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Aconitate decarboxylase 1 regulates glucose homeostasis and obesity in mice

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Keywords: obesity, metabolism, immunology, glucose control

Running Title: *Acod1 regulates glucose homeostasis and obesity*

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Word Count: 3997

Funding: This study was supported by National Institutes of Health grant F31NS108617 (T.M.V.), by National Institutes of Health grant T32-HL125242, Crohns and Colitis Foundation award 609962 (R.A.F.), and by P30 grants DK020572 (MDRC), DK089503 (MNORC), and 1U2CDK110678-01 (Mi-MMPC).

Disclosures: The authors declared no conflict of interest.

Author Contributions: Conceived and designed the study (RAF, RMM), acquired data (RAF, TMV, JS, CL), analyzed data and constructed figures (RAF, CNL), wrote the manuscript (RAF), interpreted results (DRG, CNL, RAF, RMM).

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/oby.23509

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STUDY IMPORTANCE

What is already known?

- Chronic inflammation is an important contributor in the pathophysiology of obesity and associated insulin resistance and type 2 diabetes mellitus
- The metabolite, itaconate, has important immunoregulatory and suppressive effects on inflammation

What does this study add?

- Acod1 is expressed in adipose tissue during inflammation and obesity
- Acod1 is an important regulator of glucose homeostasis during obesity
- Acod1 is critical for maintenance of body weight and glucose control with aging

How might these results change the direction of research or the focus of clinical practice?

- Regulation of immunometabolism through Acod1-itaconate action is critical to our understanding of obesity and metabolic dysfunction and could be an important therapeutic approach

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ABSTRACT

Objective
The intersection between immunology and metabolism contributes to the pathogenesis of obesity-associated metabolic diseases as well as molecular control of inflammatory responses. The metabolite, itaconate and cell permeable derivatives have robust anti-inflammatory effects, therefore we hypothesize that Acod1-produced itaconate has a protective, anti-inflammatory effect during diet-induced obesity and metabolic disease.

Methods
Wild type and $Acod1^{-/-}$ mice were subjected to diet induced obesity. Glucose metabolism was analyzed by GTT, ITT, and indirect calorimetry. Gene expression and transcriptome analysis were performed using qRT-PCR and RNA-Seq.

Results
WT and $Acod1^{-/-}$ mice on HF diet had equivalent weight gain, but $Acod1^{-/-}$ mice had impaired glucose metabolism. ITT and GTT at 12 weeks on high fat diet revealed significantly higher blood glucose levels in $Acod1^{-/-}$ mice. This was associated with significant enrichment of inflammatory gene sets and a reduction in genes related to adipogenesis and fatty acid metabolism. Analysis of naïve $Acod1^{-/-}$ mice showed a significant increase in fat deposition at 3 and 6 months age and obesity and insulin resistance by 12 months.

Conclusions
Our data show that Acod1 has an important role in the regulation glucose homeostasis and obesity under normal and high fat diet conditions that worsens significantly with age.

Keywords: diabetes, obesity, inflammation, immunometabolism, itaconate

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1. INTRODUCTION

The obesity epidemic represents a major challenge to public health because of its role in the development of type 2 diabetes mellitus and its association with adverse health consequences. Chronic, low-grade systemic inflammation is a widely recognized aspect of obesity and is a contributing factor in the development of insulin resistance and other associated co-morbidities. Adipose tissue is one of the major metabolic tissues driving systemic inflammation during obesity, and this is largely due to a significant influx of inflammatory cells as well as activation of resident immune cells. Adipose tissue macrophages are present in lean, visceral white adipose tissue depots, but are further recruited and undergo activation and proinflammatory signaling in response to excessive adipose tissue expansion during obesity (1). This occurs in coordination with a network of other innate and adaptive immune cells, and this proinflammatory response impairs adipocyte function and contributes to insulin resistance.

Metabolic regulation and immune responses during obesity and adipose tissue inflammation are critically intertwined. There is a body of literature demonstrating that proinflammatory cytokines and chemokines can promote inflammation and induce both localized and systemic insulin resistance and metabolic dysfunction (2, 3). Nutrient metabolism and intracellular metabolic pathways also have critical roles in governing immune cell function and metabolic responses in non-immune cells (4). Metabolism, particularly mitochondrial metabolism, and the production of metabolites has been shown to be critical in regulating inflammatory signaling and macrophage phenotype (5). Itaconate is a TCA-cycle derived metabolite produced by the enzyme cis-aconitate decarboxylase 1 (Acod1/Irg1), and Acod1 and itaconate are among the most highly upregulated genes and metabolites during proinflammatory macrophage activation (6). Itaconate has been shown to have anti-inflammatory and anti-oxidative effects in vitro in macrophages through multiple mechanisms including succinate dehydrogenase (SDH) inhibition, induction of ATF3 and Nrf2, and inhibition of NLRP3 inflammasome activity (6-11).

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Augmentation of itaconate signaling has also been shown to be effective in mitigating inflammation in various animal models of disease. The cell-permeable itaconate derivatives, dimethyl itaconate (DMI) and 4-octyl itaconate (4-OI) have been employed as a strategy to target immunometabolism to regulate hypoxia and inflammation in disease. Itaconate has been shown to decrease oxidative phosphorylation and ROS production by both diverting cycle intermediates towards itaconate production and by inhibiting SDH. Itaconate derivatives have been shown to be effective in mitigating injury during hypoxia responses such as cardiac ischemia (6), cerebral ischemia (12, 13), and experimental colitis (14), Hypoxia and oxidative responses mediated by HIF1α and ROS are also thought to play a role in the pathophysiology of adipose dysfunction and insulin resistance during obesity (15, 16). In addition, itaconate derivatives are effective in a wide array of inflammatory and autoimmune models of disease (17-25).

Although Acod1 and itaconate have been the focus of many studies on immune metabolism and immunoregulation, none have addressed the in vivo role in glucose metabolism, obesity or diabetes mellitus. Because of the anti-inflammatory effects of itaconate in immune cells, we hypothesized that Acod1/itaconate would have a beneficial effect on adiposity and metabolic dysfunction during DIO by dampening adipose tissue inflammatory responses. In the present study, we examined the role of endogenous itaconate in the development of obesity and diabetes using Acod1 deficient mice as a model of itaconate deficiency in the context of diet-induced obesity. Here we defined the effects of Acod1 knockout on adiposity and glucose homeostasis in both obese and aging mice.

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2. METHODS

2.1 Animals

Male Acod1$^{-/-}$ mice (Acod1$^{tm1a(KOMP)Wtsi}$) and littermate controls were on a C57BL/6N background, and myeloid Acod1$^{-/-}$ (MyAcod1$^{-/-}$: Acod1$^{fl/fl}$-*LysM*-Cre) and floxed controls were on a C57BL/6J background (6, 26). Mice were multi-housed in static cages in a temperature controlled SPF room (21-23°C) with a light:dark cycle of 12:12 h (lights on at 6 A.M.). Mice were maintained on standard laboratory chow (5L0D, LabDiet) or high fat diet (60 kcal% fat, [D12492, Research Diets]) and water ad libitum. Estimation of required sample size for all experiments were based on A priori power analysis calculations using expected standard deviations from previous experiments. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

2.2 Food consumption

Food intake was repeatedly measured in both standard static cages and metabolic cages over 2-3 day periods.

2.3 LPS-induced systemic inflammation

See supplemental methods.

2.4 Transcriptomic analysis

Total RNA was extracted from tissues using TRIzol reagent and purified using an RNeasy Mini Kit (Qiagen) with an on-column DNase digestion. RNA sequencing was performed by the University of Michigan Advanced Genomics Core, using the Lexogen QuantSeq library prep and sequenced on an Illumina NextSeq system (~10 million raw reads per sample). Data were trimmed using TrimGalore (v 0.5.0) and Aligned using STAR (v 2.6.0). Differential expression analysis was performed using the DESeq2 package in R.

