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A Synthetic ERR Agonist Alleviates Metabolic Syndrome

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d) Abbreviation:

BAT: Brown Adipose Tissue
CLAMS: Comprehensive Laboratory Animal Monitoring System
CT: Circadian Time
DDIT4: DNA Damage Inducible Transcript 4
DIO: Diet-Induced Obesity
ER: Estrogen Receptor
ERR: Estrogen Related Receptor
FAO: fatty acid oxidation
GTT: Glucose Tolerance Test
HDL: High-Density Lipoprotein
HF: High Fat
HFD: High Fat Diet
ITT: Insulin Tolerance Test
LDL: Low-Density Lipoprotein

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NMR: Nuclear Magnetic Resonance
ORO: Oil Red O
Pck1: phosphoenolpyruvate carboxykinase
PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PTT: Pyruvate Tolerance Test
RER: respiratory exchange ratio
RT-qPCR: Reverse Transcription- quantitative Polymerase Chain Reaction
TCA: TriCarboxylic Acid
TG: Triglycerides
WAT: White Adipose Tissue
WT: Wild Type
ZT: Zeitgeber time

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Abstract

Physical exercise induces physiological adaptations and is effective at reducing the risk of premature death from all causes. Pharmacological exercise mimetics may be effective in the treatment of a range of diseases including obesity and metabolic syndrome. Previously, we described the development of SLU-PP-332, an agonist for the ERRα, β and γ nuclear receptors that activates an acute aerobic exercise program. Here, we examine the effects of this exercise mimetic in mouse models of obesity and metabolic syndrome. Diet-induced obese or *ob/ob* mice were administered SLU-PP-332 and the effects on a range of metabolic parameters were assessed. SLU-PP-332 administration mimics exercise-induced benefits on whole-body metabolism in mice including increased energy expenditure and fatty acid oxidation. These effects were accompanied by decreased fat mass accumulation. Additionally, the ERR agonist effectively reduced obesity and improved insulin sensitivity in models of metabolic syndrome. Pharmacological activation of ERR may be an effective method to treat metabolic syndrome and obesity.

Significance Statement:

An estrogen receptor-related orphan receptor (ERR) agonist, SLU-PP-332, with exercise mimetic activity, holds promise as a therapeutic to treat metabolic diseases by decreasing fat mass in mouse models of obesity.

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1. Introduction

Obesity is a major health issue and is a predisposing risk factor for early mortality due to multiple diseases including cancers, cardiovascular disease, and type 2 diabetes (Booth *et al.*, 2012). Obesity is associated with the accumulation of fat in the skeletal muscle that leads to reduced metabolic efficiency and insulin resistance (Taylor, 1999). Skeletal muscle is one of the most flexible organs for fuel selection and depending on the environmental conditions (diet, exercise, etc.) it can utilize glucose or fatty acids as the main source of energy. In general, glucose is the fuel preferentially utilized by skeletal muscle and is selected for short and high-load exercise (resistance exercise), and as the duration of energy demand lengthens (long and low-load exercise) skeletal muscle switches its preference to fatty acids (aerobic exercise) (Meex *et al.*, 2010). Increased fatty acid utilization decreases fat storage in the tissues and improves overall metabolism functionality and is typically associated with improved glucose tolerance and insulin sensitivity. A key adaptation of skeletal muscle in response to aerobic exercise is increased oxidative metabolic capacity mediated by enhanced mitochondrial respiratory capacity, which allows for more efficient energy production and enhanced exercise endurance (Meex *et al.*, 2010).

The estrogen receptor-related receptors (ERRα ERRβ and ERRγ) are members of the nuclear receptors superfamily. ERRs are constitutively active orphan receptors most closely related to estrogen receptors (ERs) in terms of sequence homology but do not display the estrogen-binding properties of the ERs (Giguere *et al.*, 1988). High energy demand tissues, such as muscle, heart, or liver, display high levels of expression ERRs (Giguere *et al.*, 1988; Sladek *et al.*, 1997; Chen *et al.*, 1999). Classical ERR target genes encode proteins involved in

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fatty acid oxidation, mitochondrial biogenesis, and the TCA cycle (Audet-walsh and Giguére, 2015; Fan and Evans, 2015).

Several genetic gain- and loss-of-function mouse models of ERR have been developed in the past decade. ERRβ knock-out mice display embryonic lethality while ERRγ and ERRα KO mice display varying degrees of susceptibility to the development of heart failure (Luo *et al.*, 1997; Alaynick *et al.*, 2007; Sakamoto *et al.*, 2020). Additionally, several studies have shown a role of ERR in skeletal muscle function and endurance via modulation of mitochondrial biogenesis and lipid oxidation (Narkar *et al.*, 2011; LaBarge *et al.*, 2014). Importantly, mice over-expressing ERRγ in the skeletal muscle display an increase in oxidative muscle fibers and exhibit increased exercise endurance (Narkar *et al.*, 2001; Rangwala *et al.*, 2010).

Increased skeletal muscle oxidative capacity is associated with improved glucose tolerance and insulin sensitivity and reduced obesity (Donnelly *et al.*, 2013), thus we hypothesized that synthetic ERR agonists that increase their transcriptional activity above their very high constitutive level may hold significant utility in the treatment of metabolic syndrome.

In continuation of our efforts to develop ERR pan agonists (Shahien *et al.*, 2020), we recently reported the development of an ERR agonist, SLU-PP-332, that targets all three ERR isoforms allowing for evaluation of this unique class of agent *in vivo.* SLU-PP-332 activates all three ERRs in cell-based assays with slightly more potency at ERRα. Moreover, we previously demonstrated that SLU-PP-332 displays both plasma and muscle exposure (0.2 μM and 0.6 μM respectively) *in vivo* 6 hrs after intra-peritoneal (IP) injection (30mg/kg) (Billon *et al.*, 2023).

