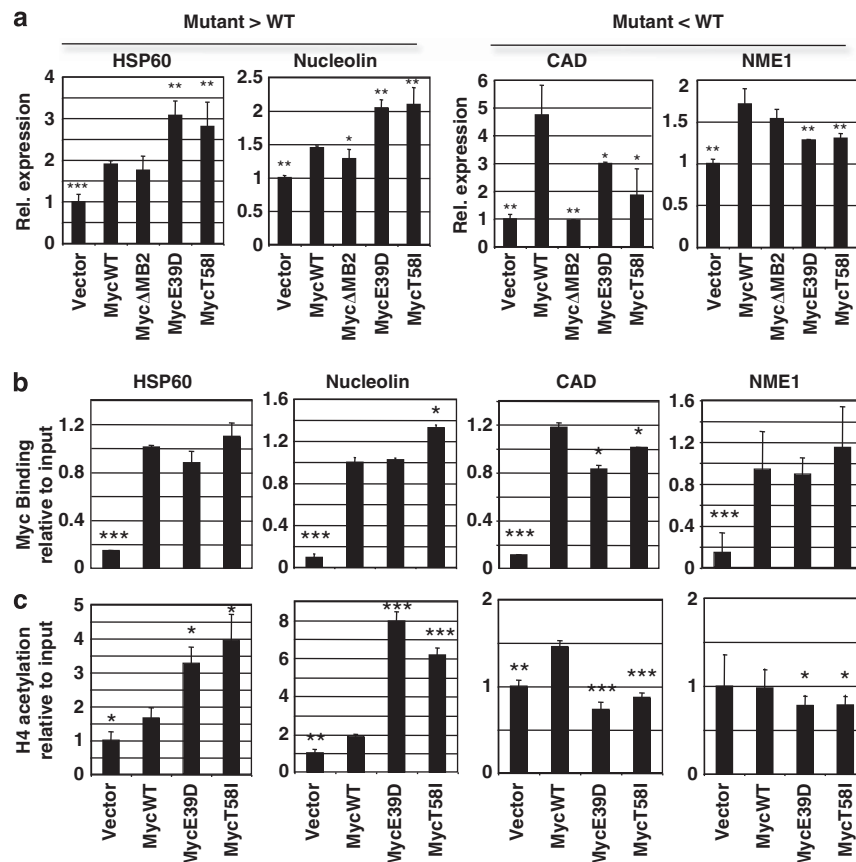


Figure 3. Changes in target gene expression between MycWT and BL-associated mutants correspond with changes in H4 acetylation, but not Myc promoter occupancy. (a) Validation of expression microarrays was performed by reverse transcription-PCR on Rat1a cell lines expressing the indicated form of Myc or vector, to confirm expression levels of known Myc-target genes *HSP60*, *Nucleolin*, *CAD*, *NME1*. The differential regulation of these target genes is consistent with relative expression levels determined by expression microarray. (b) ChIP assays using anti-Myc antibodies were performed to determine Myc binding to E boxes in the promoters of indicated target gene. (c) ChIP assay using anti-acetylated H4 antibodies were performed at the same sites to analyze histone acetylation by the indicated Myc protein. Error bars indicate s.d. over three biological replicates. Asterisk indicates significant difference compared with MycWT; Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

