THE ROLE OF THE MTOR PATHWAY IN DEVELOPMENTAL REPROGRAMMING OF HEPATIC LIPID METABOLISM AND THE HEPATIC TRANSCRIPTOME AFTER EXPOSURE TO 2,2',4,4'-TETRABROMODIPHENYL ETHER (BDE-47)

An Honors Thesis Presented

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ABSTRACT

An emerging hypothesis links the epidemic of metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD) and diabetes with chemical exposures during development. Evidence from our lab and others suggests that developmental exposure to environmentally prevalent flame-retardant BDE47 may permanently reprogram hepatic lipid metabolism, resulting in an NAFLD-like phenotype. Additionally, we have demonstrated that BDE-47 alters the activity of both mTOR complexes (mTORC1 and 2) in hepatocytes. The mTOR pathway integrates environmental information from different signaling pathways, and regulates key cellular functions such as lipid metabolism, innate immunity, and ribosome biogenesis. Thus, we hypothesized that the developmental effects of BDE-47 on liver lipid metabolism are mTOR-dependent. To assess this, we generated mice with liver-specific deletions of mTORC1 or mTORC2 and exposed these mice and their respective controls perinatally to BDE-47. We found that developmental exposure to BDE-47 permanently reprograms gene expression related to hepatic lipid metabolism, innate immunity, and other key cellular functions in an mTORC1- and 2-dependent manner. Our results also provide a hypothetical model of gene-environment interaction in which early-life BDE-47 exposure triggers life-long reprogramming of liver lipid metabolism and other key cellular functions in an mTOR-dependent manner, and indicate that modulation of the mTOR pathway by environmental chemicals such as BDE-47 may lead to long-lasting changes in liver disease susceptibility.

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List of abbreviations

ACLY – ATP citrate lyase

AKT – Also known as PKB or protein kinase B

BDE-47 – 2,2',4,4'-tetrabromodiphenyl ether

CD36 – Cluster of differentiation 36

DEPTOR – DEP-domain-containing mTOR interacting protein

FKBP12 – 12 Kda FK506-binding protein

GSEA – Gene Set Enrichment Analysis

HAT – Histone acetyltransferase

HDAC – Histone deacetylase

IGF – Insulin-like growth factor

IPA – Ingenuity Pathway Analysis

KO – Knockout

mLST8 – Mammalian lethal with sec13 protein 8 mLST8

mSIN1 – Mammalian stress-activated protein kinase interacting 1

mTOR – Mechanistic target of rapamycin

mTORC1 - Mechanistic target of rapamycin complex 1

mTORC2 - Mechanistic target of rapamycin complex 2

NAFLD – Non-alcoholic fatty liver disease

PBDE – Polybrominated diphenyl ether

PPARy – Peroxisome proliferator-activated receptor gamma

PI3K – Phosphoinositide 3-kinase

PKC – Protein kinase C

PRAS40 – Proline-rich AKT substrate 40 kDa,

Protor1/2 – Protein observed with Rictor 1 and 2

Raptor – Regulatory-associated protein of mTOR

Rictor – Rapamycin-insensitive component of mTOR

SGK1 – Serum/glucocorticoid regulated kinase 1

S6K1 – Ribosomal protein S6 kinase beta-1

TSC2 – Tuberous sclerosis complex 2

I. Experimental goals

In the liver, the comparative roles of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and mTOR complex 2 (mTORC2) in hepatic gene expression regulation and liver health are not entirely clear. Additionally, previous work suggests that the mTOR pathway may be a mechanism by which early-life exposure to toxicants such as the environmentally ubiquitous flame-retardant 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) influence liver health to increase metabolic disease susceptibility. Thus, the goal of this series of experiments was twofold: first, to advance our understanding of the comparative roles of mTORC1 and 2 in hepatic gene expression regulation using an RNA-seq approach and liver-specific genetic knockouts of *Raptor* (for mTORC1) or *Rictor* (for mTORC2). Building off this, we then looked to experimentally determine the individual roles of mTORC1 and 2 in mediating the deleterious effects of early-life exposure to BDE-47 on hepatic gene expression and lipid metabolism. To these ends, this study provides insights into mTOR-controlled hepatic gene expression regulation at the mechanistic and systems levels, the role of mTOR as a mediator of the long-term effects of early-life environmental toxicant exposure, and mechanisms by which the effects of early-life environmental toxicants can intersect with genetics to permanently alter hepatic susceptibility to disease later in life.

II. Introduction

Introduction to the mTOR pathway

Since its isolation from brain tissue, the protein kinase mTOR (mechanistic target of rapamycin) and its pathway have become known as the central regulator of growth and metabolism, capable of integrating a vast number of internal and external signals to exert a plethora of effects including cellular growth and proliferation, ribosome biogenesis, lipid and glucose metabolism, and autophagy (Laplante and Sabatini, 2009; Sabatini, 2017). mTOR exists in two complexes, named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

mTORC1 consists of mTOR, mammalian lethal with sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40), DEP-domain-containing mTOR interacting protein (DEPTOR), and regulatory-associated protein of mTOR (Raptor) (Laplante and Sabatini, 2009). This complex is downstream of protein kinase B (PKB, also known as AKT), which stimulates its activation through phosphorylation of PRAS40 and Tuberous sclerosis complex 2 (TSC2) (Laplante and Sabatini, 2009). Through this mechanism and others, mTORC1 has been demonstrated to sense signals triggered by growth factors, amino acid and energy availability, oxygen levels, DNA damage, inflammation, and other signals key to cellular growth and metabolism (Laplante and Sabatini, 2009; Sabatini, 2017; Saxton and Sabatini, 2017). When stimulated, mTORC1 activates lipid synthesis, protein synthesis (by triggering both ribosome biogenesis and mRNA translation), nucleotide synthesis, glycolysis, and mitochondrial biogenesis, while exerting an inhibitory effect on autophagy, proteasome assembly, and lysosome biogenesis (Laplante and Sabatini, 2009; Sabatini, 2017; Saxton and Sabatini, 2017; (Mao and Zhang, 2018). Overall, while mTORC1 is involved in several cellular processes, its

general role appears to be shifting between catabolic and anabolic states to stimulate or reduce growth based on the integration of signals from both intracellular and extracellular environments (Saxton and Sabatini, 2017).

On the other hand, mTORC2 consists of mTOR, mammalian stress-activated protein kinase interacting protein (mSIN1), DEPTOR, mLST8, rapamycin-insensitive component of mTOR (Rictor), and protein observed with Rictor 1 and 2 (Protor1/2) (Laplante and Sabatini, 2009; Mao and Zhang, 2018). While mTORC1 is known to sense signals from several inputs, the input signals for mTORC2 are less understood, although mTORC2 appears to primarily receives input from growth factors, most notably insulin/insulin-like growth factor (IGF) (Laplante and Sabatini, 2009; Saxton and Sabatini, 2017; Mao and Zhang, 2018). Phosphoinositide 3-kinase (PI3K) activation (downstream of the insulin receptor) stimulates localization of mTORC2 to the ribosome, triggering activation of mTORC2 (Zinzalla et al., 2011). Downstream cellular functions stimulated by mTORC2 include cell survival by inhibiting apoptosis (via AKT and serum/glucocorticoid regulated kinase 1 or SGK1), ion transport regulation (via SGK1), cell migration (via AKT and protein kinase C or PKC), and cytoskeletal remodeling (via PKC) (Laplante and Sabatini, 2009; Lang and Pearce, 2016; Saxton and Sabatini, 2017). While mTORC2 remains relatively understudied in comparison to mTORC1, mTORC2 seems to regulate cell survival and proliferation. However, additional evidence suggests that mTORC2 may play a role in some metabolic processes, such as fatty acid synthesis via ATP citrate lyase (ACLY), that are distinct from mTORC1 (Linke et al., 2017).

The defining elements of mTORC1 and 2 respectively are Raptor and Rictor, whose binding to mTOR are mutually exclusive and determine which type of complex will form (Sabatini. 2017; Saxton and Sabatini, 2017). Although these two complexes appear to have

distinct downstream effects, there is inherent cross-talk between these pathways. Ribosomal protein S6 kinase beta-1 (S6K1), one of the most well-characterized targets of mTORC1, also destabilizes insulin receptor substrate 1 (IRS1) in the insulin receptor/PI3K pathway upstream of mTORC2. Phosphorylation of AKT at Ser473 by mTORC2 is required for its full activation, and AKT indirectly activates mTORC1 (Laplante and Sabatini, 2009; Saxton and Sabatini, 2017). These interactions and many others suggest that while the two mTOR complexes are distinct in their input signals and downstream targets, they often act indirectly to regulate activity of the opposing complex and coordinate cellular processes (Zarogoulidis et al., 2014; Linke et al., 2017; Silvera et al., 2017; Luo et al., 2018).

The complexes mTORC1 and 2 are also distinct in their response to acute rapamycin exposure. Rapamycin, a natural antifungal agent whose discovery eventually led to the discovery of the mTOR pathway, triggers the binding of 12 Kda FK506-binding protein (FKBP12) to mTORC1, leading to its inhibition (Choi et al., 1996; Laplante and Sabatini, 2009; Sabatini, 2017; Saxton and Sabatini, 2017). Acute rapamycin exposure does not inhibit mTORC2, but chronic rapamycin exposure suppresses its activity (Sarbassov et al., 2006). As of now, there are no chemical methods to chronically inhibit a single mTOR complex, or acutely inhibit mTORC2 (Murray and Angus, 2017; Saxton and Sabatini, 2017). Thus, genetic methods must be used to selectively suppress activity of a single mTOR complex. Such methods include the Cre/LoxP system, which can be used to induce a conditional knockout of *Raptor* or *Rictor*, preventing formation of mTORC1 or 2 respectively (Cybulski et al., 2012).

Despite the lack of complex-specific mTOR inhibitors, current small-molecule mTOR inhibitors have nonetheless proved useful in the clinic. In particular, mTOR inhibitors have played a significant role in the clinic as both cancer therapeutics (Zarogoulidis et al., 2014;

Saxton and Sabatini, 2017), and immunosuppressants for tissue transplants (Morrisett, et al., 2002; Kasiske et al., 2008; Zimmerman et al., 2018). Beyond cancer, the mTOR pathway is also involved in the pathogenesis of neurodegenerative disorders, inflammatory diseases, type 2 diabetes, obesity, and non-alcoholic fatty liver disease (NAFLD) (Dazert and Hall, 2011; Saxton and Sabatini, 2017).

mTOR and physiological aging

All the diseases and biological functions in which mTOR plays a role are influenced by or associated with aging (Saxton and Sabatini, 2017, Papadopoli et al., 2019). Interestingly, suppression of the mTORC1 pathway has been shown to extend lifespan either by both treatment with rapamycin and caloric restriction (Saxton and Sabatini, 2017, Papadopoli et al., 2019). Although the exact mechanism by which mTORC1 inhibition increases lifespan is still unknown, it appears that mTORC1 acts upstream of many key aging-associated processes including changes in the rate of ribosome biogenesis and protein synthesis, proteotoxic/oxidative stress, decreased autophagy and other processes (Saxton and Sabatini, 2017; Papadopoli et al., 2019). The fact that mTORC1 inhibition and downstream suppression of cellular growth and metabolism lead to an increase in lifespan in many organisms is consistent with the antagonistic pleiotropy theory of aging. This theory posits that growth and metabolism processes are necessary for an organism to grow, but following development these same processes result in cellular aging and have deleterious effects on organismal health over time (Blagosklonny and Hall, 2009; Papadopoli et al., 2019).

mTOR and its role in regulation of hepatic gene expression

One of the reasons mTOR signaling affects a plethora of cellular processes is due to the broad control it exerts over gene expression. Indeed, activation of both cytoplasmic mTOR complexes, as well as nuclear activity of mTOR, influence gene expression (Laribee, 2018). Specifically, mTOR has been shown to alter the activity of various transcription factors (Laplante and Sabatini, 2013), and influence the epigenome (Laribee, 2018). Consistent with more information known about mTORC1 function, mTORC1 signaling has been shown to regulate histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity to regulate ribosomal biogenesis (Laribee, 2018). However, the role of mTORC2 in regulating gene expression, and whether it has distinct effects on the epigenome and transcriptome are even less understood than the effects of mTORC1. Thus, methods to perturb gene expression and epigenetic programming in different tissue types via the mTOR pathway (such as the Cre/LoxP system) may provide further insight into its role in growth, development, aging, and disease.

Because the liver plays an essential role in systemic metabolism, understanding mTOR signaling in this tissue is critical. mTOR signaling in the liver has been shown to play an important role in maintaining healthy liver function. Specifically, mTOR signaling is known to play a role in lipogenesis, glycogen synthesis, cholesterol synthesis, and gluconeogenesis (Mao & Zhang, 2018). Accordingly, dysregulation of mTOR signaling specifically in the liver is associated with many different kinds of age-associated liver diseases, including the metabolic syndrome, NAFLD, type II diabetes, and hepatocellular carcinoma (Kim, Kisselva, & Brenner 2016; Okuno et al, 2018; Mao & Zhang, 2018).

Despite how clear it is that mTOR has diverse functions in liver health and disease, the comparative roles of both mTOR complexes in liver gene expression regulation specifically are poorly understood. Fasting and refeeding (to suppress and then hyperactivate mTOR activity) with and without rapamycin has been used to probe the role of mTORC1 on hepatic gene expression, but no significant changes in expression of individual genes have been observed using this method, at least when changes in hepatic gene expression were analyzed via microarray (Boylan et al, 2015). However, Gene Set Enrichment Analysis (GSEA), which uses manually curated sets of genes to identify often subtle but biologically meaningful trends in the transcriptome, indicated that modulation of mTORC1 activity using fasting-refeeding may downregulate gene sets associated with the spliceosome and upregulate sets associated with nuclear-encoded mitochondrial genes, Cytochrome P450 monooxygenases, proteasomal constituents, peroxisomal proteins, and ribosomal proteins (Boylan et al, 2015). Another report (using rapamycin only) indicated that hepatic mTORC1 regulates genes involved with cholesterol synthesis and metabolism. Ingenuity Pathway Analysis (IPA), which can be used to identify affected biological functions, build networks of interacting genes and proteins, and predict upstream transcription factors whose activity are likely altered based on transcriptomic data identified *Srebf1* and *Srebf2*, as well as *Nrf2*, as regulators of these genes (Lamming et al., 2014). Overall, the current impression of the role of mTORC1 in hepatic gene expression regulation is that its effects “broad but subtle” Boylan et al (2015), and that mTORC1 may exert its influence on gene expression in the liver via epigenetic mechanisms that have yet to be fully understood.

The fasting-refeeding protocol in combination with rapamycin and hepatic *Rictor* knockout (RKO) mice has previously been used to probe the role of mTORC2 in hepatic gene

expression regulation (Lamming et al., 2014). In this experiment, mTORC2 had a statistically significant role in expression of four genes: the leptin receptor (*Lepr*), insulin-like growth factor binding protein 1 (*Igfbp1*), insulin receptor substrate 2 (*Irs2*), and glucokinase (*Gck*). Analysis of these four genes and nonsignificant genes that were altered more than 1.5-fold using IPA indicated that mTORC2 regulates liver lipid and carbohydrate metabolism, expression of cytochrome P450 monooxygenases, cholesterol esterases, and hydroxysteroid dehydrogenases (Lamming et al., 2014). Gene set enrichment analysis (GSEA) of the full list of microarray fata revealed significant upregulated gene sets in the RKO group that could be best summarized as: Ribosomal proteins, genes involved in oxidative phosphorylation and genes encoding components of the 26S proteasome (Lamming et al., 2014)..

While these reports have made significant progress in providing an understanding of mTOR-controlled gene expression regulation in the liver, differences in the design of experiments addressing the roles of mTORC1 and mTORC2 do not allow for direct comparison of the transcriptomic roles of each complex. The suppression of mTORC2 in mouse livers was permanent and achieved by a liver-specific knockout of *Rictor*, while suppression of mTORC1 by rapamycin in both mice and rats was short-term, reversible, and not tissue-specific. Furthermore, it was recently demonstrated that permanent genetic knockouts often result in significantly different phenotypes compared to transient reduction of protein activity or gene expression (El-Brolosy & Stainier, 2017). Additionally, experiments to profile the role of mTORC1 and 2 in hepatic gene expression regulation thus far have been conducted using microarrays, which may provide biased results relative recent advancements in RNA-seq given their reliance on the design of specific oligonucleotides to target known and predicted sequences in specific mRNAs (Jaksik et al., 2015). One indication of poor microarray performance in the

above studies (Lamming et al, 2014; Boylan et al, 2015) is that few significantly altered genes were identified in all experimental conditions, which contradicts the known roles of the mTOR pathway in liver metabolism and other aspects of liver physiology. Thus, revisiting the individual roles of mTORC1 and 2 in gene expression regulation using an RNA-seq approach and similar genetic methods to probe activity of each complex may yield more insights in this area.

PBDEs and metabolic toxicity

Polybrominated diphenyl ethers (PBDEs) were used as flame retardants in a wide variety of textiles, home products, and electronics (Hites, 2004). Given their widespread use, PBDEs slowly leaked into the environment worldwide, and are now ubiquitous in the environment, humans, and animals (Hites, 2004; Suvorov et al., 2018). Notably, a meta-analysis conducted in 2004 showed that over the prior 30 years PBDE concentrations in human blood, milk, and tissues had increased exponentially in Western countries (Hites, 2004). Because of their extensive half-lives, tendency to accumulate in human tissues, and potential adverse health effects such as thyroid function disruption, neurodevelopmental alterations, and reproductive changes, PBDEs were completely removed from production in the U.S. and other Western countries in 2013 (Dodson et al., 2012; Linares et al., 2015; Suvorov et al., 2018). However, despite banning the use of PBDEs, their concentration in human tissues has continued to increase or remained stable (Dodson et al., 2012).

PBDEs are highly lipophilic and accumulate in adipose tissue (Khalil et al., 2018). Additionally, the prevalence of PBDEs in dust in households leads to crawling infants and toddlers inhaling and ingesting it at a much higher rate than adults (Wilford et al., 2005). This in combination with ingesting high quantities of milk early in life results in infants having blood

plasma concentrations of BDE-47 significantly higher than their mothers (Khalil et al., 2018). However, given the environmental ubiquity and high lipophilicity of BDE-47, humans continue to be exposed throughout their lives via diet, inhalation, and other means (Dodson et al., 2012).

BDE-47 (also known as 2,2',4,4'-tetrabromodiphenyl ether) is one of the most stable breakdown products of other PBDEs, making it one of the most prevalent in the environment, and is the most prevalent one found in human milk and cord blood (Suvorov et al., 2018). During pregnancy, mobilization of lipids leads to a significant increase of BDE-47 and other PBDEs in blood that quickly crosses the placenta, which also later accumulate in breast milk (Khalil et al., 2017). Evidence from both experimental and epidemiological studies associates developmental exposure in early life to BDE-47 and chronic exposure in adulthood with the current epidemic of metabolic diseases in which dysregulation of lipid and glucose metabolism are involved, such as metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), obesity, and diabetes (Lim et al., 2008; Zhang et al., 2016; Khalil et al., 2017; Khalil et al., 2018; Yang et al., 2019).

BDE-47, mTOR, and liver health

Interestingly, perinatal exposure to low levels of BDE-47 starting on the first postnatal day (PND) of life leads to increased hepatic activity of both mTOR complexes by PND 21 in mice. Interestingly, although the activity of both mTOR complexes return to normal by postnatal week 20, cellular changes related to the mTOR pathway, including an increase in blood IGF1 and fatty acid levels, suppression of S6K1, decreased expression of the fatty acid translocase and scavenger receptor cluster of differentiation 36 (*Cd36*), and upregulation of ribosomal genes were observed in the liver (Khalil et al., 2017; Khalil et al., 2018). Additionally, expression of gene sets involved in mTORC1 signaling, ribosomal gene expression, and other pathways

downstream of mTORC1 and 2 were all found to be significantly enriched based on GSEA on both postnatal day 21 and postnatal week 20 (Khalil et al., 2017). Both mTOR complexes were also activated (mTORC1 at 3 hours, mTORC2 at 24 hours) following acute exposure to BDE-47 in HepG2 cells (Khalil et al., 2017). These results suggest that exposure to BDE-47 can activate the mTOR pathway in an acute manner, and if exposed continuously or during a critical period of development, the effects on the mTOR pathway may be long lasting and have significant implications for overall health. However, it is likely that the relationship between BDE-47 and mTOR is complex, as it has been demonstrated that while perinatal exposure to low doses of BDE-47 affects many functions regulated by the mTOR pathway as mentioned above, prenatal exposure to high doses of BDE-47 lead to the opposite effects, including downregulation of ribosomal genes, upregulation of *Cd36*, and an increase in triglyceride levels in the liver relative to blood (Khalil et al., 2018). Taken together, these results suggest that BDE-47 exposure exerts complex effects on the mTOR pathway in the liver throughout the life span, and suggests that mTOR plays a role in the liver not only in transient regulation of gene expression in response to nutrients and other short-term signals, but also in more long-lasting reprogramming of gene expression in response to environmental toxicants (Khalil et al., 2018). Probing the mechanisms behind how BDE-47 modulates the mTOR pathway, and the resulting changes in mTOR function in different tissue types, could provide novel insight into the mechanisms behind the deleterious health effects associated with BDE-47 exposure and mTOR biology.

