Published in final edited form as:
*Hepatology*. 2012 November ; 56(5): 1892–1901. doi:10.1002/hep.25819.

# Pericentral activity of AFP enhancer E3 and glutamine synthetase upstream enhancer in the adult liver are regulated by β-catenin

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## Abstract

We previously showed that mouse alpha-fetoprotein enhancer E3 activity is highly restricted to pericentral hepatocytes in the adult liver. Here, using transgenic mice, we show that the upstream enhancer of the rat glutamine synthetase gene is also active specifically in pericentral regions. Activity of both enhancers is lost in the absence of β-catenin, a key regulator of zonal gene expression in the adult liver. Both enhancers contain a single highly conserved TCF/LEF binding site that is required for responsiveness to β-catenin. We also show that endogenous AFP mRNA levels in the perinatal liver are lower when β-catenin is reduced. These data identify the first distinct zonally-active regulatory regions required for β-catenin responsiveness in the adult liver and suggest that postnatal AFP repression and the establishment of zonal regulation are controlled, at least in part, by the same factors.

### Keywords

zonal gene regulation; TCF/LEF; development; transcription; hepatocellular carcinoma

The liver performs numerous metabolic and homeostatic functions in the body, including xenobiotic metabolism, energy storage/production, urea formation, glutamine synthesis and cholesterol homeostasis (1). Many of these functions require the unique architecture of the liver, which is comprised of periportal and pericentral regions (2). Certain hepatic enzymes are expressed solely in periportal regions along this porto-centro axis, whereas other enzymes are synthesized in pericentral regions. This compartmentalization of function, or "liver zonation", enables the liver to perform multiple, and sometimes opposing, metabolic pathways in distinct hepatocyte subpopulations (3).

The pericentral expression of glutamine synthetase (GS) was the first example of zonal gene regulation in the adult liver (4). Since this discovery, numerous additional enzymes were found to exhibit zonal patterns of expression in the liver (5). It is generally believed that

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certain blood-borne compounds (i.e., oxygen, nutrients) that form a gradient along the porto-centro axis provide signals that establish the heterogeneity of gene expression in the liver, although the nature of such signals is not fully understood (6). Regardless of the stimulus, intracellular signaling pathways must link extracellular events to the nucleus to govern zonal gene regulation. An elegant study by Benhamouche, et al., demonstrated that signaling through β-catenin, a downstream activator of Wnt, governs zonal gene regulation in the adult liver (7). In the absence of Wnt signaling, cytosolic β-catenin is complexed with adenomatous polyposis coli (APC), Axin and the kinases GSK-3β and CK1. This inhibitory complex phosphorylates β-catenin at specific serine residues that mark it for ubiquitin-mediated proteolysis. In the absence of this phosphorylation (i.e., when blocked by Wnt signaling), β-catenin enters the nucleus and regulates target gene expression. Regarding zonal regulation, activated β-catenin (through expression of a non-degradable form of β-catenin or loss of APC) is associated with increased expression of GS (and other pericentral genes) and decreased expression of periportal genes in periportal regions (7–9). In contrast, blocking β-catenin signaling results in a loss of pericentral enzymes and increased periportal enzyme expression (7).

β-catenin does not bind DNA directly, but can regulate target genes through several pathways. In the canonical pathway, β-catenin controls target genes via interactions with the T Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) family of transcription factors (10). In the absence of β-catenin, TCF/LEF proteins are bound to consensus motifs and silence target genes by recruitment of co-repressors such as Groucho (11). Upon nuclear entry, β-catenin interacts with TCF/LEF proteins, dissociating repressors and recruiting the co-activators CBP/p300, leading to target gene activation (12).

Using transgenic mice, we showed previously that the activity of alpha-fetoprotein (AFP) enhancer 3 (E3), one of the three AFP enhancers, is highly restricted to a single layer of pericentral hepatocytes in the adult liver (13, 14), a pattern identical to GS and other highly restricted pericentral enzymes. AFP is expressed abundantly in the fetal liver and silenced at birth, but can be transiently reactivated during liver regeneration and is often activated in hepatocellular carcinoma (HCC)(15). Here, we show that pericentral activity of E3 is β-catenin dependent. Using transgenic mice, we also show that the upstream enhancer of the rat *GS* gene can confer pericentral activity to a linked reporter gene, and that the activity of this enhancer is also β-catenin-dependent. Both enhancers contain a highly conserved TCF site that binds TCF4 *in vitro* and mutation of these TCF sites results in a loss of β-catenin responsiveness in cultured cells. We also show that E3 activity and endogenous AFP expression in the perinatal liver are reduced in the absence of β-catenin, suggesting that β-catenin regulates AFP during liver development. These data identify the first defined zonally-regulated *cis*-acting control regions that confer β-catenin responsiveness.

## Materials and Methods

### Plasmids

E3 was excised from E3-βgl-$D^d$ (14) and cloned into pGL3-promoter (Promega) to generate E3-Luc. $RGS^e$ was amplified from rat DNA using primers $RGS^e$U and $RGS^e$L (Table I) and cloned into βgl-$D^d$ to generate $RGS^e$-βgl-$D^d$ or into pGL3-promoter to generate $RGS^e$-Luc.

Megaprimer mutagenesis (16) using primers listed in Table I generated TCF site mutations in E3-Luc and RGS$^{e}$-Luc; mutations were confirmed by DNA sequencing. TOP-Flash and FOP-Flash were provided by H. Clevers (17). Expression vectors for wild-type β-catenin, βcatS37A, and TCF4 were provided by S. Byers (18) and Chunming Lui (19).

### Cell culture and luciferase assays

Hep3B human hepatoma cells and HEK293 cells were maintained and transfections were performed as described (20). Hep3B cells were seeded into 12-well dishes and transfected in duplicate with 500 ng of luciferase reporter plasmid, 1μg of expression plasmid and 12.5 ng of renilla luciferase plasmid. To prepare nuclear extracts, HEK293 cells were seeded onto 10 cm plates and transfected with 15 μg of the Flag-TCF4 expression plasmid (or mock transfected). For both cell types, media was changed after six hours and cells harvested 48 hours later. Transfected Hep3B cells were harvested into Glo-lysis buffer (Promega Corp.) and firefly/renilla luciferase levels were determined in duplicate. All transfections were repeated at least twice. Results were analyzed using the Student's *t*-test with $p$ values $< 0.05$ considered to be statistically significant.

### Electrophoretic mobility shift assay (EMSA)

Nuclear lysates were collected from HEK293 cells using the NE-PER extraction kit (Thermo-Scientific). Protein concentrations were determined using the BCA protein assay (Pierce). Labeling of annealed oligonucleotides (Integrated DNA Technologies, Table I) with $^{32}$P and EMSAs were carried out as described (21).

