ER lipid activities induce specific morphological and transcriptional responses

Endoplasmic reticulum lipid flux influences enterocyte nuclear morphology and lipid-dependent transcriptional responses

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Abstract

Responding to a high-fat meal requires an interplay between multiple digestive tissues, sympathetic response pathways, and the gut microbiome. The epithelial enterocytes of the intestine are responsible for absorbing dietary nutrients and preparing them for circulation to distal tissues, which requires significant changes in cellular activity, including both morphological and transcriptional responses. Following a high-fat meal, we observe morphological changes in the enterocytes larval zebrafish, including elongation of mitochondria, formation and expansion of lipid droplets, and the rapid and transient ruffling of the nuclear periphery. Dietary and pharmacological manipulation of zebrafish larvae demonstrated that these subcellular changes are specific to triglyceride absorption. The transcriptional changes that occur simultaneously with these morphological changes were determined using RNASeq revealing a cohort of up-regulated genes associated with lipid droplet formation and lipid transport via lipoprotein particles. Using an Mtp (Microsomal triglyceride transfer protein) inhibitor to block beta-lipoprotein particle formation, we demonstrate that the transcriptional response to a high-fat meal is associated with the transfer of ER triglyceride to nascent beta-lipoproteins, possibly through the activation of Creb3l3/Crebh (cyclic AMP response element binding protein). These data suggest that a transient increase in ER lipids is the likely mediator of the initial physiological response of intestinal enterocytes to dietary lipid.

Introduction

The intestinal epithelium is largely composed of polarized, columnar, absorptive cells called enterocytes whose apical surfaces form the lumen of the intestine [1]. The plasma membrane at this apical surface is characterized by a highly specialized membrane domain called the brush border and is responsible for absorbing nutrients that pass through the intestinal lumen. Enterocytes respond to the presence of food by increasing their size and remodeling several organelles, including mitochondria and the endoplasmic reticulum (ER) [2, 3]. Although a great deal is known about the structure and organization of enterocytes, many cell biological details regarding how lipids are absorbed at the apical surface, partitioned into lipid droplets within the cytoplasm, and packaged into chylomicrons for delivery to blood and lymph at the basal membrane are poorly understood.

In recent years, the zebrafish has proven to be a powerful model organism for the study of vertebrate lipid metabolism, including dietary lipid processing by the intestine and liver [4-8]. Unlike in vitro studies, the larval zebrafish can be used to study the enterocyte response to feeding in the context of the signals and
organization of the intestine in vivo. Dietary nutrients (e.g., lipids, proteins, carbohydrates) can be added to zebrafish media together with labeled lipids and/or other small molecules [9-12]. In this way, a large number of larvae can be treated concurrently with different foods or pharmaceuticals, allowing for rapid and robust analyses.

The nucleus is a functionally and physically dynamic organelle which enables cells to appropriately respond to the multitude of signals required to maintain a viable multicellular organism. Between different cell types of a single organism, great diversity exists in the shape and structure of the nucleus [13]. During cell division, the nucleus undergoes dramatic changes to its organization and morphology. While the shape of the nucleus in an interphase cell is widely considered to be static, variations in nuclear shape occur in some cell types, such as neutrophils, and feature prominently in various diseases [14]. Nuclear shape changes have been associated with the transduction of cytoplasmic signals into specific transcriptional responses, although many of the molecular mechanisms regulating these responses remain to be explained [15]. We investigated whether metabolic changes associated with specific dietary nutrients might play a role in nuclear morphology.

In this study, we report that when zebrafish larvae are fed a lipid-rich diet (high in triglycerides and cholesterol), the morphology of enterocyte nuclei, mitochondria, and lipid droplets are significantly altered. The timing of these morphological changes suggests a coordination between organelles in responding to the influx of lipid. While morphological changes in mitochondria and lipid droplets during a high-fat feed have been previously reported [2, 3, 16], here we characterize a novel rapid and reversible ruffling of the nuclear envelope that is dependent upon the absorption of dietary triglyceride. This initial observation led to a fundamental question: Does the dramatic change in nuclear morphology indicate related changes in cellular physiology?

We also report the transcriptional response of the zebrafish digestive organs to an acute high-fat feed using RNASeq analysis and highlight the changes in gene expression involved in the synthesis, storage, and dispersal of lipids. These key physiological responses to a high-fat meal all stem from the endoplasmic reticulum (ER), where lipids are formed and assigned to their fates. To examine the role of lipids in the ER in the enterocyte response to a high-fat meal, we disrupted the formation of key lipoprotein particles in enterocytes, the chylomicrons, using a Microsomal triglyceride transfer protein (Mtp) inhibitor. The Mtp inhibitor blocks the lipidation of apolipoprotein B (ApoB) in the ER where it normally seeds the formation of triglyceride-loaded chyomicron particles. Inhibition of Mtp led to extreme changes in nuclear morphology, likely due to the disrupted flow of lipids from the ER. Additionally, Mtp inhibition reduced the transcription of key genes activated by high-fat feeding and targeted by the transcription factor Creb3l3 (Cyclic AMP response element binding protein; Crebhl). Our hypothesis is that triglyceride within the ER induces both cellular morphological changes and transcriptional changes that reconfigure enterocytes into lipid-processing and lipid-exporting machines.

Results

A high-fat meal induces changes in enterocyte morphology during feeding

By 6.5 days post fertilization (dpf), zebrafish larvae have a fully functional digestive system (mouth, pharynx, intestine, liver and pancreas), have completely absorbed their maternally deposited yolk, and actively hunt for exogenous food [17]. To examine the cellular responses of the intestine in response to feeding, a lipid-rich meal was administered to the larvae for 1 h and the intracellular morphology and organization of enterocytes were characterized at various times after the meal. Transmission electron microscopy (TEM) revealed striking morphological changes within the enterocytes, including elongation of mitochondria, appearance of cytoplasmic lipid droplets and changes in nuclear shape (Fig. 1). As shown in Figure 1B, enterocyte nuclei are initially round and smooth prior to feeding, but after 1h of feeding, the shape of many enterocyte nuclei became lobular and irregular. The degree of nuclear curvature was quantified using a custom algorithm implemented in MATLAB (discussed in detail in the Methods). The average nuclear curvature coefficient of variation (COV) for nuclei in the unfed state was 1.12 ± 0.17 (MEAN ± SD), whereas this increases to 1.42 ± 0.16 following 1 h of feeding as the nuclei become ruffled (Fig. 1D). For each intestinal section examined, the percentage of cells exhibiting ruffled nuclei (defined as a curvature COV greater than the mean curvature COV of unfed fish plus one standard deviation) also increased from an unfed baseline of 25.7% ± 20.7 to 61.6% ± 17.0 at 1 h of a high-fat meal (Fig. 1E). This change is transient, such that by 2 h of feeding, the percentage of ruffled nuclei and the mean degree of nuclear curvature is similar to that in the unfed larvae (Fig. 1D and 1E). There was a statistically significant difference in curvature COV and percent of cells with nuclear ruffling between groups as determined by one-way ANOVA ($F(5, 28) = 0.54, p < 0.0001$ and $F(5, 28) = 2.81, p < 0.0001$, respectively). A Tukey post-hoc test revealed that both the nuclear curvature COV and the percent of cells with nuclear...
Enterocyte nuclear ruffling is induced specifically by meals that include fat

To determine whether the observed changes in nuclear morphology are specific to absorption of dietary fat, meals of different nutrient composition were fed to the larvae (6.5 dpf) followed by TEM analysis. Our data indicate that only lipid-rich foods were able to induce nuclear ruffling (Figs. 2A and 2B; one-way ANOVA \(F(4,38) = 2.30, p < 0.0001\)). A Tukey post-hoc test revealed chicken egg yolk and artemia feeds induced a statistically significant increase in nuclear curvature COV. This ruffling followed the same reverse profile observed previously, so that at 2 h after the initiation of feeding, nuclei returned to an unruffled state in both chicken egg yolk and artemia (data not shown). The higher amount of fat in chicken egg yolk solution (1.3% lipid w/v) increased the proportion of enterocytes exhibiting ruffled nuclei, as compared to what was observed in fish fed artemia solution (0.5% lipid w/v) (Fig 2C), suggesting that there may be a threshold level of lipid in the cell that must be reached before ruffling is observed. Food solutions which contained little fat (spirulina 0.002% lipid w/v) or no fat (chicken egg white and glucose) did not induce nuclear ruffling. In these low-fat or fat-free conditions, nuclear ruffling was not simply delayed, as no evidence of ruffling was observed at 2 h after feeding was initiated (data not shown). As expected, lipid droplet formation was only observed in the chicken egg yolk- and artemia-fed fish (Fig. 2A). However, the appearance of the lipid droplets was remarkably different in animals fed these two foods; lipid droplets in the egg yolk-fed larvae were less electron dense than those in the artemia-fed fish. Given that a major site of osmium reactivity is the C=C double bonds contained within the acyl chains of complex lipids [18], the darker osmium stain observed in artemia lipid droplets would suggest a higher number of polyunsaturated acyl chains than egg yolk. In fact, egg yolk acyl chains are predominantly monounsaturated fatty acids (42% oleic; 18:1) and saturated fatty acids (28% palmitic; 16:0, 9% stearic; 18:0) [19], whereas artemia contains 28% polyunsaturated fatty acids in addition to 52% monounsaturated fatty acids [20].

