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Nocturnin regulates circadian trafficking of dietary lipid in intestinal enterocytes

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Summary

Background—Efficient metabolic function in mammals depends on the circadian clock, which drives temporal regulation of metabolic processes. Nocturnin is a clock-regulated deadenylase that controls its target mRNA expression post-transcriptionally through poly(A) tail removal. Mice lacking Nocturnin ($Noc^{-/-}$ mice) are resistant to diet-induced obesity and hepatic steatosis, yet are not hyperactive or hypophagic.

Results—Here we show that Nocturnin is expressed rhythmically in the small intestine, is induced by olive oil gavage and that the $Noc^{-/-}$ mice have reduced chylomicron transit into the plasma following the ingestion of dietary lipids. Genes involved in triglyceride synthesis, storage and chylomicron formation have altered expression and large cytoplasmic lipid droplets accumulate in the apical domains of the $Noc^{-/-}$ enterocytes. The physiological significance of this deficit in absorption is clear since maintenance of $Noc^{-/-}$ mice on diets that challenge the chylomicron synthesis pathway result in significant reductions in body weight, while diets that bypass this pathway do not.

Conclusions—Therefore we propose that Nocturnin plays an important role in the trafficking of dietary lipid in the intestinal enterocyes by optimizing efficient absorption of lipids.

Introduction

Diverse classes of organisms employ biological clocks to temporally coordinate their physiology and behavior, and to enable accurate predictions of external environmental conditions including timing of meals and subsequent nutrient uptake. Recent studies have

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demonstrated extensive links between the circadian clock and metabolic regulatory networks, and loss of clock function has serious negative effects on metabolic health (reviewed in [1]). Among humans with disrupted clocks due to jet lag or shift work, gastrointestinal disturbances are a major complaint [2] and humans exposed to simulated shift work have disrupted postprandial responses to fatty meals [3]. However, the role of the clock in regulating intestinal function is still not well understood.

One link between the circadian clock and metabolic function in mammals is the protein Nocturnin (gene name, *Ccrn4l*), which is expressed with high amplitude rhythms in many tissues, peaking during the night [4]. Nocturnin is a deadenylase; a class of exonuclease that specifically degrades the poly(A) tail of target mRNAs, usually leading to mRNA turnover or translational silencing [5, 6]. $Noc^{-/-}$ mice are resistant to diet-induced obesity and this is not due to increased physical activity, decreased food intake, or a higher metabolic rate [7]. These and other pieces of evidence suggest that these animals have deficits in lipid metabolism or uptake, presumably due to loss of rhythmic post-transcriptional regulation of genes necessary for lipid uptake, metabolism, and/or storage.

Although little is known about the mechanism by which circadian clocks control digestion, several reports suggest that they play an important role in regulating timing of digestive function to ready the correct components for maximum uptake of nutrients. The core clock genes are expressed with rhythmic profiles throughout the digestive tract [8–11] and a number of digestive processes and products are rhythmic (reviewed in [12]). These include diurnal variations in circulating lipids and proteins related to lipid uptake, circadian production of serum lipids and apolipoproteins, colonic motility, gastric emptying and rhythms in cell proliferation and radiation sensitivity. Many important genes in the small intestine and colon are expressed rhythmically [8, 9, 11, 13–15], resulting in circadian rhythms of macronutrient absorption and lipoprotein synthesis and secretion that are lost in mice with genetic disruptions of the circadian clock mechanism [11, 15].

One nutrient of primary importance is lipid. Within vertebrates, lipids are valued as a high caloric energy source, and also as conveyors of lipid soluble vitamins, such as vitamin A and vitamin E. The path that triglycerides take from the gut to the circulation involves a series of processes, beginning with emulsification and hydrolysis in the lumen, followed by uptake of hydrolyzed products by enterocytes. Short or medium chain fatty acids diffuse across the enterocyte and enter the portal vein blood while longer chain fatty acids are re-synthesized into triglyceride (TG) in the ER. The majority of the TG is either secreted as part of chylomicrons into the lymphatic system or stored in the cytosol as lipid droplets [16, 17]. Consequently, circadian regulation of this process could potentially occur at a variety of levels.

Here we demonstrate that the circadian deadenylase Nocturnin is an important link between the circadian clock and the lipid-absorption pathway in the small intestine. The lack of Nocturnin in mice causes increased retention of dietary lipids in cytoplasmic stores within the intestinal enterocytes and reduced secretion of chylomicron lipoprotein particles into the circulation. The physiological significance of this change in storage/secretion dynamics is evident by the inability of these mice to maintain their body weight on lipid-rich diets that depend on rapid lipid flux through the chylomicron secretion pathway.

