The basic-helix-loop-helix-zipper1 (BHLHZip)1 protein Max is an essential component in a transcription factor network that functions to regulate cell growth and differentiation (1, 2). Max can form DNA binding heterodimers with at least three different families of BHLHZip proteins: the Myc family of proto-oncogenes (c-Myc, N-Myc, and L-Myc), (3–5) the Mad family members (Mad1, Mxi1, Mad3 and Mad4) (6–8), and Mnt (9). Max:Mad heterocomplexes function as transcriptional activators (10–12), whereas Mad:Max (6, 7, 13) and Mnt:Max heterocomplexes function as transcriptional repressors. All three heterodimer combinations can recognize the same E-box motif (CACGTG), suggesting that they reciprocally regulate the same target genes. Several lines of evidence suggest that Mad family proteins play an important role in counteracting the growth-promoting activity of Myc. Both Mad family members and Mnt can efficiently block Myc + Ras co-transformation of rat embryo fibroblasts (6, 9, 14–16), and cause cells to be blocked in the G1 phase of the cell cycle (17, 18). In addition, the mxi1 gene maps to a region on chromosome 10q that is frequently mutated in human cancers, and mxi1 null mice show increased susceptibility to tumorigenesis (19, 20).

The opposing activities of Myc and Mad are manifest during the transition from proliferation to differentiation (2, 21). Myc mRNA and protein expression is associated with cellular proliferation and is typically down-regulated during differentiation (22, 23). By contrast, Mad1 is expressed at low levels in proliferating cells but is induced during the differentiation of several distinct cell lineages in vitro and in vivo (14, 16, 24–27). Max protein abundance is not highly regulated, suggesting that it is continually available to complex with either Myc or Mad (28, 29). Isolation of Max heterocomplexes demonstrated a shift from Myc:Max to Mad:Max heterocomplexes during chemically induced differentiation of a myeloid leukemia cell line and primary human keratinocytes (24, 30). We have proposed that this switch in heterocomplexes is important in regulating cell cycle exit during differentiation, presumably by down-regulating Myc-dependent target genes required for cell proliferation (24). These data suggest that the switch from Myc:Max to Mad:Max heterodimers has functional consequences in vivo.

It is not yet understood what biological roles are played by individual members of the Mad family and what biochemical mechanisms provide specificity to Mad family function. In vitro studies cannot distinguish functional differences between Mad family members. For instance, each family member is able to repress transcription by targeting the mSin3A corepressor complex to DNA (31–33), heterodimerizing with Max to bind CACGTG binding sites, and blocking Myc + Ras co-transformation (1). However, analysis of mad family mRNA expression patterns in developing embryos and the phenotypes of mice with null mutations in either mad1 or mxi1 suggests that the in vivo functions of the Mad family members are not completely redundant. The expression patterns of mad family and mnt mRNAs are unique and complex; although transcripts for mnt and mad3 are detected in proliferating cells, mxi1 and mad4 transcripts are expressed early in the differentiation process, and mad1 transcripts appear later in differentiation (9, 14, 16, 27). Mice null for mad1 or mxi1 are viable but show increased proliferation in precursors cell populations of the spleen and prostate; these effects were most pronounced in granulocytic cluster-forming colonies derived from mad1-deficient mice, whereas mxi1-deficient mice displayed hyperplastic growth in
plexes were resolved on 5% nondenaturing acrylamide gels in
2°C. The binding reactions contained 0.5 mM dithiothreitol), 0.4 mg ml
1-10 ng, with 0.5 ng of32P-labeled probe for 15 min at room

In situ

65 °C.

35S-labeled antisense probes against

a virion packaging

were performed by incubating recombinant proteins, typically

like Max, is a

stable, widely expressed protein. In addition, Mlx forms hetero-
dimers with Mad1 that are capable of interacting with the
mSin3A corepressor and repressing transcription. However,

unlike Max, Mlx forms heterodimers with only select members

of the Mad family (Mad1 and Mad4). We propose that Mix
diversifies the functional capabilities of the Mad family of

