Reversible Kinetic Analysis of Myc Targets In vivo Provides Novel Insights into Myc-Mediated Tumorigenesis

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Abstract

Deregulated expression of the Myc transcription factor is a frequent causal mutation in human cancer. Thousands of putative Myc target genes have been identified in in vitro studies, indicating that Myc exerts highly pleiotropic effects within cells and tissues. However, the complexity and diversity of Myc gene targets has confounded attempts at identifying which of these genes are the critical targets mediating Myc-driven tumorigenesis in vivo. Acute activation of Myc in a reversibly switchable transgenic model of Myc-mediated β cell tumorigenesis induces rapid tumor onset, whereas subsequent Myc deactivation triggers equally rapid tumor regression. Thus, sustained Myc activity is required for tumor maintenance. We have used this reversibly switchable kinetic tumor model in combination with high-density oligonucleotide microarrays to develop an unbiased strategy for identifying candidate Myc-regulated genes responsible for maintenance of Myc-dependent tumors. Consistent with known Myc functions, some Myc-regulated genes are involved in cell growth, cycle, and proliferation. In addition, however, many Myc-regulated genes are specific to β cells, indicating that a significant component of Myc action is cell type specific. Finally, we identify a very restricted cadre of genes with expression that is inversely regulated upon Myc activation-induced tumor progression and deactivation-induced tumor regression. By definition, such genes are candidates for tumor maintenance functions. Combining reversibly switchable, transgenic models of tumor formation and regression with genomic profiling offers a novel strategy with which to deconvolute the complexities of oncogenic signaling pathways in vivo. (Cancer Res 2006; 66(9): 4591-601)

Introduction

The Myc transcription factor regulates many genes implicated in diverse cellular functions, including proliferation, differentiation, death, and tissue reorganization. Disruption of the control of these processes is a mandatory feature of cancer and, consistent with this, genetic lesions in the Myc locus occur frequently in human malignancy. In general, such lesions result in the overexpressed and/or deregulated expression of Myc. However, the molecular mechanisms by which Myc promotes cellular transformation and oncogenesis remain unclear.

The pleiotropic effects of Myc function are thought to arise through its action as a transcription factor, positively or negatively regulating target effector genes. Induction of gene targets by Myc requires its dimerization with its partner protein, Max, and subsequent heterodimeric binding to canonical (CACGTG) and noncanonical DNA E-box sequences (1–4). The mechanisms by which Myc represses gene targets are less clear but likely involve functional interference via binding to and inhibiting the activity of positive regulators, such as Miz-1 (5). In attempts to define the transcriptional target genes of Myc, several genome-wide screens have been conducted using different cell types and experimental conditions (6–9). These studies have indicated the great diversity of Myc target gene function and have, cumulatively, identified well over 1,000 putative transcriptional targets. Unfortunately, the degree of overlap among these in vitro studies has been small, alluding to the critical potential role of cell type and context in determining the biological consequences of Myc action. With respect to its specific role in cancer, it has not been possible to substantiate which of these Myc targets are mission critical for tumorigenesis and which are functionally inconsequential. Importantly, to date, no systematic analysis of Myc transcriptional targets in any orthotopic tissue setting in vivo has been conducted.

MycERTAM is a regulatable fusion protein that exhibits Myc activity only in the presence of the ligand 4-hydroxytamoxifen (4-OHT). In plhs-MycERTAM transgenic mice, MycERTAM expression is targeted to pancreatic β cells via the insulin promoter (10). The β cells of plhs-MycERTAM mice exhibit uniform cell cycle entry upon systemic administration of 4-OHT; however, the net biological outcome of such acute Myc activation depends on the extent to which Myc-induced apoptosis is opposed. By itself, activation of Myc triggers massive β-cell apoptosis that overwhelms proliferation and leads to islet involution (10). In contrast, coexpression of Bcl-xL in β cells inhibits apoptosis, allowing Myc-induced proliferation to supersede death and resulting in rapid and progressive β-cell expansion (10). Upon sustained activation of MycERTAM, hyperplastic islets progressively acquire the defining characteristics of cancer, including dysplasia and dedifferentiation, angiogenesis, local invasion, and metastasis. Moreover, these features are completely dependent on continuous Myc action as evidenced by the uniform regression of tumors upon 4-OHT withdrawal (10).