Results

Nocturnin expression in the proximal intestine shows diurnal variations

$Noc^{-/-}$ mice are resistant to diet-induced obesity and yet do not have reduced food intake, increased activity, or significant changes in energy expenditure [7]. Therefore, we sought to

determine whether Nocturnin plays a role in the intestinal absorption of nutrients. We found that Nocturnin protein is expressed throughout the small intestine, with highest levels in the proximal portion (duodenum and proximal jejunum; Figure 1a), the region where the majority of lipid absorption takes place. Gene expression analysis of proximal jejunum samples collected every four hours throughout the day demonstrated that *Nocturnin* mRNA is expressed with a high amplitude rhythm, peaking in the early night at ZT12 ('ZT' refers to Zeitgeber Time in hours, where ZT0 is defined as light onset and ZT12 is defined as dark onset; Figure 1b). The central clock gene *Bmal1* is shown for comparison and is also robustly rhythmic, but peaks with an opposite phase, at ZT0. Consistent with the protein data, *Nocturnin* mRNA levels are lower in the more distal sections of the small intestine (ileum; Figure 1c, left panel). And in contrast to the high amplitude rhythms in the proximal intestine, *Nocturnin* mRNA levels did not significantly differ between ZT0 and ZT12 in the distal small intestine. *Bmal1* mRNA is expressed at higher levels at ZT0 than ZT12 in all three regions of the small intestine (Figure 1c, right panel). We have previously reported that *Nocturnin* is acutely inducible by various stimuli with "immediate early gene" induction properties [6, 18], and so we tested the effect of an olive oil gavage during the day when *Nocturnin* mRNA levels are normally low. We observed a significant acute induction of *Nocturnin* mRNA expression 2 hours after an olive oil gavage given at ZT 3 (Figure 1d). These data suggest that Nocturnin plays a role in intestinal responses to lipids.

Noc$^{-/-}$ mice absorb less triglyceride and cholesterol than *Noc*$^{+/+}$ counterparts

We examined Nocturnin's role in the postprandial TG response in fasted *Noc*$^{+/+}$ and *Noc*$^{-/-}$ mice by measuring TG levels in plasma following an olive oil gavage (Figure 2a). The plasma TG levels increase significantly following gavage in the *Noc*$^{+/+}$ mice, but not in the *Noc*$^{-/-}$ mice. To understand mechanisms for the reduced TG levels in the *Noc*$^{-/-}$ mice, we studied lipid absorption by feeding the *Noc*$^{+/+}$ and *Noc*$^{-/-}$ mice radiolabeled triglycerides and cholesterol. Plasma collected at time points over the next two hours demonstrated that the *Noc*$^{-/-}$ mice absorbed significantly less triglyceride and cholesterol (Figs. 2b and 2c) and had a significant reduction of both the HDL and non-HDL class of lipoproteins with the largest deficit in the non-HDL class (Figs. 2d and 2e) and specifically in the chylomicron/VLDL fraction (Figs. 2f and 2g). In contrast, the *Noc*$^{-/-}$ intestines retained significantly higher amounts of radiolabeled lipid than the *Noc*$^{+/+}$ intestines two hours after feeding, with increasing amounts in the more distal sections of the intestine (Figs. 2h and 2i). This is likely the result of the reduced secretion of fat taken up by the enterocytes in the proximal intestinal regions, causing excess lipids to accumulate in the distal regions. Together, these data show that lipids are absorbed inefficiently in proximal jejunum of the *Noc*$^{-/-}$ mice.

Primary enterocytes from the *Noc*$^{-/-}$ mice also have reduced lipoprotein secretion

Based on the *in vivo* data, we examined the lipid secretion from isolated enterocytes following a pulse labeling with radiolabeled oleic acid and cholesterol. As seen *in vivo*, the primary enterocyte cultures from *Noc*$^{-/-}$ mice retained more radiolabeled lipids intracellularly (Figs. 3a and 3b) and secreted less (Figs. 3c and 3d) than *Noc*$^{+/+}$ enterocytes. Density gradient fractionation analysis of the secreted oleic acid–derived lipids and cholesterol from the enterocyte culture medium indicated that the secreted lipoproteins were primarily deficient in low-density chylomicron fractions (fraction 1 and 2) in the *Noc*$^{-/-}$ cells (Figs. 3e and 3f). Therefore, consistent with the data from the *in vivo* analysis (Fig. 2), the *Noc*$^{-/-}$ enterocytes are deficient in efficiently secreting the absorbed lipid.

Decreased lipoprotein secretion in *Noc*$^{-/-}$ mice is not due to microsomal triglyceride transfer protein deficiency

Microsomal triglyceride transfer protein (MTP) is required for the processing of dietary lipid to blood soluble chylomicrons through the direct facilitation of chylomicron assembly and is

under circadian control [11, 13, 19]. Therefore, we measured the activity and protein levels of MTP in the $Noc^{+/+}$ and $Noc^{-/-}$ mice to determine whether a deficiency in this activity could explain the reduced chylomicron secretion in the $Noc^{-/-}$ intestines. Contrary to this hypothesis, MTP protein levels and activity were both higher in proximal small intestines from $Noc^{-/-}$ mice (Figs. 4a and 4b). This increase in activity was specific to the intestine, as no changes in MTP levels or activity were detected in liver samples from the same animals (Fig. 4c). However, even with this enhanced MTP activity, chylomicron secretion in the $Noc^{-/-}$ mice is still significantly reduced relative to $Noc^{+/+}$ mice (Figure 2), suggesting that some other component of this pathway is rate limiting.

