Cannabinoid receptor signaling regulates liver development and metabolism

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ABSTRACT
Endocannabinoid (EC) signaling mediates psychotropic effects and regulates appetite. By contrast, potential roles in organ development and embryonic energy consumption remain unknown. Here, we demonstrate that genetic or chemical inhibition of cannabinoid receptor (Cnr) activity disrupts liver development and metabolic function in zebrafish (\textit{Danio rerio}), impacting hepatic differentiation, but not endodermal specification: loss of \textit{cannabinoid receptor 1} (\textit{cnr1}) and \textit{cnr2} activity leads to smaller livers with fewer hepatocytes, reduced liver-specific gene expression and proliferation. Functional assays reveal abnormal biliary anatomy and lipid handling. Adult \textit{cnr2} mutants are susceptible to hepatic steatosis. Metabolic analysis reveals reduced methionine content in \textit{Cnr} mutants. Methionine supplementation rescues developmental and metabolic defects in \textit{Cnr} mutant livers, suggesting a causal relationship between EC signaling, methionine deficiency and impaired liver development. The effect of \textit{Cnr} on methionine metabolism is regulated by sterol regulatory element-binding transcription factors (Srebf), as their overexpression rescues \textit{Cnr} mutant liver phenotypes in a methionine-dependent manner. Our work describes a novel developmental role for EC signaling, whereby \textit{Cnr}-mediated regulation of Srebf and methionine metabolism impacts liver development and function.

KEY WORDS: Cannabinoid receptor, Liver development, Methionine, Zebrafish, Mouse

INTRODUCTION
The energy requirements of developing embryos both influence and depend on the growth of essential metabolic organs. The factors impacting energy consumption and metabolism during embryogenesis, however, are not well understood. Each developmental stage may require the implementation of specific metabolic programs, and defects in metabolic processing or nutrient utilization could disrupt the differentiation and function of essential organs (Shyh-Chang et al., 2013). Alterations in developmental metabolism may also impact adult organ function through direct regulation of essential acetylation and methylation processes to alter the epigenetic control of development (Shyh-Chang et al., 2013; Sinclair and Watkins, 2014). Impairment of cellular proliferation pathways can disrupt organogenesis, thereby inhibiting normal physiological functions and leading to long-term consequences for adult metabolic homeostasis. Here, we show that endocannabinoid (EC) signaling is required for normal embryonic liver development and function. Disruptions of essential liver metabolic processes contribute to the development of diabetes, obesity and chronic liver disease. Elucidating the genetic pathways and mechanisms regulating developmental metabolism might inform early intervention strategies for individuals with genetic predisposition to metabolic disease.

The EC signaling pathway has predominantly been explored for its effects in the central nervous system (CNS), such as regulating appetite, mood and pain (Castillo et al., 2012). Primary ECs are bioactive fatty acid amides and esters synthesized in the CNS and the periphery, including 2-arachidonoyl glycerol (2-AG) (Sugiura et al., 1995) and arachidonoyl ethanolamide (anandamide, AEA) (Devane et al., 1992). Their effects are mediated by the G protein-coupled receptors Cnr1 and Cnr2. Cnr1 is primarily expressed in the CNS, where it promotes appetite control (Maccarrone et al., 2010), whereas Cnr2 acts in immune cells and the gastrointestinal tract (Munro et al., 1993). Although ECs regulate appetite in the CNS, how these ligands or their receptors influence embryonic metabolism and liver function via downstream targets is unknown.

Here, we discover that \textit{cnr1} and \textit{cnr2} are required for normal hepatocyte proliferation and differentiation during zebrafish development. Disrupted EC activity leads to decreased numbers of functionally immature hepatocytes, with impaired lipase activity and biliary lipid excretion. EC signaling orchestrates crucial metabolic functions during early nutritional transitions, and aberrations in liver histology and physiology in \textit{Cnr} mutants persist in larval and adult zebrafish. Polar metabolomics analysis reveals dysregulation of methionine and its metabolic intermediates in \textit{Cnr} mutants, a process mediated by sterol regulatory element-binding transcription factors (Srebfs). Supplementation with methionine and overexpression of \textit{srebfl2} both rescue the histological and metabolic defects in mutant larvae. Furthermore, long-term exposure of \textit{cnr2\textsuperscript{−/−}} mutants to methionine improves adult liver histology, with evidence for decreased hepatic fat accumulation. Our work uncovers a previously unrecognized relationship between embryonic EC signaling, liver development and metabolic homeostasis that impacts adult liver structure and function.

RESULTS
Inhibition of signaling through \textit{cnr1} and \textit{cnr2} impairs liver development
A chemical screen to identify novel regulators of liver development (Garnaas et al., 2012) revealed that the EC agonists anandamide, linoleoyl ethanolamide, mead acid ethanolamide, tetrahydrocannabinol (all Cnr1, Cnr2 agonists) and L759,633 (a...
Cnr2 agonist) increased liver size, as determined by transgenic hepatocyte reporter fatty acid binding protein 10a (fabp10a):GFP expression at 72 h post fertilization (hpf). To confirm the screen results, embryos were exposed to Cnr modifiers (1-10 μM) from 18-72 hpf, and liver size was analyzed at 72 hpf by in situ hybridization (ISH) for fabp10a. Compared with DMSO-treated controls, the Cnr1/2 agonist O2545 (Martin, 2006), Cnr1 agonist leelamine hydrochloride (L-HCl), and the Cnr2 agonist JWH015 increased total fabp10a expression area, whereas the antagonists rimonabant (Cnr1) and AM630 (Cnr2) diminished liver size (Fig. 1A,B). Quantification of fabp10a:GFP-positive hepatocytes by fluorescence-activated cell sorting (FACS) confirmed the enhancing effect following agonist treatment (Fig. 1A1A).

Together, these assays suggest an important role for EC signaling in liver development.

