Coordinated Cross-Talk Between the Myc and Mlx Networks in Liver Regeneration and Neoplasia

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SUMMARY

The Myc and Mlx Networks show extensive crosstalk and regulate distinct but overlapping sets of transcriptional targets. The current work shows the cooperation between these 2 networks in supporting the regenerative capabilities of normal hepatocytes while also showing that the Mlx Network serves as a suppressor of spontaneous hepatic adenomatosis.

BACKGROUND & AIMS: The c-Myc (Myc) Basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factor is deregulated in most cancers. In association with Max, Myc controls target genes that supervise metabolism, ribosome biogenesis, translation, and proliferation. This Myc network crosstalks with the Mlx network, which consists of the Myc-like proteins MondoA and ChREBP, and Max-like Mlx. Together, this extended Myc network regulates both common and distinct gene targets. Here, we studied the consequence of Myc and/or Mlx ablation in the liver, particularly those pertaining to hepatocyte proliferation, metabolism, and spontaneous tumorigenesis.

METHODS: We examined the ability of hepatocytes lacking Mlx (MlxKO) or Myc-+Mlx (double KO [DKO]) to repopulate the liver over an extended period of time in a murine model of type I tyrosinemia. We also compared this and other relevant behaviors, phenotypes, and transcriptomes of the livers with those from previously characterized MycKO, ChrebpKO, and MycKO × ChrebpKO mice.

RESULTS: Hepatocyte regenerative potential deteriorated as the Extended Myc Network was progressively dismantled. Genes and pathways dysregulated in MlxKO and DKO hepatocytes included those pertaining to translation, mitochondrial function, and hepatic steatosis resembling nonalcoholic fatty liver disease. The Myc and Mlx Networks were shown to crosstalk, with the latter playing a disproportionate role in target gene regulation. All cohorts also developed steatosis and molecular evidence of early steatohepatitis. Finally, MlxKO and DKO mice showed extensive hepatic adenomatosis.

CONCLUSIONS: In addition to showing cooperation between the Myc and Mlx Networks, this study showed the latter to be more important in maintaining proliferative, metabolic, and translational homeostasis, while concurrently serving as a
Myc (Myc) is a Basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor that regulates numerous target genes, which collectively support survival, proliferation, metabolism, ribosome biogenesis and translation. Positive regulation involves Myc's direct sequence-specific DNA binding in heterodimeric association with its obligate bHLH-Zip partner, Max. This occurs at canonical E-box elements that typically reside in the vicinity of proximal promoters. Bound Myc–Max heterodimers recruit an assortment of transcription co-factors and chromatin modifiers such as histone acetylases and methyltransferases, which collectively increase chromatin accessibility, relieve transcriptional pausing, and increase the rate and efficiency of messenger RNA (mRNA) elongation. Down-regulation of these genes, often occurring during cellular quiescence or differentiation, involves a reduction in Myc levels and a shift in E-box occupancy to heterodimers now comprising Max and members of the transcriptionally repressive bHLH-Zip Mxd family, which includes Mxd1–4 and the less-related Mnt and Mga factors. Together, their binding reverses the chromatin modifications mediated by Myc–Max binding and restores transcriptional repression. Negative regulation by Myc is more indirect and involves interaction with and inhibition of positively acting transcription factors such as Miz1 and Sp1. The loss of transcriptional balance maintained by these different competing interactions is a feature of transformed cells, which often overexpress and/or otherwise deregulate Myc.

The Myc Network crosstalks and shares considerable regulatory overlap with a structurally related but distinct group of bHLH-Zip transcription factors that comprise the so-called Mlx Network. Classically believed to control target gene sets smaller and more functionally restricted than those overseen by Myc, the Myc-like equivalents of the Mlx Network include the transcription factors Carbohydrate response element (ChRE)-binding protein (ChREBP) and MondoA. Upon binding glucose and other nutrients, these cytoplasmic proteins translocate to the nucleus, heterodimerize with the Max-like protein Mlx, and bind to their target genes at carbohydrate response elements (ChREs) comprising tandem E-boxes separated by 5 nucleotides. Myc Network and Mlx Network members (collectively termed the Extended Myc Network) can bind to one another’s DNA target sequences and some genes are dually regulated by both sets of factors; however, the numbers of these genes and the degree to which their regulation is a result of binding to shared vs separate sites has not been delineated clearly. Although the Mlx Network is less widely implicated in tumorigenesis than the Myc Network, recurrent MLX gene deletions nevertheless occur in as many as 10%–20% of several human cancers, with the precise fraction correlating with the size of the deletion.

We previously explored the roles for these 2 networks in normal hepatocyte proliferation using mice lacking the enzyme fumarylacetoacetate hydrolase (FAH). These animals serve as a model for type I hereditary tyrosinemia in which FAH’s absence allows toxic tyrosine catabolites to accumulate, leading to hepatic necrosis and liver failure. Treatment with the drug 2-[2-nitro-4-trifluoromethylbenzoyl]-1,3-cyclohexanedi one (NTBC) blocks the enzyme 4-hydroxyphenylpyruvic dioxygenase, which catalyzes the second step in tyrosine catabolism, thereby preventing the accumulation of these deleterious intermediates and circumventing the lethal consequences of FAH deficiency. Immunocompromised fumarylacetoacetate hydrolase nonobese diabetic (FRG-NOD) mice thus can be used as a robust and sensitive animal model in which to evaluate the regenerative potential of any other hepatocyte population, so long as it is Fah+/+. Cells are delivered intrasplenically followed by the cyclic withdrawal and reinstatement of NTBC over several months. As recipient hepatocytes accumulate toxic tyrosine intermediates and die, they are replaced by the donor cells, which expand as much as 50- to 100-fold before eventually comprising up to 70% of the hepatic mass and allowing the recipients to achieve NTBC independence. The FAH model thus places greater proliferative demands on regenerating hepatocytes than does two-thirds partial heptectomy (PH), which represents the gold standard for liver regeneration. It also permits the simultaneous delivery of 2 or more competing populations of hepatocytes to the same recipient, thus allowing for a direct comparison of their relative proliferative rates within the identical environment.

Using this approach, we previously showed that wild-type (WT) and Myc/- (MycKO) hepatocytes possess indistinguishable regenerative potential. This is quite different from most other cases in which Myc’s loss in either non-transformed or transformed cells or tissues profoundly suppresses proliferation.

Abbreviations used in this paper: bHLH-Zip, Basic helix-loop-helix leucine zipper; ChRE, Carbohydrate response element; ChREBP, ChRE-binding protein; ChIP, Chromatin immunoprecipitation; ChREBP, Carbohydrate response element binding protein; DKO, double knockout; FAH, fumarylacetoacetate hydrolase; FRG-NOD, fumarylacetoacetate hydrolase nonobese diabetic; HB, Hepatoblastoma; HCC, Hepatocellular Carcinoma; IPA, Ingenuity Pathway Analysis; KO, knockout; LoxP, locus of X-over P1; mRNA, messenger RNA; NASH, Nonalcoholic steatohepatitis; NTBC, 2-[2-nitro-4-trifluoromethylbenzoyl]-1,3-cyclohexanedi one; PH, partial heptectomy; WT, Wild-type.
proliferation of Chrebp/- (ChrebpKO) hepatocytes is impaired significantly and MycKO × ChrebpKO hepatocytes are even more defective.25 These findings indicated that normal hepatocyte regeneration is more dependent on the Mlx Network than the Myc Network and that the 2 pathways crosstalk and rescue one another’s defects to varying degrees. At the same time, they raise questions about the possible functional redundancy of MondoA in the context of ChREBP’s loss.

