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Zebrafish Lipid Metabolism: From Mediating Early Patterning to the Metabolism of Dietary Fat and Cholesterol

Jennifer L. Anderson[*], **Juliana D. Carten**[*], and **Steven A. Farber**
Carnegie Institution for Science, Department of Embryology, Baltimore, Maryland, USA

Abstract

Lipids serve essential functions in cells as signaling molecules, membrane components, and sources of energy. Defects in lipid metabolism are implicated in a number of pandemic human diseases, including diabetes, obesity, and hypercholesterolemia. approaches for disease prevention and treatment. Numerous studies have shown that the zebrafish is an excellent model for vertebrate lipid metabolism. In this chapter, we review studies that employ zebrafish to better understand lipid signaling and metabolism.

I. Introduction

Lipids play essential roles in cells as signaling molecules, membrane components, and sources of fuel. Given their necessity for proper cellular function, it is not surprising that defects in lipid metabolism underlie a number of human diseases, including obesity, diabetes, and atherosclerosis (Joffe *et al*., 2001; McNeely *et al*., 2001; Watanabe *et al*., 2008). In 2007–08, one-third of US adults and 18% of children were classified as obese (Flegal *et al*., 2010; Ogden *et al*., 2010), with obesity and type 2 diabetes on the rise worldwide (Misra and Khurana, 2008). The globalization of the high-fat western diet and the concurrent rise in the incidence of lipid disorders has provided an impetus to better understand lipid metabolism in the context of metabolic dysfunction. This need to investigate the role of lipids in metabolic disease has brought into focus unanswered questions in the field. For instance, although the genes involved in cholesterol and fatty acid (FA) uptake in intestinal cells have been identified, their exact mechanisms of action are highly debated or largely unknown (Klett and Patel, 2004; Nassir *et al*., 2007; Nickerson *et al*., 2009; Shim *et al*., 2009; Stahl *et al*., 1999). Such gaps in our understanding of these genes and how they function hinder the development of effective therapeutics for lipid disorders and reveal a need to create better approaches to address them.

In this chapter, we will review novel approaches undertaken to study lipid signaling and metabolism using the zebrafish model organism, with an emphasis on presenting a diverse array of techniques employed to visualize lipid metabolism during various stages of zebrafish development.

[*]These two authors contributed equally to the work.

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A. The Need for Whole Animal Studies of Lipid Metabolism

In vitro studies have laid much of the groundwork for our biochemical understanding of vertebrate lipid metabolism and continue to comprise many of the studies done in the field today; however, a number of caveats arise when attempting to study lipid metabolism *in vitro*. Such studies are often performed in transformed cultured cells, such as liver HepG2, intestinal Caco2, and adipocyte 3LT3 cells. These cell lines, comprised of a single cell type, cannot duplicate the cellular heterogeneity of an entire organ, such as the intestine, which is composed of stem, enteroendocrine, immune, and goblet cells. These multiple cell types are known to influence each other through paracrine signaling that can have global effects on lipid uptake and processing. Furthermore, bile, intestinal mucus, and the gut microbiota are all known to greatly influence dietary lipid processing and absorption in the intestine (Backhed *et al.*, 2004; Field *et al.*, 2003; Kruit *et al.*, 2006; Martin *et al.*, 2008; Moschetta *et al.*, 2005; Pack *et al.*, 1996; Titus and Ahearn, 1992; Turnbaugh *et al.*, 2008), and are absent in cultured cell models. For these reasons, employing whole animal *in vivo* strategies, in addition to cultured cell work, is vital to better understand how metabolic dysfunction arises and manifests itself in an organism.

The importance of utilizing whole animal models to identify drugs to ameliorate metabolic dysfunction is exemplified by how the cholesterol absorption inhibitor ezetimibe (Zetia, Vitorin; Merck-Schering Plough) was developed (Van Heek *et al.*, 1997), and how its mechanism of action was defined (Van Heek *et al.*, 2000). Studies of bile duct-cannulated rats treated with ezetimibe revealed that the bioactive compound responsible for the diminished effect on cholesterol absorption was a glucur-onidated form of ezetimibe. Since *in vitro* studies cannot recreate the complex interplay of neural, chemical, and hormonal cues known to regulate metabolic processes *in vivo*, there is a need for whole animal approaches to study lipid metabolism as it plays out in a multicellular context.

B. Larval Zebrafish as a Model of Vertebrate Lipid Metabolism

The larval zebrafish is well suited for whole animal studies of lipid metabolism. Larval zebrafish possess many of the same gastrointestinal organs present in humans (e.g., the liver, intestine, exocrine and endocrine pancreas, and gallbladder) (Lieschke and Currie, 2007; Pack *et al.*, 1996; Schlegel and Stainier, 2006; Wallace and Pack, 2003; Wallace *et al.*, 2005) as well as the specialized cell types involved in lipid absorption and processing (e.g., intestinal enterocytes, fat-storing adipocytes, hepatocytes in the liver, and acinar cells of the pancreas) (Wallace *et al.*, 2005). These digestive organs and the cell types present in them are formed using similar developmental programs as in mammals with *hhex* (Wallace *et al.*, 2001), *pdx1* (Yee *et al.*, 2005), and *shha* (Wallace and Pack, 2003) genes playing critical roles in liver and pancreas organogenesis in zebrafish. Notch and its ligands, Delta and Jagged, play a role in the developing pancreas, both by maintaining undifferentiated precursors and regulating acinar, exocrine, and endocrine differentiation (Apelqvist *et al.*, 1999; Esni *et al.*, 2004; Hald *et al.*, 2003; Jensen *et al.*, 2000; Murtaugh *et al.*, 2003; Zecchin *et al.*, 2004). Due to the optical transparency of larvae, these organs and their multiple cell types can all be directly observed through the body wall without the need for invasive surgical manipulations.

Work from our lab and others has shown that zebrafish express many of the genes needed to transport and metabolize lipids, such as the lipoprotein gene microsomal triglyceride transfer protein (MTP) (Marza *et al.*, 2005) and the FA transport protein (*slc27a*) and acyl-CoA synthetase (*acsl*) gene families (Thisse *et al.*, 2005; Thisse *et al.*, 2004; Miyares, unpublished). Zebrafish express the putative cholesterol transporter Niemann-Pick C1-Like 1 (*npc1l1*) (Farber, unpublished) and treatment of larvae with the human drug ezetimibe, which works through an NPC1L1-dependent pathway and is used to treat

hypercholesterolemia, blocks intestinal cholesterol absorption (Clifton *et al.*, 2010). Apolipoprotein C2, a gene needed for lipoprotein assembly in humans, is expressed and required during zebrafish larval development; larvae injected with an *apoc2* morpholino exhibit an unabsorbed yolk phenotype (Pickart *et al.*, 2006). Additionally, the enzymes needed to synthesize lipid-signaling molecules, such as prostaglandins and thromboxanes, are highly conserved in zebrafish and these enzymes can be inhibited by commonly used nonsteroidal anti-inflammatory drugs (Grosser *et al.*, 2002). We have presented here only a small sampling of the numerous studies that document the homologies between human and zebrafish lipid metabolism and validate the zebrafish as a model for investigating vertebrate lipid metabolism.

II. Lipid Metabolism in Developing Zebrafish

During the first four days of development, a zebrafish embryo relies entirely on its yolk sac for the nutrients needed to sustain its growth and survival. Yolk lipids are the source of essential fat-soluble vitamins and triacylglycerol (TAG), as well as cholesterol, a required component of cell membranes and a precursor for bile acids (Babin *etal.*, 1997; Bownes, 1992; Munoz *et al.*, 1990). Lipids enter the developing embryo at the yolk and embryo interface, an area termed the yolk syncytial layer (YSL). In the YSL, lipoproteins (e.g., apoE, apoA1, apocII, and vitellogenin) and a host of lipid-modifying enzymes (e.g., Mtp) transport lipids from the yolk ball to the embryo (Babin *et al.*, 1997; Marza *et al.*, 2005). Once the circulatory system forms, yolk, hepatic, and intestinal lipids are transported by lipoproteins to specific target tissues throughout the organism via the bloodstream.