transcription factors by interacting with only a subset of Mad
proteins.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen and Cloning—Yeast two-hybrid screen-
ing was performed as described previously using a VP16 library
constructed from mRNA isolated from mouse embryos at 9.5
and 10.5 days p.c. (31, 48). 80 VP16 fusion clones were chosen for
characterization. All 80 VP16 fusion clones failed to show a
positive two-hybrid readout when tested for interaction with
LexAMad1 in a wild-type Saccharomyces cerevisiae strain.
However, when a subset of these clones was tested with Lexa-
Mad1 in a sin3 strain, all yielded β-galactosidase levels similar
to those observed when they were tested for interaction with
LexAMad1 (L12P/A16P) in a wild-type background (data not
shown). Therefore, it is likely that the 80 VP16 fusions inter-
acted with wild-type Mad1 in the two-hybrid analysis, but
these interactions were masked because endogenous yeast
Sin3p repressed the lacZ reporter gene. Full-length cDNAs
encoding human and mouse Mlx were isolated from an HL60
cDNA library and an embryonic stem cell library, respectively.
Mlx cDNAs were subcloned by standard methods into the
mammalian expression vector pRCC/CMV (Invitrogen). The mu-
tant of Mlx lacking the leucine zipper (MlxΔZI) was produced by
polymerase chain reaction amplification and lacks amino
acids 133–161. MlxΔZ shows complete deficiency for dimerization
with Mad1 (data not shown). In ΔBRMIX, Glu-84 and Gln-85 of
the basic region of Mlx were mutated to glycine and proline,
respectively. These mutations abolish Mlx DNA binding (data
not shown). VP16 fusions to Mad3, Mad4, N-Myc and L-Myc,
and LexAMax were kindly provided by Dr. Peter Hurlin (Fred
Hutchinson Cancer Research Center, Seattle, WA); VP16Mad1
and LexAMix were constructed by amplifying the Mad1 or Mlx
cDNA by polymerase chain reaction and cloning the products
into pBTM116 or pVP16 (48), respectively. Multiple tissue
cDNA by polymerase chain reaction and cloning the products
into pBTM116 or pVP16 (48), respectively. Multiple tissue
Northern blots (CLONTECH) were probed with the full-length
Mlx cDNA, which had been labeled by random priming (Life
Technologies, Inc.). Blots were washed with 0.1× SSPE (18 mM
NaCl, 1 mM NaH2PO4, 0.1 mM EDTA), 0.1% SDS for 30 min at
65 °C. In situ hybridization was performed as described with
35S-labeled antisense probes against mad1, max, and mix (27).

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs
were performed by incubating recombinant proteins, typically
1–10 ng, with 0.5 ng of32P-labeled probe for 15 min at room

temperature. The binding reactions contained 0.5× HMO.1
buffer (12.5 mM HEPES, pH 7.5, 5% glycerol, 50 mM KCl, and
0.5 mM dithiothreitol), 0.4 mg ml−1 bovine serum albumin, 8
mM dithiothreitol, and 0.08% Nonidet P-40. Protein-DNA com-
plexes were resolved on 5% nondenaturing acrylamide gels in
25 mM HEPES, pH 7.5, at 4 °C.

Protein Expression and Antibody Production—Rabbit pol-
clonal antisera specific for the N terminus and C terminus of
Mlx were generated against GST fusion proteins encoding
amino acids 1–76 or 162–244 of Mlx, respectively. Test bleeds
were assayed for specific immunoreactivity by low and high
stringency immunoprecipitation of radiolabeled in vitro trans-
labeled Mlx. The purification of recombinant GST-BHLHZip Mlx
(encoding amino acids 76–162), GST-BHLHZip-Mad3, GST-
BHLHZip-Mad4, GST-C92-Myc, Mad1, and Max was based on
previously published techniques (6, 7, 49). Max protein was
isolated from SF9 insect cells infected with a Max-expressing
recombinant baculovirus (7). All other proteins were expressed
in Escherichia coli.