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2.5 Body composition

See supplemental methods.

2.6 Glucose and insulin tolerance tests

For glucose tolerance tests, mice were fasted for 6 h (5:00 A.M.–11:00 A.M.) before receiving an intraperitoneal injection of glucose. Glucose dose (1.25 mg/g lean mass) was determined from lean body mass to avoid confounding effects from obesity.(27, 28) Blood glucose was measured at 0, 15, 30, 60, and 120 min after glucose injection using a Contour (Bayer) glucometer. For insulin measurements, plasma was collected at 0, 30 and 60 min after glucose injection and plasma insulin was assayed using an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem). For insulin tolerance tests, mice were injected with insulin (0.5 U/kg lean mass) (Humulin R; Lilly) and blood glucose was measured at 0, 15, 30, 60, and 120 min.

2.7 Gene expression analysis

Relative mRNA expression was determined using quantitative reverse transcription–polymerase chain reaction. Total RNA was extracted from tissues using TRIzol reagent and purified using an RNeasy Mini Kit (Qiagen) with an on-column DNase digestion. RNA (1 ug) was reverse transcribed to cDNA with an Applied Biosystems kit and quantitative reverse transcription–polymerase chain reaction was performed using a 7900HT fast real-time PCR system (Applied Biosystems). The relative mRNA expression was quantified by the comparative method and normalized to the housekeeping gene L32.

2.8 Flow cytometry

See supplemental methods.

2.9 Indirect calorimetry

See supplemental methods.

2.10 3T3-L1 adipocyte culture

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3T3-L1 cells were cultured to confluency and treated with preadipocyte expansion media (DMEM + 10% calf serum). After 48 h, cells were then cultured in differentiation media (DMEM + 10% FBS, 1.0 μM dexamethasone, 0.5 mM methylisobutylxanthine [IBMX], 1.0 ug/mL bovine insulin) for 2 days and then cultured in adipocyte maintenance media (DMEM + 10% FBS, 1.0 ug/mL bovine insulin) for 10 days. Differentiated 3T3-L1 adipocyte cultures were then pretreated with dimethyl itaconate (125 or 250 μM) and 4-ocytyl itaconate (250 μM) for 18 h and then stimulated with LPS (100 ng/mL) for 3 h, or palmitic acid (PA, 0.5 mM) or vehicle for 24 h. The PA-FFA-BSA conjugate (6:1 molar ratio PA:BSA) was made by dissolving PA in ethanol and then combining with FFA-BSA dissolved in DMEM. The PA:FFA-BSA mixture was sonicated to promote solvation of the PA:FFA-BSA conjugate.

2.11 Statistical analysis

A Shapiro-Wilk normality test was used to determine if data were normally distributed. For normally distributed data with equal variance, values are presented as mean ± SEM, and statistical comparison of mean values between multiple groups was performed by Student's t test, one-way ANOVA with a Tukey's post-test, or two-way ANOVA with a Tukey's post-test as indicated in the text. For normally distributed data with unequal variance, a Welch's t test was used. Data that were not normally distributed were analyzed with the nonparametric Mann-Whitney test. All statistical analysis of data was performed in GraphPad Prism (version 7; GraphPad Software, Inc). $P < 0.05$ was considered significant.

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3. RESULTS

3.1 Acod1 expression is increased during DIO but does not affect weight gain or fat deposition

To delineate the role of Acod1/itaconate during DIO, we used Acod1$^{-/-}$ mice from KOMP which employ the tm1a cassette to produce global gene inactivation. We confirmed using BMDMs a total inhibition of LPS-induced Acod1 expression in Acod1$^{-/-}$ mice (Figure 1A). Systemic inflammation by LPS treatment showed significant induction of Acod1 expression in mouse fat depots (Figure 1B). Similarly, mice on high fat diet also had significantly elevated expression of Acod1 in gWAT (Figure 1C). We detected robust induction of Acod1 expression in various tissues after induction of systemic inflammation with LPS (Figure S1A). Induction of itaconate production in high expressing tissues (liver and spleen), and deficiency in Acod1$^{-/-}$ mice, was also confirmed (Figure S1B).

To determine the role of Acod1 in the pathogenesis of diet-induced obesity and metabolic disease, WT and Acod1$^{-/-}$ mice were placed on a HF diet for 12 wk. WT and Acod1$^{-/-}$ mice gained weight at an equivalent rate and displayed similar levels of food consumption (Figure 1D-E). Although body weights of Acod1$^{-/-}$ mice were marginally greater at the end of the study (Figure 1E), this was due to a higher baseline body weight prior to administration of HF diet (discussed below). Both fat mass and lean body mass were similar between WT and Acod1$^{-/-}$ mice on HF diet (Figure 1F). Subsequent analysis of major WAT depots revealed a similar degree of gWAT and iWAT expansion in WT and Acod1$^{-/-}$ mice on HF diet (Figure 1G), although Acod1$^{-/-}$ mice had a significant increase in BAT depot compared to WT mice.

3.2 Acod1 is important in maintaining glucose homeostasis and insulin sensitivity during obesity.

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To assess whether disruption of Acod1 and itaconate function are involved in glucose metabolism during obesity, we performed GTT and ITT on WT and Acod1$^{-/-}$ mice. After 2 wk. on HF diet, no differences were detected between WT and Acod1$^{-/-}$ mice on HF diet (Figure 2A). By week 12, Acod1$^{-/-}$ mice had significantly elevated blood glucose levels during GTT compared to WT controls (Figure 2A). Acod1$^{-/-}$ mice also displayed signs of insulin resistance during ITT where they showed increased glucose levels with significant impairment of glucose disposal (Figure 2B). No statistically significant differences were detected in fasting glucose or insulin, although the mean fasting insulin levels were greater and suggestive of insulin impairment (Figure 2C).

3.3 Acod1$^{-/-}$ mice have increased inflammatory gene expression in adipose tissue

Histological analysis of adipose tissue after high fat diet did not reveal any structural or morphological differences between WT and Acod1$^{-/-}$ mice (Figure 3A). Since itaconate has anti-inflammatory effects, and Acod1 is largely upregulated in inflammatory macrophages, we analyzed the expression of inflammatory genes in adipose tissue by qRT-PCR. Acod1 deficiency selectively regulated inflammatory gene expression during DIO. We detected increases in the expression of the chemokine MCP1/CCL2 and IL1RA, but not other inflammatory cytokines such as TNFα or IL6 (Figure 3B). Acod1$^{-/-}$ mice also had a significant decrease in the insulin-sensitizing adipokine adiponectin.

We next perform RNA sequencing on eWAT from HFD fed Acod1$^{-/-}$ and WT mice and identified 1033 differentially expressed (DE) genes. Gene set enrichment analysis (GSEA) analysis showed that Acod1$^{-/-}$ mice had significant enrichment of inflammatory gene sets related to B cell activation, lymphocyte activation, interferon responses, and myeloid responses (Fig 3 C-E). Enrichment of B cell related genes in the Acod1$^{-/-}$ mice was prominent and supported by enrichment analysis in the Immgen database. WT mice had increased expression of adipogenic (Adipoq, Cd36) and fatty acid (FA) catabolic genes (Lpin1, Acat1) suggesting Acod1 may play a

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role in enhancing FA metabolism and limiting fat expansion. Although there was significant enrichment of inflammatory gene sets by transcriptomic analysis, we did not detect any differences in the percentage of circulating or infiltrated immune cells by flow cytometry (Figure S2), suggesting a potential change in functional phenotype rather than through leukocyte trafficking.