Noc$^{-/-}$ enterocytes exhibit increased lipid storage

In order to further investigate the deficiencies in lipid absorption in the $Noc^{-/-}$ mice, we examined the expression levels of genes that play a role in lipid transport, storage, or chylomicron synthesis and secretion upon olive oil gavage (Supplementary Figure S1). Of particular interest was a significant decrease in the $Noc^{-/-}$ mice in the levels of *Adipophilin* (also known as *Perilipin2*, *Adph*, *Adrp* or *Adfp*), a protein known to be associated with the periphery of cytoplasmic lipid droplets (CLDs) and thought to be involved in regulation of CLD formation and size [20, 21]. We also observed a decrease in adipose triglyceride lipase (also known as *ATGL* or *Pnpla2*), a lipase important for the depletion/degradation of CLDs in adipose and non-adipose cells [22, 23] and in *Diacylglycerol acetyltransferase 2* (*Dgat2*), but not in *Dgat1*. The Dgats are two enzymes that play non-redundant roles in the synthesis of triglycerides (TGs) for chylomicron formation and for CLD storage [24]. We also observed reductions in *Apolipoprotein A-IV* (*ApoA IV*), a protein important for the efficient transport of fat with chylomicrons, and small but significant reductions in *Apolipoprotein B* (*ApoB*), a structural protein required for chylomicron assembly. We did not detect any deficits in *ApoB* mRNA editing (data not shown). In all these cases, these changes were observed following oil gavage, while no significant change was seen between genotypes when the animals were gavaged with water. Notably, mRNAs encoding proteins involved in the secretory process such as the GTPase *Sar1b*, and a protein critical for vesicle trafficking; *Vesicle transport through interaction with t-SNAREs homolog 1A* (*Vti1a*) were not changed in the $Noc^{-/-}$ intestines. There were also no differences between genotypes in the mRNAs encoding the sterol transporters ABCG5 and ABCG8, which are involved in lipid excretion back into the gut lumen.

Since the genes that show expression changes in the $Noc^{-/-}$ enterocytes include those that encode proteins involved in the pathways leading to synthesis of TGs (DGAT2), regulation of CLD size (Adipophilin, DGAT2, ATGL) and flux into the chylomicron synthesis pathway in the endoplasmic reticulum (ApoA IV), we microscopically examined the enterocytes from mice that had been gavaged with olive oil. Oil Red O staining of sections of the duodenum revealed lipid droplets in both the $Noc^{+/+}$ and $Noc^{-/-}$ cells, however, the droplets in the $Noc^{-/-}$ intestines appeared larger (Figure 5a). Quantitative analysis of multiple sections from multiple mice confirmed that this was the case, with fewer small droplets and more numerous large droplets in the $Noc^{-/-}$ as compared to the $Noc^{+/+}$ sections (Figure 5b). Transmission electron microscopy clearly shows the morphology of these large cytoplasmic lipid droplets (Figure 5c). Together, these data implicate Nocturnin as an important regulator of intestinal lipoprotein processing, suggesting that the absence of Nocturnin increases storage of lipids and decreases formation and secretion of lipoproteins.

The changes in chylomicron secretion have significant ramifications on body weight

The idea that $Noc^{-/-}$ mice absorb TGs and cholesterol less efficiently than $Noc^{+/+}$ mice is consistent with our findings that $Noc^{-/-}$ mice remain lean on a high fat diet (40% kcal fat), although they show no increase in respiration or activity and no decrease in food intake ([7]

and Figure 6a). In contrast, feeding the $Noc^{-/-}$ mice either a standard diet (17% kcal fat; [7]), or a high carbohydrate diet (70% kcal carb; Figure 6b) or a high fat diet containing medium chain (60% C8, 40% C10) fatty acids (45% kcal fat), which can be absorbed without being packaged into chylomicrons [25] (Figure 6c), results in weight gains indistinguishable from $Noc^{+/+}$ mice. To further test the physiological significance of the reduced lipid uptake in the $Noc^{-/-}$ mice, we maintained the mice on feeding regimens that challenge the efficiency of the normal lipoprotein packaging pathways. In the first of these, we fed $Noc^{+/+}$ and $Noc^{-/-}$ mice a ketogenic diet (95% kcal fat), in which nearly all of the calories come from fat [26]. $Noc^{+/+}$ mice lose weight rapidly on this diet, but eventually stabilize, while the $Noc^{-/-}$ mice lose more weight and do not stabilize (Figure 6d). This suggests that the decreased efficiency of lipid uptake in the $Noc^{-/-}$ mice results in the inability to maintain body weight when forced to rely on a diet in which lipids are the primary energy source.

Finally, we examined the mice under restricted feeding (RF) conditions, in which standard chow was available for only 6 hours during the middle of the light period (ZT3–9). Under this regimen, both the $Noc^{+/+}$ and $Noc^{-/-}$ mice entrain to the food availability after a few days, anticipating the food with increased bouts of locomotor activity just prior to the feeding period, and have indistinguishable overall levels of activity (Figure 6e,g). The $Noc^{+/+}$ mice lose weight and then as they entrain to the new food schedule, they begin to stabilize and even regain some weight. However, as with the ketogenic diet, the $Noc^{-/-}$ mice cannot maintain their body weight on this regimen. The $Noc^{-/-}$ mice lose significantly more weight than $Noc^{+/+}$ mice during the first 2 days of RF (Figure 6f), although their food intake is the same as $Noc^{+/+}$ over this period (Figure 6h). Unlike the $Noc^{+/+}$ mice, the $Noc^{-/-}$ mice do not recover following entrainment and continue to lose weight. This is not due to an inability to entrain to the food, since anticipatory activity onset does not differ between genotypes (Figure 6i). These results suggest that even on standard chow, the reduced efficiency of dietary lipid absorption in the $Noc^{-/-}$ mice results in significant deficits in body weight maintenance when food availability is limited to a few hours during the day.

