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Iterative use of nuclear receptor Nr5a2 regulates multiple stages of liver and pancreas development

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Abstract

The stepwise progression of common endoderm progenitors into differentiated liver and pancreas organs is regulated by a dynamic array of signals that are not well understood. The nuclear receptor subfamily 5, group A, member 2 gene *nr5a2,* also known as Liver receptor homolog-1 (Lrh-1) is expressed in several tissues including the developing liver and pancreas. Here, we interrogate the role of Nr5a2 at multiple developmental stages using genetic and chemical approaches and uncover novel pleiotropic requirements during zebrafish liver and pancreas

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Author Contributions

S.N., O.W., and W.G. conceived and designed the experiments. S.N., O.W., J.H., and W.G. analyzed the resulting data. J.C.T. generated the *nr5a2* TALEN mutant. O.W. and J.H. propagated mutant lines and validated the endoderm phenotypes in the *nr5a2*^{oz3} background. O.W., S.N., and J.H. performed embryonic drug experiments. S.N., O.W., and J.W. performed morpholino experiments. S.S.B., M.C., O.W., S.N., and J.W. conducted FACS sorting and qRT-PCR. K.A. performed *in situ* hybridizations to assess Wnt signaling in *nr5a2*^{oz3} mutants. S.N. and J.W. performed morpholino injections and morpholino analysis. S.N., O.W., and W.G. wrote the manuscript. All authors reviewed and edited the manuscript.

development. Zygotic loss of *nr5a2* in a targeted genetic null mutant disrupted the development of the exocrine pancreas and liver, while leaving the endocrine pancreas intact. Loss of *nr5a2* abrogated exocrine pancreas markers such as *trypsin*, while pancreas progenitors marked by *ptf1a* or *pdx1* remained unaffected, suggesting a role for Nr5a2 in regulating pancreatic acinar cell differentiation. In the developing liver, Nr5a2 regulates hepatic progenitor outgrowth and differentiation, as *nr5a2* mutants exhibited reduced hepatoblast markers *hnf4α* and *prox1* as well as differentiated hepatocyte marker *fabp10a*. Through the first *in vivo* use of Nr5a2 chemical antagonist Cpd3, the iterative requirement for Nr5a2 for exocrine pancreas and liver differentiation was temporally elucidated: chemical inhibition of Nr5a2 function during hepatopancreas progenitor specification was sufficient to disrupt exocrine pancreas formation and enhance the size of the embryonic liver, suggesting that Nr5a2 regulates hepatic versus pancreatic progenitor fate choice. Chemical inhibition of Nr5a2 at a later time during pancreas and liver differentiation was sufficient to block the formation of mature acinar cells and hepatocytes. These findings define critical iterative and pleiotropic roles for Nr5a2 at distinct stages of pancreas and liver organogenesis, and provide novel perspectives for interpreting the role of Nr5a2 in disease.

Keywords

nuclear receptor; endoderm development; hepatopancreas progenitors; exocrine pancreas; liver

1. Introduction

The liver and pancreas develop from common progenitors in the embryonic gut endoderm through a complex series of developmental events, including the specification of progenitor cells, epithelial budding and outgrowth, and differentiation of precursors into mature cells with specialized function. How these stepwise events are regulated is not well understood. An emerging theme is that these two endodermally-derived organs share many regulatory signals during their development, including fibroblast growth factor 10, bone morphogenetic proteins (Bmps), Wnt, and prostaglandin E_2 (PGE_2) (Chung et al., 2008; Dong et al., 2007; Goessling et al., 2008; Nissim et al., 2014; Wandzioch and Zaret, 2009). Moreover, the same signals are often used iteratively to regulate different events in the progression from shared gut endoderm into diverse cell types (Goessling et al., 2008; McLin et al., 2007; Ober et al., 2006). In this work, we define multiple roles of the nuclear receptor Nr5a2 in specific stages of pancreas and liver organogenesis.

Pancreas and liver progenitors develop as distinct sub-populations of the endoderm gut tube, distinguishable by gene expression patterns prior to organ bud formation. In zebrafish, pancreas progenitors are marked as early as 14 hpf by *pancreatic and duodenal homeobox 1 (pdx1)* and liver progenitors are marked later by *prospero homeobox 1* (*prox1*) and *hematopoietically expressed homeobox (hhex*) at 24 hpf (Biemar et al., 2001; Field et al., 2003c; North and Goessling, 2011; Ober et al., 2003). A subset of pancreas progenitors acquires an endocrine fate, manifested by expression of *insulin* or other endocrine markers. Progenitors of the pancreas or liver subsequently emerge as epithelial buds from the endoderm tube. Cells of the endocrine pancreas lineage emerge at 24 hpf as the posterodorsal pancreatic bud containing a “principal islet” (Field et al., 2003a). Anterior to

this bud, the remaining pancreas progenitors in the gut activate expression of the *pancreas specific transcription factor 1a* (*ptf1a*) by 32 hpf and subsequently emerge as the anteroventral pancreatic bud visible by 40 hpf, engulfing the principal islet by 48 hpf (Field et al., 2003a; Lin et al., 2004; Zecchin et al., 2004). Likewise, liver progenitors begin budding by 28 hpf, activate expression of the *hepatocyte nuclear transcription factor 4 alpha* (*hnf4a*) by 30–34 hpf, and by 46 hpf they completely detach from the gut tube (Field et al., 2003c). Lastly, pancreas and liver progenitor cells differentiate into mature cell types with specialized function. The *ptf1a*-expressing pancreas progenitors mainly give rise to acinar cells, the functional cells of the exocrine pancreas that produce and secrete digestive enzymes, and also to pancreatic ductal epithelial cells, which contribute to the epithelial lining of the tubes that transport digestive enzymes from the acinar cells into the duodenum (Grapin-Botton, 2005). Acinar cells are marked by expression of *trypsin* at 48 hpf while pancreatic ductal epithelial cells are marked by expression of cytokeratins at 48 hpf (Yee et al., 2005). Likewise, markers of differentiated hepatocytes, including *liver fatty acid binding protein 10a* (*fabp10a*) are visible by approximately 48hpf (Her et al., 2003). After 48 hpf, the nascent pancreas and liver organs expand laterally in opposite directions from the midline (Field et al., 2003c).

In addition to these similar developmental milestones in organogenesis, the developing pancreas and liver are also often regulated by the same signaling pathways. Early in development, these signaling pathways act on a common pool of endoderm progenitors to specify them to liver or pancreas fate, and hence consequences on the pancreas and liver may be opposing. For example, between 12–20 hpf, Wnt or Prostaglandin E2 (PGE_2) activity has opposite effects, resulting in a smaller exocrine pancreas but larger liver at 72 hpf (Goessling et al., 2008; Nissim et al., 2014). Later in development after pancreas and liver progenitors have been specified, Wnt or PGE_2 activity stimulates both the exocrine pancreas and liver enlargement (Ober et al., 2006; Goessling et al., 2008; Murtaugh, 2008; Nissim et al., 2014). These effects can be due to signals affecting fate decisions of hepatopancreatic progenitors or differential impact of these signaling pathways on the pancreas and liver progenitors depending on the developmental stage during which they act. These examples highlight that a single signaling pathway can be repeatedly used over time to direct distinct developmental events.

A number of extracellular signals such as Bmps, Wnts, fibroblast growth factors (Fgfs), and PGE_2 have been demonstrated to have pleiotropic roles in pancreas and liver development (Deutsch et al., 2001; Goessling et al., 2008; McLin et al., 2007; Nissim et al., 2014; Ober et al., 2006; Rossi et al., 2001; Wandzioch and Zaret, 2009; Zaret and Grompe, 2008). In contrast, assigning multiple sequential, developmentally distinct roles to transcription factors has been more challenging due to limitations in temporal control over transcription factor activity, partially from a lack of specific inhibitors. One transcription factor, the orphan nuclear receptor NR5A2 is a candidate transcription factor that may have diverse roles in both pancreas and liver organogenesis (Fayard et al., 2004; Pare et al., 2001).

Nr5a2 is primarily expressed in the developing and mature gastrointestinal endoderm, including liver hepatocytes and exocrine pancreas cells (Bookout et al., 2006; Pare et al., 2004; Rausa et al., 1999). The *Nr5a2* promoter contains binding sites for a number of genes

that regulate early endoderm development, including the GATA factors (Fayard et al., 2004; Pare et al., 2001). Further evidence suggests that *Nr5a2* expression may be regulated by transcription factors expressed in pancreas progenitors, including *Pancreatic-duodenal Homeobox 1* (PDX1). In endoderm progenitor cells, the NR5A2 protein has been shown to promote the expression of genes involved in hepatopancreas maturation, including the hepatocyte nuclear factors (HNFs) (Pare et al., 2001). In the mature liver and pancreas, NR5A2 also regulates transcriptional networks responsible for cholesterol and bile acid homeostasis and the production of digestive enzymes (Chong et al., 2012; Fayard et al., 2004; Hale et al., 2014; Holmstrom et al., 2011). Given established pathway connections, NR5A2 is thought to function as a signal linking early endoderm development and endoderm differentiation (Fayard et al., 2004; Pare et al., 2001).

Investigations into the role of NR5A2 in endoderm organogenesis have been complicated because global loss of NR5A2 is lethal in early mouse embryogenesis at E6.5–7.5 due to gastrulation defects, before development of the liver or pancreas (Gu et al., 2005; Labelle-Dumais et al., 2006; Pare et al., 2004). However, lineage-specific knockout of *Nr5a2* has been accomplished in mouse models: selective loss of NR5A2 in already specified pancreas progenitor cells by a *Pdx1*^{early}*-Cre* results in severe reduction in exocrine and endocrine pancreas cell number, supporting a post-specification role for NR5A2 in pancreas cell fate (Fayard et al., 2004; Hale et al., 2014). Additionally, loss of *Nr5a2* in differentiated mouse hepatocytes, as driven by an *Albumin-Cre*, results in no apparent effects (Lee et al., 2008). Due to the relatively late expression of *Albumin-Cre* in differentiated hepatocytes, the requirements of NR5A2 for specification and outgrowth of hepatic progenitors and hepatic differentiation have not been elucidated. These data indicate the need for a detailed investigation of the role of NR5A2 at all stages of hepatopancreatic development.

While NR5A2 has specific functions in development and metabolism of liver and pancreas, it may also have more general functions in self-renewal and cell cycle progression of stem and progenitor cells. NR5A2 acts as a regulator of embryonic stem cell (ESC) pluripotency by maintaining the expression of ESC identity genes, including *Oct4* and *Nanog*, and is further capable of replacing OCT4 in the reprogramming of adult differentiated fibroblasts into induced pluripotent stem cells (Gu et al., 2005; Heng et al., 2010b). In mouse ESCs and intestinal crypt stem cells, NR5A2 also regulates genes involved in self-renewal and growth, including *c-Myc, n-Myc, Cyclin D1,* and *Cyclin E1*, often by functioning as a coactivator of β-catenin/Tcf4 (Botrugno et al., 2004; Heng et al., 2010a; Wagner et al., 2010). Consistent with a general role in cell cycle progression, NR5A2 also regulates the proliferation of breast cancer and pancreatic cancer cell lines (Annicotte et al., 2005; Benod et al., 2011). Reduced NR5A2 activity in *Estrogen Receptor-α+ (ERα+)* breast cancer cell lines can abrogate estradiol-induced proliferation (Annicotte et al., 2005). Furthermore, blocking NR5A2 activity in pancreatic cancer cell lines can inhibit proliferation *in vitro* through the down-regulation of *c-Myc, Cyclin D1,* and *Cyclin E1* (Benod et al., 2011). The relevance of NR5A2 activity to cancer has been strengthened by the repeated association of *NR5A2* with pancreatic ductal adenocarcinoma in genome wide association studies (GWAS) (Benod et al., 2011; Petersen et al., 2010; Ueno et al., 2015).

We have previously harnessed the zebrafish model as a tool for interrogating the iterative roles of signaling pathways at specific events in pancreas or liver development (Goessling et al., 2008; Nissim et al., 2014). To study the role of Nr5a2 during distinct developmental time windows in zebrafish, an important recent advance has been the identification of a pharmacologic antagonist (Benod et al., 2013). This molecule, named “Compound 3” (Cpd3), directly binds to the NR5A2 ligand binding domain and inhibits transcriptional activation of downstream target genes and proliferation of human cancer cell lines (Benod et al., 2013). Prior to the current study, the impact of Cpd3-mediated NR5A2 inhibition has not been investigated *in vivo*.

