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Zhx2 and Zbtb20: Novel regulators of postnatal alpha-fetoprotein repression and their potential role in gene reactivation during liver cancer

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Abstract

The mouse alpha-fetoprotein (AFP) gene is abundantly expressed in the fetal liver, normally silent in the adult liver but is frequently reactivated in hepatocellular carcinoma. The basis for AFP expression in the fetal liver has been studied extensively. However, the basis for AFP reactivation during hepatocarcinogenesis is not well understood. Two novel factors that control postnatal AFP repression, Zhx2 and Zbtb20, were recently identified. Here, we review the transcription factors that regulate AFP in the fetal liver, as well as Zhx2 and Zbtb20, and raise the possibility that the loss of these postnatal repressors may be involved in AFP reactivation in liver cancer.

Keywords

Alpha-fetoprotein; hepatocellular carcinoma; transcription; liver; development; mouse

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of cancer deaths worldwide¹. Currently, HCC is most common in Asia and sub-Saharan Africa. However, while relatively rare in Europe and North America, the incidence of HCC is increasing in the United States. Hepatitis B Virus (HBV) infection is the most common cause of HCC worldwide. Other risk factors include Hepatitis C Virus (HCV), alcohol-induced cirrhosis, metabolic disorders (i.e., Wilson's Disease and α 1-antitrypsin deficiency) and aflatoxins (in certain geographical regions). Prognosis is poor because HCC is frequently diagnosed at late stages, HCC is an aggressive cancer, and there is a lack of effective treatment options. Thus, there is a clear need for better biomarkers, improved treatment modalities, and increased understanding of HCC progression².

Historically, the most important diagnostic marker for HCC has been serum alpha-fetoprotein (AFP). While initially described in 1956 as an abundant fetal serum protein³, interest in AFP increased considerably in the early 1960s when Abelev and colleagues

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demonstrated that elevated serum AFP levels were associated with HCC⁴. Indeed, AFP was among the first oncofetal proteins to be described, i.e., proteins that are expressed during fetal development, normally absent after birth, and reactivated in tumors⁵. AFP has been used clinically since the 1970's as a diagnostic marker for HCC detection and to monitor HCC progression. While there is some debate over the clinical efficacy of AFP, it continues to be among the most widely used diagnostic biomarkers⁶. AFP expression in HCC has generated considerable interest in the basis of AFP regulation. Over the past several decades, the *cis*-acting regulatory regions of the AFP gene and the transcription factors that bind these elements have been characterized. Despite these advances, the basis for AFP reactivation in HCC remains poorly understood. Here, we will review regulatory factors that control AFP expression during development, focusing primarily on several recently identified regulators and their potential role in AFP reactivation during tumor progression.

Overview of AFP gene structure and protein function

The *AFP* gene, containing 15 exons that encode a 2.2 kb mRNA, is a member of the serum albumin gene family that consists of *albumin* (*alb*), *AFP*, *afamin* (*Afm*, also called *alpha-albumin*), *vitamin D binding protein* (*DBP*), and *AFP-related gene* (*Arg*). These genes arose from a primordial gene by a series of duplication events and exhibit a highly conserved intron-exon structure^{7,8}. The AFP protein is 609 and 605 amino acids in humans and mice, respectively. AFP functions as a serum transport protein and binds numerous molecules, including estrogen, fatty acids, bilirubin, steroids, heavy metals, various environmental compounds and certain drugs⁹. AFP has also been proposed to modulate immune function¹⁰ and to regulate intracellular signaling¹¹. However, several examples of congenital AFP deficiency have been observed in humans, with no apparent consequences. In addition, AFP-deficient mice are viable and develop normally, even though the deficient females are infertile because of an inadequate hormonal environment that blocks normal ovulation^{12,13}. Thus, AFP is not essential for fetal development, raising the possibility that some AFP functions are redundant with the functions of other albumin family members that are also expressed during fetal life. Hereditary persistence of AFP (HPAFP) is known to occur in adult humans without phenotypic consequences^{14–16}. Thus, the complete repression of AFP at birth also is not an essential process.