By 5–6 days postfertilization (dpf), the yolk is depleted and larvae must eat to acquire lipids. Both in the wild and the laboratory, zebrafish consume a lipid-rich diet (≥ 10% by weight) high in TAG, phospholipids, and sterols (Enzler *et al.*, 1974; Spence *et al.*, 2008). Prior to absorption by the intestine, these yolk lipids must be processed and solubilized by the digestive enzymes and bile that make up the intraluminal intestinal milieu. As in humans, zebrafish bile is produced by hepatocytes and secreted into an extensive network of intrahepatic ducts, which drains into the gall bladder. In response to hormonal stimulation triggered by food consumption, bile is released into the intestinal lumen to emulsify dietary fat and facilitate its absorption by intestinal enterocytes. The main components of bile are bile acids, phospholipids, and salts, which together promote the formation of micelles by inserting themselves between lipid bilayers and reducing the surface tension to allow for membrane curvature (Tso and Fujimoto, 1991; Verkade and Tso, 2001). After dietary fat is emulsified, TAG and phospholipids must be broken down by luminal lipases to release free FA or mono- and di-acylglycerols, which can then enter the specialized absorptive cells (enterocytes) that line the gut (Thomson *et al.*, 1993). The exocrine pancreas is the main source of these fat-splitting enzymes in larval zebrafish (Hama *et al.*, 2009) and known in mammals to secrete lipase- and protease-rich pancreatic juice into the gall bladder (Layer and Keller, 1999). After being emulsified and broken down, TAG can be absorbed by enterocytes, the main absorptive cell type of the zebrafish intestine. These cells are highly reminiscent of mammalian enterocytes (Buhman *et al.*, 2002), with the characteristic microvilli and basal nuclei (Fig. 1).

Following food consumption, zebrafish accumulate cytoplasmic lipid drops (LD) in their enterocytes (Walters, unpublished). From there, fats are likely burned via oxidative pathways in the mitochondria or peroxisomes or packaged into chylomicrons, which are secreted from the basolateral surface of enterocytes into lymphatic or blood vessels (Field, 2001; Levy *et al.*, 2007). In chickens, chylomicron production and secretion is highly conserved with the exception that lipoproteins are secreted from the intestine directly into the portal vein and thus are termed portomicrons (Bensadoun and Rothfeld 1972; Griffin *et*

al., 1982). This difference has led some to propose that a similar portomicron process occurs in fish (Robinson and Mead 1973); however, careful ultrastructural studies and isotopic lipid labeling experiments suggest that fish and mammals produce chylomicrons containing largely similar lipoproteins (Sire *et al.*, 1981; Skinner and Rogie, 1978). While it remains to be seen how closely the zebrafish system will model human intestinal lipoprotein metabolism, it is likely that many of the mechanisms of lipoprotein production are conserved.

III. Yolk Metabolism During Early Vertebrate Development

To mobilize lipids stockpiled in the yolk sac, rodents express the same genes required for lipoprotein production as those expressed in fully differentiated intestinal enterocytes and liver hepatocytes (e.g., MTP and the apolipoproteins apoE, apoB, and apo-A-IV and apo-A-I (Elshourbagy *et al.*, 1985; Farese *et al.*, 1996; Plonne *et al.*, 1996)). Without these genes, the rapid transport of essential nutrients from the yolk to developing embryonic tissues is impaired and development cannot proceed normally. Not surprisingly, mice null for MTP (Raabe *et al.*, 1998) and apoB (Farese *et al.*, 1996) are embryonic lethal, and DGAT-2 null mice, which lack the enzyme needed to synthesize diacylglycerol, exhibit stunted embryonic growth and die perinatally (Stone *et al.*, 2004).

The zebrafish YSL expresses a number of lipoprotein-encoding mRNAs that are also expressed later in the larval intestine and liver, including MTP (Marza *et al.*, 2005), apoC2 (Farber Lab, unpublished), intestinal FA binding protein (Sharma *et al.*, 2004), and apoE and apoA-I (Babin *et al.*, 1997). Due to the parallel gene expression patterns observed between the embryonic YSL and larval digestive organs, we hypothesize that yolk utilization during early zebrafish embryogenesis is a regulated process highly analogous to intestinal and hepatic lipoprotein-mediated lipid transport.

To study the role of lipid metabolism genes in the YSL during early zebrafish development, we utilize synthetic antisense morpholinos (MO) to attenuate gene expression and assay subsequent yolk metabolism. MOs are widely used in the zebrafish community to knock down mRNA levels (Heasman, 2002; Nasevicius and Ekker, 2000), and numerous studies have phenocopied mutants by targeting particular mRNA transcripts using this method (Dutton *et al.*, 2001; Karlen and Rebagliati, 2001; Urtishak *et al.*, 2003). By injecting MOs targeting lipid-specific genes into the yolk of 1–4 cell stage embryos, followed by yolk injection of fluorescent lipid analogs, such as BODIPY-labeled FA (Fig. 2A), we can directly assess the necessity of a given gene for yolk metabolism during early larval development. Following sequential injections of the morpholino and BODIPY-labeled FA, embryos are allowed to develop and total larval lipids are extracted at 2–3 dpf. We assay the incorporation of the fluorescent FA into metabolites using thin layer chromatography (TLC). This approach allows one to identify metabolic abnormalities in morphants that may not exhibit obvious morphological phenotypes. Furthermore, we can assay the effects of essential genes at earlier stages of larval development prior to lethality caused by insufficient transcript amounts.

We are currently using this technique to better understand how the apolipoprotein *apoc2* functions in yolk utilization during early zebrafish development. This gene was first identified in a MO screen targeting secreted proteins of the unknown function. In a screen of approximately 100 MOs, we identified one with an unab-sorbed yolk phenotype (Fig. 2B and 2C). TLC analysis revealed that these larvae exhibit metabolic defects in phospholipid production, as morphants incorporated less BODIPY-C12 into phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) (Fig. 2D and 2E). Unabsorbed yolk and phospholipid metabolic deficiency in *apoc2* morphants suggest that this gene has a function in the yolk or

YSL during zebrafish development that is unrelated to its known role in the activation of lipo-protein lipase in peripheral tissues (Jong *et al.*, 1999).

IV. Lipid Signaling During Early Zebrafish Development

It is not surprising that many lipid-signaling molecules are derived from membrane phospholipids. Such a system allows for rapid transmission of extracellular signals via membrane, and constituents to the intracellular environment to activate appropriate signaling cascades and cellular responses. The synthesis of membrane-derived signaling lipids is often dependent on phospholipases, enzymes that cleave phospholipids in response to specific cellular signals. Phospholipase A2 (PLA2) is one such enzyme that catalyzes the hydrolysis of the second fatty acyl bond of glycerophospholipids to liberate lysophospholipid and free FA, both lipid-signaling precursors. In the last decade, PLA2 activity and its products have been implicated in a wide range of cellular phenomena including inflammation, membrane remodeling, and cancer.

Lipid signaling events during early development are not well elucidated and have only been recently explored in zebrafish. Studies examining PLA2 enzymatic activity throughout larval development using whole embryo lysates revealed varying activity levels during different stages of development, with larvae exhibiting a significant PLA2 activity during somitogenesis (Fig. 3A). Pharmacological inhibition of PLA2 activity causes developmental arrest at epiboly (Farber *et al.*, 1999). Further experiments identified the primary source of phospholipase activity to be the Ca2+-dependent cytosolic PLA2 (cPLA2) type, a crucial mediator of stimulusinduced eicosanoid release. cPLA2 activity releases the polyunsaturated FA arachidonic acid from membranes, allowing it to participate in the synthesis of eicosanoids, a potent class of lipid-signaling molecules that exhibit both paracrine and autocrine influences on cells and tissues (Burke and Dennis, 2009; Clark *et al.*, 1991) and are commonly associated with provoking inflammatory and immune responses. The developmental arrest observed in cPLA2 morphants suggests that cPLA2 activity and eicosanoid signaling are essential for early embryonic patterning, pointing to a novel role for lipid signaling during embryogenesis.

Arachidonic acid can act as a substrate for a number of other lipid modifying enzymes (e.g., cyclooxygenase) that are critical for cell movements required to pattern the early zebrafish embryo. Cha *et al.* (2006) found that PGE2, an eicosanoid downstream of cyclooxygenase, is essential for the morphogenic movements of convergence and extension (Cha *et al.*, 2006; Cha *et al.*, 2005). Moreover, the arrest of epiboly that results from the inhibition of cyclooxygenase 1 (also known as Prostaglandin–endoperoxide synthases, Ptgs1) using an antisense morpholino oligonucleotide can be completely rescued by the addition of PGE2 (Speirs *et al.*, 2010) (Fig. 3B).

The importance of PGE2 during zebrafish development was further demonstrated in a small molecule screen performed in zebrafish larvae (36 hpf) by North *et al.* (2007). Using this approach PGE2 was found to impact hematopoietic stem cell proliferation as evidenced by a dramatic increase in the expression of hematopoietic markers in larvae treated with a PGE2 analog (North *et al.*, 2007) (Fig. 3C). Taken together, these data provide evidence for the importance of PLA2-derived lipid mediators during zebrafish development.