Given the consistent, synchronous, and reproducible pattern of phenotypic changes that Myc elicits and maintains in this model, the plhs-MycERTAM x RIP7-Bcl-xL double-transgenic mouse is an ideal system with which to study the genetic processes that
underlie Myc-induced tumorigenesis. We have, therefore, used this model to track the transcriptional changes that follow acute activation of Myc in pancreatic β cells in their orthotopic environment. Using genomic profiling of laser capture micro-dissected islets, we show that acute activation of Myc drives a progressively more complex pattern of expression changes over time as direct Myc targets engage secondary transcriptional programs. Interestingly, although we identified the anticipated cell cycle and metabolic genes, most of the Myc-regulated genes have not been previously reported as such in *in vitro* studies. Furthermore, numerous genes implicated in pancreatic islet development and β-cell differentiation were profoundly modulated by Myc, demonstrating that cell type is an integral determinant of the biological outcome of Myc action. Finally, by combining this activation analysis with analogous kinetic analysis of the transcriptional changes that accompany deactivation of Myc in established β-cell tumors, we have defined a discrete set of Myc target genes whose functions are implicated in the maintenance of Myc-dependent tumors.

**Materials and Methods**

**Mice, tissue sample generation, and preparation.** All mice were housed and treated in accordance with protocols approved by the committee for animal research at the University of California, San Francisco. Transgenic mice expressing switchable *pins-MycERTAM* and constitutive *RIP7-Bcl-XL* in their pancreatic β cells (*M+XL*+) have been previously described and characterized (10).

For expression microarray studies, 8- to 12-week-old *M+XL*+ mice were injected i.p. every 24 hours with 1 mg 4-OHT (early activation) or 1 mg tamoxifen (sustained activation and regression cohorts) to activate MycERTAM. Tamoxifen is metabolized in *vivo* to 4-OHT and studied in our laboratories to confirm their equivalent effects when administered to *M+XL*+ mice (data not shown). For the activation cohort, mice were sacrificed by cervical dislocation at 2, 4, 8, and 24 hours, or 21 days postinjection. Regression cohort mice were sacrificed 2, 4, or 6 days after completing 21 days of treatment. Pancreata were immediately dissected and frozen on dry ice in optimum cutting temperature medium and stored at −80°C. Organs from untreated (0 hours) *M+XL*+ littermates were obtained to act as reference controls. Pancreata from 0- and 24-hour 4-OHT-treated single transgenic *RIP7-Bcl-XL* littermates (*M−XL*+) served as additional 4-OHT-only controls. Three mice were obtained for each experimental condition and mice of both sexes were randomly included across all conditions with only controls. Three mice were obtained for each experimental condition.

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exposed to cycloheximide (10 μmol/L) for 30 minutes before 4-OHT/ethanol treatment to determine if gene regulation was dependent on functional protein synthesis. Total RNA (1 μg) from all islet and MEF samples was reverse transcribed to first strand cDNA (iScript Reverse Transcriptase kit, Bio-Rad, Hercules, CA), and gene expression levels were detected using standardized TaqMan Assays-on-Demand (Applied Biosystems, Foster City, CA) and an ABI Prism 7700 PCR sequence detector (Applied Biosystems). Samples were run in triplicate and the level of expression of each assayed gene was averaged and then quantified relative to the level of expression of β-glucuronidase.

Results

Kinetic profiling of Myc effects in vivo is feasible using the combined technologies of laser capture microdissection, RNA amplification, and expression microarrays. Using linearly amplified RNA from laser-captured transgenic islets and high-density oligonucleotide microarrays, we profiled the transcriptional consequences of Myc activation and subsequent deactivation in the β cells of M+X_I+ mice in vivo. cMycERTAM was activated in vivo by injection of 4-OHT and islets were laser captured from pancreatic frozen sections. All samples were treated independently yielding 20 to 50 ng total islet RNA per sample. Two rounds of linear amplification generated 20 to 50 μg of high-quality, biotin-labeled 2a-cRNA/sample. The overall quality of microarray data obtained for 33 independently amplified and hybridized RNA samples was excellent, with an average “present” call of 46 ± 4%, which is consistent with published results (20). Following normalization of the raw data, 9,813 probe sets were identified as being reproducibly detectable in hIns-MycERTAM–positive pancreatic islets.4 We then analyzed these probe sets for evidence of modulation by Myc.