Regulation of AFP expression

AFP, like other members of the albumin gene family, is expressed primarily in the liver. In addition, AFP is abundantly expressed in the extraembryonic visceral yolk sac and at much lower levels in the fetal gut and kidney¹⁷. Embryonic expression of the human and mouse AFP genes are similar, although the human gene is expressed at higher levels in the developing kidney¹⁸. During mouse development, AFP is expressed at low but detectable levels in the foregut endoderm, but is highly activated in hepatoblasts that form the liver bud at about embryonic day 8.5 of development¹⁹. Indeed, AFP is one of the earliest markers of the hepatocyte lineage. AFP continues to be expressed at high levels in the fetal liver but is dramatically repressed during the first several weeks after birth; this repression results in a nearly 10,000-fold reduction in hepatic AFP mRNA levels²⁰. The AFP gene remains inactive in the adult liver under normal conditions. However, as mentioned above, AFP is frequently reactivated in HCC, leading to increased serum AFP levels that can be readily monitored. Elevated AFP levels can also be associated with non-neoplastic liver disease, including viral hepatitis and drug- or alcohol-induced liver damage. Increased serum AFP has also been associated with germ cell tumors (embryonal carcinoma and teratomas) and to a lesser extent with pancreatic, stomach and kidney tumors²¹.

Studies to identify the molecular mechanisms of AFP regulation have relied extensively on cultured hepatoma cell lines in which the AFP gene is expressed. A majority of these studies have been performed in HepG2 human hepatoma cells, although other hepatic cell lines have been used. While an advantage of cell lines is that they are easily maintained and readily transfected, they cannot be used to monitor changes in AFP expression that occur during development and in a diseased liver. AFP expression has also been extensively studied in transgenic mice^{22–29}. Taken together, studies in cell lines and mice have identified five distinct regulatory elements in the mouse gene, including the promoter located within the first ~200 bp upstream of exon 1, a repressor region and three independent enhancers, E1, E2, and E3, centered ~0.8, 2.5, 5.0 and 6.5 kilobases (kb) upstream of the AFP gene, respectively^{30,31}.

A number of liver-enriched transcription factors have been identified in the past twenty years^{32–34}. Since all of these factors are variably expressed in other tissues, there is no factor that is truly liver specific. Several of these factors have been identified to bind mouse AFP regulatory elements (Table I). The AFP promoter contains two binding sites for Hepatocyte Nuclear Factor 1 (HNF1), one centered at –120 and one at –60 (Figure 1)³⁵. Two HNF1 isoforms, HNF1 α and HNF1 β , are found in the liver³⁶, and both can bind the HNF1 sites in the AFP promoter, although HNF1 α is a more potent activator³⁷. The –120 HNF1 site overlaps with a binding site for Nuclear Factor I (NFI)³⁸. NFI proteins are ubiquitously expressed, are comprised of four family members (NFIA, NFIB, NFIC, and NFIX) and can act as both transcriptional activators and repressors³⁹. Naturally occurring mutations have been identified in the two HNF1 sites in the human AFP gene that lead to continued AFP expression in the postnatal liver (hereditary persistence of AFP, or HPAFP)^{14–16,40}. In all cases, the mutations result in increased HNF1 binding, suggesting that the HNF1 binding influences postnatal AFP silencing in the adult liver. Several binding sites for CAAT/Enhancer Binding Protein (C/EBP) are also present in the AFP promoter⁴¹. The –165 region of the AFP promoter binds Fetoprotein Transcription Factor (FTF) and Nkx2.8^{42,43}. Foxa1 and Foxa2 (formerly HNF3 α and HNF3 β) can also regulate the AFP promoter through the –165 bp region, even though Foxa proteins do not directly bind this region⁴⁴. While some of these factor binding sites are highly conserved between the mouse and human AFP promoters, others are less conserved (Fig. 1). The repressor region, centered around –850, binds Foxa proteins and the p53 family members p53 and p73^{45–47}. p53 binding blocks Foxa binding and alters chromatin structure, which has led to the suggestion that p53/p73 contributes to postnatal AFP silencing. Indeed, AFP repression is slightly delayed in p53-deficient mice⁴⁶.

