WNT AND BETA-CATENIN IN HEPATIC DIFFERENTIATION: WHICH WAY DOES THE WNT BLOW?

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My thesis is dedicated to Dr. Roger L. Clem, Jr.,

whose seemingly endless patience saw me through

two lab changes and countless scientific heartbreaks,

and without whose devotion and genuine support

I would not have made it through graduate school.

Roger, you were my rock and my inspiration every step of the way.

PREFACE

I owe so much to so many people who supported me in my path to getting my Ph.D.

In pursuit of my PhD, I had the unfortunate distinction of being a student who had to switch labs not once, but twice, during my graduate career. This was neither easy nor something I would recommend to anyone. But while I ultimately benefitted from these moves, I cherish my experiences with my former mentors, both of whom I really like as people and for whose contributions to my scientific development I still have a great deal of appreciation.

Working in Paul Monga's lab was absolutely ideal for me. In the 2.5 years I was in the Monga lab, my growth as a scientist was profound. He is an expert at tailoring his mentoring to each student according to their individual needs. In my case, what he gave me was freedom to work independently, encouragement, respect, and tireless patience and enthusiasm. He is a mentor who never chastises his students, just encourages them, supports them, and cheers them on. Paul helped me learn how to think like a scientist, and, most importantly, he has made me feel confident about my abilities as one.

Despite my setbacks, I could not be more thrilled with my graduate experience. The University of Pittsburgh Biomedical Graduate Program is a place where people do groundbreaking science, but where friendly faces and happy people abound. I have made many lifelong friends here, and felt a great sense of belonging. And the Department of Pathology, an incredibly close-knit, collaborative and supportive place could not have been a more ideal place to work.

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The Wnt/beta-catenin pathway is an evolutionarily conserved signaling cascade with key roles in development and adult tissue homeostasis and is aberrantly activated in many tumors. Beta-catenin, the central component of the canonical Wnt pathway has a number of roles in liver including regulation of processes of regeneration, development and carcinogenesis. In liver development across various species, beta-catenin has been shown to undergo tight temporal regulation to modulate the processes of hepatic competence, specification and liver bud expansion through transactivation of gene targets. Conflicting observations have been reported regarding the role of beta-catenin during hepatic differentiation, however. Whereas temporal stabilization of beta-catenin in mouse hepatoblasts results in livers with excessive biliary epithelial cells (BECs) and an absence of hepatocytes, loss of beta-catenin expression in developing hepatoblasts results in failure of both BEC differentiation and hepatocyte maturation, suggesting a role for beta-catenin in promoting both processes. We now report cleavage of beta-catenin by calpain coincident with the onset of hepatocyte differentiation, generating a transcriptionally active protein that is the predominant beta-catenin species in liver until birth. The truncated beta-catenin species lacks the N-terminal 95 amino acids, is localized

at the membrane and in the nucleus of differentiating hepatocytes, and correlates with expression of hepatocyte-specific beta-catenin targets (Glutamine synthetase, Regucalcin). During this time full-length beta-catenin expression is limited to developing BECs. We propose that cleavage of beta-catenin may represent a critical mechanism regulating hepatoblast cell fate, and that N-terminally truncated beta-catenin may have a unique role in hepatocyte differentiation. In addition, we found that while full-length beta-catenin was present in embryonal hepatoblastomas, the more differentiated fetal hepatoblastoma cases contain N-terminal truncations of beta-catenin. This finding may have broad implications for hepatoblastoma progression and prognosis.

In addition, we investigate roles for Wnt-independent activation of beta-catenin by receptor tyrosine kinases during hepatic differentiation, as well as that of non-canonical, beta-catenin-independent pathways activated by Wnt family proteins. Determining the molecular program for hepatocyte differentiation is of great clinical value not only as it relates to hepatoblastoma and other liver diseases, but also to inform efforts to create fully functional hepatocytes from various stem cell types.

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LIST OF ABBREVIATIONS

Abbreviation	Term
ALD	alcoholic liver disease
APC	adenomatous polyposis coli
AVE	anterior visceral endoderm
BEC	biliary epithelial cell
ChIP	chromatin immunoprecipitation
CK19	cytokeratin 19
CKI	casein kinase 1
CMV	cytomegalovirus
DDC	3,5-diethoxycarbonyl-1,4-dihydro-collidin
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FGF	fibroblast growth factor
FZD	frizzled
GSK3β	glycogen synthase kinase 3-beta
h.p.f.	hours post-fertilization
HB	hepatoblastoma
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
HMG	high mobility group
HNF	hepatocyte nuclear factor
LDL	low density lipoprotein
LPM	lateral plate mesoderm
NAFLD	non-alcoholic fatty liver disease
PCNA	proliferating cell nuclear antigen
PMO	phosphorodiamidate morpholino oligomers
PVDF	Polyvinylidene fluoride
RALDH	retinaldehyde dehydrogenase
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STM	septum transversum mesenchyme
TGFβ	transforming growth factor beta
TNF	tumor necrosis factor

INTRODUCTION

1. WNT/BETA-CATENIN PATHWAY IN LIVER DEVELOPMENT

With roles in processes such as stem cell maintenance and renewal, cell survival, proliferation, embryo patterning, organogenesis, differentiation, cell migration, and polarity; beta-catenin regulates numerous events in liver development, homeostasis, metabolism, regeneration and carcinogenesis (Lade and Monga, 2011) (Figure 1.1.1).

Figure 1.l.1 The beta-catenin signaling pathway plays multiple roles in liver patho-physiology. Beta-catenin signaling regulates cellular proliferation, differentiation, survival, metabolism, and redox state. Through these events beta-catenin plays important roles in physiological processes such as liver development, regeneration, stem cell-assisted regeneration, and zonation (Lade and Monga, 2011). Furthermore, through regulation of metabolism, beta-catenin activity is relevant to alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), and through its role in cell proliferation and survival plays significant roles in liver cancers (Thompson and Monga, 2007; Monga, 2009a).

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The beta-catenin protein is composed of unstructured N- and C-terminal tails flanking a core of 12 Armadillo domains. Each Armadillo domain is a 42 amino acid module comprising three alpha-helical structures (Huber et al., 1997), with the overall structure assuming a right-handed superhelix with a positively charged groove spiraling along the helix (Graham et al., 2000). It is highly conserved and appears throughout the metazoan lineage (Schneider et al., 2003), playing a dual role in cells, as a participant in adherens junctions in linking the transmembrane protein E-cadherin to the actin cytoskeleton (McCrea and Gumbiner, 1991; McCrea et al., 1991; Aberle et al., 1994; Rimm et al., 1995), and also as a signaling effector through binding and derepression of TCF family transcription factors (Behrens et al., 1996; Huber et al., 1996). Regulation of beta-catenin occurs post-translationally – it is constitutively expressed in the cytoplasm, but its activity is regulated through phosphorylation events that modulate its affinity for E-cadherin (Piedra et al., 2001) and alpha-catenin (Aberle et al., 1996; Ozawa and Kemler, 1998; Piedra et al., 2003) as well as its degradation by the proteasome (Aberle et al., 1997). The signaling activity of beta-catenin is activated by extracellular binding of Wnt family proteins to membranous Frizzled (Fzd) receptors. The Wnt family contains 19 members - secreted glycoproteins activated by lipid modification that act as morphogens in the context of embryogenesis, cancer, and normal tissue physiology. Signals from secreted Wnt proteins are transduced through their transmembrane Frizzled (FZD) receptors (Bhanot et al., 1996; Wang et al., 1997), of which there are 10, and associated LDL related protein 5/6 (LRP5/6), triggering a cascade involving Disheveled (Dvl) and components of the beta-catenin degradation complex. These signals stabilize beta-catenin by triggering inactivation of its degradation complex (Cook et al., 1996; Papkoff et al., 1996; Aberle et al., 1997; Nakamura et

al., 1998; Sakanaka et al., 1999; Swiatek et al., 2004); in the absence of Wnt signals, cytoplasmic beta-catenin is phosphorylated on threonine 41 and serines 33, 37, and 45 by a protein complex composed of glycogen synthase kinase 3 beta (GSK3β) (Rubinfeld et al., 1996; Yost et al., 1996), Adenomatous Polyposis Coli (APC) (Munemitsu et al., 1995), AXIN (Ikeda et al., 1998; Nakamura et al., 1998), and casein kinase I (CKI) (Liu et al., 2002; Swiatek et al., 2004) (Figure 1.1.2).

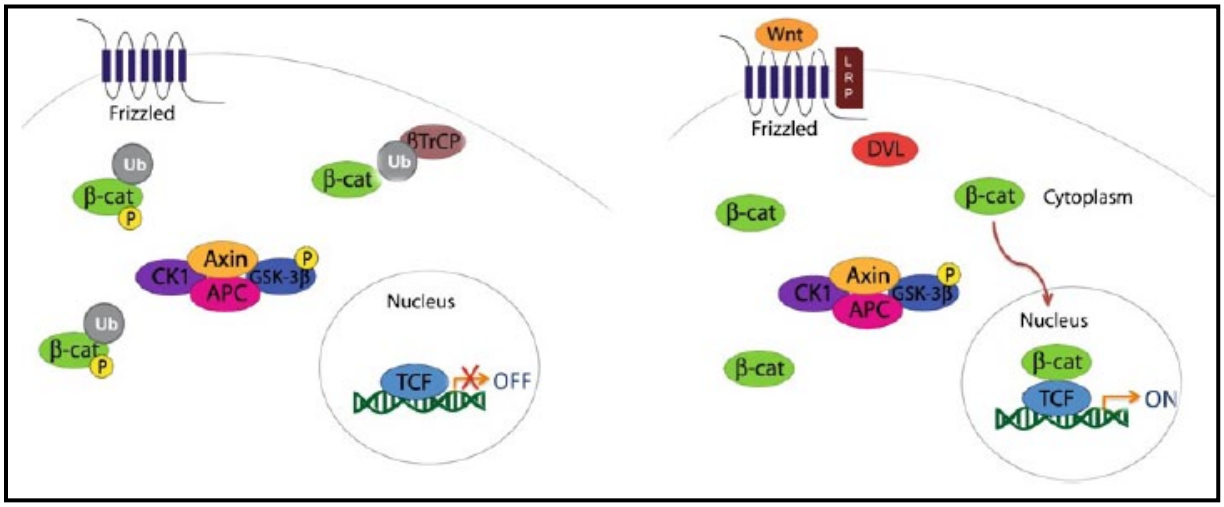


Figure 1.1.2 The canonical Wnt/beta-catenin signaling pathway. In the absence of WNT signals (left), the constitutively expressed beta-catenin signaling protein is targeted for proteasomal degradation via phosphorylation by a cytoplasmic protein complex composed of AXIN, APC, GSK3b, and CK1, and subsequent recognition by a ubiquitin ligase. When WNT ligands are present (right), they signal through Frizzled receptors and co-receptor LRP5/6 to induce inactivation of beta-catenin degradation that enable hypophosphorylated beta-catenin to translocate to nucleus to derepress TCF/LEF family transcription factors and activate expression of target genes.

Phosphorylation of these residues allows for recognition and ubiquitination by an E3 ubiquitin ligase complex including the F-box protein beta-transducin repeat containing protein βTrCP (Hart et al., 1999), and ultimate degradation by the 26S proteasome (Aberle et al., 1997) (Figure 1.1.2) A second degradation pathway for beta-catenin has recently emerged, triggered by phosphorylation of C-terminal residues 666 and 671, and subsequent recognition by the ubiquitin ligase Siah (Dimitrova et al., 2010). Emerging evidence also indicates that activation and nuclear translocation of beta-catenin can be effected by a growing number of factors other than Wnt pathway proteins, including several tyrosine kinases (Roura et al., 1999; Piedra et al., 2001; Monga et al., 2002; Sekhon et al., 2004; Lilien and Balsamo, 2005; Apte et al., 2006; Zeng et al., 2006; Berg et al., 2007; Rhee et al., 2007).

1.1.1 Beta-Catenin in development

The Wnt/beta-catenin pathway is critical throughout embryonic development beginning prior to implantation (Xie et al., 2008). In mouse and other amniotes, the fertilized zygote undergoes a series of cell divisions followed by cavitation to form a hollow ball called a blastocyst. The blastocyst implants in the maternal uterine endometrium, then flattens and elongates into a cylinder consisting of an outer cup-shaped epithelial layer called the epiblast, as well as the visceral endoderm and extra-embryonic endoderm. During gastrulation in mammals and birds, a population of epiblast cells migrates to the midline of the blastula and ingresses to form the primitive streak, ultimately giving rise to the mesoderm and definitive endoderm (Tam and Beddington, 1992; Parameswaran and Tam, 1995; Tam and Behringer, 1997). Patterning of the primitive streak by Wnt and TGFβ signaling results in an anterior

region with potential to form both the anterior mesoderm from which cardiac tissue arises as well as the definitive endoderm from which the hepatic endoderm is generated (Conlon et al., 1994; Liu et al., 1999; Brennan et al., 2001; Brennan et al., 2002; Perea-Gomez et al., 2002; Haramoto et al., 2004; Lu and Robertson, 2004; Mohamed et al., 2004; Kemp et al., 2005; Onuma et al., 2005; Barrow et al., 2007).

In mice, active beta-catenin is detectable immediately prior to gastrulation at E5.5 in the extraembryonic visceral endoderm and in a narrow region of cells in the epiblast at E6 that go on to form the primitive streak. Thereafter, beta-catenin signaling occurs both in the primitive steak and the node (Mohamed et al., 2004). Attempts to identify the Wnts responsible for activating beta-catenin during this time have revealed expression of *Wnt1* and *Sfrp1* localized to the inner cell mass of the blastocyst, *Wnt3a, -6, -7b* and *-10b* expressed throughout the blastocyst, and *Wnt9a* expression in the cells around the blastocoels cavity. Shortly before gastrulation, *Wnt2b* expression occurs in the prestreak embryo on the posterior end of the presumptive primitive streak, and its expression expands as the streak forms. In addition, WNT3 plays a critical role in driving gastrulation, as is evident by the inability of *Wnt3* mutants to form a primitive streak, maintain or produce mesoderm (Liu et al., 1999; Barrow et al., 2007). Also at this time, Wnt signaling antagonists *Sfrp1*, *Sfrp5* and *Dkk1* are expressed in partially overlapping regions in the anterior visceral endoderm through midstreak stage (Kemp et al., 2005). The expression of Wnts in the posterior visceral endoderm and Wnt antagonists in the anterior visceral endoderm establishes an anterior-posterior gradient of Wnt activity (Figure 1.1.3A). The Wnt/beta-catenin activity also promotes expression of the TGFβ family member Nodal, whose expression radiates outward from the posterior pre-gastrula epiblast, and is antagonized by proteases and Cerberus and Lefty in the anterior visceral endoderm (AVE) to

create its own gradient (Perea-Gomez et al., 2002). Through regulation of the Wnt, FGF and BMP pathways, Nodal signaling promotes the initiation gastrulation in the posterior epiblast (Haramoto et al., 2004; Onuma et al., 2005). Together these gradients promote the anterograde chemotaxis of visceral endoderm cells and thus play roles in formation of the primitive streak and establishment of the anterior-posterior axis (Conlon et al., 1994; Brennan et al., 2001; Perea-Gomez et al., 2002; Lu and Robertson, 2004). After gastrulation, Nodal promotes endoderm- and mesoderm-specific gene expression programs in a dose-dependent manner, with higher levels specifying endoderm and lower levels specifying mesoderm (Vincent et al., 2003). The definitive endoderm arises from the source of WNT-mediated Nodal expression at the anterior end of the primitive streak, and here Nodal activity promotes expression of endodermal transcription factors including GATA4-6, Eomesodermin, Mix-like proteins, and Foxa1-3, as well as Sox17 (Ang et al., 1993; Arceci et al., 1993; Laverriere et al., 1994; Suzuki et al., 1996; Gao et al., 1998; Germain et al., 2000; Aoki et al., 2002; Brennan et al., 2002; Ben-Haim et al., 2006; Hagos et al., 2007; Howard et al., 2007; Arnold et al., 2008).

Sox17, an HMG box transcription factor critical to endoderm development, partners with beta-catenin to transcribe targets including *Hnf1β*, *Foxa1* and *Foxa2* in posterior endoderm. Inhibition of Sox17 activity by expression of antisense oligos (Clements et al., 2003) or dominant negative Sox17 engrailed protein (Hudson et al., 1997) interferes with endoderm formation, and embryos with a targeted deletion of Sox17 are embryonic lethal (Kanai-Azuma et al., 2002). Furthermore, nuclear beta-catenin is detectable in early endoderm cells, and depletion of beta-catenin results in repression of Sox17 targets (Sinner et al., 2004), revealing the critical role of the interplay of these two factors in proper endoderm formation.

Embryos homozygous for a null allele of the beta-catenin gene *Ctnnb1* have been generated, and exhibit disrupted anterior-posterior axis formation manifesting as a mislocation in *cerberus-like* and *Lim1*-expressing prospective anterior visceral endoderm tissue, a lack of mesoderm and head formation, as well as an absence of expression of posterior mesoderm markers *Brachyury* and *goosecoid*, and expression of anterior markers of *Hex, Hesx1, Otx2*, and *Engrailed1 (Huelsken et al., 2000).* In addition to these and the aforementioned malformations caused by deletion of *Wnt3* (Liu et al., 1999; Barrow et al., 2007) the role of beta-catenin in embryonic patterning is further supported by studies in which beta-catenin activity is disrupted through deletion of WNT co-receptors *Lrp5* and *Lrp6* (Kelly et al., 2004) which leads to disrupted node formation, axis formation and establishment of endoderm. Furthermore, while DKK1-mediated blockade of endogenous Wnt activity inhibits mesodermal differentiation (Lindsley et al., 2006), constitutively active beta-catenin promotes premature mesodermal differentiation of epiblast cells (Kemler et al., 2004). Recent *in vitro* models using embryonic stem cells also support this role, as treatment with WNT3A or low levels of activin result in a population of cells resembling posterior primitive streak cells in gene expression pattern and developmental potential, whereas treatment with high levels of activin induce a cell population resembling anterior primitive streak (Gadue et al., 2006).

1.1.2. Beta-Catenin in endoderm patterning

Morphogenesis in the post-gastrulation embryo results in a transformation of the endoderm into a tube-like structure surrounded by mesoderm. Within this tube, precise temporal and spatial gradients of Wnt signaling (McLin et al., 2007), in conjunction with BMP ligands from the septum transversum mesenchyme (Roberts et al., 1995; Rossi et al., 2001; Tiso et al., 2002; Zhang et al., 2004; Shin et al., 2007), FGF ligands (FGF1 and FGF2) from cardiac mesoderm

(Jung et al., 1999; Zhang et al., 2004; Shin et al., 2007), and mesenchyme-derived retinoic acid (Stafford et al., 2004; Martin et al., 2005; Wang et al., 2006; Bayha et al., 2009), and FGF4 (Dessimoz et al., 2006) direct the patterning along the anterior-posterior axis necessary for hepatic competence in the ventral foregut region. FGF4 and WNT secreted by posterior mesoderm promote dose-dependent expression of Pdx1 and CdxB, which specify hindgut and midgut, respectively (Ohlsson et al., 1993; Heller et al., 1998; Wells and Melton, 2000; Ehrman and Yutzey, 2001; Kumar et al., 2003; Dessimoz et al., 2006), and suppress expression of the hepatic specification factor Hhex. (Dessimoz et al., 2006) The most anterior region of the gut tube receives the lowest level of FGF4 and WNT, as well as actively inhibiting Wnt signaling by the secretion of secreted Frizzled-related proteins (sFRPs) (Pilcher and Krieg, 2002; Finley et al., 2003), thereby inducing hepatic competence in the foregut region by allowing Hhex expression (Dessimoz et al., 2006; McLin et al., 2007; Li et al., 2008). BMP signaling also promotes Hhex expression (Zhang et al., 2002a).

Accumulating evidence supports the role of repression of Wnt/beta-catenin signaling in gut tube patterning, the first step towards hepatic competence of the foregut endoderm (Figure 1.1.3B). While forced expression of *Wnt8* or a stabilized beta-catenin construct in presumptive foregut cells of Xenopus gastrulas leads to an absence of foregut markers and foregut organ buds, antagonism of Wnt signaling by ectopic expression of *Dkk1* or overexpression of GSK3β in the posterior lateral endoderm leads to an expansion of expression of foregut markers and enlarged hepatic and pancreatic buds (McLin et al., 2007). Furthermore, inducible expression of the beta-catenin transactivation domain revealed opposing effects on liver development depending on the timing of the induction, and enabled a mapping of the time window during which repression is required. Induction of beta-catenin activity between stage 11 (midgastrula)

and stage 20 (6-7 somites) (gut patterning/hepatic competence, corresponding to approximately E7-E8.5 in mouse) leads to absence of foregut markers, induction between stages 25 and 30 (formation of liver diverticulum, approximately equivalent to E9-E9.5 in mouse) leads to normal foregut development and induction between stages 30 and 42 (liver bud expansion, approximately E9.5-E13.5 in mouse) leads to enlarged liver buds in some embryos, underscoring the precision in both timing and dose of Wnt signal necessary for proper hepatic competence and specification (McLin et al., 2007). During gut patterning/hepatic competence, beta-catenin appears to repress *Hhex* expression by interaction with Vent2, a transcription factor effector of BMP signaling, to prevent expression of liver-specific targets in regions with Wnt signaling active (McLin et al., 2007).

The inhibition of Wnt signaling necessary for hepatic specification appears to be achieved by expression of SFRP5 in the foregut epithelium, which inhibits the activity of WNT11 concomitantly expressed in nearby endodermal cells. Depletion of *sFrp5* results in both disruption of hepatic specification, as well as loss of adhesion and polarity in cells of the ventral foregut, underscoring its role in modulating both canonical Wnt signaling and non-canonical Wnt/PCP signaling. In addition, ectopic *sFrp5* expression in ventral posterior endoderm expands the foregut and represses the hindgut region, indicating that sFrp5 expression may be sufficient for specification of foregut (Li et al., 2008). While these observations were made in Xenopus, studies in zebrafish also show similar findings. High beta-catenin activity in APC homozygous and heterozygous null embryos in zebrafish led to failure of anterior endodermal organization, observed as decreased numbers of endodermal progenitors, that eventually leads to decreased hepatic and pancreatic progenitors (Goessling et

al., 2008). This is consistent with the requirement of inhibition of beta-catenin activity for proper endodermal organization, which is the first step towards hepatic competence.

Following foregut endoderm specification, activation of Wnt/beta-catenin signaling appears to be critical in shifting the fate of these endodermal progenitors to liver-specific fates, especially in zebrafish and *Xenopus*. In *Xenopus*, the Wnt ligand *Wnt2b* is expressed by the mesoderm flanking the anterior gut endoderm between stage 20 and stage 32, and expression of the secreted Frizzled-related gene *sFRP5* can be detected overlapping with *Hex*-expressing regions between stage 25 and persisting through tailbud stage 37/38 (Pilcher and Krieg, 2002), at which time the liver bud is visible. In zebrafish, expression of a novel Wnt isoform, *Wnt2bb*, is seen by *in situ* hybridization at the onset of endoderm patterning at 18 h.p.f (corresponding to E7.5 in mouse development) and persists through 52 h.p.f (postnatal day 8/9 in rodents). Mutation of *Wnt2bb*, or inhibition of its expression with an antisense morpholino oligonucleotide, results in a severe delay in liver development evident by 24 h.p.f., a time at which the liver bud is undergoing expansion (Wallace and Pack, 2003a). Injection of wild-type cells into the lateral plate mesoderm, but not the endoderm rescues this phenotype, revealing that the WNT2BB signals driving this stage of liver development are produced by the lateral plate mesoderm (LPM) (Ober et al., 2006). Additionally, use of a heat-shock inducible dominant negative *Tcf3* construct revealed that inhibition of beta-catenin activity between 16 h.p.f and 21 h.p.f resulted in an absence or substantial decrease in hepatic tissue, while fish heat-shocked at 25 h.p.f. formed livers of variable sizes (Ober et al., 2006). Further underscoring the necessity of beta-catenin inhibition during this period, animals heterozygous for wild-type APC developed a hepatic enlargement correctable by injection with a morpholino antisense oligonucleotide targeting beta-catenin or inhibition of beta-catenin activity with

dominant-negative *TCF* (Goessling et al., 2008). *APC*$^{+/-}$ embryos show defects in endoderm patterning, exhibiting an enlargement of liver buds at the expense of pancreatic buds, consistent with an expansion of the hepatic foregut region into the pancreatic domain. Furthermore, cell transplantation experiments verified that the role of beta-catenin in liver formation is cell autonomous. Temporal modulation of Wnt/beta-catenin activity through heat-shock inducible expression of *Wnt8* confirmed that while foregut specification of endoderm requires inhibition of Wnt signaling, this pathway becomes critical for hepatic specification (Goessling et al., 2008) (Figure 1.1.3C).

Interestingly, a mutation in the retinoic acid biosynthesis enzyme retinaldehyde dehydrogenase type2 (RALDH2) results in a strikingly similar delay in zebrafish liver bud formation as the *Wnt2bb* mutant, which can be partially rescued by treatment with exogenous all-trans-retinoic acid or injection of *Raldh2* mRNA. Absence of *Wnt2bb* expression in LPM was also observed in these animals, suggesting that retinoic acid may be responsible for induction of *Wnt2bb* expression (Negishi et al., 2010).

The role of beta-catenin in hepatic specification in mice has yet to be definitively addressed. Successful deletion of beta-catenin beginning at E9.5 can be achieved by breeding Foxa3-*Cre* and floxed beta-catenin expressing transgenic mice and results in beta-catenin's absence in HNF4a-expressing hepatic cells (Tan et al., 2008). While hepatic specification stage of foregut endoderm was not specifically assessed in this model, primitive liver bud observed at E9.5 was comparable in the control and beta-catenin-null livers and differences became apparent only later in development (Tan et al., 2008), as discussed in the forthcoming sections.

1.1.3. Hepatic morphogenesis

Once the foregut undergoes hepatic specification, the cells in this region undergo a morphological transition from gut-like columnar cell morphology to pseudostratified epithelia that causes thickening of the epithelium into a diverticulum (Bort et al., 2006). The diverticulum is surrounded by septum transversum mesenchyme (STM), which is lined with a basal lamina and endothelial precursors. At E9.5, migration of the cells, now bipotential hepatoblasts, occurs through matrix metalloproteinase digestion of the laminin-rich basement membrane and delamination into the STM (Margagliotti et al., 2008). After migration of hepatoblasts into the STM, liver bud expansion is mediated, in part, by an activation of Wnt signaling (Monga et al., 2003; Hussain et al., 2004; Micsenyi et al., 2004; Suksaweang et al., 2004; Tan et al., 2008). This phase consists of expansion of the liver bud through rapid proliferation of the resident cells, as well as other processes underlying hepatic morphogenesis.

Between E9.5 and E13.5, hepatic architecture begins to be established, with sinusoids (Enzan et al., 1997) and bile canaliculi appearing and partially maturing (Luzzatto, 1981). Between E9.5-E10 the liver bud separates into the cranial lobe, from which the extrahepatic and intrahepatic bile ducts arise, and caudal lobe, from which the gall bladder develops. The gall bladder and common bile duct join between E10.5 and E11.5, and the gallbladder subsequently elongates. Hepatic cords and sinusoids form around E10, and by E12.5, sinusoids decrease and the parenchymal compartment becomes predominant. Stellate cells also appear at this time, and interlobular spaces appear between liver lobes. Between E11.5 and E12.5 the left umbilical vein becomes the ductus venosus and the right vitelline vein becomes the portal vein (Crawford et al., 2010). Certain organelles, including lysosomes, Golgi and rough endoplasmic reticulum also become apparent during this period (Medlock and Haar, 1983; Vassy et al.,

1988). Hematopoietic cells colonize the expanding liver bud at E12 and recede at approximately E16 (Sasaki and Matsumura, 1986; Crawford et al., 2010).

At approximately E13.5 in mouse development, bipotential hepatoblasts begin differentiating into biliary epithelial cells (BECs) or hepatocytes, based on proximity to portal veins. Prior to differentiation, hepatoblasts express markers of both hepatocytes and BECs, such as Albumin, a-fetoprotein and CK19. BECs differentiate from hepatoblasts around portal veins under the influence of TGFb and Notch signaling, first producing a monolayer, and then a bilayer of cuboidal, CK19 positive cells (Kodama et al., 2004; Clotman et al., 2005; Loomes et al., 2007; Lozier et al., 2008). At around E17.5, ductal plate remodeling is observed, in which focal dilations emerge at points in the bilayer, become surrounded by portal mesenchyme, and undergo tubulogenesis into intrahepatic bile ducts (Lemaigre, 2003).