3.4 Adipocytes express Acod1 and have attenuated inflammatory signaling with itaconate treatment

Most reports suggest Acod1 expression is mainly from macrophages, but there is accumulating evidence for Acod1 induction in non-macrophage cell types. Injection of mice with LPS showed attenuation of Acod1 expression in myeloid Acod1 KO mice (LysM-Cre) in spleen, liver, and eWAT, however, Acod1 induction was not completely lost suggesting activation of Acod1 in LysM negative cells (Figure S3A). Stratification of eWAT and iWAT after LPS injection showed that myeloid Acod1 KO mice have a significant decrease in Acod1 induction in the adipose stromal-vascular fraction (SVF) especially compared to whole adipose tissue. This suggests that adipocyte Acod1 contributes significantly to whole tissue Acod1 expression and that there is significant retention of Acod1 expression in the SVF even after deletion from macrophages, consistent with non-myeloid sources for Acod1-itaconate in adipose tissue (Figure S3B)

To further assess the possibility that itaconate has a role in regulating adipocyte function, we cultured 3T3-L1 adipocytes and stimulated them with LPS. Acod1 expression was significantly upregulated with the addition of LPS (Figure 4A). This is consistent with a previous report showing that treatment of 3T3-L1 adipocytes with TNFα can induce Acod1expression (29). To evaluate if the cell permeable itaconate derivatives 4-OI and DMI (Figure 4B) can influence adipocyte differentiation, 3T3-L1 cells were differentiated with or without the itaconate analogues however no differences in cell proliferation or morphology were observed (Figure 4C-

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D). To further evaluate the effect of itaconate derivatives on inflammation, 3T3-L1 adipocytes were treated with LPS in the presence or absence of 4-OI or DMI. Both 4-OI and DMI significantly suppressed LPS-induced MCP1 and IL-6 expression (Figure 4E). The Nrf2 dependent gene Nqo1 (NAD(P)H Quinone Dehydrogenase 1) was also induced by DMI and 4-OI in adipocytes suggesting Nrf2 dependent suppression of inflammatory genes in adipocytes.

Palmitic acid is one of the most abundant circulating free fatty acids in serum and promotes inflammation and insulin resistance in tissues. Similar to LPS stimulation, treatment of 3T3-L1 adipocytes with palmitic acid resulted in a modest increase in Acod1 mRNA expression (Figure 4F). We also evaluated the effects of DMI and found that pretreatment with DMI (125 μM) significantly suppressed palmitic acid-induced inflammatory gene expression (Figure 4G).

3.5 Acod1$^{-/-}$ mice develop spontaneous obesity, glucose intolerance, and adipose tissue inflammation with age

While analyzing multiple cohorts of mice, we observed that the body weights of the male Acod1$^{-/-}$ mice were significantly larger than littermate WT control mice. Body weights began to diverge at 6 months of age and 12 month old Acod1$^{-/-}$ mice were markedly larger in body weight compared to WT controls (Figure 5A). The difference in body weight was due to increased fat mass in Acod1$^{-/-}$ mice based on body composition analysis (MRI) and weight of fat depots (Figure 5B). Analysis of glucose metabolism by GTT and ITT revealed that old Acod1$^{-/-}$ mice were glucose intolerant and insulin resistant compared to age matched WT controls (Figure 5C-D). This was associated with significantly increased adipocyte size in eWAT from 12mo old Acod1$^{-/-}$ mice (Figure 5E). Further analysis of Acod1$^{-/-}$ mice revealed a significant increase in both cardiac and kidney mass (Figure S4A-B). Cardiac hypertrophy in Acod1$^{-/-}$ mice was associated with increased cardiomyocyte size (Figure S4C).

RNA sequencing was performed on eWAT from 12 month old mice and PCA analysis demonstrated that the old Acod1$^{-/-}$ mice gene profiles clustered with HFD fed mice (Figure 6A).

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Compared to age matched controls, old Acod1$^{-/-}$ mice had significant enrichment of inflammatory genes related to macrophage activation (Itgax, Trem2, Spp1), lymphocyte activation, and genes in the Chen Metabolic Syndrome Network (Figure 6B-C).19 Similar to DIO mice, old Acod1$^{-/-}$ mice had increased B cell activation genes (Figure 6D) These data demonstrate that Acod1$^{-/-}$ mice develop obesity with age that leads to features of unhealthy WAT expansion and exaggerated myeloid inflammation.

3.6 Acod1$^{-/-}$ mice have increased fatty acid (FA) synthetic gene expression in adipose tissue To better understand the mechanisms of the increased weight gain in the 12 month old Acod1$^{-/-}$ mice, we used CLAMS to evaluate an independent cohort of weight matched 3 month old mice before body weight between genotypes diverged. During the CLAMS, Acod1$^{-/-}$ mice had increased food intake and increased resting energy expenditure per mouse that disappeared when normalized for lean body mass (Figure 7A-B). Activity and RER were not different between KO and WT mice. Assessment of substrate utilization demonstrated a decrease in fat oxidation and an increase in glucose oxidation in Acod1$^{-/-}$ mice compared to controls (Figure 7C). RNAseq was performed in eWAT from weight matched 3 month old chow fed Acod1$^{-/-}$ and WT mice and 1279 DE genes were identified (Figure 7D-E). eWAT from Acod1$^{-/-}$ mice had a significant increase in the expression of pathways related to adipogenesis, lipid biosynthesis, and FA synthesis (Acly, Fasn, Slc27a1, Acbp).

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4. DISCUSSION

The intersection between immune and metabolic responses is a widely recognized feature of metabolic dysfunction that occurs during both obesity and aging. Although many proinflammatory pathway which contribute to adipose tissue inflammation and insulin resistance have been identified, many of the intracellular metabolic pathways that govern immune cell function and metabolism are largely undefined.

In this study, we investigated the role of the anti-inflammatory, TCA-cycle metabolite, itaconate, during the pathogenesis of obesity and metabolic dysfunction. Through disruption of itaconate production by Acod1 deficiency, we have identified that Acod1/itaconate has a critical role in glucose homeostasis and adiposity during high fat diet and aging. We have shown that Acod1$^{-/-}$ mice have impaired glucose homeostasis during obesity, and this is associated with increased inflammatory signaling in visceral adipose tissue. Naïve Acod1$^{-/-}$ mice also developed obesity, insulin resistance, and enhanced inflammatory signatures with age, highlighting an important endogenous, immunometabolic mechanism that protects against obesity and glucose dysregulation. To our knowledge, this is the first investigation to link Acod1 and itaconate to glucose metabolism and obesity.

Although global deletion of Acod1 did not have any effect on weight gain during DIO, it did result in increased glucose intolerance and insulin resistance. Acod1$^{-/-}$ and itaconate deficiency was also associated with increased inflammation in Acod1$^{-/-}$ mice as determined by gene expression and transcriptomic analysis using qPCR and RNA-Seq. These findings are consistent with the hypothesis that Acod1 has a protective, anti-inflammatory effect during DIO through suppression of the immune response. This is also consistent with reported in vitro and in vivo studies using both genetic (Acod1$^{-/-}$) and pharmacological (itaconate derivatives: DMI, 4-OI) approaches that have shown itaconate has potent anti-inflammatory effects. Mills and colleagues found that 4-OI effectively suppressed inflammatory cytokines (Il-1β and Tnfα) in

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serum after induction of systemic inflammation (8), therefore it will also be important to further investigate whether Acod1/itaconate affects chronic systemic inflammation in our DIO model.

Acod1 is known to be most highly upregulated in macrophages, and adipose tissue macrophages are major regulators of adipose tissue inflammation. Our data show significant enrichment in inflammatory gene sets involving innate immune response, myeloid leukocyte migration, and interferon signaling and response pathways. There was also enrichment of inflammatory gene sets related to lymphocyte and B-cell activation. Although there are limited reports of Acod1 effects outside of activated macrophages, there is a growing body of literature implicating B-cells in the pathogenesis of obesity and aging (30-33). Similar to obesity, aging is also associated with chronic inflammation with B-cell infiltration that drives insulin resistance and metabolic dysfunction. This would be consistent with our findings showing significant enrichment of B-cell related genes in $Acod1^{-/-}$ mice with both DIO and aging.

