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THE MYC/MAX/MAD NETWORK AND THE TRANSCRIPTIONAL CONTROL OF CELL BEHAVIOR

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■ **Abstract** The Myc/Max/Mad network comprises a group of transcription factors whose distinct interactions result in gene-specific transcriptional activation or repression. A great deal of research indicates that the functions of the network play roles in cell proliferation, differentiation, and death. In this review we focus on the Myc and Mad protein families and attempt to relate their biological functions to their transcriptional activities and gene targets. Both Myc and Mad, as well as the more recently described Mnt and Mga proteins, form heterodimers with Max, permitting binding to specific DNA sequences. These DNA-bound heterodimers recruit coactivator or corepressor complexes that generate alterations in chromatin structure, which in turn modulate transcription. Initial identification of target genes suggests that the network regulates genes involved in the cell cycle, growth, life span, and morphology. Because Myc and Mad proteins are expressed in response to diverse signaling pathways, the network can be viewed as a functional module which acts to convert environmental signals into specific gene-regulatory programs.

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OVERVIEW OF THE NETWORK

The Max network comprises a group of transcription factors whose functions profoundly affect cell behavior (for reviews see Amati & Land 1994, Henriksson & Luscher 1996, Bouchard et al 1998, Facchini & Penn 1998). These factors possess two common attributes. First, members of the network are a subset of the larger class of proteins containing basic helix-loop-helix zipper (bHLHZ) motifs. This domain is known to mediate protein-protein interactions and DNA binding (Murre et al 1989a,b; Ferre-D'Amare et al 1994). Second, each of the members of the network utilizes its bHLHZ domains to form individual dimers with Max, itself a small bHLHZ protein (Figure 1). Association with Max results in the formation of a heterodimer possessing sequence-specific DNA binding and transcriptional activities. Max itself can homodimerize and bind DNA, but such Max homodimers appear to be transcriptionally inert (Amati et al 1992, Kretzner et al 1992). The ability to modulate transcription is derived from specific domains within the Max-interacting factors which, in turn, appear to mediate associations with specific

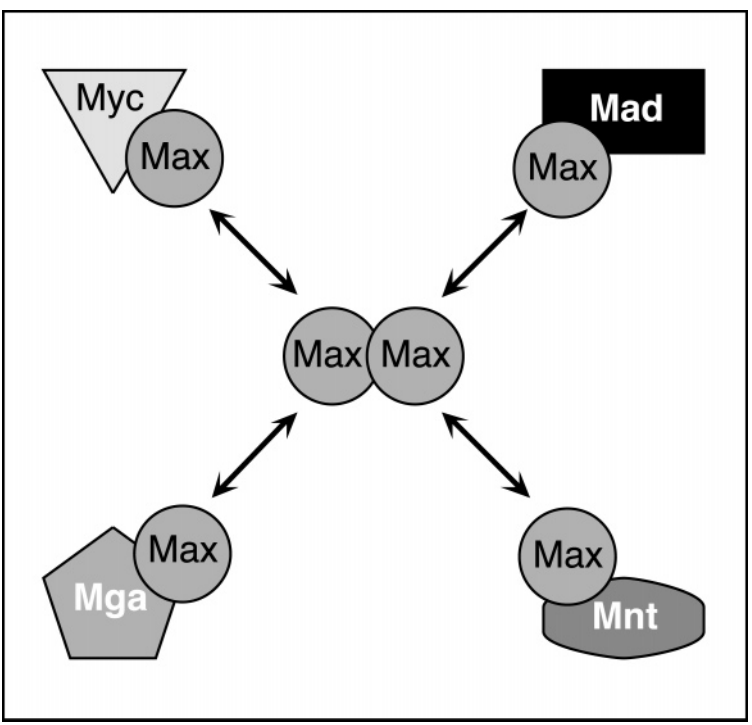

Figure 1 Max-interacting proteins. Max forms heterodimers with members of the Myc and Mad protein families as well as with the Mnt (or Rox) and Mga proteins. Each of these proteins interacts with Max through its bHLHZ domain.

coactivators or corepressors, resulting in the formation of higher-order complexes. Because Max-interacting proteins homodimerize poorly on their own and therefore bind DNA weakly, it can be argued that it is through the highly specific association with Max that the activities of these proteins are manifested. In general, Max-interacting proteins have short half-lives and their biosynthesis is highly regulated. Max, on the other hand, is stable and constitutively expressed, suggesting that the regulation of the network is largely dependent on the abundance of the Max-associated transcription factors (Blackwood et al 1992, Berberich et al 1992).

Our understanding of the Max network grew out of research on the *myc* oncogene family. *myc* was originally defined as an oncogene (v-*myc*) transduced by a number of avian retroviruses capable of potently inducing neoplastic disease. Subsequently c-*myc*, the cellular homolog of v-*myc*, was identified and eventually shown to be a member of a family of proto-oncogenes comprising c-*myc*, N-*myc*, and L-*myc*. These genes are considered proto-oncogenes in the sense that alterations in their structure and expression have been linked to a wide variety of human and other animal cancers (for reviews see Hayward et al 1982, Cole 1986, Cory 1986, Magrath 1990, DePinho et al 1991, Nesbit et al 1999). The proteins (Myc) encoded by *myc* family genes are predominantly localized in the cell nucleus, and their expression generally correlates with cell proliferation. When it was shown that the N-terminal region of Myc could function as a transcriptional activation domain (Kato et al 1990) and that the C-terminal region possessed homology to bHLHZ proteins (Murre et al 1989a), it was widely assumed that Myc proteins would form homo- or heterodimers, bind DNA, and function as transcriptional activators. Because neither dimerization nor specific DNA binding could be readily demonstrated for Myc, except at high protein concentrations, a search for novel Myc interactors was initiated, leading to the identification of Max. Max was shown to interact specifically with all Myc family proteins, and the resulting heterocomplexes recognize the hexameric DNA sequence CACGTG (belonging to the larger class of sequences known as E-boxes, CANNTG) at concentrations at which binding by either partner alone is undetectable (Blackwood & Eisenman 1991, Prendergast et al 1991). Importantly, Myc requires Max to activate transcription of genes containing E-box binding sites (Amati et al 1992). Furthermore, Myc has been shown to repress transcription at certain target promoters (Li et al 1994). The transcription activation function of Myc is mediated at least in part by recruitment of a histone acetyltransferase (HAT) (McMahon et al 2000).

The fact that Max is expressed in the absence of Myc led to the question of whether Max might have additional dimerization partners. This prompted a search for new Max-interacting proteins by the use of expression cloning and two-hybrid screens. In this manner, two novel, but related, Max-interacting bHLHZ proteins were discovered—Mad1 and Mxi1 (Ayer et al 1993, Zervos et al 1993)—followed later by Mad3 and Mad4 (Hurlin et al 1995b). These four proteins, considered to compose the Mad protein family, behave much like Myc in that they have only weak homodimerization and DNA-binding capacities but readily heterodimerize

with Max and bind the E-box consensus sequence. However, in contrast to Myc, which activates transcription at promoters proximal to E-box sites, the Mad-Max heterodimers act as transcriptional repressors at the same binding sites. Each of the Mad proteins acts as a repressor by associating with the mSin3 corepressor complex (for reviews see McArthur et al 1998, Schreiber-Agus & DePinho 1998). Also, in contrast to Myc, expression of Mad family proteins appears closely linked to terminal differentiation (Ayer & Eisenman 1993, Larsson et al 1994, Vastrik et al 1995, Cultraro et al 1997, Queva et al 1998).

Max interacts with at least two other bHLHZ proteins in addition to the Myc and Mad family members–Mnt (also called Rox) and Mga. Both of these proteins possess transcriptional activities which appear to be context dependent. Thus Mnt, similar to the Mad family proteins, recruits the mSin3 corepressor complex and represses transcription in some, but not all, cell types (Hurlin et al 1997, Meroni et al 1997). Mga contains two functional DNA-binding domains, a bHLHZ region, which interacts with Max, and a Brachyury or T-box domain. Activation of transcription by Mga at T-box binding sites depends on binding of Max to the distal bHLHZ domain, suggesting that dimerization with Max displaces a repressor or induces a conformational change in Mga (Hurlin et al 1999).

Although yet other Max-interacting proteins are likely to be identified, the basic outlines of the Max network are emerging. Max is a stable, ubiquitously expressed protein with little transcriptional activity of its own. The ability of Max to heterodimerize with several distinct groups of highly regulated proteins (outlined in Figure 1) results in transcriptional activation or repression directed at specific sets of target genes. Furthermore, the different complexes may have antagonistic properties whose functions play out at the level of chromatin structure. The involvement of these proteins in key biological events suggests that network function may be critical for growth and development. Indeed, the recent report that targeted deletion of Max results in very early embryonic lethality in mice underscores the importance of Max-dependent functions (Shen-Li et al 2000). In this review we concentrate on the transcriptional activities manifested through the Max network, with particular emphasis on recent findings relating to the function of Myc and Mad family proteins.

MYC

Biological Functions of Myc

The intense scrutiny with which *myc* has been studied over the last 15 years derives mainly from its apparent involvement in a wide range of cellular processes including proliferation, differentiation, and tumorigenesis (see above). In the following sections we summarize the evidence for *myc*'s role in both normal and abnormal cellular behavior, with special attention to the effects of deregulated expression of *myc*.

Induction by Mitogenic Signals One of the most compelling ideas about *myc* is that it functions to drive proliferation in response to diverse signals. This notion arises from several broad lines of evidence which will be briefly reviewed here. First, *myc* family genes are broadly expressed during embryogenesis, and targeted deletions of c-*myc* or N-*myc* genes in mice lead to lethality in midgestation embryos (see below; Sawai et al 1991, Moens et al 1992, Charron et al 1993, Davis et al 1993, Moens et al 1993, Sawai et al 1993, Stanton et al 1993, Hatton et al 1996). Second, there is a strong correlation between *myc* expression and proliferation. This probably applies to all *myc* family genes but has been most extensively documented for c-*myc*. Myc expression is known to be induced in many cell types by a wide range of growth factors, cytokines, and mitogens (see, for example, Kelly et al 1983, Armelin et al 1984, Morrow et al 1992, Shibuya et al 1992, Sato et al 1993). The increase in Myc levels has been shown to occur through both transcriptional and post-transcriptional mechanisms (for a review see Spencer & Groudine 1991) and appears to occur as an immediate early response to most mitogenic factors, suggesting that the *myc* regulatory region is a nexus for multiple growth signal response pathways. Regulation of translation initiation also occurs upon mitogenic stimulation (West et al 1998). For a number of receptors (including those for interleukin-2, macrophage colony-stimulating factor, Epo, epidermal growth factor, platelet-derived growth factor, and antigens), it can be argued that induction of *myc* is a necessary, but probably not sufficient, component of the mitogenic response (Roussel et al 1991, Shibuya et al 1992, Barone & Courtneidge 1995). Moreover, a failure to induce *myc* in response to mitogenic signaling inhibits quiescent cells from entering the cycle (Roussel et al 1991, Barone & Courtneidge 1995). In contrast, ligands such as transforming growth factor beta and gamma interferon, which in some settings act to inhibit proliferation, also cause rapid down-regulation of c-*myc* expression (Pietenpol et al 1990, Ramana et al 2000). Thus Myc expression strongly correlates with growth and proliferation.

Myc Overexpression The idea that Myc plays a critical role in the proliferative process is also consistent with results from experiments involving its ectopic expression in a variety of different cell types under a range of conditions. In these experiments, Myc expression is uncoupled from its normal physiological regulation; it is overexpressed and cannot be down-regulated. Myc overexpression in cycling cells has been reported to reduce requirements for growth factors, block exit from the cell cycle, accelerate cell division, and increase cell size (Sorrentino et al 1986, Stern et al 1986, Karn et al 1989, Johnston et al 1999, Iritani & Eisenman 1999). In the absence of survival factors, c-Myc overexpression elicits a proliferative response but leads to apoptosis through a mechanism at least partly dependent on the Arf-Mdm2-p53 pathway (Askew et al 1991, Evan et al 1992, Harrington et al 1994, Hermeking & Eick 1994, Wagner et al 1994, Zindy et al 1998). Experiments with primary murine embryo fibroblasts (MEFs) demonstrate that loss of p53 or Arf greatly attenuates c-Myc-induced apoptosis and permits cells to survive crises and proliferate continuously in the absence of serum

(Zindy et al 1998). In some cell types, *myc* requires coexpression of other genes (such as *ras*, *jun*, and *fos*) in order to drive entry into S phase (Roussel et al 1991, Leone et al 1997).

