Zebrafish mutants provide insights into apolipoprotein B functions during embryonic development and pathological conditions

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Apolipoprotein B (ApoB) is the primary protein of chylomicrons, VLDLs, and LDLs and is essential for their production. Defects in ApoB synthesis and secretion result in several human diseases, including abetalipoproteinemia and familial hypobetalipoproteinemia (FHBL1). In addition, ApoB-related dyslipidemia is linked to nonalcoholic fatty liver disease (NAFLD), a silent pandemic affecting billions globally. Due to the crucial role of ApoB in supplying nutrients to the developing embryo, ApoB deletion in mammals is embryonic lethal. Thus, a clear understanding of the roles of this protein during development is lacking. Here, we established zebrafish mutants for 2 apoB genes: apoBa and apoBb.1. Double-mutant embryos displayed hepatic steatosis, a common hallmark of FHBL1 and NAFLD, as well as abnormal liver laterality, decreased numbers of goblet cells in the gut, and impaired angiogenesis. We further used these mutants to identify the domains within ApoB responsible for its functions. By assessing the ability of different truncated forms of human ApoB to rescue the mutant phenotypes, we demonstrate the benefits of this model for prospective therapeutic screens. Overall, these zebrafish models uncover what are likely previously undescribed functions of ApoB in organ development and morphogenesis and shed light on the mechanisms underlying hypolipidemia-related diseases.

Introduction

Apolipoprotein B (ApoB) is the primary structural component of atherogenic lipoproteins, such as chylomicrons, VLDLs, and LDLs, and is essential for their assembly (1). Elevated levels of LDL are widely recognized as a cardiovascular risk factor, and abundant data point to chemically modified LDL and ApoB as triggers of most of the features of the pathobiology of atherosclerosis. Besides the important role of ApoB in lipid metabolism, a large body of data, accumulated during the past years, has revealed new roles for lipoproteins as signaling mediators in various cell types, operating at different levels and through various classic and nonclassic mechanisms (2).

Two APOB isoforms, APOB100 and APOB48, are present in humans and mice, which are encoded by a single gene and generated by RNA editing (3). Defects in ApoB synthesis and secretion, resulting from mutations in the ApoB, microsomal triglyceride transfer protein (MTP), and proprotein convertase subtilisin/kexin type 9 genes, lead to abetalipoproteinemia (ABL) and familial hypobetalipoproteinemia (FHBL; refs. 4, 5), two disorders characterized by low or absent levels of ApoB and LDL-cholesterol in plasma. ABL and homozygous FHBL display similar clinical symptoms, including steatorrhea, neurological dysfunction, ophthalmologic abnormalities, and fatty liver. Since both disorders also carry a reduced risk of cardiovascular diseases (CVDs), research into the underlying molecular mechanisms could uncover attractive targets for lipid-lowering therapies in patients with hypercholesterolemia.

In addition to the involvement of ApoB in atherosclerosis and CVD, ApoB-related dyslipidemia is linked to another global epidemic, nonalcoholic fatty liver disease (NAFLD), which affects around 20%–30% of the adult population in Western countries (6). NAFLD results in steatosis, the accumulation of lipid in hepatocytes (7), which can lead to liver malfunction, cirrhosis, and, in some cases, hepatocellular carcinoma (8). Defects in production and/or secretion of ApoB lipoproteins are highly linked to hepatic steatosis (9). In particular, the impaired synthesis of VLDL observed in patients with FHBL, which results in triglyceride accumulation in the liver, represents one among many causes of NAFLD in humans (10). The crucial role...
ApoB plays in various pathological conditions has prompted the generation of several genetically modified mouse models (11, 12). However, because APOB is expressed in the yolk sac during early embryonic development, where it facilitates the supply of nutrients to the developing embryo, ApoB-knockout mice die at gestational stage 9.5 to 10.5, thereby precluding the study of the roles of this protein during embryogenesis.

The close relationship between zebrafish and human apolipoprotein expression and function (13, 14) makes the former an attractive animal model for studying the repertoire of ApoB functions during embryonic development. In addition, the well-documented ability of zebrafish to develop hyperlipidemia (15, 16) and lipoprotein oxidation (17, 18) motivated the study of the cellular and molecular mechanisms linking lipoproteins and CVD in this animal. Here, we established mutants for 2 zebrafish apoB genes: apoBa and apoBb.1. We found that double-mutant embryos displayed lipid accumulation in hepatocytes, a common hallmark of FHBL1 and NAFLD. In addition, we detected early developmental defects, including abnormal liver laterality, decreased numbers of goblet cells in the gut, and impaired angiogenesis in double apoB mutants. Interestingly, we found altered Notch signaling to underlie some of the observed phenotypes. To identify the domains within ApoB associated with the antiangiogenic function, we assessed the ability of truncated forms of the human APOB (APOB25 and APOB34) to rescue the zebrafish mutant phenotypes. We found that only mutant embryos injected with APOB34 displayed a significant rescue of the vascular phenotypes, suggesting that the antiangiogenic activity of ApoB is contained between the 25 to 34 N-terminus remnant of the APOB protein. Altogether, these results uncover previously unappreciated functions of ApoB during early organ development and highlight the potential of these newly established apoB zebrafish mutants as models for studying human pathologies associated with hypolipidemia, as well as for related drug screens.