Expression of cnr1 and cnr2 is conserved in the zebrafish CNS at 24, 48 and 72 hpf (Fig. S1B) (Lam et al., 2006; Watson et al., 2008). Between 48 and 72 hpf, cnr1 expression is also enriched in the liver, whereas cnr2 is more broadly expressed, including the hepatic region, as determined by ISH (Fig. S1B). FACS-sorted fabp10a:GFP hepatocytes revealed strong cnr1 expression by qRT-PCR (Fig. S1C). By contrast, cnr2 expression was present in the hepatic region, but undetectable in the isolated hepatocyte fraction, a finding supported by previously established expression in whole liver, specifically the non-parenchymal liver cells, and its paracrine signaling functions in the liver (Juliën et al., 2005; Teixeira-Clerc et al., 2010). Consistent with the chemical modulation studies, liver development was negatively impacted in both cnr1 and cnr2 morphants, each exhibiting smaller livers and hepatocyte numbers at 72 hpf (Fig. S2A,B).

To further validate the role of Cnr activity during hepatogenesis, cnr1−/− and cnr2−/− knockout zebrafish were generated using transcription activator-like effector nucleases (TALENs) (Sander et al., 2011) targeting the first exon of cnr1 and cnr2 (Fig. S2C,D): cnr1−/− and cnr2−/− mutants survived to adulthood, were fertile and exhibited no gross morphological defects or developmental delays (Fig. S2E). Compared with wild-type (WT) embryos and heterozygotes, fabp10a expression was significantly decreased in homozygous Cnr mutants at 72 hpf (Fig. 1C), as confirmed and quantified by liver morphometrics after ISH (Fig. 1D), FACS analysis of fabp10a:GFP embryos (Fig. 1E), and qRT-PCR for fabp10a (Fig. 1F). cnr1−/−; cnr2−/− double mutants were also viable and exhibited liver phenotypes indistinguishable from single mutants by fabp10a expression and histology (Fig. 1G, Fig. S2F), suggesting a lack of compensation between the receptors, consistent with their different cellular expression. Together, these data confirm that both cnr1 and cnr2 are necessary for normal hepatogenesis.

Additional markers of differentiated hepatocytes, namely group-specific component (vitamin D binding protein) (gc), secreted immunoglobulin domain 4 (sid4) and transferrin-a (tfa) at 72 hpf confirmed the validity of the findings (Fig. S3A-D). We examined endodermal and mesodermal organs to assess liver specificity: in cnr1−/− and cnr2−/− mutants, intestinal development was disrupted, with decreased fabp2 expression at 72 hpf (Fig. S4A); by contrast, exocrine pancreas development (trypsin at 72 hpf) was unaffected, as were kidney (pax2a) and heart (cmel2; also known as myl7) (not shown). Importantly, expression of the endoderm marker foxa3 and the hepatic progenitor markers hhex and proxl was unchanged in Cnr homozygous mutants, heterozygotes, or morphants at 48 and 72 hpf (Fig. 2A,B, Fig. S3E-G and Fig. S4B). EC signaling therefore impacts differentiation and/or proliferation of committed hepatocytes or hepatic progenitors, whereas hepatoblasts are properly specified from early endoderm.

To further delineate the temporal importance of EC signaling and the target cell population during endoderm formation and hepatic differentiation, embryos were exposed to Cnr agonists from 24-48 hpf (hepatoblast formation) or 48-72 hpf (hepatocyte differentiation and proliferation). Only exposure from 48-72 hpf impacted liver size (Fig. 2C,D) at 72 hpf, supporting a role in hepatocyte differentiation and/or expansion, consistent with our observation of the timing of hepatic cnr1 and cnr2 expression.

**cnr1−/− and cnr2−/− mutants exhibit disrupted hepatocyte proliferation**

We examined later time points to determine if alterations in hepatocyte differentiation persisted beyond initial liver growth stages: defects in total liver cell number and liver expression area were still observed at 96 hpf in cnr1−/− and cnr2−/− mutants, but were less pronounced by 120 hpf (Fig. S4C-E). Mutants did not exhibit increased TUNEL staining at 96 hpf, excluding cell death as a contributor (Fig. S4F). EdU incorporation assays at 72, 96 and 120 hpf in fabp10a:GFP embryos (Fig. 2E,F) revealed reduced proliferation at 72 hpf in cnr1−/−; fabp10a:GFP and cnr2−/−; fabp10a:GFP embryos, indicating that loss of EC signaling results in hepatocyte proliferation defects. However, by 96 hpf, there is no detectable difference in EdU incorporation rate, although the liver remains smaller in the mutant until normalization of liver size at 120 hpf. Given the gradual normalization of liver size in Cnr mutants by 120 hpf in the absence of hyperproliferation, presumably delayed or suboptimal differentiation and subsequent cellular remodeling of existing hepatic progenitors must occur.

To assess additional morphological defects, hepatic cellular morphology was examined by histology at 120 hpf. cnr1−/− and cnr2−/− hepatocytes have increased cytoplasmic staining and a decreased nucleus-to-cytoplasm ratio compared with WT, whereas overall hepatocyte size remains unchanged (Fig. 3A). These data indicate that, despite apparent recovery of the domain of fabp10a expression at 120 hpf, histological features remain abnormal in Cnr mutant larvae, consistent with a persistent impact on hepatocyte differentiation.

To confirm whether EC signaling affects the maturation of other hepatic cell types, we examined the biliary network using the Notch reporter tp1bglob:eGFP (Parsons et al., 2009). Morpholino-mediated knockdown of cnr1 or cnr2 decreased biliary development, including branching, as visualized by confocal microscopy at 96 hpf (Fig. 3B). The biliary marker 2F11 revealed compact and evenly distributed biliary epithelial cells in larval sections at 120 hpf; by contrast, cnr1−/− and cnr2−/− mutants showed impaired biliary tree formation, with a clustered and less uniform appearance (Fig. 3C).
Fig. 1. Chemical and genetic modulation of endocannabinoid (EC) signaling alters liver size and cell number during zebrafish development.

