A Large Scale Genetic Analysis of c-Myc-regulated Gene Expression Patterns*

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The myc proto-oncogenes encode transcriptional regulators whose inappropriate expression is correlated with a wide array of human malignancies. Upregulation of Myc enforces growth, antagonizes cell cycle withdrawal and differentiation, and in some situations promotes apoptosis. How these phenotypes are elicited is not well understood, largely because we lack a clear picture of the biologically relevant downstream effectors. We created a new biological system for the optimal profiling of Myc target genes based on a set of isogenic c-myc knockout and conditional cell lines. The ability to modulate Myc activity from essentially null to supraphysiological resulted in a significantly increased and reproducible yield of targets, and revealed a large subset of genes that respond optimally to Myc in its physiological range of expression. The total extent of transcriptional changes that can be triggered by Myc is remarkable and involves thousands of genes. Although the majority of these effects are not direct, many of the indirect targets are likely to have important roles in mediating the elicited cellular phenotypes. Myc activated functions are indicative of a physiological state geared towards the rapid utilization of carbon sources, the biosynthesis of precursors for macromolecular synthesis, and the accumulation of cellular mass. In contrast, the majority of Myc repressed genes are involved in the interaction and communication of cells with their external environment, and several are known to possess anti-proliferative or anti-metastatic properties.
The Myc protein is a member of the basic region/helix-loop-helix/leucine zipper (b/HLH/Zip) family of transcriptional regulators, and is capable of exerting both transactivation and transrepression activities (1,2). Transactivation is mediated by binding as an obligate heterodimer with the b/HLH/Zip factor Max to the consensus sequence CA(C/T)GTG (the E box) (3). Transrepression is less well understood (4,5). In either mode Myc is a weak transcriptional regulator, exerting most of its effects within the 2-5 fold range. In a general sense, the upregulation of Myc strongly enforces proliferation and growth, antagonizes cell cycle withdrawal and differentiation, and in some situations promotes apoptosis (6-8). In agreement, the downregulation of Myc results in the attenuation of both cell division and cell growth, as well as protection against some apoptotic processes (9-13). Despite extensive research, the specific mechanisms by which these highly evident biological endpoints are achieved are not well understood. This is largely due to the fact that a comprehensive list of biologically relevant Myc target genes has not yet been defined.

A wide variety of techniques have been employed in the hunt for Myc targets, ranging from differential expression screens, promoter analysis and informed guesswork (14-16) to the modern methods of microarray profiling, SAGE, and chromatin immunoprecipitation (17-23). This search has been complicated by several factors. First, Myc's weak transcriptional effects present significant experimental challenges. Second, by all recent indications the total set of Myc targets may be very large. Third, not all E boxes are bound by Myc, and transient transfection studies do not adequately reflect regulation in a chromosomal context. Fourth, comparing tumor cells expressing amplified Myc with non-deregulated counterparts is complicated by the non-
isogenic nature of the cells. A widely used approach has been to compare cell lines engineered to
overexpress ectopic Myc with parental cells (17,19,21,23). However, it is questionable to what
extent this approach can detect genes that respond optimally to physiological changes in Myc
expression.

In an attempt to circumvent the latter problem we have generated some time ago c-myc
null cells that were derived by gene targeting from an immortalized but otherwise
nontransformed rat fibroblast cell line (9). To date, the c-myc<sup>−/−</sup> cells have been used in two
limited profiling experiments that examined the expression of 4400 rat (18) and 6355 mouse (24)
cDNAs and ESTs in spotted glass slide microarray formats. To create a new biological system
for the optimal profiling of Myc target genes, we have reconstituted c-myc<sup>−/−</sup> cells with the
conditionally-active, tamoxifen-specific c-Myc–estrogen receptor fusion protein (MycERTM)
(25). These new cell lines allow the modulation of Myc activity from essentially null to
supraphysiological.

To achieve maximum consistency in expression profiling we sought a simple
experimental regimen in which the only changing parameter was the expression of c-Myc and in
which a change in c-Myc status elicited and clear and significant change in phenotype. We chose
to use randomly cycling, exponential phase cultures, and we developed conditions such that cells
experience a constant environment and are in a balanced, steady state of growth for significant
periods of time. Under these conditions c-myc null cells display a pronounced phenotype: a 2-3-
fold reduction in macromolecular synthesis accompanied by a commensurate slowing of the cell
cycle (9). Most importantly, we showed that under these conditions both c-myc<sup>++</sup> and c-myc<sup>−/−</sup>
cultures cycle uniformly, namely, that there are no cohorts of differentially cycling or non-
cycling cells within a given culture (26).
Expression profiling using a total of 81 Affymetrix GeneChip arrays was performed in three experiments (Fig. 1). First, we compared c-myc<sup>+/+</sup> (TGR), c-myc<sup>–/–</sup> (HO), and c-myc<sup>–/–</sup> cells reconstituted with a constitutive c-myc transgene (HOmyc3). This revealed the total number of genes that respond to a sustained loss of c-Myc under exponential growth conditions. Second, c-myc<sup>–/–</sup> cells reconstituted with the conditional c-myc<sub>ER</sub> transgene (HOmycER) were stimulated with tamoxifen (OHT) and data were collected during a 16 h time course. This revealed the kinetics of the responses to Myc activation. Finally, the time course of induction with OHT was performed in the presence of cycloheximide, revealing a subset of direct transcriptional targets of c-Myc. All experiments, including the growth of cells and preparation of RNA, were performed on three separate occasions (independent biological replicates) and all data were subjected to a statistical analysis of significance.
MATERIALS AND METHODS

Cell lines and culture conditions—TGR-1 is a hprt- subclone of the Rat-1 cell line (27). HO15.19 (referred to as HO) is a homozygous c-myc null derivative of TGR-1 generated by gene targeting (9). HOMyc3 was derived from HO15.19 by constitutively expressing murine c-myc cDNA using a retroviral vector (10). HOMyc3 cells express c-Myc protein at 3-4-times the level seen in TGR cells. HOMycER12 and HOMycER104 were derived in the same fashion to express a c-Myc–estrogen receptor fusion protein, MycER (25). MycER is a hybrid protein consisting of the entire c-Myc polypeptide at its N-terminus and the ligand (estrogen)–binding domain of the human estrogen receptor at the C-terminus. In the MycER construct used here the estrogen–binding domain has been mutated to be specific for the agonist 4-hydroxy-tamoxifen (OHT). Retroviral vectors were packaged in BOSC cells (28) and supernatants were used to infect HO15.19 cells. Colonies were selected with 120 µg/ml hygromycin (Calbiochem), ring cloned, and expanded into clonal cell lines. The mRNA encoding the MycER protein is thus expressed constitutively from the viral LTR promoter, and the activity of this promoter is not affected by OHT. OHT is instead believed to elicit a conformational change in MycER that allows the protein to become biologically active. All cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 10% calf serum (CS, Hyclone) at 37°C in an atmosphere of 5% CO₂, except BOSC cells, which were supplemented with 10% fetal bovine serum (FBS, Hyclone). Great care was taken that cultures were cycling asynchronously and were in rapid and exponential phase of growth (26). Briefly, cells were
always split at subconfluent densities (<50%) and at relatively low dilution (1:10 for c-myc\(^{\text{+/+}}\) and 1:4 for c-myc\(^{\text{--}}\) cells). Cultures can thus be maintained continuously at densities of 10 to 50% confluence (to avoid any contact inhibition), and the relatively frequent passaging (every 3-4 days) and media changes maintain a rapid growth rate. This regimen was followed for a minimum of 2 passages before cells were harvested for other experiments. MycER was activated with 200 nM OHT (Sigma). Dose-response studies showed that 200 nM OHT is saturating for the activation of MycER. OHT was dissolved in absolute ethanol at 1 mM and stored at -80°C. Mock-treated cultures received vehicle (ethanol) at a final concentration of 0.02%. Protein synthesis was inhibited with 20 µg/ml cycloheximide (Sigma) which was added 30 min. prior to the addition of OHT. BrdU labeling and flow cytometry were performed as described (9), except that the Vectastain Elite ABC\(^{\text{TM}}\) and Novared\(^{\text{TM}}\) kits (Vector Laboratories) were used for histochemical staining.