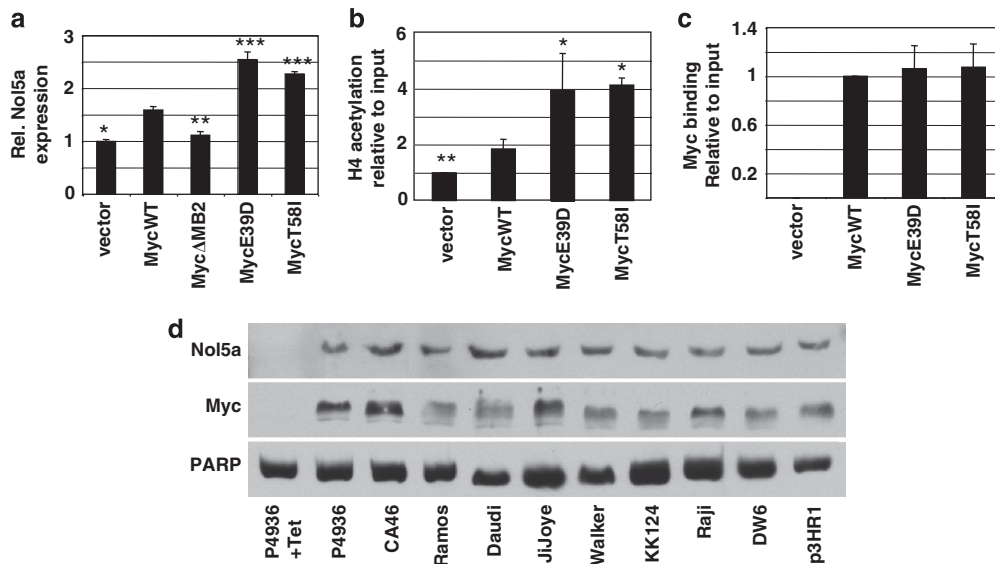


Figure 4. The nucleolar protein gene, *Nol5a*, is differentially induced and acetylated by MycE39D and MycT58I, and highly expressed in Burkitt's lymphoma (BL) cell lines. **(a)** The relative expression level of *Nol5a* in Rat1A fibroblasts engineered to express MycWT or the indicated BL-associated mutations was determined by quantitative PCR. **(b)** ChIP assays were performed at the same site in the *Nol5a* promoter using anti-acetylated H4 antibodies to determine relative acetylation by the indicated Myc proteins. **(c)** ChIP assays were performed using anti-Myc antibodies to determine Myc binding at a conical E box in the *Nol5a* promoter. **(d)** P493-6 human B cells express high levels of Myc, which can be repressed by tetracycline. After 72 h treatment with 0.1 μ g/ml tetracycline or vehicle, nuclear lysates for P493-6 cells as well as indicated BL cell lines were prepared and analyzed by western blots using an anti-c-myc and anti-Nop56 antibodies. Error bars (s.d.) represent three biological replicates. Asterisk indicates significant difference compared with MycWT; Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Rat1a fibroblasts in response to MycWT (Figure 4a). *Nol5a* expression was further elevated in cells expressing MycE39D and MycT58I. Histone acetylation on the *Nol5a* gene was higher in cells expressing the Myc mutants compared with those expressing MycWT, correlating with *Nol5a* expression levels (Figure 4b). In accord with the earlier examples of Myc target genes, the Myc mutants and MycWT exhibit equivalent recruitment to the E-box closest to the *Nol5a* transcription initiation site (Figure 4c). *Nol5a* expression was also elevated in response to the activation of Myc expression in P493-6 cells and in BL cell lines (Figure 4d).

Nol5a overexpression is required for Myc-mediated transformation To explore a functional role for *Nol5a* in Myc transformation, *Nol5a* expression was carefully titrated using small interfering RNAs (siRNAs). As expected, nearly complete depletion of *Nol5a* expression resulted in severely reduced proliferation rates and increased cell death (not shown), consistent with its essential role in ribosome biogenesis.^{39–41} However, when levels of *Nol5a* were reduced by 50% in Rat1a/Myc cells, equivalent to levels in cells not overexpressing Myc, proliferation rates were unaffected and cells remained viable (Figure 5a and Supplementary Figure 3). We next examined how the reduction of *Nol5a* expression would affect the transformation potential of MycWT, MycE39D and MycT58I. Soft agar colony growth confirmed a substantial increase in transformation in cells overexpressing MycWT, MycE39D and MycT58I. Upon depletion of *Nol5a*, there was significant loss in transformation (Figure 5b). In summary, cell transformation by Myc is dependent on Myc-induced *Nol5a* expression, whereas a maximal cell proliferation rate only requires vector control levels of *Nol5a* expression.