The above results demonstrating changes in mTOR pathway activity and related alterations in lipid metabolism and ribosomal gene expression in the liver following early-life exposure to BDE-47 provide evidence for the mTOR pathway as a mediator of the deleterious metabolic health effects associated with BDE-47 later in life (Khalil et al., 2017; Khalil et al.,

2018). In a similar manner, chronic suppression of mTOR using rapamycin to prevent rejection during kidney transplants leads to a significant risk for hyperlipidemia (Morrisett, et al., 2002; Kasiske et al., 2008), the risk of which tends to increase with age (Aslam, Haque, Lee, & Foody, 2009). Before these studies, mTORC1 was known to regulate expression of lipid synthesis genes and *Cd36* (Laplante and Sabatini, 2013). Furthermore, recent evidence suggests that mTORC2 regulates lipid synthesis via ACLY and peroxisome proliferator-activated receptor gamma (PPARy) (Guo et al., 2019). Whether mTORC1 and 2 have distinct roles in regulating liver lipid metabolism in the presence and absence of BDE-47 remains to be seen, as some studies on the differential roles of mTORC1 and 2 in this area have provided seemingly conflicting results (Caron et al., 2015). However, it does appear that BDE-47 modulates the ratio of total triglycerides between the blood and liver (blood-liver lipid balance) in a dose-dependent manner through changes in mTOR pathway activity (Khalil et al., 2017; Khalil et al., 2018). BDE-47 metabolites have also been shown to influence AKT Ser437 phosphorylation in testis tissue (Zhang et al., 2013), providing additional evidence that BDE-47 is an mTOR modulator. This change in AKT phosphorylation may take place in the liver as well during BDE-47 exposure. Beyond regulating liver lipid metabolism, it also remains to be seen which long-lasting effects of BDE-47 exposure on the hepatic transcriptome are dependent on one or both mTOR complexes, how BDE-47 dose influences these changes, and how these transcriptomic changes are maintained throughout the life span.

III. Methodology

Achieving a liver-specific knockout of mTORC1 and 2 using a Cre/LoxP system

Given our interest in probing the independent roles of mTORC1 and 2 in hepatic gene expression regulation and in mediating the effects of early-life BDE-47 exposure in the liver, we used mouse models with a liver-specific deactivation mTOR complexes generated through a genetic knockout of their defining components: *Raptor* for mTORC1 and *Rictor* for mTORC2. To generate mTORC1 liver-specific knockout mice, females with a transgene containing the Cre recombinase controlled by a serum albumin (Alb) specific promoter (Stock #: 003574, The Jackson Laboratory) were crossed with homozygous floxed mutant male mice possessing loxP sites flanking exon 6 of the *Raptor* gene (Stock #: 013188, The Jackson Laboratory) to obtain F_1 animals heterozygous for both the *Raptor* floxed allele and the Alb-Cre transgene. All of these mice were mostly of C57BL/6 background. F_1 females were backcrossed with the first generation of *Raptor* floxed males, resulting in F_2. F_2 mice were genotyped to identify offspring homozygous for floxed *Raptor* lacking the Alb-Cre transgene, and offspring homozygous for floxed *Raptor* and heterozygous for the Alb-Cre transgene. These 2 groups of animals were then crossed with each other, and pregnant females were assigned to two groups. Upon delivery, nursing dams were exposed to either vehicle only or 1.0 mg/kg body weight BDE-47 dissolved in tocopherol stripped corn oil via daily feeding from the tip of a pipette in a volume of 1 µl/gram body weight from the day of delivery through postpartum day 21, as done in previous studies in our lab (Khalil et al., 2017; Khalil et al., 2018)

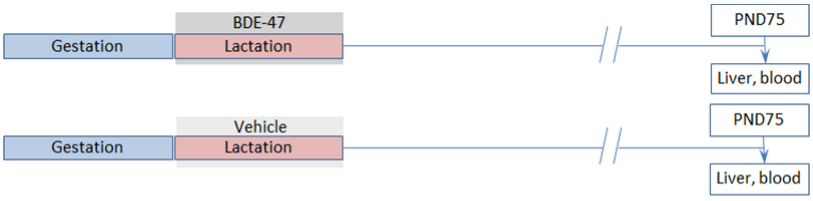

Figure 1. A Visual Representation of our Exposure Paradigm. All animals were exposed perinatally to BDE-47 or vehicle only via lactating dams to simulate one of the most common routes of exposure for infants. Liver and blood samples were collected on postnatal day 75. Only males were used for subsequent analyses.

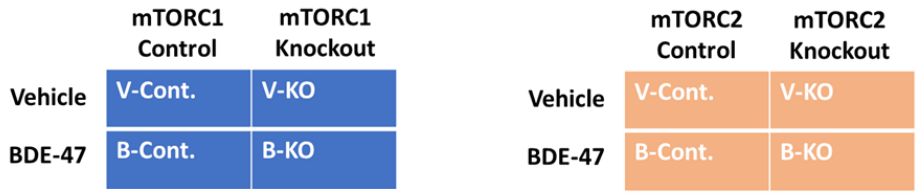

Figure 2. A Visual Representation of the Groups of Animals Used for this Series of Experiments. Groups shown in blue were the result of a *Cre-Loxp* breeding scheme to generate a liver-specific knockout of mTORC1 (via deletion of *raptor*) with the appropriate control, while groups shown in red were the result of a *Cre-Loxp* breeding scheme to generate a liver-specific knockout of mTORC2 (via deletion of *rictor*).

(Figure 1). Nursing (and thus exposed) F_3 offspring of exposed dams were comprised of two different genotypes: both were homozygous for floxed *Raptor* but differed regarding the presence or absence of the Alb-Cre transgene. Mice with Alb-Cre had a constitutively inactivated mTORC1 in their livers, and the second genotype lacking Alb-Cre was used as a control.

F_3 offspring were genotyped and euthanized on postnatal day 75, approximately 5 weeks after the last exposure to BDE-47. The same procedure was conducted to generate mTORC2 liver-specific knockout F_3 mice, with the only difference being that in the first cross, homozygous floxed mutant mice were used that possess loxP sites flanking exon 11 of the *Rictor* gene (Rapamycin-insensitive companion of mTOR, the defining component of mTOR complex, Stock #: 020649, The Jackson Laboratory). Upon euthanasia via cervical dislocation, liver and blood samples were collected. Liver samples were snap frozen in liquid nitrogen. Blood samples were centrifuged at 3000g at 4 ℃ for 10 minutes, and serum was collected. Serum samples were placed in a -80 ℃ freezer for long-term storage. All procedures and protocols were approved by the University of Massachusetts Amherst IACUC. Only males were used for the following analysis.

It should be noted that the genotype of "mTORC1 control" mice are $raptor^{flox/flox}Alb^{0}$, "mTORC1 knockout" mice are $raptor^{flox/flox}Alb^{Cre}$ (with a hepatocyte-specific deletion of mTORC1), "mTORC2 control" mice are $rictor^{flox/flox}Alb^{0}$, and "mTORC2 knockout" mice are

rictor$^{flox/flox}$*Alb*^{Cre} (with a hepatocyte-specific deletion of mTORC2). While all mice are genetically modified, the terms "mTORC1 control" and "mTORC2 control" for mice lacking Cre recombinase with floxed *Raptor* or *Rictor* respectively were used to prevent confusion when differentiating between the two types of control animals used, one in each breeding scheme. Figure 2 presents a visual representation of all groups used. In the remainder of the text, the breeding scheme to generate mice with a liver-specific deletion of mTORC1 and their controls, followed by perinatal exposure of these mice to BDE-47, is referred to as the "mTORC1 experiment" (Figure 2, in blue). Likewise, the breeding scheme to generate mice with a liver-specific deletion of mTORC2 and their respective controls, followed by perinatal exposure of these mice to BDE-47, is referred to as the "mTORC2 experiment" (Figure 2, in red)

Lipid profile analysis

Triglyceride levels were analyzed in serum samples and liver extracts using the Triglyceride Colorimetric Assay Kit (Cat. # 10010303, Cayman Chemical Company, Ann Arbor, MI) in accordance with the manufacturer's instructions.

mTORC1/2 activity analysis

Using western blotting, we analyzed changes in mTOR activity using rabbit antibodies for the phosphorylated forms of the most well-characterized targets of mTORC1 (S6K1 at Thr389) (Cell Signaling, Cat. # 9202 for S6K1, # 9205 for p-S6K1) and mTORC2 (AKT at Ser473) (Cell Signaling, Cat. # 4691 for AKT, # 4060 for p-AKT). Antibodies for corresponding total protein were used to control for protein expression (though these blots could not be completed due to the coronavirus pandemic), and β-actin (Cell Signaling, Cat. # 4970) was used as a loading control. Western blot analyses were performed after extracting total protein from

male livers from all groups using T-PER reagent, quantifying total protein using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Cat. #23227), separating the proteins on 4-20% SDS-PAGE gels (Bio-Rad, Cat. # 456-1094) and transferring them onto a nitrocellulose membrane under wet conditions. Proteins were visualized using goat anti-rabbit secondary antibody conjugated with HRP (Abcam, Cat. # ab6721), and Pierce ECL enhanced chemiluminescence reagent (ThermoFisher Scientific, Cat. # 32106).

Gene expression analysis

The liver transcriptome for males in all groups (N=3 per exposure/genotype group in mTORC1 experiment and N=4 per exposure/genotype group in mTORC2 experiment) was analyzed using an RNA-seq approach as done previously (Khalil et al., 2017; Khalil et al., 2018). In brief, total RNA was isolated from liver samples using TRIzol reagent (Invitrogen) and RNA concentration was quantified using a NanoDrop 1000 instrument (Thermo Fisher Scientific). RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). Liver RNA samples with integrity values >9 were used for library preparation. An RS-122-2101-TruSeq Stranded mRNA LT-SetA kit (Illumina) was used to isolate intact poly(A)+ RNA from 4 μg of total RNA and construct strand-specific libraries with multiplexing indexes. The quality and purity of the libraries were assessed using the Agilent 2100 Bioanalyzer. The concentration of the libraries was measured using Qubit 3.0 fluorometer (Life Technologies). High-throughput sequencing (75 bp, single-end) was performed using the NextSeq500 sequencing system (Illumina) in the Genomic Resource Laboratory of the University of Massachusetts, Amherst. cDNA libraries were single-end sequenced in 76 cycles using a NextSeq 500 Kit v2 (FC-404-2005, Illumina) in one multiplex run. RNA-Seq reads were aligned to the mm10 reference genome using the TopHat package, and transcribed genomic regions were assembled into

transcripts using a DEseq2 package. All RNA-Seq experiments were conducted in accordance with standards, guidelines, and best practices for RNA-seq developed by the ENCODE consortium. The lists of genes with relative expression values were imported to genomic tools for cluster and enrichment analysis.

Gene expression analysis

Metascape Ontology (Express Analysis) was used to determine changes in ontological clusters of genes associated with different biological functions between groups for all genes differentially expressed with an FDR q-value less than 0.05. To compare the effects of BDE-47 exposure in control and knockout mice, we plotted corresponding gene expression values against each other in Tableau. We then used thresholds of 1.5-fold change to identify fields in a coordinate plane containing the following groups of genes: Altered in comparison A (such as vehicle-exposed control vs. BDE-47 exposed control) but not B (such as vehicle-exposed knockout vs. BDE-47 exposed knockout), altered in comparison B but not A, altered in opposite directions in A and B, and altered in similar directions in A and B. These gene groups were then analyzed using Metascape (express analysis).

Gene set enrichment analysis (GSEA) was used to investigate coordinated changes in expression of gene sets associated with biological functions between groups using entire gene lists. To illustrate the differential roles of mTORC1 and 2 on changes in gene set expression, gene sets significantly enriched by mTORC1 and/or 2 knockout (Normalized enrichment score (NES) greater than or equal to an absolute value of 1.7 and an FDR q-value of 0.25 or less, per Broad Institute GSEA recommendations) were plotted using Tableau. Reference lines were generated to indicate which gene sets met or exceeded a NES with an absolute value of 1.7 in

both mTORC1 and mTORC2 knockouts, and data was exported via crosstab to Microsoft Excel to provide a list of which gene sets and their dependency on mTORC1 and 2. A similar analysis was conducted when comparing the effect of BDE-47 exposure in mTORC1 control and knockout animals, as well as when comparing the effect of BDE-47 exposure in mTORC2 control and knockout animals. Sort lists of genes were also analyzed using IPA to identify affected canonical pathways, generate networks of affected genes, and identify potential upstream transcription factors and nuclear receptors.

IV. Results part 1: the comparative roles of mTORC1 and 2 in liver gene expression

Liver-specific deletion of mTORC1 or 2 increases the activity of the other complex at PND75

Protein immunoblot of whole liver tissue (*N*=2 per group) collected from male mice on PND75 confirmed that developmental liver-specific knockout of *Raptor* or *Rictor* decreases phosphorylation of well-characterized targets of mTORC1 (S6K1 at Thr389) and mTORC2 (AKT at Ser473) respectively (Figure 1). Notably, liver-specific deletion of *Rictor* abolished any visible phosphorylation of AKT at Ser473, while liver-specific deletion of *Raptor* decreased levels of visible S6K1 Thr389 phosphorylation significantly but not entirely. This indicates that our liver-specific knockouts were likely effective and may suggest that while only mTORC2 phosphorylates AKT at Ser473, there are other enzymes that phosphorylate S6K1 at Thr389 in addition to mTORC1. Additionally, it appears that liver-

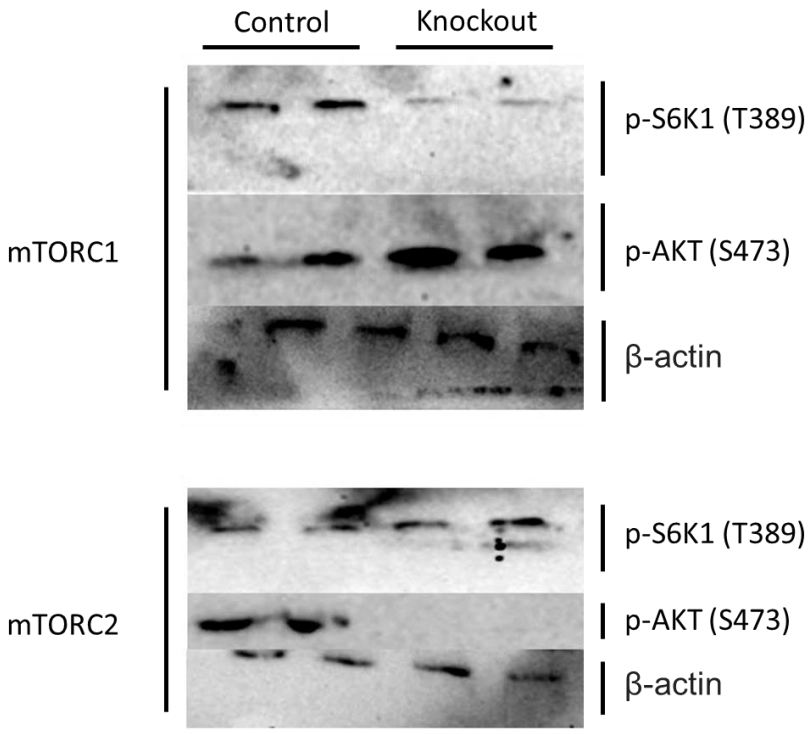

Figure 3. Western Blots to Characterize Phosphorylation Activity in Mice with a Liver-Specific Deletion of mTORC1 or 2. Western blots were conducted for mTORC1 and 2 knockouts animals and their respective control controls exposed to vehicle only. β-actin was used as a loading control.

specific deletion of *Raptor* increases mTORC2 activity, while liver-specific deletion of *Rictor* likely increases the activity of mTORC1. However, given that due to the coronavirus outbreak we were unable to obtain western blots of total S6K1 and AKT before labs were required to close, these results should be taken with caution.

Liver-specific deletion of mTORC1 or 2 did not significantly alter blood-liver balance of triglycerides

To assess the effect of mTOR function on hepatic triglyceride levels, we measured circulating and hepatic triglyceride levels *(N*=2 for the mTORC1 control and knockout, *N*=3 for the mTORC2 control and knockout). We found no statistically significant differences in blood or serum triglyceride levels in mice with a liver-specific deletion of mTORC1 or 2 compared to controls.

Metascape reveals similar and different biological functions regulated by mTORC1 and 2 at the transcriptional level

To identify systems-level effects of mTOR pathway perturbation, we next analyzed the genes that were significantly affected by mTORC1 (347 genes in total, FDR q-value < 0.05), as well as genes affected by mTORC2 knockout (104 genes in total, FDR q-value < 0.05) were analyzed using Metascape, a publicly available web tool to characterize transcriptomic and genetics datasets based on gene list annotations and enriched ontology terms (Zhou et al., 2019). Results for mTORC1 (Figure 4A) indicate that mTORC1 knockout significantly alters expression of genes involved in a wide variety of biological functions, most commonly functions involved in lipid metabolism (such as “metabolism of steroids,” “monocarboxylic acid metabolic

Figure 4. Metascape Reveals Ontology Terms Regulated by mTORC1 or 2. Ontology terms associated with genes significantly affected by mTORC1 are presented in **A** and indicate that mTORC1 plays a role in regulating expression of genes associated with lipid metabolism, innate immunity, cell growth, and many other key biological functions in the liver. Ontology terms enriched for genes significantly affected by mTORC2 knockout are shown in **B** and appear to be primarily involved in lipid metabolism, innate immunity, and ion transport. Common enriched terms were "monocarboxylic acid metabolic process," "triglyceride metabolic process," "PPAR signaling pathway," and "response to stillbenoid," "integration of energy metabolism" and "neutrophil degranulation." All other enriched terms for mTORC1 or 2 were unique for each knockout.

process," "regulation of small molecule metabolic process," "cytochrome P450 - arranged by substrate type," "triglyceride metabolic process," "fatty acid metabolism," and "PPAR signaling"), innate immunity (such as "regulation of chemokine production," "acute inflammatory response," "neutrophil degranulation," "extrinsic apoptotic pathway via death domain receptors," and "regulation of mast cell activation"), and cell cycle processes (such as "regulation of mitotic cell cycle, "cyclin D1-associated complex," "cell cycle arrest" and "positive regulation of cell growth"). Other notable processes generally affected by mTORC1 knockout include energy metabolism ("positive regulation of oxidative

phosphorylation" and "integration of energy metabolism"), protein processing and amino acid metabolism ("negative regulation of proteolysis," "negative regulation of protein processing," "serine family amino acid metabolic process," and "negative regulation of protein secretion"), cell death ("positive regulation of cell death," "negative regulation of anoikis," "apoptosis," "extrinsic apoptotic signaling pathway via death domain receptors," and "negative regulation of neuron death"), and xenobiotic metabolism ("cytochrome P450 - arranged by substrate type," and "response to toxic substance"). Additionally, the terms "regulation of type B pancreatic cell proliferation" and "response to hypoxia" were enriched as well.

Metascape results for mTORC2 knockouts revealed that, deletion of mTORC2 affected expression of genes involved in a narrower variety of cellular processes (Figure 4B). Enriched terms for mTORC2 knockout included terms involved in lipid metabolism (such as "monocarboxylic acid metabolic process," "monocarboxylic acid biosynthetic process," "regulation of small molecule metabolic process," "triglyceride metabolic process," "steroid metabolic process," "PPAR signaling pathway," and "lipid catabolic process"), innate immunity (such as "monocyte differentiation," "cell chemotaxis," "neutrophil degranulation," "regulation of reactive oxygen species biosynthetic process," "interleukin-1 secretion," and "positive regulation of NIK/NK-kappaB signaling"), and ion transport ("cellular transition metal ion homeostasis" and "transport of bile salts and organic acids, metal ions and amine compounds"). Notably, the terms "maturity onset diabetes of the young," and "regulation of cellular response to growth factor stimulus" were also enriched. While the majority of ontology terms enriched due to mTORC1 and 2 were unique between the two knockouts, there were a portion of similar terms. These included terms involved in lipid metabolism ("monocarboxylic acid metabolic process," "triglyceride metabolic process," "PPAR signaling pathway"), "response to

stillbenoid," "integration of energy metabolism" and "neutrophil degranulation" (Figure 4 A and B). Additionally, deletion of both mTORC1 and mTORC2 knockouts in enrichment of similar terms involved in temperature response: "positive regulation of cold-induced thermogenesis" for mTORC1 knockout and "response to temperature stimulus" for mTORC2 knockout.

Comparative analysis of the roles of mTORC1 and 2 at the transcriptomic level identifies novel independent, similar, and opposing regulatory functions for both complexes

The above analysis indicates that the mentioned genes and ontology terms are affected by deletion of mTORC1 and/or mTORC2, but does not indicate the direction in which these genes and ontology terms are regulated by each complex. Thus, it is entirely possible that terms present in the above Metascape results for both complexes (Figure 4A and 4B) are regulated in similar or opposing directions due to knockout of mTORC1 and/or 2. It is also difficult to identify which ontology terms present in the results for knockout of one mTOR complex are truly independent of the other complex. This is especially true given the results of our protein immunoblots, which reveal that knockout of one mTOR complex increases the activity of the other (Figure 3). As such, we elected to parse through genes significantly affected by mTORC1 and 2 to obtain these results using a novel comparison analysis in combination with Metascape.

