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Zinc Fingers and Homeoboxes 2 (Zhx2) Regulates Sexually Dimorphic *Cyp* Gene Expression in the Adult Mouse Liver

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The mammalian cytochrome P450 (*Cyp*) gene family encodes a large number of structurally related enzymes that catalyze a variety of metabolic and detoxification reactions. The liver is the primary site of Cyp expression in terms of expression levels and number of expressed genes, consistent with this organ's essential role in metabolism of endogenous and xenobiotic compounds. Many *Cyp* genes exhibit sexually dimorphic expression. For example, Cyp2a4 is expressed significantly higher in the adult liver of female mice compared to male mice. An exception to this pattern is seen in BALB/cJ mice, where male hepatic Cyp2a4 mRNA levels are substantially elevated compared to male mice of other strains. The Zinc fingers and homeoboxes 2 (Zhx2) protein governs the silencing of several genes in the postnatal liver, including α-fetoprotein, H19, and glypican 3. Zhx2 also regulates numerous hepatic genes that govern lipid homeostasis. We previously showed that the *Zhx2* gene is mutated in BALB/cJ mice, which led us to consider whether elevated male hepatic Cyp2a4 levels in this strain are due to this Zhx2 mutation. Using mice with a conditional Zhx2 deletion, we show here that the absence of Zhx2 in hepatocytes results in increased Cyp2a4 expression in adult male liver. We extend this finding to show that additional *Cyp* genes are disregulated in the absence of Zhx2. We also show that mRNA levels of Cyp2a4 and several other female-biased *Cyp* genes are increased, and male-biased Cyp4a12 is decreased in mouse liver tumors. These data indicate that Zhx2 is a novel regulator of sex-biased *Cyp* gene expression in the normal and diseased liver.

Key words: Transcription; Development; Hepatocyte; Regeneration; Knockout mice; Cancer

INTRODUCTION

The cytochrome P450 (*Cyp*) supergene family is one of the largest and most diverse gene families in eukaryotes[1,2]. *Cyp* genes encode structurally related enzymes that catalyze a variety of metabolic reactions, including metabolism of steroid-based hormones, lipids, drugs, and environmental chemicals[3]. In humans, mutations in *CYP* genes contribute a variety of metabolic diseases[4], and polymorphisms in *CYP* genes are a major contributor to variations in susceptibility to xenobiotics[5,6]. The *Cyp* supergene family arose through numerous duplication events[7]. In vertebrates, the number of *Cyp* genes and pseudogenes varies dramatically between different species. Analysis of genome databases suggests that humans contain 57 functional *CYP* genes and 58 *CYP* pseudogenes, whereas mice contain 102 functional *Cyp* genes and 88 *Cyp* pseudogenes[7].

Cyp genes exhibit a variety of expression patterns. Some *Cyp* genes are expressed in multiple tissues, whereas others are more highly restricted to one or several tissues[8–10]. The liver has the highest level of *Cyp* expression, which is not surprising considering the liver's important role in metabolism and because ingested xenobiotics enter the liver before circulation elsewhere in the body. Numerous drugs and xenobiotics can induce *Cyp* gene expression, and much of this is governed by members of the nuclear receptor family, including the constitutive androstane receptor, aryl hydrocarbon receptor, pregnane X receptor, and peroxisome proliferator activated receptor α[11]. As with structural polymorphisms in Cyp enyzmes, variation in Cyp enzyme expression and induction in response to xenobiotics can lead to different responses to these agents. These variations must be considered when comparing the metabolism and toxicity

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of drugs and environmental chemicals between different individuals as well as between different species such as mice and humans[12].

A common feature of many vertebrate *Cyp* genes is sexually dimorphic expression[13]. Many *Cyp* genes are expressed primarily in males, whereas numerous other *Cyp* genes show a female-biased pattern of expression[9]. The degree of sex-biased expression varies considerably. Sex differences in circulating growth hormone (GH) levels play an important role in this sexually dimorphic expression[14]. In the liver, the transcription factors STAT5a/b, hepatocyte nuclear factor 4α (HNF4α), Bcl6, and Cux2 also contribute to sex-biased expression[15–19].

A dramatic example of sexually dimorphic expression is seen with the murine *Cyp2a4* gene. In normal adult mice, Cyp2a4 is expressed abundantly in female livers with very low hepatic expression in adult males[20]. Curiously, Cyp2a4 expression in adult male BALB/cJ mice is substantially increased[21]. Elevated Cyp2a4 mRNA levels in BALB/cJ males is an autosomal recessive trait. It was speculated that a mutation in a transcriptional repressor resulted in derepression of Cyp2a4 in adult BALB/cJ male liver[21].

Several years ago, we identified zinc fingers and homeoboxes 2 (Zhx2) as a regulator of gene expression in the postnatal liver. Several Zhx2 target genes, including α-fetoprotein (*AFP*), *H19*, and glypican 3 (*Gpc3*) are expressed abundantly in the fetal liver and dramatically repressed in the first several weeks after birth[22,23]. In contrast to other strains of mice, AFP, H19, and Gpc3 continue to be expressed in the adult liver of BALB/cJ mice[22–24]. The continued expression of these genes in adult BALB/cJ liver, where AFP mRNA levels are 10- to 20-fold higher than other mouse strains, is an autosomal recessive trait regulated by a locus on chromosome 15[25]. Using positional cloning, we found that elevated AFP, H19, and Gpc3 mRNA levels in BALB/cJ livers were due to a hypomorphic mutation in the *Zhx2* gene that dramatically reduces Zhx2 levels[23,26]. Interestingly, when placed on a high-fat diet, BALB/cJ mice have less serum triglycerides and reduced atherosclerotic lesions compared to other mouse strains on the same diet[27]. This strain difference is also due to the *Zhx2* mutation, and several genes involved in lipid/cholesterol homeostasis are disregulated in BALB/cJ livers[28]. Taken together, these data indicate that Zhx2 is an important regulator of numerous genes in the postnatal liver.