A. Lipid Modifications Influence Primordial Germ Cell Migration

In addition to uncovering a role for lipid signaling during the early movements of gastrulation, work on the zebrafish has shown that lipid modifications influence another critical cellular movement during early embryonic development: primordial germ cell (PGC) migration. PGC migration in zebrafish embryos can be visualized in embryos as early as

80% epiboly by injecting *in vitro* transcribed, capped *GFP-nanos* mRNA (which consists of the coding sequence of GFP fused to the 3′UTR of the *nanos* gene) into zebrafish embryos at the one-cell stage. *GFP-nanos* message and protein are stabilized preferentially in PGCs such that they maintain their fluorescence throughout early development, facilitating the detailed study of PGC migratory behavior (Doitsidou *et al.*, 2002).

Because lipid metabolism is highly conserved across vertebrates, human drugs can be used to block particular steps in metabolic pathways to determine what pathway or metabolites are necessary for a developmental process, such as PGC migration. In *Drosophila*, loss of 3-hydroxyl-3-methylglutaryl-CoA reductase (HMGCoAR) results in PGC migration defects (Van Doren *et al.*, 1998). Similarly, studies in zebrafish have shown that pharmacologic inhibition of HMGCoAR by atorvastatin (Lipitor) results in abnormal development and PGC migration (Thorpe *et al.*, 2004). HMGCoAR is a rate-limiting step in the synthesis of cholesterol and is the target of statins (Fig. 4). Zebrafish embryos soaked in statins, either mevinolin (Lovastatin) or simvastatin (Zocor), exhibit developmental arrest, blunted axis elongation, misshapen somites, and head and axial necrosis (Fig. 5). Additionally, PGC migration is profoundly perturbed. Embryos treated with a more hydrophobic statin (Lipitor) exhibit PGC migration defects and only mild morphologic abnormalities.

To determine which downstream products of HMGCoAR mediate these developmental defects, a “block and rescue” approach was taken through injection of putative biochemical pathway intermediates downstream of HMGCoAR following statin-mediated inhibition. Injection of mevalonate, the product of HMGCoAR's reduction of ß-hydroxy-ß-methylglutaryl-CoA, completely rescued all the phenotypes associated with the statin treatment (Fig. 6). Similar experiments using intermediates downstream of mevalonate (Fig. 7) indicated that the prenylation pathway, responsible for adding polyunsaturated lipids to proteins, was likely mediating the effect of statins on PGC migration. High doses of the selective farnesyl transferase inhibitors L-744 or FTI-2153 (Crespo *et al.*, 2001; Sun *et al.*, 1999) had no effect on PGC migration. However, embryos treated with geranylgeranyl transferase I (GGTI) inhibitor (GGTI-2166) exhibited a strong PGC migration phenotype with only a slight disruption of the notochord. These data suggest that protein prenylation, specifically by GGTI, is required for correct PGC migration (Thorpe *et al.*, 2004).

V. Visualizing Lipid Metabolism in Larval and Adult Zebrafish

A. Lipophilic Dyes

Lipophilic dyes, known as lysochromes, are one of the first tools used to visualize lipids in cells. These dyes are capable of labeling a variety of lipids and lipidcontaining structures including TAG, FA, and lipoproteins. Dyes, such as oil red O (ORO), sudan black B, and nile red, were initially used to label LDs in tissue sections and cultured cells and continue to be used today. Marza and colleagues (Marza *et al.*, 2005) utilized sudan black B to identify LDs in histological sections of fed adult zebrafish. The authors found that feeding a high-fat meal increased the expression of MTP in intestinal epithelial cells. This protein is required for proper assembly and secretion of hepatic and intestinal ApoB-containing lipoproteins, chylomicrons, and very low-density lipoproteins (VLDL) (Gordon *et al.*, 1995). Their observation that LDs are coincident with an upregulation of MTP expression is consistent with MTP's known function in humans (Marza *et al.*, 2005).

Lysochromes can also be used to visualize endogenous lipid stores in whole fixed zebrafish to generate an overall picture of neutral lipid localization during development. Schlegel and Stainier used ORO to assess the consequence of MTP knockdown (via MO) on lipid absorption in whole zebrafish larvae (Schlegel and Stainier, 2006). MTP morphants exhibited decreased yolk consumption and an inability to absorb dietary neutral lipids,

resulting in death by 6 dpf Although lysochromes such as sudan black B and ORO consistently label neutral lipids in tissue sections and fixed larvae, fixation techniques are laborious and staining procedures have been shown to cause artificial fusion of adjacent LDs and mislocalization of the LD marker, adipose differentiation-related protein (Adrp/Perilipin2) (Fukumoto and Fujimoto, 2002). More recent techniques to visualize LDs have focused on staining these drops *in vivo*.

Greenspan *et al*. (1985) first showed the utility of nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) to label intracellular LD in live cultured peritoneal macrophages and smooth muscle cells. Nile red is an uncharged heterocyclic molecule that only fluoresces in a hydrophobic environment. Labeled neutral lipids fluoresce a yellow-gold to red color, depending on their relative hydrophobicity, with no detectable damage or deformation of dye-infused tissues (Fowler and Greenspan, 1985). More recently, Jones *et al*. (2008) used nile red to visualize neutral lipid deposits in live zebrafish larvae. They initially demonstrated that daily exposure of larvae to nile red-containing embryo media for 4 days (from 3 to 7 dpf) consistently labeled lipid-rich tissues. The authors then sought to test the effects of known pharmacological inhibitors of triglyceride metabolism on total larval lipid content. Treatment with nicotinic acid, a potent pharmacological inhibitor of adipocyte lipolysis (Carlson, 1963), resulted in an increase in total triglyceride content and decreased cholesterol levels. Treatment with resveratrol, a compound known to inhibit FA synthase (Tian, 2006), resulted in a decrease in total triglyceride content as detected by nile red staining. Total triglyceride content was further decreased when resveratrol was supplemented with norepinephrine (Jones *et al*., 2008).

While fluorescent dyes and stains are useful for identifying lipid deposits in cells and tissues, issues arise regarding the distribution and affinity properties of these compounds. Nonspecific labeling of tissues devoid of lipid deposits may be observed and staining and washing procedures must then be carefully optimized to minimize this effect. Additionally, nile red staining does not distinguish between FA and cholesterol *in vivo*, although some discrimination based on staining intensity of tissue sections is possible (Fowler and Greenspan, 1985).

B. BODIPY Fatty Acid Analogs

The wide variety of fluorescent lipid analogs commercially available allows one to fully exploit the optical clarity of zebrafish larvae to study lipid metabolism. One type of analog widely used in cultured cells to visualize lipid dynamics is BODIPY-labeled FA. These analogs consist of an acyl chain of variable length attached to the BODIPY (4,4-difluoro-4-bora-3a, 4a-diaza-S-indacene) fluorescent moiety. First synthesized by Treibs and Kreuzer in 1968 (Treibs and Kreuzer, 1969), the BODIPY fluorophore possesses a number of advantageous qualities including high photostability, strong and narrow wavelength emission in the visible spectrum, and an overall uncharged state (Monsma *et al*., 1989; Pagano *et al*., 1991).

To administer BODIPY FA analogs to live zebrafish larvae, we developed a feeding assay that utilizes liposomes to create a suspension of relatively hydrophobic FA analogs in embryo media (Carten, unpublished). Following a short liposome feed, digestive organ structure and metabolic function can be assessed, as the fluorescent FAs accumulate readily throughout numerous larval organs and tissues. With this assay, we have observed that different chain length FAs (short, medium, and long) accumulate in distinct patterns throughout digestive organs and tissues, with each chain length suited to visualize particular larval organs and cellular structures. Long chain fatty acids (LCFA) appear in cytoplasmic LD in enterocytes and hepatocytes. Short chain fatty acids (SCFA) accumulate primarily in the hepatic and pancreatic ducts and are particularly suited to illuminate ductal networks.

Medium chain fatty acids (MCFA) accumulate in LDs throughout a wide range of cell types, as well as ductal and arterial networks, and reveal the subcellular structures of multiple cell types. Because this feeding assay enables the rapid assessment of digestive function and FA metabolism in live zebrafish larvae, it has the potential for use in genetic and pharmacologic screens to identify genes involved in FA metabolism and potential drug targets.

C. BODIPY Cholesterol

In addition to FA analogs, a number of sterol analogs are available for use with cultured cells and zebrafish larvae (e.g., NBD-cholesterol, BODIPY-cholesterol). Initially created to visualize cholesterol partitioning into membranes, these analogs were found to preferentially enter into liquid-disordered domains, making them less useful for studies of sterol trafficking in cells (Li *et al.*, 2006). To address these limitations a new BODIPY-tagged cholesterol analog was synthesized with a modified fluorophore linker (Li and Bittman, 2007). Recent studies utilizing the improved BODIPY-cholesterol analog found it to partition into the cholesterol-rich liquid-ordered membrane domain (Ariola *et al.*, 2009) and interact with membranes in ways similar to native sterols, making it a powerful new tool for imaging sterol trafficking in live cells (Marks *et al.*, 2008).