In this study, we use genetic and pharmacologic approaches to show that Nr5a2 is repeatedly used to regulate both hepatopancreatic specification and differentiation. In zebrafish, *nr5a2* is expressed in the anteroventral pancreas and liver buds during outgrowth from the gut endoderm tube. Zebrafish *nr5a2* null mutants exhibit abrogated exocrine pancreas and liver development, while endocrine pancreas development is largely unaffected. In the pancreas, markers of differentiated acinar cells are lost, while exocrine pancreas progenitor markers *ptf1a* and *pdx1* appear unchanged, consistent with a role for *nr5a2* in regulating progenitor cell differentiation. Additionally, *prox1* expression is reduced in a subset of pancreatic progenitors whose contribution to pancreas development is largely undefined. A loss of the *prox1* expressing progenitors in the pancreatic region of the $nr5a2^{oz3}$ mutants may have an impact on pancreas development. In the liver, *nr5a2* regulates both progenitor outgrowth and differentiation as mutants demonstrate a reduction in hepatoblast and differentiated hepatocyte markers.

Importantly, our study is the first *in vivo* study of Nr5a2 pharmacologic antagonism with Cpd3, defining distinct temporal requirements for Nr5a2 activity in both the pancreas and liver. First, we demonstrate that Nr5a2 regulates the commitment of bipotent hepatopancreas progenitor cells to distinct hepatic or pancreatic lineages. The loss of Nr5a2 activity during the specification of hepatopancreas progenitor cells is sufficient to disrupt the formation of the mature exocrine pancreas and expand the mature liver, potentially through the preferential commitment of progenitors to the hepatic lineage at the expense of the pancreatic lineage. Second, we demonstrate that Nr5a2 is necessary for the expansion and differentiation of the liver and pancreas. Inhibition of Nr5a2 activity with chemical antagonist during differentiation reduces the size of both organs. Taken together, our results reveal that Nr5a2 is a transcription factor with multiple sequential and developmentally distinct roles in hepatopancreatic development.

2. Materials and Methods

2.1 Zebrafish husbandry

Zebrafish were maintained according to Institutional Animal Care and Use Committee protocols (HMS 04626) and in compliance with NIH guidelines. Transgenic lines *Tg(fabp10a:GFP)*, *Tg(elastase:GFP)*, *Tg(fabp10a:DsRed)*, *Tg(prox1a:Citrine)*, *Tg(sox17:eGFP)*, and *Tg(trypsin:GFP)* were previously described (Bagnat et al., 2007; Bussmann and Schulte-Merker, 2011; Her et al., 2003; Nissim et al., 2014; Wan et al., 2006).

2.2 Generation of nr5a2$^{+/oz3}$ mutants

Custom TALEN constructs were designed to target the first constitutively present exon of *nr5a2* and constructed by the University of Utah mutagenesis core facility (Cermak et al., 2011). Linearized plasmids were transcribed with mMessage mMachine Sp6 transcription kit for injection of 150 pg each. Mutagenesis was confirmed by reduced *BsrI* cleavage within the spacer between TALEN binding sites. Founder mutants were identified using high resolution melt analysis by progeny with melt curves that deflect from WT, using primers (F: 5′ ACTCTTATGTTTTCAGCCCCACAGTTT 3′) and (R: 5′ TCACAGGTCAGCAACCCATAGTGAT 3′) (Dahlem et al., 2012). PCR fragments from deflecting progeny were subsequently cloned into pCRII-TOPO vectors; inserts were Sanger sequenced for confirmation. Heterozygous progeny were raised to adulthood and genotyped prior to subsequent matings. *nr5a2*$^{+/oz3}$ adult heterozygotes were identified by PCR amplification using primers (F: 5′ ACGAACCTCATAACACATGACAGCCA 3′), (R: 5′ AGCTCTCACAGGTCAGCAACCCATA 3′) and subsequent *BsrI* restriction enzyme digestion. Mutants were identified by the destruction of a *BsrI* cut site. The *nr5a2*^{oz3} carriers were outcrossed for at least two generations prior to analysis.

2.3 Embryonic zebrafish expression studies

In situ hybridization was conducted on paraformaldehyde-fixed embryos using standard protocols (http://zfin.org/ZFIN/SD/ThisseProtocol.html) and established probes (Nissim et al., 2014). *nr5a2* probe was generated from zebrafish cDNA using the following primers: 5′ TGTAAGGGCTTCTTCAAGCGC 3′ (forward) and 5′ GGAGAACAGTGTCTGGTCAGCC 3′ (reverse). *hnf4α* probe was generated from zebrafish cDNA using the following primers: 5′ CGCAGTGTACGCAAAAACCA 3′ (forward) and 5′ GGTGAGCGTGAGGTGCTTCATT 3′ (reverse). *wnt2* probe was generated from zebrafish cDNA using the following primers: 5′ ATGAACTTTTTGCCAAATGGAA 3′ (forward) and 5′ TCAGGACTGGGTTTTGCAGG 3′ (reverse). Changes in *trypsin, fabp10a, pdx1, ptf1a, prox1,* and *hnf4α* expression were scored in images using ImageJ to quantify pancreas, liver, endoderm, or progenitor population size. Volumetric analysis was performed using 3D reconstruction of confocal stacks in ImageJ.

2.4 Morpholino Injections

Morpholino (GeneTools) knockdown was performed as previously described (North et al., 2007), utilizing 2 nanoliters of 100 μM *nr5a2* morpholino (5′ TCACTCTCAAAACTACTGGACATTT 3′).

2.5 Chemical Treatments

Zebrafish embryos were exposed to 100 μM (or 200 μM when specified) pharmacologic Nr5a2 antagonist, 1-(3′-(1-(2-(4-Morpholinyl)ethyl)-1H-pyrazol-3-yl)-3-biphenylyl)ethanone (Cpd3, ChemBridge) (Benod et al., 2013) at the specified time points. Embryo water containing 100 μM Cpd3 was replaced at 24-hour intervals. DMSO carrier content for 100 μM treatments was 0.04%, and 0.08% for 200 μM treatments.

2.6 Fluorescence activated cell sorting (FACS)

For hepatocyte cell isolations, *Tg(fabp10a:GFP)* embryos (72 hpf) were incubated in 50μg/mL Liberase™ (Roche) at 37ºC for 1.5 hours, manually dissociated with a p1000 pipette, strained through a 30μm mesh filter, and suspended in 1% fetal bovine serum. SYTOX Red Dead Cell Stain (ThermoFisher) was added to the cell suspension to detect nonviable cells. Cell suspensions were separated into GFP+ and GFP− fractions using a BD FACSAria II SORP flow cytometer (Goessling et al., 2008). For *elastase:GFP* cell counting, whole fluorescent embryos were manually dissociated in 0.25% trypsin for 20 minutes and analyzed for GFP fluorescence by flow cytometry (> 100,000 cells were analyzed per sample; n = 10–20 per treatment).

2.7 Total RNA isolation and production of antisense RNA

Cells isolated by FACS (approximately 200,000) were processed using the RNAqueous-Micro Total RNA Isolation Kit (ThermoFisher) according to the manufacturer's protocol. DNase I (Ambion) processing was performed on total cellular RNA according to manufacturer's protocol. Amplified cDNA was prepared using the Ovation Pico WTA System V2 (NuGEN) in the Molecular Genetics Core Facility at Beth Israel Deaconess Medical Center. RNA was isolated from whole embryos using TRIzol® Reagent and treated with the TURBO DNA-free DNAse kit (Life Technologies); cDNA was generated using iScript cDNA synthesis reagents (Bio-Rad).

2.6 qRT-PCR

qRT-PCR was performed on amplified cDNA libraries using the SYBR Green Supermix (Bio-Rad) and relative expression levels were calculated using the ΔΔCt method. Expression was normalized to *ef1α*. See Supplementary Table 1 for primer sequences.

2.8 TUNEL staining

TUNEL staining was performed on paraformaldehyde fixed embryos using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon International). Apoptosis signal was developed using the ImmPACT DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories).

2.9 Wholemount *immunohistochemistry*

Embryos were fixed overnight in 4% paraformaldehyde at 4ºC and permeabilized in acetone at −20ºC. Embryos were incubated with Anti-phospho-histone H3 (Ser10) Antibody (EMD Millipore) overnight and again with secondary antibody Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam).

3. Results

3.1 nr5a2 is expressed in the developing endoderm of zebrafish embryos

Previous work has shown that *nr5a2* is expressed in the developing digestive organs (Lin et al., 2000), and we demonstrate here that transcripts also appear to be maternally provided and are present in embryonic blastomeres and their derivatives until the end of gastrulation (Figure S1A, Figure 1A). Later in the endoderm, *nr5a2* transcripts are first detected in the

liver bud and anteroventral pancreas bud by 30 hpf and 36 hpf, respectively (Figure 1A) (Bertrand et al., 2007; Lin et al., 2000). Expression of *nr5a2* was detected in the liver bud during budding stages II and III when the liver bud emerges and separates from the gut tube, and persisted through hepatocyte differentiation, which begins at approximately 44 hpf. *nr5a2* expression also appeared during the outgrowth of the developing pancreas; however, *nr5a2* expression remained restricted to a portion of differentiated pancreas acinar cells (60–72 hpf) until approximately 84 hpf, when it was expressed in most exocrine pancreas cells (Figure 1A). Fluorescence activated cell sorting (FACS)-isolated GFP+ pancreatic acinar cells and hepatocytes from the *Tg(trypsin:GFP)* and *Tg(fabp10a:GFP)* reporter lines, respectively, confirms that *nr5a2* is expressed in isolated acinar cells and hepatocytes, and that *nr5a2* expression is specifically enriched in GFP+ hepatocytes relative to GFP- cells isolated from *Tg(fabp10a:GFP)* embryos (Figure 1B–C). These results demonstrate that *nr5a2* is expressed in gut endoderm during hepatopancreatic development, and that *nr5a2* is present in the endoderm during critical stages of hepatopancreatic progenitor specification and differentiation.

3.2 nr5a2 is required for exocrine pancreas development

The requirement of *nr5a2* in zebrafish pancreas development was initially assessed by morpholino-mediated knockdown. *nr5a2* morphants exhibited impaired exocrine pancreas formation, as assessed by *in situ* hybridization for *trypsin* at 96 hpf (Figure 1D). Liver formation was also inhibited by morpholino-mediated knockdown, as assessed by fluorescence microscopy of the *Tg(fabp10a:DsRed)* reporter line (Figure S1B). Histological analysis of controls and *nr5a2* morphant embryos confirmed the absence of exocrine pancreas structure (Figure 1E). Furthermore, section *in situ* hybridization for *trypsin* in control and *nr5a2* MO injected embryos revealed that *nr5a2* morphants lack mature acinar cells (Figure 1E). To further confirm that *nr5a2* is required for the formation of exocrine pancreas cells, *nr5a2* MO was injected into *elastase:GFP* reporter embryos followed by FACS analysis to quantify the number of GFP-labeled exocrine pancreas cells present in pooled embryo groups. *elastase* is expressed in the exocrine pancreas following *trypsin* expression, and is a marker of mature acinar cells (Mudumana et al., 2004; Wan et al., 2006). *nr5a2* morphants contained a significantly reduced quantity of elastase GFP+ cells (Figure 1F). These findings suggest a conserved impact of *nr5a2* function in exocrine pancreas formation.

To genetically test whether *nr5a2* is required for the formation and maturation of the endoderm, we generated zebrafish mutants using TALEN-based genome editing (Cermak et al., 2011; Sander et al., 2011) resulting in an early frameshift due to a 2-basepair insertion within exon 3 (Figure 2A). The mutation disrupts the protein sequence prior to major functional domains (DNA-binding domain and ligand binding domains), thereby generating a likely null allele (Figure 2A) (Lin et al., 2000). Heterozygous *nr5a2*$^{+/oz3}$ fish were viable and fertile through adulthood. *nr5a2*$^{oz3/oz3}$ homozygous mutants were embryonic lethal at 8–9 days post fertilization (dpf). Homozygous *nr5a2*$^{oz3/oz3}$ and heterozygous *nr5a2*$^{+/oz3}$ embryos are grossly morphologically indistinguishable from wildtypes during early embryogenesis. The earliest visible phenotype appears upon examination of endoderm development by *in situ* hybridization or fluorescent reporter lines, as described below.

Expression of markers for the mature exocrine (*trypsin*) and endocrine (*insulin*) pancreas in wildtype, *nr5a2*$^{+/oz3}$ and *nr5a2*$^{oz3/oz3}$ embryos revealed that genetic loss of *nr5a2* completely disrupted the development of the mature exocrine pancreas at 72 and 84 hpf, while leaving the endocrine pancreas largely intact (Figure 2B–E). Quantification of exocrine pancreas size by area of *trypsin* expression (84 and 120 hpf) demonstrated that *nr5a2*$^{oz3/oz3}$ embryos have a significantly smaller exocrine pancreas than *nr5a2*$^{+/+}$ siblings (Figure 2C–D). Additionally, heterozygous nr5a2$^{+/oz3}$ embryos have a variably penetrant, but statistically significant, reduced exocrine pancreas phenotype, which ranges from completely normal to fully absent (Figure 2C,D). The heterozygous exocrine pancreas phenotypes suggest that *nr5a2* is haploinsufficient for reliable patterning.

