Zhx2 (zinc fingers and homeoboxes 2) regulates major urinary protein gene expression in the mouse liver

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The mouse major urinary proteins (Mups) are encoded by a large family of highly related genes clustered on chromosome 4. Mups, synthesized primarily and abundantly in the liver and secreted through the kidneys, exhibit male-biased expression. Mups bind a variety of volatile ligands; these ligands, and Mup proteins themselves, influence numerous behavioral traits. Although urinary Mup protein levels vary between inbred mouse strains, this difference is most pronounced in BALB/c mice, which have dramatically low urinary Mup levels; this BALB/c trait had been mapped to a locus on chromosome 15. We previously identified Zhx2 (zinc fingers and homeoboxes 2) as a regulator of numerous liver-enriched genes. Zhx2 is located on chromosome 15, and a natural hypomorphic mutation in the BALB/c Zhx2 allele dramatically reduces Zhx2 expression. Based on these data, we hypothesized that reduced Zhx2 levels are responsible for lower Mup expression in BALB/c mice. Using both transgenic and knock-out mice along with in vitro assays, our data show that Zhx2 binds Mup promoters and is required for high levels of Mup expression in the adult liver. In contrast to previously identified Zhx2 targets that appear to be repressed by Zhx2, Mup genes are positively regulated by Zhx2. These data identify Zhx2 as a novel regulator of Mup expression and indicate that Zhx2 activates as well as represses expression of target genes.

Mups are 18- to 19-kDa proteins of the lipocalin superfamily that, as their name implies, are secreted in the urine at high concentrations (1, 2). As with other lipocalin proteins, Mups have the ability to bind small organic molecules such as pheromones. Although these small molecules have the ability to influence sexual and social behavior, including male aggression, Mup proteins themselves can also influence behaviors in rodents (3–6).

In mice, Mups are encoded by a multigene family comprised of 21 genes and 21 pseudogenes that are tightly clustered on chromosome 4 (7–9). Mup genes can be separated into two classes: six class A Mup genes that are 84–92% identical to each other at the DNA level and 15 highly related class B Mup genes that exhibit >97% identity (9). Mup genes are transcribed abundantly in the liver and at somewhat lower levels in secretory tissues, including the salivary, lachrymal, and mammary glands (10). They are also transcribed at higher levels in males and, as with other genes that exhibit this sex-biased expression, are influenced by testosterone, growth hormone and thyroxine (1, 11, 12). Although Mup genes are among the most highly expressed genes in the mouse liver, the factors that regulate Mup transcription are not known.

The zinc fingers and homeoboxes 2 (Zhx2) gene belongs to a small gene family found only in vertebrates that also includes Zhx1 and Zhx3 (13–15). All three Zhx genes have the same unique structure, with the entire coding region on an unusually large third exon (16). The proteins encoded by these genes are predicted to contain two C2-H2 zinc fingers and four (or five) homeodomains; these domains suggest that Zhx proteins are involved in DNA binding (16). The initial phenotype associated with the mouse Zhx2 gene was a mouse strain-specific difference in adult liver α-fetoprotein (AFP) levels. The AFP gene is expressed abundantly in the fetal liver, normally silenced at birth, and remains off in the adult liver but can be reactivated during liver regeneration and in hepatocellular carcinoma (HCC) (17, 18). A unique feature of AFP expression is seen in BALB/c mice, in which adult liver AFP mRNA levels are roughly 10- to 20-fold higher than what is seen in other strains, including other highly related BALB/c substrains (18, 19). This persistent AFP expression in BALB/c mice is due to a hypomorphic mutation in the BALB/c Zhx2 gene (20, 21), which is located on chromosome 15 (22, 23). This mutation in BALB/c mice is due to the insertion of a mouse endogenous retroviral element into Zhx2 intron 1 that dramatically reduces Zhx2 mRNA levels (20, 21). Several other developmentally silenced genes, including H19, Glypican 3 (Gpc3), and Lipoprotein lipase, are also targets of Zhx2 based on their elevated expression in the adult liver of BALB/c mice compared with other...
Zhx2 and hepatic Mup expression