Pharmaceutical manipulation of dietary lipid absorption links triglyceride metabolites (monoglyceride/free fatty acid) to nuclear ruffling

Egg yolk is composed of a heterogeneous mixture of lipids that includes phospholipids, cholesterol, and TAG [21, 22]. To determine whether a particular lipid constituent was inducing changes in nuclear morphology, we treated larvae with pharmaceutical drugs that inhibited the uptake of cholesterol or TAG metabolites in conjunction with the previously described high-lipid meal (Fig. 2D). To test the hypothesis that dietary
cholesterol was mediating nuclear ruffling, cholesterol absorption was blocked pharmacologically using the Npc1l1 inhibitor, ezetimibe [23-25]. Consistent with previous studies [11, 26], ezetimibe treatment efficiently inhibited the uptake of fluorescent cholesterol analog (TopFluor-Cholesterol, Avanti Polar Lipids) into the enterocytes of treated animals (data not shown). Importantly, ezetimibe treatment had no effect on feeding (data not shown). Notably, zebrafish treated with ezetimibe prior to a high-fat meal formed intestinal cytoplasmic lipid droplets similarly to their untreated and vehicle-treated counterparts (Fig. 2F). However, nuclear ruffling was no different in degree or timing than the untreated and vehicle-treated controls (Fig. 2E), indicating dietary cholesterol is not responsible for the observed nuclear ruffling.

To evaluate the role of TAG byproducts produced in the intestinal lumen following a high-fat meal, Orlistat was utilized to prevent the action of pancreatic lipases, which subsequently blocks enterocyte absorption of TAG metabolites of triglycerides (fatty acids and monoglycerides) and cholesterol esters [27]. As was the case with ezetimibe, orlistat did not alter the total amount of food consumed (data not shown). As evidence of the drug’s effectiveness in the larval zebrafish, treated animals failed to form cytoplasmic lipid droplets (Fig. 2F), due to a lack of absorbed fatty acids required for the synthesis of TAG within the ER and the subsequent formation of cytoplasmic lipid droplets [28]. Furthermore, orlistat-treated fish exhibited attenuated nuclear ruffling, suggesting that monoglycerides/free fatty acids are the dietary trigger that induces nuclear ruffling (Fig. 2E).

A high-fat meal alters the transcription of genes involved in lipid transport, storage, and metabolism
The morphological changes observed in enterocytes are indicative of a rapid and robust cellular response to an influx of dietary fatty acids and other TAG metabolites. As the cell exits a resting state, transcriptional changes must occur that allow for the enterocyte to upregulate lipid export through lipoprotein particle secretion as well as intracellular lipid storage capacity [29]. The rapid morphological responses observed in our studies indicated that an early time point (1 h post feeding) would capture the initial cellular response to dietary fat. We performed RNASeq analysis of zebrafish larval (6.5 dpf) gut RNA from animals fed with either chicken egg white or egg yolk (one and four hours post meal) to identify genes that were specifically changed by a high-fat meal and not by feeding. In addition, we performed RNASeq with 6.5-dpf zebrafish guts prior to feeding (unfed controls) as a reference. The results of three separate experiments were utilized to compare the induction or repression of transcripts under each condition and at specific time points (unfed controls were used to determine baseline transcription levels) (Table S1). This allowed us to separate genes induced or repressed specifically by high-fat meals from those that were induced or repressed by feeding in general (Fig. 3A). Conclusions based on RNASeq data must be determined by a combined examination of fold change and RPKM (Reads Per Kilobase of transcript per Million mapped reads) of genes of interest (Table S1). Fold changes presented are on a log2 scale, and significant fold changes (p < 0.05) between 1.0 and -1.0 were excluded from the analysis. RPKM’s below 3.4 (two times the highest RPKM value of nanos (1.73) across the three experimental groups) were considered artifacts and excluded as well.

As expected the expression of genes involved in metabolism, chromatin organization, translation, cellular structure, proliferation, and immunity were altered regardless of the type of food (Fig. 3A). Examination of the genes specifically upregulated by high-fat feeding revealed an enrichment in lipoprotein synthesis and transport genes (apoA-IVb.3, apoA-IVa, tm6sf2, cubn, apoA-IVb.2, apoBa, apoEa, apoEb, angiptl4), lipid droplet synthesis and maintenance (plin2, gos2, plin3, cidec), and sterol and fatty acid processing (hmgcs1, elovl5, acot, sqa, fdl, acbd3, ehp) (Fig. 3B). Genes involved in sterol and triglyceride synthesis (nr1d1, fgl19, znf451, rdh14a, insig1) as well as genes involved in the response to starvation (flcn, atg9a, prr5, ulk1a) were largely down-regulated, consistent with the consumption of food rich in fat and cholesterol. The high-fat expression pattern of apolipoprotein transcription during these early time points is consistent with previously published results [30]. The genes most robustly upregulated in response to the high-fat meal are involved in apolipoprotein synthesis, lipid droplet processing (plin2), triglyceride signaling (creb313a), sterol metabolism (hmgcs1) and fatty acid metabolism (elovl5, acot). These transcriptional increases were verified by qRT-PCR (Fig. 3C). Many of these highly upregulated genes (hmgcs1, elovl5, creb313a, insig1, apoBa) localize to the ER, where absorbed lipid metabolites are targeted for processing, storage, and export.

Inhibition of ER lipid flux increases nuclear ruffling and attenuates the transcriptional response to a high-fat meal
The RNASeq and TEM results both demonstrate a rapid and robust shift in enterocyte morphology and physiology in response to dietary fatty acids. Specifically, the enterocyte increases its propensity for lipid storage (TAG synthesis and lipid droplet synthesis)
and export (lipoprotein synthesis). Both of these processes start at the smooth ER, where fatty acids are incorporated into TAG within the ER membrane bilayer (Fig. 4C). ER TAG can then either bud off to form lipid droplets or bind Mtp for transfer to ApoB and subsequent secretion within a mature lipoprotein [29]. Chylomicron secretion is regulated primarily by lipid availability in the ER for lipoparticle assembly, a process initiated when Mtp offloads its TAG to ApoB as it is passing through the ER translocon [31]. In order to explore the specific role of ER TAG on the morphological and transcriptional changes observed in enterocytes, an inhibitor of Mtp (lomitapide) [32, 33] was administered to block both lipoprotein synthesis and ER TAG flux. Our data indicate that disrupting these critical enterocyte functions enhanced and prolonged the degree of nuclear curvature (Fig. 4A, B). Notably, nuclear ruffling has not been observed in cells of other digestive organs known to produce lipoprotein particles (such as the liver) under any feeding conditions. While treatment with the Mtp inhibitor did not alter mtp expression, it did reduce the responsive expression of a large number of genes important for apolipoprotein synthesis (Fig. 4D). Mtp inhibition also dampened the responsive expression of plin2 and hmgcs1, key regulators of lipid droplets and TAG processing, respectively (Fig 4D).

Discussion

Dramatic morphological changes of the nucleus, mitochondria, and lipid droplets are induced by a high-fat meal. TEM has been a mainstay approach to visually examining fat absorption, transport, and packaging in the intestine because of the ease with which lipid droplets can be detected [34]. Classical ultrastructural studies examining the effect of feeding on mammalian enterocyte subcellular structures are consistent with our observations in zebrafish larvae. Several prior studies described the effect of an oil gavage on rodent enterocyte morphology (increases in lipid droplets, expansion of smooth endoplasmic reticulum (SER) and Golgi) [34-36]. Although few authors comment on nuclear morphology in these studies, a reexamination of these TEM images clearly reveals the unique nuclear ruffling of enterocytes. Buschman and Manke (1981) compared the ruffling of the enterocyte nuclei in fasted and fat-fed hamster by subjective examination of the overlay of traced nuclei [2, 3]. They determined that high-fat feeding does indeed alter the peripheral ruffling of the nucleus, though they observed nuclear ruffling in both fed and unfed states. Modest nuclear ruffling was also observed in the enterocytes of 1.5-year old zebrafish adults after a 90-hour fast (data not shown). We predict that the discrepancy between nuclear ruffling in fasted adults and larvae may be due to developmental differences in the intestine combined with the absence of adipose tissue in 6.5-dpf zebrafish larvae. Adults have stored lipids in their adipose tissue that can be used to elevate circulating free fatty acids in the fasted state, whereas larvae would require lipid synthesis or metabolism to acquire new lipids in the absence of dietary lipid. The absence of fat tissue may amplify the physiological and morphological responses in larval enterocytes to dietary lipid. Further examination of the enterocyte response to high-fat feeding at different ages in zebrafish will determine the effect of age and adipocyte lipid storage.