Nol5a enhances MycWT-induced transformation and tumor growth

Expression of the Myc mutants increases *Nol5a* expression to a level above that induced by MycWT. We wanted to investigate whether this hyperactivation had functional significance. To this end, we experimentally increased *Nol5a* levels above those found

in MycWT-expressing cells by ectopically expressing *Nol5a* in MycWT/Rat1a cells. We confirmed that ectopic expression of *Nol5a* did not enhance endogenous or exogenous MycWT expression (Figure 6a). Cells were plated in soft agar, and colonies were counted and measured (Figure 6b). As described earlier, expression of MycWT allowed cells to grow into robust colonies in soft agar. We found that further increasing the expression of *Nol5a* in MycWT-expressing cells enhanced cell transformation, producing significantly larger colonies, as depicted in colony images and diameter measurements (Figures 6b and c). Increased *Nol5a* expression alone was not sufficient to permit cell growth in soft agar (Figure 6b). The increased colony size was not due to any increase in the cell proliferation rate of cells expressing MycWT and elevated *Nol5a* (Supplementary Figure 3b) or in rRNA processing (Supplementary Figure 4). In summary, expression of *Nol5a* at levels above those induced by MycWT, as found in response to the BL-associated Myc mutants, is functionally significant and can enhance MycWT-induced cell transformation.

As *Nol5a* expression enhances MycWT-induced cell transformation in the soft agar assay, we wanted to expand upon these assays and measure *in vivo* tumor growth. We injected Rat1a cells expressing MycWT, MycWT plus *Nol5a* or vector control subcutaneously into the flanks of nude mice (Figures 6d and e). Vector control injections resulted in no tumors or nearly undetectable tumors (0.1 g or below), whereas all mice injected with cells expressing MycWT or MycWT and *Nol5a* produced sizeable tumors. We compared the mean mass of each of the groups, 1.20 and 2.14 g, respectively, and determined that the mass of MycWT/*Nol5a* tumors was significantly higher than those produced from MycWT (P -value > 0.001). Therefore, *Nol5a* enhances Myc-induced tumor formation similarly to oncogenic transformation assays in cell culture.

DISCUSSION

We have been interested in a detailed understanding of how BL-associated Myc mutations contribute to cancer. The most

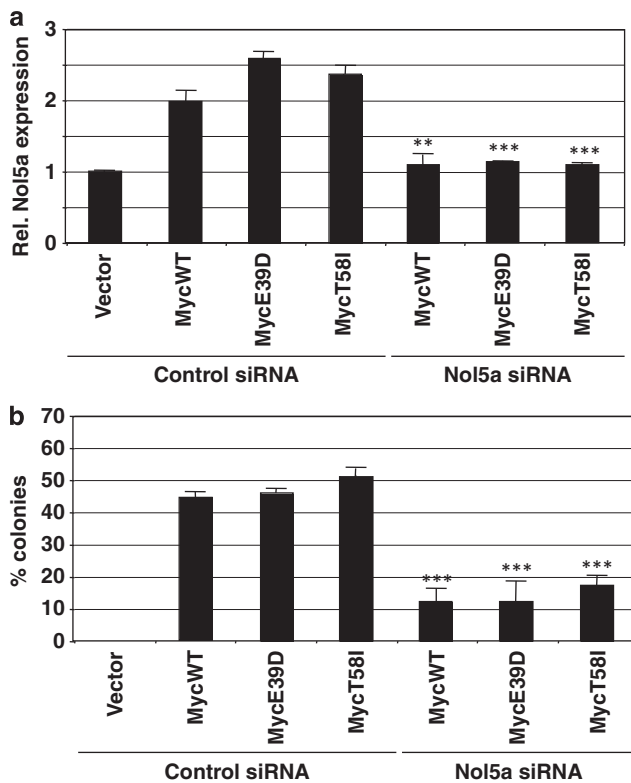


Figure 5. Myc-induced Nof5a overexpression is necessary for MycWT and BL mutant cellular transformation. **(a)** Rat1A fibroblast cells expressing the indicated proteins were treated with control or Nof5a siRNA. After 48 h, RNA was obtained and quantitative PCR was performed to determine relative levels of Myc-induced Nof5a. Nof5a levels were titrated to levels equivalent to cells containing control vector. **(b)** Cells that expressed Nof5a at equivalent levels as vector cells were replated and assessed for their ability to grow in soft agar. Error bars (s.d.) represent three biological replicates. Asterisk indicates significant difference between control and Nof5a siRNA-treated cells; Student's *t*-test, ** $P \leq 0.01$, *** $P \leq 0.001$.