Figure 1. Low Mup expression in BALB/cJ liver is corrected in hepatocyte-specific Zhx2 transgenic mice. Livers were removed from age-matched adult male mice (4–6 mice/group). Total RNA was prepared, and mRNA levels were analyzed by RT-qPCR using primers described in supplemental Table 2. Zhx2, AFP, and Mup transcript levels were normalized against ribosomal L30 protein mRNA. A, hepatic Zhx2 levels are dramatically reduced in BALB/cJ mice compared with BALB/c mice (Zhx2 WT) and restored to wild-type levels in BALB/cJ mice expressing a hepatocyte-specific TTR-Zhx2 transgene (BALB/cJ + Zhx2 TG). AFP is derepressed in BALB/cJ mice but restored to near wild-type levels in TTR-Zhx2 transgenic mice. Zhx2 and AFP levels in BALB/c mice were set at 1. B, the mRNA levels for Mup20 and class B Mups (using a primer pair that detects all three liver-expressed class B Mups) are dramatically reduced in BALB/cJ mice and restored to wild-type levels in TTR-Zhx2 transgenic mice. Zhx2 and AFP levels in BALB/c mice were set at 1. **, p < 0.01; ***, p < 0.001.

strains (24–26). These in vivo data, as well as in vitro studies, indicate that Zhx2 represses target gene expression in the adult liver (23, 27, 28).

Variation in Mup protein levels is observed in different strains of mice (29). This difference is most pronounced in BALB/cJ mice, which express Mups at considerably lower levels than what is seen in other strains, including other BALB/c substrains (30). This reduced Mup expression in BALB/cJ mice was mapped to a locus on mouse chromosome 15 (30). The dramatic reduction in urinary Mup levels in BALB/cJ mice led us to investigate whether this trait was due to the Zhx2 mutation. Using BALB/c substrains, transgenic models, and recently developed Zhx2 knock-out mice, our data indicate that a number of genes in the liver are targets of Zhx2 regulation. However, in contrast to previous Zhx2 targets, which are elevated when Zhx2 levels are reduced, Mup expression decreases when Zhx2 levels are lower. Using transient transfections and ChIP, we show that Zhx2 directly binds and activates Mup promoters and that this activation is governed primarily through the Zhx2 homeodomain region. These data provide further insight into Mup expression during liver development and expand our understanding of Zhx2 regulation of target genes in the adult liver.

Results
Reduced hepatic Mup gene expression in BALB/cJ mice is due to the Zhx2 mutation

Urinary Mup levels in BALB/cJ male mice are dramatically lower than in other mouse strains (30). Another trait unique to the BALB/cJ substrain is elevated AFP expression in the adult liver, which, as we showed previously, is due to a hypomorphic mutation in the BALB/cJ Zhx2 gene (20). To test whether the BALB/cJ Zhx2 mutation is responsible for reduced Mup protein levels, the expression of several Mup genes was analyzed by RT-qPCR in the adult male liver of the BALB/cJ substrain, which have a wild-type Zhx2 gene, and the BALB/cJ substrain. Studies were performed in male mice because Mups are synthesized at much higher levels in male than in female mice. Of the 21 Mup genes, we focused our analysis on the class A Mup20 and Mup3 and class B Mup7, Mup10, and Mup19 genes (using Mouse Genome Informatics nomenclature) because these exhibit the highest expression in the liver (9). Zhx2 mRNA levels in BALB/cJ mice were roughly 4% of that seen in BALB/c mice, and, as expected, AFP mRNA levels were over 6-fold higher (Fig. 1A). Mup20 mRNA levels were dramatically lower in BALB/cJ, being only ~1% of the level in age-matched adult BALB/cJ liver. In contrast to Mup20, expression of the class A Mup3 gene was not affected by the Zhx2 mutation. Expression of the highly related class B Mup genes in BALB/cJ was reduced to 0.3% of the levels seen in BALB/c mice (Fig. 1B).