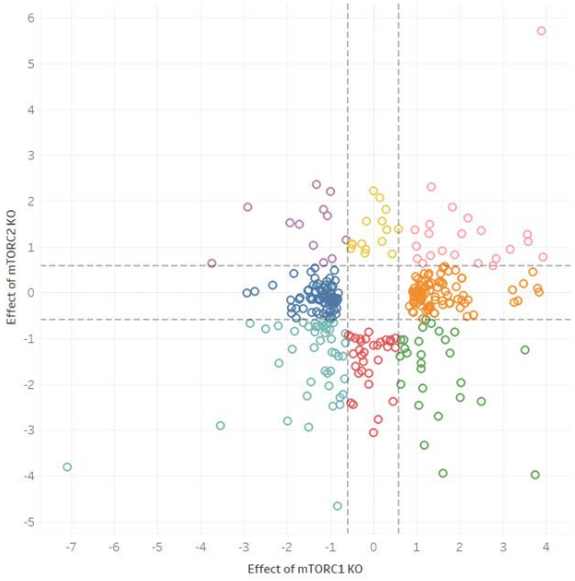

Figure 5. Comparison Analysis Reveals Similar and Differential Regulatory Roles for mTORC1 and 2 at the Transcriptional Level. The $\log_2$(ratio) of each gene significant in at least one of the two comparisons is plotted for both mTORC1 and mTORC2 comparisons ("control" versus respective knockout). Grey dashed lines perpendicular to an axis mark a 1.5-fold ($\log_2$(ratio)= 0.59 or -0.59) change in expression due to the knockout of an mTOR complex. These dashed lines were used to generate each colored group for subsequent analysis. Genes considered to be upregulated by mTORC1 knockout only are shown in orange, while those downregulated by mTORC1 knockout only are shown in blue. Genes considered to be upregulated by mTORC2 knockout only are shown in yellow, while genes downregulated by mTORC2 knockout only are shown in red. Genes upregulated by mTORC1 knockout and downregulated by mTORC2 knockout are in green, while genes downregulated by mTORC1 knockout and upregulated by mTORC1 knockout are down in purple. Genes that were upregulated by both mTORC1 and 2 knockout are in pink, while those downregulated by both knockouts are in teal.

Log_2(ratio) values for genes in the mTORC1 knockout comparison (mTORC1 control versus mTORC1 knockout), were plotted against log_2(ratio) values for genes in the mTORC2 knockout comparison (mTORC2 control versus mTORC2 knockout). Only genes that were statistically significant in at least one comparison were included in our analyses, while genes with less than 1.5-fold change (log_2(ratio) = 0.59 or -0.59) in both comparisons were excluded. These genes were then divided into eight groups based on whether they were altered at least 1.5-fold by the mTORC1 knockout, mTORC2 knockout, or both, as well as the directions in which the fold change occurred for each comparison. This analysis revealed a multitude of genes that fell into four groups: genes dependent on mTORC1 only (orange and blue, Figure 5), genes dependent on mTORC2 only (yellow and red, Figure 5), genes regulated in opposite directions by mTORC1 and 2 (green and purple, Figure 5), and genes regulated in similar directions by both complexes (pink and teal, Figure 5). Each of these four sets of genes were analyzed via Metascape to clearly differentiate the hepatic regulation of liver gene expression by both of these complexes.

Metascape analysis for genes dependent on mTORC1 only (Figure 6) revealed results that were like Figure 4A with some notable differences. Significantly enriched ontology terms in this category were again related to lipid metabolism (“Cholesterol biosynthesis,” “small molecule biosynthesis,” “regulation of small molecule metabolic process,” and “lipid homeostasis”), innate immunity (“regulation of chemokine production,” “inflammatory response,” “neutrophil degranulation,” and “platelet degranulation”), cell cycle (“negative regulation of cell proliferation, negative regulation of cell cycle”), and protein processing (“regulation of protein maturation,” and “HSP90 chaperone cycle for steroid hormone receptors (SHR)”). Additionally, the terms “response to stilbenoid” and “response to hypoxia” were enriched here as well. New results for this analysis included “adaptive immune system,”

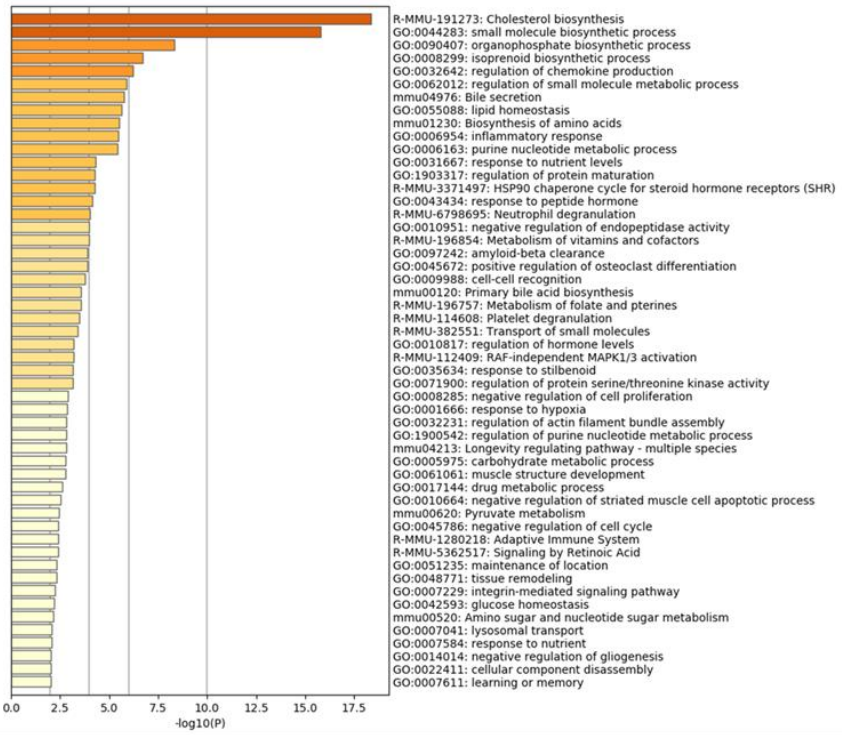

Figure 6. Metascape Results for Genes Dependent on mTORC1 Only. Genes for which expression is dependent on mTORC1 in the liver are primarily involved in lipid metabolism, the immune system, cell cycle regulation, protein processing, sugar metabolism, and other key biological functions.

"longevity regulating pathway - multiple species," terms involved in glucose metabolism ("carbohydrate metabolic process," and "pyruvate metabolism"), "signaling by retinoic acid" and "regulation of actin filament bundle assembly." Notably, the terms "PPAR signaling," "regulation of type B pancreatic cell proliferation," "positive regulation of cold-induced thermogenesis," and many apoptosis-related terms present in Figure 4A were absent from the enrichment results of Figure 6.

For the sake of brevity, as this group of genes was larger than all other resulting groups, a list of all genes dependent on mTORC1 only can be found in Table A1 in the appendix. Selected genes dependent on mTORC1 knockout only are shown in Table 1. Upregulated genes are involved in functions such as lipid metabolism, immune system function, xenobiotic metabolism, cell cycle regulation, and cytoskeletal structure. Downregulated genes are associated with cytoskeletal structure, autophagy regulation, xenobiotic metabolism, cell adhesion, immunity lipid metabolism, and small molecule/ion transport.

Table 1. Selected genes dependent on mTORC1 only.

Genes upregulated by mTORC1 KO		Genes downregulated by mTORC1 KO	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Apoa4	apolipoprotein A-IV	Tedc2	tubulin epsilon and delta complex 2
Gstp3	Glutathione S-transferase pi 3	Depp1	DEPP1 autophagy regulator
C1qb	complement component 1, q subcomponent, beta polypeptide	Cd24a	CD24a antigen
Ccl6	chemokine (C-C motif) ligand 6	Cyp4f14	cytochrome P450, family 4, subfamily f, polypeptide 14
Ccnd1	cyclin D1	Cyp7b1	cytochrome P450, family 7, subfamily b, polypeptide 1
Cd5l	CD5 antigen-like	Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1
Cd9	CD9 antigen	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1
Cd63	CD63 antigen	Cyp51	cytochrome P450, family 51
Cd83	CD83 antigen	Elovl3	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3
Cd84	CD84 antigen	Gstp1	glutathione S-transferase, pi 1
Cybb	cytochrome b-245, beta polypeptide	Il6ra	interleukin 6 receptor, alpha
Igsf11	immunoglobulin superfamily, member 11	Ldlr	low density lipoprotein receptor
Lpl	lipoprotein lipase	Lpin1	lipin 1
Rarb	retinoic acid receptor, beta	Scap	SREBF chaperone
Tlr12	toll-like receptor 12	Slc13a2	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2

Tuba8	tubulin, alpha 8	Slc17a8	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8
Tubb2a	tubulin, beta 2A class IIA	Slc22a18	solute carrier family 22 (organic cation transporter), member 18
Tubb2b	tubulin, beta 2B class IIB	Slc37a4	solute carrier family 37 (glucose-6-phosphate transporter), member 4
		Slc39a4	solute carrier family 39 (zinc transporter), member 4
		Slco2a1	solute carrier organic anion transporter family, member 2a1

For Metascape results of genes dependent on mTORC2 only (Figure 7), there were significantly fewer enriched ontology terms. These enriched terms were primarily related to lipid metabolism ("unsaturated fatty acid metabolic process," and " "lipid transport"), and innate immunity ("neutrophil degranulation "regulation of symbiosis, encompassing a mutualism through parasitism," and "regulation of reactive oxygen species metabolic process"). Interestingly, "transmembrane receptor protein tyrosine kinase signaling pathway" was the most significantly enriched ontology term. Notable terms absent from this analysis that were present in Figure 4B include "PPAR signaling" and other terms related to glucose and lipid metabolism.

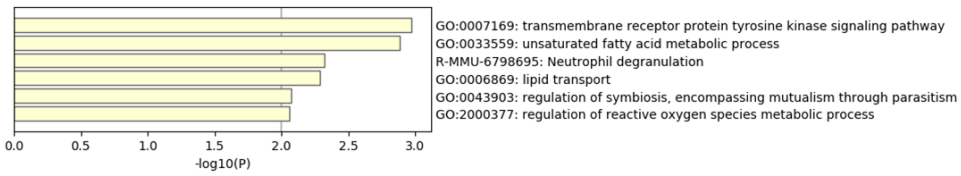

Figure 7. Metascape Results for Genes Dependent on mTORC2 Only. Genes for which expression is dependent on mTORC2 in the liver are primarily involved in lipid metabolism, the immune system, xenobiotic metabolism, and ion transport.

All genes dependent on mTORC2 only are listed in Table 2. Genes upregulated by mTORC2 knockout were primarily related to lipid metabolism, xenobiotic metabolism, ribosome biogenesis, cell cycle, and innate immunity. Genes downregulated by mTORC2 deletion were also involved in innate immunity and lipid metabolism, as well as in xenobiotic metabolism, ion transport, and more.

Table 2. Genes dependent on mTORC2 only.

Genes upregulated by mTORC2 KO		Genes downregulated by mTORC2 KO	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	2510009E07Rik	RIKEN cDNA 2510009E07 gene
B930025P03Rik	RIKEN cDNA B930025P03 gene	Acot11	acyl-CoA thioesterase 11
Cish	cytokine inducible SH2-containing protein	Apcs	serum amyloid P-component
Cyp4a32	cytochrome P450, family 4, subfamily a, polypeptide 32	Atp11a	ATPase, class VI, type 11A
Fabp5	fatty acid binding protein 5, epidermal	Cdc42ep1	CDC42 effector protein (Rho GTPase binding) 1
Gbp11	guanylate binding protein 11	Chic1	cysteine-rich hydrophobic domain 1
Gstt2	glutathione S-transferase, theta 2	Clstn3	calsyntenin 3
Igfbp2	insulin-like growth factor binding protein 2	Nat8f5	N-acetyltransferase 8 (GCN5-related) family member 5
Inmt	indolethylamine N-methyltransferase	Col15a1	collagen, type XV, alpha 1
Nlrp12	NLR family, pyrin domain containing 12	Cxcl1	chemokine (C-X-C motif) ligand 1
Nrep	neuronal regeneration related protein	Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1

Rn45s	45S pre-ribosomal RNA	Dnajc12	DnaJ heat shock protein family (Hsp40) member C12
Rpl22l1	ribosomal protein L22 like 1	Epc1	enhancer of polycomb homolog 1
Socs2	suppressor of cytokine signaling 2	Mir1893	microRNA 1893
Zyg11a	zyg-11 family member A, cell cycle regulator	Frk	fyn-related kinase
		Gca	grancalcin
		Gm4956	predicted gene 4956
		Hipk3	homeodomain interacting protein kinase 3
		Mir1902	microRNA 1902
		Insc	INSC spindle orientation adaptor protein
		Lgalsl	lectin, galactoside binding-like
		Lrg1	leucine-rich alpha-2-glycoprotein 1
		Mfsd2a	major facilitator superfamily domain containing 2A
		Mir5113	microRNA 5113
		Tnrc6b	trinucleotide repeat containing 6b
		Nat8	N-acetyltransferase 8 (GCN5-related)
		Oasl1	2'-5' oligoadenylate synthetase-like 1
		Robo1	roundabout guidance receptor 1
		Saa3	serum amyloid A 3
		Slc10a2	solute carrier family 10, member 2

Slc30a10	solute carrier family 30, member 10
Slc41a2	solute carrier family 41, member 2
Spp1	secreted phosphoprotein 1
Treh	trehalase (brush-border membrane glycoprotein)
Tsc22d1	TSC22 domain family, member 1

Metascape analysis of genes that were regulated in opposing directions by mTORC1 and 2 deletion revealed that these genes are primarily associated with two general categories. The first was response to cellular stressors, such as xenobiotics ("cellular response to xenobiotic stimulus"), temperature changes ("temperature homeostasis"), and fluid shear stress ("fluid shear stress and atherosclerosis"), as well as general transcriptional regulation in response to stress ("regulation of DNA-templated transcription in response to stress"). The second category was expression regulation of protein cascades in which both mTORC1 and 2 are integral components. Ontology terms and pathways in this category include "negative regulation of cellular response to growth factor stimulus," "positive regulation of protein kinase activity," "regulation of wound healing," and "fluid shear stress and atherosclerosis."

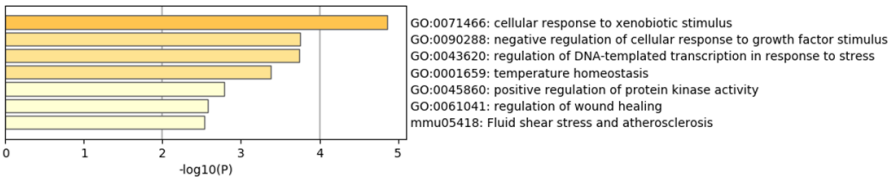

Figure 8. Metascape Results for Genes Inversely Dependent on mTORC1 and 2. Genes for which mTORC1 and 2 play opposing roles in gene expression regulation are primarily associated with responses to cellular stresses such as changes in temperature or xenobiotics, as well as ontology terms and pathways associated with mTOR signaling itself ("fluid shear stress and atherosclerosis," "negative regulation of cellular response to growth factor stimulus," and "positive regulation of protein kinase activity").

All genes oppositely changed by deletion of mTORC1 and 2 are listed in Table 3. Interestingly, there were approximately twice as many genes upregulated by deletion of mTORC and downregulated by deletion of mTORC2 as there were genes that responded in the opposite manner. Notable genes in this first category included *Cd36*, the regulator of WNT/beta-catenin signaling *Tcim*, multiple cytochrome p450 genes (*Cyp2b10*, *Cyp26a1*, *Cyp46a1*), the phosphatase *Dusp1*, the glutathione sulfotransferase *Gsta1*, and the chemoattractant *Cxcl14*. Some notable genes that were regulated in the opposite direction, that is, downregulated in response to mTORC1 deletion and upregulated in response to mTORC2 deletion, included the smooth muscle actin *Acta2*, the transcriptional regulator *Egr1*, and *Mir671*, a miRNA whose expression varies significantly between the alcoholic and non-alcoholic forms of fatty liver disease (Estep et al., 2010).

Table 3. Genes transcriptionally regulated by mTORC1 and 2 in opposing directions.

Genes upregulated by mTORC1 KO, downregulated by mTORC2 KO		Genes downregulated by mTORC1 KO, upregulated by mTORC2 KO	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Tcim	transcriptional and immune response regulator	Acta2	actin, alpha 2, smooth muscle, aorta
Gpat3	glycerol-3-phosphate acyltransferase 3	Adgrf1	adhesion G protein-coupled receptor F1
Cd36	CD36 molecule	Chpf2	chondroitin polymerizing factor 2
Cdcp1	CUB domain containing protein 1	Mir671	microRNA 671
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	Egr1	early growth response 1

Cib3	calcium and integrin binding family member 3	Grem2	gremlin 2, DAN family BMP antagonist
Cxcl14	chemokine (C-X-C motif) ligand 14	Cnmd	chondromodulin
Cyp2b10	cytochrome P450, family 2, subfamily b, polypeptide 10	Mcm10	minichromosome maintenance 10 replication initiation factor
Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1	Onecut1	one cut domain, family member 1
Cyp46a1	cytochrome P450, family 46, subfamily a, polypeptide 1	Rgs16	regulator of G-protein signaling 16
Dusp1	dual specificity phosphatase 1	Serpina4-ps1	serine (or cysteine) peptidase inhibitor, clade A, member 4, pseudogene 1
Enc1	ectodermal-neural cortex 1	Serpinb1a	serine (or cysteine) peptidase inhibitor, clade B, member 1a
Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	Tbc1d30	TBC1 domain family, member 30
Gpc1	glypican 1		
Gsta1	glutathione S-transferase, alpha 1 (Ya)		
Lad1	ladinin		
Lcn2	lipocalin 2		
Lrtm1	leucine-rich repeats and transmembrane domains 1		
Mir8104	microRNA 8104		

Ppm1h	protein phosphatase 1H (PP2C domain containing)
Mmd2	monocyte to macrophage differentiation-associated 2
Mt1	metallothionein 1
Mtnr1b	melatonin receptor 1B
Olfm3	olfactomedin 3
Ppl	periplakin
Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5
Sult1e1	sulfotransferase family 1E, member 1
Tceal8	transcription elongation factor A (SII)-like 8

Lastly, Metascape analysis of genes for which mTORC1 and 2 have similar regulatory roles (Figure 9) appear to be primarily associated with metabolism of lipids such as cholesterol and fatty acids ("monocarboxylic acid metabolic process," "small molecule biosynthetic process," "regulation of small molecule metabolic process," "cholesterol metabolic process," "lipid biosynthetic process," "fatty acid biosynthetic process," and "PPAR

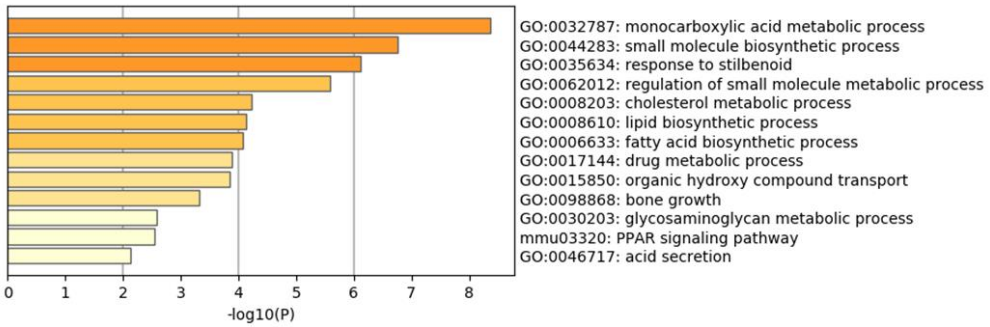

Figure 9. Metascape Results for Genes Similarly Altered in Expression by mTORC1 and 2 Knockouts. Genes for which mTORC1 and 2 have similar regulatory roles appear to be primarily associated with metabolism of lipids such as cholesterol and fatty acids and regulation of extracellular matrix components ("bone growth," and "glycosaminoglycan metabolic process").

signaling"), ribonucleotide metabolism ("monocarboxylic acid metabolic process," "small molecule biosynthetic process," "regulation of small molecule metabolic process") as well as and regulation of extracellular matrix components ("bone growth," and "glycosaminoglycan metabolic process"). Additionally, ontology terms related to genes similarly regulated by both mTOR complexes also include "response to stillbenoid," "organic hydroxy compound transport," "drug metabolic process," and "acid secretion."

All genes regulated in similar directions by deletion of mTORC1 and 2 are listed in Table 4. Notable genes upregulated by both knockouts include acyl-CoA thioesterases *Acot1*, *2*, and *3*, the calcium-binding growth regulator *Cgref1*, multiple cytochrome P450 genes (*Cyp2a4*, *Cyp2b9*, and *Cyp4a14*), the beta-defensin *Defb1*, the leptin receptor *Lepr*, the growth regulator gene *Myc*, the ubiquitin-conjugating enzyme *Ube2c*, and the ubiquitin-specific peptidase *Usp18*. Notable genes downregulated in both knockouts include the aquaporin *Aqp8*, the collagen and extracellular matrix protein *Col27a1*, the glucokinase gene *Gcn*, the protein kinase *Mapk15*, the liver lipogenesis regulator *Srebf1*, and the thyroid-hormone-inducible hepatic protein *Thrsp*.

Table 4. Genes upregulated by mTORC1 and 2 KO in similar directions.