The loss of mature exocrine pancreas markers was not specific to the *trypsin* gene, as mutants also exhibited *carboxypeptidase A (carbA)* expression deficits in the exocrine pancreas with similarly variable penetrance in *nr5a2*$^{+/oz3}$ embryos (Figure S1C). *In situ* hybridization for *trypsin* expression in the newly differentiated exocrine pancreas confirms that *trypsin+* cells fail to form in the *nr5a2*$^{oz3/oz3}$ embryos all together (Figure S1D). Cell death may be an unlikely explanation for the nr5a2 mutant phenotype, because TUNEL labeling appears normal through development in these mutants and morphants, and proliferation is also largely unchanged in the early embryo (Figure S2A–D). Further, the endocrine pancreatic lineages may remain unaffected in *nr5a2*$^{oz3/oz3}$ mutants, and consistent with this, *nr5a2* is not co-expressed in endocrine β-cells (Figure S3A). These results demonstrate the requirement of *nr5a2* for normal exocrine pancreas development and reveal that optimal exocrine pancreas development is sensitive to *nr5a2* gene dose.

3.3 Nr5a2 is required for liver development

Given our previous findings that the same developmental pathways co-regulate pancreas and liver formation (Goessling et al., 2008; Nissim et al., 2014), we examined whether liver development is affected in *nr5a2* mutants. *nr5a2*$^{oz3/oz3}$ homozygous mutants displayed severely impaired expression of the mature hepatocyte marker *fabp10a*. Liver size, calculated by *fabp10a* expression area, at 72 and 96 hpf was significantly reduced, with a subset of *nr5a2*$^{+/oz3}$ heterozygous embryos also exhibiting reduced liver size (Figure 2F–G). Given that *nr5a2* regulates aspects of cholesterol and bile acid homeostasis (Paré et al., 2004), and may therefore impact the expression of genes involved in lipid transport, we confirmed that a reduction in liver size was not specific to the *fabp10a* hepatocyte marker. Expression of an additional hepatocyte marker, *transferrin-a,* was also reduced in the *nr5a2*$^{oz3/oz3}$ embryos, as assessed by *in situ* hybridization (Figure S3B). Furthermore, *in situ* hybridization for *fabp10a* at earlier stages of liver differentiation revealed that *fabp10a+* hepatocytes fail to form in *nr5a2*$^{oz3/oz3}$ embryos (Figure S3C). These results demonstrate that *nr5a2* is required for the development of differentiated hepatocytes.

3.4 Expression of prox1, but not ptf1a and pdx1, is reduced in pancreas progenitors by nr5a2$^{oz3/oz3}$

The observed reduction in exocrine pancreas size may result from defects in pancreas progenitor cell formation or from defects in differentiation. We therefore sought to determine whether standard exocrine pancreas progenitors marked by *pdx1* and *ptf1a* (Tiso

et al., 2009) were impacted in *nr5a2*$^{oz3/oz3}$ mutants. *pdx1* expression appeared normal in pancreas progenitors during early morphogenesis (26 hpf), and this progenitor expression also appeared relatively normal by 48 hpf (Figure 3A–D); similarly, *ptf1a* expressing progenitors were also largely unaffected at 48 hpf (Figure 3E).

We also examined the expression of *prox1*, a less commonly used hepatopancreas progenitor marker, thought to regulate the differentiation of pancreas progenitors and the expansion of the exocrine pancreas (Wang et al., 2005; Westmoreland et al., 2012). Expression of *prox1* is reduced in the pancreas bud of *nr5a2*$^{oz3/oz3}$ and *nr5a2*$^{+/oz3}$ embryos at 48 hpf (Figure 4A). However, at 30 hpf, *prox1* expression in the pancreas bud of *nr5a2*$^{oz3/oz3}$ embryos appears normal, suggesting that Nr5a2 regulates *prox1* expression only during specific stages of bud formation or progenitor differentiation (Figure S3D). Furthermore, the expression of *prox1* in the pancreas does not recover in the mutants by the 96 hpf time point (Figure S4A–B). These results indicate that while *nr5a2* is not required for the expression of conventional progenitor markers *pdx1* and *ptf1a*, *nr5a2* is required for the maintenance, but not the initiation, of *prox1* expression in the pancreas bud.

3.5 Nr5a2 is required for emergence of an hnf4α and prox1 expressing liver bud

Given the observed impact of loss of *nr5a2* on differentiated hepatocytes, we examined hepatoblast marker expression in *nr5a2* mutants to assess the role of *nr5a2* in hepatic progenitor formation. *nr5a2* is known to transcriptionally activate the hepatoblast marker *hnf4α in vitro,* suggesting that *hnf4α* expression would be reduced in *nr5a2*$^{oz3/oz3}$ homozygotes (Kyrmizi et al., 2006; Pare et al., 2001). *nr5a2*$^{oz3/oz3}$ embryos had a reduced expression size of hepatic progenitor markers *hnf4α* and *prox1* in the liver bud at 48 hpf (Figure 4A–D). We next examined whether other hepatoblast markers were affected in the *nr5a2*^{oz3} mutants, including *hhex.* We observed that the size of *hhex* expression in the liver bud was also reduced, though less substantially, in the *nr5a2*$^{oz3/oz3}$ mutants (Figure 4E). These results demonstrate that *nr5a2* is required for the formation of a liver bud that contains an appropriately sized population of *hnf4α* and *prox1* expressing hepatocyte progenitors, and further indicate that *nr5a2* may be required for the formation of the subsets of hepatic progenitors labeled by these markers.

Reduced expression of *prox1* and *hnf4α*, as well as subtle differences in budding (as revealed by pdx1 expression) in *nr5a2*$^{oz3/oz3}$ embryos by 48 hpf, suggest that defects in liver and pancreas budding may underlie the mature liver and pancreas phenotypes observed in *nr5a2*$^{oz3/oz3}$ mutants. To evaluate this possibility, we examined the expression of *foxa3*, a global marker for the developing gut tube, liver bud, and pancreas buds (Field et al., 2003c). Homozygous *nr5a2*$^{oz3/oz3}$ mutants exhibited altered gut looping and disrupted liver and pancreas budding at 32 hpf (Figure 4F). At 48 hpf, the outgrowth of the liver bud and the fusion of the pancreas buds were delayed in both *nr5a2*$^{oz3/oz3}$ homozygotes and *nr5a2*$^{+/oz3}$ heterozygous embryos (Figure 4G). These findings point to a role for *nr5a2* in gut morphogenesis, including gut looping and the outgrowth of the liver and pancreas buds.

3.6 Nr5a2 chemical antagonist disrupts exocrine pancreas development in vivo

Cpd3 is a known chemical antagonist of human NR5A2 that directly binds to the NR5A2 ligand binding domain and renders NR5A2 transcriptionally inactive *in vitro* (Benod et al., 2013). We exposed zebrafish embryos to Cpd3 to determine whether Cpd3 has the ability to disrupt Nr5a2-mediated developmental events and produce phenotypes consistent with the *nr5a2*$^{+/oz3}$ or *nr5a2*$^{oz3/oz3}$ mutants. Treatment of developing embryos after gastrulation (starting at 12 hpf) with 100 μM Cpd3 significantly disrupted the formation of the mature exocrine pancreas, as assessed by *trypsin* expression area at 72 hpf (Figure 5A–B). Cpd3 reduced the average pancreas size, but did not ablate exocrine pancreas formation as severely as observed in the mutants, suggesting partial loss of Nr5a2 at doses utilized, consistent with the effects observed for *nr5a2*^{oz3} haploinsufficiency. Further corroborating the *nr5a2*$^{oz3/oz3}$ mutants, the Cpd3-treated embryos formed *insulin*-expressing endocrine pancreas cells normally (Figure 5C). The *islet1* endocrine pancreas lineages, including β-cells (*insulin)* and δ-cells *(somatostatin),* were not visibly affected by chemical Nr5a2 inhibition (Figure 5C, Figure S5A–B). Similar to the *nr5a2*$^{oz3/oz3}$ mutant, *pdx1*-expressing pancreas progenitors were also unaffected by Cpd3 treatment (Figure 5D–E). Lastly, we assessed whether heterozygote *nr5a2*$^{+/oz3}$ fish were sensitized to the Nr5a2 chemical antagonist Cpd3. Heterozygotes were more sensitive to the chemical antagonist and showed a statistically significant reduction in pancreas size in response to Cpd3 treatment relative to *nr5a2*$^{+/oz3}$ controls and Cpd3 treated *nr5a2*$^{+/+}$ embryos (Figure 5F, Figure S5C). Collectively, these data demonstrate that chemical inhibition of Nr5a2 over the duration of pancreas development results in similar phenotypes to those observed in heterozygous *nr5a2* mutants and suggests specificity of the inhibitor *in vivo*.

3.7 Nr5a2 is required at multiple stages of pancreas and liver development

In order to determine when Nr5a2 is temporally required for hepatopancreas development, embryos from the Tüpfel long fin (TL) background were exposed to Cpd3 during time windows that correspond to distinct pancreas and liver developmental events (Figure 5G). For the pancreas studies, we performed Nr5a2 antagonist treatments over the periods of posterodorsal and ventral bud specification (12 – 32 hpf), ventral bud formation and emergence (33 – 50 hpf), exocrine pancreas differentiation and expansion (50 – 96 hpf), and the period of endoderm-specific *nr5a2* expression (28 – 96 hpf) (Figure 5G). Antagonism of Nr5a2 activity in the 12 – 32 hpf and 50 – 96 hpf windows, but not the 33 – 50 hpf window, reduced the size of the exocrine pancreas (Figure 6A, Figure S6A). Despite presenting with a reduced exocrine pancreas size, the embryos in the 12 – 32 hpf treatment group initiated exocrine differentiation along a standard timeline (Figure 6B–C). At 44 hpf, approximately 50% of fish in the control and treatment groups had *trypsin*-expressing exocrine pancreas cells (Figure 6B). By 56 hpf, the size of the *trypsin* bud was also consistent across the control, 12 – 32 hpf, and 33 – 50 hpf treatment groups (Figure 6C). Furthermore, 12 – 32 hpf treatment did not alter the size of the pancreas progenitor pool marked by *pdx1* at 36 hpf (Figure S6).

Quantification of the exocrine pancreas size across all treatment groups at 72 and 96 hpf confirmed that a reduction in Nr5a2 activity during 12 – 32 hpf and 50 – 96 hpf was sufficient to reduce the size of the *trypsin*-expressing exocrine pancreas (Figure 6D–E).

Importantly, loss of Nr5a2 during ventral bud formation and emergence (33 – 50 hpf) did not impact acinar cell expansion or differentiation (Figure 6A,D–E). We hypothesize that the loss of Nr5a2 activity between 12 – 32 hpf reduces the competence of the total exocrine pancreas progenitor pool, thereby impacting exocrine pancreas expansion and differentiation beyond the 56 hpf time point. Furthermore, reduced exocrine pancreas size in the 50 – 96 hpf treatment group reveals a separate and distinct role for Nr5a2 during exocrine pancreas differentiation or expansion.

Next, we performed Cpd3 exposures to examine critical windows of Nr5a2 function for liver formation. Timed drug experiments were employed to reduce Nr5a2 activity during important stages of liver development, including liver progenitor and bud specification (12 – 32 hpf) as well as liver differentiation and outgrowth (50 – 72 hpf). Unexpectedly, area measurements performed on *fabp10a* expression demonstrated that loss of Nr5a2 during liver specification (12 – 32 hpf) produced a larger *fabp10a*-expressing liver when measured at 72 hpf (Figure 7B). Given that loss of Nr5a2 activity from 12 – 32 hpf reduces the exocrine pancreas size and expands the size of the liver, Nr5a2 may be implicated in hepatic-versus-pancreatic commitment or the specification of bipotent hepatopancreas progenitor populations. To evaluate this possibility, we examined the expression of hepatoblast markers at 36 hpf following 12 – 32 hpf Cpd3 treatment using *in situ* hybridization. Similar to the pancreas progenitor findings following 12 – 32 treatment, there were no notable differences in the size of the *prox1* or *hnf4a* hepatoblast populations (Figure S6B). Furthermore, the size of the liver bud by confocal analysis of the *Tg(sox17:eGFP)* line in control and 12 – 32 hpf treated embryos was largely unchanged at 36 hpf (Figure S6C). Although no obvious differences in the size of the hepatoblast population or liver bud were observed, progenitors may have an intrinsically enhanced ability to respond to inductive cues, possibly through epigenetic mechanisms. Additionally, Nr5a2 may regulate proliferation in hepatoblast and pancreas progenitor cell populations, thereby contributing to increased liver and reduced pancreas size.