We now have explored the relationship between the Myc and Mlx Networks further by generating 2 additional mouse strains. In the first (hereafter referred to as MlxKO), deletion of the Mlx gene functionally inactivates the entire Mlx Network, including any potential rescue by MondoA that might have existed in ChrebpKO mice.25 The second mouse strain contains a double knockout (DKO) of both Myc and Mlx that further inactivates the Extended Myc Network. We show that hepatocytes from both strains, but particularly the latter, are profoundly compromised in repopulating the livers of Fahh/- recipients. They also show markedly attenuated expression of genes that are direct targets for both the Myc and Mlx Networks and that control mitochondrial structure and function, ribosomal biogenesis, and more general aspects of mRNA processing and translation. Older mice of both groups also develop steatosis akin to that previously described in MycKO, ChrebpKO, or MycKO × ChrebpKO mice. Finally, and unexpectedly, more than one third of older MlxKO and DKO mice develop multifocal hepatic adenomas occasionally associated with small regions of hepatocellular carcinoma (HCC). These results further support the idea that the Myc and Mlx Networks crosstalk and cooperatively regulate a range of pathways related to energy metabolism, lipid balance, translation, and proliferation. Finally, they show a heretofore unsuspected role for the Mlx Network as a suppressor of benign hepatic adenomatosis.44

Results

Repopulation by MlxKO and DKO Hepatocytes Is Severely Compromised

Donor mouse strains used for competitive hepatocyte repopulation studies carried homozygous floxed alleles of the Mlx and/or Myc genes (Figure 1A and B and Table 1) and expressed an albumin promoter–driven tamoxifen-inducible estrogen receptor and Cre recombinase (CreER).25,45 Five daily intraperitoneal injections of tamoxifen were sufficient to allow inactivation of each allele by the time hepatocytes were transplanted 3–4 months later (Figure 1C).

Using FRG-NOD mice as recipients, we previously showed that WT donor hepatocytes can outcompete an equal number of ChrebpKO hepatocytes whereas WT and MycKO hepatocytes compete equally.25,35,37 Suspecting that MlxKO hepatocytes would be even more defective, and to emphasize this, we delivered a total of 3 × 10^5 donor hepatocytes intrasplenically into recipient mice at an approximately 1.6 WT:MlxKO ratio (Figure 2A and B). After 24–28 weeks of NTBC cycling, a number of recipients had died and no survivors had achieved NTBC independence, possibly as a result of the deliberate under-representation of WT hepatocytes in the initial inoculum. Indeed, quantification of the total donor population in the surviving recipients indicated that it comprised only 2%–46% of all hepatocytes, which is both lower and more variable than typically achieved when mice receive larger numbers of replication-competent donor cells (Figure 2C).25,37 Despite this low-level reconstitution, the surviving donor hepatocytes were nearly all WT despite their initial minority status (P < .001) (Figure 2D).

Although Myc deletion alone does not confer a replicative disadvantage to hepatocytes, Chrebp deletion does and is exacerbated further by the concurrent inactivation of Myc.25 This suggests that the Myc and Mlx Networks are redundant and compensate for one another under certain circumstances. Because MycKO × ChrebpKO hepatocytes still express MondoA,25 we asked whether its redundancy might mask more prominent phenotypes. We therefore compared the replicative potential of a mixed population of WT and DKO hepatocytes (1:10 ratio) in which the latter cells have functionally inactivated both ChREBP and MondoA as a consequence of Mlx deletion. This experiment achieved a somewhat greater rate of transplant success, with more animals surviving and with recipient livers eventually containing more than 50% donor hepatocytes (Figure 2E). As before, however, virtually all of these were of WT origin (P < .001) (Figure 2F).

To determine more directly which KO population was more proliferatively challenged, an additional competitive transplant experiment was performed using a 1:1 input ratio of MlxKO and DKO donor hepatocytes. Overall survival again was low, no animals achieved NTBC independence and less than 2% of hepatocytes isolated from recipients were of donor origin (Figure 2G). However, despite their own inherent replicative compromise, MlxKO hepatocytes showed an overwhelming survival advantage (Figure 2H) and comprised nearly 95% of the recovered donor population (P < .001). Together, these results argue that, in a highly demanding, long-term model of liver regeneration,25,37,46,47 Mlx loss and the ensuing functional inactivation of ChREBP and MondoA markedly compromise donor hepatocyte proliferation and/or survival, with the additional loss of Myc strongly reinforcing the defect.

Overlapping Transcriptional Dysregulation in MlxKO and DKO Livers Primarily Involves Genes With Roles in Mitochondrial Structure and Function and Translation

Before comparing whole-transcriptome profiles of WT, MlxKO, and DKO livers, we confirmed that the latter 2 evidenced the predicted dysregulation of their direct target genes. For this, gene set enrichment analysis24 was performed on 3 collections of direct Myc target genes from the Molecular Signatures Data Base C2 collection (MSigDB) (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp) and a 154-member panel of MondoA/ChREBP/Mlx direct target genes from the Qiagen Ingenuity Pathway Analysis (IPA) data set (Table 2). In the first case, 2 of the 3 Myc
Figure 1. Strategies for the quantification of total donor and recipient hepatocytes and donor subpopulations. (A) Relevant regions of the murine Myc locus before and after CreER-mediated recombination showing the location of LoxP sites flanking coding exons 2 and 3 (red triangles).25,37 (B) Relevant regions of the Mlx locus before and after CreER-mediated recombination showing the location of LoxP sites flanking coding exons 3 and 6.8 (C) Verification of MlxKO and DKO. (A–C) Four to 5 weeks after CreER activation, DNA from the indicated livers was assessed for the presence of intact or recombined Myc and Mlx alleles. Hepatocytes then were used for transplant studies. Low levels of nonexcised genes likely originated from the nonhepatocyte populations present in the liver.25,37