Discussion

These studies demonstrate that the circadian clock-controlled gene, Nocturnin, plays an important role in the normal utilization of dietary lipids, providing a new link between the circadian timing system and this important metabolic process. Our data support the idea that the deficiency in nutrient uptake is due to decreased flux of dietary lipids going through the enterocyte lipoprotein synthesis and secretion pathway and increased storage in CLDs. This is reflected in the significant weight loss by the $Noc^{-/-}$ mice relative to their wild-type ($Noc^{+/+}$) counterparts when maintained on a ketogenic diet, but not when maintained on a high carbohydrate diet or on high fat diets containing medium chain fatty acids which can be transported into the plasma without assembly into chlomicrons [25]. Our studies using radiolabeled lipids proved that the reduced lipid mobilization capacity is happening at the level of the enterocyte in the $Noc^{-/-}$ mice, most likely at the level of regulation of resynthesis and storage of TGs, upstream of the lipoprotein assembly process in the endoplasmic reticulum. The reduced lipid secretion is not due to a decrease in the critical chylomicron assembly mediator, MTP, since MTP activity levels are significantly increased in the intestines of these mice. There is evidence that MTP may be up-regulated by fatty acids [27] and therefore we interpret this to be a compensatory response to the increased lipid content present in these cells, consistent with our observation that this up-regulation does not occur in the non-steatotic livers of the $Noc^{-/-}$ mice (Figure 4c). Previous studies reported that the amount of lipid found in the enterocyte can be manipulated by changes in bile presence [28]. Although there is a small change in bile acid pH in the $Noc^{-/-}$ mouse (Supplementary Fig. S2), emulsification of lipids and transport across the luminal surface

are probably not major components of the *Noc*$^{-/-}$ lipid malabsorption phenotype since primary enterocyte experiments demonstrated that *Noc*$^{-/-}$ cells could sequester lipid within these cells and Oil Red O and TEM studies demonstrate that there is significant accumulation of lipid mass in the intestine, residing in large lipid droplets inside the cells near the apical border. Therefore, although lipid uptake by these cells is not limiting, secretion of lipid from the *Noc*$^{-/-}$ enterocytes is attenuated compared to *Noc*$^{+/+}$ enterocytes.

Nocturnin's robust rhythmicity suggests that it is an important mediator of circadian control of intestinal lipid absorption, presumably through circadian post-transcriptional control of mRNA decay and/or translation. A recent study [11] demonstrated a clear role for the clock in these pathways and revealed that significantly more TG and cholesterol could be absorbed into the circulation at night as compared to day, and the lower daytime levels of radiolabeled lipids in the plasma could be accounted for by increased retention of counts in the intestine, similar to our observations in the *Noc*$^{-/-}$ mice. These differences were not simply due to time of food intake since these rhythms were lost in *Clock* mutant mice [11]. The clock has previously been shown to regulate nuclear receptors such as LXR, RXR, PPARa and SREBP in other metabolic tissues [29, 30] and these receptors have known roles in cholesterol and fatty acid absorption and metabolism [31]. Furthermore, intestinal expression of many mRNAs encoding key enzymes involved in TG synthesis and storage and chylomicron assembly are regulated by both the circadian clock and food, while a few were only regulated by food [11]. Interestingly, the genes that are changed in the *Noc*$^{-/-}$ enterocytes, as well as *Nocturnin* itself, fall into the class that are both clock and food regulated, providing another link between these important processes.

It is clear from our data that Nocturnin is required for optimal dietary lipid absorption into the circulation. The reduced secretion from the *Noc*$^{-/-}$ enterocytes results in excess fat accumulation in CLDs within the enterocytes and increased transport of fat to the distal regions of the intestine under conditions of high fat feeding. Increased CLD size has also been observed in other mice with defects in the TG re-synthesis or chylomicron assembly pathways [32–34], presumably reflecting the need to store the excess lipids that accumulate due to the reduced secretion rate. However, a more active and regulated role for CLDs in this process cannot be ruled out. Although CLDs were originally thought to be inert intracellular sites of TG storage, they have recently been shown to be dynamic organelles with organized structures and changing composition involved in highly regulated lipid storage/mobilization [21, 35, 36] and lipid droplet proteins are regulated in a dynamic fashion in enterocytes in response to acute and chronic high fat diets, suggesting that droplet size and function are under careful modulation [20, 37]. Whether active regulation of CLD size and function contributes in a significant way to the flux of lipids going through the lipoprotein pathways in enterocytes is not known, but chylomicrons secreted from enterocytes can contain TGs from stored cytoplasmic pools rather than from the most recently ingested lipids [37–42] and it has recently been suggested that the postprandial TG response may be modulated by regulatory steps that control CLD size and therefore dictate the balance between storage and secretion in these cells, rather than simply reflecting the rate of direct transit of dietary lipids [24].

The lipid absorption defects seen in the *Noc*$^{-/-}$ mice are mild relative to chylomicron assembly deficiencies such as seen in mice with reduced expression of MTP or intestinal ApoB [43–45] or in some human disorders such as Abetalipoproteinemia (Bassen-Kornzweig syndrome) or chylomicron retention disease (Anderson's disease) [46, 47]. In each of these cases the constitutive alteration of chylomicron assembly had severe health consequences. In contrast, the *Noc*$^{-/-}$ mice are able to form and secrete chylomicrons but do so less efficiently. As such, when this system is unchallenged, such as on ad lib standard diet, or high carbohydrate diet, a weight phenotype is not observed. On a high fat "western-

style" diet, they have a lean phenotype, but this develops slowly over several weeks. But, when forced to rely almost solely on fat as in the ketogenic diet, or when forced to obtain their daily energy in a short period of time (such as on RF) this deficit in lipid absorption into the circulation has more profound effects on body weight. It is likely that the reduced uptake of dietary lipids is a major causative factor in at least some aspects of the $Noc^{-/-}$ lean phenotype and is consistent with the $Noc^{-/-}$ mouse's resistance to weight gain on high fat diets, their lower constitutive body temperature, decreased lipid deposits in the liver and smaller white adipocytes [7]. However, Nocturnin is widely expressed in many metabolically relevant tissues where it has been implicated in other processes, such as in the stimulation of PPARγ transcriptional activity and adipogenesis [48]. Therefore, the relative contribution of the changes in the small intestine to the whole body lean phenotype in the $Noc^{-/-}$ mice will have to wait for the testing of mice with intestine-specific ablation of Nocturnin function.