(A) Quantification of liver size in DMSO-treated and Cnr agonist/antagonist-treated WT embryos from 18-72 hpf. Agonists (O2545, L-HCl, JWH015) increased, whereas antagonists (rimonabant, AM630) decreased, liver size. **P<0.01, DMSO versus L-HCl or rimonabant; *P<0.05, DMSO versus O2545, JWH015 or AM630. (B) Representative ISH images for fabp10a in zebrafish embryos after treatment with cannabinoid drugs from 18-72 hpf. (C) Representative ISH images showing decreased fabp10a expression in cnr1−/− and cnr2−/− mutants compared with WT and heterozygous siblings. (D) Quantification of liver size by measurement of fabp10a expression area after ISH in WT (Tu), heterozygous, and homozygous cnr1−/− and cnr2−/− mutants at 72 hpf. Results represent one independent experiment of triplicate experiments yielding similar results. ****P<0.0001, for cnr1+/− versus cnr1−/− and for cnr2+/− versus cnr2−/−. (E) FACS quantification of fabp10a:GFP hepatocyte number in WT compared with cnr1−/− and cnr2−/− mutants at 72 hpf. The fraction of GFP+ cells was normalized to controls. n=3 pooled samples of ten embryos. *P<0.05. (F) Quantification of fabp10a expression in WT and Cnr mutants by qRT-PCR at 72 hpf. n=3 pooled samples of 20 embryos. *P<0.05. (G) ISH for fabp10a in cnr1−/−; cnr2−/− double mutants at 72 hpf. All error bars show mean±s.e.m. P-values by one-way ANOVA. Scale bars: 0.2 mm.
Fig. 2. Hepatocyte proliferation is disrupted in Cnr mutants without impairment of hepatic progenitors. (A) ISH for hhex at 48 hpf in WT (16 abnormal/89 observed), cnr1+/− (6/28 abnormal), cnr2+/− (5/32 abnormal), cnr1−/− (19/70 abnormal) and cnr2−/− (7/35 abnormal) embryos, revealing no significant differences in the hepatic progenitor population. (B) foxa3 ISH at 48 hpf in WT (19/96 abnormal), cnr1+/− (4/29 abnormal), cnr2+/− (5/30 abnormal), cnr1−/− (12/54 abnormal) and cnr2−/− (4/40 abnormal) embryos, showing no differences in the endoderm population. (C) Relative frequency of liver size distribution of zebrafish embryos treated with Cnr agonists during different stages of liver development, as assessed by fabp10a expression at 72 hpf. Treatment with the Cnr1/2 agonist O2545 (1 μM) or the Cnr2 agonist JWH015 (1 μM) only from 48-72 hpf increased liver size, whereas earlier treatment had no effect. Normal liver size, red; small liver, blue; large liver, green. n>30 embryos. Chi-squared analysis. O2545, ***P<0.001 control versus 48-72 hpf treatment; JWH015, *P<0.05 control versus 48-72 hpf treatment. (D) Treatment with JWH015 from 48-72 hpf increases fabp10a expression in zebrafish embryos at 72 hpf. (E) Confocal projections of fabp10a:GFP livers at 72 hpf after EdU incorporation. EdU-positive hepatocytes are represented by colocalization (arrows) of red EdU staining and hepatocyte-specific GFP expression. (F) FACS quantification of EdU-positive cells as a percentage of the GFP+ population at 72, 96 and 120 hpf. At 72 hpf there is less EdU incorporation compared with controls, indicating a decreased proliferation rate. At 96 and 120 hpf, there is no difference in the proliferation rate. Mean±s.e.m., n=3-8 samples of ten pooled embryos. One-way ANOVA with Holm-Sidak’s multiple comparison test. At 72 hpf, *P<0.01 for control versus cnr1−/−; fabp10a:GFP and **P<0.05 for control versus cnr2−/−; fabp10a:GFP. Scale bars: 0.2 mm in A,B,D; 20 μm in E.
Fig. 3. Livers in Cnr mutants eventually develop to normal size but continue to exhibit architectural and metabolic defects. (A) H&E-stained transverse sections through 120 hpf larvae reveal altered hepatocyte morphology in cnr1−/− and cnr2−/− mutants, with more dense cytoplasm and decreased nucleus-to-cytoplasm ratios compared with WT, but no difference in hepatocyte size. Liver is outlined (white dashed line) and inset shows hepatocytes at higher magnification. (B) Confocal projections at 96 hpf in cnr1 or cnr2 morphants in the tp1glob:eGFP Notch transgenic background, showing smaller liver size and decreased biliary branching and bile duct formation versus the control (6/10 cnr1 morphants, 6/8 cnr2 morphants). (C) Transverse sections of 120 hpf larvae stained with the biliary marker 2F11 and DAPI show an abnormal biliary tree in cnr1−/− (6/8 abnormal) and cnr2−/− (3/4 abnormal) livers. Liver region is outlined. (D) Confocal microscopy of livers in WT, cnr1−/− and cnr2−/− larvae at 6 dpf, showing metabolism of BODIPY C5 throughout the biliary tree. cnr1−/− larvae have decreased biliary branching (n=6/8 with phenotype of this representative image), while cnr2−/− livers are characterized by lipid deposits (arrows, n=7/10 abnormal), as compared with WT (0/12). (E) PED6 lipase reporter assay in cnr1−/− (n=18/40 abnormal) and cnr2−/− (n=7/24 abnormal) mutants demonstrates decreased fluorescence compared with controls (n=0/24 abnormal) at 120 hpf. Fisher’s exact test. cnr1−/− versus WT, P<0.0001; cnr2−/− versus WT, P=0.0094. Arrow marks the gallbladder, which does not fluoresce in the mutants. Scale bars: 0.1 mm in A,C; 20 μm in B,D; 0.2 mm in E.
cnr1−/− and cnr2−/− mutants demonstrate differential susceptibility to metabolic injury