frequently studied mutation has been MycT58A, which can have a modest effect on Myc protein levels through reduced turnover. However, we show that BL-associated mutations enhance cell transformation independently of loss of T58 phosphorylation or increases in Myc expression. All Myc mutants investigated have increased soft agar colony size. Thus, the effect of T58 mutations appear to be allele-specific, with some leading to increased half-life and others unchanged from MycWT. These observations are consistent with previous findings that Myc mutations do not alter Myc protein levels in a murine B-lymphoma model.¹⁷ Thus, the mutations contribute to qualitative rather than quantitative changes in Myc protein biochemistry and activity.

To understand how the BL-associated Myc mutants increase cell transformation, we used expression microarrays to investigate the endogenous gene response to these mutants. We focused our study on MycE39D, which retains T58 phosphorylation, and on MycT58I, which lacks the T58 phosphorylation site, to determine whether this phosphorylation status qualitatively effected target gene expression. We found that the MycE39D mutation increases the number of Myc target genes by approximately 50%, but the T58I mutation had no effect on the absolute number of activated or repressed genes at each threshold. Therefore, a global increase or decrease in gene expression is not necessary for increased cell transformation. However, analysis of the transcriptome of

MycE39D and MycT58I-expressing cells revealed that these point mutants had a significant effect on the profile of responsive genes compared with MycWT. From gene clustering and SAM analysis, MycWT, MycE39D and MycT58I were found to regulate a subset of the same genes, but a large proportion of the BL-associated Myc mutant responsive genes are significantly different from those regulated by MycWT. The surprising finding was that MycE39D and MycT58I converge to regulate an almost identical set of target genes that is distinct from MycWT. Thus, in this system, subtle mutations in the Myc transactivation domain substantially alter the responsive genes, and blocking T58 phosphorylation is not responsible for altering the Myc transcriptome. Recent publications suggest a model for Myc function as a 'transcriptional amplifier' of all active genes in a cell.^{42,43} Although this model can account for the broad effect that Myc has on genome-wide transcription, it does not account for the allele-specific differences in individual gene expression quantitated in our study.

Myc promotes increased transcription by increasing histone acetylation at target gene promoters.^{12,32,33} To gain insight into the mechanism by which the BL-associated point mutations were altering the Myc transcriptome, we investigated Myc recruitment and histone acetylation at four well-established Myc target genes. The BL-associated mutations did not influence Myc recruitment to the genes tested, but we found significant changes in histone acetylation that paralleled changes in mRNA levels. We suggest that the BL-associated mutations induce similar conformational changes in the Myc transactivation domain from that of MycWT, which results in altered recruitment of chromatin-modifying and transcription-promoting complexes.

We found that over 2000 genes were differentially regulated between MycWT and the Myc mutants. Although it is possible that many of these genes are collectively responsible for the increased transforming activity of the mutants, we focused our attention on a single differentially regulated gene, Nof5a/Nop56. Nof5a is a nucleolar protein that is a component of a ribonuclear protein complex, which methylates rRNA and regulates rRNA processing, although it may also have other functions.^{39–41} Our attention was drawn to Nof5a because its expression has been found to be highly associated with BL and serves as a molecular marker for diagnosis,³⁸ suggesting a potentially biological role for its differential expression in these tumors. In addition, Nof5a has been shown to be upregulated in two additional B-cell lymphomas, diffuse B-cell lymphoma and chronic lymphocytic leukemia.^{44,45} Nof5a has previously been found to be a Myc-target gene by several global analyses of gene expression, including in B cells.^{46–49} We show that Nof5a expression is also high in BL cell lines and induced in response to expression of MycWT in B cells and in fibroblasts. Nof5a is further increased by the MycE39D and MycT58I mutations. The higher levels of Nof5a expression induced by MycE39D and MycT58I, above that induced by MycWT, is functionally significant because it enhanced MycWT-induced transformation in two different assays. This demonstrates that increased Nof5a expression by the BL-associated Myc mutants is critical for enhanced oncogenicity.