Studies done in the zebrafish have found that BODIPY-cholesterol localizes to the yolk of developing zebrafish larvae (Holtta-Vuori *et al.*, 2008). Ongoing work in the Farber lab is examining the localization of BODIPY-cholesterol in zebrafish larval intestinal enterocytes after a high-fat meal and comparing its localization to that of LD (revealed by BODIPY-labeled FA). Recent data suggest that the initial uptake of sterol and of FA segregate into nonoverlapping compartments (Walters, unpublished). These types of experiments will ultimately enable the development of a clearer model for the uptake and trafficking of dietary lipid in intestinal enterocytes.

D. Fluorescent Reporters of Lipid Metabolism

The ability to perform forward genetic studies in zebrafish by mutagenizing the entire genome and screening for particular phenotypes has made this vertebrate model popular (Driever *et al.*, 1996; Haffter *et al.*, 1996). Mutagenesis methods commonly utilized by the zebrafish community include gamma-ray irradiation to generate large deletions and translocations (Fisher *et al.*, 1997), soaking founder fish in mutagenic chemicals such as ethylnitrosourea (ENU) to generate point mutations, (Driever *et al.*, 1996; Haffter *et al.*, 1996), and retrovirus- or transposon-mediated gene insertions (Chen and Farese, 2002; Ivics *et al.*, 2004). We perform an ongoing ENU-mutagenesis screen to search for mutations that perturb lipid processing. To screen for mutants defective in lipid processing, we soak zebrafish larvae in fluorescent lipid reporters that are swallowed and allow lipid processing to be visualized *in vivo* (Farber *et al.*, 2001).

One fluorescent reporter we routinely use in our screen is the phosphoethanolamine analog PED6, [N-((6-(2,4-dinitro-phenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-*sn*-glycerol-3-phosphoethanolamine] (Fig. 8A). Following ingestion, PED6 is cleaved by phospholipase A_2 (PLA_2), resulting in the release of the fluorescent labeled acyl chain (Farber *et al.*, 1999) (Fig. 8C). When zebrafish larvae (5 dpf) are immersed in media containing PED6, bright green fluorescence is observed in the intestine, gall bladder, and liver (Fig. 8E). We also utilize the sterol analog 22-NBD-cholesterol (22-[N-(7-nitronbenz-2-oxa-1,3-diazol-4-yl) amino]-23,24-bisnor-5-cholen-3-ol) (Fig. 8B) to visualize cholesterol absorption in live larvae and screen for mutants (Fig. 8F). This reagent is different from PED6 in that it continuously fluoresces and due to its hydrophobicity it is slightly more difficult to administer via feeding.

Because PED6 and NBD-cholesterol provide rapid readouts for digestive organ morphology and lipid processing, we used them to perform the first forward genetic physiologic screen in zebrafish to identify new genes that regulate lipid metabolism. One mutation identified with these lipid reporters, *fat-free (ffr)*, was a recessive lethal mutation. Although *ffr* mutants appeared morphologically normal, they exhibited significantly diminished fluorescence in their intestine and the gall bladder following PED6 treatment (Fig. 9) (Farber *et al.*, 2001). *ffr* larvae were further characterized using NBD-cholesterol to determine if the uptake and trafficking of sterol-like molecules was also impaired. In contrast to wild-type larvae, which accumulated NBD-cholesterol in their gall bladders within a few hours of feeding, *ffr* mutants were unable to concentrate NBD-cholesterol in their gall bladders. This observation suggests that *ffr* larvae have a significant defect in bile secretion and/or transport. When *ffr* mutants were incubated with BODIPY FL-C5, a medium chain FA analog, they had nearly normal digestive organ fluorescence. Because this medium chain length FA is less hydrophobic and is not dependent on emulsifiers, such as bile, for absorption, this further suggests that the *ffr* mutation attenuates biliary synthesis or secretion (Ho *et al.*, 2003).

To better characterize the metabolic defects of *ffr* larvae, we immersed mutants in media containing radioactive oleic acid (250 mCi/mmol, 3 h), followed by whole embryo lipid extraction and TLC analysis. We found that *ffr* larvae have significantly reduced radioactivity incorporated into phosphatidylcholine (PC) fraction ($p<0.05$) in comparison to wild-type larvae (Fig. 10). PC is a major component of bile and its overall reduction in *ffr* mutants is consistent with impaired liver lipid synthesis.

The positional cloning of *ffr* indicated that this gene encodes a protein with no known function. Further sequence analysis revealed *ffr* to be well conserved from invertebrates to mammals. Further studies found *ffr* to regulate a number of cellular processes including Golgi structure/maintenance, protein sorting, vesicle trafficking, and intestinal lipid absorption and processing (Ho *et al.*, 2006). Recent in *vivo* and *in vitro* work suggests that *ffr* is a novel Arf GTPase family effector that regulates the phospholipase D (PLD) activity. *ffr* mutants have excess PLD, leading to higher levels of phosphatidic acid (Wang *et al.*, 2006), which is known to increase membrane curvature (Begle *et al.*, 2009). The excessive membrane curvature in *ffr* mutants may cause abnormal Golgi-vesicle trafficking of nutrient transporters to the cell surface, leading to higher extracellular blood glucose levels and impaired lipid metabolism (Liu *et al.*, 2010).

After identifying the molecular nature of the zebrafish *ffr* mutation, the human ortholog was located by BLAST searches of public databases. While the human gene was present in these databases, there was no data on its possible function. This example demonstrates the power of forward genetic methods in zebrafish to assign functions to mammalian genes. Other reports of forward genetic screens demonstrate the utility of PED6 and similar synthetic analogs to identify genes involved in digestive processes. For example, Wantanabe *et al.* used PED6 to identify genes that regulate liver morphogenesis and bile synthesis in Medaka fish (Watanabe *et al.*, 2004).

VI. Triple Screen: Phospholipase, Protease and Swallowing Function Assays

Although PED6 has proven successful in identifying abnormal lipase activity in zebrafish mutants, there can be significant variability in the digestive tract fluorescence observed between wild-type siblings (Fig. 11A). Such variable labeling can make the use of PED6 in genetic screens difficult, as an increase or decrease in fluorescence cannot be attributed solely to mutations and may reflect interindividual differences in reporter ingestion. To address this issue, it was reasoned that PED6 could be used in conjunction with other

reporters of digestive function to create a physiologically relevant readout of *in vivo* digestive processes.

Hama *et al.* (Hama *et al.*, 2009) recently demonstrated that concurrent feeding of PED6, EnzChek protease reporter (Invitrogen Inc.), and nonhydrolyzable microspheres allows one to monitor lipase, protease, and swallowing activities, respectively, in larval zebrafish. Since the three reporters fluoresce at distinct wavelengths, simultaneous viewing of all three signals is possible (Figs. 11B and 11C). The EnzChek protease reporter consists of the phosphoprotein casein labeled with multiple red or green BODIPY fluorophores. The proteolytic cleavage of the quenched reporter generates highly fluorescent casein fragments, with total fluorescence proportional to enzyme activity (Jones *et al.*, 1997). EnzChek has been previously used to detect the activity of metallo, serine, acid and thiol proteases in a number of biological systems (Haugland and Kang, 1988; Jones *et al.*, 1997; Menges *et al.*, 1997; Sarment *et al.*, 1999). Unlike PED6 and EnzChek, which report enzymatic function, nonhydrolyzable microspheres assess swallowing and intestinal lumen integrity.

Administering this screening cocktail to larval zebrafish revealed a correlation between the intestinal protease and phospholipase activity, consistent with the hypothesis that the variance observed in PED6 fluorescence was partly due to differing amounts of PED6 consumed by each larva (Fig. 11D). This work demonstrated that the ratio of PED6 to EnzChek fluorescence can serve as a readout of the digestive function, since the ratio in individual larvae is unaffected by differences in reporter ingestion.