### Mouse studies

The E3-βgl-D$^{d}$ transgenic mice were described (14). RGS$^{e}$-βgl-D$^{d}$ founder mice were generated at the University of Kentucky Transgenic Mouse facility. Mice were screened for the transgene by PCR analysis of tail DNA. Mice containing *Albumin-Cre* (*Alb-Cre*)(22) and the floxed β-catenin (*Ctnnb1)* gene (23) were purchased from The Jackson Labs. Standard breeding was performed to obtain mice of the appropriate genotype. Oligonucleotides and PCR conditions for screening genetically modified mice are available upon request. All mouse experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee, following guidelines established by the NIH.

### Immunohistochemistry

Frozen livers were sectioned at 10 μm thickness. For transgene analysis, slides were incubated overnight with FITC-conjugated anti-H2-D$^{d}$ monoclonal antibodies (BD-Pharmingen 553579). For β-catenin and GS, antibodies against active β-catenin (Millipore 05-665) and GS (Sigma G2781) were used. Staining details are available upon request.

### Hydrodynamic Tail Vein Injections/Flow Cytometry

Eight-week old E3-βgl-D$^{d}$ mice were injected with 2.5 ml of 0.9% saline containing 50 μg of plasmid (24). After 48 hours, mice were sacrificed and livers harvested. Livers, along with 5 ml of RPMI containing 10% FBS, were placed in a stomacher bag and compressed for 60 seconds using Stomacher-80 Laboratory blender (Seward Lab Systems). Cells were

transferred to 15 ml conical tubes along with DNaseI (160U/ml) and collagenase (400U/ml) and incubated at 37°C for 20 min, passaged through a 40 micron strainer, centrifuged, and washed with 2 ml ice-cold PBS with 0.1% BSA/0.1% Azide (PBS-BSA-Az). Hepatocytes were purified by percoll centrifugation, and stained with FITC-anti-H2-$D^d$ or control IgG followed by flow cytometry at the University of Kentucky Flow Cytometry Facility.

### RNA analysis

RNA was prepared using 2 rounds of Trizol (Life Technologies) and processed into cDNA using qscript (Quanta Biosciences). Quantitative PCR was carried out using Sybr Green (Quanta Biosciences) using the Bio-Rad MyiQ thermal cycler. AFP and β-catenin mRNA levels were normalized to ribosomal gene L30, using primers shown in Table I.

## Results

### The upstream GS enhancer exhibits pericentral activity in transgenic mice

Pericentral GS expression was first described in 1983 (4, 25), and GS continues to be the most extensively studied pericentral gene. Several elements controlling GS activity have been identified in tissue culture cells, including a single upstream enhancer centered 2.2 kb upstream of exon 1 and several regulatory regions within the first intron (26–30). Previous studies indicated that the 3.2 kb region upstream of the rat GS gene (−3150 to +59) could confer pericentral expression of a linked reporter gene in transgenic mice (31). To test whether the upstream enhancer of the rat GS gene exhibited pericentral activity by itself, this enhancer was cloned as a 400 bp fragment {as defined in (21) and which will be referred to as $RGS^e$} and fused to the heterologous human β-globin promoter linked to the mouse H-$2D^d$ reporter gene (βgl-$D^d$). We have used βgl-$D^d$ extensively to monitor gene expression in cells and transgenic mice; this cassette is inactive in all mouse tissues but is highly responsive to linked enhancers (14). Furthermore, transgenes with βgl-$D^d$ fused to the albumin enhancer are expressed throughout the adult liver, demonstrating that the β-globin promoter can be activated in all adult hepatocytes (unpublished data). Several founder mice containing the $RGS^e$-βgl-$D^d$ transgene were generated. Immunofluorescence staining using anti-$D^d$ antibodies indicated that transgene expression was restricted to a single layer of pericentral hepatocytes in the adult liver (Fig. 1). This pericentral activity is identical to the expression of endogenous GS and to E3- βgl-$D^d$ transgenes, which we previously showed to exhibit pericentral expression in the adult liver (13, 14). This data demonstrates that the upstream GS enhancer, $RGS^e$, is sufficient to confer highly restricted pericentral activity in the adult liver in the absence of other GS regulatory regions.

### E3 and $RGS^e$ enhancer activities are dependent on β-catenin

Numerous genes expressed in pericentral regions are controlled by β-catenin signaling, with active β-catenin being found only in pericentral hepatocytes of the adult liver (7). Since E3 activity is highly restricted to this population of hepatocytes, we predicted that pericentral activity of this enhancer would co-localize with active β-catenin. To test this, we co-stained liver sections of adult E3-βgl-$D^d$ mice with antibodies against $D^d$ and active (unphosphorylated) β-catenin (Fig. 2A). Consistent with previous studies, we found active β-catenin localized to pericentral hepatocytes (7). E3-βgl-$D^d$ expression and active β-catenin

overlap in these pericentral cells. This co-localization of $D^d$ and active β-catenin supports the possibility that active β-catenin is required for E3 activity.

The phosphoryation and subsequent degradation of β-catenin accounts for its absence in non-pericentral hepatocytes. In contrast to the wild-type protein, a mutant form of β-catenin in which the serine at position 37 is changed to alanine (βcatS37A) cannot be phosphorylated and is less susceptible to degradation (18). Previous studies showed that adenovirus-mediated βcatS37A overexpression resulted in increased GS expression throughout the liver lobule (8). We used hydrodynamic tail-vein injection to express βcatS37A in the liver of adult E3-βgl-$D^d$ mice. Three days after injection, hepatocytes were purified and analyzed by flow cytometry with anti-$D^d$ antibodies. In control mice, roughly 3% of hepatocytes, presumably representing pericentral cells, stained positive for $D^d$ (Fig. 2B). In contrast, 30% of hepatocytes expressed $D^d$ in livers from βcatS37A-injected mice. This provides evidence that AFP enhancer E3 can be activated in non-pericentral hepatocytes by constitutively active β-catenin.

If β-catenin is required for the activity of pericentral enhancers, its absence should result in the loss of enhancer activity in the adult liver. To explore this possibility, we crossed E3-βgl-$D^d$ and $RGS^e$-βgl-$D^d$ transgenic mice to mice that were homozygous for a floxed allele of the β-*catenin* gene (*Ctnnb1*) and expressed the *Alb-Cre* transgene. Previous studies showed that *Alb-Cre* transgene expression leads to a loss of β-catenin in essentially all hepatocytes of adult mice (32). Expression of both E3-βgl-$D^d$ (Fig. 3A) and $RGS^e$-βgl-$D^d$ (Fig. 3B) transgenes was completely absent in hepatocytes lacking β-catenin ($\beta Cat^{\Delta liv}$), whereas both transgenes continued to be expressed in hepatocytes of $\beta Cat^{fl}$ mice that did not contain *Alb-Cre*. As expected, endogenous GS proteins were also absent in β-catenin deficient livers (Fig. 3).