Hepatoblasts not adjacent to portal veins instead differentiate into hepatocytes and arrange into cords lined by sinudoidal epithelial cells and bile canaliculi. Once hepatoblasts are committed to the hepatocyte lineage and undergo further expansion, they begin acquiring the functions of a mature hepatocyte, a process referred to as hepatocyte maturation. This process is gradual, and eventually mature hepatocytes appear around E17 stage as highly polarized epithelial cells with abundant glycogen accumulation. This process of progressive hepatocyte differentiation or maturation appears to be prompted by oncostatin M secreted by hematopoietic cells (Kamiya et al., 1999; Matsui et al., 2002), as well as glucocorticoid hormones (Strick-Marchand and Weiss, 2002; Michalopoulos et al., 2003) and HGF (Michalopoulos et al., 2003) (Figure 1.1.3E). Oncostatin M, possibly regulated by TNFα (Kamiya and Gonzalez, 2004), appears to elicit metabolic maturation of hepatocytes by activation of JAK/STAT signaling

through its receptor gp130 (Kamiya et al., 1999) and promotes adhesion and polarity through upregulation of the tight junction protein claudin-2 (Imamura et al., 2007) and the adherens junction protein E-cadherin (Battle et al., 2006). Glucocorticoids may act to suppress growth and induce expression of critical hepatocyte transcription factors HNF4α (Li et al., 2000) and C/EBPα (Tomizawa et al., 1998; Michalopoulos et al., 2003; Yamasaki et al., 2006). Many other factors have demonstrated roles in liver cell differentiation, including transcription factors HNF1α, HNF1β, HNF6/the Onecut (OC) factors, HNF4α, c/ebpα, Jagged/Notch, TGFβ, TNFα, Foxa1–3, Hhex, GATA4/6, nuclear hormone receptors and extracellular matrix interactions (Rastegar et al., 2000; Clotman et al., 2002; Coffinier et al., 2002; Jacquemin et al., 2003; Parviz et al., 2003; Friedman et al., 2004; Kamiya and Gonzalez, 2004; Kodama et al., 2004; Odom et al., 2004; Clotman et al., 2005; Zhao et al., 2005; Cheng et al., 2006; Kyrmizi et al., 2006; Yamasaki et al., 2006; Hunter et al., 2007; Margagliotti et al., 2007; Watt et al., 2007; Gkretsi et al., 2008). Recent work has also revealed a role for microRNA expression in liver development, with miR-30a family microRNAs critical for biliary morphogenesis (Hand et al., 2009), and miR-495 and miR-218 responsible for regulating expression of HNF-6 and OC-2 (Simion et al., 2010).

1.1.4. Role of beta-catenin activity in hepatic morphogenesis

The role of beta-catenin during the process of hepatic morphogenesis is quite pleiotropic and highly temporal. Beta-catenin is initially required for expansion of hepatoblasts during early stages of hepatic morphogenesis and is later important for proper specification of hepatoblasts to BECs as well as hepatocyte maturation (Micsenyi et al., 2004; Day et al., 2005; Tan et al., 2008).

Compelling evidence exists for the role of beta-catenin during liver bud expansion in mice. In developing livers between days E10.5 to E18.5, beta-catenin protein levels peak at E10-E12 and then gradually decline. Nuclear/cytoplasmic beta-catenin staining also peaks at E10-E12 and decreases gradually, and correlates with percentage of PCNA positive cells, consistent with a role for beta-catenin in driving hepatoblast proliferation (Micsenyi et al., 2004). Also, infection of chick liver primordium at E3 with a viral construct expressing an N- and C-terminally truncated, constitutively active beta-catenin leads to hepatomegaly at E15, as well as a high nuclear-to-cytoplasmic ratio and hepatoblast-like morphology in targeted cells, disrupted architectural organization, loosened cell-cell contacts, decreased glycogen storage, and cytoplasmic rather than membranous localization of E-cadherin, highlighting the function of beta-catenin in expansion of liver progenitors (Suksaweang et al., 2004). Conversely, expression of the WNT antagonist *Dkk-1* or a dominant-negative LEF1 to inhibit beta-catenin activity produced undersized livers with decreased cell proliferation, increased apoptosis, disrupted cord structures and sinusoids, and reduced E-cadherin expression and glycogen storage capacity (Suksaweang et al., 2004). In zebrafish (Goessling et al., 2008) as well as Xenopus (McLin et al., 2007), Wnt signaling also promotes the growth and differentiation of liver progenitors after liver specification.

Embryonic day 10 livers cultured *ex vivo* in the presence of serum-free, WNT3A-conditioned medium show biliary hyperplasia and impaired hepatocyte differentiation. Culturing in WNT3A-conditioned serum-free medium plus sFRP-1 resulted in a decreased proliferation and survival as well as morphological defects. Growth in serum-containing medium rescued liver phenotypes, as did addition of HGF to serum-free WNT3A conditioned medium, suggesting

that while beta-catenin is sufficient for BEC differentiation, both MET and beta-catenin activity are required for hepatocyte differentiation (Hussain et al., 2004) (Figure 1.1.3E).

A similar culture system in which embryonic day 10 livers were cultured in the presence of antisense phosphorodiamidate morpholino oligomers (PMO) targeting beta-catenin for 72 hours resulted in a substantial decrease in organ mass owing to a pronounced decrease in proliferation and significant increase in apoptosis, implicating beta-catenin in both hepatoblast expansion and survival(Monga et al., 2003). In addition, PMO-treated livers showed similar expression of albumin as well as staining with Hep-Par, a marker of hepatocyte lineage (the urea cycle enzyme carbamoyl phosphate synthetase 1) (Butler et al., 2008), relative to control-treated and untreated livers. Interestingly though, the Hep-Par+ albumin+ cells also exhibited c-kit staining, suggesting a lack of differentiation. PMO treated livers also exhibited a lack of CK19 positive cells, and a marked increase in c-kit positive cells (marker of hepatoblasts) around ducts.

Furthermore, mice exhibiting premature beta-catenin activation in hepatoblasts via conditional deletion of APC exhibit excessive differentiation of hepatoblasts to biliary epithelial cells. Transplantation of cells from APC-null embryonic livers into adult mouse liver leads to formation of fully differentiated ducts, lending *in vivo* support to the role of beta-catenin in promoting biliary epithelial cell specification from hepatoblasts (Decaens et al., 2008). It is important to note, however, that APC deletion may have effects other than stabilization of beta-catenin, since APC is involved in regulation of mitosis (Bahmanyar et al., 2009; Caldwell and Kaplan, 2009), asymmetrical stem cell division (Graham et al.), cell polarity (Barth et al., 2008), and motility (Etienne-Manneville, 2009).

Finally, more compelling evidence that absence of beta-catenin activity leads to failure of hepatoblast expansion and defects in both biliary specification and hepatocyte maturation comes from the generation of a mouse line with a hepatoblast-specific (FoxA3-*Cre* driven) *Ctnnb1* deletion (Tan et al., 2008). Embryos possessing this deletion die between E16-E17 with undersized livers due to decreased hepatic cell proliferation and increased apoptosis likely resulting from impaired regulation of oxidative stress. Intriguingly, these animals possess an apparent defect in hepatoblast differentiation, with parenchymal cells exhibiting the high nuclear-to-cytoplasmic ratio and unpolarized morphology characteristic of uncommitted E13/14 stage hepatoblasts. Furthermore, knockout livers show a deficit both in BECs and in expression of the transcription factors *C/ebpα* and HNF4α characteristic of hepatocyte differentiation. Absence of hepatic beta-catenin also leads to decreased expression of a-fetoprotein, albumin (*Alb*), cyclin-D1, the adherens junction protein E-cadherin, the tight junction protein ZO-2 (*Tjp2*), as well as beta-catenin targets glutamine synthetase (*Glul*), regucalcin (*Rgn*), *Egfr*, cytochrome p450 oxidases *Cyp2e1* and *Cyp1a2*, Glutathione-S-transferase *Gsta3*, *Gsto1*, and *Gstm1*, ornithine aminotransferase (*Oat*), and Leukocyte cell-derived chemotaxin 2 (*Lect2*), as well as numerous other proteins detected in mature hepatocytes. The resident immature cells also show elevated levels of 4-hydroxynonenal and malondialdehyde indicative of increased oxidative stress, possibly owing to the decreased expression of glutathione-S-transferases (Tan et al., 2008).

Together, the above findings point to a highly temporal and rather pleiotropic role for beta-catenin in hepatic morphogenesis (See Figure 1.1.3 for summary). It plays a key role in the expansion of the hepatic bud directly through regulation of cell proliferation and indirectly through modulation of cell survival via regulation of oxidative stress. In addition, it plays an

important and temporal role in the commitment of hepatoblasts to biliary epithelial cells, and eventually also directs the process of progressive differentiation of the hepatocytes, possibly through regulation of independent sets of targets of the Wnt pathway or through regulation of cell adhesion and/or polarity. However, as contradictions exist regarding the precise function of beta-catenin in hepatic differentiation/maturation, this subject deserves extensive exploration, an endeavor we have undertaken. The results of our experimentations along these lines are reported and discussed in Chapter 2.

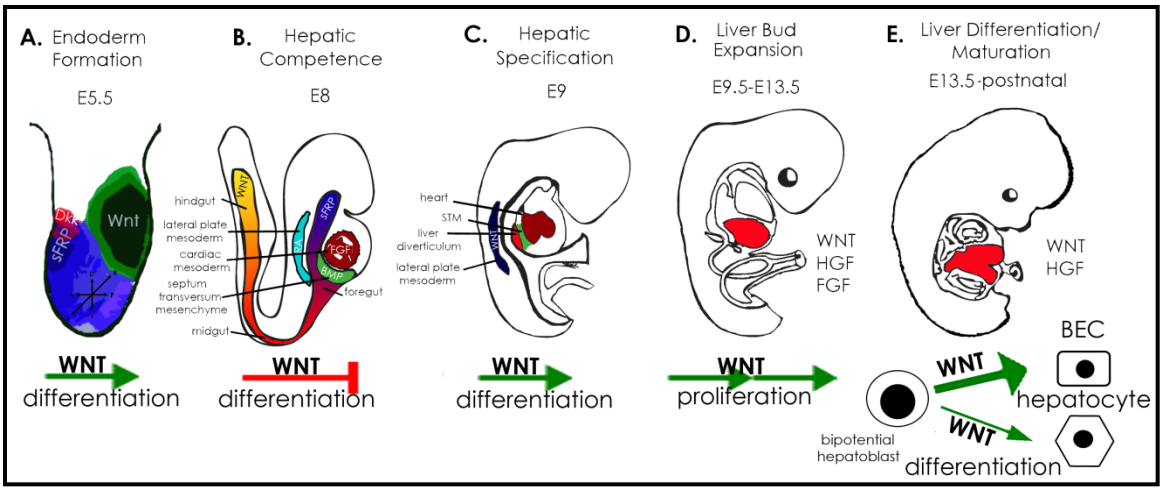


Figure 1.1.3 Temporal role and regulation of beta-catenin during prenatal hepatic development. A. In the prestreak embryo at E5.5, a gradient of Wnt activity controlled by regional expression of Wnts and Wnt antagonists (sFRP and Dkk) promotes gastrulation, with the region expressing Wnt ultimately giving rise to the anterior visceral endoderm from which the gut tube derives. **B.** During gut tube patterning around E8, suppression of WNT activity through enhanced sFRP5 expression promotes hepatic competence in the ventral foregut endoderm. At this time, FGF from the developing heart and BMP signals from the septum transversum mesenchyme also contribute to hepatic competence, along with retinoic acid emanating from the lateral plate mesoderm. **C.** Wnt signaling resumes activity immediately after foregut patterning by E9, during which time the hepatic endodermal cells undergo a morphological transition from columnar to pseudostratified resulting in thickening into the early liver bud. In zebrafish, Wnt2bb, a downstream target of retinoic acid activity in the lateral plate mesoderm, is critical for proper timing of liver development, however the responsible Wnt ligand in mouse has not yet been identified. **D.** Wnt/beta-catenin activity, in conjunction with HGF/MET activity and FGF, promotes expansion of the bipotential hepatoblasts comprising the liver bud. **E.** Wnt/beta-catenin activity appears to be essential for both biliary epithelial cell (BEC) differentiation and hepatocyte differentiation from hepatoblasts. While WNT activity promotes BEC differentiation, the combination of WNT + HGF appears to lead to hepatocyte differentiation.

1.1.5 Wnt and Frizzled expression in developing liver

Also of relevance to beta-catenin activity in the liver are the Wnts and Frizzleds acting as upstream effectors/regulators of this activity. Depending on context, Wnt activation of Fzds can activate either canonical, beta-catenin mediated signaling or beta-catenin independent, non-canonical Wnt signaling pathways (Wnt/Ca^{2+} and Wnt/planar cell polarity) that actively suppress canonical Wnt/beta-catenin activity (Mikels and Nusse, 2006). In addition, there are secreted frizzled related proteins (sFRPs) that inhibit Wnt signaling by binding to Wnt and Frizzled proteins and blocking their activation (Rattner et al., 1997; Hoang et al., 1998; Uren et al., 2000; Jones and Jomary, 2002; Kawano and Kypta, 2003) and are involved in regulating beta-catenin activity.

After hepatic specification, many *Wnt* genes have been reported to be expressed in the liver including *Wnt1*, *Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt9b*, *Wnt10a* and *Wnt10b* (Bi et al., 2009). *Fzds 1-10* are also expressed, with *Fzd 2, 3, 5, 6, 7, 8* most prominent prenatally (Bi et al., 2009). Wnt inhibitors *SFrp2, 3* and *5* mRNA are high at E12.5 and E14.5 and then decline with the onset of differentiation, but while expression of *sFrp1* and *4* also decreases as differentiation occurs, their mRNA expression level remains significant throughout pre- and post-natal development (Bi et al., 2009), perhaps indicative of a role in postnatal liver zonation.

In adult mouse liver, *Wnt4* is highly expressed by sinusoidal endothelial cells (SECs), stellate cells, and biliary epithelial cells (BECs), and *Wnt9b* is expressed by both SECs and BECs (Zeng et al., 2007). Adult hepatocytes express *Fzd2, 4, 7 and 8*, as well as producing *Wnt5b* (Zeng et al., 2007). Immunohistochemistry to detect WNTs expressed around the developing

chick liver detected WNT3A and WNT8B in the localized liver growth zones, as well as diffuse peripheral and central WNT5A and WNT11 staining (Suksaweang et al., 2004).

Evidence for the role of sinusoidal endothelial cells in secreting Wnt signals necessary for liver development emerged when the generation of a *Vegfr2*-null mouse deficient of endothelial cells was found to exhibit failure of both hepatoblast migration from the liver bud and hepatocellular cord formation (Matsumoto et al., 2001). In the developing chick, the liver buds form around, and eventually envelop, a vein running through the septum transversum (the ductus venosus) that secretes a chemoattractant called Neurturin (Tatsumi et al., 2007). Thereafter, hepatoblasts move radially within the septum transversum mesenchyme to arrange into hepatocellular cords lined by sinusoidal endothelial cells, which are now known to mediate this process through secretion of WNT9A (Matsumoto et al., 2008).

1.1.6 Non-canonical Wnt pathways and non-Wnt mediated β-catenin activity

The beta-catenin-independent "non-canonical" Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+} signaling also utilize Wnt and Fzd proteins, but tend to suppress rather than activate beta-catenin (Bernard et al., 2008; Yuzugullu et al., 2009) by enhancing its degradation in a GSK3β-independent mechanism involving APC and the ubiquitin ligase Siah (Topol et al., 2003; Dimitrova et al., 2010). Wnt ligands are separated into two classes based on their biological activities (Du et al., 1995; Torres et al., 1996): "canonical" Wnt1 class ligands, including WNTs 1, 3, 3A and 8 that signal through beta-catenin and induce secondary axis formation in embryos, and "non-canonical" Wnt5a class ligands including WNT4, 5A, 5B and 11 that do not. Though while Wnt5a class ligands have been historically designated as "non-canonical" Wnts, emerging evidence suggests that it is actually the particular Frizzled protein,

and not its Wnt ligand, that dictates whether canonical or non-canonical signaling occurs downstream (Mikels and Nusse, 2006) and that all Wnts may be able to activate all pathways (Kestler and Kuhl, 2008).

The Wnt/Ca^{2+} pathway involves activation of PLC downstream of a Wnt binding to a non-canonical Frizzled (Ahumada et al., 2002; Ma and Wang, 2007), which leads to intracellular calcium release, (Kohn and Moon, 2005); and activation of calcium-activated signaling proteins including PKC (Sheldahl et al., 2003), Cdc42 (Habas et al., 2003; Schlessinger et al., 2007; Schlessinger et al., 2009), which promotes leading edge actin assembly to enable cell motility (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003), CAMKII, (Ishitani et al., 2003) and also the phosphatase calcineurin, which then further activates the transcription factor NF-AT (Saneyoshi et al., 2002).

The Wnt/planar cell polarity pathway was discovered in Drosophila, in which it controls the spatial orientation of cuticular bristles within the fly epidermis (Gubb and Garcia-Bellido, 1982; Nubler-Jung et al., 1987). Wnts binding to Frizzled proteins capable of activating this pathway promote membrane translocation of Disheveled, which activates small GTPases Rho and Rac (Fanto et al., 2000) to regulate cytoskeletal remodeling and activate JNK (Boutros et al., 1998; Yamanaka et al., 2002).

While the role of non-canonical Wnt signaling in liver development has not been fully characterized, some relevant observations have been made. Wnt/PCP pathway activity promotes elongation of dorsal meso-endoderm during gastrulation in conjunction with FGF signals, and regulates gut elongation in *Xenopus* (Li et al., 2008) and gut tube fusion in zebrafish via wnt4a, silberblick/wnt11, and wnt11-related (Matsui et al., 2005). In addition, the

foregut-secreted sFrp5 that regulates beta-catenin activity during gut patterning also inhibits Wnt11-mediated PCP activity in order to allow proper foregut morphogenesis (Li et al., 2008). While these findings illustrate some contributions of non-canonical Wnt signaling to liver development, a better assessment of Wnt and Frizzled expression at the protein level and their effect on beta-catenin activity and contributions to liver development remains to be done. In chapter 4 of this thesis, I report the results of an analysis of expression of Wnts and Fzds potentially involved in non-canonical Wnt signaling during hepatic differentiation.

1.1.7 Non-Wnt mediated regulation of beta-catenin activity

In addition to aforementioned mechanisms of regulation of beta-catenin activity, others also exist. Phosphorylation of several tyrosine residues by both receptor and non-receptor tyrosine kinases alters the activity of beta-catenin, including Y86, 142, 654, 670 and others. Phosphorylation of Y654 by kinases such as c-SRC, BCL-ABL, MET, EGFR and ERBB2 decreases the affinity of beta-catenin for E-cadherin, leading to disassembly of the adherens junctions complex, decreased cell-cell adhesion, and translocation of beta-catenin into the nucleus to promote cell proliferation (Hoschuetzky et al., 1994; Shibata et al., 1996; Roura et al., 1999; Monga et al., 2002; Apte et al., 2006; Zeng et al., 2006; Coluccia et al., 2007). Phosphorylation of residue Y142, which decreases binding affinity for alpha-catenin, can occur via FYN, FER or MET activity (Piedra et al., 2003; Brembeck et al., 2004)., and there is also evidence of phosphorylation of Y489 by ABL, which may regulate cadherin affinity and nuclear translocation of beta-catenin (Rhee et al., 2007).

Another receptor tyrosine kinase, fibroblast growth factor receptor (FGFR), is capable of activation of beta-catenin and, in fact, multiple lines of evidence support a role for FGF/FGFR

signaling in promotion of liver bud expansion (Jung et al., 1999; Kelly et al., 2001). Recent work has revealed that FGF signals from embryonic stellate cells peak during liver bud expansion, correlating with beta-catenin activity in hepatoblasts, and treatment of *ex vivo* cultured whole E10 mouse livers with FGF leads to a marked increase in cytoplasmic/nuclear beta-catenin staining along with an increase in membranous beta-catenin (Sekhon et al., 2004). Additionally, FGFR activation is seen during liver bud expansion (Sekhon et al., 2004)(Micsenyi et al., 2004), and *ex vivo*-cultured E12.5 *TOPGAL*$^{+/+}$ mouse livers show striking increases in β-galactosidase activity upon treatment with FGF10, consistent with nuclear translocation and canonical beta-catenin activity (Berg et al., 2007). Finally, *Fgf10* and *Fgfr2b* knockout mice show decreased liver size and increased apoptosis relative to wild-type embryos, similar to the phenotypes described in liver models with beta-catenin activity inhibited (Berg et al., 2007; Tan et al., 2008), lending support to the idea that FGFR regulation of beta-catenin may be critical in liver bud expansion.

Of note, while FGF/FGFR signaling also regulates hepatic competence in early foregut endoderm (Jung et al., 1999; Serls et al., 2005; Shin et al., 2011), it appears to inhibit beta-catenin activity during this time (Shin et al., 2011).

Another growth factor capable of Wnt-independent activation of beta-catenin is hepatocyte growth factor (HGF) (Monga et al., 2002), the ligand for the receptor tyrosine kinase MET (Naldini et al., 1991a; Naldini et al., 1991b). Immunofluorescently labeled MET and beta-catenin co-localize at the membrane in normal adult rat liver, and co-immunoprecipitation analysis reveals that beta-catenin-MET complexes exist at the membrane of hepatocytes independently of beta-catenin-E-cadherin complexes. HGF has been shown to promote increased nuclear translocation and transcriptional activation by beta-catenin concomitant with

decreased GSK3beta activity (Papkoff and Aikawa, 1998), suggesting HGF/MET signaling may also regulate beta-catenin stabilization. Such an effect was also observed in primary hepatocytes in culture, along with a decrease in MET-beta-catenin association by co-immunoprecipitation, effects not seen with a dominant-negative MET with deleted tyrosine kinase domain (Monga et al., 2002). Mutation of various beta-catenin tyrosine residues pinpoints Y654 and Y670 as MET phosphorylation targets critical for inducing nuclear translocation, and non-phosphorylatable Y654F/Y670F mutants were shown to be deficient in TCF binding, and cell cycle entry (Zeng et al., 2006).

Further investigation of the relationship between MET activation and beta-catenin activity comes from a study in which naked plasmid DNA expressing HGF under the control of the CMV promoter was hydrodynamically injected into tail veins of mice. These mice developed hepatomegaly characterized by decreased MET-beta-catenin association and increased beta-catenin nuclear translocation relative to empty vector-injected mice. Most tellingly, reproducing this experiment in beta-catenin hepatic null mice revealed no hepatomegaly, but, rather, persistence of the decreased liver weight-to-body weight ratio that characterizes these animals, confirming that HGF-induced stimulation of liver growth depends on beta-catenin activity (Apte et al., 2006).

A previously described study revealed that E10 *ex vivo* cultured liver buds exhibit biliary hyperplasia in serum-free, WNT3A containing medium, but underwent normal growth and morphogenesis in the presence of WNT3A and HGF (Hussain et al., 2004). During liver bud expansion, HGF reportedly activates MET in the mouse embryo (Micsenyi et al., 2004), raising the possibility that alone, or in conjunction with one of the many WNTs expressed during this time, it is responsible for activation of beta-catenin activity. Furthermore, the respective

knockouts of MET and beta-catenin have a notable similarity in hepatic phenotypes (Schmidt et al., 1995; Tan et al., 2008). These findings prompted us to explore whether phosphorylation of beta-catenin plays a role in liver bud expansion, which will be discussed in Chapter 3.

1.1.8. Calpain regulation of beta-catenin activity

Finally, in addition to modification of beta-catenin activity by phosphorylation to regulate its stabilization, turnover, and binding partner affinity and localization, emerging evidence points to proteolytic alteration by calpain as an additional means of regulation. Calpains are members of a family of cytosolic intracellular Ca^{2+}-dependent cysteine proteases that are defined by a shared similarity to human μ-calpain. They are notable among proteases in that they tend to modify the function of their substrates rather than degrade them. There are 15 calpain genes in mammals, with roles in regulation of cell motility, morphology, membrane repair, apoptosis and signal transduction (Sorimachi et al., 2010). Calpains μ-calpain (calpain-I) and m-calpain (calpain-II) are the best studied members of this protein family, and both are ubiquitously expressed and exist as heterodimers between a small regulatory subunit and a larger catalytic subunit. Though no evidence exists of differences in substrate specificity between m- and μ-calpain, they do exhibit important difference in terms of functional regulation. In fact, the m- and μ- designations came from observations of differences in calcium requirements for activity, with μ -calpain appearing to require micromolar (μM) calcium levels and m- requiring millimolar (mM) concentrations. It now appears, however, that m-calpain is regulated *in vivo* by phosphorylation and localization rather than by high calcium levels (Shiraha et al., 1999; Shiraha et al., 2002; Smith et al., 2003; Glading et al., 2004; Leloup et al., 2010). In particular, while ERK phosphorylation of m-calpain on serine 50 promotes activation, phosphorylation by

PKA at ST369/370 inhibits membrane localization and therefore membrane activity (Leloup et al., 2010). Calpain activity is also regulated via autolysis of its N-terminus, an event that occurs subsequent to initial activation and results in a decreased calcium dependence (Hathaway et al., 1982). Further autolysis appears to result in decreased activity (Li et al., 2004). Calpain substrates include the well-known tumor suppressor p53, cell adhesion components such as paxillin, talin, focal adhesion kinase, and β-integrin, cell cycle regulators cyclin D1 and p35, as well as proteins associated with apoptosis including caspase-3, caspase-12, Bax, and Bcl-xl, and the signaling protein PKC (Goll et al., 2003).

Processing of beta-catenin by calpain has been noted in several studies (Li and Iyengar, 2002; Rios-Doria et al., 2004; Benetti et al., 2005; Kramerova et al., 2006; Abe and Takeichi, 2007). In some cases, calpain is purported to promote degradation, and, hence, loss of beta-catenin activity (Benetti et al., 2005). In 2005, Benetti et al. reported that siRNA knockdown of calpain increases the pool of free, transcriptional beta-catenin and that increasing calpain activity using a dominant-negative Gas2 construct induces beta-catenin degradation (Benetti et al., 2005) In other studies, however, the effect of calpain on beta-catenin appears to be an enhancement of activity. Rios-Doria et al. found expression of a 75-kDa proteolytic fragment of beta-catenin in metastatic prostate cancer specimens as well as several prostate and breast cancer cell lines whose presence was correlated with calpain activity in these samples. Treatment of cells with ionomycin, a calpain activator, led to generation of the 75-kDa fragment via cleavage of the N-terminus of beta-catenin, as well as its nuclear translocation. Expression of a beta-catenin fragment lacking the first 132 N-terminal amino acids revealed that N-terminally truncated beta-catenin is capable of activating TCF-dependent transcriptional activity over baseline levels, but at a lower level than a truncated protein lacking amino acids

29-48 (Rios-Doria et al., 2004). Similarly, in 2007 Abe et al. published their finding that activation of NMDA-receptors on hippocampal neurons promotes cleavage of beta-catenin by calpain to a 75-kDa fragment. This event occurs *in vitro* via application of glutamate to neurons in culture, but also in mice exposed to novel environments. This 75-kDa beta-catenin fragment was capable of activating TCF/LEF dependent gene transcription, was resistant to degradation via the proteasome via GSK3β and was shown by peptide sequencing to be truncated at amino acid 95 (Abe and Takeichi, 2007).

At this time, very little is known about the role of calpain in liver development. Mice lacking either global calpain activity due to a deletion of the *Capn4* gene (Arthur et al., 2000; Zimmerman et al., 2000; Tan et al., 2006b), or m-calpain activity (Dutt et al., 2006) exhibit embryonic lethality, precluding an assessment of specific developmental roles, though *Capn4* null mice were noted to possess defects in both vasculogenesis and erythropoiesis (Tan et al., 2006b). Mu-calpain null mice have also been created; these animals are viable and appear normal except for a reduction in platelet aggregation and clot retraction without affect on bleeding times (Azam et al., 2001). Roles for calpain have been established in the differentiation of many cell types, including muscle cells, osteoblasts, keratinocytes and adipocytes (Cottin et al., 1994; Saito et al., 1994; Sparatore et al., 1994; Barnoy et al., 1996; Moraczewski et al., 1996; Barnoy et al., 1997; Grynspan et al., 1997; Murray et al., 1997; Garach-Jehoshua et al., 1998; Ueda et al., 1998; Patel and Lane, 1999; Patel and Lane, 2000; Yajima and Kawashima, 2002; Moyen et al., 2004; Kramerova et al., 2006; Liang et al., 2006; Li and Xie, 2007; Shimada et al., 2008), establishing a firm precedence for calpain as a pro-differentiation factor. In adipocytes, calpain processing is responsible for calpain-dependent transcriptional activation of C/EBP alpha (Patel and Lane, 1999; Patel and Lane, 2000), a

protein that also regulates many liver specific genes (Johnson, 1990; Liu et al., 1991; Rastegar et al., 2000; Westmacott et al., 2006). In addition, it has also been reported that calpain cleavage of a related liver specific transcription factor, C/EBPβ, occurs in developing liver (Welm et al., 1999). Additionally, calpains have also been implicated in PI3K/Akt independent regulation of insulin-stimulated glycogen synthesis in the hepatic HepG2 cell line (Meier et al., 2007), a process characteristic of mature hepatocytes. The recent generation of a loxP/Cre conditional *Capn4* targeted mouse (Tan et al., 2006b) has now made possible tissue-specific and temporal deletion of calpain activity, and reports using these mice will make important contributions to elucidating calpain's many roles including in developing liver.