To examine whether abnormal lipid handling during larval development alters susceptibility to pathological lipid accumulation, we utilized an established alcohol-induced steatosis model (Passeri et al., 2009); zebrafish exposed to 2% ethanol from 96-120 hpf develop steatosis at an incidence of 30-60%, as visualized by whole-mount Oil Red O (ORO) staining. cnr1−/−, but not cnr2−/−, mutants are protected from steatosis, with hepatic ORO staining detectable in only 19% of ethanol-exposed cnr1−/− larvae (Fig. S5A,B). These findings were confirmed by treatment with Cnr modulators (Fig. S5C) and in cnr1 and cnr2 morphants (Fig. S5D), and are consistent with reports on the differential role of Cnr in steatosis pathogenesis (Jen et al., 2008; Louvet et al., 2011). Furthermore, hand2, a marker of zebrafish hepatic stellate cells, is usually upregulated in response to liver injury (Yin et al., 2012). Consistent with the ORO findings, hand2 induction was observed in cnr2−/−, but not cnr1−/−, larvae at 120 hpf following ethanol exposure (Fig. S5E,F), consistent with the observed differential expression of cnr1 and cnr2 in different cell types.

To determine the impact of Cnr activity on the clinical problem of nutritionally induced hepatic steatosis, embryos were exposed to 3% egg yolk solution from 96-120 hpf, mimicking a high-fat diet. This treatment induces steatosis in ~65% of WT larvae, but in only 24% of cnr1−/− larvae (Fig. S5G,H); similar to ethanol exposure, Cnr1 and Cnr2 modulation had opposing effects on egg yolk-induced steatosis (Fig. S5I). These findings imply that developmental disruption of EC signaling affects lipid homeostasis and susceptibility to metabolic insult, which may directly affect adult organ function.

cnr1−/− and cnr2−/− mutants have altered metabolism in adulthood

To determine the long-term impact of the observed developmental and metabolic defects on organ homeostasis and lipid metabolism, adult mutant livers were examined. Levels of the endogenous Cnr ligands 2-AG and AEA were unaltered in liver tissue from cnr1−/− and cnr2−/− mutants compared with controls at 6 months, as determined by liquid chromatography/tandem mass spectrometry (Fig. S6A), consistent with findings in adult male Cnr1 knockout mice (Mukhopadhya et al., 2011). Ratios of liver mass to body mass in 6-month-old WT and cnr1−/− and cnr2−/− mutants were comparable (Fig. S6B). By contrast, liver histology revealed cholestasis and inflammatory infiltrates in cnr1−/− mutants. cnr2−/− mutants demonstrated abnormal cellular morphology with increased cytoplasm-to-nucleus ratio, suggesting spontaneous steatosis on a regular diet in the absence of metabolic injury induction (Fig. 4A), further indicating differential action of the receptors in different cell populations. Biliary marker expression was also severely diminished in cnr1−/− and cnr2−/− mutants, consistent with the embryonic biliary phenotype (Fig. 4B). To reveal systemic consequences of reduced Cnr activity and altered lipid metabolism, triglyceride levels were assessed in adult zebrafish serum, revealing significantly elevated levels in cnr2−/− mutants compared with age-matched cnr1−/− mutants and WT (Fig. 4C,D). The abnormal liver architecture and metabolism in adult Cnr mutants indicates that Cnr-regulated alterations in liver development and function can negatively impact adult homeostasis, causing ongoing disruptions in global lipid metabolism.

Defects in EC signaling affect methionine metabolism

To define the metabolites most severely affected by disrupted EC signaling, we performed mass spectrometry-based metabolomic analysis on adult livers, detecting over 290 polar metabolites (Yuan et al., 2012). Metabolites clustered according to genotype without gender differences, indicating significant changes in individual metabolites from cnr1−/− and cnr2−/− livers. Methionine pathway intermediates were significantly altered: levels of methionine, homocysteine, S-adenosylhomocysteine, cysteine, homoserine, serine and S-adenosylmethionine were reduced (Fig. 5A, S6C,D), suggesting dysregulated methionine metabolism.

To further characterize the effects on methionine metabolism in cnr1−/− and cnr2−/− mutants, expression of key metabolic enzymes was examined by qRT-PCR at 120 hpf. S-adenosylhomocysteine hydrolase (ahcy) and spermidine synthase (srn) were downregulated in mutants, whereas methylenetetrahydrofolate reductase (mthfr) was upregulated (Fig. 5B). Analysis of RNA sequencing data from adult male Cnr1 knockout mouse livers (Mukhopadhya et al., 2011) revealed conserved downregulation of most methionine metabolism pathway enzymes (Fig. 5C). As methionine generates methyl groups for several physiological and molecular functions, we examined global protein methylation. Methylated protein levels, based on methyl-lysine detection, were diminished in cnr1−/− and cnr2−/− whole embryo lysates at 120 hpf (Fig. 5D). These data indicate dysregulated methionine metabolism in cnr1−/− and cnr2−/− mutants, which could be the direct result of loss of EC activity or a reflection of altered hepatocyte function.