Myc levels rapidly diminish during the terminal differentiation of many cell types, and enforced expression of Myc inhibits or modulates terminal differentiation of myoblasts (Miner & Wold 1991), erythroleukemia cells (Coppola & Cole 1986, Birrer et al 1989), adipocytes (Freytag 1988), B lymphoid cells (Thompson et al 1987), and myeloid cells (Larsson et al 1988) among others (for a review see Henriksson & Luscher 1996). There is some evidence that Myc may not directly interfere with the programmed expression of differentiation genes but rather is incompatible with the cell cycle exit required for terminal differentiation (La Rocca et al 1989, Miner & Wold 1991). However, not all differentiation events involve cell cycle arrest, and there are indications that Myc may play a role in advancing cells along pathways of epidermal and hematopoietic differentiation (Gandarillas & Watt 1997; B Iritani & RN Eisenman unpublished data). Perhaps Myc is important for changes in cell growth and metabolism that are required for lineage commitment (see below).

Myc Deregulation in Tumors The ability of overexpressed Myc to facilitate proliferation and inhibit terminal differentiation fits well with the fact that tumors of diverse origins contain genetic rearrangements involving *myc* family genes. These rearrangements include retroviral transductions, amplifications, and chromosomal translocations, as well as viral insertions, and in general are thought to increase *myc* expression levels and prevent *myc* turnoff rather than alter the function of the Myc protein through mutation (for reviews see Hayward et al 1982, Cole 1986, Cory 1986, Eisenman 1989, Magrath 1990, DePinho et al 1991, Nesbit et al 1999). Indeed, many of the genomic alterations in *myc* result in increased *myc* mRNA levels through increased transcription initiation, decreased transcription attenuation, and augmented stability of the *myc* messenger RNA (see Spencer & Groudine 1991). However, although mutations in the Myc protein are relatively rare, they nonetheless do occur, particularly in retrovirus-transduced *myc* genes and in translocated *myc* genes (Gaidano et al 1991, Bhatia et al 1993). It has been suggested that these mutations influence Myc's transactivation ability by altering binding to inhibitors such as the retinoblastoma protein (Rb) or p107 (Gu et al 1994) or by modulating the effects of serine and threonine phosphorylation required for full transcriptional activity (Seth et al 1993, Gupta et al 1993). Another possibility is that the transcriptional effects of mutations are secondary to alterations in Myc protein degradation. Myc family proteins have short half-lives, on the order of 20–30 min (Hann & Eisenman 1984), and, although some instances of stabilization have been detected (Chen et al 1989, Spotts & Hann 1990, Shindo et al 1993), it was reported earlier that in most tumor-associated Myc proteins, stability was not consistently affected (Luscher & Eisenman 1987, 1988). More recently, it has been discovered that Myc degradation is carried out through the ubiquitin-mediated proteosome pathway (Ciechanover et al 1991, Gross-Mesilaty et al 1998, Salghetti

et al 1999) and that many tumor-related mutations in Myc result in significant stabilization of the protein (Salghetti et al 1999, Gavine et al 1999). Furthermore, activated Ras, an oncoprotein that collaborates with Myc in the transformation of primary cells, has also been reported to induce stabilization of Myc protein (Sears et al 1999). The molecular consequences of Myc stabilization are unknown, but the heightened stability must contribute to an overall increase in Myc protein levels and is likely to exacerbate the transcriptional effects of Myc. In this regard it is intriguing that the highly conserved region called Myc box II (see below), which is essential for transformation and at least some of Myc's transcriptional activities, has also been shown to regulate Myc protein turnover (Flinn et al 1998, Salghetti et al 1999). There is also evidence that c-Myc translation can be regulated by the switch from an apparently inefficient cap-dependent mechanism to a cap-independent internal ribosome entry site within the 5′ untranslated region (Stoneley et al 1998, Johannes et al 1999). This has been shown to occur during apoptosis as well as serum stimulation and has been suggested to also increase the rate of Myc translation during transformation (Stoneley et al 2000).

The idea that the tumor-associated Myc mutations serve to activate Myc is attractive. However, a recent study systematically examining the effects of these mutations on Myc function in tissue culture cells has revealed that they have little if any effect on transformation, proliferation, apoptosis, or target gene expression (Chang et al 2000). Indeed the most common Myc mutation found in Burkitt's lymphomas, T58I, confers decreased transforming activity, with no evident change in apoptosis compared with wild-type Myc. These results may reflect important differences between in vitro transformation assays and in vivo tumorigenesis. Furthermore, the maintenance of apoptotic function in these Myc mutants may simply come from the ability of tumor cells to circumvent Myc-induced cell death by other mechanisms such as loss of p53 or Arf activities (see below).

Studies of Myc's role in oncogenesis indicate that augmented Myc levels can arise through transcriptional, post-transcriptional, and post-translational mechanisms. Several recent studies using murine models of epithelial and hematopoietic transformation have demonstrated that high *myc* levels are continuously required to maintain the tumorigenic phenotype (Felsher & Bishop 1999a, Pelengaris et al 1999). Because, as described above, Myc overexpression in tissue culture cells can result in extended proliferation, it seems likely that similar effects occur in tumor cells and that secondary mutations in other genes cooperate with *myc* to generate overt tumors (Cory & Adams 1988, Harris et al 1988). Another, not mutually exclusive role for Myc may be to induce genomic instability (Mai et al 1996, Felsher & Bishop 1999b, Kuschak et al 1999, Mushinski et al 1999). Although the mechanism for this has not been established, instability might generate cooperating mutations in other genes.

A large number of genes that cooperate with *myc* in mouse models of lymphomagenesis have been identified (for a review see Jonkers & Berns 1996). While in many cases the functions of these cooperating genes are unknown, recent work suggests that an important subset is likely to act by abrogating the apoptotic

function of Myc. This notion had surfaced earlier when it was found that the antiapoptotic protein Bcl-2 inhibited Myc-induced apoptosis in tissue culture cells and also promoted lymphomagenesis in mice in collaboration with Myc (Strasser et al 1990, Bissonnette et al 1992, Fanidi et al 1992, McDonnell & Korsmeyer 1991). More recent experiments have demonstrated that in mice carrying a c-*myc* transgene whose expression is directed to lymphoid cells (Eμ-*myc* mice; Harris et al 1988) the *myc*-overexpressing progenitor cells exhibit high rates of spontaneous apoptosis and contain an intact Arf-Mdm2-p53 checkpoint pathway (Eischen et al 1999). However, lymphomas derived from these same mice display spontaneous inactivation of the p53 pathway through mutation or loss of p53 or Arf or via elevation of Mdm2 levels (Eischen et al 1999), nicely echoing the findings in MEFs (Zindy et al 1998) and in K562 cells, where Myc has also been shown to antagonize the effects of p53 (Ceballos et al 2000). As expected from these results, *myc*-transgenic mice with hemizygous or null *ARF* alleles show greatly accelerated lymphomagenesis (Eischen et al 1999), as do Eμ-*myc* mice lacking p53 (Schmitt et al 1999). The importance of Arf and apoptosis in *myc*-induced lymphomagenesis was strikingly underscored by experiments demonstrating that the protein encoded by the cooperating oncogene *bmi1* is a member of the Polycomb class of repressors that acts to suppress expression from the *Ink4a* locus, which encodes Arf and $p16^{INK4a}$ (Jacobs et al 1999b). Moreover, loss of one or both *bmi1* alleles inhibits lymphomagenesis in Eμ-*myc* mice by increasing Arf-dependent apoptosis (Jacobs et al 1999a). This work represents perhaps the clearest example of a mechanism through which Myc collaborates with other genes during tumor evolution. It is likely that other cooperating oncogenes will similarly act to evade the apoptosis-inducing activity of deregulated *myc* (for a review see Hueber & Evan 1998).

Targeted Deletions of Myc Family Genes The role played by the *myc* gene family in proliferation, apoptosis, and differentiation has naturally raised the issue of the degree to which *myc* is involved in embryonic development. Studies along these lines have involved both targeted deletions of *myc* family genes and overexpression of *myc* transgenes (for reviews see Davis & Bradley 1993, Morgenbesser & DePinho 1994). Loss of c- and N-*myc* clearly leads to embryonic lethality (Sawai et al 1991, Moens et al 1992, Charron et al 1993, Davis et al 1993, Sawai et al 1993, Stanton et al 1993), while deletion of L-*myc* produces no detectable phenotype (Hatton et al 1996). An important result emerging from the N-*myc* deletions is that the dose of N-*myc* determines the severity of the phenotype produced. Thus N-*myc* null mutations result in embryonic lethality at day 11.5 of gestation, with dramatic abnormalities in the heart, liver, kidney, limb bud, lungs, and nervous systems—all tissues in which N-*myc* is normally expressed (Sawai et al 1991, Charron et al 1993, Stanton et al 1993, Sawai et al 1993). On the other hand, an N-*myc* hypomorphic mutant and a compound heterozygote producing increasing doses of N-*myc* survive for significantly longer times and display more-restricted effects on specific organs (Moens et al 1992, 1993; Nagy et al 1998). This work makes it clear that variations in Myc levels have important consequences.

Compared with the very early embryonic lethality observed for the Max knockout (Shen-Li et al 2000), the longevity of the different *myc*-null mutants is intriguing. Of course Max, in addition to dimerizing with Myc, also interacts with Mad, Mnt, and Mga proteins (and potentially others; Shors et al 1998), and therefore its loss might be expected to have more severe consequences. Furthermore, in the single *myc* knockouts, compensation by other family members is likely (double and triple *myc* family knockouts have yet to be reported). Indeed a very recent report demonstrates that N-*myc* apparently can replace c-*myc* in mouse development (Malynn et al 2000). Nonetheless, the single knockouts reveal to us that extensive proliferation can occur in the absence of c-, N-, or L-*myc* and suggest that the relationship between *myc* function and proliferation is more complex than envisaged from the tissue culture experiments described above. Indeed, an immortalized cell line with somatic disruption of c-*myc* and no detectable expression of N- or L-*myc* is capable of dividing, although at a significantly reduced rate compared to wild-type parental cells (Mateyak et al 1997). In addition, several studies have indicated that both c- and N-*myc* are expressed in certain nondividing cell types (Downs et al 1989, Craig et al 1993, Wakamatsu et al 1993). Other work has also indicated that cell cycle entry can be uncoupled from Myc expression (Coughlin et al 1985, Mehmet et al 1997). In addition, studies employing *myc*-transgenic mice indicate that *myc* overexpression correlates with increased cell size or hypertrophy in specific tissues (Jackson et al 1990, Iritani & Eisenman 1999). Together these studies raise the possibility that the requirement for *myc* expression during proliferation is not absolute and that its relationship to cell division may be indirect.

DNA Binding and Transcriptional Activities of Myc-Max Heterodimers

The complex biology of *myc* as outlined above brings us to the question of how a single protein can affect so many aspects of cell behavior. Part of the answer may lie in the relationship of *myc* to the network of interacting proteins within which it functions. It is evident that we need to understand Myc's molecular function and how this function impacts directly on the cell. In the following sections we review recent data on the DNA binding and transcriptional activities ascribed to Myc as well as on the genes believed to be regulated through these activities.

