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APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development

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ABSTRACT

Developmental signaling pathways hold the keys to unlocking the promise of adult tissue regeneration, and to inhibiting carcinogenesis. Patients with mutations in the *Adenomatous Polyposis Coli (APC)* gene are at increased risk of developing hepatoblastoma, an embryonal form of liver cancer, suggesting that Wnt affects hepatic progenitor cells. To elucidate the role of *APC* loss and enhanced Wnt activity in liver development, we examined *APC* mutant and wnt inducible transgenic zebrafish. $APC^{+/-}$ embryos developed enlarged livers through biased induction of hepatic gene programs and increased proliferation. Conversely, $APC^{-/-}$ embryos formed no livers. Blastula transplantations determined that the effects of APC loss were cell autonomous. Induction of wnt modulators confirmed biphasic consequences of wnt activation: endodermal pattern formation and gene expression required suppression of wnt signaling in early somitogenesis; later, increased wnt activity altered endodermal fate by enhancing liver growth at the expense of pancreas formation; these effects persisted into the larval stage. In adult $APC^{+/-}$ zebrafish, increased wnt activity significantly accelerated liver regeneration after partial hepatectomy. Similarly, liver regeneration was significantly enhanced in $APC^{Min/+}$ mice, indicating the conserved effect of Wnt pathway activation in liver regeneration across vertebrate species. These studies reveal an important and time-dependent role for wnt signaling during liver development and regeneration.

Introduction

Patients with *APC* mutations develop multiple colonic polyps and eventually colon cancer (Kinzler et al., 1991). Approximately 1% of people who carry an *APC* mutation develop an embryonal form of liver cancer, hepatoblastoma (Hirschman et al., 2005), which is thought to originate from hepatic progenitor cells; there is a 1000-fold greater risk in these patients than the general population to develop this type of liver cancer. This suggests that increased β-catenin activity caused by *APC* loss influences the earliest steps of hepatic development and that its dysregulation leads to neoplasia. Furthermore, alterations in the Wnt signaling pathway have been found in a significant fraction of cases of hepatocellular carcinoma (*β-catenin* 20%, *Axin* 10%) and cholangiocarcinomas (*β-catenin* 8%, *APC* 12%, *Axin* 42%) (Taniguchi et al., 2002; Tokumoto et al., 2005), indicating that Wnt acts on several cell types in the liver to induce carcinogenesis. As these data suggest that manipulation of Wnt activity may be therapeutically beneficial to patients with liver disease, we sought to characterize the effects of *APC* loss and increased wnt activity during liver development and regeneration.

Wnt signaling and its main transcriptional mediator, β-catenin, play important roles in controlling tissue patterning, cell fate decisions, and proliferation in many embryonic contexts, including organ development and differentiation (Clevers, 2006). In the absence of Wnt signaling, β-catenin is phosphorylated through the destruction complex consisting of APC, Axin, and Glycogen Synthase Kinase (GSK) 3β, and targeted for degradation. Binding of Wnt ligand to cell surface receptors allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus, where it modulates target gene expression.

The role of wnt signaling in endodermal development was initially described in *Caenorhabditis elegans* (Lin et al., 1995), and is highly evolutionarily conserved (Heasman et al., 2000). Early embryonic

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lethality of mice with homozygous deletion of *β-catenin* initially precluded an analysis of the role of Wnt/β-catenin signaling in vertebrate endodermal development (Haegel et al., 1995). Inducible inactivation of *β-catenin* subsequently revealed a requirement of wnt/β-catenin signaling for intestinal development (Ireland et al., 2004). APC^{Min} homozygous mutant mice, with dysregulated β-catenin activity, fail to form distal visceral endoderm and are embryonic lethal (Chazaud and Rossant, 2006; Moser et al., 1995). Heterozygote mice are viable and develop intestinal neoplasia as adults (Su et al., 1992). The effects of heterozygous *APC* loss on wnt/β-catenin activity and liver development have not been studied in detail.

The liver develops from anterior endodermal progenitor cells. In the zebrafish, endodermal progenitors fated to become liver are identifiable between the 18 somite stage (16 h post fertilization (hpf)) and 24 hpf as a thickening in the anterior endoderm and a restriction of previously pan-endodermal gene expression (Field et al., 2003). As the endoderm develops further, the liver primordium appears as a prominent bud extending to the left from the midline over the yolk sac. At 48 hpf the liver expresses mature markers such as *liver fatty acid binding protein (lfabp)* (Her et al., 2003). Hepatic growth continues through the embryonic and larval stages as the zebrafish liver further differentiates (Wallace and Pack, 2003). The morphogenesis of the endoderm is organized differently in other vertebrate species, such as *Xenopus* and mice (Zorn and Wells, 2007). The molecular mechanisms, identified to date, that initiate and control liver development, however, appear to be well conserved across vertebrate species (Stainier, 2002).

Biochemical analysis of embryonic murine livers and knockdown studies in liver cultures suggested a role for β-catenin in hepatocyte proliferation (Micsenyi et al., 2004; Monga et al., 2003). More recently, the zebrafish *wnt2bb* mutant *prometheus (prt)* demonstrated that mesodermally derived wnt signaling plays a critical role in regulating liver growth during embryogenesis (Ober et al., 2006). Homozygous *prt* mutants showed decreased expression of early liver genes *hhex* and *prox1* at 24 hpf, and have delayed liver formation. In contrast, McLin et al. demonstrated the need for wnt repression in the anterior endoderm for proper liver development in *Xenopus* (McLin et al., 2007). It remains to be determined whether these findings are mutually exclusive, suggesting species-specific variations in signaling requirements, or whether they reflect changing temporal requirements during liver development. Supporting this paradigm, previous studies have revealed alterations in β-catenin levels between pre- and postnatal liver development (Apte et al., 2007; Micsenyi et al., 2004).

During liver regeneration, Wnt signaling is activated shortly after liver resection (Monga et al., 2001). Furthermore, both morpholino knockdown and conditional ablation of β-catenin in mice results in decreased regeneration following partial hepatectomy (Sekine et al., 2007; Sodhi et al., 2005; Tan et al., 2006). To date, however, it has not been shown whether enhanced activation of wnt signaling confers a growth advantage that would accelerate liver regeneration.

To characterize the role of progressive *APC* loss and increased levels of wnt/β-catenin signaling during liver development and generation, we utilized *APC* mutant zebrafish (Hurlstone et al., 2003). The heterozygous mutants are at increased risk of developing intestinal, pancreas and liver tumors in adulthood (Haramis et al., 2006). The liver tumor histology resembles that of hepatoblastomas, suggesting an effect of wnt activation on hepatic progenitor cells. We found that there is a cell autonomous differential response to wnt activation: $APC^{+/-}$ mutants exhibited increased embryonic liver size, while $APC^{-/-}$ status led to an absent liver and embryonic death. Furthermore, we demonstrated biphasic effects of wnt activation during hepatogenesis; early wnt induction led to diminished endoderm formation and failure to specify liver, while wnt activation in mid-somitogenesis influenced endodermal progenitor fate decisions, resulting in increased liver size and decreased pancreas formation. These data suggest that β-catenin signaling affects both endodermal and hepatic progenitor cells. We also demonstrated that enhanced wnt/β-catenin activation accelerates liver regeneration, and that this response is conserved throughout vertebrate species. Our study reveals the WNT signaling pathway as an attractive pharmacological target to manipulate hepatic progenitor cells and to accelerate liver regeneration in humans.

Materials and methods

Zebrafish husbandry

Zebrafish were maintained according to IACUC protocols. The *lfabp:GFP*, *gut:GFP*, *ptf1α:GFP*, *hs:wnt8-GFP*, *hs:dnTCF-GFP*, and *hs:dkk-GFP* transgenic lines were described previously (Dorsky et al., 2002; Her et al., 2003; Lewis et al., 2004; Ober et al., 2003; Pisharath et al., 2007; Stoick-Cooper et al., 2007; Weidinger et al., 2005). Genotyping for *APC* mutants was performed as described; the wild-type and mutant bands can be distinguished in a single reaction for each sample (Hurlstone et al., 2003).

Heat-shock modulation of wnt signaling

Embryonic heat-shock experiments were conducted at 38 °C for a duration of 20 min. Genotype was determined by the presence of GFP fluorescence at 3 h post heat-induction; sorted non-fluorescent (wild-type) siblings were used as controls.

Morpholino knockdown

Morpholinos (GeneTools) directed against zebrafish *β-catenin* (Lyman Gingerich et al., 2005) or mismatched controls were injected into zebrafish embryos at the one-cell stage at a concentration of 40 μM; injection experiments were replicated ≥3 times.

Blastula transplantation

Embryos were harvested, pronased, and manually dechorionated. 50–100 blastomeres were removed from a donor embryo at the 1000-cell stage and injected at the blastoderm margin of a recipient. Matched donors and recipients were arrayed in multi-well plates until further analysis and genotyped as described for *APC*.

In situ hybridization

Paraformaldehyde-fixed embryos were processed for in situ hybridization using standard zebrafish protocols (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). The following RNA probes were used to detect alterations in endodermal development: *GFP*, *lfabp*, *sterol carrier protein*, *transferrin*, *foxa3*, *sox17*, *hhex*, *prox1*, *pdx1*, *insulin*, *trypsin*, and *ifabp*. Changes in expression compared to wild-type controls are reported as the # altered / # scored per genotype; a minimum of 3 independent experiments of $n \geq 25$ embryos were conducted per analysis.

Immunohistochemistry

Zebrafish embryos, adults and en-bloc abdominal sections as well as resected murine livers were fixed with paraformaldehyde, paraffin embedded and cut in 10 μm serial step-sections for histological analysis. Hematoxylin/eosin staining was performed on alternate sections using standard techniques. Antibodies to β-catenin (1:100, BD 61054), TUNEL (Chemicon International), BrdU (1:2000, Sigma BU-33), and PCNA (1:80, Calbiochem PC10) were visualized by DAB and counterstained with hematoxylin or methylene green. Cell counts were quantified in 5 corresponding sections/genotype for each stain.