Cell Culture, Immunoprecipitation, and Luciferase Assays—

Immunoprecipitations were performed as described previously
(24). SDS-polyacrylamide electrophoresis gels were transferred
to polyvinylidene difluoride membranes and visualized with
ECL (Amersham Pharmacia Biotech) using the manufacturer’s
protocol. Recombinant retrovirus expressing Mlx was made by
filling in a HindIII/XbaI fragment containing the Mlx-coding
region and adding ClaI linkers. The linked fragment was cloned in
to the ClaI site of pSRα-MSV-TKCD8 (50). Virus was
produced by transfecting 293 cells with the retroviral construct
and a plasmid encoding an amphotrophic helper virus contain-
ing a virion packaging ψ2 sequence (51, 52). Supernatants
containing retrovirus were collected 36 h after transfection and

suggested that mSin3A and mSin3B

were identified (31). In

Saccharomyces cerevisiae

using Mad1 as bait, only the corepressors mSin3A and mSin3B
were identified (31). In S. cerevisiae, LexAMad1 can repress
transcription via interaction with the endogenous SIN3p co-
pressor complex (35), suggesting that mSin3A and mSin3B
were identified because they could displace the endogenous
corepressor. Furthermore, because repression by the
mSin3A-HDAC1 complex is dominant over activation by VP16
(36), other proteins may have interacted with the LexAMad1
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RESULTS

Identification of Mad1-interacting Proteins Using a Modified
Two-hybrid Strategy—In a previous yeast two-hybrid screen
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For luciferase assays, NIH3T3 cells grown in Dulbecco’s
modified Eagle’s medium supplemented with 10% bovine calf
serum (Hyclone), glutamine, and penicillin-streptomycin were
seeded onto 6-well dishes at 3 × 105 cells in 2 ml of medium/
well. Twenty-four h after seeding, cells were transfected using
Superfect (Qiagen) in triplicate. Each transfection contained
400 ng of luciferase reporter, 100 ng CMV-β-galactosidase, 1 μg
of expression construct, and carrier DNA to a total of 5 μg of
DNA. Cell lysates were prepared 24 h after transfection. Lu-
ciferase and β-galactosidase activities were assayed according
to manufacturers’ instructions (Promega, Tropix). To normalize
for transfection efficiency, luciferase values were divided by
β-galactosidase activity values. Errors reported are the S.E.
calculated from experiments performed in triplicate. The luci-
erase reporter pGL3-CM2 was constructed by inserting four
copies of the E-box-containing sequence CCCAGTCGCAGCT-
GTGTTAGG between the Sac1 and BgII sites of pGL3-pro-

To elucidate potential functional differences between the
Mad family of transcription factors, we have identified addi-
tional members of this transcriptional network that interact
with Mad1. Here we present the isolation and characterization
of Mlx, a novel Max-like BHLHZip protein. Mlx, like Max, is a
stable, widely expressed protein. In addition, Mlx forms het-

unlike Max, Mlx forms heterodimers with only select members

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bait but failed to score positively due to a simultaneous inter-

the splenic white pulp and prostatic epithelium (20, 34). To-
gether these findings indicate that the activity of Mad family
transcriptional repressors may be differentially regulated de-
pending on the cell type or stage in the cellular differentiation
program.

Unlike Max, Mlx forms heterodimers with only select members

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action between the bait and the endogenous SIN3p corepressor (Fig. 1A). To identify additional Mad1-interacting proteins, we performed a yeast two-hybrid interaction screen using a fusion between the LexA DNA binding domain and a Mad1 protein that has proline substitutions (L12P/A16P) in the SID (Sin3-interaction domain) as bait. These mutations eliminate the interaction between Mad1 and mSin3 proteins or SIN3p (31, 35, 36) but leave the remainder of Mad1 functionally intact (Fig. 1A). We reasoned that this bait might allow the detection of Mad1-interacting proteins other than mSin3A and mSin3B.

Approximately 8 × 10^6 primary transformants were obtained using a VP16 fusion library made from day 9.5 and 10.5 p.c. mouse embryo cDNA and tested for growth on plates lacking histidine in the presence of 25 mM 3-aminotriazole. Eighty positive clones were chosen for further characterization. Based on the high degree of primary amino acid sequence homology to Max and on the functional characterization presented below, we have termed the protein Max-like protein X (Mlx). We cloned full-length cDNAs encoding Mlx from both human and mouse and identified a putative Drosophila melanogaster Mlx homologue in the expressed sequence tag data base (LD05774 Berkeley Drosophila Genome Project). The translation products of human and mouse Mlx are highly conserved over their entire length, differing at only four amino acid positions in their 244-amino acid open reading frames. A search of the data base revealed that our clone is identical to a previously described mouse Mlx homologue (max), which is roughly 7 amino acids longer than the other members of the Max network. Outside of the BHLHZip domain, Mlx displays little similarity to Max, to other members of the Max network. Furthermore, these results demonstrate that under certain circumstances, the spectrum of protein interactions uncovered by the two-hybrid methodology can be strongly influenced when the bait interacts with endogenous proteins.