Results

**Generation of zebrafish apoB mutants.** Three apoB genes are detected in the zebrafish genome: apoBa, apoBb.1, and apoBb.2 (14, 19). Two days postfertilization (dpf) embryos displayed strong expression of apoBa and apoBb.1 mRNA in the yolk syncytial layer (YSL), whereas apoBb.2 expression was undetectable (Figure 1, A, B, and E). At 5 dpf, apoBa expression became restricted to the liver (Figure 1C, arrowhead), and apoBb.1 was detected mostly in the YSL and the intestine (Figure 1D). To investigate the role of ApoB during embryonic development, we generated zebrafish carrying mutations in the apoBa and apoBb.1 genes. sgRNAs targeting exon 5 of the apoBb.1 gene (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.130399DS1) were injected along with cas9 mRNA into 1-cell-stage Tg(fli1:EGFP) embryos (20). F0 injected fish were raised to adulthood and screened for germline transmission. We identified 2 different mutants, including an 18 bp insertion containing a premature in-frame stop-codon (Supplemental Figure 1A), which was used throughout this study. To target the apoBa gene, we used the transcription activator-like effector nuclease (TALEN) technology. mRNA-encoding left and right TALENs designed to hit exon 3 of the apoBa gene (Supplemental Figure 1B) were injected into 1-cell-stage Tg(fli1:EGFP) embryos. We identified 3 mutations, out of which we chose to focus on an 8 bp deletion in the TALENs’ target site, introducing a premature stop-codon after 70 amino acids (Supplemental Figure 1B). Both apoBa and apoBb.1 mutants displayed strong decreases of the respective mRNA levels (Figure 1E). In addition, Western blot analysis demonstrated complete absence of ApoB in apoBb.1 but not in apoBa mutants (Supplemental Figure 1C), confirming previous data showing that ApoBb.1 is the predominant isoform (~95% abundance; ref. 14).

**Characterization of the mutant phenotypes.** apoBa homozygous mutants were viable, fertile, and morphologically indistinguishable from their WT siblings (Figure 1, F and H, and Supplemental Figure 2A). Moreover, lipid distribution was normal in these mutants, as shown by Oil Red O (ORO) staining (ref. 15 and Figure 1, G and I). Similarly, injection of apoBa antisense morpholino oligonucleotides (MOs) did not elicit any noticeable phenotypes (Supplemental Figure 2, D and E). In contrast, apoBb.1 homozygous mutants presented with several defects. First, they were easily identified due to their dark yolk (Figure 1J and Supplemental Figure 2B), indicative of impaired lipid absorption and aberrant accumulation of lipid droplets in the YSL (15, 21). This was also verified by ORO staining, which showed a strong reduction in circulating lipid levels (Figure 1K). Injection of apoBb.1 MOs into WT embryos fully phenocopied the mutant phenotype (Supplemental Figure 2F). However, no effects were observed following injection of apoBb.1 MOs into apoBb.1 mutants (Supplemental Figure 2, G and H), confirming the MO specificity. The central role of the apoBb.1 isoform was further corroborated by the decreased viability of the apoBb.1 mutants (Supplemental Figure 2L). In addition, we noticed that the few apoBb.1<sup>−/−</sup> animals that survived by approximately 60 dpf were significantly thinner than their WT counterparts (Supplemental Figure 2M).
Although ApoBb.1 represents the predominant ApoB isoform, we suspected that the presence of ApoBa in apoBb.1 mutants could compensate for the lack of its functions. Therefore, we generated apoBa apoBb.1 double homozygous mutants (Figure 1L and Supplemental Figure 2C). These animals displayed a darker yolk and complete absence of lipids in circulation (Figure 1M). Moreover, the lack of both ApoB isoforms led to lethality at 6 to 8 dpf, with the larvae exhibiting profound yolk and pericardial edema, as well as a curved trunk (Figure 1, N and O). Finally, injection of apoBb.1 MOs into apoBa homozygous mutants or vice versa (apoBa MOs into apoBb.1–/– embryos) recapitulated the phenotypes of apoB double mutants (Supplemental Figure 1C and Supplemental Figure 2, I–K).

Figure 1. apoB mutants feature severe hypolipidemia. (A–D) Whole mount in situ hybridization (WISH) on 2 dpf zebrafish embryos showing strong expression of apoBa (A) and apoBb.1 (B) in the YSL (napoBa = 7; napoBb.1 = 6). At 5 dpf apoBa expression is enriched in the liver (C) and apoBb.1 in the YSL and the intestine (D) (napoBa = 3; napoBb.1 = 3). (E) Semiquantitative PCR for the apoB genes in the different mutants. (F–M) Transmitted light images of 2 dpf embryos showing dark yolk in apoBb.1+/− (J) and apoBa–/− apoBb.1+/− (L) but not in WT (F) or apoBa–/− (H) mutant embryos. Three dpf embryos stained with Oil Red O (ORO) show decreased lipid levels in apoBb.1 mutant (K), as compared with WT (G) and apoBa–/− (I), embryos. (M) apoBa apoBb.1 double mutants display complete absence of lipids in circulation. (N and O) Transmitted light images at 6 dpf demonstrate severe malformations, unabsorbed yolk, and pronounced edema in apoBa apoBb.1 double mutants as compared with WT siblings. (P and Q) Cholesterol (P) and TG (Q) levels in the different apoB mutants compared with WT controls at 3 dpf. All measurements were carried out in deyolked embryos. n = 3.  nembryos/sample = 20. The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. Scale bar: (A–D; F–M) 100 μm, (N and O) 1 mm. *P < 0.05, **P < 0.01, ***P < 0.001. P < 0.05 (considered significant versus control group).
We then carried out a lipid profile analysis of the different mutants. In order to accurately measure yolk-to-body ApoB-dependent transport, we utilized deyolked embryos. As seen in Figure 1P, we found no differences in cholesterol levels between apoB<sup>−/−</sup> and apoB<sub>b.1</sub>−/− individual mutants. In contrast, these were strongly reduced in both apoBa apoB<sub>b.1</sub> double mutants and apoBa<sup>−/−</sup> apoB<sub>b.1</sub>MO, as compared with WT siblings (Figure 1P). Interestingly, triglyceride (TG) measurements revealed a slightly different picture (Figure 1Q). While in apoBa<sup>−/−</sup> mutants TG levels were similar to those of WT siblings, levels were strongly decreased in apoB<sub>b.1</sub> mutants (Figure 1Q). In addition, TG levels were significantly reduced in both apoBa apoB<sub>b.1</sub> double mutants and in apoB<sub>b.1</sub>−/− apoB<sub>b.1</sub>MO (Figure 1Q).