Caspase assay

Single embryos ($n=5$ per genotype, two independent experiments) were manually dissociated in lysis buffer and centrifuged. Supernatant (100 μl) was used for the Caspase-Glo 3/7 assay according to manufacturer's protocol (Promega). DNA isolated from the cell pellet was used to confirm *APC* genotype.

Confocal microscopy

GFP transgenic zebrafish embryos were embedded in 1% low melting point agarose containing 0.04 mg/ml Tricaine-S in glass-bottom culture dishes. Microscopy was performed using a Zeiss LSM Meta confocal microscope. A minimum of 10 embryos per genotype were imaged over 3 independent experiments.

Flow cytometry analysis

Whole individual fluorescent embryos were manually dissociated in 0.9% PBS and analyzed for % GFP or dsRed positive fluorescence by flow cytometry; 20,000 cells were analyzed per sample ($n=10$–20/genotype). Genotyping for *APC* was performed by PCR on excess cells following analysis.

qPCR

qPCR was performed using primer sets for *cyclind1*, *cmyc*, *foxa3*, *sox17*, *hhex*, *prox1*, *pdx1*, and *lfabp* (Supplementary Table 1). Embryos were treated as described, and RNA was extracted from pooled cohorts ($n=50$) of whole embryos. qPCR (60 °C annealing temperature) was performed using SYBR Green Supermix on the iQ5 Multicolor RTPCR Detection System (BioRad) and relative expression levels were determined. At least 3 independent experiments with 3–5 samples were performed for each gene.

Liver resection

Following administration of tricaine anesthetic (0.04 mg/ml), 1/3 partial hepatectomy of the adult zebrafish liver was performed under brightfield imaging on a dissection microscope. The incision was made using microdissection scissors posterior to the heart on the left lateral portion of the abdomen. Forceps were utilized to resect the entire length of the inferior liver lobe. Ultrasound analysis was performed using a Vevo770 high-frequency ultrasound machine (Visualsonics, Toronto) as described (Goessling et al., 2007). Murine liver resections were performed as described (Greene and Puder, 2003).

Results

APC loss and β-catenin levels affect liver development

To determine the effects of β-catenin dysregulation on liver organogenesis, we utilized zebrafish that carry a mutation in the *APC* gene, an essential component of the β-catenin destruction complex. $APC^{+/-}$ zebrafish were crossed into a *lfabp:GFP* reporter line, and liver size was assessed by fluorescence microscopy. At 72 hpf, $APC^{+/-}$*; lfabp:GFP* embryos showed a dramatic increase in liver size (265 altered/297 scored), compared to wild-type siblings (Figs. 1A, B, D). Flow cytometric analysis of GFP+ cells in $APC^{+/-}$*; lfabp:GFP* mutants revealed a three-fold increase in hepatocytes per embryo, as compared to wild-type controls (Figs. 1E, F, H; $APC^{+/+}$ 1.00±0.37%; $APC^{+/-}$ 2.65±0.62%; $APC^{-/-}$- 0.20±0.10%; ANOVA, $n=10$, $p<0.00001$); genotyping of GFP+ cells demonstrated the presence of both the wild-type and mutant APC allele in the livers of $APC^{+/-}$ embryos confirming this phenotype was specific to the heterozygous *APC* state and not due to somatic loss of heterozygosity (LOH) at the *APC* allele. In contrast, no *GFP* expression could be detected in homozygous $APC^{-/-}$ mutant embryos (134/134) at any stage of development (Figs. 1C and G); $APC^{-/-}$ embryos exhibit a myriad of developmental defects as reported previously, however, they are viable to 120 hpf+, significantly past the initiation of *lfabp:GFP* expression in the maturing liver (Hurlstone et al., 2003; Nadauld et al., 2006). To ensure that the observed phenotypic changes in liver development were not due to a variation in the expression of *lfabp*, we performed in situ hybridization for other liver-specific transcripts, *sterol carrier protein* and *transferrin* (data not shown), and obtained similar results. Hepatocyte nuclei counts in corresponding histological sections of 72 hpf embryos corroborated the differential effects of *APC* loss on liver development (Figs. 1I–L). The hepatocyte numbers consistently showed significant three-fold increases in the $APC^{+/-}$ embryos (93.3±20.4 vs. 39.7±3.1; *t*-test, $n=5$, $p=0.004$). Overall hepatocyte morphology, however, showed no differences between wild-type and $APC^{+/-}$ samples, indicating that cell number alone was responsible for the increased liver size. In serial sections through $APC^{-/-}$ mutants, no hepatocytes could be detected. These data demonstrate that progressive loss of *APC* does not have linear effects on liver development.

β-catenin levels mediate the liver phenotypes in APC mutants

As APC regulates the availability of β-catenin in the nucleus, we next used immunohistochemistry to examine the cellular content and localization of β-catenin within hepatocytes in incrosses of $APC^{+/-}$ mutants at 72 hpf (Figs. 2A–D). In wild-type embryos hepatocytes exhibited primarily membrane-bound β-catenin (Fig. 2A). The livers of $APC^{+/-}$ heterozygous mutants, however, demonstrated a 4-fold and 5-fold increase in cytoplasmic and nuclear β-catenin, respectively (Figs. 2B and D; cytoplasmic: $APC^{+/-}$, 39.4±13.4% vs. wt, 9.8±3.8%; $n=5$, $p=0.0006$; nuclear: $APC^{+/-}$, 20.8±8.2% vs. wt, 4.3±1.8%; $n=5$, $p=0.0012$). The absence of liver formation precluded an assessment of β-catenin distribution in hepatocytes of $APC^{-/-}$ embryos; the endodermal tissue, however, exhibited intense β-catenin staining (Fig. 2C), consistent with complete impairment of APC-mediated destruction.

To determine whether increased β-catenin causes both the enlarged liver in $APC^{+/-}$ embryos and the failure of liver development in $APC^{-/-}$ mutants, we reduced β-catenin levels through a morpholino antisense oligonucleotide (MO) strategy (Supplementary Figs. 1A–C). We injected *β-catenin* (Lyman Gingerich et al., 2005) or mismatched control MOs into progeny of an $APC^{+/-}$ incross, performed in situ hybridization for *lfabp*, scored the liver phenotypes, and then genotyped each embryo (Figs. 2E–I). Injection of the control MO did not alter the enlarged liver of $APC^{+/-}$ heterozygotes or the absence of liver in $APC^{-/-}$ homozygotes (Fig. 2E left panel). Injection of a low dose of *β-catenin* MO did not change the gross morphology of any of the progeny of the $APC^{+/-}$ incross. However, 75% (94/126) of $APC^{+/-}$ heterozygotes injected with β-catenin MO now displayed a normal, not enlarged, liver phenotype (Figs. 2E right panel, F and G). In addition, 43% (10/23) of $APC^{-/-}$ homozygotes injected with *β-catenin* MO now showed *lfabp* expression (Figs. 2E right panel, H and I). Although expression of *lfabp* was rescued, these $APC^{-/-}$ embryos still exhibited other severe developmental defects and were not viable beyond 120 hpf. These data suggest that enhanced wnt activity resulting from loss of APC acts through β-catenin to cause both the enlarged liver phenotype of $APC^{+/-}$ heterozygotes and the loss of liver in $APC^{-/-}$ homozygotes.

APC loss leads to altered endodermal proliferation and apoptosis

Wnt/β-catenin signaling is known to mediate effects on cellular proliferation and apoptosis in a variety of tissues (Alonso and Fuchs, 2003; Pinto et al., 2003; Reya et al., 2003). To determine whether the increase in total hepatocyte numbers in the $APC^{+/-}$ embryos was due to increased proliferative activity, we examined BrdU incorporation at 72 hpf (Figs. 3A–D). $APC^{+/-}$ embryos had a significant increase of BrdU-positive cells per liver compared to wild-type (Figs. 3A, B, D; $APC^{+/-}$ 33.8±12.6% vs. wt 11.3±7.4%; $n=5$, $p=0.016$). BrdU incorporation in $APC^{-/-}$ embryos was high in the endodermal region, but the lack of liver tissue precluded further analysis in hepatocytes (Fig. 3C). Aberrant wnt signaling due to *APC* loss in the intestine and developing brain leads first to a block in differentiation and later to apoptosis (Chenn and Walsh, 2002; Sansom et al., 2004). To assess whether the failure of $APC^{-/-}$ embryos to develop hepatocytes resulted in apoptotic cell death, we evaluated TUNEL staining at 24 and 72 hpf. At 24 hpf, no significant cell death was seen in the endoderm of $APC^{-/-}$ mutants (Supplementary Figs. 2A–C). By 72 hpf, numerous TUNEL+ cells were found along the entire length of the endoderm in $APC^{-/-}$ mutants (Fig. 3G), including the area where liver development failed to occur. Wild-type and $APC^{+/-}$ mutants continued to show minimal apoptotic activity (Figs. 3E and F). Similarly, caspase activity, a marker of apoptosis, was markedly elevated in $APC^{-/-}$ embryos compared to wild-types at 72 hpf (Fig. 3H; ANOVA, $n=10$/genotype, $p<0.00001$). Together, these data demonstrate that apoptosis is likely the consequence of improper specification or differentiation of endodermal progenitor cells.