Methionine rescues liver development defects in Cnr mutants

To define a direct relationship between abnormal methionine metabolism and the observed liver phenotypes in cnr1−/− and cnr2−/− mutants, embryos were exposed to methionine metabolism intermediates. Treatment with methionine or cysteine, but not other amino acids (glycine, homocysteine), at physiological concentrations (100 μM) from 24-72 hpf rescued the liver size defect in cnr1−/− and cnr2−/− mutants as assessed by fabp10a ISH (Fig. 6A,B), while not affecting expression in controls. Methionine-treated cnr1−/− and cnr2−/− larvae demonstrated improved liver histology, with normal cytosol staining and decreased nucleus-to-cytoplasm ratios comparable to WT hepatocellular organization (Fig. 6C). To determine if methionine treatment likewise impacted lipid deposition and biliary morphogenesis, cnr2−/− mutants were treated with methionine beginning at 24 hpf, followed by administration of BODIPY C5 fluorophore at 6 dpf. Methionine-exposed cnr2−/− mutants exhibited increased biliary branching and improved lipid handling, with decreased fatty accumulation (Fig. 6D). Furthermore, treatment with methionine or cysteine protected cnr2−/− mutants, but not cnr1−/− or WT, from ethanol-induced steatosis (Fig. 6E,F). These findings indicate that early methionine supplementation can prevent many of the morphological hepatobiliary and metabolic defects seen with aberrant EC signaling.

To further corroborate that reduced methionine content negatively impacts liver development, we examined a previously characterized methionine metabolism disruption: Alcy functions to break down S-adenosylhomocysteine into adenosine and homocysteine, and ahcy mutant zebrafish display hepatic steatosis and liver degeneration, followed by larval death (Matthews et al., 2012). Consistent with the ORO findings, alcy expression was ∼50% lower in cnr1−/− and cnr2−/− mutants compared with controls at 120 hpf (Fig. 6D). These data indicate dysregulated methionine metabolism in cnr1−/− and cnr2−/− mutants, which could be the direct result of loss of EC activity or a reflection of altered hepatocyte function.
Morpholino knockdown of *ahcy* reduces liver size, as reflected in *fabp10a* expression, at 72 hpf. Exposure of morphants to methionine from 24-72 hpf rescued liver growth, demonstrating that disrupted methionine metabolism can directly impact liver development during hepatogenesis (Fig. S6E,F).

Finally, to determine the long-term impact of methionine exposure, *cnr2*−/− mutants were treated daily with methionine either until larval stages or for 2 months: with long-term intervention, liver histology was improved towards a WT appearance in H&E staining, as compared with untreated *cnr2*−/− controls or those exposed to methionine during embryonic stages alone, suggesting a lack of steatosis (Fig. 6G). These rescue studies indicate the important role of methionine in preventing histological and direct or subsequent metabolic consequences of *cnr2* loss.

**Srebfs mediate the effects of EC signaling**

To identify potential mediators between methionine metabolism and EC signaling, we employed a candidate gene approach: prior studies in *C. elegans* showed that genes involved in one-carbon metabolism are regulated by Srebfs (Walker et al., 2011). Zebrafish homologs *srebf1* and *srebf2* are expressed in endoderm and developing liver beginning at 48 hpf, as determined by ISH and confirmed by qRT-PCR of FACS-sorted *fabp10a:GFP* hepatocytes (Fig. S6G-I). *srebf1* and *srebf2* expression, as determined by qRT-PCR, increased at 72 hpf in *cnr2*−/− mutants, indicating that Srebfs mediate the effects of EC signaling.
PCR, is downregulated in cnr1−/− and cnr2−/− mutants at 120 hpf and remains low in adult livers (Fig. 7A); low Srebf gene expression is also seen in Cnr1−/− male mouse livers (Fig. 7B), indicating conserved regulation by Cnr signaling. MO-mediated srebf1 and srebf2 knockdown decreased liver size at 72 hpf, which cannot be restored by Cnr agonists (Fig. S7A,B). By contrast, overexpression of srebf1 and srebf2 mRNA rescued fabp10a expression in cnr1−/− and cnr2−/− mutants (Fig. 7C,D), together indicating Srebf1/2 functions downstream of Cnr signaling. Further examination of mRNA-injected cnr1−/− and cnr2−/− larvae by H&E staining revealed that srebf1, but not srebf2, overexpression resulted in normalized liver histology, comparable to WT hepatic organization (Fig. S7C); these data suggest distinct roles of Srebf1 and Srebf2 targets in hepatic maturation. Additionally, overexpression of neither srebf1 nor srebf2 could rescue the aberrant BODIPY C5 lipid processing phenotypes of cnr2−/− larvae (Fig. S7D), probably owing to the non-cell-autonomous role of Cnr2 in hepatic development.