Identification and Expression Profile of a Max-like BHLHZip Protein—One of the 80 positive clones isolated in the two-hybrid screen encoded a novel BHLHZip protein with significant sequence similarities to the BHLHZip domain of Max and was chosen for further characterization. Based on the high degree of primary amino acid sequence homology to Max and on the functional characterization presented below, we have termed the protein Max-like protein X (Mlx). We cloned full-length cDNAs encoding Mlx from both human and mouse and identified a putative Drosophila melanogaster Mlx homologue in the expressed sequence tag data base (LD05774 Berkeley Drosophila Genome Project). The translation products of human and mouse Mlx are highly conserved over their entire length, differing at only four amino acid positions in their 244-amino acid open reading frames. A search of the data base revealed that our clone is identical to a previously described cDNA, transcription factor-like 4 (TCFL4), which was originally cloned in a screen for mRNAs differentially expressed in intestinal epithelium (37). Based on our functional characterization, we propose that the Mlx nomenclature be adopted. The predicted amino acid sequence of Mlx is conserved at all of the positions that define the BHLHZip class of transcription factors, and the consensus amino acids are shown at the bottom of the diagram. Lowercase letters indicate conservative amino acid changes. The 13 amino acids of the basic region are numbered. Amino acids His-5, Glu-9, and Arg-13, which direct binding of this class of BHLHZip proteins to the CACGTG E-box, are conserved in Mlx.
Mlx (Fig. 3A). A protein of approximately 30 kDa was immunoprecipitated from high stringency extracts of SW480 colon carcinoma cells. This protein was not detected in immunoprecipitates using preimmune serum. Furthermore, the immunoprecipitated protein is similar in apparent molecular mass to the one produced in 293 cells transfected with an expression vector encoding Mlx. Together, these data confirm that the immunoprecipitated 30-kDa protein is Mlx. A survey of other cell lines showed that HL60, P19, and PC12 cells also contained detectable Mlx protein, whereas NIH3T3 and 293 cells did not (data not shown). These data are consistent with the in situ hybridization and Northern-blotting results and suggest that Mlx protein expression is widely distributed although not ubiquitously expressed like Max.

Max is a stable protein with a half-life of at least 6 h (39). In contrast, c-Myc and Mad1 turn over very rapidly, with half-lives of approximately 20 min (24, 40). To determine the stability of Mlx, we measured its half-life in NIH3T3 cells that had been transduced with a Mlx-expressing retrovirus. Because Mlx is labeled poorly in vivo using [35S]methionine (data not shown), it was not possible to determine its half-life by performing a pulse-chase experiment. Therefore, the stability of Mlx was determined by blocking protein synthesis with cycloheximide and measuring the amount of Mlx that remained at specific time points after cycloheximide addition. Mlx protein was detected by immunoprecipitation and Western blotting following 0, 2, 4, 6, and 12 h of cycloheximide treatment and then determined by autoradiography. Therefore, the stability of Mlx, we measured its half-life in NIH3T3 cells that had been transduced with a Mlx-expressing retrovirus. Because Mlx is labeled poorly in vivo using [35S]methionine (data not shown), it was not possible to determine its half-life by performing a pulse-chase experiment. Therefore, the stability of Mlx was determined by blocking protein synthesis with cycloheximide and measuring the amount of Mlx that remained at specific time points after cycloheximide addition. Mlx protein was detected by immunoprecipitation and Western blotting following 0, 2, 4, 6, and 12 h of cycloheximide treatment and then determined by autoradiography.

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The heterocomplexes and free probe are indicated. Complexes were resolved on a nondenaturing 5% acrylamide gel. The binding site, was incubated with the indicated recombinant proteins.

To further examine functional similarities between Max and Mlx, we determined whether Mlx has intrinsic transcriptional properties and whether Mad1:Mlx heterocomplexes repress transcription in a manner similar to Mad1:Max. These experiments show that, like Max, Mlx is able to repress transcription as transcriptional repressors. These data suggest that Mlx itself lacks intrinsic transcriptional activation or repression domains but, like Max, is able to mediate transcriptional repression by Mad1.