**Molecular biology procedures**—The c-myc, full length mycER (25), and deletion mutant of mycER missing amino acids 106-143 (29) cDNAs were cloned into the HpaI site of the pLXSH retroviral vector (30) using standard procedures (31). Total RNA for Northern hybridization and microarray analysis was isolated using Trizol reagent (Life Technologies). Total RNA for quantitative real-time RT-PCR (qPCR) was isolated using the RNAqueous-4PCR kit (Ambion). Northern hybridization was performed using the formaldehyde gel method and \(^{32}\)P-labeled probes were synthesized using the random oligo labeling method from gel-purified restriction or PCR fragment templates as described (32). qPCR was performed using the Applied Biosystems Prism 7700 Sequence Detector and software. Primers were designed using Primer Express software (Applied Biosystems) for amplification of 100 bp fragments. cDNA was generated using the TaqMan reverse transcription kit and amplified using the SYBR green PCR.
and RT-PCR kits (Applied Biosystems). Amplification efficiencies were determined by serial
dilution of template cDNA for each gene. All samples were run in triplicate. Glyceraldehyde
phosphate dehydrogenase (Gapdh) was used as the internal standard. Gapdh was used because
microarray profiling showed that the signals for six distinct Gapdh probe sets were equivalent
between TGR, HO, and HOMyc3 cell lines under our exponential phase culture conditions.
Protein samples were prepared by lysing whole cells in radioimmune precipitation assay (RIPA)
buffer (33) supplemented with protease inhibitors. Immunoblotting was performed as described
(10,34). The following antibodies were used: c-Myc, Upstate Biotechnology (cat # 06-340),
neomycin phosphotransferase II, 5'-3' Inc. (cat # 7-511721), actin, Sigma (cat # A5316).
Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch.
Signals were visualized using the ECL reagent (Amersham).

*Microarray analysis*—Target cRNA was prepared and hybridized (45°C, 16 h) to
GeneChip rat U34 arrays according to the manufacturer's directions (Affymetrix). Hybridized
arrays were washed and stained using the GeneChip fluidics station 400 and scanned using the
Agilent GeneArray scanner. Signals were analyzed using Microarray Suite 5.0 software
(Affymetrix). Data was normalized using a set target intensity of 1500, published to a database
using MicroDB 3.0, and analyzed in Data Mining Tool 3.0 software (Affymetrix). Analysis of
each cell line and/or condition was based on three biological replicates (RNAs prepared from
independent experiments performed at different times). The replicates were used to calculate
means and standard deviations for the expression values of all probe sets for each cell line and/or
condition. Probe sets were considered present if they received a present call in two out of the
three biological replicates. Pair-wise comparisons between cell lines and/or conditions were
made using the students T test (p<0.05).
Analysis of experiment 1 (Fig. 1). Myc-influenced expression patterns were assigned to four categories: 1) activated by Myc, 2) repressed by Myc, 3) activated by overexpression of Myc, and 4) repressed by overexpression of Myc (see Fig. 4 for examples). Probe sets were categorized based on the following criteria. 1) Activated by Myc: ratio of average signal intensity between HOmyc3 and HO and/or between TGR and HO was $\geq 2$. 2) Repressed by Myc: ratio of average signal intensity between HOmyc3 and HO and/or between TGR and HO was $\leq 0.5$. 3) Activated by Myc overexpression: HO/Myc3 average intensity values were significantly greater than those of TGR and those of HO (p<0.05), TGR and HO average intensity values were not statistically different (p<0.05), and the ratio of average intensity values between TGR and HO was less than 1.4. Fourteen probe sets were moved from category 1) to category 3) based on visual inspection (in these cases, the ratio of average intensity values between HO and HOmyc3 was greater than or equal to twice the ratio of average intensity values between HO and TGR. 4) Repressed by Myc overexpression: HOMyc3 average intensity values were significantly less than those of TGR and those of HO (p<0.05), TGR and HO average intensity values were not statistically different (p<0.05), and the ratio of average intensity values between TGR and HO was greater than 0.7. Nine probe sets were moved from category 2) to category 4) based on visual inspection (in these cases, the ratio of average intensity values between HO and HOmyc3 was less than or equal to half the ratio of average intensity values between HO and TGR).

Analysis of experiment 2 (Fig. 1). Probe sets were considered responsive to OHT if, at any given time point, they displayed statistically significant (p<0.05) differences between OHT and vehicle treated replicates and the fold change between the means was $\geq 1.5$. Of the 535 probe sets on the U34A chip which were identified in the initial comparison of TGR, HO and HOmyc3
cell lines (Experiment 1, Fig. 1), 142 probe sets were OHT responsive by the above criteria. An additional 460 probe sets satisfied the OHT inducibility test (Experiment 2, Fig. 1) but failed the 2-fold induction limit set in the comparison of TGR, HO and HOmyc3 cells (Experiment 1, Fig. 1). 76 probe sets were recovered from this list and designated as c-Myc targets if the inducibility in the TGR, HO and HOmyc3 comparison (Experiment 1, Fig. 1) was \( \geq 1.5 \). The resultant 218 MycER-responsive probe sets represent 180 nonredundant genes. 75 of the 180 genes (41%) identified in HO/mycER12 cells satisfied the same statistical criteria in HOmycER104 cells. Of the remaining 105 genes, 49 (27%) were already deregulated by the high basal Myc activity in HO/mycER104 cells, 37 (21%) behaved qualitatively similarly in HOmycER104 cells but failed the T test, and 19 (10%) failed the fold-change test or behaved anomalously. Since the MycER protein is capable of eliciting low Myc activity even in the absence of OHT, we also asked whether this “leakiness” could mask potential responses to Myc activation if, for example, probe sets were already maximally induced/repressed prior to the addition of OHT. Probe sets were considered leaky if the average intensity values in HOmycER12 cells were statistically different (\( p<0.05 \)) and greater (for Myc activated genes) or smaller (for Myc repressed genes) than the average intensity values in HO cells. Probe sets that were leaky, nonresponsive to OHT, unable to respond to elevated levels of Myc (nonresponsive to OHT in HOmycER104 cells and/or not overexpressed in HOmyc3 versus TGR cells), and expressed above a threshold intensity value of 500 in TGR cells comprised less than 10% of the probe sets identified in experiment 2 (Fig. 1).

Analysis of experiment 3 (Fig. 1). Probe sets were considered to be direct Myc targets if differences in expression between samples treated with cycloheximide plus OHT and those treated with cycloheximide alone at any time point were statistically significant (\( p<0.05 \)) and had a magnitude of \( \geq 1.5 \). Probe sets were classified as indirect targets of Myc if differences in
expression between samples treated with cycloheximide alone were not statistically different (p<0.05) from the untreated control, and if differences in expression at any time point between samples treated with cycloheximide plus OHT and cycloheximide alone were not statistically significant (p<0.05).

To assess the effect of OHT alone on RNA expression (in the absence of the MycER transgene) TGR and HO cells were treated with OHT for 16 h (or vehicle for the same time period), RNA was extracted, and subjected to microarray analysis. A total of 288 probe sets out of the total 8799 probe sets on the U94A chip were affected by OHT by a factor of ≥2 in either TGR or HO cells. 2 of the OHT-affected probe sets are on the list of 180 genes reported in Table 1. However, these probe sets were affected by OHT only in HO cells and not in TGR cells. The genes affected by OHT alone in HO cells are alpha mannosidase II (M24353) and cytosolic Na/K transporting ATPase, B subunit (AA859920). The expression of these genes was clearly affected in a comparison of TGR, HO and HOmyc3 cells in the absence of OHT, however, since part of their response in HOmycER cells may be due to the effect of OHT alone, further examination may reveal them to be indirect Myc targets.
RESULTS

Complementation of c-myc<sup>−/−</sup> cells with a conditional c-mycER transgene—The c-mycER cDNA (25) was introduced into c-myc<sup>+/+</sup> cells using the retrovirus vector LXSH. Selection with hygromycin in the absence of OHT resulted in an approximately equal mixture of slowly and rapidly growing colonies. Cells in the slow growing colonies displayed a flattened and spread-out morphology typical of c-myc<sup>−/−</sup> cells, while cells in the rapidly growing colonies had the fibroblastic morphology characteristic of the parental c-myc<sup>+/+</sup> cells. Expression of a c-mycER transgene containing an internal deletion of Myc sequences (MycER∆106-143) did not generate any fast growing colonies. The fast growing colonies are thus likely to be the result of low levels of Myc activity elicited by the MycER transgene even in the absence of OHT. Colonies of both types were picked and expanded into cell lines. Two representative clones were chosen for further study: HOmycER12, derived from a slow growing colony, and HOmycER104, derived from a rapidly growing colony.

As expected, HOmycER104 cells expressed higher levels of MycER protein than HOmycER12 cells (Fig. 2A). Several assays were performed to examine the functionality of the c-mycER transgene. Since c-Myc is known to repress its own promoter, we examined the expression of the Neo mRNA, which is encoded by one of the c-myc gene targeting vectors and was placed under control of the endogenous c-myc promoter by the homologous recombination event. Treatment of both HOmycER12 and HOmycER104 cells with OHT clearly reduced the expression of the Neo mRNA (Fig. 2B). In contrast, repression of Neo by OHT was not observed.
in the HO or HOmycERΔ106-143 cell lines (Fig. 2B, legend). Since expression of Myc accelerates the cell cycle and thus increases the fraction of cells in S phase (9), we measured S phase content of HOmycER12 and HOmycER104 cultures using BrdU labeling (Fig. 3). The percentage of cells in S phase was increased from 32% to 48% in HOmycER12 cells and from 42% to 57% in HOmycER104 cells after a 24 h treatment with OHT.