Zhx2 is a member of a small gene family that also contains *Zhx1* and *Zhx3*[29]. Zhx proteins contain two amino-terminal C_2-H_2 zinc fingers and four (or five, in the case of Zhx1) carboxy-terminal homeodomains[30,31]. In vitro studies suggest that Zhx proteins function as transcriptional repressors[29]. This, along with the fact that the *Zhx2* gene is mutated in BALB/cJ mice, led us to investigate whether the mutation in *Zhx2* could account for elevated Cyp2a4 expression in adult male BALB/cJ liver. Using a novel mouse model in which *Zhx2* is deleted specifically in hepatocytes, our data indicate that Cyp2a4 is a target of Zhx2 repression. Furthermore, we demonstrate that numerous other female-biased *Cyp* genes exhibit increased expression in male livers in the absence of Zhx2, although these levels are still considerably lower than what is seen in female liver. We also find that expression of female-biased Cyp2a4, Cyp2b13, and Cyp2b9 increases in liver tumors, whereas mRNA levels of male-biased Cyp4a12 decrease in liver tumors. These data demonstrate that Zhx2 is an important regulator of sex-specific Cyp enzyme expression in the adult liver.

MATERIALS AND METHODS

Mice

All mice were housed in the University of Kentucky Division of Laboratory Animal Research (DLAR) facility in accordance with Institutional Animal Care and Use Committee (IACUC)-approved protocols. All mice had ad libitum access to food and water and were maintained on a 12:12-h light/dark cycle.

Generation of Hepatocyte-Specific Zhx2 *Knockout* ($Zhx2^{\Delta hep}$) *Mice*

Breeding pairs of C57BL/6 (BL/6) mice with a floxed Zhx2 allele ($Zhx2^{fl}$) were obtained from the Knock-Out Mouse Project (KOMP) Repository at the University of California–Davis. In these mice, exon 3 has been flanked by *loxP* sites. Since the entire Zhx2 coding region is found on exon 3, deletion of this exon results in the loss of the entire Zhx2 protein-coding region. These mice were crossed with BL/6 mice expressing the *Cre* recombinase driven by the liver-specific albumin enhancer/promoter (Alb-Cre) (#003574; Jackson Laboratory). Breeding was performed to obtain mice that were homozygous for the floxed Zhx2 allele with or without the *Alb-Cre* transgene (designated as $Zhx2^{\Delta hep}$ and $Zhx2^{fl}$, respectively). DNA was prepared from tail biopsies obtained when mice were 2 weeks of age, and polymerase chain reaction (PCR) genotyping for the Zhx2 alleles (wild-type and floxed) and *Alb-Cre* transgene was carried out with ThermoStart Master Mix (Thermo Scientific) as described[32] using primers shown in Table 1. Mice were maintained under normal conditions until they were euthanized at various time points by CO_2 asphyxiation for tissue harvest.

Developmental Time Point Studies

Female C3H/HeJ (C3H) mice were bred to male BL/6 mice (both strains have wild-type Zhx2 alleles; The Jackson Laboratory), and female mice were monitored for vaginal plugs to estimate the time of fertilization. For embryonic day 17.5 (e17.5), pregnant females were

Table 1. Primer Sequences

Gene	Forward (5′)	Reverse (5′)
Genotyping		
Cre recombinase	ACCTGAAGATGTTCGCGATTATCT	ACCGTCAGTACGTGAGATATCTT
Zhx2 floxed allele	GGACCGAATCTCACTATTTAACTCA	ACAACGGGTTCTTCTGTTAGTCC
RT-qPCR		
AFP	CCGGAAGCCACCGAGGAGGA	TGGGACAGAGGCCGGAGCAG
CYP1A1	CCGGCATTCATCCTTCGT	GCCATTCAGACTTGTATCTCTTGTG
CYP1A2	ATCCTTTGTCCCCTTCACCAT	GGGAATGTGGGAAGCCATTCA
CYP2A4	GGAAGACGAACGGTGCTTTC	TTC CCA GCA TCA TTC TAA GA
CYP2A5	GGA AGACGAACG GTG CTT TT	TTC CCA GCA TCA TTC GAA GC
CYP2B9	CCTCGACTACATTGCCCATAG	GTTCTGGTGATGGAACTCTGTG
CYP2B13	GCTTTTCTACCCTTCTCCACAG	ATGTCCTTAGAAGCAACAGGGC
CYP2C40	TGGAAGAGGAAGGATTCCGG	TCACTGTGAAGACCCTTGTGG
CYP2D9	AGAAGTCTCTGGCTTAATTCCTG	GTGGTCCTATTCTCAGTCAACAC
CYP2D10	GAAGGTCTTCCAAGGTCAGAAG	CAGCATTCCCCTTTACCTTCTC
CYP3A16	GATGCCCTCTTTTTGGTTCTGTTGGC	TCAGGTTGGAATTCTTCAGGCTCTGG
CYP3A25	TCCTCTTCACCGAAATCCTGAG	TCCTGGGTCCATTTCCAAAGG
CYP4A10	TCCTTAATGACCCTAGACACTG	TGAAAGATATTCCTCACACGGG
CYP4A12	ATCCTTCTCGATTTGCACCAGG	TTCATCGCAAACTGTTTCCCAATG
CYP7A1	GGGCTGTGCTCTGAAGTTCGG	CACAGAGCATCTCCCTGGAGGG
CYP8B1	CAGAGAAAGCGCTGGACTTC	GGCCCCAGTAGGGAGTAGAC
CYP39A1	TGGCTCCTGGCGCTGTTTGAG	TGGACTGTATTGACGTGTTTCCGTCT
L30	ATGGTGGCCGCAAAGAAGACGAA	CCTCAAAGCTGGACAGTTGTTGGCA
Zhx2	AGGCCGGCCAAGCCTAGACA	TGAGGTGGCCCACAGCCACT

euthanized by CO_2 asphyxiation at 17.5 days postconception, and fetuses were removed. For postnatal time points, mice were killed at the designated times after birth. The e17.5 fetuses and postnatal day 7 (p7) mice were euthanized by decapitation; mice at later postnatal time points were euthanized by CO_2 asphyxiation. Livers from mice at all time points were isolated, frozen, and stored at −80°C.