After validating the triple screening method, Hama *et al.* (2009) demonstrated the utility of this assay in evaluating the role of the exocrine pancreas in the digestive function. The exocrine pancreas secretes many of the gastric lipases and proteases needed for the breakdown and subsequent uptake of nutrients (Layer and Keller, 1999). Previous work has shown that morpholino knockdown of the *ptf1a* transcription factor can selectively prevent exocrine pancreas development (Lin *et al.*, 2004). Analysis of *ptf1a* mutants (5 dpf) using the triple screen found that these larvae retain normal levels of lipase activity yet have reduced protease activity. Further investigation revealed that there is a marked increase in pancreatic phospholipase A2 (groups IB and III) expression between 5 and 6 dpf, supporting the notion that the exocrine pancreas is not the main source of phospholipase activity in 5 dpf fish. However, 6 dpf *ptf1a* mutant larvae exhibit decreased amounts of both protease and lipase activity, suggesting the exocrine pancreas begins providing gastric lipases after 5 dpf.

The regulation of phospholipase and protease activity was also examined using the triple screening method. The peptide hormone Cholecystokinin (CCK) facilitates digestion by causing the secretion of gastric enzymes into the small intestine after food consumption (Raybould, 2007). Release of CCK into the circulatory system activates the CCK receptor A (CCK-RA) in the exocrine pancreas. Larvae (5 dpf) treated with CCK-RA antagonist showed a reduction in protease activity but had unaffected lipase activity. Much like the *ptf1a* mutants, 6 dpf larvae had lower levels of both protease and lipase activity. Not surprisingly, the effect of CCK-RA antagonist was abolished in *ptf1a* morphants. This work suggests that CCK signaling regulates zebrafish secretion of exocrine pancreas-derived intestinal proteases earlier in development (5 dpf) and phospholipases later (6 dpf).

The utility of employing multiple reagents to screen for the digestive function using larval zebrafish has been demonstrated by others in the field. Recent work from Clifton *et al.* utilized a multi-tiered approach to identify and describe novel compounds that inhibit dietary lipid absorption (Clifton *et al.*, 2010). In the initial screen, larvae (5 pdf) were incubated in PED6 after compound exposure and assayed for a reduction in gall bladder fluorescence. Results were validated in adult fish and inactive or toxic compounds were

removed from the study. Further narrowing was done through the use of fluorescent microspheres to confirm swallowing, as previously described. Absorption of fluorescent cholesterol (NBD-cholesterol) and FA analogs (SCFA BODIPY-C5, LCFA BODIPY-C16) was also assayed to better understand the mechanism of action for seven potentially novel compounds and two known lipid absorption inhibitors, orlistat and ezetimibe. Interestingly, the authors found that ezetimibe was found to reduce metabolism of both PED6 and BODIPY-C16 in addition to NBD-cholesterol. Five of the seven compounds identified in the initial PED6 screen had comparable results. AM1–43, a dye commonly used to visualize endocytosis, and the EnzChk protease assay were used to screen for physiological disruptions, while the lipophilic dye ORO was used to determine whether reduced gall bladder fluorescence was due to reduced lipid processing. AM1–43 staining after ezetimibe treatment was significantly reduced, supporting a disruptive role in endocytosis, perhaps more pronounced than previously described. Those with reduced AM1–43 staining underwent further testing to determine whether cholesterol synthesis was normal: larvae were pretreated with a reagent that extracts membrane cholesterol (methyl-ß-cyclodextran;MßC) and observed for recovery. By using a series of well-established assays, three compounds were identified for testing in mammals and ezetimibe was implicated as playing a larger role in intestinal endocytic dynamics than previously thought. These studies further establish the zebrafish system as a useful and translatable model for testing and prioritizing new compounds for follow-up studies in mammals.

As with any *in vivo* system, there is inherent variability (developmental timing, intestinal microenvironment), which will result in signal variation. Therefore, caution should be taken to minimize this variability by carefully scoring digestive organ morphology of larvae prior to and after reporter ingestion, discarding sickly larvae that lack swim bladders or exhibit developmental delay, and using properly staged larvae.

VII. Zebrafish Models of Human Dyslipidemias

Recently, an adult zebrafish model for early atheroschlerosis has been developed through administration of a high-cholesterol diet (HCD) (Stoletov *et al.*, 2009). After receiving a 4% cholesterol diet from 5 weeks postfertilization (wpf) to 13–17 wpf, fish developed hypercholesterolemia, as demonstrated by their four-fold plasma total cholesterol levels. No weight gain or increase in TAG was observed. Lipoprotein profiles of HCD-fed fish showed, in addition to the wild-type control HDL fraction, strong fractions for LDL and VLDL. Antibody studies showed that the fish had oxidized plasma lipoproteins, correlating to the development of atherosclerotic lesions. Staining of sections indicated early lesions of atherosclerosis (fatty streaks) while antibody (human L-plastin) staining implicated macrophage infiltration. Live larvae were used to visualize myeloid cell and lipid accumulation, endothelial cell defects, and increased PLA2 activity, signs of early atherosclerosis. This novel model has been used in transplant studies to demonstrate *in vivo* the requirement of toll-like receptor-4 in macrophage lipid uptake.

The zebrafish has also emerged as a model of glucose metabolism and thus, diabetes, allowing the role lipids play in the onset and pathologies of both subtypes of this disease to be examined. Diabetes was once studied primarily in the context glucose uptake and metabolism; however, the causal effects of dietary lipids on type 2 diabetes are now being examined more closely. Larval zebrafish contain insulin-secreting beta cells that are triggered to release insulin in response to elevated blood glucose and FA levels, much like in humans. Work done by Elo *et al.* has shown that the exposure of larval and adult zebrafish to high glucose levels results in hyperglycemia (Elo *et al.*, 2007). Furthermore, treatment with the antidiabetic compounds Glipizide and Metformin reduces blood glucose levels in adult zebrafish and lowers the expression of phosphoenolpyruvate carboxykinase, which is

regulated by insulin and catalyzes the rate-limiting step of gluconeogenesis, in larval zebrafish (Elo *et al.*, 2007).

VIII. Summary

In this, chapter we have presented novel approaches undertaken using the zebrafish to investigate lipid metabolism and signaling. The optical transparency of zebrafish embryos and larvae can be fully exploited by using transgenic lines, fluorescent reporters, and lipid analogs to visualize metabolic events. Furthermore, the high genetic conservation across lipid signaling and metabolic pathways allows pharmacological regents to be utilized to study the importance of lipids during early development. The high fecundity, small size, and genetic tractability of these organisms make them ideal for high-throughput screening efforts to identify genes involved in lipid metabolism and signaling and thus identify potential therapeutic targets for human diseases.

Despite the immense potential of the zebrafish as a genetic and therapeutic screening tool, these organisms are currently underutilized by the academic and pharmaceutical research communities. While we have discussed some of the exceptions here, many studies use the zebrafish to investigate developmental events such as pattern formation and early neurogenesis, with few taking advantage of the system to study digestive physiology and lipids. We advocate the increased use of zebrafish in metabolic studies and fully expect a continued upward trend in the use of this organism as its contributions to our understanding of human disease become more apparent.

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Abbreviations

LCFA	long chain fatty acid
LD	lipid drop
MCFA	medium chain fatty acid
MTP	microsomal triglyceride transfer protein
SCFA	short chain fatty acid
TAG	triacylglycerol

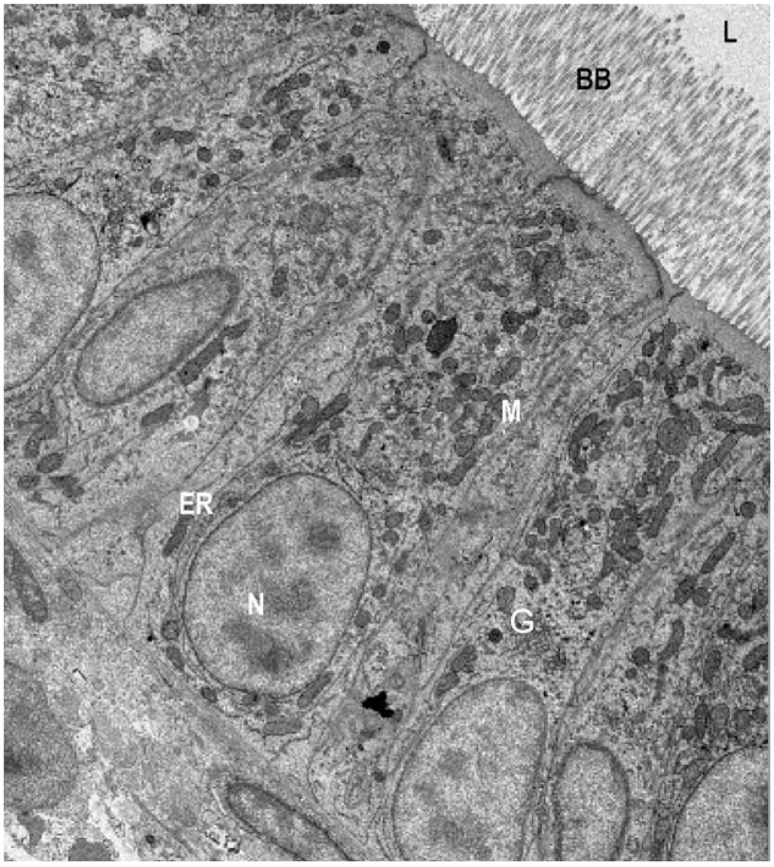

Fig. 1.