Due to the difficulty in obtaining large numbers of double homozygous mutants from apoBa<sup>−/−</sup> × apoB<sub>b.1</sub>−/− crosses, and having verified that the apoB<sub>b.1</sub> MO fully recapitulates the mutant phenotype, we decided to use apoBa<sup>−/−</sup> apoB<sub>b.1</sub>MO embryos in most experiments of this study.

Defective organ development in apoB mutants. Next, we analyzed the development of the liver and the intestine, the main organs responsible for ApoB synthesis, assembly, and secretion in vertebrates (3). We took advantage of the fact that, in contrast to ApoB-null mice, apoBa<sup>−/−</sup> apoB<sub>b.1</sub>−/− double mutants survive beyond the initial stages of formation of the digestive system to investigate the role of ApoB during normal formation of these organs. To examine liver morphology, we used Tg(2.8<sub>fabp10a</sub>:EGFP)<sup>−/−</sup>, a well-established transgenic reporter expressing EGFP under the regulation of the fatty acid-binding protein 10A (<sub>fabp10a</sub>) promoter (22). Injection of apoB<sub>b.1</sub> MOs into Tg(fabp10a:EGFP) apoBa<sup>−/−</sup> embryos resulted in significantly reduced liver size at 4 dpf (Figure 2A and Supplemental Figure 3A), which was not due to apoptotic cell death, as confirmed by TUNEL staining (Figure 2B). Similar results were obtained following in situ hybridization for <sub>fabp10a</sub> (Figure 2C), suggesting a defect in hepatocyte specification, differentiation, and/or proliferation. To distinguish between these possibilities, we analyzed the expression of prospero homeobox 1a (<sub>prox1a</sub>), a transcription factor essential for hepatocyte differentiation and liver development (23, 24). As seen in Figure 2D, <sub>prox1a</sub> hepatic progenitors were normally detected to the left of the midline at 2 dpf, in phenotypically normal apoB<sub>b.1</sub>−/− embryos. By contrast, loss of both apoBa and apoB<sub>b.1</sub> caused embryos to develop bilateral livers (Figure 2D, arrowheads), suggesting an early role for ApoB in controlling liver laterality. This defective positioning of hepatoblasts was still detected at 4 dpf, as depicted by the expression of transferrin (Tfα), an additional liver-specific marker (ref. 25 and Figure 2E). Interestingly, these defects were specific for the liver because we did not detect laterality problems in gut looping (Figure 2F, arrowheads, and Supplemental Figure 3B) or in early left-right asymmetry markers, such as lefty1 (not shown). These results indicate that hepatoblast specification is initiated normally in apoB-mutant embryos but that liver formation begins to be affected after 2 days of development.

Liver organogenesis in zebrafish involves 2 main phases: first, specification and migration of hhex<sup>+</sup> and prox1<sup>+</sup> hepatoblasts to form the liver bud on the left side of the embryo, and second, hepatoblast differentiation into hepatocytes and biliary epithelial cells (BECs), followed by massive cell proliferation (26). To check whether the development of the intrahepatic biliary network is also affected in apoB mutants, we used the Tg(EPV-Tp1-Mnu.Hbb:EGFP)<sup>−/−</sup> reporter (12xNRE:EGFP), which consists of 12 repeats of Notch-responsive elements driving EGFP expression (27), because Notch signaling in hepatic progenitor cells was shown to be both required and sufficient for biliary specification (28). At 5 dpf, the Tg(12xN-RE:EGFP) positive intrahepatic biliary network was highly branched in WT, apoBa<sup>−/−</sup>, and apoB<sub>b.1</sub>−/− larvae (Figure 2G), as opposed to apoBa<sup>−/−</sup> apoB<sub>b.1</sub>MO that displayed largely reduced Notch activity. Taken together, the observed phenotypes suggest that, while ApoB is not required for initial hepatocyte specification, it appears to play an important role in hepatoblast positioning (i.e., budding and/or migration), proliferation, and segregation between hepatocytes and BECs.