Upregulated in both knockouts		Downregulated in both knockouts	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Acot1	acyl-CoA thioesterase 1	2900076A07Rik	RIKEN cDNA 2900076A07 gene
Acot2	acyl-CoA thioesterase 2	Mir1839	microRNA 1839
Acot3	acyl-CoA thioesterase 3	Abca3	ATP-binding cassette, sub-family A (ABC1), member 3
Cgref1	cell growth regulator with EF hand domain 1	Acly	ATP citrate lyase

Chmp4c	charged multivesicular body protein 4C	Acpp	acid phosphatase, prostate
Cidec	cell death-inducing DFFA-like effector c	Aqp8	aquaporin 8
Clec2h	C-type lectin domain family 2, member h	Capn8	calpain 8
Cln6	ceroid-lipofuscinosis, neuronal 6	Col27a1	collagen, type XXVII, alpha 1
Cyp2a4	cytochrome P450, family 2, subfamily a, polypeptide 4	Csad	cysteine sulfinic acid decarboxylase
Cyp2b9	cytochrome P450, family 2, subfamily b, polypeptide 9	Dct	dopachrome tautomerase
Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14	Dpy19l3	dpy-19-like 3 (C. elegans)
D17H6S56E-5	DNA segment, Chr 17, human D6S56E 5	Extl1	exostoses (multiple)-like 1
Defb1	defensin beta 1	Fasn	fatty acid synthase
Dsg1c	desmoglein 1 gamma	Fitm1	fat storage-inducing transmembrane protein 1
Gadd45b	growth arrest and DNA-damage-inducible 45 beta	Gck	glucokinase
Lepr	leptin receptor	Gm12718	predicted gene 12718
Meg3	maternally expressed 3	Gna14	guanine nucleotide binding protein, alpha 14
Mir1906-1	microRNA 1906-1	Hcn3	hyperpolarization-activated, cyclic nucleotide-gated K+ 3
Mir770	microRNA 770	Hes6	hairy and enhancer of split 6
Myc	myelocytomatosis oncogene	Kcnk5	potassium channel, subfamily K, member 5
Nipal1	NIPA-like domain containing 1	Klf13	Kruppel-like factor 13
Scn3a	sodium channel, voltage-gated, type III, alpha	Maoa	monoamine oxidase A

Slc25a30	solute carrier family 25, member 30	Mapk15	mitogen-activated protein kinase 15
Ube2c	ubiquitin-conjugating enzyme E2C	Me1	malic enzyme 1, NADP(+)-dependent, cytosolic
Usp18	ubiquitin specific peptidase 18	Mid1ip1	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish))
		Moxd1	monooxygenase, DBH-like 1
		Pklr	pyruvate kinase liver and red blood cell
		Ppp1r3b	protein phosphatase 1, regulatory subunit 3B
		Rcan2	regulator of calcineurin 2
		Saa1	serum amyloid A 1
		Saa2	serum amyloid A 2
		Scara5	scavenger receptor class A, member 5
		Scd1	stearoyl-Coenzyme A desaturase 1
		Serpina12	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12
		Serpine2	serine (or cysteine) peptidase inhibitor, clade E, member 2
		Slc6a9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9
		Slc15a5	solute carrier family 15, member 5

Smpd3	sphingomyelin phosphodiesterase 3, neutral
Snhg11	small nucleolar RNA host gene 11
Srebf1	sterol regulatory element binding transcription factor 1
Syde2	synapse defective 1, Rho GTPase, homolog 2 (C. elegans)
Syt3	synaptotagmin III
Thrsp	thyroid hormone responsive
Tiam2	T cell lymphoma invasion and metastasis 2
Tmie	transmembrane inner ear
Tsc22d3	TSC22 domain family, member 3

Overall, dependency analysis revealed a multitude of biological functions that are dependent on mTORC1 only, dependent on mTORC2 only, regulated similarly by both complexes, and regulated in opposing directions by both of these complexes. Based on the above dependency analysis, there were 287 genes that appeared to be "dependent" on mTORC1 in some manner, and 162 genes that were "dependent" on mTORC2. Of these genes, there were 112 that were dependent on both mTORC1 and mTORC2, though the direction in

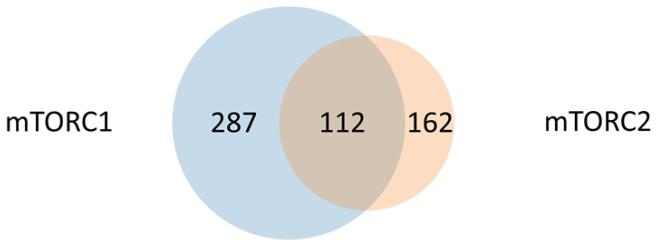

Figure 10. Overlap of Genes whose Expression was Altered by mTORC1 and mTORC2 Knockouts Filtered by Dependency Analysis. Dependency analysis revealed that 287 genes were dependent on mTORC1 (blue circle), while 162 were dependent on mTORC2 (orange circle). There were 112 genes whose expression was altered by knockout of both mTORC1 and 2 (brown overlap).

which each overlapping gene was regulated as a result of each mTOR complex knockout was not necessarily the same for both complexes (Figure 10). These results suggest that mTORC1 and 2 have roles in hepatic gene expression regulation that partially overlap.

Gene set enrichment analysis reveals novel independent, similar, and opposing regulatory functions for both complexes at the systems level

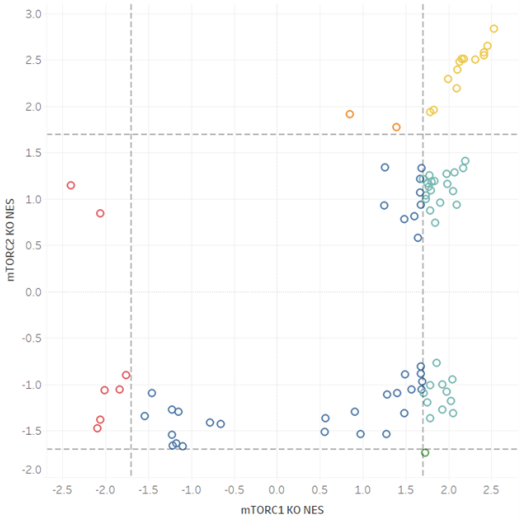

Figure 11. Comparison of Significantly Enriched GSEA Gene Sets for mTORC1 and 2 Knockouts. Gene set enrichment results are presented for all gene sets with an FDR q-value of 0.25 or less for at least one of the two knockouts. That crossed the threshold normalized enrichment score (NES) of 1.7 or -1.7 for one or both knockouts were considered significant. Gene sets upregulated by mTORC1 KO only are shown in teal, gene sets downregulated by mTORC1 KO only are shown in red, gene sets upregulated by mTORC2 KO only are shown in orange, gene sets that were upregulated by both mTORC1 and 2 KO are shown in yellow, and the one gene set upregulated by mTORC1 KO and downregulated by mTORC2 KO is shown in green. Gene sets that did not pass the threshold NES of 1.7 or -1.7 for either comparison are shown in blue.

To complement our analysis of the short list of genes significantly affected by mTORC1 and mTORC1 deletion, we used gene set enrichment analysis (GSEA) to identify subtle trends in expression of specific gene groups which may have biological meaning. This was done using the full list of RNA-seq data for both mTORC1 and mTORC2 deletion, as compared to analyzing only significantly differentially expressed genes. Per Broad Institute GSEA recommendations, we used an FDR q-value of 0.25 and a normalized enrichment score (NES) of less than -1.7 or greater than 1.7 as the cutoff for significance. The Hallmark, Reactome, and Kegg gene set collections were used for this analysis. By comparing the NES of each gene set between sequencing datasets for the mTORC1 knockout and mTORC2 knockout, we were able to identify and compare the potential regulatory roles that mTORC1 and 2 may have at the systems level in terms of specific genes sets and cellular pathways in the liver (Figure 11). Table 5 presents the full list of significant gene sets and their relative dependencies on mTORC1 and 2. Interestingly, most significant gene sets appear to be dependent on mTORC1 only. Gene sets positively

enriched due to mTORC1 knockout only are primarily related to cell cycle regulation (such as "HALLMARK_E2F_TARGETS," "REACTOME_CELL_CYCLE_CHECKPOINTS," REACTOME_MITOTIC_METAPHASE_AND_ANAPHASE, and "REACTOME_APC_C:CDC20_MEDIATED_DEGRADATION_OF_CYCLIN_B"), actin cytoskeletal regulation ("REACTOME_RHO_GTPASE_EFFECTORS," and "REACTOME_RHO_GTPASES_ACTIVATE_FORMINS"), cellular adhesion ("REACTOME_L1CAM_INTERACTIONS"), and immunity/inflammation ("HALLMARK_ALLOGRAFT_REJECTION," "HALLMARK_INFLAMMATORY_RESPONSE," "KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION," "REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL," and others). Interestingly, gene sets associated with cellular stress and senescence "REACTOME_DNA_DAMAGE_TELOMERE_STRESS_INDUCED_SENESCENCE" and "REACTOME_THE_ROLE_OF_GTSE1_IN_G2_M_PROGRESSION_AFTER_G2_CHECKPOINT" were positively enriched as well. In summary, it appears that mTORC1 knockout upregulates gene sets associate with cell cycle regulation, inflammation/innate immunity, cell adhesion/actin cytoskeleton dynamics, and cell senescence/apoptosis functions (that may occur via P53).

Gene sets negatively enriched by mTORC1 knockout only are primarily associated with liver lipid metabolism ("HALLMARK_BILE_ACID_METABOLISM," "HALLMARK_CHOLESTEROL_HOMEOSTASIS," "REACTOME_ACTIVATION_OF_GENE_EXPRESSION_BY_SREBF_SREBP,"

"REACTOME_CHOLESTEROL_BIOSYNTHESIS," and "REACTOME_REGULATION_OF_CHOLESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF"). Additionally, the gene sets "HALLMARK_XENOBIOTIC_METABOLISM," and "KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM" were downregulated due to mTORC1 knockout only as well.

Interestingly, only two gene sets were upregulated by mTORC2 knockout only ("HALLMARK_MYC_TARGETS_V2" and "HALLMARK_OXIDATIVE_PHOSPHORYLATION"), while no gene set was significantly downregulated as a result of mTORC2 knockout only. However, there was a trend of several gene sets with an FDR q-value of 0.25 or less towards our threshold for negative enrichment for mTORC2 knockout, which may have some sort of biological meaning (Figure 11, marked in blue).

The only gene set that was significantly upregulated by mTORC1 knockout and significantly downregulated by mTORC2 knockout was the gene set "REACTOME_INTERLEUKIN_4_AND_INTERLEUKIN_13_SIGNALING." Unlike the results for specific genes in our Metascape dependency analysis, there were no gene sets downregulated by mTORC1 knockout and upregulated by mTORC2 knockout.

While no gene sets were downregulated by deletion of both mTORC1 or mTORC2, a multitude of gene sets were upregulated by both. These gene sets were primarily related to ribosomal biogenesis (such as "KEGG_RIBOSOME" and "REACTOME_RRNA_PROCESSING"), translation (such as "REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDIN

G_COMPLEX_AND_EIFS_AND_SUBSEQUENT_BINDING_TO_43S," and "REACTOME_INFLUENZA_INFECTION,"), nonsense-mediated decay ("REACTOME_NONSENSE_MEDIATED_DECAY_NMD" and "REACTOME_NONSENSE_MEDIATED_DECAY_NMD_INDEPENDENT_OF_THE_EXON_JUNCTION_COMPLEX_EJC"), SLIT/ROBO signaling ("REACTOME_REGULATION_OF_EXPRESSION_OF_SLITS_AND_ROBOS" and "REACTOME_SIGNALING_BY_ROBO_RECEPTORS"), and selenoamino acid metabolism ("REACTOME_SELENOAMINO_ACID_METABOLISM").

Table 5. GSEA results for mTORC1 and 2 knockouts.

Gene Set	mTORC1 KO NES	mTORC2 KO NES	Result
KEGG_RIBOSOME	2.526	2.832	
REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDING_COMPLEX_AND_EIFS_AND_SUBSEQUENT_BINDING_TO_43S	1.994	2.293	
REACTOME_EUKARYOTIC_TRANSLATION_INITIATION	2.409	2.546	
REACTOME_INFLUENZA_INFECTION	2.154	2.508	
REACTOME_NONSENSE_MEDIATED_DECAY_NMD	2.313	2.501	
REACTOME_NONSENSE_MEDIATED_DECAY_NMD_INDEPENDENT_OF_THE_EXON_JUNCTION_COMPLEX_EJC	2.451	2.646	
REACTOME_REGULATION_OF_EXPRESSION_OF_SLITS_AND_ROBOS	2.099	2.192	Upregulated in both mTORC1 and mTORC2 KO
REACTOME_RRNA_PROCESSING	2.105	2.395	
REACTOME_RRNA_PROCESSING_IN_THE_NUCLEUS_AND_CYTOSOL	2.182	2.511	
REACTOME_SELENOAMINO_ACID_METABOLISM	2.130	2.476	
REACTOME_SIGNALING_BY_ROBO_RECEPTORS	1.828	1.963	
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	2.412	2.580	
REACTOME_TRANSLATION	1.788	1.935	
REACTOME_INTERLEUKIN_4_AND_INTERLEUKIN_13_SIGNALING	1.725	-1.734	Upregulated in mTORC1 KO, downregulated in mTORC2 KO
HALLMARK_ALLOGRAFT_REJECTION	1.976	-1.081	
HALLMARK_E2F_TARGETS	2.168	1.335	Upregulated in mTORC1 KO only
HALLMARK_G2M_CHECKPOINT	2.092	0.934	
HALLMARK_IL6_JAK_STAT3_SIGNALING	1.707	-1.095	

HALLMARK_INFLAMMATORY_RESPONSE	1.754	-1.195	
HALLMARK_INTERFERON_GAMMA_RESPONSE	1.861	-0.769	
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.787	-1.365	
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	1.925	-1.274	
REACTOME_APC_C:CDC20_MEDIATED_DEGRADATION_OF_CYCLIN_B	1.778	1.251	
REACTOME_CELL_CYCLE_CHECKPOINTS	1.802	1.181	
REACTOME_CELL_CYCLE_MITOTIC	1.767	1.132	
REACTOME_CHROMOSOME_MAINTENANCE	1.737	1.000	
REACTOME_CONDENSATION_OF_PROPHASE_CHROMOSOMES	2.041	-0.948	
REACTOME_DNA_DAMAGE_TELOMERE_STRESS_INDUCED_SENESCENCE	1.930	-0.999	
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	2.053	-1.315	
REACTOME_INHIBITION_OF_THE_PROTEOLYTIC_ACTIVITY_OF_APC_C_REQUIRED_FOR_THE_ONSET_OF_ANAPHASE_BY_MITOTIC_SPINDLE_CHECKPOINT_COMPONENTS	1.752	1.167	
REACTOME_INTERLEUKIN_10_SIGNALING	2.029	-1.180	
REACTOME_KINESINS	1.848	0.746	
REACTOME_L1CAM_INTERACTIONS	1.789	0.877	
REACTOME_M_PHASE	1.734	1.041	
REACTOME_MITOTIC_METAPHASE_AND_ANAPHASE	1.833	1.194	
REACTOME_MITOTIC_PROMETAPHASE	1.976	1.270	
REACTOME_MITOTIC_SPINDLE_CHECKPOINT	2.070	1.285	
REACTOME_NUCLEOSOME_ASSEMBLY	1.906	0.957	
REACTOME_PHOSPHORYLATION_OF_THE_APC_C	1.984	1.164	
REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE	1.706	1.214	
REACTOME_RESOLUTION_OF_SISTER_CHROMATID_COHESION	2.199	1.411	
REACTOME_RHO_GTPASE_EFFECTORS	1.786	-1.005	
REACTOME_RHO_GTPASES_ACTIVATE_FORMINS	2.053	1.083	
REACTOME_THE_ROLE_OF_GTSE1_IN_G2_M_PROGRESSION_AFTER_G2_CHECKPOINT	1.791	1.092	
HALLMARK_BILE_ACID_METABOLISM	-2.063	0.842	
HALLMARK_CHOLESTEROL_HOMEOSTASIS	-1.759	-0.904	
HALLMARK_XENOBIOTIC_METABOLISM	-1.834	-1.052	
KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM	-2.006	-1.067	Downregulated in mTORC1 KO only
REACTOME_ACTIVATION_OF_GENE_EXPRESSION_BY_SREBF_SREBP	-2.063	-1.377	
REACTOME_CHOLESTEROL_BIOSYNTHESIS	-2.398	1.149	
REACTOME_REGULATION_OF_CHOLESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF	-2.091	-1.476	
HALLMARK_MYC_TARGETS_V2	1.392	1.774	

HALLMARK_OXIDATIVE_PHOSPHORYLATION	0.848	1.915	Upregulated in mTORC2 KO only

Ingenuity Pathway Analysis reveals novel predicted upstream regulators and a regulatory network of mTOR-controlled transcription factors.

To identify transcription regulators potentially involved in mediating the statistically significant changes in gene expression observed in our mice with a liver-specific deletion of mTORC1 or mTORC2, we analyzed the two short lists of differentially expressed genes using the upstream analysis function of IPA (Table 6). We elected to only analyze our gene expression data for associated upstream transcription factors and nuclear receptors. This analysis indicated that as a result of mTORC1 knockout, 12 transcription regulators were predicted to be activated upstream of our observed changes in liver gene expression (such as STAT1, JUN, CREB1, PPARA, E2F3, NFKB2, and RARA), while 13 transcription regulators were predicted to be inhibited (such as SREBF1, STAT5B, TP73, TRIM24, and SREBF2). As a result of mTORC2 knockout, there were 10 transcription regulators predicted to be inhibited (such as NR1H3, PPARD, RORA, STAT5B, and HNF1A), but no predicted activated regulators. This observation is consistent with the general trend observed in Metascape and GSEA data that mTORC1 deletion has a wider effect on hepatic gene expression regulation in the liver compared to mTORC2 deletion. Notably, there was only one predicted transcription factor that overlapped between the mTORC1 and mTORC2 knockout datasets: a key element of JAK/STAT signal transduction involved in cytokine and growth factor signaling (Nadeau, Hwa, and Rosenfeld, 2011).

Next, we took the predicted activated and inhibited transcription factors for both mTOR complex knockouts and used the grow function of IPA to generated a predicted network of transcription regulators downstream of mTORC1 (Figure 12A) and mTORC2 (Figure 12B) using the predicted transcription factors for each knockout as input. Interestingly, while all the predicted transcription factors from upstream analysis were distinct between deletions of mTORC1 and mTORC2 except for STAT5B, the growth function of IPA included this transcription factor in the network for mTORC1 only, and included the transcription regulator CEPBA in this same network, despite being predicted as an upstream regulator for mTORC2 only. Likewise, the transcription regulator SIRT2 was included in the mTORC2 network, despite being predicted as an upstream regulator for mTORC1 knockout only during upstream analysis. The two predicted regulatory networks in Figure 12 intersect at SREBF1, but many regulators included in these two networks downstream of SREBF1 are distinct for each mTOR complex.

Table 6. IPA predicted transcription factors and nuclear receptors for mTORC1 and mTORC2 knockouts.