Murine Model of Hepatocellular Carcinoma

Female C3H mice were bred to male BL/6 mice and monitored until pups were born. At 14 days of age, the male B6C3F1 offspring were injected with either diethylnitrosamine (DEN; $n=16$; Sigma-Aldrich) or PBS ($n=5$) at a dose of 10 µl/g body weight[33]; these F1 mice were used since they have been shown to be highly susceptible to DEN-induced liver tumors[34]. Mice were weaned at 21 days of age and maintained under normal conditions for 36 weeks, and then euthanized by CO_2 asphyxiation. Livers were removed and examined for hepatocellular carcinoma (HCC) development. Livers from DEN-injected mice had numerous tumors, which were dissected, snap frozen, and stored at −80°C. Livers from age-matched PBS-injected littermates, which were tumor free, were removed, frozen, and stored at −80°C.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Approximately 100 mg of frozen liver tissue was homogenized in 1 ml of RNAzol RT (#R4533; Sigma-Aldrich), and mRNA was extracted according to the product technical bulletin. Rehydrated RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific), and cDNA was synthesized from 1 µg of RNA by the iScript cDNA Synthesis Kit (#170-8891; Bio-Rad) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) reactions using SYBR Green PCR Supermix (#172-5275; Bio-Rad) were performed using a CFX96 Touch Real-Time PCR Detection System, and results were analyzed with the CFX Manager software (Bio-Rad). All samples were analyzed in duplicate. Oligonucleotide primer sequences are listed in Table 1. The qPCR Ct values were normalized to the ribosomal protein L30 or Sfrs4 and reported as "normalized expression" of the indicated gene. L30 was used because we have found that hepatic L30 mRNA levels remain stable at different developmental times; Sfrs4 was used for tumor studies since it is more stable than L30 in these samples[35]. Data shown as fold change were normalized to L30 or Sfrs4, and then normalized to wild-type or control values calculated using the ΔΔCt method[36].

Immunohistochemistry

Dissected mouse tissues were embedded in OCT media, snap frozen, and stored at −80°C. Sections (10 µm) were cut using a Micron HM505 N cryostat, placed onto glass slides, air dried for 10 min, and then fixed in 4% paraformaldehyde for 10 min. Slides were washed twice in cold 1× PBS buffer and then blocked with the appropriate

serum-blocking buffer at room temperature for 1 h. Slides were incubated overnight at 4°C with primary anti-Zhx2 antibody (1:250; #96083; Abcam), washed twice with 1× PBS, and incubated with TRITC-conjugated secondary antibody (1:200; #4010-13; Southern Biotech) for 1.5 h at room temperature. Slides were mounted (Dako mounting media; #2013-05), covered, and imaged with a Nikon Eclipse 80i upright microscope with NiS Elements software.

Statistical Analysis

All values within a group were averaged and plotted as mean ± standard deviation; p values were calculated between two groups using Student's t-test. Developmental gene expression analysis was calculated using two-way analysis of variance (ANOVA) plus Bonferroni post hoc test. Sexually dimorphic gene expression data were analyzed by one-way ANOVA plus Tukey post hoc test. A value of $p \leq 0.05$ was considered significant. Data were graphed and analyzed using GraphPad Prism 6 software.

RESULTS

Zhx2 *Is Absent in Hepatocytes of* Zhx2$^{\Delta hep}$ *Mice*

Previous studies to investigate Zhx2 function in vivo used BALB/cJ mice, which have a natural hypomorphic Zhx2 mutation that reduces, but does not eliminate, Zhx2 expression[23,26]. To understand better the role of Zhx2 in hepatocytes, mice with a floxed *Zhx2* allele were bred with *Alb-Cre* transgenic mice to generate mice that were homozygous for the floxed *Zhx2* allele and *Alb-Cre*$^{+}$ (*Zhx2*$^{\Delta hep}$); floxed homozygous littermates that did not contain the *Alb-Cre* transgene (*Zhx2*^{fl}) were used as controls. Zhx2 mRNA levels in the liver of adult male *Zhx2*$^{\Delta hep}$ mice were ~90% lower than that in age-matched *Zhx2*^{fl} control mice (Fig. 1A). To confirm the loss of Zhx2, hepatic AFP mRNA levels were also analyzed in *Zhx2*$^{\Delta hep}$ and *Zhx2*^{fl} adult mice. Similar to what is seen in BALB/cJ mice, AFP levels are roughly eightfold higher in *Zhx2*$^{\Delta hep}$ mice compared to those in *Zhx2*^{fl} controls (Fig. 1B); similar differences were seen in female *Zhx2*$^{\Delta hep}$ and *Zhx2*^{fl} mice (data not shown). Immunofluorescence staining with anti-Zhx2 antibodies demonstrated that Zhx2 is present

