Minireview

Making decisions through Myc

Sergio Nasi*, Roberta Ciarapica, Richard Jucker, Jessica Rosati, Laura Soucek

Centro Acidi Nucleici CNR, Dipartimento di Genetica e Biologia Molecolare, Università La Sapienza, P.le A. Moro 5, 00185 Rome, Italy

Received 15 December 2000; accepted 9 January 2001

First published online 19 January 2001

Edited by Gianni Cesareni

Abstract c-Myc is a transcriptional regulator involved in carcinogenesis through its role in growth control and cell cycle progression. Here we attempt to relate its role in stimulating the G1–S transition to the ability to affect functioning of key cell cycle regulators, and we focus on how its property of modulating transcription of a wide range of target genes could explain its capacity to affect multiple pathways leading to proliferation, apoptosis, growth, and transformation.

Key words: Oncogene; G1; Cell cycle; Transcription; Repression; Chromatin modelling

1. Introduction

c-*myc* has been the object of ongoing interest since its identification as a cellular homolog of the transforming element in the oncogenic retrovirus MC29. The c-Myc protein is a critical component for the control of normal cell growth, and is the best studied member of a family that includes N-Myc, L-Myc, S-Myc and B-Myc. The family seems to have evolved, in part, to facilitate differential expression patterns, since N-Myc was shown to functionally replace c-Myc, when expressed from the c-*myc* locus [1]. Altered *myc* activity by translocation, amplification, overexpression, and mutation is widespread in tumor cells; some of the best evidence for the importance of altered Myc expression for multi-step carcinogenesis comes from recent studies with inducible transgenes. Enforced expression of Myc in either skin or hematopoietic lineages in transgenic mice leads to neoplastic premalignant and malignant phenotypes, respectively, but when Myc expression is turned off, spontaneous regression of the neoplastic changes occurs [2,3]. In recent years there has been a revival of interest in the biology of Myc, as its role in cell cycle regulation and the relevance of Myc-related apoptosis in cancer came into focus. Also, the realization that Myc works in a complex with Max to directly regulate transcription stimulated considerable attention in mechanisms and targets of transcriptional regulation, in relationship to Myc biological function.

*Corresponding author. Fax: (39)-6-499 12500.
E-mail: s.nasi@caspur.it

2. Myc biological activities

Myc function is not clarified from classical knock-out studies, which demonstrated that both N-*myc* and c-*myc* are essential for embryonic survival beyond day 10; Myc null embryos are smaller, retarded in development, and show pathological abnormalities in various organs [4]. Since there may be cross-compensation, it is not clear whether c- or N-Myc are necessary for proliferation of the embryonic stem (ES) cells utilized for generating the embryos or for embryo cells before day 10. Consistent with the central role of Max in the Myc network, Max−/− embryos die at an earlier stage, around day 6, coincident with loss of maternal storage of Max proteins [5]. Apparently ES cells can proliferate without Max. Generation of conditional *myc* knock-out mice is expected to illuminate the function of Myc in proliferation and development of distinct tissues. c-*myc* ablation in fibroblasts or in B lineage cells has suggested that the c-Myc protein is dispensable for survival, but is required for proliferation upon stimulation with serum or cytokines, and that it is linked to apoptotic pathways (I. Moreno de Alboran and A. Trumpp, personal communication).

Myc is a strong inducer of proliferation, and its role in cell cycle control has been intensively investigated as it is believed to be critical for the oncogenic properties. However, it is still unclear how altered cell cycle control by Myc contributes to oncogenesis. Studies with rodent cell lines favored the view that deregulated Myc expression drives cells inappropriately through the cell cycle, leading to uncontrolled proliferation characteristic of neoplastic cells. Studies with normal primary fibroblasts and the analysis of transgenic mice expressing a regulable Myc showed instead that Myc alone cannot sustain the division cycle and suggested that a G2 checkpoint must be eliminated for Myc to initiate continuous proliferation and tumorigenesis. Upon Myc protein induction, normal fibroblasts are stimulated to pass through G1 and S phase, but they are arrested in G2; this checkpoint is compromised by the absence of p53 or its effector p21. G2-arrested cells frequently become aneuploid, possibly due to inappropriate reinitiation of DNA synthesis. In this regard, Myc is different from other oncoproteins like Ras, Raf, and E2F1, whose continuing activity causes a G1 block. Conditional Myc expression in keratinocytes of transgenic mouse epidermis drives extensive proliferation, while proliferation in derma is blocked by p53 (reviewed in [6]). In human epidermal keratinocytes, c-Myc initially drives proliferation, and subsequently differentiation, concomitant with activation of the p53 G2 checkpoint [7]. In *Drosophila*, Myc overexpression was able to pre-

0014-5793/01/$20.00
PII: S0014-5793(01)02118-4

vent a G1 block, but cells were still arrested in G2 by patterning signals from the Wingless pathway [8]. It is unclear how sustained Myc expression activates the G2 checkpoint in mammalian cells, but its capacity to indirectly cause induction of ARF, and, consequently to stabilize p53 may be involved. There is evidence that inactivation of the ARF/Mdm2/p53 pathway is required for immortalization through overexpression of Myc. The ability of Myc to activate telomerase reverse transcriptase (hTERT), the rate-limiting enzyme in the telomerase complex, is clearly insufficient to explain its capacity to promote immortalization, since cells are prevented from further proliferation unless the cell cycle checkpoint is abrogated. In mouse embryo fibroblasts and during in vivo lymphomagenesis Myc activity appears to favor immortalization indirectly, by promoting the selection of mutant cells that inactivate the ARF/Mdm2/p53 pathway [9].

A connection between Myc deregulated expression and control of G2 progression is also observed when dMyc (*Drosophila* Myc) was overexpressed in developing wings: a G1 shortening caused by Myc induction was compensated by a G2 increase, with no diminished cell proliferation [8]. The major effect of dMyc was to increase the growth rate, leading to a greater cell mass, rather than to an increase in cell number. Since increase in cell mass (growth) and increase in cell number (proliferation) are tightly coordinated in most tissues [10], these data suggest either that Myc functions in coordinating growth and proliferation or that its impact on cell proliferation may be secondary to its role in control of growth. Although various experiments confirmed that Myc is involved in control of growth in mammals as well, they failed to show the uncoupling between growth and proliferation observed in flies following perturbation of Myc levels. In conditional overexpression or knock-out studies, c-*myc* appeared to be essential for proliferation of several mouse tissues, without discernible effects on cellular size ([6] and A. Trumpp, personal communication). The small size of c-*myc*-deficient embryos or conditionally deficient mice is likely due to the concomitant reduction of growth and proliferation rates, and, clearly, much is to be learned about the coordination of the two processes.

The fact that Myc controls growth, besides controlling cell cycle progression, was also independently suggested by the decrease in RNA and protein synthesis rates observed in c-*myc* null fibroblasts [11], which can still proliferate, although at a significantly impaired rate. These cells have a three times slower cell cycle, and both the G1 and G2 phases are significantly lengthened, whereas duration of the S phase is unaffected. The rates of RNA and protein accumulation, as well as protein degradation, are markedly reduced in a way that exactly matches the lengthening of the cell cycle, so that c-*myc* null cells have the same size, rRNA, and protein content as their c-*myc*+/+ parents.