Hepatic steatosis, the accumulation of lipid within hepatocytes, is a critical step in the pathogenesis of several human diseases, including alcoholic liver disease and NAFLD. While NAFLD is mostly associated with hyperlipidemia (29), defective synthesis and secretion of ApoB lipoproteins can also lead to hepatic steatosis, as observed in patients with FHBL1 (9, 30). To investigate whether the different apoB mutants develop steatosis, we utilized Nile red staining, which labels polar lipids in red and neutral lipids in green (31, 32). As expected, liver lipid stores were nearly undetected in WT and apoBa<sup>−/−</sup> mutants (Figure 2H), as opposed to apoB<sub>b.1</sub>MO, which featured excessive accumulation of lipid droplets in hepatocytes (Figure 2H). Interestingly, lipid droplet accumulation was less severe in the livers of apoBa<sup>−/−</sup> apoB<sub>b.1</sub>MO embryos (Figure 2H), most probably due to the impaired YSL lipoprotein production (Figure 2H, arrowheads). These results were confirmed by H&E staining of histological sections (Supplemental Figure 3C, arrowheads).
Figure 2. apoB mutants exhibit defective liver development and steatosis. (A) Confocal images at 4 dpf of apoBa–/– and apoBa+– apoBb.1MO embryos in Tg(-2.8fabp10a:EGFP)as3 background, showing smaller liver and decreased expression of fabp10a EGFP in apoBa–/– apoBb.1MO embryos (nWT = 8, napoBa–/– apoBb.1MO = 7/7). (B) No apoptotic cells are detected in the liver of apoBa–/– or apoBa+– apoBb.1MO embryos, following TUNEL staining (nWT = 6, napoBa–/– apoBb.1MO = 5/5). (C) WISH showing expression of fabp10a at 4 dpf, depicting decreased liver size in apoBa–/– apoBb.1MO embryos (nWT = 7, napoBa–/– apoBb.1MO = 13). (D and E) WISH with riboprobes against prox1a at 2 dpf (D) (nWT = 4, napoBa–/– apoBb.1MO = 7/9) and tfa at 4 dpf (E) (nWT = 7, napoBa–/– apoBb.1MO = 5/6) shows bilateral liver buds in apoBa–/– apoBb.1MO embryos as opposed to apoBa–/– mutants, which display a single liver bud located to the left of the midline. (F) Expression of forkhead box a2 (foxa2) at 2 dpf remains unchanged in apoBa–/– apoBb.1MO embryos as compared with apoBa–/– siblings. (G) Confocal images of 5 dpf Tg(12xNRE:Egfp) embryos with outlined livers, showing decreased Notch signal in the bile ducts of apoBa–/– apoBb.1MO embryos (nWT = 4, napoBa–/– apoBb.1MO = 8, napoBb.1–/– apoBb.1MO = 6, napoBa–/– apoBb.1MO = 20). (H) Confocal images of Nile red staining at 4 dpf depicting neutral lipid accumulation in apoBb.1MO and apoBa–/– apoBb.1MO embryos. Red staining labels polar lipid; green fluorescence highlights neutral lipids (nWT = 5, napoBa–/– = 8, napoBb.1–/– = 5, napoBa–/– apoBb.1MO = 9). Scale bar: (A, B, G, and H) 50 μm, (C–F) 100 μm.
Because of the close relationship between liver and intestine formation, we tested whether ApoB depletion also affects gut development. The intestine is responsible for the absorption of lipids and their secretion in the form of chylomicrons into lymphatic vessels. Analysis of foxa2 (ref. 33; Figure 2F, arrowheads; and Supplemental Figure 3B) and fatty acid binding protein 2 (fabp2) (ref. 25 and Figure 3A) expression revealed normal development of the intestinal tube in individual mutants at 2 dpf. However, by 4 dpf we noticed that the gut appeared somewhat reduced in size in apoBa–/– apoBb.1MO embryos (Figure 3B). Yet, the

**Figure 3.** Gut development is impaired in apoB mutants. (A and B) WISH showing the spatial expression of fabp2 at 2 (A) and 4 (B) dpf. (C-E) Confocal images at 4 dpf of WT (C), apoBa–/– apoBb.1MO (D), and mtpMO (E) embryos in the TgBAC(cldn15la-GFP) reporter background, stained with phalloidin (red). Enlarged lumen caliber (arrow) is detected in apoBa–/– apoBb.1MO and mtpMO embryos, as quantified (F) (n = 2, nWT = 17, napoBa–/– apoBb.1MO = 15, nmtpMO = 16). The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. *P < 0.05. (G and H) Confocal images of the guts of 4 dpf apoBa–/– and apoBa–/– apoBb.1MO embryos stained with Nile red, show no lipid accumulation. (I and J) Bright-field images of 5 dpf embryos stained with Alcian blue showing reduced number of goblet cells in apoBa–/– apoBb.1–/– double mutants (J) as compared with WT embryos (I), as quantified (K) (n = 3, nWT = 30, napoBa–/– apoBb.1–/– = 27, napoBa–/– apoBb.1MO = 27). The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. ****P < 0.0001. (L) Confocal images at 5 dpf of WT and apoBa–/– apoBb.1MO embryos in the Tg(12xNRE:Egfp) reporter background. (M) Quantification of number of cells displaying active Notch signaling in the guts of WT and apoBa–/– apoBb.1MO embryos. The data are shown as the mean ± SEM, calculated using 2-tailed Student’s t test. Scale bar: (A–E, G, and H) 50 μm, (I, J, and L) 100 μm. ***P < 0.01. P < 0.05 (considered significant versus control group).
effects of ApoB depletion on gut formation appeared to be less severe than the effects observed in the liver. We also inspected the guts of the different mutants by mating the fish with the TgBAC(cldn15la-GFP)pelos tall reporter, which labels tight junctions in the intestine. We detected slightly enlarged lumens in the guts of apoBa−/− apoBb.1+/− embryos (Figure 3, C, D, and F) as compared with WT siblings. Similar effects were observed following injection of mtp MOs (Figure 3, C, E, F). These defects were corroborated by direct inspection of H&E staining of histological sections (Supplemental Figure 3D). Although at this stage of development the intestine is known to be folded and to contain polarized epithelial cells with a microvillus brush border (ref. 35 and Supplemental Figure 3D), in apoBa−/− apoBb.1−/− embryos, the intestinal epithelium appeared thin and poorly folded, and the cells had only few scattered microvilli as compared with their WT counterparts (Supplemental Figure 3, D and E, blue arrowheads). Also, intestine-specific deletion of Mtp or ApoB in mice renders small and large intestines with enlarged calibers (36, 37), mostly due to fat accumulation in the villus enterocytes, fully recapitulating the phenotypes reported for abetalipoproteinemia (38).