## β-catenin regulates E3 and $RGS^e$ through conserved TCF/LEF sites

β-catenin does not bind DNA directly but can regulate target gene expression through several mechanisms. In the canonical pathway, nuclear β-catenin replaces repressors interacting with bound TCF/LEF factors with coactivators. To explore further the control of E3 and $RGS^e$ by β-catenin, we searched for TCF sites in these two defined enhancers. AFP enhancer E3 contains a strong TCF/LEF site located towards the 3' end of this 340 bp element. Previous studies had identified a single TCF/LEF site in the 3' end of $RGS^e$ (33); our analysis indicates that this is the only TCF/LEF site in this 400 bp enhancer (33). The TCF sites in these two enhancers are highly conserved, suggesting that they are important for the activities of these elements (Table II).

EMSAs were used to determine whether the conserved TCF/LEF sites in E3 and $RGS^e$ could bind TCF4. Nuclear extracts were prepared from HEK293 cells that were mock-transfected or transfected with a FLAG-tagged TCF4 expression plasmid. Using extracts from TCF4-transfected cells, TCF4 binding could be detected using radiolabeled oligonucleotides containing the conserved TCF sites from E3 and $RGS^e$ (Fig. 4A and 4B, respectively); these complexes were not present in mock-transfected cell extracts. In both cases, oligonucleotides containing a consensus TCF site, as well as unlabeled self-fragments, could effectively compete for TCF4 binding. In contrast, mutant forms of these

oligonucleotides could no longer act as cold competitors. The ability of anti-FLAG antibodies to supershift the bands with E3 and RGS$^{e}$ oligonucleotides confirmed the presence of TCF4 in these complexes. When the consensus TCF oligonucleotide was used as a radiolabeled probe, wild-type E3 and RGS$^{e}$ oligonucleotides could effectively compete for binding whereas mutant forms of these oligonucleotides could no longer compete (data not shown).

TCF4 regulation of E3 and RGS$^{e}$ was explored further using transient transfections. Both enhancers were linked to pGL3-Luciferase and transfected into Hep3B cells along with wild-type β-catenin or βcatS37A. Hep3B cells were used since they have low endogenous β-catenin levels. TOP-Flash and FOP-Flash vectors, which contain 3 copies of consensus or mutated TCF sites, respectively, upstream of the minimal c-fos promoter, were included as controls (17). As expected, TOP-Flash responded to increasing βcatS37A, as determined by normalized luciferase levels, whereas FOP-Flash was unresponsive to co-transfected βcatS37A (Fig. 5). Similar results were seen when wild-type β-catenin was transfected, although the extent of TOP-Flash activation was less than that seen with βcatS37A (data not shown). Both E3-Luc and RGS$^{e}$-Luc were activated by βcatS37A (Fig. 5) and to a lesser extent by β-catenin (data not shown). Enhancers containing mutated TCF/LEF sites were also assayed for responsiveness to β-catenin. Mutation of the TCF4 site in both enhancers resulted in increased basal activity compared to wild-type enhancers, which is likely due to a release of repression from TCF4-associated co-repressors. The mutant E3-Luc reporter gene showed a ~2-fold induction in response to βcatS37A (Fig. 5) or β-catenin (data not shown); this modest activation was less than the wild-type enhancer and did not change with increasing amounts of βcatS37A. RGS$^{e}$-Luc with the mutated TCF4 site showed no responsiveness to βcatS37A or β-catenin (Fig. 5 and data not shown).

### Control of E3 activity and endogenous AFP expression by β-catenin in perinatal livers

In contrast to the pericentral expression seen in the adult liver, E3-βgl-D$^{d}$ transgenes are expressed in all hepatocytes in the fetal liver (14). A gradual loss of E3 activity in periportal hepatocytes occurs during the perinatal period, which led us to consider whether E3 activity during this time is also dependent on β-catenin. To test this, we monitored E3-βgl-D$^{d}$ expression by immunofluorescence at postnatal day 1 (p1) in βCat$^{\Delta liv}$ mice (Fig. 6A). We chose p1 rather than prenatal timepoints since *Alb-Cre* expression begins later during fetal development and we wanted to allow maximal time for Cre to delete the floxed *Ctnnb1* allele (22). In the presence of β-catenin, E3-regulated transgenes are zonally expressed, although activity is not yet fully restricted to a single layer of hepatocytes (Fig. 6A). Similarly to what was seen in the adult liver, E3-βgl-D$^{d}$ transgenes were not expressed in p1 βCat$^{\Delta liv}$ livers; the small number of cells that still express D$^{d}$ likely represents hepatocytes in which the *Ctnnb1* gene had not yet been deleted (Fig. 6A). Since endogenous AFP expression in the developing liver requires the AFP enhancer region (34), we also analyzed hepatic AFP mRNA levels in p1 βCat$^{\Delta liv}$ mice. While AFP mRNA levels varied between mice, we found a significant reduction in AFP mRNA levels when β-catenin levels were low (Fig. 6B). This data suggests that β-catenin is required for normal AFP expression in the developing liver.

## Discussion

The compartmentalization of function enables the adult liver to carry out a variety of different and in some cases opposing functions. Previous studies showed that the β-catenin signaling pathway has an important role in regulating zonally-expressed genes in the adult liver, although the mechanism by which β-catenin regulates target genes is not fully understood. Here, we have shown that two defined enhancer elements that exhibit pericentral activity in the adult liver, E3 and RGS$^e$, are regulated by β-catenin. This was accomplished by demonstrating overlapping E3 activity and active β-catenin expression in the adult liver, increased activation of E3-regulated transgenes in βcatS37A-overexpressing livers, and a loss of E3- and RGS$^e$-regulated transgene expression in the absence of β-catenin. Furthermore, we identified evolutionarily conserved TCF/LEF sites in these enhancers that are required for β-catenin responsiveness. We also showed that endogenous AFP expression is reduced in the perinatal liver in the absence of β-catenin, indicating that this pathway also contributes to developmental AFP regulation.