Though the cause of the robust and rapidly-reversible nuclear ruffling described here is still unclear, we hypothesize that the initial influx of dietary fat may transiently increase lipids in the ER and associated nuclear envelope, thereby altering the rigidity of the nuclear membrane. As the cell upregulates lipid export and storage, ER lipid homeostasis may be restored along with nuclear curvature. The ruffling of the nuclear periphery might also indicate dramatic modifications and reorganization of the nuclear lamina and associated chromatin. This reorganization could prepare the cell to exit a resting state and enter into a state of high metabolic, storage, and export activity.

Within 1/2 h of feeding a high-fat meal, a robust morphological response of mitochondrial elongation was observed. Alterations in mitochondrial morphology were previously noted by Jasper and Bonk (1968) in rat mucosal strips exposed to exogenous amino acids [37]. Furthermore, mitochondrial fusion can be promoted by increased metabolic demand [38]. The dramatic shifts in mitochondrial morphology observed here in the larval zebrafish intestine are consistent with enhanced fusion driven by the metabolic demands of rapid lipid influx.

The correlation between the number of lipid droplets and the degree of nuclear ruffling at 1 h of a high-fat meal suggests a connection between the nucleus, lipid droplets, and the ER that plays a predominant role in early lipid droplet formation. Upon high-fat feeding, lipid droplets form and subsequently expand, so that the number of lipid droplets starts to decline at 2 h post feed, while the total area occupied by lipid droplets remains stable to 3 h. One possible hypothesis is that small lipid droplets initially form throughout the cytoplasm, grow, and then fuse to form larger lipid droplets. While no direct observations of fusion events were noted, these data are consistent with a prior study in cultured cells where 15% of all lipid droplets are engaged in fusion events at any given moment [39]. These data are also consistent with a model of two pools of lipid droplets: one with enzymes
that synthesize TAG and the other associated with lipases that result in TAG loss. Assuming that the cell is absorbing fatty acids/monoglyceride (MAG) at a steady rate, the stability of enterocyte lipid droplet area can be explained by a shift in the enterocyte’s physiology to drive lipid export (through lipoprotein synthesis and secretion) and to promote lipid oxidation.

The timing and profile of the nuclear, mitochondrial, and lipid droplet morphological changes of a second high-fat feed closely mirrored morphological changes in the initial high-fat meal. Therefore, these morphological responses are not simply a consequence of the first exposure of the intestine to exogenous food, but are more likely a typical enterocyte response to high-fat feeding in larvae.

By manipulating the amount of lipid absorbed by enterocytes after a meal by altering the diet, we identified the meal’s lipid content as a key indicator of nuclear ruffling. Meals that did not contain lipid (glucose and egg white solutions) did not induce nuclear ruffling or lipid droplet formation. For meals that did contain lipid (egg yolk, artemia, and spirulina solutions), there appeared to be a threshold level of lipid absorption that must be reached before ruffling and lipid droplets are observed. For example, no nuclear ruffling was observed in spirulina-fed fish (0.002% lipid w/v), but nuclear ruffling was observed in both larvae fed egg yolk solution (1.3% lipid w/v) and artemia solution (0.5% lipid w/v). Further, the proportion of enterocytes exhibiting ruffled nuclei was higher in larvae fed chicken egg yolk solution than larvae fed artemia solution. Future examination of the total lipid content of enterocytes after meals with various levels of lipid would provide more insight into this possible explanation.

Pharmaceutical manipulation of dietary lipid absorption linked triglyceride metabolites in particular to nuclear ruffling. Blocking the absorption of dietary cholesterol with ezetimibe treatment prior to a high-fat meal had no effect on the timing or magnitude of nuclear ruffling and lipid droplet formation. However, blocking the absorption of dietary monoglycerides and free fatty acids using orlistat attenuated nuclear ruffling and lipid droplet formation. Therefore the same high-fat egg solution capable of inducing rapid and robust morphological changes in enterocytes cannot induce a response when treated with orlistat. We hypothesize that the acute lipid flux within the ER resulting from monoglyceride/fatty acid absorption and subsequent ER TAG synthesis 1) plays a role in altering the nuclear periphery, which results in a ruffled nuclear appearance, or 2) induces a re-organization of the nuclear periphery as the cell transitions from a resting state (fasted) to a transcriptionally responsive state (fed), which results in a ruffling of the nuclear periphery.

The transcriptional profile of enterocytes rapidly responds to a high-fat meal, with changes in gene expression occurring as early as 1 hour after feeding. Genes involved in lipid transport, storage, signaling, and metabolism were most responsive to a high-fat meal. This change in transcriptional profile indicates that under high-fat feeding conditions, regulation of lipid is paramount to the enterocyte response. Specifically, the enterocyte increases its propensity for lipid storage (TAG synthesis and lipid droplet synthesis) and export (lipoprotein synthesis), likely to both protect the enterocytes from the toxic effects of free fatty acids and to ensure that dietary lipids are distributed throughout the body.

Analysis of the genes whose expression changed in response to a high-fat meal indicates the activity of multiple lipid-induced signaling proteins (insulin, pparg, srebhp, creb3l3) and pathways, which converge on the ER as absorbed lipid metabolites are targeted to this organelle. Future work examining the interplay between these pathways in the intestine would enhance fields largely focused on the function of these pathways in other metabolic tissues (i.e., the liver).

Among the highest upregulated genes, the enrichment of ER-localized genes involved in lipid processing, signaling, and export indicated a critical role of this organelle in the enterocyte response to absorbed dietary lipid. Pharmaceutical inhibition of ER lipid packaging into apolipoproteins using an Mtp inhibitor enhanced the magnitude and duration of nuclear ruffling. The increased nuclear ruffling seen when lipoprotein synthesis and export is blocked is consistent with our hypothesis that increased levels of lipid in the ER result in changes to the rigidity of the nuclear envelope. The presumptive increase of TAG within the ER caused by blocking lipid transport through chylomicrons could be responsible for this increased level of nuclear ruffling. These data are consistent with reports from humans with abetalipoproteinemia and mice with Mtp deficiency showing slightly more ruffled nuclei as compared with control [40, 41]. However, few of the published electron micrographs images include enterocyte nuclei, making it difficult to draw definitive conclusion as to the effect of lipoprotein retention on mammalian enterocyte nuclear morphology.

Nuclear ruffling has not been observed in cells of other digestive organs known to produce lipoprotein particles (such as the liver) under any feeding conditions. The
unique rapid influx of lipid to the intestinal lumen and subsequently to the enterocytes may have a stronger effect on nuclear morphology than the gradual change in lipid flux experienced by other tissues. Future intestinal studies will clarify this potential connection between lipid flux in the ER and changes to the structure and/or organization of the nuclear periphery.

Notably, genes that had responded to the acute high-fat meal but were dampened by Mtp inhibitor treatment, such as apoA-IV, closely mirror the affected genes identified in longer-term atherogenic feeding studies in mice lacking the Creb3l3 transcription factor, a member of the CREB3 (cyclic AMP response element binding protein) family of bZIP transcription factors [42]. Further, Creb3l3 knockout mice fasted for 24 h show a similar misregulation of genes involved in lipid transport and triglyceride metabolism in both liver and intestine [43]. Creb3l3 is an ER-tethered transcription factor activated by saturated fatty acids, insulin, and/or inflammatory stimuli whose expression is restricted to the liver and intestine of vertebrates, including zebrafish [42, 44-46]. Prior studies have demonstrated that Creb3l3 strongly upregulates apoA-IV, a gene which is primarily expressed in the intestine [30] where it can expand chylomicron size to increase gross lipid export [47-51]. Zebrafish have multiple apoA-IV genes that exhibit rapid increase in expression in the intestine after high-fat feeding (Fig. 3B) [30]. We propose that the reduction in apoA-IV expression that we observe under Mtp inhibition is due to a reduction in Creb3l3 processing and activity, as observed by Cheng et al. (submitted) [52]. Cheng et al. (submitted) show that TAG flux in the hepatocyte ER controls apoB-VLDL particle assembly, which regulates Creb3l3 proteolytic processing, which in turn promotes the upregulation of apoA-IV expression to enhance the efficient export of lipid [52]. In our hands, Mtp inhibition also dampened the responsive expression of plin2 and hmgcs1, key regulators of lipid droplets and TAG processing, respectively (Fig 4D). Taken together, these findings support our hypothesis that TAG flux in the enterocyte ER stimulates a robust cellular response that coordinates lipid processing, storage, and export from the cell.