Table 1. Quantitative Polymerase Chain Reaction Primers and Probes Used to Quantify Each Allele Shown in Figure 1A and B and Other Necessary Genes as Indicated

| Name       | Sequence of primers and probes                                      | GenBank accession number | Location     | Target          |
|------------|---------------------------------------------------------------------|--------------------------|--------------|-----------------|
| Fah-/-     | Forward: 5′-GGGAGGATTGGAAGACAATAG-3′                               | KF947529                 | 4896-4917    | bGH_PA_terminator |
|            | Reverse: 5′-ATTCTTCTGCCTCGTGAACATA-3′                              | NM_010176                | 657-635      | FAH             |
|            | Probe: 5′/56-FAM/CTTCTGAGC/ZN/GCCA                                | KF947529                 | 4950-4973    | bGH_PA_terminator |
| GFP        | Forward: 5′-AGGTCTTCAAGCGGCTACC-3′                                 | MT776902                 | 302-319      | eGFP            |
|            | Reverse: 5′-AGGAGTGAGACGGTGCTGAC-3′                               | MT776902                 | 393-376      | eGFP            |
|            | Probe: 5′/56-FAM/TCAGCTGC/ZN/GCCA                                 | MT776902                 | 346-366      | eGFP            |
| Mlx WT     | Forward: 5′-TGGGCAATGAGCTAGTCTCA-3′                               | NC_000077.7              | 100980807-100980825 | Mlx     |
|            | Reverse: 5′-AGGAGTACAGGAGGTAGTAC-3′                               | NC_000077.7              | 100980911-100980952 | Mlx     |
|            | Probe: 5′/56-FAM/CGATCCACGC/ZN/GCCA                                | NC_000077.7              | 100980856-100980879 | Mlx     |
| Mlx-/-(MlxKO) | Forward: 5′-CACAGGTAGGACGCAATCA-3′                              | NC_000077.7              | 100979147-100979166 | Mlx     |
|            | Reverse: 5′-GGATGAGGATTCTGTAATC-3′                                | NC_000077.7              | 100980990-100980969 | Mlx     |
|            | Probe: 5′/56-FAM/CACAGCTCAGC/ZN/GCCA                              | NC_000077.7              | N/A          | Loxp site       |
| Myc WT     | Forward: 5′-GGGAATATCCACTACTCTACTT-3′                              | NC_000081.7              | 61858492-61858513 | Myc     |
|            | Reverse: 5′-GATTAGACTCCAGGTTGCA-3′                                | NC_000081.7              | 61858613-61858626 | Myc     |
|            | Probe: 5′/56-FAM/TAAGAAAGC/ZN/GCCA                                | NC_000081.7              | 61858549-61858572 | Myc     |
| Myc-/-(MycKO) | Forward: 5′-TGATGACGCGCGTCCGTA-3′                              | NC_000081.7              | 61858570-61858588 | Myc     |
|            | Reverse: 5′-CAGAAAGGCAAAGACTCCAG-3′                               | NC_000081.7              | 61863536-61863515 | Myc     |
|            | Probe: 5′/56-FAM/TCAGCAAGC/ZN/GCCA                                | NC_000081.7              | 61858624-61858629 | Myc+loxp site |
Figure 2. WT hepatocytes outcompete MlxKO and DKO hepatocytes in repopulation assays. (A) The extended Myc Network. Top: Myc Network, comprising Myc, Max, Mxd1–4, Mnt, and Mga1 and their consensus E-box binding site. Bottom: Mlx Network and its E-box–related but more complex ChoRE binding site.25,26 Mix interacts with the nutrient-regulated positive factors ChREBP and MondoA or the negative factors Mxd1, Mxd4, and Mnt.2,13,24 The latter cross-talk with the Myc Network (dotted arrow). (B) Hepatocyte transplantation strategy. Isolated Fah+/+ WT or KO hepatocytes were mixed at the desired ratio and injected intrasplenically into FRG-NOD Fah−/− mice maintained continuously on NTBC. NTBC cycling was continued until mice achieved NTBC independence or for 24–28 weeks, at which time total hepatocytes were isolated and the fractional representation of the total donor and recipient populations was determined (Figure 1). The contribution of each donor set then was further determined and compared with that of the input inoculum. (C) After intrasplenic injection of 3 × 10^5 donor hepatocytes comprising a 1:6 ratio of WT and MixKO cells, NTBC cycling was continued for 24–28 weeks in the 3 animals that survived, with none achieving NTBC independence. Hepatocyte DNA was isolated from the transplanted animals and the percentage of recipient and total donor cells was determined. (D) DNA from panel C was used to determine the ratio of the WT and MixKO donor populations. DNA from an aliquot of hepatocytes at the time of transplant was used to confirm the input donor cell ratio. (E) Transplants performed in FRG-NOD Fah−/− mice using inocula containing an approximate 1:10 ratio of WT:DKO cells. Hepatocytes isolated after 24–28 weeks of NTBC cycling showed that, on average, 52.5% of hepatocytes were composed of donor cells. (F) Hepatocyte DNA from panel E was used to determine the fraction of WT and MixKO donor hepatocytes. Lane 1 shows the approximate 1:10 ratio of the initial input inoculum. (G) Transplants performed in FRG-NOD Fah−/− mice using inocula containing an approximate 1:1 ratio of MixKO and DKO cells. Total hepatocytes isolated after 24–28 weeks of NTBC cycling were evaluated for the fractional representation of total donor and recipient populations showing that, on average, less than 2% of the total hepatocyte mass was of donor origin. (H) Fractional make-up of the donor population from panel G. Lane 1 shows the approximate 1:1 ratio of the input population.

target gene sets also were enriched significantly in MixKO liver RNA sequencing profiles, indicating as previously shown that some Myc-regulated genes also are responsive to Mlx Network inactivation (Figure 3A).13,24,25 The broader and more pronounced enrichment of these transcripts in DKO livers indicated that the Myc Network further contributes to the Mix Network–mediated regulation of these targets as expected. In the second case, MondoA/ChREBP/ Mix target genes were enriched significantly in both MixKO and DKO livers (Figure 3B). These results confirmed that Myc and/or Mlx inactivation was associated with both unique and shared responses of each network’s respective target genes.

To assess the effect of progressive dismantling of the extended Myc Network on target gene sets, volcano plots were used to compare individual gene expression profiles in the earlier-described livers and previously described MycKO, ChrebpKO, and MycKO × ChrebpKO livers (GEO accession number: GSE114634).25 In the latter 3 cases, fewer than 30 differences were identified relative to normal livers from age-
matched animals (differential expression, ≥1.5-fold and q < 0.05), whereas MlxKO and DKO livers showed up to 60-fold more differences (Figure 3C). This more pronounced gene dysregulation again suggested that the combined loss of ChREBP and MondoA eliminated all redundant functions from the Mix Network, thereby allowing a much larger complement of gene expression differences to be shown. The relatively few differences between MixKO and DKO expression profiles (Figure 3D and insert) was consistent with the notion that, at least in the normal nonproliferating liver, the Mix Network contributes more to regulating both the direct and indirect targets of both networks. The differences between the DKO vs MixKO groups in Figure 3D thus were comparable with those between WT and MycKO groups shown in Figure 3C.