Structure The crystal structure of Max homodimers makes it clear why both dimerization with Max and E-box recognition require the intact bHLHZ regions of each partner. The three-dimensional structure demonstrates that the bHLHZ domains of both Max monomers associate to generate a novel four-helix bundle fold (Figure 2, see color insert) (Ferre-D'Amare et al 1993, Brownlie et al 1997). Although Myc-Max and Mad-Max structures are not yet available, there is little reason to suspect that the basic folds are substantially different from those of Max-Max. In Max homodimers, helices 1 and 2 contribute to the hydrophobic

TABLE 1 An alignment of the basic regions of E-box-binding proteins[a]

	1	2	3	4	(5)	6	7	8	(9)	10	11	12	(13)
c-Myc	K	R	R	T	H	N	V	L	E	R	Q	R	R
N-Myc	R	R	R	N	H	N	I	L	E	R	Q	R	R
L-Myc	K	R	K	N	H	N	F	L	E	R	K	R	R
dMyc	K	R	N	Q	H	N	D	M	E	R	Q	R	R
Max	K	R	A	H	H	N	A	L	E	R	K	R	R
dMax	K	R	A	H	H	N	A	L	E	R	R	R	R
Mxl-1	A	R	E	Q	H	N	A	L	E	R	R	R	R
Mad-1	S	R	S	T	H	N	E	M	E	K	N	R	R
Mxi-1	N	R	S	T	H	N	E	L	E	K	N	R	R
Mad-3	G	R	S	V	H	N	E	L	E	K	R	R	R
Mad-4	N	R	S	S	H	N	E	L	E	K	H	R	R
Mdl-1	S	R	T	A	H	N	E	L	E	K	T	R	R
Mnt	T	R	E	V	H	N	K	L	E	K	N	R	R
Mga	Y	R	R	T	H	T	A	N	E	R	R	R	R
Mlx	R	R	R	A	H	T	Q	A	E	Q	K	R	R
USF	R	R	A	Q	H	N	E	V	E	R	R	R	R
TFE-3	K	K	D	N	H	N	L	I	E	R	R	R	R
MyoD	R	R	K	A	A	T	M	R	E	R	R	R	L

[a]Amino acids are assigned to one of four groups and shaded as follows: positive (R, K, H) in white boxes: negative (D, E) in black; charged polar (S, T, Y, N, Q) in light gray; and hydrophobic (G, A, V, I, L, C, M, F, W, P) in dark gray. The circled residues (5, 9, 13) make specific base pair contacts in the Max cocrystal structure.

core which stabilizes the dimer structure, while the leucine zipper regions, which are C-terminal extensions of helix 2, form a coiled coil. The zipper interactions involve hydrogen bonds and salt bridges that are likely to determine dimerization specificity (Figure 2, *right side*) (Amati et al 1993a, Ferre-D'Amare et al 1993, Brownlie et al 1997, Soucek et al 1998).

The Max structure also shows that DNA binding is mediated primarily by the basic region, which comprises an N-terminal extension of helix 1 of each monomer. Table 1 compares the basic regions of all known Max network proteins as well as several other bHLHZ proteins. Each basic region makes four specific DNA base contacts within the E-box as well as numerous phosphate backbone contacts. The loop and the N terminus of helix 2 also contact the phosphate backbone. All

E-box sequences contain, at the end of each half-site, a CA which is contacted by a conserved glutamic acid present in all bHLHZ (residue 9 in Table 1), as well as in several E-box-binding bHLH proteins (such as MyoD and E47). A basic region arginine residue in Max network proteins, as well as USF and TFE3, contacts the internal G of each half-site and thus distinguishes these proteins which bind CACGTG from those such as MyoD and E47 which have polar or hydrophobic residues in this position and recognize CAGCTG (Table 1, residue 13) (Dang et al 1992, Ferre-D'Amare et al 1993).

Myc-Max heterodimers also recognize noncanonical sites, variations of the canonical E-box containing core TG or CG dinucleotides (Blackwell et al 1993). In addition, nucleotides immediately flanking the E-box, as well as methylation of CpG within the E-box, can affect Myc-Max binding (Halazonetis & Kandil 1991, Prendergast & Ziff 1991, Fisher et al 1993, Solomon et al 1993). Most of the early studies identifying Myc-Max DNA binding preferences were carried out in vitro. It is somewhat reassuring, then, that recent compilations of candidate in vivo recognition sites for Myc-Max (see below) have confirmed binding to both canonical and noncanonical sites, as well as a preference for GC residues immediately flanking the E-box (for a review see Grandori & Eisenman 1997). Furthermore, many putative Myc-Max target genes contain multiple binding sites, suggesting the possibility that interaction between individual heterodimers facilitates binding, thereby increasing selectivity. In fact, cooperative binding of Myc-Max has been reported for the ornithine decarboxylase (ODC) promoter in vitro (Walhout et al 1997). Myc-Max binding is also likely to be influenced by the proximity of other transcription factors; for example, AP2 negatively affects Myc-Max DNA binding (Gaubatz et al 1995), and E-box sites have been shown to be transiently occupied in vivo by both USF and Myc (Boyd & Farnham 1999a). Other Max network proteins, such as Mnt, and some Mad family members may be normally coexpressed with Myc and therefore may compete for available Max and for Myc-Max binding sites (Hurlin et al 1997). Indeed, Mnt and USF constitute major E-box binding activities in nuclear extracts from proliferating cells (Sommer et al 1998). Because Myc-Max binding sites occur approximately every 1 kb in the mammalian genome, it seems likely that Myc-Max heterodimers recognize a restricted subset of potential binding sites. The resulting affinity for a particular E-box might depend on the nature of the flanking sequences, the number and spacing of sites, the methylation status of the E-box, and the presence or absence of other transcription factors competing for these same sites. It has been suggested that the interaction of Myc-Max with the target sequences in the cell may be a stochastic process (Boyd & Farnham 1999a) which in some sense integrates the local transcriptional environment.

Transcription Activation and the Myc Box II Riddle When introduced into cells, Myc can activate transcription of synthetic reporter genes containing promoter-proximal E-boxes in both yeast and mammalian cells (Amati et al 1992, Kretzner et al 1992, Gu et al 1993). In addition, Myc stimulates natural E-box-containing promoters or sequences derived from putative Myc target genes (Benvenisty et al

1992, Bello-Fernandez et al 1993, Gaubatz et al 1994, Jones et al 1996). This transcriptional activity appears to require two regions of Myc: the C-terminal bHLHZ domain and the N-terminal transactivation domain comprising the first 143 amino acids (Figure 3, see color insert) (Kato et al 1990, Amati et al 1992, Kretzner et al 1992). The implication from these results is that Myc heterodimerizes with the ubiquitously expressed endogenous Max to permit sequence-specific DNA binding followed by Myc-dependent activation of transcription. This is consistent with many studies in which Myc bHLHZ mutations, leading to the loss of association with Max and/or decreased DNA binding, serve to abrogate Myc's transcriptional activation and biological activities (see, for example, Crouch et al 1990, Freytag et al 1990, Smith et al 1990, Crouch et al 1993, Blackwood et al 1994).

Further evidence supporting the necessity of Myc-Max interaction for transcriptional activation comes from experiments exchanging HLHZ domains of Myc and Max as well as altering the dimerization specificity of the zipper regions so that the mutant proteins dimerize with each other but not with their wild-type counterparts. These altered-specificity Myc and Max proteins fail to function on their own but are dependent on each other for stimulating transcription, proliferation, transformation, and apoptosis (Amati et al 1993a,b). Interestingly, artificial Myc homodimers generated through altered dimerization specificity-inducing mutations are deficient in biological function and transcriptional activation, suggesting that an interaction with Max is required for Myc-Max DNA binding or transcriptional activity. The dependence on Max for Myc function is consistent with other studies demonstrating that Myc-Max complexes can be detected in cells and that Max alone has little transcriptional activity (Blackwood et al 1992, Kato et al 1992). Taken together, these experiments suggest a strong link between Myc-Max heterodimerization and transcriptional activation. While it seems likely that Myc's transactivation is crucial to its function, these studies offer no formal proof.

The region of Myc responsible for gene activation (the transactivation domain, or TAD) was initially defined by using fragments of c-Myc protein fused to the DNA binding domain of the yeast Gal4 protein (Kato et al 1990). These experiments mapped transcriptional activation to a segment lying between amino acids 1 and 143, which encompasses two regions, Myc box I (MBI; from approximately amino acids 45–63) and Myc box II (MBII; approximately amino acids 128–143) (see Figure 3), containing sequences highly conserved among the different Myc family proteins throughout evolution (see Atchley & Fitch 1995). An N-terminal region containing both MBI and MBII (amino acids 41–143) exhibits the highest transcriptional activity of any of the N-terminal Myc fusion proteins (Kato et al 1990). In this context, deletion of either MBI or MBII, as well as at least part of the sequence lying between them, diminishes transcriptional activation potential 10- or 50-fold, respectively. In biological assays, MBI deletion was found to attenuate only Myc transforming activity while an MBII deletion completely abolished it (Stone et al 1987, Li et al 1994).

Although MBII is clearly required for Myc's transforming function, its role in transcription has been controversial. This is because deletion of MBII from the

full-length c-Myc protein has little effect on activation in transient assays using either synthetic reporter genes or promoters derived from putative Myc target genes (Li et al 1994, Brough et al 1995). On the other hand, a careful study of the E-boxes in the promoter of α-prothymosin, a putative Myc target gene, shows that deletion of MBII eliminates Myc-induced activation at sites distal to the promoter while activation of more-proximal sites does not require MBII (Desbarats et al 1996). However, this detailed analysis has not been performed on the promoters of other target genes. Other work bearing on MBII and transactivation involves a naturally occurring variant of Myc, Myc-S (residues 101–439 of wild-type Myc), which lacks the majority of the transactivation domain but retains MBII. Myc-S has repression activity but does not appear to function as a transactivator in transient assays (Xiao et al 1998). Nonetheless, Myc-S can induce the expression of a number of endogenous Myc target genes (C Grandori, manuscript submitted). The correlation between the biological functions and the transcriptional activities of MBII is controversial because MBII has been shown to be important for Myc-induced repression (see below). If MBII is not required for activation, then we might well conclude that cell transformation by Myc is more dependent on gene repression than on activation. Perhaps one problem is that assays using synthetic reporter genes, while capable of demonstrating the intrinsic activation or repression functions of transcription factors, may not faithfully reproduce the chromatin context characteristic of endogenous target genes. Indeed, the finding that MBII interacts with a coactivator complex with HAT activity argues for MBII's involvement in chromatin-dependent activation (McMahon et al 2000; for a review see Grunstein 1997).

Mechanisms of Myc Transactivation The initial excitement generated by the discoveries that Myc heterodimerizes with Max, binds DNA, and activates transcription soon gave way to the disappointing realization that Myc's ability to transactivate both engineered reporters and putative endogenous target genes was relatively weak (generally ranging from 3- to 10-fold transactivation) when assayed in mammalian cells. In general, transactivation domains function by facilitating recruitment of the basal transcription machinery either directly or indirectly. In nearly all cases, TAD function involves interactions with other proteins. Although the N-terminal Myc TAD has been shown to associate with a number of proteins (reviewed by Sakamuro & Prendergast 1999), few provide obvious clues to the mechanism of Myc-induced activation. A striking exception has been the recent identification of a novel nuclear cofactor called TRRAP as a Myc-binding protein. TRRAP was identified by using Myc-TAD as an affinity reagent to isolate interacting proteins and was shown to require MBII for binding (Brough et al 1995, McMahon et al 1998). The TRRAP sequence is homologous to the ATM/PI-3 kinase family, although amino acid changes in the active-site region suggest that TRRAP is unlikely to possess kinase activity (McMahon et al 1998). Concomitantly, studies of the yeast *Saccharomyces cerevisiae* identified a protein highly related to TRRAP as a component of the SAGA complex, a molecular assembly

containing the HAT GCN5 and other components which facilitate TBP positioning (Grant et al 1997, Saleh et al 1998).

Recently, a mammalian counterpart of SAGA has been identified (Martinez et al 1998). While it is still unclear whether this entire complex is associated with the Myc TAD, a recent report indicates that HAT activity coimmunoprecipitates with Myc protein both in vitro and in vivo (McMahon et al 2000). The recruitment of the TRRAP-GCN5 complex places Myc, and specifically the MBII region of Myc, among the group of transcriptional activators. When considered with earlier work indicating that Mad-Max and Mnt-Max dimers repress transcription through recruitment of a corepressor complex containing histone deacetylase (HDAC) activity (see below; for a review see Schreiber-Agus & DePinho 1998), these findings produce a satisfyingly symmetrical view of Myc and Mad functions and immediately suggest that Myc and Mad antagonism stems from the opposing enzymatic activities directed toward histone modification. Although many of the Mad proteins are induced during terminal differentiation, when Myc is downregulated, there is also evidence of Mad family and Mnt expression in proliferating and quiescent cells (Pulverer et al 2000). One possible explanation for this is that the Myc-Max complex exhibits weak transactivation activity because its major role is not to fully activate targets but rather to augment the accessibility of regions of chromatin that have closed through the action of Mad- or Mnt-associated deacetylases. The region of chromatin opened through Myc would permit subsequent binding and activation by constitutive transcription factors (such as USF). This view of Myc-Max as a derepressor has received some support from recent work on the cyclin D2 promoter. Induction of a conditional Myc can activate expression of the endogenous D2 gene, although Myc is inactive in transient assays with the D2 promoter. When Mad-Max is used to repress the promoter, then Myc can induce its expression (Bouchard et al 1999). Interestingly, induction of cyclin D2 mRNA can also be achieved by treatment of cells with a HDAC inhibitor. This suggests that Myc may function most efficiently in a context of repression.