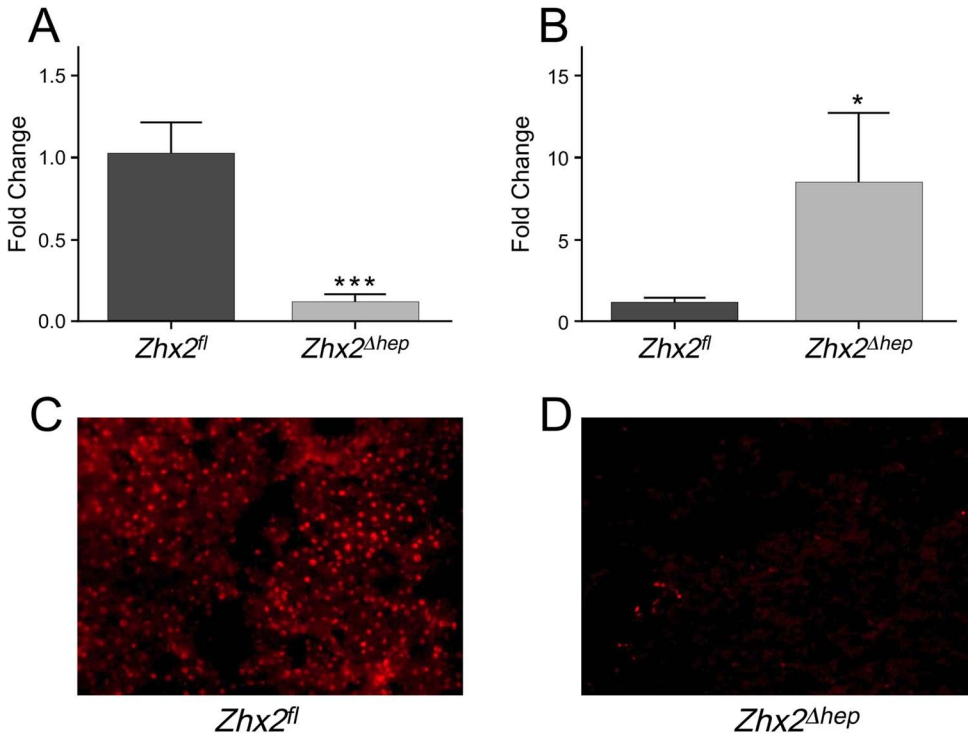

Figure 1. *Zhx2* is absent in hepatocytes of *Zhx2*$^{\Delta hep}$ mice. (A, B) Total liver RNA was prepared from adult male *Zhx2*$^{fl/fl}$, *Alb-Cre*$^{+}$ (*Zhx2*$^{\Delta hep}$; $n=5$) and *Zhx2*$^{fl/fl}$, *Alb-Cre*$^{-}$ (*Zhx2*^{fl}; $n=5$) mice. Reverse transcriptase quantitative PCR (RT-qPCR) was used to monitor levels of Zhx2 (A) and AFP (B), which were normalized to L30 mRNA levels. Zhx2 and AFP mRNA levels in *Zhx2*^{fl} mice were set to 1.0, and fold change in *Zhx2*$^{\Delta hep}$ mice is shown. Zhx2 levels decrease ninefold, whereas AFP levels increase eightfold in *Zhx2*$^{\Delta hep}$ mice. *$p<0.05$; ***$p<0.001$. (C, D) Frozen adult liver sections were incubated with anti-Zhx2 antibody followed by TRITC-conjugated secondary antibody. Nuclear Zhx2 staining is evident in control *Zhx2*^{fl} liver (C) but absent from hepatocytes in *Zhx2*$^{\Delta hep}$ liver, although Zhx2 staining is still evident in nonparenchymal cells (D).

in hepatocyte nuclei in control $Zhx2^{fl}$ mice (Fig. 1C). In contrast, Zhx2 protein is not detectable in hepatocytes in adult $Zhx2^{\Delta hep}$ mice, although Zhx2 continues to be found in nonparenchymal cells (Fig. 1D). These data confirm that Zhx2 is absent in adult $Zhx2^{\Delta hep}$ hepatocytes. Zhx2 mRNA levels are lower in the livers of whole-body Zhx2 knockout mice than in $Zhx2^{\Delta hep}$ livers (data not shown), consistent with Zhx2 expression in nonparenchymal cells. Furthermore, Zhx2 mRNA levels in adult $Zhx2^{fl}$ adult mice are the same as those in wild-type BL/6 mice, indicating that the intronic *loxP* sites do not alter normal Zhx2 mRNA processing (data not shown).

Cyp2a4 mRNA Levels Are Elevated in Male Zhx2$^{\Delta hep}$ *Liver*

Cyp2a4 is normally expressed at much higher levels in the adult livers of female mice compared to adult male liver. However, Cyp2a4 mRNA levels are substantially increased in BALB/cJ male mice but are still lower than what is seen in female adult liver[21]. To test whether this male-specific increase is due to the Zhx2 mutation, hepatic Cyp2a4 mRNA levels were analyzed in $Zhx2^{\Delta hep}$ and $Zhx2^{fl}$ mice. In 8-week-old control $Zhx2^{fl}$ mice, Cyp2a4 mRNA levels are ~1,000-fold lower in male liver compared to female liver, as expected (Fig. 2A and Table 2). Cyp2a4 mRNA levels are increased nearly eightfold in age-matched $Zhx2^{\Delta hep}$ male mice, but still at levels considerably lower than those in $Zhx2^{fl}$ female mice, whereas Cyp2a4 levels remain essentially the same in $Zhx2^{\Delta hep}$ and $Zhx2^{fl}$ females (Fig. 2A). These data indicate that Zhx2 contributes significantly to the low Cyp2a4 mRNA levels in adult male mice. Interestingly, Cyp2a5, which is highly related to Cyp2a4 but shows a much more modest female-biased expression in the liver, exhibits only a 3.1-fold increase in $Zhx2^{\Delta hep}$ male mice (Fig. 2B and Table 2).

Zhx2 *Regulates Multiple Female-Biased* Cyp *Gene Expression in Adult Male Mice*

A number of Cyp enzymes in addition to Cyp2a4 exhibit sex-biased expression, with some being expressed at higher levels in females and others in males. Since Cyp2a4 mRNA levels increased in male $Zhx2^{\Delta hep}$ mice, we evaluated the expression of other hepatic Cyp enzymes in the presence or absence of Zhx2. In addition to Cyp2a4 and Cyp2a5, six *Cyp* genes previously shown to be expressed at higher levels in female mice, to varying degrees, were examined. In general, the loss of hepatic Zhx2 led to increased expression of these female-biased *Cyp* genes in adult male liver (Fig. 3A and Table 2). The increase was robust in several cases (over 33-fold for Cyp2b13) and more modest for other enzymes. Expression of these six *Cyp* genes was generally not affected by the loss of Zhx2 in female $Zhx2^{\Delta hep}$ mice, except for a modest decrease in Cyp4a10 and a more robust decrease in Cyp7a1 mRNA levels. Interestingly, Cyp7a1 is the rate-limiting enzyme that converts cholesterol to bile acids, so alterations in its expression likely affects cholesterol metabolism. This increase in female-biased *Cyp* genes in male livers is similar to what was seen with Cyp2a4. A different pattern was seen with several male-biased Cyp enzymes (Fig. 3B and Table 2).