The intestinal enterocytes of zebrafish and mammals exhibit a high degree of morphological similarity. Electron micrograph of enterocytes from a larval zebrafish (6 dpf). Zebrafish enterocytes exhibit the typical characteristics of mammalian polarized intestinal cells including apical microvilli, which extend into the intestinal lumen (L) and form the brush border, as well as basal nuclei (N). Organelles and subcellular details including mitochondria (M), Golgi bodies (G), and endoplasmic reticulum (ER) are apparent throughout the enterocytes. For comparison, see mouse EMs in Buhman *et al.* (2002).

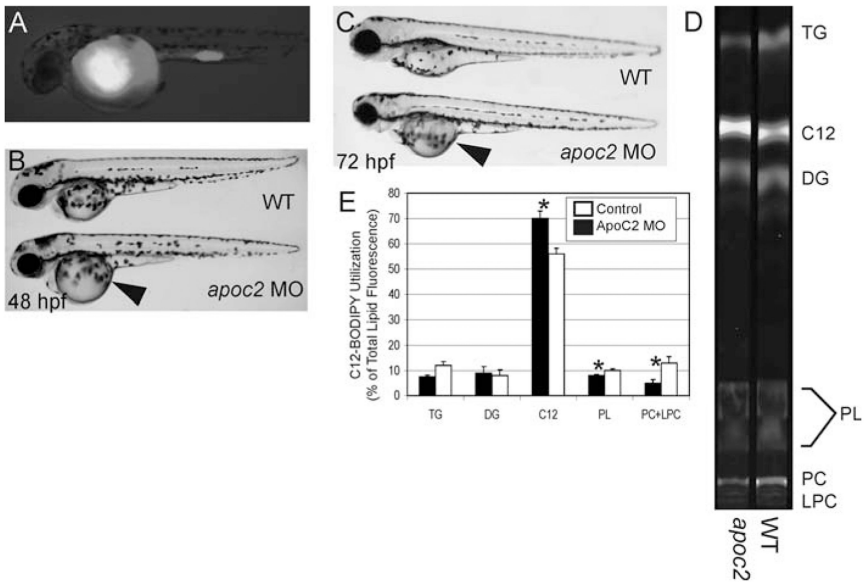

Fig. 2.

BODIPY lipid analogs enable studies of yolk metabolism during early zebrafish development. To assay the function of *apoc2* during zebrafish development, embryos were injected with an *apoc2* morpholino at the 1–4 cell stage followed by injection of a fluorescent fatty acid (BODIPY-C12) at 24 h postfertilization (hpf). (A) At 48 hpf, embryos injected with BODIPY-C12 retain the fluorescent analog primarily in their yolk. (B, C) *apoc2* morphants exhibit an enlarged yolk phenotype (arrowhead) at 48 and 72 hpf, indicating that *apoc2* is necessary for yolk utilization during larval development. (D, E) To determine the metabolic consequences of *apoc2* deficiency, 1 dpf wild-type and *apoc2* morphant larvae were injected with BODIPY-C12 and assayed using fluorescent thin layer chromatography (TLC) 2 days later. (D) TLC analysis shows that BODIPY-C12 is incorporated primarily into triacylglycerol (TAG), diacylglycerol (DG), and phosphatidylcholine (PC) in both wild-type and *apoc2* morphant larvae. (E) *apoc2* morphants exhibit defects in PC and lysophosphatidylcholine (LPC) metabolism. Total lipid fluorescence was quantified from TLC plates run with total lipids extracted from wild-type and *apoc2* morphants. Triacylglycerol (TAG), diacylglycerol (DG), BODIPY C12:0 (C12), phospholipids (PL), PC, and LPC.* $p < 0.05$.

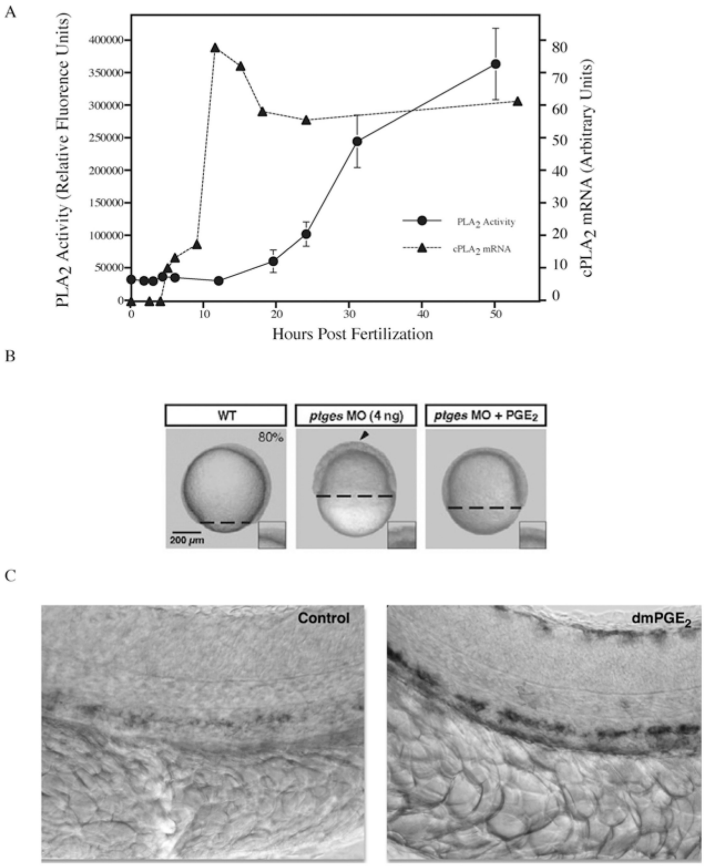

Fig. 3.

Phospholipase activity is required for proper zebrafish development. (A) Cytosolic phospholipase A2 (cPLA2) mRNA levels and PLA2 enzymatic activity were quantified from whole embryo lysates during early stages of zebrafish development. cPLA2 expression peaks during somitogenesis (10 h) while enzymatic activity steadily increases as development proceeds. PLA2 activity is required to generate prostaglandins. (B) Blocking prostaglandin production during early zebrafish development through inhibition of prostaglandin–endoperoxide synthase (Ptgs1) via morpholino knockdown results in developmental arrest at epiboly. Developmental arrest can be rescued by adding back the exogenous enzyme product (PGE2). Reproduced with permission by *Development* (Speirs *et al.*, 2010). (C) Embryos exposed to the PGE2 analog (16,16-dimethyl-PGE2; dmPGE2) exhibit increased expression of *runx11* and *cmyb1* as evidenced by *in situ* hybridization. These genes are expressed in the ventral wall of the dorsal aorta in a region analogous to the mammalian aorta–gonad–mesonephros and are required for mammalian hematopoietic stem cell development. Reprinted by permission from Macmillan Publishers Ltd.: *Nature* (North *et al.*, 2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* 447, 1007–11, copyright 2007.

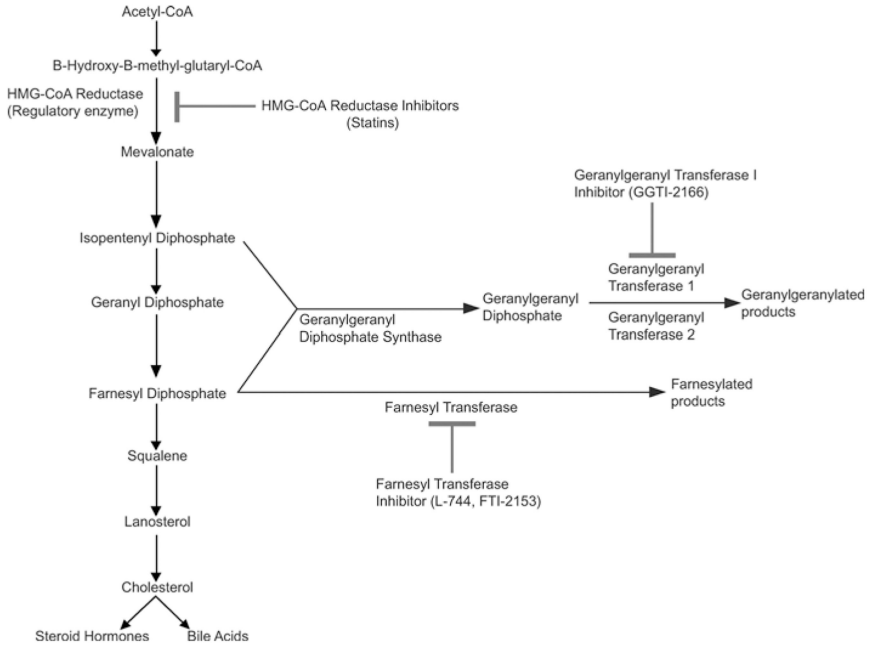

Fig. 4.