Myc has also been linked to an SWI/SNF-like ATP-dependent chromatin remodeling complex. A recent report showed that Myc can interact in vivo with the SNF5 homolog INI1 through Myc's bHLHZ domain and that putative dominant interfering forms of INI1 and BRG1/hSNF2 can block Myc transactivation (Cheng et al 1999).

Because INI1 is a strong candidate tumor suppressor (Versteege et al 1998), its potential role as a positive mediator of Myc function is somewhat puzzling. A major unanswered question is whether INI1 acts to displace Max from the HLHZ region or forms a ternary complex with Myc and Max. Perhaps binding of the INI1 complex to Myc alters Myc's target specificity or transcriptional activity. In any event, it is intriguing to imagine that Myc recruits both histone-modifying and chromatin-remodeling activities.

A number of other interesting proteins have been identified as being capable of associating with Myc and possibly influencing activation (or repression). For most of these proteins, functional information is limited, and it still remains to be

determined whether interactions occur when the partners are expressed at physiological levels. (For a recent review of Myc-interacting proteins see Sakamuro & Prendergast 1999.)

Transcriptional Repression For a number of years it has been observed that high levels of Myc expression in transformed cell lines correlate with down-regulation of specific mRNAs. These mRNAs include those encoding cell surface proteins such as the class I HLA molecules in melanoma cells, the $\alpha_3\,\beta_1$ integrin in neuroblastomas, and the LFA-1 ($\alpha_L\,\beta_2$ integrin) cell adhesion protein in transformed B cells as well as H-ferritin (Versteeg et al 1988, Inghirami et al 1990, Barr et al 1998, Wu et al 1999b). More recently a DNA element required for Myc-mediated repression has been demonstrated to lie within the promoters of repressed target genes, indicating that Myc repression is likely mediated at the transcriptional level (Li et al 1994, Amundson et al 1998). These studies have raised two major questions: first, what is the mechanism of Myc repression and, second, what roles do repression and activation play in Myc's biological functions? Do repression and activation cooperate to facilitate Myc function, and are they antagonistic or separable independent activities? The question of mechanism again leads to the highly conserved MBII region. MBII is required for repression of the growth arrest gene *gas1* and for the down-regulation of the C/EBPα and the albumin promoter in transient assays (Li et al 1994, Lee et al 1997). However, the region of Myc-spanning residues 96–106 appears to be required for down-regulation of the cyclin D1 mRNA (Philipp et al 1994) (Figure 3). Taken at face value, these studies indicate that there are at least two regions involved in repression and that these regions are contained within the transcriptional activation domain.

Evidence suggesting that Myc repression may require both recognition of a specific DNA sequence and interaction with specific proteins has accumulated. A number of Myc-repressed targets contain a subclass of initiator elements (INRs; consensus, YYCAYYYYY, where Y is a pyrimidine base) which are usually, but not invariably, present at TATA-less promoters. Other Myc-repressed genes, such as *gadd45*, do not contain INR sequences; rather, repression appears to be mediated by a GC-rich region that is potentially recognized by WT1 and p53 (Amundson et al 1998). INR elements are recognized by TFIID (Verrijzer et al 1995) as well as a number of regulatory proteins, such as the transcription initiation factor TFII-I, YY-1, and the POZ domain protein MIZ-1. Interestingly, the last three proteins have been reported to associate with the bHLHZ region of Myc (Roy et al 1993, Shrivastava et al 1993, Peukert et al 1997). While there has been little follow-up on the initial reports involving Myc interaction with TFII-I, the association of MIZ-1 with Myc has been recently confirmed and shown to promote stabilization of Myc by inhibiting its ubiquitin-dependent degradation (Salghetti et al 1999). Perhaps a stable Myc-MIZ1 interaction blocks the ability of MIZ-1 to initiate transcription at INR-containing and other promoters (Peukert et al 1997). Similarly, high Myc levels in the cell are thought to sequester YY-1 and prevent transcription of one of its target genes (Zhao et al 1998), although another report suggests that the effects

of YY-1 on Myc are indirect (Austen et al 1998). There is also evidence that a naturally occurring Max protein variant (dMax) lacking a basic region and helix 1 can interact with c-Myc to block its transcriptional activation function but promote its repression activity (FitzGerald et al 1999).

Perhaps the common theme underlying Myc repression is a loss of Myc E-box binding function, for example by displacement of Max by other bHLHZ binding proteins, permitting Myc to associate with and sequester positively acting transcription factors (e.g. Miz-1). Here the role of MBII in recruiting TRRAP-HAT might involve not acetylation of chromatin at E-box sites but rather an inhibitory acetylation of the sequestered transcription factors. Indeed, there are several reports of acetylation altering protein specificity and activity (Gu & Roeder 1997, Kehle et al 1998).

What is the relative importance of Myc's activation and repression functions? Unfortunately, an unequivocal answer to this question is not available. Recent data from DNA microarray experiments attempting to determine the effects of Myc overexpression on global gene expression profiles have demonstrated that multiple genes are both activated and repressed by Myc, with the majority being activated (O'Hagan et al 2000, Coller et al 2000). However, the relative importance of activated and repressed genes cannot be accurately assessed at this point. Nonetheless, it seems likely that both activation and repression are required for Myc biological function. Only when we learn more about the molecular details of Myc's transcriptional functions will we be able to generate mutations permitting us to distinguish the consequences of activation vs repression.

Transcriptional Targets of Myc-Max

The realization that Myc is a transcription factor suggested that its biological effects could best be understood in terms of the genes whose activities are modulated by Myc-Max heterodimers. This has led to an effort by many laboratories to identify genes that are regulated by Myc and to link these genes to specific cellular functions.

Myc Target Genes: a Link to Biological Function The identification of Myc target genes has proven to be a more difficult task than initially anticipated, for several reasons. First, the hexameric Myc-Max binding sequences, as well as the INR sequence thought to target Myc repression, occur far too frequently in the genome to permit definitive identification of Myc-regulated genes on the basis of their presence alone. Moreover, other bHLHZ proteins are known to interact with these E-box sites. Second, identification of Myc-regulated genes has been bedeviled by the comparatively weak transactivation and repression activities of Myc, which frequently make it difficult to effectively separate signal from background. Third, because Myc expression alone is frequently capable of affecting cellular behavior, it is often difficult to distinguish between genes that are direct targets of Myc and those whose expression is modulated as a result of secondary events following cell cycle entry, apoptosis, or transformation. Fourth, because

Myc may cooperate with other mitogen-induced factors to regulate target genes, the expression of Myc alone may not provide an accurate assessment of targets.

In spite of these problems, work over the last several years has led to the tentative identification of an already large, and still growing, number of target genes. These genes have been defined by a number of approaches, including inference, differential expression screens, and immunoprecipitation of Myc-Max-associated chromatin (for reviews see Grandori & Eisenman 1997, Dang 1999, Boyd & Farnham 1999b). Most recently, analysis of the expression profiles of a large number of genes by using cDNA or oligonucleotide microarrays has been employed to delineate Myc targets (O'Hagan et al 2000, Coller et al 2000). Many of these approaches have used the Myc-ER system, which allows inducible activation of Myc function in the absence of protein synthesis as a means of distinguishing between primary and secondary consequences of Myc (Eilers et al 1989, Littlewood et al 1995). Another important method for identification of Myc-Max target genes has come from the recent optimization of chromatin cross-linking and immunoprecipitation procedures (Boyd et al 1998). This approach has not only characterized *CAD* (encoding the enzyme carbamoyl-phosphate synthetase/aspartate carbamoyl/tranfora/dihydrooratase) as a gene bound and activated by Myc-Max but also demonstrated the transient occupancy of the E-box binding site by Myc and USF (Boyd & Farnham 1999a). It seems likely that this procedure will provide the most definitive assessment of in vivo occupancy at specific Myc-Max target genes in the future.

As a result of these multiple approaches, the list of genes whose expression is modulated by Myc has been rapidly growing. However, a major issue concerns the verification of reported targets. For the reasons mentioned above it has been difficult to obtain compelling evidence for all of the targets, and a number have been disputed in the literature (Mol et al 1995, Bush et al 1998). This problem will become exacerbated as additional targets are identified en masse with DNA microarrays. Nonetheless, the larger challenge is now to discern patterns of gene expression that will better define Myc function and provide molecular clues to its known activities. In the following sections we attempt to reconcile Myc biological functions with a number of its putative targets (Figure 4). [For a more complete compilation of Myc target genes see Dang (1999)].

Cell Cycle Expression of exogenous Myc in cultured fibroblasts promotes S-phase entry and shortens G_1, while activation of a conditional Myc is sufficient to drive quiescent cells into the cell cycle (Eilers et al 1989, Karn et al 1989). These studies, together with the observation that Myc activation induced a rapid increase in cyclin- dependent kinase (Cdk) activity and Rb phosphorylation, prompted the search for potential target genes among cell cycle-regulatory components. The first proposed direct Myc cell cycle target was the *cdc25A* gene, encoding a phosphatase thought to control the phosphorylation and activation state of Cdk2 (Galaktionov et al 1996). However, it has been argued that activation of the *cdc25A* gene by Myc is indirect in some cell lines, and, furthermore, Cdc25A is apparently not limiting

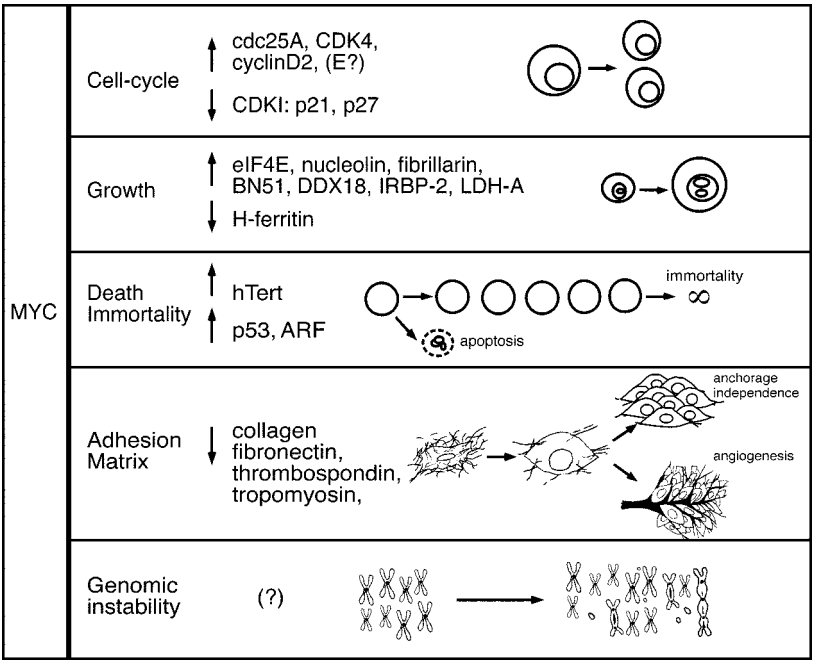

Figure 4 Myc biological functions and related target genes. Myc functions and examples of target genes believed to be involved in these pathways are indicated. Target gene expression is either increased (upward arrow) or decreased (downward arrow). For a more comprehensive list of Myc target genes see Dang 1999.

for Myc activation of cyclin/Cdk complexes (Steiner et al 1995, Perez-Roger et al 1997). Recently, several independent studies have indicated that the genes encoding cyclin D2 and Cdk4 are directly activated by Myc and that expression of the Cdk inhibitor $p21^{CIP1}$ is repressed (Bouchard et al 1999, Perez-Roger et al 1999, Hermeking et al 2000, Coller et al 2000). A major result of the induction by Myc of cyclin D2 is its sequestration of the $p27^{KIP1}$ Cdk inhibitor, permitting unfettered activity of the cyclin E/Cdk2 complex. Together with the repression of $p21^{CIP1}$ the net effect of Myc overexpression would be the prolonged activation of cyclin E/Cdk2 irrespective of the cell cycle phase (Pusch 1997, Coller et al 2000). Induction of Cdk4 by Myc is also likely to increase the independence of the cell cycle from mitogenic signals. Cdk4 has been shown to partly rescue *myc*-null Rat1a cells (Hermeking et al 2000). However, these cells were previously reported to have normal levels of Cdk4 protein (Mateyak et al 1999).