Further analysis using cell-specific ablation models will be critical to delineate critical cell types producing and responding to itaconate. In addition to the potential role of immune cells within adipose tissue, skeletal muscle and liver are also critically involved in regulation of glucose homeostasis. In skeletal muscle cells, dimethyl itaconate was found to mitigate palmitate-induced insulin resistance through suppression of inflammation (34). In our studies, palmitic acid resulted in a modest, but significant upregulation of Acod1 expression, and DMI was able to effectively suppress palmitic acid-induced inflammatory gene expression. Futures studies will be important to test whether itaconate can mitigate palmitic acid-induced insulin resistance through regulation of inflammatory signaling. Itaconate derivatives have been shown to suppress liver inflammation in models of disease (35-38), and it is unknown whether this has any impact on glucose homeostasis. Our data in 3T3-L1 cells also implicates a possible role for Acod1 in adipocytes as either a potential producer of itaconate or as an effector cell. Overall, our results suggest a novel role for Acod1 in immune cells with evidence suggestive of innate immune cells and B cells as effectors or primary responders to the action of Acod1-itaconate.

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Acod1$^{-/-}$ mice had alterations in energy substrate utilization with a decrease in fatty acid oxidation and increase in glucose oxidation. These observations suggest that loss of Acod1/itaconate relieves inhibition of glycolysis and promotes glucose oxidation at the expense of fat oxidation. Changes in substrate utilization were associated with increases in genes related to adipogenesis, lipid biosynthesis, and fatty acid synthesis. This suggests a tonic role for Acod1 limiting fatty acid deposition in adipose tissue under chow diet conditions as a potential mechanism for the age induced obesity. Thus, in lean mice, tonic Acod1 expression and itaconate production protects against age-induced obesity by counteracting lipid deposition in adipocytes either by promoting lipolysis, suppressing lipogenesis, or promoting adipogenesis. With DIO or aging, the expression of Acod1 in myeloid cells, B cells, and/or adipocytes and other non-immune cell types limits the inflammatory response in adipose tissue and protects against insulin resistance.

The various mechanisms by which itaconate exerts its anti-inflammatory effects are thought to have a role in dampening immune activation to limit host tissue damage. Although Acod1 is increased in adipose tissue during DIO and has an important role in controlling insulin resistance, this response is not able to adequately suppress inflammation or modulate other potentially direct mechanisms (i.e. – adipocyte function) to maintain insulin sensitivity. While it is important to have mechanisms to negatively regulate immune cell activation, these endogenous mechanisms are not sufficient to inhibit inflammation and compensate for the excess lipid and fatty acid alterations that result in chronic inflammation and adipose tissue dysfunction. However, the anti-inflammatory mechanisms of endogenous itaconate can be potentially exploited with administration of exogenous itaconate derivatives to achieve a therapeutic effect.

LIMITATIONS OF THE STUDY AND FUTURE DIRECTIONS:

We have identified a new connection between immunometabolism and metabolic dysfunction, but it remains to be determined whether this pathway can be manipulated to protect

against disease. Numerous studies have employed the use of cell permeable itaconate derivatives (DMI and 4-OI) to augment itaconate signaling and modulate and suppress inflammation during various models of disease. Future studies will be focused on determining if supplementation with exogenous itaconate derivatives can suppress inflammation and mitigate metabolic dysfunction. However, these derivatives are esterified to enhance cell permeability, and they do not fully recapitulate the endogenous effects of itaconate as they do not get metabolized to itaconate. Therefore, while beneficial and potentially useful as a therapeutic agent, it is still critical to understand the role of endogenous itaconate signaling using genetic tools to manipulate the metabolic system.

As discussed above, it will also be important to delineate the itaconate-producing and itaconate-responsive cell types during DIO and insulin resistance. Acod1-itaconate effects may be a result of intracellular action or could reflect a more complex intercellular communication involving itaconate secretion and uptake. Further analysis of isolated cell types to define changes in both Acod1 and itaconate during aging and DIO will also be important for delineating the critical cell types regulating these physiological and pathophysiological processes. Although itaconate production is the only known function of Acod1, it will be important to confirm the role of itaconate in this Acod1 deficiency model by correlating phenotypic changes with alterations in itaconate levels.

SUMMARY

In summary, our data advance our understanding of the Acod1/itaconate pathway in immunometabolism and show that it protects against metabolic dysfunction in DIO and age. This is critical to our understanding of the pathogenesis of metabolic dysfunction that occurs with obesity and aging, because while many of the pro-inflammatory mechanisms are well defined, we know very little about anti-inflammatory, immunometabolism pathways that can be targeted for therapeutic intervention. Attenuating low-grade, systemic inflammation during

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obesity through modulation of immunometabolism may have therapeutic potential to regulate glucose homeostasis and prevent metabolic dysfunction.

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ACKNOWLEDGEMENTS

We would like to thank Dr. Michael Diamond (Washington University School of Medicine, St. Louis, MO) for sharing the Acod1 knockout mice that were used in this study.

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FIGURE LEGEND

Figure 1. Acod1 is increased in adipose tissue during inflammation and obesity. (A) tm1a construct from KOMP used to generate Acod1$^{-/-}$ mice. BMDMs from Acod1$^{-/-}$ mice treated with LPS show efficient knockout. Effect of LPS (B) and HFD (C) on Acod1 expression by qPCR in adipose tissue. (D) Acod1$^{-/-}$ mice on HFD gain the same amount of weight as WT controls. (E) Body weight after 12 wk. on high fat diet, and food consumption. (F) Total fat mass and lean mass measured by MRI at 12 wk. on HF diet (G) Fat depot mass at 12 wk. on HF diet. N = 4-10. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 2. Acod1 deficiency impairs glucose metabolism during obesity. (A) GTT at 2 and 12 wk. on HFD and AUC for 12 wk GTT data (B) ITT at 12 wk. on HFD and AUC for non-normalized ITT data (C) Fasted blood glucose and insulin levels. N = 6-11. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 3. Increased inflammatory gene expression in eWAT of obese Acod1$^{-/-}$ mice. (A) eWAT histology at 12 wk. on HFD. (B) qRT-PCR analysis of inflammatory genes from eWAT. RNA seq (Quantseq) was performed on eWAT from DIO WT and Acod1$^{-/-}$ mice. (C) Volcano plots of DE genes. Inflammatory (black) and Interferon responsive (blue) genes are highlighted. (D) Enriched pathways in HFD eWAT (GSEA analysis; all padj<0.05). (E) Heatmaps of B cell and FA metabolic genes. (F) GSEA of IFNA dependent genes. N = 4-6. *P < 0.05, **P < 0.01.

Figure 4. 3T3-L1 adipocytes express Acod1 and have attenuated inflammatory signaling with itaconate treatment. (A) Acod1 expression in LPS treated 3T3-L1 adipocytes. (B) Structure of itaconate and cell permeable derivatives. (C) Cell proliferation of 3T3-L1 adipocytes measured by MTT assay after adipocyte differentiation. (D) Photomicrograph of 3T3-L1 cells pre and post-differentiation. (E) mRNA gene expression of MCP1, IL-6 and Nqo1 from 3T3-L1 adipocytes treated with LPS in the presence and absence of itaconate derivatives. (F) Acod1 expression in 3T3-L1 adipocytes after 24 h palmitic acid (PA) treatment (G) mRNA gene expression of inflammatory genes in 3T3-L1 adipocytes treated with palmitic acid after 12 h pretreatment with DMI. n = 3-6 per group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5. Acod1$^{-/-}$ mice develop spontaneous obesity and impaired glucose metabolism with age. (A) Body weight time course in WT and Acod1$^{-/-}$ mice (B) Time course of eWAT, iWAT, and BAT fat pad mass (C) GTT and (D) ITT in naïve 12 mo. Acod1$^{-/-}$ mice and AUC of non-normalized ITT data (E) Adipocyte size in 12 mo. Acod1-/- mice. N = 4-7 per group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 6. Old Acod1$^{-/-}$ mice have increased inflammatory gene expression. RNA seq of eWAT from 12 mo. old mice. (A) PCA analysis of ND, HFD, and old WT and Acod1-/- mice. (B) Volcano plot of DE genes between 12 mo. old WT and Acod1-/- mice. (C) Most enriched pathways from DE genes (GSEA) in 12 mo. old WT and Acod1-/- mice. (padj<0.05). (D) B cell activation module (all padj<0.05). N = 3-4 per group.