To determine whether Mad1:Mlx heterocomplexes require the same molecular interactions to repress transcription as Mad1:Max, we tested Mad1-dependent repression in the presence of Mlx mutants that either lack the leucine zipper (ΔLZ), which prevents interaction of Mlx with Mad1 in the two-hybrid assay (data not shown), or contain point mutations in the basic region (ΔBR) that abolish its DNA binding activity. Neither of these mutant Mlx molecules were able to repress transcription with Mad1 (Fig. 4C). Therefore, for Mlx to function as a transcription factor, it must be complexed with a binding partner, and this complex must directly bind DNA.

Mad1:Max and Mnt:Max heterocomplexes repress transcription by recruiting a large multiprotein complex containing mSin3 and histone deacetylase 1 and 2 (HDAC1 and HDAC2) to DNA (32, 33, 45). To test whether Mad1:Mlx complexes also require the mSin3A-HDAC corepressor to be functional repressors, we utilized a mutant of Mad1, Mad1(L12P/A16P), which is unable to interact with mSin3A/HDAC corepressor complex. Compared with wild-type Mad1, Mad1(L12P/A16P) was unable to repress transcription when cotransfected with Mlx or Max (Fig. 4D), indicating a requirement for the mSin3A heterocomplex in Mad1:Mlx transcriptional repression. These experiments show that, like Mad1:Max, Mad1:Mlx represses transcription as a sequence-specific DNA binding heterodimer that recruits the mSin3A/HDAC corepressor complex.

Restricted Dimerization between Mlx and a Subset of Max Network Proteins—If Max and Mlx are functionally indistinguishable in vitro, how might Mlx diversify the functions of the Mad family in vivo? Given its similarity to Max in its BHLHZip region, it seemed possible that Mlx would interact with all members of the Mad family. Using directed two-hybrid assays, Mad family proteins can interact with endogenous SIN3p to repress transcription via their SID (Fig. 4A), and hence, all of the Mad-VP16 fusions used in the directed two-hybrid assay lacked the SID. In yeast, LexAMlx showed an interaction with only VP16Mad1 and VP16Mad4 but not with other members of the Max network or with itself (Fig. 5A). As expected, LexAMlx showed an interaction with Mad1, Mxi1, Mad3, Mad4, L-Myc or N-Myc VP16 fusion proteins, demonstrating that all the VP16 fusions were expressed and functional. The inability to detect an interaction...
between LexAMlx and VP16Mlx confirms the finding shown in Fig. 4A that Mlx, like Max, forms homodimers poorly.

We next tested whether Mlx could form heterocomplexes capable of specific DNA binding with other Max network proteins by EMSAs. As assayed by EMSA, Mlx could form CACGTG binding heterodimers with Mad1 and Mad4 (Fig. 5B, compare lane 2 with lanes 6 and 12). In contrast, no differences in DNA binding were observed when Mlx was incubated in the presence of Max, Mad3, and c-Myc (Fig. 5B, compare lane 2 with lanes 3, 9, and 15). Similar to previously published results, Max showed heterodimerization and specific DNA binding with members of the Max network. The directed two-hybrid assay and EMSA demonstrate that Mlx is more restricted than Max in the protein partnerships it can form with members of the Max network.

**DISCUSSION**

To identify new regulatory partner proteins for Mad1, we devised a yeast two-hybrid screen using a LexAMad1 fusion incapable of binding SIN3p. In a previous two-hybrid screen, only the PAH2 domains of the mSin3A/B corepressors were isolated. This was unexpected as cDNAs encoding Max are represented in the library (data not shown). The small number of positives in the original screen arose because they could compete with endogenous SIN3p for binding to the LexAMad1 bait and counteract transcriptional repression of the reporter gene mediated by SIN3p (35). The strategy described here was adopted to search for additional binding partners that were not found in the original screen. Our current screen identified a known Mad1 binding partner, Max (data not shown), and new potential partner proteins for Mad1.