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We have shown that frequent pharmacological activation of ERR provides similar improvements to repeated bouts of aerobic exercise associated with increased skeletal muscle oxidative capacity (Billon *et al.*, 2023). Moreover, a recent study has shown that ERRα activation via a synthetic agonist improved fatty liver disease *in vivo* (Mao *et al.*, 2022). In this study, we examined the ability of SLU-PP-332 to modulate energy expenditure *in vivo.* We observed an increased resting energy expenditure in mouse models of obesity/metabolic syndrome that was associated with increased fatty acid oxidation leading to improvements in glucose tolerance and decreasing adiposity.

2. Materials and Methods

2.2. Mice

Male mice were obtained from Jackson Laboratories (Bar Harbor, ME). All procedures were approved and conducted per the Saint Louis University and Washington University Animal Care Use Committees.

2.2. General mouse studies

For all experiments, 8-10 male C57BL6/J or *ob/ob* mice per group (12 weeks of age for chow) were administered SLU-PP-332 at 50 mg/kg (i.p., b.i.d.) or vehicle for 28 days or 12 days (for the *ob/ob*). Dose and frequency of dosing we chose based on our previously published data (Billon *et al.*, 2023). The ~1-month dosing regimen was selected based on our previous experience with monitoring the effects of exercise mimetics in WT and DIO mice (Solt et al. 2012). Studies with the *ob/ob* mice were shortened to 12 days due to reduced tolerance of twice per day ip administration. At the termination of the experiment, tissues were collected and gene expression analysis was completed by real-time qPCR using methods. Food intake and body weight were monitored daily. Body composition was measured before initiation and at the termination of the experiments using NMR (Bruker BioSpinLF50). Plasma was collected for

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triglyceride and cholesterol measurements as well. All b.i.d. dosing was performed with dosing occurring at ZT0 and ZT12.

2.3. Diet-induced obesity model

Twenty-week-old male C57BL6/J were maintained on a high-fat diet (20% carbohydrate, 60% fat) for 8 weeks (average weight = 38g). The mice were maintained on the high-fat diet for the duration of the experiment, which included SLU-PP-332 administration for 28 days (50 mg/kg, b.i.d., i.p.).

For acute insulin response, mice were dosed for 7 days (50 mg/kg, b.i.d., i.p.), fasted for 7h, and then acutely dosed with insulin (0.75U/kg of fat-free mass) and sacrificed 30 minutes after. Upon termination of the study, tissue samples were collected for gene expression as assessed by real-time qPCR. Blood samples were collected for further metabolite screening.

2.4. Comprehensive laboratory animal monitoring system (CLAMS)

The Columbus Instruments (Columbus, OH) CLAMS system was used to assess the metabolic parameters of mice. Male C57BL6/J (10 or 20 weeks of age for chow or HFD respectively) were housed individually in metabolic cages on a 12 h day–night cycle, fed with either a normal chow or high-fat diet. Mice were acclimated for 5 days in the CLAMS unit prior to administration of SLU-PP-332 or vehicle (10% tween, 10% DMSO, 80% PBS). The administration of SLU-PP-332 was continued for 10 days. Eight animals were included per group. The hourly or average values during light and dark periods were calculated. Two-way ANOVA followed by the Bonferroni post-test was used to calculate the p-value.

2.5. Glucose tolerance test

After a 6-hour fast, vehicle and SLU-PP-332 treated mice (n=8) were injected IP with glucose (2g/kg of fat-free mass) (Sigma-Aldrich, St Louis MO, USA) to examine glucose tolerance. Blood was collected by tail snip and glucose was measured before the injection (t=0 min) and 15 min, 30 min, 60 min, and 120 min post-injection using a OneTouch Ultra®2 glucometer.

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2.6. Lipid assays

Plasma triglycerides (TGs), total cholesterol, and liver enzymes were assessed using an Analox instrument (GM7 MicroStat, St Louis University School of Medicine).

2.7. Real-Time PCR (RT-PCR)

Total RNA extraction from mouse tissues was performed using Trizol reagent methods (Invitrogen). The RNA samples were reverse transcribed using the qScript cDNA kit (Quanta). All samples were run in duplicates and the analysis was completed by determining $\Delta\Delta$Ct values. The reference gene used was 36B4, a ribosomal protein gene.

2.8. Statistical analysis

Data are expressed as mean +/- SEM. Student's test or Two-way ANOVA was used to calculate statistical significance. $P<0.05$ was considered significant.

3. Results

3.1. SLU-PP-332 induces fatty acid metabolism *in vivo*.

Increased whole-body fatty acid oxidation is a characteristic metabolic adaptation to aerobic exercise and aerobic training (Egan and Zierath, 2013). Previously, we demonstrated that SLU-PP-332 induces an acute aerobic exercise genetic program and increased exercise endurance (Billon *et al.*, 2023). Here, we began by assessing if SLU-PP-332 administration would result in metabolic adaptations typically observed with aerobic training. To monitor both acute and chronic effects of drug administration, we examined the effect of SLU-PP-332 (b.i.d, 50mg/kg) on metabolic parameters in C57Bl/6 mice using a comprehensive laboratory animal monitoring system (CLAMS). Mice were treated with either SLU-PP-332 or vehicle for 28 days. To avoid any interference of ERR with thermogenesis, mice were housed at thermoneutrality (Gantner *et al.*,