IPA profiling of the differentially expressed transcripts in DKO livers (and by extension MixKO livers) showed that 6 of the top 7 most affected pathways were those with roles in mRNA translation and its control, energy metabolism, and mitochondrial structure and function (Figure 3E). The seeming exception (coronavirus pathogenesis pathway) contained numerous ribosomal protein transcripts whose dysregulation accounted for this pathway’s inclusion.
Collectively, these findings agreed with previous reports in livers, liver cancers, and other cell types showing roles for the extended Myc pathway in the earlier-described processes.\textsuperscript{1,2,13,17,18,24,25,37,47,51-54} Gene expression profiles compiled from the pathways shown in Figure 3E showed the down-regulation of numerous transcripts encoding proteins involved in translation and mitochondrial structure and function in MycKO and MlxKO livers and an even greater degree of down-regulation in DKO livers (Figure 3F and G).\textsuperscript{15}

To explore the potential co-regulation of direct target genes by the Myc and Mlx Networks, we obtained data from the most current version of the Encyclopedia of DNA Elements database (https://www.encodeproject.org)\textsuperscript{55} and focused on the HepG2 HCC cell line, which was deemed the most relevant to the current work. CRISPR editing had inserted in-frame 3xFLAG epitope tags into the 3' end of the endogenous MYC or MLX coding regions, thus allowing all Chromatin immunoprecipitation (ChIP) sequencing studies to be performed under identical conditions with the same anti-FLAG antibody. The results were trimmed using a default setting that enumerated only those binding sites within ±2.5 kb of the transcriptional start site of each gene, thereby maximizing the likelihood of functional relevance. In this way, we identified 4152 genes that bound only Myc, 748 that bound only Mlx, and 2433 that bound both factors at 6047 sites, 5267 of which overlapped, either entirely or partially (Figure 3H). Thirty-seven percent of Myc target genes (2433 of 6658) also bound Mlx, whereas 76% of Mlx target genes (2433 of 3181) also bound Myc, thus indicating that Mlx target genes are twice as likely to also bind Myc. Fifty percent of the Myc and Mlx binding site peaks mapped to within 65 bp of one another and 75% mapped to within 170 bp, thus indicating that the 2 factors either bound to the same E-box or ChoRE, or to more than 1 element in such close proximity that their individual peaks could not be resolved simply by examining the ChIP sequencing footprints (Figure 3I).

To confirm the earlier-described results and obtain greater resolution and characterization of Myc and Mlx binding, we analyzed the sequences flanking the sites of maximum factor binding (ie, the ChIPseq binding peaks). The earlier-described 6047 binding sites were merged to 2863 distinct sites for motif analysis. A total of 1220 of these (42.6%) contained consensus E-boxes for Myc-Max and 714 contained consensus ChoREs\textsuperscript{56} (Figure 3J). A total of 45.2% of the sites contained neither E-boxes nor ChoREs, despite the presence of prominent Myc and/or Mlx footprints, thereby indicating either that the motifs did not conform to the conservative consensus sequences used in our search or that Myc and Mlx binding was indirect as a result of association with other DNA binding factors.

Remarkably, the E-boxes and ChoREs in Figure 3J showed a nonrandom distribution and tended to reside within close proximity of factor-binding peaks. The consensus binding sites located closest to or at the peak centers tended to be those whose adjacent sequences contained either the fewest numbers of additional motifs or tightly clustered ones. This suggested that many ChIP sequencing peaks represent the integrated signal of multiple variably overlapping and unresolvable individual binding sites and thus do not necessarily directly overlie a particular site. Collectively, these findings confirm the presence of multiple E-boxes and/or ChoREs within the majority of common target genes as well as direct evidence of Myc’s binding to ChoREs and Mlx’s binding to E-boxes in select cases. They further suggest a means by which the apposition of multiple binding sites within some genes could allow for the simultaneous binding of different combinations of factors as well as their direct interaction and crosstalk.

Cataloging the functions of the 2433 common genes shown in Figure 3H using the IPA and MitoProteome databases and a bespoke collection of previously published genes\textsuperscript{25,51,57,58} showed that many could be categorized as supporting mitochondrial and ribosomal structure and function (Figure 3K).\textsuperscript{25,57} Thus, the common Myc- and Mlx-bound target genes in human HepG2 cells faithfully reflect both the current transcript changes and those previously documented in MycKO and/or ChrebKO murine livers and hepatoblastomas.\textsuperscript{55-57} Interestingly, the 4152 genes bound only by Myc and the 748 genes bound only by Mlx were in somewhat different IPA categories than the common genes (Figure 3K). For example, Myc-specific genes also were involved in more restricted and/or unique functions such as transforming growth factor-β signaling, cell cycle, and oxidative phosphorylation. Similarly, Mlx-specific genes also tended to belong to distinct functional categories such as those related to retinoic acid signaling, xenobiotic metabolism, fatty acid β-oxidation, and the tricarboxylic acid (TCA) cycle.

Given the extended Myc Network’s dynamic nature (Figure 2A), the potential for different members to bind multiple closely neighboring sites with different affinities (Figure 3J), and their ability to either augment or antagonize one another’s transcriptional impact, we hypothesized that Myc and Mlx binding alone (Figure 3I and J) would not necessarily predict target gene expression levels. We further hypothesized that the transcriptional impact on any individual target gene ultimately would reflect the entire extended network’s integrated action.\textsuperscript{59} We thus examined the expression of the 2433 common Myc and Mlx target genes (Figure 3H) in 371 human HCCs using data from The Cancer Genome Atlas. Target gene expression could be categorized into 4 groups (designated A–D) that correlated with 4 patterns of extended Myc Network member expression (groups 1–4) (Figure 3L). Two of the tumor groups also showed significant differences in survival (Figure 3M). Together with the results of Figure 3J, these findings support the idea that the binding of Myc, Mlx, or any other extended Myc Network factor to a target gene likely reflects only 1 aspect of the complex and integrative interplay among other network members that collectively dictates the gene’s expression level and downstream biological consequences.\textsuperscript{20}

Myc, particularly when it is overexpressed by tumor cells, promotes the Warburg effect by up-regulating genes encoding glucose transporters and glycolytic enzymes.\textsuperscript{12,25,30,53,60-65} However, none of these showed altered expression in MycKO livers (Table 3). In contrast, the
progressive inactivation of the extended Myc Network was associated with the down-regulation of 3 transcripts encoding rate-limiting transporters or enzymes, including the major hepatocyte glucose transporter Glut2/Slc2a2 and the glycolytic enzymes liver-type phosphofructokinase (Pfk) and liver-type pyruvate kinase (Pklr). Glut2/Slc2a2 also is required for the proper regulation of glucose-sensitive genes and for glucose-stimulated insulin secretion.64,66 These findings were consistent with the previous IPA showing that genes comprising a glycosis-related set were co-bound by Myc and Mlx (Figure 3K).