Nof5a is an essential gene for cell viability, and it has been demonstrated to be rate limiting for cell proliferation in yeast and mammalian cells.^{39,50} Notably, a screen for genes that promote a shortened G1 phase of the cell cycle in yeast identified Nop56/Sik1 as an important mediator of cell cycle progression.⁵¹ We also found that severe Nof5a depletion inhibited cell proliferation. However, when we titrated the levels of Nof5a in Myc-expressing cells to levels found in vector control cells, cell transformation was inhibited but there was no effect on cell proliferation. Therefore, Nof5a is rate limiting for biological effects over a broad range of expression. A basal level of Nof5a is necessary and sufficient for cell proliferation; however, the MycWT-induced increase in Nof5a is necessary for cell transformation, and further Nof5a increases enhance cell transformation.

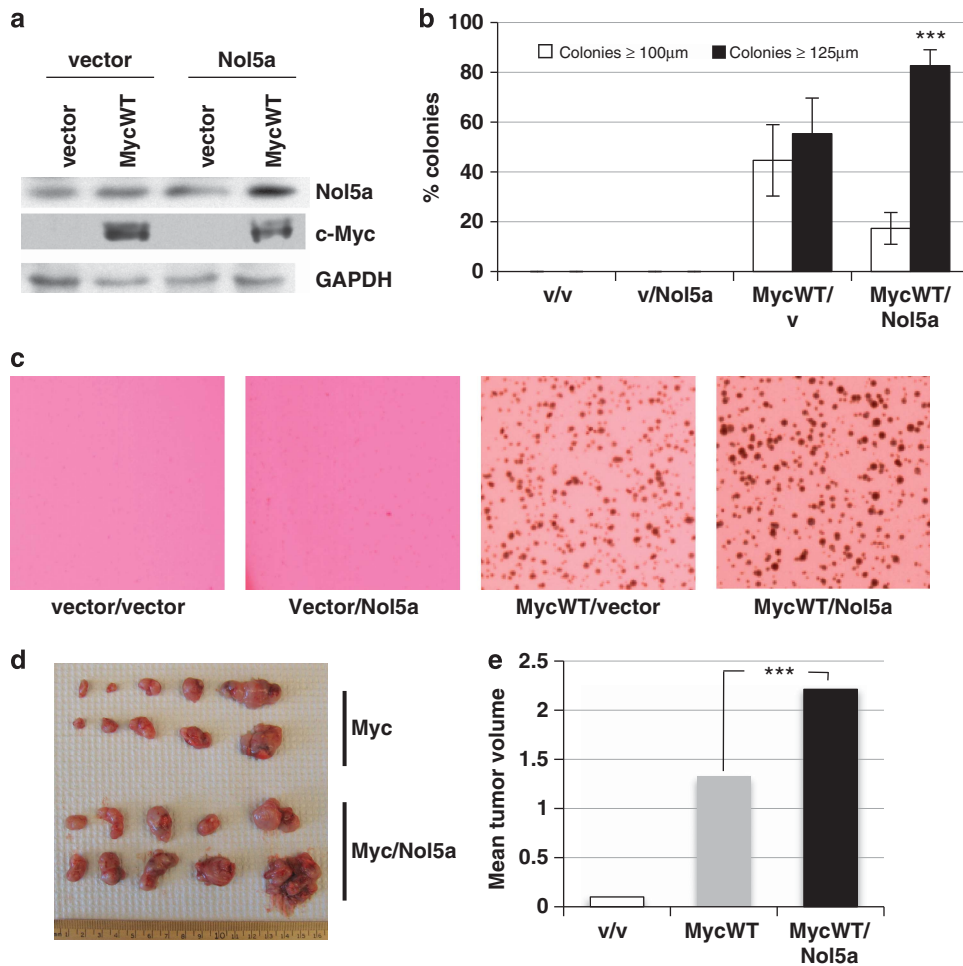


Figure 6. Nol5a synergizes with Myc WT to increase cellular transformation and tumor size. **(a)** Rat1A fibroblast was engineered to express control vector, Nol5a and/or MycWT. Lysates were assayed by western blot using anti-Nol5a (Sigma), anti-Myc and anti-GAPDH. **(b)** Cells expressing the indicated vectors were plated in soft agar and cellular transformation was measured by colony formation and scored for size after 7 days. Colonies were scored as $<100\mu\text{m}$ or $>125\mu\text{m}$ using a reticule; Student's *t*-test, $P \leq 0.001$. **(c)** Representative soft agar plates expressing the indicated vectors 7 days after plating. **(d)** Rat1A cells engineered to express vector ($n = 10$), MycWT ($n = 10$) or MycWT/Nol5a ($n = 10$) were injected subcutaneously into the flanks of nude mice and allowed to form tumors. The tumors were extracted after 3 weeks of growth. **(e)** Individual tumors were weighed and mean mass for each tumor type was calculated. The mass of tumors induced by MycWT, and MycWT/Nol5a were determined to be significantly different; Student's *t*-test, $***P \leq 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Nol5a joins the emerging group of nucleolar proteins, which are essential for ribosome biogenesis and which have also been found to promote cell transformation and tumorigenesis.^{52,53} Myc proteins are recognized to regulate expression of a large number of genes involved in ribosome biogenesis^{47,54–56} including NPM1⁵⁷ and DKC1,⁵⁸ as well as rRNA synthesis itself.