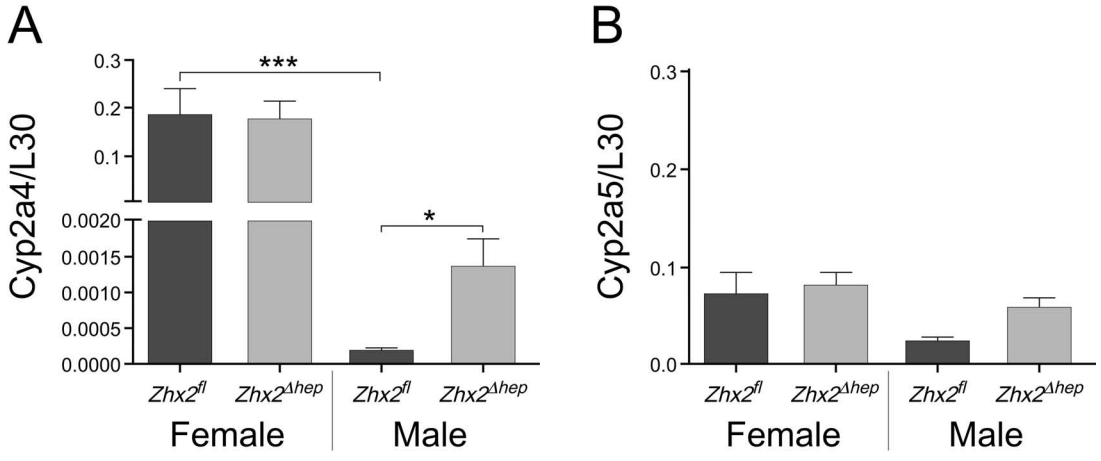

Figure 2. *Cyp2a4* and Cyp2a5 mRNA levels are elevated in adult liver of male $Zhx2^{\Delta hep}$ mice. Total liver RNA was prepared from 8-week-old male and female $Zhx2^{\Delta hep}$ and control $Zhx2^{fl}$ mice. RT-qPCR was used to monitor levels of Cyp2a4 mRNA (A) and Cyp2a5 (B), normalized to L30 mRNA levels. Cyp2a4 levels are eightfold higher in male livers from $Zhx2^{\Delta hep}$ mice ($n=9$) compared to $Zhx2^{fl}$ male mice ($n=5$). Expression of Cyp2a5, which is highly related to Cyp2a4, shows a modest increase in male $Zhx2^{\Delta hep}$ mice that is not significant. Cyp2a4 and Cyp2a5 mRNA levels are the same in female $Zhx2^{\Delta hep}$ ($n=8$) and $Zhx2^{fl}$ ($n=4$) mice. $*p<0.05$; $***p<0.001$.

Table 2. Sex-Biased *Cyp* Expression in Female and Male *Zhx2*$^{\Delta hep}$ and *Zhx2*^{fl} Mouse Liver

	Published Bias	Female/Male Ratio in *Zhx2*^{fl} Mice	Male *Zhx2*$^{\Delta hep}$/*Zhx2*^{fl} Ratio	Female *Zhx2*$^{\Delta hep}$/*Zhx2*^{fl} Ratio
Female biased				
Cyp2b13	Female[9]	10,936	33.7	1
Cyp2a4	Female[9]	981	7.9	1
Cyp2b9	Female[9]	770	6.8	1
Cyp4a10	Female[9]	7.1	5.4	0.4
Cyp39a1	Female[8]	1.5	4.8	1.8
Cyp2a5	Female[9]	3.2	3.1	1.1
Cyp2c40	Female[9]	5.8	1.5	1.2
Cyp7a1	n/a	7.0	1.2	0.1
Male biased				
Cyp4a12	Male[9]	0.002	0.9	0.6
Cyp2d9	Male[9]	0.4	0.9	0.9
Cyp8b1	Male[10]	0.6	1.2	0.9
Unbiased				
Cyp3a25	Unbiased[9]	0.6	1	1.1
Cyp2d10	Unbiased[9]	0.6	0.8	0.9
Cyp3a16	Female[9]	0.8	2.1	1

Analysis of liver mRNA expression levels of 14 *Cyp* genes examined (female biased, male biased, and unbiased) is categorized based on sex differences seen in adult *Zhx2*^{fl} mice. Ratios are based on data shown in Figures 2 and 3. A previous report indicated Cyp3a16 is a female-specific *Cyp* gene, but this was not seen in our study. n/a, not available.

Two that were analyzed, Cyp8b1 and Cyp2d9, showed little change in either sex in *Zhx2*$^{\Delta hep}$ liver compared to *Zhx2*^{fl} liver, whereas a third, Cyp4a12, was only slightly reduced in female *Zhx2*$^{\Delta hep}$ mice. Three Cyp enzymes that are expressed at roughly equal levels in both sexes, Cyp3a25, Cyp2d10, and Cyp3a16, show little change in expression in response to the loss of Zhx2 (Fig. 3C and Table 2).

Cyp2a4, Cyp2b13, and Cyp4a12 mRNA Levels Are Developmentally Regulated in the Postnatal Liver

Previously identified Zhx2 targets, including AFP, H19 and Gpc3, are expressed abundantly in the fetal liver and silenced after birth but do not exhibit sex-biased expression. This led us to investigate developmental changes in the expression of female-biased Cyp2a4 and Cyp2b13 and male-biased Cyp4a12 in both sexes. Since Zhx2 is involved in male-specific silencing of female-biased Cyps, we also monitored changes in Zhx2 mRNA levels in male and female mice. At e17.5, Zhx2 levels are higher in female than in male fetuses (Fig. 4A). Zhx2 mRNA levels in female mice remain steady through p7, exhibit a slight increase at p14 and another modest increase at p28. In contrast, male liver Zhx2 mRNA levels exhibit a slow but steady increase between e17.5 and p28, but then show a dramatic ~7-fold increase between p28 and p56 (Fig. 4A). In both sexes, Cyp2a4 levels are very low in the e17.5 liver and show a gradual increase during the first 3 weeks after birth (Fig. 4B). The sex bias in Cyp2a4 levels is first evident at p28; Cyp2a4 levels remain relatively steady in male mice at this time point but increase over 10-fold in female mice. Cyp2a4 levels remain high 8 weeks after birth in female livers. However, in male liver, Cyp2a4 levels exhibit a dramatic reduction after p28, decreasing to barely detectable levels by p56. Thus, the dramatic difference in hepatic Cyp2a4 mRNA levels between male and female mice is established between p28 and p56, indicating that male-specific Cyp2a4 silencing is associated with sexual maturity that occurs between 4 and 8 weeks after birth. A somewhat similar pattern is seen with Cyp2b13. Expression of Cyp2b13 increases in both sexes between e17.5 and p7. Cyp2b13 levels gradually increase between p14 and p56 in females and decrease in male mice during the same period, although the most dramatic change in male mice occurs between p28 and p56 (Fig. 4C). Zhx2 also shows a significant increase in male mice between p28 and p56, which supports the possibility that Zhx2 contributes to Cyp2a4 and Cyp2b13 silencing in the adult male liver. The developmental changes in male-biased Cyp4a12 mRNA levels are different than what is seen with female-biased Cyps (Fig. 4D). Cyp4a12 levels are low but found at equal levels in male and female livers at e17.5. Levels in both sexes remain low until p28, at which time Cyp4a12 mRNA levels increase dramatically in male livers.