Table 3. Relative Expression Levels of Transcripts for Rate-Limiting Factors in the Glycolytic Pathway

| Liver genotype | Glucose transporter 2 (Slc2a2) | Phosphofructokinase-liver type (Pfk) | Pyruvate kinase L/R (Pklr) |
|----------------|---------------------------------|--------------------------------------|--------------------------|
| WT             | 1.00                            | 1.00                                 | 1.00                     |
| MycKO          | 1.19 (q = 1)                    | 0.89 (q = 1)                         | 1.62 (q = 0.66)          |
| ChrebpKO       | 0.30 (q = 5.0 x 10^-6)          | 1.07 (q = 1)                         | 0.30 (q = 2.0 x 10^-7)   |
| MycKO x ChrebpKO | 0.41 (q = 0.02)                  | 0.95 (q = 1)                         | 0.29 (q = 7.0 x 10^-3)   |
| MlxKO          | 0.10 (q = 8.3 x 10^-10)         | 0.81 (q = 0.44)                      | 0.19 (q = 1.0 x 10^-10)  |
| DKO            | 0.13 (q = 3.9 x 10^-9)          | 0.63 (q = 0.01)                      | 0.16 (q = 2.4 x 10^-16)  |

Loss of the Extended Myc Network Members Causes Steatosis

Consistent with findings that the Myc and Mlx Networks both impact pathways involved in carbohydrate and lipid metabolism (Figure 3K),1,2,7,24,25,37,67,68 young MycKO, ChrebpKO, and MycKO x ChrebpKO mice develop steatosis.25,37,69 However, these studies did not determine if this was progressive or if the dual compromise of the Myc and Mlx Networks increased its severity. We therefore examined the livers of older (14–16 mo) MycKO, ChrebpKO, MycKO x ChrebpKO, MlxKO, and DKO mice to evaluate the extent of lipid imbalance. Relative to WT livers, all KO livers showed more intense Oil Red O staining but did not differ significantly from one another (Figure 4A–F). They also contained more total triglycerides than livers from younger MycKO, ChrebpKO, and MycKO x ChrebpKO mice.37,69 (Figure 4G). These findings suggest that steatosis appears earlier in MlxKO and DKO mice, with MycKO mice eventually achieving a similar degree of severity.

In further support of the earlier-described conclusions, we also found evidence for enrichment of a 163-member gene set
associated with human nonalcoholic fatty liver disease (NAFLD) in all cohorts except Chrebp KO (https://www.wikipathways.org/instance/WP4396_r98945) (Figure 4H). Thus, despite the fact that the causes of steatosis in the earlier-described mice and human beings differ considerably, in most cases there is considerable similarity in the disease-related gene expression profiles.70

Finally, we performed IPA to identify additional disease-related pathways that sometimes are dysregulated in NAFLD70 and in all cases found several relating to lipid synthesis/metabolism and peroxisome proliferator-activated receptor (PPAR) activation (Table 4). While noting little histologic evidence for inflammatory cell infiltrates or fibrosis in our KO livers, several of these pathways were in fact associated with nonalcoholic steatohepatitis (NASH) (Table 4). Although these transcriptome-based findings may represent early evidence of actual NASH, the altered expression of inflammatory markers also could be indicative of the immune function changes that accompany Myc dysregulation in nonhepatic tissues.37,71 We believe the most conservative interpretation of the earlier-described results is that the steatosis accompanying the loss of most extended Myc Network members is progressive and eventually leads to a mild NASH-like picture as indicated by significantly altered molecular markers of this state, but little documentable histopathologic change.

**MlxKO and DKO Mice Develop Age-Related Hepatic Adenomatosis and Occasional HCC**

Unexpectedly, 36% of MlxKO and DKO animals (15 of 42) of both sexes developed multiple small- to medium-sized hepatic neoplasms, which were never observed in WT, MycKO, ChrebpKO, or MycKO x ChrebpKO mice (Figure 5A and B).25,37 These were mostly well-differentiated and/or myxoid-type tumors with numerous...
balloon cells, nuclear enlargement, and microvesicular steatosis. A minority also showed small foci of well-differentiated HCC, which sometimes is associated with hepatic adenomas in human beings (Figure 5C). Despite our previous transplant studies having been performed with different input ratios of WT and KO hepatocytes, their outcomes were consistent with those reported here and allowed us to extend our conclusions regarding the relative importance of the Myc and Mlx networks.

### Discussion

Most previous investigations into Myc’s role in hepatic regeneration have relied on the PH model and yielded conflicting results that likely reflected differences in how and when regeneration was assessed and quantified. The short time frame over which this process occurs and the dependency on separate groups of mice may have further contributed to disparate outcomes. Because post-PH hepatocytes require fewer than 2 divisions to replace the missing mass, the model also poses a comparatively modest regenerative challenge. Indeed, even this low number overestimates the actual contribution made by dividing hepatocytes given that approximately half the response to PH involves hypertrophy of the liver remnant plus replicative contributions by nonhepatocyte populations such as endothelial, Kupfer, and stellate cells. In contrast, the FAH model is associated with a more sustained and robust 50- to 100-fold expansion of pure populations of transplanted hepatocytes. It also provides a well-defined point at which a stable level of regeneration can be assessed and a means by which competing donor populations within the same liver can be simultaneously distinguished and quantified after their delivery at any desired and preselected ratio. Using this approach, we previously showed that the long-term proliferation of otherwise normal hepatocytes requires ChREBP but not Myc, although the loss of both factors was additive. Even more pronounced interdependencies were seen during HB tumorigenesis, with HB growth impaired markedly in both MycKO and ChrebpKO livers, and even more so in MycKO × ChrebpKO livers. These findings implied a means of communication between the Myc and Mlx Networks, with each one being able to rescue, at least partially, defects in the other. They also showed that the requirement for Myc becomes progressively more critical as proliferative demand increases, thus emphasizing its strong contextual dependency (Figure 6).

Despite our previous transplant studies having been performed with different input ratios of WT and KO hepatocytes, their outcomes were consistent with those reported here and allowed us to extend our conclusions regarding the relative importance of the Myc and Mlx networks in the development of hepatic adenomas.

### Table 4: IPA Profiling of KO Livers

| IPA diseases and functions | Cohort | P value |
|---------------------------|--------|---------|
| Activation of PPAR in Liver cells | MycKO | <.01 |
| ChrebpKO | <.01 |
| MycKO × ChrebpKO | <.01 |
| MixKO | <.01 |
| DKO | <.01 |
| Lipid synthesis | MycKO | <10^-5 |
| ChrebpKO | <10^-5 |
| MycKO × ChrebpKO | <10^-5 |
| MixKO | <10^-5 |
| DKO | <10^-5 |
| Fatty acid metabolism | MycKO | <10^-5 |
| ChrebpKO | <10^-5 |
| MycKO × ChrebpKO | <10^-5 |
| MixKO | <10^-5 |
| DKO | <10^-5 |
| Liver inflammation | MycKO | <.01 |
| ChrebpKO | <.01 |
| MycKO × ChrebpKO | <.01 |
| MixKO | <.01 |
| DKO | <.01 |
| Fibrosis of the liver | MycKO | <.01 |
| ChrebpKO | <.01 |
| MycKO × ChrebpKO | <.01 |
| MixKO | <.01 |
| DKO | <.01 |
| Hepatic steatosis | MycKO | <.01 |
| ChrebpKO | <.01 |
| MycKO × ChrebpKO | <.01 |
| MixKO | <.01 |
| DKO | <.01 |