Experimental Procedures

Zebrafish
All procedures were approved by the Carnegie Institution Animal Care and Use Committee (Protocol #139). For all experiments, WT (AB background) embryos were collected from natural spawning, staged, and raised in zebrafish embryo media (EM) as previously described [53].

Preparation of zebrafish meals
For all feeding solutions, a total volume of 20 mL was prepared. The high-fat vesicle feeding solution was prepared similarly to previous reports [12, 54]. Briefly, a 5% egg yolk emulsion was made with frozen aliquots of chicken egg yolk resuspended in zebrafish EM. The egg yolk emulsion was pulse sonicated (Sonicator Ultrasonic Processor 6000, Misonix Inc.) for 10 seconds using a ¼-inch tapered microtip (output intensity: 3W, 1s on/off, 5 s total processing time), passed through a strainer, pulse sonicated for an additional 30 s, then vortexed for 30 s. The artemia feed was prepared as 1% (w. /v.) shell-free artemia (INVE Aquaculture) in zebrafish EM and sonicated for 5 s for uniformity. The spirulina solution was prepared as 0.5% (w./v.) spirulina powder (Salt Creek Inc.) in EM and mixed by vortexing. The glucose solution was prepared as a 5% (w./v.) glucose in EM and mixed by vortexing. A 10% egg white solution was made with frozen aliquots of chicken egg white resuspended in EM and sonicated for 5 s.

Feeding Zebrafish
To improve feeding rates, all feeding assays were performed in 35 mm petri dishes kept in an incubated orbital shaker set to 29°C and 30 RPM (Incu-Shaker Mini H1000-M; Benchmark Scientific). For assays measuring transcriptional responses to feeding, 30-40 larvae were placed in 5 mL sonicated egg yolk emulsion to feed. For TEM experiments, 70-100 larvae were placed in 5 mL sonicated egg yolk emulsion to feed. For all feeding procedures, after 50 minutes in the feeding solution, larvae were washed 2X in embryo media, anesthetized with tricaine, and examined for full intestines to verify that they had fed. For certain foods (egg yolk, artemia, spirulina), feeding was determined visually by the color of the intestine. For other foods (glucose and egg white), feeding was determined by an expansion of the intestinal lumen and the presence of bile. After verifying feeding, larvae that were going to continue past the 1 h mark of the feeding time course were placed in EM and returned to the incubated shaker for the duration of their time course.

Transmission Electron Microscopy
For TEM, unfed larvae and larvae with “full” intestines were removed and fixed at different times after feeding. After 2 EM rinses, and anesthetization using tricaine, larvae were fixed in a 3% glutaraldehyde, 1% formaldehyde, 0.1 M cacodylate solution for 1-3 h. Heads and tails were trimmed, and swim bladders deflated prior to embedding in 2% low-melt agarose. Post-fixation was done in 1% osmium tetroxide + 1% potassium ferricyanide in cacodylate solution for 1 h. This was followed by washes in water, cacodylate, and water again (2 x 10 min each), and then incubation in
0.05 M maleate pH 6.5 for 10 min. Samples were then En Bloc stained with 0.5% uranyl acetate in maleate for 2 h at room temperature or at 4°C overnight. After two 15 min washes with water, samples were dehydrated through graded ethanol dilution (35% 2 x 15 min, 50% 15 min, 75% 15 min, 95% 15 min, 100% 3 x 15 min). Samples were then washed with propylene oxide three times before incubation in 1:1 propylene oxide:resin (Epon 812 epoxy) and evaporated overnight, followed by two 2 h washes in 100% resin, and a final embedding in 100% resin at 50°C followed by 70°C. Sections were made with a Reichert Urectcut-S (Leica Microsystems), mounted on naked 200 thin mesh grids, and stained with lead citrate. Images were captured with a Phillips Tecnai 12 microscope and recorded with a Gatan multiscan CCD camera using Digital Micrograph software.

Drug treatments
For all treatments, vehicle or drug were added to the 5% egg yolk emulsions directly after sonication, prior to vortexing. For Orlistat treatment, larvae were pre-treated overnight with vehicle (0.1% ethanol, 5% egg yolk emulsion in EM), 0.1% ethanol in EM, and 1 h before feeding with 0.5 mM Orlistat (Alli, 60 mg capsules, GlaxoSmithKline), and a final embedding in 100% resin at 50°C followed by 70°C. Sections were made with a Reichert Ultracut-S (Leica Microsystems), mounted on naked 200 thin mesh grids, and stained with lead citrate. Images were captured with a Phillips Tecnai 12 microscope and recorded with a Gatan multiscan CCD camera using Digital Micrograph software.

Quantification of Coefficient of Variation of nuclear curvature in electron micrographs
To define the curvature of nuclei under different feeding conditions and drug treatments, data were collected from several sets of larvae from at least 2 independent experiments per condition examined. TEM images of a complete transverse section of a larval intestine were divided into four equal quadrants, and one quadrant was randomly selected for further analysis. Random quadrant selection was automated using MetaMorph software (Molecular Devices, LLC). Nuclei in the selected quadrant were then segmented by hand with the aid of a Wacom Intuos4 tablet (Wacom Technology Corporation). The experimental condition of the images was blinded for the analyses in Figure 1. Nuclear outlines were binarized (MetaMorph) and then fed analyzed for curvature with a custom algorithm implemented in MATLAB (The Mathworks) using the Image Processing toolbox, as follows. The starting point is a segmented image of the nucleus. The command “bwboundaries” was used to obtain the boundary of the segmented nucleus - using the default 8-connected neighborhood. This leads to a set of pixels

\[ z_i = (x_i, y_i), \quad i = 1, \ldots, n \]

where \( n \) is the number of pixels in the boundary. For each of these points, we then:

1. Find the osculating circle at \( z_i \) along the boundary, by calculating the radius, \( r_i \), and the center \( c_i \). To compute the radius and center that define the osculating circle at index \( i \), we take approximately 5% of the pixels on the boundary before and after index \( i \):

\[ \hat{Z}_i = \{ z_{i-m}, z_{i-m+1}, \ldots, z_{i-1}, z_i, z_{i+1}, \ldots, z_{i+m-1}, z_{i+m} \} \]

where \( m = n / 20 \). We fit the circle to these 2m + 1 points by minimizing the geometric error (sum of squared distances from the points to the fitted circle) using nonlinear least squares. This is based on the work of Gander and colleagues [55] and uses the implementation “fitcircle,” available from MATLAB Central file exchange [56]. Compute the magnitude of the curvature at \( z_i \) as the inverse of the radius \( k_i = 1 / r_i \). If the computed radius is infinite (NaN) then the curvature is set to be zero; otherwise, we take the inverse of the radius.

2. Set the sign of the curvature by determining whether the center of the osculating circle is inside or outside the cell boundary. To this end, we compute a short line from the point \( z_i \) in the direction of the circle center \( c_i \). This cannot be too long or it might go through the boundary on the other side of the cell. We used lines three pixels long. If this point is inside the cell, then the curvature is negative; otherwise, it is positive. After these calculations, we have a set of local curvatures \( \{ k_i \}_{i=1}^{n} \) at each of the points along the boundary. Finally, we compute the mean

\[ \mu(k) = \frac{1}{n} \sum_{i=1}^{n} k_i, \]

standard deviation

\[ \sigma(k) = \left( \frac{1}{n-1} \sum_{i=1}^{n} (k_i - \mu(k))^2 \right)^{1/2}, \]
and coefficient of variation of the local curvatures
\[ c_v = \frac{\sigma(\kappa)}{\mu(\kappa)}. \]
This presents a measure of the variation of local curvature around the mean. Note that for a perfectly round cell, the standard deviation is zero and hence so is \( c_v \). Normalization for the mean allows us to compare nuclei of varying size.

**RNA Extraction from larval guts**
A feeding time course was undertaken with 6.5-dpf larval zebrafish. 5% egg yolk emulsion (high-fat) feeds and 10% egg white feeds were prepared as described above. At the appropriate time points, digestive organs (intestine, liver, pancreas) were dissected from 10 anesthetized larval zebrafish and immediately transferred into 30 uL RNALater (Ambion). The samples were stored at -20°C, thawed on ice, and RNA was extracted using an RNAqueous Micro Kit (Ambion) and stored at -80°C.