To confirm that reduced Mup expression in BALB/cJ mice was due to the Zhx2 mutation, we analyzed Mup expression in TTR-Zhx2 transgenic BALB/cJ mice, which express Zhx2 specifically in hepatocytes. Hepatic Zhx2 and AFP mRNA levels in these transgenic mice are found at roughly the same levels as seen in BALB/c mice, consistent with previous studies (Fig. 1A) (20). The presence of the TTR-Zhx2 transgene in BALB/cJ mice restored Mup20 and class B Mup expression to levels that were roughly 45% and 86% of that seen in BALB/c mice, respectively, whereas Mup3 mRNA levels were not substantially affected by the TTR-Zhx2 transgene (Fig. 1B). Because Mups are synthesized primarily in the liver, these data indicate that the low urinary Mup levels seen previously in BALB/cJ mice are due to reduced hepatic Mup20 and class B Mup mRNA levels. The dramatic reduction in Mup expression in BALB/cJ mice and restoration of near wild-type Mup mRNA levels in TTR-Zhx2 BALB/cJ mice demonstrate that Zhx2 is required for normal Mup expression in the adult liver. In contrast to previously identified Zhx2 targets that are negatively regulated by Zhx2, Mup20 and class B Mup mRNA levels are positively regulated by Zhx2.

Mup expression is reduced in Zhx2 knock-out mice

Our studies in BALB/c substrains indicated that Zhx2 is required for normal Mup expression in the adult liver. Studies were also performed in Zhx2 knock-out mice in which the entire Zhx2 protein-coding region in exon 3 is flanked by loxP sites; Cre-mediated excision therefore removes the entire Zhx2 reading frame (31). These mice were bred to E2a-Cre and Alb-Cre transgenic mice to obtain whole-body (Zhx2KO) and hep-
Hepatic Mup mRNA levels are reduced in Zhx2 knock-out mice compared with Zhx2fl mice. Livers were removed from age-matched adult male mice (4–6 mice/group). Total RNA was prepared, and mRNA levels were analyzed by RT-qPCR using primers shown in supplemental Table 2. Zhx2, AFP, and Mup transcript levels were normalized against ribosomal L30 protein mRNA. A, hepatic Zhx2 levels are dramatically reduced in Zhx2^∆hep mice and not detectable in Zhx2^KO mice. B, AFP mRNA levels are increased similarly in the livers of both Zhx2^∆hep mice and Zhx2^KO mice. C, Mup20 and class B Mup (Mup7, Mup10, and Mup 19) mRNA levels are reduced to a greater extent in Zhx2^KO mice than in Zhx2^∆hep mice, whereas a reduction in Mup3 levels is only seen in Zhx2^KO mice. Data are presented as mean ± S.D. **, *p < 0.01; ***, *p < 0.001.

Figure 3. Zhx2 positively regulates Mup protein levels. Livers and urine were obtained from age-matched adult male mice that were homozygous for the Zhx2^fl, Zhx2^∆hep, or Zhx2^KO alleles (4 mice/group, each lane represents a single mouse). A and B, liver cell lysates (A) and urine (B) were analyzed by Western blotting using a pan-Mup antiserum. Hepatic Mup protein levels are moderately reduced in Zhx2^∆hep livers and dramatically reduced in Zhx2^KO livers. Blots were reprobed for β-actin to control for variations in protein loading. The predominant urinary Mup proteins showed a reduction in Zhx2^∆hep and Zhx2^KO mice that was similar to that seen in liver. The faster-migrating, lower-abundance Mup protein (encoded by the divergent Mup17 gene) was found in urine and not responsive to changes in Zhx2 levels. C, total urine was collected and clarified, and urinary proteins were separated by 15% SDS-PAGE and stained with LabSafe Gel Blue. Levels of the predominant urinary Mup proteins were reduced in Zhx2^∆hep mice and, to a greater extent, in Zhx2^KO mice. The slower-migrating band (asterisk) served as a loading control.