APC loss results in altered endodermal progenitor development

The previous results in the *APC* mutants suggested that wnt signaling may affect endodermal and hepatic progenitor cells. To determine the effects of progressive *APC* loss on these cells, *foxa3* expression was examined at 24 and 48 hpf. Compared to wild-type siblings (Figs. 4A and D), $APC^{+/-}$ embryos exhibited enlarged liver buds

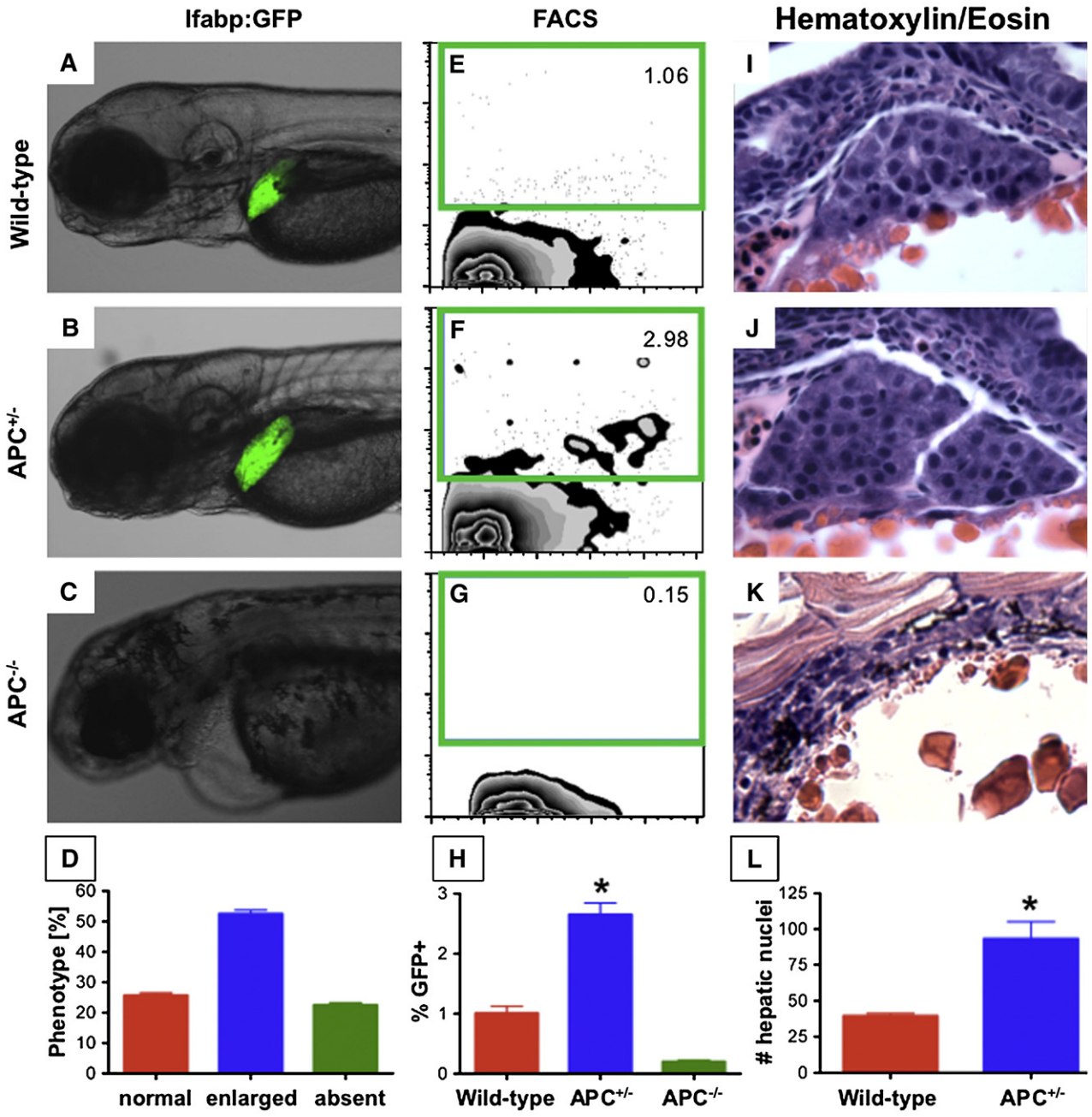

Fig. 1. *APC* loss has differential effects on liver development. Zebrafish embryos were analyzed at 72 hpf. (A–C) Fluorescence microscopy of progeny of an $APC^{+/-}$; *lfabp:GFP* incross revealed differences in liver size. (D) Graphic representation of the liver phenotypes (5 independent clutches; $n=584$) shows a Mendelian distribution. (E–G) Total hepatocytes per embryo were quantified by flow cytometry for GFP in each *APC* genotype (green gate) and confirmed the differential effects of *APC* loss on liver cell number ($APC^{+/+}$ 1.00±0.37% (% GFP+ hepatocytes of 20,000 total embryo cells counted±SD); $APC^{+/-}$ 2.65±0.62%; $APC^{-/-}$ 0.20±0.10%; ANOVA, $n=10$, $p<0.00001$); (H) $APC^{+/-}$ embryos have significantly more hepatocytes than wild-type controls, while $APC^{-/-}$ embryos have no GFP+ hepatocytes. (I–K) H+E liver sections (10 μm) from wild-type, $APC^{+/-}$, and $APC^{-/-}$ embryos (40×) corroborated the effects of *APC* mutations; (L) $APC^{+/-}$ embryos had increased hepatocytes per section compared to controls (93.3±20.4 vs. 39.7±3.1; *t*-test, $n=5$, $p=0.004$).

and diminished pancreatic buds (Figs. 4B and E; 35/49). $APC^{-/-}$ embryos failed to pattern endoderm, including the liver bud, appropriately (Figs. 4C and F; 23/25). Similar results were found using the hepatic progenitor marker *hhex* (Supplementary Figs. 3A–C) and by in vivo fluorescence analysis of $APC^{+/-}$; *gut:GFP* incross progeny at 48 hpf (Figs. 4G–I). Quantitation of endodermal progenitors by FACS analysis of $APC^{+/-}$; *gut:GFP* embryos demonstrated increased GFP+ cells compared to wild-type siblings, and decreased numbers in $APC^{-/-}$; *gut:GFP* siblings (Fig. 4J; wt 1.56±0.063%; $APC^{+/-}$ 0.99±0.099%; $APC^{-/-}$ 0.19±0.089%; ANOVA, $n=10$/genotype, $p<0.00001$). These findings were confirmed by qPCR at 48 hpf for *foxa3*, *hhex* and *lfabp* (Fig. 4K). These data imply that both endodermal patterning and cellular proliferation are regulated by *APC* loss and that wnt/β-catenin signaling influences both endodermal and hepatic development.

wnt signaling has biphasic effects on endodermal and liver development

To determine how wnt activity exerts opposing effects on endodermal differentiation and liver growth during development, we used inducible transgenic zebrafish expressing *wnt8*. wnt activation at the 1 somite (10 hpf) stage caused significant cardiac edema, reduced body length, and absence of liver formation, reminiscent of $APC^{-/-}$ mutants (Figs. 5A and B; 49/52). In contrast, induction of *wnt8* at 10 somites (14 hpf) resulted in markedly enlarged livers compared

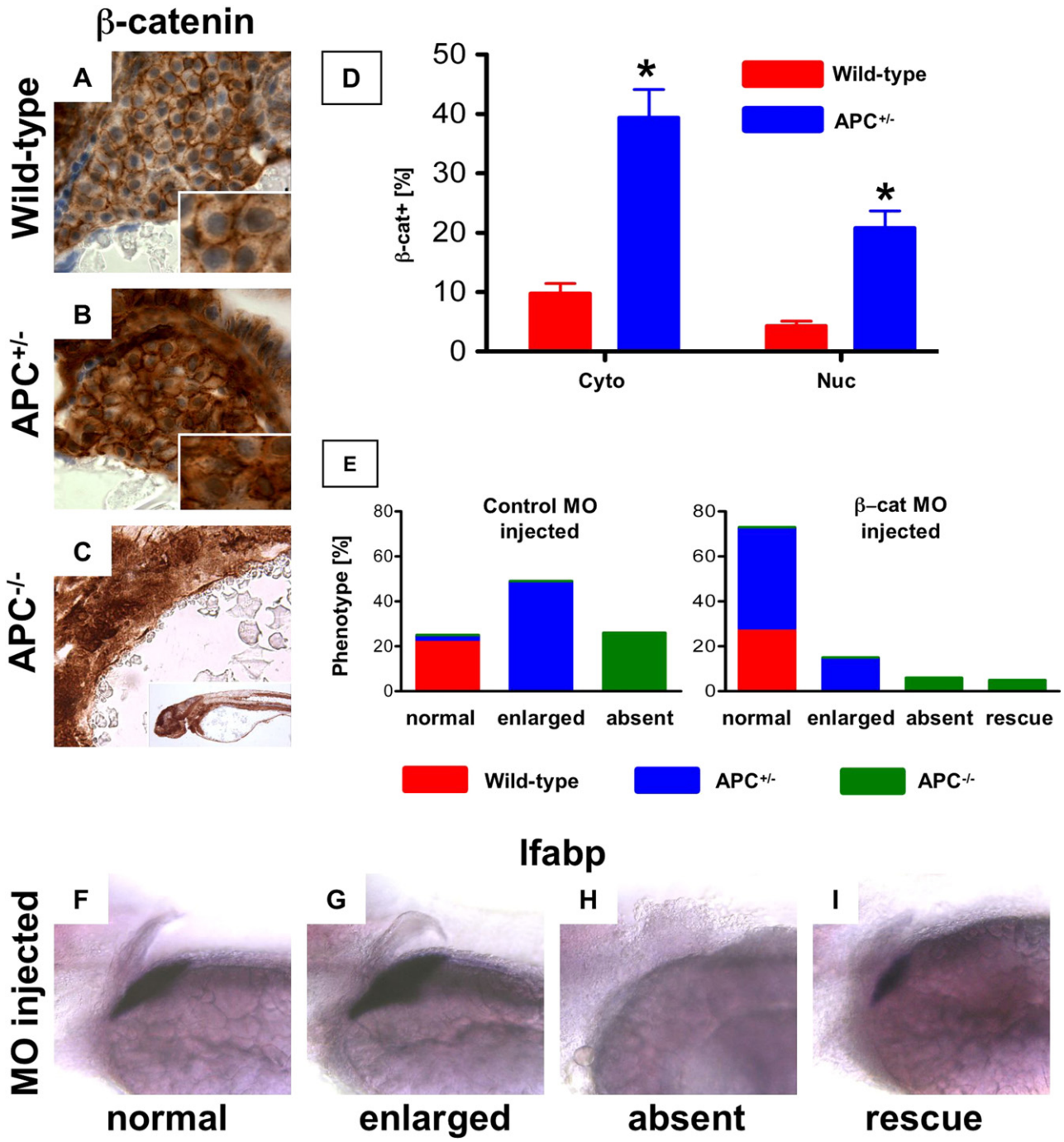

Fig. 2. β-catenin levels mediate differential liver phenotypes in *APC* mutant zebrafish. (A–D) IHC for β-catenin at 72 hpf (40×, close-up as inset) revealed an increase in both cytoplasmic (*APC*$^{+/-}$: 39.4±13.4% (% of hepatocytes with cytoplasmic β-catenin±SD) vs. 9.8±3.8%; $n=5$, $p=0.0006$) and nuclear staining (20.8±8.2% vs. 4.3±1.8%; $n=5$, $p=0.0012$) in the hepatocytes of *APC*$^{+/-}$ embryos compared to wild-type. While liver-specific IHC could not be performed in the *APC*$^{-/-}$embryos, β-catenin staining was widely positive in the region of the endoderm. (E–I) MO (40 μM) injected in the progeny of an *APC*$^{+/-}$ incross at the one-cell stage revealed a shift in liver phenotype distribution. (E) A graphical depiction of the shift in distribution of liver phenotypes with correlated genotypes in β-catenin MO injected embryos compared to controls. Distribution of control MO phenotypes (genotype): 59/234 normal (92% *APC*$^{+/+}$, 8% *APC*$^{+/-}$), 114/234 big (*APC*$^{+/-}$), 61/234 absent (*APC*$^{-/-}$); β-catenin MO phenotypes: 156/211 normal (40% *APC*$^{+/+}$, 60% *APC*$^{+/-}$), 32/211 big (*APC*$^{+/-}$), 13/211 absent and 10/211 rescue (*APC*$^{-/-}$). (F–I) Representative *lfabp* in situ hybridization phenotypes observed in β-catenin MO injected embryos at 72 hpf.