We next examined the expression of several known c-Myc target genes in HOmycER12 cells in the absence and presence of OHT (Fig. 2C). Activation of MycER resulted in the upregulation of CAD mRNA and downregulation of gadd45 mRNA, while the expression of ODC mRNA was essentially unchanged or only weakly activated. The same behavior has been documented for these genes by comparing TGR and HO cells under similar exponential growth conditions (35). In control experiments, OHT had no effect on the expression of CAD, gadd45 or ODC mRNAs in either TGR or HO cells (Fig. 2C, legend). We therefore conclude that HOmycER12 cells in the absence of OHT resemble c-myc<sup>−/−</sup> (HO) cells and that addition of OHT elicits a c-myc<sup>+/+</sup> (TGR)-like phenotype.

In preparation for experiments using OHT induction in the presence of cycloheximide we examined the effect of cycloheximide on the expression of the MycER protein (Fig. 2D). Expression of the MycER protein declined rapidly and was barely detectable at 8 h after cycloheximide and OHT addition. Since c-Myc protein is known to be unstable, with a half life estimated in the range of 30–40 min (36), the turnover of the MycER protein after addition of cycloheximide was not unexpected. This observation unfortunately limits the utility of cycloheximide as a tool to investigate direct action of Myc to targets that respond within a relatively short time frame.
Genes differentially expressed in c-myc−/− cells—Total RNA was extracted from exponentially cycling cultures of TGR, HO and H0myc3 cells and expression profiling was performed using Affymetrix U34 rat GeneChips. Each cell line was grown on three separate occasions and each of the corresponding RNAs (total of 9 RNA samples comprising 3 biological replicates) was hybridized to the three available Affymetrix rat GeneChips (U34A, U34B, U34C; experiment 1 in Fig. 1). 5,732 probe sets displayed statistically significant differences (p<0.05) between TGR and HO cells and/or between HO and H0myc3 cells. Adopting an expression differential cutoff of ≥ 2-fold between the means of TGR and HO and/or HO and H0myc3 reduces the number of probe sets to 1,527. These probe sets were then grouped into four categories according to their patterns of expression: 599 probe sets (39%) were categorized as activated by Myc, 695 probe sets (46%) as repressed by Myc, 94 (6%) as activated by overexpression of Myc, and 87 (6%) as repressed by overexpression of Myc. Representative examples of each functional category are shown in Fig. 4. The remaining 52 probe sets (3%) exhibited patterns of expression whose biological relevance to c-myc is not clear. To ascertain the accuracy of the microarray analysis, we examined the mRNA expression levels of 7 Myc–activated, 6 Myc–repressed and 4 unaffected genes using qPCR. In 17 out of 17 cases the qPCR data confirmed the microarray results. Since the U34A GeneChip contains most of the known rat genes it was used in subsequent experiments.

Kinetic analysis of c-Myc target gene regulation—Exponentially cycling cultures of H0mycER12 cells were treated with either OHT or vehicle (ethanol) and samples were collected 2, 4, 8 and 16 h after treatment (experiment 2 in Fig. 1). The zero time point reference sample was harvested at the time of drug addition, resulting in 9 total RNA samples. The time course experiment was performed on three separate occasions to obtain three biological replicates for a
total of 27 RNA samples. Of the 611 probe sets differentially expressed on the U34A chip in the TGR, HO and HOmyc3 comparison (experiment 1 in Fig. 1), 218 were responsive to OHT in experiment 2. Due to some redundancy present on the chips, the 218 probe sets responsive to MycER correspond to 180 unique genes or EST clusters. They were further categorized according to their kinetics of induction as early–, mid–, or late–responding if the change in expression was first evident at 2 to 4 h, 8 h, or 16 h after OHT addition, respectively. Finally, within these categories genes were grouped according to general function (Table 1).

Representative induction profiles are shown in Fig. 5. The HOmycER104 cell line was also profiled in a time course of OHT induction with samples collected 0, 8, and 16 h after treatment. There was a high degree of concordance between the HOmycER12 and HOmycER104 data sets, thus providing additional verification (Table 1).

Direct targets of c-Myc—Next, we sought to determine which of the 180 unique genes and ESTs that we identified as MycER-responsive may be direct targets. Exponentially cycling cultures of HOmycER12 cells were treated with either OHT plus cycloheximide, or cycloheximide alone, and samples were collected 4, 8 and 16 h after treatment (experiment 3 in Fig. 1). The zero time point reference sample did not contain either drug. As previously, the time course experiment was performed in three biological replicates. 21 out of 180 OHT-responsive genes were designated as direct targets. Interestingly, all 21 were in the Myc-activated category. In addition, we identified 24 activated genes and 16 repressed genes which appeared to be bona fide indirect targets. It is well documented that cycloheximide alone can strongly influence gene expression. These effects have the potential of significantly masking the influence of Myc on the expression of bona fide target genes, and underscore the importance of doing cycloheximide only controls at all time points. Indeed, all the genes that failed the criteria of a direct or indirect target
showed significant induction or repression due to cycloheximide alone. In these cases we do not believe that a clear distinction between a direct and an indirect target can be made.

Representative plots of time courses in various categories are shown in Fig. 5, and the data for all 180 OHT-responsive genes is summarized in Table 1.
DISCUSSION

The three Affymetrix U34 GeneChips provide the most extensive coverage of the rat genome available (26,261 probe sets, 20,691 unique genes and EST clusters). The data set presented here is thus the most comprehensive analysis of Myc-influenced gene expression profiles to date. In total, we identified 1,527 probe sets differentially expressed by 2-fold or more. Given that 43% of all U34 probe sets were expressed in Rat-1 fibroblasts, ~14% of the active transcriptome is responsive to Myc within the 2-fold differential expression cutoff. Since the U34 chips cover approximately half of the rat genome, ~3,000 probe sets (~2,400 genes) can be estimated to be Myc-responsive in this cell type. However, if the differential expression criterion is relaxed to statistical significance only, the Myc-responsive transcriptome becomes greater than 50% of all active genes.

The significantly increased yield of Myc responsive genes achieved in this study is due primarily to our ability to modulate Myc expression from almost zero to supraphysiological. This is clearly evidenced by the fact that a previous report (17) which relied on ectopic MycER expression in a normal (c-myc\textsuperscript{+/+}) cell background identified only 36 MycER responsive targets from a total of 6,416 genes surveyed. Both studies used Affymetrix technology and very similar criteria for data analysis. It is thus clear that while a subset of Myc targets can respond to elevated Myc levels, the great majority of responses occur in the range of physiological expression.

Given the wide-spread effects of Myc on gene expression, it is noteworthy that only 36% of differentially expressed probe sets responded acutely to Myc activation in the HOMycER12
22% of the MycER-responsive genes have been identified in previous studies. One limitation of the MycER activation regimen is the response time of repressed genes, since any observable effects depend on the turnover of the pre-existing mRNA. However, Myc repressed genes comprised 45% of the OHT-responsive set and 51% of the total pool of differentially expressed probe sets, indicating that the extent to which Myc-repressed genes are being underestimated in the 16 h OHT time course is likely to be minor.

The most likely explanation for the large fraction of MycER-nonresponsive genes is that they represent longer term adaptive responses to the loss of c-Myc function. Although the existence of direct targets with delayed responses cannot be ruled out, the fact that many genes respond rapidly argues that factors other than Myc are likely to account for the slow kinetics. Indeed, the list of OHT-responsive genes includes many indirect targets, demonstrating that even indirect effects can be rapid enough to score in the 16 h time course. The morphological changes that accompany the addition of OHT to HOmycER12 cells are slow, taking effect between 24 and 48 h. It is not unreasonable that the extensive adjustments of cellular physiology that take place in response to loss and/or gain of Myc activity would extend over one or even several cell cycles. It is clear that the great majority of these changes are reversible, since 95% of probe sets (on all 3 chips) that are differentially expressed in c-myc<sup>−−</sup> (HO) cells relative to c-myc<sup>++</sup> (TGR) cells are reverted to a c-myc<sup>++</sup> pattern of expression in the HOmyc3 cell line.

Although the activation of MycER with OHT in the presence of cycloheximide has been frequently used to differentiate direct and indirect action of Myc, our data indicate that this method has limited resolution. The major problems are the short life span of the MycER protein, and the presence of significant changes in gene expression caused by cycloheximide alone. Of the 180 OHT-responsive genes, 119 (66%) were significantly affected by cycloheximide alone.
Of the remaining genes, 21 were activated and direct, 24 were activated and indirect, and 16 were repressed and indirect. No directly repressed genes were apparent. One possible explanation is that the resolution of the cycloheximide methodology becomes critically limiting when the short half life of MycER and the effects of cycloheximide are superimposed on the slow response of many repressed genes. Another possibility is that repression by Myc depends on interaction with other proteins, such as Miz-1, and that this interaction, or the activity of the interacting proteins, is masked by cycloheximide artifacts.