2016). No difference in total body weight was observed after 28 days of treatment with SLU-PP-332 (b.i.d, 50mg/kg) (Fig. 1a and Supplementary Fig. 1a) but a decrease in fat mass gain was observed using NMR (Fig. 1b). As expected for mice maintained at thermoneutrality, the interscapular brown adipose tissue (BAT) depot was small and SLU-PP-332 treatment did not alter BAT mass (Supplementary Fig. 1b). No difference in daily food intake or lean mass was observed over the 28 day dosing period (Supplementary Figs. 1c & 1d). Only relatively minor changes in plasma cholesterol and liver enzyme levels were noted in SLU-PP-332-treated mice compared to vehicle-treated (Fig. 1c & 1d). There was no effect on locomotor activity in the mice as well (Fig. 1e). Within 2h following the first dose of SLU-PP-332 in the mice we observed an acute decrease in the respiratory exchange ratio (RER) indicative of a shift in fuel utilization towards lipids (Fig. 1f). The lower RER was maintained during the length of the dosing during both light and dark periods and as shown in Fig. 1g. The amount of fatty acid oxidized in drug vs. vehicle-treated mice was calculated using Frayn's equation (Frayn, 1983) and SLU-PP-332 treatment increased fatty acid oxidation (FAO) by 25% compared to vehicle-treated animals (Fig. 1h). Reciprocally, a decrease in carbohydrate utilization was observed (Fig. 1i). An increase in energy expenditure was observed (Fig. 1j) but there was no difference in food intake (Fig.1k). No change in white adipocyte size was observed between vehicle and SLU-PP-332 treated mice (Fig.1l)

3.2. SLU-PP-332 does not alter glucose metabolism *in vivo*.

SLU-PP-332 did not affect fed or fasted blood glucose levels (Fig. 2a) or insulin levels (Fig. 2b). We also assessed the potential effect of SLU-PP-332 glucose metabolism by performing insulin

sensitivity and glucose/pyruvate tolerance tests in the same mice described above. Three weeks of SLU-PP-332 treatment did not affect glucose tolerance (Fig. 2c). ERRs have been suggested to be involved in gluconeogenesis (Kim *et al.*, 2012), but we observed no significant alteration in pyruvate tolerance in response to SLU-PP-332 suggesting hepatic glucose output was not affected (Fig. 2d). We also examined pancreatic islet structure and observed no distinctions between drug and vehicle treatment groups (Fig.2e). Consistent with this observation, the expression of the rate-limiting enzyme of gluconeogenesis, *phosphoenolpyruvate carboxykinase (Pck1),* was not significantly altered with SLU-PP-332 treatment (Supplementary Fig. 1e). Although there were no indications of hepatic steatosis in the WT mice, SLU-PP-332 treatment did cause a reduction of hepatic triglycerides (Supplementary Fig. 1f). We next assessed the effect of SLU-PP-332 in muscle metabolism *in vivo*. Mice treated with SLU-PP-332 displayed increased muscle pyruvate (Fig. 2f) and decreased glycogen content (Fig. 2g) compared to the vehicle-treated group. We tested *in vivo* glucose uptake using fluorescently labeled 6-deoxyglucose. Mice treated with SLU-PP-332 displayed increased glucose uptake by the quadricep muscle after 2 weeks of treatment (Fig. 2h) compared to the vehicle-treated group. These data indicate that SLU-PP-332 induced an alteration in preference for fuel utilization consistent with physiological adaptation to exercise that results in increased whole-body fatty acid oxidation (review in Astorino and Schubert, 2018) and also suggest that ERR agonists may hold utility in the treatment of metabolic diseases.

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3.3. SLU-PP-332 reduces fat mass and improves glucose metabolism in several mouse models of obesity.

Based on the exercise mimetic activity and alterations in energy metabolism we observed in mice treated with SLU-PP-332, we hypothesized that such compounds may have a beneficial effect in models of obesity/metabolic syndrome. We utilized both the diet-induced obesity (DIO) and *ob/ob* mouse models to examine the effects of SLU-PP-332 on various metabolic parameters. For the DIO model, we initiated a study with 20-week-old C57BL6 mice that had been maintained on a high fat (HF) diet for 8 weeks (20% carbohydrate 60% fat) before initiation of SLU-PP-332 treatment and housed at thermoneutrality. The mice were maintained on the HF diet throughout the SLU-PP-332 treatment (50mg/kg, i.p., b.i.d.). We noted a progressive weight loss in both DIO and *ob/ob* mice treated with SLU-PP-332 (Supplementary Fig. 2a) and after 28-days of treatment the drug treated mice weighed ~12% less (Fig. 3a). After 28-days of treatment, vehicle-treated mice had gained ~5g of fat mass while drug treated mice had gained less than 0.5g of fat mass (Fig. 3b). As expected for mice maintained at thermoneutrality, the interscapular brown adipose tissue (BAT) depot was small and SLU-PP-332 treatment did not alter BAT mass (Supplementary Fig. 2b). No significant differences in food intake were observed during the duration of the treatment period (Supplementary Fig. 2c). There were no significant changes in lean mass (Supplemental Fig. 2d). In addition to the decrease in adiposity, we also observed a decrease in plasma total cholesterol, high density lipoprotein (HDL) and triglycerides (TG) but no change in low density lipoprotein (LDL) (Fig. 3c). There were also no increases in liver enzyme levels (Fig. 3d). Fasting plasma glucose and insulin

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levels were lower in SLU-PP-332-treated animals, but no differences were observed in fed blood glucose levels (Fig. 3e & 3f).

We next assessed whether SLU-PP-332 treatment affected whole-body fuel selection and energy expenditure using the CLAMS. As observed for chow-fed mice, SLU-PP-332 treatment reduced the RER (Fig. 3g), indicating an increase in fatty acid oxidation (Fig. 3h) and reduced glucose oxidation (Fig. 3i) compared to vehicle-treated mice. No changes were observed in locomotor activity (Fig. 3j). RER was similarly reduced by SLU-PP-332 treatment to that observed with mice on a regular chow diet (Supplementary Fig. 2e). Resting energy expenditure was significantly higher in the SLU-PP-332 treatment group (Fig. 3k & 3l). Additionally, SLU-PP-332-treated mice displayed improved glucose tolerance (Fig. 3m) although there was no change in an insulin tolerance test (Supplemental Fig. 2f left). There was also no effect on pyruvate tolerance or hepatic *pck1* expression (Supplemental Fig. 2g & h) and there were no significant changes in pancreatic histology (Supplementary Fig. 2i). Since the SLU-PP-332 treated mice did not gain fat mass, we examined the effect of the ERR agonist on white adipose tissue (WAT). Paraffin-sections of visceral WAT show a significant decrease of adipocytes size in SLU-PP-332 treated mice when they were maintained on the HF diet (Fig. 3n). We also noted a decrease in hepatic steatosis (Fig. 3o) and a decrease in hepatic triglycerides (Fig. 3p) consistent with increased lipid utilization. Given that conflicting data regarding insulin sensitivity (decreased fasting plasma glucose levels combined with lower fasting plasma insulin and a decrease in adipocyte size but no alteration in the insulin tolerance test), we assessed the effect of SLU-PP-332 treatment on AKT phosphorylation in skeletal muscle (quadricep) in response to insulin. We

observed only a trend towards an increase in AKT phosphorylation in skeletal muscle from DIO mice (8 weeks on HF diet prior to 7 days SLU-PP-332 administration) (Supplemental Fig. 2j).