Each of the three independent mouse AFP enhancers, E1, E2 and E3, are roughly 300 bp in length⁴⁸. E1 and E2 are similar, suggesting they arose from a duplication event. E2 is present in all species whereas E1 is found only in rodents⁴⁹; E1 has been shown to bind C/EBP⁵⁰. E3 is distinct from the other enhancers and has been the most extensively studied. It contains a nuclear receptor binding site as well as sites for Foxa (and also HNF6) and C/EBP⁴⁹. Transgenic mice studies indicate that all three enhancers are active in both the fetal and adult liver²⁵. Interestingly, while all three enhancers function in all hepatocytes in the fetal liver, their activities differ after birth. E1 and E2 are active in all hepatocytes but exhibit a gradual decrease in activity in a pericentral – periportal direction. In contrast, E3 is active in only 1–2 layers of hepatocytes surrounding the central vein²⁵; this absence of E3 activity in all but this small percentage of cells is due to active repression in non-pericentral hepatocytes⁵¹.

Most of the studies to define transcription factor binding sites in the AFP gene have used the mouse rather than the human gene. The mouse and human promoter regions are highly conserved, so similar binding factors likely contribute to regulating these genes.

Interestingly, although the E3 C/EBP site is conserved, the Foxa and nuclear receptor sites in this enhancer differ between human and mice⁴⁹.

Postnatal regulators of AFP

Most studies on AFP regulation have focused on factors found in the fetal liver, where AFP is expressed abundantly, rather than in the adult liver, where the AFP gene is silent. While increased AFP expression in HCC compared to normal adult liver could be due to increased positive regulation, it is also possible that the loss of repressive factors could lead to AFP reactivation. As mentioned earlier, p53/p73 negatively controls the AFP repressor region and the AFP enhancer E3 is actively repressed in non-pericentral hepatocytes. These data provide evidence that AFP silencing in the adult liver is due, at least in part, to active repression. Over the past several years, two novel factors that are involved in postnatal AFP silencing have been identified. These factors, Zinc-fingers and homeoboxes 2 (Zhx2) and Zinc finger and BTB domain containing 20 (Zbtb20), were identified using natural and targeted mutations in mice, respectively^{52,53}. In addition, a gene whose product has not yet been identified, Alpha-fetoprotein regulator 2 (Afr2), controls AFP reactivation during liver regeneration²⁰. These three factors provide an opportunity to understand better the basis for postnatal AFP repression, and may lead to new information about AFP reactivation in HCC. This section will provide an overview of our understanding of these AFP regulators.

Zhx2

Since our understanding of postnatal AFP silencing has been limited for many years, it is somewhat ironic that the first insight into AFP repression came from mouse studies performed more than thirty years ago. In 1977, Roushlatti and colleagues reported that adult serum AFP levels were 10- to 20-fold higher in BALB/cJ mice than in a number of other strains⁵⁴. This elevated AFP expression is a recessive, single gene trait, which they called *Regulator of Alpha-fetoprotein (Raf)* that was subsequently renamed *Alpha-fetoprotein regulator 1 (Afr1)*. Tilghman and colleagues showed that adult BALB/cJ mice had higher levels of steady-state hepatic AFP mRNA and this difference was seen in the adult liver but not in the fetal liver²⁰. In screening for cDNAs that were expressed in fetal liver but not the adult liver, Pachnis, et al, identified H19, a large non-coding mRNA that has become a model of genomic imprinting, as a second Afr1 target⁵⁵. *Afr1* was mapped to chromosome 15 (Chr 15), and was therefore unlinked to *AFP* (mouse Chr 5) and *H19* (mouse Chr 17)⁵⁶. These studies suggested that Afr1 is a repressor of AFP and H19 in the adult liver, and that a mutation in the BALB/cJ *Afr1* allele results in incomplete postnatal repression of AFP and H19 expression. More recently, the cell-surface proteoglycan Glypican 3 (Gpc3) was identified to be another target of Afr1⁵⁷.

Perincheri, et al., identified Afr1 by positional cloning and showed that it was the *Zhx2* gene⁵³. A mouse endogenous retroviral element (MERV) was found in the first intron of the BALB/cJ *Zhx2* gene⁵⁸. Transcription initiates correctly in the BALB/cJ *Zhx2* allele, but a majority of the primary transcript is spliced to the MERV element. Since a low level of wild-type *Zhx2* splicing occurs in BALB/cJ mice, this natural mutation is a hypomorph rather than a null mutation. Expression of a *Zhx2* transgene in hepatocytes, by linking a *Zhx2* cDNA to a transthyretin enhancer/promoter cassette, resulted in complete repression of AFP and H19 in the adult BALB/cJ liver. In addition, *Zhx2* levels normally increase after birth, concomitant with AFP repression. Taken together, these data indicate that *Zhx2* is indeed required for normal AFP silencing in postnatal hepatocytes.