Figure 7. Non-obese Acod1$^{-/-}$ mice have altered energy substrate utilization. 3 mo. old chow diet fed weight matched WT and *Acod1*$^{-/-}$ mice analyzed by (A) CLAMS for food intake, (B) energy expenditure, (C) fat, and glucose oxidation. (D) Enriched pathways in chow fed WT and *Acod1*$^{-/-}$ mice (GSEA; padj<0.05). (E) Heatmap of DE genes related to FA synthesis. N = 5 per group. *P < 0.05.

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Figure 1.

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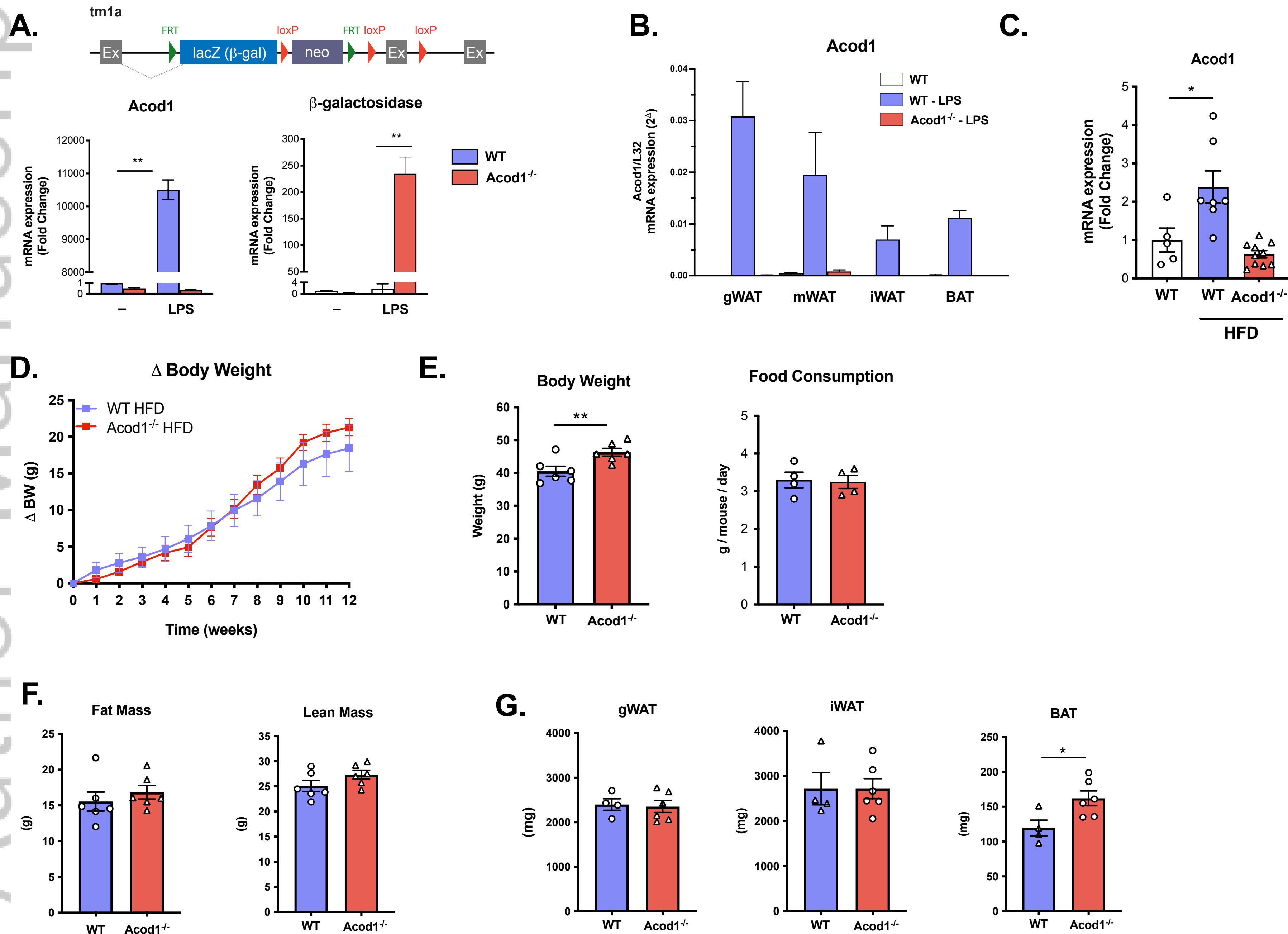

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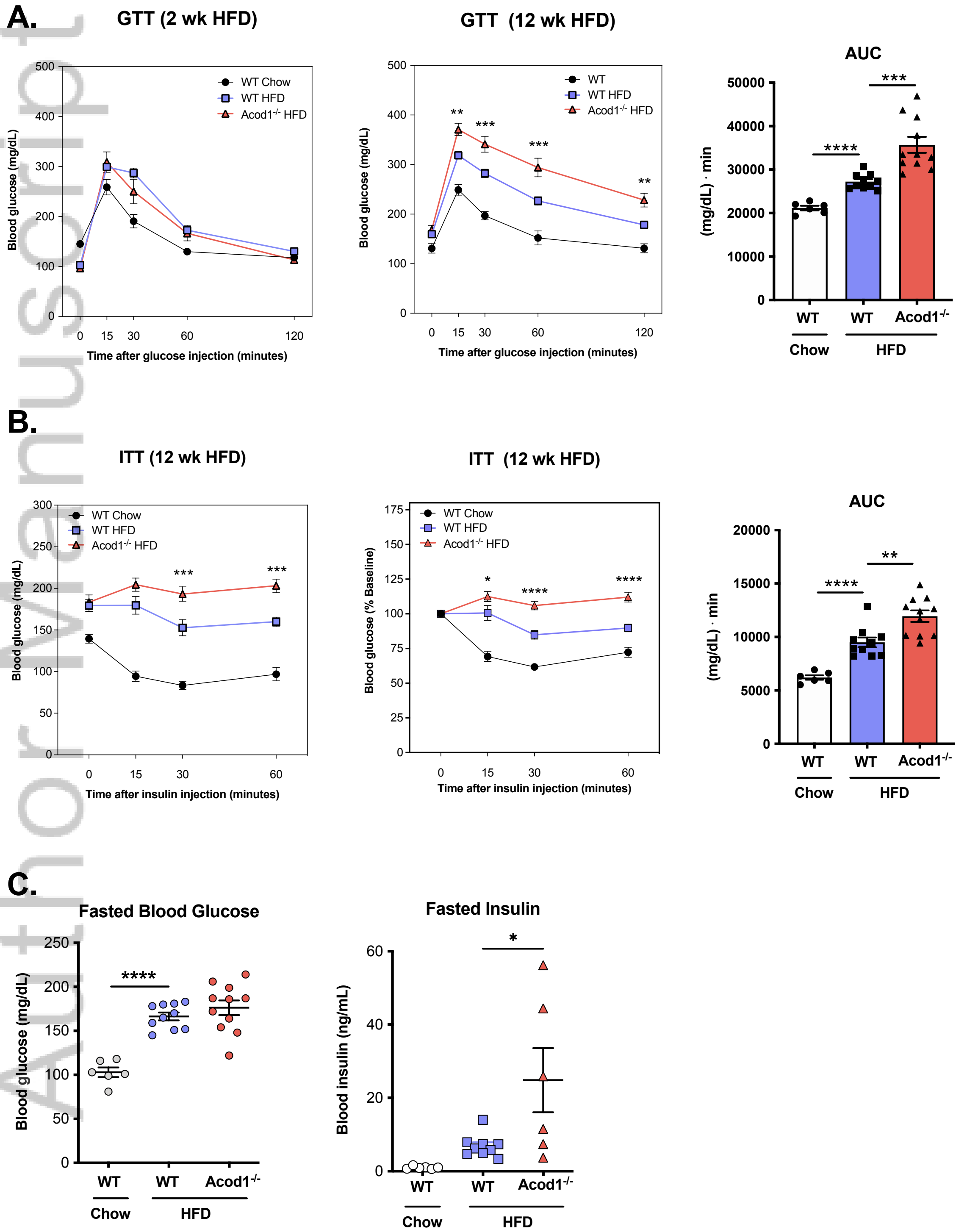