How do these Myc-induced changes affect cell cycle entry and progression? Activation of conditional Myc has been shown to be quickly followed by induction of cyclin E (Steiner et al 1995, Leone et al 1997, Perez-Roger et al 1997). Increased cyclinE/Cdk2 activity has been shown to shorten G_1, while increased Cdk4 and

Cdk2 activities would be expected to result in Rb hyperphosphorylation. Release of E2F from Rb and the direct induction of *E2F2* by Myc may further contribute to cell cycle progression (Sears et al 1997). It is of interest that a number of E2F target genes contain closely spaced E2F binding sites and E-box sequences. Examples include the genes encoding cyclin E and DNA polymerase α and the *ORC1* gene, among others (C Grandori, unpublished observations). While synergy between E2F and Myc at the transcriptional level has not been reported, functional cooperation between Myc and E2F might be expected to accelerate S-phase entry.

Although the group of cell cycle-related Myc targets could in principle explain the proliferative response to Myc, it is unlikely that they are sufficient to account for the broad effects of Myc on cellular functions.

Cell Growth An important aspect of cell proliferation for organisms ranging from yeasts to mammals is the capacity of the cell to increase in size and to coordinate this growth with division (for a review see Neufeld & Edgar 1998). The notion that Myc influences cell growth first arose from an observed correlation between Myc and the expression of the rate-limiting translation initiation factors eIF4E and eIF2α (Rosenwald et al 1993). Subsequently it was demonstrated that the eIF4E promoter harbors functional Myc-Max binding sites (Jones et al 1996). Indeed, cells lacking c-*myc* exhibit markedly decreased rates of protein and ribosomal RNA synthesis, resulting in reduced growth. Because these cells also divide more slowly, their size does not differ from that of wild-type parental cells (Mateyak et al 1997).

A recent analysis using *dmyc*, the *Drosophila* ortholog of vertebrate *myc* (Gallant et al 1996, Schreiber-Agus et al 1997), has demonstrated its direct role in cell growth (Johnston et al 1999). A series of mutations resulting in diminished *dmyc* expression resulted in smaller but developmentally normal flies. In the wing imaginal disk, *dmyc*-mutant cells were smaller but their cell cycle distribution was unaffected. By contrast, *dmyc* overexpression resulted in larger cells without a significant change in the division rate, although the G_1 fraction was decreased and the G_2 fraction was increased. Interestingly, overexpression of *Drosophila* cyclin E causes similar changes in cell cycle distribution but generates smaller cells, as does cyclin E in mammalian cells and dE2F in *Drosophila* cells (Ohtsubo & Roberts 1993, Neufeld et al 1998, Johnston et al 1999). The ability of *dmyc* to influence cell size independently of changes in cell division suggests that it may directly regulate components of the cell growth machinery.

Overexpression of mammalian Myc also produces larger cells. This has been demonstrated in Eμ-*myc* mouse-derived B cells at different developmental stages, as well as in a B-cell line in which induction of c-*myc* results in cell growth in the absence of cell cycle progression (Iritani & Eisenman 1999, Schuhmacher et al 1999). Given these findings, it is hardly surprising that several genes reported to be direct targets of Myc appear to be involved in ribosome biogenesis and protein synthesis. These include, in addition to eIF4E and eIF2α, the genes encoding the nucleolar proteins nucleolin, BN51, fibrillarin, and RNA helicase DDX18 as

well as ribosomal proteins (Grandori et al 1996, Greasley et al 2000, Coller et al 2000). Interestingly, *Pitchoune*, a *Drosophila* homolog of the DDX18 helicase gene, has also been reported to be a *dmyc* target gene, and loss-of-function mutations produce a severe growth arrest at the first larval instar stage (Zaffran et al 1998). Much remains to be learned about the function of *DDX18* and whether it, or any of these putative targets, encodes proteins rate limiting for growth (for a review see Schmidt 1999).

Because of Myc's growth-stimulatory effects, there is also a strong possibility that this protein will satisfy the requirement of growing cells for metabolites by increasing the levels of metabolic enzymes. Myc directly regulates the gene for lactate dehydrogenase A, one of the glycolytic enzymes whose levels are frequently increased in human cancers (Shim et al 1997). Up-regulation of the lactate dehydrogenase A gene has been demonstrated to be necessary for anchorage-independent growth and Myc-dependent apoptosis in response to glucose deprivation (Shim et al 1998). The metabolic pathway involved in maintaining intracellular iron concentrations is also influenced by Myc, which down-regulates the H-ferritin gene via an INR element in its promoter while stimulating expression of iron-binding protein 2 (Wu et al 1999b). These functions are essential for Myc-induced transformation in tissue culture.

In summary, several lines of evidence demonstrate that at least some aspects of Myc function are likely to be mediated through regulation of genes involved in cell growth and metabolism. In the yeast *S. cerevisiae*, growth has been shown to be regulated by genes upstream of those controlling cell division. However, in vertebrate cells, coordination between these processes is not well understood (Neufeld & Edgar 1998, Schmidt 1999). In *Drosophila* imaginal disks, transgenic mice, and some mammalian culture systems, Myc stimulates growth without having major effects on division. However, in most cultured cells, Myc appears to stimulate both growth and division. Perhaps, dependent on context, Myc coordinates these processes.

Death, Immortality, and Genetic Instability Although cell cycle and growth regulation are likely to be important in both the normal and oncogenic functions of Myc, it is clear that Myc also exerts profound effects on the cell that probably cannot be explained solely by changes in growth and cycling. These effects include apoptosis, immortalization, and genetic instability.

We have already discussed the key role that abrogation of Myc-induced apoptosis has been demonstrated to play in tumor formation. Apoptosis has been shown to require Myc-Max interaction, and there is evidence that phosphorylation of the Myc TAD influences the anti-apoptotic effect of at least one survival factor (Amati et al 1993a, Chang et al 2000). However, to date there is scant evidence concerning the Myc target genes involved. One important pathway appears to involve the release of cytochrome *c* from mitochondria (Juin et al 1999), which is independent of both p53 and CD95 (Fas) signaling pathways previously implicated in Myc-induced cell death (Hermeking et al 1994, Wagner et al 1994, Hueber et al 1997).

How Myc causes cytochrome *c* release remains a mystery. Thus far several genes encoding mitochondrial proteins, including a cyclophilin and a heat shock protein, have been found to be up-regulated by Myc (Coller et al 2000), but their functions in the context of apoptosis have not been explored. Other Myc targets such as ODC and lactate dehydrogenase A (LDH-A) have also been implicated in Myc-mediated apoptosis (Packham & Cleveland 1994, Shim et al 1997), but no direct link to mitochondrial function has been demonstrated under apoptotic conditions. Interestingly, a recent study has shown that growth factor withdrawal correlates with a loss of outer mitochondrial membrane permeability, which ultimately leads to cytochrome *c* release and apoptosis (Vander Heiden et al 2000). This finding raises the possibility that Myc's effects on cell growth and metabolism (perhaps through target genes encoding ODC and LDH-A, among others) may provide a link to mitochondrial function and cell death.

Myc has long been thought to be involved in cell immortalization (Simm et al 1994, Rao & Anderson 1997, Zindy et al 1998). In fact, somewhat paradoxically, apoptosis and immortality may be connected if one considers that mouse fibroblasts overexpressing Myc can achieve immortality following rescue from apoptosis through loss of p53 or Arf (Zindy et al 1998, Eischen et al 1999). Whereas overexpression of Myc appears to favor establishment of immortal cells from murine cell types other than fibroblasts, the much lower efficiency of immortalization of human cells indicates that multiple steps are likely to be required (Sedivy et al 1998, Hahn et al 1999). Among these is the up-regulation of telomerase activity, an event necessary to prevent the successive shortening of telomeres with each cell division, thought to be a major trigger of cellular senescence. Telomerase activity in human cells correlates tightly with cell immortalization and transformation (Holt & Shay 1999). It has now been reported that the *hTert* gene, which encodes the rate-limiting enzyme in the telomerase complex, is a target of Myc. This was shown in human mammary epithelial and B cells (Wang et al 1998, Wu et al 1999a) but not in other cells, e.g. up-regulation of *hTert* could not be detected upon activation of conditional Myc-ER in human primary fibroblasts (C Grandori, unpublished observations). Overexpression of *hTert* efficiently immortalizes human fibroblasts, but other cell types, such as keratinocytes, require inactivation of tumor suppressor genes in order to achieve immortality (Kiyono et al 1998). Thus although Myc overexpression can extend the life span of human mammary epithelial cells through activation of *hTert*, it is unclear whether these cells are truly immortal. It will be important to understand just how far Myc will push cells along the pathway to immortalization and the role that hTert plays in this effect.

Another characteristic of many cancer cells is their accumulation of genetic abnormalities, which include chromosomal alterations, gene amplifications, and nucleotide changes, a phenomenon that is referred to as genomic instability (Lengauer et al 1998). While a propensity toward genomic instability has short-term detrimental effects on cell proliferation and, paradoxically, can limit tumorigenesis in mouse models, it can also favor tumor evolution, depending on the genetic background (Chin et al 1999, Greenberg et al 1999). In humans, a number of

genetic disorders that predispose individuals to cancer are caused by alterations of genes involved in DNA repair or in the maintenance of genome integrity. More commonly, however, tumors exhibit gross chromosomal changes in the form of aneuploidy, translocations, and/or gene amplifications (Lengauer et al 1998). The possibility that Myc plays a role in genomic instability stems from the observation that overexpression of Myc causes the rapid development of chromosomal abnormalities, including increased ploidy as well as gene amplification (Mai et al 1996, Felsher & Bishop 1999b, Kuschak et al 1999, Mushinski et al 1999). Furthermore, when Myc is overexpressed in the presence of a mitotic block, apoptosis occurs in primary cells while DNA endoreplication is observed in established cell lines (Li & Dang 1999). How can the observed genomic instability in Myc-overexpressing cells be explained? One possibility is that Myc is involved in driving DNA replication directly (as proposed much earlier but not confirmed; Iguchi-Ariga et al 1987, Gutierrez et al 1988). Alternatively, Myc may trigger instability by decreasing the length of G_1 (through its effects on growth and/or cell cycle targets), thereby permitting premature S-phase entry. Accelerated initiation of S phase has been shown to result in genomic instability in yeast and mammalian cells due to replication of unrepaired intrinsic or extrinsic DNA damage, replication in the presence of insufficient nucleotide pools, or failure to activate enough replication origins to complete DNA synthesis prior to mitosis (for a review see Paulovich et al 1997). In this model, Myc-induced genetic instability would be a consequence of its effects on growth and G_1 progression.

Cell Adhesion and Angiogenesis Among the genes that are repressed by Myc, several encode proteins involved in cell adhesion, cytoskeletal structure, and the extracellular matrix (Inghirami et al 1990, Tikhonenko et al 1996, Barr et al 1998, O'Hagan et al 2000, Coller et al 2000). So far, with the exception of the role of thrombospondin, the mechanism underlying the decrease in steady-state mRNA detected for these genes has not been explored in detail. Thrombospondin is a secreted protein with potent antiangiogenic effects. Medium from Myc-overexpressing cells contains decreased thrombospondin levels and promotes neovascularization, a process necessary for tumor cell survival in vivo (Ngo et al 2000). Promotion of angiogenesis by sustained expression of Myc has also been shown in a transgenic-mouse model and in chicken bursal cells (Pelengaris et al 1999, Brandvold et al 2000). In this instance, an increased production of the secreted vascular endothelial growth factor has been shown to correlate with Myc-induced angiogenesis. However, in established tumor cell lines, down-regulation of vascular endothelial growth factor as well as decreased tumorigenicity has been observed as a response to increased *myc* expression levels (Barr et al 2000). While much remains to be learned about the relationship of *myc* to angiogenesis, it is nonetheless clear that *myc* influences the ability of tumors to induce an angiogenic response.

While Myc alone cannot transform primary cells, activation of a conditional Myc-ER in primary fibroblasts rapidly leads to the down-regulation of mRNAs for

cytoskeletal and extracellular matrix proteins (Coller et al 2000), cellular changes long known to be associated with transformation. Perhaps the marked decrease in extracellular-matrix components is also due to the ability of Myc to induce expression of specific metalloproteinases (for a review see Giambernardi et al 1998). Overall these observations indicate that Myc may exert a profound effect on cellular structure and architecture which, in vivo, may favor invasion, angiogenesis, and unrestricted growth.

Perspectives The identification of Myc target genes, together with the progress made in understanding Myc's biological effects, is allowing recognition of functional pathways (Figure 4). The pace of discovery is likely to accelerate with the increasing use of DNA microarray technology. However, given the relatively small transcriptional effects and possible cell type dependence of most target genes, it will clearly be necessary to process large amounts of information and compare results from different studies to provide compelling evidence of Myc function through regulation of specific genes. Doubt over individual targets is likely to persist (see Cole & McMahon 1999), and therefore chromatin precipitation assays might best be employed to verify the most promising candidates. Furthermore, genetic studies in both mice and *Drosophila* may permit a test of pathways as well as identification of new targets and functions. Defining the molecular pathways through which Myc functions will be important in understanding not only the function of an oncogene involved in many human cancers but also the molecular mechanisms controlling normal cellular behavior.