Similar to the *nr5a2*$^{+/oz3}$ heterozygote embryos, the 28 – 72 hpf and 12 – 72 hpf treated embryos had a significantly reduced liver size relative to their control counterparts. These findings suggested a second and later role for Nr5a2 in the differentiation of specified progenitors (Figure 7A–B). As expected, reduced Nr5a2 activity during the subsequent stages of liver differentiation and outgrowth (50 – 72 hpf) significantly reduced the size of the *fabp10a* liver at 72 hpf, demonstrating that Nr5a2 activity during these stages is required for hepatic maturation (Figure 7A–B). We performed Cpd3 treatment (50 – 72 hpf) on *Tg(prox1a:Citrine)* reporter embryos and observed a similarly significant reduction in the size of the *prox1+* liver bud in Cpd3 treated fish (Figure 7C–D). Additionally, confocal volumetric analysis and 3D volumetric reconstruction of livers from the *Tg(fabp10a:GFP)* reporter fish line demonstrate that the Cpd3 treatment reduced the total volume of the embryonic liver at 72 hpf (Figure 7E–F). These experiments further confirm a separate stage-dependent role for Nr5a2 during liver differentiation and outgrowth.

Wnt/β-catenin signaling has a role in multiple stages of hepatic development, including the specification, proliferation, and differentiation of hepatic progenitors (Decaens et al., 2008; Goessling et al., 2008; McLin et al., 2007; Ober et al., 2006; Poulain and Ober, 2011; Tan et

al., 2008). In particular, the mesodermal-derived *wnt2* signal has been shown to regulate hepatoblast proliferation (Poulain and Ober, 2011). We assessed expression of *wnt2* in *nr5a2*^{oz3} mutants and found no changes compared to wildtype embryos (Figure S7A). Furthermore, treatment of embryos with Cpd3 at 50 – 72 hpf did not impact the expression of Wnt/β-catenin target genes assayed by qPCR (Figure S7B). These data suggest that the impact of *nr5a2* on hepatic development occurs through a mechanism independent of Wnt/β-catenin signaling.

4. Discussion

In this study, we characterize the requirement of Nr5a2 activity in zebrafish pancreas and liver development. Zygotic loss of *nr5a2* disrupted the formation of the mature exocrine pancreas and liver. Analysis of hepatopancreas progenitor marker expression in *nr5a2* null mutants reveals that Nr5a2 is required for the expansion and organization of endoderm progenitors as well as expression of hepatoblast markers (Figure 8). Timed inhibition of Nr5a2 using a chemical antagonist also reveals that Nr5a2 is required for the differentiation of both the liver and pancreas, as well as for the specification of the hepatopancreas progenitors (Figure 8). This study is the first to demonstrate that Nr5a2 is employed at multiple stages throughout hepatopancreas development to achieve distinct developmental milestones. We present evidence of several novel roles for Nr5a2 in endoderm development, including in the regulation of liver development and the specification of common endoderm progenitors to the hepatic or pancreatic fate. Furthermore, we accomplish the first *in vivo* inhibition of Nr5a2 with the chemical antagonist Cpd3 and demonstrate that it can be used to modify the specification and differentiation of hepatopancreas progenitors.

4.1 Nr5a2 regulates hepatopancreas morphogenesis and hepatopancreas progenitor specification upstream of pancreas and liver differentiation

It has been demonstrated that NR5A2 is required for exocrine pancreas differentiation in mice; however, roles for NR5A2 in hepatopancreas morphogenesis and progenitor specification have been unexplored due to the embryonic lethality of *Nr5a2*$^{-/-}$ mice (Hale et al., 2014; Labelle-Dumais et al., 2006; Paré et al., 2004). The *nr5a2*$^{oz3/oz3}$ global knockout fish that we have generated survive through stages of hepatopancreas progenitor formation and differentiation, enabling the evaluation of novel roles for Nr5a2 in these stages of endoderm development. The *nr5a2* mutant zebrafish displays defects in several events prior to hepatopancreas differentiation, including disrupted expression of hepatic and pancreas progenitor markers such as *prox1* and *hnf4α,* and delayed liver and pancreas budding.

First, delayed morphogenesis of the endoderm was identified by examination of the pan-endoderm marker *foxa3* in the *nr5a2*$^{oz3/oz3}$ mutants. The pancreas and liver buds failed to emerge from the gut tube and expand on a normal developmental timescale in the homozygous mutants. We propose that altered morphogenesis of the liver and pancreas buds could contribute to the pancreas and liver defects observed in the *nr5a2*$^{oz3/oz3}$ mutant. For example, liver bud outgrowth in *nr5a2*$^{oz3/oz3}$ embryos is likely required for the emergence of the *hnf4α*- and *hhex*-expressing hepatoblasts. Therefore, when loss of Nr5a2 function reduces the size of the *foxa3* expressing liver bud, the total population of hepatoblast cells

may be similarly diminished. Future studies should seek to clarify the impact of a loss of *nr5a2* on the migration and outgrowth of pancreas and liver progenitors that give rise to the mature endodermal organs, with attention to the impact of *nr5a2* on proliferation in these populations.

Although Nr5a2 was not required for initial expression of standard pancreas progenitors markers *pdx1* and *ptf1a, prox1* expression in the pancreas progenitors was largely absent at 48 hpf. Additionally, *prox1* and *hnf4α* had reduced expression in the liver bud. The loss of progenitor marker expression in the *nr5a2*$^{oz3/oz3}$ mutants during liver and pancreas budding may be intricately coupled to the hepatopancreas morphogenesis defects. PROX1 is thought to control the migration of hepatoblast cells in the liver bud, and genetic ablation of *Prox1* in the mouse leads to a failure of liver bud outgrowth (North and Goessling, 2011; Seth et al., 2014; Sosa-Pineda et al., 2000). Furthermore, loss of *Prox1* in mice disrupts pancreas morphology, morphogenesis, and growth through control of pancreatic branching and tip progenitor differentiation (Wang et al., 2005; Westmoreland et al., 2012). We therefore posit that *prox1* deficiency visible at 48 hpf in the hepatopancreas progenitors of *nr5a2*$^{oz3/oz3}$ embryos may be partially responsible for the defects in liver and pancreas outgrowth, as well as the reduced size of the mature pancreas and liver structures. Loss of *hnf4α* expression in the liver bud may similarly underlie failed hepatocyte differentiation.

Importantly, our study identifies a general need to evaluate the role of hepatopancreas progenitor markers in the formation of the mature pancreas and liver. Although markers such as *prox1, ptf1a, pdx1, hhex,* and *hnf4α* identify populations of hepatopancreas progenitor cells, delineation of the functional roles of these genes in endoderm organ development is incomplete. Identifying the functions of *prox1* and *hnf4α* in zebrafish, for example, will provide a greater understanding of the mechanisms by which genetic *nr5a2* inactivation leads to a diminished liver size.

4.2 Nr5a2 controls hepatopancreas progenitor specification and priming

Timed drug studies with the Nr5a2 antagonist Cpd3 discovered a previously unidentified role for Nr5a2 in pancreas versus liver progenitor specification. Inactivation of Nr5a2 during early pancreas progenitor specification was sufficient to disrupt downstream exocrine pancreas formation and promote excess liver formation. There are several possible mechanisms that could explain the reduced exocrine pancreas and expanded liver size following Cpd3 treatment during hepatopancreas progenitor specification. One possibility we propose is that Nr5a2 activity could restrict liver expansion or progenitor differentiation during the 12 – 32 hpf specification window, while simultaneously promoting pancreas commitment and expansion. In a normal developmental context, Nr5a2 is expressed primarily in the liver bud prior to 32 hpf, where it may regulate the speed of liver growth and differentiation. Further studies are needed to explore the possibility that temporally restricted loss of Nr5a2 activity can differentially impact proliferation or differentiation rate in the developing liver and pancreas.

Another possibility is that Nr5a2 may be directly involved in the priming of hepatopancreas progenitors. Inhibition of Nr5a2 activity between the 12–32 hpf window significantly reduced the size of the exocrine pancreas at 72 and 96 hpf, despite unaltered expression of

progenitor markers *pdx1* or *ptf1a* and normal timing of exocrine pancreas differentiation. In our proposed progenitor priming model, the presence of Nr5a2 in pancreas or hepatic progenitor cells would establish epigenetic landscapes or transcriptional programs that enable progenitors to respond to future developmental cues (Figure 8). This model could partially explain why pancreas progenitors form, yet fail to respond to differentiation cues. Recent studies suggest that pancreatic progenitors adopt poised enhancer states during gut tube formation, and that these poised states can pre-program cells to activate pancreas-specific transcriptional programs in response to pancreas induction cues (Wang et al., 2015). Smaller exocrine pancreas size following Cpd3 treatment from 12 – 32 hpf may result because the pancreas progenitors have an altered developmental potential and a reduction in their developmental competence to become mature pancreatic acinar cells. Similarly, we hypothesize that the expanded liver following Cpd3 treatment from 12 – 32 hpf could occur because shared hepatopancreas progenitors develop a heightened competence to differentiate into hepatic lineages. Specifically, we hypothesize that Nr5a2 normally acts as a repressor of hepatic fate in a subset of shared hepatopancreas progenitors during specification stages by restricting the response to differentiation cues, and that a loss of Nr5a2 is sufficient to release this repression. Further studies are needed in order to elucidate the role of Nr5a2 in hepatopancreas progenitor priming and specification events.

4.3 Novel developmental roles for Nr5a2 inform studies on Nr5a2-dependent disease

In addition to its function in embryonic development, NR5A2 also impacts human and murine cancer development. A genome wide association study (GWAS) has implicated NR5A2 as a risk modulator for pancreatic ductal adenocarcinoma (PDAC) (Murtaugh, 2014; Petersen et al., 2010). Interestingly, acinar-to-ductal metaplasia, in which acinar cells lose their mature markers and transdifferentiate into ductal-like pancreas cells, is thought to be a critical step in PDAC formation (Murtaugh, 2014; von Figura et al., 2014). A loss of *Nr5a2* in the mature exocrine pancreas using lineage-specific knockout disrupts acinar cell identity and can accelerate Kras-mediated PDAC precursor lesions (von Figura et al., 2014). Given the role of NR5A2 in regulating hepatopancreas progenitor development and acinar cell differentiation, we speculate that understanding the developmental roles of NR5A2 may elucidate the association of NR5A2 in cellular transformation and oncogenesis. Importantly, as a nuclear receptor that can bind ligands that modify its function, NR5A2 may offer a viable therapeutic target for cancers, including PDAC. Our study demonstrates that Cpd3 treatment blocks exocrine pancreas and hepatocyte differentiation, indicating that the drug can be used to modulate involvement of NR5A2 in cellular identity *in vivo.* Pharmacologic modulation of NR5A2 *in vivo* through antagonism or agonism may therefore have broad application for the treatment of endoderm-derived cancers and other disease processes impacted by NR5A2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Annicotte JS, Chavey C, Servant N, Teyssier J, Bardin A, Licznar A, Badia E, Pujol P, Vignon F, Maudelonde T, Lazennec G, Cavailles V, Fajas L. The nuclear receptor liver receptor homolog-1 is an estrogen receptor target gene. Oncogene. 2005; 24:8167–8175. [PubMed: 16091743]

Bagnat M, Cheung ID, Mostov KE, Stainier DYR. Genetic control of single lumen formation in the zebrafish gut. Nature Cell Biology. 2007; 9:954–960. [PubMed: 17632505]

Benod C, Carlsson J, Uthayaruban R, Hwang P, Irwin JJ, Doak AK, Shoichet BK, Sablin EP, Fletterick RJ. Structure-based discovery of antagonists of nuclear receptor LRH-1. The Journal of Biological Chemistry. 2013; 288:19830–19844. [PubMed: 23667258]

Benod C, Vinogradova MV, Jouravel N, Kim GE, Fletterick RJ, Sablin EP. Nuclear receptor liver receptor homologue 1 (LRH-1) regulates pancreatic cancer cell growth and proliferation. Proceedings of the National Academy of Sciences. 2011; 108:16927–16931.

Bertrand S, Thisse B, Tavares R, Sachs L, Chaumot A, Bardet PLL, Escrivà H, Duffraisse M, Marchand O, Safi R, Thisse C, Laudet V. Unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. PLoS Genetics. 2007; 3:2085–2100.