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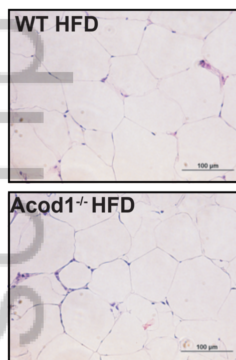

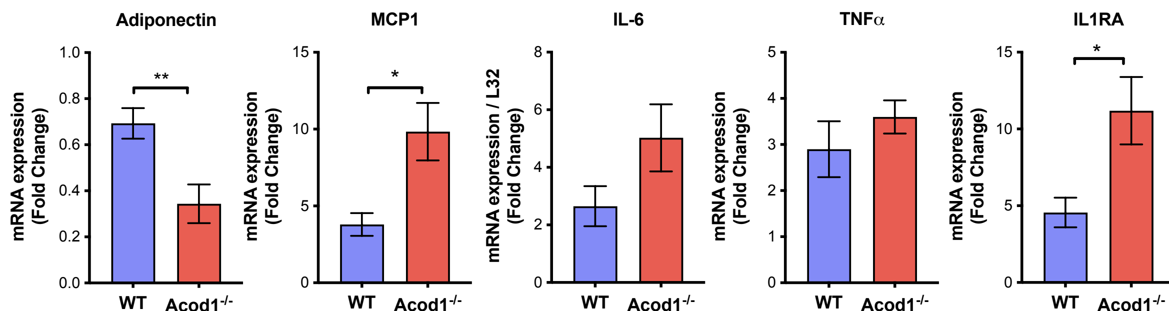

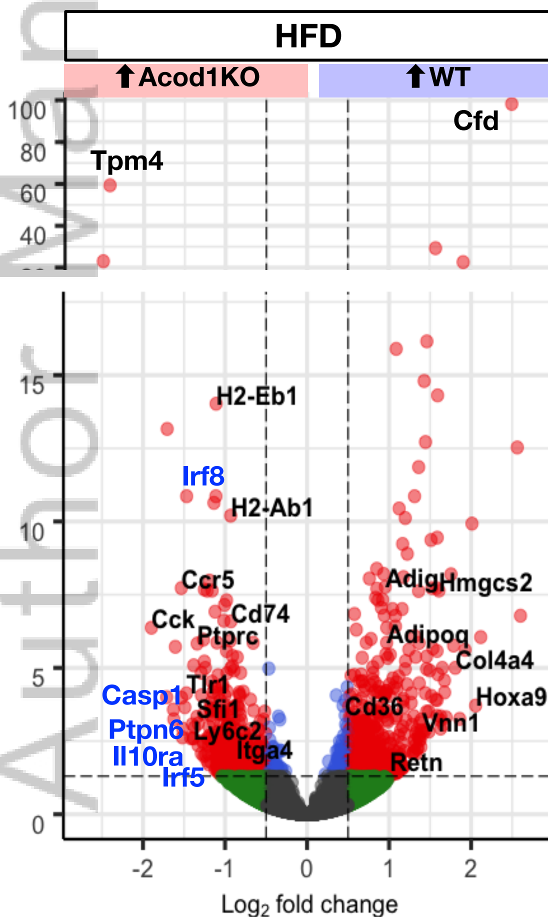

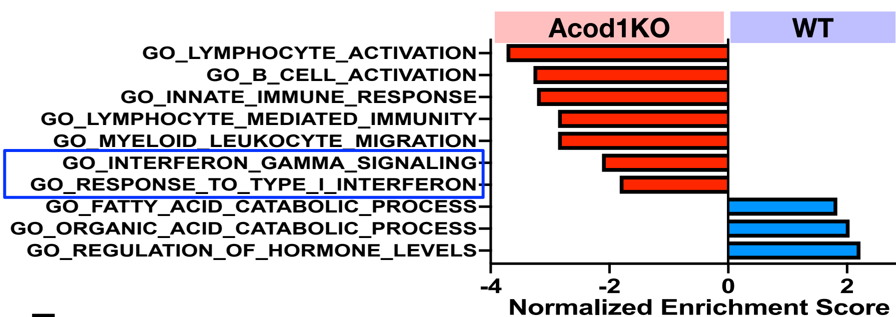

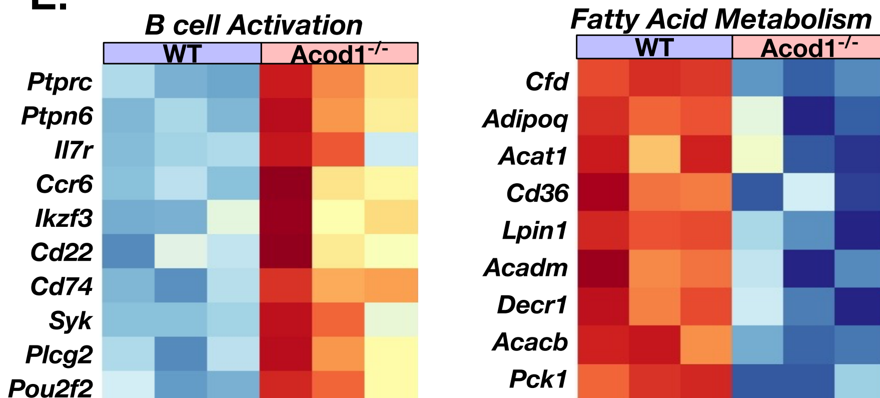

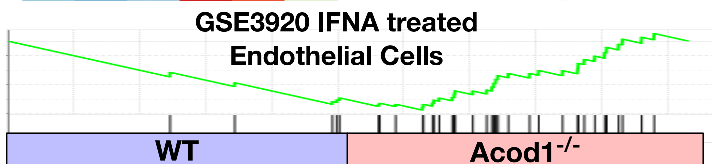

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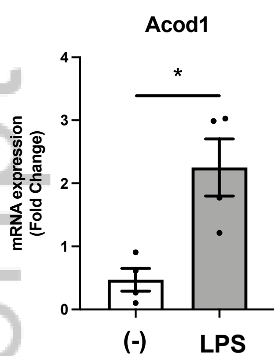

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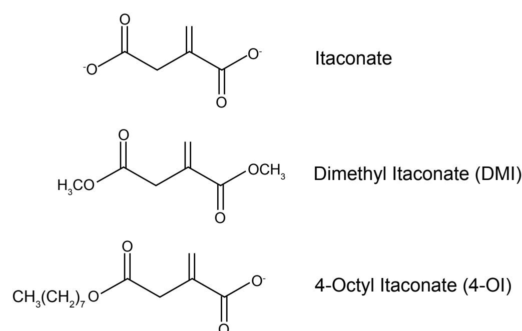

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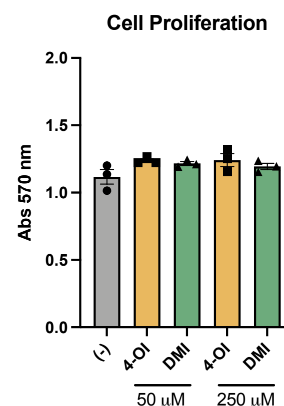