Predicted upstream regulators for mTORC1 KO			Predicted upstream regulators for mTORC2 KO		
Name	Direction	Zscore	Name	Direction	Zscore
STAT1	Activated	2.288	ZBTB20	Inhibited	-2.449
JUN	Activated	2.08	NR1H3	Inhibited	-2.432
CREB1	Activated	2.28	PPARD	Inhibited	-2.464
PPARA	Activated	2.655	RORA	Inhibited	-2.219
HOXC8	Activated	2.412	CEBPA	Inhibited	-2.342
FOXM1	Activated	2.516	MLX	Inhibited	-2.207
MYBL2	Activated	2.77	CEBPB	Inhibited	-2.375
E2F3	Activated	2.707	ETV5	Inhibited	-2.236
MITF	Activated	2.853	**STAT5B***	**Inhibited**	**-2.052**
NFKB2	Activated	2.219	HNF1A	Inhibited	-2.153
RARA	Activated	2.281			
MTPN	Activated	2.19			
SIRT2	Inhibited	-2.646			
ZFP36	Inhibited	-2.592			
HOXA10	Inhibited	-2.137			
SREBF1	Inhibited	-2.428			
CDKNZA	Inhibited	-2.08			
STAT5B*	**Inhibited**	**-2.674**			
TP73	Inhibited	-3.048			
HNF4A	Inhibited	-2.056			
NCOA2	Inhibited	-3.037			
MAF	Inhibited	-2.425			
TRIM24	Inhibited	-2.219			

KDM5B	Inhibited	-2.273
SREBF2	Inhibited	-3.287

*STAT5B was the one transcription factor predicted to be inhibited due to deletion of both mTORC1 and mTORC2

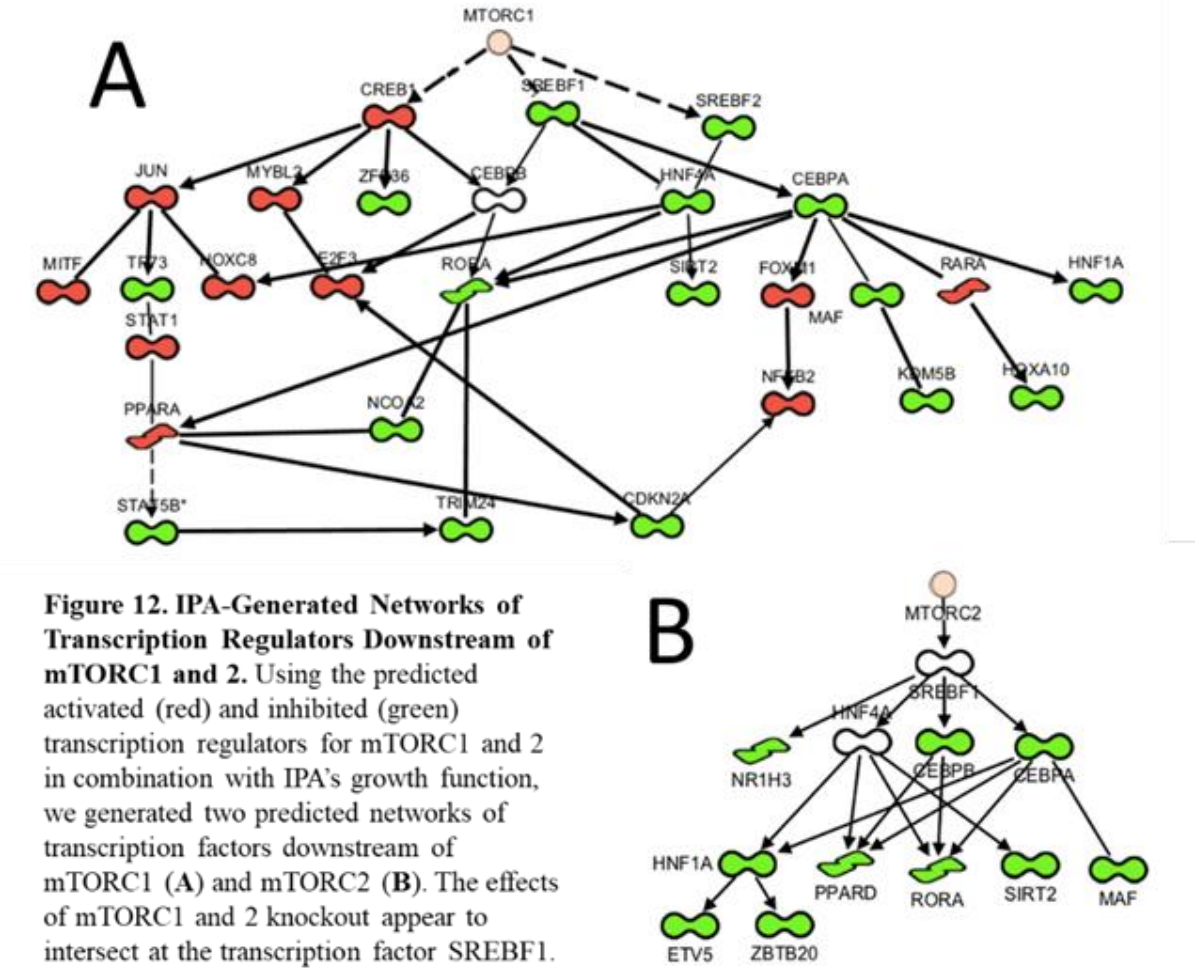

Figure 12. IPA-Generated Networks of Transcription Regulators Downstream of mTORC1 and 2. Using the predicted activated (red) and inhibited (green) transcription regulators for mTORC1 and 2 in combination with IPA's growth function, we generated two predicted networks of transcription factors downstream of mTORC1 (**A**) and mTORC2 (**B**). The effects of mTORC1 and 2 knockout appear to intersect at the transcription factor SREBF1.

Ingenuity Pathway analysis identifies similar and dissimilar associations with diseases, biological functions, and toxicological responses at the systems level for mTORC1 and mTORC2

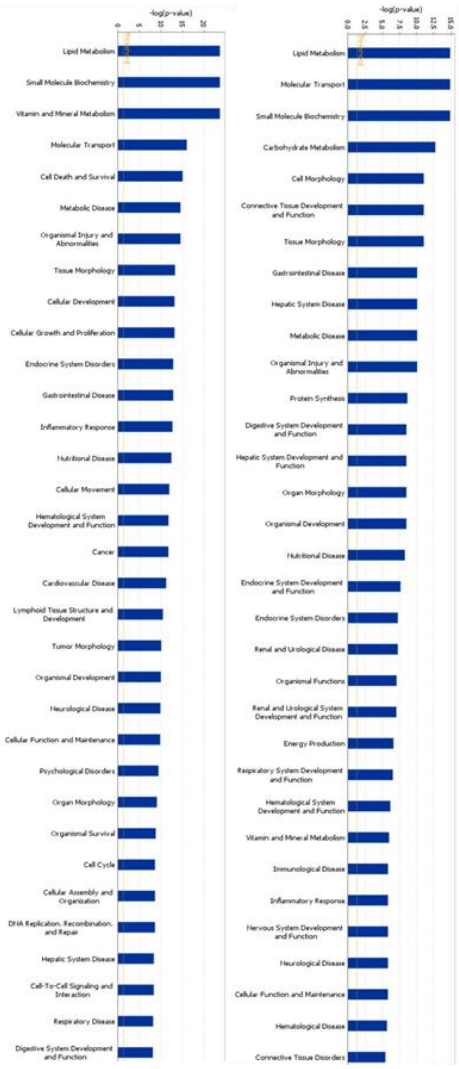

Figure 13. IPA Disease and Biological Functions for mTORC1 Knockout (Left) and mTORC2 Knockout (Right). Many associated diseases and biological functions were common between our two sets of differentially expressed genes, such as metabolic disease, lipid metabolism, small molecule biochemistry, and molecular transport. However, there were some distinct functions for both mTORC1 (such as cell death and survival, cellular development, cellular growth and proliferation, and others) and mTORC2 (carbohydrate metabolism, cell morphology, connective tissue development and function, and others).

In addition to using IPA to understand the possible regulatory network associated with our observed gene expression changes in response to mTOR complex knockout, we also utilized the platform to identify potential diseases, biological processes, and toxicological functions associated with observed changes in gene expression as a result of mTOR complex knockout. IPA predicted a multitude of diseases and biological functions associated with both sets of mTOR complex knockout gene expression data (Figure 13). The most significant of these for deletion of mTORC1 and mTORC2 was lipid metabolism. Other diseases and biological functions predicted for both gene sets were small molecule biochemistry, vitamin and mineral metabolism, molecular transport, metabolic disease, tissue morphology, organismal injury and abnormalities, endocrine system disorders, gastrointestinal disease, inflammatory response, nutritional disease, hematological system development and function, digestive system development and function, organismal development, neurological disease, cellular function and

maintenance, organ morphology, hepatic system disease, and digestive system development and function. Furthermore, consistent with our gene expression data, several common terms exhibited higher enrichment for mTORC1 relative to mTORC2 (the most notable were lipid metabolism, small molecule biochemistry, and vitamin and mineral metabolism).

Top diseases and biological functions predicted to be affected due to mTORC1 deletion only (Figure 13, left) include cell death and survival, cellular growth and proliferation, cellular movement, cancer, cardiovascular disease, lymphoid tissue structure and development, tumor morphology, psychological disorders, organismal survival, cell cycle, Cellular assembly and organization, DNA replication, recombination comma and repair, cell-to-cell signaling and interaction, and respiratory disease.

Top predicted diseases and biological functions associated with mTORC2 deletion only (Figure 13, right), included carbohydrate metabolism, cell morphology, connective tissue development and function, protein synthesis, hepatic system development and function, endocrine system development and function, renal and urological disease, organismal functions, renal and urological system development and function, energy production, respiratory system development and function, immunological disease, nervous system development and function, hematological disease, and connective tissue disorders.

IPA also predicted many toxicological functions to be associated with gene expression changes observed as a result of both liver-specific mTOR complex deletions (Figure 14). Common predicted toxicological functions between mTORC1 and 2 knockouts include liver

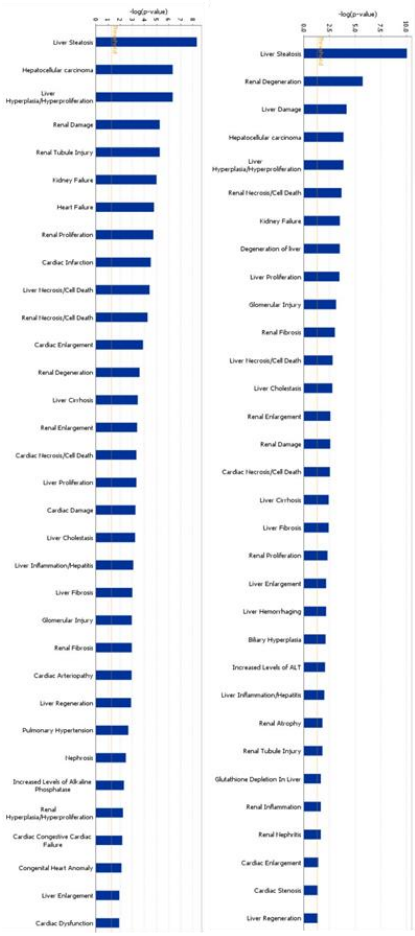

Figure 14. IPA Toxicological Functions for mTORC1 Knockout (Left) and mTORC2 Knockout (Right). Many associated toxicological functions were common between our two sets of differentially expressed genes, such as liver steatosis, hepatocellular carcinoma, and liver hyperplasia/hyperproliferation. However, there were also some distinct toxicological functions for both mTORC1 (such as heart failure, cardiac infarction, renal necrosis/cell death, and others) and mTORC2 (renal degeneration, liver damage, kidney failure, and others).

steatosis (the most highly enriched term for both knockouts), hepatocellular carcinoma, liver hyperplasia/hyperproliferation, renal damage, renal tubule injury, kidney failure, renal proliferation, liver necrosis/cell death, renal necrosis/cell death, renal degeneration, cardiac enlargement, glomerular injury, liver cirrhosis, renal enlargement, cardiac necrosis/cell death, liver proliferation, liver cholestasis, liver inflammation/hepatitis, liver fibrosis, renal fibrosis, liver regeneration, and liver enlargement.

Top toxicological functions predicted due to deletion of mTORC1 only (Figure 14, right) included heart failure, cardiac infarction, renal necrosis/cell death, cardiac damage, cardia arteriopathy, pulmonary hypertension, nephrosis, increased levels of alkaline phosphatase, renal hyperplasia/hyperproliferation, cardiac congestive heart failure, congenital heart anomaly, and cardiac dysfunction. Top predicted toxicological functions due to deletion of mTORC2 only (Figure 14, right) only included renal degeneration, liver damage, kidney failure, degeneration of liver, liver hemorrhaging, biliary hyperplasia, increased levels of ALT, renal atrophy, renal tubule injury, glutathione depletion in liver, renal inflammation, and renal nephritis.

V. Results part 2: The role of mTORC1 in mediating the long-term effects of BDE-47 on the liver

Exposure to BDE-47 is not associated with altered mTORC1 activity at PND75

To gain insight into the relationship between BDE-47 exposure and activity of mTORC1, we conducted protein immunoblots of whole liver tissue collected on PND75 from male mice (*N*=2 per group) exposed to BDE-47 (Figure 15) in the mTORC1 experiment. Interestingly, while perinatal exposure to BDE-47 did not cause visible changes in mTORC1 or mTORC2 activity at PND75 in mTORC1 control mice, it is possible that exposure to BDE-47 in mice with a liver-specific deletion of mTORC1 may lead to increased S6K1 phosphorylation at Thr389 and decreased phosphorylation of AKT at Ser473 (Figure 15). These results suggest early-life exposure to BDE-47 may decrease the effect of deletion of mTORC1 on S6K1 phosphorylation and decrease the mTORC1 knockout- induced increase in mTORC2 activity as well. However, given the quality of the only western blots we were able to obtain before the coronavirus outbreak, and the lack of blots for total AKT or total S6K1, this remains purely

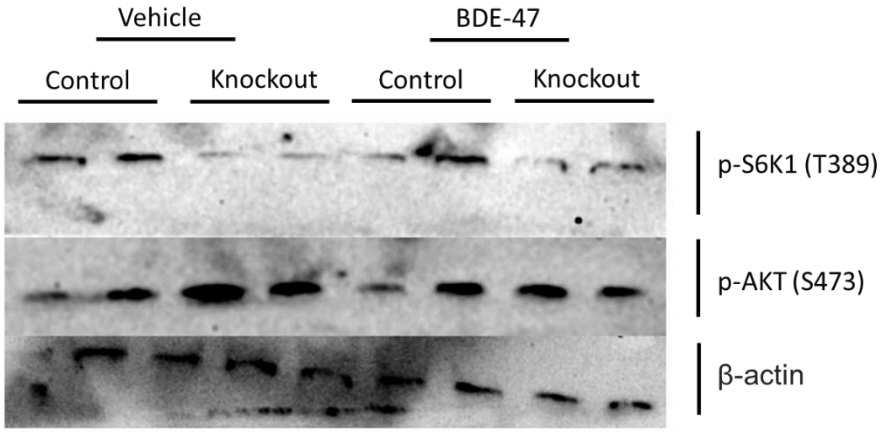

Figure 15. Western Blots to Characterize the Relationship Between Early-Life BDE-47 Exposure and mTORC1 Activity. Western blots were conducted for mice in the mTORC1 experiment (both control and knockout) who had been exposed to either BDE-47 or vehicle only. Beta-actin was used as a loading control.

speculative. Regardless, it is clear that perinatal exposure to BDE-47 in the mTORC1 experiment does not alter activity of mTORC1 or 2 at PND75 in control mice.

BDE-47 exposure does not significantly alter blood-liver balance of triglycerides

We next assessed triglyceride levels (N=2 male animals per group) and found no statistically significant differences in blood or serum triglycerides between BDE-47 exposed and vehicle-exposed mTORC1 control mice, as well as between BDE-47 exposed and vehicle-exposed mice with a liver-specific mTORC1 deletion. As such, at least in this experiment, it does not appear that BDE-47 exposure alters the blood-liver balance of triglycerides, nor does mTORC1 knockout abolish or permit any significant BDE-47-induced changes in blood-liver triglyceride balance.

Metascape reveals effects of BDE-47 exposure that are mTORC1-dependent at the transcriptomic level, and indicates possible synergy between mTORC1 deletion and BDE-47 exposure

Short lists of genes significantly altered due to BDE-47 exposure in mTORC1 control mice (52 genes in total, FDR q-value < 0.05), as well as genes significantly altered in mTORC1 knockouts (38 genes in total, FDR q-value < 0.05), were analyzed via Metascape to identify systems-level effects of BDE-47 exposure in mTORC1 control mice and mTORC1 knockout mice (Figure 16). Ontology terms associated with the effect of BDE-47 exposure on liver gene expression in mTORC1 control animals (Figure 16A) appear to be related to a wide variety of biological functions, such as innate immunity (“cellular response to exogenous dsRNA,” “natural killer cell mediated cytotoxicity,” “regulation of cytokine production,” and “regulation of interleukin-1 production”), cell cycle regulation (“positive regulation of mitotic cell cycle”),

lipid metabolism (“glycerophospholipid biosynthesis”), and ion homeostasis (“divalent inorganic cation homeostasis”). Interestingly, the ontology terms “response to leptin,” “locomotor rhythm,”, “neurotransmitter biosynthetic process,” “smooth muscle contraction,” “cellular component assembly involved in morphogenesis,” “negative regulation of secretion by cell,” “epithelial cell differentiation,” “kidney development,” and “carbohydrate transport” were enriched as well. Ontology terms associated with changes in gene expression due to BDE-47 exposure in mTORC1 KO mice are presented in Figure 16B. These terms are primarily associated with lipid metabolism (“steroid metabolism,” “lipid biosynthetic process,” and “regulation of lipid biosynthetic process”). Investigating the associated sub-terms that produced the enriched term “intracellular receptor signaling pathway” revealed that sub-terms contributing to enrichment are involved in xenobiotic metabolism and hormone-associated responses and metabolism. Enriched terms also included cellular development-related terms such as “skeletal muscle cell differentiation, “pattern specification process,” and “skin development.” Two terms were also associated with cell growth within the adaptive and innate immune systems respectively: “T cell activation” and “response to leukemia

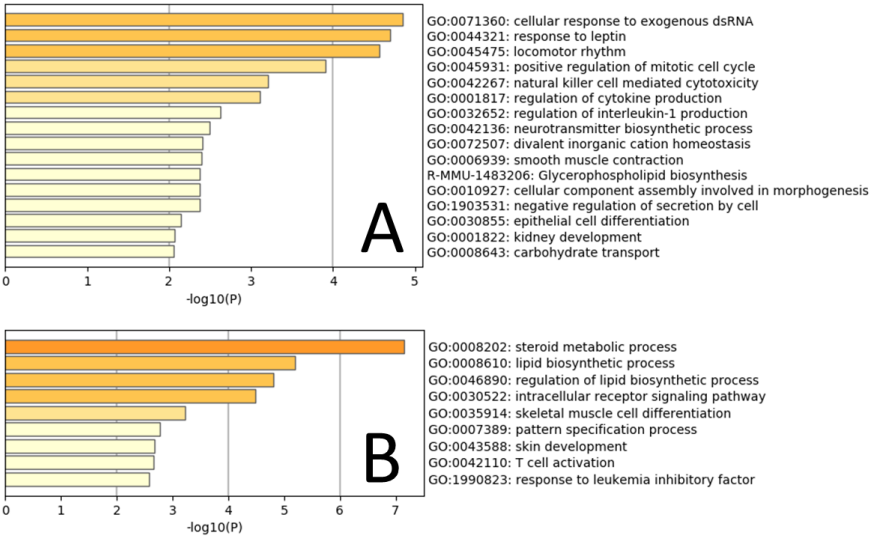

Figure 16. Metascape Reveals Ontology Terms Enriched Due to BDE-47 Exposure in the Presence and Absence of mTORC1 in the mTORC1 Experiment. Ontology terms associated with the transcriptomic effects of BDE-47 in the liver in the presence and absence of mTORC1 are shown in **A** and **B** respectively. Ontology terms associated with the effect of BDE-47 exposure in mTORC1 control animals appear to be related to a wide variety of biological functions, such as innate immunity, cell cycle regulation, lipid metabolism, and ion homeostasis. Ontology terms associated with the effects of BDE-47 exposure in mTORC1 knockouts appear to be primarily associated with lipid metabolism, innate immunity, and cellular development.

inhibitory factor." Interestingly, there were no ontology terms commonly enriched for both mTORC1 control and mTORC1 knockout mice, suggesting that the effects of BDE-47 exposure in the mice used for the mTORC1 experiment are mTORC1-dependent.

Comparative analysis of the effect of BDE-47 exposure in control and knockout mice in the mTORC1 experiment parses through mTORC1-dependent and mTORC1-independent effects of BDE-47 exposure.

The above analyses indicate that there is a strong relationship between mTORC1 activity and perinatal BDE-47 exposure. However, to fully understand how deletion of mTORC1 mediates the effect of BDE-47 exposure, we parsed through genes significantly affected by exposure to BDE-47 in mTORC1 control and knockout mice using the same type of comparison analysis that was used to identify the comparative roles of mTORC1 and 2 in liver gene expression regulation (Figure 5). In this case, the $\log_2$(ratio) of expression of genes in the comparison identifying the effect of BDE-47 exposure in mTORC1 control mice (vehicle-exposed mTORC1 control versus BDE-47 exposed mTORC1 control) were plotted against those indicating the effect of BDE-47 exposure in mTORC1

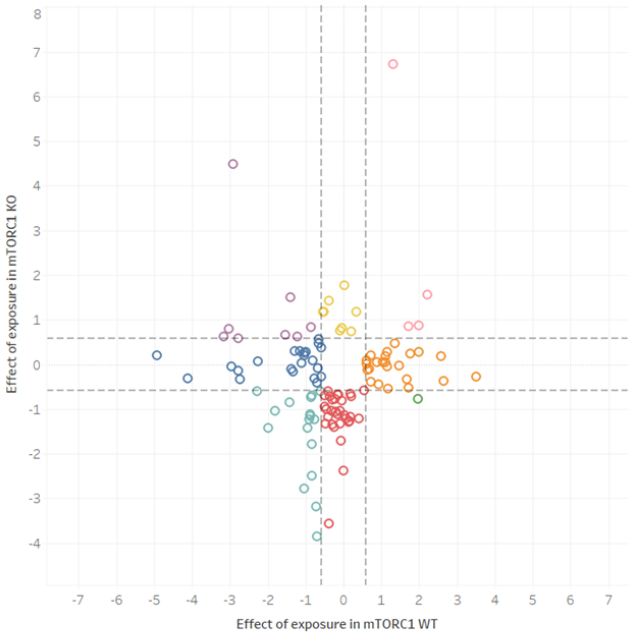

Figure 17. Comparison Analysis Reveals mTORC1 Dependent and Independent Effects of Early-Life BDE-47 Exposure in the mTORC1 Experiment. Grey dashed lines perpendicular to an axis mark a 1.5-fold ($\log_2$(ratio)= 0.59 or -0.59) change in expression due to BDE-47 exposure in a comparison. These dashed lines were used to generate each colored group for subsequent analysis. Genes considered to be upregulated by BDE-47 in control mice only are shown in orange, while those downregulated by BDE-47 exposure in control mice only are shown in blue. Genes considered to be upregulated by BDE-47 exposure in mTORC1 knockout only are shown in yellow, while genes downregulated by BDE-47 exposure in mTORC1 knockout only are shown in red. Genes upregulated by BDE-47 exposure in control mice and downregulated by BDE-47 exposure in mTORC1 knockout mice are in green, while genes downregulated by BDE-47 exposure in control mice and upregulated by BDE-47 exposure in mTORC1 knockout mice are in purple. Genes that were upregulated by BDE-47 exposure in both control and mTORC1 knockout mice are in pink, while those downregulated by BDE-47 exposure in both types of mice are in teal.

knockout mice (vehicle-exposed mTORC1 knockout versus BDE-47 exposed mTORC1 knockout). Genes were statistically significant in at least one comparison. Genes with less than 1.5-fold change ($\log_2$(ratio) = 0.59 or -0.59) in both comparisons were excluded from this analysis. Genes were then divided into eight groups based on whether they were altered at least 1.5-fold by BDE-47 exposure in mTORC1 controls, BDE-47 exposure in knockouts, or both, as well as the directions in which the fold change occurred for each comparison. This analysis revealed many genes that fell into four groups: genes altered by BDE-47 exposure in mTORC1 control mice only (orange and blue, Figure 17), genes altered by BDE-47 exposure in mTORC1 knockout mice only (yellow and red, Figure 17), genes regulated in opposite directions by BDE-47 exposure in mTORC1 controls and knockout (green and purple, Figure 17), and genes regulated in similar directions by BDE-47 exposure in both controls and knockouts (pink and teal, Figure 17). Each of these four sets of genes were analyzed via Metascape to clearly differentiate between the mTORC1-dependent and independent effects of perinatal BDE-47 exposure on liver gene expression. Upon visual inspection, most genes are altered by BDE-47 exposure in either mTORC1 control or knockout mice only, rather than in both.