Sex-Biased Cyp Enzymes Are Disregulated in a Mouse Model of HCC

A hallmark of previously identified Zhx2 targets is that they are frequently reactivated in HCC and other

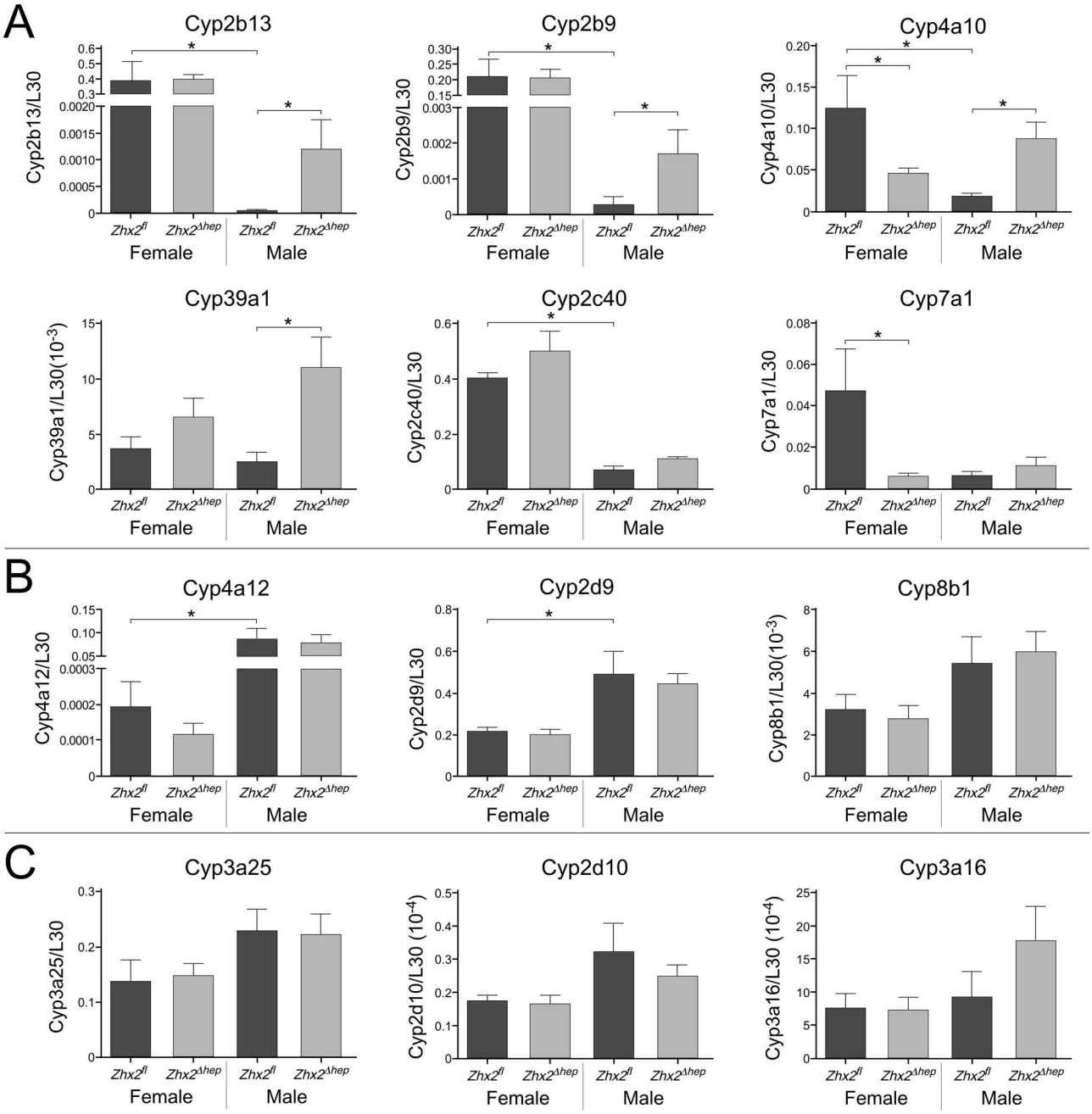

Figure 3. Zhx2 represses female-biased *Cyp* genes in adult male mice. Liver mRNA was analyzed by RT-qPCR in adult male and female $Zhx2^{fl}$ and $Zhx2^{\Delta hep}$ littermates (male: $Zhx2^{fl}$ $n=5$, $Zhx2^{\Delta hep}$ $n=9$; female $Zhx2^{fl}$ $n=4$, $Zhx2^{\Delta hep}$ $n=8$). (A) Six *Cyp* genes characterized as being more abundant in females were analyzed. Four genes, Cyp2b13, Cyp2b9, Cyp4a10 and Cyp39a1, increased significantly in male $Zhx2^{\Delta hep}$ mice; a more modest increase was observed with Cyp2c40 and Cyp7a1, which was the only female-biased Cyp analyzed that decreased in female $Zhx2^{\Delta hep}$ liver. (B) Three *Cyp* genes typically more abundant in males were analyzed. There is no difference in expression in male $Zhx2^{\Delta hep}$ mice compared to $Zhx2^{fl}$ controls. Female $Zhx2^{\Delta hep}$ mice exhibited slightly reduced Cyp4a12 expression. (C) The absence of Zhx2 does not significantly change expression of *Cyp* genes that are expressed equally in males and females. $^{*}p<0.05$.