The *Zhx2* protein in humans and mice is 837 and 836 amino acids in length, respectively (Fig. 1). *Zhx2* belongs to a small family of proteins, with *Zhx1* and *Zhx3*, which are found only in vertebrates. They all share a similar gene and protein structure⁵⁹. *Zhx1* and *Zhx2* are

tightly linked to each other on mouse Chr 15 (Chr 8 in humans), whereas *Zhx3* is on Chr 20. These three genes have an unusual structure in that the entire protein coding regions are found on one unusually large internal exon. These proteins contain two C2H2 zinc fingers and four or five homeodomains, which suggest nucleic acid binding. *Zhx1* was originally identified in a yeast 2-hybrid screen for NF-YA-interacting proteins; another lab identified *Zhx1* by immunoscreening a bone marrow stromal cell cDNA library^{60,61}. *Zhx2* and *Zhx3* were subsequently identified in a yeast 2-hybrid screen for *Zhx1*-interacting proteins^{62,63}. All three *Zhx* proteins can form homodimers and heterodimers with each other and with NF-YA. Structural studies suggest that *Zhx2* homeodomain 2 has an unusual conformation⁶⁴.

Zhx2 and other family members have been called transcriptional repressors based on modest (up to two-fold) activity in a one-hybrid assay^{62,63}. Similar repressive effects have been seen in other tissue culture models^{65,66}. However, this modest level of repression is unlikely to fully represent *Zhx2* activity *in vivo*, since, in BALB/cJ mice, the reduction of *Zhx2* results in about a twenty-fold increase in AFP and H19 expression. Transgenic mouse studies showed that *Zhx2* works through the 250 bp mouse AFP promoter, which are consistent with tissue culture studies indicating that *Zhx2* acts through the human AFP promoter^{24,66}. The fact that the AFP promoter can confer *Zhx2* regulation on a heterologous reporter gene suggests that *Zhx2* functions at the level of transcription. However, nuclear run-on studies by Vacher, et. al,²⁸ and confirmed by us (L. Morford, B.T.S., and M.L.P, unpubl. obs.) showed that RNA polymerase II loading differences could not account for the difference in steady state AFP and H19 mRNA levels, suggesting that *Zhx2* acts at a post-transcriptional level. One model to account for these seemingly contradictory results is that *Zhx2* acts at the post-transcriptional level in a promoter-dependent manner. The coupling of transcription and post-transcriptional steps of gene expression is supported by multiple *in vitro* studies^{67,68}. Further analysis is warranted to elucidate this potentially novel mechanism by which *Zhx2* regulates target gene expression.

More recent experiments suggest that AFP, H19 and *Gpc3* are not the only targets of *Zhx2* regulation. A quantitative trait locus (QTL) on mouse Chr 15 called *Hyperlipidemia 2* (*Hyplip2*) controls the extent of atherosclerosis and serum triglycerides when mice were fed a high fat diet; BALB/cJ mice have reduced serum triglycerides and atherosclerotic plaques compared to other mouse strains⁶⁹. Recently, the *Hyplip2* phenotype in BALB/cJ mice was shown to be due to the *Zhx2* mutation⁷⁰. This study identified Lipoprotein lipase (*Lpl*) as a target of *Zhx2*, and there are likely other enzymes involved in hepatic control of lipid homeostasis that are controlled by this factor. Interestingly, *Lpl*, like AFP, H19 and *Gpc3*, is highly expressed in fetal liver, repressed at birth, and reactivated in HCC⁷¹.