Myc activity is also known to affect apoptosis and differentiation [12]. Myc may favor an initial commitment from proliferation to differentiation [7]; however, Myc levels are down-regulated during terminal differentiation and enforced Myc expression usually inhibits terminal differentiation, possibly by interfering with cell cycle exit. Evidence suggests that Myc sensitizes cells to a variety of apoptotic triggers rather than directly inducing apoptosis by itself (reviewed in [13]). This biological activity is mediated through cytochrome *c* release but requires other apoptotic signals such as those of CD95/Fas and p53. The ability of Myc to concomitantly induce proliferation and apoptosis in certain tissues can be considered a fail-safe mechanism against the unrestrained growth of a cell with a single proliferative lesion [6].

Its ability to affect such different aspects of cell behavior can be in part related to the finding that Myc participates in a network of interacting proteins (Myc, Max, Mnt/Rox, Mga, and the Mad family members Mad1, Mxi1/Mad2, Mad3, and Mad4), all containing a basic helix-loop-helix–zipper motif (bHLHZip) involved in dimerization and DNA binding. Max holds a central position, since the other network proteins depend on heterodimerization with Max in order to bind DNA and regulate gene transcription (see [14] for review). All dimers recognize in vitro the same E box binding site CACGTG, but have distinct transcriptional activities. Myc/Max dimers weakly activate transcription of promoters proximal to E box sites; Max/Mad dimers repress transcription from the same binding sites, by recruiting a Sin3–histone deacetylase complex. Overexpression of Myc/Max dimers induces proliferation or apoptosis, while Max/Mad and Max/Mnt complexes cause cell growth arrest and differentiation [15,16]. The equilibria among the various dimers are mainly controlled through extracellular signal-induced modifications in Myc or Mad expression levels, the Max concentration remaining constant. *Mad* gene expression, which is usually low in proliferating cells, is induced during cell differentiation, while c-*myc* transcription, which is regulated by tyrosine kinase signalling and is rapidly turned on upon mitogenic stimulation, is repressed [12,15]. The network can be viewed as a functional module which acts to convert environmental signals into specific gene-regulatory programs [14], and its basic features are conserved in *Drosophila*, and possibly in *Caenorhabditis elegans*, where only Max and Mad homologs were reported [17].

3. Control of Myc expression in G1

c-*myc* is regulated by specific growth signals in a cell cycle-dependent manner, and its expression is tightly controlled at different levels (Fig. 1). The relationship between c-*myc* expression and progression in G1 is evident when tissue culture cells such as fibroblasts or lymphocytes are stimulated to enter the cell cycle. Transcription of c-*myc*, absent in quiescent cells, is strongly induced within 1 h upon addition of growth factors and cytokines like platelet-derived growth factor (PDGF), colony-stimulating factor (CSF), epidermal growth factor, interleukin (IL) 7, IL-2, antigen, or other mitogens; Myc is then present at a low level throughout the cell cycle [18]. Perhaps not surprisingly, also enforced Tert expression activates c-*myc* [19]. On the other hand, Myc transcription is negatively controlled by cAMP, transforming growth factor β (TGFβ), interferon γ, the cyclin-dependent kinase inhibitor p21, the β-catenin/APC pathway, and other proliferation inhibitors [20]. The v-Abl tyrosine kinase turns on c-*myc* through a pathway involving Ras, Raf, and E2F proteins [21], while the APC tumor suppressor inhibits induction of c-*myc* by the Tcf-4 transcription factor [20]. The increase in Myc protein observed as cells enter the G1 phase reflects both an increase in transcription and mRNA stability, and a concomitant stabilization of the protein [18]. Myc has an half-life of only 30 min in growing cells and is destroyed via the ubiquitin/26S proteasome pathway; its stability is controlled by two

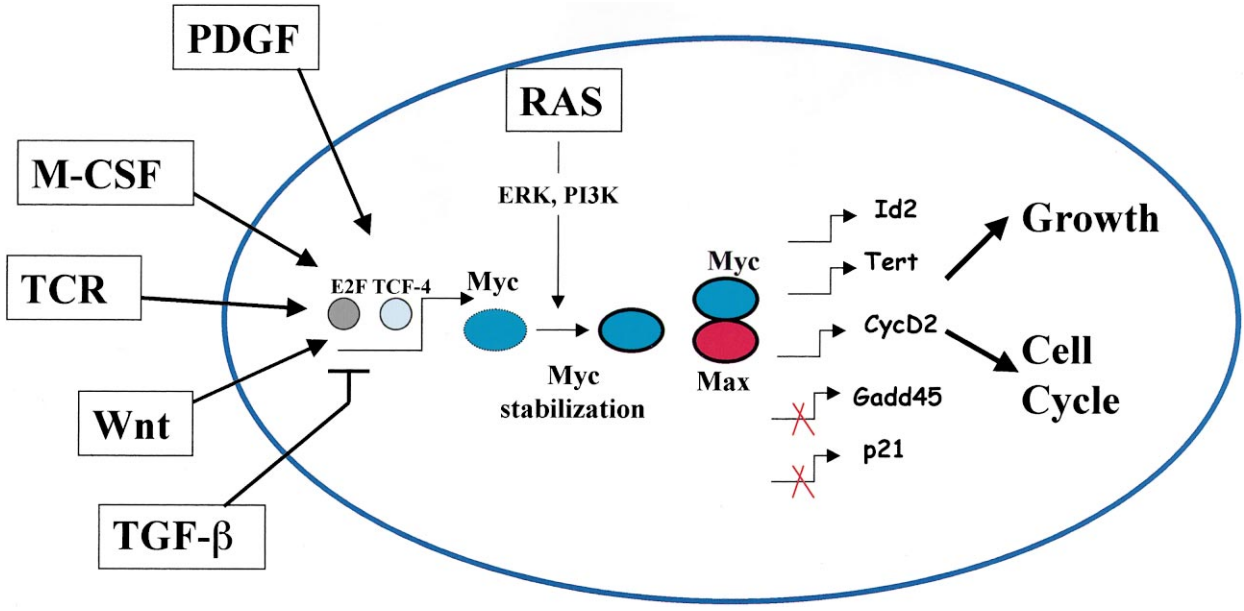

Fig. 1. Simplified model of signals modulating Myc expression, and its consequences on growth and proliferation. c-*myc* transcription is rapidly induced by a variety of growth factors and mitogenic stimuli, and is repressed by TGF-β and other proliferation inhibitors. The increase in Myc protein observed as cells enter the G1 phase reflects an increase in mRNA synthesis, and a concomitant protein stabilization dependent on Ras-induced phosphorylations via the ERK and PI3K pathways. Induction and repression of key regulators by Myc can lead to coordinated growth and cell proliferation. PDGF: platelet-derived growth factor; M-CSF: multi-colony-stimulating factor; TCR: T cell receptor; TGF-β: transforming growth factor-β; ERK: extracellular signal-regulated kinase; PI3K: phosphoinositide 3-kinase.

adjacent N-terminal phosphorylation sites, Ser-62 and Thr-58 [22]. Phosphorylation of Ser-62 stabilizes Myc, while phosphorylation of Thr-58, which is dependent on prior Ser-62 phosphorylation, promotes its degradation. The transient accumulation of Myc in G1 is ensured by the interplay of the two Ras effector pathways Raf/ERK and PI3K/AKT, operating at different times. Activation of Ras signalling stabilizes Myc via ERK phosphorylation of Ser-62 and via the inhibition of GSK-3 activity by the AKT pathways; at later times, when Ras signaling weakens, AKT activity declines and GSK-3 phosphorylates Thr-58, destabilizing Myc. Interestingly, ERK phosphorylation also stabilizes c-Jun, which contains a sequence of six amino acids that exactly matches that surrounding the two phosphorylation sites in Myc [22].