to heat-shocked wild-type controls (Fig. 5C; 141/173). Transient wnt activation at time points from 24 to 48 hpf produced a moderate increase in liver size at 72 hpf (Supplementary Figs. 4A–D). Global inhibition of wnt signaling by induction of *dnTCF* (Supplementary Figs. 4E–J) or *dkk* (data not shown) severely diminished liver size compared to controls.

The expression of hepatocyte-specific genes such as *lfabp* begins at ~44 hpf. Liver specification, however, occurs between 18 somites (16 hpf) and 24 hpf. The results obtained in the heat-shock embryos suggest that the wnt-mediated effects on liver development originated prior to the formation of the mature organ at, or slightly before, the stage at which the fate of endodermal progenitors is determined. To investigate the effects of wnt induction on the endodermal progenitor cell population, expression of the pan-endodermal marker *foxa3* was analyzed at 24 and 48 hpf. Induction of *wnt8* at 1 somite (10 hpf) resulted in a failure of *foxa3* positive cells to extend along the anterior–posterior axis at 24 hpf (Figs. 5D and E; 36/45) and in a lack of liver and pancreatic bud formation at 48 hpf (Figs. 5G and H; 45/61). In

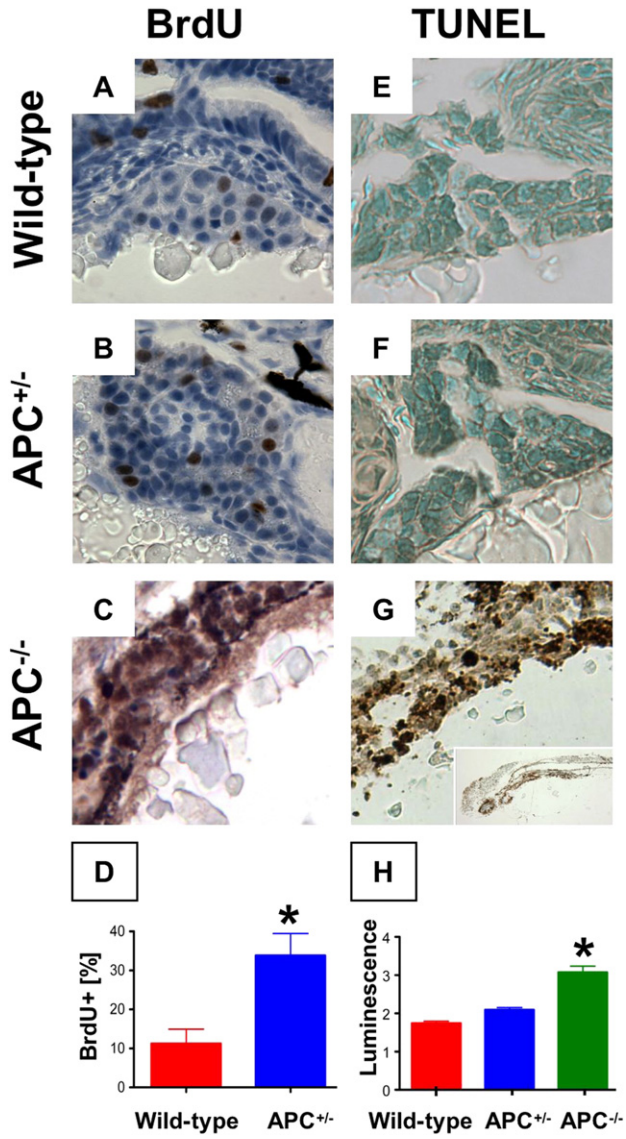

Fig. 3. Endodermal proliferation and cell death are dependent on β-catenin levels. (A–D) BrdU incorporation in liver sections corresponding to Figs. 1I, J was significantly upregulated in $APC^{+/-}$ embryos (33.8 ± 12.6% vs. 11.3 ± 7.4%; $n=5$, $p=0.016$, 40 x), and also in the endodermal region of $APC^{-/-}$ embryos. (E–H) TUNEL staining demonstrated no apoptosis in wild-type and $APC^{+/-}$ embryos, while a high number of TUNEL+ cells were found in the endoderm of $APC^{-/-}$ embryos. (L) Caspase-3 and 7 activity was significantly increased in $APC^{-/-}$ mutants compared to wild-type and $APC^{+/-}$ (ANOVA, $n=10$/genotype, $p<0.00001$). Significant differences are indicated with an asterisk (*).

contrast, a significant increase in the number of *foxa3* positive cells was observed at 24 hpf following heat-shock activation at 10 somites (14 hpf; Fig. 5F; 52/67), leading to a markedly enlarged liver bud and a diminished pancreatic anlage at 48 hpf (Fig. 5I; 52/78).

To analyze the molecular mechanisms by which wnt activation regulates endoderm development, qPCR was performed at 24 hpf following *wnt8* induction at 1 or 10 somites (Fig. 5J). The well-known wnt target genes *cyclind1* and *cmyc* were induced following *wnt8* activation at both 1 and 10 somites (10 and 14 hpf), confirming upregulation of wnt activity. Expression of the pan-endodermal genes *foxa3* and *sox17* was severely diminished after *wnt8* induction at 1 somite (10 hpf), and increased after heat-shock at 10 somites (14 hpf); the hepatic progenitor markers *hhex* and *prox1* were similarly affected. *Pdx1* expression, as a marker of pancreatic progenitors, was diminished in response to *wnt8* induction at both 1 and 10 somites (10 and 14 hpf). These results were confirmed by in situ hybridization for *hhex* and *pdx1* (Supplementary Figs. 4K–P). Taken together, these results demonstrate that early wnt induction inhibits the proliferation and organization of endodermal progenitors, while later induction of wnt promotes the specification and growth of hepatic progenitors.

APC loss affects liver formation in a cell autonomous manner

To determine whether the effects of APC loss on liver formation were cell autonomous, we conducted blastula transplant experiments. First, progeny of an incross of $APC^{+/-}$; *lfabp:GFP* fish were used as donors for blastula transplants into wild-type recipients, and the presence of GFP+ hepatocytes was assessed at 72 hpf (Fig. 6A). Both wild-type and $APC^{+/-}$derived donor blastomeres contributed to liver formation (Figs. 6B and C; wt 4 GFP+/28 examined; $APC^{+/-}$ 12/38), whereas $APC^{-/-}$derived donor blastomeres did not (0/15, Fisher's exact test, $p=0.025$). Using a reciprocal transplantation strategy (Fig. 6D), we found that wild-type donor blastomeres contributed to liver formation in wild-type and $APC^{+/-}$ hosts at 72 hpf (Figs. 6E and F). Wild-type blastomeres injected into $APC^{-/-}$ mutants also gave rise to *lfabp*-expressing hepatocytes, which were unable to recapitulate the formation of an entire liver, but aligned into cord-like structures (Fig. 6G). Together, these data indicate that APC has a cell autonomous role in modulating wnt activity during liver formation.

β-catenin activation in APC mutants results in altered endodermal fate

As all populations of endodermal progenitors appeared to be affected by *APC* loss or wnt activation, mature endodermal organs were examined for consequences of this early alteration in development. *Insulin* and *trypsin* expression, indicative of endocrine and exocrine pancreas differentiation, respectively, were decreased in $APC^{+/-}$ embryos at 72 hpf (Supplementary Figs. 5A, B, D, E). In $APC^{-/-}$ mutants, *insulin* expression was reduced, but still detectable, while *trypsin* expression was virtually absent (Supplementary Figs. 5C and F). The effect of *APC* loss on differentiated intestine as marked by expression of *intestinal fatty acid binding protein (ifabp)* was similar to the liver: $APC^{+/-}$ embryos had increased *ifabp* staining compared to wild-type, while $APC^{-/-}$ embryos failed to express *ifabp* (Supplementary Fig. 5G–I). Induction of *wnt8* at 10 somites (14 hpf) had similar effects on each endodermal organ but resulted in more disorganized patterning, especially of the pancreas (data not shown). These data demonstrate that nascent wnt/β-catenin signaling regulates endodermal development prior to organ specification, and that this effect mediates a shift in the differentiation of endodermal progenitors towards liver at the expense of pancreatic tissue. In addition, early excess wnt/β-catenin activation leads to a failure of endodermal specification and proliferation that results in elevated endodermal cell death and the inability to develop mature endodermal organs.

wnt/β-catenin signaling enhances hepatocyte growth

In order to determine if elevated wnt activity continued to influence not only the specification and proliferation of endodermal progenitors, but had persistent effects on the growth of mature cell populations in the larval stage, we assessed liver size by in vivo confocal microscopy in *lfabp:GFP* larvae at 144 hpf. Additionally, we quantified the differences in total cell number between wild-type and $APC^{+/-}$ fish by FACS. Here, we determined that differences in liver size persist between wild-type and $APC^{+/-}$ larvae (Supplementary Figs. 6A and B), however, the change in hepatocyte number (Supplementary Fig. 6C; 1.5-fold) is smaller than seen at 72 hpf (3-fold). In addition, using *ifabp:dsRed* and *ptf1α:GFP* fish, we found that total cell number in both the intestine and pancreas also remained altered in larval stages (Supplementary Figs. 6D–I). These effects on cell number may simply persist from the earlier influence of wnt activity on endodermal progenitor proliferation or could reflect a role of wnt in regulating the growth of mature endodermal populations.