¹⁹ Furthermore, ribosome biogenesis is the only cellular process in which the genes harbor conserved Myc/Max-binding sites from primitive metazoans to humans.³⁶ Although the only known function of Nol5a is in ribosome biogenesis, it may also have an extraribosomal function that remains to be discovered.⁵⁹ Nucleophosmin, which is frequently mutated in human cancer, offers a precedent for a novel non-nucleolar function as it is essential for ribosome biogenesis but also has a number of additional roles including regulating the activity and stability of Myc, p53 and ARF, and in centrosome duplication.^{57,60} Nevertheless, the activity of Nol5a in oncogenic transformation strengthens the link between Myc overexpression and ribosome biogenesis and suggests that the protein synthetic capacity of cancer cells may be crucial for Myc-induced tumor formation.

MATERIALS AND METHODS

Cell culture and stable transfections

Rat1a fibroblast, *myc*^{-/-} fibroblast (HO15.19)³⁰ and retroviral packaging Phoenix cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. BL lines were maintained in Iscove's modified dulbecco's medium (IMDM)/10% fetal bovine serum. c-Myc repressible P493-6 cells were maintained in RPMI/10% fetal bovine serum and exogenous c-Myc was repressed by treatment with 0.1 $\mu\text{g/ml}$ tetracycline for 72 h. Retroviral expression of HO15.19 and Rat1a cells was performed using Phoenix retroviral transduction system.

Western blots and immunoprecipitation

Rat1a fibroblast, *myc*^{-/-} fibroblast and BL cell lines were lysed in F-buffer (10 mM Tris pH 7.05, 50 mM NaCl, 30 mM Na pyrophosphate, 5 mM ZnCl, 10% glycerol, 0.1% Triton). Cell extracts were normalized for protein content and either immunoprecipitated or whole-cell lysates run for western blotting analysis. Myc was immunoprecipitated in HO cells using either monoclonal anti-Myc (C33, Santa Cruz, Santa Cruz, CA, USA) or anti-pyo (AFC-115P, Covance, Denver, PA, USA), and western blots performed with polyclonal anti-Myc (N262, Santa Cruz) for total Myc. To determine the T58 phosphorylation status, 293 cells were transiently transfected, lysed in RIPA

buffer (Pierce, Rockford, IL, USA) with phosphatase inhibitors (Sigma, St Louis, MO, USA), and western blots performed using anti-phospho-Myc (T58/S62, Santa Cruz), which recognizes both phosphorylated residues. Whole-cell lysates were analyzed using polyclonal anti-Myc (N262), anti-Nop56 (BL lines; C19, Santa Cruz) and anti-Nol5a (Rat1A lines; AV40645, Sigma) and anti-tubulin or anti-GAPDH controls (Santa Cruz).