Among Myc activated genes, the relative proportion of direct and indirect targets was approximately equal (21% and 24%, respectively). Since there are no obvious reasons why this relationship should not also hold for genes subject to cycloheximide effects, by extrapolation we can expect ~45 direct targets among the 180 OHT-responsive genes. Since 38% of Myc-responsive probe sets were found on the U34A chip, and the set of 3 chips covers approximately half of the rat genome, we can expect ~235 directly activated Myc targets in a rat fibroblast under exponential growth conditions.

While our simple and easily controlled experimental design greatly facilitates expression profiling, there are several reasons why it may be underestimating the total spectrum of Myc regulated genes. First, a gene may not be affected equally by Myc under all growth conditions. For example, the induction of Myc following serum stimulation of quiescent cells could contribute significantly to the regulation of genes that may respond only weakly under balanced, steady state growth conditions. Second, some genes may only be able to respond to Myc during a specific segment of the cell cycle. Third, cell line- or cell type–specific effects are also likely to be encountered. Fourth, some genes are detected poorly or not at all by the current U34 probe sets (e.g., p15 (Ink4b), p27 (Kip1), and Cdk7).
MycER-responsive genes identified in our profiling screen have diverse functions (Table 1). The largest single category on the Myc activated list (22 out of 101 genes) are enzymes involved mostly in carbon assimilation, anabolic pathways, and energy metabolism. Only 6 of these have been previously reported (17,21,37,38). What is striking is the preponderance of enzymes that catalyze the first committed and regulated steps of major pathways, such as glycolysis, biosynthesis of purines, pyrimidines, polyamines, fatty acids, phospholipids, S-adenosylmethionine (a key molecule in one carbon transfer reactions), creatine (important, as creatine phosphate, in short term energy storage), and NADPH (needed in most reductive anabolic reactions). In addition to being rate limiting and regulated allosterically, many of the genes are regulated transcriptionally and their activation is correlated with rapid growth and proliferation.

Also prominent on the Myc activated list are functions that positively impact protein synthesis, including proteins involved in the synthesis and processing of rRNA, the biogenesis of ribosomes, and translation initiation and elongation factors. 17 genes fall in this category, including RNA polymerase I, 8 of which have been previously reported. The expression of 13 ribosomal proteins was decreased in c-myc<sup>−/−</sup> (HO) cells, but none were OHT responsive in MycER cells, indicating that they are likely to be indirect targets. However, since many were weakly inducible at late times in MycER104 cells, it is possible that ribosomal protein genes can also respond to very high Myc levels, such as those found in tumor cells (20).

The appearance of several protein folding functions on the Myc activated list, both cytoplasmic and mitochondrial, is also consistent with an increased capacity for protein biosynthesis. Notable among these are the mitochondrial chaperones and chaperonins prohibitin, BAP-37, Hsp60, Hsp10, and GrpE, all of which have been previously identified as possible Myc
targets. Interestingly, chaperones have been shown to have important roles in the control of apoptosis. The GroEL/GroES homologs Hsp60/Hsp10 have proapoptotic effects involving caspase-3 activation (39,40). In contrast, Hsp27 and alpha B crystallin, identified as Myc repressed in our analysis, have been shown to function as negative effectors of apoptosis through their ability to sequester cytochrome c from Apaf-1 (41), and inhibit the maturation of caspase 3 (42), respectively. We also identified a component of the mitochondrial permeability transition pore complex as a Myc repressed gene.

In a general sense, the majority of Myc activated metabolic functions are indicative of a physiological state geared towards the rapid utilization of major carbon sources and the biosynthesis of precursors for the synthesis of DNA, RNA, proteins and lipids. Combined with the upregulation of the machinery for protein synthesis and folding these changes would promote the accumulation of cellular mass, which is required to support ongoing cellular proliferation. Myc also impacts the expression of key G1 phase cell cycle regulators, raising the question as to which functions, metabolic or cell cycle, are the primary effectors. The preponderance of genes that promote metabolism and cell growth, as well as the documentation of increasing numbers of bona fide direct Myc targets in this category, makes it very unlikely that all these effects are secondary. Indeed, preliminary evidence indicates that both metabolic and cell cycle functions may be equally important: overexpression of an enzyme involved in one carbon metabolism (serine hydroxymethyl transferase) or a cell cycle regulator (Cdk4) both partially rescued the slow growth phenotype of c-myc<sup>−/−</sup> cells (43,44).

The Myc-repressed genes stand in stark contrast to the Myc activated targets: metabolic and protein synthesis functions are absent, and the list is dominated by genes involved in cell adhesion, cell-cell contact, extracellular matrix synthesis and modification, and vesicular
 trafficking. In particular, the latter category has not been previously identified as Myc-responsive. This list includes genes involved in vesicular transport of secreted proteins, such as the calcium binding protein P22, cellubrevin, the secretory carrier membrane protein1, the small G protein ARF2, and phospholipase D. In addition, proteins involved in vesicle docking and fusion, including Rab10, the Rab effector GM130, and the Rab GDP-dissociation inhibitor were found to be repressed by Myc. In a general sense, a significant fraction of Myc repressed genes are involved in the interaction and communication of cells with their external environment. It is especially interesting to note that several of these targets have been shown to possess tumor suppressor and anti-metastatic properties. By repressing genes involved in vesicular trafficking and cellular adhesion inappropriate Myc expression may thus create a permissive environment for aggressive tumor cell invasion.

Although much work will be needed to sort out direct and indirect targets, and to fully integrate the functions of the activated and repressed genes, this study has significantly expanded our appreciation of the impact of Myc on cellular physiology, and has revealed a number of intriguing novel candidates for drug targets. The total extent of transcriptional changes that can be triggered in response to Myc activity is remarkable, and it should be noted that many of the indirect targets are likely to have important roles in mediating the elicited cellular phenotypes.
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1The abbreviations used are: CAD, trifunctional enzyme carbamoyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase; CHX, cycloheximide; EST, expressed sequence tag; MycER, c-Myc–estrogen receptor fusion protein; ODC, ornithine decarboxylase; OHT, 4-hydroxy-tamoxifen; qPCR, quantitative real-time reverse transcription polymerase chain reaction; SAGE, serial analysis of gene expression.
Table 1. Genes Responsive to Myc Expression