Mup expression is developmentally activated in the postnatal liver

Previously identified Zhx2 targets are expressed abundantly in the fetal liver and repressed within the first 4 weeks after...
birth. Because Mup genes appear to be positively regulated by Zhx2, we predicted that Mup expression would increase during postnatal liver development. Steady-state Mup20, Mup3, and class B Mup mRNAs are barely detectable at embryonic day 17.5 (E17.5) and remain at very low levels up to postnatal day 14 (P14; Fig. 4 and supplemental Table 1). Expression of all these Mups sharply increase at P21. By 8 weeks after birth, expression of all Mups analyzed had increased 105-fold over embryonic levels. During this same time, AFP mRNA levels decrease 105-fold, consistent with previous studies (18), whereas Zhx2 mRNA levels increased 35-fold (Fig. 4 and supplemental Table 1). Interestingly, Mup20 and the class B Mup genes exhibit a substantial increase between P28 and P56, the same period when Zhx2 shows the greatest increase.

**Zhx2 activates Mup expression in vitro**

The *in vivo* mouse data indicate that Zhx2 positively regulates Mup expression in the adult liver. We asked whether this regulation could be observed *in vitro*, which would provide a system to mechanistically explore how Zhx2 controls Mup expression. To determine whether Zhx2 can activate endogenous Mup genes, we transiently expressed a Zhx2-GFP fusion protein in the AML12 mouse hepatocyte cell line. After 48 h, cells were visualized by fluorescence, and total RNA was extracted. Fluorescence microscopy indicated that roughly 30–40% of transfected cells expressed Zhx2-GFP and that the fusion protein was localized to the nucleus (supplemental Fig. 2), as had been observed previously (14, 28). RT-qPCR shows that endogenous Mup20, Mup3 and class B Mup mRNA levels increase in Zhx2-GFP-transfected cells compared with control empty vector (pcDNA3.1 alone)-transfected cells (Fig. 5). In contrast, expression of Transthyretin (TTR), which is not affected by the loss of Zhx2 in BALB/cJ mice (24), does not change in Zhx2-GFP-transfected AML12 cells (Fig. 5).

To determine whether Zhx2 activated expression through the Mup promoter region, a 1.4-kb region containing the Mup20 promoter (−1373 to +32) was inserted into the pGL4.14[luc2/Hygro] luciferase reporter gene to generate Mup20p-luc. Plasmids encoding Zhx2 and Mup20p-luc were co-transfected into AML12 cells and HepG2 human hepatoma cells along with a Renilla luciferase vector to control for variations in transfection efficiency. The control pGL4.14 plasmid was not affected by Zhx2. However, co-transfected Zhx2 increased luciferase levels 2.3-fold and 1.5-fold from Mup20p-luc in HepG2 and AML12 cells, respectively (Fig. 6A). To further investigate the region(s) of the Mup20 promoter responsive to Zhx2, a series of promoter deletion constructs were inserted into pGL4.14 and co-transfected with Zhx2 in HepG2.
cells. Promoter fragments extending −1.4 and 1.0 kb upstream of Mup20 exon 1 were activated by Zhx2, whereas further deletions resulted in non-responsiveness to Zhx2 (Fig. 6B). This indicates that the Zhx2-responsive element of the Mup20 promoter region resides between −1000 and −787.

**Zhx2 activates the Mup20 promoter through the homeodomain region**

Zhx2 is generally considered to be a transcriptional repressor. However, our transfection data indicate that Zhx2 can activate the Mup20 promoter. This positive regulation of Mup genes is consistent with our mouse studies showing decreased Mup levels when Zhx2 is reduced. To further explore this regulation, the Gal4 DNA binding domain was fused to the entire Zhx2 coding region to generate Gal4DBD-Zhx2; the Gal4DBD will tether Zhx2 to Gal4 binding sites (UAS). This fusion protein was co-transfected into HEK293 cells with Mup20p-luc or Mup20p-luc fused to 5 tandem Gal4 binding sites (UAS). As controls, luciferase expression vectors containing the SV40 promoter, with or without the UAS, were also co-transfected with Gal4DBD-Zhx2. The presence of the UAS increased Gal4DBD-Zhx2 responsiveness of Mup20p nearly 3-fold and repressed SV40p roughly 3.4-fold. Data are presented as mean ± S.D. **, p < 0.01; ***, p < 0.001.