The second metabolic disease model we utilized to assess the effects of SLU-PP-332 was the *ob/ob* model. Three-month-old male *ob/ob* mice maintained on a regular chow diet and maintained at thermoneutrality were administered SLU-PP-332 (50mg/kg, i.p., b.i.d.) or vehicle for 12 days. Although total body weight only displays a trend towards a decrease after 12 days of administration of the drug (Fig. 4a), adiposity was significantly decreased as well as liver weight (Fig. 4b & 4c) even though food intake was unaltered (Fig. 4d). In a manner similar to what was observed in WT and DIO mice, we noted a decrease in RER within 2h of administration of SLU-PP-332 (Figs. 4e & 4f). Fatty acid oxidation was increased with drug treatment while carbohydrate oxidation was lower (Figs. 4g & 4h). Resting energy expenditure was elevated (Fig. 4i). The mice displayed substantial hepatic steatosis that was reduced by SLU-PP-332 treatment (Fig. 4j). These data demonstrating the efficacy of pharmacological activation of ERR in two mouse models of metabolic disease/obesity suggests that such compounds may be useful in treating metabolic diseases.

4. Discussion

Obesity and fat accumulation are associated with a higher risk of diabetes and cardiovascular diseases (Hill and Wyatt, 2013). Lipid accumulation in the muscle induces insulin resistance and impairs glucose uptake (Callahan *et al.*, 2017) while exercise and decreased caloric intake (negative energy balance) are known to decrease fat accumulation and improve glucose and insulin signaling (Heath *et al.*, 1983).

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The ERRs play essential roles in the regulation of energy metabolism and fuel selection. Loss of ERRα or ERRγ function results in reduced muscle oxidative function and reduced functional endurance (Perry *et al.*, 2014; Yoshihara *et al.*, 2016). Therefore, pharmacological activation of ERRs may provide beneficial metabolic effects associated with increased skeletal muscle activity for the treatment of metabolic diseases.

Our data clearly suggest that such compounds may hold utility in the treatment of diseases such as type 2 diabetes and obesity where exercise is typically prescribed. Obesity and fat accumulation are associated with a higher risk of development of diabetes and cardiovascular diseases (Hill and Wyatt, 2013). Lipid accumulation in the muscle induces insulin resistance and impairs glucose uptake (Callahan *et al.*, 2017) while exercise and decreased caloric intake are known to decrease fat accumulation and improve glucose and insulin signaling (Heath *et al.*, 1983). WT mice maintained either on the normal or high-fat diet and *ob/ob* mice were resistant to gaining fat mass when administered SLU-PP-332. Importantly, SLU-PP-332 treatment increases resting energy expenditure that mimics the recovery phenotype after exercise, but without increasing physical activity (Gillette *et al.*, 1994).

The beneficial effects of SLU-PP-332 on the metabolic profile could not necessarily have been predicted based on conflicting data with regard to the role of ERRα. For example, it has been reported that ERRα-null mice have reduced fat mass and are resistant to diet-induced obesity (Luo *et al.*, 2003). Additionally, selective ERRα *inverse* agonists have been characterized that display anti-diabetes activity (Patch *et al.*, 2011, 2017). In both of these cases, it appears that several ERRα target genes are actually elevated rather than reduced suggesting that there may be some engagement of compensatory mechanisms. Interestingly, these ERRα-null mice

also display reduced muscle oxidative capacity; an effect that is also induced by C29, a synthetic ERRα *inverse* agonist (Patch *et al.*, 2011) These contradictory effects, at least in terms of a potential treatment for metabolic diseases, are also aggravated by the potential for an ERRα *inverse* agonist to induce heart failure (Huss *et al.*, 2007). Thus, based on our data with SLU-PP-332 along with the preponderance of the genetic gain- and loss-of-function data, ERR agonists are more likely to be beneficial in the treatment of metabolic disease.

The relative balance of ERRα vs. ERRγ agonist activity of a therapeutic compound may be important to avoid potential side effects. Although there has been considerable redundancy in function described, distinct regulatory roles of the ERR isoforms have been demonstrated as well, such as their effects on gluconeogenesis. ERRα suppresses whereas ERRγ activates phosphoenolpyruvate carboxykinase 1 (*Pck1)* gene expression, providing a mechanism where the isoforms have opposing effects on gluconeogenesis (Herzog *et al.*, 2006; Kim *et al.*, 2012). These observations are consistent with increased *Pck1* expression observed in the ERRα-null mice (Perry *et al.*, 2014) as well as the ability of the ERRγ *inverse* agonist GSK5182 to suppress gluconeogenesis (Kim *et al.*, 2012). These effects of GSK5182 appear to conflict with the report that an ERRβ/γ agonist increases muscle oxidative function (Rangwala *et al.*, 2010). We note that with SLU-PP-332 there is no change in *Pck1* expression or pyruvate tolerance suggesting no effect on hepatic glucose output (Supplementary Figs. 1e, 2f and g, Fig. 2f) This might be due to competing effects of ERRα and ERRγ that effectively cancel the effect on these genes or it may be due to alterations in the gluconeogenic response to whole body alterations in metabolic activity. The fact that SLU-PP-332 exhibits a degree of preference for ERRα over ERRγ may help to eliminate the potential side effect of induction of gluconeogenesis.