| Genes activated by Myc | Accession | Encoded gene product | Function | E/M/L^1 | Fold change | D/I/X^4 | ER104^5 gPCR^6 |
|-----------------------|-----------|----------------------|----------|----------|-------------|---------|---------------|
|                       |           |                      |          |          | T/H^2       | M/H^3   |               |
| Metabolic enzymes     |           |                      |          |          |             |         |               |
| D2639                 | Hexokinase_type II | Metabolism (glycolysis) | E | 1.4 | 1.6 | D |
| *M767                 | Fatty acid synthase | Metabolism (fatty acid) | E | 1.3 | 1.5 | X |
| *D10853               | Phosphoribosyl pyrophosphate amidotransferase | Metabolism (nucleotide) | E | 1.9 | 2.6 | X |
| A008131               | S-Adenosylmethionine decarboxylase | Metabolism (polyamine) | E | 1.7 | 1.5 | X |
| J03585                | Guanidinoacetate methyltransferase | Metabolism (creatine synthesis) | E | 2.2 | 2.8 | X + |
| *M58040               | Transferrin receptor | Iron transport | E | 6.0 | 7.6 | X | 5.6 |
| S70011                | Sideroflexin 1 | Iron transport (mitochondrial) | E | 6.6 | 9.9 | X + |
| J05571                | S-adenosylmethionine synthetase | Metabolism (one carbon) | EO | 1.1 | 1.6 | X |
| AA799700              | Selenophosphate synthetase 2 | Metabolism (selenocysteine) | EO | 1.2 | 2.5 | X + |
| A059508               | S-Adenosylmethionine decarboxylase | Metabolism (polyamine) | M | 1.9 | 3.0 | X + |
| AA859981              | Myo-inositol monophosphatase A2 | Metabolism (phosphoinositide) | M | 1.8 | 2.4 | X + |
| *AB0007768            | CAD | Metabolism (nucleotide) | M | 4.0 | 6.1 | X + 2.1 |
| U07201                | Asparagine synthetase | Metabolism (amino acid) | M | 1.7 | 1.8 | X |
| AI230228              | Similar to phosphoserine aminotransferase | Metabolism (amino acid) | M | 2.8 | 5.1 | X |
| D10262                | Choline kinase | Metabolism (lipid) | MO | 1.0 | 2.4 | X + |
| *L25387               | Phosphofructokinase C | Metabolism (glycolysis) | L | 3.6 | 5.0 | I |
| D13921                | Acetyl-CoA acetyltransferase (mitochondrial) | Metabolism (carbon utilization) | L | 1.4 | 1.5 | X + |
| AA891785              | Isoxurate dehydrogenase (NADP, mitochondrial) | Metabolism (energy) | L | 2.3 | 2.0 | X + |
| AA979466              | Adenylyl kinase 2 | Metabolism (energy) | L | 1.4 | 1.8 | X + |
| *X13527               | Fatty acid synthetase (acyl carrier protein) | Metabolism (fatty acid) | L | 1.7 | 2.2 | X + |
| AA799779              | Dihydroxyacetonephosphate acyltransferase | Metabolism (lipid) | L | 1.3 | 1.7 | X + |
| U57042                | Adenosine kinase | Metabolism (nucleoside salvage) | L | 2.2 | 2.2 | X + |
| Ribosome biogenesis    |           |                      |          |          |             |         |               |
| AF025424              | RNA polymerase I (127 kDa subunit) | Ribosome biogenesis | E | 1.5 | 1.7 | X + |
| AA799724              | RNA polymerase I (RPA16 subunit) | Ribosome biogenesis | E | 1.6 | 1.9 | I + |
| *AA998882             | Nucleolar phosphoprotein Nop140 | Ribosome biogenesis | M | 1.7 | 5.0 | D + 3.6 |
| AA9892562             | Nucleolar protein NAP57 | Ribosome biogenesis | M | 1.5 | 1.8 | I |
| *J04943               | Nucleolusam-related protein B23 | Ribosome biogenesis | M | 1.8 | 2.4 | I |
| *U21719               | DEAD-box RNA helicase DDX21, nucleolar | Ribosome biogenesis | L | 2.8 | 3.9 | D + |
| AA891759              | Similar to DEAD-box RNA helicase | Ribosome biogenesis | L | 1.4 | 1.7 | X + |
| *M55017               | Nucleolin | Ribosome biogenesis | L | 2.2 | 2.6 | D + 1.4 |
| Protein synthesis     |           |                      |          |          |             |         |               |
| A031019               | Initiation factor eIF-2B alpha | Protein synthesis | E | 1.4 | 1.5 | D + |
| U38253                | Initiation factor eIF-2B gamma | Protein synthesis | E | 1.7 | 2.0 | D + |
| *AA875205             | Initiation factor eIF3, subunit 9 | Protein synthesis | E | 1.6 | 2.1 | I + |
| *M98327               | tRNA-valine synthetase | Protein synthesis | M | 1.9 | 2.5 | D |
| *AA891689             | Mitochondrial ribosomal protein L38 | Protein synthesis | M | 2.0 | 2.6 | I + |
| AA866234              | Highly similar to EF-Tu elongation factor | Protein synthesis | MO | 1.3 | 2.0 | I + |
| *J02646               | Initiation factor eIF2-α | Protein synthesis | L | 1.6 | 1.5 | X + |
| AA891553              | Initiation factor eIF3, subunit 7 | Protein synthesis | L | 1.8 | 2.1 | I |
| U62635                | Ribosomal protein L23-related protein | Protein synthesis | L | 3.4 | 2.6 | X |
| Protein folding       |           |                      |          |          |             |         |               |
| *U75392               | BAP-37 (chaperone) | Protein folding (mitochondrial) | E | 2.0 | 2.6 | X + |
| *AA858640             | Hsp60 chaperonin | Protein folding (mitochondrial) | M | 2.0 | 2.4 | D |
| *A170613              | Hsp10 chaperonin | Protein folding (mitochondrial) | M | 1.6 | 2.2 | X + 3.2 |
| *A169631              | Protein folding (mitochondrial) | Protein folding (cytosolic) | M | 1.6 | 2.3 | X + |
| *AA799545             | TCP-1 chaperonin, gamma subunit | Protein folding (mitochondrial) | M | 1.5 | 1.8 | X + |
| *U62940               | GrpE stress-inducible chaperone | Protein folding (mitochondrial) | L | 1.3 | 1.9 | I |
| Protein degradation   |           |                      |          |          |             |         |               |
| M61142                | Metalloendopeptidase | Protein degradation | E | 2.5 | 2.6 | X + |
| U38379                | Gamma-glutamyl hydrolase | Protein degradation | E | 2.2 | 1.8 | X + |
| M19647                | Kallikrein 1 serine protease | Protein degradation | M | 1.8 | 2.4 | X |
| AB012759              | Prolyl endopeptidase | Protein degradation | M | 1.4 | 1.7 | X |
| AA891445              | SKD3, member of Clp/Hsp 104 ATPase family | Protein degradation | M | 1.6 | 2.9 | I + |
| A176351               | Tripeptidylpeptidase II | Protein degradation | L | 2.1 | 2.5 | X |
| Protein glycosylation, transport, ion transport |           |                      |          |          |             |         |               |
| AF087431              | Glucosidase 1 | Protein glycosylation | E | 1.6 | 1.8 | D + |
| AB006451              | Inner membrane translocase TIM23 | Protein transport (mitochondrial) | E | 1.2 | 1.6 | I + |
| AF061242              | Inner membrane translocase subunit TIM9B | Protein transport (mitochondrial) | EO | 1.1 | 2.7 | I |
| Accession | Encoded gene product | Function | E/M 1 Fold change | T/H 2 | M/I 3 | ER104 4 | qPCR 5 |
|-----------|----------------------|----------|-------------------|-------|-------|---------|--------|
| D13985    | Chloride channel     | Ion channel | M | 1.5 | 2.5 | I | + |
| AA859920  | Cytosolic Na/K transporting ATPase, B subunit | Ion pump | M | 2.0 | 1.8 | I | + |
|           | **Vesicular trafficking** |          | | | | | |
| AA875098  | Small G protein ARF3 | Vesicular trafficking | E | 1.9 | 3.5 | X | + |
| U40999    | UNC-119 (C. elegans) homolog | Vesicular trafficking | EO | 1.0 | 1.6 | X | + |
| AA798879  | Moderately similar to synaptogyrin 1 | Vesicular trafficking | L | 2.2 | 4.0 | I | + |
|           | **DNA replication, repair, chromatin assembly** | | | | | | |
| *AJ222691 | DNA polymerase delta catalytic subunit | DNA replication | M | 1.9 | 2.3 | X | + |
| *AF062594 | Nucleosome assembly protein | Chromatin assembly | M | 1.7 | 1.4 | D | + |
| U60882    | Protein arginine N-methyltransferase | Histone methylation | M | 1.8 | 2.6 | I | + |
| *D44495   | APEX nuclease | DNA repair | L | 2.8 | 4.2 | D | 4.8 |
| AA894030  | Similar to tyrosyl-DNA phosphodiesterase | DNA repair | L | 2.1 | 2.1 | I | + |
|           | **Transcription** | | | | | | |
| D45254    | CNBP transcription factor | Transcription | E | 1.7 | 2.2 | D | |
| AA875084  | Transducin-like enhancer of split 1 | Transcription | E | 2.3 | 3.5 | X | + |
| *AB002406 | Tip49 DNA helicase | Transcription | E | 1.8 | 2.2 | D | 2.9 |
| AA893611  | Moderately similar to Mxi1 | Transcription | EO | 1.2 | 2.3 | X | |
| AA799412  | Similar to estrogen-related receptor alpha | Transcription | EO | 0.9 | 2.1 | X | |
| *X62875   | HMGI/Y | Transcription | M | 1.0 | 1.9 | D | |
| AI229637  | Myb-binding protein (p160) | Transcription | M | 1.7 | 2.3 | I | + |
| Z71925    | RNA polymerase II (polypeptide G) | Transcription | M | 1.3 | 2.2 | I | + |
| *D28557   | RYB-α Y-box binding protein | Transcription | L | 1.7 | 1.5 | D | |
| L01267    | General transcription initiation factor TFIIIF | Transcription | LO | 1.1 | 1.7 | X | |
|           | **mRNA processing, stability** | | | | | | |
| AF044910  | SMN protein | RNA processing | M | 1.4 | 1.7 | X | + |
| M12156    | hRNP A1 | RNA processing | L | 2.5 | 3.2 | I | + |
| AA875102  | RUXE snRNP | RNA processing | L | 1.9 | 2.0 | I | |
| U21718    | PAI-1 mRNA binding protein | mRNA stability | L | 1.8 | 1.9 | I | |
|           | **Signaling, cell cycle** | | | | | | |
| AA892789  | Moderately similar to receptor for cholecystokinin | Signaling | E | 1.6 | 2.4 | X | + |
| *AI178135 | Complement component 1 | Signaling | E | 2.5 | 3.4 | D | |
| X82445    | Nuclear distribution gene C homolog | Proliferation | E | 1.3 | 1.6 | X | + |
| *AI231547 | FBK4 FK506-binding protein | Signaling | M | 1.6 | 1.7 | I | |
| *U82591   | RCL | Transformation/uncertain | M | 2.3 | 3.4 | X | + |
| AA874794  | Nerve growth factor receptor associated protein 1 | Signaling/apoptosis | M | 2.0 | 2.8 | I | |
| *L11007   | Cdk4 | Cell cycle | M | 1.5 | 2.0 | X | |
| *D14015   | Cyclin E | Cell cycle | M | 1.5 | 2.4 | X | |
| D30735    | ALR hepatic growth factor | Signaling/proliferation | LO | 1.1 | 1.6 | X | + |
|           | **Uncertain/unknown** | | | | | | |
| AA639158  | Weakly similar to yeast initiation factor IF3Y | Uncertain | E | 4.2 | 5.4 | D | + |
| AA8007739 | Weakly similar to KT12 yeast protein | Uncertain | E | 1.0 | 1.5 | D | + |
| AA891838  | Weakly similar to C. elegans F10E7.5.p | Unknown | E | 1.5 | 1.9 | I | + |
| H31976    | Weakly similar to C. elegans T06E6.1.p | Unknown | E | 1.6 | 2.4 | X | |
| AA799475  | Hypothetical protein | Unknown | E | 1.6 | 1.5 | X | + |
| AA859856  | Hypothetical protein | Unknown | E | 1.6 | 2.3 | X | |
| AA859996  | Similar to hypothetical human protein | Unknown | EO | 1.3 | 1.9 | X | + |
| AA892598  | Hypothetical GTP binding protein | Unknown | M | 1.7 | 2.2 | D | |
| AA875126  | Hypothetical protein | Unknown | M | 2.8 | 4.4 | D | + |
| AI639093  | Hypothetical protein | Unknown | M | 1.5 | 1.6 | D | + |
| AA891322  | Weakly similar to nucleolin | Uncertain | L | 1.7 | 1.8 | X | |
| U44948    | Cysteine-rich protein 2 | Unknown | L | 3.2 | 4.4 | X | |
| AI639411  | Similar to human hypothetical protein | Unknown | L | 1.6 | 1.5 | X | + |
| AA892399  | Hypothetical protein | Unknown | L | 1.9 | 2.3 | X | + |