Metascape analysis for which the changes in expression due to BDE-47 exposure occur only in the presence of mTORC1 (Figure 18) indicated these genes are primarily associated with innate immunity ("recycling pathway of L1," "prolactin signaling," and "negative regulation of cytokine production"), lipid metabolism

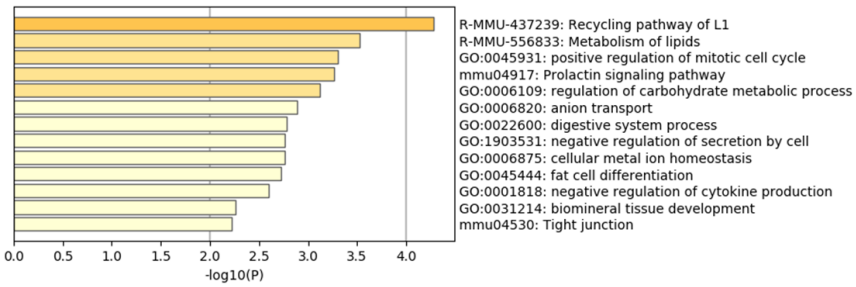

Figure 18. Metascape Results for Genes for which Deletion of mTORC1 Abolished Effects of BDE-47 Exposure in the mTORC1 Experiment. Genes for which mTORC1 deletion abolishes the effect of BDE-47 exposure are primarily associated with innate immunity, lipid metabolism, cell cycle regulation, cell adhesion and motility, ion transport, and cellular differentiation.

("metabolism of lipids" and "fat cell differentiation"), cell cycle regulation ("positive regulation of mitotic cell cycle" and "prolactin signaling"), cell adhesion and motility ("recycling pathway of L1" and "tight junction") ion transport ("anion transport," "cellular metal ion homeostasis," and "tight junction"). The terms "regulation of carbohydrate metabolic process" and "biomineral tissue development" were also enriched within the Metascape results.

All individual genes for which deletion of mTORC1 abolished effects of BDE-47 exposure are listed in Table 7. Notable genes that are upregulated by BDE-47 exposure only when mTORC1 is present include the gene encoding Cyclin D1, *Ccnd1*, the cytochrome P450 gene *Cyp17a1*, the fatty acid binding protein gene *Fabp2*, the glucokinase gene *Gcn*, *Lpin1* (which mediates signaling between mTORC1 and downstream transcription factors involved in cholesterol synthesis), the cell cycle, apoptosis, and inflammation regulator *Nr4a1*, and the retinol binding protein *Rbp1*. Notable genes downregulated due to BDE-47 exposure in mTORC1 control animals only include the autophagy regulator *Depp1*, the aquaporin *aqp8*, the cytochrome P40 *Cyp4a14*, the regulator of cell proliferation *Gas6*, the myosin light chain *Myl9*, the phosphate transporter *Slc17a8*, and the tubulin genes *Tubb2a* and *Tubb2b*.

Table 7. Genes for which mTORC1 deletion abolishes effects of BDE-47 exposure.

Upregulated by BDE-47 exposure in mTORC1 control mice only		Downregulated by BDE-47 exposure in mTORC1 control mice only	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Hectd2os	Hectd2, opposite strand	2900076A07Rik	RIKEN cDNA 2900076A07 gene
1810053B23Rik	RIKEN cDNA 1810053B23 gene	Mir1839	microRNA 1839

Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	Depp1	DEPP1 autophagy regulator
Ccnd1	cyclin D1	Acacb	acetyl-Coenzyme A carboxylase beta
Mir3962	microRNA 3962	Aqp8	aquaporin 8
Clec2h	C-type lectin domain family 2, member h	Atp11a	ATPase, class VI, type 11A
Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1	Cd24a	CD24a antigen
Ercc2	excision repair cross-complementing rodent repair deficiency, complementation group 2	Clic5	chloride intracellular channel 5
Mir343	microRNA 343	Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14
Fabp2	fatty acid binding protein 2, intestinal	Ddhd1	DDHD domain containing 1
Fam81a	family with sequence similarity 81, member A	mir5131	Micro RNA 5131
Fkbp5	FK506 binding protein 5	Ezr	ezrin
Gck	glucokinase	Gask1a	golgi associated kinase 1A
Lpin1	lipin 1	Gas6	growth arrest specific 6
Mt1	metallothionein 1	Hspb1	heat shock protein 1
Mtnr1b	melatonin receptor 1B	Krt19	keratin 19

Nr4a1	nuclear receptor subfamily 4, group A, member 1	Lama5	laminin, alpha 5
Rabggtb	Rab geranylgeranyl transferase, b subunit	Myl9	myosin, light polypeptide 9, regulatory
Snord45c	small nucleolar RNA, C/D box 45C	Ppp1r10	protein phosphatase 1, regulatory subunit 10
Rbp1	retinol binding protein 1, cellular	Prom1	prominin 1
Scara5	scavenger receptor class A, member 5	Slc17a8	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8
Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	Smpd3	sphingomyelin phosphodiesterase 3, neutral
Tff3	trefoil factor 3, intestinal	Tspan8	tetraspanin 8
Tsku	tsukushi, small leucine rich proteoglycan	Tubb2a	tubulin, beta 2A class IIA
Usp2	ubiquitin specific peptidase 2	Tubb2b	tubulin, beta 2B class IIB
Zbtb16	zinc finger and BTB domain containing 16		

Metascape analysis for genes for which deletion of mTORC1 permits an effect of BDE-47 exposure, meaning that altered expression of these genes occurred only in the absence of mTORC1 (Figure 19) produced ontology terms that are associated with a wide variety of functions, including innate immunity ("acute-phase response," which included the sub-terms "acute-phase response," "acute inflammatory response," and "neutrophil degranulation"), lipid metabolism ("lipid biosynthetic process," and "glycerolipid biosynthetic process), protein metabolism ("protein digestion and absorption"), different developmental processes ("connective tissue development" and "lung development"), organismal homeostasis ("multicellular organism homeostasis," included the sub-terms "multicellular organism homeostasis," "cold-induced thermogenesis," "regulation of cold-induced thermogenesis," "adaptive thermogenesis," and "temperature homeostasis"). Terms associated with specific types of signaling cascades were also present: "adenylate cyclase-modulating G protein-coupled receptor signaling pathway" and "positive regulation of ERK1 and ERK2 cascade." Lastly, the terms "response to metal ion," "learning or memory," "tissue remodeling," and "gland morphogenesis" were also enriched.

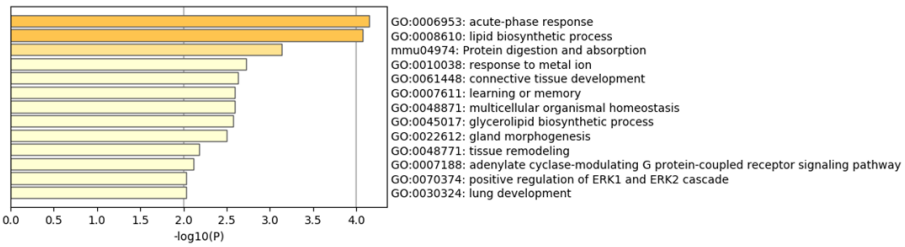

Figure 19. Metascape Results for Genes for which Deletion of mTORC1 Permitted an Effect of BDE-47 Exposure mTORC1 Experiment. Genes for which mTORC1 deletion is permissive of an effect of BDE-47 exposure are primarily associated with a wide variety of functions including innate immunity, lipid metabolism, ion transport, and developmental processes.

The individual genes for which mTORC1 deletion permits an effect of BDE-47 exposure are listed in Table 8. Notable genes upregulated by BDE-47 exposure in mTORC1 knockout mice only include the circadian rhythm-controlled hydrolase *Noct*, the insulin-like growth factor binding protein *Igfbp5*, and the scavenger receptor *Marco*. Genes downregulated due to BDE-47

exposure in mTORC1 knockout mice only include *Rnase4*, the cytokine *Cxcl1*, the cytochrome P450 enzymes *Cyp7b1* and *Cyp39a1*, the fatty acid elongase *Elovl6*, the immune system, tumor suppressor, and immediate-early gene *Fos*, the orosomucoids *Orm1*, *Orm2*, and *Orm3*, and the thyroid-hormone responsive lipid metabolism/lipogenesis regulator *Thrsp*. Interestingly, it appears that the majority of genes altered due to BDE-47 exposure in mTORC1 knockout mice are downregulated.

Table 8. Genes for which mTORC1 deletion permits effects of BDE-47 exposure.

Upregulated by BDE-47 exposure in mTORC1 knockout mice only		Downregulated by BDE-47 exposure in mTORC1 knockout mice only	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Noct	nocturnin	Adgrf1	adhesion G protein-coupled receptor F1
Chpf2	chondroitin polymerizing factor 2	Ang	angiogenin, ribonuclease, RNase A family, 5
Mir671	microRNA 671	Rnase4	ribonuclease, RNase A family 4
Col1a1	collagen, type I, alpha 1	Arrdc3	arrestin domain containing 3
Ccn2	cellular communication network factor 2	Atxn1	ataxin 1
Gpnmb	glycoprotein (transmembrane) nmb	C730036E19Rik	RIKEN cDNA C730036E19 gene
Igfbp5	insulin-like growth factor binding protein 5	Cxcl1	chemokine (C-X-C motif) ligand 1
Marco	macrophage receptor with collagenous structure	Cyp7b1	cytochrome P450, family 7, subfamily b, polypeptide 1
Slc25a24	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 24	Cyp39a1	cytochrome P450, family 39, subfamily a, polypeptide 1

Dclk3	doublecortin-like kinase 3
Dct	dopachrome tautomerase
Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)
Fabp5	fatty acid binding protein 5, epidermal
Fam47e	family with sequence similarity 47, member E
Fgl1	fibrinogen-like protein 1
Fos	FBJ osteosarcoma oncogene
Foxq1	forkhead box Q1
Gbp11	guanylate binding protein 11
Gm4956	predicted gene 4956
Lin7a	lin-7 homolog A (C. elegans)
Mme	membrane metallo endopeptidase
Msmo1	methylsterol monoxygenase 1
Mup9	major urinary protein 9
Orm1	orosomucoid 1
Orm2	orosomucoid 2
Orm3	orosomucoid 3
Phlda1	pleckstrin homology like domain, family A, member 1

Rgs16	regulator of G-protein signaling 16
Rmrp	RNA component of mitochondrial RNAase P
Saa4	serum amyloid A 4
Slc3a1	solute carrier family 3, member 1
Sucnr1	succinate receptor 1
Tfrc	transferrin receptor
Thrsp	thyroid hormone responsive

Only one ontology term was enriched for genes altered in different directions in response to BDE-47 exposure in mTORC1 controls and knockouts: "response to organic cyclic compound." Notably, only three genes contributed to enrichment of this term: The G-protein alpha subunit *Gnai1*, the protein arginine *N*-methyltransferase *Prmt2*, and the lymphocyte antigen protein *Ly6d*. All three of these genes were downregulated due to BDE-47 exposure in mTORC1 control mice, but upregulated due to BDE-47 exposure in mTORC1 knockout mice. See Table 9 for additional genes in this category. The only gene upregulated due to BDE-47 exposure in mTORC1 controls and downregulated in mTORC1 knockouts was the circadian rhythm-associated transcription regulator *Ciart*.

Table 9. Genes for which mTORC1 deletion leads to an inverse effect of BDE-47 exposure.

Downregulated by BDE-47 exposure in mTORC1 control mice, upregulated by BDE-47 exposure in mTORC1 KO mice		Upregulated by BDE-47 exposure in mTORC1 control mice, downregulated by BDE-47 exposure in mTORC1 KO mice	
Gene Symbol	Gene Name	Gene Symbol	Gene Name

Acta2	actin, alpha 2, smooth muscle, aorta	Ciart	circadian associated repressor of transcription
Cfap69	cilia and flagella associated protein 69		
Erdr1	erythroid differentiation regulator 1		
Fam83b	family with sequence similarity 83, member B		
Gnai1	guanine nucleotide binding protein (G protein), alpha inhibiting 1		
Ly6d	lymphocyte antigen 6 complex, locus D		
Mir678	microRNA 678		
Prmt2	protein arginine N-methyltransferase 2		
Pls1	plastin 1 (I-isoform)		

Lastly, Metascape analysis of genes for which effects of BDE-47 exposure are similar in mTORC1 control and knockout mice (Figure 20) are, based on enriched ontology terms, associated with lipid metabolism (“steroid metabolic process,” “regulation of steroid metabolic process,” and “monocarboxylic metabolic process”), immunity (“inflammatory response” and “lymphocyte differentiation”), cellular development and differentiation (“pattern specification process” and “lymphocyte differentiation”), and glucose response and metabolism (“response to glucose”). Additionally, the ontology term “transmembrane receptor protein serine/threonine kinase signaling pathway” was enriched as well.

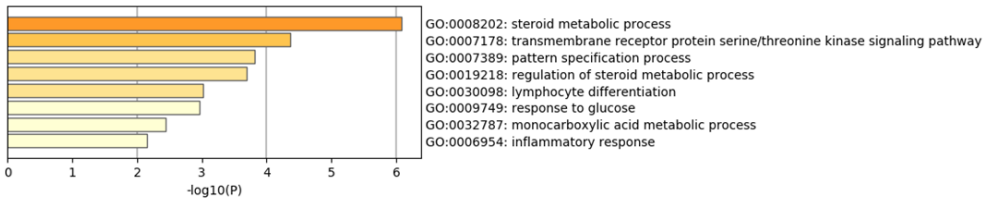

Figure 20. Metascape Results for Genes for which Effects of BDE-47 Exposure are Independent of mTORC1. Genes for which mTORC1 status does not influence the effect of BDE-47 exposure are primarily related to lipid metabolism, innate immunity, cellular development, and other processes.

Specific genes upregulated in response to early-life BDE-47 BDE-47 exposure in both mTORC1 control and mTORC1 knockout mice include the fatty acid transporter *Mfsd2a*, as well as the micro RNA genes *Mir369*, *Mir410*, *Mir412*, *Mirg*, and *Mir8104*. There were notably more genes downregulated in both mTORC1 controls and knockouts in response to BDE-47 exposure. Notable genes in this category include they cytochrome P450 proteins *Cyp26a1* and *Cyp26b1*, the transcription factor *Egr1*, the insulin-like growth factor *Igf2*, and the hepatic acute-phase proteins *Saa1* and *Saa2*. See Table 10 for a summary of all genes affected by BDE-47 exposure similarly in mTORC1 controls and knockouts.

Table 10. Genes for which effect of BDE-47 exposure is similar in mTORC1 control and knockout mice.

Upregulated by BDE-47 exposure in control and KO mice		Downregulated by BDE-47 exposure in control and KO mice	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Asns	asparagine synthetase	Bcl6	B cell leukemia/lymphoma 6
Mfsd2a	major facilitator superfamily domain containing 2A	Nat8f5	N-acetyltransferase 8 (GCN5-related) family member 5
Mir369	microRNA 369	Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1
Mir410	microRNA 410	Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1
Mir412	microRNA 412	Dancr	differentiation antagonizing non-protein coding RNA

Mirg	miRNA containing gene	Snora26	small nucleolar RNA, H/ACA box 26
Mir8104	microRNA 8104	Egr1	early growth response 1
Ppm1h	protein phosphatase 1H (PP2C domain containing)	Ehf	ets homologous factor
		Fst	follistatin
		Nckap1	NCK-associated protein 1
		Mir675	microRNA 675
		Igf2	insulin-like growth factor 2
		Me1	malic enzyme 1, NADP(+)-dependent, cytosolic
		Nrep	neuronal regeneration related protein
		Onecut1	one cut domain, family member 1
		Saa1	serum amyloid A 1
		Saa2	serum amyloid A 2
		Serpina12	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12
		Slc41a2	solute carrier family 41, member 2

Gene set enrichment analysis indicates that mTORC1 knockout permits significant effects of BDE-47 exposure at the gene set level in the mTORC1 experiment

To complement our analysis of short lists of genes from the mTORC1 experiment using Metacape, we again used GSEA to understand subtle purturbations to gene sets within the transcriptome that may have biological meaning undetectable by analyzing only the short-list of significantly altered genes between groups. Per Broad Institute GSEA recommendations, we used an FDR q-value of 0.25 and a normalized enrichment score (NES) of less than -1.7 or greater than 1.7 as the cutoff for significance. The Hallmark, Reactome, and Kegg gene set collections were used for this analysis. By comparing the NES of each gene set between sequencing datasets for the effect of BDE-47 exposure in mTORC1 control mice versus mTORC1 knockout mice, we were able to identify the potential relationship between mTORC1 and BDE-47 exposure in this mouse model in terms of specific genes sets and cellular pathways in the liver (Figure 21). Table 11 presents the full list of significant gene sets and their relative enrichment due to BDE-47 exposure in mTORC1 control and knockout mice. Interestingly, while both upregulated and

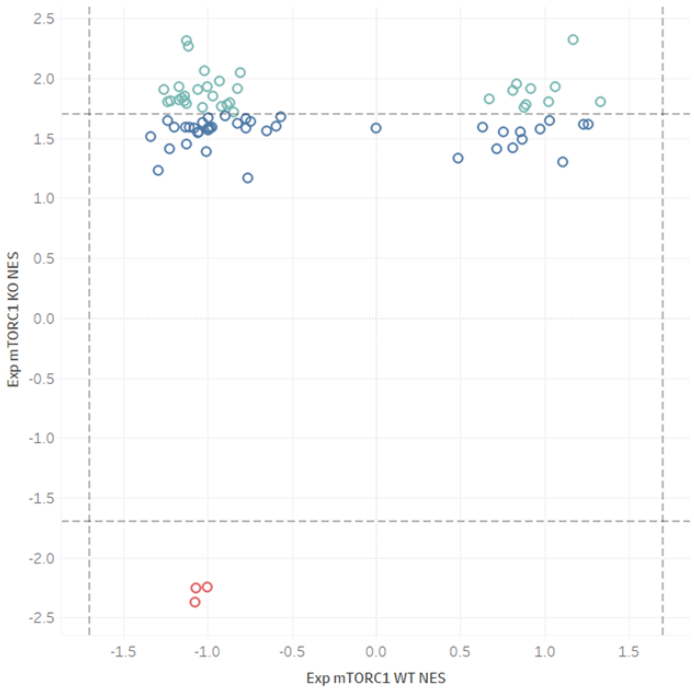

Figure 21. Comparison of Significantly Enriched GSEA Gene Sets In BDE-47 Exposed mTORC1 Control and Knockout Mice. Gene set enrichment results are presented for all gene sets with an FDR q-value of 0.25 or less for at least one of the two knockouts. Gene sets that crossed the threshold NES of 1.7 or -1.7 for one or both knockouts were considered significant. Interestingly, no gene sets were significantly enriched in either the positive or negative direction due to BDE-47 exposure in mTORC1 control mice. Gene sets upregulated by BDE-47 exposure in mTORC1 knockout mice only are shown in teal, while gene sets downregulated by BDE-47 exposure in mTORC1 KO mice only are shown in red. Gene sets that did not pass the threshold NES of 1.7 or -1.7 for either comparison are shown in blue.

downregulated genes were observed due to BDE-47 exposure in mTORC1 control models, there were no GSEA gene sets significantly enriched due to BDE-47 exposure in mTORC1 control mice. However, there were a significant number of gene sets enriched due to BDE-47 exposure in mTORC1 knockout mice. Upregulated gene sets in this category included sets associated with immunity ("HALLMARK_ALLOGRAFT_REJECTION,"
"HALLMARK_INFLAMMATORY_RESPONSE,"
"KEGG_CHEMOKINE_SIGNALING_PATHWAY,"
"KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION,"
"KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY,"
"KEGG_PRIMARY_IMMUNODEFICIENCY,"
"KEGG_HEMATOPOIETIC_CELL_LINEAGE,"
"REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES,"
"REACTOME_COSTIMULATION_BY_THE_CD28_FAMILY,"
"KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION,"
"REACTOME_INFLAMMASOMES," "REACTOME_INTERLEUKIN_10_SIGNALING,"
"REACTOME_ROS_AND_RNS_PRODUCTION_IN_PHAGOCYTES" and
"REACTOME_SCAVENGING_BY_CLASS_A_RECEPTORS"), cell cycle
("HALLMARK_MITOTIC_SPINDLE,"
"REACTOME_MITOTIC_SPINDLE_CHECKPOINT"), extracellular matrix, epithelial-mesenchymal transition, and cell motility
("HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION,"
"REACTOME_ASSEMBLY_OF_COLLAGEN_FIBRILS_AND_OTHER_MULTIMERIC_STRUCTURES,"

“REACTOME_COLLAGEN_BIOSYNTHESIS_AND_MODIFYING_ENZYMES,
REACTOME_COLLAGEN_CHAIN_TRIMERIZATION,”
“REACTOME_COLLAGEN_FORMATION,”
“REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX,”
“REACTOME_DISEASES_ASSOCIATED_WITH_O_GLYCOSYLATION_OF_PROTEINS,”
“REACTOME_ECM_PROTEOGLYCANS,”
“REACTOME_ELASTIC_FIBRE_FORMATION,”
“REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS,”
“REACTOME_LAMININ_INTERACTIONS,”
“REACTOME_MOLECULES_ASSOCIATED_WITH_ELASTIC_FIBRES,”
“REACTOME_O_LINKED_GLYCOSYLATION,”
“KEGG_DILATED_CARDIOMYOPATHY,”
“REACTOME_SYNDECAN_INTERACTIONS,” and
“REACTOME_SIGNALING_BY_RHO_GTPASES”). The term
“REACTOME_SIGNALING_BY_PDGF” was also positively enriched. Lastly, three gene sets associated with cholesterol and lipid metabolism
(“REACTOME_CHOLESTEROL_BIOSYNTHESIS,”
“REACTOME_METABOLISM_OF_STEROIDS,” and
“REACTOME_REGULATION_OF_CHOLESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF
”) were negatively enriched due to BDE-47 exposure in mice lacking mTORC1.