In summary, the ERR agonist SLU-PP-332 functions as an exercise mimetic inducing an acute aerobic exercise program that leads to myriad physiological adaptations that are associated with exercise including increased skeletal muscle oxidative fibers, increased fatty acid oxidation, and enhanced exercise endurance. Although much of the exercise mimetic activity may be associated with targeting ERR in the skeletal muscle, additional target tissues such as the liver may also play a role. In obese mice SLU-PP-332 reduced fat mass and improved glucose tolerance, suggesting a potential use of ERR agonists in the treatment of a range of metabolic diseases including obesity and type 2 diabetes.

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Data availability statement:

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Author contributions:

Participated in research design: Billon and Burris.

Conducted experiments: Billon, Schoepke, and Avdagic

Contributed to new reagents or analytic tools: Chatterjee, Butler, Elgendy, and Walker.

Performed data analysis: Billon, Butler, and Burris.

Wrote or contributed to the writing of the manuscript: Billon and Burris.

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Footnotes:

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Figure legends

Figure 1. SLU-PP-332 improves muscle function and increases fatty acid metabolism *in vivo*. Results from a 28-day SLU-PP-332 dosing regimen in C57Bl6 mice on a normal chow diet. Mice were kept at thermoneutrality and also assess in a CLAMS. Body weight **(a)**, fat mass gain **(b)**, blood lipid profile **(c)** (total cholesterol, HLD, and TG), and liver enzymes **(d)** of three-month-old males treated with vehicle (white bar, n=8) or SLU-PP-332 (black bar, n=8) for 28 days. Results from the CLAMS of the same animals treated with vehicle (white bar, n=8) and SLU-PP-332 (black bar, n=8) under chow diet during the day (solid bar) and night (shaded bar) over the 5 days at thermoneutrality are illustrated in **(e)** through **(j).** Locomotor activity (**e**); respiratory

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exchange ratio (RER) (**f** & **g**); fatty acid oxidation (FAO) (**h**); carbohydrate oxidation (**i**) in these mice is illustrated. Energy expenditure (**j**) and average food consumption (**k**) over the 28 days from the same mice. In **(f)** arrow points to the first dosing. **(l)**, Hematoxylin and Eosin staining of white adipose tissue from vehicle (left) or SLU-PP-332 (right) treated animals (n=4) and quantitation of adipocytes size. * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

Fig 2. SLU-PP-332 does not improve glucose metabolism *in vivo* in chow-fed mice

Fed and fasted blood glucose levels **(a)** of the animals treated with vehicle (white bar, n=8) and SLU-PP-332 (black bar, n=8) maintained on a chow diet (fed) or after 8 h of fasting (fast). Blood insulin level **(b)** of the animals treated with vehicle (white bar, n=8) and SLU-PP-332 (black bar, n=8) under chow diet (fed) after 28 days of treatment. IP glucose **(c)** and pyruvate **(d)** tolerance tests from vehicle (open circle/ white bar) or SLU-PP-332 (black square/ bar) treated animal (n=8/ group) under chow diet. The area under the curve is represented on each graph. **(e)** Hematoxylin and Eosin staining of the pancreas from vehicle (left) or SLU-PP-332 (right) treated animals (n=4). Muscle pyruvate **(f)** and glycogen **(g)** content from animals treated with vehicle (white bar, n=8) and SLU-PP-332 (black bar, n=8) under chow diet (fed) after 28 days of treatment. **(h)** *In vivo* muscle glucose uptake from vehicle (white bar, n=4) or SLU-PP-332 (black bar, n=4) treated mice for 15 days.

Fig 3. SLU-PP-332 increases fatty acid metabolism in a diet-induced obesity mouse model.

Body weight **(a)**, fat mass gain **(b)**, blood lipid profile **(c)** (total cholesterol, HDL, LDL, and TG), and liver enzymes **(d)** of DIO males treated with vehicle (gray bar, n=7) or SLU-PP-332 (black bar, n=7) for 28 days. Mice were kept at thermoneutrality and fed with an HFD. Blood glucose (**e**) and Insulin (**f**) levels of animals treated with vehicle (gray bar, n=7) and SLU-PP-332 (black bar, n=7) under HFD (fed) or after 8 h of fasting (fast). Only fasting insulin levels are shown. **(g-l)** Indirect calorimetry measurement from the same animals treated with vehicle (gray bar, n=7) and SLU-PP-332 (black bar, n=7) during the day (solid bar) and night (shaded bar) over 5 days. Respiratory exchange ratio (RER) **(g)**; Fatty acid oxidation **(h)**; carbohydrate oxidation **(i)**, activity and **(j),** and energy expenditure **(k)**. **(l)** Energy expenditure difference between before and after dosing normalized by food intake and body weight from the same animals. Glucose tolerance test (GTT) **(m)** from the same animals treated with vehicle (gray bar, n=7) and SLU-PP-332 (black bar, n=7) maintained on an HFD. The area under the curve for the GTT is also shown. WAT (**n**) from mice dosed with vehicle (gray bars, n=7) or SLU-PP-332 (black bar, n=7) maintained on an HFD. The bar graph represents the average size of adipocytes (vehicle: white and SLU-PP-332: black bar). Liver from these mice stained with Oil Red O **(o)** and liver triglyceride **(p)** content from mice administered vehicle (gray bars, n=7) or SLU-PP-332 (black bar, n=7) under HFD. **(q)** Liver gene expression from mice under chow diet (solid bar, n=8) or fed with HFD (striped bar, n=7), treated with vehicle (white bar) or SLU-PP-332 (black bar for 28 days.* $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.

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Figure 4. SLU-PP-332 increases fatty acid metabolism and energy expenditure in *ob/ob* mice.