Biemar F, Argenton F, Schmidtke R, Epperlein S, Peers B, Driever W. Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. Developmental Biology. 2001; 230:189–203. [PubMed: 11161572]

Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell. 2006; 126:789–799. [PubMed: 16923397]

Botrugno OA, Fayard E, Annicotte JSS, Haby C, Brennan T, Wendling O, Tanaka T, Kodama T, Thomas W, Auwerx J, Schoonjans K. Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. Molecular Cell. 2004; 15:499–509. [PubMed: 15327767]

Bussmann J, Schulte-Merker S. Rapid BAC selection for tol2-mediated transgenesis in zebrafish. Development. 2011; 138:4327–4332. [PubMed: 21865323]

Cermak T, Doyle EL, Christian M, Wang L. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Research. 2011; 39:1–11. [PubMed: 20805246]

Chong HK, Biesinger J, Seo Y-KK, Xie X, Osborne TF. Genome-wide analysis of hepatic LRH-1 reveals a promoter binding preference and suggests a role in regulating genes of lipid metabolism in concert with FXR. BMC Genomics. 2012:13. [PubMed: 22233093]

Chung WSS, Shin CH, Stainier DY. Bmp2 signaling regulates the hepatic versus pancreatic fate decision. Developmental Cell. 2008; 15:738–748. [PubMed: 19000838]

Dahlem TJ, Hoshijima K, Jurynec MJ, Gunther D, Starker CG, Locke AS, Weis AM, Voytas DF, Grunwald DJ. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genetics. 2012; 8

Decaens T, Godard C, de Reyniès A, Rickman DS, Tronche F, Couty JPP, Perret C, Colnot S. Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology (Baltimore, Md). 2008; 47:247–258.

Deutsch G, Jung J, Zheng M, Lora J, Zaret KS. A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development. 2001; 128:871–881. [PubMed: 11222142]

Dong PD, Munson CA, Norton W, Crosnier C, Pan X, Gong Z, Neumann CJ, Stainier DY. Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. Nature Genetics. 2007; 39:397–402. [PubMed: 17259985]

Fayard E, Auwerx J, Schoonjans K. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends in Cell Biology. 2004; 14:250260.

Field HA, Dong PD, Beis D, Stainier DY. Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. Developmental Biology. 2003a; 261:197–208. [PubMed: 12941629]

Field HA, Ober EA, Roeser T, Stainier DYR. Formation of the digestive system in zebrafish. I. Liver morphogenesis. Developmental Biology. 2003c:279–290. [PubMed: 12645931]

Goessling W, North TE, Lord AM, Ceol C, Lee S, Weidinger G, Bourque C, Strijbosch R, Haramis A-P, Puder M, Clevers H, Moon RT, Zon LI. APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development. Developmental Biology. 2008:161–174.

Grapin-Botton A. Ductal cells of the pancreas. The International Journal of Biochemistry & Cell Biology. 2005; 37:504–510. [PubMed: 15618005]

Gu P, Goodwin B, Chung AC, Xu X, Wheeler DA, Price RR, Galardi C, Peng L, Latour AM, Koller BH, Gossen J, Kliewer SA, Cooney AJ. Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. Molecular and Cellular Biology. 2005; 25:3492–3505. [PubMed: 15831456]

Hale MA, Swift GH, Hoang CQ, Deering TG. The nuclear hormone receptor family member NR5A2 controls aspects of multipotent progenitor cell formation and acinar differentiation during pancreatic organogenesis. Development (Cambridge, England). 2014; 141:3123–3133.

Heng JC, Feng B, Han J, Jiang J, Kraus P, Ng JH, Orlov YL, Huss M, Yang L, Lufkin T, Lim B, Ng HH. The Nuclear Receptor Nr5a2 Can Replace Oct4 in the Reprogramming of Murine Somatic Cells to Pluripotent Cells. Cell Stem Cell. 2010a; 6:167–174. [PubMed: 20096661]

Heng JC, Feng B, Han J, Jiang J, Kraus P, Ng JH, Orlov YL, Huss M, Yang L, Lufkin T, Lim B, Ng HH. The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell. 2010b; 6:167–174. [PubMed: 20096661]

Her GM, Chiang CCC, Chen WYY, Wu JLL. In vivo studies of liver-type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (Danio rerio). FEBS letters. 2003; 538:125–133. [PubMed: 12633865]

Holmstrom SR, Deering T, Swift GH, Poelwijk FJ, Mangelsdorf DJ, Kliewer SA, MacDonald RJ. LRH-1 and PTF1-L coregulate an exocrine pancreas-specific transcriptional network for digestive function. Genes & Development. 2011:1674–1679. [PubMed: 21852532]

Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. Genes & Development. 2006; 20:2293–2305. [PubMed: 16912278]

Labelle-Dumais C, Jacob-Wagner M, Pare JF, Belanger L, Dufort D. Nuclear receptor NR5A2 is required for proper primitive streak morphogenesis. Developmental dynamics : an official publication of the American Association of Anatomists. 2006; 235:3359–3369. [PubMed: 17075876]

Labelle-Dumais C, Jacob-Wagner M, Paré JF, Bélanger L, Dufort D. Nuclear receptor NR5A2 is required for proper primitive streak morphogenesis. Developmental Dynamics. 2006; 235:3359–3369. [PubMed: 17075876]

Lee YK, Schmidt DR, Cummins CL, Choi M, Peng L, Zhang Y, Goodwin B, Hammer RE, Mangelsdorf DJ, Kliewer SA. Liver receptor homolog-1 regulates bile acid homeostasis but is not essential for feedback regulation of bile acid synthesis. Molecular endocrinology. 2008; 22:1345–1356. [PubMed: 18323469]

Lin JW, Biankin AV, Horb ME, Ghosh B, Prasad NB, Yee NS, Pack MA, Leach SD. Differential requirement for ptf1a in endocrine and exocrine lineages of developing zebrafish pancreas. Developmental Biology. 2004; 274:491–503. [PubMed: 15570689]

Lin W, Wang HW, Sum C, Liu D, Hew CL, Chung B. Zebrafish ftz-f1 gene has two promoters, is alternatively spliced, and is expressed in digestive organs. The Biochemical Journal. 2000; 348(Pt 2):439–446. [PubMed: 10816440]

McLin VAA, Rankin SA, Zorn AM. Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. Development (Cambridge, England). 2007; 134:2207–2217.

Mudumana SP, Wan H, Singh M. Expression analyses of zebrafish transferrin, ifabp, and elastaseB mRNAs as differentiation markers for the three major endodermal organs: liver, intestine, and exocrine pancreas. Developmental Dynamics. 2004; 230:165–173. [PubMed: 15108321]

Murtaugh LC. The what, where, when and how of Wnt/beta-catenin signaling in pancreas development. Organogenesis. 2008; 4:81–86. [PubMed: 18953422]

Murtaugh LC. Putting GWAS to the functional test: NR5A2 and pancreatic cancer risk. Gut. 2014; 63:535–536. [PubMed: 23759730]

Nissim S, Sherwood RI, Wucherpfennig J, Saunders D, Harris JM, Esain V, Carroll KJ, Frechette GM, Kim AJ, Hwang KL, Cutting CC, Elledge S, North TE, Goessling W. Prostaglandin E2 regulates liver versus pancreas cell-fate decisions and endodermal outgrowth. Developmental Cell. 2014; 28:423–437. [PubMed: 24530296]

North TE, Goessling W. Chapter 10 – Endoderm Specification, Liver Development, and Regeneration. Methods in Cell Biology. 2011; 101:205–223. [PubMed: 21550446]

North TE, Goessling W, Walkley CR, Lengerke C. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature. 2007; 447:1007–1011. [PubMed: 17581586]

Ober EA, Field HA, Stainier DYR. From endoderm formation to liver and pancreas development in zebrafish. Mechanisms of Development. 2003:5–18. [PubMed: 12490292]

Ober EA, Verkade H, Field HA, Stainier DY. Mesodermal Wnt2b signalling positively regulates liver specification. Nature. 2006; 442:688–691. [PubMed: 16799568]

Paré JF, Malenfant D, Courtemanche C, Jacob-Wagner M, Roy S, Allard D, Bélanger L. The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis and is regulated by a DR4 element. Journal of Biological Chemistry. 2004; 279:21206–21216. [PubMed: 15014077]

Paré JF, Roy S, Galarneau L, Belanger L. The mouse fetoprotein transcription factor (FTF) gene promoter is regulated by three GATA elements with tandem E box and Nkx motifs, and FTF in turn activates the Hnf3beta, Hnf4alpha, and Hnf1alpha gene promoters. The Journal of Biological Chemistry. 2001; 276:13136–13144. [PubMed: 11145965]

Petersen GM, Amundadottir L, Fuchs CS, Kraft P, Stolzenberg-Solomon RZ, Jacobs KB, Arslan AA, Bueno-de-Mesquita HB, Gallinger S, Gross M, Helzlsouer K, Holly EA, Jacobs EJ, Klein AP, LaCroix A, Li D, Mandelson MT, Olson SH, Risch HA, Zheng W, Albanes D, Bamlet WR, Berg CD, Boutron-Ruault MCC, Buring JE, Bracci PM, Canzian F, Clipp S, Cotterchio M, de Andrade M, Duell EJ, Gaziano JM, Giovannucci EL, Goggins M, Hallmans G, Hankinson SE, Hassan M, Howard B, Hunter DJ, Hutchinson A, Jenab M, Kaaks R, Kooperberg C, Krogh V, Kurtz RC, Lynch SM, McWilliams RR, Mendelsohn JB, Michaud DS, Parikh H, Patel AV, Peeters PH, Rajkovic A, Riboli E, Rodriguez L, Seminara D, Shu XOO, Thomas G, Tjønneland A, Tobias GS, Trichopoulos D, Van Den Eeden SK, Virtamo J, Wactawski-Wende J, Wang Z, Wolpin BM, Yu H, Yu K, Zeleniuch-Jacquotte A, Fraumeni JF, Hoover RN, Hartge P, Chanock SJ. A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. Nature Genetics. 2010; 42:224–228. [PubMed: 20101243]

Poulain M, Ober EA. Interplay between Wnt2 and Wnt2bb controls multiple steps of early foregut-derived organ development. Development. 2011; 138:3557–3568. [PubMed: 21771809]

Rausa FM, Galarneau L, Belanger L, Costa RH. The nuclear receptor fetoprotein transcription factor is coexpressed with its target gene HNF-3beta in the developing murine liver, intestine and pancreas. Mechanisms of Development. 1999; 89:185–188. [PubMed: 10559496]

Rossi JM, Dunn NR, Hogan BL, Zaret KS. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes and Development. 2001; 15:1998–2009. [PubMed: 11485993]

Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, Yeh JRJR. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nature Biotechnology. 2011; 29:697–698.

Seth A, Ye J, Yu N, Guez F, Bedford DC, Neale GA, Cordi S, Brindle PK, Lemaigre FP, Kaestner KH, Sosa-Pineda B. Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment. Development (Cambridge, England). 2014; 141:538–547.

Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. Nature Genetics. 2000; 25:254–255. [PubMed: 10888866]

Tan X, Yuan Y, Zeng G, Apte U, Thompson MD, Cieply B, Stolz DB, Michalopoulos GK, Kaestner KH, Monga SP. Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. Hepatology (Baltimore, Md). 2008; 47:1667–1679.

Tiso N, Moro E, Argenton F. Zebrafish pancreas development. Molecular and Cellular Endocrinology. 2009; 312:24–30. [PubMed: 19477220]

Ueno M, Ohkawa S, Morimoto M, Ishii H. Genome-wide association study-identified SNPs (rs3790844, rs3790843) in the NR5A2 gene and risk of pancreatic cancer in Japanese. Nature Scientific Reports. 2015; 5

von Figura G, Morris JP, Wright CV, Hebrok M. Nr5a2 maintains acinar cell differentiation and constrains oncogenic Kras-mediated pancreatic neoplastic initiation. Gut. 2014; 63:656–664. [PubMed: 23645620]

Wagner RT, Xu X, Yi F, Merrill BJ, Cooney AJ. Canonical Wnt/catenin regulation of liver receptor homolog 1 mediates pluripotency gene expression. Stem Cells. 2010; 28:1794–1804. [PubMed: 20734354]

Wan H, Korzh S, Li Z, Mudumana SP, Korzh V, Jiang YJJ, Lin S, Gong Z. Analyses of pancreas development by generation of gfp transgenic zebrafish using an exocrine pancreas-specific elastaseA gene promoter. Experimental Cell Research. 2006; 312:1526–1539. [PubMed: 16490192]

Wandzioch E, Zaret KS. Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. Science. 2009; 324:1707–1710. [PubMed: 19556507]

Wang A, Yue F, Li Y, Xie R, Harper T, Patel NA, Muth K, Palmer J, Qiu Y, Wang J, Lam DK, Raum JC, Stoffers DA, Ren B, Sander M. Epigenetic priming of enhancers predicts developmental competence of hESC-derived endodermal lineage intermediates. Cell Stem Cell. 2015; 16:386–399. [PubMed: 25842977]

Wang J, Kilic G, Aydin M, Burke Z, Oliver G. Prox1 activity controls pancreas morphogenesis and participates in the production of “secondary transition” pancreatic endocrine cells. Developmental Biology. 2005:182–194. [PubMed: 16122728]

Westmoreland JJ, Kilic G, Sartain C, Sirma S, Blain J. Pancreas-specific deletion of Prox1 affects development and disrupts homeostasis of the exocrine pancreas. Gastroenterology. 2012; 142:999–1009. [PubMed: 22178591]

Yee NS, Lorent K, Pack M. Exocrine pancreas development in zebrafish. Developmental Biology. 2005; 284:84–101. [PubMed: 15963491]

Zaret KS, Grompe M. Generation and regeneration of cells of the liver and pancreas. Science. 2008; 322:1490–1494. [PubMed: 19056973]

Zecchin E, Mavropoulos A, Devos N, Filippi A, Tiso N, Meyer D, Peers B, Bortolussi M, Argenton F. Evolutionary conserved role of ptf1a in the specification of exocrine pancreatic fates. Developmental Biology. 2004; 268:174–184. [PubMed: 15031114]

- Zygotic loss of *nr5a2* disrupts development of exocrine pancreas.
- Nr5a2 regulates acinar differentiation downstream of progenitor cell formation.
- Nr5a2 regulates *hnf4a* and *prox1* expression in hepatic progenitors.
- Nr5a2 is required for the formation of differentiated hepatocytes.
- Nr5a2 antagonism reveals roles at multiple stages of hepatopancreas development.