These findings suggest that the circadian clock in the enterocytes regulates the postprandial TG response, at least in part, by controlling the relative flux of dietary lipids that are either diverted into transient storage within the enterocytes or into the chylomicron assembly process for secretion into the plasma. Our data support the idea that high levels of Nocturnin during the night normally contribute to the maximization of lipid absorption through the chylomicron pathway. In its absence, this pathway is less optimal and lipids accumulate in cytosolic droplets resulting in inefficient utilization of dietary lipids by the animal. It is worth noting that mice lacking Nocturnin do not accumulate measurable excess lipids in their feces (data not shown). Therefore gastrointestinal tract-specific inhibition of Nocturnin enzyme activity may be an attractive therapeutic strategy to prevent obesity in humans through reduction of dietary fat absorption without the negative side effects of currently available compounds such as olestra.

Experimental Procedures

Animals and Diets

Animal experiments were conducted according to relevant national and international guidelines and following the protocols approved by the Institutional Animal Care and Use Committees. Eight-week old male $Noc^{-/-}$ and $Noc^{+/+}$ mice on a congenic C57BL/6J background (N9 or N10) were maintained on a 12:12 LD cycle and fed ad lib unless otherwise stated. Olive oil gavage and restricted feeding procedures and compositions of the diets are in Supplementary Experimental Procedures.

Small Intestine Histology

Following olive oil gavage, 3 cm segments of intestine starting 1 cm distal to the pyloric valve were taken for further histological analysis by light and electron microscopy as described in Supplemental Experimental Procedures.

Plasma TG measurements and Lipid Absorption Studies

For postprandial TG measurements, blood was collected from tail bleeds at various time points following the gavage as indicated and TG levels were measured from plasma using a *Serum Triglyceride Determination Kit* (Sigma, St. Louis, MO). Short-term in vivo lipid absorption studies were done as described in Supplemental Experimental Procedures based on methods from [49–51]. Lipoproteins carrying TG and cholesterol were identified from primary enterocytes from $Noc^{-/-}$ and $Noc^{+/+}$ mice as described in Supplementary Experimental Procedures. MTP activity measurements were done as previously described [52, 53] (Supplementary Experimental Procedures).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Nocturnin mRNA is rhythmic in the small intestine and is induced by dietary lipids
- Mice lacking Nocturnin have increased accumulation of lipid in enterocytes
- Rates of chylomicron secretion are reduced in Nocturnin knockout mice
- Nocturnin knockout mice are unable to maintain body weight under dietary challenges

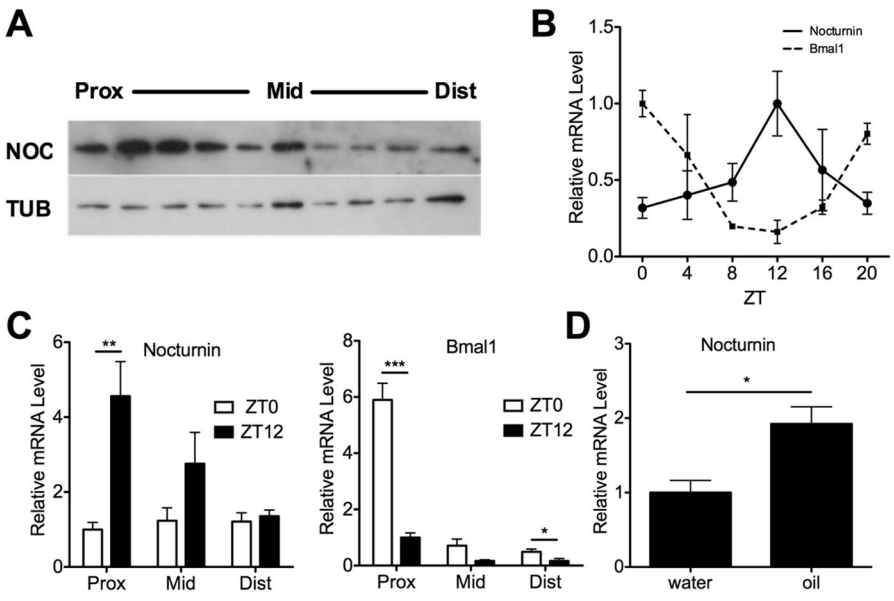

Figure 1. Nocturnin is expressed rhythmically in the small intestine and is induced by lipid feeding