1.1.9. Postnatal beta-catenin activity in liver

Like other liver phenomena, postnatal liver growth and development is also dependent on beta-catenin activity. Extensive cell proliferation occurs in the liver after birth, in conjunction with a substantial increase in beta-catenin protein and nuclear translocation. Increased *Ctnnb1* gene expression occurs at postnatal day 15 (p15), and a cyclic pattern of GSK3β activation and inactivation occurs during postnatal development, with GSK3β inactivation occurring at p10 and p20, and activation occurring at p5 and p20. Beta-Catenin colocalizes with E-cadherin during postnatal development, but association with MET does not occur until P20. In mice with an albumin-Cre driven, hepatocyte-specific deletion of beta-catenin occurring by postnatal day 15, liver weight/body weight ratios are significantly decreased relative to wildtypes between day 15 and day 20, and this decrease persists throughout the lives of the animals (Apte et al., 2007). Postnatally, beta-catenin is localized mostly at the hepatocyte membrane with some additional cytoplasmic staining in centrizonal hepatocytes, though nuclear beta-catenin localization by immunohistochemistry may be underappreciated due to the harsh antigen

retrieval methods required to detect it in mouse liver and, therefore, immunoblot analysis of nuclear extracts may be a more reliable means of such detection. Virtually all other cells contained in an adult liver such as BECs, endothelial cells and stellate cells express beta-catenin, although the role of Wnt signaling in such cells is only beginning to be investigated (Zeng et al., 2007).

1.1.10. Zonal regulation of liver metabolism

In the adult liver, hepatocytes are not equivalent, with position along the portocentrovenular axis within a liver lobule dictating expression of metabolic genes involved in drug metabolism, carbohydrate metabolism, ammonia detoxification, and bile production and secretion. Wnt/beta-catenin has now been identified to be playing a key role in this phenomenon. Complementary localization patterns exist for active beta-catenin, expressed perivenously, and its negative regulator APC, expressed periportally. Induction of liver-specific deletion of *Apc* or inhibition of Wnt signaling by virally mediated overexpression of *Dkk1* reveals that beta-catenin activity controls zonal expression of metabolism genes (Benhamouche et al., 2006). Shortly after *Apc* deletion, livers show extension of several perivenously localized enzymes, including the beta-catenin targets glutamine synthetase (*Glul*) and the glutamine transporter *Glt1* (Benhamouche et al., 2006). Likewise, expression of periportal enzymes involved in ammonia and urea metabolism, Glutaminase2 (*Gls2*), Arginase1 (*Arg1*), Carbamoyl phosphate synthetase I (*Cps I*) and Phosphoenolpyruvate carboxykinase 1 (*Pck1)* were suppressed by *Apc* deletion, and additional defects were observed in nitrogen metabolism. Moreover, mice expressing activated mutant forms of beta-catenin, either transgene expression of ΔN131-beta-catenin or adenovirally infected S37A-beta-catenin, showed similar metabolism gene expression perturbations to *Apc* null mice. Infection of wild-type mice with an adenovirus

encoding the Wnt pathway inhibitor Dickkopf-1 (*Dkk1*) produces a massive conversion of perivenous hepatocytes to periportal hepatocytes (Benhamouche et al., 2006). Furthermore, deletion of beta-catenin, but not *c-myc*, results in a loss of GS expression and an extension of expression of the periportally-expressed carbamoylphosphate synthetase I (CPS I) around the central vein, implicating beta-catenin in c-myc independent suppression of expression of periportal metabolic genes (Burke and Tosh, 2006). Finally, while spontaneous differentiation of liver stem cells leads to their expression of periportal enzymes, stabilization of beta-catenin in these cells by treatment with the GSK3β inhibitor 6-bromoindirubin-3'-oxime (BIO) switches their gene expression profile to that of a perivenous hepatocyte.

ChIP analysis of perivenously expressed *GS* and *Cyp1a1* and periportally expressed *Gls2* and *H19* revealed that the level of binding of TCF family member LEF1 to HNF4a binding sites on promoters regulates their transcription. The ChIP results suggest a model in which beta-catenin–induced LEF1 activation regulates LEF1 inhibition of HNF4α repressive activity, with LEF1 binding to and displacing repressive HNF4α at its consensus sites on promoters in perivenous hepatocytes with active Wnt/beta-catenin activity. For periportal genes *Gls2* and *H19*, during transcriptional repression seen in the context of active Wnt/beta-catenin pathway activity HNF4α is displaced, concomitant with the recruitment of LEF1 only to its own consensus sites (Colletti et al., 2009).

Very recently, beta-catenin was also shown to regulate expression of both phase I drug-metabolizing Cytochrome p450 (Cyp) enzymes and phase II glutathione-s-transferases in perivenous hepatocytes (Loeppen et al., 2005; Tan et al., 2008; Giera et al., 2010).

1.1.11 Role of beta-catenin in adult hepatic progenitors or oval cell biology

Wnt/beta-catenin signaling is a key player in the process of liver regeneration across various species. Utilization of models such as surgical resection of the liver or toxicant-induced hepatic injury reveals that beta-catenin activation appears to be necessary for optimum regeneration of the remnant liver. Additionally, Wnt/beta-catenin signaling has also been uncovered in the progenitor cell-mediated regeneration of the liver that occurs only in response to regenerative stimulation in a context that prevents hepatocyte proliferation. This has been observed in response to chronic or acute liver damage, both experimentally and clinically (Knight et al., 2005; Hu et al., 2007; Apte et al., 2008; Yang et al., 2008). Several signaling pathways have now been shown to play an important role in emergence, expansion and differentiation of these transiently amplifying progenitor cells, also referred to as oval cells (Erker and Grompe, 2007).

In a model of oval cell activation induced by treatment with 2-acetylaminofluorine followed by 2/3 partial hepatectomy (PHx) in rats, increased presence of active nuclear and cytoplasmic beta-catenin in oval cells between 5 and 10 days post-pHx correlated with increases in oval cell proliferation (Apte et al., 2008; Yang et al., 2008). Increased WNT1 and FZD2 expression appeared at these times as well, coinciding with decreased Wnt pathway inhibitors WIF1 and GSK3β (Apte et al., 2008). More recently, a role for WNT1 was also identified in the differentiation of the oval cells to hepatocytes, such that shRNA-mediated *Wnt1* suppression led to not only decreased oval cell proliferation but also affected their differentiation to mature hepatocytes (Williams et al., 2010). Thus, as in development, the role of Wnt signaling in oval cell proliferation and differentiation may be highly temporal and context development.

Oval cell activation in mice is also observed in animals fed a diet containing 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). This treatment leads to biliary and hepatocyte injury followed by atypical ductular proliferation and oval cell response (Preisegger et al., 1999; Thompson et al., 2010a). Wnt signaling in oval cells after DDC injury has also been investigated through the use of hepatocyte-specific beta-catenin conditional null mice (Tan et al., 2006a) (Figure 1.1.4). Treatment of beta-catenin null mice with DDC resulted in a decreased number of cells positive for the oval cell marker A6 relative to treatment of wild-type animals (Apte et al., 2008). In another study, quantitative reverse transcription polymerase chain reaction and *in situ* hybridization identified upregulation of several *Wnts* in DDC-treated animals around portal triads and areas of atypical ductular response, with most pronounced increases in *Wnt 7a, 7b, 9b 10a and 11* mRNA levels. DDC-treatment of the TOPGAL transgenic beta-catenin reporter mouse strain confirmed the increase in beta-catenin/TCF activity in oval cells/atypical ductular cells. Moreover, increased active beta-catenin, and beta-catenin/TCF reporter activity were observed in oval cells isolated from livers of DDC-fed mice in response to purified WNT3A (Hu et al., 2007). Finally, in 2008, Yang et al. administered naked plasmid DNA encoding a stabilized S37Y beta-catenin to mice via naked tail vein injection. This method, known to produce high DNA expression in mouse liver, resulted in the appearance of a higher number of A6-positive cells in response to DDC treatment, thus providing additional support for the role of beta-catenin in promoting oval cell activation (Yang et al., 2008).

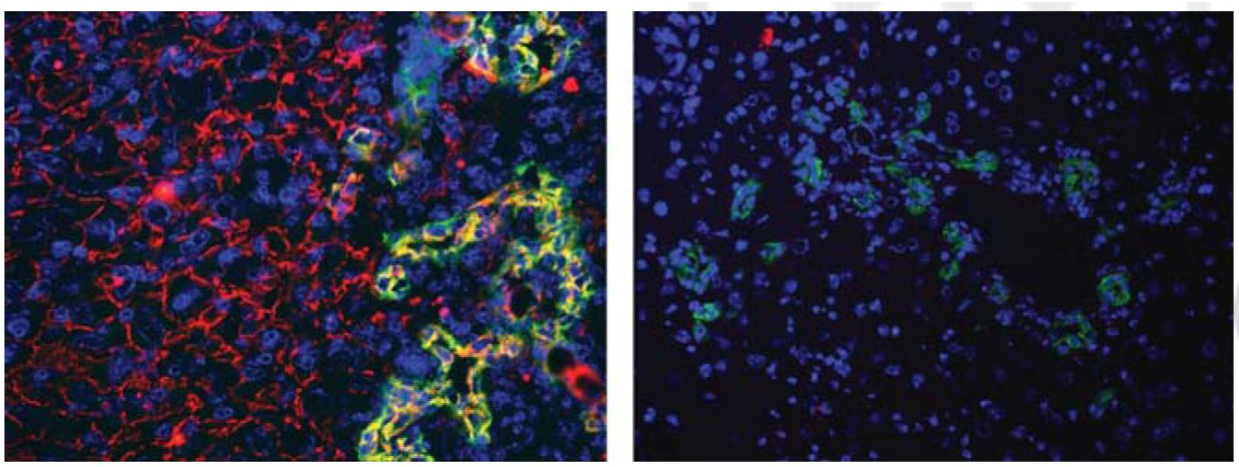


Figure 1.1.4 Beta-Catenin in oval cells after 15-days of DDC-exposure. Control mice fed DDC diet for 15 days, display beta-catenin (red) and A6 (green) colocalization (yellow) in oval cells (arrowhead) in liver sections by immunofluorescence. Liver sections from beta-catenin conditional null mice fed DDC diet for the same time lack beta-catenin (red) and show a dramatic decrease in numbers of A6-positive cells (green) (arrowhead).

Interestingly, cells staining with an OV6 oval cell antibody and exhibiting nuclear/cytoplasmic beta-catenin localization were detected in sections of cirrhotic livers and hepatocellular carcinomas from human patients, suggesting beta-catenin drives oval cell expansion in human liver pathologies as well. Additionally, OV6+ cells isolated from human liver cancer cell lines show enrichment for the epithelial stem cell marker CD133+ as well as several other progenitor/stem cell markers, as well as exhibiting increased tumorigenicity in nude mice, and resistance to chemotherapy drugs. Treatment with a stabilizer of beta-catenin produces enrichment of these cultures for OV6+ cells, highlighting a potential role for beta-catenin in oval cell expansion in liver tumors (Yang et al., 2008). These observations are significant since

a subset of hepatocellular cancers may have cancer stem cell origin (Sell and Leffert, 2008) and Wnt/beta-catenin signaling, which is independently implicated in this form of tumor-type due to mutations in beta-catenin gene, may be contributing to hepatocarcinogenesis in more than one way (Monga, 2009a). More recently, strong activation of Wnt/beta-catenin signaling was also detected in proliferating hepatic progenitors evident in acute necrotizing hepatitis (Spee et al., 2010).

1.1.12 Beta-catenin in Hepatoblastoma

Hepatoblastoma is a childhood liver cancer affecting 1.5 out of every million children born in the U.S. Its incidence is on the rise, and little is known about its etiology (Bulterys M, 1999). This cancer is most prevalent in those under 3 years of age, and is more common in males and white patients (Hartley et al., 1990; Vos, 1995). Emerging evidence suggests it arises from hepatic stem cells during development (Ruck and Xiao, 2002; Fiegel et al., 2004; Lopez-Terrada et al., 2009).

Originally, hepatoblastomas were categorized histologically as either epithelial or mixed epithelial/mesenchymal (Stocker, 1994). Later, within the epithelial classification, they were further classified into 4 subtypes: fetal, embryonal, macrotrabecular, and anaplastic/small-cell undifferentiated. While embryonal tumors appear to be composed of cells resembling bipotential, undifferentiated hepatic stem cells, those classified as fetal resemble late prenatal immature hepatocytes lacking lobular architecture. Tumors designated small cell undifferentiated contain small cells with dense nuclei and scant cytoplasm. Macrotrabecular hepatoblastomas are distinguished by their arrangement into cell plates, similar to the pattern typically seen in adult hepatocellular carcinoma (Schnater et al., 2003). Currently, however, most pathologists merely classify tumors as embryonal or fetal (Schnater et al., 2003). Some

reports indicate that disease prognosis is correlated with differentiation status, with fetal status associated with better outcome than epithelial, and small cell undifferentiated predicting the poorest outcome (Haas et al., 1989). However, disagreements persist regarding the relationship between differentiation status and prognosis (Gonzalez-Crussi et al., 1982; Conran et al., 1992; von Schweinitz et al., 1994; von Schweinitz et al., 1997), though clinically fetal hepatoblastomas are treated differently than embryonal (Rowland, 2002).

Specific chromosomal abnormalities have been detected in hepatoblastomas, including gains of chromosome 1q, 2, 6q, 8q, 17q, 20 and X, and losses on chromosomal region 4q (Balogh et al., 1997; Schneider et al., 1997; Weber et al., 2000; Kumon et al., 2001; Buendia, 2002; Terracciano and Tornillo, 2003; Terracciano et al., 2003a; Terracciano et al., 2003b; Stejskalova et al., 2009; Terada et al., 2009). The majority of hepatoblastoma cases possess mutations in *Ctnnb1* (Blaker et al., 1999; Koch et al., 1999; Jeng et al., 2000; Wei et al., 2000; Udatsu et al., 2001), the gene encoding beta-catenin, strongly implicating it in pathogenesis. Mutations in *APC* leading to increased beta-catenin activity have also been detected in some hepatoblastomas (Oda et al., 1996), and an increased risk for hepatoblastoma is associated with familial adenomatous polyposis, which is characterized by defects the APC gene (Garber et al., 1988; Hughes and Michels, 1992; Giardiello et al., 1996; Montalto et al., 2000; Cetta et al., 2003; Hirschman et al., 2005). Despite this, absence or presence of beta-catenin mutations does not correlate with prognosis. Survival time, does, however, correlate with nuclear localization of beta-catenin (Park et al., 2001). Intriguingly, HGF/MET activation can induce WNT-independent nuclear translocation of beta-catenin, so this may be one of the ways it promotes hepatoblastoma growth (Monga et al., 2002).

Recent work by Lopez-Terrada et al. suggests that Wnt/beta-catenin activity can also be used to classify tumors and thus may be useful prognostically. This group reported that large deletions in CTNNB1 tend to be observed only in pure fetal cases, and that the beta-catenin target genes CCND1 and GLUL are upregulated in these cases as well. Small-cell undifferentiated tumors, however, were more likely to be negative for GLUL expression. Notch expression was higher relative to Wnt expression in fetal cases, and lower in small cell tumors. Together these data support a model in which Wnt activation correlates with the less differentiated embryonal hepatoblastomas and Notch activation correlates with fetal histology. Another pathway implicated in hepatoblastoma development, the HGF/MET pathway, is capable of inducing Wnt-independent nuclear translocation of beta-catenin (Miyazaki et al., 1992; Kuniyasu et al., 1996; von Schweinitz et al., 1998; von Schweinitz et al., 2000; Ranganathan et al., 2005). Activation of beta-catenin may be one mechanism by which this pathway promotes tumor growth.

In summary, roles for beta-catenin have been established for nearly every aspect of liver biology, including embryogenesis, organogenesis, regeneration, zonation and maintaining tissue and organ homeostasis. It has critical responsibilities in the regulation of multiple cellular functions, including lineage specification, differentiation, proliferation, survival, maintenance of redox state, morphogenesis and metabolism (Thompson and Monga, 2007). Multiple means of regulation of beta-catenin activity exist, and understanding them and the contexts in which they operate is critical to understanding normal liver physiology as well as abnormal liver states.

1.1.13 Conclusion

Over the past decade, evidence supporting the role of the Wnt/beta-catenin in liver biology, and specifically in liver development, has rapidly accumulated. While the canonical Wnt/beta-catenin pathway has received the most attention, Wnt-independent functions are also being identified, as are roles for beta-catenin-independent Wnt signaling pathways. Given that these pathways, collectively, can affect every major developmental process, including cell growth, differentiation, migration, and polarity, it is perhaps unsurprising that they play fundamental and highly conserved roles in so many aspects of development. Abundant evidence points to highly temporally regulated activation of these pathways in the various stages of liver development, underscoring the precisely timed and spatially regulated activation of these major processes necessary for proper organ development. Beginning with its critical role in gastrulation, the very first differentiation step in development, and reemerging in control of foregut endoderm specification, hepatic specification of the foregut, regulation of hepatoblast proliferation and differentiation, as well as postnatal liver zonation, facultative stem cell activation and homeostasis, beta-catenin is indispensible to the liver in all stages of life. In addition, abnormal beta-catenin activity is often seen in pathological liver conditions such as hepatoblastoma, as well as hepatocellular carcinomas, cholangiocarcinomas, focal nodular hyperplasias, hepatic adenomas, liver fibrosis and steatohepatitis (Thompson and Monga, 2007; Behari et al., 2009).

A comprehensive understanding of the Wnt signals and beta-catenin activities regulating proper liver development and physiology is critical. Defects in the canonical beta-catenin

pathway characterize many hepatocellular carcinomas, and a better insight into these pathologies is crucial for their effective treatment. Mapping out the mechanisms responsible for hepatocyte differentiation holds other promise as well: the potential to replace patient liver tissue lost to disease. Understanding the liver development program could allow us to create functional hepatocytes *ex vivo* from autologous patient stem cells for use in cell-based therapies or to serve as models of genetic diseases, or could make it possible to differentiate a patient's endogenous liver stem cells *in vivo*, or to enhance or modify the activity of existing hepatocytes. In pursuit of a better understanding of hepatocyte differentiation, we have focused our efforts on three areas: the role of beta-catenin in hepatic differentiation and hepatoblastoma (Chapter 2), the role of MET/beta-catenin interactions in liver bud expansion (Chapter 3), and the role of non-canonical Wnt signaling in late liver development (Chapter 4).

CHAPTER 2. ROLE OF BETA-CATENIN IN HEPATIC DIFFERENTIATION

2.1. Introduction

Applications of hepatocytes derived from stem cells can range from modeling human disease and toxicity screening tools to cell therapy and regenerative medicine and thus are of high significance (Dalgetty et al., 2009). While many attempts have been made to differentiate stem cells from various sources into fully functional hepatocytes suitable for patient transplantation, even the most effective protocols produce cells that retain expression of a-fetoprotein (AFP), a marker of undifferentiated cells, and lack expression of many key cytochrome p450 enzymes critical to mature liver function, revealing that our understanding of this development program is incomplete (Navarro-Alvarez et al., 2009).

A key pathway known to guide liver development is the Wnt/beta-catenin pathway. Wnt/beta-catenin signaling is instrumental in liver development and physiology, beginning with its role in patterning the foregut endoderm from which the liver arises (Pilcher and Krieg, 2002; Finley et al., 2003; McLin et al., 2007; Goessling et al., 2008; Li et al., 2008). After gastrulation and gut tube formation, a gradient of Wnt signaling participates in hepatic induction by patterning the gut along the anterior-posterior axis, with the anteriormost foregut from which the liver arises experiencing an inhibition of Wnt signaling (Pilcher and Krieg, 2002; Finley et al., 2003; McLin et al., 2007; Goessling et al., 2008; Li et al., 2008). Shortly thereafter, activation of Wnt signaling becomes critical to subsequent liver development (Pilcher and Krieg, 2002; Wallace

and Pack, 2003b; Ober et al., 2006; Goessling et al., 2008). The bipotential hepatoblasts that form the nascent liver bud, undergo expansion mediated, in part, by an activation of Wnt signaling (Monga et al., 2003; Hussain et al., 2004; Micsenyi et al., 2004; Suksaweang et al., 2004; Tan et al., 2008). The importance of the Wnt/beta-catenin pathway in liver persists throughout the life of an adult organism, as it plays roles in zonal metabolism (Benhamouche et al., 2006; Burke and Tosh, 2006; Colletti et al., 2009; Giera et al., 2010), hepatic stem cell maintenance (Tan et al., 2006a; Hu et al., 2007; Apte et al., 2008; Yang et al., 2008; Spee et al., 2009; Thompson et al., 2010b; Williams et al., 2010) and liver regeneration (Monga et al., 2001; Sodhi et al., 2005; Sekine et al., 2006; Tan et al., 2006a; Sekine et al., 2007; Apte et al., 2009; Nejak-Bowen et al., 2010), and its dysregulation is observed in many liver cancers (Monga, 2009b).

While compelling evidence supports the role of beta-catenin in proliferation of hepatoblasts during liver bud expansion, seemingly conflicting reports exist on the role of beta-catenin in hepatoblast differentiation into biliary epithelial cells and hepatocytes. Premature activation of beta-catenin via targeted deletion of APC in the livers of developing mice results in pronounced biliary differentiation of hepatoblasts at the cost of hepatocytes (Decaens et al., 2008), consistent with a role for beta-catenin activity in promoting hepatoblast differentiation into BECs. Hepatoblast-specific, FoxA3-*Cre* driven beta-catenin deletion leads to not only defects in biliary specification of hepatoblasts but also maturation of hepatocytes (Tan et al., 2008). Embryos possessing this particular beta-catenin deletion die late in gestation, with livers exhibiting abnormalities beginning at approximately embryonic day 13, when hepatoblast differentiation starts to occur. Knockout livers appear to arrest at this stage, composed of cells exhibiting the high nuclear to cytoplasmic ratio and unpolarized morphology

reminiscent of uncommitted E13/14 stage hepatoblasts. These knockout livers also show an absence of bile ducts and also expression of the hepatocyte-specific transcription factors *C/ebpα* and HNF4α, as well as reduced expression of several other hepatocyte markers (Tan et al., 2008). This indicates that beta-catenin activity is necessary for both BEC and hepatocyte differentiation, but it is unclear what mechanism could account for these observations.

In addition, the role of beta-catenin in hepatoblastoma has been reported (Blaker et al., 1999; Koch et al., 1999; Jeng et al., 2000; Wei et al., 2000; Takayasu et al., 2001; Taniguchi et al., 2002; Ranganathan et al., 2005; Cairo et al., 2008; Curia et al., 2008; Armengol et al., ; Lopez-Terrada et al., 2009), and mutations and deletions in exon 3 of *CTNNB1*, the region where beta-catenin is targeted for degradation by the proteasome, are present in 50-90% of hepatoblastomas (Koch et al., 1999; Wei et al., 2000; Taniguchi et al., 2002; Curia et al., 2008; Lopez-Terrada et al., 2009). Hepatoblastomas are characterized on the basis of morphology as either fetal or embryonal types, with fetal exhibiting greater differentiation and better prognosis (Weinberg and Finegold, 1983), and a gene expression pattern resembling that of late prenatal liver (E14.5-E18.5), while embryonal HBs have an expression profile more like earlier (E11.5-E12.5) liver specimens. While embryonal hepatoblastomas often reveal in-frame *CTNNB1* deletions of exon 3 (codons 5-80) or point mutations targeting nucleotides between codons 27 and 41, fetal hepatoblastomas have been shown to have larger deletions, often missing exon 3 as well as part of exon 4 (Lopez-Terrada et al., 2009). Upregulation of beta-catenin targets Glutamine synthetase (*GLUL*) and CyclinD1 (*CCND1*) at the mRNA level has been observed in both embryonal and fetal HBs (Takayasu et al., 2001; Cairo et al., 2008; Lopez-Terrada et al., 2009), suggesting that partial deletion of exon 4 and differentiation status are associated with differences beta-catenin target gene expression.

In this study, we describe a novel, truncated 75-kDa beta-catenin species that appears in developing liver whose localization and appearance in hepatocytes coincides with hepatocyte maturation. Moreover, we demonstrate that this form of beta-catenin is produced post-translationally via proteolytic cleavage of its N-terminal 95 amino acids by calpain, that it localizes to the membranes and nuclei of hepatocytes in late fetal liver, and that biliary epithelial cells instead express full-length beta-catenin at this time. Thus, for the first time we demonstrate that calpain-mediated cleavage of beta-catenin occurs during normal liver development and may be responsible for modulating its function, and that the truncation of beta-catenin in exon 4 may produce a species with a unique role in promoting expression of genes involved in hepatocyte differentiation from hepatoblasts in developing liver as well as in hepatoblastomas.

2.2 Materials and Methods

2.2.1 Immunoblotting

Whole livers isolated from wild-type C57BL/6 mice at various developmental time points (E12.5, E14.5, E16.5, E17.5, E18.5 and adult) were pooled (n>3) and snap frozen in NP-40 buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl) to promote lysis, then allowed to thaw and lyse on ice. Equal protein amounts, as assessed by BCA assay, were subjected to SDS-PAGE and blotted onto PVDF membrane, then blocked in 5% nonfat milk in TBST, 5% BSA in TBST, or 5% fish gelatin in TBST. Thereafter, membranes were incubated overnight in the appropriate primary antibody diluted in blocking solution, washed in TBST, incubated with secondary antibody (Millipore) diluted 1:30,000-1:80,000 in blocking, washed, and signal visualized using ECL. Antibodies used as follows: TCF4,, Millipore 05-511, 1:250; FAK, Cell

Signaling, #3285, 1:1000; mu-calpain, 1:500, Cell Signaling #2556; cyclin D1, LabVision 1:200, RB-9041-P; Axin2, 1:1000, Santa Cruz sc-8567, glutamine synthetase, 1:200, Santa Cruz sc-9067; actin, 1:500, Millipore MAB1501.

2.2.2 Northern blotting

Messenger RNA was isolated from E12.5, E14.5 E16.5, E17.5, and E18.5 livers using Poly(A)Purist™ Kit (Ambion). Samples were then pooled, and northern blot analysis performed according to standard methods, using a full-length, radiolabeled beta-catenin cDNA probe.

2.2.3 RT-PCR

Whole livers isolated from wild-type C57Bl/6 mice at various developmental time points (E12.5, E14.5, E16.5, E17.5, E18.5 and adult) were pooled (n>3) and total RNA extracted with Trizol (Invitrogen) according to manufacturer's instructions. SuperScript III (Invitrogen) was used to synthesize first strand cDNA from 1 μg total DNase-treated RNA with oligo dT_{20} primers according to manufacturer's instructions. The cDNA was used as the template for RT-PCR performed with primers complementary to the 5' UTR (5'-AAG CCC TCG CTC GGT GG-3') and 3' UTR (5'-CTGAACCATTTCTATAACCGCATCTGTTG-3') and SYBR Green PCR Master Mix reagent (SuperArray Bioscience).

2.2.4 Cell fractionation studies

Nuclear/cytoplasmic fractions and membrane fractions were extracted using the NE-PER kit and MEM-PER kit (Pierce), respectively, according to manufacturer's instructions. Equal

protein amounts were then boiled in SDS gel loading buffer, loaded onto polyacrylamide gels and subjected to SDS-PAGE.

2.2.5 Immunoprecipitation studies

500 μg of liver lysates in NP-40 buffer were diluted to 700 μl in NP-40 buffer containing protease/phosphatase inhibitors. For beta-catenin IP, 20 μl of agarose beads pre-conjugated to rabbit anti-beta-catenin antibody (Santa Cruz, sc-1496-R AC) were added and incubated on an inverter for 1 hour at 4 degrees. For E-cadherin and TCF4 IPs, 2 μg antibody (TCF4: Millipore, E-cadherin: BD Biosciences 610182) was added to tube, incubated on inverter 1 hour at 4 degrees, and then 20 μl Protein A/G Plus agarose beads (Santa Cruz, sc-2003) added and incubated at 4 degrees for one hour on inverter. All reactions were then spun to collect beads, supernatant removed, and beads washed 4 times in 800 μl NP-40 buffer, then beads were boiled in 1x SDS loading buffer for loading on gels.

2.2.6 Immunostaining

To prepare tissue for immunohistochemistry, whole livers (or whole embryos in the case of E12.5 embryos) were fixed for 2 days in 10% buffered formalin, followed by 70% ethanol prior to paraffin embedding. Four to five micron thick paraffin sections were deparaffinized, antigen retrieval performed by microwaving in citrate buffer (10 mM citric acid, pH 6.0) for 12 minutes, cooled to room temp, and endogenous peroxidase activity quenched by treatment for 7 minutes with 3% H_2O_2. Tissue was then blocked by Large Ultra V Block (Labvision) for 5 minutes, followed by incubation in primary antibody diluted in TBST containing 5% serum from the species in which the secondary antibody was raised (Normal Donkey Serum or

Normal Goat Serum, Jackson ImmunoResearch) overnight at 4 degrees. Sections were washed in PBS, then incubated in 1:500 dilution of biotinylated secondary antibody (Millipore) at RT for 30 minutes, washed, and then developed using DAB and counterstained in Shandon Instant Hemotoxylin.

2.2.7 TOPFLASH assay

HEK293 cells (~50% confluent) were fed with fresh medium (antibiotic free, freshly made MEM + 10% FBS) just prior to transfection. DNA (2 μg, 800 ng beta-catenin (WT, Δ95 beta-catenin or empty vector, 800 ng TOPFLASH DNA, 400 ng Renilla) in 96 well containing 200 μl OptiMEM, then added 6 μl FUGENE (3:2 ratio) and incubated for 15 min. 25 μl of transfection cocktail was added to each well (in triplicate) of 24 well plate containing Hek293s and swirled to mix. 24 hours after transfection, cells were washed 3X in 1X PBS, and then lysed in 1X cell lysis buffer for 15 minutes at room temperature on rocker. 20 μl of cell lysates were mixed with 100 μl TOPFLASH reagent (Promega) in a glass vial, swirled for 1 second to mix, and read on a luminometer (EG&G Berthold Lumat LB 9507). Luciferase activity was then quenched and Renilla expression detected by addition of 100 μl STOP-GLO reagent. TOPFLASH values were calculated as ratios of Luciferase signal to Renilla signal.