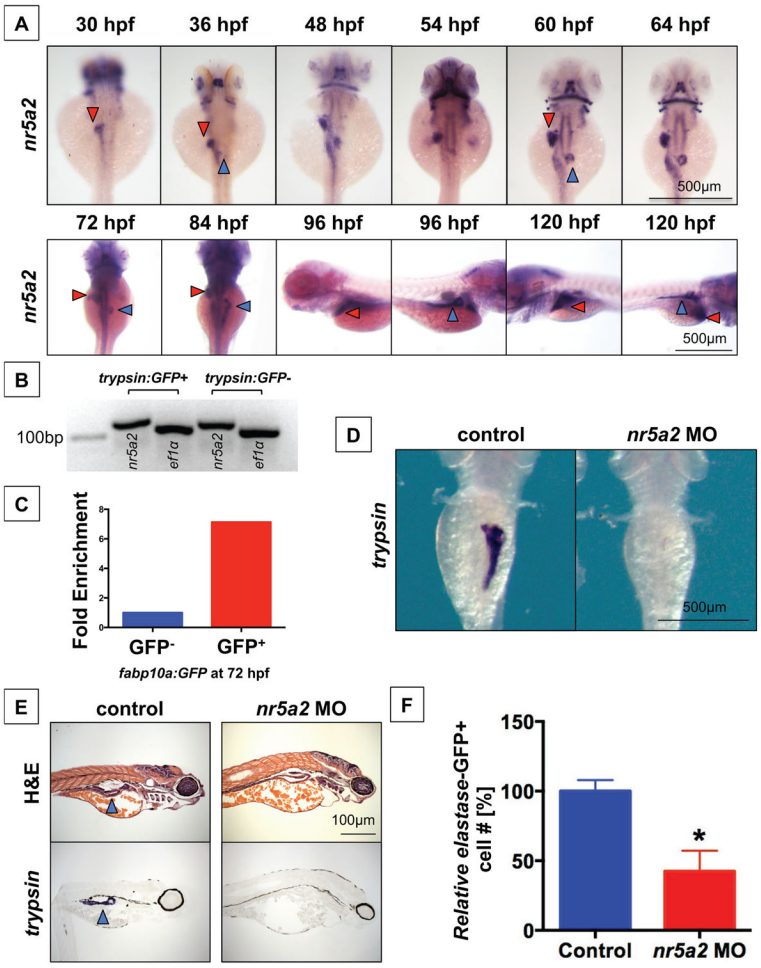

Figure 1. *nr5a2* is expressed in the developing endoderm and is required for exocrine pancreas formation

A) Expression pattern of *nr5a2* in the developing endoderm by *in situ* hybridization. *nr5a2* appears in the developing liver bud (red arrow) by 30 hpf and the pancreas bud (blue arrow) by 36 hpf. After 84 hpf, it is expressed in the mature liver (red arrow) and exocrine pancreas (blue arrow).

B) Expression of *nr5a2* in FACS isolated acinar cells from the *Tg(trypsin:GFP)* line at 72 hpf by qRT-PCR.

C) Expression of *nr5a2* in FACS isolated hepatocytes from pooled *Tg(fabp10a:GFP)* embryos at 72 hpf by qRT-PCR (fold enrichment displayed as an average of technical triplicates).

D) Splice-site morpholino (MO) knockdown of Nr5a2 reduces the size of the exocrine pancreas by *in situ* hybridization for *trypsin* at 96 hpf.

E) *nr5a2* MO injected embryos (96 hpf) have a reduced exocrine pancreas size (blue arrow) by histology and section *in situ* hybridization for *trypsin* (n = 5).

F) FACS quantification of GFP+ cells (% of control) from *elastase*:GFP reporter fish at 96 hpf demonstrates that MO knockdown of *nr5a2* reduces the number of exocrine pancreas cells (n = 20 per treatment; $p < 0.05$; unpaired t-test).

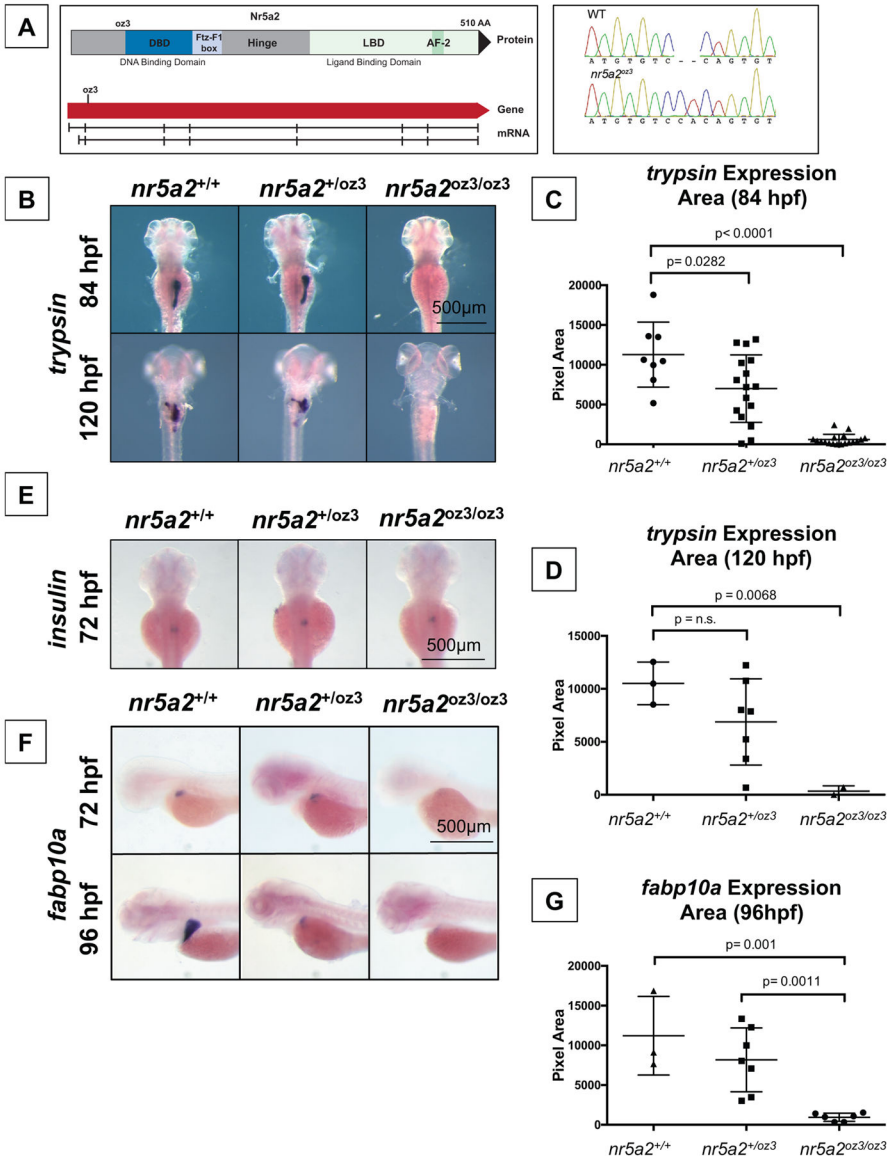

Figure 2. Genetic modulation of Nr5a2 disrupts the formation of the mature exocrine pancreas and liver

A) The *oz3* genetic loss of function mutation is a two base pair insertion within exon 3 of the *nr5a2* transcript, before the DNA-binding domain and ligand-binding domain.

B) *trypsin in situ* hybridization at 84 and 120 hpf reveals that *nr5a2*$^{oz3/oz3}$ embryos do not develop a mature exocrine pancreas.

C) Quantification of exocrine pancreas size in *nr5a2*$^{+/+}$, *nr5a2*$^{+/oz3}$, and *nr5a2*$^{oz3/oz3}$ embryos by *trypsin* area calculations. *nr5a2*$^{oz3/oz3}$ and *nr5a2*$^{+/oz3}$ embryos have a significantly reduced exocrine pancreas size relative to *nr5a2*$^{+/+}$ siblings at 84 hpf (n > 8 per group; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{+/oz3}$ ($p = 0.0282$; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{oz3/oz3}$, $p < 0.0001$; unpaired t-test).

D) Quantification of exocrine pancreas size in *nr5a2*$^{+/+}$, *nr5a2*$^{+/oz3}$, and *nr5a2*$^{oz3/oz3}$ embryos by *trypsin* area calculations. At 120 hpf, *nr5a2*$^{oz3/oz3}$ have a significantly reduced

exocrine pancreas size relative to *nr5a2*$^{+/+}$ siblings (n > 2 per group; p = 0.0068; unpaired t-test).

E) *In situ* hybridization for *insulin* demonstrates that *nr5a2*$^{+/+}$, *nr5a2*$^{+/oz3}$, and *nr5a2*$^{oz3/oz3}$ embryos develop an endocrine pancreas.

F) *In situ* hybridization for *fabp10a* reveals that *nr5a2*$^{oz3/oz3}$ embryos have a reduced liver size at 72 and 96 hpf.

G) Quantification of liver size by *fabp10a* area calculations demonstrates that *nr5a2*$^{oz3/oz3}$ embryos have a significantly reduced liver size relative to *nr5a2*$^{+/+}$ and *nr5a2*$^{+/oz3}$ categories (n > 3 per group; p = 0.001; unpaired t-test).

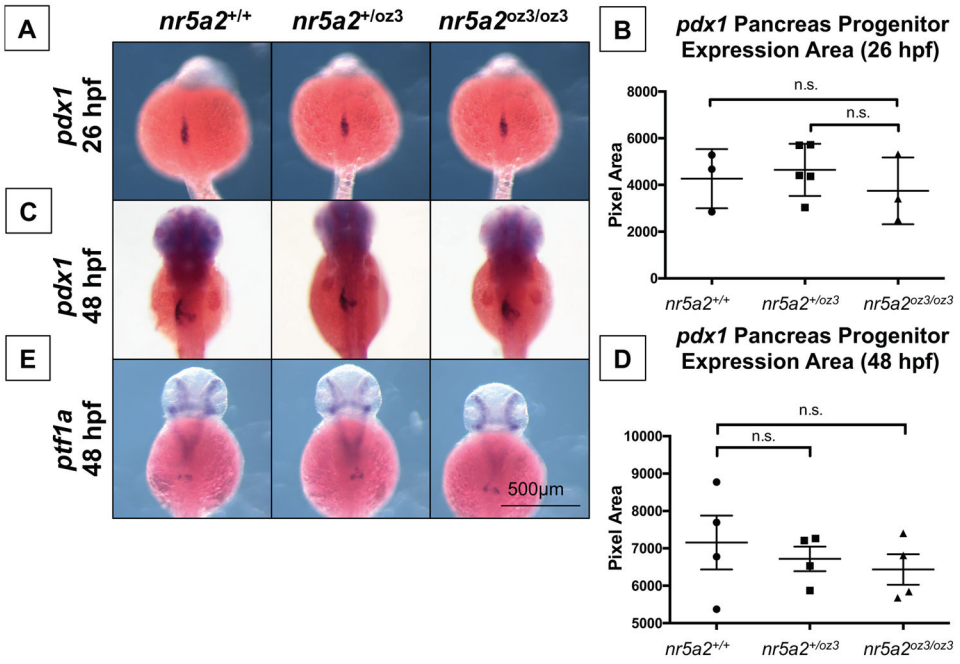

Figure 3. Nr5a2 is not required for the formation of *pdx1* and *ptf1a* pancreas progenitors

A) The area of *pdx1* pancreas progenitors is similar across *nr5a2*$^{+/+}$, *nr5a2*$^{+/oz3}$, and *nr5a2*$^{oz3/oz3}$ embryos at 26 hpf.