Body weight **(a)**, fat mass **(b)**, liver weight **(c),** and daily food intake **(d)** of three-month-old *ob/ob* male mice treated with vehicle (white bar, n=8) or SLU-PP-332 (black bar, n=8) for 15 days. Mice were maintained at thermoneutrality and fed a chow diet. Results from indirect calorimetry **(e)**, RER **(f)**, fatty acid oxidation **(g)**, carbohydrates oxidation **(h**), resting energy expenditure **(i)** of the same animals treated with vehicle (white bar, n=8), and SLU-PP-332 (black bar, n=8) maintained on an HFD during day and night over 15 days at thermoneutrality. Liver histology **(j)** Hematoxylin and Eosin and Oil Red O (ORO) staining of frozen liver sections from mice treated with vehicle or SLU-PP-332 for 15 days * $p<0.05$.**$p<0.01$, ***$p<0.001$.

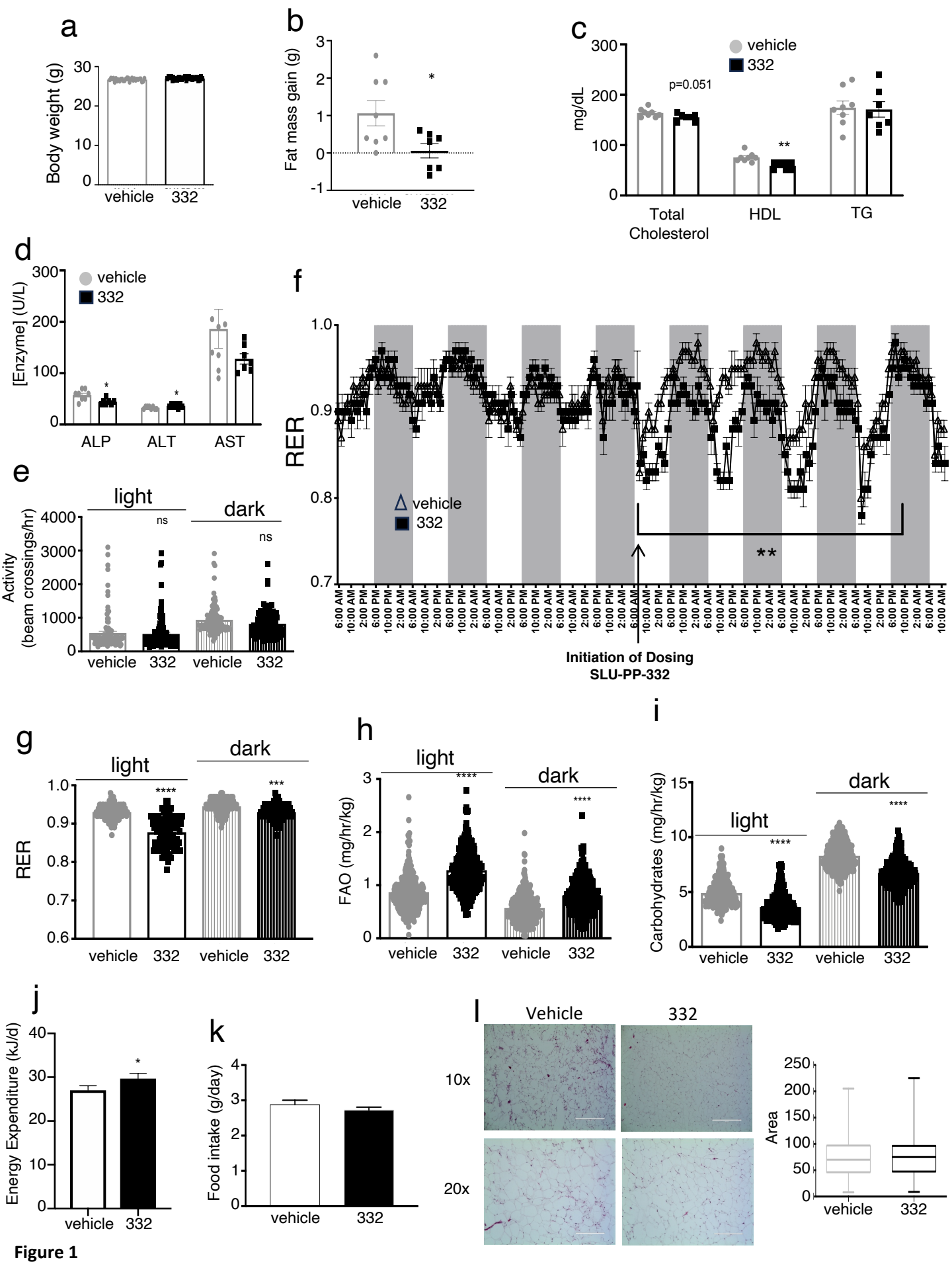
a
Body weight (g)
vehicle
332
b
Fat mass gain (g)
vehicle
332
c
vehicle
332
p=0.051
mg/dL
Total Cholesterol
HDL
TG
d
[Enzyme] (U/L)
vehicle
332
ALP
ALT
AST
e
light
dark
ns
Activity (beam crossings/hr)
vehicle
332
f
RER
vehicle
332
Initiation of Dosing
SLU-PP-332
g
light
dark
RER
h
light
dark
FAO (mg/hr/kg)
i
light
dark
Carbohydrates (mg/hr/kg)
j
Energy Expenditure (kJ/d)
k
Food intake (g/day)
l
Vehicle
332
10x
20x
Area