The region including the two sites appears to be relevant for Myc oncogenic properties, since it is a mutational hotspot for *myc* alleles in Burkitt's lymphomas, AIDS lymphomas, and mouse plasmacytomas, with the majority of mutations affecting Thr-58 [23]. However, most of the mutations, with the exception of a lymphoma-derived Thr58Ala mutation, do not affect either Myc apoptosis or transformation of tissue culture cells.

4. Control of G1 progression by Myc

It has been well documented that ectopic Myc expression induces quiescent fibroblasts to enter the cell cycle, reduces G1 duration, and promotes S phase entry; conversely, down-regulation of Myc expression through an antisense approach has antiproliferative effects [24]. The simple idea that a critical gene exists, whose activation explains Myc activity on the cell cycle, is unrealistic. As a matter of fact, attempts to complement the growth defect in c-*myc* null fibroblasts showed that no single gene, except c-*myc* itself or N-*myc*, was able to restore a normal cell cycle [25]. On the other hand, only the simultaneous deletion of p107, Rb, and p130 prevents Myc from stimulating proliferation. It is clear that a complex web of interactions connects c-Myc to the Rb-regulated restriction point, a critical decision point in the mammalian cell cycle (Fig. 2). This network appears to be flexible and robust, so that disruption of a single component is unable to prevent Myc action and, conversely, no single target gene can substitute for Myc function.

Myc facilitates G1 exit by positively modulating cyclin/CDK (cyclin-dependent kinase) complexes, by negatively modulating the CDK inhibitors p27 and p21, and by interfering with Rb/E2F activity. A plethora of mechanisms have been described, mostly derived from overexpression studies, whose physiological relevance is not always supported by genetic information (Fig. 3).

4.1. Cyclin E complexes

Myc can bypass the p16/Rb growth inhibitory pathway downstream of Rb activation, with effects indistinguishable from those of cyclin E, indicating that activation of cyclin E–Cdk2 kinase complexes is an important step in Myc-induced proliferation. Activation of c-Myc in quiescent fibroblasts leads to the rapid induction of cyclin E–Cdk2 kinase activity, while dominant-negative mutant alleles or somatic c-*myc* deletion suppress cyclin E–CDK2 activity and may cause G1 arrest [26]. Although Myc was reported to stimulate the cyclin E protein synthesis rate and to be involved, together with E2F, in transcriptional regulation of the cyclin E promoter [27], it appears that the activation of cyclin E–Cdk2 complexes is largely mediated by an action of Myc upstream of cyclin E.

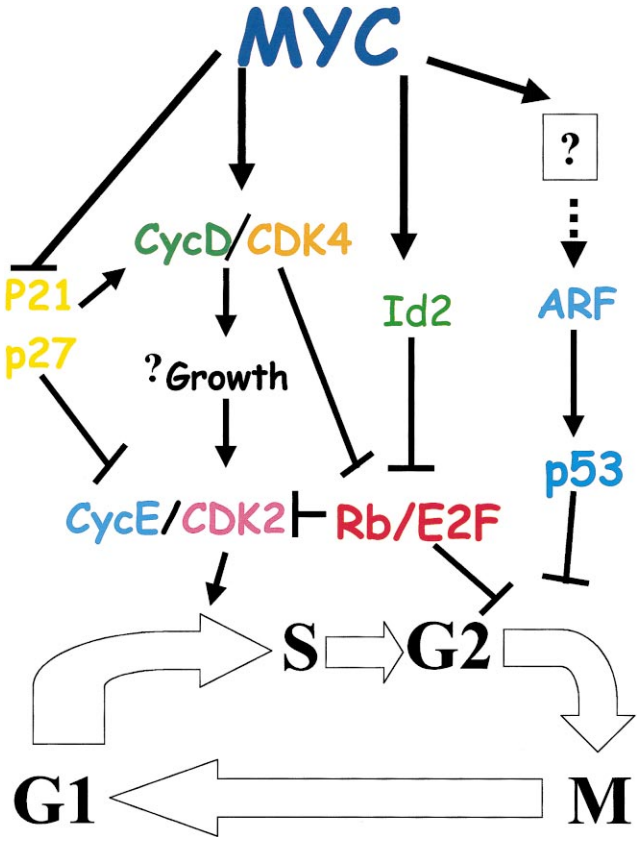

Fig. 2. Schematic representation of Myc-activated and -repressed pathways affecting cell cycle progression in mammalian cells.

4.2. p27 and p21 (kip/cip proteins)

A clue to the mechanism of cyclin E complex activation by Myc came from the observation that Myc rescues a block imposed by the Cdk inhibitors p27 and p21. Upon activation of Myc, p27 is rapidly and transiently sequestered by cyclin D2–Cdk4 complexes, therefore dissociating from cyclin E–Cdk2 complexes and allowing their activity [28]. Besides affecting p27 and p21 activity through sequestration by cyclin D2–Cdk4 complexes, Myc was recently shown to directly repress the expression of p21 [29], and possibly p27 (G. Sonenshein, personal communication), and to induce expression of Cul1 [30]. Cul1 is a critical component of the ubiquitin ligase complex SCF–SKP2, which promotes selective proteolysis of p27; SKP2-mediated ubiquitination appears to depend on phosphorylation of p27, by cyclin E–Cdk2 complexes. Since degradation of p27 occurs at the G1/S transition, Cul1 regulation may be a critical point through which Myc promotes G1 exit.

However important, inhibition of p27 does not appear to be the only rate-limiting cell cycle target of Myc since p21/p27-deficient fibroblasts are still sensitive to cell cycle regulation by Myc. However, mice deleted for genes of the three pocket proteins (Rb, p107, p130) are insensitive, indicating that Myc activates cyclin E by directly targeting Rb family members [31].

4.3. Cyclin D complexes

Furthermore, genetic evidence from Myc or cyclin D null cells strongly supports that cyclin D–Cdk complexes are targets for Myc-induced proliferation [26,32]. A 12-fold reduction in the activity of these complexes is the largest defect in a c-*myc*−/− cell line during the G0–S transition; mouse embryo fibroblast cells lacking either D1 or D2 are unable to activate cyclin E complexes through overexpressed Myc. Moreover, cyclin D2-deficient primary fibroblasts undergo accelerated senescence in culture and are not immortalized by Myc [32]. Recently, independent studies showed that Myc induces an increase in the amount of cyclin D1, cyclin D2, and CDK4. Induction of cyclin D1 and D2 leads to sequestration of the inhibitors p27 and p21. Myc up-regulates D2 mRNA, while D1 appears to be affected post-transcriptionally, and D3 is unaffected by Myc; up-regulation of cyclin D2 in response to activation of Myc occurs by derepression of a Mad/Max-mediated inhibition of the D2 promoter [32]. An increase in CDK4 mRNA levels following activation of conditional Myc was observed in human umbilical vein cord cells, but not in Rat-1 cells [33], and the CDK4 protein did not appear to be limiting for cell cycle progression in c-*myc* null fibroblasts [26]. Myc was also found to repress the expression of p15ink4b, an inhibitor of cyclin D–Cdk4 complexes (M. Eilers, personal communication).