Cholesterol biosynthesis and protein lipidation are highly conserved in vertebrates. Due to the high genetic conservation of these pathways, human drugs can be used to block specific biochemical steps to determine the roles that cholesterol and protein lipidation play in zebrafish development. Reprinted from *Development Cell* with permission from Elsevier (Thorpe *et al.*, 2004).

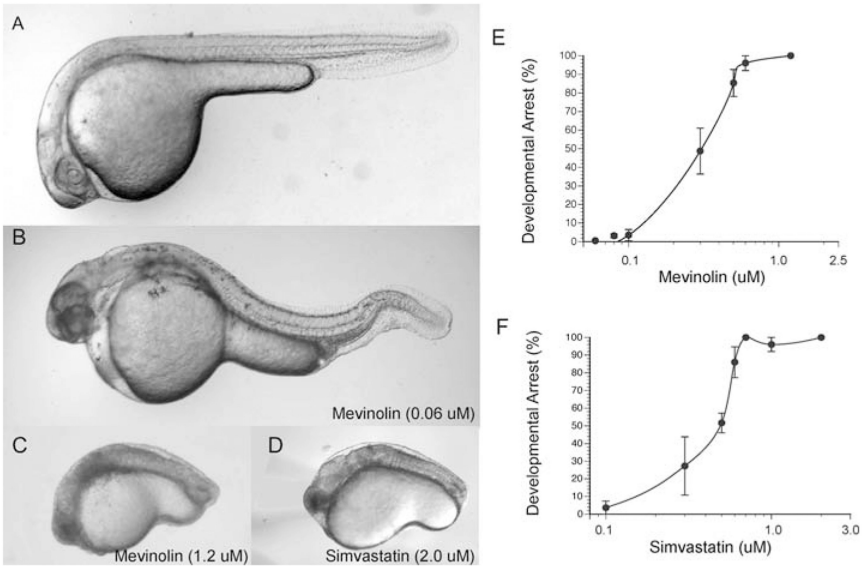

Fig. 5.

Embryos treated with statins exhibit developmental defects. In comparison to untreated embryos (A), embryos soaked in a low dose of mevinolin (0.06 μM). (B) exhibit mild developmental defects, as evidenced by tail kinks. (C) Exposure to higher doses of mevinolin (1.2 μM) results in blunted axis elongation, necrosis and developmental arrest. (D) Simvastatin treated embryos (2.0 μM) exhibit similar developmental defects. (E) Dose response of mevinolin on developmental arrest (mean + SEM, $n = 3$). (F) Dose response of simvastatin on developmental arrest (mean + SEM, $n = 3$). Reprinted from *Development Cell* with permission from Elsevier (Thorpe *et al.*, 2004).

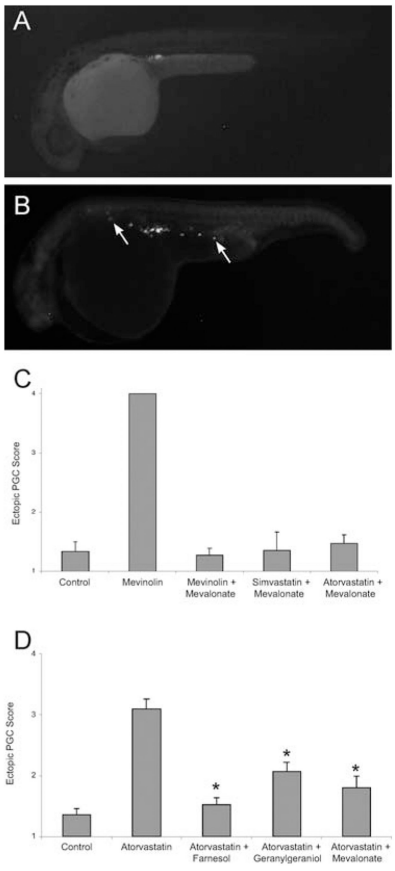

Fig. 6.

Isoprenoid intermediates rescue the developmental defects caused by statin treatment. Embryos at early cell stages were injected with mevalonate and then soaked overnight in mevinolin, simvastatin, or atorvastatin. (A) Embryos treated with mevinolin show severe developmental defects at 24 hpf. (B) Embryos injected with the isoprenoid intermediate mevalonate (1–16 cell stages) and then treated with mevinolin exhibit normal morphology. (C) Mevalonate injection rescues the somatic defects observed in embryos treated with different statins. Embryo morphology was scored at 24 hpf. Data represent the MEAN ± SEM from 3–4 experiments. Reprinted from *Development Cell* with permission from Elsevier (Thorpe *et al.*, 2004).

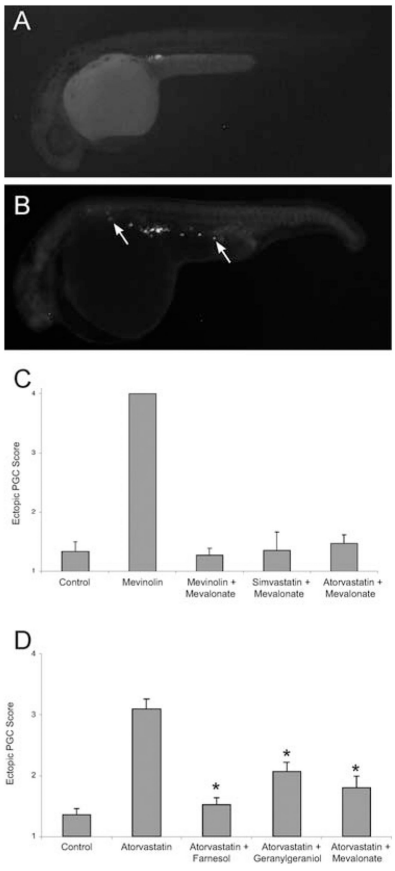

Fig. 7.

Statin treatment causes abnormal primordial germ cell (PGC) migration in zebrafish embryos. Compared to wild-type (A), embryos treated with statins (B) display ectopic PGCs. The arrows indicate ectopic PGCs that have failed to migrate to the developing gonad. (C) The PGC migratory defect observed following statin treatment is prevented by injections of isoprenoid intermediates. Embryos at early stages (1–16 cell) were injected with gfp-nos mRNA and mevalonate and then soaked overnight in mevinolin, simvastatin or atorvastatin. At 24 hpf, embryos were scored for ectopic PGCs, with a score of 1 indicating a wild-type single gonadal cluster and score of 4 indicating no discernable PGC cluster. Data represent the mean ± SEM from 3–4 experiments. (D) The PGC migratory defect observed following statin treatment is prevented by increasing the levels of isoprenoid synthesis intermediates. Embryos injected at the 1–16 cell stage with farnesol, geranylgeraniol or mevalonate and then soaked overnight with atorvastatin (10 μM) show normal PGC migration. Data represents MEAN ± SEM, $*p < 0.01$ difference from atorvastatin alone. Reprinted from *Development Cell* with permission from Elsevier (Thorpe *et al.*, 2004).

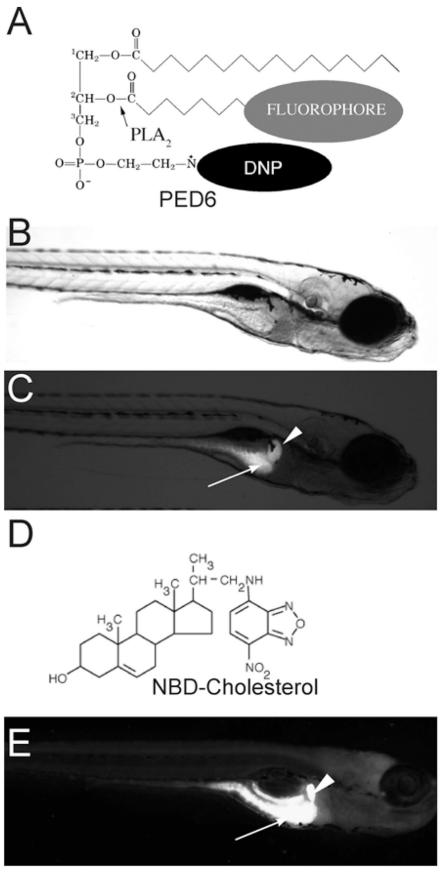

Fig. 8.