To further examine the direct relationship between Srebf and methionine in liver development, we examined their epistatic impact: methionine exposure did not rescue the liver size defects in Srebf morphants (Fig. S7E), probably owing to multiple downstream effects of Srebf during development. To delineate the dependence on methionine of Srebf-mediated rescue of liver defects in Cnr mutants, methionine metabolism was disrupted by ahcy knockdown in the context of Srebf overexpression: here, the Srebf-induced rescue of liver size in cnr1−/− and cnr2−/− embryos was inhibited (Fig. 7E-G). These modified epistatic analyses indicate that Srebf acts downstream of Cnr activity and upstream of methionine metabolism during liver development; however, the
Fig. 6. Treatment with methionine and cysteine can rescue liver development defects in Cnr mutants. (A) Scatter plot of liver morphometric measurements of cnr1−/− and cnr2−/− mutants after 100 μM methionine or cysteine versus DMSO exposure from 24-72 hpf. Results shown represent one independent experiment of triplicate experiments yielding similar results. Mean±s.e.m.; n>15. One-way ANOVA. ***P<0.001, cnr1−/− methionine versus control; **P<0.01, cnr2−/− methionine versus control; *P<0.05, cnr2−/− cysteine versus control. (B) fabp10a expression at 72 hpf in cnr1−/− embryos after DMSO, methionine or cysteine exposure from 24-72 hpf. (C) H&E staining of transverse sections of 120 hpf larvae shows that treatment of cnr1−/− and cnr2−/− mutants with methionine partially restores WT liver histology. cnr1−/−, 6 abnormal/9; cnr1−/−+methionine, 5 normal/7; P=0.065 (Chi-square). cnr2−/−, 6 abnormal/9; cnr2−/−+methionine, 6 normal/8; P=0.043 (Chi-square). (D) Confocal microscopy images of livers in cnr2−/− larvae after BODIPY C5 incorporation with and without methionine treatment. Extensive biliary branching is observed in WT larvae, and decreased branching with lipid and cholestatic deposition in cnr2−/− mutants (7 abnormal/10). Treatment of cnr2−/− mutants with methionine causes increased biliary branching and decreased lipid accumulation (2 abnormal/6). (E) Percentage of larvae with fatty liver after ethanol-induced liver injury and with methionine or cysteine treatment for cnr1−/− and cnr2−/− mutants at 120 hpf. n>30 samples. Fisher’s exact test. *P<0.05 and **P<0.01 for methionine and cysteine versus DMSO. (F) ORO stain in cnr2−/− embryos at 120 hpf after 2% ethanol exposure alone or co-treated with 100 μM methionine or cysteine from 96-120 hpf. Liver region is outlined. (G) H&E staining of liver sections in 2-month-old male zebrafish. cnr2 morphant liver histology appears similar to that of WT; cnr2−/− and cnr1−/−; cnr2−/− double mutants have decreased cytoplasmic hepatocyte staining. Early methionine treatment of cnr2−/− mutants had no effect on liver histology; however, daily methionine altered histological appearance towards WT features, with increased cytoplasmic staining and decreased cytoplasm-to-nucleus ratios. n=5/group. Scale bars: 0.2 mm in B,F; 0.1 mm in C; 20 μm in D; 50 μm in G.
Fig. 7. EC signaling regulates methionine metabolism via Srebf in Cnr mutants. (A) srebf1 and srebf2 expression in pooled WT, cnr1−/− and cnr2−/− zebrafish embryos at 120 hpf, and in adult livers, quantified by qRT-PCR. Data analyzed using the ΔΔCt method. Mean±s.e.m.; n=3 pooled biological samples of 20 embryos each. One-way ANOVA. ****P<0.0001, ***P<0.001 and **P<0.01 for srebf1 or srebf2 expression in mutants versus WT. (B) Srebf1 and Srebf2 expression in male Cnr1−/− mice. n=6. (C,D) Scatter plot of liver morphometric measurements (C) and fabp10a ISH (D) at 72 hpf in cnr1−/− and cnr2−/− mutants with and without overexpression of srebf1 and srebf2 mRNA. Results shown represent one independent experiment of triplicate experiments which yielded similar results. Mean±s.e.m.; n>15. One-way ANOVA. ***P<0.001 for cnr1−/− control versus srebf1 or srebf2 overexpression; **P<0.01 for cnr2−/− control versus srebf1 overexpression; ****P<0.0001 for cnr2−/− control versus srebf2 overexpression. (E-G) Scatter plot of liver morphometric measurements (E,F) and live imaging (G) of cnr1−/−; fabp10a:GFP and cnr2−/−; fabp10a:GFP at 72 hpf with overexpression of srebf1 and srebf2 in the presence and absence of concurrent ahcy morpholino knockdown. Results represent one independent experiment of triplicate experiments yielding similar results. Mean±s.e.m.; n>15. Student’s t-test. *P<0.05 in cnr1−/− background (E); **P<0.01 in cnr2−/− background (F). Scale bars: 0.2 mm.
inability to reach linear correspondence across all assays suggests that Cnr and Srebf have additional downstream mediators and targets that are independent of methionine and that contribute to the complex hepatobiliary maturation and metabolic phenotypes.

**DISCUSSION**

In this study, we characterize the requirement for Cnr1 and Cnr2 activity in normal zebrafish liver development and function. Cnr loss impairs hepatocyte differentiation and proliferation and alters biliary development and function, but spares earlier stages of endoderm specification. Even though cnr1 and cnr2 are expressed in different liver cell types at 72 hpf, EC signaling is required for the establishment and maintenance of proper hepatic physiology and lipid metabolism during embryogenesis. Srebf and methionine act downstream of Cnr signaling and are responsible for several of the phenotypes observed in cnr1−/− and cnr2−/− mutants. This important interaction demonstrates a novel role for EC signaling during liver development that has functional consequences for metabolic homeostasis in the adult organ.

**EC signaling through Cnr is required for normal liver development**

Our results demonstrate that the lack of a normal hepatocyte population during hepatogenesis impedes proper liver physiology throughout life. Although important for optimal liver function, cnr1 and cnr2 are not essential, as cnr1−/−; cnr2−/− double mutants reach adulthood and are fertile. EC signaling has previously been implicated in promoting trophoblast cell lineage differentiation (Sun et al., 2010) and neural development (Palazuelos et al., 2012; Psychoyos et al., 2012); one indication that Cnr signaling may also affect endodermal development and/or metabolic function was observed in Cnr1−/− ob/ob double-mutant mice, which exhibit growth retardation and exacerbated glucose intolerance (Li et al., 2013). The developmental phenotypes observed in Cnr mutant zebrafish support previously described liver abnormalities in Cnr knockout mice: the impact of Cnr activity on embryonic liver maturation might explain the delayed or aberrant hepatocyte proliferation and recovery of liver mass seen after partial hepatectomy in adult Cnr1 and Cnr2 knockout mice (Teixeira-Clerc et al., 2010), as the recapitulation of proliferation-promoting developmental pathways has been shown to be important during liver regeneration (Goessling et al., 2008; Nissim et al., 2014; Yin et al., 2012).