Global gene expression profiling in pancreatic islets following Myc activation in vivo reveals complex and dynamic changes. To define immediate early transcriptional responses to Myc, we first identified those genes modulated within 24 hours of acute Myc activation. Statistical analysis of microarray software (17) defined 293 probe sets as significantly altered in this early activation cohort. Expression of eight probe sets was also altered following 4-OHT treatment of control MycERTAM transgene-negative M–X_I+ mice, indicating that they represent genes that can be directly modulated by 4-OHT. These eight probe sets were excluded from further analysis, leaving 285 probe sets whose expression in pancreatic β cells in vivo was significantly altered within 24 hours of acute Myc activation (Supplementary Table S1). Supervised hierarchical clustering of the 285 significantly regulated probe sets shows the dynamic nature of Myc-mediated gene regulation, even over such a short time period (Fig. 1A).

To define Myc-dependent effects in established β cell tumors, we expanded our data analysis to include samples obtained from mice exposed to tamoxifen for 21 days. M+X_I+ transgenic islets exposed to sustained Myc activity exhibit all the phenotypic characteristics of malignant β-cell tumors (10). Consistent with our published results, histologic sections confirmed that the islets isolated from these mice were massive, proliferative, and angiogenic (data not shown). Kinetic expression profiling of this sustained activation cohort, revealed that 2,532 probe sets were significantly modulated by Myc over the course of 21 days (Supplementary Table S2).

Supervised hierarchical clustering of the 2,532 probe sets shows significant elaboration and evolution of the dynamic changes observed in the early activation cohort (Fig. 1B). Many of the probe sets that were initially induced within 24 hours of Myc activation were repressed in established islet tumors and vice versa. The dramatic transcriptional differences apparent in 24-hour and 21-day islets underscore the profound effect exerted by sustained Myc activation.

Myc activation in vivo induces synchronous cell cycle entry and modulates previously identified in vitro targets. Consistent with its potent mitogenic action, cell cycle genes figure prominently among in vivo Myc targets (21). We monitored S-phase entry of β cells in vivo by BrdUrd incorporation following acute MycERTAM activation. This showed that onset of β cell cycle entry is extremely synchronous, with S-phase entry of β cells evident in all islets within 16 to 24 hours (Fig. 2A).

Of the 285 significant early activation probe sets, 36 (representing 34 unique genes) are designated “cell cycle genes” by gene ontology classification of biological function (Fig. 2B; ref. 22). The synchrony of cell cycle entry we observe upon MycERTAM activation is reflected in the kinetics of expression of key cell cycle regulatory