(**a**) Mouse small intestine was harvested at ZT 12, dissected into 10 equally sized portions, from proximal to distal, and NOCTURNIN (NOC) protein expression was measured by western blot. TUBULIN (TUB) was used as a loading control. Prox, proximal; Mid, middle; and Dist, distal regions of the intestine. (**b**) Proximal sections of the small intestine (approximately the upper third) were collected from mice at different circadian times throughout the day as shown. *Nocturnin* and *Bmal1* mRNA expression were assessed by quantitative RT-PCR (n=3 per time point, means ± SEM) (**c**) Small intestines were collected from mice at ZT0 and ZT12 and dissected into 3 equally sized portions called “proximal” (prox), “middle” (mid) and “distal” (dist). *Nocturnin* and *Bmal1* mRNA expression were measured as described in (b). (n=4 per time point, means ± SEM) asterisks denote statistically significant differences in gene expression between sample times: * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ by Student’s T-test. (**d**) A gavage of olive oil or water was administered at ZT 3 followed by isolation of the proximal small intestine 2 hours later at ZT 5. *Nocturnin* mRNA expression was measured as in (b) (n=5 per treatment, means ± SEM) and asterisks denote statistically significant differences (* $P < 0.05$) between treatments. The *beta-2-microglobin* (B2M) mRNA was used for normalization (**b–d**).

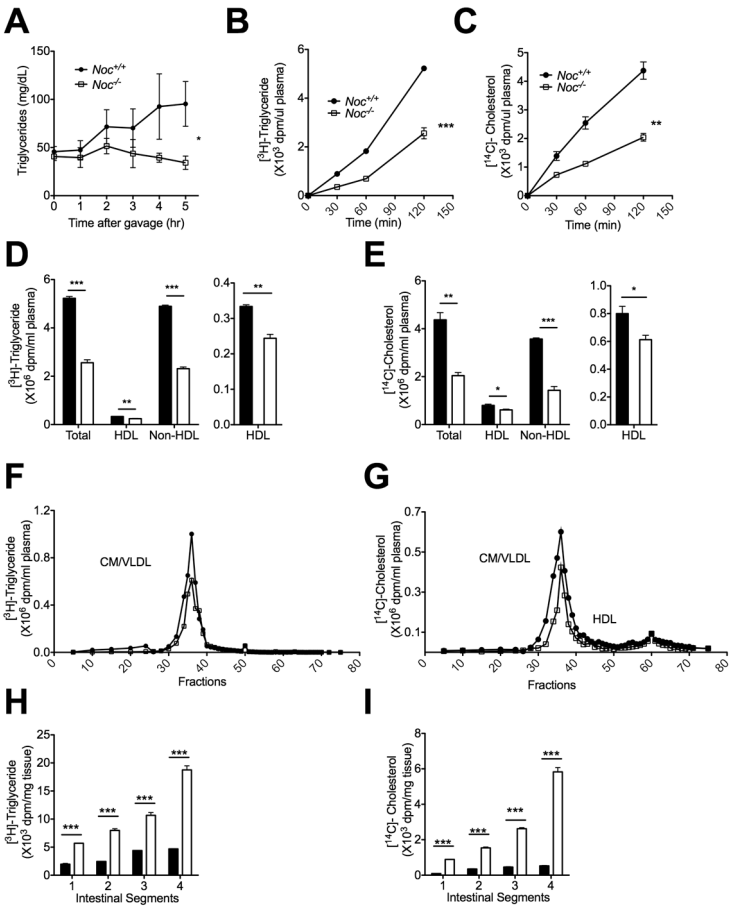

Figure 2. *Noc*$^{-/-}$ mice have deficits in triglyceride and cholesterol transport into blood

(a) *Noc*$^{+/+}$ and *Noc*$^{-/-}$ mice were given an olive oil gavage and then plasma was collected at various timepoints following the gavage, as shown. Total TG content was measured from each plasma sample. (**b** and **c**) Mice were fed radiolabeled TG and cholesterol and plasma was collected at various times post-gavage and assayed for radioactivity. Shown are the plasma levels of [^{3}H]-triglyceride (**b**) or [^{14}C]-cholesterol (**c**) after an oral gavage. (**d – g**) Plasma samples from **(b)** and **(c)** were fractionated into total, HDL and non-HDL plasma fractions (**d** and **e**) or separated into different lipoprotein density fractions by FPLC (**f** and **g**). To the right of each graph, the HDL fractions are replotted with expanded y-axes for better visualization. (**h** and **i**) Graphs show the level of [^{3}H]-triglyceride (**h**) and [^{14}C]-cholesterol (**i**) that remained within the intestine after an oral gavage. Segment 1 is proximal and Segment 4 is distal part of intestine. All the graphs are means ± SEM, and asterisks denote statistically significant differences between genotypes: * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ by linear mixed effects model **(a)**, repeat measures ANOVA (**b** and **c**) or Student's T-test (**d**, **e**, **h**, **i**). Samples are n=6 per genotype **(a)** and n=3 for both genotypes per time point (**b** and **c**), or pooled (**d**, **e**, **h**, **i**).