Figure 1

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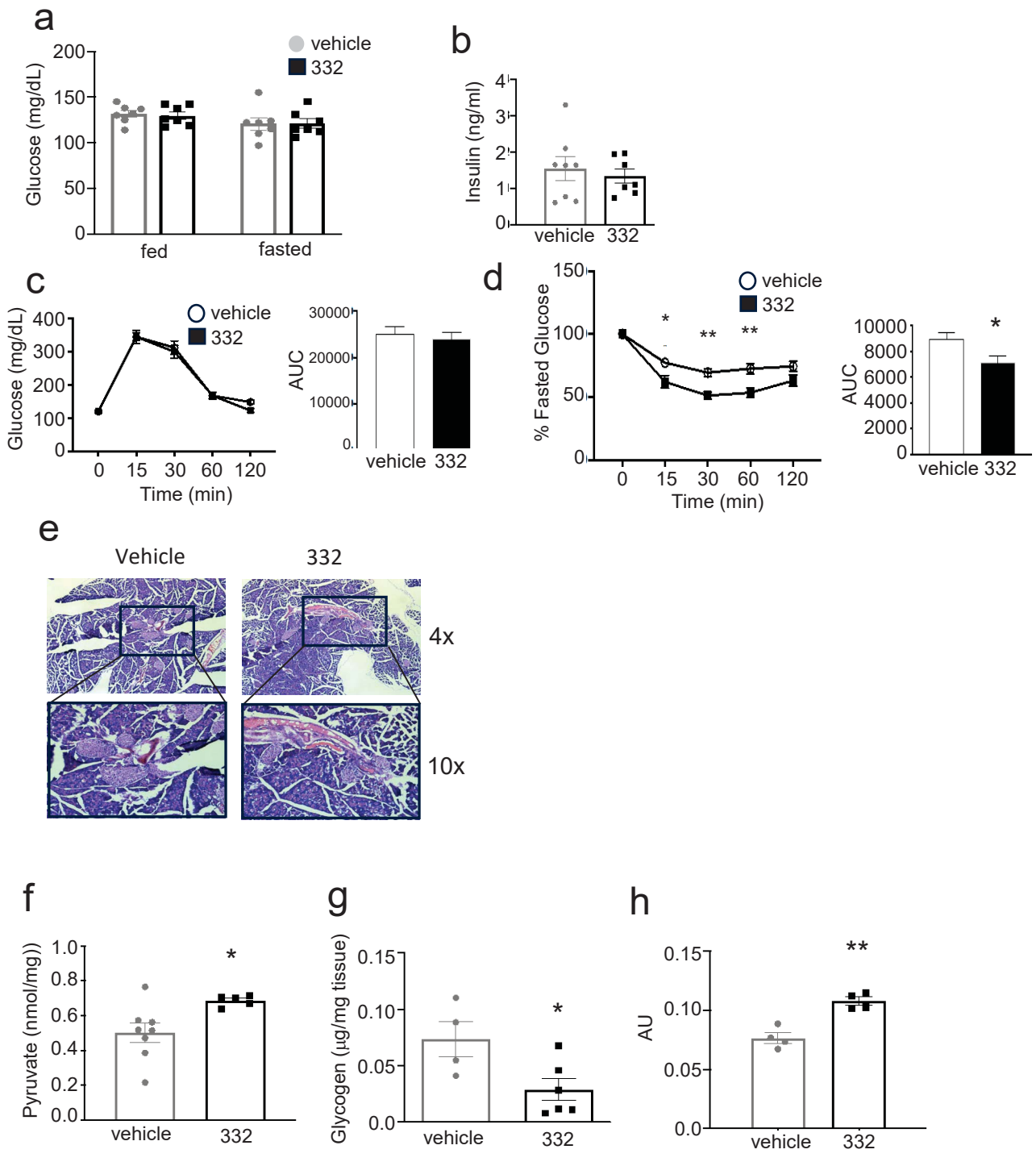

Figure 2

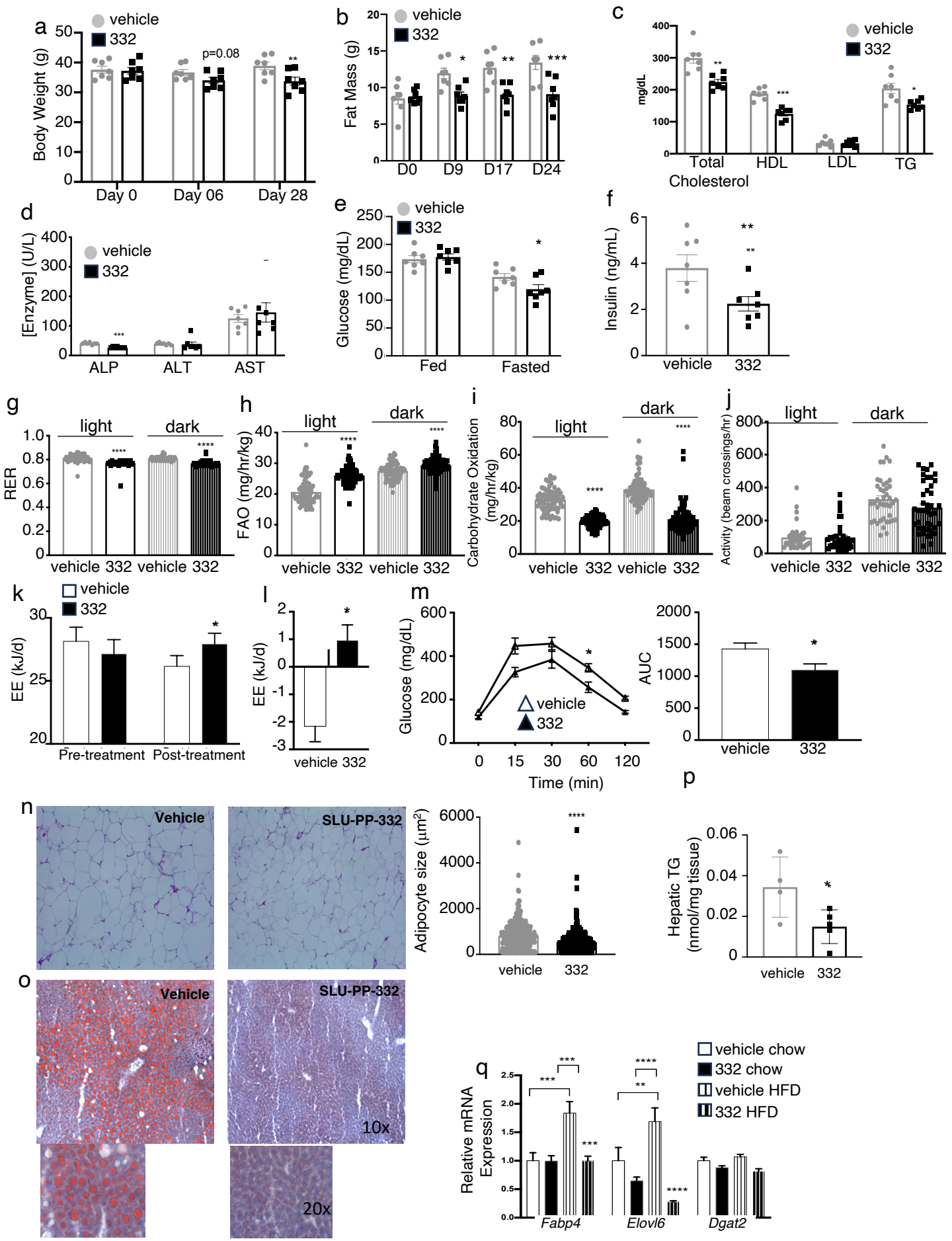
a
vehicle
332
Body Weight (g)
p=0.08
Day 0
Day 06
Day 28
b
Fat Mass (g)
D0
D9
D17
D24
c
mg/dL
Total Cholesterol
HDL
LDL
TG
d
[Enzyme] (U/L)
ALP
ALT
AST
e
Glucose (mg/dL)
Fed
Fasted
f
Insulin (ng/mL)
g
RER
light
dark
h
FAO (mg/hr/kg)
i
Carbohydrate Oxidation (mg/hr/kg)
j
Activity (beam crossings/hr)
k
EE (kJ/d)
Pre-treatment
Post-treatment
l
m
Time (min)
AUC
n
Vehicle
SLU-PP-332
Adipocyte size (μm²)
p
Hepatic TG (nmol/mg tissue)
o
10x
20x
q
Relative mRNA Expression
vehicle chow
332 chow
vehicle HFD
332 HFD
Fabp4
Elovl6
Dgat2