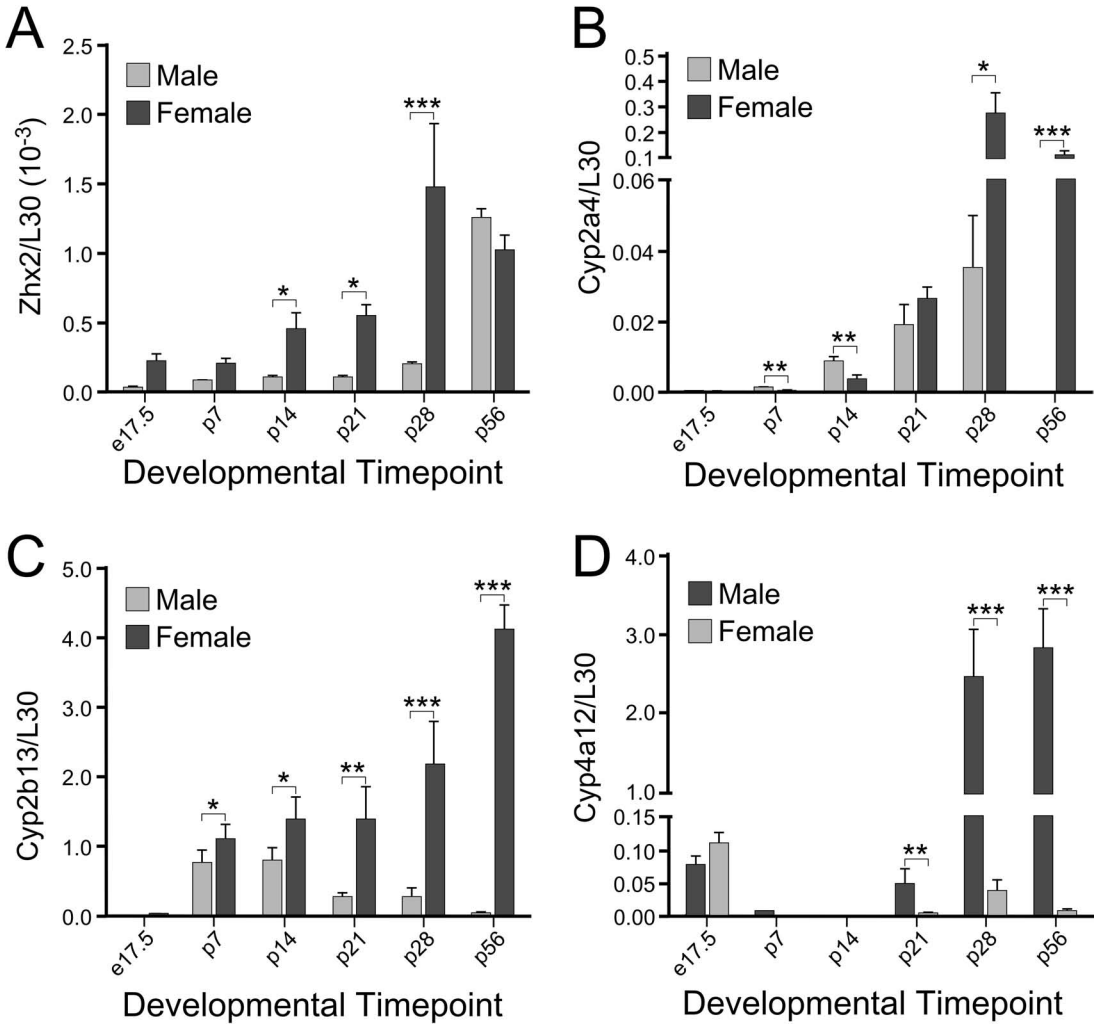

Figure 4. Developmental changes in Zhx2, Cyp2a4, Cyp2b13, and Cyp4a12 expression are different in male and female mice. Hepatic Zhx2 and Cyp mRNA levels, at e17.5 and various postnatal time points (n=4–10/group), were analyzed in male and female B6C3F1 mice by RT-qPCR and normalized to L30. (A) Female Zhx2 mRNA levels show a modest increase between p7 and p14 and again between p21 and p28. Male Zhx2 mRNA levels increase gradually between e17.5 and p28 and a more dramatic (~7-fold) increase between p28 and p56. (B) Cyp2a4 mRNA levels increase between e17.5 and p21 in male and female livers. Between p21 and p28, female hepatic Cyp2a4 levels increase over 10-fold and remain high at p56. In contrast, male hepatic Cyp2a4 levels decrease dramatically between 4 and 8 weeks of age to levels that are barely detectable. (C) Cyp2b13 mRNA levels increase between e17.5 and p7 in male and female livers. Between p14 and p56, female hepatic Cyp2b13 levels gradually increase; male Cyp2b13 levels decrease over this same period to levels that are barely detectable by p56. (D) Cyp4a12 mRNA levels are expressed at low but equal levels in both male and female livers at e17.5. Levels drop in both sexes after birth and remain very low in female liver. In male liver, Cyp4a12 levels increase over 50-fold between p21 and p28 and remain high at p56. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

liver cancers. This led us to ask whether female-biased *Cyp* genes, which are repressed by Zhx2 in male mice, are also activated in liver tumors. Male B6C3F1 mice were injected with DEN or PBS. After 36 weeks, mice were euthanized and livers were removed. Livers of DEN-injected mice had numerous tumors, whereas the livers of PBS-injected mice were tumor free. RNA was prepared from dissected tumors and the control PBS-injected liver and analyzed by RT-qPCR. Cyp2a4 levels were dramatically higher in the tumors compared to the control liver (Fig. 5), indicating that Cyp2a4 is activated in liver cancer. Expression of Cyp2b9 and Cyp2b13 was also increased in liver tumors, whereas the female-biased Cyp2b5 expression was unchanged in tumors. In contrast to the majority of female-biased Cyps, the male-biased *Cyp4a12* gene exhibited a substantial decrease in liver tumor samples compared to normal adult liver (Fig. 5).

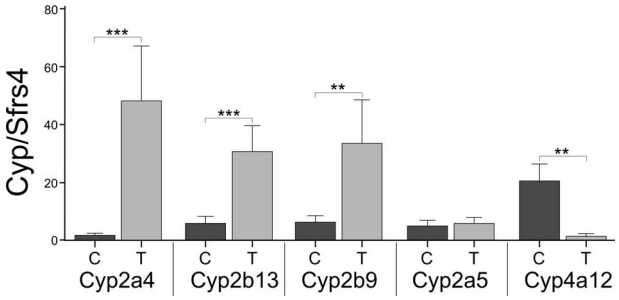

Figure 5. Cyp mRNA levels are altered in mouse liver tumors. B6C3F1 male mice were injected with PBS ($n=5$) or DEN ($n=16$) at p14 to induce HCC tumor formation. Livers were harvested 36 weeks postinjection. The DEN-injected mice had massive liver tumors, whereas no tumors were present in the PBS-injected livers. Levels of female-biased Cyp2a4, Cyp2b13, and Cyp2b9 increased significantly in tumors (T, gray bars) compared to PBS-injected livers (C, black bars); female-biased Cyp2a5 showed no change in tumors. In contrast, the male-biased Cyp4a12 decreased in tumor samples compared to normal liver. $^{**}p<0.01$; $^{***}p<0.001$.