### Genes repressed by Myc

**Vesicular trafficking**

| Accession | Encoded gene product | Function | E/M 1 Fold change | T/H 2 | M/I 3 | ER104 4 | qPCR 5 |
|-----------|----------------------|----------|-------------------|-------|-------|---------|--------|
| L12381    | Small G protein ARF2 | Vesicular trafficking | E | 0.6 | 0.4 | X | 0.6 |
| E07296    | N-acetylglucosamine transferase-I | Vesicular trafficking | M | 0.8 | 0.6 | I | |
| AA944423  | cis-Golgi matrix protein GM130 | Vesicular trafficking | M | 0.8 | 0.7 | I | |
| X74402    | Rab GDI α (GDP-dissociation inhibitor) | Vesicular trafficking | M | 0.7 | 0.5 | I | |
| U40628    | Rab10 | Vesicular trafficking | MO | 0.9 | 0.5 | X | |
| U39875    | Calcium binding protein p22 | Vesicular trafficking | L | 0.7 | 0.5 | X | |
| S63830    | Cellubrevin | Vesicular trafficking | L | 0.7 | 0.6 | X | + |
| AI073164  | Secretory carrier membrane protein 1 | Vesicular trafficking | L | 0.8 | 0.7 | X | + |
| Accession | Encoded gene product | Function | $E/M^1$ | Fold change | D/I/X$^4$ | ER104$^5$ | qPCR$^6$ |
|-----------|---------------------|----------|--------|------------|--------|--------|--------|
| AF084186  | Alpha-fodrin (nonerythroid spectrin) | Vesicular trafficking | L | 0.6 | 0.4 | X |
| M24353    | Alpha-mannosidase II | Golgi processing enzyme | L | 0.3 | 0.2 | X |
| AI171167  | Annexin IV | Vesicular trafficking | LO | 0.9 | 0.5 | X |
| D42137    | Annexin V | Vesicular trafficking | LO | 1.1 | 0.6 | X |
| X86086    | Annexin VI | Vesicular trafficking | LO | 1.1 | 0.4 | X |
|           | **Membrane dynamics, recycling** | | | | | | |
| U45749    | Synaptotagmin | Membrane recycling | E | 0.7 | 0.5 | X |
| AA892373  | Syntenin | Membrane dynamics | M | 0.6 | 0.4 | I |
| AA799580  | Moderately similar to junctophilin 2 | Membrane dynamics | M | 0.7 | 0.4 | I |
|           | **Cytoskeletal architecture** | | | | | | |
| AA892649  | Aminobutyric acid receptor associated protein | Cytoskeletal architecture | M | 0.8 | 0.7 | X |
| U75404    | Ssecks 322 | Cytoskeletal architecture | M | 0.3 | 0.2 | I |
| X74800    | Myosin, unconventional Myr2 I heavy chain | Motor protein | M | 0.7 | 0.3 | X |
| AI180288  | Caldesmon 1 | Actin polymerization | LO | 0.7 | 0.4 | I |
|           | **Extracellular matrix** | | | | | | |
| *AJ005394 | Collagen, type V, alpha 1 | Extracellular matrix | L | 0.6 | 0.3 | X |
| AI104225  | Laminin chain beta 2 | Extracellular matrix | L | 0.6 | 0.5 | I |
| AI234060  | Lysyl oxidase (has tumor suppressor activity) | Extracellular matrix modification | L | 0.7 | 0.4 | X |
| Z78279    | Procollagen, type I, alpha 1 | Extracellular matrix | LO | 1.0 | 0.4 | X |
|           | **Cell-matrix adhesion, motility** | | | | | | |
| AF097593  | Cadherin 2, type 1 | Cell-matrix adhesion | L | 0.7 | 0.5 | X |
| AF017437  | Integrin-associated protein | Cell-matrix adhesion | L | 0.6 | 0.4 | X |
| *AA948313 | Osteonectin | Cell-matrix adhesion | L | 0.7 | 0.4 | X |
| AA600005  | Platelet endothelial tetraspan antigen-3 | Cell motility | L | 0.4 | 0.6 | X |
| *AA874848 | Thy-1 cell surface glycoprotein | Surface glycoprotein/signaling | M | 0.6 | 0.4 | X |
| AA892921  | Moderately similar to human sorcin | Ion channel binding protein | M | 0.7 | 0.6 | I |
| X07648    | Amyloidogenic glycoprotein | Cell-cell contact | L | 0.4 | 0.4 | X |
| L02896    | Glypican 1 | Surface glycoprotein | M | 0.6 | 0.4 | X |
| AA799520  | Integral membrane protein 2B | Cell-cell contact/apoptosis | L | 0.6 | 0.6 | X |
| AA892918  | Moderately similar to tight junction protein 1 | Cell-cell contact | L | 0.7 | 0.4 | X |
| X14323    | Fc receptor subunit p51 | Cell surface receptor for IgG | L | 0.6 | 0.5 | I |
| X56541    | Membrane-spanning proteoglycan NG2 | Surface glycoprotein | LO | 1.0 | 0.6 | X |
|           | **Protease inhibitors** | | | | | | |
| X76985    | Latexin | Carboxypeptidase A inhibitor | M | 0.7 | 0.4 | X |
| X56729    | Calpastatin | Calpain inhibitor | M | 0.6 | 0.5 | X |
| AI008888  | Cystatin beta | Cysteine proteinase inhibitor | M | 0.7 | 0.6 | X |
| AI169037  | TIMP-1 | Extracellular protease inhibitor | L | 0.6 | 0.6 | X |
| S27594    | TIMP-2 | Extracellular protease inhibitor | L | 0.5 | 0.3 | X |
| AA849769  | Follistatin-related protein | Serine protease inhibitor | L | 0.6 | 0.3 | X |
| AA875037  | Moderately similar to human serpin B9 | Serine protease inhibitor | LO | 0.7 | 0.2 | I |
| AA892486  | Lysosomal alpha-glucosidase (acid maltase) | Carbohydrate degradation | M | 0.5 | 0.3 | I |
| M32016    | LAMP-2, lysosome-associated membrane protein | Lysosome function/metastasis | L | 0.6 | 0.6 | X |
| M95768    | N-acetylglutamate (lysosomal) | Glycoprotein degradation | LO | 0.8 | 0.4 | X |
|           | **Protein folding/apoptosis** | | | | | | |
| *AA998683 | Hsp27 chaperone | Protein folding/apoptosis | L | 0.8 | 0.7 | X |
| *M55534   | AlphaB-crystallin chaperone | Protein folding | L | 0.1 | 0.0 | X |
|           | **Apoptosis** | | | | | | |
| U14647    | Caspase (IL1 beta converting enzyme) | Protein degradation/apoptosis | M | 0.2 | 0.3 | X |
| *J05122   | Mitochondrial permeability transition pore protein | Apoptosis | L | 0.7 | 0.6 | X |
|           | **Oxidative stress response** | | | | | | |
| AI179610  | Heme oxygenase 1 | Oxidative stress response | M | 0.5 | 0.6 | X |
| *D15069   | Adrenomedullin (cytokine) | Oxidative stress response | M | 0.4 | 0.4 | X |
|           | **Signaling** | | | | | | |
| U02553    | PTPase, non-receptor type 16 | Signaling | E | 0.4 | 0.4 | X |
| AA818593  | Phosphatidate phosphohydrolase type 2 | Signaling | LO | 0.8 | 0.3 | X |
| AB000778  | Phospholipase D | Signaling/Vesicular trafficking | L | 0.9 | 0.5 | X |
| D88666    | Phosphatidylethanolamine-specific phospholipase A1 | Signaling/metastasis | L | 0.5 | 0.4 | X |
| *L02529   | Frizzled homolog (cytokine) | Signaling | L | 0.6 | 0.2 | X |
| M81642    | Thrombin receptor | Signaling | L | 0.7 | 0.4 | X |
| X71898    | Urinary plasminogen activator receptor 2 | Signaling | L | 0.7 | 0.7 | X |
| AI113289  | PTPase | Signaling | L | 0.7 | 0.6 | X |