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4 The data discussed in this article have been deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE4356.
The transcriptional profile of murine pancreas during development has been characterized, providing a suite of genetic fingerprints that define the developing organ at various stages of differentiation (25). Adult islets can be distinguished from less differentiated pancreas by the differential expression of 217 mature islet-specific genes that are, in the main, markers of fully differentiated β cells (25). One hundred eighty-nine of 217 mature islet genes are represented in our M+X+ islet data set by 218 probe sets and expression of 89 (47%) of these was altered as a consequence of Myc activation (Supplementary Table S4). The vast majority (73 of 89) of these islet genes were down-regulated by Myc and, in the case of 25 genes, sustained Myc activity repressed expression by ≥2-fold (Fig. 3A). Interestingly, several islet-specific genes, including Gad1, Ptpn11, Slc2a2, and the key transcriptional regulator of β-cell-specific gene expression Ipfi (Pdx1), were repressed within the first 2 to 4 hours of Myc activation, intimating that they may be direct targets of Myc (Supplementary Table S4). qRT-PCR analysis of three well-characterized β cell genes, Th, Slc2a2, and Ins2, which encode tyrosine hydroxylase, facilitated glucose-transporter 2 (glut2), and insulin, respectively, corroborated the microarray data (Fig. 3B). To establish whether this gene repression leads to loss of the differentiated β-cell phenotype, we assessed the influence of Myc activation on expression of the mature β-cell proteins insulin, Glut2, and Isl-1. Expression of all three β-cell markers was suppressed upon Myc activation, whereas none was affected by 4-OHT treatment of MycERTAM-negative M–X+ mice (Fig. 3C). Finally, to show that β-cell gene repression and dedifferentiation were a consequence of Myc activity, we assessed expression in established β-cell tumors 2, 4, and 6 days following acute deactivation of Myc. Consistent with our previously published observations (10), acute deactivation of Myc in these β-cell tumors resulted in tumor regression and reappearance of mature β-cell markers insulin, Glut2, and Isl1 (data not shown). Genomic profiling of these regressing islet tumors showed that reappearance of the mature β-cell phenotype was heralded by the

**Figure 2.** Myc activation in β cells in vivo induces rapid and synchronous cell cycle entry and sustained proliferation. A, activation of Myc in double-transgenic (M–X+β) pancreatic β cells by systemic administration of 4-OHT induced synchronous S-phase entry in β cells in all islets of the pancreas by ~16 to 24 hours, as evidenced by BrdUrd-positive staining (green immunofluorescence). Analogous 4-OHT treatment of MycERTAM-negative, M–X+ mice induced no S-phase transition. B, hierarchical gene cluster of 41 probe sets (38 genes) with gene ontology functions of cell cycle or proliferation, which were regulated in the first 24 hours of MycERTAM activation. C, qRT-PCR analysis of cyclin D1 (Ccnd1) and cyclin B2 (Ccnb2) expression in laser-captured islets confirmed the sequential modulation observed in the microarray data. Columns, fold change; bars, SD.

**Cyclin D1 gene (Ccnd1), whose product is pivotal for G1 phase progression, was induced within 2 hours of Myc activation, whereas expression of the S-phase cyclin A (Cena2a) and mitotic cyclin B1 (Ccnb1) and cyclin B2 (Ccnb2) genes peaked subsequently (Fig. 2B).** Induction of genes encoding the G2-M transition regulatory phosphatase Cdc25c and the mitotic regulator kinase Cdk1 (Cdc2) paralleled induction of the mitotic cyclins. This sequential pattern of cyclin induction was confirmed by qRT-PCR (Fig. 2C).

Next, we used an established database (21) to determine which of our significant in vivo targets had been previously labeled as Myc targets in vitro.5 Perhaps surprisingly, only 50 of 285 early activation target probe sets (equivalent to 47 unique genes) have been previously identified as Myc targets (Supplementary Fig. S1). In the sustained activation cohort, we identified 303 designated Myc target genes among our list of 2,532 regulated probe sets (Supplementary Table S3). Thus, kinetic profiling of Myc target genes in pancreatic β cells in vivo corroborates in vitro studies but also identifies multiple novel targets with diverse biological roles.

**Myc represses mature β-cell genes and results in global islet dedifferentiation.** Activation of Myc in many normal and neoplastic cell types is associated with an undifferentiated phenotype. It remains unclear whether this inverse relationship between proliferation and differentiation is merely correlative or represents some obligate exclusivity in the two biological programs. In β cells, it has been noted that ectopic expression of Myc represses insulin expression (10, 23, 24). To assess whether Myc activation more globally suppresses terminal β-cell differentiation, we analyzed our microarray database to determine the effect of Myc activation on the expression of mature β-cell-specific genes.

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5 http://www.myccancergene.org/site/mycTargetDB.asp.
reexpression of β-cell-specific genes (Fig. 3D). Thus, sustained Myc activity in pancreatic β cells represses the expression of mature β-cell genes, leading to loss of differentiation, whereas subsequent deactivation of Myc rapidly restores β-cell gene expression. These results indicate that the process of Myc-mediated tumorigenesis involves profound changes in differentiation status, many of which arise from modulation of tissue-specific genes.