DISCUSSION

Previous studies demonstrated that Zhx2 contributes to the silencing of fetal genes in the postnatal mouse liver[22–24,37]. Here, we provide the first evidence that Zhx2 also contributes to sex-biased gene expression in the adult liver by repressing female-biased *Cyp* genes in the adult male liver, indicating a link between developmental and sex-biased hepatic gene expression. Although Zhx2 contributes to the silencing of female-biased *Cyp* genes in male livers and of fetal-expressed genes, there are fundamental differences in these types of repression. Female-biased *Cyp* genes are derepressed in adult male hepatocytes in the absence of Zhx2, whereas expression of these genes is, in most cases, not altered in female *Zhx2*$^{\Delta hep}$ livers. In contrast, hepatic AFP, H19, and Gpc3 mRNA levels are elevated in both male and female mice when Zhx2 is deleted, indicating that Zhx2 represses these targets in both sexes. Furthermore, the temporal pattern of postnatal changes in sex-biased Cyps and previously identified Zhx2 targets are dramatically different. AFP, H19, and Gpc3 are abundantly expressed in the fetal liver, and mRNA levels of these genes decrease dramatically during the first 4 weeks after birth[22–24,37]. However, female-biased Cyp2a4 and Cyp2b13 are not expressed at e17.5 in the livers of either sex. Expression of both Cyps increases in male and female liver within the first several weeks after birth, but continues to increase in female livers and decrease in male livers. These changes are gradual for Cyp2b13 but more dramatic for Cyp2a4, with hepatic Cyp2a4 mRNA levels exhibiting a significant increase in females between p21 and p28 and a striking reduction in males between p28 and p56, ultimately leading to the ~1,000-fold difference in Cyp2a4 levels seen between 8-week-old male and female mice. Thus, sex differences in Cyp2a4 and, to a lesser extent with Cyp2b13, do not occur when fetal genes are being repressed but rather when mice are reaching sexual maturity. Variations in the cross talk between Zhx2 and other factors/signaling pathways could possibly account for differences in the silencing of fetal-expressed and sex-biased genes.

Previously identified Zhx2 targets, including AFP, H19, and Gpc3, are frequently activated in HCC in mice and humans[38]. Cyp2a4, Cyp2b13, and Cyp2b9 are also activated in DEN-induced mouse liver tumors, although the female-biased Cyp2a5, which does show a modest increase in Zhx2-deficient male livers, did not increase in tumors. Interestingly, the male-biased *Cyp4a12* gene, which does not appear to be a target of Zhx2, is decreased in liver tumors compared to normal adult liver. Similar changes in these *Cyp* genes in livers tumors were recently reported[39]. CYP2A6, a human CYP expressed in the liver that has the highest sequence homology to mouse Cyp2a4, is frequently upregulated in human HCC samples[40]. Taken together, these data suggest a potential role for Zhx2 in HCC progression via expression of target genes. Consistent with this possibility, nuclear Zhx2 protein levels are reduced in human HCC samples compared to nontumor liver tissue, suggesting that differential Zhx2 localization could account for the lack of repression of Zhx2 targets in HCC[41] (K. T. Creasy and B. T. Spear, unpublished observations). Since HCC is more common in males than in females in both humans and mice[42–44], it is interesting to consider whether Zhx2, through the control of its targets in a sex-biased manner, may contribute to this difference.

Most female-biased *Cyp* genes we analyzed remain unchanged in *Zhx2*$^{\Delta hep}$ females but are derepressed in the liver of adult male *Zhx2*$^{\Delta hep}$ mice, although Cyp mRNA levels are still considerably lower than what is found in wild-type adult female liver. In general, male-biased and unbiased *Cyp* genes do not respond to changes in Zhx2. Cyp2a5, which is highly related to Cyp2a4, shows a less pronounced female-biased expression and is less responsive to changes in Zhx2 expression. Interestingly, *Cyp* genes that exhibit the strongest female bias also have the greatest increase in *Zhx2*$^{\Delta hep}$ male mice. This is most evident with Cyp2b13, which exhibits a ~10^4 female bias in *Zhx2*^{fl} mice and increases over 30-fold in male livers in the absence of Zhx2 (Table 2). The three Cyps that show the greatest change in the absence of Zhx2 in male liver, Cyp2b13, Cyp2a4, and Cyp2b9, are tightly clustered on mouse chromosome 7; whether this indicates coordinated regulation will require further investigation. Consistent with our studies in knockout mice, these three Cyps were also found to be increased in BALB/cJ adult male liver[28].

Sex-biased *Cyp* gene expression is attributed to STAT5 activation via GH signaling[15,45]. STAT5b is necessary for expression of male-specific *Cyp* genes, whereas both

STAT5a and STAT5b are required for female *Cyp* gene patterning[16]. GH is secreted in a pulsating manner in males, while females have more steady GH circulation[17]. HNF4α, a key regulator of numerous hepatic genes, is necessary for the normal expression of multiple sex-biased *Cyp* genes in the liver[46,47]. A current model that integrates HNF4α with GH signaling suggests the pulsating spike in GH in male mice activates STAT5b, and possibly HNF4α, increasing transcription of male-specific *Cyp* genes. The continuous GH levels in females fail to fully activate STAT5b and therefore may not be sufficient to activate male *Cyp* genes; increased HNF6 and Foxa2 activity in females due to continuous GH signaling may contribute to female-biased Cyp expression[47,48]. Cux2 activates female-biased *Cyp* genes in female liver, whereas Bcl6 represses these genes in male liver[18,19]. Our studies indicate that Zhx2 contributes, in part, to the active repression of female-biased *Cyp* genes in the male liver. Whether this is due to interactions between Zhx2 and Bcl6 or other factors involved in GH/Stat5b signaling will require further studies. We have found that Stat5b, Cux2, Bcl6, Stat5a, and Stat5b mRNA levels are not altered in male or female adult liver of *Zhx2*$^{\Delta hep}$ mice (unpublished data), so regulation of these genes by Zhx2 cannot account Zhx2 repression of female-biased genes in male mice.

In conclusion, our studies expand considerably the number of hepatic genes controlled by Zhx2 and indicate that Zhx2 represses female-biased genes in adult male livers. Other studies indicate that lipoprotein lipase (*Lpl*), elongation of very long chain fatty acids-like 3 (*Elovl3*), and murine major urinary protein (*Mup*) genes are also targets of Zhx2 and misregulated in HCC (manuscript in preparation). This expanding number of Zhx2 targets, along with liver functions associated with these targets, provides further evidence that Zhx2 is an important regulator of hepatic gene expression in normal and diseased liver. Zhx2 is also expressed in other tissues, and at least some Zhx2 targets are also misregulated in whole-body Zhx2 knockout mice (unpublished observations), suggesting that Zhx2 has a role in controlling gene expression in multiple tissues. The floxed Zhx2 mice described here will be essential to fully understand the role of Zhx2 in health and disease.

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