2.2.8. *In vitro* calpain assay

Recombinant GST-beta-catenin (Millipore) was diluted to 22.5 nM in calpain reaction buffer (10 mM HEPES pH 8, 2 mM DTT, 1 mM EDTA) in 1.5 ml Eppendorf tubes coated with Silicote (Sigma), and added to a 2.5 nM dilution of recombinant calpain (Calbiochem #208718) in the same buffer, incubated at room temperature for 15 minutes, and boiled in

SDS buffer for 5 minutes to stop reaction. Identical reactions were performed in the presence of 10 mM EGTA to inhibit calpain, and also in the absence of calpain as negative controls for beta-catenin cleavage by calpain. These samples were loaded onto gels and immunoblots performed using anti-beta-catenin antibody (1:500 BD Biosciences #610154).

2.2.9 Calpain activity assay

Freshly isolated E14.5, E18.5 and adult livers were pooled and lysed in NP-40 buffer in the absence of protease inhibitors. Protein concentrations were measured by BCA assay, and diluted in Hepes/DTT/EDTA buffer 10mM HEPES (pH 7.2), 10mM DTT and 1mM EDTA. Diluted lysate was added to an equal amount of prepared Calpain-GLO reagent (Promega), incubated 15 min, and luminescence measured on a plate reader (Biotek Synergy HT) Hepes/DTT/EDTA buffer containing 45 nM calpain (Calbiochem #208718) was used as a positive control, and lysates mixed with Calpain-GLO reagent in the presence of EGTA were used as a negative control to ensure specificity of assay for calpain activity.

2.2.10 Animal studies

Timed pregnant animals obtained from Charles River were injected intraperitoneally at E13.5 with either 12 mg/kg MDL28170 (Sigma) or vehicle (DMSO, Sigma) and embryonic livers removed 2-3 hours later, lysed in NP-40 buffer containing 2X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), protein concentration measured by BCA assay, and equal amounts of liver from DMSO-treated and MDL28170-treated animals loaded onto gels for SDS-PAGE/immunoblot analysis.

2.3 Results

2.3.1 Expression of a truncated beta-catenin species correlates with hepatoblast differentiation. To begin investigating the role of beta-catenin in hepatoblast differentiation, we immunoblotted for beta-catenin on liver lysates from mice at embryonic days E12.5, E14.5, E16.5, E17.5 and E18.5, and also samples taken from adult (>28 days) mouse livers. While immunoblot analysis reveals the presence of full-length (~97 kDa) beta-catenin at E12.5 and E14.5, we also observed a truncated, 75 kDa beta-catenin species beginning at approximately embryonic day 12. The truncated species increases during development concurrently with the decrease in full-length protein, and exists as the predominant form of beta-catenin from E16.5 until birth. (Figure 2.3.1A).

2.3.2 Truncated beta-catenin is not the result of alternative splicing. In order to determine whether the shorter beta-catenin species is the result of alternative splicing or post-translational proteolytic cleavage, we performed northern blot and RT-PCR analyses. A northern blot on pooled mRNA from embryonic days E12.5, E14.5, E16.5, E17.5 and E18.5 using a full-length, radiolabeled beta-catenin probe reveals a single mRNA transcript size for *Ctnnb1* (Figure 2.3.1B). Likewise, RT-PCR on cDNA from E14 (both full-length and truncated beta-catenin expressed), E18 (predominantly truncated beta-catenin expressed) and adult liver (predominantly full-length beta-catenin expressed) using primers against the 5'UTR and 3'UTR of *Ctnnb1* produces a single PCR product consistent with the expected full-length transcript length of 3565 bp. (Figure 2.3.1C).

2.3.3 Truncated beta-catenin is lacking 95 N-terminal amino acids. Next we sought to establish which region of beta-catenin was absent in the truncated species. Immunoblotting of

embryonic liver samples with antibodies against various beta-catenin epitopes (amino acids 571-781, pY654, AA1-18, pY142, AA29-49) reveals that the truncated species retains AA 571-781, pY654, and pY142, but not AA1-18 and AA29-49 (Figure 2.3.1D). Tandem mass spectrometry peptide sequencing of immunoprecipitated 75-kDa beta-catenin from E18.5 mouse livers detects AA96 as N-terminal-most amino acid, as well as inclusion of amino acids 96-125, 134-151, 159-170, 486-495, 535-541, 550-564, 647-660, and 673-683 (Figure 2.3.1E). Thus, we have identified a 75-kDa truncated beta-catenin, which is present during normal liver development and lacks N-terminal 95 amino acids.

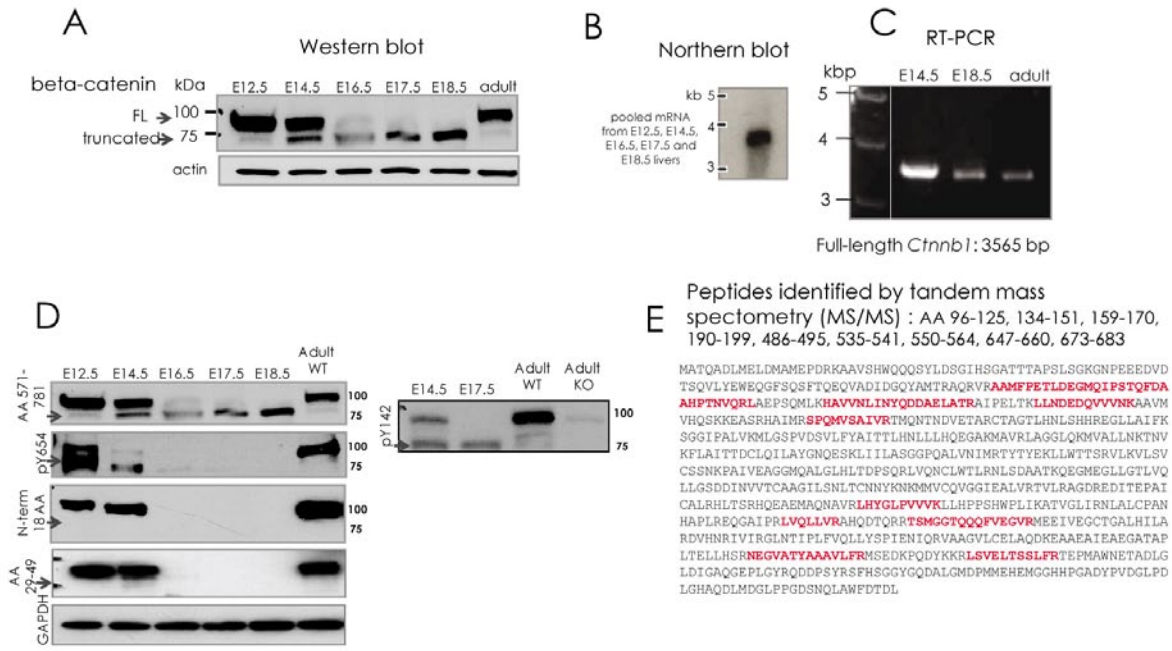


Figure 2.3.1 A truncated beta-catenin species produced by post-translational cleavage of the N-terminal 95 amino acids is produced in developing liver during hepatoblast differentiation. (A) Immunoblot analysis reveals a truncation of beta-catenin from 97 kDa to ~75 kDa occurring as liver develops. (B) Northern blot on pooled mRNA from E12.5, E14.5, E16.5, E17.5, and E18.5 livers probed with a radiolabeled, full-length *Ctnnb1* probe reveals only one beta-catenin transcript at the expected size of ~3565 bp. (C) RT-PCR on cDNA from E14.5, E18.5, and adult using primers against the 5'UTR and 3'UTR of the *Ctnnb1* transcript livers also indicates the presence of one mRNA species coding for beta-catenin. (D) Immunoblots on liver lysates from E12.5, E14.5, E16.5, E17.5 and E18.5 using antibodies against different beta-catenin epitopes shows that that antibodies targeting amino acids 571-781, phospho-Y654 and phospho-Y142 bind to the truncated beta-catenin species, but those targeting amino acids 1-18 or 29-49 do not. (E) Tandem mass spectrometry on truncated beta-catenin protein immunoprecipitated from pooled E18.5 liver lysate detected peptides comprising amino acids 96-125, 134-151, 159-170, 190-199, 486-495, 535-541, 550-564, 647-660, and 673-683.

2.3.4 Truncated beta-catenin is present at membranes and in nuclei of hepatocytes in late fetal liver. Cell fractionation experiments reveal the presence of truncated beta-catenin in both the membrane and nuclear fractions (Figure 2.3.2A). In addition, co-immunoprecipitation experiments indicate an association of truncated beta-catenin with both its adherens junctions binding partner E-cadherin and its nuclear binding partner TCF4 (Figure 2.3.2B). Immunohistochemistry on serial E18.5 liver sections using antibodies against the N-terminus (to detect full-length beta-catenin) and C-terminus of beta-catenin (to detect full-length plus truncated beta-catenin) reveal full-length beta-catenin expression limited to the nucleus and cytoplasm of bile duct cells, while the anti-C-terminal antibody detects those regions but also abundant membranous and nuclear staining in hepatocytes (Figure 2.3.2.C).

2.3.5 Presence of Δ95 beta-catenin correlates with expression of a subset of liver-specific beta-catenin targets. Next we were interested in determining whether the truncated beta-catenin was transcriptionally active, or, possibly acting as a dominant negative. Therefore, we investigated expression of known, liver-specific targets of beta-catenin during the times when these different species were observed. Intriguingly, immunoblot analysis across the same embryonic time points (E12.5, E14.5, E16.5, E17.5, E18.5, adult) for cyclin D1, axin, regucalcin, and glutamine synthetase revealed a correlation between full-length beta-catenin expression and that of cyclin D1 and axin, and also revealed that expression of other beta-catenin targets (regucalcin, glutamine synthetase) correlated with expression of truncated beta-catenin (Figure 2.3.2.D). While glutamine synthetase (GS) activity is also expressed in adult liver, when truncated beta-catenin is less abundant, some truncated beta-catenin is present during that time. Since we believe it is acting as a transcription factor, it is reasonable that

small amounts of truncated beta-catenin may be promoting high expression of glutamine synthetase, perhaps in a zonal fashion, and/or that post-translational regulation of GS in adult liver promotes increased abundance at the protein level. Overall, our results suggest that truncated beta-catenin may be involved in transcription of a subset of target genes involved in hepatocyte maturation.

2.3.6 Δ95 beta-catenin possesses enhanced capacity to promote TCF activity in response to Wnt stimulation. Next, to determine the ability of Δ95 beta-catenin to interact with TCF and regulate transactivation of TCF targets, transient transfections were performed in HEK293 cells, which were then treated with conditioned media either from L cells transfected with control or *Wnt3a* expression plasmid. Δ95 beta-catenin shows an enhanced capacity to promote TCF activity in the nucleus relative to the full-length protein, demonstrating transcriptional superiority of Δ95 beta-catenin (Figure 2.3.2E).

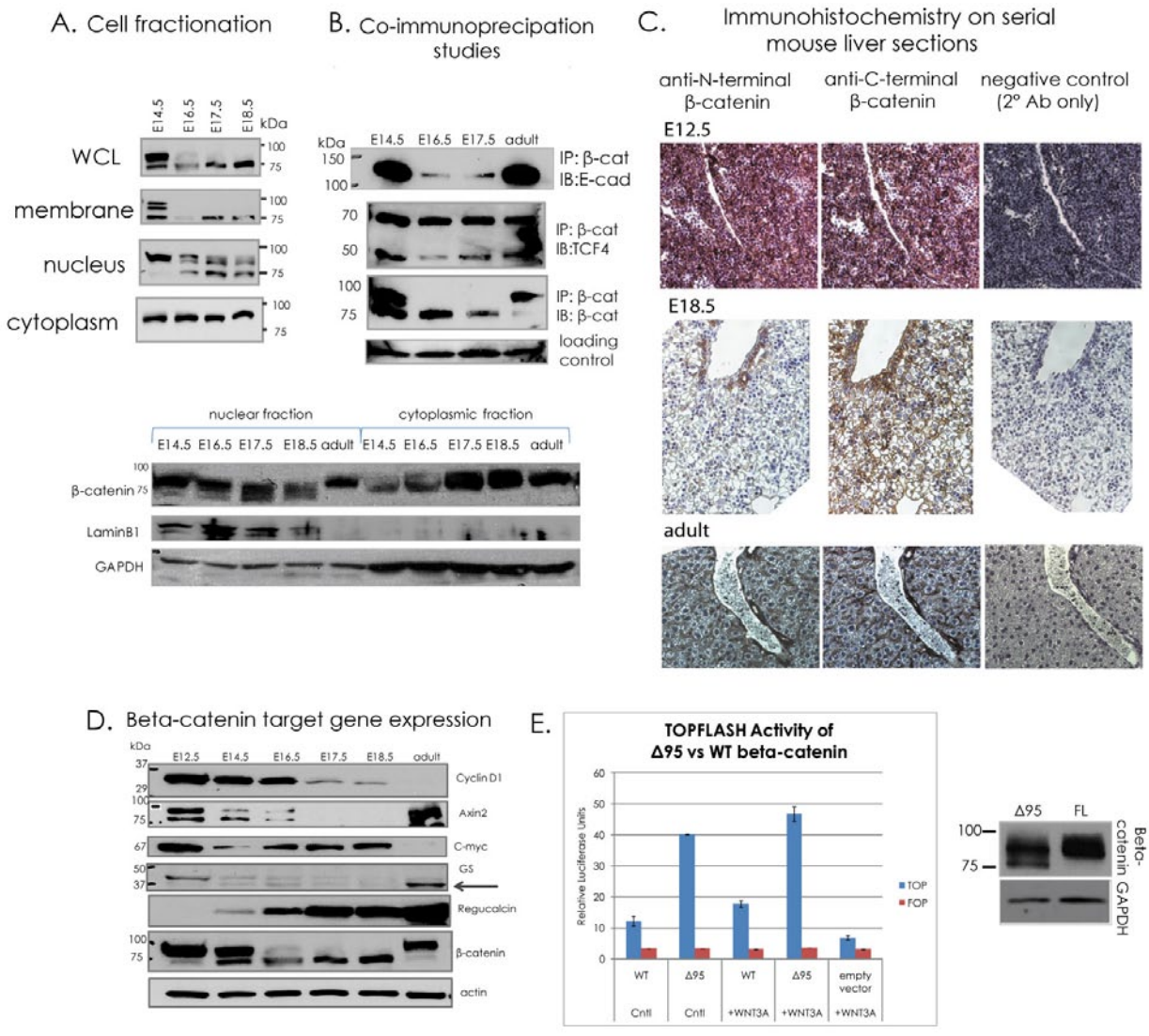

A. Cell fractionation
B. Co-immunoprecipation studies
C. Immunohistochemistry on serial mouse liver sections
E14.5
E16.5
E17.5
E18.5
kDa
WCL
membrane
nucleus
cytoplasm
IP: β-cat
IB:E-cad
IP: β-cat
IB:TCF4
IP: β-cat
IB: β-cat
loading control
nuclear fraction
cytoplasmic fraction
adult
β-catenin
LaminB1
GAPDH
anti-N-terminal β-catenin
anti-C-terminal β-catenin
negative control (2° Ab only)
E12.5
E18.5
adult
D. Beta-catenin target gene expression
Cyclin D1
Axin2
C-myc
GS
Regucalcin
β-catenin
actin
E.
TOPFLASH Activity of Δ95 vs WT beta-catenin
Relative Luciferase Units
TOP
FOP
WT
Δ95
WT
Δ95
empty vector
Cntl
Cntl
+WNT3A
+WNT3A
+WNT3A
Δ95
FL
Beta-catenin
GAPDH

Figure 2.3.2 Several lines of evidence suggest that truncated beta-catenin may play a role in hepatocyte differentiation. (A) Cell fractionation studies reveal truncated beta-catenin present in membrane and nuclear fractions of liver during E16.5, E17.5, and E18.5. Fractionation efficacy was assessed (below) by probing nuclear and cytoplasmic fractions for the nuclear fraction component LaminB1 and the cytoplasmic component GAPDH. (B) Co-immunoprecipitation indicates that truncated beta-catenin binds both E-cadherin and TCF4. (C) Immunohistochemistry with antibodies against N-terminus of beta-catenin, to detect full-length beta-catenin, and C-terminus, to detect both forms of beta-catenin, reveal that at E18.5 truncated beta-catenin (regions positive with anti-C-terminal, but not anti-N-terminal antibody) is located in the nuclei and at membranes of hepatocytes. Full-length beta-catenin expression is limited to biliary epithelial cells only at this time. Staining of E12.5 and adult sections reveal similar staining patterns with both antibodies, consistent with detection of the full-length form predominant at these times (D) Presence of full-length truncated beta-catenin correlates with expression of a subset of beta-catenin targets (cyclinD1, Axin2) and presence of truncated beta-catenin correlates with expression of others (glutamine synthetase, regucalcin). (E) Δ95 beta-catenin activates TOPFLASH at a higher level than wild-type beta-catenin in HEK293 cells. (WT beta-catenin vs Δ95 beta-catenin: Cntrl medium $p<0.0003$. WNT3A medium $p<0.003$, student's t-test). Western blot (right) indicates that increased TOPFLASH activity of Δ95 beta-catenin is not due to accumulation of the protein.

2.3.7 Calpain is the protease responsible for truncation of beta-catenin in developing liver. Since it has been reported that calpain cleavage of the N-terminal 95 amino acids leads to generation of a 75-kDa, truncated beta-catenin product (Rios-Doria et al., 2004; Abe and Takeichi, 2007), we explored whether calpain might be responsible for producing this cleavage during development. Western blot revealed appearance of m-calpain at E12.5 that increased dramatically between E14.5 and E18.5, and coincided with the presence of 75-kDa species of

beta-catenin (Figure 2.3.3A). However, because calpain activity is regulated post-translationally, we performed a calpain activity assay on lysates from freshly isolated embryonic livers at E14.5 and E18.5 as well as adult liver. High activity of calpain was indeed observed during development (Figure 2.3.3B). Activity was highest at E14.5, approximately when truncated beta-catenin begins to be prominently produced, is appreciable at E18.5 when truncated form is still significant. Calpain activity is lowest in the adult liver where truncated beta-catenin is scarce as well. As another way to assess calpain activity, we probed embryonic liver lysates for focal adhesion kinase, a known calpain target, and found cleavage of this protein paralleling that of beta-catenin (Figure 2.3.3C). To demonstrate the feasibility of cleavage of beta-catenin by calpain, we treated beta-catenin protein with recombinant calpain *in vitro* in a cell-free assay. Such treatment produced a cleavage product of similar molecular weight to the truncated species observed in development (Figure 2.3.3D).

2.3.8 Inhibition of calpain activity in developing embryos prevents the production of truncated beta-catenin. Finally, in order to directly test the role of calpain in producing truncated beta-catenin during *in vivo* development, we injected timed-pregnant female mice at E13.5 stage of gestational development with the specific calpain inhibitor MDL28170 or DMSO. Three hours after injection, the livers from embryos were assayed for beta-catenin by western blot. A marked absence of beta-catenin cleavage was observed in calpain inhibitor- and not DMSO-injected group, proving calpain to be the post-translational modifier of beta-catenin during physiological hepatic development. To verify efficacy of calpain inhibitor, we examined focal adhesion kinase cleavage following injection of inhibitor and DMSO. We

observed a dramatic decrease in the cleavage of focal adhesion kinase as indicated by a gain of its uncleaved higher molecular weight form after the inhibitor and not DMSO treatment (Figure 2.3.3E).

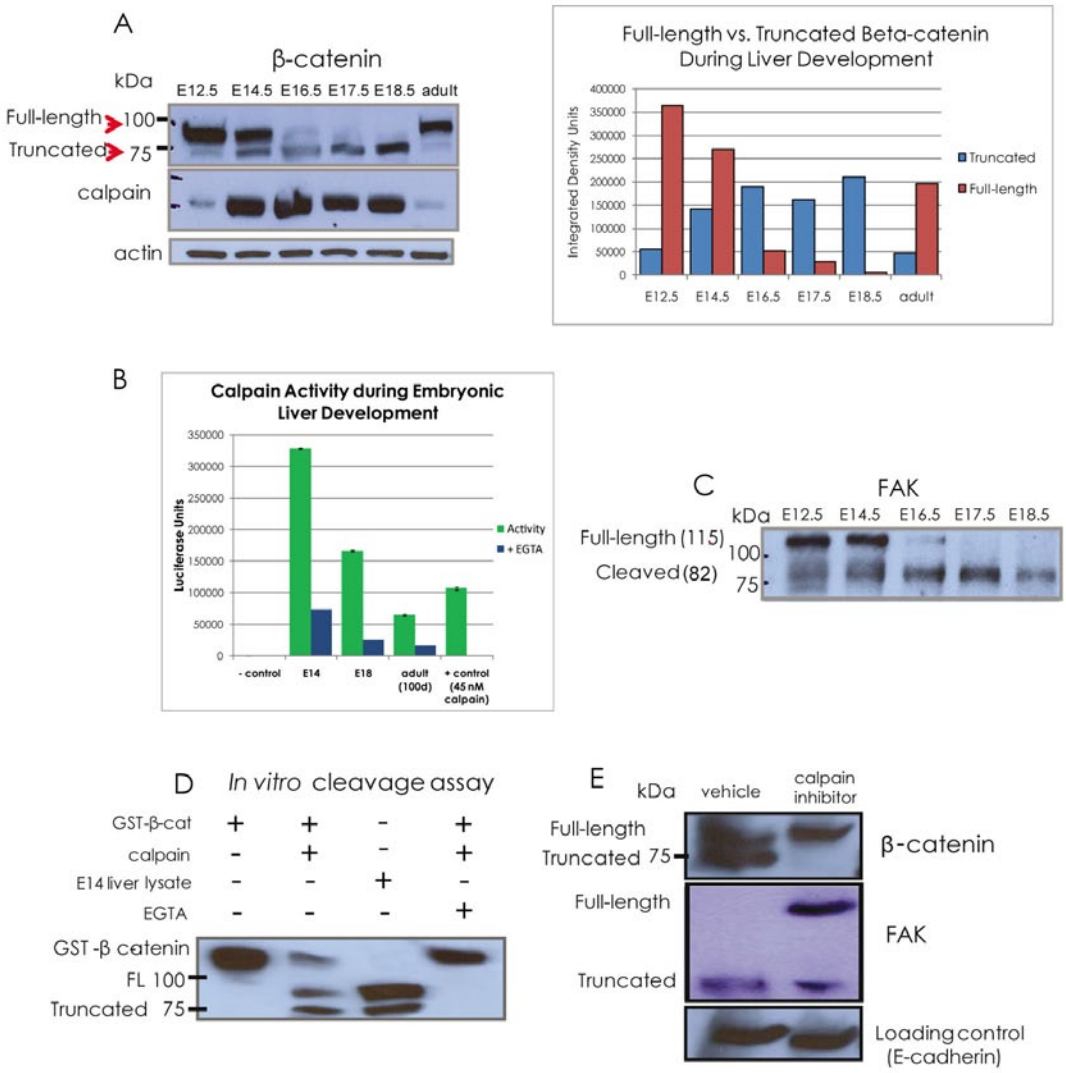


Figure 2.3.3 Calpain cleavage of beta-catenin is responsible for production of the truncated beta-catenin species. (A) Immunoblot analysis indicates that calpain is expressed in developing liver and that its expression level is highest at times when beta-catenin cleavage is observed. (B) Calpain-GLO activity assay detects active calpain during liver development, with activity highest at E14.5, when significant beta-catenin cleavage appears, and persisting at E18.5 when the truncated beta-catenin is the predominant form present. Calpain activity is lowest, but still present in adult samples when minimal beta-catenin cleavage is observed. No calpain activity is seen in samples lacking addition of liver lysates or purified calpain (far left), and positive control for calpain (45 nM purified enzyme added) is positive for calpain activity (far right). In each case, addition of EGTA, a known calpain inhibitor, results in significant quenching of calpain activity. (C) Immunoblot for the known calpain substrate focal adhesion kinase (FAK) showing a cleavage pattern paralleling that of beta-catenin provides further evidence that calpain is active in developing liver. (D). *In vitro* protease assay combining purified calpain and recombinant GST-tagged beta-catenin produces a beta-catenin cleavage pattern identical to that seen in developing liver. This cleavage is inhibited by EGTA, a known inhibitor of calpain activity. (E). Livers from embryos of timed pregnant mice at 13.5 days post-coitus (dpc) injected intraperitoneally with the calpain inhibitor MDL28170 (12.5 mg/kg) show a lack of truncated beta-catenin production relative to vehicle treated animals. Immunoblot performed in parallel for focal adhesion kinase shows that the calpain-mediated cleavage of FAK is inhibited as well.

2.3.9 Beta-catenin species differentiate histological subtypes of hepatoblastoma. While beta-catenin genetic mutations and interstitial deletions are rampant in HB, we speculated that its normal allele should still yield a full-length or truncated beta-catenin species based on the

stage of hepatoblasts composing a tumor. Therefore we investigated, whether N-terminally deleted beta-catenin expression observed normally in late liver development would correlate with fetal HBs in patients, and full-length beta-catenin expression observed during early liver development would correlate with embryonal HB. We stained HB samples (n=16) with antibodies against the N-terminus and C-terminus of beta-catenin and scored them as positive or negative for each, as well as scoring them histologically as fetal or embryonal (Table 2.3.1). These observations were next assessed for correlations using the Fisher's Exact test (Table 2.3.2). We found that tumors positive for N-terminal beta-catenin and C-terminal beta-catenin (corresponding to full-length beta-catenin expression) were invariably embryonal (8/8) while those positive for C-terminal beta-catenin but negative for N-terminal beta-catenin (corresponding to an N-terminally truncated beta-catenin) were always of the fetal subtype (7/7), ($p=0.00016$ for correlation between embryonal/fetal and N-terminal beta-catenin staining). This clearly indicates the utility of N- and C-terminal specific beta-catenin antibodies to distinguish embryonal from fetal HB.

Case No	Histologic subtype	C-terminal Nuclear &/or Cytoplasmic (+/-)	N-terminal Nuclear &/or Cytoplasmic (+/-)
HB-83B	E	+	+
HB-85	E	+	+
HB-70A	E	+	+
HB-50A	E	+	+
HB-24	E	+	+
HB-2B	E - 87%	+	+
HB-3B	E	+	+
HB-39	E treated	+	+
HB-84B	F	+	-
HB-83B	F	+	-
HB-85	F	-	-
HB-50A	F	+	-
HB-25	F	+	-
HB23	F	+	-
HB14	F	+	-
HB-41A	F	+	-

Table 2.3.1 Immunohistochemistry for N-terminal and C-terminal beta-catenin-directed antibodies in hepatoblastoma patients. A correlation exists between absence of N-terminus of beta-catenin and fetal gene expression histologic subtype in hepatoblastoma.

STAINING		HISTOLOGICAL SUBTYPE		TOTAL CASES
		EMBRYONAL	FETAL	
N-term +	C-term +	8	0	8
N-term -	C-term +	0	7	7
		8	7	15
				P=0.0002

Table 2.3.2 Fisher Exact test for correlation studies of b-catenin immunohistochemistry in hepatoblastomas.

2.4 Discussion

The results presented here highlight the existence of a novel, 75-kDa species of beta-catenin that is produced via proteolytic cleavage of the N-terminal 95 amino acids by calpain and whose expression and localization point to a role in the promotion of hepatocyte vs. BEC cell fate from differentiating hepatoblasts. We observed the truncated beta-catenin species localized to the membranes and nuclei of hepatocytes, whereas full-length beta-catenin became limited to the nuclei and cytoplasm of biliary epithelial cells. While further work to characterize the specific function of truncated beta-catenin in differentiating hepatocytes is ongoing in our laboratory, we believe at this time that full-length beta-catenin promotes BEC differentiation, and that calpain is activated in cells fated to become hepatocytes, leading to production of the truncated beta-catenin species that promotes the process of hepatocyte differentiation and maturation (Figure 2.3.4).

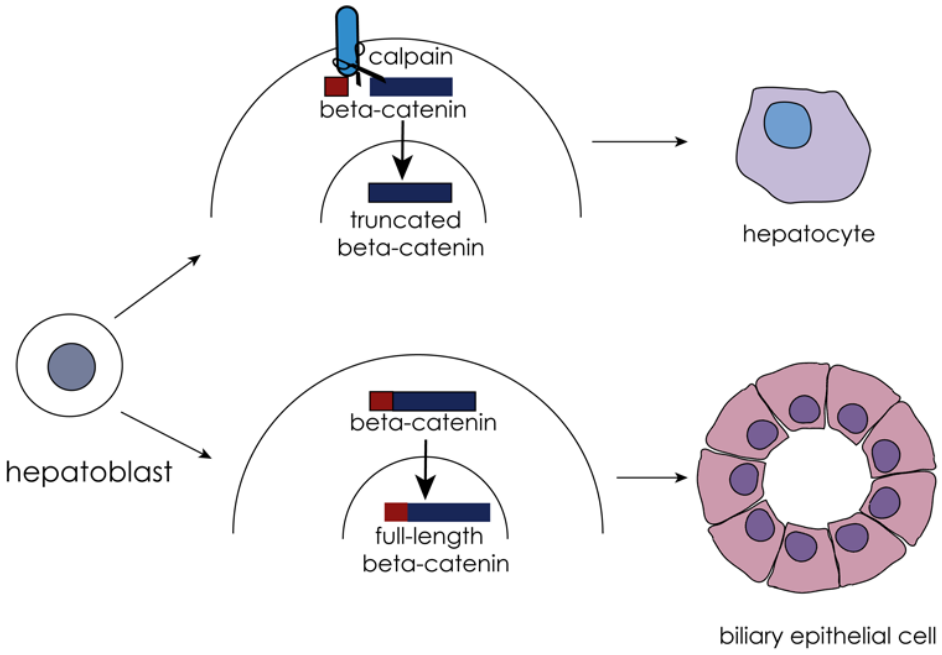


Figure 2.3.4 Model. Hepatoblasts in which beta-catenin is truncated by calpain become hepatocytes, while those in which full-length beta-catenin is retained become biliary epithielial cells.