Figure 6. A region of the Mup20 promoter between −787 and −1000 is responsive to Zhx2. A, HepG2 cells and AML12 cells were transfected with pGL4 control or Mup20p-Luc reporter genes along with pcDNA3.1 E.V. or the Zhx2 expression vector; Renilla luciferase was also included to control for variations in transfection efficiency. After 48 h, cells were harvested, and lysates were analyzed for firefly/Renilla luciferase. pGL4 was not responsive to Zhx2, whereas the Mup20 promoter was activated by Zhx2 in both HepG2 and AML12 cells. B, a series of Mup20 promoter deletion constructs were transfected with E.V. or Zhx2 in HepG2 cells and analyzed as described in A. Mup20 promoter fragments extending to −1373 and −1000 were responsive to co-transfected Zhx2, whereas fragments extending to −787, −588, −365, and −181 were no longer responsive. Data are presented as mean ± S.D., **, p < 0.01; ***, p < 0.001.

Figure 7. Gal4DBD-Zhx2 activates the Mup20 promoter and represses the SV40 promoter when tethered by UAS motifs. HEK293 cells were co-transfected with Gal4DBD-Zhx2 along with luciferase expression vectors containing the Mup20 promoter (Mup20p), Mup20p and 5× UAS copies (UAS-Mup20p), SV40 promoter (SV40p), or SV40p and 5× UAS copies (UAS-SV40p). After 48 h, cells were harvested, and lysates were analyzed for firefly/Renilla luciferase. The presence of the UAS increased Gal4DBD-Zhx2 responsiveness of Mup20p nearly 3-fold and repressed SV40p roughly 3.4-fold. Data are presented as mean ± S.D. **, p < 0.01; ***, p < 0.001.
Zhx2 and hepatic Mup expression

To determine whether Zhx2 directly binds Mup promoters in vivo, ChIP was performed using livers from Zhx2fl and Zhx2KO adult male mice. We focused on Mup20 because our transfections localized Zhx2 binding to a region between −1000 and −787 (Fig. 6B). The TTR promoter was used as a control because the TTR gene is not a Zhx2 target. Although the Zhx2 ChIP signal for the TTR promoter was ~2-fold higher than the IgG controls, this binding was the same in the KRNQOT region of Zhx2fl and Zhx2KO liver and thus considered background (Fig. 9). However, using primers that span the −884 to −759 region of the Mup20 promoter, an ~4.4-fold enrichment of Zhx2 binding to the Mup20 promoter was observed in Zhx2fl liver compared with the Zhx2KO liver (Fig. 9). A similar pattern was observed using primers for the class B Mup promoters (the primers will amplify Mup7, Mup10, and Mup19 promoters; data not shown). These data indicate that Zhx2 can directly bind Mup promoters in vivo.