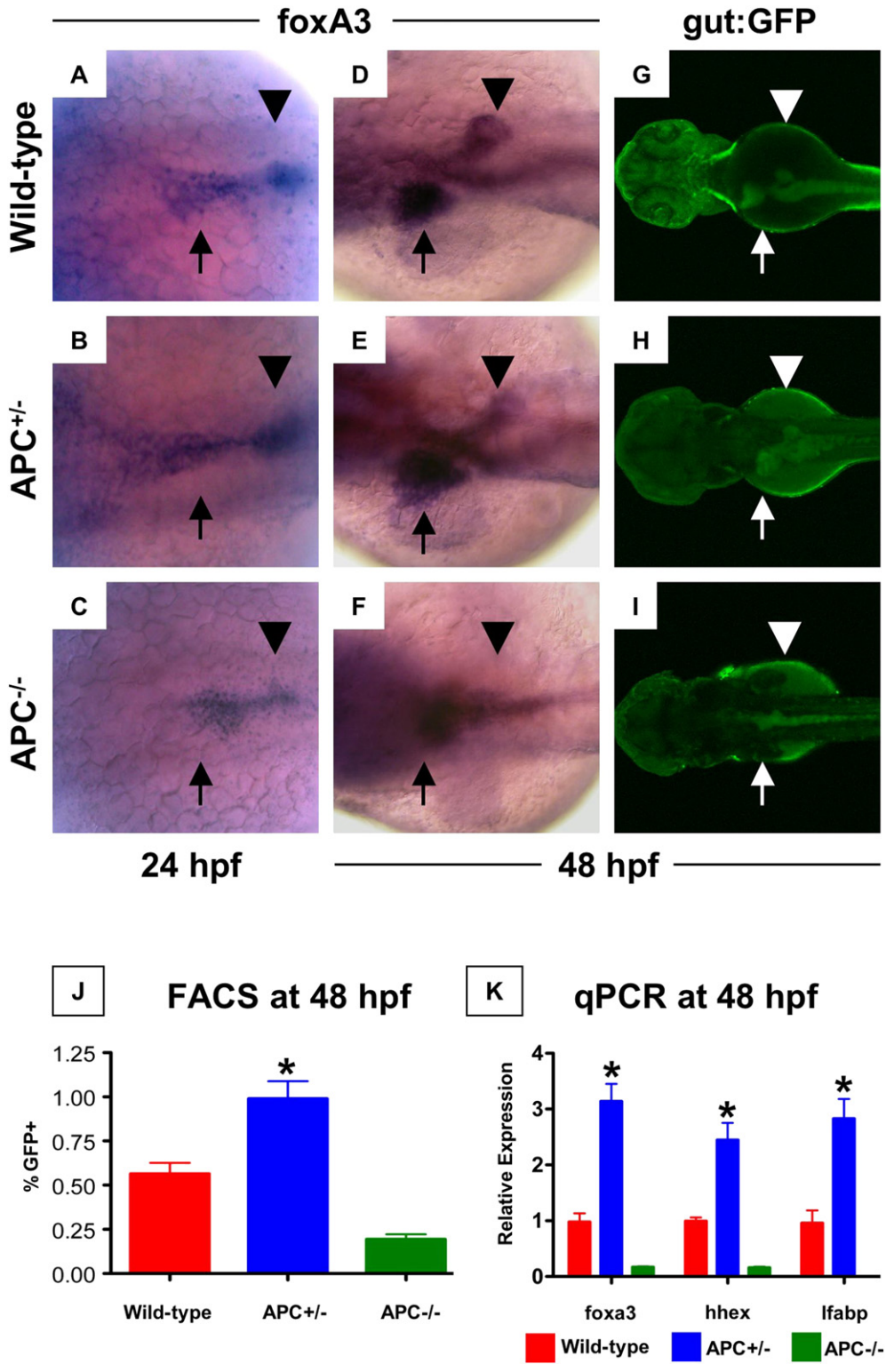

Fig. 4. *APC* loss affects endodermal and hepatic progenitor cells. (A–C) In situ hybridization for *foxa3* revealed changes in endodermal progenitor organization in *APC* mutant embryos as early as 24 hpf. (D–F) By 48 hpf, this led to a progressive increase in hepatic and corresponding decrease in pancreatic buds in the $APC^{+/-}$ embryos (35 altered/49 scored), while the $APC^{-/-}$ embryos failed to develop an organized endodermal pattern (23/25). (G–I) In vivo confocal fluorescence imaging of *gut:GFP* transgenic embryos at 48 hpf revealed similar effects on endodermal patterning. (J) FACS analysis demonstrated a doubling in the number of gut:GFP+ (green gate) endodermal progenitor cells in $APC^{+/-}$ embryos compared to wild-type controls; GFP+ cells were severely diminished in $APC^{-/-}$ embryos ($APC^{+/+}$ 1.56±0.063%; $APC^{+/-}$ 0.99±0.099%; $APC^{-/-}$ 0.19±0.089% of 20,000 cells analyzed; ANOVA, $n=10$/genotype, $p<0.00001$). (K) qPCR analysis confirmed the increased expression levels of *foxa3*, *hhex* and *lfabp* in $APC^{+/-}$ (blue) embryos and the depressed/absent expression in $APC^{-/-}$ (green) embryos at 48 hpf (ANOVA, $n=10$/category, $p<0.05$).

To confirm that wnt/β-catenin signaling could also mediate an effect on the growth of differentiated hepatocytes, *wnt8* expression was induced at 48 hpf. This resulted in a 2-fold increase in liver size both by confocal microscopy of *lfabp:GFP* fish (data not shown) and in GFP+ cells by FACS at 72 hpf (Supplementary Fig. 6J). *wnt8*-induced embryos still possessed increased numbers of GFP+ hepatocytes at

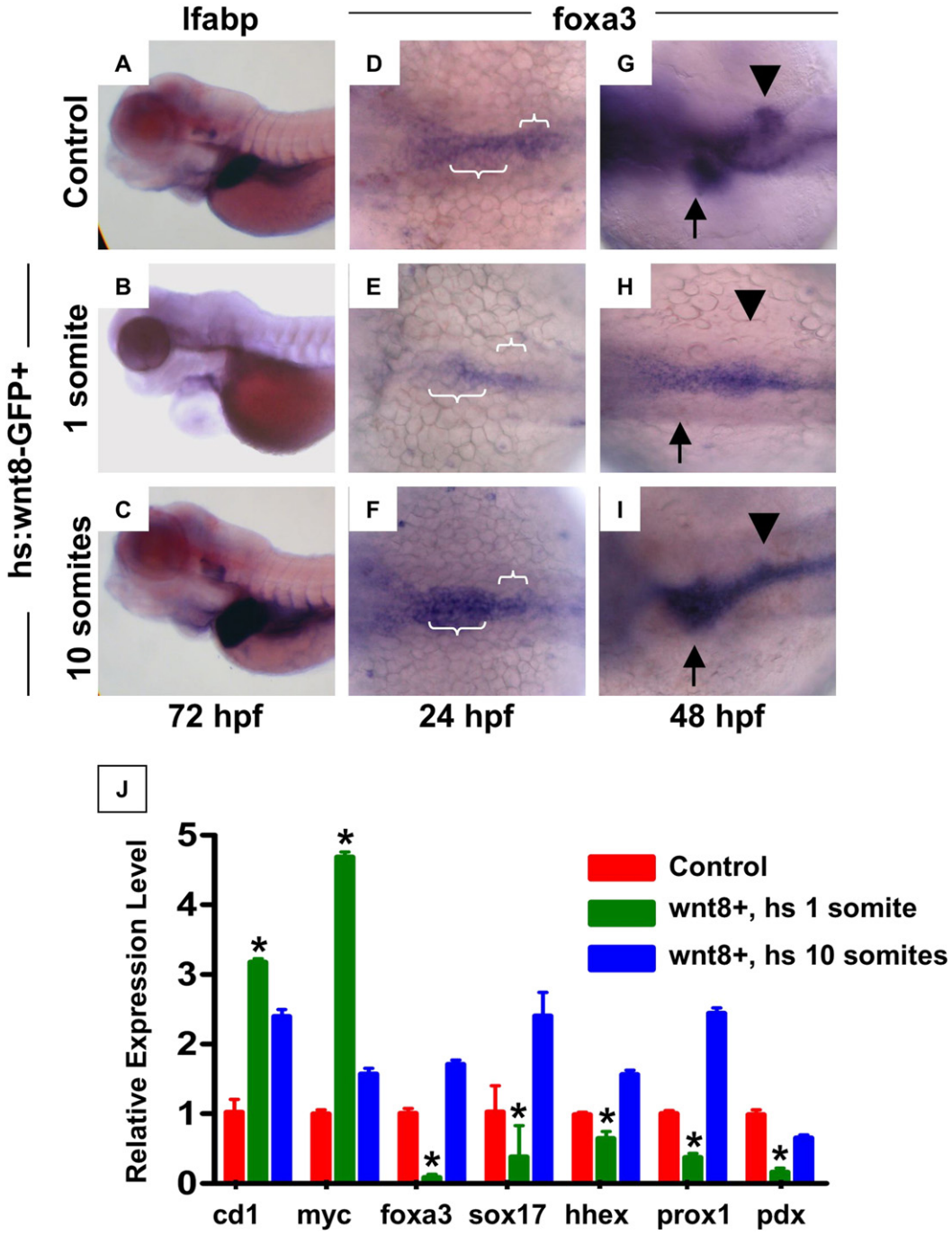

Fig. 5. wnt signaling regulates endoderm development prior to liver specification. Heat-shock inducible *wnt8-GFP* or control embryos were incubated for 20 mins at 38 °C at various time points between 50% epiboly and 48 hpf, and were analyzed for alterations in liver size at 72 hpf by in situ hybridization for *lfabp*. (A, B) Induction of *wnt8* at 1 somite (10 hpf) led to grossly abnormal embryos, with cardiac edema and absent livers (49 altered/ −52 scored). (C) Induction of *wnt8* at 10 somites (14 hpf) resulted in a markedly enlarged livers compared to controls (141/173). (D–F) *foxa3* expression at 24 hpf following heat-shock at 1 somite (10 hpf) revealed a failure of cells to converge at the midline and expand in the a–p direction (36/45); heat-shock at 10 somites (14 hpf) led to enhanced *foxa3* expression (52/67). (G–I) Expression of *foxa3* at 48 hpf revealed a failure to form organized liver and pancreatic buds after wnt8 induction at 1 somite (10 hpf) (45/61); wnt8 induction at 10 somites (14 hpf) led to an increased liver and decreased pancreatic anlage (52/78). (J) qPCR analysis of the expression levels of wnt target (*cyclind1, cmyc*), endodermal (*foxa3, sox17*), hepatic (*hhex, prox1*) and pancreatic (*pdx1*) progenitor genes at 48 hpf in controls (red) and following wnt8 induction at either 1 somite (10 hpf; green) or 10 somites (14 hpf; blue). All changes were statistically significant compared to controls (ANOVA, $n=10$, $p<0.05$).