In contrast, we did not detect lipid accumulation in enterocytes of apoBa−/− apoBb.1−/−:mtpMOor ApoBKOembryos (Figure 3, G and H), suggesting that the enlarged lumen phenotype and thinner epithelia are most likely derived from impaired nutrient absorption, as previously described (39).

Finally, Alcian blue staining at 5 dpf highlighted a significantly reduced number of goblet cells in apoBa−/− apoBb.1−/− mutant guts (Figure 3, I–K). Previous reports have shown that inhibition of Notch signaling directs the specification of intestinal cells toward a secretory fate (40), leading to increased numbers of goblet cells. We therefore evaluated the levels of Notch activation in the guts of WT and mutant embryos using the 12NRE:GFP reporter. As seen in Figure 3, L and M, we detected increased numbers of Notch+ cells in the guts of apoBa−/− apoBb.1+/− embryos as compared with WT siblings, pointing to alteration in Notch signaling as the mechanism underlying goblet cell differentiation. Altogether, our results indicate that ApoB deficiency results in impaired liver development accompanied by hepatic steatosis and disrupted intestinal architecture.

ApoB depletion affects vascular development. Previous studies, including our own, have demonstrated that ApoB-containing lipoproteins have an inhibitory effect on vascular growth (2, 15, 16, 41). We therefore decided to analyze the involvement of the ApoB isoforms in the development of the vascular system by assessing the early vasculature of the different mutants. We first focused on the subintestinal vessels (SIVs), a plexus located in the posterior cerebral vein (PCeV) at 4 dpf (Supplemental Figure 4, A and F–H), and in the dorsal trunk (Supplemental Figure 4, B–E), confirming the MO specificity. Similar hyperangiogenic behaviors were detected in apoBa−/− apoBb.1MOs into WT embryos (Figure 4, G and ref. 15), were clearly detected following injection of apoBb.1MOs into WT embryos (Figure 4, E, F, H, and I). However, no effects were observed upon injection of apoBb.1MOs into WT embryos (Supplemental Figure 4, B–E), confirming the MO specificity. Similar hyperangiogenic behaviors were detected in the posterior cerebral vein (PCeV) at 4 dpf (Supplemental Figure 4, A and F–H), and in the dorsal trunk (Supplemental Figure 4, A and I–K) of apoBa−/− apoBb.1MOlarvae, suggesting that impaired angiogenesis is a general consequence of ApoB depletion that becomes more pronounced as development proceeds.

We have previously shown that ApoB lipoproteins induce ectopic angiogenesis by downregulating the expression of the VEGF decoy receptor Vegfr1/Flt1 (15). Accordingly, deletion of both membraneous and soluble forms of Flt1 fully phenocopied this phenotype (27). Given that loss of Flt1 has been shown to result in impaired Notch signaling (43), and that Flt1−/− mutant sprouts are less likely to retract (44), we decided to investigate whether this mechanism is activated in apoB mutants. Live imaging of the 12xNRE:EGFP reporter revealed clear activation of Notch signaling in the ectopic sprouts that failed to retract in apoBa−/− apoBb.1+/− embryos (Figure 4, J and K). In contrast, no ECs displaying active Notch signaling were detected in the SIV plexus of WT embryos at comparable developmental stages (i.e., 5 dpf), when the vessels have acquired their final, quiescent stage. Taken together, these results reinforce and expand our previous findings by showing that not only Flt1 but also Notch signaling act downstream of ApoB to modulate angiogenesis.