We have presented a characterization of a new Max-like BHLHZip protein and Mad1 binding partner that we have named Mlx. Mlx shares numerous biochemical and physiological characteristics with Max, suggesting similarities in their function. Mlx and Max proteins are stable, with half-lives greater than 6 h, which contrasts with the short half-lives of their heterodimeric partners, the Myc and Mad transcription factors. This finding suggests that the formation of active Max- or Mlx-containing heterocomplexes will be limited by the synthesis and degradation of their heterodimeric partners. Furthermore, the mRNAs encoding each protein are abundant and expressed in both fetal and adult tissues, suggesting that Max and Mlx are constitutively available to bind to their heterodimeric partners. Both Max and Mlx require heterodimerization with another BHLHZip protein for high affinity binding to the CACGTG E-box elements. To repress transcription, both proteins must form heterocomplexes with Mad1 that are capable of binding DNA, and they must recruit the mSin3-3HDAC complex.

The data we present suggest that Mad1:Max and Mad1:Mlx heterodimers are very similar in their biochemical properties. However, the lack of homology between Mlx and Max outside their DNA binding and dimerization domains suggests that the heterodimers will have nonredundant functions. One mechanism by which such functional diversity might be achieved is through targeting different promoters. The loop domains within the BHLHZip proteins Max, upstream stimulatory factor, and PH04 have been shown to make contacts with the DNA phosphate backbone outside the CACGTG core (42, 46, 47). We hypothesize that the longer loop of Mlx might similarly make contacts outside the core, allowing Mlx-containing heterocomplexes to recognize sites different from Max-containing heterocomplexes. In support of this idea, our binding site selection experiments revealed a difference in the sequences flanking the CACGTG core preferred by each heterodimer. We are currently investigating the contribution of CACGTG flanking sequences to target gene selection in vivo. Alternatively, differences in the functions of the two heterodimers could occur by other mechanisms such as cell type-specific factors that facilitate discrimination of E box flanking sequences in vivo. Finally, it is possible that the function of Mlx may partially overlap with that of Max with regard to Mad1 and Mad4 activity.

Mad1 was cloned as a Max-binding protein. Therefore, it has been suggested that Mad1 dimerization with Max is sufficient to explain the effects of Mad1 overexpression, such as cell cycle arrest (18, 24). However, there is little evidence that the biological activity of Mad1 relies solely on dimerization with Max or that the Mad family in general is dedicated only to the direct opposition of Myc activity. The biological functions of Mad1 could be mediated through heterodimeric partners other than Max. We propose that Mad1 function and the activity of other Mad family members may be regulated by dimerization with other partner such as Mlx. Mad1:Mlx dimers may allow the Mad1 repressor to function in the absence of Max or to function differently in particular cell types or in specific stages of the cellular differentiation program.

Given the similarity between Mlx and Max in their dimerization domains, it was surprising that both two-hybrid and EMSAs revealed interactions only between Mlx and Mad1 or Mad4. This restricted dimerization between Mlx and Mad1 and Mad4 implies that these two Mad family members may be more similar in function to one another than they are to Mxi1 and Mad3. Detailed analysis of the spatial and temporal expression patterns of Mxi1 and Mad3 show that these two family members are expressed in proliferating cells and early in the differentiation process. In contrast, Mad4 and in particular Mad1 are expressed later during differentiation. Therefore, Mxi1 and Mad3 may regulate aspects of the differentiation pathway distinct from those regulated by Mad1 and Mad4. However, if Max is continuously available in these tissues, what is the function of Mlx? Although other mechanisms are possible, it seems most likely that Mad1:Max and Mad4:Mlx heterodimers will regulate a unique subset of downstream target genes whose expression is required for the later stages of differentiation. We postulate that these targets either are not recognized by Mad1:Max and Mad4:Max heterocomplexes or that under some circumstances Max, although expressed, is not available for heterodimerization with some or all of its partners.

Finally, the similarities between Max and Mlx suggest that Mlx may function as a common dimerization partner of a new transcription factor network. In support of this hypothesis, we have recently identified a novel family of BHLHZip proteins that interact with Mlx. These new BHLHZip proteins function as transcriptional activators, demonstrating that, like the Max network, the Mlx network will have both positive and negative components. We are currently examining the function of the Mlx network in controlling aspects of cell growth and differentiation.

**Acknowledgments**—We thank Kathryn Coulter, Barbara Graves, Elizabeth Leibold, Jennifer Logan, and Andrew Thorburn for critically reading the manuscript.

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