**RNASeq Sample Preparation and Analysis**
Triplicate samples were independently prepared from pairwise crosses fed each food type. RNA sample purity was verified with the Agilent RNA 6000 Pico Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA libraries were constructed from poly(A)-selected RNA using the Illumina TruSeq RNA Sample Prep Kit v2 (Illumina) following the LS (low sample throughput) option. Six samples were run per lane on an Illumina HiSeq2000 for a 50 base pair plus indexing run. Refseq annotation for zebrafish was obtained from Ensembl. RNASeq reads were mapped to zebrafish genome (Zv9) by TopHat [57] using the Refseq annotation as reference. All reads were trimmed to 50 base pairs before mapping. Reads falling on each gene were counted using custom scripts. Differentially-expressed genes were called using edgeR [58].

**RT-PCR Sample Preparation and Analysis**
Guts were collected from 10 larvae per experimental group and pooled together. Samples were collected in triplicate, each from a different AB cross. cDNA was constructed from RNA extracted from dissected larval guts with the iScript cDNA Synthesis Kit (BioRad). Triplicate RT-PCR samples were prepared using cDNA, SsoAdvanced Universal SYBR Green Supermix (BioRad), and gene specific primers. The BioRad CFX96.5 Real-Time System was used to run the RT-PCR, with 45 cycles: 95°C 15 sec, 59°C 20 sec, 72°C 20 sec. The BioRad CFX Manager 3.0 software platform was used to analyze the results. Gene expression was quantified using the \( \Delta \Delta CT \) method [59, 60] using 18S as the reference gene. The following apolipoprotein and 18s primer sequences were developed and validated in a previous publication [36]:
- apoA-IVa: (F: GACCGAGCTCAAGCCTTATG; R: GACCCAGCTCAAGCCTTATG)
- apoA-IVb.1: (F: GAGTTCCAGGAAACTGTTA TG; R: TCGTACAGAGATGCTGTTA TG)
- apoA-IVb.2: (F: TGTGTTGCTTTTCCAACCTTGCT; R: TCACTTGGACGGTTCCCTCG)
- apoA-IVb.3: (F: TGAAGGTCTTTTGGGTTGCTC; R: AATGGATTTCCCTTGGGTTT)
- ApoA-ia: (F: CCAATTTCCTTGGGAGGCTGAT; R: CAACCTGGGTGAGGATGCT)
- ApoEa: (F: GCAGAAGCTCTGGAGCTCAAGCCAACACAG; R: AATGGATTTCCCTTGGGTTT)
- acot (F: CATGTGAATGAGGATAAC ATGT; R: TCAACAGAAGCCAGGTCT)
- creb3la (F: AAGCCCTTACAGACCGAAC; R: TGGCCGTTTACTGATCCATT)
- elolv5 (F: AATGGGCTGGGTGCTTATCCC; R: TGGCCGTTTACTGATCCATT)
- hmgcs1 (F: GCTGAGACAATGAAGCTCAGA; R: CATGGATCTGCTGCTGCT)
- plin2 (F: TTCACTAAATGGGTGAGGCTA; R: CACCACACATGTGCTGCTGCT)
- Mtp: (F: GAGGCCACGGCTGGACTTCAG; R: TTGACACCAGTCTTCTGAGAAG)

**Statistics**
Unless otherwise indicated, one-way analysis of variance (ANOVA) with significance set at \( p < 0.05 \) was used to compare differences between experimental conditions after determining homogeneity of variances across samples using the Bartlett’s test. Tukey’s multiple comparisons test was used for post-hoc analysis. Results of the multiple comparison tests are indicated by letters (significant differences between conditions fell into families) or asterisks depending on the experimental results. All error bars represent standard deviation. The GraphPad Prism software platform was used to compile all statistical analyses (GraphPad Prism version 6.0b for Mac, GraphPad Software).
Author Contributions: EMZ was the lead author and responsible for the primary experimental design and analysis. MHW performed some RT-PCR, data analysis and contributed to the writing and editing of the manuscript. XZ performed RNA Seq analysis. PAI designed, wrote and optimized the Matlab curvature script. MAS designed TEM methodology, preparation and imaging. MAS designed and wrote two metamorph journals to complement the Matlab script. JLA assisted with statistical analysis and edited the manuscript. YZ provided expertise and guidance with experimental design and edited the manuscript. SAF oversaw all aspects of the experiments, provided advice on experimental design and wrote the manuscript with EMZ.

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References

1. Creamer, B. (1974). Intestinal structure in relation to absorption. Biomembranes 4A, 1-42.
2. Buschmann, R.J., and Manke, D.J. (1981). Morphometric analysis of the membranes and organelles of small intestinal enterocytes. II. lipid-fed hamster. J Ultrastruct Res 76, 15-26.
3. Buschmann, R.J., and Manke, D.J. (1981). Morphometric analysis of the membranes and organelles of small intestinal enterocytes. I. Fasted hamster. J Ultrastruct Res 76, 1-14.
4. Anderson, J.L., Carten, J.D., and Farber, S.A. (2011). Zebrafish lipid metabolism: from mediating early patterning to the metabolism of dietary fat and cholesterol. Methods Cell Biol 101, 111-141.
5. Asaoka, Y., Terai, S., Sakaida, I., and Nishina, H. (2013). The expanding role of fish models in understanding non-alcoholic fatty liver disease. Dis Model Mech 6, 905-914.
6. Fang, L., Liu, C., and Miller, Y.I. (2014). Zebrafish models of dyslipidemia: relevance to atherosclerosis and angiogenesis. Translational research: the journal of laboratory and clinical medicine 163, 99-108.
7. Holta-Vuori, M., Salo, V.T., Nyberg, L., Brackmann, C., Enejder, A., Panula, P., and Ikonen, E. (2010). Zebrafish: gaining popularity in lipid research. The Biochemical journal 429, 235-242.
8. Schlegel, A., and Stainier, D.Y. (2007). Lessons from "lower" organisms: what worms, flies, and zebrafish can teach us about human energy metabolism. PLoS Genet 3, e199.
9. Farber, S.A., Pack, M., Ho, S.Y., Johnson, I.D., Wagner, D.S., Dosch, R., Mullins, M.C., Hendrickson, H.S., Hendrickson, E.K., and Halpern, M.E. (2001). Genetic analysis of digestive physiology using fluorescent phospholipid reporters. Science 292, 1385-1388.
10. Hama, K., Provost, E., Baranowski, T.C., Rubinstein, A.L., Anderson, J.L., Leach, S.D., and Farber, S.A. (2009). In vivo imaging of zebrafish digestive organ function using multiple quenched fluorescent reporters. Am J Physiol Gastrointest Liver Physiol 296, G445-453.
11. Clifton, J.D., Lucumi, E., Myers, M.C., Napper, A., Hama, K., Farber, S.A., Smith, A.B., 3rd, Huryn, D.M., Diamond, S.L., and Pack, M. (2010). Identification of novel inhibitors of dietary lipid absorption using zebrafish. PLoS One 5.
12. Carten, J.D., Bradford, M.K., and Farber, S.A. (2011). Visualizing digestive organ morphology and function using differential fatty acid metabolism in live zebrafish. Developmental biology.
13. Webster, M., Witkin, K.L., and Cohen-Fix, O. (2009). Sizing up the nucleus: nuclear shape, size and nuclear-envelope assembly. J Cell Sci 122, 1477-1486.
14. Worman, H.J., Ostlund, C., and Wang, Y. (2010). Diseases of the nuclear envelope. Cold Spring Harb Perspect Biol 2, a000760.
15. Queisser, G., Wiegert, S., and Bading, H. (2011). Structural dynamics of the cell nucleus: basis for morphology modulation of nuclear calcium signaling and gene transcription. Nucleus 2, 98-104.
16. Murphy, S., Martin, S., and Parton, R.G. (2009). Lipid droplet-organelle interactions; sharing the fats. Biochim Biophys Acta 1791, 441-447.
17. Wallace, K.N., Akhter, S., Smith, E.M., Lorent, K., and Pack, M. (2005). Intestinal growth and differentiation in zebrafish. Mech Dev 122, 157-173.
18. Stoeckenius, W., and Mahr, S.C. (1965). Studies on the Reaction of Osmium Tetroxide with Lipids and Related Compounds. Lab Invest 14, 1196-1207.
19. Skellon, J.H., and Windsor, D.A. (1962). The fatty acid composition of egg yolk lipids in relation to dietary fats. Journal of the Science of Food and Agriculture 13, 300-303.
20. Chakraborty, R.D., Chakraborty, K., and Radhakrishnan, E.V. (2007). Variation in fatty acid composition of Artemia salina nauplii enriched with microalgae and baker’s yeast for use in larviculture. J Agric Food Chem 55, 4043-4051.
21. Husbands, D.R. (1970). The composition of triglycerides from liver, egg yolk and adipose tissue of the laying hen. The Biochemical journal 120, 365-371.
22. Jones, D. (1969). Variations in the cholesterol content of egg yolk. Nature 221, 780.
23. van Heek, M., Austin, T.M., Farley, C., Cook, J.A., Tetzloff, G.G., and Davis, H.R. (2001). Ezetimibe, a potent cholesterol absorption inhibitor, normalizes combined dyslipidemia in obese hyperinsulinemic hamsters. Diabetes 50, 1330-1335.