Cyclin D–Cdk4 and –Cdk6 serve two functions: a catalytic one causing the initial Rb phosphorylation in G1, and a second function involving sequestration of the cip/kip proteins. Cyclin D–kinase and cyclin E–kinase complexes compete for binding to p21 and p27, which have opposite effects on the two complexes. They inhibit cyclin E complexes, while they are essential for cyclin D complex assembly. The p27/p21 sequestration function of D cyclins appears to be crucial for activation of cyclin E–Cdk2 and stimulation of cell proliferation by Myc, while the kinase activity associated with D cyclins is not limiting [32]. An interesting possibility is that the cyclin D–Cdk4 kinase activity may have a role in other Myc responses, such as cell growth stimulation, since in *Drosophila* these complexes directly affect both the cell cycle, via Rb, and growth, via unknown targets [34].

4.4. Cdc25a

The Cdc25a phosphatase is involved in the progression from G1 to S phase and is thought to control activity of cyclin E–Cdk2 complexes. It is unclear whether direct upregulation of Cdc25a mRNA by Myc represents a critical step for Myc regulation of the cell cycle since, in certain contexts, Cdc25a appears not limiting for cyclin E–Cdk2 complex activation. *Cdc25a* transcription appears to be indirectly regulated by Myc in some cell lines, for instance through the inactivation of Rb family proteins, which repress the Cdc25a promoter [35].

4.5. Rb and Id2

The HLH protein Id2 is largely increased in neuroblastomas, possibly as an epigenetic mechanism to bypass the tumor suppressor function of Rb. Id2 induction by Myc [36] may represent a physiologically relevant component of the circuit connecting Myc and Rb, as two phenotypic hallmarks of Myc activation, the ability to promote cell cycle reentry in the absence of growth factors and the ability to cooperate with Ras to transform fibroblasts, are dependent on the presence of Id2. This protein physically associates with active, hypophosphorylated forms of the pocket proteins. Experiments with genetically modified MEFs (mouse embryo fibroplasts) showed that Myc must induce Id2 to overcome a Rb block on

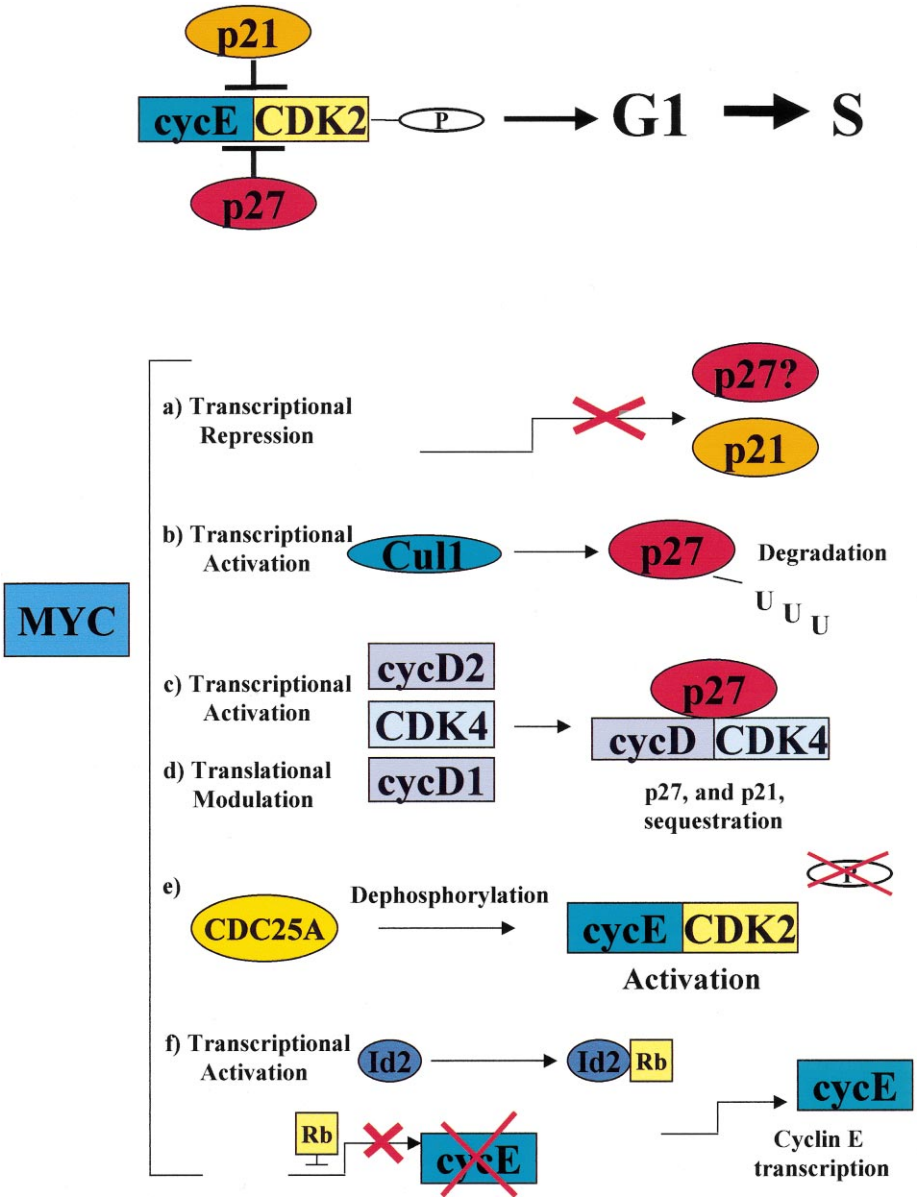

Fig. 3. Myc targets for control of G1 progression. G1 progression is controlled by the CDK complexes cyclin D–Cdk4 (or Cdk6) and cyclin E–Cdk2: both are rate-limiting for the G1–S transition (reviewed in [55]). Myc may affect G1 progression through multiple mechanisms: (a) direct repression of p21, and possibly p27, gene transcription [29]; (b) induction of *cul1*, a cullin family gene encoding a critical component of the ubiquitin ligase SCF–SKP2, which promotes proteolysis of p27 [30]; (c) increase in CDK4 mRNA levels [33]; (d) induction of cyclin D2 mRNA and cyclin D1 protein synthesis rate [32]. Besides facilitating the activation of cyclin E–Cdk2 complexes through p21 and p27 sequestration, cyclin D–Cdk4 complexes may have a direct role in promoting cell growth [34]; (e) up-regulation of Cdc25a mRNA, encoding a phosphatase involved in activation of cyclin E–Cdk2 complexes [14] (Myc may affect Cdc25a transcription also indirectly, by inactivation of the pocket proteins; see f); (f) transcriptional induction of Id2, which may facilitate cyclin E transcription by inactivating Rb [36].

cell cycle progression; if the Id2-Rb pathway is removed, other Myc targets are sufficient to promote cell cycle progression. The Id2 mRNA is directly upregulated by Myc transcription factors: c-Myc binding to the Id2 promoter in vivo occurs after stimulation with serum of quiescent fibroblasts [36].

5. Regulation of transcription

The current model is that Myc exerts its biological activities by regulating gene expression at the transcriptional level. This poses the identification of Myc target genes as a central question, together with a second one: how can Myc, as a single protein, have so many functions? In a sense, Myc resembles a nuclear counterpart of the Ras oncoprotein, recruited by different modules to signal a multiplicity of responses. By analogy, Myc may be viewed as a nodal element of one, or possibly more, functional units that implement instructions from a variety of inputs within the nucleus. Any given outcome would critically depend on the particular interactions established within a network of Myc interacting proteins, in a dynamic way.