120 hpf by FACS analysis, although this difference was no longer readily apparent by gross examination of *lfabp* expression. Heat-shock induced inhibition of wnt signaling at 48 hpf demonstrated that wnt was required for optimal liver growth; both *dkk* (data not shown) and *dnTCF* embryos had reduced liver size compared to controls by in situ hybridization at 72 hpf (Supplementary Fig. 4J). These data demonstrate that wnt signaling continues to be important in the proliferation of differentiated hepatocytes.

Activated wnt signaling enhances liver regeneration

In order to evaluate the continued role of wnt activity in regulating hepatocyte growth, we performed liver resections in adult zebrafish. Zebrafish have a trilobar liver; after 1/3 partial hepatectomy by removal of the inferior lobe, >95% of wild-type zebrafish recover, and their livers regenerate entirely within 7 days. To determine whether excess wnt activation provides a regenerative advantage, we resected

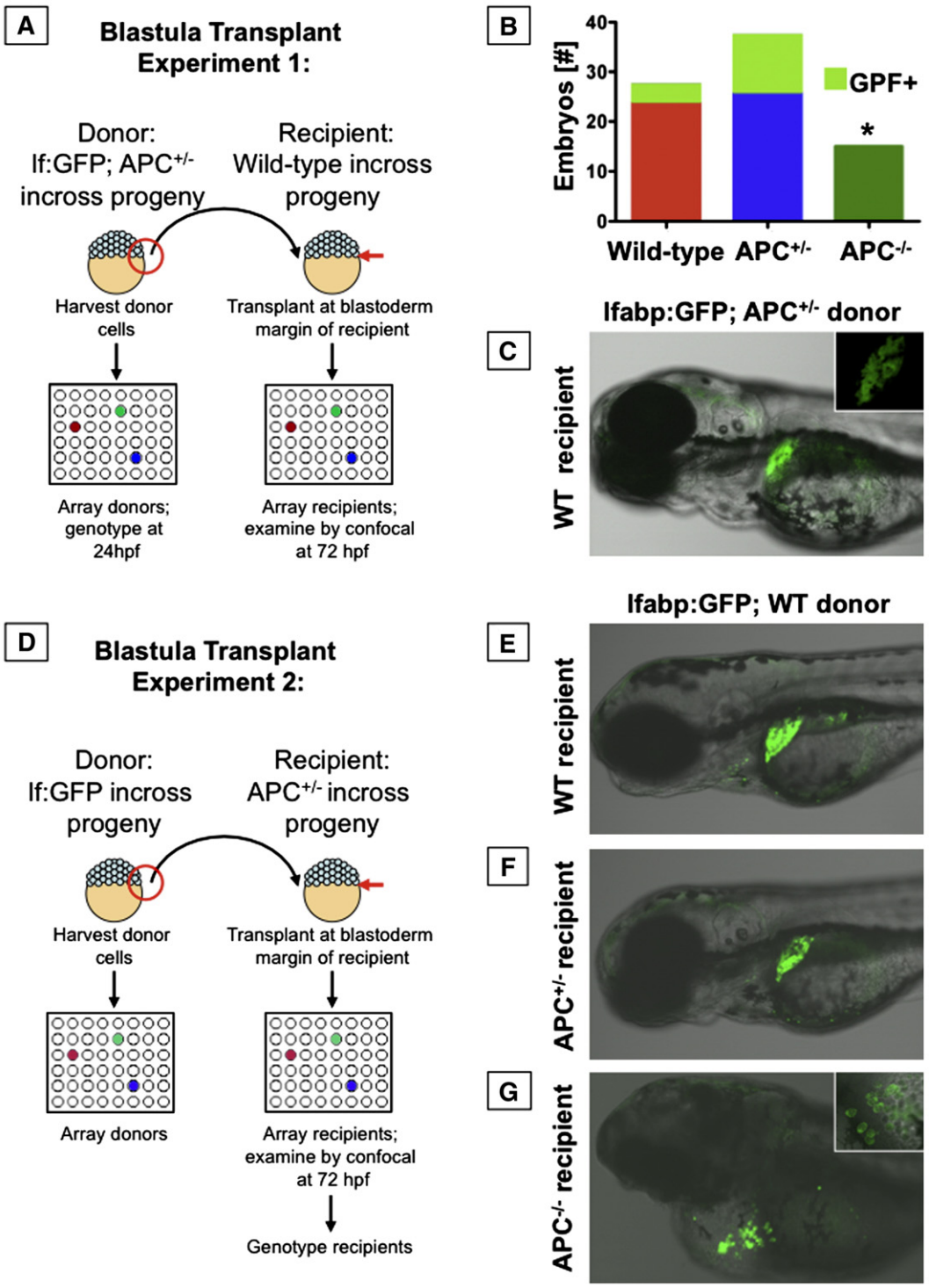

Fig. 6. *APC* has cell autonomous effects on endoderm development. Blastula transplant experiments; embryos were analyzed at 72 hpf. (A) Schematic depiction of blastula transplant experiments with each *APC* genotype as donor. (B) Graphic summary of the number of recipient embryos that received each donor genotype; the fraction of embryos that showed donor contribution to the liver is highlighted in light green. No $APC^{-/-}$ donor cells contributed to liver formation (Fisher's exact, $p=0.025$). (C) Mosaic livers showed green hepatocytes interspersed with unlabeled cells. (D) Schematic depiction of blastula transplant experiments with different *APC* genotypes as recipients. (E, F) *lfabp:GFP* donor cells transplanted into both wild-type or $APC^{+/-}$ hosts gave rise to mosaic livers. (G) In an $APC^{-/-}$ host, *lfabp:GFP* hepatocytes developed, but could not rescue liver development. The inset shows formation of chains of hepatocytes near the heart and around the yolk sac. Significant differences are indicated with an asterisk (*).

livers in $APC^{+/-}$ mutant and heat-shocked *wnt8* transgenic fish and measured hepatic regeneration by analysis of en-bloc resected livers and histology. In each, we observed accelerated liver growth compared to wild-type controls during the early stages of liver regeneration (Figs. 7A–C; Supplementary Figs. 7A–I). However, by 7 days, the liver in wild-type, $APC^{+/-}$ mutant, and wnt8 transgenic fish had each fully regenerated to baseline organ size. Inhibition of β-catenin signaling in *dnTCF* transgenics severely impaired liver regeneration, confirming the requirement of wnt/β-catenin signaling in this process (Fig. 7D; Supplementary Figs. 7J–L). We quantified these observations using the liver lobe:remnant length ratios of the regenerating inferior lobe at 1, 3, 5 and 7 days post resection (dpr) (Fig.