THE MAD PROTEIN FAMILY

Biological Functions of Mad

The Mad1, Mxi1, Mad3, and Mad4 proteins (here called the Mad family) were all identified in expression cloning screens through their ability to bind Max specifically (Ayer et al 1993, Zervos et al 1993, Hurlin et al 1995b). Each of the *mad* genes has a distinct chromosomal location (Edelhoff et al 1994, Hurlin et al 1995b), and their protein coding sequences are related to each other both within and outside their conserved bHLHZ domains (the overall similarity among family members ranges from 56–72%) (Hurlin et al 1995b). In their biochemical behavior, the Mad proteins are similar to the Myc family members in that they homodimerize poorly but form specific heterodimers with Max which can be detected in vitro and in vivo. Furthermore, Mad-Max dimers recognize the same CACGTG E-box sequence as do Myc-Max dimers (Ayer et al 1993, Zervos et al 1993, Hurlin et al 1995b), although more recently a distinct specificity for flanking sequences has been posited (O'Hagan et al 2000; see below). However, transcription assays show that Mad, in sharp contrast to Myc, acts to repress E-box-dependent expression of synthetic reporter genes (Ayer et al 1993, Hurlin et al 1995b, Schreiber-Agus et al

1995). Experiments employing a dominant interfering form of Max indicate that Max is required for both repression by Mad and activation by Myc, thus underscoring Max's pivotal role in the transcriptional functions of both Mad and Myc family proteins (Figure 1) (Ayer et al 1993). These findings suggest that Myc-Max and Mad-Max might have overlapping target genes and potentially antagonistic functions, at least when considering Mad as a repressor and Myc solely as an activator.

Mad Expression Studies of *mad* family gene expression demonstrate a clear correspondence between terminal differentiation and increased *mad* mRNA levels in a wide range of cell types, including chondrocytes, colonic epithelia, epidermal keratinocytes, adipocytes, and motor neurons, as well as erythroid and myeloid hematopoietic cells (Ayer & Eisenman 1993; Zervos et al 1993; Larsson et al 1994; Gandarillas & Watt 1995; Hurlin et al 1995a,b; Pulverer et al 2000; Vastrik et al 1995; Lymboussaki et al 1996; Cultraro et al 1997; Foley et al 1998; Quéva et al 1998). In situ hybridization analyses of embryonic tissues during mouse development indicate distinct yet overlapping expression of *mad* family RNAs in skin, bone, colon, neural tube, brain, retina, and thymus (Hurlin et al 1995a,b; Quéva et al 1998). These tissues display stratification between dividing and differentiating cells and confirm previous reports that c-*myc* and N-*myc* expression is restricted primarily to the proliferating precursor cell layer (see *myc* section above and Downs et al 1989, Schmid et al 1989, Hirning et al 1991). Both *mxi1* and *mad4* transcripts can also be detected at low levels in the precursor layer but at increased levels in differentiating cells. Expression from the *mad1* gene appears to be restricted to cells late in the differentiation process. Perhaps most intriguing is *mad3* expression, which is confined to the S phase of proliferating cells (Hurlin et al 1995b, Quéva et al 1998, Pulverer et al 2000).

The correlation between *mad* gene expression and differentiation has been confirmed by biochemical studies using cells that can be induced to undergo terminal differentiation in vitro. Both *mad1* and *mxi1* transcripts are detected very rapidly in hematopoietic cell lines after treatment with chemical inducers of differentiation (Ayer & Eisenman 1993, Zervos et al 1993, Larsson et al 1994). The induction of *mad1* RNA corresponds to the appearance of the Mad1 protein, which was shown, as was Myc, to be a nuclear phosphoprotein with a relatively short half-life. Importantly, the increase in Mad1 protein levels is accompanied by a switch from Myc-Max complexes to Mad-Max complexes following induction of differentiation (Ayer & Eisenman 1993, Hurlin et al 1995a). Further evidence that the switch from *myc* to *mad* expression is strongly linked to differentiation was provided by experiments examining mouse tissues and human keratinocytes expressing human papillomavirus E6 and E7 transforming genes. In these cells, where differentiation is compromised, *myc* down-regulation is delayed and *mad* induction is reduced or abrogated (Hurlin et al 1995b, Lymboussaki et al 1996).

There is also evidence that some *mad* family genes are expressed during proliferation and in nondifferentiated quiescent cells (Zervos et al 1993, Hurlin et al

1995b, Quéva et al 1998, Pulverer et al 2000). One possibility is that these proteins are differentially regulated to antagonize or modulate the effects of Myc during distinct periods of cell proliferation and differentiation. The Mnt repressor is also thought to act in this manner (Hurlin et al 1997). In addition, these proteins may have target genes distinct from those regulated by Myc (see below).

Mad Blocks Proliferation and Inhibits Transformation As a means of understanding the biological role of Mad family members, a number of laboratories have examined the effects of ectopically overexpressed Mad proteins in tissue culture and in mice. Nearly all these studies have found that *mad1* gene overexpression interferes with the proliferation of nontransformed cells and blocks cooperative transformation by Myc and Ras (Lahoz et al 1994, Cerni et al 1995, Chen et al 1995, Koskinen et al 1995, Vastrik et al 1995, Roussel et al 1996, McArthur & Eisenman 1997, Quéva et al 1999). Inhibition of Myc/Ras transformation of primary cells was also observed upon independent overexpression of Mxi1, Mad3, and Mad4. Furthermore, Mxi1 was shown to block the growth of glioblastoma cell lines (Lahoz et al 1994, Cerni et al 1995, Chen et al 1995, Hurlin et al 1995a, Wechsler et al 1997). An important point is that the capacity of Mad to inhibit proliferation is tightly linked to its transcriptional function. Thus Mad1 mutants that do not bind DNA, associate with Max, or contain a functional repression domain (see below) are inactive in biological assays (Hurlin et al 1995a, Koskinen et al 1995, Schreiber-Agus et al 1995, Roussel et al 1996, Cultraro et al 1997). There is one report indicating that the DNA binding function of Mad family proteins can be negatively regulated by association with a widely expressed bZip protein, Mmip1 (Gupta et al 1998). It remains to be seen whether other functions of Mad are subject to regulation. Although the ability of Mad proteins to inhibit proliferation and transformation is more or less consistent with the idea that they antagonize Myc function, these experiments do not prove this. In fact, although controversial (see, for example, Lahoz et al 1994, Cerni et al 1995), it has been argued that the inhibition of E1a/Ras-dependent transformation by Mad occurs through a Myc-independent pathway.

Because of the link between Mad expression and differentiation, several attempts have been made to determine whether ectopic *mad* expression might be sufficient to trigger differentiation. However, in two cell types which express endogenous Mad1 during differentiation, ectopic *mad1* either failed to facilitate differentiation (keratinocytes; P Gallant & RN Eisenman, unpublished data) or inhibited differentiation (3T3L1 cells; Pulverer et al 2000). In 3T3L1 cells, ectopic Mad1 inhibited the proliferative burst required for the cells to differentiate, indicating that the precise timing of Mad expression is likely to be crucial. For murine erythroleukemia (MEL) cells, differentiation of only about 25% of the *mad1*-transfected population was observed, indicating that Mad is a weak inducer of differentiation in these cells (Cultraro et al 1997). Together these findings suggest that Mad protein function is more likely to cause cell cycle arrest than to induce a specific program of gene expression resulting in differentiation. In some highly

committed cells, such as murine erythroleukemia (MEL) cells, proliferation arrest itself may be sufficient to drive differentiation. In this sense, Mad proteins would be quite distinct from transcription factors, like MyoD, which induce cell-specific differentiation programs (Davis et al 1990).

The negative effects of Mad family members on cell proliferation in tissue culture are consistent with recent results from studies of mice in which a *mad1* transgene under the control of the β-actin promoter is ubiquitously overexpressed throughout development (Quéva et al 1999). Surviving mice showed reduced body and organ weights but no evident morphogenetic abnormalities. However, both erythroid and myeloid progenitor cells derived from the bone marrow of the *mad1*-transgenic mice were less numerous and showed decreased proliferative capacity. MEFs prepared from the *mad1*-transgenic mice exhibited a greater than threefold increase in doubling time as well as delayed cell cycle reentry after G_0 arrest compared to their wild-type littermates. These experiments, together with data showing up-regulation of the Rb family protein p130 and decreased cyclin D1/Cdk activity, suggest that overexpression of Mad1 leads to a quiescent state, due at least in part to an inability to inactivate Rb family proteins.

Targeted Deletions of mad1 and mxi1 While the Mad overexpression studies described in the preceding section argue that Mad proteins possess an intrinsic ability to suppress cell proliferation, they nonetheless rely on extraordinarily high levels of expression in cell types in which endogenous *mad* genes are normally either present in low levels or not expressed at all. Therefore, it is of some interest to compare the overexpression studies with the converse approach to analyzing gene function, that of generating targeted deletions of specific genes through homologous recombination in embryonic stem cells. To date, phenotypes for homozygous *mad1* (Foley et al 1998) and *mxi1* (Schreiber-Agus et al 1998) knockout mice have been described (for a recent review comparing these knockouts see Foley & Eisenman 1999).

Targeted deletion of both *mad1* alleles resulted in viable and fertile mice that appeared phenotypically normal and did not spontaneously develop tumors (Foley et al 1998). While the *mad1*-null mice possessed normal numbers of mature nonhematopoietic and hematopoietic cell types, it was discovered, using in vitro clonogenic assays, that bone marrow granulocytic progenitor cells at the cluster-forming cell stage were significantly delayed in their ability to exit the cell cycle prior to differentiation into mature granulocytes. In addition, the cluster-forming cells showed increased sensitivity to apoptosis-inducing conditions. This is consistent with the decreased sensitivity to apoptosis in hematopoietic cells from *mad1*-transgenic mice (Quéva et al 1999). Together these results indicate that the normal function of Mad1 is to limit proliferation of granulocyte progenitor cells at a stage prior to differentiation. A paradox arising from the results with the *mad1* knockout mouse is that its phenotype is restricted to the granulocytic lineage despite the fact that Mad1 is normally expressed during differentiation of many other cell types. It is possible that the activities of other Mad family proteins

are functionally redundant and act to compensate for the loss of Mad1 in other tissues. In this regard, it is of interest that in the *mad1* knockout mice both *mxi1* and *mad3* transcripts are strongly up-regulated in tissues in which they are not normally expressed (Foley et al 1998). The notion that *mad* family genes may be cross-regulating is reminiscent of similar negative cross-regulation thought to limit *myc* gene expression (Leder et al 1983, Penn et al 1990, Stanton et al 1993).

In contrast to the findings with *mad1* knockout mice, *mxi1*-null mice exhibit hyperplasia within multiple tissues (Schreiber-Agus et al 1998). In vitro, both MEFs and T cells from *mxi1*-null mice showed an accelerated proliferative response to mitogenic stimuli compared with control mice, and the MEFs were more sensitive to transformation by Myc and Ras. Treatment with the chemical carcinogen 9,10-dimethyl-1,2-benzanthracene resulted in significantly higher mortality of *mxi1*-null mice than of control mice due to squamous cell carcinomas. In addition, the *mxi1*-null mice, when bred in a $p16^{INK4a}$-null background, displayed accelerated lymphomagenesis and fibrosarcoma development. These findings indicate that Mxi1 plays a broad role in limiting cell proliferation and that its loss results in a significantly increased propensity toward tumor formation.

Despite the differences in overt phenotype between the *mxi1*- and *mad1*-null mice, specific cell types from both of these mice exhibit a partial deregulation of their ability to exit the cell cycle. The results are consistent with a role for Mad1 and Mxi1 in restraining cell proliferation. The increased proliferative capacity of committed progenitor cells prior to differentiation and their augmented apoptotic response are at least superficially similar to the effects observed in cells overexpressing Myc (La Rocca et al 1989, Packham & Cleveland 1995). Several of the phenotypes exhibited by the *mxi1*-null mice, such as B-cell lymphomas and degenerative changes in the kidney, could also potentially result from deregulated *myc* expression (Magrath 1990, Lanoix et al 1996). Interestingly, there is evidence suggesting that Mxi1 inhibits Myc expression (Lee & Ziff 1999). Taken together, these results raise the possibility that the phenotypes of the *mad1*- and *mxi1*-null mice are secondary to deregulation of Myc function. If so, then these findings suggest that a balance between Myc and Mad levels may be an important determinant of whether, and when, cells can leave the proliferative state. Nevertheless, it is clear that null alleles in either *mad1* or *mxi1* alone are insufficient to block terminal differentiation in many tissues. It will be of some interest, then, to generate and examine mice with deletions in multiple *mad* family genes.