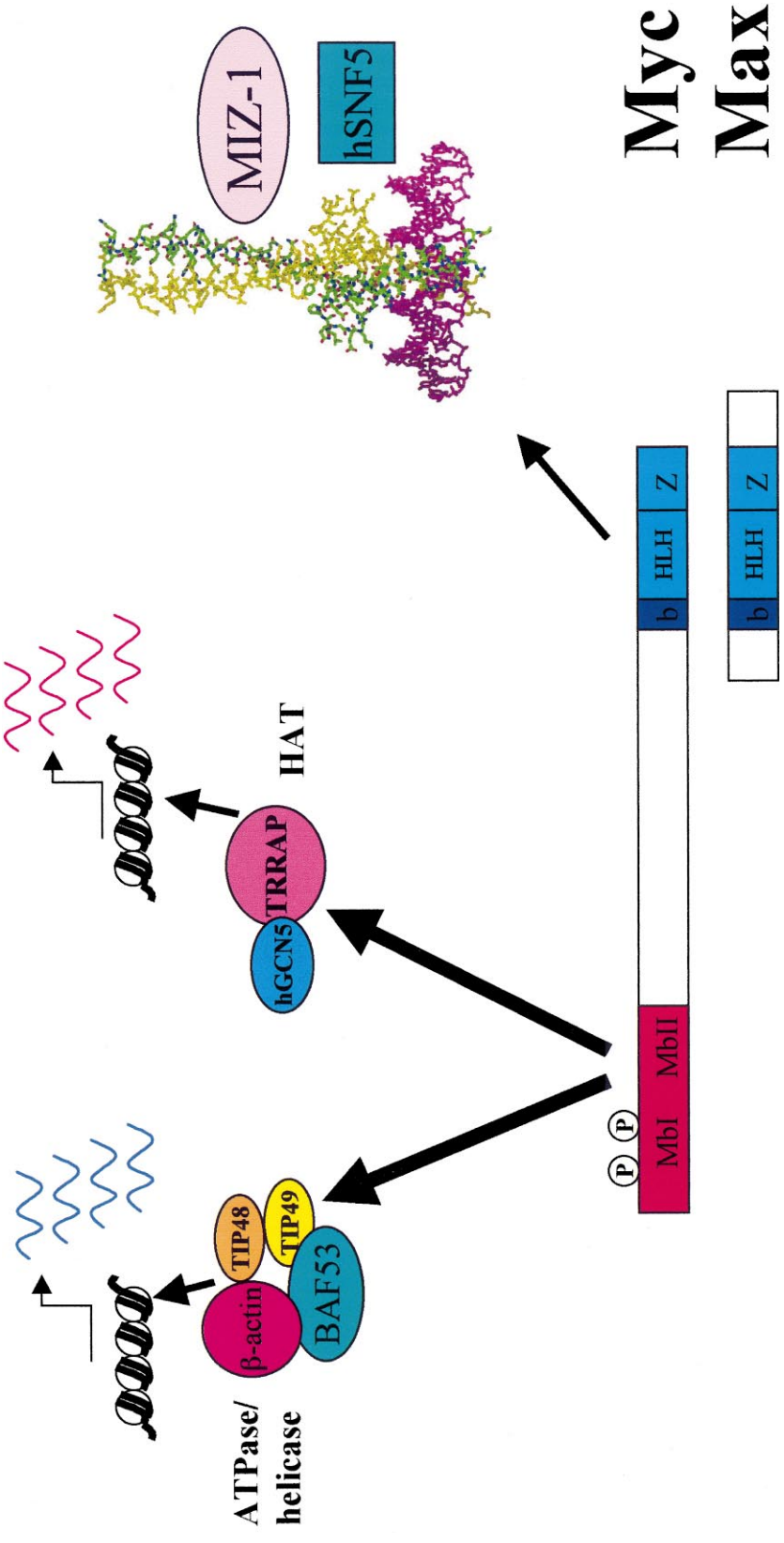

Fig. 4. c-Myc association with proteins modulating its transcriptional activity. MbI: Myc box I (aa 42–66); MbII: Myc box II (aa 133–147); b: basic region (aa 352–366); HLH: helix-loop-helix (aa 367–410); Z: leucine zipper (aa 411–439). MbI is a mutational hotspot in Burkitt's lymphoma, and is a target of phosphorylations that may affect Myc activity and stability. MbII binds to several factors implicated in chromatin remodelling. TIP48 and TIP49, found in association with BAF53 and β-actin, display ATPase/helicase activity, while TRRAP is able to recruit the hGCN5 histone acetylase; different targets could be affected by the two complexes. A bHLHZip model structure of a Myc/Max dimer bound to DNA is shown on the right; Myc is colored by atom type (green: C; red: O; blue: N), while Max is in yellow [38]. The Myc bHLHZip domain can interact with Miz-1, possibly inhibiting its transcriptional activation function, and with a component of the ATP-dependent chromatin remodelling complex SWI-SNF [45,46].

It is clear that two regions of the Myc protein are essential for its biological functions: the C-terminal bHLHZip domain, and an N-terminal 'activation' domain (Fig. 4).

The bHLHZip domain consists of two α-helices, basic region plus helix 1 and helix 2 plus leucine zipper, separated by a loop of variable length. The Max dimer crystal structure showed that bHLHZip domains associate in a characteristic four-helix bundle fold, a structure shared by the bHLH protein family [37]. The two basic regions diverge from the bundle to contact DNA; three amino acids in the basic region contact DNA bases, while the loop and the N-terminus of helix 2 bind to the phosphate backbone. Helices 1 and 2 of each monomer form a hydrophobic core that stabilizes the dimeric structure, while the leucine zipper regions form a coiled coil; the zipper interactions determine dimerization specificity [38]. The Myc/Max dimer structure is very similar to Max dimers (Nair, personal communication). Myc interaction with Max appears to be required for binding to the E box and for transcriptional activation, since artificial Myc homodimers are unable to bind the core sequence and are defective in biological function [38]. How specific recognition of a Myc/Max target gene is achieved is not fully understood. The target E box sequence CACGTG defined by in vitro selection is very frequent in the genome, occurring approximately every 4000 bp; target sequences become even more frequent, one every 1000 bp, if one takes into account the non-canonical E boxes shown to be associated with Myc/Max in vivo [39]. Some specificity for DNA target recognition comes from the nucleotides flanking the core sequence, since Myc/Max dimers show a preference for GC residues immediately adjacent to the E box [30,39]. Moreover, structural studies indicate that the sequence specifically recognized by a Max dimer, and likely by a Myc/Max heterodimer, is eight nucleotides long, as it includes the core sequence plus the two adjacent nucleotides. Interaction between the loop and nucleotides outside the E box may further contribute to target recognition specificity, as suggested by the observation that a single lysine residue in the loop of the bHLH protein Deadpan greatly affects DNA binding [40]; the corresponding lysine in Max contacts the DNA phosphate backbone in a region outside the E box. Cooperativity of multiple E boxes within a promoter or combinatorial mechanisms involving association of an E box with the target sequence for another transcription factor may also affect binding affinity or specificity for subsets of target genes. Accessibility of target sequences is also likely to play a role: methylation of CpG within the consensus E box may affect DNA binding [41], and the binding sites can be obstructed by abundant bHLHZip proteins like Mnt and USF that recognize the same target sequence [14]. Although target recognition by Myc/Max and Max/Mad dimers appears very similar in vitro, there are differences in the basic regions of Myc and Mad; the third amino acid residue, which varies among Myc, Mad, and Max basic regions, was reported to give specificity for recognition of nucleotides flanking the E box. Myc binds inefficiently when the flanking nucleotide is A or T, while Mad appears to have little specificity; so Myc/Max and Mad/Max appear to have both common and specific targets [30].