Zbtb20

Zbtb20 (also known as DPZF, HOF, and *Zfp288*) was initially described in a screen of genes expressed in human dendritic cells⁷². *Zbtb20* is a member of a large family of proteins that contain an N-terminal BTB (Broad complex, tramtrack, *bric-a-brac*) domain, involved in protein-protein interactions, and multiple C-terminal Kruppel-like C2H2 zinc fingers (four in the case of *Zbtb20*) that mediate interactions with nucleic acids (Fig. 1). Proteins in this family often act as transcriptional repressors but in some cases can activate target genes. The main isoforms of *Zbtb20* in humans and mice are 741 and 733 amino acids in length. The liver-specific deletion of *Zbtb20*, accomplished by crossing *Zbtb20* floxed mice with Albumin-Cre transgenic mice, resulted in the persistence of AFP expression in adult mouse livers⁵². This continued expression was not dependent on cell proliferation; hepatocytes were equally quiescent in the adult livers of wild-type and hepatocyte-deleted *Zbtb20* mice. Furthermore, adult livers appeared normal in the absence of *Zbtb20*, indicating that this protein was not essential for normal liver development. However, *Zbtb20* is required for

normal glucose homeostasis⁷³. Transient co-transfections indicate that Zbtb20 regulates AFP expression through a region in the promoter between -162 and +27⁵². Consistent with this, EMSAs indicated that the zinc finger domain of Zbtb20 bound directly to a region of the AFP promoter between -108 and -53. Furthermore, hepatic Zbtb20 expression increases during the first month after birth in a manner that parallels AFP repression. Taken together, this data is consistent with Zbtb20 being an important repressor of AFP in the postnatal mouse liver.

Afr2

In addition to being reactivated in HCC, AFP is also transiently reactivated during liver regeneration. In contrast to most strains of mice where AFP reactivation is readily apparent, C57BL/6 mice show minimal AFP reactivation²⁰. This strain-specific difference in AFP induction is governed by a gene called *Alpha-fetoprotein regulator 2* (*Afr2*). The *Afr2^b* allele in C57BL/6 mice is co-dominant with the *Afr2^a* allele found in other mouse strains⁵⁵. Although *Afr2* has been mapped to mouse Chr 2, the *Afr2* gene has not yet been identified⁷⁴. *Afr2* regulates both AFP and H19 and we have identified Gpc3⁵⁷ and Lpl (H. Ren and B.T.S., unpubl. obs.) to be two additional *Afr2* targets. Interestingly, these four genes are targets of both *Zhx2* and *Afr2* and all four are frequently reactivated in HCC, suggesting that *Afr2* may be involved in liver cancer progression. However, this possibility has not been formally tested. It is interesting that C57BL/6 mice are resistant to hepatocarcinogenesis compared to most other strains, it is not known if *Afr2* contributes to this difference, which is likely due to polymorphisms at multiple loci⁷⁵.

Postnatal regulators and HCC

The role of *Zhx2* and Zbtb20 in postnatal AFP repression raises the possibility that these factors could be involved in AFP reactivation in HCC. Specifically, one model would predict that the loss of these repressors would lead to elevated AFP expression. Although not much is known about *Zhx2* and Zbtb20, including the mechanism by which they control target gene expression, several lines of evidence suggest that these factors may be involved in HCC gene expression changes. Lv, et al. performed methylation-sensitive restriction fingerprinting to identify sequences that were differentially methylated between HCC and adjacent non-tumor tissues⁷⁶. This analysis identified the *Zhx2* promoter as being hypermethylated in HCC at a significantly higher frequency than surrounding tissue. They also showed that *Zhx2* expression was silenced in HCC compared to normal liver, and this silencing was correlated with increased *Zhx2* promoter methylation. Although the sample number was small in this study, it did suggest that the loss of *Zhx2* expression correlated with AFP reactivation. However, it should be noted that another study suggested that *Zhx2* was increased in liver cancer⁷⁷. This second study relied on immunohistochemistry, which is likely to be less reliable than the molecular study performed by Lv, et al.⁷⁶. Data linking Zbtb20 and HCC is more limited. However, a recent transposase-based mutagenesis screen in mice identified Zbtb20 as one of 19 loci that were frequently mutated in HCC⁷⁸. Unfortunately, this screen did not determine whether the mutations were activating or inactivating. (It should also be noted that this screen did not score for mutations on Chromosome 15, the location of *Zhx2*). In addition, microarray analysis indicated that Gpc3 is upregulated in Zbtb20-deficient livers⁷³. While it will be necessary to identify the product of the *Afr2* locus to evaluate its expression and potential role in liver cancer, it is interesting that all the targets of *Zhx2* are also targets of *Afr2*. Overall, these studies provide compelling evidence to explore further the role of these factors in AFP reactivation and other events during liver tumor progression.