Table 11. GSEA results for effect of BDE-47 exposure in mTORC1 controls vs mTORC1 knockouts.

Gene Set	Exp mTORC1 WT NES	Exp mTORC1 KO NES	Result
HALLMARK_ALLOGRAFT_REJECTION	1.171	2.321	
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	-1.02	2.064	
HALLMARK_INFLAMMATORY_RESPONSE	-0.807	2.048	
HALLMARK_MITOTIC_SPINDLE	0.881	1.759	
KEGG_CHEMOKINE_SIGNALING_PATHWAY	-1.152	1.836	
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.916	1.909	
KEGG_DILATED_CARDIOMYOPATHY	-0.868	1.798	
KEGG_HEMATOPOIETIC_CELL_LINEAGE	1.024	1.8	
KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION	-1.127	1.784	
KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	0.89	1.779	
KEGG_PRIMARY_IMMUNODEFICIENCY	0.832	1.952	
REACTOME_ASSEMBLY_OF_COLLAGEN_FIBRILS_AND_OTHER_MULTIMERIC_STRUCTURES	-1.134	1.852	
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	-0.969	1.848	
REACTOME_COLLAGEN_BIOSYNTHESIS_AND_MODIFYING_ENZYMES	-0.823	1.914	
REACTOME_COLLAGEN_CHAIN_TRIMERIZATION	-0.927	1.976	
REACTOME_COLLAGEN_FORMATION	-1.003	1.931	
REACTOME_COSTIMULATION_BY_THE_CD28_FAMILY	0.814	1.898	Upregulated due to BDE-47 exposure in mTORC1 KO only
REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX	-1.171	1.927	
REACTOME_DISEASES_ASSOCIATED_WITH_O_GLYCOSYLATION_OF_PROTEINS	-1.26	1.909	
REACTOME_ECM_PROTEOGLYCANS	-1.123	2.31	
REACTOME_ELASTIC_FIBRE_FORMATION	-1.134	1.81	
REACTOME_INFLAMMASOMES	1.064	1.927	
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	-1.114	2.267	
REACTOME_INTERLEUKIN_10_SIGNALING	-0.845	1.717	
REACTOME_LAMININ_INTERACTIONS	-1.059	1.909	
REACTOME_MITOTIC_SPINDLE_CHECKPOINT	1.333	1.805	
REACTOME_MOLECULES_ASSOCIATED_WITH_ELASTIC_FIBRES	-1.169	1.817	
REACTOME_O_LINKED_GLYCOSYLATION	-1.236	1.799	
REACTOME_ROS_AND_RNS_PRODUCTION_IN_PHAGOCYTES	-0.882	1.776	
REACTOME_SCAVENGING_BY_CLASS_A_RECEPTORS	0.674	1.83	
REACTOME_SIGNALING_BY_PDGF	-1.219	1.808	
REACTOME_SIGNALING_BY_RHO_GTPASES	-0.916	1.767	
REACTOME_SYNDECAN_INTERACTIONS	-1.031	1.759	

REACTOME_CHOLESTEROL_BIOSYNTHESIS	-1.069	-2.253	
REACTOME_METABOLISM_OF_STEROIDS	-1.075	-2.371	Downregulated due to BDE-47 exposure in mTORC1 KO only
REACTOME_REGULATION_OF_CHOLESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF	-1.001	-2.247	

Ingenuity Pathway Analysis reveals unique predicted upstream regulators due to BDE-47 exposure for mTORC1 control and knockout mice

To identify regulators of transcription potentially involved in mediating the statistically significant changes in gene expression observed in our BDE-47 exposed mTORC1 control and knockout mice, we analyzed the two short lists of differentially expressed genes using Ingenuity Pathway Analysis (IPA) upstream regulator analysis. We elected to only analyze our gene expression data for associated upstream transcription factors and nuclear receptors. Interestingly, results predicted only one transcription factor for each gene expression dataset. For the effect of BDE-47 exposure in mTORC1 control mice, IPA predicted that the ATP-dependent chromatin remodeler SMARCA4 (also known as BRG1), a key component of the SWI/SNF complex, was inhibited due to BDE-47 exposure (Z-score -2.563). For the effect of BDE-47 exposure in mTORC1 knockout mice, this transcription factor was not inhibited; the only reported transcription factor for this set of gene expression data was the estrogen receptor ESR1, which was inhibited (Z-score -2.563). It is interesting to note that the predicted transcription factors affected by BDE-47 exposure in mTORC1 control vs knockout mice are different, suggesting that mTORC1 may possibly help “direct” the effect of early-life BDE-47 to reprogram affect different regions of the liver transcriptome. We also used the grow function of IPA to generate a network linking mTORC1, mTORC2, SMARCA4, and ESR1 using upstream analysis data, but IPA failed to generate any connections.

Ingenutiy pathway analysis identifies similar and dissimilar associations with diseases, biological functions, and toxicological responses at the systems level due to BDE-47 exposure in mTORC1 control and knockout mice

In addition to using IPA to understand the possible regulatory network associated with our observed gene expression changes in response to early-life BDE-47 exposure in control and mTORC1 knockout mice, we again utilized the platform to identify potential diseases, biological processes, and toxicological functions associated with observed changes in gene expression as a result of BDE-47 exposure in these two sets of gene expression data. IPA predicted a plethora of diseases and biological functions associated with BDE-47 exposure in both mTORC1 control and knockout mice (Figure 22). Disease and biological functions significantly enriched in both datasets included connective tissue development and function, organismal injury and abnormalities, organ morphology, cellular movement, gastrointestinal disease, hepatic system development and function, molecular transport, small molecule biochemistry, lipid metabolism, neurological disease, nervous system development and function, immunological disease, organismal development, reproductive system development and function, cell-to-cell signaling and interaction, cell growth and proliferation, and cancer.

Associated diseases and biological functions enriched due to BDE-47 exposure in mTORC1 control animals (Figure 22, left) only include cell morphology (most significant term), tissue morphology, inflammatory response, cell death and survival, metabolic disease, endocrine system disorders, renal and urological system development, and function, cellular assembly and organization, cellular compromise, renal and urological disease, hematological system development and function, nutritional disease, nutritional disease, and cardiovascular disease.

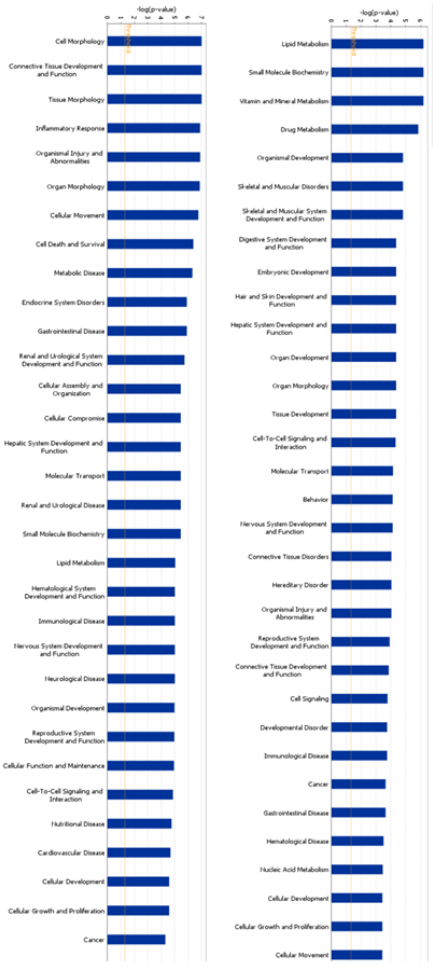

Figure 22. IPA Disease and Biological Functions for BDE-47 Exposed Control (Left) and mTORC1 Knockout (Right) mice in the mTORC1 Experiment. Many associated diseases and biological functions were common between our two sets of differentially expressed genes, such as connective tissue development and function, organismal injury and abnormalities, and lipid metabolism. However, there were some distinct functions for both BDE-47 exposure in mTORC1 controls (such cell morphology, inflammatory response, cell death and survival, and others) and BDE-47 exposure in mTORC1 knockouts (such as vitamin and mineral metabolism, drug metabolism, skeletal and muscular disorders, and others).

Terms enriched due to BDE-47 exposure in mTORC1 knockout mice (Figure 22, right) only include vitamin and mineral metabolism, drug metabolism, skeletal and muscular disorders, skeletal and muscular system development and function, digestive system development and function, embryonic development, hair and skin development and function, organ development, tissue development, behavior, connective tissue disorders, hereditary disorders, cell signaling, developmental disorder, nucleic acid metabolism, and cellular development.

We used IPA to also predict toxicological functions associated with observed expression changes in these two datasets (Figure 23). Common predicted toxicological functions between both mTORC1 control and mTORC1 knockout mice exposed to BDE-47 included liver hyperplasia/hyperproliferation, liver damage, and increased levels of AST.

Toxicological functions predicted due to BDE-47 exposure in mTORC1 control mice only included renal necrosis/cell death, heart failure, liver regeneration, renal degeneration, glomerular injury, Liver

hemorrhaging, cardiac dilation, cardiac dysfunction, cardiac enlargement, degeneration of the liver, cardiac infarction, kidney failure, Cardiac congestive cardiac failure, cardiac arrhythmia , renal degradation, renal atrophy, renal inflammation, renal nephritis, renal fibrosis, renal enlargement, cardia fibrosis, liver inflammation/hepatis, renal damage, renal tubule injury, liver enlargement, cardiac stenosis, congenital heart anomaly, hepatocellular carcinoma, liver necrosis/cell death, and nephrosis. Interestingly, there were only seven toxicological function terms that were significantly enriched due to BDE-47 exposure in mTORC1 knockout mice. Four of these terms were present for this group of significantly expressed genes only: liver cirrhosis, liver cholestasis, liver proliferation, and increased levels of ALT.

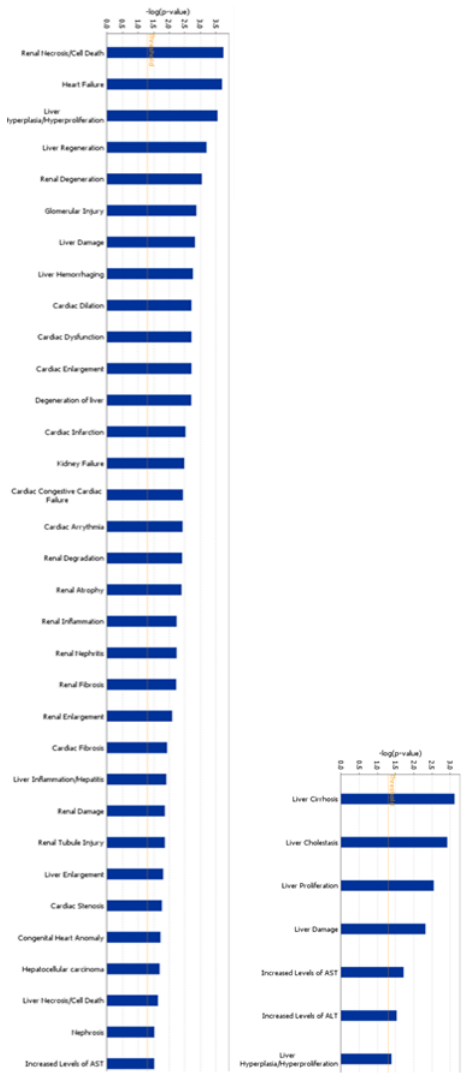

Figure 23. IPA Toxicological Functions for BDE-47 Exposed Control (Left) and mTORC1 Knockout (Right) mice in the mTORC1 Experiment. Three associated toxicological functions were common between our two sets of differentially expressed genes: liver hyperplasia/hyperproliferation, liver damage, and increased levels of AST. However, there were many distinct functions for BDE-47 exposure in mTORC1 controls (such renal necrosis/cell death, heart failure, liver regeneration, and others). There were four unique toxicological terms enriched due to BDE-47 exposure in mTORC1 knockouts: liver cirrhosis, liver cholestasis, liver proliferation, and increased levels of ALT.

VI. Results part 3: the role of mTORC2 in mediating long-term effects of BDE-47 on the liver

Exposure to BDE-47 is not associated with altered mTORC2 activity at PND75

To gain insight into the relationship between BDE-47 exposure and mTORC2 signaling, we first assessed mTORC1 and 2 activity using protein immunoblotting of whole liver tissue (PND75) from male mTORC2 control and mTORC2 knockout mice after BDE-47 exposure (*N*=2 per group, Figure 24). While perinatal exposure to BDE-47 did not lead to changes in mTORC1 or mTORC2 activity at PND75 in mTORC2 control mice, it is possible that exposure to BDE-47 in mice with a liver-specific deletion of mTORC2 increased S6K1 phosphorylation at Thr389 relative to knockouts exposed to vehicle only. These results suggest that it is possible that early-life BDE-47 exposure increases activity of mTORC1 by a small degree in mTORC2 knockout mice relative to mTORC2 knockout mice exposed to vehicle only. Given the quality of the only western blots we were able to obtain before the coronavirus outbreak, and the lack of blots for total AKT or total S6K1, this remains purely speculative. Regardless, based on this protein blot it is highly likely that perinatal exposure to BDE-47 in the mTORC2 experiment does not alter activity of mTORC1 or 2 at PND75.

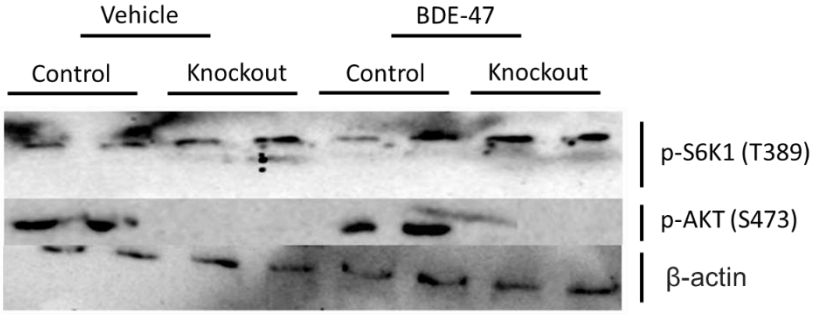

Figure 24. Western Blots to Characterize the Relationship Between Early-Life BDE-47 Exposure and mTORC2 Activity. Western blots were conducted for mice in the mTORC2 experiment (both control and knockout) who had been exposed to either BDE-47 or vehicle only. Beta-actin was used as a loading control.

BDE-47 exposure significantly alters the blood-liver balance of triglycerides and is mTORC2 dependent in the mTORC2 experiment

Comparing triglyceride levels between mTORC2 control and mTORC2 knockout mice exposed to BDE-47 (*N*=3 male animals per group) revealed that serum triglyceride levels were significantly higher in exposed control mice exposed to BDE-47 (p = 0.01, Figure 25). Interestingly, this effect of BDE-47 exposure was abolished due to mTORC2 knockout. These results suggest that, at least for mice used to characterize the role of mTORC2 in mediating BDE-47 exposure, BDE-47 alters the blood-liver balance of triglycerides and that this effect is mTORC2-dependent.

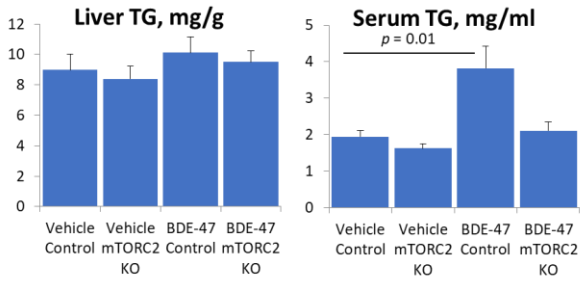

Figure 25. BDE-47 Exposure Significantly Increases Serum Triglycerides in the mTORC2 experiment and is mTORC2 dependent. Quantification of liver and serum triglycerides revealed that serum triglyceride levels were significantly higher in exposed control mice relative to controls. This effect of BDE-47 exposure was abolished due to mTORC2 knockout. It should be noted that no statistically significant change in serum or liver triglycerides due to BDE-47 exposure was detected in the mTORC1 experiment.

Metascape reveals effects of BDE-47 exposure that are mTORC2 dependent and mTORC2 independent at the transcriptomic level

To identify systems-level effects of BDE-47 exposure in mTORC2 control and mTORC2 knockout mice, we took the short lists of genes significantly altered due to BDE-47 exposure in control mice (227 genes in total, FDR q-value < 0.05), as well as genes significantly altered in mTORC2 knockouts (48 genes, FDR q-value < 0.05), and analyzed them using Metascape (Figure 27). Ontology terms associated with the effect of BDE-47 exposure on liver gene expression in mTORC2 control animals (Figure 26A) are related to many different biological functions, such as a wide variety of "muscular" processes ("muscle system process," "muscle

organ development," "sarcomere organization," "muscle contraction," "muscle adaptation," "skeletal muscle contraction," "striated muscle adaptation," "cardiac ventricle morphogenesis"), lipid metabolism ("monocarboxylic acid metabolic process," "PPAR signaling pathway," "sterol metabolic process," and others), innate immunity ("acute-phase response," "binding and uptake of ligands by scavenger receptors," "platelet degranulation," and others), glucose metabolism ("glycolysis (Embeden-Meyerhof pathway), glucose => pyruvate," "glucose homeostasis" "pyruvate metabolism," and others), ion and molecular transport ("succinate transport," "organic anion transport," "sarcoplasmic reticulum calcium ion transport" and more), and cellular stress ("negative regulation of reactive oxygen species metabolic process," "response to reactive oxygen species," and "regulation of transcription from RNA polymerase II promoter in response to stress"). Additionally, enriched terms demonstrated to be mTOR-dependent or upstream of mTOR signaling were present as well, such as "response to stillbenoid," and "regulation of insulin-like growth factor receptor signaling pathway."

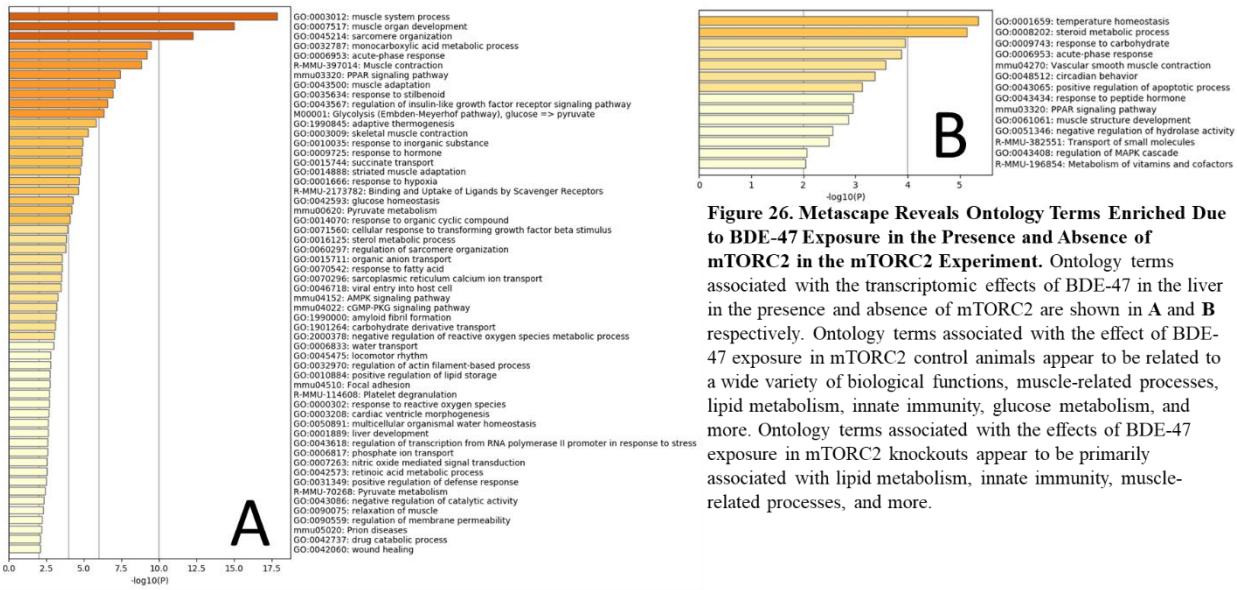

Figure 26. Metascape Reveals Ontology Terms Enriched Due to BDE-47 Exposure in the Presence and Absence of mTORC2 in the mTORC2 Experiment. Ontology terms associated with the transcriptomic effects of BDE-47 in the liver in the presence and absence of mTORC2 are shown in **A** and **B** respectively. Ontology terms associated with the effect of BDE-47 exposure in mTORC2 control animals appear to be related to a wide variety of biological functions, muscle-related processes, lipid metabolism, innate immunity, glucose metabolism, and more. Ontology terms associated with the effects of BDE-47 exposure in mTORC2 knockouts appear to be primarily associated with lipid metabolism, innate immunity, muscle-related processes, and more.