In order to verify that the observed vascular phenotypes indeed derive from the absence of ApoB lipoproteins, we attempted to rescue the defects by intravascularly injecting Dil-labeled LDL into apoBa−/− apoBb.1+/− embryos (Supplemental Figure 4L) as previously described (15, 17). As seen in Figure 4, L–N, a single injection of Dil-LDL was sufficient to revert the excessive angiogenesis phenotype, as opposed to oleic acid
Figure 4. apoB mutants display hyperangiogenic phenotypes. (A) Schematic representation of the zebrafish embryonic vasculature, with red square marking the subintestinal vessels (SIVs). (B–G) Confocal images at 3 dpf showing ectopic sprouts (arrowheads) arising in the SIVs of apoBa−/− apoBb.1−/− double mutants (E), and apoBa−/− apoBb.1MO (F) and stalticate (stl) mutants (G), but not in WT (B), apoBa−/− (C), or apoBb.1−/− (D) animals. (H and I) Quantification of the number (H) and length (I) of ectopic sprouts at 3 dpf. n = 3, nWT = 45, napoBa−/− = 55, napoBb.1−/− = 23, napoBa−/− apoBb.1−/− = 31, napoBa−/− apoBb.1MO = 40, napoBa−/− apoBb.1−/− apoBb.1MO = 21. The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. ****P < 0.0001, **P < 0.01. (J and K) Confocal images at 5 dpf of apoBa−/− and apoBb.1MO embryos in the Tg(12xNRE:Egfp) reporter background. White arrowheads in apoBa−/− apoBb.1MO embryos point to endothelial cells (ECs) with active Notch signaling, in ectopic sprouts that failed to retract. n = 2, napoBa−/− = 10, napoBa−/− apoBb.1MO = 12. (L and M) Alkaline phosphatase (AP) staining of the SIVs at 3 dpf, showing inhibition of ectopic sprouting following intravascular injection of Dil-LDL into apoBa−/− apoBb.1MO. (N) Quantification of number and length of ectopic sprouts following intravascular injection of Dil-LDL into apoBa−/− apoBb.1MO embryos. (O) Quantification of number and length of ectopic sprouts following intravascular injection of Dil-LDL into apoBa−/− apoBb.1MO embryos treated with OA, as quantified (P) (n = 3, napoBa−/− apoBb.1MO = 20, napoBa−/− apoBb.1MO+6μg OA = 19, napoBa−/− apoBb.1MO+10μg OA = 21, napoBa−/− apoBb.1MO+20μg OA = 22). The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. Scale bar: (B–G) 50 μm, (J–O) 100 μm. P < 0.05 (considered significant versus control group).
In particular, we find that apoBa–/– apoBb.1MO strong hypolipidemia. Finally, deletion of both isoforms resulted in severe phenotypes and lethality. Accordingly, while the absence of apoBa development, deficiency for assessing the physiological activity of potential therapeutic agents.

in circulation in vivo, despite it being produced and secreted into the blood (45, 48), due to fast clearance. The discrepancy between the in vivo and the in vitro results may derive from differences in the mobility and clearance of the truncated forms of APOB in vivo. Specifically, it has been shown that APOB25 is not present with human APOB34 or APOB25, which have been previously shown to undergo proper lipidation and secretion (45, 46), into HEK293 cells (Supplemental Figure 4M). We then transferred the media containing the different truncated forms to human umbilical vein endothelial cells (HUVECs) and examined their ability to generate tubes. LDL, which we previously showed inhibits tube formation in culture (16), was used as positive control. Interestingly, both APOB25 and APOB34 forms inhibited angiogenesis in this assay, suggesting that the antiangiogenic effect of ApoB is conveyed by the 25% (N-terminus) of the protein (Figure 5, A–E).

Discussion

In this work, we generated zebrafish mutants for 2 ApoB isoforms: apoBa and apoBb.1. Similar to ApoB-null mice, zebrafish carrying mutations in both apoBa and apoBb.1 demonstrate early lethality. However, in contrast to the mammalian models, the external fertilization and development of zebrafish allowed us to investigate previously unappreciated roles of ApoB during embryogenesis.

Three apoB genes are present in the zebrafish genome, namely apoBa, apoBb.1, and apoBb.2. During early development, apoBa and apoBb.1 are highly expressed, whereas apoBb.2 is absent (14). Interestingly, despite the strong expression of apoBa mRNA, its protein levels are almost 20-fold lower than those of ApoBb.1 (14). Accordingly, while the absence of apoBa did not elicit any noticeable phenotypes, apoBb.1 depletion caused strong hypolipidemia. Finally, deletion of both isoforms resulted in severe phenotypes and lethality.

Our results uncover the roles for ApoB during development of the liver, one of its main producing organs. In particular, we find that apoBa−/− apoBb.1MO embryos display similar phenotypes to those caused by retinoic acid (RA) deficiency (49), including decreased liver volumes and liver bud bilaterality. Zebrafish embryos treated with the Raldh inhibitor DEAB, or injected with MOs against the 3 RA receptors (raraa, ranab, ranac, and ranga), were shown to display smaller livers because of impaired hepatocyte proliferation. In contrast, RA addition to the fish water resulted in massive proliferation of hepatocytes and enlarged livers (49). In addition, downregulation of the RA receptor rargb led to bilateral liver buds and intrahepatic biliary defects, similar to those displayed by double apoB mutants. While most RA circulation is mediated through retinol binding protein 4, it has been shown that ApoB-containing lipoproteins (e.g., LDL and VLDL) also participate in RA shuttling (50). Therefore, the possibility exists that complete depletion of ApoB leads to RA unavailability, which in turn results in liver bilaterality and reduced hepatocyte and BEC proliferation.

The absence of both apoB isoforms resulted in low levels of TGs and cholesterol in circulation, accompanied by massive lipid droplet accumulation in hepatocytes, and liver steatosis. These phenotypes, reminiscent of human NAFLD, recapitulate part of the clinical manifestations of FHBL1, making the apoBa−/− apoBb.1−/− mutant zebrafish an advantageous model for the study of this disorder and for the identification of potential treatments.