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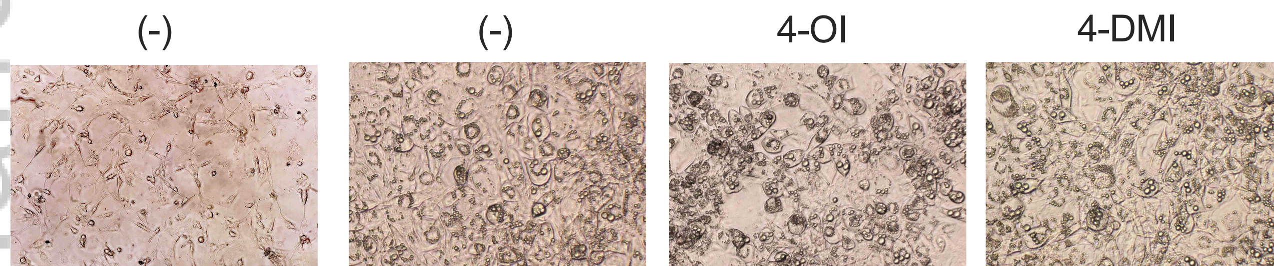

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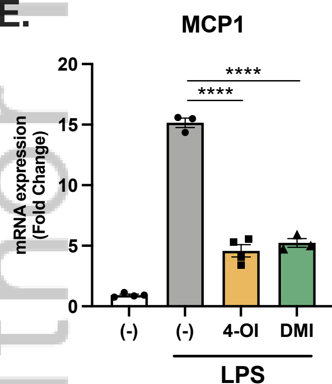

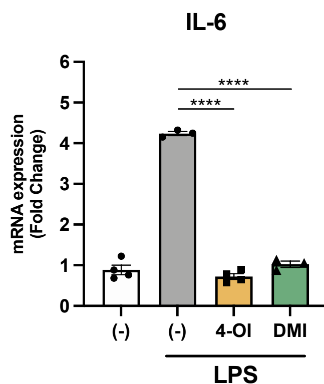

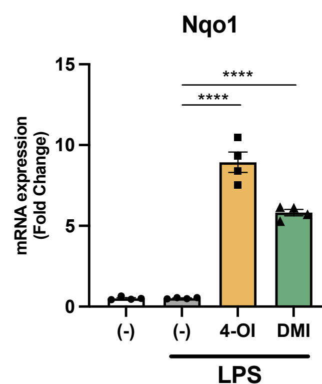

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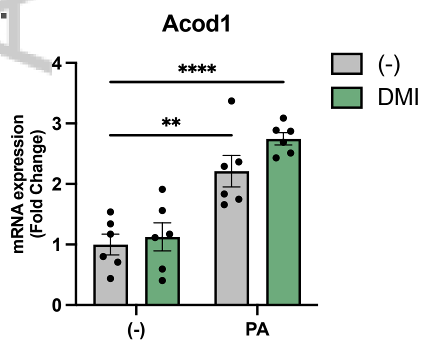

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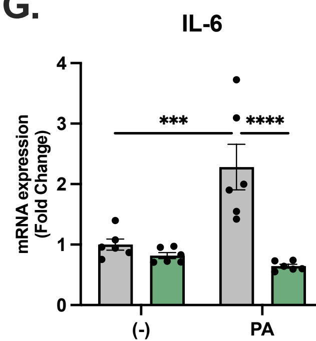

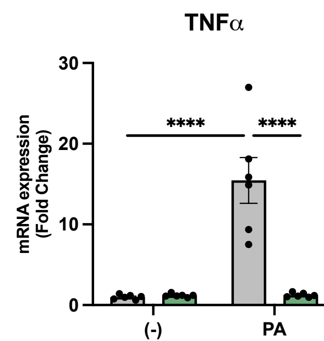

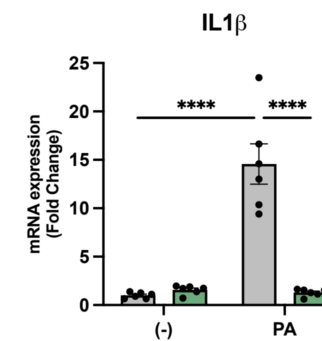

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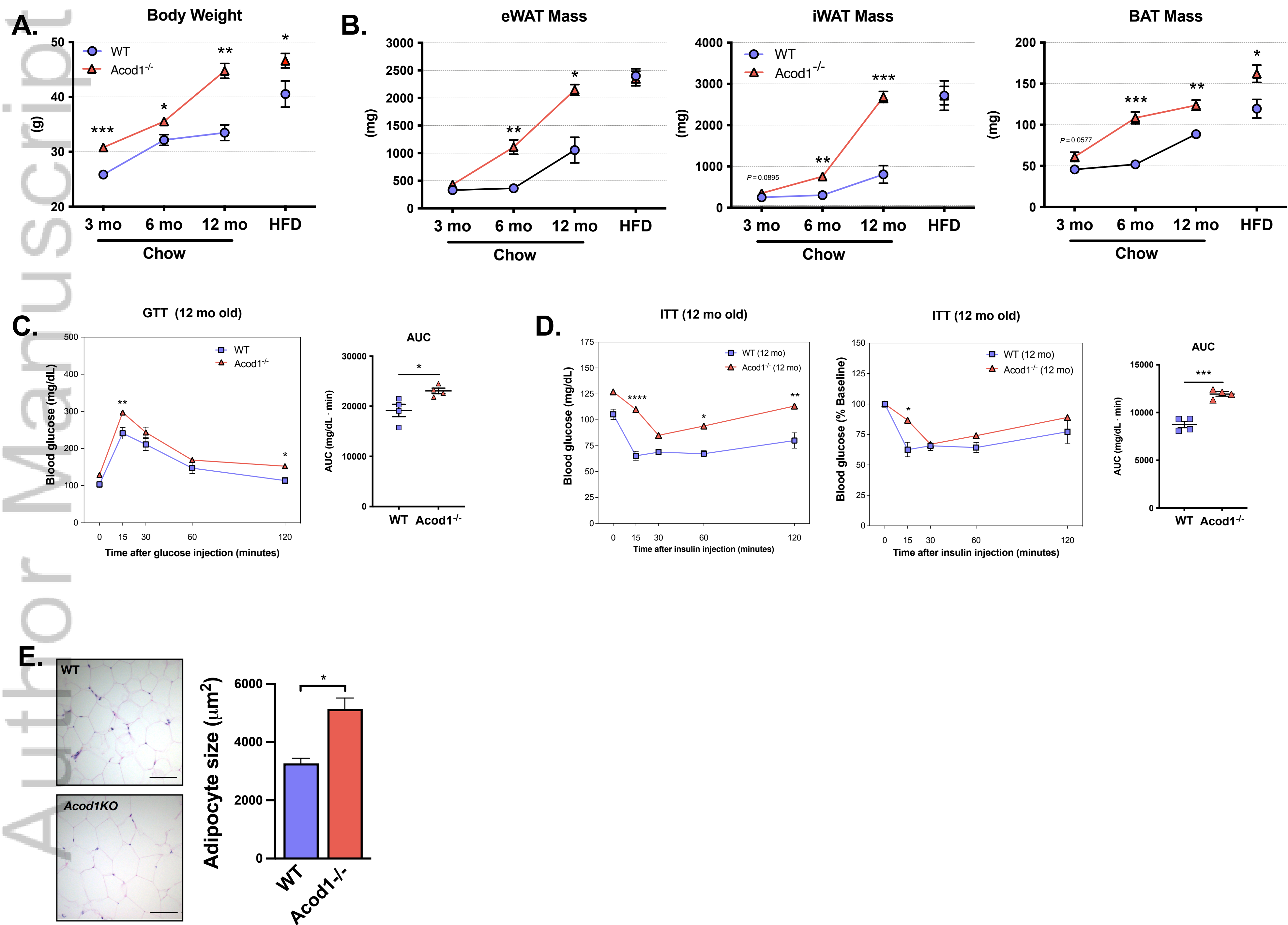