24. Altmann, S.W., Davis, H.R., Jr., Zhu, L.J., Yao, X., Hoos, L.M., Tetzloff, G., Iyer, S.P., Maguire, M., Golovko, A., Zeng, M., et al. (2004). Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science 303, 1201-1204.

25. Garcia-Calvo, M., Lisnock, J., Bull, H.G., Hawes, B.E., Burnett, D.A., Braun, M.P., Crona, J.H., Davis, H.R., Jr., Dean, D.C., Detmers, P.A., et al. (2005). The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci U S A 102, 8132-8137.

26. Baek, J.S., Fang, L., Li, A.C., and Miller, Y.I. (2012). Ezetimibe and simvastatin reduce cholesterol levels in zebrafish larvae fed a high-cholesterol diet. Cholesterol 2012, 564705.

27. Hogan, S., Fleury, A., Hadvary, P., Lengsfeld, H., Meier, M.K., Triscari, J., and Sullivan, A.C. (1987). Studies on the antiobesity activity of tetrahydrolipstatin, a potent and selective inhibitor of pancreatic lipase. Int J Obes 11 Suppl 3, 35-42.

28. Farese, R.V., Jr., and Walther, T.C. (2009). Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 139, 855-860.

29. Sturley, S.L., and Hussain, M.M. (2012). Lipid droplet formation on opposing sides of the endoplasmic reticulum. Journal of lipid research 53, 1800-1810.

30. Otis, J.P., Zeituni, E.M., Thierer, J.H., Anderson, J.L., Brown, A.C., Boehm, E.D., Cerchione, D.M., Ceasrine, A.M., Avraham-David, I., Tempelhof, H., et al. (2015). Zebrafish as a model for apolipoprotein biology: comprehensive expression analysis and a role for ApoA-IV in regulating food intake. Dis Model Mech 8, 295-309.

31. Iqbal, J., and Hussain, M.M. (2009). Intestinal lipid absorption. Am J Physiol Endocrinol Metab 296, E1183-1194.

32. Cuchel, M., Bloedon, L.T., Szapary, P.O., Kolansky, D.M., Wolfe, M.L., Sarkis, A., Millar, J.S., Ikewaki, K., Siegelman, E.S., Gregg, R.E., et al. (2007). Inhibition of microsomal triglyceride transfer protein in familial hypercholesterolemia. N Engl J Med 356, 148-156.

33. Robl, J.A., Sulsky, R., Sun, C.Q., Simpkins, L.M., Wang, T., Dickson, J.K., Jr., Chen, Y., Magnin, D.R., Taunk, P., Slusarchyk, W.A., et al. (2001). A novel series of highly potent benzimidazole-based microsomal triglyceride transfer protein inhibitors. J Med Chem 44, 851-856.

34. Cardell, R.R., Jr., Badenhausen, S., and Porter, K.R. (1967). Intestinal triglyceride absorption in the rat. An electron microscopical study. J Cell Biol 34, 123-155.

35. Jersild, R.A., Jr. (1966). A time sequence study of fat absorption in the rat jejunum. Am J Anat 118, 135-162.

36. Palay, S.L., and Karlin, L.J. (1959). An electron microscopic study of the intestinal villus. II. The pathway of fat absorption. J Biophys Biochem Cytol 5, 373-384.

37. Jasper, D.K., and Bronk, J.R. (1968). Studies on the physiological and structural characteristics of rat intestinal mucosa. Mitochondrial structural changes during amino acid absorption. J Cell Biol 38, 277-291.

38. Mishra, P., and Chan, D.C. (2016). Metabolic regulation of mitochondrial dynamics. J Cell Biol 212, 379-387.

39. Bostrom, P., Rutberg, M., Ericsson, J., Holmdahl, P., Andersson, L., Frohman, M.A., Boren, J., and Olofsson, S.O. (2005). Cytosolic lipid droplets increase in size by microtubule-dependent complex formation. Arterioscler Thromb Vasc Biol 25, 1945-1951.

40. Bouma, M.E., Beucler, I., Pessaah, M., Heinzmann, C., Lusis, A.J., Naim, H.Y., Ducastelle, T., Leluyer, B., Schmitz, J., Infante, R., et al. (1990). Description of two different patients with abetalipoproteinemia: synthesis of a normal-sized apolipoprotein B-48 in intestinal organ culture. Journal of lipid research 31, 1-15.
41. Hamilton, R.L., Wong, J.S., Cham, C.M., Nielsen, L.B., and Young, S.G. (1998). Chylomicron-sized lipid particles are formed in the setting of apolipoprotein B deficiency. Journal of lipid research 39, 1543-1557.

42. Zhang, C., Wang, G., Zheng, Z., Maddipati, K.R., Zhang, X., Dyson, G., Williams, P., Duncan, S.A., Kaufman, R.J., and Zhang, K. (2012). Endoplasmic reticulum-tethered transcription factor cAMP responsive element-binding protein, hepatocyte specific, regulates hepatic lipogenesis, fatty acid oxidation, and lipolysis upon metabolic stress in mice. Hepatology 55, 1070-1082.

43. Lee, J.H., Giannikopoulos, P., Duncan, S.A., Wang, J., Johansen, C.T., Brown, J.D., Plutzky, J., Hegele, R.A., Glimcher, L.H., and Lee, A.H. (2011). The transcription factor cyclic AMP-responsive element-binding protein H regulates triglyceride metabolism. Nat Med 17, 812-815.

44. Omori, Y., Imai, J., Watanabe, M., Komatsu, T., Suzuki, Y., Kataoka, K., Watanabe, S., Tanigami, A., and Sugano, S. (2001). CREB-H: a novel mammalian transcription factor belonging to the CREB/ATF family and functioning via the box-B element with a liver-specific expression. Nucleic Acids Res 29, 2154-2162.

45. Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D.T., Back, S.H., and Kaufman, R.J. (2006). Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. Cell 124, 587-599.

46. Thisse, B., and Thisse, C. (2004). Fast Release Clones: A High Throughput Expression Analysis. ZFIN Direct Data Submission (http://zfin.org).

47. Barbosa, S., Fasanella, G., Carreira, S., Liarena, M., Fox, R., Barreca, C., Andrew, D., and O’Hare, P. (2013). An orchestrated program regulating secretory pathway genes and cargos by the transmembrane transcription factor CREB-H. Traffic 14, 382-398.

48. Weinberg, R.B., Gallagher, J.W., Fabritius, M.A., and Shelsness, G.S. (2012). ApoA-IV modulates the secretory trafficking of apoB and the size of triglyceride-rich lipoproteins. Journal of lipid research 53, 736-743.

49. Karathanasis, S.K., Yunis, I., and Zannis, V.I. (1986). Structure, evolution, and tissue-specific synthesis of human apolipoprotein AIV. Biochemistry 25, 3962-3970.

50. Elshourbagy, N.A., Walker, D.W., Boguski, M.S., Gordon, J.I., and Taylor, J.M. (1986). The nucleotide and derived amino acid sequence of human apolipoprotein A-IV mRNA and the close linkage of its gene to the genes of apolipoproteins A-I and C-III. J Biol Chem 261, 1998-2002.

51. VerHague, M.A., Cheng, D., Weinberg, R.B., and Shelsness, G.S. (2013). Apolipoprotein A-IV expression in mouse liver enhances triglyceride secretion and reduces hepatic lipid content by promoting very low density lipoprotein particle expansion. Arterioscler Thromb Vasc Biol 33, 2501-2508.

52. Cheng, D., Xu, X., Simon, T., Boudyguina, E., Deng, Z., VerHague, M., Lord, C., Brown, J.M., Lee, A.H., Shelsness, G.S., et al. (2016). Very Low Density Lipoprotein Assembly is Required for cAMP Responsive Element-Binding Protein H Processing and Hepatic Apolipoprotein A-IV Expression. Submitted.

53. Westerfield, M. (1995). The Zebrafish Book, 3rd Edition, (Eugene: University of Oregon).

54. Semova, I., Carter, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A., and Rawls, J.F. (2012). Microbiota regulate intestinal absorption and metabolism of Fatty acids in the zebrafish. Cell Host Microbe 12, 277-288.