Comparison of genomic profiles during β-cell tumor formation and regression identifies genes whose modulation is exquisitely dependent on continual Myc activity. Myc-induced β-cell tumorigenesis arises through induction of β-cell proliferation, dedifferentiation, and angiogenesis. Subsequent Myc deactivation induces proliferative arrest, redifferentiation, and collapse of vascular integrity, indicating that tumor regression is essentially a reversal of the processes that drive tumor formation (10). Comparison of the regression cohort expression profiles with those of stable tumors identified 1,431 probe sets that were significantly altered during the onset of tumor regression (Supplementary Table S5). Hierarchical clustering of these genes identifies two distinct categories: 1,000 probe sets that were rapidly down-regulated upon Myc deactivation and 431 probe sets that were induced (Supplementary Fig. S2). To identify which Myc effectors may be involved in maintaining established β-cell tumors, we compared the 2,532 probe sets that were significantly regulated upon Myc activation with the 1,431 regression probe sets modulated by Myc deactivation. This comparison revealed 428 probe sets common to both cohorts, 257 of which were regulated in opposing directions during tumor initiation (Myc on) and regression (Myc off; Fig. 4; Table 1). Thus, regulation of these genes (either positive or negative) was, like maintenance of the β-cell tumors, dependent on sustained Myc activity. Of the 257 “candidate tumor maintenance” probe sets, 154 (equivalent to 132 genes) were both induced upon Myc activation and repressed upon deactivation (type A genes), whereas 103 probe sets (representing 101 genes) were repressed by Myc activation and then reexpressed when Myc was deactivated (type B genes; Table 1). The diverse biological functions of these candidate tumor maintenance genes are apparent from their gene ontology designations (Table 1A).

To identify which of the 257 probe sets are likely to be direct Myc targets, we determined which were regulated immediately upon Myc activation or deactivation. Myc-induced β-cell tumorigenesis arises through induction of β-cell proliferation, dedifferentiation, and angiogenesis. Subsequent Myc deactivation induces proliferative arrest, redifferentiation, and collapse of vascular integrity, indicating that tumor regression is essentially a reversal of the processes that drive tumor formation (10). Comparison of the regression cohort expression profiles with those of stable tumors identified 1,431 probe sets that were significantly altered during the onset of tumor regression (Supplementary Table S5). Hierarchical clustering of these genes identifies two distinct categories: 1,000 probe sets that were rapidly down-regulated upon Myc deactivation and 431 probe sets that were induced (Supplementary Fig. S2). To identify which Myc effectors may be involved in maintaining established β-cell tumors, we compared the 2,532 probe sets that were significantly regulated upon Myc activation with the 1,431 regression probe sets modulated by Myc deactivation. This comparison revealed 428 probe sets common to both cohorts, 257 of which were regulated in opposing directions during tumor initiation (Myc on) and regression (Myc off; Fig. 4; Table 1). Thus, regulation of these genes (either positive or negative) was, like maintenance of the β-cell tumors, dependent on sustained Myc activity. Of the 257 “candidate tumor maintenance” probe sets, 154 (equivalent to 132 genes) were both induced upon Myc activation and repressed upon deactivation (type A genes), whereas 103 probe sets (representing 101 genes) were repressed by Myc activation and then reexpressed when Myc was deactivated (type B genes; Table 1). The diverse biological functions of these candidate tumor maintenance genes are apparent from their gene ontology designations (Table 1A).

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following MycERTAM activation. Thirty maintenance probe sets were modulated within 24 hours of Myc activation (Fig. 5A) and 10 were altered >1.5-fold within 2 to 4 hours (Fig. 5B). Of these, six were induced (BC037006, Eif4ebp1, Fzd9, Gmip, St6galnac4, and Zbtb16), whereas four were repressed (5730592L21, Dap, Derl2, and Sec23b; Fig. 5B; Table 1B). Although only 3 of these 10 genes have been previously reported to be Myc targets (Gmip, St6galnac4, and Sec23b), by sequence analysis all six type A genes contain canonical Myc:Max binding sites in their promoters (data not shown), supporting the idea that these are direct Myc targets. To further validate these targets, we selected three type A genes (Fzd9, Eif4ebp1, and Gmip) and one type B gene (Dap) for analysis by qRT-PCR. These studies confirmed Myc-mediated regulation of all four genes in β cells in vivo (Fig. 5C).