Our data indicate that the canonical pathway involving β-catenin and TCF4 contributes to E3 and RGS$^e$ activity in cultured cells. The 340 bp E3 and 400 bp RGS$^e$ enhancers were both found to contain a single, highly conserved consensus TCF/LEF site that could bind TCF4. Both enhancers were activated by wild-type and the constitutively active S37A variant of β-catenin. This data provides strong evidence that the TCF/LEF sites in E3 and RGS$^e$ are essential for β-catenin-mediated regulation. When the TCF/LEF sites of E3 and RGS$^e$ were mutated, their basal activities increased in the absence of co-transfected β-catenin. Since TCF/LEF factors can bind the Groucho family of co-repressors in the absence of β-catenin, it is not surprising that we saw a de-repression of enhancer activity when TCF/LEF proteins and associated co-repressors could no longer bind their cognate sites. These results raise the question whether these co-repressors also contribute to zonal gene regulation in the adult liver. In this regard, it is interesting that overexpression of the Groucho-related co-repressor Grg3 in H2.35 liver cells reduced endogenous AFP mRNA levels (35). At least one groucho-related protein, Grg5, is expressed in the adult mouse liver (36, 37).

Mouse AFP enhancer E3 was originally defined as a 340 bp element by deletion analysis (38). Since then, research has focused on the 5’ end of E3 since it contains three important *cis*-acting sites in close proximity, one that binds Foxa and HNF6 proteins, a second that binds C/EBP proteins, and a third site that binds several orphan nuclear receptors, including COUP-TFs, RORα, Rev-erbα and Rev-erbβ (39, 40). In contrast to the 5’ end of E3, the role of the 3’ end has remained elusive. The TCF/LEF site identified here represents the fourth important factor binding site in E3 and the first functional site in the 3’ end of this enhancer. In contrast to E3, RGS$^e$ has not been well characterized. Purification of rat liver nuclear proteins bound to RGS$^e$ identified STAT5 and TCF (33); the TCF site identified in this earlier report is the one analyzed here.

While our studies demonstrate an important role for β-catenin in the zonal activity of E3 and RGS$^e$, they cannot rule out a role for other factors in the pericentral activity of these enhancers. Using transgenic mice, we have found that mutating the E3 orphan receptor site

resulted in increased E3 activity throughout the adult liver, suggesting that orphan receptors bound to this site might repress E3 activity in non-pericentral hepatocytes (JEB, ELC and BTS, manuscript in preparation). Consistent with this result, deleting the orphan receptor HNF4α gene in adult hepatocytes led to elevated expression of GS and other pericentral genes in periportal regions (41). This study, which also identified an HNF4α site in the mouse GS upstream enhancer, argues that HNF4α suppresses GS and other pericentral genes in periportal regions. This is consistent with studies in RLSC mouse liver cells showing a correlation between HNF4α expression and a periportal phenotype (42). How orphan receptors contribute to zonal control, and the possible interplay between these factors and β-catenin, will require further investigation.

The ability of β-catenin to control E3 activity raises the question of whether it also regulates AFP expression during liver development and in HCC. When the β-catenin gene was deleted early during hepatogenesis, AFP mRNA levels were reduced 4-fold (43). However, because liver development was severely disrupted in this study, changes in AFP mRNA could not be clearly attributed to direct or indirect effects of the absence of β-catenin. We found that AFP mRNA levels were significantly reduced in p1 livers when *Alb-Cre* was used to delete the β-*catenin* gene late during gestation. Since liver development occurs normally in these mice, our data indicates that β-catenin is required for normal developmental AFP expression. Several clinical studies have evaluated β-catenin and AFP levels in human HCC samples and have found no association between elevated AFP and β-catenin (44–46). In contrast, many pericentral genes, including GS, are highly expressed in liver tumors where β-catenin is activated (8, 47). Thus, AFP reactivation during hepatocarcinogenesis is likely due to mechanisms that do not require β-catenin.

E3 is active in all hepatocytes in the fetal liver, and pericentral expression of E3-regulated transgenes is established during the perinatal period in a periportal-pericentral direction. This developmental transition is similar to what is seen with many pericentral enzymes, including GS, which are also expressed in all hepatocytes throughout the fetal liver. Interestingly, there are parallels between AFP shut-off and establishment of pericentral gene expression; postnatal AFP silencing occurs in a periportal-pericentral direction with pericentral hepatocytes being the last cells to express AFP before the gene is completely silenced (48). Our data indicating that the activity of zonal enhancers in the adult liver and AFP expression in the perinatal liver are both regulated by β-catenin is consistent with the idea that postnatal AFP silencing and establishment of pericentral gene expression are controlled by similar factors. Future studies on the regulation of well-defined zonally active enhancers will further elucidate this unique aspect of hepatic gene regulation.

## Acknowledgements

This work is supported by Public Health Service Grant DK-074816 to B.T.S.

The authors thank members of the Spear lab, Catherine Mao and Martha Peterson for helpful discussions and review of the manuscript, Drs. Steven Byers, Hans Clevers, Catherine Mao and Chunming Lui for plasmids, and Dr. Jeffrey Davidson for assistance with TCF/LEF site alignments.

## Abbreviations

| | |
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| **GS** | glutamine synthetase |
| **APC** | adenomatous polyposis coli |
| **TCF/LEF** | T Cell Factor/Lymphoid Enhancer Factor |
| **AFP** | alpha-fetoprotein |
| **HCC** | hepatocellular carcinoma |
| **RGS$^{e}$** | rat glutamine synthetase upstream enhancer |
| **βgl** | β-globin |
| **EMSA** | electrophoretic mobility shift assay |

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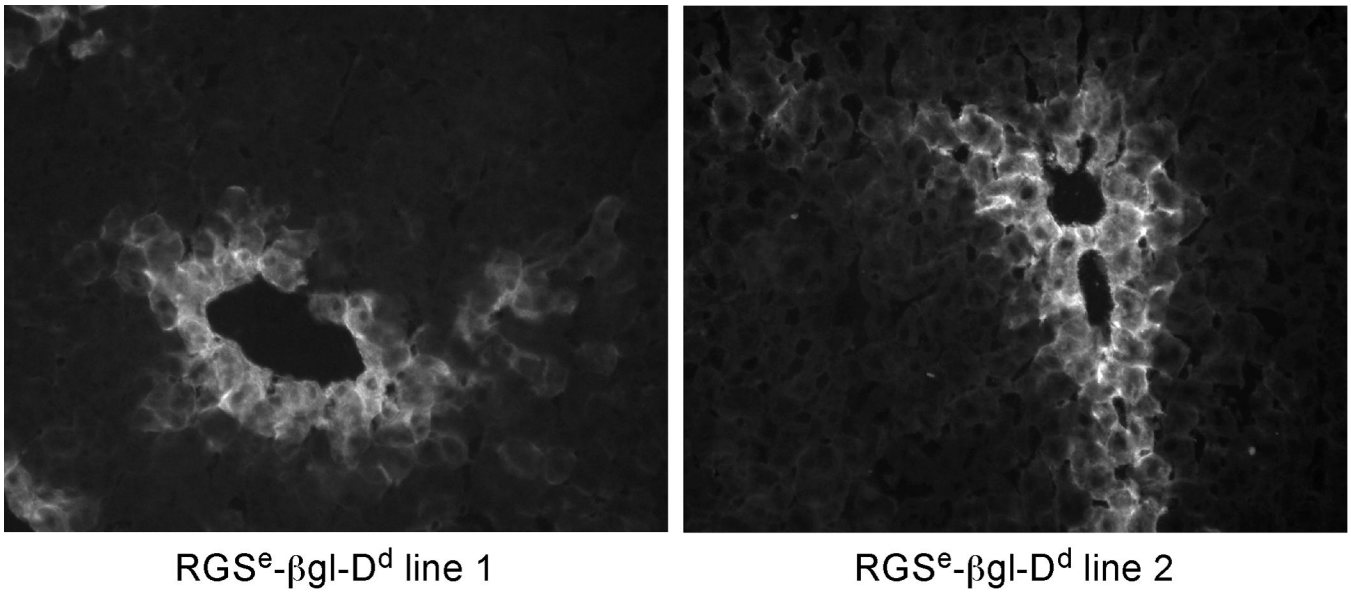