Discussion

The Mup multigene family is ideal to study aspects of mammalian gene regulation, including coordinated control of multiple related genes, sex-biased expression, and tissue-specific expression. Most mammalian species have a single Mup gene (humans have only one single non-functional Mup pseudogene), whereas mice and rats have 21/21 and 9/13 clustered Mup genes/pseudogenes, respectively (9). In mice, Mups are expressed primarily in males. Although different Mups are expressed in different tissues and at different levels, the liver is the primary site of Mup synthesis. In fact, Mups are among the most highly expressed genes in the adult male mouse liver, with ~5% of the total mRNA being transcribed from this gene family (11). However, the basis for Mup expression in the liver or other organs is poorly understood. Here we show that Zhx2 is required for robust Mup expression in the adult male liver, although the liver-enriched Mup genes (Mup20, Mup3, and the class B Mup7, Mup10, and Mup19) exhibit differential responsiveness to Zhx2. Mup20 mRNA levels in BALB/cJ and Zhx2KO mice are ~1–2% the levels of age-matched wild-type controls. A similar pattern is seen with hepatic class B Mups; mRNA levels are less than 4% in BALB/cJ and Zhx2KO mice compared with control mice. Mup3 expression appears to be less responsive to changes in Zhx2; no difference in hepatic Mup3 mRNA levels were seen in BALB/cJ and BALB/c mice, whereas a modest reduction was seen in Zhx2KO mice. Curiously, in contrast to the in vivo data, Mup3 is equally, if not more responsive, than Mup20 and class B Mups in Zhx2-transfected AML12 cells. One possible explanation for this differential response is that the chromatin (histone modification, DNA methylation) of Mup3 in the intact liver and AML12 cells is different. A second possibility is that Mup3 is expressed in non-parenchymal cells (i.e. non-hepatocytes) in the liver. Along these lines, all Mup genes examined were reduced to a greater extent in Zhx2KO livers than in Zhx2het livers, suggesting that Mup genes might be expressed in non-parenchymal cells or that these cells influence Mup expression in hepatocytes. Although our analysis has focused on the liver, which is the major site of Mup synthesis, certain Mups are expressed in other mouse tissues, including the lachrymal, salivary, and mammary glands (10). Zhx2 is also ubiquitously expressed (20). The role of Zhx2 in controlling expression of Mups and other target genes in these glands and other non-hepatic tissues is ongoing.
The mechanism by which Zhx2 controls target gene expression is not fully understood. A consensus Zhx2 binding site has not been identified, and it is not known which of the genes that are dysregulated in the absence of Zhx2 are direct or indirect targets. However, Zhx2 binds to and represses the Cyclin A and Cyclin E promoters (28), whereas the transfection and ChIP data presented here show that Zhx2 binds directly to and activates Mup promoters. Deletion analysis indicates that a region of the Mup20 promoter between −1000 and −787 is the target of Zhx2 activation. Further analysis of this region should help identify a specific Zhx2 binding site. It will be interesting to determine whether this site is also found in other Zhx2 targets, including those that are repressed by Zhx2. Having found that the Zhx2 homeodomain region is responsible for Mup promoter activation, studies with hybrid proteins can further localize the activation domains within Zhx2.

The ability of Zhx2 to positively or negatively regulate target genes is of particular interest to us. The initial analysis of human ZHX2 utilized cell culture systems to analyze ZHX2 function (14). These studies included co-transfections with Zhx2-Gal4DBD fusion proteins and SV40 promoter-luciferase reporter constructs linked to UAS. These studies indicated that Zhx2-Gal4DBD, when tethered to the UAS, could repress the SV40 promoter (14). We repeated this experiment and obtained the same result (Fig. 7). However, using this same system, we found that the presence of the UAS increases Gal4DBD-Zhx2 responsiveness of the Mup20 promoter nearly 3-fold over Zhx2 alone (Fig. 7). These data indicate that Zhx2 activation or repression of target promoters is context-dependent, i.e. requires gene-specific interactions with other factors and/or chromatin remodeling complexes. Although other factors that directly control Mup genes have not been identified, expression of several Mups decreases dramatically in HNF4α knock-out mice and decrease to a lesser extent in Stat5b-deficient mice (33).

Interestingly, positively regulated Mups and genes that are negatively regulated by Zhx2 exhibit similar but opposite expression patterns under multiple conditions, including development, liver regeneration, and in HCC. For example, steady-state AFP mRNA levels in mouse liver decrease over 105-fold within the first 4 weeks after birth. In contrast, expression of hepatic Mups increases 104- to 105-fold during this same period. AFP and other negatively regulated Zhx2 targets are transiently activated in regenerating liver, whereas Mup expression is decreased during this period.5 Because AFP and all other previously identified Zhx2 targets are often activated in HCC, we would predict that Mups would be repressed in liver tumors. This possibility is supported by an earlier mouse study (34). Our preliminary analysis also indicates that expression of some, but not all, Mups are lower in mouse HCC samples compared with normal adult liver.5

Our initial interest in Zhx2 regulation of Mup genes came from the observation that urinary Mup levels are dramatically reduced in BALB/cJ mice (30). Having shown a direct relationship between Zhx2 and Mup expression raises the possibility that Zhx2 might influence Mup-associated traits. Mups are known to influence a variety of behaviors in mice, including male-male aggression. Zhx2 is mutated in BALB/cJ but not in other BALB/c substrains.6 BALB/cJ mice exhibit higher fighting behavior compared with the highly related BALB/cN substrain, and this trait is controlled by a single locus (35, 36). A genome-wide analysis of loci associated with male-male aggression was performed using F2 offspring of a BALB/cJ (high aggression) X A/J (low aggression) cross (37). This genetic study identified three loci that influence aggression, including one tightly associated with the D15Mit96 microsatellite marker, which is also tightly linked to Zhx2 (23). Future studies can determine whether Zhx2 influences behavior in mice and whether this influence is associated with altered Mup expression.