Tumor Suppression by Mxi1 Because of the well-established involvement of Myc in many types of cancer, an obvious corollary of the idea that Mad proteins antagonize Myc function and suppress proliferation is that they may act to limit the generation of tumor cells. Interestingly, *mxi1* has been mapped to a chromosomal region known to be altered in several tumor types (Edelhoff et al 1994). Furthermore, deletion, mutation, and loss of heterozygosity have been reported for *mxi1* in prostate cancers (Eagle et al 1995, Prochownik et al 1998). These latter findings have not been widely confirmed or extended (see, for example, Bartsch et al 1996,

Edwards et al 1997), and one can question on theoretical grounds whether cells with highly deregulated Myc would gain much selective advantage from the loss of Mxi1. Nonetheless, the increased tumorigenic potential of mice lacking *mxi1* suggests that it can function as a tumor suppressor and that further study of this issue is warranted.

Transcriptional Repression by Mad

Mad family proteins heterodimerize with Max and bind to E-box sequences in DNA. Mad-Max dimers repress transcription of synthetic reporter genes in an E-box-dependent fashion. Importantly, this repression function is closely tied to the ability of Mad proteins to inhibit cell proliferation (for a review see Schreiber-Agus & DePinho 1998). Recently, a great deal of information has emerged on the mechanism of Mad repression.

Mad Function Correlates with Sin3 Binding Because it seemed likely that transcriptional repression would involve interaction with other proteins, Mad1 and Mxi1 were used as "bait" in yeast two-hybrid screens, and both were found to interact with orthologs of ySin3p (Ayer et al 1995, Schreiber-Agus et al 1995), which has long been known as a transcriptional corepressor in the yeast *S. cerevisiae* (Wang et al 1990, Vidal et al 1991). All Mad family proteins, as well as the Max binding protein Mnt, interact with the mammalian Sin3 proteins (Hurlin et al 1995a, 1997). Although ySin3p does not bind DNA, it is capable of repressing transcription of a reporter gene when tethered to a heterologous DNA binding domain (Wang & Stillman 1993). This led to the idea that by functioning as the DNA binding component, Mad (as a heterodimer with Max) represses transcription by recruiting mammalian orthologs of ySin3p. Two highly related (although distinct) forms of Sin3 isolated from mouse were named mSin3A and mSin3B. Alternatively spliced forms of mSin3A and mSin3B are also detected (Figure 5*B*, see color insert) (Ayer et al 1995, Schreiber-Agus et al 1995, Koipally et al 1999).

Secondary-structure prediction shows that ySin3p contains four paired amphipathic helices (PAHs) thought to mediate protein-protein interactions (Wang et al 1990). All four PAH domains are conserved in mSin3A and mSin3B, and it is PAH2 which has been definitively demonstrated to interact with Mad proteins (Ayer et al 1995, Schreiber-Agus et al 1995). The conserved region in Mad family proteins which associates with mSin3 is located near their N termini and has been designated the SID (for mSin3 interaction domain). A peptide consisting of as few as 13 amino acids (residues 8–20 in Mad1) (Figure 5*A*) is capable of binding mSin3 and of repressing transcription as a fusion protein with Gal4. Mutation of conserved residues within the SID destroys interaction with mSin3A, although it is unclear whether this loss of binding is due to disruption of amino acid contacts with Sin3 or the observed loss of helicity within the SID (Ayer et al 1996, Eilers et al 1999). The same SID mutations in the context of full-length Mad1 render the mutant incapable of repressing transcription, inhibiting

Myc/Ras cotransformation, or blocking Myc-dependent cell cycle entry (Ayer et al 1996, Roussel et al 1996). The SID domain is highly conserved in all Mad family members and the related Max binding protein Mnt (Hurlin et al 1995b, 1997).

In Mxi1 the SID lies within a region which is alternatively spliced to generate two isoforms, termed Mxi-SR and Mxi-WR for weak repressor and strong repressor, respectively, of Myc/Ras transformation. Mxi-SR encodes full-length Mxi, while Mxi-WR lacks 36 amino acids at its N terminus, which includes the SID. While the physiological relevance of the two spliced forms remains an open question, it is clear that Mxi-SR is capable of inhibiting transformation by Myc and Ras while Mxi-WR is not, establishing another link between mSin3 binding and Mxi function in vivo (Schreiber-Agus et al 1995). In fact, mSin3A is capable of substituting for the SID domain altogether since a fusion protein consisting of SID-deleted Mxi fused to full-length mSin3A is able to inhibit Myc/Ras transformation (Rao et al 1996). A final piece of evidence linking Sin3 to Mad1 function comes from yeast studies. *S. cerevisiae* strains lacking y*Sin3* are viable, although they show changes in gene expression. In these mutant cells, a Gal4-Mad fusion protein cannot repress transcription (Kasten et al 1996).

mSin3A and mSin3B are very stable, with half-lives in excess of 6 h, compared with 20 min for Mad1 (McArthur et al 1998). Both proteins also appear to be ubiquitously expressed (Rao et al 1996), making it unlikely that mSin3 abundance would be limiting for Mad function. The only reported differential regulation of mSin3A and mSin3B expression was noted in neurons undergoing apoptosis after treatment with okadaic acid (Korhonen et al 1998).

Interaction of Sin3 with Histone Deacetylases The findings summarized above demonstrate that the specific association of Mad proteins with the Sin3 corepressor is essential for the ability of Mad to repress transcription. However, it leaves open the question of exactly how Sin3 proteins mediate repression. The answer appears to be that Sin3 proteins from yeasts to mammals are involved in modification of chromatin structure through their association with HDACs.

The initial evidence that Sin3 might play a role in rearranging chromatin structure derived from yeast studies. ySin3 (originally named Rpd1) was isolated in a screen for genes that rescue expression of the HO gene in yeast lacking the HO activator *Swi5*. *Swi5* encodes a DNA binding protein, subsequently shown to be part of the Swi/SNF chromatin remodeling complex (Stillman et al 1988) (for a review see Armstrong & Emerson 1998). The absence of Sin3 abrogates the requirement for Swi5, suggesting that Sin3 might repress transcription by affecting chromatin structure (Vidal et al 1991). Another gene isolated from the same screen was *Rpd3* (Vidal & Gaber 1991). Loss of either Sin3 or Rpd3 results in the same phenotype, therefore suggesting that they function as part of the same pathway. However, because no function for Rpd3 was apparent, the genetic interaction with ySin3 added little information. All this changed when it was discovered that yeast Rpd3 and its mammalian homologs are HDACs (Taunton et al 1996, Yang et al 1996). It was quickly shown that mSin3A copurifies with active HDAC1 and

HDAC2 and that a ternary complex comprising Mad1, mSin3, and HDAC2 could be isolated (Hassig et al 1997, Laherty et al 1997). In addition, it was shown that Mad repression is sensitive to HDAC inhibitors and that HDAC activity can be immunoprecipitated from cells along with Mad1 (Alland et al 1997, Hassig et al 1997, Laherty et al 1997). This mechanism of transcriptional repression has been highly conserved through evolution, since Sin3 and Rpd3 were shown to be part of the same complex recruited by the yeast repressor Ume6 (Kadosh & Struhl 1997).

Further studies defined the highly conserved region between PAH3 and PAH4 as the histone deacetylase interaction domain (HID). A Gal4-HID fusion protein is capable of repressing transcription when bound to a heterologous promoter (Laherty et al 1997). Interestingly, a Gal4-Sin3 fusion protein lacking the HID domain is still capable of repressing transcription, as is the short form of mSin3B that lacks a HID, suggesting a second, possibly HDAC-independent, repression function (Alland et al 1997, Laherty et al 1997). Although HDAC1 and HDAC2 are involved in Sin3 transcriptional repression, mSin3A is unable to bind the recently isolated HDAC3, -4, -5, or -6 (Grozinger et al 1999). The conservation of the Sin3-HDAC complex from humans to yeasts, which lack Myc and Mad homologs, suggests that Sin3 function is an ancient mechanism that has been adopted for use by multiple individual transcriptional repressors. The association of Mad with an HDAC-associated corepressor suggests that Mad repression occurs through deactylation of histone tails within nucleosomes, leading to formation of a relatively inaccessible, and therefore repressive, chromatin structure. It is also possible that Mad-associated HDAC activity can deacetylate other transcription factors or even Mad itself. The targets of HDAC repression remain to be established.

mSin3 Forms a Multiprotein CoRepressor Complex mSin3 has a highly modular structure, as demonstrated by the ability of individual PAH domains and the HID to function independently of one another. It is therefore no surprise that mSin3 is capable of multiple protein-protein interactions in addition to those with Mad and HDAC. Many of the proteins interacting with mSin3 are, like Mad, DNA binding repressors involved in differentiation and other biological functions (Figure 5*C*) (for a review see Knoepfler & Eisenman 1999). Some of these proteins, such as that encoded by the proto-oncogene Ski, as well as the related protein Sno, may impinge on Mad function. Ski mutants (including v-Ski), which lack an mSin3 interaction domain, have a dominant-negative effect on Mad transcriptional repression, suggesting that both Ski and Mad simultaneously bind the Sin3-HDAC complex, although this has yet to be directly demonstrated (Nomura et al 1999). In addition, cells lacking *ski* overexpress ornithine decarboxylase, a Mad1 target gene, again consistent with a role for Ski in Mad function. As more and more Sin3-interacting proteins are reported, it is becoming clear that Sin3 is a central player in the function of many diverse types of transcriptional repressors (Knoepfler & Eisenman 1999). Doubtless, multiple Sin3-containing complexes occur in cells; whether these complexes are functionally heterogeneous remains to be determined.

Common Target Genes of Myc and Mad

Although Mad as a transcriptional repressor has been shown to oppose many of the biological functions of Myc, it is unclear to what extent this antagonism reflects overlapping target genes. Resolution of this question is hampered by many of the same problems inherent in defining Myc targets as well as by the gaps in our understanding of how any facet of Myc or Mad biology relates to specific target genes. Nonetheless, preliminary work is beginning to shed some light on the question of target gene overlap. Although there are differences between the basic regions of Mad and Myc (see Table 1), Myc-Max and Mad-Max heterodimers bind the consensus E-box with similar affinities in vitro (Ayer et al 1993, Sommer et al 1998). Furthermore, Mad-Max recognizes the same noncanonical E-boxes as does Myc-Max (Blackwell et al 1993; DE Ayer, unpublished data). These findings suggest a high degree of similarity between Myc and Mad in terms of DNA recognition specificity in vitro. However, more recently a Myc protein in which the basic region had been replaced by that from Mxi1 [Myc(Mxi1-BR)] was shown to transactivate with a broader E-box-flanking sequence preference than Myc. Furthermore, DNA microarray analysis of the expression patterns induced by Myc and Myc(Mxi1-BR) showed that these proteins influenced transcription of both overlapping and distinct genes (O'Hagan et al 2000). However, some caution in interpreting results from experiments involving domain replacement within a region such as the bHLHZ may be in order because there may be an influence on the basic region by the rest of the HLHZ (Davis & Weintraub 1992) (note that DNA phosphate backbone contacts are made between the loop and H2 regions in Max homodimers). Indeed, in vitro selection experiments with randomized oligonucleotides demonstrate that full-length Mad selects the same consensus E-box and flanking sequences as Myc (LP James, unpublished data), a result not predicted from the studies with Myc(Mxi1-BR).

There also remains the perplexing issue of how Mad function affects the targets of Myc repression. If Mad truly antagonizes every Myc target, then Mad should activate or block repression of target genes normally repressed by Myc. There is no evidence yet available on this issue.

In summary, while work to date suggests that Myc and Mad target both overlapping and nonoverlapping genes, much remains to be discovered. It will be particularly interesting to compare the structures of Myc-Max and Mad-Max heterodimers bound to DNA.

THE MYC/MAX/MAD NETWORK IS A TRANSCRIPTION MODULE

The research summarized in this review has resulted in the identification and detailed analysis of the individual components of the Myc/Max/Mad network (Figure 6, see color insert). One danger of this basically reductionist approach is that the multitude of components and details often obscure the larger view.