In addition to liver abnormalities, ApoB and MTP deficiency resulted in disrupted intestinal architecture. In particular, the guts were poorly folded and displayed enlarged lumen calibers, resembling phenotypes displayed
Double apoB mutants feature an increased number of goblet cells. Interestingly, it has been shown that RA negatively regulates goblet cell differentiation (52), whereas inhibition of Notch signaling directs specification of intestinal cells toward a secretory fate (40) leading to increased numbers of goblet cells. Our results indeed demonstrate increased activation of Notch signaling in the intestines of apo-Ba–/– apoBb–/– mutants, supporting the idea that ApoB affects goblet cell differentiation through activation of Notch targets. Downstream of Notch, the transcription factor Kruppel-like factor 4 (Klf4) has been shown to

Figure 5. Truncated forms of human APOB inhibit angiogenesis in apoB mutants. (A–D) Tube formation assay on HUVECs untreated (A, C, and D) or treated with LDL (B) plus conditioned medium from HEK293 cells cotransfected with (A and B) MTP+GFP, (C) MTP+APOB25, and (D) MTP+APOB34. Red dashed line outlines vascular tube structure. (E) Quantification of total number of tubes following different treatments (n = 3, nMTP+GFP = 4, nMTP+GFP+LDL = 4, nMTP+APOB25 = 5, nMTP+APOB34 = 4). The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. **P < 0.01. (F–I) Confocal images at 4 dpf showing reduction in the number and length of ectopic sprouts in Tg(fli1:EGFP) apoBa–/– apoBb.1MO embryos injected with apo14:APOB25 (I) as compared with uninjected apoBa–/– (F), uninjected apoBa–/– apoBb.1MO (G), and apoBa–/– apoBb.1MO injected with apo14:APOB25 (H). White arrowheads point to ectopic sprouts. (J–M) Quantification of the number and length of ectopic sprouts in apo14 APOB25 (J and K) (n = 3, napoBa–/– = 47, napoBa–/– apoBb.1MO = 54, nepapoBa–/– apo14:APOB25 = 54) and apo14 APOB34 (L and M) (n = 3, napapoBa–/– = 47, napapoBa–/– apoBb.1MO = 51, napapoBa–/– apo14:APOB34 = 55) injected embryos. The data are shown as the mean ± SEM, calculated using ANOVA followed by Tukey’s multiple-comparison test. Scale bar (F–I): 50 μm. ****P < 0.0001, P < 0.05 (considered significant versus control group).
be required for terminal differentiation of goblet cells (53). Expression of klf4a was shown to be controlled by Notch signaling (i.e., embryos treated with the γ-secretase inhibitor DAPT display increased klf4a expression in the intestine, while decreased klf4a expression and reduction in goblet cell number were observed in embryos injected with Notch intracellular domain mRNA; ref. 54). On the other hand, it has been demonstrated that the RA/RARa axis negatively modulates klf4a expression, thereby impacting goblet cell differentiation as well. In the future it will be interesting to investigate whether ApoB is involved in the interplay between the Notch and RA/RARa pathways in the developing intestine.

Finally, the phenotypes detected in the liver and intestine of apoB mutants could result from the absence of fat-soluble vitamins, such as vitamins E and A, and their RA metabolites, whose absorption and transport throughout the body rely mostly on ApoB, in the form of chylomicrons (55, 56).

Previous studies have demonstrated the strong impact of lipoproteins on EC behavior (15, 17, 57, 58); yet, the mechanisms by which ApoB affects these cells are not fully understood. The double apoB mutant exhibited a marked angiogenic phenotype characterized by excessive sprouts in the SIVs, PCeV, and arterial intersomitic vessels. This angiogenic effect may result from a combination of factors related to ApoB deficiency, such as lipid raft density, lack of fat-soluble vitamins, and activation of downstream signaling pathways in ECs. Interestingly, the angiogenic defects were observed only following complete depletion of circulating ApoB lipoproteins. Although apoBh.1 mutants featured severe hypolipidemia, they did not display a strong angiogenic phenotype, suggesting that small amounts of ApoB are sufficient to maintain healthy control over the angiogenic process and further confirming the specific effects of the ApoB protein on EC behavior.

Our structure-function analyses revealed that APOB34, a truncated form of human APOB lacking the LDL receptor binding domain, is able to rescue the angiogenic phenotype in double apoB mutants. These results raise the hypothesis that an LDL receptor–independent pathway might regulate the effects of ApoB on ECs. Recently, it has been shown that LDL can bind the TGF-β receptor activin receptor-like kinase 1 (ALK1; ref. 59), which is also involved in lipoproteins’ transcytosis (60). Moreover, ALK1 has been shown to exert antiangiogenic effects via upregulation of VEGFR1 (61). Interestingly, stl mutants display reduced expression of vegfr1, leading to excessive angiogenesis (15). Therefore, it seems feasible that APOB induction of ALK1 activity leads to upregulation of vegfr1 and inhibition of angiogenesis.

Overall, our newly generated apoB mutants can serve as a model for studying human pathologies associated with hypolipidemia, as well as for related drug screens. Moreover, understanding the molecular mechanisms by which ApoB regulates angiogenesis will provide potential new therapeutic targets for the treatment of vascular pathologies ranging from cancer to ischemic heart disease.

**Methods**

Zebrafish husbandry and transgenic lines. Tg(fli1:EGFP)y1 (15), TgBAC(cldn15a-GFP)y1010 (34), Tg(fli1:dsRed)y1113 (62), Tg(fiy1:dsRed)y1110 (63), Tg(fli1:9a_cFls:GFP)y13 (63), Tg(EFV:Tp1-Mmu.Hbb:EGFP)y13 (27), and Tg(f2.8sib-p10a:EGFP)y12 (22) have been previously described.

To generate apo14:APOB25-IRESmCherry and apo14:APOB34-IRESmCherry, the zebrafish apo14 promoter (47) was cloned into pDONR5-P1R (Invitrogen, Thermo Fisher Scientific), human APOB25 and APOB34 (45) were cloned into pDONR221 (Invitrogen, Thermo Fisher Scientific), and the IRESmCherry sequence was cloned into pDONRP2R-P3 (Invitrogen, Thermo Fisher Scientific), using Gateway BP Clonase II (Invitrogen, Thermo Fisher Scientific, 11789-020). The 3 vectors were then transferred into pDestTol2pa2 (62) using a Gateway LR reaction (Invitrogen, Thermo Fisher Scientific, 12538-120). The plasmids were injected along with Tol2 transposase mRNA into 1-cell-stage embryos (62).