NOTE. Relevant NAFLD-associated IPA in livers from the indicated KO groups compared with WT livers. Because so few gene expression differences existed in MycKO, ChrebpKO, and MycKO × ChrebpKO livers (Figure 3C), the criteria for differential expression were relaxed to include those genes with more than 1.2-fold differences and P values < .05.
Networks in liver regeneration. For example, our studies comparing WT and ChrebptKO hepatocytes used an input inoculum comprising 62% of the latter population that was reduced by more than half after competitive repopulation.24 Our current results in which MixKO hepatocytes comprised approximately 84% of donor cells but only approximately 4% of the final population (Figure 2D) provided strong evidence that the concurrent functional inactivation of both ChREBP and MondoA confers an even more profound proliferative disadvantage. This could be a direct effect resulting from the concurrent loss of ChREBP and MondoA binding to their respective target genes, either individually or collaboratively with Myc, thereby eliminating any possibility of rescue of 1 factor by another (Figure 2A).24,26–29 A non-mutually exclusive indirect effect that allowed Mxd1, Mxd4, and Mnt to suppress Myc target genes more effectively by increasing their association with Max also remains possible (Figure 2A). The relative importance of these 2 models could vary among different target genes at different times during repopulation or in different liver compartments.
replacement of tus.25,37 This is supported by the progressive deterioration of glycolytic enzymes that collectively are responsible for the Warburg effect as well as other metabolic pathways and functions that support increased energy demands and rapid growth.2,3,24,40,53,65,69,78 Despite the rapid growth that occurs in response to the overexpression of Myc and mutant forms of β-catenin and YAP127A,25,51,73 The Mix Network, which supports this rapid growth, is proposed to contribute to tumor suppression as well (Figure 5).

We also previously showed that the combined loss of Myc and ChREBP suppressed regeneration more than the KO of either individual gene, thereby corroborating previous evidence for internetwork crosstalk.2,3,13,24,25 In 1 such study, performed with nearly equal contributions of WT and MycKO × ChrebpKO donor hepatocytes, the latter was reduced to 7.5% after repopulation.25 Although impaired markedly, the residual proliferative activity of these cells could have reflected the redundant function of MondoA (Figure 2A), which is supported by 2 separate aspects of the current work. The first was the approximately 45-fold repopulation advantage of WT hepatocytes over DKO hepatocytes, whereas the second was the approximately 10-fold repopulation advantage of MixKO hepatocytes over DKO hepatocytes (Figure 2F and H). Collectively, our current results indicate that both the Myc and Mix Networks play distinct as well as redundant roles in normal hepatocyte replication. However, much of the proliferative drive needed to sustain hepatocyte expansion in FAH mice is subsumed by the Mix Network regardless of the Myc Network’s status.25,37 This is supported by the progressive deterioration of repopulation potential as the Extended Network is gradually dismantled (Figure 2C–H).25

In proliferatively quiescent cells or organs such as the liver, Myc usually is expressed at low levels and regulates relatively few genes in contrast to Mix (Figures 3C and 5E).25,37,78 Myc’s contribution to genome-wide transcription therefore may be better appreciated in tumors where its overexpression can activate genes that are otherwise non-physiologic targets owing to their low-affinity binding sites.10,11,25,68 Another plausible explanation for the seemingly modest transcriptional consequences of Myc loss in some normal tissues is that at least some Myc target gene expression is maintained by the Mix Network with redundant contributions being made by MondoA and/or ChREBP.25,37,78 This is best appreciated in livers and tumors when the Myc and Mix Networks are either individually or concurrently inactivated (Figure 3C).25

In tumors, the Myc Network positively regulates most of the genes encoding glycolytic enzymes and strongly contributes to the Warburg effect,5,12,46,51,53,61,65,80 as it does in rapidly growing fibroblasts in vitro.40,80 In contrast, our transcriptomic studies have not shown such widespread roles for the Myc and Mix Networks in maintaining glycolysis in vivo (Table 3 and Figure 3K), which may reflect Myc’s low-level expression, the relative proliferative quiescence of the normal liver, and its greater reliance on fatty acid oxidation as an energy source.25,46,51,73 Nonetheless, among the most down-regulated genes in MixKO and/or DKO livers were Glut2/Slc2a2, Pfkl, and Pklr, whose encoded proteins are rate-limiting for glucose uptake and glycolysis. In rat insulinoma cells, the Pklr proximal promoter binds both ChREBP and Myc, with the former interacting with a ChoRE element and the latter binding elsewhere.24,25 These results suggest that, in normal liver, glucose uptake and oxidation are more reliant on the Mix Network (Figure 6) whereas, in response to Myc-driven transformation or normal proliferation, more extensive transcriptional regulation of glucose uptake and its oxidation is achievable.4,14,23,25,51,81 This could have the additional benefit of maximizing glycolytic efficiency and sustaining cell division when microenvironmental glucose and oxygen supplies were limiting and nutrient-dependent functions of MondoA and ChREBP were attenuated.52

Coordination in direct and/or functionally related Myc and Mix Network target genes were identified in MixKO and DKO livers but were more pronounced in the latter (Figure 3A–D).25,37 Some have been shown previously
to support protein translation and its control as well as mitochondrial structure and function (Figure 3E–G). As was seen for individual glycolysis-related transcripts (Table 3), the collective expression of these sets became increasingly compromised as the extended Myc Network was progressively inactivated. The dramatic up-regulation of these pathways that accompanies tumorigenesis in WT livers also had been shown to be attenuated in response to Myc and/or ChREBP inactivation and to correlate with impaired rates of tumor growth. We provided a mechanistic underpinning for the coordinated response of the relevant gene sets associated with these pathways by showing that, in HepG2 cells, 37% of Myc's direct target genes also bind Mlx, while 76% of direct Mlx target genes also bind Myc (Figure 3H). Many of the previously mapped binding sites for these 2 factors overlapped and/or contained multiple E-boxes and/or ChoREs (Figure 3I and J). Although this sometimes made it difficult to attribute Myc or Mlx binding precisely to a specific motif within a factor's ChIP sequencing footprint, the collective binding landscape suggested a model for target gene regulation that accommodates this and all other observations. The model accounts for the fact that many sites containing only E-boxes coincided with Mlx binding peaks whereas many ChoRE-only sites coincided with Myc binding peaks (Figure 3J). This indicated that crosstalk between the Myc and Mlx Networks occurs by virtue of shared common binding sites as previously suggested. The nonrandom distribution of E-boxes and ChoREs around Myc and Mlx binding peaks (Figure 3J) also suggests that more than one such site could be occupied at any given time, that binding might be cooperative, and that the composition of the bound factors, their interactions, with each other and differential protein–DNA affinities are dynamic and serve to fine-tune the target gene's transcriptional output. Although we examined only Myc and Mlx binding, these motifs also could bind other extended Myc Network members such as those between Max and Mxd proteins, which would not have been detected with our ChIP sequencing analysis. Whether closely spaced Mlx sites contained ChREBP or MondoA elements also potentially could determine if, when, and the degree to which a gene was responsive to metabolic substrate-mediated regulation. Finally, the 4 groups into which the expression patterns of the 2433 common Myc + Mlx direct target genes in HCCs could be compiled correlated with the patterns of extended Myc Network transcript expression and, in 2 cases, with significant survival differences (Figure 3L and M). In future studies, it will be important to determine the degree to which different neighboring heterodimeric combinations of extended Myc Network members either cooperate with or antagonize one another under different conditions in different cell types.