55. Gander, W., Golub, G.H., and Strebel, R. (1994). Least-squares fitting of circles and ellipses. BIT Numerical Mathematics 34, 558-578.

56. Brown, R. (2007). fitcircle.m. (Matlab Central: File Exchange).

57. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14, R36.

58. Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139-140.
59. Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3, 1101-1108.

60. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

61. Miyares, R.L., de Rezende, V.B., and Farber, S.A. (2014). Zebrafish yolk lipid processing: a tractable tool for the study of vertebrate lipid transport and metabolism. Dis Model Mech 7, 915-927.

62. Bligh, E., and Dyer, W. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-918.
**FIGURE LEGENDS**

**Figure 1: A high-fat meal induces changes in enterocyte morphology during feeding.**
A) Time course of 6.5-dpf zebrafish larvae fed high-fat meal composed of an emulsion of 5% chicken egg yolk. Electron micrographs show representative images of cellular changes in intestinal epithelial cells prior to feeding (unfed) and at 0.5 h, 1 h, 1.5 h, 2 h, 3 h after feeding begins. N, nucleus; LD, lipid droplet; BB, brush border. B) Enlargement of enterocyte nuclei (boxed) at indicated time points of a high-fat meal. Nuclear ruffling is indicated by white arrowheads. C) Enlargement of a portion of apical cytoplasm (boxed) of enterocytes at given time points of a high-fat meal. Examples of rounded and elongated mitochondria are indicated with red and yellow arrowheads, respectively. D) Quantification of the average curvature COV of nuclei at each time point. Each dot represents an average of ≥ 10 nuclei from a single fish. There was a statistically significant difference between groups as determined by one-way ANOVA (F(5, 28) = 0.54, p < 0.0001). A Tukey post-hoc test revealed that the average curvature COV at the 1h time point was statistically significantly higher than all other time points, and that there were no statistically significant differences between any other time points. E) Quantification of the percentage of cells exhibiting ruffled nuclei at each time point. Each dot represents an average of ≥ 10 nuclei from a single fish. There was a statistically significant difference between groups as determined by one-way ANOVA (F(5, 28) = 2.81, p < 0.0001). A Tukey post-hoc test revealed that the percentage of cells with ruffled nuclei at the 1h time point was statistically significantly higher than all other time points, and that there were no statistically significant differences between any other time points. F) Quantification of the frequency of elongated mitochondria at each time point. Two cells from each fish were analyzed. There was a statistically significant difference between groups as determined by one-way ANOVA (F(5, 64) = 1.56, p < 0.0001). A Tukey post-hoc test revealed that mitochondrial elongations at the 0 h time point was statistically significantly lower than all other time points, and that there were no statistically significant differences between any other time points. G) Quantification of the number of LD per cell at each time point. Each dot represents the number of LD in a single cell; two cells were analyzed per fish. There was a statistically significant difference between groups as determined by one-way ANOVA (F(5, 62) = 4.01, p < 0.0001). A Tukey post-hoc test revealed that the number of LD increased significantly within 30 minutes of eating a high-fat meal, peaking at 1 h then decreasing with time. H) Quantification of the portion of the apical cytoplasmic area that is comprised of LD at each time point. Two cells from each fish were analyzed. Log10 adjustment was used to reduce the effect of outliers. There was a statistically significant difference between groups as determined by one-way ANOVA (F(5, 64) = 3.60, p < 0.0001). A Tukey post-hoc test revealed that the portion of the total apical cytoplasmic area taken up by LD increased significantly upon feeding, but did not change as the high-fat feeding time course progressed, so that the LD area at 1 h post feeding was not statistically significantly different than the LD area at 3 h post feeding. I-L) Quantification of subcellular changes observed in intestinal epithelial cells prior to re-feeding (unfed) and at 1 h and 2 h after the re-feeding begins. Measured changes include curvature COV of enterocyte nuclei (I), morphology of mitochondria (J), and lipid droplet number and size (K and L, respectively). Quantification protocols match those used in C, E, F, and G. Statistically significant differences between groups were determined by one-way ANOVA with a Tukey post-hoc test for multiple comparisons. Similar trends were observed in both the first (A-H) and second (I-L) high-fat feedings.

**Figure 2: Lipid metabolites trigger enterocyte nuclear ruffling and lipid droplet production.**
A) Time courses of 6.5-dpf zebrafish larvae fed various diets. Electron micrographs show representative images of cellular changes in enterocytes at 1h after feeding began. N, nucleus; LD, lipid droplet; BB, brush border. Yellow arrowheads indicate nuclear ruffling. White arrowheads indicate mitochondria. Scale bar = 10 µm. Insets highlight differences in lipid droplets between egg yolk and artemia fed larvae. B) Quantification of nuclear curvature COV observed in intestinal epithelial cells at 1 h after initiation of feeding of the indicated food. Each dot represents an average of at least 10 nuclei from a single fish. Curvature COV measurements were normalized to 1 h egg yolk feed. Statistical analysis was conducted with the raw data. There was a statistically significant difference between groups as determined by one-way ANOVA (F(4,38) = 2.30, p < 0.0001). A Tukey post-hoc test revealed egg yolk and artemia feeds induced a statistically significant increase in nuclear curvature COV. In contrast, spirulina, egg white, and glucose feeds exhibited nuclear curvature COV similar to the unfed control. C) Analysis of the profile of curvature COV of enterocyte nuclei from fish fed egg yolk or artemia solutions. Nuclei were binned based on their curvature COV measurement. 155 nuclei were analyzed and graphed for each condition. Chi-Square analysis demonstrates a significant difference in the nuclear ruffling between the two treatment groups (p < 0.0001). D) Schematic mode of action of drugs that block dietary lipid absorption by enterocytes. E) Quantification of nuclear curvature COV observed in treated intestinal epithelial cells at 1 h of a high-fat meal. Each dot represents an average of at least 10 nuclei from a single fish. Curvature COV measurements...
were normalized to untreated larvae at 1 h of a high-fat meal. Statistical analysis was conducted with the raw data. There was a statistically significant difference between groups as determined by one-way ANOVA ($F(7,74) = 1.07, p < 0.0001$). A Tukey post-hoc test revealed a significant increase in nuclear curvature COV in the untreated, vehicle-treated, and Ezetimibe-treated fish. In contrast, fish treated with Orlistat exhibited nuclear curvature COV similar to the unfed control. 

F) 6.5-pf zebrafish larvae treated with Vehicle, Orlistat, or Ezetimibe and fed a high-fat meal for 1 h. Electron micrographs show representative images of cellular changes in enterocytes from the indicated treatment groups at 1 h after feeding began. Yellow arrowheads indicate nuclear ruffling. Scale bar = 10 µm.

Figure 3: A high-fat meal induces a unique transcriptional response.
A) Overview of the genes induced in larval guts upon feeding 6.5-dpf zebrafish a high-fat and a fat-free meal, as assayed by RNASeq. Samples were taken prior to feeding (unfed) and at 1 h and 4 h after feeding began. The transcript reads from unfed fish were treated as the baseline for each group. The total number of genes whose expression changed only with a high-fat diet (Induced/Repressed by feeding high-fat) or with both types of food (Induced/Repressed by feeding) are indicated in the center of the diagrams. Genes are grouped by function. B) Gene families with a role in metabolism that were specifically induced or repressed by a high-fat meal are shown. The table indicates the fold change of expression (log2) over unfed controls, as determined by RNASeq analysis. The table is color-coded to indicate induction (orange) or repression (purple). Statistical analysis of the fold changes for each condition are in Table S1. C) RT-PCR validation of high-fat meal induction of the most robustly changed genes from several gene categories involved in lipid storage and metabolism. Relative expression is shown, normalized to an unfed control for each respective treatment using the ΔΔCT method. There was a statistically significant difference between groups as determined by one-way ANOVA ($F(4,15) = 17.50, p < 0.002$; $creb3l3a (F(4,15) = 2.49, p < 0.02$; $hmgcs1 (F(4,15) = 2.32, p < 0.006$; $elolv5 (F(4,15) = 3.67, p < 0.04$; $acot (F(4,15) = 0.42, p < 0.006$). A Dunnett’s post-hoc test revealed significant differences between unfed controls and certain time points of a high-fat feed. Time points with a statistically significant increase in expression over unfed controls are indicated by an asterisk.