Perhaps a more integrated view of the network is to envision it as a group of proteins whose expression and functions coordinate basic cellular responses with external cues. Myc and Mad family proteins are rapidly synthesized in response to an extraordinarily wide variety of mitogenic and differentiation signals, respectively. Through their interactions with Max, DNA, coactivator/corepressor complexes, and other proteins, Myc and Mad appear to alter the chromatin structure of a set of target genes. Of the proteins in the network, only the levels of Myc and Mad are highly regulated, while Max, along with the coactivator and corepressor complexes, appears to be both abundant and stable. This suggests that the assembly of higher-order transcription complexes on the specific target genes must be almost wholly dependent on the mechanisms regulating expression of *myc* and *mad* gene products. While much remains to be learned about the nature and relative importance of the individual gene targets, initial evidence suggests that many of them are directly involved in the cell cycle, growth, life span, and morphology. The network then can be viewed as an intermediary between extrinsic or intrinsic signal pathways and their complex cellular responses.

One way to think about the Myc/Max/Mad network is as a functional module. Modules, as applied to biological systems, have been recently defined as relatively autonomous functional units consisting of diverse components (Hartwell et al 1999). The module's discrete functions arise from the interactions among these components. Signal transduction pathways, ribosomes, and splicing machinery can be considered examples of such modular units. Interacting transcription factors, such as the proteins involved in circadian rhythm (Dunlap 1999), the NF-κB system (Mercurio & Manning 1999), and the Myc/Max/Mad network discussed here, can also be considered as modular units. In general, modules are thought to incorporate certain design principles that render them both robust and flexible. These include feedback, response to simultaneous signals, and "exploration" with selection over short time scales. In addition, it would be expected that while individual modules have distinct functions, there would exist some form of communication and coordination between them. Although we do not know whether the Myc/Max/Mad network truly conforms to these principles, it clearly shows aspects of feedback (e.g. cross-regulation of Myc and Mad family members), response to multiple signals, and possibly exploration (in terms of the stochastic nature of its interaction with binding sites) (Boyd et al 1998, Boyd & Farnham 1999a). The recent descriptions of Mlx and Mga may point the way to intermodule communication. Mlx, a bHLHZ protein that specifically recognizes Mad1 and Mad 4 but does not associate with Max, may connect Mad function to another bHLHZ network (Billin et al 1999). Mga, a bHLHZ protein that interacts with Max, also contains a T-box DNA binding domain common to a large class of proteins involved in embryonic development (Hurlin et al 1999). Mga therefore may permit coordination between the Myc/Max/Mad network and the developmental programs controlled by the T-box proteins. Furthermore, the interaction of Max network proteins with coactivators and corepressors, and the association of these latter complexes with yet other transcription factors, may also constitute a form of intermodule communication.

Another aspect of modules is that their overall function may not necessarily be understood from the functions of their individual components. If this is true for the Myc/Max/Mad network, it implies that we still have a great deal more to learn.

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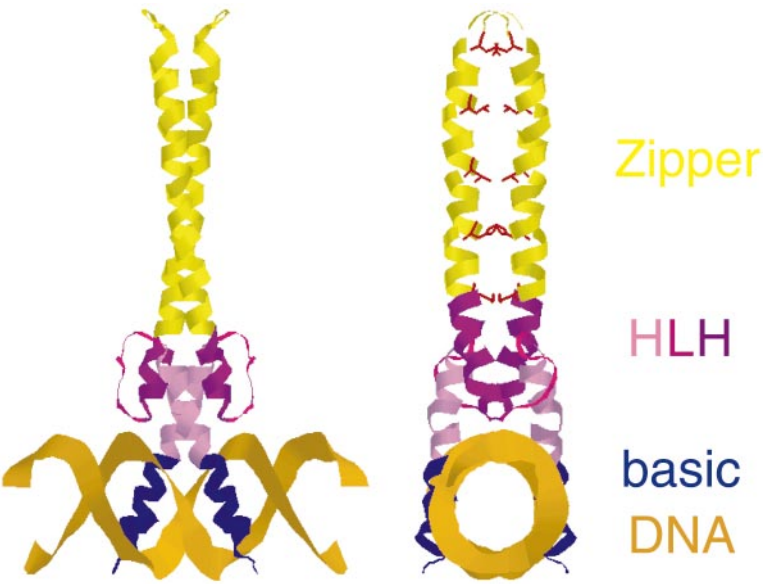

Figure 2 Structure of a Max homodimer bound to DNA. This figure is based on the published crystal structure (Ferre-D' Amare et al 1993) and reconstructed from the co-ordinates deposited in the protein database (pdb file: 1an2). Individual regions were colored as follows: basic region, *blue;* helix 1-loop-helix 2, violet-purple-magenta; leucine zipper, yellow; DNA, orange. The interactions between hydrophobic residues in the leucine zipper are shown on the right in red. Note that the basic region is continuous with helix 1 while the leucine zipper is continuous with helix 2. The basic region contacts individual base pairs directly. Additional contacts are made between the DNA phosphate backbone and the helix 2 and loop regions of Max.

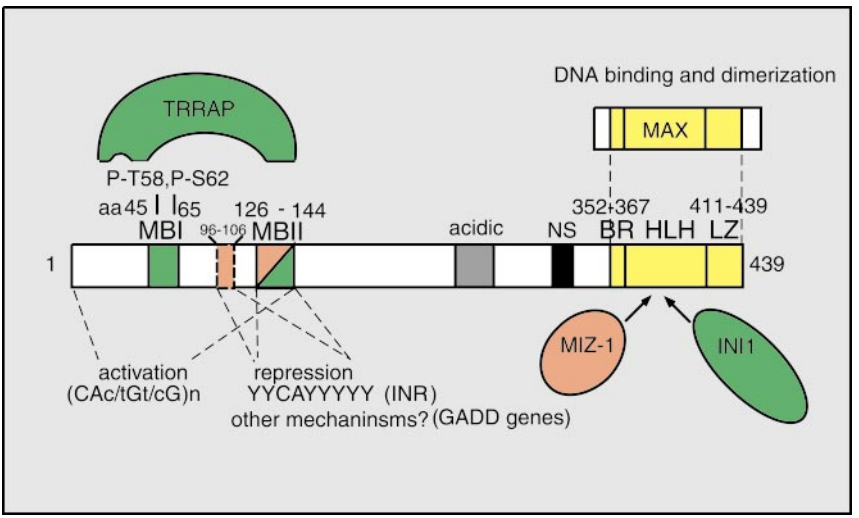

Figure 3 c-Myc organization and association with proteins important in transcriptional activity. MBI, Myc box 1; MBII, Myc box 2; NLS, nuclear localization signal; BR, basic region; HLH, helix-loop-helix; LZ, leucine zipper. The amino acid boundaries of each domain are indicated above Myc. Domains and proteins implicated in activation are shown in green, and those purportedly involved in repression are in red. The DNA element involved in transcriptional activation by Myc-Max heterodimers includes the canonical and the preferred noncanonical sites. INR is the INR thought to mediate Myc transcriptional repression. Miz-1 and INI1 are protein interacting with the Myc bHLHZ and thought to be involved in repression/turnover and chromatin remodeling, respectively. TRRAP binds the N-terminal region of Myc. See the text for details.

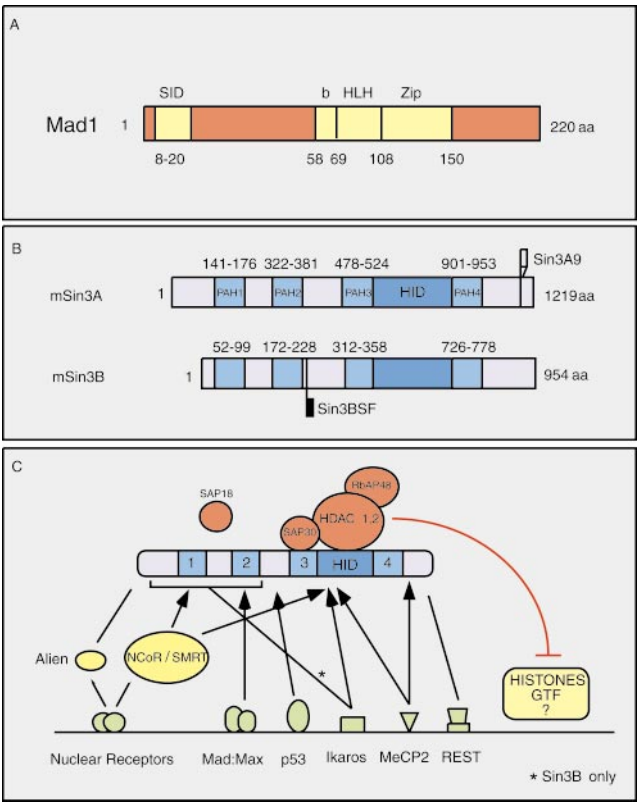

Figure 5 Mad1 organization and the components of Mad repression. A, Schematic representation of Mad1 functional domains. SID, Sin3 Interaction Domain; b, basic region; HLH, Helix-loop-Helix; Zip, Zipper domain. The amino-acid boundaries are shown below. All Mad family proteins have a similar overall organization. B, Sin3 structural domains, Paired-amphipathic helices 1-4 are shown with their respective amino acid boundaries; HID, histone deacetylase interaction domain. Also shown are alternative spliced forms of mSin3A and mSin3B. mSin3A9 contains a 9 amino-acid insertion at position 1205. The short form of mSin3B, mSin3BSF, contains a change of frame which incorporates 19 unique amino acids and an in-frame stop codon at position 274. C, Sin3 interacting proteins. Many diverse repressors inhibit transcription by recruiting the Sin3/HDAC complex. Individual repressors (in green) and corepressors (yellow) are shown, with their specific sites of interaction with Sin3. Lines without arrow heads indicate an interaction with no specific region defined. The Sin3/HDAC complex represses transcription by deacetylating histones, and possibly other proteins such as general transcription factors (GTF) or as yet undiscovered targets (?).

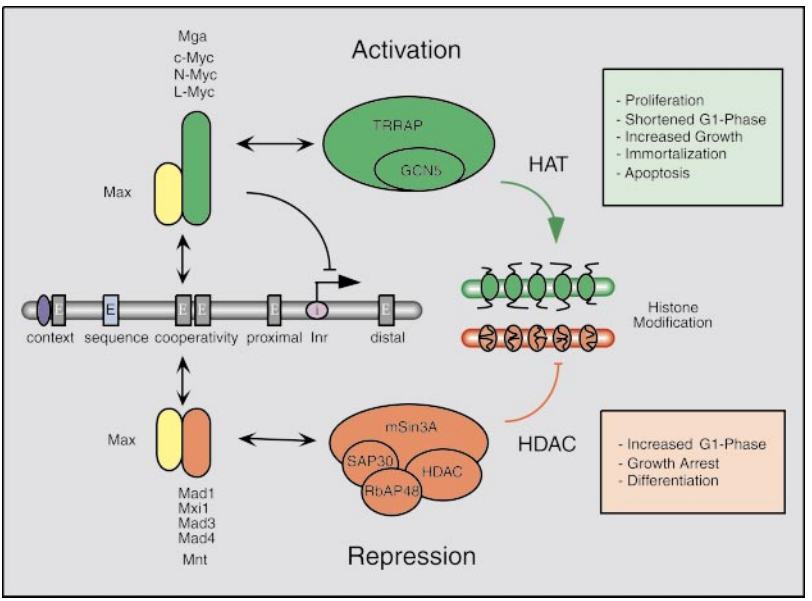

Figure 6 Model of the protein interactions and functions of the Myc/Max/Mad transcription network. Myc-Max and Mad-Max (along with Mnt-Max and Mga-Max) complexes bind to DNA to E-boxes. Binding can be affected by the context, sequence, cooperativity, and location of the E-boxes. Myc-Max heterodimers activate transcription by recruiting HAT's via TRRAP. This leads to the acetylation of histone tails and the opening of local chromatin structure. Additionally, Myc-Max appears to repress transcription through Inr elements via an undefined mechanism. As a result of these activities at target genes, Myc affects proliferation, cell cycle, growth, immortalization, and apoptosis. When deregulated, Myc cooperates with other oncogenes to cause a variety of cancers. Mad-Max and Mnt-Max heterodimers repress transcription by recruiting HDAC's via mSin3A. This leads to the deacetylation of histone tails and the closing of local chromatin structure. As a result of target gene repression, Mad causes an increased cell doubling time, growth arrest, and the maintenance of differentiation. Mga-Max also appears to activate and repress transcription, but its protein interactions have not been fully characterized.