The identification of Zhx2 began with the observation that AFP (19) as well as other fetally expressed genes (24, 25) continue to be expressed in the postnatal BALB/cJ liver. Zhx2 also controls the expression of hepatic genes that govern lipid homeostasis, and altered Zhx2 expression leads to changes in serum lipid levels (26). In this regard, it is interesting that two human genome-wide association studies have identified SNPs in Zhx2 that associate with carotid intima media thickness, a subclinical measure of atherosclerotic lesions (38, 39). More recently, we have found that female-biased Cyp genes are repressed by Zhx2 in the male liver, demonstrating that Zhx2 contributes to sex-biased gene expression (31). However, many of these previously identified targets exhibit modest changes in expression in the absence of Zhx2. AFP levels are elevated ~10-fold in the absence of Zhx2 but still repressed about 104-fold compared with what is found in the fetal liver. Female-biased Cyps are expressed at higher levels in adult male Zhx2-deficient livers but still expressed at significantly lower levels than what is seen in adult female liver. In contrast, expression of Mup20 and the class B Mup genes in the adult liver is highly dependent on Zhx2. This strong association between Zhx2 and hepatic Mup expression suggests that Mups will provide a more robust system to explore the mechanism by which Zhx2 regulates target gene expression.

In summary, the data presented here provide further evidence that Zhx2 is an important regulator of gene expression in the adult liver, including developmental regulation, sex-biased expression, and changes in gene expression during liver disease, including HCC. Many of the genes that are dysregulated in the absence of Zhx2 are associated with metabolism. Although Mups are most frequently associated with behavior, there is evidence that Mups can regulate lipid and glucose metabolism in mice (40). Because Zhx2 is expressed ubiquitously, it is likely that it also regulates genes in other tissues.

**Experimental procedures**

**Mice and treatments**

All mice were housed in the University of Kentucky Division of Laboratory Animal Research facility in accordance with Institutional Animal Care and Use Committee-approved protocols. C3H/HeJ (C3H), BALB/cJ, and C57BL/6J (BL/6) mice

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5 J. Jiang and B. T. Spear, unpublished observations.

6 M. Al-Kafajy and B. T. Spear, unpublished data.
were purchased from The Jackson Laboratory; BALB/c mice, which have a wild-type Zhx2 allele, were obtained from Charles River. The TTR-Zhx2 transgenic mice (on a BALB/c background) express Zhx2 from a hepatocyte-specific transthyretin promoter-enhancer cassette (41), were generated by the University of Kentucky Transgenic Mouse Facility, and were described previously (20). To generate Zhx2 knock-out mice, exon 3, which encodes the entire Zhx2 coding region, was flanked by two flox sites to generate a Zhx2 floxed allele (Zhx2\(^{fl}\)) in C57BL/6 mice (31). The Zhx2\(^{fl}\) mice were crossed with Alb-Cre mice (The Jackson Laboratory, 003574) to generate hepatocyte-specific Zhx2 knock-out mice (Zhx2\(^{2\text{Cjt}}\)) or crossed with E2a-Cre mice (The Jackson Laboratory, 003724) to generate whole-body Zhx2 knock-out mice (Zhx2\(^{2\text{Cjt}}\)). For developmental time point studies, female C3H mice were bred to male BL/6 mice (both strains have wild-type Zhx2 alleles), and female mice were monitored for vaginal plugs to estimate the time of fertilization. For E17.5, pregnant females were killed by CO\(_2\) asphyxiation at 17.5 days post-conception, and fetuses were removed. For postnatal time points, mice were killed by CO\(_2\) asphyxiation at the designated times after birth. Purification of parenchymal hepatocytes and non-parenchymal cells is described in the supplemental Experimental Procedures.