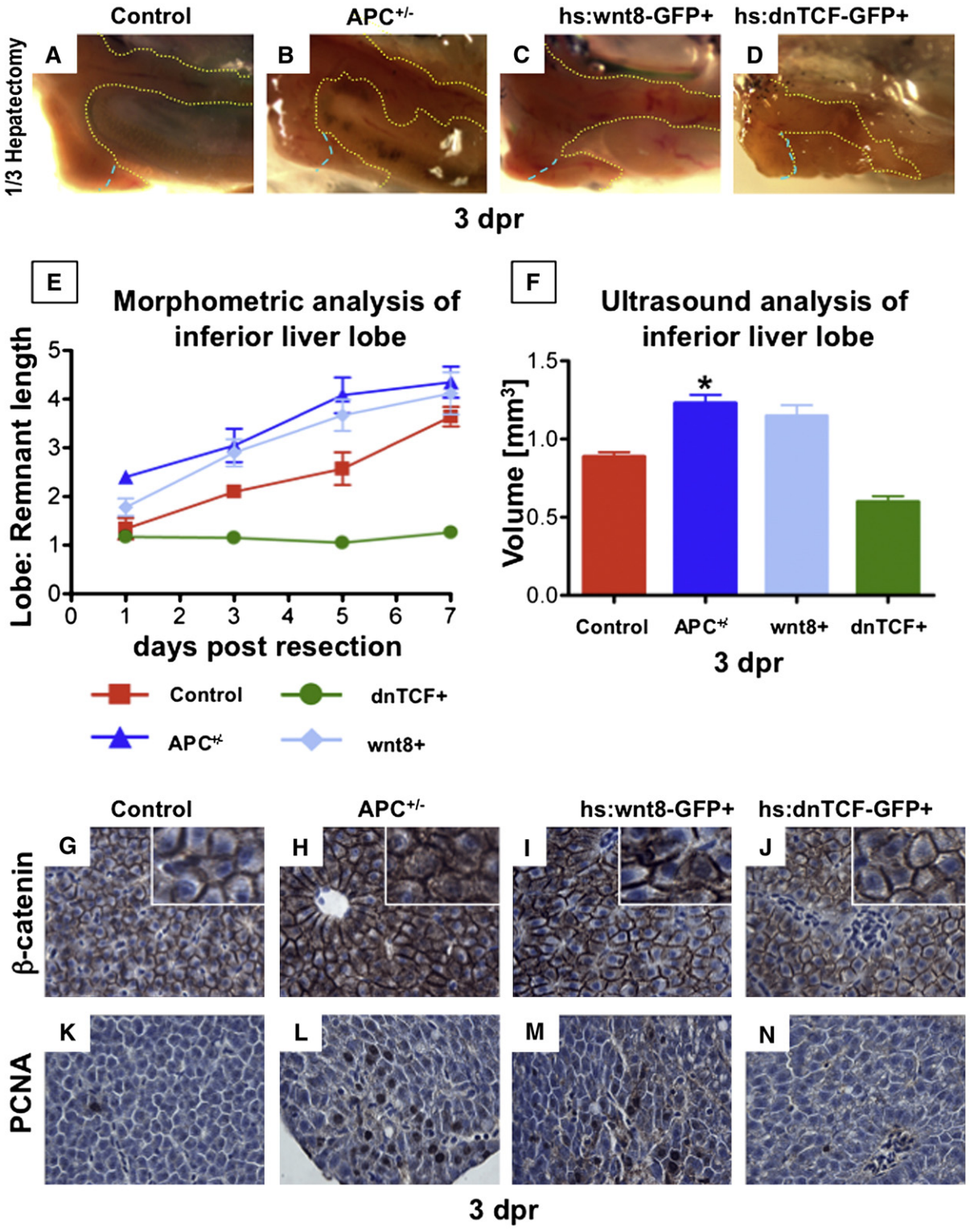

Fig. 7. Liver regeneration following partial hepatectomy in adult zebrafish is enhanced by wnt activation. (A–D) En-bloc dissection of $APC^{+/-}$ and *hs:wnt8* (induced from 6 to 18 hpr) fish at 3 dpr revealed enhanced regeneration, while inhibition of signaling by *dnTCF* (induced from 6 to 18 hpr) resulted in severely impaired regenerative capacity. (E) Quantitative analysis of the liver lobe:remnant ratios in zebrafish at days 1, 3, 5 and 7 post resection reveals enhanced regenerative kinetics caused by wnt/β-catenin activation in $APC^{+/-}$ (blue triangle) and *wnt8* (light blue diamond) fish, while *dnTCF* (green circle) inhibits liver regeneration. (F) In vivo volume measurements of the inferior liver lobe by high-frequency ultrasound at 3 dpr confirmed the regenerative advantage in $APC^{+/-}$ (blue) and *wnt8* (light blue) fish and the decreased regenerative capacity in *dnTCF* fish (green) (ANOVA, $n=3$/genotype, $p<0.05$). (G–J) IHC in these fish at 3 dpr revealed increased cytoplasmic and nuclear β-catenin staining in $APC^{+/-}$ and *hs:wnt8* fish. (K–N) Hepatocyte proliferation measured by PCNA staining was increased in $APC^{+/-}$ and *wnt8* fish, but absent in *dnTCF* fish. Significant differences are indicated with an asterisk (*). (O) Enhanced liver regeneration caused by activation of wnt/β-catenin signaling. $APC^{Min/+}$ status mediates a growth advantage during murine liver regeneration compared to wild-type controls. Liver weight:body weight ratios measured at postoperative days 1, 3 and 5 revealed a significant increase at postoperative day 3 ($32.1 \pm 2.32 \times 10^{-3}$ vs. $29.6 \pm 1.51 \times 10^{-3}$, $n=10$–14/time point/genotype, $p=0.003$) in $APC^{Min/+}$ mice. (P–S) IHC of wild-type murine livers demonstrated primarily pericentral β-catenin activity at baseline, and the subsequent upregulation, nuclear localization, and periportal concentration of β-catenin following 2/3 partial hepatectomy (left insets: 40× magnified view of periportal area). (T–W) In the livers of $APC^{Min/+}$ mice, there was moderate β-catenin staining in both pericentral and periportal areas in sham-operated controls (T). Marked enhancement of β-catenin activity occurred following resection (U–W). BrdU incorporation (right insets) increased in wild-type mice following liver resection, peaking at 3 dpr. This activity was enhanced and accelerated in $APC^{Min/+}$ mice.

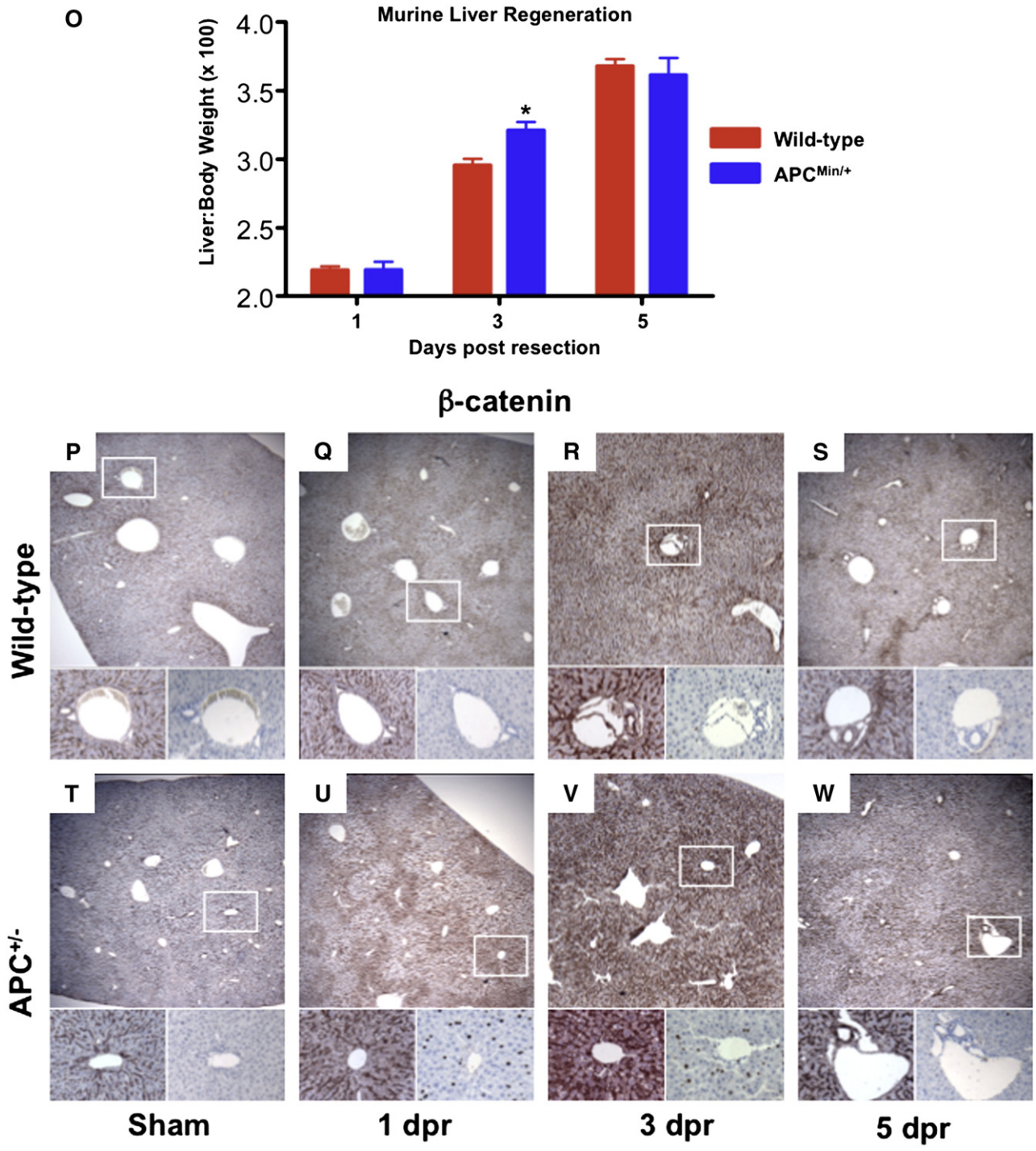

Fig. 7 (*continued*).

7E), and in vivo by high-frequency ultrasound (Goessling et al., 2007) measurements of the inferior liver lobe volume (Fig. 7F). Additionally, nuclear and cytosolic β-catenin levels (Figs. 7G–J) as well as PCNA staining (Figs. 7K–N) were increased in zebrafish with elevated wnt signaling and enhanced regeneration, and absent in *dnTCF* fish. These experiments highlight the persistent importance of wnt/β-catenin signaling in liver homeostasis and growth throughout the lifetime of the zebrafish.

To determine if the effect of wnt activation on liver regeneration is conserved across vertebrate species, we performed 2/3 partial hepatectomies in $APC^{Min/+}$ mice. Assessment of liver:body weight ratios revealed a significantly increased regenerative capacity in the $APC^{Min/+}$ mice compared to wild-type controls at 3 dpr (Fig. 7O; $n=10$–14/time point/genotype, $p=0.003$). In wild-type unresected livers, baseline β-catenin activity was mainly found in the pericentral region (Fig. 7P). Following resection, β-catenin localization progressively shifted to the periportal area from 1 to 3 dpr (Figs. 7Q and R), and then returned to the pericentral region by 5 dpr (Fig. 7S). In $APC^{Min/+}$ mice, β-catenin levels were increased at baseline, in both the pericentral and periportal regions (Fig. 7T). β-catenin was dramatically upregulated around the periportal areas from 1 to 3 dpr (Figs. 7U and V) and diminished by 5 dpr (Fig. 7W). We observed the highest proliferative

activity by BrdU incorporation (Figs. 7P–W, bottom right inset) and nuclear β-catenin staining (bottom left inset) in the periportal regions in both wild-type and $APC^{Min/+}$ mice at 1 to 3 dpr. These data demonstrate that wnt activation also enhances the kinetics of mammalian liver regeneration.