**Figure 1. Expression of RGS$^{e}$-βgl-D$^{d}$ transgenes is restricted to hepatocytes directly surrounding the central vein in adult liver**

The rat glutamine synthetase upstream enhancer centered at −2.2 kb (RGS$^{e}$) was amplified as a 400 bp fragment and fused to βgl-D$^{d}$. Offspring of two different transgenic founders containing RGS$^{e}$-βgl-D$^{d}$ were sacrified at ~8 weeks of age. Frozen sections were prepared and stained with a monoclonal FITC-anti-D$^{d}$ antibody. D$^{d}$ expression was restricted to hepatocytes directly surrounding the central veins. The same pattern of staining was observed with lines from two other RGS$^{e}$-βgl-D$^{d}$ founder mice (data not shown). Magnification, 20X.

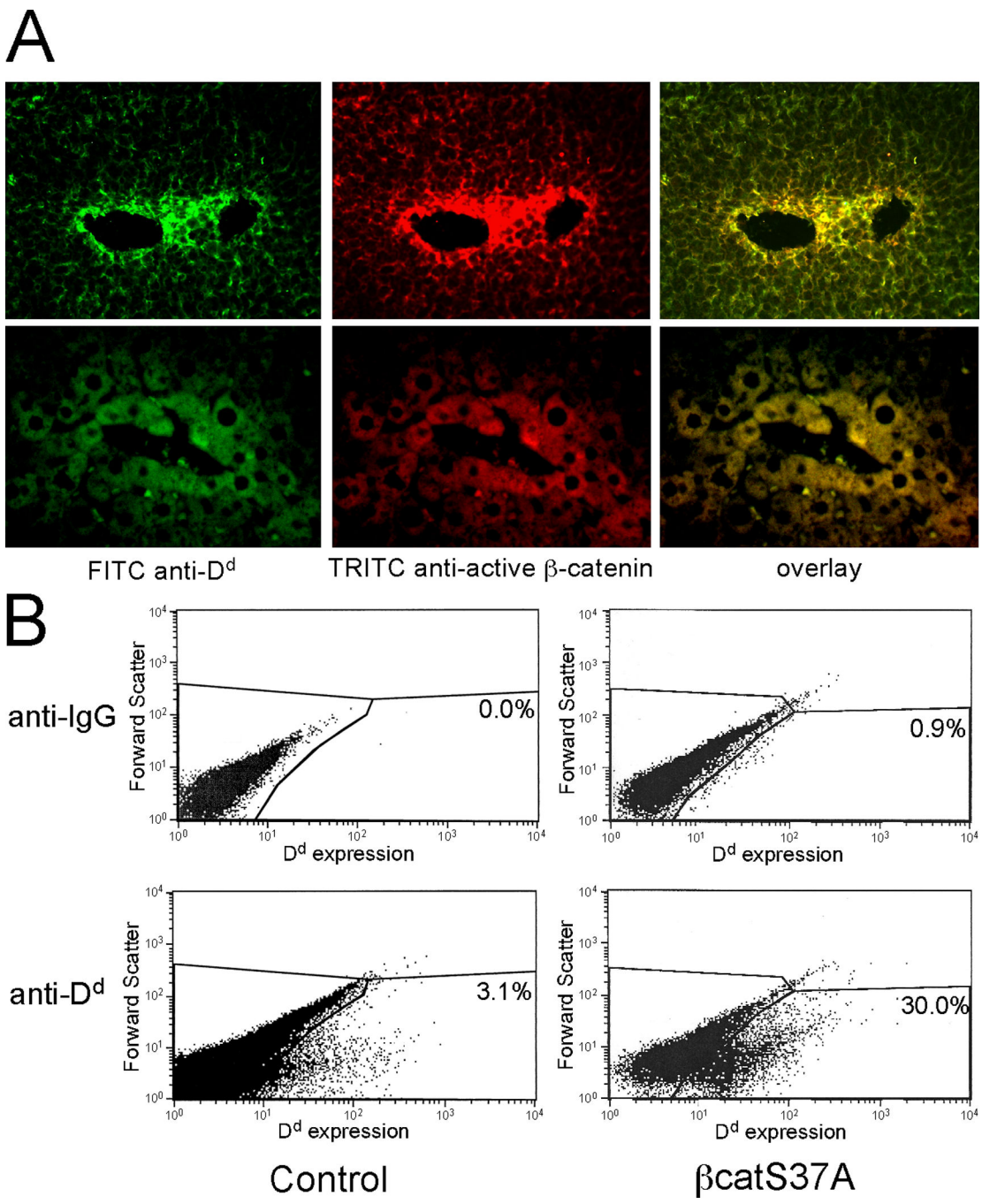


**Figure 2. E3-βgl-D$^d$ transgene expression and active β-catenin are co-localized in pericentral hepatocytes**

***(A)*** Transgenic mice containing E3-βgl-D$^d$ were sacrificed at ~8 weeks of age. Sections were stained for D$^d$ expression (left panel, green) and active β-catenin (middle panel, red) as described. Overlay of these (right panel, yellow) demonstrates co-location of D$^d$ and active β-catenin in the same population of hepatoctyes. ***(B)*** Hydrodynamic tail-vein injection was used to transfer control plasmid (pcDNA3.1, left panels) and the constitutively active βcatS37A (right panels) into adult E3-βgl-D$^d$ mice. After two days, hepatocytes were isolated, stained with control IgG antibodies (upper two panels) or FITC-anti-D$^d$ antibodies

(lower two panels). The percentage of cells gated as positive for $D^d$ expression (those in the lower right area of each panel) are shown.