1. $E/M$ represents the fold change in expression between two groups.
2. $T/H$ represents the fold change in protein levels.
3. $M/H$ represents the fold change in mRNA levels.
4. $D/I/X$ indicates the direction of change; + indicates upregulation, - indicates downregulation.
5. $ER104$ indicates the expression ratio.
6. $qPCR$ indicates qPCR results.
| Accession | Encoded gene product                                      | Function             | E/M/L | Fold change | D/I/X | ER104 | qPCR  |
|-----------|----------------------------------------------------------|----------------------|-------|-------------|-------|--------|-------|
| D38222    | Protein tyrosine phosphatase-like protein                | Signaling            | L     | 0.5         | 0.5   | I      | +     |
| X13412    | Fik receptor type protein tyrosine kinase               | Signaling            | L     | 0.8         | 0.7   | I      | +     |
| D37951    | MIBP-1 c-Myc intron binding protein                      | Transcription        | E     | 0.8         | 0.5   | X      | +     |
| U14746    | von Hippel-Lindau protein                               | Transcription        | M     | 0.7         | 0.5   | X      |       |
| L26292    | Kruppel-like factor 4                                   | Transcription        | L     | 0.4         | 0.2   | X      |       |
| AA799498  | Natriuretic factor (hormone)                            | Osmoregulation       | M     | 0.2         | 0.0   | X      | +     |
| S55427    | Gas-3 homolog                                           | Growth arrest        | M     | 0.8         | 0.4   | X      | +     |
| *AA944007 | Nucleobindin                                            | Calcium homeostasis  | L     | 0.4         | 0.3   | X      | +     |
| AA875428  | Hypothetical protein                                    | Unknown              | E     | 0.6         | 0.4   | X      |       |
| AA891797  | Weakly similar to Pea15 astrocyte phosphoprotein        | Unknown              | M     | 0.5         | 0.3   | X      |       |
| AA894212  | Weakly similar to C. elegans hypothetical protein       | Unknown              | M     | 0.7         | 0.6   | X      |       |
| AA893454  | Hypothetical protein                                    | Unknown              | MO    | 0.9         | 0.5   | X      |       |
| D42116    | 5i2 antigen, clone 17                                   | Unknown              | MO    | 1.0         | 0.6   | I      |       |
| AA800882  | Similar to human hypothetical protein                   | Unknown              | L     | 0.7         | 0.4   | X      |       |
| AA875032  | Similar to human hypothetical protein                   | Unknown              | L     | 0.7         | 0.6   | X      |       |
| AA859885  | Hypothetical protein                                    | Unknown              | L     | 0.5         | 0.3   | X      |       |
| AA866432  | Hypothetical protein                                    | Unknown              | L     | 0.6         | 0.4   | X      |       |
| AA894297  | Hypothetical protein                                    | Unknown              | L     | 0.9         | 0.5   | X      |       |
| A1639149  | Hypothetical protein                                    | Unknown              | L     | 1.0         | 0.6   | I      |       |

*Designates genes identified in previous screens.

1 Kinetics of response to MycER activation: early (E), middle (M), or late (L) if the change in expression was first evident at 2 to 4 h, 8 h, or 16 h after OHT addition, respectively. O designates that the gene responded to Myc overexpression only.

2 Fold change in target gene expression between c-myc<sup>+/+</sup> (TGR) and c-myc<sup>−/−</sup> (HO) cells expressed as the ratio of TGR divided by HO average intensities.

3 Fold change in target gene expression between Myc-reconstituted c-myc<sup>−/−</sup> (HOmyc3) and c-myc<sup>−/−</sup> (HO) cells expressed as the ratio of HOmyc3 divided by HO average intensities.

4 Response to MycER activation with OHT in the presence of cycloheximide was used to evaluate direct versus indirect mode of action: D, direct target; I, indirect target; X, inconclusive target.

5 Response confirmed in HOmycER104 cell line indicated as (+).

6 Response confirmed by qPCR. Values given are the fold change in target gene expression between c-myc<sup>+/+</sup> (TGR) and c-myc<sup>−/−</sup> (HO) cells expressed as the ratio of TGR divided by HO qPCR values.
FIG. 1. **A schematic representation of expression profiling experiments.** Salient points of experimental design are indicated, as well as an overall summary of the obtained data. In experiment 1 each RNA sample was used to interrogate 3 chips (U34A, U34B and U34C) for a total of 27 chips.

FIG. 2. **Characterization of c-myc<sup>−/−</sup> cell lines reconstituted with the conditional c-mycER transgene.** A, Expression levels of c-Myc and MycER proteins. The indicated cell lines were harvested in exponential phase of growth and analyzed by immunoblotting (key for cell line abbreviations: Myc3, HOmyc3; ER12, HOmycER12; ER104, HOmycER104; ERA, HOmycERΔ106-143). Where indicated, OHT was included in the culture medium for 16 h prior to harvest. The MycERΔ protein contains an internal deletion (amino acids 106-143) and thus migrates faster than MycER. Actin was used as the loading control. X-band: a cross-reacting protein of unknown identity. B, Repression of knocked-in Neo mRNA by MycER. HOmycER12 and HOmycER104 cells were harvested in exponential phase of growth and analyzed by qPCR. Where indicated, OHT was included in the culture medium for 16 h prior to harvest. Gapdh was used to normalize for equal input of RNA. Repression of Neo mRNA by the 16 h OHT treatment was 1.7±0.22-fold in the HOmycER12 cell line, and 3.8±1.1-fold in the HOmycER104 cell line. To demonstrate that the effect of OHT on Neo mRNA expression is dependent on MycER, HO and HOmycERΔ106-143 cells were treated with OHT for 16 h (or propagated in parallel without OHT), RNA was harvested, and analyzed by qPCR. All data were normalized to Gapdh, and are expressed relative to the no OHT condition, which was set to a value of 1.0 for each cell line.
The values in the presence of OHT were: HO, 0.97±0.17; HOMycERΔ106-143, 0.86±0.11. C, The effect of MycER on expression of gadd45, CAD and ODC mRNAs. HOMycER12 cells were harvested in exponential phase of growth and analyzed by Northern hybridization. Where indicated, OHT was included in the culture medium for 48 h prior to harvest. Gapdh was used as the loading control. To demonstrate that the effect of OHT on gadd45, CAD, and ODC mRNA expression is dependent on MycER, TGR and HO cells were treated with OHT for 48 h (or propagated in parallel without OHT), RNA was harvested, and analyzed by qPCR. All data were normalized to Gapdh, and are expressed relative to the no OHT condition, which was set to a value of 1.0 for each gene and cell line. The values in the presence of OHT were: CAD: TGR, 0.95±0.20; HO, 1.14±0.15; gadd45: TGR, 1.07±0.13; HO, 1.11±0.12; ODC: TGR, 0.93±0.13; HO, 1.45±0.11. D, Expression of MycER protein in the presence of cycloheximide. HOMycER12 cells in exponential phase of growth were treated with OHT and cycloheximide (CHX). Note that cycloheximide was added 30 min prior to OHT. Samples were harvested at the indicated times and analyzed by immunoblotting. Actin was used as the loading control.

**FIG. 3.** Proliferation of c-myc<sup>−/−</sup> cell lines reconstituted with MycER. The fraction of cells in S phase was determined using pulse labeling with BrdU. The indicated cell lines were pulsed for 30 min during exponential phase of growth, and BrdU incorporation was visualized using in situ immunocytochemical methods. Where indicated, OHT was included in the culture medium for 24 h prior to harvest. c-myc<sup>+/+</sup> (TGR) and c-myc<sup>−/−</sup> (HO) cells processed as above (in the absence of OHT) were used as controls. OHT does not affect BrdU incorporation of TGR or HO cells (data not shown).
FIG. 4. Categories of Myc target genes. A, Myc activated target. Expression is reduced in c-myc<sup>−/−</sup> (HO) cells but is approximately equivalent in c-myc<sup>+/+</sup> (TGR) and Myc overexpressing (HOmyc3) cells. U75392 corresponds to the prohibitin 2 gene. B, Myc repressed target. Expression is increased in c-myc<sup>−/−</sup> (HO) cells but is approximately equivalent in c-myc<sup>+/+</sup> (TGR) and Myc overexpressing (HOmyc3) cells. S81478 corresponds to an oxidative stress inducible protein tyrosine phosphatase gene. C, Target activated by Myc overexpression. Expression is increased in Myc overexpressing (HOmyc3) cells but is approximately equivalent in c-myc<sup>+/+</sup> (TGR) and c-myc<sup>−/−</sup> (HO) cells. D10262 corresponds to the choline kinase gene. D, Target repressed by Myc overexpression. Expression is decreased in Myc overexpressing (HOmyc3) cells but is approximately equivalent in c-myc<sup>+/+</sup> (TGR) and c-myc<sup>−/−</sup> (HO) cells. Z78279 corresponds to the type I procollagen alpha 1 gene.