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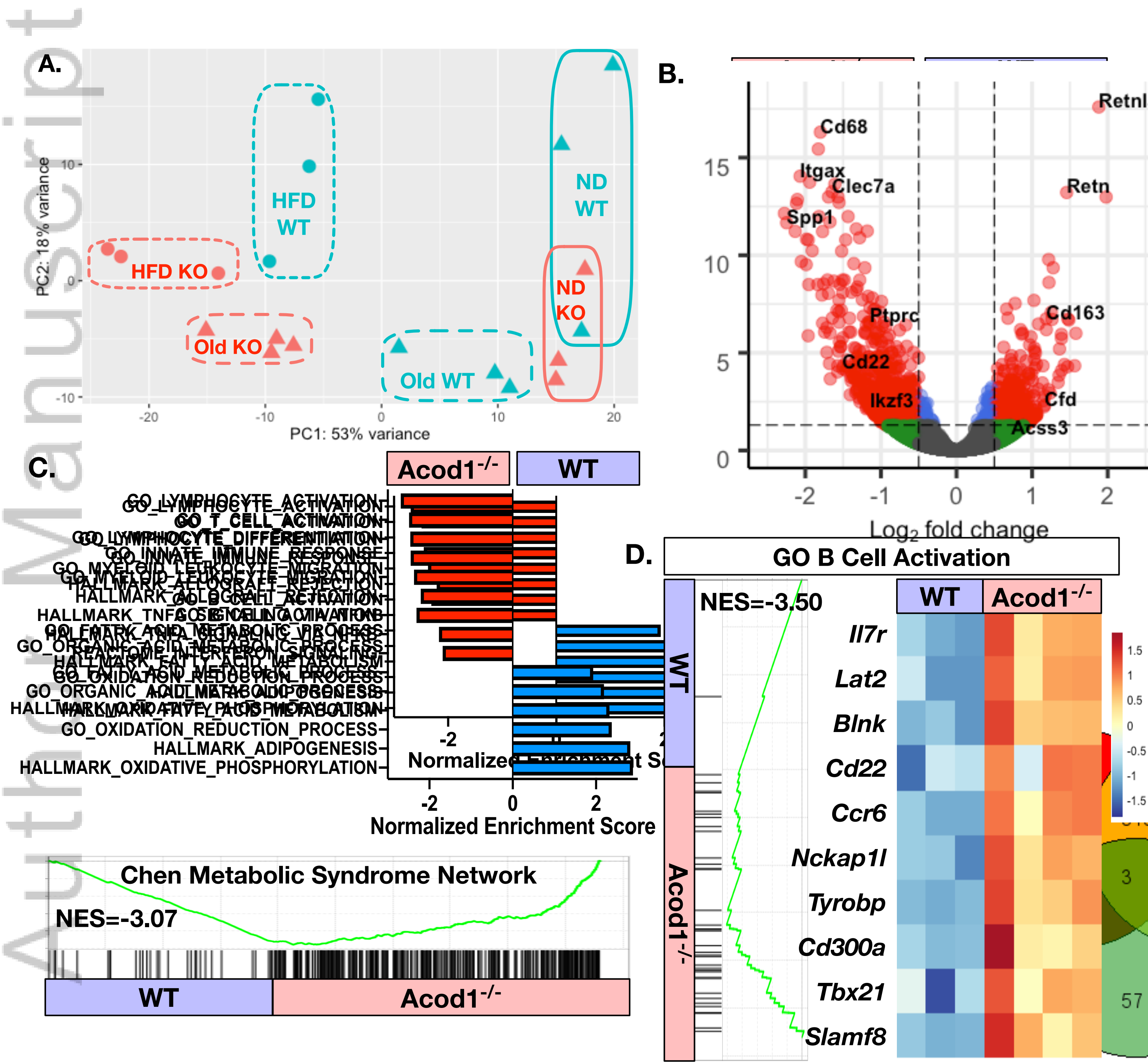

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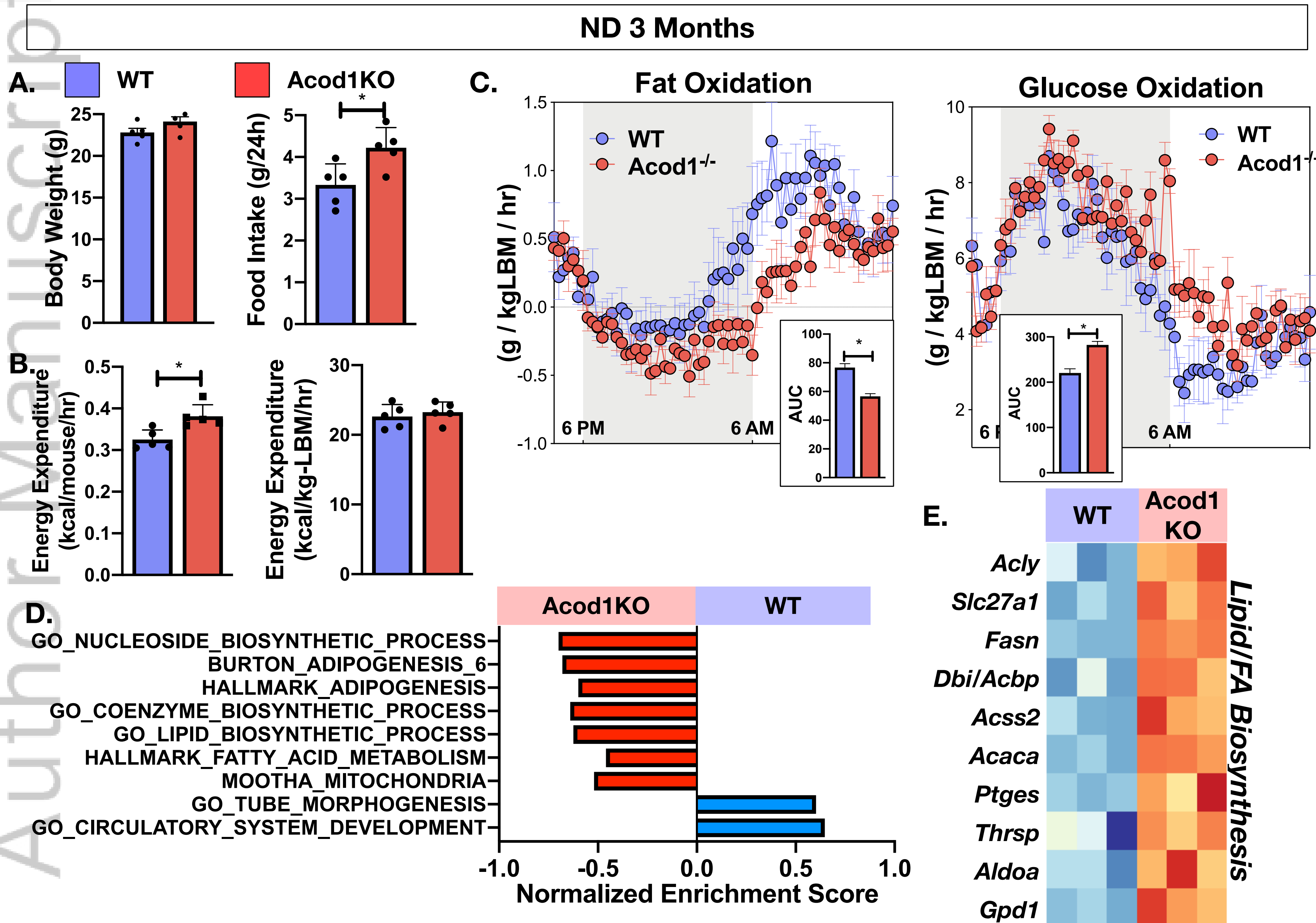

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