B) No significant differences in *pdx1* progenitor population size identified by a quantification of *pdx1* expression area by genotype (p > 3 per group; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{+/oz3}$, p = 0.6746; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{oz3/oz3}$, p = 0.6606; unpaired t-test).

C) *pdx1* pancreas progenitors are formed at 48 hpf in *nr5a2*$^{+/oz3}$ and *nr5a2*$^{oz3/oz3}$ embryos; however, subtle difference in *pdx1* progenitor budding is noted with variable penetrance across the *nr5a2*$^{oz3/oz3}$ population.

D) No significant differences in the size of the *pdx1* pancreas progenitor pool were observed by quantification of expression area at 48 hpf (n > 3 per group; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{+/oz3}$, p = 0.6024; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{oz3/oz3}$, p = 0.4178; unpaired t-test).

E) *ptf1a* progenitors are formed in *nr5a2*$^{+/oz3}$ and *nr5a2*$^{oz3/oz3}$ embryos by 48 hpf.

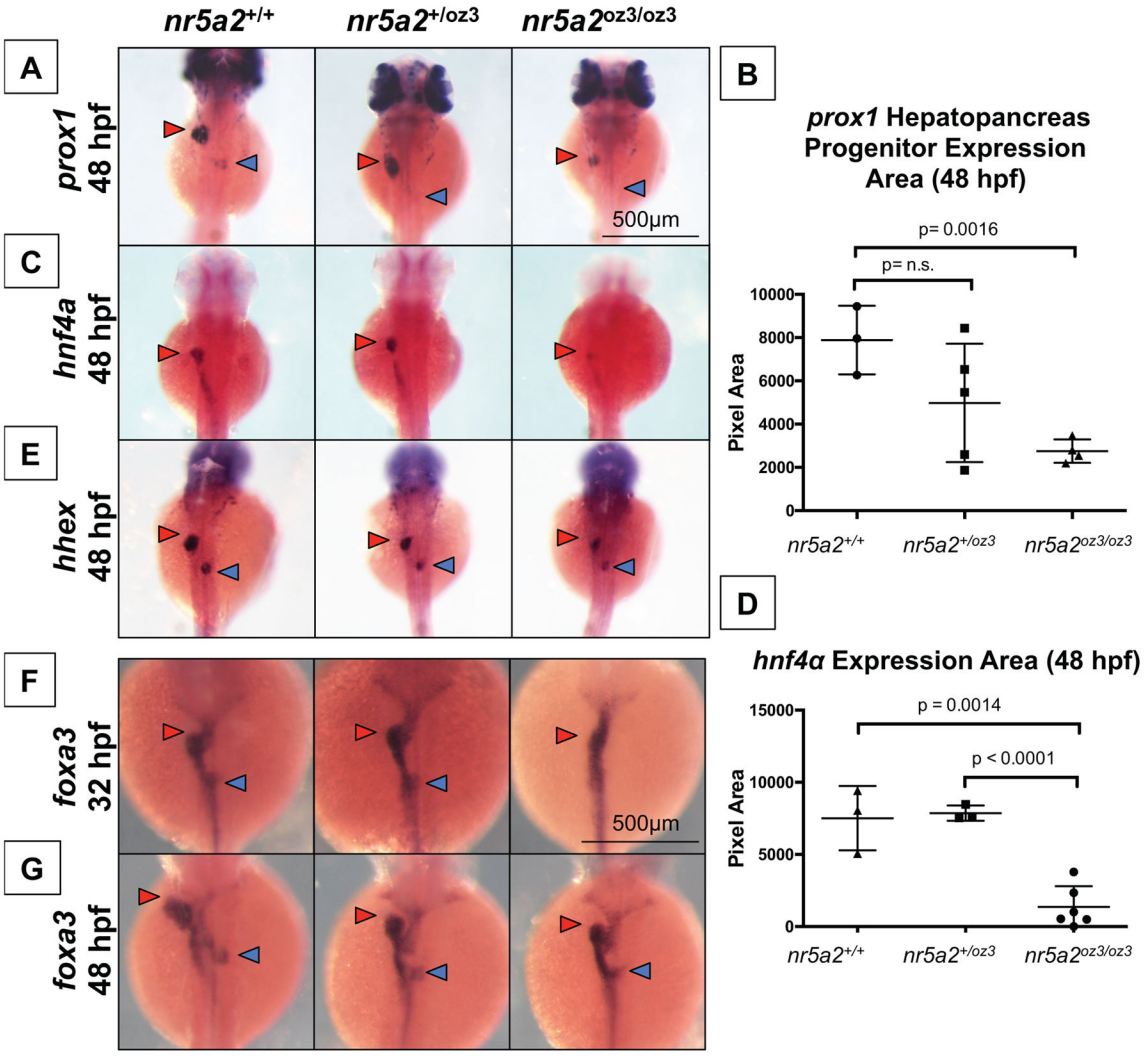

Figure 4. A subset of hepatopancreas progenitors require *nr5a2* for specification

A) *nr5a2*$^{oz3/oz3}$ mutants have reduced *prox1* expression in the liver (red arrow) and pancreas (blue arrow) buds relative to *nr5a2*$^{+/+}$ controls at 48 hpf.

B) Quantification of *prox1* expression area in the combined liver and pancreas buds (n > 3 per group; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{+/oz3}$, p = 0.1502; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{+/oz3}$, p = 0.0016; unpaired t-test).

C) *In situ* hybridization reveals consistent decreased *hnf4α* expression in the hepatoblasts (red arrow) of *nr5a2*$^{oz3/oz3}$ embryos.

D) Quantification of *hnf4α* expression area in the intestine and liver buds shows significant expression reduction in *nr5a2*$^{oz3/oz3}$ embryos relative to *nr5a2*$^{+/+}$ and *nr5a2*$^{+/oz3}$ groups (n > 3 per group; for *nr5a2*$^{+/+}$ vs. *nr5a2*$^{oz3/oz3}$, p = 0.0014; for *nr5a2*$^{+/oz3}$ vs. *nr5a2*$^{oz3/oz3}$, p < 0.0001; unpaired t-test).

E) The size of the *hhex* liver bud (red arrow) is reduced in the *nr5a2*$^{oz3/oz3}$ embryos compared to *nr5a2*$^{+/+}$ siblings, while the pancreas bud (blue arrow) is largely unaffected.

F) *In situ* hybridization for *foxa3* endoderm marker consistently demonstrates delayed gut looping, liver (red arrow), and pancreas (blue arrow) budding in the *nr5a2*$^{oz3/oz3}$ embryos at 32 hpf.

G) The sizes of the *foxa3* liver (red arrow) and pancreas (blue arrow) buds are consistently reduced in the *nr5a2*$^{+/oz3}$ and *nr5a2*$^{oz3/oz3}$ mutant populations at 48 hpf.

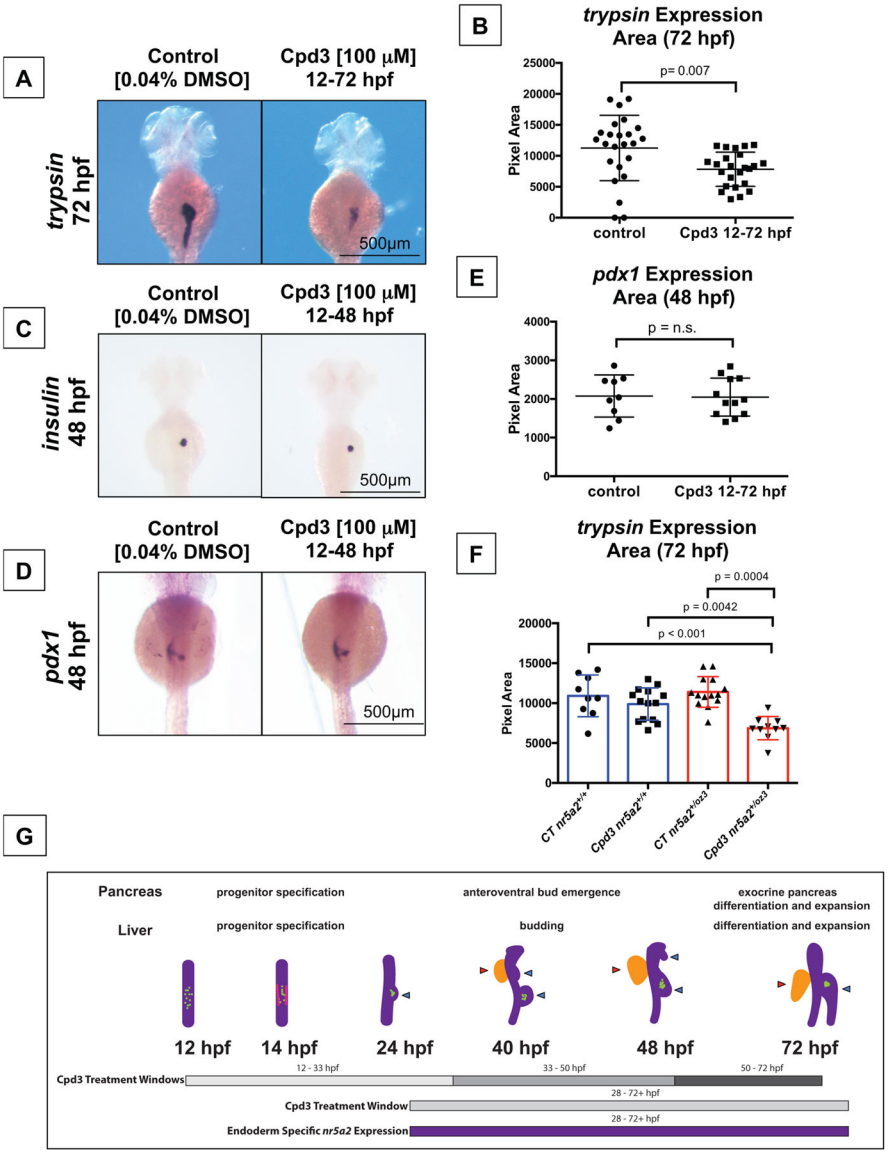

Figure 5. Nr5a2 chemical antagonist Cpd3 impacts exocrine pancreas development *in vivo*

A) The Nr5a2 antagonist (Cpd3) produces reduced exocrine pancreas sizes that mimic the *nr5a2*$^{+/oz3}$ heterozygotes when treated from 12–72 hpf.

B) Quantification of exocrine pancreas size by area of *trypsin* expression at 72 hpf. Cpd3 significantly reduces exocrine pancreas size compared to vehicle (0.04% DMSO) treated controls (n = 24 per group; p = 0.007; unpaired t-test).

C) Loss of Nr5a2 activity by Cpd3 treatment does not visibly impact the formation of the *insulin* expressing β-cells.

D) Cpd3 treatment does not impact the size of the *pdx1* pancreas progenitor pool at 48 hpf.

E) Quantification of *pdx1* pancreas progenitor expression area at 48 hpf in control and Cpd3 treated groups (n > 9 per group; p = 0.9089; unpaired t-test).

F) Quantification of exocrine pancreas size by area of *trypsin* expression at 72 hpf. Heterozygote *nr5a2*$^{+/oz3}$ embryos show heightened sensitivity to 100 μM Cpd3 treatment

relative to the *nr5a2*$^{+/+}$ sibling controls (n > 10 per group; for control *nr5a2*$^{+/+}$ vs. Cpd3 treated *nr5a2*$^{+/oz3}$, p < 0.001; for control *nr5a2*$^{+/oz3}$ vs. Cpd3 treated *nr5a2*$^{+/oz3}$, p = 0.0004; for Cpd3 treated *nr5a2*$^{+/+}$ vs. Cpd3 treated *nr5a2*$^{+/oz3}$, p = 0.0042; ordinary one-way ANOVA with Tukey's multiple comparisons test).

G) Drug treatment windows to examine the impact of Nr5a2 loss of function on distinct stages of pancreas and liver development, including progenitor specification, bud emergence, and organ differentiation and expansion. At 12 hpf, *insulin* expressing β-cells form within the gut tube (green dots). By 14 hpf, *pdx1* expressing pancreas progenitors appear in two stripes (pink lines) An endocrine cell cluster (green) forms (beginning at approximately 24 hpf) and the dorsoventral pancreas bud emerges from the gut tube. By 40 hpf, the dorsoventral and anteroventral buds (blue arrows) have emerged from the gut tube, along with the liver bud (red arrow). Post 50 hpf, the liver and pancreas buds differentiate and expand. Figure adapted (Tiso et al., 2009).