Finally, to determine if Myc-dependent regulation of key maintenance genes is dependent on cell type, we assessed the effects of acute MycERTAM activation in primary MEFs in vitro. Evaluation of the aforementioned three type A genes and one type B gene revealed that Eif4ebp1 was not measurably expressed in MEFs, Gmip was unaffected by Myc status, and Dap was repressed to an insignificant degree (data not shown). However, Fzd9 was significantly induced following Myc activation in MEFs and this occurred even in the presence of cycloheximide, suggesting that Fzd9 is likely to be a direct transcriptional target of Myc (Fig. 5D). Such data are consistent with the emerging notion that Myc targets comprise both genes that are universally regulated and genes that are specific to certain cell lineages and/or environmental circumstances.

Discussion

Understanding how genes implicated in cancer conspire to disrupt normal cell and tissue biology is one of the most intractable problems in contemporary biology. The Myc oncoprotein exemplifies this problem: Originally identified through its role in promoting cell proliferation, its mechanism of action involves the coordinated regulation of a multitude of biological properties mediated through a myriad of target genes (7–9, 21, 26–31). This makes determining the extent to which these various genes contribute to Myc oncogenic potential virtually impossible to determine. It is also apparent that cell type, genetic background, and experimental context can all influence the downstream consequences of Myc activation, leading some to propose that Myc may act as a global transcriptional regulator (6).

Notwithstanding such a “global” role in biology, the mechanisms by which Myc drives and maintains tumorigenesis are likely to be more restricted. However, the problem remains that as Myc exerts its inexorable tumorigenic effect, a legion of secondary genetic programs evolve. Dissecting which of these is cause or consequence of Myc action has proven very difficult, as has identifying
which, if any, of its direct gene targets act as linchpins of Myc oncogenic action. In this article, we have addressed the problem of objectively defining what attributes of Myc are required for the maintenance of tumors by combining a switchable in vivo genetic model with a global genomic analysis of Myc-dependent transcription. In this way, we have, for the first time, correlated Myc oncogenic action with the underlying transcriptional changes that accompany tumorigenesis. In particular, the reversibility of our switchable model has enabled us to define a highly restricted cadre of Myc target genes that are candidates for mediating tumor maintenance function of Myc in vivo.

Not surprisingly, cell cycle genes figure prominently among our Myc targets. Ccnd1, the gene encoding cyclin D1, has been previously identified as a Myc target in vitro and our in vivo data support the induction of Ccnd1 by Myc. Because cell growth must accompany cell cycle progression, it is also noteworthy that genes encoding several members of the AKT-mTOR nutrient use and response pathway are also modulated by Myc. These include...
### Table 1. Putative tumor maintenance genes (Cont’d)

**B. Ten early target genes implicated in tumor maintenance in pIns-MycER\textsuperscript{TAM}/RIP7-Bcl-\textsubscript{x}\textsubscript{L} transgenic mice**