Myc and/or ChREBP inhibition are widely associated with lipid accumulation, which stems from an over-reliance on fatty acid oxidation and a resulting increase in lipid uptake that exceeds the amount necessary to satisfy energy demands (Figure 4). Young mice with hepatocyte-specific loss of Chrebp or combined Myc + Chrebp loss also accumulate more neutral lipids than do those with isolated Myc loss. Although we did not serially follow these animals, our findings suggest that, early in life, the partial or complete inactivation of the Mlx Network promotes a more rapid genesis of steatosis than inactivation of Myc alone. Over time, however, lipid accumulation equalizes, with little differences among the various KO groups being discernible in older individuals (Figure 4G). KO livers dysregulated many of the same gene sets and/or IPA pathways that have been described in NASH and its progression in human beings (Figure 4H and Table 4), thereby further supporting the mechanistic relatedness of the various factors responsible for this state. Because KO livers also showed molecular evidence of incipient NASH, it will be important in future work to determine whether these features become more pronounced with age and whether histologic findings of inflammation and fibrosis eventually emerge.

An unanticipated finding was the development of hepatic adenomas in more than one third of MlxKO and DKO mice (Figure 5A and B). This incidence likely represents an underestimate because animals older than 14–16 months were not investigated and microscopic adenomas may have been overlooked in some instances. That similar neoplasms did not appear in WT, MycKO, ChrebpKO, or MycKO × ChrebpKO mice makes it likely that complete Mlx Network inactivation is a prerequisite for their development. Although these neoplasms bear the hallmarks of actual human adenomas, several features suggest more aggressive and malignant predilections, despite the lack of Myc expression in most. These include their multifocality, their occasional HCC-like features, and their robust Ki-67 expression (Figure 5C and D). In contrast, human adenomas, although well known for their occasional conversion to HCC, typically are few in number and tend to show only modestly higher Ki-67 expression. Molecular features suggestive of more aggressive behavior in our adenomas include the dysregulation of 15 of 22 transcripts that we recently identified as predicting inferior outcomes in human HBs and more than a dozen other human cancer types (Figure 5H).

Recurrent MLX gene deletions are associated with at least 8 human cancer types and provide further reason to implicate the Mlx Network in the pathogenesis of hepatic adenomatosis. Genetic suppressors of hepatic adenomas and other benign tumors such as meningiomas, neurofibromas, and uterine fibroids are well documented but are distinct from more notorious counterparts such as TP53, RB, PTEN, APC, and BRCA1/2 that are associated with malignant tumors. However, the role of Mlx and its members also may be more indirect and nuanced. For example, NASH (Figure 4) is a known predisposing factor for the development of both adenomas and HCC and we are currently unable to determine how it might affect tumorigenesis in MlxKO mice. On the other hand, the failure of MycKO, ChrebpKO, or MycKO × ChrebpKO mice to develop adenomas or HCCs, despite their equally pronounced steatosis as well as the fact that high-fat diets can actually suppress hepatic tumor growth, argues for a more direct
role for the Mlx Network in adenoma suppression. It will be of interest to determine whether MlxKO mice are more susceptible to transformation by other oncogenic stimuli even though the emergence of the ensuing tumors may be delayed and their subsequent growth slowed.

In summary, we have shown that the Mlx Network engages in considerable biological and molecular crosstalk with the Myc Network and plays a more substantive role in long-term liver regeneration. Both networks, but the former in particular, alter the expression of numerous genes responsible for broad aspects of translation and energy generation by both aerobic and anaerobic pathways. The majority of Myc and Mlx targets are co-regulated or at least bound by both factors, which appear to share many of the same binding sites, often lying in close proximity to one another. The actual expression of these target genes further correlates with the expression patterns of all 13 members of the extended Myc Network, thereby suggesting complex interactions and interdependent crosstalk at their sites of binding. Mechanistically, the defects that ensue in KO cells as a result of compromising these genes reflect an inability to maintain energy production and translation at levels commensurate with their proliferative demands. This is particularly acute in the neoplastic setting where tumor growth, but not induction, may be severely compromised. The presumptive energy dysequilibrium that arises as a consequence of perturbing either or both of the networks likely is addressed by the increased uptake and storage of fatty acids, leading to eventual steatosis. The hepatic adenomatosis and occasional HCC seen in response to Mlx Network compromise suggests that the tumor suppressor–activity of the Mlx Network counters the more pro-oncogenic tendencies of Myc overexpression. Our findings emphasize the elaborate orchestration of the Extended Myc Network in balancing energy demands and metabolism with normal and neoplastic proliferation.

Materials and Methods

Animal Studies

All breeding, care, husbandry, and procedures were approved by The University of Pittsburgh Department of Laboratory and Animal Resources and the Institutional Animal Care and Use Committee, with standard animal chow and water provided ad libitum. C57BL6 mice expressing green fluorescent protein (GFP) (C57BL/6-Tg[UBC-GFP]30Scha, MGI:3057178) have been described previously and were used as a source of WT hepatocytes because of the ease with which the GFP gene could be identified. C57BL6 c-myloxp/Loxp mice (B6.129S6-Myctm2Fwa, MGI:2178233) have been described previously and were obtained as a gift from I. Moreno de Alboran. The generation of mice bearing a 1717-bp deletion spanning exons 3–6 of the Mlx locus (MlxKO mice) (Figure 1B) also has been described recently. Transgenic mice expressing a fusion protein comprising the hormone-binding domain of CreER and under the control of the albumin promoter that allows CreER to be activated in hepatocytes after tamoxifen exposure (B6.129S2-Albm1(cre/ErT2)jme, MGI:3052812) were a kind gift from Dr Frank Gonzalez (Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute). The latter mice were bred to homozygosity with MloxP/LoxP mice or MycLoxP/LoxP × MloxP/LoxP mice. At weaning, mice were subjected to 5 daily intraperitoneal injections of tamoxifen (75 mg/kg) each in corn oil (Sigma-Aldrich, St. Louis, MO). Several weeks later, hepatocytes were harvested as previously described. An aliquot of these was used for DNA isolation and to quantify the extent of Myc and/or Mlx knockout (Figure 1). The remainder of the MycKO, CrebpKO, or MycKO × CrebpKO hepatocytes then were combined in the indicated proportions with WT hepatocytes and a total of 3 × 105 cells were injected intrasplenically into Fah−/− FRG-NOD mice (Yecuris, Inc., Tualatin, OR) (ChrebP mice: B6.129S6-MLxip1tm1Ku, MGI:3043871; Fah mice: NOD.Cg-Rag1tm1Mom Fahtm1Mww Il2rgtm1Wjl MGI:5485380). All animals were maintained on 8 mg/L NTBC (Ark Pharm, Libertyville, IL) in their drinking water. After 4 days, NTBC was discontinued until mice lost approximately 20% of their body weight. NTBC then was reinstated until mice regained their age-appropriate weight. NTBC cycling was continued either until mice had become NTBC-independent (at least 20 weeks after transplantation) or until week 28 in those cases in which NTBC independence was not achieved. Hepatocyte DNAs then were isolated from recipients and the TaqMan-based approaches shown in Table 1 and Figure 1 were used again to determine the donor:recipient ratio and the relative contribution of each donor population. Polymerase chain reactions were performed in a volume of 12 μL with 50 ng of genomic DNA. Conditions for amplification were 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 60 seconds.