Figure 3

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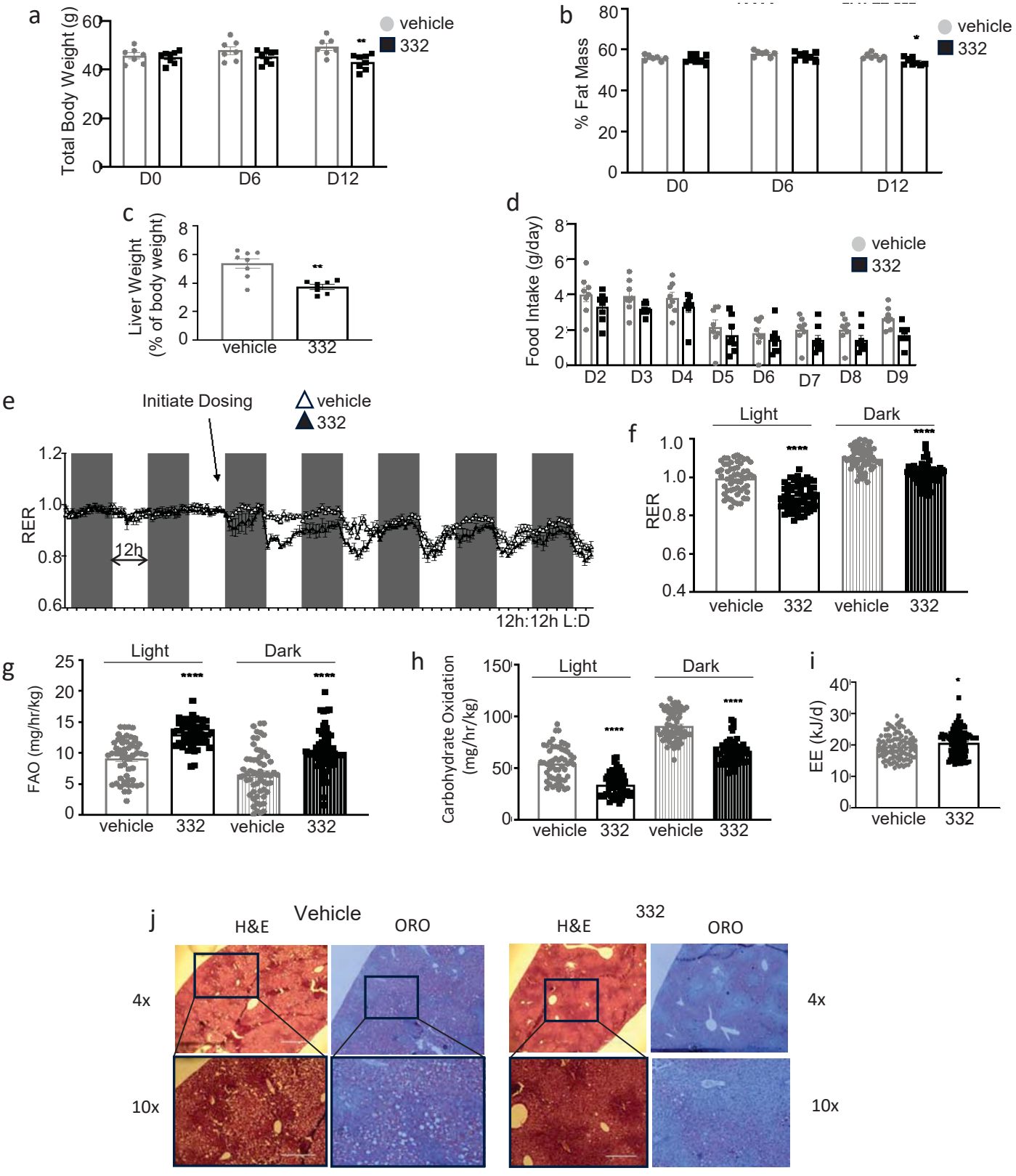

Figure 4