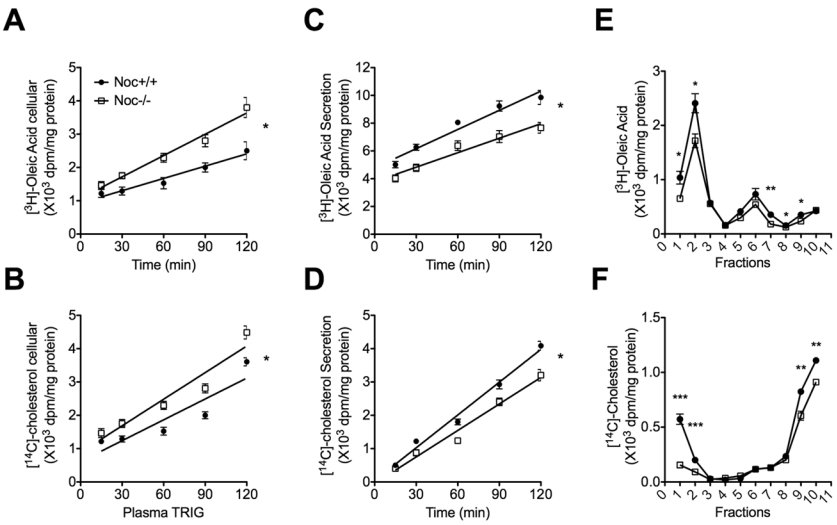

Figure 3. Primary enterocyte cultures from *Noc*$^{-/-}$ mice exhibit decreased lipoprotein secretion

Primary enterocytes were isolated from Noc+/+ and Noc −/− mice and cultured with radiolabeled oleic acid or cholesterol, washed and chased for 2 hours in the presence of 1.5mM oleic acid-containing micells. (**a** and **b**) Shown is intracellular content of [^{3}H]-Oleic acid (**a**) and [^{14}C]-Cholesterol (**b**) in primary enterocytes. (**c** and **d**) and secretion of [^{3}H]-Oleic acid (**c**) and [^{14}C]-Cholesterol (**d**) from primary enterocytes. (**e** and **f**) The radioactivity of [^{3}H]-Oleic acid (**e**) and [^{14}C]-Cholesterol (**f**) in conditioned culture medium was separated into different lipoprotein density fractions by density gradient ultracentrifugaion. All the graphs are means ± SEM (n=3), and asterisks in (**a, b, c,** and **d)** denote statistically significant differences (* $P < 0.05$) between genotypes by repeat measures ANOVA, and asterisks in (**e** and **f)** denote statistically significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) by Student's T-test.. Slopes of regression lines are as follows: **(a)** WT: 0.012 +/− 0.002; KO: 0.021+/− 0.002; **(b)** WT: 0.021 +/− 0.003; KO: 0.027+/− 0.003; **(c)** WT: 0.046 +/− 0.004; KO:. 0.034 +/− 0.004; **(d)** WT: 0.033 +/− 0.001; KO: 0.026 +/− 0.002.

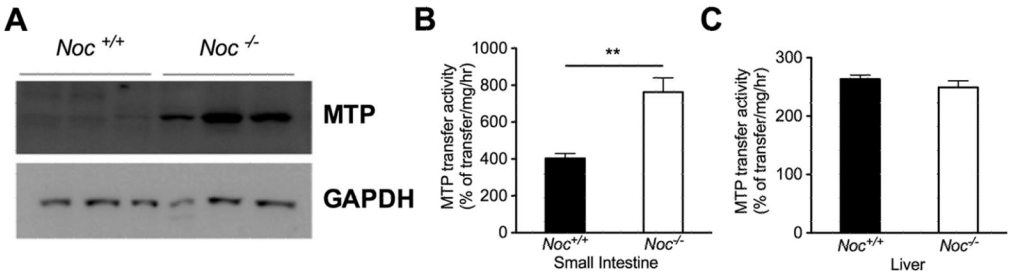

Figure 4. Activity and abundance of MTP protein is significantly upregulated in the small intestine of *Noc*$^{-/-}$ mice

(**a**) The proximal portions of small intestines from *Noc*$^{+/+}$ and *Noc* $^{-/-}$ mice were collected at ZT12 after a 24-hr fast. MTP protein levels were measured by western blot. Shown are results from three individual mice for each genotype. (**b** and **c**) Extracts from intestine (**b**) or liver (**c**) were assayed for MTP activity. All the graphs are means ± SEM. (n=4 for both genotypes). Asterisks denote statistically significant differences (** $P < 0.01$) between genotypes by Student's T-test.