Our findings shed new insights into common metabolic diseases such as hepatic steatosis, obesity and diabetes, which have complex genetic causes. Patients with genetic variants or deficiencies of CNRI, CNR2, the enzyme fatty acid amidase hydrolase (FAAH), which breaks down ECs, and methionine metabolism enzymes such as AHCY, PEMT, GNMT, MTHFR and CBS, have a higher likelihood of developing metabolic abnormalities or liver disease (Baric et al., 2004; Coppola et al., 2014; Feng et al., 2010; Floreani et al., 2010; Mato et al., 2008; Sipe et al., 2005). Our work provides evidence that the developmental defect itself and/or a persisting metabolic abnormality may significantly impact adult liver homeostasis. These findings offer therapeutic opportunities for early or long-term interventions that might minimize disease phenotypes later in life. For example, modulation of peripheral Cnr alone can impact nutrient utilization and response to excessive nutrient overload, and prior studies have utilized the Cnr1 antagonist rimonabant for obesity treatment. Our studies further suggest that normal Cnr2 activity, present not in hepatocytes but in another liver cell population, is likewise important for optimal liver and metabolic function.

**Importance of methionine for development and hepatic function**

Methionine metabolism generates S-adenosylmethionine (SAMe), which is required for multiple cellular functions, including as a methyl donor for nucleic acid, phospholipid, and protein methylation processes during epigenetic regulation. Methionine is also crucial for protein synthesis as an amino acid, and is therefore essential for cellular proliferation and function. Mammalian models for studying methionine metabolism enzymes also reveal the importance of this pathway for early development: knockout of methionine synthase (Mtn) leads to post-implantation embryonic death, while loss of Mthfr causes smaller body size and growth retardation (Chen et al., 2001; Swanson et al., 2001). Correlations between diet and environmental exposure leading to long-term metabolic dysregulation are established in mammals, with maternal diet impacting DNA methylation in offspring (Ozanne, 2014). Specifically, reducing methionine in the mammalian diet disrupts proper DNA methylation, leading to increased adiposity (Sinclair et al., 2007), while lack of proper embryonic nutrition can lead to DNA methylation defects and aberrant adult metabolism (Radford et al., 2014). Even paternal diet low in protein can affect cytosine methylation and gene expression in the mouse embryo, impacting hepatic lipid metabolism, including Srebf function (Carone et al., 2010). The developmental and adult phenotypes observed in the Cnr mutants might at least in part be mediated by abnormalities in methionine metabolism during development.

Methionine metabolism enzymes are primarily expressed in the liver, and abnormal methionine metabolism has been linked to hepatic steatosis: a methionine-choline-deficient diet has long been associated with hepatic steatosis in mouse models (Best et al., 1936). Zebrafish ahcy mutants, defective in metabolizing S-adenosylhomocysteine into adenosine and homocysteine, exhibit steatosis and liver degeneration (Matthews et al., 2009), while mouse knockouts for the Mat1a, Pemt, Gnmt, Cbs and Mthfr enzymes develop steatohepatitis and hepatocellular carcinoma, potentially via decreased methylation or AMP-activated protein kinase inhibition (Mato et al., 2008). Intriguingly, SAMe supplementation appears to prevent hepatocellular carcinoma in rodent models (Pascale et al., 1992). Our long-term methionine treatment of cnr2−/− mutants improves histological abnormalities, further emphasizing the importance of proper methionine levels for hepatic structure and function.

**EC signaling regulates methionine metabolism via Srebf**

Cnr1 and Cnr2 are G-protein coupled receptors that inhibit adenylyl cyclase and decrease cyclic AMP levels while stimulating MAP kinase, resulting in downstream activation of the ERK, FAK, JNK or PI3K/AKT pathways to control cell fate (McAllister and Glass, 2002; Pagotto et al., 2006). Although methionine adenosyltransferase (Mat1a) can regulate ERK and PI3K/AKT (Chen et al., 2004; Frau et al., 2013; Tomasi et al., 2010), these pathways have additional upstream activators besides ECs, and a direct relationship between Cnr signaling and methionine metabolism has not previously been established. Srebf are established downstream targets of EC signaling (Jeong et al., 2008). Although defects in EC signaling or methionine metabolism components are independently associated with hepatic steatosis, fibrosis or liver cancer (Huang et al., 2011; Mato et al., 2008; Pisanti et al., 2013), here we highlight a previously unknown pathway between EC signaling, Srebf and methionine metabolism that impacts liver development and function. Further studies are necessary to define the cell types involved and whether this
regulation involves autocrine, paracrine or other signaling mechanisms.

Srebfs promote fatty acid and cholesterol uptake and synthesis and can act downstream of EC signaling in the liver, with levels that can be regulated in zebrafish and mice by administration of the EC anandamide (Migliarini and Carnevali, 2008; Pai et al., 2013). In mice, modulation of dietary methionine can alter Sreb expression by feedback induction (Aissa et al., 2014). Similarly, expression of methionine metabolism genes in C. elegans not only depends on SREBF1 (SBP-1), but also feeds back to control SREBF1 and affect lipid metabolism (Walker et al., 2011). In our studies, overexpression of sreb1f and sreb2f in cma1−/− and cma2−/− zebrafish embryos restores normal liver size. However, whereas liver growth could be rescued in Cmr mutants with methionine, this was not possible in sreb1f and sreb2f morphants. This is likely to indicate that the impact of Sreb knockdown goes beyond methionine regulation. Srebfs can directly regulate virtually all enzymes involved in the biosynthesis of cholesterol (Horton et al., 2003), which, among other functions, is a central component of cell membranes and required for cell proliferation. This conclusion is also supported by the complete or partial embryonic lethality of Srebf knockout mice (Shimano et al., 1997). However, our epistatic analysis performed in the context of Sreb overexpression demonstrates a dependence on methionine metabolism downstream of Srebf to mediate the effect of Cmr signaling on liver development.

In summary, in an unbiased screen for novel regulators of liver development in zebrafish we have uncovered pathways that can reveal insights into developmental metabolism and adult liver homeostasis with implications for mammalian species. We define a novel role for EC signaling that integrates and coordinates the organism’s metabolic needs by regulating appetite in the CNS, while also promoting liver proliferation and metabolic function, achieved in part through the regulation of methionine metabolism.