**Cloning of reporter genes and expression vectors**

The Mup20 promoter region (−1373 to +32) was amplified from BL/6 mouse genomic DNA. The purified PCR product was cloned into the pGEM-T Easy vector (Promega) and confirmed by sequencing. This 1.4-kb fragment was excised and recloned into the pGL4.14[luc2/Hygro] luciferase vector (Promega). The resulting plasmid was designated Mup20p-luc. This plasmid was cloned into the pGEM-T Easy vector (Promega) and co-transfected with 250 ng of luciferase reporter plasmid (pGL4/H11001) or pGL4/H11002 empty vector control (E.V.) with or without UAS elements, HEK293 cells were seeded at 10^5/well in 24-well cell culture plates and transfected as described above. Cell lysates were prepared after 48 h using the passive lysis buffer (Promega). The firefly/luciferase levels were determined in duplicate using the Dual-Luciferase reporter assay system (Promega). All transfections were done in duplicate and repeated three times.

**RT quantitative real-time PCR**

RNA was prepared from frozen tissues (~100 mg) or AML12 cells using RNAzol RT reagent (Molecular Research Center) according to the instructions of the manufacturer. One microgram of RNA was reverse-transcribed to cDNA using the iScript\textsuperscript{TM} cDNA synthesis kit (Bio-Rad). qPCR reactions were prepared with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and amplified in a Bio-Rad CFX96 real-time PCR system. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA), and their sequences are shown in supplemental Table 2. The qPCR Ct values were normalized to ribosomal protein L30 levels and reported as normalized expression of the indicated gene using the ΔCt method (43). L30 was used because we found that L30 mRNA levels remain stable across different developmental time points (44). In all cases, primers spanned introns so that amplicons from cDNA and any contaminating genomic DNA would be of different lengths and readily detected by melting curve analysis of completed PCR reactions.

**Analysis of Mup protein levels in mouse liver and urine**

Mup protein levels in liver and urine from Zhx2\(^{fl}\), Zhx2\(^{2\text{Cjt}}\), or Zhx2\(^{2\text{Cjt}}\) mice were analyzed by Western blotting. Liver protein lysates were prepared in radioimmune precipitation assay.
buffer, and protein concentrations of lysates were determined using a Bradford protein assay reagent (Bio-Rad). Urine was also collected from adult male Zhx2+/+, Zhx2+/loxP or Zhx2loxPloxP mice and clarified by centrifugation at 6700 × g for 4 min. Total liver protein or urine for each sample was resolved by electrophoresis using 15% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% dry milk, analysis of Mup proteins was performed using a goat anti-mouse Mup polyclonal antiserum (45, 46) with minor changes. Mouse livers were harvested, homogenized in cell lysis buffer (5 mM PIPES (pH 8), 85 mM KCl, 0.5% Igepal, and proteinase inhibitors) followed by centrifugation. Chromatin fragmentation was performed by sonication (Fisher Scientific sonic dismembrator, model 500) in nucleus lysis buffer (50 mM Tris-HCl (pH 8), 10 mM EDTA, 0.5% SDS, and proteinase inhibitors). ChIP was performed using antibodies (rabbit anti-Zhx2, made for us by Bethyl Labs) and rabbit IgG with the Magna ChIP HiSens kit (Millipore) using antibodies (rabbit anti-Zhx2, made for us by Bethyl Labs) and rabbit IgG with the Magna ChIP HiSens kit (Millipore) following the instructions of the manufacturer. ChIP DNA samples were analyzed by quantitative PCR using SsoAdvancedTM Universal SYBR Green Supermix (Bio-Rad) and a Bio-Rad CFX96 real-time PCR system. Primers used for ChIP are shown in supplemental Table 2.

Statistical analysis

All values within a group were averaged and plotted as mean ± S.D. p values were calculated between two groups using Student’s t test. p ≤ 0.05 was considered significant. Data were graphed and analyzed using GraphPad Prism 6 software.

Author contributions—J.J. conducted the majority of the experiments, analyzed data, and wrote the first draft of the paper. K. T. C., and J. P. conducted additional experiments. M. L. P. provided ideas, evaluated data, and edited the manuscript. B. T. S. provided the initial idea for the experiments, supervised the experimental work, and wrote the final manuscript.

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