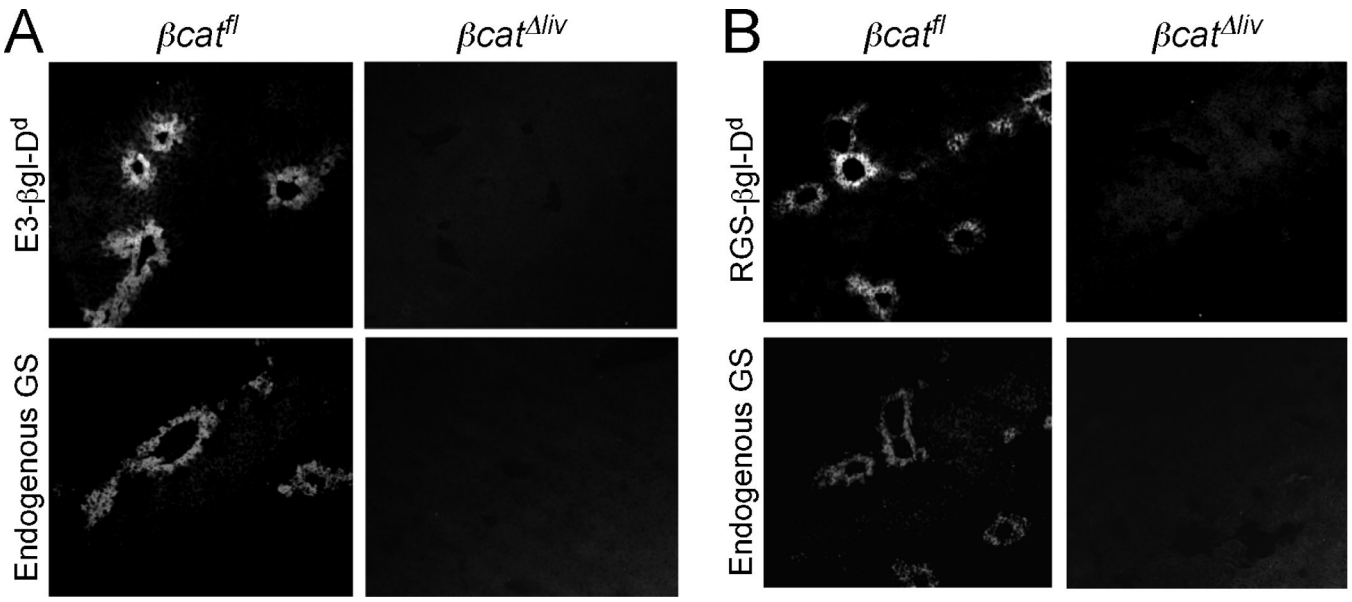


**Figure 3. E3-βgl-D$^{d}$ and RGS$^{e}$-βgl-D$^{d}$ expression is lost in the absence of β-catenin in adult hepatocytes**

Several rounds of breeding were performed to obtain that contained E3-βgl-D$^{d}$ (***Panel A***) or RGS$^{e}$-βgl-D$^{d}$***(Panel B)*** transgenes, were homozygous for the floxed *Ctnnb1* allele and contained (βcat$^{\Delta liv}$; right panels) or did not contain (βcat$^{fl}$; left panels) the *Alb-Cre* transgene. Frozen adult liver sections were stained with FITC-anti-D$^{d}$ antibodies (upper panels) or TRITC-anti-GS (lower panels). In the presence of β-catenin, D$^{d}$-containing transgenes and the endogenous GS gene were expressed in pericentral hepatocytes. In the absence of β-catenin, no transgene-derived D$^{d}$ or endogenous GS expression was observed.