Several lines of evidence support a role for beta-catenin in hepatocyte differentiation. Livers of mice with a hepatoblast-specific beta-catenin deletion exhibit failures in both hepatocyte as well as biliary epithelial cell differentiation (Tan et al., 2008), suggesting beta-catenin is critical for differentiation of both cell types. In developing human livers, WNT3A expression is seen in the parenchyma of second trimester livers, correlating with early hepatocyte differentiation (Hay et al., 2008). Furthermore, while a hepatic progenitor cell line established from day E14.5 mouse liver cells can differentiate into mature hepatocytes via treatment with dexamethasone, this differentiation is inhibited by the Wnt/beta-catenin pathway antagonist

SFRP3, lending further support to the role of beta-catenin in hepatocyte differentiation (Bi et al., 2009).

Beta-catenin lacks DNA binding property and relies on other transcription factors to dictate target gene expression for a specific biological response. One possible model to account for our observations is that truncation of beta-catenin could promote its association with a transcription factor involved in expression of genes involved in hepatocyte differentiation. Interactions between beta-catenin and other transcription factors have been reported, including HIF1a (Kaidi et al., 2007), FOXO (Liu et al., ; Essers et al., 2005) c/ebpα (Kennell et al., 2003) Smad3/4 (Labbe et al., 2000; Nishita et al., 2000; Hussein et al., 2003; Lei et al., 2004; Rodriguez-Carballo et al., 2010), Sox17 (Hudson et al., 1997; Kanai-Azuma et al., 2002; Clements et al., 2003; Sinner et al., 2004), RXRa (Xiao et al., 2003; Dillard and Lane, 2008), and other nuclear receptors (Mulholland et al., 2005). Another possibility is that truncated beta-catenin could recruit a different co-activator, leading to a switch in gene targets, as is seen in embryonic stem cells (Miyabayashi et al., 2007). While histone deacetylases CBP and p300 have often been regarded as interchangeable in function, increasing evidence points to unique roles for each in promoting expression of specific genes in conjunction with beta-catenin. Emerging work suggests that CBP/beta-catenin interaction is critical for expression of a subset of beta-catenin/TCF targets including (surviving, cyclin D1, axin2, hnkd) but not others (c-jun, fra-1, EphB2, Brachury T) (Teo and Kahn). Furthermore, it appears that the a switch from CBP to p300 recruitment may underlie differentiation of several cell types, including neural, cardiac, myogenic, and hematopoietic (Teo and Kahn). How truncated beta-catenin may differentially interact with other proteins when compared to full-length form to determine genetic expression of specific targets will be necessary for future functional characterization.

While abundant support exists for the role of calpain in differentiation of various cell types, including muscle cells, osteoblasts and adipocytes (Barnoy et al., 1997; Murray et al., 1997; Garach-Jehoshua et al., 1998; Ueda et al., 1998; Patel and Lane, 1999; Yajima and Kawashima, 2002; Moyen et al., 2004; Li and Xie, 2007) at this time no role has been identified for calpain in hepatocyte differentiation. Interestingly, it appears that beta-catenin is a substrate for calpain in differentiating muscle cells (Kramerova et al., 2006), potentially lending precedence to such a phenomenon in liver development. Unfortunately, the calpain inhibitor we employed that crosses the placenta has a very short half-life (2 hours) and does not provide inhibition long enough for us to investigate effects on phenotype. Additional studies to directly address the role of calpain-mediated beta-catenin truncation in liver development are ongoing.

The existence of a beta-catenin species with a role in hepatocyte differentiation also has relevance to hepatoblastoma, a childhood liver malignancy arising from derived from hepatic stem cells during development (Ruck and Xiao, 2002; Fiegel et al., 2004; Cairo et al., 2008). Hepatoblastomas often harbor monoallelic mutations or deletions affecting the N-terminus of beta-catenin. In many cases they are missing exon 3 and/or part of exon 4, producing a truncated beta-catenin resembling the species described herein (Koch et al., 1999; Jeng et al., 2000; Wei et al., 2000; Taniguchi et al., 2002). However, it remains undetermined whether the product of the two mechanisms differ or relate in their functions. But based upon the product of one preserved normal allele of the beta-catenin gene in HB, we were able to distinguish embryonal from the fetal subtype. The tumors positive for full-length beta-catenin (N-terminal-positive) implied a lack of calpain-mediated truncation of the product of normal beta-catenin allele irrespective of presence of any mutations or deletions in the second beta-catenin allele,

thus indicating embryonal hepatoblasts comprising a HB. *Au contraire*, a complete lack of N-terminal staining of a HB implied calpain-mediated cleavage of the product of normal beta-catenin allele irrespective of mutations or deletions in the other allele and hence identified fetal stage of hepatoblasts contained within a HB. Again, it is worth iterating that the functional consequences secondary to beta-catenin in a HB are dependent on the mutations and deletions evident in beta-catenin gene, however, we exploited the product of normal beta-catenin allele that has capability to undergo temporal modulation similar to normal development, as a diagnostic modality to identify HB subtypes, which may vary in phenotype, prognosis and treatment.

We observed full-length beta-catenin associated with a more embryonal histology (resembling E12.5-E14.5 liver) and truncated beta-catenin associated with fetal histology (resembling E14.5-E18.5),consistent with a pro-differentiation role for N-terminally truncated beta-catenin. Our findings suggest that presence or absence of the N-terminus of beta-catenin in hepatoblastomas by IHC may be a useful as a prognostic test.

CHAPTER 3. ROLE OF MET PHOSPHORYLATION OF BETA-CATENIN DURING LIVER DEVELOPMENT

3.1 Introduction

3.1.1 HGF/MET signaling

In addition to beta-catenin, HGF signaling through its receptor MET appears to play a role in hepatocyte differentiation. HGF was discovered independently in several systems and was shown to promote a diverse array of context-specific effects, including cell migration (Stoker et al., 1987; Gherardi et al., 1989; Morimoto et al., 1991), growth (Miyazawa et al., 1989; Nakamura et al., 1989; Rubin et al., 1989; Zarnegar and Michalopoulos, 1989; Morimoto et al., 1991), morphogenesis (Montesano et al., 1991) and inhibition of tumor growth (Shima et al., 1991) before molecular characterization revealed a single protein to be behind these effects (Behrens et al., 1991; Naldini et al., 1991a; Weidner et al., 1991). Structurally, HGF is related to the blood protein plasminogen, which is the precursor of the clotting protein plasmin. Along with apolipoprotein, HGF and plasminogen share an N-terminal plasminogen activation peptide domain, a serine protease modality, and "kringle" domains – loop-in-a-loop structures formed by disulfide linkages between four cysteine residues (Donate et al., 1994). Cleavage of the 728 amino acid HGF-precursor produces an α-chain comprised of residues 1–494 and a β-chain comprised of residues 495–728. Heterodimerization of these chains produces an active ligand capable of activating its tyrosine kinase receptor.

The receptor for HGF, MET, is itself a heterodimer consisting of a 50-kDa extracellular ligand-binding subunit linked to a 140-kDa transmembrane subunit containing an intracellular kinase

domain. The active, 185-kDa dimer undergoes autophosphorylation at residues Y1234 and Y1235 to activate its kinase domain (Ferracini et al., 1991; Longati et al., 1994). Phosphorylation of residues Y1349 and Y1356 allows MET to recruit various SH2/SH3-domain containing signaling and adaptor proteins (Ponzetto et al., 1994), including growth factor receptor-bound protein 2 (GRB2), Shc transforming protein 1 (Shc), GRB2-associated binding protein 1 (GAB1), Signal transducer and activator of transcription 3 (STAT3), phosphatidylinositol 3-kinase (PI3-Kinase), phospholipase C gamma (PLCγ), as well as SRC, V-crk sarcoma virus CT10 oncogene homolog (avian) (CRK) and the related Crk-like (CRKL) (Ponzetto et al., 1994; Furge et al., 2000), to produce context-specific downstream effects.

Recruitment of different signaling proteins by MET-bound adaptors allows for the activation of a constellation of signaling pathways. One example is the activation of the RAS-MAPK and PI3-Kinase/AKT pathways that promote cell migration, invasion and branching morphogenesis (Royal and Park, 1995; Xiao et al., 2001; Grotegut et al., 2006). Another is the CRK/CRKL recruitment of C3G and DOCK180 to activate RAP1- and RAC1-mediated regulation of cytoskeletal rearrangement, cell spreading and cell adhesion (Knudsen et al., 1994). A third example is the induction of tubule morphogenesis and anchorage-independent cell growth promoted by STAT3 (Boccaccio et al., 1998; Zhang et al., 2002b). In addition to these mechanisms that activate signaling, GRB can also induce ubiquitinylation and targeting of MET for proteasomal degradation by recruitment of the ubiquitin ligase CBL (Jeffers et al., 1997; Petrelli et al., 2002).

3.1.2 Role of HGF/MET pathway activity in liver biology

Though its name suggests its primary role in the liver is to promote hepatocyte growth, HGF itself can actually inhibit proliferation of transformed rat liver cells *in vitro* (Knudsen et al., 1994) and *in vivo* (Xiao et al., 2001), and further work has revealed that expression of MET rather than HGF is critical for mitogenesis (D'Errico et al., 1996). Of note, the earliest discovery of HGF came from studies in which it was isolated from the conditioned medium of cultured rat hepatocellular carcinoma cells as a factor that had mitogenic effects on normal rat hepatocytes. *c-Met* was first detected as an oncogene in a osteosarcoma cell line transformed by carcinogen treatment (Rhim et al., 1975; Cooper et al., 1984) until further characterization revealed the protein in question to be a chromosomally-rearranged fusion between MET and TPR (Park et al., 1986; Tempest et al., 1986) missing the MET residue Y1003 that is necessary for its negative regulation (Park et al., 1986). However, though overexpression of HGF or MET is seen in some hepatocellular carcinomas (HCCs) (Boix et al., 1994; Suzuki et al., 1994; Noguchi et al., 1996; Kiss et al., 1997; Ljubimova et al., 1997; Ueki et al., 1997; Tavian et al., 2000), further studies assessing the effect of activation of this pathway on transformed cells *in vivo* or *in vitro* conversely suggest anti-tumorigenic effects. Such effects including growth inhibition (Liu et al., 1995; Conner et al., 1997; Kiss et al., 1997) and promotion of apoptosis (Kiss et al., 1997) though no correlation is observed between MET expression and tumor size (Boix et al., 1994; D'Errico et al., 1996; Okano et al., 1999). Interestingly, mice lacking *c-Met* in hepatocytes showed a paradoxical increase in HCC in response to chemical hepatocarcinogenesis (Takami et al., 2007). Critically, the patient studies do not address whether *c-Met* mutations exist in the analyzed samples that could affect downstream signaling outcomes. In some cases, rather than playing a causative role in HCC development, the

upregulation of these proteins may be secondary to tissue damage or cellular stress associated with cancer. HGF is among the first proteins upregulated after liver injury, and active MET increases concordantly (Horimoto et al., 1995), as well as the protease responsible for its activation (Mars et al., 1995). While the mechanism leading to these changes is still being elucidated, hypoxia promotes transcriptional activation of MET (Pennacchietti et al., 2003) (Matsumoto et al., 1995), as do inflammatory mediators such as IL-1 α, IL-6 and TNF-α (Moghul et al., 1994) and prostaglandins (Matsumoto et al., 1995), and this may account for the increases seen in cancers. Given their upregulation in response to liver injury and ability to promote hepatocyte growth it is no surprise that extensive research has revealed HGF and MET to be critical in supporting liver regeneration. Of note, MET also can inhibit apoptosis by binding the Fas receptor and hindering the trimerization necessary for its activation (Wang et al., 2002), another function that would facilitate a role in responding to tissue injury.

3.1.3 MET in liver development

During development, HGF and MET are expressed more highly in liver than any other tissue (Zarnegar and Michalopoulos, 1995). HGF is secreted by stellate cells (Ramadori et al., 1992; Schirmacher et al., 1992) in the liver as well as sinusoidal endothelial cells (LeCouter et al., 2003), and the receptor MET expressed by hepatocytes, biliary epithelial cells and hepatic progenitors (Prat et al., 1991; Alison et al., 1993; Hu et al., 1993; Matsumoto et al., 1994; Suzuki et al., 2000) Both *HGF*$^{-/-}$ and *c-Met*$^{-/-}$ mice die *in utero*, and these mice exhibit decreased liver parenchymal cell mass and a failure of hepatocyte differentiation (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995), pointing to a role for HGF/MET signaling in prenatal liver growth. Additional work suggests that HGF/MET signaling also inhibits

apoptosis in fetal liver (Moumen et al., 2007). The role of HGF signaling in hepatocyte differentiation has been further characterized in *in vitro* studies demonstrating that it is critical in not only the proliferation and survival, but also the differentiation of embryonic hepatic stem cells into hepatocytes (Suzuki et al., 2000; Suzuki et al., 2002; Minguet et al., 2003; Suzuki et al., 2003) as well as *in vitro* differentiation of virtually any embryonic or adult stem/progenitor cells into hepatocytes (Snykers et al., 2009). Treatment of a population of purified putative hepatic stem cells with HGF induced albumin expression and led to the appearance of hepatoblast-like cells which could then be further differentiated into cells resembling hepatoblasts with oncostatin M (OSM) treatment (Suzuki et al., 2003). Evidence exists that HGF may specifically inhibit hepatoblast differentiation along the biliary epithelial lineage (Kinoshita and Miyajima, 2002; Suzuki et al., 2003), though, intriguingly, evidence also exists for a role for HGF in transdifferentiation of hepatocytes to biliary epithelial cells *in vitro* (Block et al., 1996).

3.1.4 MET phosphorylation of beta-catenin

MET phosphorylation of beta-catenin at residue Y654 induces its translocation to the nucleus along with activation of target gene expression, some of which promote cell cycle entry (Monga et al., 2001; Monga et al., 2002; Zeng et al., 2006). In the widely-utilized 2/3 partial hepatectomy (pHx) rodent model of liver regeneration, hepatocytes have been shown to increase utilization of existing HGF stores between 0 and 3 hours after hepatectomy, and then, once these are depleted, to drastically increase their synthesis from hour 3 to hour 21 after pHx (Pediaditakis et al., 2001). Activation of MET by tyrosine phosphorylation is seen between 30-60 minutes after partial hepatectomy (Stolz et al., 1999), and mice with hepatocyte-specific

(Huh et al., 2004) or conditional *c-Met* deletions (Borowiak et al., 2004) exhibit severe impairments in regeneration. While MET clearly has a critical role in many aspects of liver physiology, the precise context-specific outcomes of MET activity are still being investigated.

We have found high levels of phosphorylation of Y654 during liver bud expansion between E12.5 and E14.5 in embryonic mice, suggesting a role for phosphorylation of this residue in promoting liver growth. Furthermore, we have found MET to be expressed and active in mouse liver at these stages, and associated with beta-catenin. Intriguingly, published reports indicate that *HGF* and *c-Met* knock-out mice die late in gestation, shortly before the stage at which lethality occurs in hepatoblast-specific beta-catenin knock-out mice. Both knockouts exhibit decreased parenchymal cell mass and reduced liver size, as well as increased hepatocyte apoptosis. Based on these data, we hypothesized that MET promotes liver growth by phosphorylating membrane beta-catenin at residue Y654.

3.2 Materials and methods

Methods for Figure 3.3.1 are reported in Schmidt et al. 1995 and Tan et al. 2008. For generation of hepatoblast-specific beta-catenin knockout mice, homozygous floxed–*Ctnnb1* mice were bred to Foxa3-Cre mice and the offspring carrying a floxed *Ctnnb1* allele and Foxa3-Cre were bred to homozygous floxed *Ctnnb1* mice to produce Hep-*Ctnnb1*-/- mice.

3.2.1 Genotyping

Heads from eighteen embryos obtained from two crosses of *c-Met*$^{+/-}$ mice were digested overnight in tail buffer (10mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0, 50mM NaCl,0.5% SDS + 20 μg/ml proteinase K) at 55° C. Samples were spun for 1 min at 14,000 rpm, supernatant

transferred to new tube, and DNA was precipitated by addition of 3M NaOAc in cold 100% ethanol for 2 hours at -80, spun 15 min at 14,000 rpm, washed with 95% ethanol, air-dried and resuspended in 100 µl nuclease-free water. 2 µl of DNA were then simplified by PCR using primers against *c-Met*. PCR products were then resolved on 1.5% agarose gels in TAE.

3.2.2 Mouse models

Embryonic liver samples were isolated from timed pregnant, wild-type C57BL6 mice purchased from Charles River Laboratories. *c-Met* heterozygotes were a generous gift from Dr. Wendy Mars at the University of Pittsburgh. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh in accordance with NIH guidelines.

3.2.3 Immunoblotting

Eighteen E12.5 embryos were harvested from two crosses of *c-Met*$^{+/-}$ mice, livers isolated, and homogenized and lysed on ice in NP-40 buffer for 1 hour. Equal protein amounts, as assessed by BCA assay, were subjected to SDS-PAGE on a 5% Tris-HCl gel and blotted onto PVDF membrane, then blocked in 5% BSA in TBST. Thereafter, membrane was cut and the section including proteins >100 kDa incubated overnight in a mixture of 1:200 mouse anti-MET (Santa Cruz Biotechnologies, sc-8057) and 1:200 mouse anti-MET (Cell Signaling, 25H2), washed in TBST, incubated with HRP-conjugated secondary antibody (goat anti-mouse, Millipore) diluted 1:60,000 in blocking, washed, and signal visualized using SuperSignal West Pico Enhanced Chemiluminescent Substrate (Pierce). Membrane containing proteins <100 kDa was incubated overnight at 4 degrees in 1:500 mouse anti-phospho-Y654 beta-catenin (Abcam,

ab24925) in 5% BSA in TBST, washed in TBST, incubated with HRP-conjugated secondary antibody (Millipore) diluted 1:60,000 in blocking, washed, and signal visualized using SuperSignal West Pico Enhanced Chemiluminescent Substrate (Pierce). Membrane was stripped using Restore Western Blot Stripping Buffer (Pierce) and reprobed with 1:500 mouse-anti-beta-catenin antibody (BD Biosciences, #610154) overnight at 4 degrees, incubated with HRP-conjugated goat anti-mouse secondary antibody (Millipore) diluted 1:60,000 in blocking, washed, and signal visualized using SuperSignal West Pico Enhanced Chemiluminescent Substrate (Pierce).

3.3 Results

Both beta-catenin and HGF/MET have been shown to be active in developing liver (Micsenyi et al., 2004; Tan et al., 2008) Intriguingly, previous work indicates that hepatoblast-specific beta-catenin knockouts show some intriguing similarities to the *HGF/c-Met* knockout phenotype, including late prenatal lethality, failure of hepatoblasts to differentiate, decreased parenchymal cell mass and loosened cell-cell adhesions (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995; Tan et al., 2008) (Figure 3.3.1).

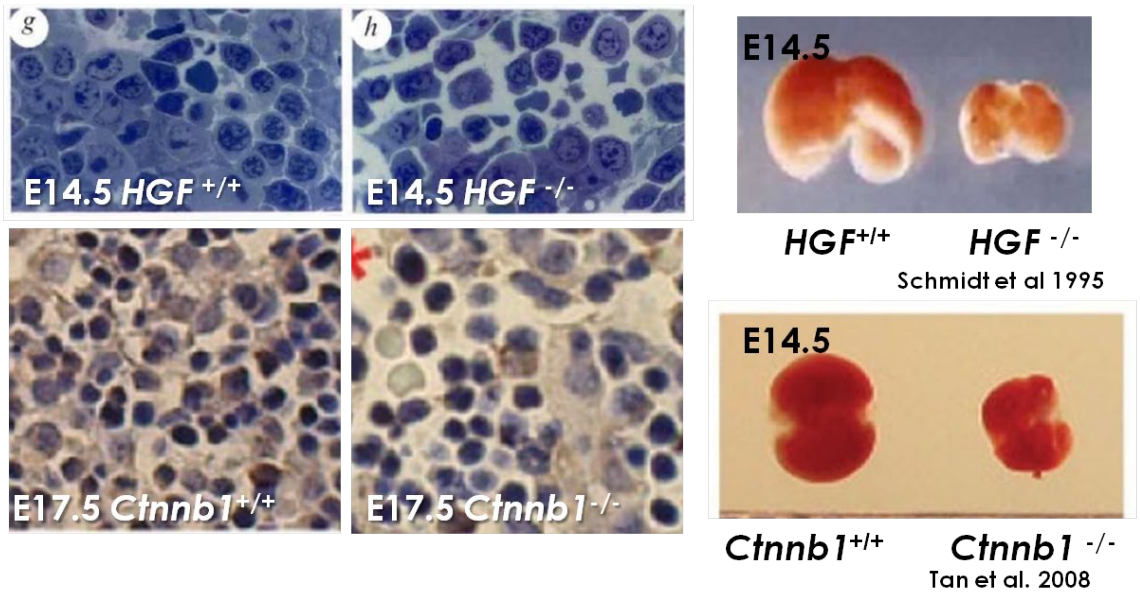


Figure 3.3.1 Beta-catenin and HGF knockouts exhibit embryonic lethality and result in similar phenotypes. Comparison of immunohistochemistry (left) and gross appearance of livers (right) from beta-catenin and HGF knockout mice reveals phenotypic similarities, including loosened cell-cell junctions (left), and decreased liver parenchyma (right).

3.3.1 MET is active and present in liver during times when beta-catenin Y654 phosphorylation is present.

Given previous evidence that the HGF/MET pathway is active in developing liver, we sought to explore whether it was phosphorylating beta-catenin prenatally. Western blot analysis of embryonic liver lysates reveals HGF, active MET, and Y654-phosphorylated beta-catenin present between E12.5-E16.5, consistent with HGF activation of MET causing beta-catenin

phosphorylation during this period of liver bud expansion (Figure 3.3.2). Therefore we moved on to explore whether MET and beta-catenin are associated during this time.

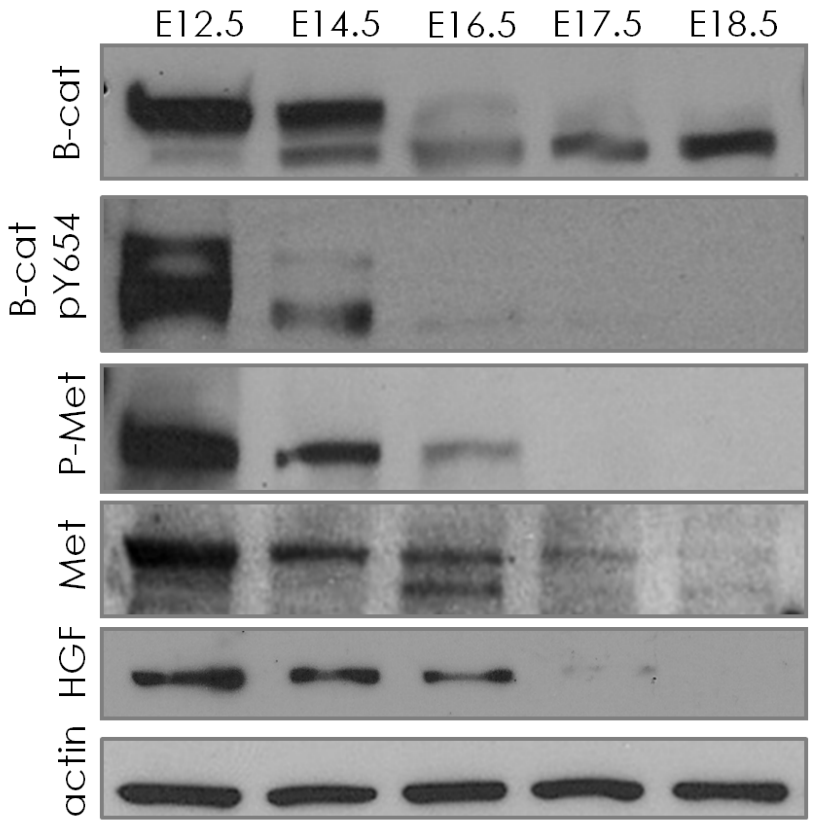


Figure 3.3.2 Active MET is present when beta-catenin Y654 phosphorylation occurs Western blot analysis of embryonic liver lysates reveals HGF, active MET, and Y654-phosphorylated beta-catenin present between E12.5-E16.5, consistent with HGF activation of Met causing beta-catenin phosphorylation during this period of liver bud expansion.

3.3.2 MET is associated with beta-catenin during times when beta-catenin Y654 phosphorylation is present.

In order to investigate beta-catenin/MET interactions, we immunoprecipitated beta-catenin from embryonic mouse liver lysates at the designated time points, and probed these samples for MET (Figure 3.3.3).

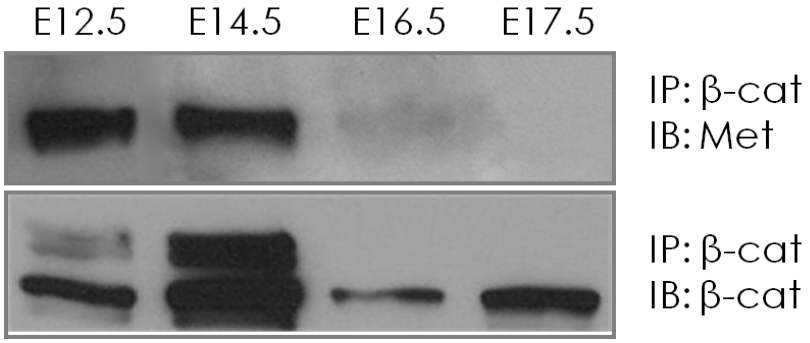


Figure 3.3.3 MET is associated with beta-catenin during times when beta-catenin Y654 phosphorylation is present. Co-immunoprecipitation evidence for beta-catenin and MET interaction between E12.5 and E14.5. Beta-catenin was immunoprecipitated from the embryonic mouse livers at the designated time points, and samples probed for MET. Beta-catenin pY654 western blot on these time points is shown for reference.

3.3.3 *c-Met* knockouts do not show absence of beta-catenin Y654 phosphorylation.

Crosses between *c-Met* heterozygotes (+/-) produced 4 global *c-Met* knockouts animals (Figure 3.3.4, top). Western blot analysis of phospho-Y654 beta-catenin from *c-Met* expressing

animals and *c-Met* KOs indicates that this residue is phosphorylated even in the absence of *c-Met* (Figure 3.3.4, bottom).

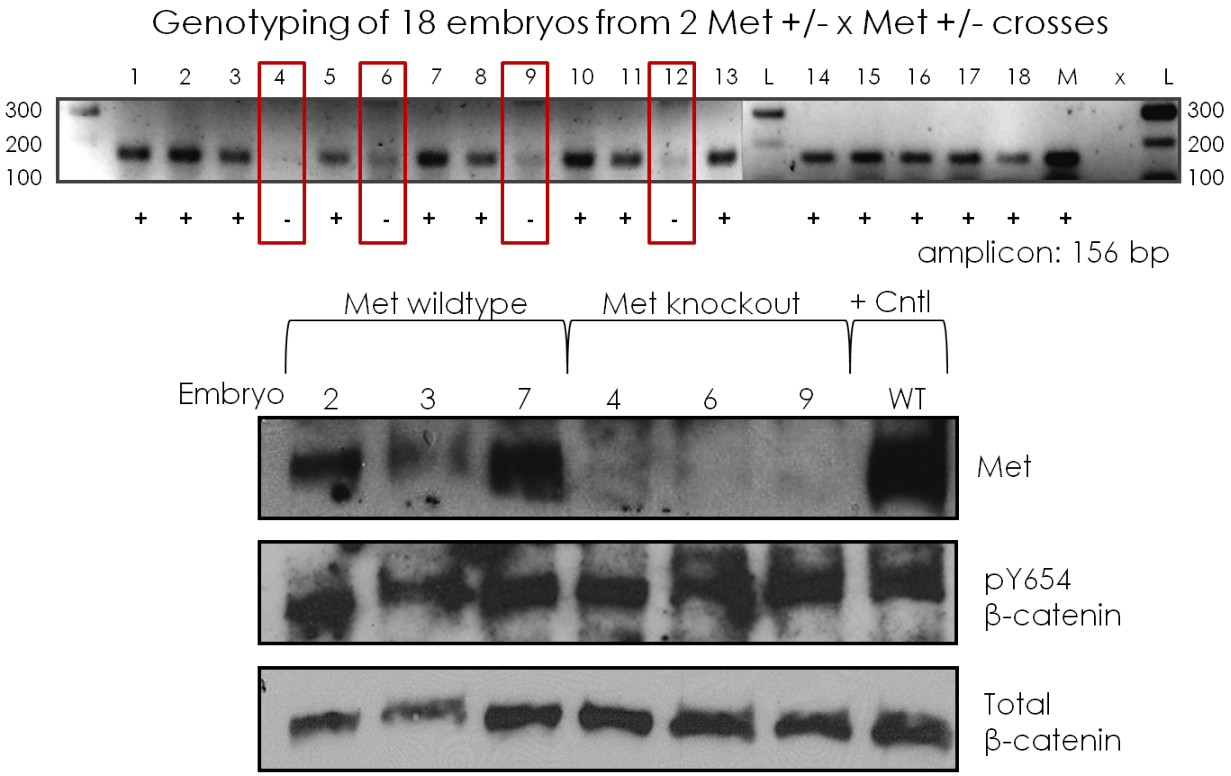


Figure 3.3.4 Met knockouts do not show absence of beta-catenin Y654 phosphorylation. A. Crosses between Met heterozygotes (+/-) produced 4 global Met knockouts animals. B. Western blot analysis of phospho-Y654 beta-catenin from livers of Met expressing animals and Met KOs at E12.5 indicates that this residue is phosphosphorylated even in the absence of Met. Liver lysate from wild-type C57/Bl6 at E12.5 was used as positive control for Y654.