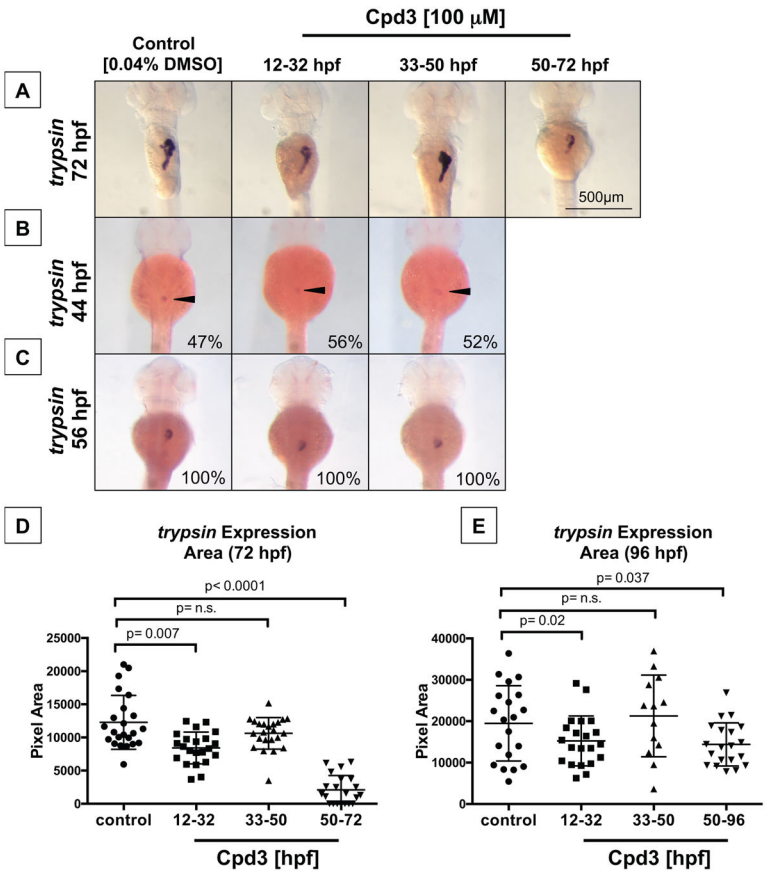

Figure 6. Two temporally defined roles for Nr5a2 during pancreas development

A) Timed Cpd3 treatment reduces *trypsin* expression at 72 hpf for the 12 – 32 hpf and 50 – 72 hpf treatment windows, but not during the 33 – 50 hpf window.

B) Control (n = 22/46), 12–32 hpf (n = 19/36), and 33–50 hpf (n = 14/25) treated fish all have the same percentage of fish with differentiated exocrine pancreas cells at 44 hpf, indicating that drug treatments do not alter the timing of initial exocrine cell differentiation.

C) Control (n = 12/12), 12–32 hpf (n = 11/11), and 33–50 hpf (n = 14/14) treated fish all have similarly sized *trypsin* cell clusters at 56 hpf, demonstrating that initial exocrine pancreas differentiation is unaffected by previous *nr5a2* inactivation.

D) Quantification of exocrine pancreas size (by area of *trypsin* expression at 72 hpf) demonstrates that the 12 – 32 hpf and 50 – 96 hpf Cpd3 treatment groups have significantly reduced exocrine pancreas sizes relative to controls. Exocrine pancreas size in the 33 – 50 hpf treatment group was not significantly altered by treatment (n > 21 per group; for control vs. 12 – 32 hpf, p = 0.007; for control vs. 33 – 50 hpf, p = 0.09; for control vs. 50 – 72 hpf, p < 0.0001; unpaired t-test).

E) Quantification of exocrine pancreas size by area of *trypsin* expression demonstrates that antagonist-induced exocrine pancreas reduction persists through 96 hpf for the 12 – 32 hpf and 50 – 96 hpf treatment categories. The size of the pancreas in 33 – 50 hpf treatment categories remains unchanged relative to the vehicle treated controls (n > 13 per group; for control vs. 12 – 32 hpf, p = 0.02; for control vs. 33 – 50 hpf, p = 0.5929; for control vs. 50 – 72 hpf, p = 0.037; unpaired t-test).

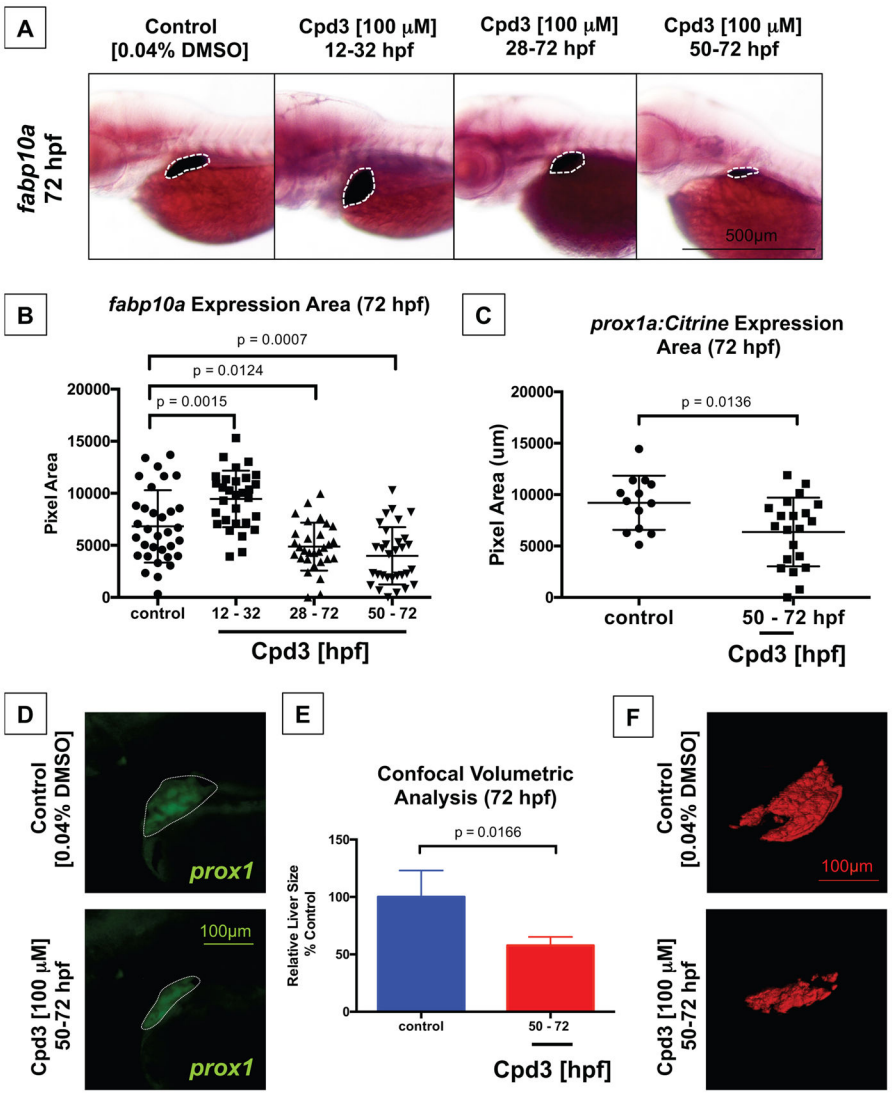

Figure 7. Liver differentiation and outgrowth depends on Nr5a2 activity

A) Cpd3 treatment reduces the size and alters the position of the *fabp10a* liver.

B) Quantification of liver size by area of *fabp10a* expression at 72 hpf. Cpd3 treatment from 12 – 32 hpf enhances the size of the mature liver relative to vehicle (DMSO) treated controls (n > 27; p = 0.0015; unpaired t-test). Treatment from 28 – 72 hpf and 50 – 72 hpf reduces the median liver size relative to vehicle (DMSO) treated controls (n > 27; p = 0.0124; p = 0.0007; unpaired t-test).

C) Quantification of liver bud size by area of fluorescence expression in the *Tg(prox1a:Citrine)* reporter line at 72 hpf. Cpd3 treatment from 50–72 hpf significantly reduces the size of the *prox1+* liver (n > 13; p = 0.0136).

D) Fluorescent microscopy images of the embryonic liver in *Tg(prox1a:Citrine)* reporters following vehicle (DMSO) or Cpd3 treatment from 50 – 72 hpf.

E) Confocal volumetric analysis of liver volume in control (DMSO) and Cpd3 treated (50 – 72 hpf) *Tg(fabp10a:GFP)* lines at 72 hpf demonstrates that Cpd3 treatment significantly reduces the volume of the liver (n > 3; p = 0.0166).

F) Representative 3D renderings of 72 hpf GFP+ livers from the *Tg(fabp10a:GFP)* line that were exposed to control DMSO or Cpd3 treatment from 50 – 72 hpf. Cpd3 (50 – 72 hpf) livers are visibly smaller than the control livers.

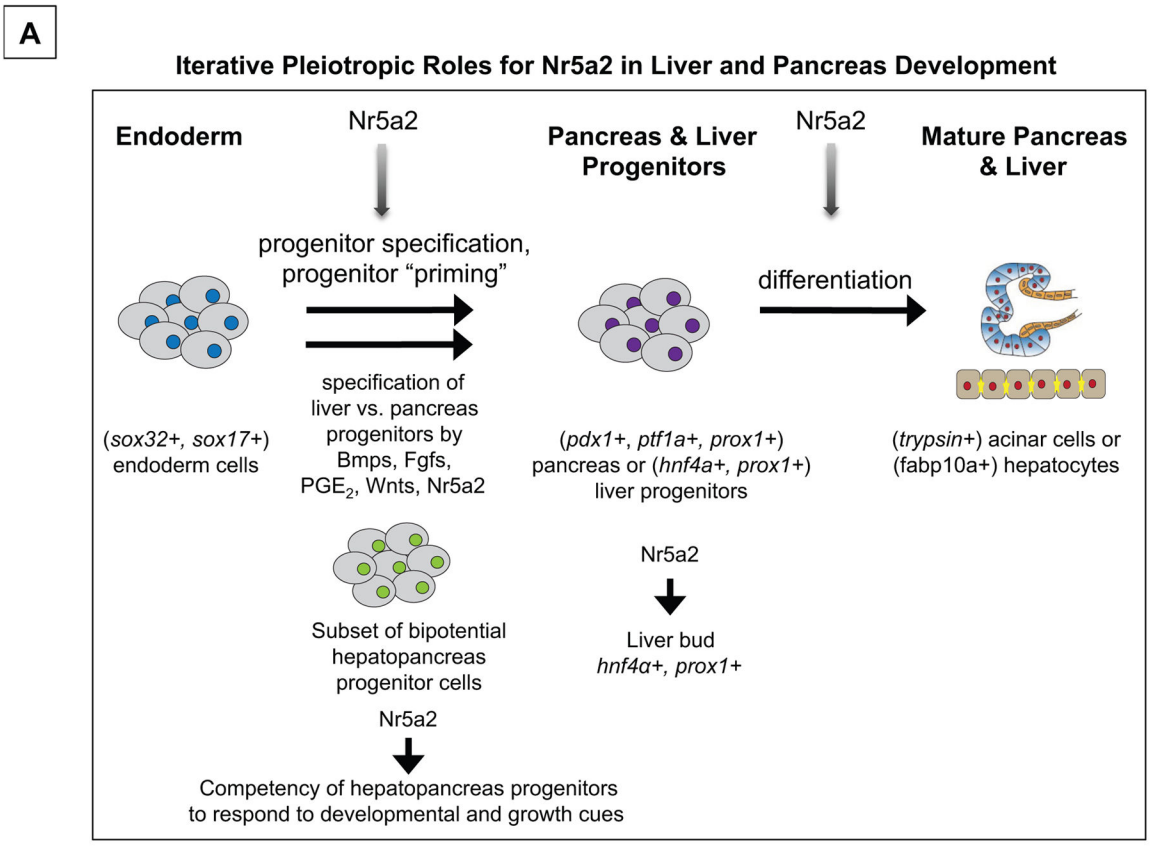

Figure 8. Pleiotropic roles for Nr5a2 in liver and pancreas development

A) Model for Nr5a2 function during multiple stages of hepatopancreas specification and differentiation. We postulate that Nr5a2 initially functions during stages of hepatopancreas progenitor specification, possibly acting to prime progenitors to competently receive differentiation cues, or to regulate their commitment to the hepatic and pancreatic lineages. Later, Nr5a2 is required for the expression of hepatoblast markers, including *hnf4α* and *prox1,* which may be crucial for liver bud outgrowth and differentiation. Finally, Nr5a2 is required for the differentiation of pancreas and liver progenitors (following 50 hpf) into mature acinar cells and hepatocytes, respectively.