Microarray studies of HCC gene expression

Multiple microarray analyses of global gene expression changes between HCC samples and normal liver tissue have been performed^{79–82}. Despite variations among the microarray platforms used, these studies consistently identified subclasses of HCC based on gene expression signatures. About two-thirds of HCC reactivate AFP expression and the gene expression signatures of AFP+ vs AFP- tumors are distinct^{82,83}. While multiple changes in liver-enriched transcription factors have been reported, consistent differences aren't always seen and the relevance to these in initiating or driving HCC progression is not clear^{79,80,84,85}. However, the tumor suppressor p53 is often mutated in HCC; since p53 is also involved in AFP repression, the loss of this protein may contribute to AFP de-repression⁸⁶. While some studies suggest that p53 mutations correlate with AFP activation⁸⁷, other studies have found no association between p53 mutations and elevated AFP⁸⁸. The postnatal repressors *Zhx2* and *Zbtb20*, being genes more recently identified, were not always present on the microarrays and thus data on them is limited. Microarray analyses can identify a set of consistently altered genes to serve as a “gene expression signature” as a way to classify tumors and this can be related to prognosis. They can also inform functional gene expression studies. However, limitations of microarray analyses, such as incomplete gene sets and the correlative nature of the data, also highlight the importance of other approaches, such as genetic and biochemical experiments, to identify and study the relevance of individual genes in the process of HCC formation and progression.

Conclusions

Numerous studies have convincingly demonstrated a strong correlation between AFP activation and HCC. It is not yet clear if AFP reactivation actively contributes to cell growth and HCC progression, or whether AFP is simply a benign marker of this disease. In either case, understanding AFP gene regulation pathways activated in HCC should provide important insights into this disease. Recent studies identifying *Zhx2* and *Zbtb20* as postnatal repressor of AFP provides new avenues to understand better AFP regulation in the adult liver which, in turn, will help elucidate the basis for global changes in gene expression during HCC. These studies could also identify additional biomarkers of HCC that will be of value for better prognostic and diagnostic strategies. There is also the potential that increased understanding of the signaling pathways and factors that govern AFP reactivation will provide new targets for novel treatment strategies.

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Figure 1.

Alignment of the mouse and human AFP promoters and locations of major transcription factor binding sites. The upper rows represent the mouse sequence, the lower rows represent human sequence; vertical lines indicate conserved nucleotides. The arrows represent the sites of transcription initiation (designated +1), nucleotides in italics correspond to the 5' end of exon 1. Locations of factor binding sites are shown. While most of these sites are conserved between mouse and human, the C/EBP sites, distal Nkx2.8 site (-176 to -170), as well as the 3' end of the NFI site, are less conserved.

Figure 2. Diagram of the 836 amino acid Zhx2 (top) and 733 amino acid Zbtb20 (bottom) mouse proteins showing predicted motifs. The cross-hatched regions indicate the C2H2 zinc fingers, stippled regions in Zhx2 indicate homeodomains, and dark grey region in Zbtb20 indicates the BTB (Broad complex, tramtrack, *bric-a-brac*) domain.

Table I

Transcription Factor Binding Sites in AFP gene

<u>Region</u>	<u>Site</u>	<u>Factor</u>	<u>Sequence</u>	<u>Reference</u>
Promoter	−62 to −50	HNF1	GTTACTAGTTAAC	35,37
	−77 to −68	C/EBP	GTTTGCTCAC	35
	−113 to −105	C/EBP	ATTGCCTAA	35,41
	−120 to −106	NFI	TTGGCAAATTGCCTA	35,38,41,89
	−128 to −116	HNFI	GTTAATTATTGGC	35,38,41,89
	−156 to −162	Nkx2.8	TGAAGGA	90
	−176 to −170	Nkx2.8	TGAAGTG	90
	−164 to −154	FTF	TGTCCTTGAAC	42,90
	−172 to −156	Foxa	?	44
	−108 to −53	Zbtb20	?	52
	−250 – +1	Zhx2	?	53
Repressor	−853 to −844	p53/p73	AAACATGTCT	45
	−849 to −855	Foxa	TGTTTGC	45
	−1,010 to −838	Afr2	?	47
Enhancer E3	−6572 to −6563	C/EBP	GTTGCCCAAT	49,91
	−6618 to −6609	Foxa/HNF6	TTGACTTTGA	49,91
	−6644 to −6649	Nuclear Receptor	AGGTCA	49,91