3.4 Conclusions

MET activity correlates temporally with beta-catenin Y654 phosphorylation in liver during embryonic days E12-5-E16.5, and MET is associated with beta-catenin during this time, Also, knockouts of both MET and beta-catenin exhibit somewhat similar phenotypes, However, detection of normal levels of pY654 beta-catenin in *c-Met*$^{-/-}$ embryos points to an another tyrosine kinase phosphorylating beta-catenin during this time such as c-src, Bcl-Abl, EGFR, ErbB2, or FGFR, all of which are capable of phosphorylating this residue (Hoschuetzky et al., 1994; Shibata et al., 1996; Roura et al., 1999; Monga et al., 2002; Sekhon et al., 2004; Apte et al., 2006; Zeng et al., 2006; Coluccia et al., 2007). However, another possibility is that the phospho-Y654 beta-catenin antibody we used lacked specificity. Immunoprecipitations on lysates from MET knockouts using an anti-phosphotyrosine antibody, followed by immunoblotting the immunoprecipitated samples with a beta-catenin antibody is an alternative approach that may offer a clearer answer to the question of whether Met is phosphorylating beta-catenin during liver development.

CHAPTER 4. NON-CANONICAL WNT SIGNALING IN DEVELOPING LIVER

4.1 Introduction

4.1.1 Non-canonical Wnt signaling

In addition to the canonical Wnt/beta-Catenin signaling pathways, beta-catenin activity can also be modulated by the non-canonical Wnt signaling pathways: the Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+} signaling pathways. While these cascades commence with the binding of Wnts to Frizzled receptors, they do not signal through beta catenin, and, in fact, actively suppress canonical beta-catenin activity (Bernard et al., 2008; Yuzugullu et al., 2009) in a mechanism involving APC and targeting for proteasomal degradation by the ubiquitin ligase Siah (Topol et al., 2003; Dimitrova et al., 2010). Wnt ligands have historically been classified according to their biological activities (Du et al., 1995; Torres et al., 1996): with Wnt1 class ligands (including WNT1, -3, -3A and -8) capable of signaling through beta-catenin and inducing secondary axis formation in embryos, and Wnt5a class ligands (WNT4, -5A, 5B and 11) activating beta-catenin independent signaling that does not induce secondary axes. However, recent work revealed that Frizzleds, and not Wnts, control whether canonical or non-canonical signaling occurs downstream (Mikels and Nusse, 2006; Kestler and Kuhl, 2008). A comprehensive characterization has yet to be done on the role of non-canonical Wnt signaling in liver development, but, given the capacity of these pathways to regulate beta-catenin activity, such an analysis is warranted.

4.1.2 Wnt/Ca^{2+} pathway

Wnt/Frizzled ligand-receptor complexes that activate Wnt/Ca^{2+} signaling lead to the activation of PLC, PDE and p38 via heterotrimeric G-proteins (Ahumada et al., 2002; Ma and Wang, 2007), and this, in turn, triggers a release of intracellular calcium stores (Kohn and Moon, 2005); and further activation of PKC (Sheldahl et al., 2003), which becomes phosphorylated and undergoes translocation to the membrane. In addition, the increased calcium concentration promotes remodeling of the actin cytoskeleton via the small GTPase Cdc42 (Habas et al., 2003; Schlessinger et al., 2007; Schlessinger et al., 2009) which becomes activated at one edge and stimulates actin assembly to create membrane protrusions and enable cell motility (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003). Calcium also activates CAMKII, which signals through MAPK pathway kinases TAK1 and NLK (Ishitani et al., 2003) and also the phosphatase calcineurin, which promotes nuclear localization and activity of the transcription factor NF-AT (Saneyoshi et al., 2002) (Figure 4.1.1).

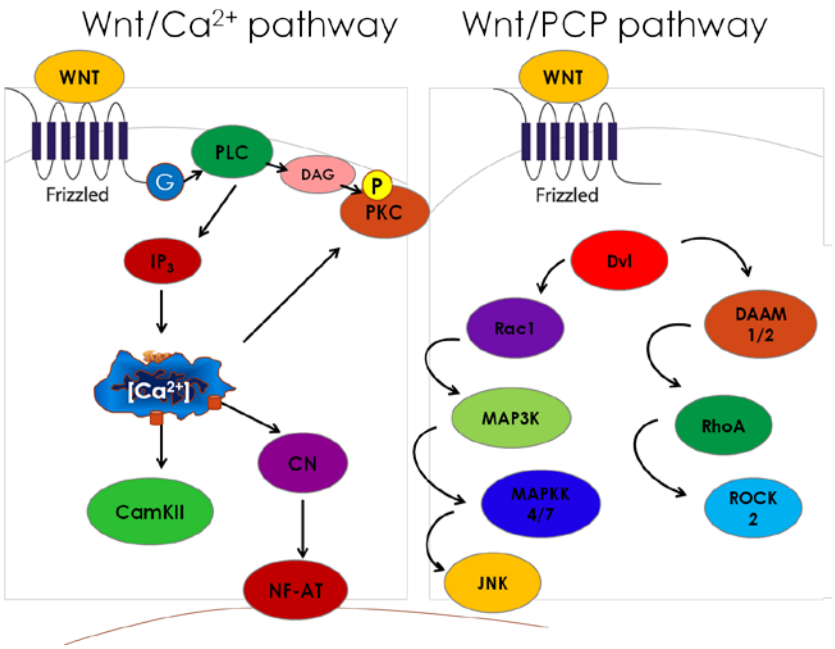


Figure 4.1.1 Non-canonical Wnt signaling pathways. In the Wnt/Calcium pathway (left), Wnt binding to Fzd activates PLC through a G-protein intermediate, leading to IP_3 release of intracellular calcium (Ca^{2+}) stores. This increase in cytoplasmic calcium activates CAMKII, PKC and calcineurin. Calcineurin then dephosphorylates the transcription factor NF-AT, permitting nuclear translocation and target gene expression. In the Wnt/PCP pathway (right), Wnt binding to Fzd activates Dvl, which transduces the signal through Rac1 to promote cell survival through MAPK pathway and ultimately JNK. Dvl also signals through Daam1 to Rho and ROCK to promote cytoskeletal rearrangement. Both of these pathways suppress the canonical Wnt pathway.

4.1.3 Wnt/PCP pathway

Planar polarity is the existence of any non-apicobasal polarity in the spatial organization of a two-dimensional tissue sheet. This phenomenon was first observed in the uniform orientation of cuticular bristles formed by insect epidermal cells (Nubler-Jung et al., 1987), which is coordinated by frizzled (Fz) in Drosophila (Gubb and Garcia-Bellido, 1982). To summarize,

activation of this pathway occurs via binding of secreted Wnts (Wg in flies) to Frizzled. Frizzled then recruits Disheveled to the membrane where it activates downstream signaling components, including small GTPases Rho and Rac (Fanto et al., 2000). Rho and Rac regulate cytoskeletal remodeling and also activate JNK (Boutros et al., 1998; Yamanaka et al., 2002) (Figure 4.1.1).

4.1.4 Non-canonical Wnt signaling in developing liver

While very little is known about non-canonical Wnt signaling during liver development , a few reports on the subject do exist. In Xenopus, gut elongation is mediated by the Wnt/PCP pathway (Li et al., 2008) and regulation of gut tube fusion in zebrafish involves wnt4a, silberblick/wnt11, and wnt11-related (Matsui et al., 2005). In addition, the sFrp5 secreted by the foregut that regulates beta-catenin activity during gut patterning also inhibits Wnt/PCP activity mediated by Wnt11, a process required for proper foregut morphogenesis (Li et al., 2008). Finally, an analyses of and *Fzd* expression in developing liver (Bi et al., 2009) revealed expression of nearly every Wnt and Fzd at the mRNA liver, including those involved in non-canonical signaling. A similar analysis performed on adult liver found expression of *Wnt4* by sinusoidal endothelial cells, stellate cells and biliary epithelial cells, as well as expression of *Fzd4, and Wnt5b* by hepatocytes (Zeng et al., 2007). While it is impossible to conclude anything about the function of these pathways in liver without analysis at the protein level, a limited immunohistochemical analysis in developing chick liver detected diffuse peripheral and central WNT5A and WNT11 staining (Suksaweang et al., 2004), raising the possibility of some contribution to liver development. At any rate, a more thorough analysis of the expression, localization, and activity of non-canonical signaling proteins is necessary.

4.2 Materials and Methods

4.2.1 Mouse models

Embryonic liver samples were isolated from timed pregnant, wild-type C57BL6 mice purchased from Charles River Laboratories. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh in accordance with NIH guidelines.

4.2.2 Immunoblotting

Whole livers isolated from wild-type C57BL/6 mice at various developmental time points (E12.5, E14.5, E16.5, E17.5, E18.5 and adult) were pooled (n>3) and snap frozen in NP-40 buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl plus 2x protease inhibitor + phosphatase inhibitor (Sigma) to promote lysis, then allowed to thaw and lyse on ice. Equal protein amounts, as assessed by BCA assay, were subjected to SDS-PAGE and blotted onto PVDF membrane, then blocked in 5% fish gelatin in TBST. Thereafter, membranes were incubated overnight in the appropriate primary antibody diluted in blocking solution, washed in TBST, incubated with HRP-conjugated Donkey anti-Goat secondary antibody (Millipore) diluted 1:60,000 in blocking, washed, and signal visualized using ECL. Antibodies used as follows: R&D Systems Goat anti-Mouse WNT4 (R&D Systems, AF475), WNT5A (R&D Systems, AF645), WNT11 (R&D Systems, AF2647), FZD3 (R&D Systems, AF1001), FZD4 (R&D Systems, AF194), FZD6 (R&D Systems, AF1526), Thr-286-CAMKII (Cell Signaling, #3361), pan-phospho-PKC (Cell Signaling, #9371) NFAT (Santa Cruz Biotechnology, sc-

7294), phospho-RhoA (Santa Cruz Biotechnology,sc-32954), Ser19-phospho-MLC (Cell Signaling, #3675).

4.2.3 RT-PCR

Whole livers isolated from wild-type C57Bl/6 mice at various developmental time points (E12.5, E14.5, E16.5, E17.5, E18.5 and adult) were pooled (n>3) and total RNA extracted with Trizol (Invitrogen) according to manufacturer's instructions. SuperScript III (Invitrogen) was used to synthesize first strand cDNA from DNase-treated RNA with oligo dT_{20} primers according to manufacturer's instructions. The cDNA was used as the template for qPCR using the SYBR Green PCR Master Mix reagent (SuperArray Bioscience) according to manufacturer's instructions and the appropriate primers (Table 4.2.1):

Table 4.2.1 Primer sequences used for Wnt/Fzd qPCR

Wnt4-F	GCAAACGGAACCTTGAGGTGATG		Fzd3-F	GGGTTGGAAGCAAAAAGACA
Wnt4-R	TGCAAAGGCCACACCTGCTG		Fzd3-R	CTCCCTGCTTTGCTTCTTTG
Wnt5a-F	CCCAGTCCGGACTACTGTG		Fzd4-F	GCTACAACGTGACCAAGATGCCC
Wnt5a-R	CACTGGTGCTGCTATGTCAAA		Fzd4-R	TGAAAGGCACATGCCACCG
Wnt6-F	TTCGGGGATGAGAAGTCAAG		Fzd6-F	GGCTGAAGGTCATTTCCAAG
Wnt6-R	CGGCACAGACAGTTCTCCTC		Fzd6-R	TGAACAGGCAGAGATGTGGA
Wnt7a-F	GACAAATACAACGAGGCCGT		Ror2-F	ACGGCAGGTGAAGTGGAAGATTC
Wnt7a-R	GGCTGTCTTATTGCAGGCTC		Ror2-R	TTCAGCCACCGCACATTGG
Wnt11-F	TGCTTGACCTGGAGAGAGGT		GAPDH-F	GTCTCCTGCGACTTCAAC
Wnt11-R	AGCCCGTAGCTGAGGTTGT		GAPDH-R	TCATTGTCATACCAGGAAATGAGC

4.2.4 Immunostaining

To prepare tissue for immunohistochemistry, whole livers (or whole embryos in the case of E12.5 embryos) were fixed for 2 days in 10% buffered formalin, followed by 70% ethanol prior to paraffin embedding. Four to five micron thick paraffin sections were deparaffinized, antigen retrieval performed by microwaving in citrate buffer (10 mM citric acid, pH 6.0) for 12 minutes, cooled to room temp, and endogenous peroxidase activity quenched by treatment for 7 minutes with 3% H_2O_2. Tissue was then blocked by Large Ultra V Block (Labvision) for 5 minutes, followed by incubation in primary antibody diluted in TBST containing 5% serum from the species in which the secondary antibody was raised (Normal Donkey Serum or Normal Goat Serum, Jackson ImmunoResearch) overnight at 4 degrees Celsius. Sections were washed in PBS, then incubated in 1:500 dilution of biotinylated secondary antibody (Millipore?) at RT for 30 minutes, washed, and then developed using DAB and counterstained in Shandon Instant Hemotoxylin.

4.3 Results

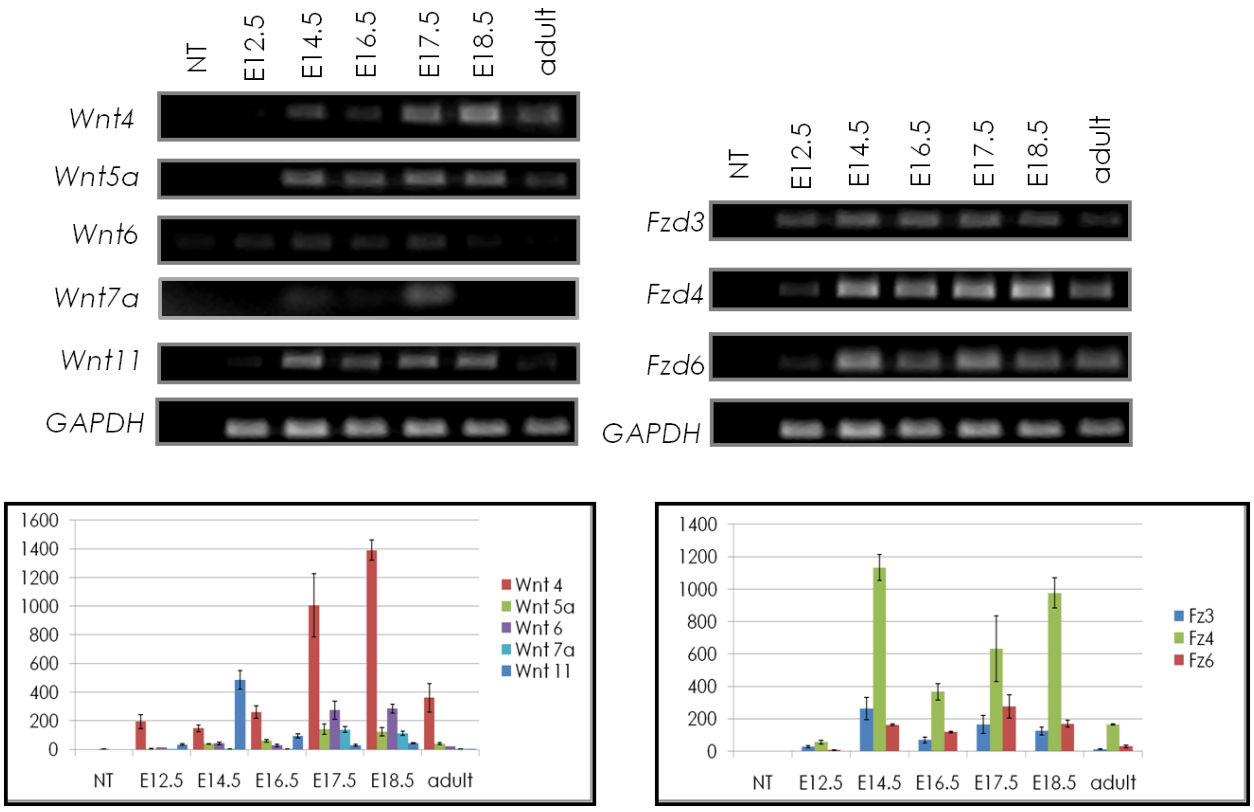


Figure 4.3.1 Non-canonical Wnt and Fzd mRNAs are expressed in developing liver. Results of qPCR performed on cDNA from developing liver to quantitate mRNA expression of Wnts and Fzds implicated in non-canonical Wnt signaling (*Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt11, Fzd3, Fzd4* and *Fzd6*) reveal expression of every mRNA measured in varying levels and temporal expression patterns.

4.3.1 Non-canonical Wnts and Fzds mRNAs are expressed in developing liver. All of the mRNAs of Wnts and Frizzled with potential roles in non-canonical Wnt signaling *(Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt11, Fzd3, Fzd4* and *Fzd6)* that we included in our assessment were expressed at the mRNA level in developing liver, though to differing extents (Figure 4.3.1). In

particular, *Wnt4* and *Fzd4* were expressed at the highest levels, and *Wnt6 Wnt7a* and *Fzd3* were expressed at the lowest levels. Based on these results, we decided to assess expression of some of these at the protein level, and chose WNT4, WNT5A, WNT11, FZD3, FZD4 and FZD6 to analyze by western blot and immunohistochemistry.

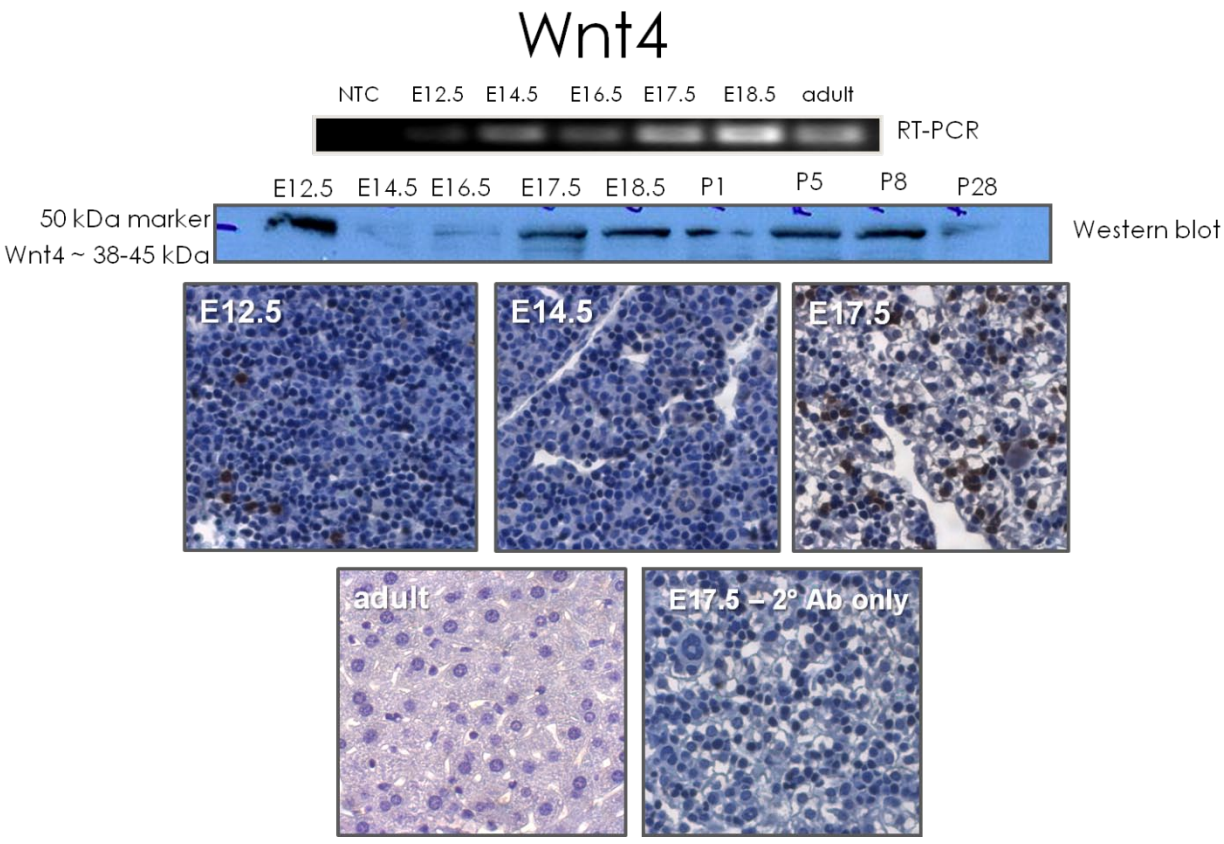


Figure 4.3.2 WNT4 is expressed by hematopoietic cells in developing liver Results of quantitative PCR analysis on cDNA made from mRNA from developing liver time points using primers specific to *Fzd4* (top). Immunoblot analysis on protein lysates from developing liver time points using anti-FZD4 antibody (middle). Immunohistochemistry on paraffin-embedded, formalin-fixed embryonic liver sections using anti-FZD4 antibody.

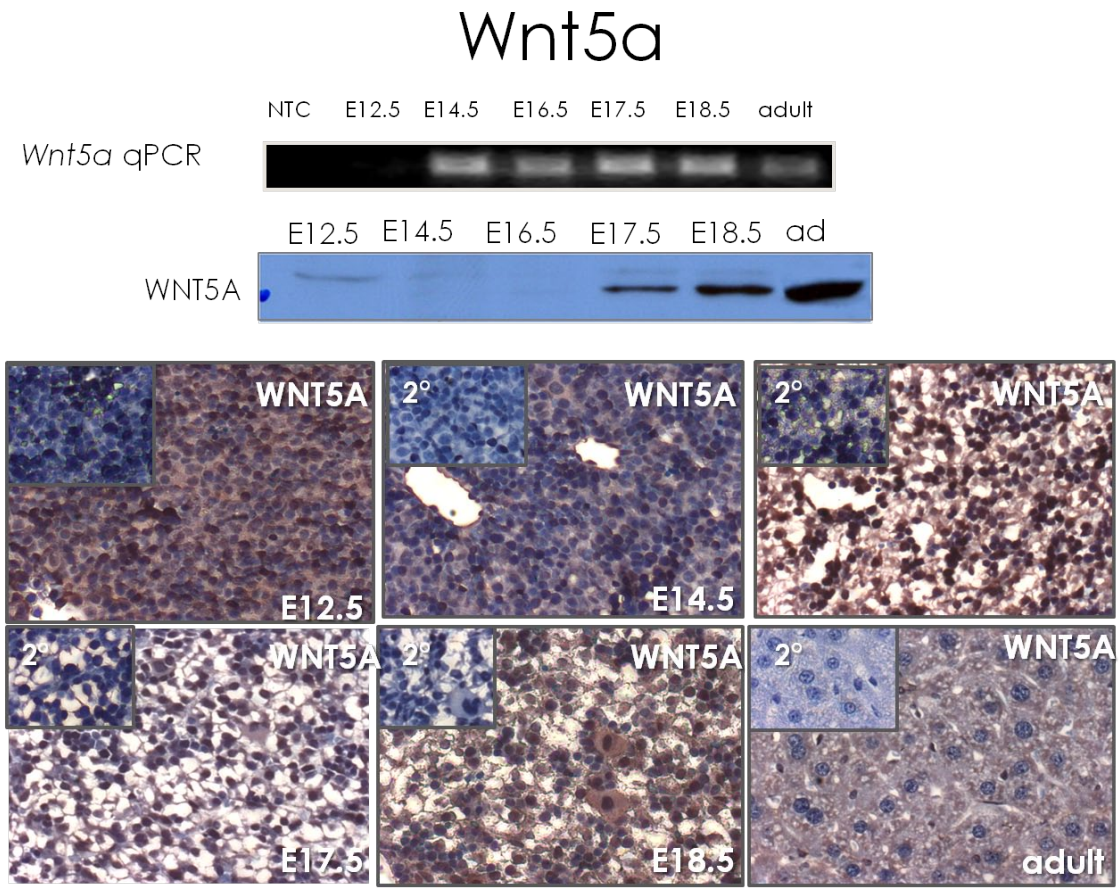


Figure 4.3.3 WNT5A does not show specific staining in developing liver. Quantitative PCR analysis of *Wnt5a* mRNA from developing liver time points. (top) Anti-WNT5A antibody was used to perform immunoblot analysis on protein lysates from developing liver time points (middle) and immunohistochemistry on paraffin-embedded, formalin-fixed embryonic liver sections (bottom).

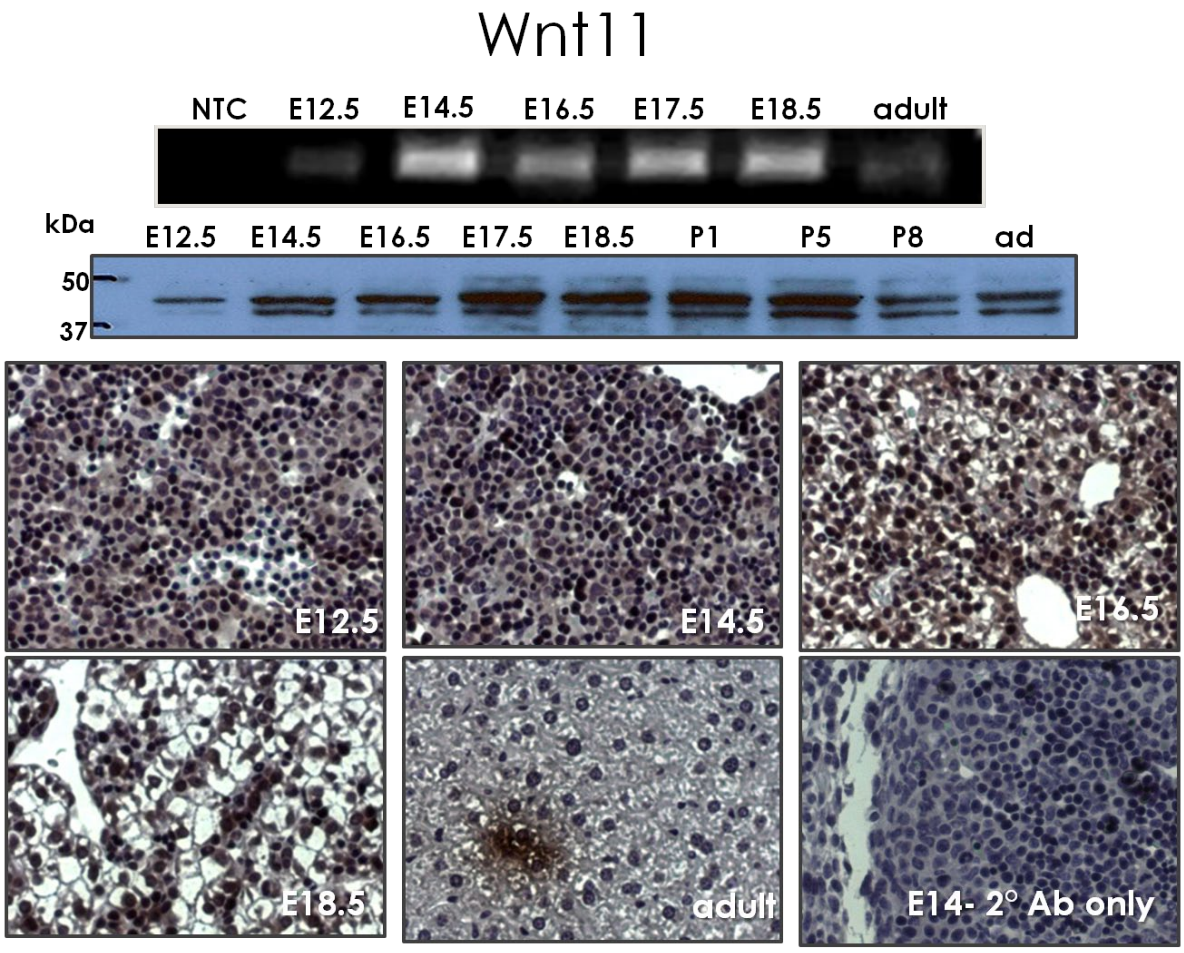


Figure 4.3.4 WNT11 is expressed by western blot, but staining appears faint/non-specific in developing liver. Quantitative PCR amplification of cDNA made from mRNA from developing liver time points using primers specific to *Wnt11* (top). Immunoblot (middle) and immunohistochemistry (bottom) on developing livers using anti-WNT11 antibody.