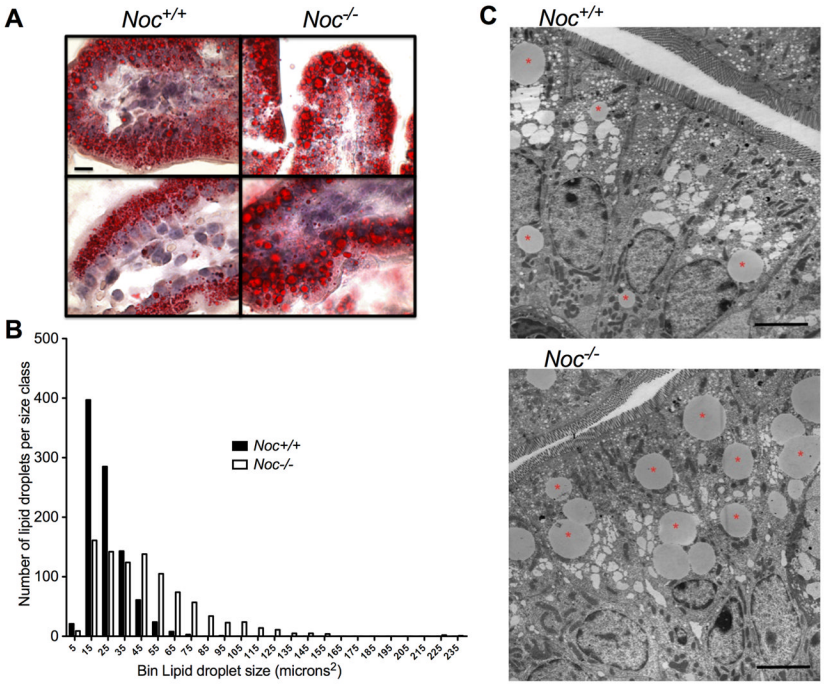

Figure 5. Lipid accumulates in larger droplets in the *Noc*$^{-/-}$ enterocytes

(**a**) Representative pictures of intestines stained with Oil-Red O (*Noc*$^{+/+}$ left, and *Noc*$^{-/-}$ right) two hours after olive oil gavage (ZT5). Examples from 2 different mice are shown for each genotype. All images were taken at the same magnification and the bar in the upper left panel represents 10 μm. (**b**) Oil-Red O stained sections were analyzed by counting the number of oil droplets in different size "bins". Shown is a histogram detailing number and size of lipid droplets stained with Oil-Red O in proximal small intestine (n=7 mice per genotype). The values on the x-axis refer to the largest size oil droplet assigned to that bin (for example, "5" refers to the number of oil droplets between 0–5 microns). (**c**) Representative TEM images of *Noc*$^{+/+}$ (top) and *Noc*$^{-/-}$ (bottom) in proximal small intestine (n=4 mice per genotype). Samples were taken at ZT14, two hours after an olive oil gavage. Asterisks denote large CLDs. Scale bar represents 5μm.

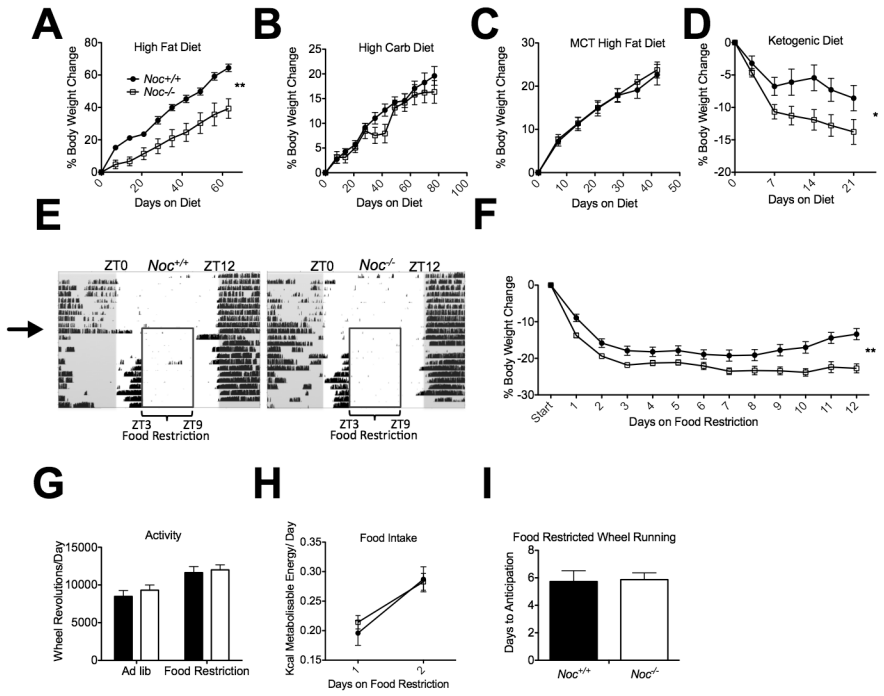

Figure 6. *Noc* $^{-/-}$ mice cannot maintain body weight on diets that challenge the lipoprotein secretion pathway

Body weights were recorded from *Noc*$^{+/+}$ and *Noc* $^{-/-}$ mice maintained on (**a**) a high fat diet (*Noc*$^{+/+}$ n=8, *Noc*$^{-/-}$ n=6), (**b**) a high carbohydrate diet (*Noc*$^{+/+}$ n=14, *Noc*$^{-/-}$ n=10), (**c**) a medium-chain triglyceride high fat diet (*Noc*$^{+/+}$ n=13, *Noc*$^{-/-}$ n=19) and (**d**) a ketogenic diet (*Noc*$^{+/+}$ n=14, *Noc*$^{-/-}$ n=17). Shown are percent body weight change (means +/− SEMs). (**e**) Representative actograms of continuous running wheel recordings of adult male *Noc*$^{+/+}$ (left) or *Noc*$^{-/-}$ (right) mice. Each horizontal line represents 24 hours and each day is plotted blow the previous day. Mice are individually housed in 12:12 Light-Dark cycle (white and gray background, respectively) with *ad lib* access to food for 7 days, followed by restriction of food to 6 hours in the middle of the day (insert box). Black hash marks indicate wheel-running behavior. (**f**) Percent body weight change (means ± SEM) over 12 days of food restriction (*Noc*$^{+/+}$ n=29–35, *Noc*$^{-/-}$ n=31–37 for each time point, Linear Mixed Effect model of repeat measures ANOVA F=8.1, p<0.01 for genotype). (**g**) Overall activity levels (wheel revolutions/day) before and during food restriction. **(h)** Energy consumed per day while on food restriction (*Noc*$^{+/+}$ n=35, *Noc*$^{-/-}$ n=37 for each time point). (**i**) Days to anticipation of food presentation as measured by sustained wheel running under food restriction diet (*Noc*$^{+/+}$ n=15, *Noc*$^{-/-}$ n=15).