<table>
<thead>
<tr>
<th>Probeset ID</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Ref Seq</th>
<th>EBOX*</th>
<th>Present in database</th>
<th>(a) GO biological process</th>
<th>(b) Published role in cancer</th>
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<tr>
<td>96518_at</td>
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<td>(b) No</td>
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<tr>
<td>100636_at</td>
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<td>Eif4ebp1</td>
<td>NM_007918</td>
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<td>No</td>
<td>(a) Insulin receptor signaling pathway, negative regulation of protein biosynthesis, negative regulation of translational initiation, regulation of protein biosynthesis, regulation of translation, and regulation of translational initiation</td>
<td>(b) Yes (45)</td>
</tr>
<tr>
<td>99841_at</td>
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<td>XM_284144</td>
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<td>No</td>
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<td>(b) Yes (46)</td>
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<tr>
<td>93647_at</td>
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<td>NM_198101</td>
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<td>Yes</td>
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<td>(b) No</td>
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<td>96682_at</td>
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<td>NM_011373</td>
<td>Yes</td>
<td>Yes</td>
<td>(a) Protein amino acid glycosylation</td>
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<td>92202_g_at</td>
<td>Zinc finger and BTB domain containing 16</td>
<td>Zbtb16</td>
<td>XM_134826</td>
<td>Yes</td>
<td>No</td>
<td>(b) Yes (44)</td>
<td>(a) Skeletal development, negative regulation of cell proliferation, embryonic pattern specification, anterior, posterior pattern formation, embryonic limb morphogenesis, forelimb morphogenesis, positive regulation of apoptosis, regulation of transcription, negative regulation of transcription, and DNA-dependent, male germ-line stem cell division</td>
</tr>
<tr>
<td>160119_at</td>
<td>RIKEN cDNA 5730592L21 gene</td>
<td>5730592L21Rik</td>
<td>NM_029720</td>
<td>Yes</td>
<td>No</td>
<td>(b) Yes (43)</td>
<td>(a) —</td>
</tr>
<tr>
<td>93842_at</td>
<td>Death-associated protein</td>
<td>Dap</td>
<td>NM_146057</td>
<td>No</td>
<td>No</td>
<td>(b) No</td>
<td>(a) Apoptosis and induction of apoptosis by extracellular signal</td>
</tr>
<tr>
<td>160769_at</td>
<td>Der1-like domain family, member 2</td>
<td>Der12</td>
<td>NM_033562</td>
<td>No</td>
<td>No</td>
<td>(b) No</td>
<td>(a) —</td>
</tr>
<tr>
<td>98944_at</td>
<td>SEC23B (Saccharomyces cerevisiae)</td>
<td>Sec23b</td>
<td>NM_019787</td>
<td>No</td>
<td>Yes</td>
<td>(b) Yes (48)</td>
<td>(a) Endoplasmic reticulum to Golgi transport, intracellular protein transport, and transport</td>
</tr>
</tbody>
</table>

*MYC:MAX binding site determined by promoter sequence analysis using Matinspector Software (http://www.genomatix.de).

\textsuperscript{1}http://www.myccancergene.org/site/mycTargetDB.asp.
Eif4ebp1, which encodes the eukaryotic translation initiation factor 4 binding protein 1, and is one of only 10 early Myc targets that we identify as candidate mediators of tumor maintenance. In addition, the inhibitor of mTOR, tuberin (Tsc2), is rapidly and continuously downregulated following MycERTAM activation whereas expression of the S6 kinase (Stk6) gene is induced 5-fold at 24 hours (Supplementary Table S1). Our data lend support to recent in vitro studies implicating Myc in the regulation of the AKT-mTor pathway (32) and confirm that the effects of Myc on cell cycle and proliferation are intimately linked to cell growth and survival.

Despite substantial evidence for direct Myc-mediated induction of the ARF-p53 pathway both in vitro and in vivo (33, 34), we found no evidence for this relationship in \( M^+X_L^+ \) mice. Neither Cdkn2a, which encodes both p19ARF and p16Ink4a, nor key p53 target genes Cdkn1a (p21) or Mdm2 were induced during the first 24 hours of Myc activation. We suspect that this lack of p53 activation may be due to the influence of Bcl-x\(_L\) overexpression in our model. It has been proposed that overexpression of Bcl-x\(_L\) may limit Myc induction of p19ARF, thereby suppressing p53-mediated cell cycle arrest and apoptosis (35). Consistent with this, we find that \( p19^{ARF} \) is induced as expected following in vivo activation of MycERTAM in \( \beta \) cells lacking overexpression of Bcl-x\(_L\). Thus, in addition to suppressing apoptosis, Bcl-x\(_L\) may cooperate oncogenically with Myc by abrogating Myc-induced activation of the ARF-p53 pathway. Ongoing work in our laboratory is directed toward elucidating this issue.