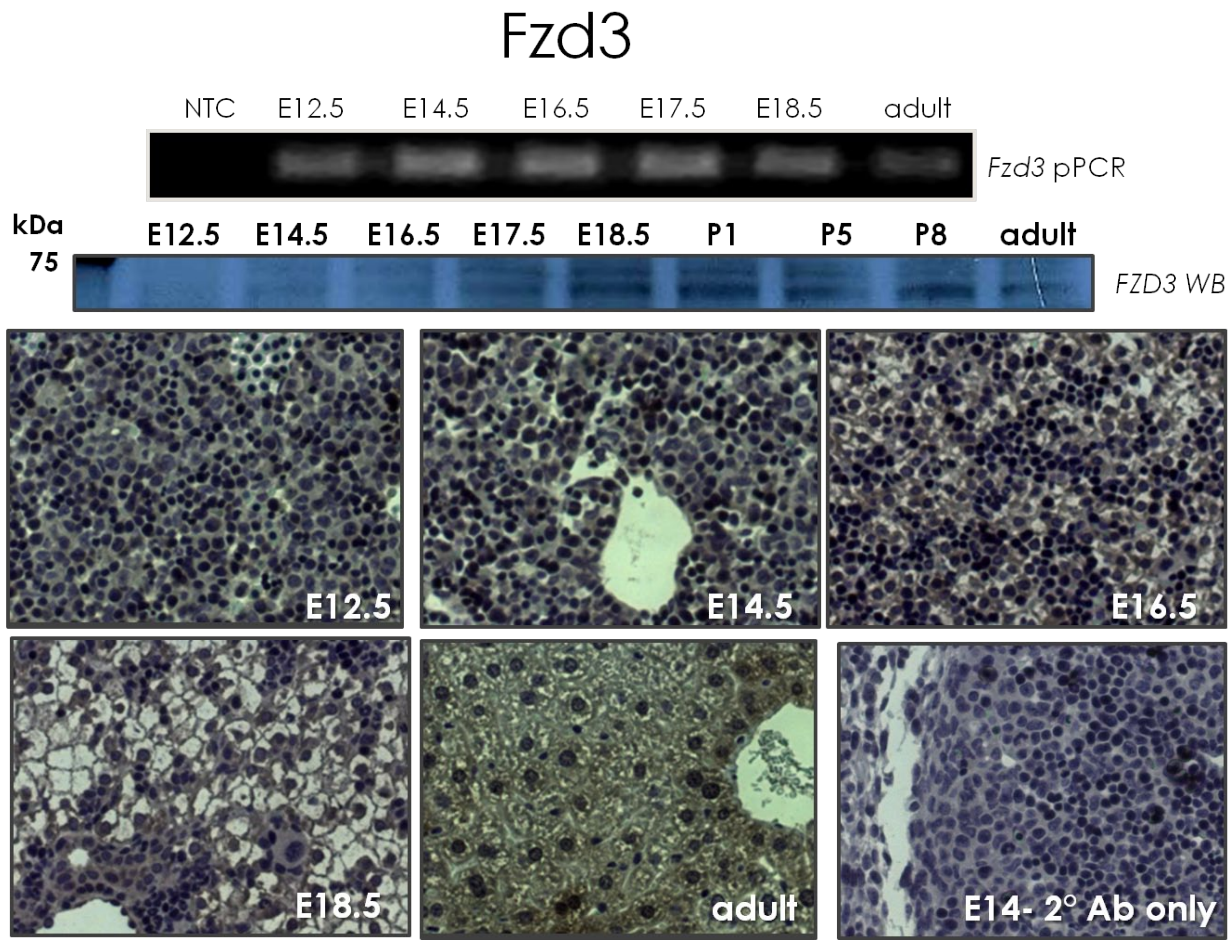


Figure 4.3.5 Frizzled 3 does not show parenchymal expression in developing liver. Quantitative PCR performed on cDNA made from mRNA from developing liver time points using primers specific to *Fzd3* (top). Immunoblot analysis on protein lysates from developing liver time points using anti-FZD3 antibody (middle). Immunohistochemistry on paraffin-embedded, formalin-fixed embryonic liver sections using anti-FZD3 antibody.

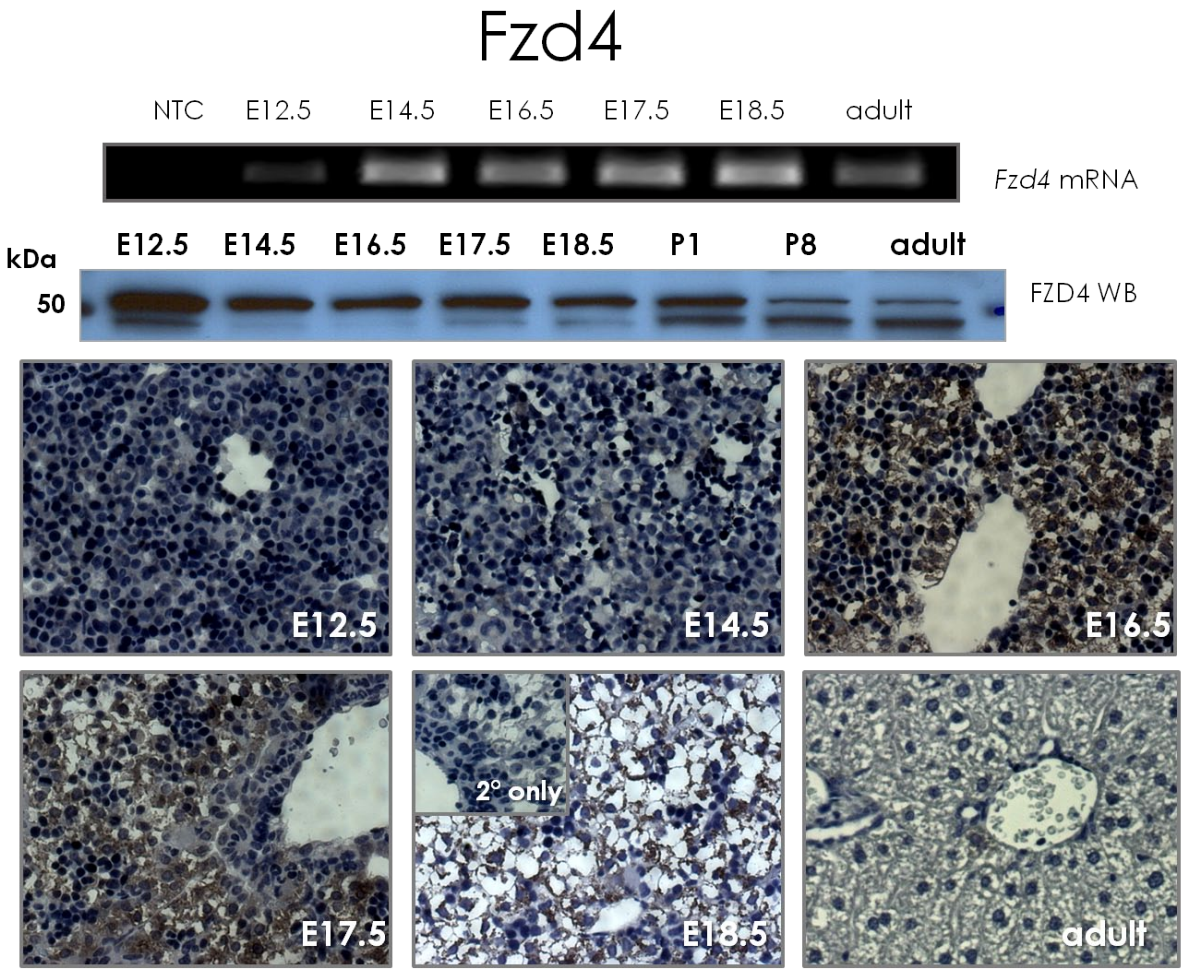


Figure 4.3.6 Frizzled 4 is expressed on differentiating hepatocytes. Quantitative PCR analysis of *Fzd4* mRNA from developing liver time points. (top) Anti-FZD4 antibody was used to perform immunoblot analysis on protein lysates from developing liver time points (middle) and immunohistochemistry on paraffin-embedded, formalin-fixed embryonic liver sections (bottom).

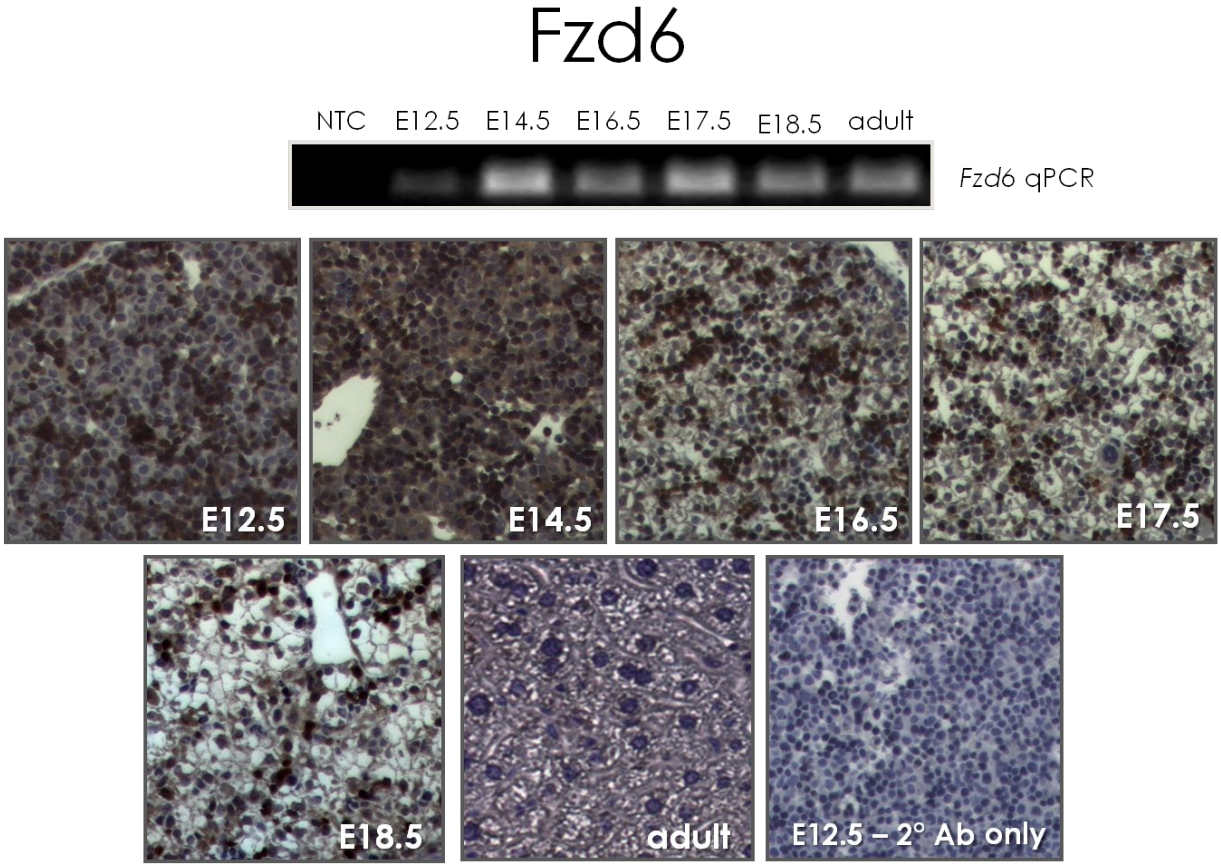


Figure 4.3.7 Frizzled 6 does not show parenchymal expression in developing liver. Results of quantitative PCR analysis on cDNA made from mRNA from developing liver time points using primers specific to *Fzd6* (top) Immunohistochemistry on paraffin-embedded, formalin-fixed embryonic liver sections using anti-FZD6 antibody.

4.3.2 Non-canonical Wnt and Fzd protein expression/localization in developing liver.

Expression and localization of the Wnts and Fzds assessed in this study are summarized in Table 4.3.1. Of the Wnts and Frizzleds whose expression level and staining pattern we assessed by western blot and immunohistochemistry, we observed that while WNT5A (Figure 4.3.3),

WNT11 (Figure 4.3.4) and FZD3 (Figure 4.3.5) expression were detectable by western blot, immunohistochemistry did not detect specific staining. Though it is possible this was due to suboptimal staining conditions, we did observe specific staining patterns for WNT4 (Figure 4.3.2) and FZD4 (Figure 4.3.6). FZD6, though not detectable by western blot, did appear localized to a subset of cells in developing liver, but the staining was nuclear, which is not consistent with correct localization for a Frizzled protein. However, the localization of this protein appears to be to the hematopoietic compartment, consistent with reports of its role in B cell differentiation in fetal liver, occurring between E12-E18 (Palacios and Samaridis, 1992). Either way, it seems reasonable to conclude that it is not playing a role in hepatic differentiation (Figure 4.3.7).

Frizzled 4 mRNA becomes prominently expressed beginning at E14.5, and persists through development, decreasing in adult animals. At the protein level, FZD4 is seen across all stages, though, interestingly, a lower band appears at E12.5, mostly disappears, and is then seen again in late prenatal liver, beginning to be faintly expressed at E17.5 and then prominent after birth and in adults. Immunohistochemical staining for FZD4 in formalin-fixed liver sections reveals robust signal for FZD4, clearly localized to membranes of emerging hepatocytes and absent from adjacent hematopoietic cells, bile duct cells and endothelial cells, suggesting a role in differentiating hepatocytes (Figure 4.3.6). Given the fact that the times when membrane Frizzled is seen are the same times a single band is seen by western blot, one possibility is that the lower band represents a degradation product resulting from Frizzled 4 being expressed at the protein level but turned over and not targeted to membranes.

As for a ligand potentially activating FZD4, the best candidate in our assessment was WNT4, which increases in mRNA level during hepatocyte differentiation and appears to exhibit a specific staining pattern in developing liver, notably to cells in the nonparenchymal, possibly hematopoietic compartment. As noted previously, Wnt1 class ligands (Wnt 1, 3, 3a and 8) may also activate non-canonical Fzd receptors (Louis et al., 2008; van Amerongen and Nusse, 2009), so it remains a possibility that a Wnt we did not investigate is responsible for binding to FZD4. Furthermore, WNT4 plays a key role in thymopoesis (Louis et al., 2008), and so it may be acting within the hematopoietic compartment to this end. Given that our goal was to uncover evidence of non-canonical Wnt signaling in hepatic differentiation, however, the localization of FZD4 to differentiating hepatocytes gave us grounds to move forward and look for evidence of activation of Wnt/Ca^{2+} or Wnt/PCP signaling.

Protein	Localization
WNT4	hematopoietic
WNT5A	non-specific
WNT11	non-specific
FZD3	not present
FZD4	hepatocyte
FZD6	hematopoietic

Table 4.3.1 Localization of non-canonical WNTs and FZDs in developing liver. This table summarizes the localization by immunohistochemistry of Wnts and Frizzleds potentially expressed during liver development.

4.4 Outcomes of non-canonical Wnt signaling

Given our detection of the expression of many Wnt and Frizzled genes implicated in non-canonical Wnt signaling at both the mRNA and protein level in developing liver, we sought to determine whether these proteins were, in fact, activating the Wnt/Ca^{2+} or Wnt/planar cell polarity pathway. The set of Wnt ligands and Frizzled receptors implicated in these pathways is almost identical, and while there are exceptions, like the involvement of WNT6 specifically in Wnt/Ca^{2+} signaling or WNT7A in Wnt/PCP activity, no evidence exists that these cannot also activate the other pathway. Therefore, detection of a high level of expression of specific ligands or receptors was not sufficient to warrant investigation of one pathway at the exclusion of the other. Instead, it was necessary to investigate outcomes specific to each pathway. The elevation of intracellular calcium levels triggered by the Wnt/Ca^{2+} pathway leads to a number of outcomes, including phosphorylation of the kinase CAMKII on threonine 286, phosphorylation and membrane translocation of protein kinase C (PKC), and nuclear translocation of the transcription factor NF-AT. Phosphorylation of CAMKII and PKC are detectable by immunoblot on protein lysates, and the localization of these signals to specific hepatic cell types can be determined by immunohistochemistry on paraffin-embedded liver sections, as can nuclear localization of NF-AT.

Outcomes of the Wnt/planar cell polarity pathway, on the other hand, are more challenging to detect. Following activation of this pathway, complexes form between Dvl, Daam1 and RhoA, as well as Dvl and Rac. Attempting to immunoprecipitation these complexes may not give definitive results, as suboptimal immunoprecipitation conditions or highly transient

interactions may lead to false negatives. Therefore, we chose to focus on Ser-19-MLC phosphorylation. Though, while this phosphorylation is induced as result of the Wnt/PCP pathway, it can occur as a result of other cellular events as well.

While some outcomes we are able to investigate here are not specific to the non-canonical Wnt pathways, and positive results are therefore not definitive evidence of activation of these pathways, evidence of these outcomes serve as grounds for further investigation by other means, and negative results can provide grounds for ruling out a role for one or both pathways in liver development.

NONCANONICAL Wnt PATHWAY	INCREASED EXPRESSION OF WNT/FZ	END-RESULT
Wnt/Ca2+	Wnt5a, 11, 4, 6 Fz3, 4, 6	Thr-286-Phospho CamKII
		Pan-Phospho-PKC
		Memb. Translocation of PKC
		Nuclear Translocation of NFAT
PCP Pathway	Wnt5a, 11, 4, 7a Fz3, 4, 6	a. Dvl-Daam1-RhoA complex b. Ser-19-phospho-MLC
		a. Dvl-Rac complex b. Ser-63-phospho-JNK

Table 4.4.1 Measureable outcomes of non-canonical Wnt signaling pathways. Known ligands and receptors involved in activating the Wnt/calcium pathway (Wnt5a, 11, 4, and 6 and Frizzled 3, 4 and 6) and Wnt/Planar Cell Polarity pathway (Wnt5a, 11, 4, and 7a and Frizzled 3, 4 and 6) are not specific to one pathway and assessment of downstream events in these pathways are critical for determining whether the pathways are active. Wnt/calcium pathway activity results in phosphorylation of CamKII on threonine 286, and phosphorylation of PKC, as well as localization of PKC to the membrane and of the transcription factor NF-AT to the nucleus. Activation of the planar cell polarity pathway, on the other hand, leads to formation of complexes between Dvl-Daam1 and RhoA, as well as Dvl and Rac, as well as phosphorylation of myosin light chain (MLC) on serine 19 and phosphorylation of c-Jun N-terminal kinase on serine 63.

We sought to investigate each of the outcomes of the Wnt/Ca^{2+} and Wnt/Planar Cell Polarity pathway (Table 4.4.1) in turn, in order to assess the potential contribution of non-canonical Wnt pathways to prenatal liver development.

4.4.1 PKC membrane localization is not observed in hepatocytes. Increased calcium concentration downstream of Wnt/Ca^{2+} pathway activation results in phosphorylation of the signaling kinase PKC, which undergoes membrane translocation when phosphorylated. Though some phospho-PKC can be observed in developing liver by immunohistochemistry and immunoblot (Figure 4.4.1), the signal is limited to the hematopoietic compartment and therefore is not the result of a contribution of Wnt/Ca^{2+} signaling to hepatic differentiation.

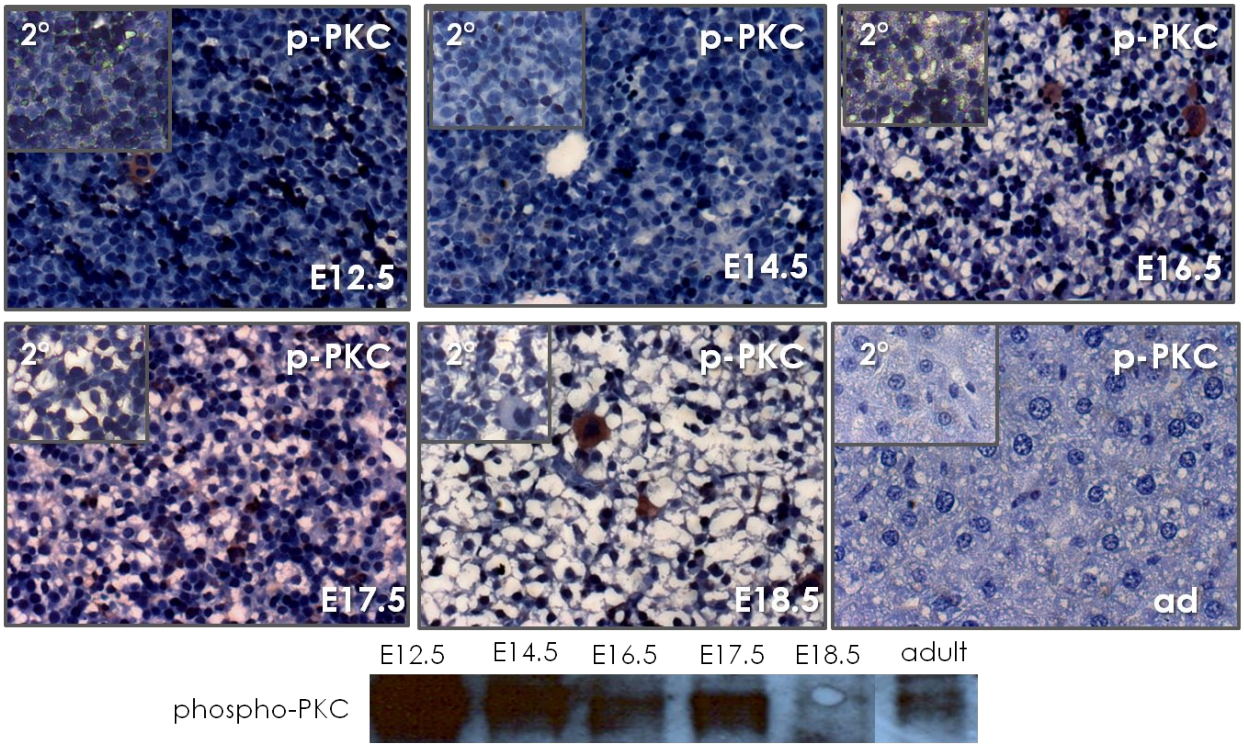
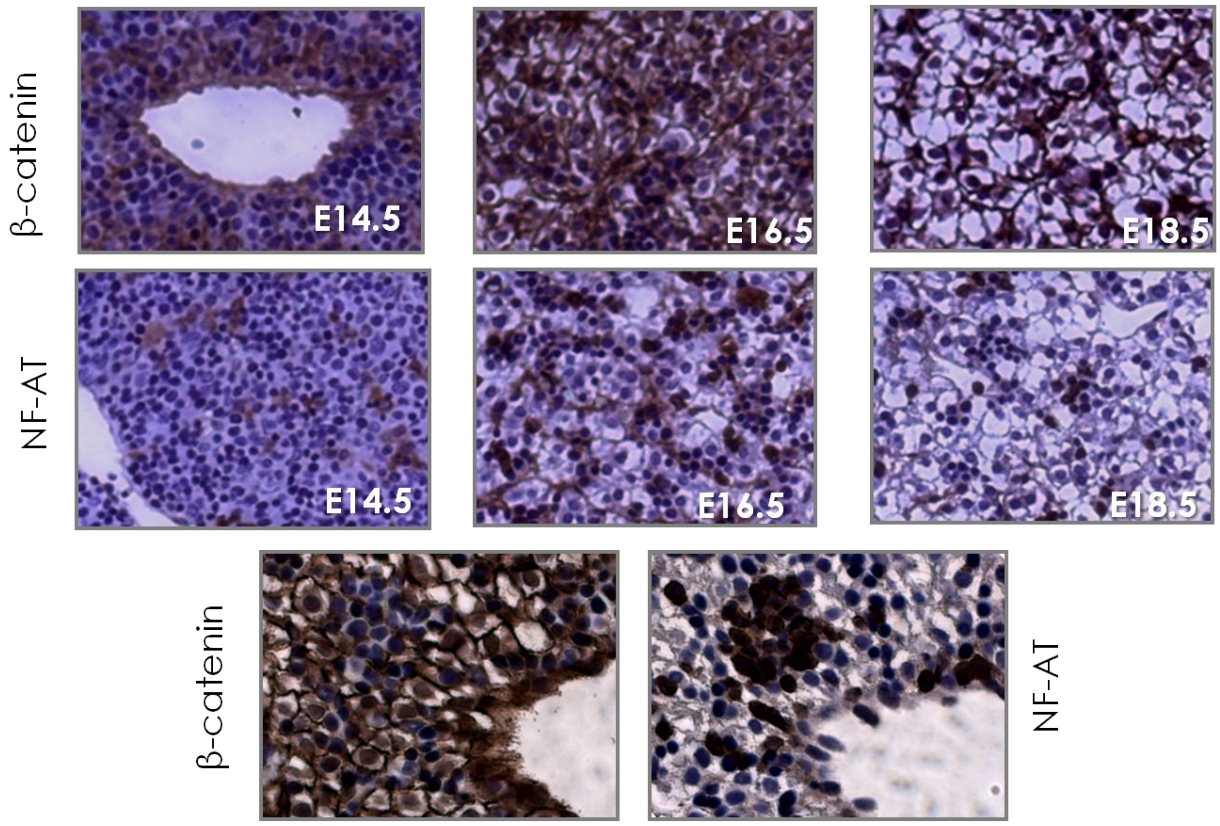


Figure 4.4.1 PKC membrane localization is not observed in hepatocytes. Immunostaining for phospho-PKC of paraffin embedded E12.5, E14.5, E16.5, E17.5, E18.5 and adult liver sections (top) reveals staining limited to hematopoietic cells. Immunoblot analysis (bottom) shows signal at early stages of development, which decreases at E18.5 and is weak in adult tissue.

4.4.2 NF-AT nuclear localization is not observed in the liver parenchyma during late liver development. While prominent cytoplasmic and nuclear NF-AT staining can be observed via immunohistochemistry in developing liver sections, staining of serial sections (bottom) with beta-catenin (localized to hepatocyte membranes) and NF-AT reveals that the staining is specific to hematopoietic cells (Figure 4.4.2). Therefore, this does not provide evidence that the Wnt/Ca^{2+} pathway is active in parenchymal cells in developing liver.



4.4.3 pThr286-CAMKII expression is undetermined in developing liver. While immunoblotting for pThr286-CAMKII suggests it is present during development, and increased at E18.5 and in adult liver tissue, staining for pThr286-CAMKII on paraffin sections results in spurious nuclear signal (Figure 4.4.3). CAMKII, active or not, has never been reported to be nuclear, and therefore this is most likely the result of non-specific antibody binding. In adult

tissue, though, some non-parenchymal cell type, possibly Kupffer cells, appear to be staining positive for pThr286-CAMKII in a possibly specific manner. In conjunction with other evidence, it seems safe to conclude that Wnt/Ca^{2+} activity does not play a role in hepatocyte differentiation.

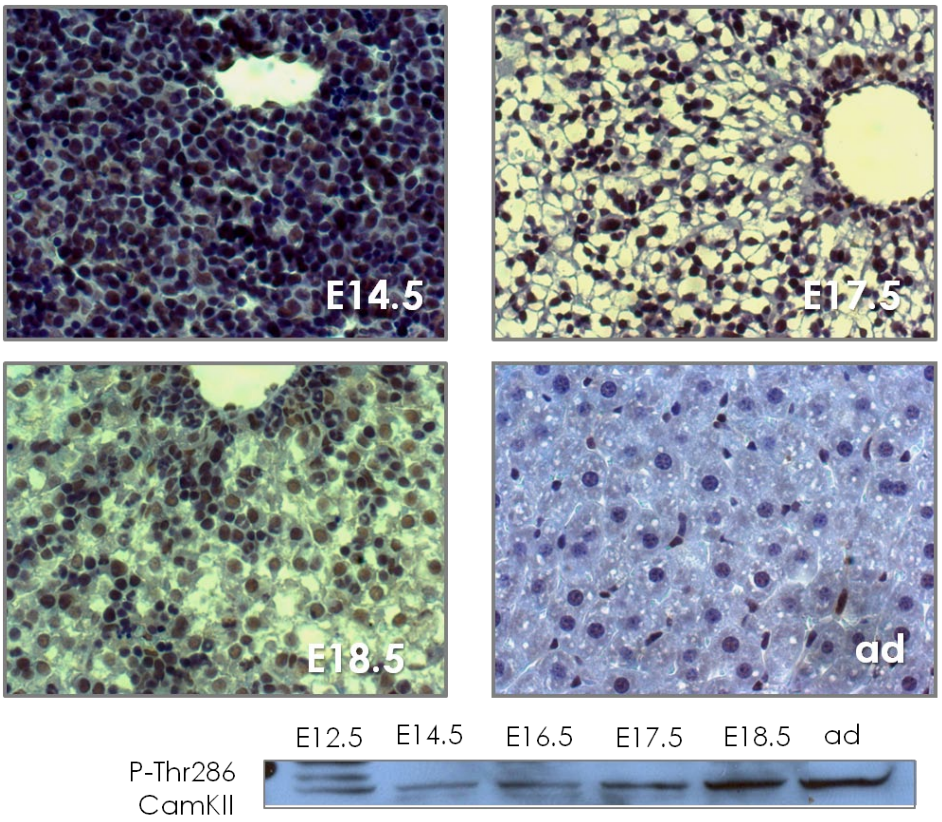


Figure 4.4.3 pThr286-CamKII expression is undetermined in developing liver. While immunoblot analysis (bottom) indicates presence of some pThr286-CamKII, immunohistochemistry (top) results in non-specific and nuclear staining only.