PED6 and NBD-cholesterol visualize lipid uptake in larval zebrafish. (A) The chemical structures of PED6. The BODIPY-labeled acyl chain of PED6 is normally quenched by the dinitrophenyl group at the *sn*-3 position. Upon PLA2 cleavage at the *sn*-2 position, the BODIPY-labeled acyl chain is unquenched and can fluoresce. Bright field (B) and fluorescent (C) images of 5 dpf larva following soaking in PED6 for 6 h. PED6 labeling reveals lipid processing in the gall bladder (arrowhead) and intestine (arrow). (D) The chemical structure of NBD-cholesterol. The NBD-cholesterol analog contains a NDB fluorophore where the alkyl tail at the terminal end of cholesterol would normally reside. (E) Soaking zebrafish larvae (5 dpf) in NBD-cholesterol (3 mg/ml, solubilized with fish bile) for 2 h visualizes cholesterol uptake in the gall bladder (arrowhead) and intestine (arrow).

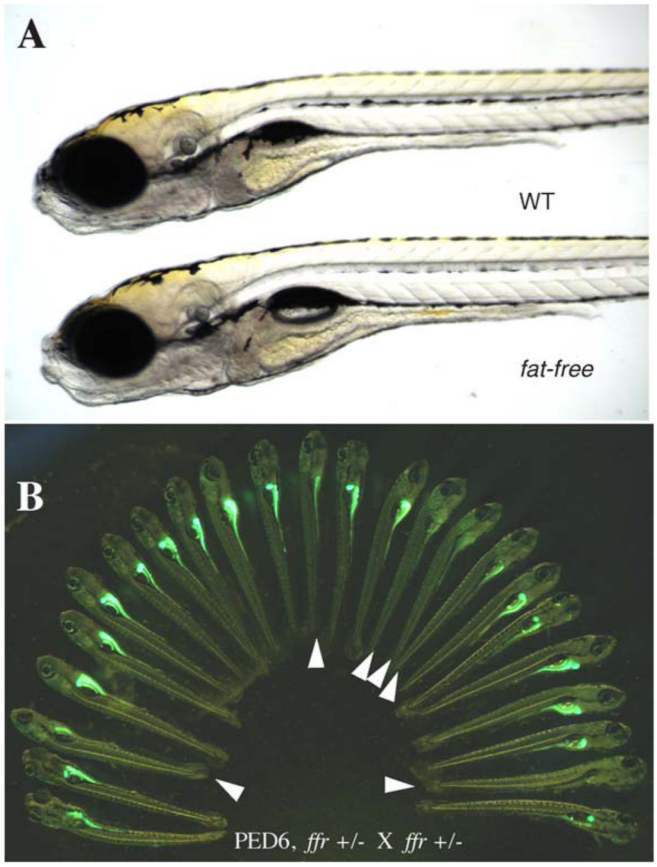

Fig. 9.

PED6 labeling of *fat-free(ffr)* larvae reveals defective lipid processing. (A) *ffr* larvae appear morphologically normal in comparison to wild-type siblings (6 dpf). (B). *ffr* mutants have diminished intestinal and gall bladder fluorescence when labeled with PED6, indicating abnormal lipid processing.

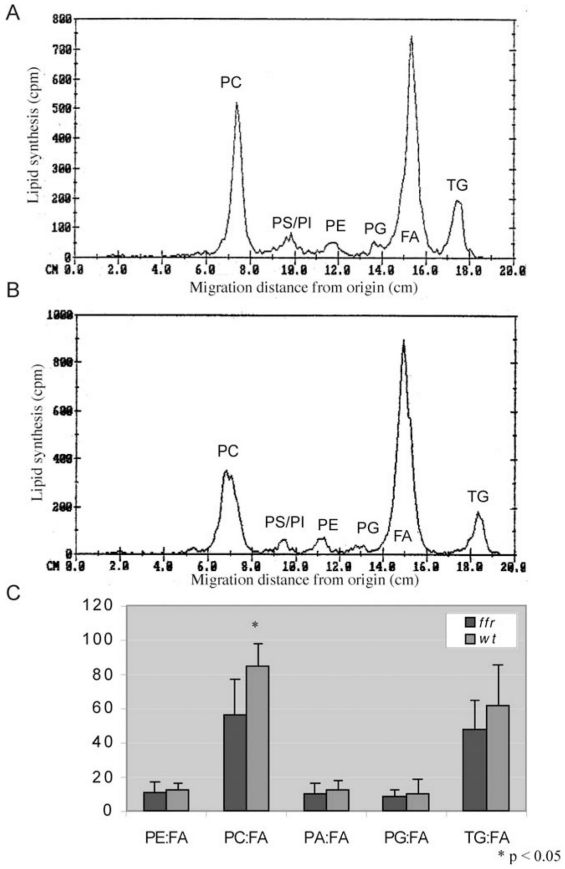

Fig. 10.

ffr larvae have abnormal phospholipid metabolism as shown by thin layer chromatography (TLC). To identify the metabolic defects in *ffr* larvae, mutants and wild-type larvae (4 dpf) were incubated with radioactive oleic acid (C18:1) for 20 h, followed by total lipid extraction and radioactive TLC analysis. (A, B) Chromatograms generated from scans of TLC plates run with total lipids extracted from wild-type (A) and *ffr* (B) larvae. They-axis reflects radioactivity counts (cpm; 2–3% of actual activity detected for ^{3}H); the *x*-axis shows various oleic acid metabolites determined by the migration distance from the start of the TLC plate measured in centimeters (cm). The major metabolites derived from oleic acid (here FA) are PC, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and TAG. (C) Comparison of relative FA metabolites between *ffr* and wild-type larvae reveals that *ffr* mutants have significantly decreased PC production (n = 9, mean ± SD). Reprinted from *Methods in Enzymology* with permission from Elsevier (Ho *et al.*, 2003).

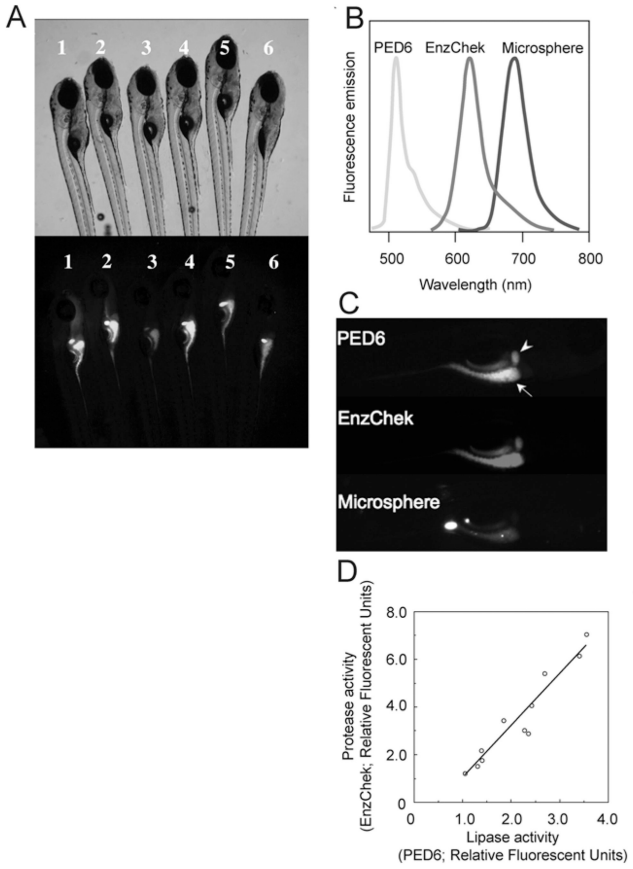

Fig. 11.

Concurrent feeding of PED6, EnzChek, and fluorescent microspheres enables the assessment of larval digestive function. To correct for interindividual variability observed in wild-type siblings (6 dpf) labeled with PED6 (A), multiple reporters of the digestive function can be used. (B) PED6, EnzChek and microspheres each fluoresce at distinct wavelengths, allowing simultaneous screening for lipase and protease activities, and swallowing function, respectively. (C) PED6 and EnzChek signal in the gall bladder (arrowhead) and intestine (arrow) of wild-type zebrafish. Microspheres in the intestine indicate normal ingestion. (D) Intestinal protease and phospholipase activity correlate, validating the use of the ratio of PED6 to EnzChek signal as readout of the digestive function. Figure from Hama *et al.* (2008), *Am. J. Physiol. Gastrointest. Liver Physiol.*, used with permission from *Am. Physiol. Soc.*