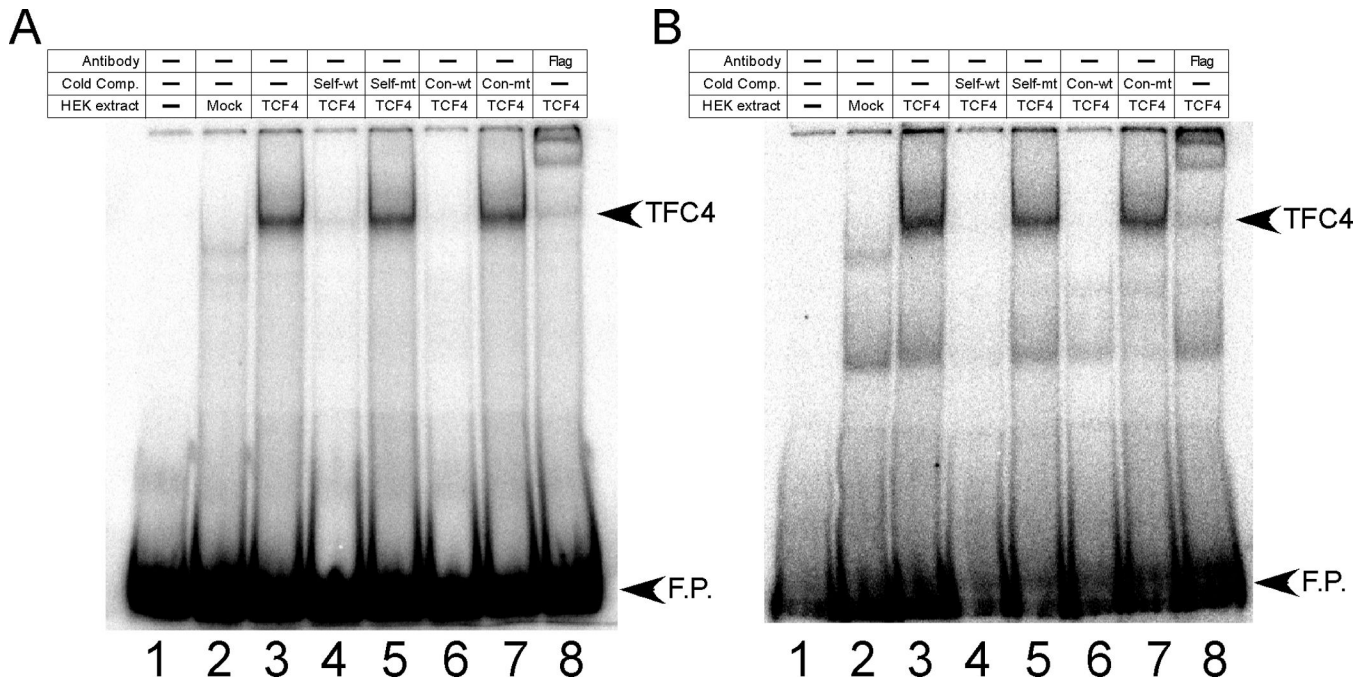


**Figure 4. TCF4 binds TCF sites found in E3 and RGS$^{e}$**

Radiolabeled oligonucleotides corresponding to TCF sites from E3 (Panel A) or GS upstream enhancer (Panel B) were used in EMSAs containing no extract (lane 1) or extracts from HEK293 cells that were mock-transfected (lane 2) or transfected with a FLAG-tagged TCF4 expression vector (lanes 3–8). Samples contained no cold competitor (lane 3) or 100-fold excess of cold competitors {oligonucleotides containing the TCF site of E3 (Panel A) or RGS$^{e}$ (panel B) that is wild-type (self-wt, lane 4) or mutant (self-mt, lane 5) or oligonucleotides containing a consensus TCF site that is wild-type (Con-wt, lane 6) or mutant (con-mt, lane 7)} or anti-FLAG antibody (lane 8). Bands corresponding to free probe (F.P.) and TCF4-DNA complex (TCF4) are designated. With both probes, the wild-type versions of the cold competitors can effectively compete for binding to TCF4 whereas the corresponding mutant forms of these oligonucleotides could not compete. The addition of the FLAG antibody resulted in a supershifted complex. Sequences of wild-type and mutant oligonucleotides are shown in Table I.

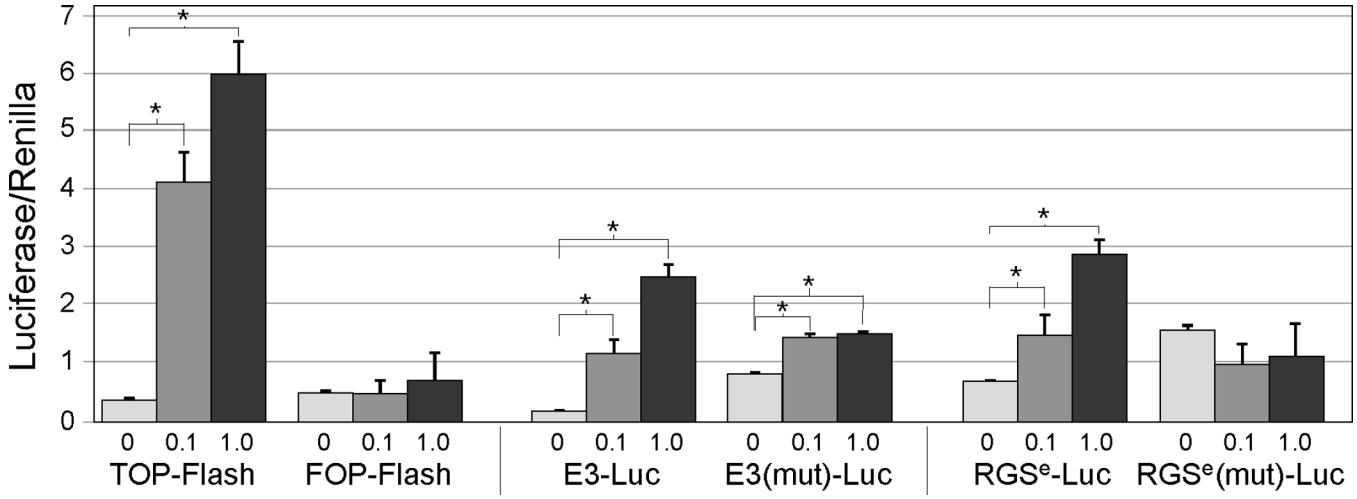


**Figure 5. β-catenin activates E3 and RGS$^e$ through their conserved TCF sites**

Hep3B cells were transfected with luciferase reporter constructs (TOP-Flash, FOP-Flash and E3-Luc and RGS$^e$-Luc with wild-type and mutated TCF sites), pcDNA alone (0 μg) or in conjunction with increasing amounts of βcatS37A (0.1, 1.0 μg), and Renilla luciferase. Cells were harvested after 48 hours and luciferase levels were determined; firefly luciferase was normalized to renilla. TOP-Flash, E3-Luc and RGS$^e$-Luc were activated by βcatS37A in a dose-dependent manner; FOP-Flash and RGS$^e$-Luc with a mutated TCF site did not respond wherease E3-Luc with a mutated TCF site showed a modest response to βcatS37A. An asterisk (*) indicates significance over cells transfected with pcDNA alone ($p < 0.05$).