Generation of zebrafish mutants. The apoBh.1 CRISPR guide was designed with CHOPCHOP; potential off-target sequences were assessed using the MIT CRISPR Design site. Oligonucleotides synthesized for the guide sequence GGACTAGTGTGGTCTTTGAC were cloned into the BsmBI sites of the pT7-eRNA plasmid (Addgene plasmid 46759). Cas9 mRNA was generated from pCS2-nCas9n (64) using mMESSAGE mMachine T7 ULTRA kit (Ambion, AM1345). Cas9 mRNA (250 ng/μL) and guide RNAs (100 ng/μL) were coinjected into 1-cell-stage Tg(fli1:EGFP)y1 transgenic embryos. For genotyping, genomic DNA was extracted at 24 hours postfertilization (hpf), amplified with 5′-TACTCTGTGAATGCCAGACAGG and 5′-TCGAGACACCAAGCAGAAACA (184 bp) primers, and analyzed on a 3% agarose gel. apoBa TALENs left — TACAGCTAACTCTAGAA — and right — TCGCCAGtacaaac plasmids were generated as described (65) and transcribed using mMESSAGE mMachine T7 ULTRA kit (Ambion, AM1345). mRNAs (250 ng each) were coinjected into 1-cell stage. For genotyping, genomic DNA was extracted at 24
μg of RNA per reaction was reverse-transcribed using following standard procedures (15). A total of 1 homogenized in TRIzol (Invitrogen, Thermo Fisher Scientific, 15596026) and processed for RNA extraction stereoscope under ×10 original magnification; each side of the animal was scored separately. (PCeV, trunk) or AP staining (SIVs). Quantitation of the SIVs’ phenotype was done using a grid lens in a Leica.

Total RNA isolation and semiquantitative reverse transcription PCR. A pool of 10–20 embryos/sample was homogenized in TRIzol (Invitrogen, Thermo Fisher Scientific, 15596026) and processed for RNA extraction following standard procedures (15). A total of 1 μg of RNA per reaction was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, 4368814). To measure relative changes in mRNA transcripts, we used the following primers: apoBb.1

5′-TATCAGTACACGAGCAGAGCCA and 5′-TCGTTACTAAGGCTTGGCC-3′, 5′-CAGGAAAGGCTCTCTATGAA-3′; apoBb.1 5′-GCTGAGTGTAGTGCACTGCAATG-3′, 5′-ATCAACAGTGGGTTCAGACCCCTT-3′; foxa2 5′-GTGTTACACCCCTGGTGACCCA-3′, 5′-CAGTATGGAGCGCATTGGCCT-3′; fabp2 5′-TGGAAGGTCAAGGCGACATGAG-3′, 5′-TACCCCGTTGTCCCTTGCGT-3′; fta 5′-TCTGAGGCTGGAATTACCTCATA-3′, 5′-TAAACCTAGACCCCTTACAGCGA-3′.

Assessment of vascular phenotypes. Larvae were assayed for SIV ectopic sprouting using fluorescence imaging (PCEv, trunk) or AP staining (SIVs). Quantification of the SIVs’ phenotype was done using a grid lens in a Leica stereoscope under ×10 original magnification; each side of the animal was scored separately.

OA and LDL treatments. OA (MilliporeSigma, O7501) was dissolved in ethanol and added to the fish water with fatty acid–free BSA (MilliporeSigma, A8806), at 6 to 20 μg/mL concentration. Dil-LDL (Invitrogen, Thermo Fisher Scientific, L3482) was injected intravascularly at 2 dpf, as described (15).
For treatment, conditional medium collected from $MTP+GFP$, $MTP+GFP+LDL$ (100 μg/mL) (BT-903), $MTP+APOB25$, and $MTP+APOB34$ transfected HEK293 cells was added. Following 18-hour incubation, 30,000 cells were seeded on Matrigel (BD, 356231) for 8 hours and cultured with the appropriate conditional medium ($MTP+GFP$, $MTP+GFP+LDL$, $MTP+APOB25$, and $MTP+APOB34$). Cells were then fixed in 4% PFA and imaged using a bright-field microscope. Nine fields/well were acquired per experiment. Total tube numbers were quantified using ImageJ. For each experiment, values were normalized to the nontreated well.

Statistics. All data are reported as mean values ± SEM and were analyzed using Prism 5 software (GraphPad Software). Comparison of 2 samples was done by unpaired 2-tailed Student’s t test. Statistical significance for 3 or more samples was calculated via 1-way ANOVA followed by post hoc Tukey’s for multiple comparisons. A P value less than 0.05 was considered significant. In box-and-whisker plots, box indicates 25th to 75th percentiles, line indicates median, and whiskers represent min and max.

Study approval. Zebrafish were raised by standard methods and handled according to, and following approval by, the Weizmann Institute Animal Care and Use Committee (68).

Author contributions
HT designed and conducted all experiments, analyzed data, and cowrote the manuscript; IAD conducted HUVEC experiments; NM managed fish work and conducted zebrafish experiments; and KY initiated and directed the study, designed experiments, analyzed data, and cowrote the paper with input from all authors.

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