Myc status has a profound influence on embryonic development (36, 37), in part through its ability to disrupt differentiation programs. In this study, acute activation of Myc triggered a widespread change in \( h \)-cell phenotype, suppressing expression of many genes that are signatures of terminally differentiated \( h \)-cells. The observed association between Myc-ER\(_TAM\) activation and loss of \( h \)-cell differentiation was substantiated by the rapid reappearance of such markers upon deactivation of Myc. Whether these...
differentiation genes are direct targets of the Myc:Max complex will need to be determined by further studies. These findings parallel recent observations of Myc-induced hepatic tumors that undergo differentiation upon oncogene deactivation (38). Abrupt or deregulated expression of intrinsic developmental pathways may, therefore, be an integral mechanistic component of Myc-induced tumorigenesis. It is noteworthy that several of the genes rapidly repressed by Myc in our study are tissue-specific and cannot, therefore, be universal Myc targets. However, other genes not restricted in their expression to pancreas (e.g., Eif4ebp1, Dap, and Gmip) were nonetheless regulated by Myc in β-cells in vivo yet not in fibroblasts in vitro. Defining the precise role of Myc targets, be they direct or indirect, in tumorigenesis will thus require careful study in biologically relevant cell types and environments in vivo.

By directly comparing the transcriptional fingerprints of islets during tumor formation and regression, we were able to identify 257 probe sets (233 genes) whose expression was inversely regulated during Myc activation and subsequent deactivation. Because Myc is required to maintain the tumors it induces, this cadre of genes must include those whose functions are required for maintenance of β-cell tumors. Functional classification of genes within this "tumor maintenance" cohort identifies many targets of potential interest. Among the type A genes, whose sustained expression requires sustained Myc activity, are the cytokinesis regulator Ect2 and the activator protein transcription factor member c-Jun, both of which have been implicated in tumorigenesis (39, 40). Another type A gene is Epas1, implicated in hypoxia-induced angiogenesis (41) and potentially involved in maintaining β-cell tumor vasculature. The designated biological functions of these type A genes are extremely diverse and underscore the idea that tumor maintenance involves multiple processes that evolve over time. For example, whereas cell cycle and proliferation genes make up over 30% of early activation gene targets, they represent only 10% of the identified maintenance gene cohort.

The kinetics of regulation of the 233 candidate maintenance genes indicates that the great majority are indirect consequences of Myc action rather than direct target genes. By confining our search to those genes that were rapidly modulated by Myc, we identified a subset of 10 genes that share the correct dynamics to be putative direct transcriptional targets. Most of these genes (i.e., Dap, Derl2, Eif4ebp1, Fzd9, St6galnac4, and Zbtb16) possess both requisite regulatory Myc recognition elements in their promoters and have already been implicated elsewhere in various aspects of cancer (42–48). Of note, the Wnt receptor–encoding gene, Fzd9, was also found to be regulated by Myc in cultured MEFs. Wnt signaling is critical to normal development and is frequently deregulated in human cancer, largely through constitutive activation of β-catenin (49). Fzd9 knockout mice show significant abnormalities in hematologic development, principally secondary to a failure of B-cell self-renewal (50). Definitive proof that Fzd9 is a direct transcriptional target of Myc and the extent to which it mediates essential aspects of Myc function will need to be resolved by direct experimentation. To that end, we have begun studies to determine the effect of acute MycER\textsuperscript{TA} activation on the islets of Fzd9 knockout mice. Demonstration of impaired tumor formation in these mice will confirm the critical role of this Wnt receptor in Myc-induced transformation of pancreatic β cells in vivo.

In summary, we have, for the first time, defined, in real time, the Myc-induced transcriptional changes that occur during tumor initiation and regression in an orthotopic tissue in vivo. We are currently extending this approach to other inducible tumor model systems to elucidate which of the identified Myc targets are β cell-specific and which are more universal. By unraveling the complexities of the tumorigenic process in vivo, we hope to identify genes and pathways that will be strategic targets for the focused development of novel, targeted anticancer strategies.

Acknowledgments

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References

20. Luzzi V, Mahadevappa M, Raja R, Warrington JA,


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