Figure 4: Treatment with an Mtp inhibitor alters the enterocyte response to a high-fat meal.
A) Representative electron micrographs of 6.5-dpf untreated zebrafish larvae and larvae treated with Vehicle or Mtp inhibitor and fed a high-fat meal indicating cellular changes in enterocytes as a result of treatment. Yellow arrowheads indicate nuclear ruffling. Scale bar = 2 µm. B) Quantification of nuclear curvature COV observed in the TEM experiment. Each dot represents an average of at least 10 nuclei from a single fish. There was a statistically significant difference between groups as determined by one-way ANOVA ($F(4,15) = 0.42, p < 0.006$). A Tukey post-hoc test revealed a statistically significant increase in nuclear curvature COV in all fed treatment groups, when compared to the Mtp Inhibitor-treated unfed control. The increased nuclear curvature COV observed with feeding Mtp Inhibitor treated fish was significantly higher at 1 h and was maintained at least 2 h into the time course. C) Analysis of the profile of curvature COV of enterocyte nuclei from fish treated with vehicle or with Mtp inhibitor. Nuclei were binned based on their curvature COV measurement. 145 nuclei were analyzed and graphed for each condition. Chi-Square analysis demonstrates a significant difference between the nuclear ruffling of the two treatment groups ($p < 0.0001$). D) RT-PCR of cDNA extracted from 6.5-dpf zebrafish guts dissected at 2 h after feeding a high-fat meal with either vehicle treatment or Mtp inhibitor treatment. Relative expression is shown, normalized to an unfed control for each respective treatment using the ΔΔCT method. Statistical analysis was conducted using the unpaired student’s t-test to identify differences between vehicle-treated and Mtp inhibitor-treated groups. Asterisks indicate $p < 0.05$. E) Schematic of lipid flux in the enterocyte after a high-fat meal. TAG is broken down into fatty acids and monoglyceride in the intestinal lumen. These metabolites are then absorbed by enterocytes and reconstituted into TAG in the ER, where it has two primary fates: 1) packaging into lipid droplets for short term storage (<16 h) or 2) incorporation into ApoB and ApoA-IV labeled chylomicrons for export to distal tissues. Creb3l3 is activated by a high-fat meal, leading to its transport from the ER to the Golgi, where it is cleaved and translocated into the nucleus to serve as a transcription factor. We hypothesize that the TAG lipiddation and translocation of ApoB may serve as the activating signal that triggers the movement of Creb3l3 from the ER to the Golgi.

Supplemental Table: Transcriptional response in larval guts upon feeding

[View Document]
**Average Nuclear Curvature COV**

**Hours of high-fat feed**

**Number of LD per cell**

**Area of LD / Total apical cytoplasmic area (log10)**

**Hours of second high-fat feed**
A. Egg Yolk, Artemia, Spirulina, Egg White, Glucose

B. Average Nuclear Curvature COV Normalized to 1h Egg Yolk

C. Number of Nuclei

D. Orlistat, Intestinal Lumen

E. Average Curvature COV Normalized to 1/1 Egg Yolk

F. Untreated, Vehicle, Orlistat, Ezetimibe
A

Up-regulation

Induced by feeding high-fat

167

Lipoproteins (9)
Lipid Droplet (4)
Fatty Acid (10)
Sterol (7)
Phospholipid (3)
Respiration (4)
Insulin Signaling (3)
Non-lipid (11)

Induced by feeding

111

Lipoproteins (2)
Fatty Acid (2)
Sterol (4)
Phospholipid (2)
Respiration (2)
Insulin Signaling (2)
Nuclear Receptor (2)
Vitamin D (2)
Non-lipid (4)

Down-regulation

Repressed by feeding high-fat

146

Lipoproteins (1)
Fatty Acid (3)
Sterol (8)
Sphingolipid (1)
Triglyceride (2)
Insulin Signaling (5)
Nuclear Receptor (2)
Non-lipid (12)
Starvation Response (5)

Repressed by feeding

46

Lipoproteins (1)
Sterol (2)
Phospholipid(1)
Insulin Signaling (2)
Nuclear Receptor (1)
Vitamin D (1)
Non-lipid (3)

B

Genes altered by high-fat feeding

| Lipoprotein | HF 1h LF 1h HF 4h LF 4h | Sterol | HF 1hLF 1h HF 4h LF 4h | Respiration | HF 1hLF 1h HF 4h LF 4h |
|-------------|--------------------------|--------|------------------------|-------------|------------------------|
| apoA-Vb.3   | 3.5 0.4 3.6 0.5          | hmgcs1 | 2.2 0.7 0.3 0.1        | cycsb       | 1.6 0.8 0.4 0.5        |
| apoA-Va     | 2.9 0.8 2.2 0.6          | sqlea  | 2.0 0.1 -0.5 -0.1     | abc10       | 1.4 0.6 0.6 0.3        |
| tm6sf2      | 2.1 0.0 1.5 -0.1         | fdps   | 1.3 -0.6 -0.5 -0.4    | cyp3c1/2    | 0.6 -0.3 1.3 0.1      |
| cubn        | 1.5 -0.1 1.9 -0.5        | acbd3  | 1.2 0.8 0.3 0.2       | pin2        | -1.3 -0.9 -0.6 -0.2   |
| apoA-Vb.2   | 1.4 0.0 1.9 0.5          | ebb    | 1.2 -0.2 -0.1 -0.2    | pin1        | -0.3 1.0 -1.6 0.4     |
| apoBa       | 1.2 0.5 0.0 -0.1         | nrd1d  | -1.0 -0.4 -0.5 0.0    | wu:fb99g09  | 1.4 0.8 0.0 0.2       |
| apoEa       | 1.2 -0.1 1.9 0.1         | wu:fb99g09 | -1.2 -0.4 0.3 0.0    | ipk         | 1.3 0.5 1.2 0.3       |
| apoEb       | 1.1 0.0 1.6 0.2          | fgf19  | -1.3 0.8 -0.7 0.0     | mem86b      | 0.8 0.0 1.4 0.1       |
| angpt1/4    | 0.5 -0.9 1.7 -0.3        | znf451 | -1.4 -0.7 -0.6 -0.2   | pla2g12a    | -0.2 0.3 0.0 0.2      |
| ace         | 0.0 0.8 -1.9 -0.3        | rhd14a | -2.3 -0.7 -0.2 -0.1   | insig1      | -0.3 1.0 -2.0 0.4     |

| Lipid droplet | HF 1h LF 1h HF 4h LF 4h | Fatty acid | HF 1h LF 1h HF 4h LF 4h | Phospholipid | HF 1h LF 1h HF 4h LF 4h |
|---------------|--------------------------|------------|--------------------------|--------------|--------------------------|
| plin2         | 5.5 -1.3 4.3 -0.7        | elov5      | 1.6 -0.3 2.4 -0.1        | tcn          | -1.2 -0.2 -0.2 -0.1     |
| g0s2          | 1.7 0.0 4.3 0.6          | acot       | 1.2 0.6 1.3 0.3          | atg9a        | -1.2 -0.7 0.3 -0.1     |
| plin3         | 1.7 0.4 2.2 0.4          | hadhaa     | 1.0 0.0 2.6 0.2          | prr5         | -1.4 -0.3 0.3 -0.2     |
| cidec         | 1.4 0.2 0.6 0.1          | fabp6      | 0.9 -0.3 1.5 -0.1        | ulk1a        | -1.4 -0.6 -0.2 0.1     |

| Signaling     | HF 1h LF 1h HF 4h LF 4h | Digestive organ function | HF 1h LF 1h HF 4h LF 4h |
|---------------|--------------------------|--------------------------|--------------------------|
| jun           | 1.6 0.9 0.0 0.2          | inhbe                    | 5.6 0.5 3.0 0.0          |
| creb331a      | 1.2 0.3 1.5 0.4          | tppose15                 | 0.2 0.3 -1.4 -0.5        |
| igf2b         | 1.2 0.8 -0.3 0.0         | pdpda                    | 0.0 0.5 1.7 0.4          |
| irs4          | -1.2 -0.7 0.0 -0.3       | klf15                    | -1.5 0.4 -0.8 0.1        |
| si:ch73-360o10.1 | -1.3 -0.7 -0.3 -0.2   |                          |                          |
| pik3ip1       | -1.9 -0.7 -0.5 0.1       |                          |                          |
| igfbp1a       | -2.1 -0.9 0.0 -0.1       |                          |                          |

| Metabolism    | Redox                     | Chromatin                | Translation             | Hsp          | Cell Structure         | Transporters  | Ubiquitination |
|---------------|----------------------------|--------------------------|-------------------------|--------------|------------------------|---------------|---------------|
Figure 4

A

1h Untreated 1h Vehicle Unfed 1h 2h

Mtp Inhibitor Treatment

B

Average Nuclear Curvature COV of Nuclei

C

Number of Nuclei

p< 0.0001

D

Expression at 2h after initiation of feed (relative to unfed controls)
Figure 4E
Endoplasmic reticulum lipid flux influences enterocyte nuclear morphology and lipid-dependent transcriptional responses
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