Myc behaves as a weak activator and a weak repressor of transcription on different targets, and a unifying view of its transcriptional activity is not yet possible. DNA binding is clearly required, but the mechanism whereby Myc recruitment to a promoter results in transcriptional regulation is unclear. Work on the cyclin D2 promoter suggested that activation by Myc might work mainly through derepression; full transcriptional activation would depend on other sequence-specific factors. The transcriptional regulatory activities reside in the first 143 amino acids of Myc, which include the two evolutionarily conserved regions MbI and MbII (Myc box I and II; Fig. 4). The identification of factors bound to MbII is providing an insight into the mechanism of transcriptional regulation by Myc. One such factor is TRRAP, which is necessary for cell transformation by Myc and has homology to the PI3K/ATM family, although it lacks kinase activity. The yeast TRRAP is a subunit of SAGA and NuA4 chromatin remodelling complexes; in mammalian cells, Myc can recruit the histone acetylase GCN5 through TRRAP, indicating that it may be involved in chromatin-dependent activation [42]. In fact, Myc/Max association with the cyclin D2 promoter is correlated with hyperacetylation, while acetylation is decreased upon Max/Mad binding following differentiation stimuli (B. Lüscher, personal communication). However, whether Myc induces histone acetylation of a target promoter or whether it binds to promoters that are already acetylated is far from being established. Studies on the Myc target genes *cad*, which displays cell cycle-dependent Myc binding, and *tert* showed that c-Myc or N-Myc binding does not influence the amount of acetylated histones on the promoter ([43] and P. Farnham, personal communication). It is possible that, on these targets, TRRAP has no significant role and Myc may affect initiation or elongation by modifying the RNA polymerase II activity. MbII also associates with Tip48 and Tip49 [44], two ATPases/helicases involved in oncogenic transformation and apoptosis by Myc, but not in cell cycle progression. Other proteins have been associated with the Myc activation domain, but the significance of the interaction remains to be elucidated (see [45] for review). Modulation of chromatin structure likely implicates the bHLHZip domain as well, which directly interacts with hSNF5, a component of the SWI/SNF ATP-dependent chromatin remodelling complex [46]; the c-Myc interaction with an ATPase associated with the SWI/SNF complex was also observed (B. Amati, personal communication).

The mechanism of transcriptional repression by Myc is even more elusive and is likely to differ among different promoters, as there is no consensus on the specific DNA sequence involved or on interactions with specific proteins. Initiator elements, which are present in some of the Myc-repressed genes, bind proteins like YY-1 and Miz-1 that may interact with the Myc bHLHZip domain [14,45]. On the other hand, repression might be achieved indirectly, through deactivation of a positive regulator. Formation of a Myc–Miz-1 complex at the initiator element was found to correlate with the ability of an overexpressed Myc to prevent p15ink4b induction by Miz-1 (M. Eilers, personal communication).

6. Target genes?

A systematic identification of Myc-regulated genes may represent the most valuable information to understand how intracellular pathways involved in proliferation, apoptosis, growth, and transformation are affected by Myc's activity. This task has been elusive for a number of reasons, not least the fact that ectopic Myc expression usually induces only a two- to three-fold change in transcription of proposed targets. The criteria for defining a Myc target gene are also contro-

Table 1
Summary of genes directly regulated by Myc

Gene		Criteria
Induced genes		
Cell cycle		
CCND2 (cyclin D2)	cell cycle regulator [29]	Microarray
GOS2 (lymphocyte G0/G1 switch gene 2) [29]		Microarray
CksHs2 homolog of yeast cdks binding protein [29]		Microarray
Cdk4	cell cycle regulator [33]	SAGE
Id2	cell cycle regulator [36]	Knock-out studies; chromatin Ip
Cdc25A, phosphatase	cell cycle regulator [49]	Promoter studies
HGF (hepatoma-derived growth factor)	growth factor [29]	Microarray
Death, immortality		
API2	inhibitor of apoptosis [30]	Microarray
TRAP1 (tumor necrosis factor receptor-associated protein) [29]		Microarray
TERT (telomerase reverse transcriptase) chromosome integrity [30,56]		Northern
Growth, metabolism, adhesion, etc.		
ODC (ornithine decarboxylase)	polyamine biosynthesis [29,48,49]	Microarray
IARS (isoleucine tRNA synthetase) [29]		Microarray
AHCY (*S*-adenosylhomocysteine hydrolase) [29]		Microarray
ASS (argininosuccinate synthetase) [29]		Microarray
ECA39	amino acid transport [48,49]	RNA subtraction hybridization
Cad	pyrimidine biosynthesis [43,48,49]	*myc*−/− cells; chromatin Ip
GRPE	molecular chaperone [30]	Microarray
EST highly similar to GRPE protein homolog precursor [29]		Microarray
FABP5 (psoriasis-associated fatty acid binding protein) [29]		Microarray
SLC16A1 (solute carrier family 16) [29]		Microarray
UMPS	metabolism [30]	Microarray
TFRC (transferrin receptor)	iron metabolism [29]	Microarray
IRP2 (iron regulatory protein 2)	iron metabolism [54]	RNA subtraction hybridization
LDH-A, glucose metabolism [52]		Representational difference analysis
GLUT1 (glucose transporter)	glucose metabolism [53]	Run-on, Northern, results in *myc*−/− cells
PFK (phosphofructokinase)	glucose metabolism [53]	Northern and results in *myc*−/− cells
Enolase	glucose metabolism [53]	Northern and results in *myc*−/− cells
TOP1 (topoisomerase)	DNA modification [30]	Microarray
DDX18 (MrDb, dead-box helicase)	RNA helicase [30,39,49]	Microarray
SNRPD1 (small nuclear ribonucleoprotein) [30]		Microarray
Fibrillarin	rRNA metabolism [29]	Microarray
Nucleolin	rRNA metabolism [29,51]	Microarray
BN51	RNA polymerase III cofactor [51]	Run-on and Northern
BLMH	drug resistance [30]	Microarray
RPIA [30]		Microarray
CTIP	transcription corepressor [30]	Microarray
EIF5A (eukaryotic translation initiation factor 5A)	protein synthesis [29]	Microarray
EIF4E (translation initiation factor 4E)	protein synthesis [14,48,49]	Northern and promoter studies
EIF2α (translation initiation factor 2α)	protein synthesis [14,48]	Northern and promoter studies
Cul1, component of the ubiquitin ligase SCF^{SKP2} complex [30]		Microarray
ZRP1,	cytoskeletal protein [30]	Microarray
FKBP52 (FK506 binding protein)	immunophilin [29]	Microarray
PPIF (peptidyl-prolyl *cis-trans* isomerase F)	immunophilin [29]	Microarray
α-Prothymosin [47,48]		Promoter studies
C/EBPα (CCAAT enhancer binding protein)	transcription factor [48]	Promoter studies
HMG-I/Y	chromatin binding protein [57]	Promoter studies
Repressed genes		
Cell cycle		
CCNG2	cell cycle regulator [30]	Microarray
CHES1	cell cycle checkpoint regulator [30]	Microarray
CDKN1A (p21)	cyclin-dependent kinase inhibitor [29]	Microarray
(p27, cyclin-dependent kinase inhibitor)		(G. Sonenshein, personal communication)
GADD45	related to growth arrest [48,49]	*myc*−/− cells, promoter studies
Gas1 (membrane-associated protein)	related to growth arrest [30]	Microarray
CTGF (connective tissue growth factor)	growth factor [29]	Microarray
IGF2R (IGF2 receptor)	growth factor receptor [30]	Microarray
PDGFRA (PDGF receptor α)	growth factor receptor [29]	Microarray
Myc	transcription factor [30]	Microarray
Growth, metabolism, adhesion, etc.		
H-ferritin (heavy subunit of ferritin) [54]		RNA subtraction hybridization
THBS1 (thrombospondin 1)	angiogenesis [30]	Microarray
COL3A1 (α-1 type 3 collagen)	adhesion molecule [29]	Microarray
FN1 (fibronectin 1)	adhesion molecule [29]	Microarray
TPM1 (tropomyosin α chain) (skeletal muscle) [29]		Microarray