4.4.4 Phosphorylation of Ser19-MLC is observed in developing liver. Given the nature of the outcomes of Wnt/PCP signaling (Table 4.4.1), activation of this process is harder to detect. Therefore, the only outcome we were able to measure was phosphorylation of myosin light chain (MLC) on Serine 19, an event that is seen during cytoskeletal remodeling in PCP (Winter et al., 2001). While we did observe this phosphorylation, it is a poor specific indicator of this pathway, since it can occur during many other processes as well. In addition, this increase could be due to an overall increase in MLC expression, which cannot be determined from our data. Since the real outcome of PCP is cell polarization, the proper line of experimentation for investigating its role in liver development would be to knock out *Fzd4* in developing liver and assess effects on cell and tissue polarity, a strategy our lab may employ in future experiments.

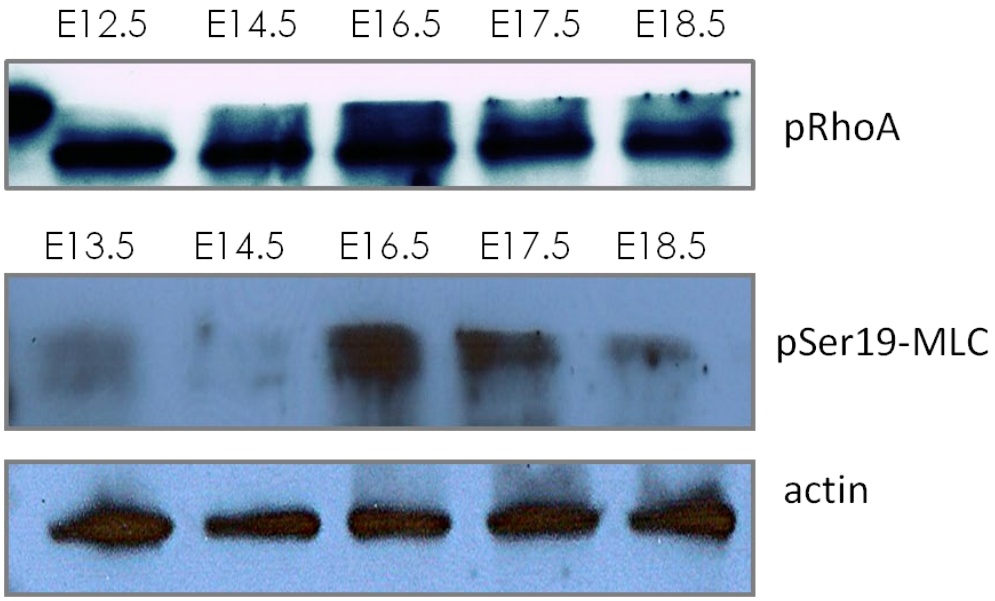


Figure 4.4.4 Phosphorylation of Ser19-MLC is observed in developing liver. Western blot analysis for phospho-Serine19-MLC on lysates from developing liver reveals phosphorylated MLC during hepatic differentiation.

4.5

Discussion

The evidence we have accumulated in this study suggests that while Wnt/Ca^{2+} pathway is not active during liver development (except in the hematopoietic compartment), it remains a possibility that Wnt/PCP activity, possibly mediated by FZD4 on hepatocyte membranes, is active. It is possible that the architecture of the mature liver is facilitated by PCP activity promoting correct planar organization of hepatocytes. Developing transgenic animals capable of conditionally deleting *Fzd4* in developing liver and assessing the effects of this deletion on hepatic cell and tissue polarity would allow for a more definitive evaluation of this possibility. Another approach to address this issue is the overexpression of Frizzled4 by hepatic cells in culture, which leads to activation of downstream signaling pathways even in the absence of Wnt ligands, or treatment of cells in culture with a Wnt/Frizzled fusion protein that is constitutively active. Any of these approaches would allow for an assessment of the affect of FZD4 activity on hepatocytes.

CHAPTER 5. OVERALL DISCUSSION AND CONCLUSIONS

5.1 Rationale for studies

Breakthroughs in inducing pluripotency in differentiated human cells may make possible the creation of healthy, immunotolerant cells of any type from a patient's own tissue, potentially allowing the treatment of patients without the limitation of donor organ availability. However, current efforts to produce fully differentiated hepatocytes from stem cells result in hepatocytes that fail to express many cytochrome p450 enzymes critical to mature liver function and retain expression of the immature hepatocyte marker alpha-feto protein, revealing that our understanding of this differentiation program is incomplete (Navarro-Alvarez et al., 2009). While roles for beta-catenin have been established in liver during all stages of life (Lade and Monga, 2011), conflicting reports exist regarding its role in hepatic differentiation. While transgenic mice expressing overactive beta-catenin in developing liver exhibit biliary epithelial cell differentiation but no hepatocytes (Decaens et al., 2008), transgenic mice deficient in beta-catenin in developing liver lack hepatocytes (Tan et al., 2008). While both reports are consistent with a role for beta-catenin in biliary epithelial cell differentiation, it is confounding that both presence and absence of beta-catenin results in failure of hepatocyte differentiation.

5.2 Summary of findings

In our investigations of liver development, we have made a number of observations regarding the regulation of beta-catenin. During the period of liver bud expansion between embryonic days 12.5 (E12.5) and 14.5 (E14.5), full-length beta-catenin expression predominates and correlates with expression of beta-catenin targets involved in proliferation (Axin2, cyclinD1).

Thereafter, from E16.5 to birth, a 75-kDa truncated beta-catenin species becomes the predominant form, is present at the membrane as well as the nucleus of maturing hepatocytes, and its expression coincides with beta-catenin targets associated with hepatocyte differentiation. During this period, full-length beta-catenin is a minor component of the total beta-catenin, and appears be mostly limited to emerging bile duct cells by immunohistochemistry. Further experimentation has revealed that truncated beta-catenin is produced by proteolytic cleavage of the N-terminus of beta-catenin by calpain, revealing a novel form of regulation of beta-catenin generating a specialized species precisely correlating with hepatic differentiation. We believe that cleavage of beta-catenin by calpain may represent a previously unappreciated event in hepatocyte differentiation, and understanding the mechanism generating it could represent the key missing element in hepatocyte differentiation protocols. Furthermore, the conflicting results showing that both stabilization of (full-length) beta-catenin and deletion of beta-catenin in liver result in a failure of hepatocyte development could be explained by presence of a special beta-catenin species promoting hepatocyte differentiation. (Though, given the dynamic role of beta-catenin in liver development it may also be related to a difference in the timing of Cre expression. Finally, our observations lead us to believe that a truncated beta-catenin species can be detected in fetal hepatoblastomas similar to the one we observed in developing liver. We observed full-length beta-catenin during days E12.5-E14.5 in developing liver correlated with expression of cyclin D1, and truncated beta-catenin from E14.5-E18.5 correlated with expression of glutamine synthetase, and also found that full-length beta-catenin was associated with a more embryonal histology (resembling E12.5-E14.5 liver) and cyclin D1 expression in hepatoblastoma, and truncated beta-catenin associated with fetal histology (resembling E14.5-E18.5) and higher glutamine synthetase

expression. This is consistent with a pro-differentiation role for N-terminally truncated beta-catenin in promoting expression of hepatocyte-specific genes like glutamine synthetase and a better prognosis, and a pro-proliferation role for full-length beta-catenin via promotion of cyclin D1 expression, associated with an embryonal morphology and worse prognosis.

5.3 N-terminal deletions of beta-catenin in cancer

Beta-catenin species lacking the N-terminal exon 3 are generally believed to be stabilized, oncogenic forms of the protein due to the presence of residues in exon 3 that allow the protein to be targeted for proteasomal degradation by the GSK3β/APC/AXIN/CKI complex (Munemitsu et al., 1995; Rubinfeld et al., 1996; Yost et al., 1996; Aberle et al., 1997; Ikeda et al., 1998; Nakamura et al., 1998; Hart et al., 1999; Liu et al., 2002; Swiatek et al., 2004). Deletions and mutations involving the exon 3 degradation box of beta-catenin have been reported in many cancers, including colorectal, endometrial, gastric, hepatocellular, ovarian, prostate, thyroid and endometrial carcinomas, as well as hepatoblastomas, medulloblastomas, Wilm's tumors (kidney), melanomas and pilomatricomas (Polakis, 2000). In liver, "activating" mutations of beta-catenin have often been detected in hepatocellular carcinomas and hepatoblastomas (Blaker et al., 1999; Koch et al., 1999; Jeng et al., 2000; Wei et al., 2000; Takayasu et al., 2001; Taniguchi et al., 2002; Ranganathan et al., 2005; Cairo et al., 2008; Curia et al., 2008; Armengol et al., ; Lopez-Terrada et al., 2009), and , in the case of hepatoblastomas, mutations or deletions affecting the N-terminus of beta-catenin are present in 50-90% of hepatoblastomas (Koch et al., 1999; Wei et al., 2000; Taniguchi et al., 2002; Curia et al., 2008; Lopez-Terrada et al., 2009). Hepatoblastomas are characterized histologically based on resemblance to embryonal (liver bud stage) or fetal (differentiation stage), and while exon 3

deletions or point mutations are often seen in the more aggressive and less differentiated embryonal phenotype, fetal hepatoblastomas often possess beta-catenin alleles lacking exon 3 as well as part of exon 4 (Lopez-Terrada et al., 2009). It is confounding that mutations and deletions in the N-terminus of beta-catenin that prevent proteasomal degradation, which would be assumed to produce the same functional outcome of beta-catenin overactivity, produce different hepatoblastoma phenotypes with different prognoses. This suggests a function for the N-terminus besides providing a means of regulating beta-catenin activation and stability.

5.4 Reassessing of the oncogenic nature of N-terminally deleted beta-catenin

While in hepatocellular carcinoma, nuclear/cytoplasmic beta-catenin accumulation is associated with exon 3 deletions and mutations (Terris et al., 1999) poorly differentiated morphology (Endo et al., 2000), higher proliferative activity (Inagawa et al., 2002), and poor prognosis, (Endo et al., 2000; Wong et al., 2001; Inagawa et al., 2002) emerging evidence from several cancer types suggests that these relationships do not hold true in every cancer type (Lucero et al.). While melanomas often exhibit nuclear/cytoplasmic localization of beta-catenin, they are rarely found to contain exon 3 mutations or deletions (Omholt et al., 2001; Pollock and Hayward, 2002; Giles et al., 2003; Chien et al., 2009). In fact, several studies have reported that nuclear beta-catenin correlates with better prognosis in several cancers, including melanomas (Kageshita et al., 2001; Maelandsmo et al., 2003; Bachmann et al., 2005; Chien et al., 2009), medulloblastomas (Ellison et al., 2005; Kool et al., 2008; Fattet et al., 2009), prostate (Horvath et al., 2005), ovarian (Gamallo et al., 1999) and colorectal carcinomas (Elzagheid et al., 2008), possibly pointing to undiscovered non-oncogenic nuclear roles for beta-catenin, such as in promoting differentiation. Furthermore, the idea that exon 3 mutations and deletions produce a

non-degradable beta-catenin is called into question by the recent discovery of a second degradation pathway for beta-catenin, in which phosphorylation of C-terminal residues 666 and 671 triggers recognition by the ubiquitin ligase Siah and subsequent proteasomal degradation (Dimitrova et al., 2010).

Further insights into functions of N-terminally deleted beta-catenin species may be gleaned from strategies utilizing a conditionally-expressed, N-terminally-deleted form to assess the role of beta-catenin in development of a specific tissue or cell type.

5.5 Pro-differentiation roles for N-terminally deleted beta-catenin

A number of studies exist which report on the effect of expression of a conditional, N-terminally deleted beta-catenin in a developing tissue. While beta-catenin is widely recognized for its role in promoting proliferation and stem cell maintenance and renewal during development (Cho et al., ; Chenn and Walsh, 2002; Megason and McMahon, 2002; Reya et al., 2003; Willert et al., 2003), in some cases, the result of such expression is premature differentiation rather than overproliferation. Expression of a beta-catenin species lacking the N-terminal 89 amino acids (Δ89 beta-catenin) in the luminal epithelial cells of the developing mammary gland of mice results in precocious lobuloalveolar development and differentiation, consistent with a cell-type-specific, pro-differentiation function of N-terminally truncated beta-catenin (Imbert et al., 2001). Intriguingly, this phenotype is different from that induced by Wnt overexpression in the mammary gland (which would presumably result in stabilization of full-length beta-catenin) which induces in ductal hyperbranching (Tsukamoto et al., 1988; Lane and Leder, 1997). Additionally, targeting expression of a Δ57 beta-catenin construct to basal myoepithelial cells produces both precocious lateral bud formation, as well as

hyperproliferation and premature differentiation of luminal epithelium during pregnancy (Teuliere et al., 2005), suggesting roles for beta-catenin in all three processes. Premature differentiation was not seen in every case of N-terminally-deleted beta-catenin expression, however. Expression of Δ89 beta-catenin in the small intestine resulted in increased cell division in undifferentiated cells in the proliferative compartment as well as increased apoptosis and an increase in E-cadherin at adherens junctions, but no observable changes in cell fate outcomes (Wong et al., 1998). Still, it is compelling that a precedent exists for N-terminally deleted beta-catenin species promoting differentiation into a particular cell type, as we believe the truncated beta-catenin is doing in developing liver.

5.6 Beta-catenin as binary cell fate switch

In addition, many studies have found beta-catenin acting as a binary cell fate switch during differentiation, in promoting differentiation of a specific cell type from a bipotent or multipotent progenitor. In developing neural crest in zebrafish, while injection of mRNA encoding cytoplasmic beta-catenin leads to pigment-cell formation at the expense of glial cells and neurons and glia, inhibition of the Wnt pathway by injection of mRNAs encoding a truncated form of the transcription factor Tcf-3 or a dominant-negative Wnt promotes neuronal fates at the expense of pigment cells (Dorsky et al., 1998; Hari et al., 2002; Lee et al., 2004). Beta-catenin is also involved in the cell fate switch between neural retina and retinal pigment epithelium (Fujimura et al., 2009; Westenskow et al., 2009). Similarly, while deletion of beta-catenin in skin stem cells promotes differentiation of epidermis at the expense of follicular keratinocytes (Huelsken et al., 2001). Likewise, ectopic canonical Wnt signaling leads to enhanced ossification and suppression of chondrocyte formation from mesenchymal stem cells,

and chondrocytes are produced at the expense of osteoblasts in mice expressing a conditionally-deleted beta-catenin mesenchymal precursor cells (Day et al., 2005; Hill et al., 2005; Hu et al., 2005; Rodda and McMahon, 2006). Interestingly, in this system deletion of Lrp5 produces a postnatal osteoblast defect but no skeletal malformations (Kato et al., 2002), indicating that the role of beta-catenin in osteoblast differentiation is independent of canonical signaling. Though it is unclear from this study how this is occurring, such an effect would be expected if beta-catenin were being "activated" by calpain cleavage.

5.7 Joint requirement for calpain activity and beta-catenin in differentiation of some mesenchymal cells

Calpain plays a known role in differentiation of osteoblasts, muscle cells and adipocytes (Barnoy et al., 1997; Murray et al., 1997; Garach-Jehoshua et al., 1998; Ueda et al., 1998; Patel and Lane, 1999; Yajima and Kawashima, 2002; Moyen et al., 2004; Li and Xie, 2007) and it appears that beta-catenin is, a substrate for calpain in differentiating muscle cells (Kramerova et al., 2006). Given that both beta-catenin and calpain are critical in promoting differentiation of osteoblasts (Murray et al., 1997; Yajima and Kawashima, 2002; Shimada et al., 2008) (Day et al., 2005; Hill et al., 2005; Hu et al., 2005; Rodda and McMahon, 2006), it is tempting to hypothesize that beta-catenin might be one of calpains targets in these cells, as well as adipocytes, where beta-catenin has been shown to form a complex with TCF4 and c/ebpα during differentiation. In light of our findings, it would be worthwhile to take a closer look at whether the truncated, 75-kDa form of beta catenin we detected in differentiating hepatocytes is also present in these tissues.

5.8 Non-TCF/Lef family transcription factor binding partners of beta-catenin

Cell fate specifications via TCF/Lef-independent beta-catenin activity, or via recruitment of additional factors to beta-catenin/TCF complexes have also been implicated in differentiation of various tissues.

In addition to TCF/Lef family transcription factors, beta-catenin has been shown to interact with a number of other transcription factors. For example, as previously mentioned, beta-catenin interaction with Sox17 is critical in endoderm development and promotes expression of *Hnf1β*, *Foxa1* and *Foxa2* in posterior endoderm. (Hudson et al., 1997; Kanai-Azuma et al., 2002; Clements et al., 2003; Sinner et al., 2004). Beta-catenin also binds to FOXO family transcription factors rather than TCF/Lef proteins under conditions of oxidative stress (Essers et al., 2005) or to FOXO3 during nutrient starvation to regulate hepatic metabolism (Liu et al.), and binds to HIF1a during hypoxic conditions (Kaidi et al., 2007). Other transcription factor binding partners include RXRa, which, when bound to beta-catenin promotes its proteasomal degradation (Xiao et al., 2003; Dillard and Lane, 2008), as well as other nuclear receptors (Mulholland et al., 2005).

In the pituitary gland, beta-catenin regulates cell fate by switching its interaction from TCF/Lef to the tissue-specific transcription factor Prop1 (Olson et al., 2006). In differentiating melanocytes, beta-catenin is known to bind to and activate the transcription factor Mitf, and promote a switch from expression of canonical Wnt signaling-regulated genes toward Mitf-specific targets (Schepsky et al., 2006). A full-length, constitutively active beta-catenin inhibited adipogenesis and stimulated osteoblast differentiation from multipotent embryonic fibroblasts, expression of an N-terminally truncated beta-catenin (Δ90 beta-catenin) with

reduced TOPFLASH activity relative to wild-type was capable of promoting both osteogenic and adipogenic differentiation (Salazar et al., 2008). A beta-catenin possessing a larger N-terminal deletion, Δ151 beta-catenin, was impaired in its ability to promote differentiation into either cell type. Cells expressing a C-terminally truncated mutant missing the TCF/Lef transactivation domain, like those expressing the full-length mutant, were capable of promoting osteogenic or adipogenic differentiation from multipotent embryonic fibroblasts. Likewise, mice expressing a "gain-of-function" ΔN-beta-catenin lacking the first 89 amino acids, as well as those expressing a "loss-of-function" ΔNΔC-deleted beta-catenin lacking the N-terminal 87 amino acids and the C-terminal 99 amino acids (preventing its association with TCF/Lef factors) in epidermis exhibited the same hyperfollicular phenotype (DasGupta et al., 2002). While the authors of this study concluded that the ΔNΔC-deleted beta-catenin was displacing endogenous beta-catenin at the membrane, allowing it to translocate into the nucleus, another possibility could be that this factor was activating a differentiation program in skin mediated by binding to a non TCF/Lef transcription factor. In all of these cases, beta-catenin makes a switch (or appears to make a switch) from binding to TCF family transcription factors to binding other transcription factors to activate a specific differentiation program. These studies do not reveal what triggers this switch, but truncation of beta-catenin by calpain could be responsible.

Finally, there are also cases in which the beta-catenin/TCF complex recruits an additional transcription factor to promote differentiation. In differentiating adipocytes, a transcriptional complex is formed comprising beta-catenin, TCF4, and c/ebpα, plus the co-activator p300 (Kennell et al., 2003). C/ebpa is of interest due to the fact that it is involved in expression of liver-specific genes (Johnson, 1990; Liu et al., 1991; Rastegar et al., 2000; Westmacott et al., 2006), and we have preliminary results indicating it interacts with beta-catenin in developing

liver (data not shown). Interestingly, the context in which it was shown to bind to beta-catenin, adipocyte differentiation, also requires calpain activity (Patel and Lane, 1999; Patel and Lane, 2000; Li and Xie, 2007), presenting the possibility that the beta-catenin in this complex is the calpain-cleaved form.

Another possible transcription factor that could promote hepatocyte differentiation in conjunction with beta catenin is Smad3/4. Beta-catenin forms a complex with Smad3/Smad4 and TCF4 in multiple cell types (Labbe et al., 2000; Nishita et al., 2000; Hussein et al., 2003; Lei et al., 2004). This complex has been shown to activate expression of genes containing adjacent TCF and Smad binding sites (Labbe et al., 2000; Letamendia et al., 2001; Lei et al., 2004) and to trigger cell fate decisions in both ectoderm (Hussein et al., 2003; Rodriguez-Carballo et al., 2010) and in mesenchymal stem cells (Rodriguez-Carballo et al., 2010). While BMP and Wnt/beta-catenin signaling, as well as FGF, participate in hepatic competence (Ober et al., 2006), some reports suggest TGFbeta signaling is involved in either hepatocyte differentiation (Mfopou et al., 2010)or BEC differentiation (Ader et al., 2006; Limaye et al., 2008). Compellingly, mice heterozygous for both Smad2 and Smad3 ($Smad2^{(+/-)}$; $Smad3^{(+/-)}$) died at midgestation, with hypoplastic livers and anemia, pointing to a critical, dose-dependent role for TGF-beta signaling in liver development. Hepatocytes in mutant mice exhibited abnormal adhesive properties reminiscent of beta-catenin and HGF/MET knockouts. Additionally cultured hepatocytes from these mice could be rescued with HGF treatment. (Weinstein et al., 2001). Of note, BMP activity can activate calpains during osteoblasts differentiation (Murray et al., 1997), raising the intriguing possibility that this pathway could also account for the calpain activity occurring during hepatocyte differentiation.

In addition to the possibility that truncated beta-catenin may recruit specific differentiation-associated transcription factors, transcription of differential beta-catenin targets by truncated beta-catenin could involve a switch in interaction with histone acetyltransferases, such as the switch from beta-catenin association with CBP to p300 during embryonic stem cell differentiation (Miyabayashi et al., 2007).

5.9 Function of the N-terminal tail of beta-catenin

In light of the abundant evidence indicating that loss of the N-terminus affects beta-catenin function, and does not always produce a pro-oncogenic protein, a question naturally arises about what functions the N-terminal tail could serve. Crystallographic analyses of beta-catenin have concluded that the N-terminal tail is unstructured, and may interact dynamically with the armadillo repeat region that comprises the core of the protein (Xing et al., 2008). Indeed, some previous work suggested that the N-terminal tail binds the armadillo repeat domain (Castano et al., 2002) and regulates interactions with binding partners including E-cadherin and alpha-catenin. Deletion of the N-tail at amino acid 120 effectively blocking E-cadherin association but enhancing alpha-catenin binding (Castano et al., 2002). However, work in cells expressing a Δ107 deleted beta-catenin revealed that the mutant protein was able to associate with E-cadherin but unable to associate with alpha-catenin (Oyama et al., 1994). Others have proposed that both the N-and C-terminal tails are critical in separating the function of beta-catenin from the closely related plakoglobin (Solanas et al., 2004) and that the first 26 amino acids of beta-catenin block its incorporation into the desmosome by decreasing affinity for desmoglein (Wahl et al., 2000).

The difference in phenotype between hepatoblastomas with a complete deletion of exon 3 (amino acids 5-80, predominantly embryonal) versus those with a deletion encompassing exon 3 and part of exon 4 (predominantly fetal) suggests the presence of important residues or motifs in exon 4 (Lopez-Terrada et al., 2009) Also, given that the truncated beta-catenin species in developing liver lacks amino acids 1-95, of particular interest is whether there are any residues with known functions in the region between amino acid 80 and 95. Of note, there is a phosphorylatable tyrosine at amino acid 86 whose regulation is emerging. Both SRC and BCL-ABL phosphorylate beta-catenin on Y86 and Y654 preventing its interaction with, and phosphorylation by, the degradation complex, thus promoting its stabilization and nuclear translocation (Roura et al., 1999; Coluccia et al., 2007; Simoneau et al., 2010). Likewise, the tyrosine phosphatase SHP-1, which dephosphorylates beta-catenin at Y86 and Y654, both inhibits nuclear beta-catenin activity by preventing its association with TBP and promotes GSK3β-dependent beta-catenin degradation (Simoneau et al., 2010).

The idea that a truncated beta-catenin species may play a role in differentiation of a particular cell type is supported in the literature. While further work must be done to directly characterize the function of the 75-kDa truncated beta-catenin species observed in developing liver, there is much evidence to support our model that truncation of the N-terminal 95 amino acids by calpain imparts a novel function, allowing beta-catenin to activate a hepatocyte differentiation program in hepatoblasts.

5.10 HGF/MET and Wnt/beta-catenin interactions in developing liver

In exploring what factors regulate hepatic differentiation, as well as what may be triggering the cell-type switch in beta-catenin species, one possibility was MET. Wnt-independent activation of beta-catenin by the receptor tyrosine kinase MET has been observed in liver and other tissues (Monga et al., 2002; Apte et al., 2006). MET is capable of interacting with beta-catenin at the membrane and phosphorylating it on tyrosines 654 and 670 to promote its detachment from E-cadherin and translocation from the adherens junction to the nucleus (Zeng et al., 2006). HGF/MET signaling is active in developing liver (Hu et al., 1993) and has been shown to promote hepatocyte differentiation *in vitr*o (Kamiya et al., 2001; Hussain et al., 2004). We have found high levels of both HGF and active MET as well as phosphorylation of beta-catenin Y654 during liver bud expansion between E12.5 and E14.5. This occurs immediately preceding the appearance of truncated beta-catenin, and there is a disproportionate level of phosphorylation of the truncated beta-catenin at these stages relative to the total amount of truncated beta-catenin. Intriguingly, *HGF* and *c-Met* knock-out mice die at late gestation, at approximately the same stage at which lethality occurs in hepatoblast-specific beta-catenin knock-out mice. Both knockouts exhibit decreased parenchymal cell mass and reduced liver size, as well as increased hepatocyte apoptosis. Our observations are consistent with phosphorylation of beta-catenin by MET promoting the production of truncated beta-catenin. This may occur either through downstream calcium release activating calpain or by tyrosine phosphorylation of beta-catenin promoting its recognition and cleavage by calpain. This interaction between MET and beta-catenin is critical for normal liver development.

Testing this hypothesis by assaying beta-catenin Y654 phosphorylation in developing liver, however, revealed similar levels of p-Y654 beta-catenin in MET KO livers. This indicates that either MET is not responsible for this phosphorylation or another RTK is compensating for loss of MET. Indeed, FGFR and EGFR are both expressed in liver during liver bud expansion and are able to phosphorylate beta-catenin on Y654 (Roura et al., 1999; Sekhon et al., 2004).

5.11 Contribution of non-Canonical Wnt signaling to liver development

One possibility we considered was that non-canonical Wnt signaling could be promoting the cleavage of beta-catenin observed in developing liver, possibly through calcium release and activation of calpain, similar to what has been observed in the differentiation of cardiomyocytes (Abdul-Ghani et al., 2010). We detected mRNA expression of potential non-canonical Wnt signaling components as well as positive immunostaining for WNT4 from the hematopoietic compartment. In addition, we observed FZD4 on the membranes of differentiating hepatocytes, and significant expression of WNT4, WNT5A and WNT11 by Immunoblotting. However, we did not detect clear evidence of activation of either Wnt/Ca^{2+} pathway or Wnt/PCP pathway. Though multiple lines of evidence lead us to conclude that Wnt/Ca^{2+} pathway signaling is active in hematopoietic cells, Wnt/PCP activity is harder to detect and therefore it is difficult to say conclusively whether this pathway is active.

WNT11 and WNT4 are involved in promoting Wnt/PCP pathway activity during various stages of liver development, including promoting gastrulation and gut tube elongation in Xenopus, (Li et al., 2008) and gut tube fusion in zebrafish (Matsui et al., 2005). Expression of *Wnt4* by sinusoidal endothelial cells and biliary epithelial cells and of *Fzd4* by hepatocytes has been shown by our lab in adult mouse liver (Zeng et al., 2007) and WNT5A and WNT11 have

been detected in developing chick liver (Suksaweang et al., 2004). While it remains a possibility that the Wnt/PCP pathway is promoting cell polarization in differentiating hepatocytes mediated by Wnt binding to FZD4, investigation of this possibility would require an in vivo knockdown or deletion of the appropriate signaling components, a line of experimentation our lab is not prepared to carry out at this time.

5.11 Future Directions

We believe that cleavage of beta-catenin may represent a critical mechanism regulating hepatoblast cell fate, and that N-terminally truncated beta-catenin may have a unique role in hepatocyte differentiation. In addition, we found that N-terminal truncations of beta-catenin in hepatoblastomas promote a more differentiated, fetal histology and promote expression of beta-catenin targets involved in hepatocyte differentiation rather than proliferation, similar to the function of the truncated beta-catenin in developing liver. Our work may help determine the molecular program for hepatocyte differentiation, which is of great clinical value not only as it relates to hepatoblastoma and other liver diseases, but also to inform efforts to create fully functional hepatocytes from various stem cell types.

Further work is required to uncover the mechanism by which the 75-kDa truncated beta-catenin species observed in developing liver may activate a hepatocyte differentiation program. In particular, a comparison of the RNA expression pattersn of hepatocytes transfected with truncated beta-catenin relative to full-length would be valuable in assessing the precise role this the truncated species plays in hepatic differentiation, and our lab plans to undertake such experiments in the future.

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