siRNA knockdowns

Nol5a or scrambled siRNA (Ambion/Life Technologies, Grand Island, NY, USA) was transfected in a six-well plate according to manufacturer's instructions. Cells were harvested and replated for soft agar 48 h post transfection. RNA and protein were harvested for analysis 48 h post transfection. Error bars (s.d.) represent three biological replicates and significance was determined using Student's *t*-test.

Protein stability

c-Myc expressing *myc*^{-/-} cells were treated with cycloheximide for up to 120 min. Cells were then lysed using F-buffer, immunoprecipitated and blotted as described above. Band intensity was determined by ImageJ image processing software. Results are from two independent experiments.

Real-time PCR

Reverse transcription-PCR was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. For each experiment, RNA from two independently established cell lines was analyzed. Quantitative PCR was performed by mixing complementary DNA, appropriate primers and iQ SYBR Green Supermix (Bio-Rad) and analyzed on a C1000 Thermal Cycler (Bio-Rad). Data were analyzed using Bio-Rad CFX Manager 2.0 software. Error bars (s.d.) represent three biological replicates and significance was determined using Student's *t*-test.

ChIP

ChIPs were performed using the Upstate Chip kit (Millipore, Billerica, MA, USA). The following antibodies were used: polyclonal anti-Myc (N-262; Santa Cruz), anti-Acetyl-H4 (Upstate) and control IgG (Santa Cruz). Error bars (s.d.) represent three biological replicates and significance was determined using Student's *t*-test.

Transformation assays

Soft agar assays were performed as described in Cowling *et al.*⁶¹ Seven days after plating, the longest diameter of 100 colonies from duplicate wells was measured using a reticule. After pretreated with Nol5a or control siRNA, 100 colonies per well from duplicate wells were measured 3 days after plating, and the size (μ m) of colonies over three reticule divisions (75 μ m) in diameter were recorded. Differences in colony size are represented by percentage of colonies greater than 125 μ m. Assays were repeated in duplicate wells over three independent experiments with similar results. Significance was determined using Student's *t*-test.

Microarrays

RNA was harvested on two independent occasions from two independent cultures of *myc*-null fibroblasts expressing vector control, MycWT, Myc Δ MB11, MycE39D and MycT58I using Trizol, followed by phenol chloroform extraction. RNA integrity and concentration was verified by gel electrophoresis. RNA was analyzed on Agilent 4 \times 44K Whole Rat genome microarrays as described in Cowling *et al.*⁶¹ RNA was labeled using the oligo-dT primed linear-amplification protocol according to manufacturer's instructions. Only genes with a signal 50% above background and with at least 70% good data across the arrays were considered for further analysis. The data were centered to the average of the two independent vector-only arrays and filtered for average changes in signal for either MycWT or Myc mutants that were above the threshold indicated.

Supervised SAM analysis, GO term analysis and Student's *t*-tests

SAM³¹ was performed using MeV 4.0 software (Dana-Farber Cancer Institute, Boston, MA, USA). To identify the genes that are most significantly different between the different cell types, we employed two-class SAM analysis with a false discovery rate of 1%. Only those clones

in which both replicate spots were found significant were selected. GO term analysis (David, NIAID) was performed on genes upregulated 1.5-fold over vector for subsets representing MycWT only, BL-associated mutants only or commonly regulated genes. All other statistical significance was determined using non-paired Student's *t*-test with Graphpad Prism 6.0b (Graphpad, La Jolla, CA, USA).

Tumorigenicity assay

1×10^6 cells in 100 μ l phosphate-buffered saline were injected subcutaneously in the flanks of 6-week-old female nude mice (Charles River Laboratories, Wilmington, MA, USA) $N = 10$ for all cell types injected. Tumors were excised after 3 weeks and weighed and measured. Significance in mean tumor volume was determined using Student's *t*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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