Table 1 (*continued*)

Gene		Criteria
EST moderately similar to dithiolethione-inducible gene 2 [29]		Microarray
P2R4	phosphatase [30]	Microarray
p311 (neuronal protein) [29]		Microarray
A2M (α-2-macroglobulin) [29]		Microarray

Most targets are defined on the basis of Myc overexpression. Only genes whose regulation by Myc was insensitive to the presence of cycloheximide (immediate targets) or whose promoter regulation was studied in greater detail are included in the list. Some of the genes spotted through microarrays were previously identified by other techniques.

versial: finding a Myc–Max binding site on a promoter and showing that it confers a Myc transcriptional response in transient assays is not sufficient; additional evidence for specificity, the correlation of expression kinetics with those of Myc, and the demonstration that the endogenous gene can be induced by Myc are important (reviewed in [47,48]). Since Myc stimulates proliferation and cell growth, the levels of many genes are predicted to change indirectly as a consequence of these processes; requirement of de novo protein synthesis is an important criterion for discriminating among direct and indirect targets. Initially, target genes were identified by differential hybridization or by guessing. Many have been validated by way of induction of an overexpressed Myc–ER (a Myc fusion to a mutated estrogen receptor); there is not a general consensus that this is a valid criterion, since overexpression may cause artifacts. The alternative approach to look at gene expression changes in Myc null cells did not settle the issue, as it was shown that only two genes (*cad* and *gadd45*) out of 11 proposed Myc targets were deregulated in c-Myc null Rat-1 fibroblasts [49]. In vivo chromatin immunoprecipitation is also an important element for target validation. Of course the most important discriminant is to establish a role of the 'target' as mediator of Myc biological activities.

Genome-wide expression analysis through cDNA or oligonucleotide microarrays is expected to clarify the connections between transcriptional activity and biological functions of Myc. Changes in gene expression upon tamoxifen activation of a Myc–ER chimera have been investigated in human fibroblasts, with microarrays of 5272 and 6416 sequences [29,30]. Altogether, 56 genes were found to be directly activated or repressed; no large effect on activation or repression of any of the monitored genes was observed, in accordance with the relatively weak transcriptional activity of Myc. This indicates that the number of genes directly modulated by Myc in these experimental conditions would be above 500 on a genome basis, a figure that might be underestimated, as it is unclear how efficiently low-abundance mRNAs, such as TERT, are detected. The number of 'targets' could grow to even more confusing dimensions when different culture conditions, favoring for instance apoptosis rather than proliferation, are taken into account. As a matter of fact, microarray profiling performed in cells deprived of serum has revealed targets different from those identified in growth medium, possibly since Myc acts as an apoptosis inducer in this case [50].

From a survey of the putative direct targets identified so far (Table 1), it is not evident that Myc may be at the top of a specific regulatory cascade since, paradoxically, it appears difficult to identify a cellular function not affected by Myc.

Cell growth stimulation by Myc is in agreement with the role that many target genes have in ribosome biogenesis, protein synthesis, and generally in cell metabolism. They include translation initiation factors (EIF4E, EIF5A), the three nucleolar proteins fibrillarin, BN51, and nucleolin, ribosomal proteins (Rps11), the RNA helicase MrDb (DDX18), and ODC (polyamine biosynthesis) [14,29,30,51]. Myc influences the metabolic pathways involved in glucose uptake and iron homeostasis, respectively, through upregulation of the glycolytic genes LDH-A (lactate dehydrogenase), GLUT1 (glucose transporter), PFK (phosphofructokinase), enolase A, and through upregulation of IRP2 (iron regulatory protein 2) and repression of H-ferritin [52–54].

Repression of genes involved in cytoskeletal structure and cell adhesion, such as fibronectin, collagen, tropomyosin, and the antiangiogenic factor thrombospondin, may contribute to altered shape and adhesiveness of transformed cells.

Several cell cycle-related genes were detected from microarrays, SAGE, or knock-out studies as novel targets for activation (CksHs2, Cdk4, Id2) or repression (p21, Ches1, and CCNG2), besides old Myc targets like cyclin D2, Gadd45, and Gas1.

The presence of genes potentially involved in apoptosis (AP12, TRAP1) among direct Myc targets in growing cells is insufficient to explain how Myc affects cell death. By gene expression profiling in conditions that favor apoptosis in a myeloid cell line, other transcripts were identified as regulated by Myc, either directly or indirectly [50]. Up-regulated transcripts included those for Ca^{2+} channel β_3 subunit, platelet factor 4 precursor, myeloperoxidase, extracellular matrix protein 1, CD9, SM-20, Spi-2 protease inhibitor; genes encoding GATA binding protein 2, β_2-integrin and chemokine (C-C) receptor 2 are, instead, among the down-regulated genes.

Growth factors, receptors, transcriptional regulators, DNA topoisomerase I, HMGI/Y, and other genes whose activity is difficult to rationalize in relation to Myc are also in the expanding list of direct Myc targets.

Acknowledgements: Our work was supported by grants from AIRC and Telethon.

References

[1] Malynn, B.A., de Alboran, I.M., O'Hagan, R.C., Bronson, R., Davidson, L., DePinho, R.A. and Alt, F.W. (2000) Genes Dev. 14, 1390–1399.
[2] Felsher, D.W. and Bishop, J.M. (1999) Mol. Cell. 4, 199–207.
[3] Pelengaris, S., Littlewood, T., Khan, M., Elia, G. and Evan, G. (1999) Mol. Cell 3, 565–577.
[4] Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R. and Bradley, A. (1993) Genes Dev. 7, 671–682.
[5] Shen-Li, H., O'Hagan, R.C., Hou Jr., H., Horner II, J.W., Lee, H.W. and DePinho, R.A. (2000) Genes Dev. 14, 17–22.
[6] Pelengaris, S., Rudolph, B. and Littlewood, T. (2000) Curr. Opin. Genet. Dev. 10, 100–115.
[7] Dazard, J.E., Piette, J., Basset-Seguin, N., Blanchard, J.M. and Gandarillas, A. (2000) Oncogene 19, 3693–3705.