MATERIALS AND METHODS

Zebrafish husbandry

Zebrafish were maintained according to standard Institutional Animal Care and Use Committee guidelines.

Generation of cma1−/− and cma2−/− mutants

TALE repeat arrays (designed using ZiFiT Targeter, http://zifit.partners.org/ZiFiT/) targeting cma1 and cma2 genes using the REAL system were cloned into TALEN vectors (Sander et al., 2011). TALEN mRNA pairs were injected into one-cell stage Tubingen (Tu) WT embryos, and somatic mutation rates from pooled embryos were analyzed. Adult founders were identified by assessing clutches for heterozygous progeny. Heterozygotes were raised to adulthood, genotyped, and outcrossed for at least three generations to reduce TALEN off-target effects.

In situ hybridization

ISH was conducted on paraformaldehyde-fixed embryos using standard protocols (Thisoe and Thisse, 2008). Probes for sreb1f and sreb2f were generated as described in the supplementary Materials and Methods. Changes in fabp10a, sihd, gc, tfa, hhex, foxa3, proxl and fabp2 expression were scored using ImageJ (NIH) to quantify liver, endoderm or progenitor population size in images. ANOVA was conducted for comparison of liver sizes across multiple groups. cma1, cma2, hand2, sreb1f and sreb2f expression patterns were assessed throughout development. Other expression patterns were scored as ‘small’, ‘normal’ or ‘large’ within a population distribution, consistent with the quantitative size distribution. The percentage of the total population that was altered was calculated for each genotype/treatment group.

Fluorescence-activated cell sorting

fabp10a-GFP embryos were incubated in 50 µg/ml liberase (Roche) at 37°C for 1.5 h, manually dissociated, and stained through a 35 µm nylon mesh filter. For cell quantification, GFP+ cells were counted using a BD FACSaria II flow cytometer. For cell sorting, GFP+ and GFP− fractions were separated using a BD FACSaria II SORP flow cytometer (Goessling et al., 2008).

qRT-PCR

RNA extracted from pooled embryos or adult livers using Trizol was treated using the TURBO DNA-free DNase kit (Life Technologies); cDNA was generated using Script cDNA synthesis reagents (Bio-Rad). Cells isolated by FACS (~200,000) were processed using the RNeasy-Micro Total RNA Isolation Kit (Thermo Fisher) according to the manufacturer’s protocol. Amplified cDNA was prepared using the Ovation Pico WTA System V2 (NuGEN). All qRT-PCR was performed using SYBR Green Supermix (Bio-Rad) and relative expression levels were calculated using the ΔΔCt method. Primers are listed in Table S1.

EdU incorporation and TUNEL staining

EdU incorporation was conducted using the Click-IT Plus EdU Alexa Fluor 647 Flow Cytometry and Imaging Kit (Life Technologies). Embryos were incubated on ice for 10 min, then incubated with 400 µM EdU for 2 h at 28°C, and labeled following the manufacturer’s protocol. EdU-positive cells were visualized by confocal microscopy or processed using the above cell sorting procedure. TUNEL staining for apoptotic cells is described in the supplementary Materials and Methods.

Chemical exposure

Chemicals used are listed in Table S3 and were utilized at the indicated concentrations.

Oil Red O staining

Whole-mount Oil Red O staining was conducted as previously described (Passeri et al., 2009). Embryos were scored based on the presence of red lipid droplets in the liver. Fisher’s exact test was used to compare changes in liver phenotype.

Histology and immunohistochemistry

Adult zebrafish midsections and embryos were processed for Hematoxylin and Eosin (H&E) staining using standard protocols, and examined together with Dr Jason Hornick, a board-certified pathologist. Immunohistochemistry was performed using 2F11 antibody and FITC fluorescent secondary antibody (see Table S4).

Analysis of lipid metabolism in live zebrafish embryos

Embryos were exposed to 0.1 µg/ml PED6 (Farber, 2001) at 12 hpf for 6 h, washed, and then imaged by fluorescence microscopy. BODIPY C5 fluorophore (Invitrogen) was utilized to observe lipid metabolism and digestive organ morphology in vivo. Embryos were incubated in 6.4 µM BODIPY C5 in 5% egg yolk solution at 6 dpf for 4 h, followed by confocal microscopy.

Serum lipid tests

Adult zebrafish blood was collected by tail incision (Baba et al., 2013). Serum triglyceride and cholesterol concentrations were analyzed using micropalte-based enzyme activity kits (Pointe Scientific).
Polar metabolomics
Quantitative polar metabolomics profiling was performed after extraction from zebrafish livers on AB/SCIEX 5000 QTRAP LC/MS/MS instrumentation, using previously described protocols (Yuan et al., 2012).

EC measurements
The tissue levels of ECs were measured as described in the supplementary Materials and Methods.

Western blotting
Proteins extracted from pooled larvae populations at 120 hpf were resolved using SDS-PAGE, and methylated lysine was detected with specific primary antibody and HRP-conjugated secondary antibody, as listed in Table S4.

mRNA injection
srebf1 and srebf2 were amplified from WT zebrafish cDNA using the primers (5'-3'): srebf1, AAGAGCCATCCGAGGACATG and GTGTCAGGTGATGGTGAGCG; srebf2, TGTTGAGTGCAGCGAGTGAGCT GGTGATGCAGCTGGTG. Polyadenylated mRNA was transcribed using the mMESSAGE mMACHINE Transcription Kit (Ambion), and injected into one-cell stage zebrafish embryos at 100 pg/ nl.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
L.Y.L. and W.G. conceived and designed the experiments and analyzed the data. L.Y.L. performed all zebrafish experiments. T.E.N. and W.G. conducted the original polar metabolomics assessment; Dr Ruma Banerjee for helpful discussions; and staff of the Beth-Israel Deaconess Medical Center Zebrafish Facility for zebrafish husbandry.

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