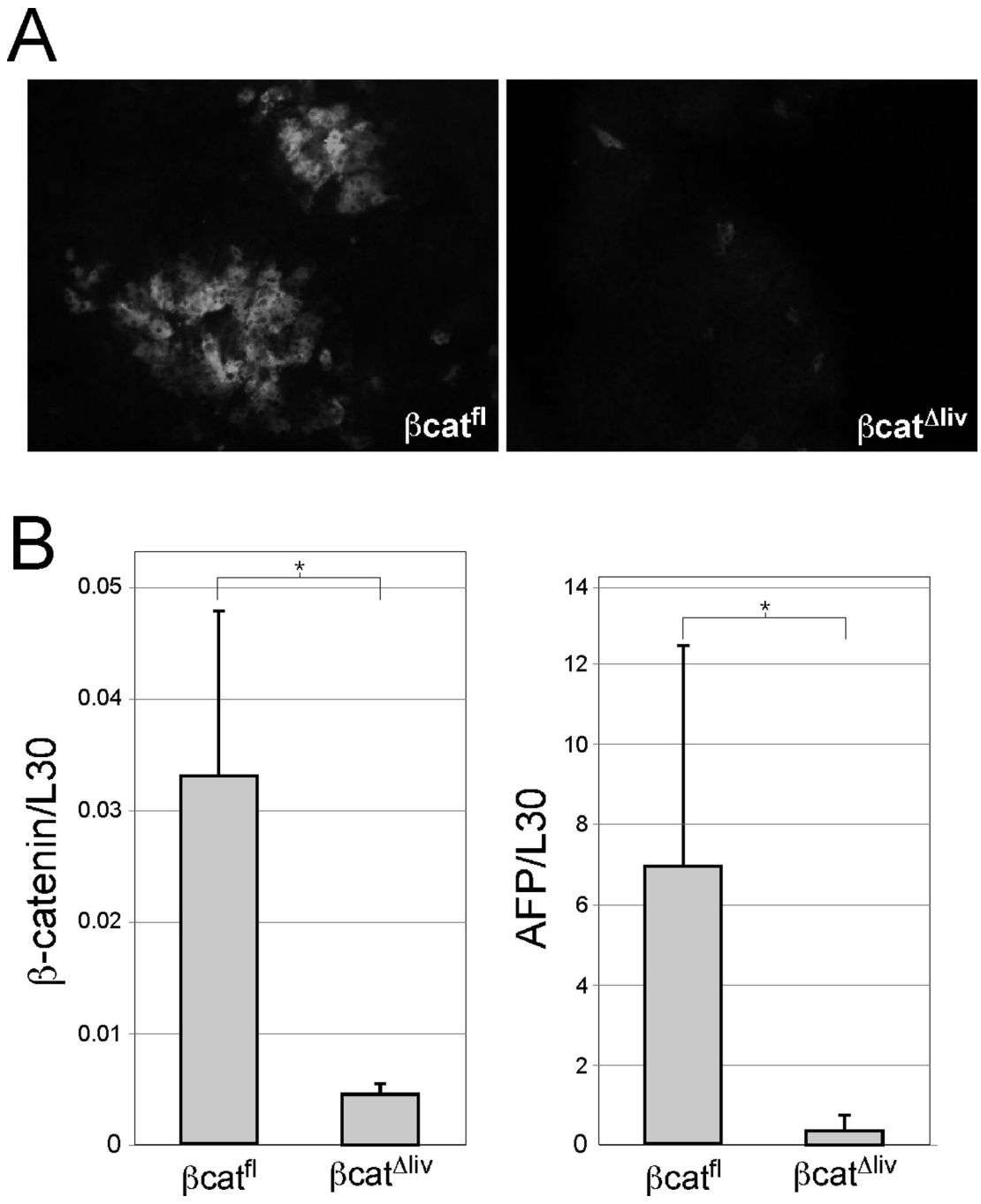


**Figure 6. β-catenin is required for E3 activity and AFP expression in the perinatal liver**

Livers were removed from E3-βgl-$D^d$ at postnatal day 1 (p1) that did ($\beta cat^{fl}$) or did not ($\beta cat^{\Delta liv}$) express β-catenin in hepatocytes. ***(A)*** The p1 livers were cryosectioned and stained with FITC-anti-$D^d$ antibodies. E3-regulated transgenes were expressed in pericentral cells from β-catenin-positive livers, although expression is not as highly restricted as it is in adult livers. Transgene expression in $\beta Cat^{\Delta liv}$ p1 livers was dramatically reduced. Magnification, 20X. ***(B)*** Total RNA was prepared from p1 livers from two independent litters and analyzed for β-catenin (left panel) and AFP (right panel) expression (normalized to L30) by real-time

RT-PCR. Levels of both transcripts are significantly reduced in $\beta cat^{\Delta liv}$ livers (n= 7) compared to transcript levels in $\beta cat^{fl}$ livers (n=5). An asterisk (*) indicates a significant difference between $\beta cat^{fl}$ and $\beta cat^{\Delta liv}$ cohorts ($p < 0.05$).

**Table I**

Oligonucleotides used in this study

| | |
|---|---|
| RGSeU: | 5’-GCTAGGATCCAAGCTTCTTGTTTACCCCTG |
| RGSeL: | 5’-TCAAGGATCCGAGTTTCAGATGGCAGCTTC |
| mE3 TFCU: | 5’-AGATAAAATTCCTTTGATGAAGGAAAA |
| mE3 TFCL: | 5’-TTTTCCTTCATCAAAGGAATTTTATCT |
| mE3 TFC$^{Mut}$U: | 5’-AGATAAAATTCC**CGG**GATGAAGGAAA |
| mE3 TFC$^{Mut}$L: | 5’-TTTTCCTTCATC**CCG**GGAATTTTATCT |
| RGS$^{e}$ TFCU: | 5’-CATGGAAGGATCAAAGCAAGCCTGC |
| RGS$^{e}$ TFCL: | 5’-GCAGGCTTGCTTTGATCCTTCCATG |
| RGS$^{e}$ TFC$^{Mut}$U: | 5’-CATGGAAGGA**C**C**CGG**GCAAGCCTGC |
| RGS$^{e}$ TFC$^{Mut}$L: | 5’-GCAGGCTTGC**CCG**G**G**TCCTTCCATG |
| Control TCFU: | 5’-GGTACTGGCCCTTTGATCTTTCTGG |
| Control TCF L: | 5’-CCAGAAAGATCAAAGGGCCAGTACC |
| Control TCF$^{Mut}$U: | 5’-GGTACTGGCC**GGG**GATCTTTCTGG |
| Control TCF$^{Mut}$L: | 5’-CCAGAAAGATC**CCC**GGGCCAGTACC |
| AFP5’: | 5’ CCGGAAGCCACCGAGGAGGA |
| AFP3’: | 5’ TGGGACAGAGGCCGGAGCAG |
| bCat5’: | 5’ CTCTTCAGGACAGAGCCAATG |
| bCat3’: | 5’ ATGCTCCATCATAGGGTCCA |
| L305’: | 5’ ATGGTGGCCGCAAAGAAGACGAA |
| L303’: | 5’ CCTCAAAGCTGGACAGTTGTTGGCA |

Underlined sequences indicate the TCF binding motif. Nucleotides in bold indicate mutations that were incorporated to eliminate TCF binding.

**Table II**

TCF sites in AFP E3 and GS upstream enhancer

**AFP E3 (3′ – 5′)**

| | | | |
|---|---|---|---|
| Mouse: | gttttccttc | ATCAAAGG | aattttatct |
| Rat: | ttttccctcc | ATCAAAGG | aattttatct |
| Gui.Pig: | catttcctca | ATCAAAAG | gactttactt |
| Cat: | tcattccttt | ATCAAAGA | gattttgcct |
| Dog: | tcattccttc | ATCAAAGG | gattttgcct |
| Lemur: | gttttccttc | ATCAAAGG | gattttattt |
| Rhesus: | gtttcccttt | ATCAAAGG | gatcttgtcc |
| Chimp: | atttcacttt | ATCAAAGG | gatcttgccc |
| Human: | atttcacttc | ATCAAAGG | gatcttgtcc |

**GS upstream enhancer (5′ – 3′)**

| | | | |
|---|---|---|---|
| Mouse: | acatgaaagg | ATCAAAGC | aaatccgctt |
| Rat: | acatgaaagg | ATCAAAGC | aagcctgctt |
| Gui.Pig: | acatgaaagg | ATCAAAGC | aaatccattt |
| Cat: | acatgaatgg | ATCAAAGC | aaatccattt |
| Dog: | acatgaatgg | ATCAAAGC | aaatccattt |
| Lemur: | acatgaacgg | ATCAAAGC | gaatctattt |
| Rhesus: | acatgaatgg | ATCAAAGC | aaatccattt |
| Chimp: | acatgaatgg | ATCAAAGC | aaatccattt |
| Human: | acatgaatgg | ATCAAAGC | aaatccattt |

TCF consensus: A/T A/T C A A A G (G) where middle “A” is indispensable and (G) improves LEF binding (55). Residues that are different from the mouse are highlighted in grey.