[8] Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N. and Gallant, P. (1999) Cell 98, 779–790.
[9] Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J. and Roussel, M.F. (1998) Genes Dev. 12, 2424–2433.
[10] Neufeld, T.P. and Edgar, B.A. (1998) Curr. Opin. Cell Biol. 10, 784–790.
[11] Mateyak, M.K., Obaya, A.J., Adachi, S. and Sedivy, J.M. (1997) Cell Growth Differ. 8, 1039–1048.
[12] Henriksson, M. and Luscher, B. (1996) Adv. Cancer Res. 68, 109–182.
[13] Prendergast, G.C. (1999) Oncogene 18, 2967–2987.
[14] Grandori, C., Cowley, S.M., James, L.P. and Eisenman, R.N. (2000) Annu. Rev. Cell Dev. Biol. 16, 653–699.
[15] Ayer, D.E. and Eisenman, R.N. (1993) Genes Dev. 7, 2110–2719.
[16] Hurlin, P.J., Steingrimsson, E., Copeland, N.G., Jenkins, N.A. and Eisenman, R.N. (1999) EMBO J. 18, 7019–7028.
[17] Yuan, J., Tirabassi, R.S., Bush, A.B. and Cole, M.D. (1998) Oncogene 17, 1109–1118.
[18] Spencer, C.A. and Groudine, M. (1991) Adv. Cancer Res. 56, 1–48.
[19] Wang, J., Hannon, G.J. and Beach, D.H. (2000) Nature 405, 755–756.
[20] He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B. and Kinzler, K.W. (1998) Science 281, 1509–1512.
[21] Zou, X., Rudchenko, S., Wong, K. and Calame, K. (1997) Genes Dev. 11, 654–662.
[22] Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K. and Nevins, J.R. (2000) Genes Dev. 14, 2501–2514.
[23] Chang, D.W., Claassen, G.F., Hann, S.R. and Cole, M.D. (2000) Mol. Cell. Biol. 20, 4309–4319.
[24] Steiner, M.S., Anthony, C.T., Lu, Y. and Holt, J.T. (1998) Hum. Gene Ther. 9, 747–755.
[25] Nikiforov, M.A., Kotenko, I., Petrenko, O., Beavis, A., Valenick, L., Lemischka, I. and Cole, M.D. (2000) Oncogene 19, 4828–4831.
[26] Mateyak, M.K., Obaya, A.J. and Sedivy, J.M. (1999) Mol. Cell. Biol. 19, 4672–4683.
[27] Santoni-Rugiu, E., Falck, J., Mailand, N., Bartek, J. and Lukas, J. (2000) Mol. Cell. Biol. 20, 3497–3509.
[28] Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D. and Amati, B. (1996) EMBO J. 15, 6595–6604.
[29] Coller, H.A., Grandori, C., Tamayo, P., Colbert, T., Lander, E.S., Eisenman, R.N. and Golub, T.R. (2000) Proc. Natl. Acad. Sci. USA 97, 3260–3265.
[30] O'Hagan, R.C., Schreiber-Agus, N., Chen, K., David, G., Engelman, J.A., Schwab, R., Alland, L., Thomson, C., Ronning, D.R., Sacchettini, J.C., Meltzer, P. and DePinho, R.A. (2000) Nature Genet. 24, 113–119.
[31] Berns, K., Martins, C., Dannenberg, J.H., Berns, A., te Riele, H. and Bernards, R. (2000) Oncogene 19, 4822–4827.
[32] Perez-Roger, I., Kim, S.H., Griffiths, B., Sewing, A. and Land, H. (1999) EMBO J. 18, 5310–5320.
[33] Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., Dang, C.V., Sedivy, J.M., Eick, D., Vogelstein, B. and Kinzler, K.W. (2000) Proc. Natl. Acad. Sci. USA 97, 2229–2234.
[34] Meyer, C.A., Jacobs, H.W., Datar, S.A., Du, W., Edgar, B.A. and Lehner, C.F. (2000) EMBO J. 19, 4533–4542.
[35] Wu, L., Goodwin, E.C., Naeger, L.K., Vigo, E., Galaktionov, K., Helin, K. and DiMaio, D. (2000) Mol. Cell. Biol. 20, 7059–7067.
[36] Lasorella, A., Noseda, M., Beyna, M. and Iavarone, A. (2000) Nature 407, 592–598.
[37] Ferre-D'Amare, A.R., Prendergast, G.C., Ziff, E.B. and Burley, S.K. (1993) Nature 363, 38–45.
[38] Soucek, L., Helmer-Citterich, M., Sacco, A., Jucker, R., Cesareni, G. and Nasi, S. (1998) Oncogene 17, 2463–2472.
[39] Grandori, C., Mac, J., Siebelt, F., Ayer, D.E. and Eisenman, R.N. (1996) EMBO J. 15, 4344–4357.
[40] Winston, R.L. and Gottesfeld, J.M. (2000) Chem. Biol. 7, 245–251.
[41] Prendergast, G.C. and Ziff, E.B. (1991) Science 251, 186–189.
[42] McMahon, S.B., Wood, M.A. and Cole, M.D. (2000) Mol. Cell. Biol. 20, 556–562.
[43] Eberhardy, S.R., D'Cunha, C.A. and Farnham, P.J. (2000) J. Biol. Chem. 275, 33798–33805.
[44] Wood, M.A., McMahon, S.B. and Cole, M.D. (2000) Mol. Cell 5, 321–330.
[45] Sakamuro, D. and Prendergast, G.C. (1999) Oncogene 18, 2942–2954.
[46] Cheng, S.W., Davies, K.P., Yung, E., Beltran, R.J., Yu, J. and Kalpana, G.V. (1999) Nature Genet. 22, 102–105.
[47] Cole, M.D. and McMahon, S.B. (1999) Oncogene 18, 2916–2924.
[48] Dang, C.V. (1999) Mol. Cell. Biol. 19, 1–11.
[49] Bush, A., Mateyak, M., Dugan, K., Obaya, A., Adachi, S., Sedivy, J. and Cole, M. (1998) Genes Dev. 12, 3797–3802.
[50] Nesbit, C.E., Tersak, J.M., Grove, L.E., Drzal, A. and Choi, H. (2000) Oncogene 19, 3200–3212.
[51] Greasley, P.J., Bonnard, C. and Amati, B. (2000) Nucleic Acids Res. 28, 446–453.
[52] Shim, H., Dolde, C., Lewis, B.C., Wu, C.S., Dang, G., Jungmann, R.A., Dalla-Favera, R. and Dang, C.V. (1997) Proc. Natl. Acad. Sci. USA 94, 6658–6663.
[53] Osthus, R.C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L.A. and Dang, C.V. (2000) J. Biol. Chem. 275, 21797–21800.
[54] Wu, K.J., Polack, A. and Dalla-Favera, R. (1999) Science 283, 676–679.
[55] Sherr, C.J. and Roberts, J.M. (1999) Genes Dev. 13, 1501–1512.
[56] Wang, J., Xie, L.Y., Allan, S., Beach, D. and Hannon, G.J. (1998) Genes Dev. 12, 1769–1774.
[57] Wood, L.J., Mukherjee, M., Dolde, C.E., Xu, Y., Maher, J.F., Bunton, T.E., Williams, J.B. and Resar, L.M.S. (2000) Mol. Cell. Biol. 20, 5490–5502.