For gene expression profiling, the earlier-described MycLoxP/LoxP, MloxP/LoxP, MycLoxP/LoxP × MloxP/LoxP mice were bred to B6.129-Gt(Rosa)26Sortm1(cre/Ert2)Byj mice (MGI:3699244), which express CreER under the control of the ubiquitously expressed ROSA26 promoter (Jackson Labs, Bar Harbor, ME). Excisional inactivation of each locus was initiated at the time of weaning and confirmed as described earlier. Liver RNAs then were obtained from mice that were the same age as those used for hepatocyte transplants (~5 mo).

Triglyceride Assays

Total lipid was extracted from approximately 50 mg liver using the Folch et al method. Total triglyceride content then was determined as described previously using the Free Triglyceride Reagent (Sigma-Aldrich, Inc). Total triglyceride was extracted from approximately 50 mg liver using the Folch et al method. Total triglyceride content then was determined as described previously using the Free Triglyceride Reagent (Sigma-Aldrich, Inc).

Histology, Immunohistochemistry, and Immunoblotting

Fresh tissues sections were immediately fixed in formalin, embedded in paraffin, and stained with H&E as previously described. Oil Red O staining and immunohistochemistry on snap-frozen sections also were performed as previously described. Tissue samples for

Histology, Immunohistochemistry, and Immunoblotting

Fresh tissues sections were immediately fixed in formalin, embedded in paraffin, and stained with H&E as previously described. Oil Red O staining and immunohistochemistry on snap-frozen sections also were performed as previously described.
immunoblotting were disrupted in sodium dodecyl sulfate–polyacrylamide gel electrophoresis lysis buffer containing protease and phosphatase inhibitors but lacking β-mercaptoethanol or bromophenol blue as previously described.25,46,51,73 Protein quantification was performed using the bicinchoninic acid (BCA) reagent according to the supplier’s directions (Thermo Fisher Scientific, Rockford, Illinois). After β-mercaptoethanol (1%) and bromophenol blue (10%) addition, samples were boiled for 5 minutes, dispensed into small aliquots, and stored at -80°C until analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and semidry transfer to PVDF membranes (Sigma-Aldrich) was performed as previously described.25,46,51,73 Antibodies used for immunoblotting included rabbit monoclonals directed against Mlx and Myc (B5570 and 13987; Cell Signaling Technologies, Inc, Danvers, MA) and a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (G8795; Sigma-Aldrich). A mouse monoclonal anti–Ki-67 antibody used for immunohistochemistry also was from Cell Signaling Technologies (#12202). Horseradish-peroxidase secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Ki-67 immunostain quantification was performed using the ImageJ Immunohistochemistry Image Analysis Toolbox (https://imagej.nih.gov/ij/plugins/ihc-toolbox/index.html; National Institutes of Health, Bethesda, MD). All antibodies were used at the dilutions recommended by the suppliers. Immunoblots were developed using an enhanced chemiluminescent assay kit as directed by the supplier (SuperSignal West Pico Plus; Thermo-Fisher, Inc, Waltham, MA).

RNA Sequencing and ChIP Sequencing Experiments

RNAs were purified from 5 replicate tissues from each group of mice using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD), followed by DNase digestion.5,37 RNA concentration and integrity was confirmed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) and only those samples with RIN values greater than 8.5 were used for sequencing. All subsequent analyses were performed as previously described.25,73 Sample preparation and sequencing was performed on a NovaSeq 600 instrument (Illumina, Inc, San Diego, CA) by Novogene, Inc (Sacramento, CA) and raw data were deposited in the National Center for Biotechnology Information GEO database (accession number: GSE181371). Data sets from previous RNA sequencing studies of MycKO, ChrebpKO, and MycKO × ChrebpKO mice and mutant forms of β-catenin+YAP8127A HBs are available from the GEO database sets GSE114634 and GSE130178.25,73 Differential gene expression was assessed by 3 different comparisons, namely EdgeR, CLC Genomics Workbench version 21 (Qiagen), and DeSeq2, as previously described.25,47 When low-abundance reads (counts per million <1) were encountered for both comparisons, they were eliminated. Reads from FASTQ files were mapped to the GRCm38.p6 mouse reference genome using STAR (https://github.com/alexdobin/STAR/releases) version 2.7.5. BAM-formatted output was analyzed and transcript abundance was determined by featureCounts (http://bioinf.wehi.edu.au/featureCounts). Where necessary, IPA (Qiagen) was used to classify transcripts into pathways whose significance was adjusted for false discovery using the Bonferroni–Hochberg correction. We further used gene set enrichment analysis48 to identify alterations of functionally related groups of transcripts from the MSigDB C2 collection (v.7.4) (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp) or from the MitoProteome database (http://www.mitoproteome.org). Volcano plots were generated using the R software package ggplot2 (https://ggplot2.tidyverse.org), with significant differences between samples being defined as having fold differences greater than 1.5 and false discovery rates less than 0.05. Heat maps were generated using the ComplexHeatmap package (version 2.6.2; https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html). Statistical analyses were performed with R software v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism v9.00 (GraphPad Software, Inc, San Diego, CA).

To analyze ChIP sequencing data, we explored binding Myc and Mlx to their target gene sequences in 2 different HepG2 cell lines that had been modified using CRISPR so as to introduce 3 × FLAG tags at the C-termini of each protein. This allowed ChIP sequencing to be performed under identical conditions with a single anti-FLAG antibody. The results were downloaded from the Encyclopedia of DNA Elements website (https://www.encodeproject.org) and analyzed using ChIPpeakAnno version 3.13 and the annotation database TxD.H. Hapians. UCSC.hg38.knownGene (R package version 3.13.0).90 Only binding sites residing within ±2.5 kb of the transcriptional start of each target gene were considered for the current analysis.25,109 Overlap between Myc and Mlx binding regions was obtained using the findOverlapsOfPeaks function (set maxgap = 0; minoverlap = 0). Venn diagrams were used to show unique and overlapping binding sites. FIMO (version 5.4.1) from the MEME software suite was used to identify E-boxes and ChoREs most closely associated with ChIP sequencing peaks. Categorization of genes associated with bound peaks was performed using previously described collections of functionally related genes or those from the IPA and MitoProteome databases (Qiagen, Inc, and http://www.mitoproteome.org).

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