FIG. 5. Patterns of MycER responsiveness in the absence and presence of cycloheximide. A., Myc activated, early responding, direct target. Expression is inducible with OHT, and is statistically significant at both the 2 and 4 h (and subsequent) time points (upper panel). Expression of this gene is affected by cycloheximide alone, but the effect is small (~2–fold), and activation by OHT plus cycloheximide is both statistically significant and ≥ 1.5–fold in comparison to the cycloheximide only treatment (lower panel). AI178135 is complement component 1, q subcomponent binding protein. B, Myc activated, mid responding, indirect target. Expression is inducible with OHT, but is not statistically significant at the 2 h and 4 h time points (upper panel). Expression is not affected by cycloheximide alone, and the difference between OHT plus cycloheximide and cycloheximide only treatments is not statistically different at any time point (lower panel). U60882 is an arginine N-methyltransferase. C, Myc repressed,
early responding target, showing a strong cycloheximide effect. Expression is repressible with OHT, and is statistically significant at the 4 h and later time points (upper panel). Expression is strongly affected by cycloheximide alone (almost 10–fold at 4 and 8 h time points). The difference between OHT plus cycloheximide and cycloheximide only treatments is not statistically different at any time point (lower panel). D15069 is adrenomedullin. D, Myc repressed, early responding target, showing a weak cycloheximide effect. Expression is repressible with OHT, and is statistically significant at the 4 h and later time points (upper panel). Expression is weakly affected by cycloheximide alone (~2–fold at 4 and 8 h time points). The difference between OHT plus cycloheximide and cycloheximide only treatments is not statistically different at any time point (lower panel). This case illustrates the limitations due to the combined effects of cycloheximide treatment and the short half life of MycER. It could be argued that the repression due to OHT (~2–fold) should be discernable on top of a cycloheximide effect of approximately the same magnitude, and that this gene should thus be classified as an indirect target. However, repression at 4 h is relatively weak, while the cycloheximide effect is at its strongest. Repression improves at the 8 h time point, but at this time virtually no MycER is present in the cells. U02553 is a non-receptor type 16 protein tyrosine phosphatase.
### Results

26,261 probe sets on U34A, U34B, U34C chips

5,732 differentially expressed probe sets (p<.05)

1,527 differentially expressed probe sets (≥2-fold)

695 repressed by Myc

94 activated by overexpression of Myc

87 repressed by overexpression of Myc

599 activated by Myc

611 probe sets on U34A chip from Exp’t 1

218 Myc-responsive probe sets in ER12

180 nonredundant genes/ESTs from Exp’t 2

67 repressed by Myc

11 activated Myc overexpression

12 repressed Myc overexpression

### Cell lines

- TGR, HO, HOmyc3

### Experimental set-up

- exponential growth

- 3 replicates = 9 RNA samples

- 611 probe sets on U34A chip from Exp’t 1

- 218 Myc-responsive probe sets in ER12

- 180 nonredundant genes/ESTs from Exp’t 2

### Results

- 5,732 differentially expressed probe sets (p<.05)

- 1,527 differentially expressed probe sets (≥2-fold)

- 695 repressed by Myc

- 94 activated by overexpression of Myc

- 87 repressed by overexpression of Myc

- 599 activated by Myc

- 611 probe sets on U34A chip from Exp’t 1

- 218 Myc-responsive probe sets in ER12

- 180 nonredundant genes/ESTs from Exp’t 2

### Experiment 1: profiling c-myc +/+ and −/− cells

- exponential growth

- 3 replicates = 9 RNA samples

- 611 probe sets on U34A chip

- 218 Myc-responsive probe sets

- 90 activated by Myc

- 67 repressed by Myc

- 11 activated Myc overexpression

- 12 repressed Myc overexpression

### Experiment 2: response to conditional Myc

- HOmycER12, HOmycER104

- time course of tamoxifen (OHT) treatment

- ER12: 0, 2, 4, 8, 16 h –OHT

- 2, 4, 8, 16 h +OHT

- ER104: 0 h –OHT

- 8, 16 h +OHT

- 3 replicates = 36 RNA samples

- 52 other

- 87 repressed by Myc

- 11 activated Myc overexpression

- 67 repressed by Myc

- 12 repressed Myc overexpression

### Experiment 3: response in presence of cycloheximide

- HOmycER12

- OHT induction in presence of cycloheximide

- 4, 8, 16 h +CHX –OHT

- 4, 8, 16 h +CHX +OHT

- 3 replicates = 18 RNA samples

- 180 nonredundant genes/ESTs from Exp’t 2

- 21 direct (D), 40 indirect (I),

- 119 inconclusive (X) targets

- activated by Myc: 21 D, 22 I, 47 X

- repressed by Myc: 13 I, 54 X

- activated by Myc overexp: 2 I, 9 X

- repressed by Myc overexp: 3 I, 9 X
Figure 2

A

Cell line treatment
ERΔ ER12 ER104 TGR HO Myc3 OHT
MycER MycERΔ
Actin

B

Relative expression
Cell line ER12 ER104 OHT

C

Cell line treatment ER12 ER12 OHT
Gadd45 ODC Gapdh
CAD

D

treatment (h) 0 0.5 1 1.5 2 2.5 8 OHT CHX
MycER Actin
Figure 3
Figure 4

Fold change

A. Activated
B. Repressed
C. Over-activated
D. Over-repressed

TGR
HO
HOMyc3
Myc activated genes
Myc activated genes
AA891759

Similar to DEAD-box RNA helicase

M55017

Nucleolin

AI031019

Initiation factor eIF-2B alpha

U38253

Initiation factor eIF-2B gamma

AA875205

Initiation factor eIF3, subunit 9

M98327

tRNA-valine synthetase

Mitochondrial ribosomal protein L38

ER12 -/+ OHT

CHX, CHX + OHT

104 -/+ OHT

Similar to DEAD-box RNA helicase

M55017

Nucleolin

AI031019

Initiation factor eIF-2B alpha

U38253

Initiation factor eIF-2B gamma

AA875205

Initiation factor eIF3, subunit 9

M98327

tRNA-valine synthetase

Mitochondrial ribosomal protein L38

ER12 -/+ OHT

CHX, CHX + OHT

104 -/+ OHT
Myc activated genes

- Similar to tyrosyl-DNA phosphodiesterase
- CNBP transcription factor
- Transducin-like enhancer of split 1
- Tip49 DNA helicase
- Moderately similar to MX1
- Similar to estrogen-related receptor alpha
- HMG I/Y
Myc activated genes

U44948

CHX, CHX + OHT

AI639411

ER12 -/+ OHT

AA892399

ER12 -/+ OHT

ER12 -/+ OHT

CHX, CHX + OHT

CHX, CHX + OHT

Cysteine-rich protein 2

Similar to human hypothetical protein

Hypothetical protein
Myc repressed genes
| Gene                        | Expression Levels |
|-----------------------------|-------------------|
| Mitochondrial permeability transition pore protein | J05122 |
| Heme oxygenase              | AI179610          |
| Adrenomedullin              | D15069            |
| PTPase, oxidative stress-inducible | S81478 |
| PTPase, non-receptor type 16 | U02553            |
| Phosphatidate phosphohydrolase type 2 | AA818593 |
| Phospholipase D             | AB000778          |
Phosphatidylserine-specific phospholipase A1

Frizzled homolog (cytokine)

Thrombin receptor

Urinary plasminogen activator receptor 2

PTPase

Protein tyrosine phosphatase-like protein

Flk receptor type protein tyrosine kinase
| Gene ID   | Description                                      | Expression Levels |
|-----------|--------------------------------------------------|-------------------|
| D37951    | MIBP-1 c-Myc intron binding protein              | 200, 400, 600     |
| U14746    | von Hippel-Lindau protein                        | 0, 200, 400       |
| L26292    | Kruppel-like factor 4                            | 0, 200, 400       |
| AA799498  | Natriuretic factor (hormone)                     | 0, 200, 400       |
| S55427    | Gas-3 homolog                                    | 0, 200, 400       |
| AA944007  | Nucleobindin                                     | 0, 200, 400       |
| AA875428  | Hypothetical protein                             | 0, 200, 400       |

**Note:** The table above shows the expression levels for various genes under different conditions. The levels are represented in arbitrary units (AU).
A large scale genetic analysis of c-Myc-regulated gene expression patterns
Brenda C. O’Connell, Ann F. Cheung, Carl P. Simkevich, Wanny Tam, Xiaojia Ren, Maria K. Mateyak and John M. Sedivy

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