Discussion

Identification of the molecular mechanisms controlling liver development will assist in our understanding of the biological basis of hepatic tumor formation and will provide targets for therapeutic manipulation. Because defects in the Wnt pathway are prevalent in both primitive (Hirschman et al., 2005) and differentiated (Taniguchi et al., 2002; Tokumoto et al., 2005) hepatic neoplasms, we sought to uncover the role of wnt signaling in regulating both liver specification and growth. Through analysis of *APC* mutant zebrafish with dysregulated β-catenin levels, we demonstrated that wnt activity needs to be precisely regulated for proper liver development and maintenance of tissue homeostasis. Furthermore, we reconciled conflicting data on the role of wnt in liver development through analysis of inducible *wnt8* transgenic zebrafish, revealing a biphasic regulation of β-catenin during endoderm development. Additionally, we demonstrated that activated wnt/β-catenin signaling in $APC^{+/-}$ zebrafish and $APC^{Min/+}$ mice led to enhanced regenerative capacity after partial hepatectomy, indicating that the role of wnt signaling to enhance organ regeneration is evolutionarily conserved. Our findings may help to identify new therapies in the treatment of liver cancer and liver failure.

Regulation of wnt signaling is important during endoderm development

Progressive loss of functional APC did not have a linear effect on liver development during embryogenesis. We demonstrate for the first time that not only complete absence of functional APC, but also heterozygous *APC* loss led to enhanced β-catenin signaling in vivo, as indicated by increased cytoplasmic and nuclear β-catenin accumulation. In the $APC^{+/-}$ embryos, this resulted in higher hepatocyte numbers and increased organ size; there was no evidence for loss of the wild-type *APC* allele. This developmental advantage is evident shortly after the specification of hepatic progenitors and appears to be due to enhanced cyclin D1-mediated cellular proliferation. Conversely, the complete absence of β-catenin regulation and degradation in the $APC^{-/-}$ mutants caused the primitive endoderm to fail to organize and properly differentiate into the various endodermal lineages; this is consistent with the original description of the homozygous mutant that formation of endodermal primordia was initiated but severely impaired (Hurlstone et al., 2003). The homozygous $APC^{-/-}$ phenotype was evident as early as 24 hpf by in situ hybridization for *foxA3* and was dramatically illustrated by the complete lack of all mature endodermal lineages by 72 hpf. We speculate that the significant apoptosis observed by 72 hpf is a secondary effect of the failure of the endoderm to organize and specify organs. In support of this conclusion, whole mount TUNEL staining demonstrated very little programmed cell death in the endoderm at 24 hpf. Our findings are consistent with recent work on the differential effects of various clinically relevant APC mutations on mouse embryonic stem cell differentiation (Kielman et al., 2002); here, complete absence of β-catenin regulation resulted in a severe differentiation blockade and further pointed to levels of nuclear β-catenin as the central mediator of the observed phenotypes. Our findings are further supported by recent observations in homozygous $APC^{Min/Min}$ mouse embryos which fail to induce expression of *Hex* and lack distal visceral endoderm (Chazaud and Rossant, 2006). Divergent dose-dependent effects of Wnt/β-catenin signaling have been described in several organ systems. For example, β-catenin signaling is known to induce proliferation of hematopoietic stem cells, and to enhance the success of hematopoietic stem cell transplants (Trowbridge et al., 2006). It is also required for the differentiation and growth of lymphoid cell types. Excessive levels of Wnt signaling, however, inhibit hematopoietic stem cell function and the ability to differentiate (Kirstetter et al., 2006; Reya et al., 2003).

Both wnt repression and activation are required during different phases of liver development

Using heat-shock inducible transgenic fish, we demonstrated the importance of temporal regulation of wnt activity in endodermal progenitors during embryonic development. These data revealed a critical period during early endoderm development (1 somite, 10 hpf) when excess wnt activity led to a failure to expand and organize endodermal progenitors (*foxa3*, *sox17*). This caused decreased numbers of hepatic (*hhex*) and pancreatic (*pdx1*) progenitors, a failure to develop differentiated hepatocytes and, finally, apoptosis. In contrast, *wnt8* activation at 10 somites (14 hpf) led to enhanced expression of *foxa3*, *sox17*, *hhex*, and *prox1* at 24 hpf, and to a significant increase in liver size by 72 hpf. Recent work has highlighted the importance of wnt signaling in liver development (Apte et al., 2007; McLin et al., 2007; Monga et al., 2003; Ober et al., 2006; Suksaweang et al., 2004). McLin et al. demonstrated that wnt repression in the anterior endoderm during early somitogenesis is required for liver development (McLin et al., 2007). Using *Xenopus* embryo studies, they showed that experimental repression of wnt activity in the posterior endoderm led to ectopic liver formation, and that β-catenin activity repressed *hhex*. In contrast, Ober et al. reported a zebrafish mutant, *prt*, with a mutation in *wnt2bb* that shows the requirement of wnt activity in liver formation (Ober et al., 2006). In these mutants, a subset of hepatic progenitors could still be specified, as demonstrated by *hhex* and *prox1* staining. Due to the lack of mesodermally derived wnt2bb, however, these hepatoblasts did not proliferate, causing the reduced liver phenotype at 52 hpf. Our data help to integrate these seemingly contradictory findings by illustrating the biphasic role of wnt signaling in establishing the liver progenitor pool, and by identifying genes affected by wnt activity in liver formation. This biphasic requirement of wnt signaling during liver development is consistent with other studies showing tight temporal regulation of wnt activity during the course of cell-type-specific differentiation (Christian and Moon, 1993; Dequeant et al., 2006; Manisastry et al., 2006).

APC loss has a cell autonomous effect on hepatogenesis

As previously demonstrated, wnt2bb is mesodermally derived and required for liver development in a non-cell autonomous fashion (Ober et al., 2006). Our blastula transplant experiments revealed that the effects of *APC* loss are cell autonomous. This indicates that, although the wnt signals are mesodermally derived, the effect of wnt activity on liver development is mediated within the hepatic progenitor cells. We found that wild-type cells can form hepatocytes in a cell autonomous manner in an $APC^{-/-}$ environment, even when elevated β-catenin levels in the surrounding embryo may prevent the formation of an organ matrix. These results further expand our understanding of the effects of wnt signaling on hepatic progenitors.

wnt signaling affects endodermal progenitor fate

Wnt signaling can alter the developmental fate of unspecified endodermal progenitors, and, when activated, shifts the distribution to liver-specific cell fates. Most notably, excess wnt signaling at 10 somites (14 hpf) inhibited the development of both the endocrine

and exocrine pancreas. Our studies establish wnt/β-catenin signaling as an important negative regulator of early pancreas formation. Decreased expression levels of *pdx1* at 24 hpf revealed effects of wnt activation on pancreatic progenitor cells, resulting in decreased organ size at 72 hpf that persists into the larval stage. One explanation for our findings is that *wnt8* induction at 1 somite (10 hpf) disrupts the expansion and organization of the primitive endoderm (as marked by *foxa3* and *sox17*), resulting in decreased *hhex* and *pdx1+* progenitors. *wnt8* induction at 10 somites (14 hpf), meanwhile, targets the specification and proliferation of hepatic and pancreatic progenitor cells. Hepatic progenitor growth may be promoted by wnt activation, while pancreatic progenitor growth is inhibited. Alternately, if bipotential hepato-pancreatic progenitors exist, biased pressure to differentiate into liver cell fates may effectively diminish the number of progenitors available to produce the pancreas. The existence of such bipotential cells has been suggested through embryo explant analysis (Deutsch et al., 2001). Endodermal lineage tracing analysis in zebrafish embryos, however, does not provide a clear answer; while the majority of blastomeres injected with a vital dye develop into populations of either liver or pancreatic progenitors, up to 20% can give rise to both lineages (Warga and Nusslein-Volhard, 1999). A reciprocal response between liver and pancreatic development was also described in response to sonic hedgehog (shh) signaling (Wallace and Pack, 2003): mutant zebrafish with disruption in the *shh* pathway (*syu*) developed an enlarged liver and a severe reduction in pancreas; together with our data this argues favorably that a population of bipotent cells likely exists and plays a significant role in the developmental regulation of organ size. Recent data demonstrate that pancreatic progenitor cell numbers determine mature organ size, while liver size is independent of the number of hepatic progenitor cells during development (Stanger et al., 2007). It will be important to determine whether mature liver and pancreas maintain a population of these multipotent progenitors that could be manipulated therapeutically.

Activated wnt/β-catenin signaling enhances liver regeneration

The adult liver can fully regenerate after substantial injury. The zebrafish is a well-established model for investigating regenerative processes (Akimenko et al., 1995; Stoick-Cooper et al., 2007). Recently, Sadler et al. introduced partial hepatectomy in zebrafish as a method for studying the effects of cell cycle mutations on liver regeneration (Sadler et al., 2007). Here, we present a similar approach, resulting in 1/3 partial hepatectomy through removal of the inferior liver lobe. Wnt signaling was activated after resection, and inhibition of wnt/β-catenin signaling severely altered liver regrowth. This is consistent with previously published studies in murine systems (Monga et al., 2001; Sekine et al., 2007; Sodhi et al., 2005; Tan et al., 2006). More significantly, we demonstrated that β-catenin activation in vivo enhances the rate of liver regeneration. However, we consistently observed that wnt activation did not cause unlimited regeneration or overgrowth of the liver in our assay, as both wild-type and wnt-activated zebrafish (*APC*$^{+/-}$, *hs:wnt8*) regenerated their livers back to baseline within 7 days. This implies that while wnt activity is required for optimal organ regeneration (Sekine et al., 2007; Sodhi et al., 2005; Tan et al., 2006) and further can accelerate the regenerative process by enhancing hepatocyte proliferation, other factors that remain to be defined must have a role in regulating terminal organ size in the regenerative setting. Our study suggests that transient manipulation of the wnt pathway may be an attractive way to enhance liver regeneration in patients after liver resection or during recovery from acute liver failure induced by toxins such as acetaminophen. Following severe injury, increased wnt/β-catenin signaling may enhance hepatic stem cell proliferation and increase the potential of the liver to regenerate.

Author contributions

WG, TEN, and AML performed all embryonic zebrafish assays. WG, TEN, CC, and CB completed blastula transplants. WG and TEN conducted the zebrafish liver resections. SL, RS, and MP performed murine liver resections. GW, APH, HC, and RTM provided fish lines. WG, TEN, and LIZ wrote the manuscript. All authors provided input on and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.05.526.

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