Ontology terms associated with the effect of BDE-47 exposure in the liver transcriptome in mTORC2 knockout mice (Figure 26B) are primarily associated with lipid metabolism ("steroid metabolic process" and "PPAR signaling pathway), innate immunity ("acute-phase response"), and muscle-associated processes ("vascular smooth muscle contraction" and "muscle structure development"). Additionally, enriched terms were present related to apoptosis ("positive regulation of apoptotic process"), molecular transport ("transport of small molecules"), carbohydrate metabolism ("response to carbohydrate"), hydrolase activity ("negative regulation of hydrolase activity"), MAPK signaling ("regulation of MAPK cascade"), circadian rhythm ("circadian behavior"), and vitamin metabolism ("metabolism of vitamins and cofactors"). Interestingly, the top enriched term was "temperature homeostasis." It is very clear that knockout of mTORC2 abolished a significant portion of the effect of BDE-47 exposure in mTORC2 control models, indicating that many of the effects of BDE-47 exposure are mTORC2-dependent.

Comparative analysis of the effect of BDE-47 exposure in control and knockout mice in the mTORC2 experiment parses through mTORC2-dependent and mTORC2-independent effects of BDE-47 exposure.

The above analyses indicate that there is a strong relationship between mTORC2 activity and perinatal exposure to BDE-47. However, to fully understand how mTORC2 mediates the effect of BDE-47 exposure, we parsed through genes significantly affected by BDE-47 exposure in mTORC2 control mice and mice lacking mTORC2 using the same type of comparison analysis that was used to identify the comparative roles of mTORC1 and mTORC2 in hepatic gene expression regulation (Figure 5) and the relationship between mTORC1 activity and BDE-47 exposure (Figure 17). In this case, the $\log_2$(ratio) of expression of genes the comparison

identifying the effect of BDE-47 exposure in control mice (vehicle-exposed mTORC2 control versus BDE-47 exposed control) were plotted against those indicating the effect of BDE-47 exposure in mTORC2 knockout mice (vehicle-exposed mTORC2 knockout versus BDE-47 exposed mTORC2 knockout). Genes were statistically significant in at least one comparison. Genes with less than 1.5-fold change (log_2(ratio) = 0.59 or -0.59) in both comparisons were excluded from this analysis. Genes were then divided into eight groups based on whether they were altered at least 1.5-fold by BDE-47 exposure in mTORC2 controls, BDE-47 exposure in mTORC2 knockouts, or in both, as well as the directions in which the fold change occurred for each comparison (Figure 27). This analysis revealed many genes that fell into four groups: genes altered by BDE-47 exposure in control mice only (orange and blue, Figure 27), genes altered by BDE-47 exposure in mTORC2 knockout mice only (yellow and red, Figure 27), genes regulated in opposite directions by BDE-47 exposure in mTORC2 controls and knockouts (green and purple, Figure 27), and genes regulated in similar directions by BDE-47 exposure in both mTORC2 controls and mTORC2 knockouts (pink and teal, Figure 27). Each of these four sets of genes were analyzed via Metascape to clearly differentiate between mTORC1-dependent and independent effects of

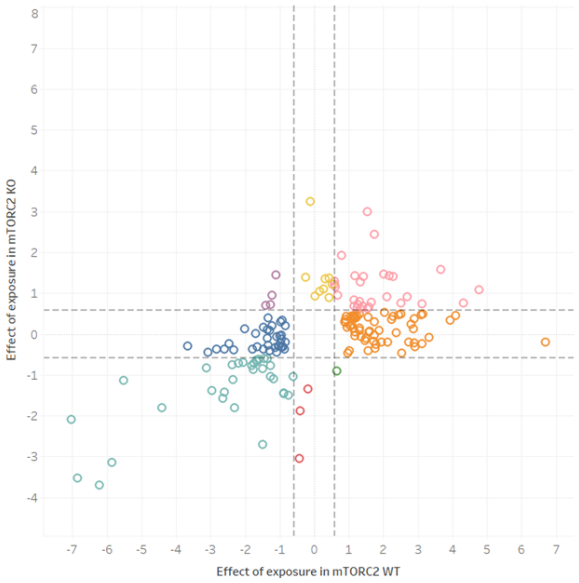

Figure 27. Comparison Analysis Reveals mTORC2 Dependent and Independent Effects of Early-Life BDE-47 Exposure in the mTORC2 Experiment. Grey dashed lines perpendicular to an axis mark a 1.5-fold (log_2(ratio)= 0.59 or -0.59) change in expression due to BDE-47 exposure in a comparison. These dashed lines were used to generate each colored group for subsequent analysis. Genes considered to be upregulated by BDE-47 in control mice only are shown in orange, while those downregulated by BDE-47 exposure in control mice only are shown in blue. Genes considered to be upregulated by BDE-47 exposure in mTORC2 knockout only are shown in yellow, while genes downregulated by BDE-47 exposure in mTORC2 knockout only are shown in red. Genes upregulated by BDE-47 exposure in control mice and downregulated by BDE-47 exposure in mTORC2 knockout mice are in green, while genes downregulated by BDE-47 exposure in control mice and upregulated by BDE-47 exposure in mTORC2 knockout mice are in purple. Genes that were upregulated by BDE-47 exposure in both control and mTORC2 knockout mice are in pink, while those downregulated by BDE-47 exposure in both types of mice are in teal.

early-life BDE-47 exposure on liver gene expression. These results suggest that while several genes are altered due to BDE-47 exposure via mTORC2 in mTORC2 control mice, several genes are altered due to BDE-47 exposure independently of mTORC2.

Metascape analysis for genes for which deletion of mTORC2 abolishes the effect of BDE-47 exposure (Figure 28) yielded terms that are primarily associated with glucose and pyruvate metabolism ("pyruvate metabolism," and "glucose homeostasis), muscle-associated processes ("muscle organ development," "muscle adaptation," and "striated muscle contraction"), innate immunity ("positive regulation of inflammatory response," "acute-phase response," and "cellular response to interleukin-1"), and lipid metabolism ("lipid homeostasis"). Additionally, terms were present related to hormone and IGF signaling ("response to hormone," and "regulation of insulin-like growth factor receptor signaling pathway"), organismal homeostasis ("multicellular organismal homeostasis"), viral entry ("regulation of viral entry into host cell"), membrane permeability ("regulation of membrane permeability"), liver development ("liver development"), and organic ion transport ("organic anion transport").

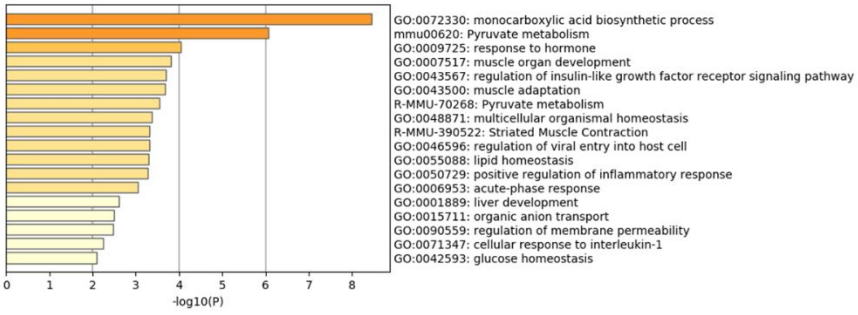

Figure 28. Metascape Results for Genes for which Deletion of mTORC2 Abolished Effects of BDE-47 Exposure in the mTORC2 Experiment. Genes for which mTORC2 deletion abolishes the effect of BDE-47 exposure are primarily associated with glucose and pyruvate metabolism, muscle-associated processes, innate immunity, and lipid metabolism.

Individual genes for which mTORC2 deletion abolished the effect of BDE-47 exposure are presented in Table 12. Notable genes upregulated due to BDE-47 exposure in mTORC2 control mice only include the aquaporin *Aqp8*, the cytochrome P450 enzyme Cyp2c23, the fatty

acid binding proteins *Fabp2* and *Fabp4*, the insulin-like growth factor binding protein *Igfbp5*, the immediate-early gene *Jun*, the skeletal muscle myosin *Mylpf*, and the tubulins *Tubb2a* and *Tubb2b*. Notable genes downregulated due to BDE-47 exposure in mTORC2 control mice only include the RNase *Rnase4*, the circadian-associated repressor of transcription *Ciart*, the cytochrm P450 enzyme *Cyp39a1*, the insulin-like growth factor binding protein *Igfp2*, the interleukin 1 receptor *Il1r1*, and the ubiquitin-specific peptidase *Usp2*.

Table 12. Genes for which mTORC2 knockout abolishes the effect of BDE-47 exposure.

Upregulated by BDE-47 exposure in mTORC2 control only		Downregulated by BDE-47 exposure in mTORC2 control only	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Hectd2os	Hectd2, opposite strand	1810053B23Rik	RIKEN cDNA 1810053B23 gene
Acaca	acetyl-Coenzyme A carboxylase alpha	Ang	angiogenin, ribonuclease, RNase A family, 5
Acacb	acetyl-Coenzyme A carboxylase beta	Rnase4	ribonuclease, RNase A family 4
Aldoa	aldolase A, fructose-bisphosphate	Atp11a	ATPase, class VI, type 11A
Aqp8	aquaporin 8	Bcl3	B cell leukemia/lymphoma 3
Arrdc3	arrestin domain containing 3	Cd5l	CD5 antigen-like
Asb2	ankyrin repeat and SOCS box-containing 2	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta
Atxn1	ataxin 1	Ciart	circadian associated repressor of transcription
Btbd19	BTB (POZ) domain containing 19	Cyp39a1	cytochrome P450, family 39, subfamily a, polypeptide 1

Car3	carbonic anhydrase 3	Dnajc12	DnaJ heat shock protein family (Hsp40) member C12
Cldn1	claudin 1	Enho	energy homeostasis associated
Clec2h	C-type lectin domain family 2, member h	Fam81a	family with sequence similarity 81, member A
Cryab	crystallin, alpha B	Gfra1	glial cell line derived neurotrophic factor family receptor alpha 1
Cyp2c23	cytochrome P450, family 2, subfamily c, polypeptide 23	Gse1	genetic suppressor element 1, coiled-coil protein
Des	desmin	Hipk3	homeodomain interacting protein kinase 3
Dpt	dermatopontin	Mir1902	microRNA 1902
Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	Hpx	hemopexin
Eno3	enolase 3, beta muscle	Ifitm2	interferon induced transmembrane protein 2
Fabp2	fatty acid binding protein 2, intestinal	Igfbp2	insulin-like growth factor binding protein 2
Fabp4	fatty acid binding protein 4, adipocyte	Il1r1	interleukin 1 receptor, type I
Fam13a	family with sequence similarity 13, member A	Isyna1	myo-inositol 1-phosphate synthase A1
Fbxo32	F-box protein 32	Kcnk5	potassium channel, subfamily K, member 5
Fhl1	four and a half LIM domains 1	Lbp	lipopolysaccharide binding protein
Gbp11	guanylate binding protein 11	Ly6e	lymphocyte antigen 6 complex, locus E
Gsn	gelsolin	Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)

Gys1	glycogen synthase 1, muscle	Marco	macrophage receptor with collagenous structure
Hspb6	heat shock protein, alpha-crystallin-related, B6	Ppl	periplakin
Igfbp5	insulin-like growth factor binding protein 5	Rbp1	retinol binding protein 1, cellular
Inmt	indolethylamine N-methyltransferase	Saa3	serum amyloid A 3
Jun	jun proto-oncogene	Saa4	serum amyloid A 4
Ldhb	lactate dehydrogenase B	Sh3bp2	SH3-domain binding protein 2
Lpl	lipoprotein lipase	Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3
Me1	malic enzyme 1, NADP(+)-dependent, cytosolic	Sult5a1	sulfotransferase family 5A, member 1
Mgp	matrix Gla protein	Tff3	trefoil factor 3, intestinal
Mir331	microRNA 331	Tmprss2	transmembrane protease, serine 2
Vezt	vezatin, adherens junctions transmembrane protein	Tsc22d3	TSC22 domain family, member 3
Mir5625	microRNA 5625	Tsku	tsukushi, small leucine rich proteoglycan
Slc35f6	solute carrier family 35, member F6	Usp2	ubiquitin specific peptidase 2
Mylpf	myosin light chain, phosphorylatable, fast skeletal muscle		
Nexn	nexilin		
Nrep	neuronal regeneration related protein		
Onecut1	one cut domain, family member 1		

Oxct1	3-oxoacid CoA transferase 1
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4
Pfkm	phosphofructokinase, muscle
Pkm	pyruvate kinase, muscle
Ptp4a3	protein tyrosine phosphatase 4a3
Rgs16	regulator of G-protein signaling 16
Rilpl1	Rab interacting lysosomal protein-like 1
Serpina4-ps1	serine (or cysteine) peptidase inhibitor, clade A, member 4, pseudogene 1
Serpina12	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12
Slc17a8	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8
Slc25a4	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4
St5	suppression of tumorigenicity 5
Sucnr1	succinate receptor 1
Timp2	tissue inhibitor of metalloproteinase 2
Tmem38a	transmembrane protein 38A
Tnnt1	troponin T1, skeletal, slow
Tpm1	tropomyosin 1, alpha
Tubb2a	tubulin, beta 2A class IIA

Tubb2b	tubulin, beta 2B class IIB

Interestingly, there were only three ontology terms enriched for genes altered by BDE-47 exposure in mTORC2 knockouts only, which were comprised of only a small subset of the total genes in this gene group: "heart development" (*Id1*, *Slc9a1*, *Dusp6*, *Dipk2a*), "positive regulation of apoptotic process" (*G0s2*, *Slc9a1*, *Dusp6*, *Klf11*), and "regulation of growth" (*Slc9a1*, *Dusp6*, *Osgin1*). The number of genes affected by BDE-47 exposure in mTORC2 knockout mice only was much smaller than those in mTORC2 control mice only. Genes upregulated due to BDE-47 exposure in mTORC2 knockout mice only include the G0/G1 switch gene *G0s2* and the oxidative stress-induced growth inhibitor *Osgin1*, while genes downregulated due to BDE-47 exposure in mTORC2 knockout mice only include the microRNAs *Mir192* and *Mir671*. A full list of genes affected by BDE-47 exposure in mTORC2 knockout mice is presented below in Table 13.

Table 13. Genes for which mTORC2 knockout permits the effect of BDE-47 exposure.

Upregulated by BDE-47 exposure in mTORC2 KO only		Downregulated by BDE-47 exposure in mTORC2 KO only	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Dipk2a	divergent protein kinase domain 2A	Atp6v0d2	ATPase, H+ transporting, lysosomal V0 subunit D2
Dusp6	dual specificity phosphatase 6	Chpf2	chondroitin polymerizing factor 2
G0s2	G0/G1 switch gene 2	Mir671	microRNA 671
Glce	glucuronyl C5-epimerase	Mir192	microRNA 192
Mir5133	microRNA 5133		
Id1	inhibitor of DNA binding 1, HLH protein		

Klf11	Kruppel-like factor 11
Marcks	myristoylated alanine rich protein kinase C substrate
Slc9a1	solute carrier family 9 (sodium/hydrogen exchanger), member 1
Osgin1	oxidative stress induced growth inhibitor 1

There were only five genes regulated in opposing directions in mTORC2 control and knockout mice due to BDE-47 exposure. The one gene upregulated due to BDE-47 exposure in mTORC2 control mice and downregulated in mTORC2 knockouts was the cytochrome P450 enzyme *Cyp8b1*. The four genes downregulated due to BDE-47 exposure in mTORC2 controls but upregulated due to BDE-47 exposure in mTORC2 knockouts were the cytochrome p450 enzyme *Cyp26b1*, the metabolic regulator *Angptl8*, the monooxygenase *Moxd1*, and the major urinary protein *Mup5*.

Metascape analysis of genes for which the effects of BDE-47 exposure are independent of mTORC2 (Figure 29) are primarily associated with innate immunity ("acute-phase response," "regulation of response to wounding," and "platelet degranulation"), lipid metabolism ("steroid metabolic process," and "PPAR signaling pathway"), and ion transport ("organic anion transport," "cellular metal ion homeostasis," and

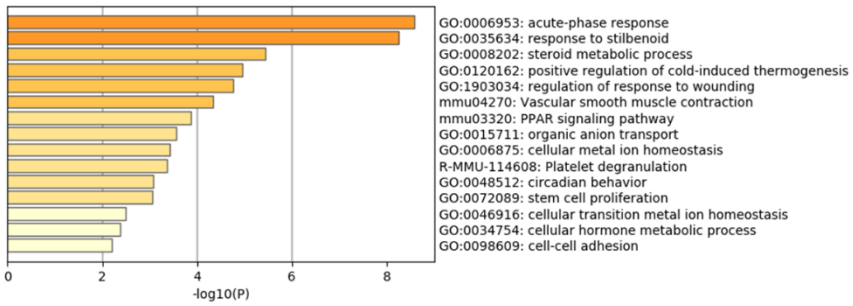

Figure 29. Metascape Results for Genes for which Effects of BDE-47 Exposure are Independent of mTORC2. Genes for mTORC2 status does not influence the effect of BDE-47 exposure are primarily associated with innate immunity, lipid metabolism, and ion transport.

"cellular transition metal ion homeostasis"). Additionally, the terms "response to stillbenoid," "positive regulation of cold-induced thermogenesis," "vascular smooth muscle contraction," "circadian behavior," "stem cell proliferation," "cellular hormone metabolic process," and "cell-cell adhesion" were also enriched.

The full list of individual genes that were similarly affected by BDE-47 exposure independently of mTORC2 are presented in Table 14. Notable genes that are upregulated by BDE-47 exposure independent of mTORC2 include *Cd36*, the autophagy regulator *Depp1*, the smooth muscle actin *Acta2*, the antigens *CD24a* and *CD34*, the transcription factor *Egr1*, and the insulin-like growth factor binding protein *Igfbp6*. Notable genes downregulated by BDE-47 independently of mTORC2 include the cytochrome P450 enzymes *Cyp2c38*, *Cyp4a14*, *Cyp*7a1, and *Cyp17a1*, the leptin receptor *Lepr*, and the orosomucoids *Orm1*, *Orm2*, and *Orm3*.

Table 14. Genes for which the effects of BDE-47 exposure are independent of mTORC2.

Upregulated by BDE-47 exposure independent of mTORC2		Downregulated by BDE-47 exposure independent of mTORC2	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Depp1	DEPP1 autophagy regulator	Apcs	serum amyloid P-component
Acta2	actin, alpha 2, smooth muscle, aorta	Apoa4	apolipoprotein A-IV
Calcrl	calcitonin receptor-like	Asns	asparagine synthetase
Cav1	caveolin 1, caveolae protein	Noct	nocturnin
Cd24a	CD24a antigen	Cxcl1	chemokine (C-X-C motif) ligand 1
Cd34	CD34 antigen	Cyb561	cytochrome b-561
Cd36	CD36 molecule	Cyp2c38	cytochrome P450, family 2, subfamily c, polypeptide 38

Cox7a1	cytochrome c oxidase subunit 7A1	Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14
Cpe	carboxypeptidase E	Cyp7a1	cytochrome P450, family 7, subfamily a, polypeptide 1
Cyp2c55	cytochrome P450, family 2, subfamily c, polypeptide 55	Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1
Dct	dopachrome tautomerase	Fgl1	fibrinogen-like protein 1
Dsg1c	desmoglein 1 gamma	Fkbp5	FK506 binding protein 5
Egr1	early growth response 1	Hp	haptoglobin
Gdf15	growth differentiation factor 15	Itih3	inter-alpha trypsin inhibitor, heavy chain 3
Gm4956	predicted gene 4956	Lcn2	lipocalin 2
Zfp982	zinc finger protein 982	Lepr	leptin receptor
Igfbp6	insulin-like growth factor binding protein 6	Lrg1	leucine-rich alpha-2-glycoprotein 1
Junb	jun B proto-oncogene	Mfsd2a	major facilitator superfamily domain containing 2A
Krt19	keratin 19	Mmd2	monocyte to macrophage differentiation-associated 2
Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	Mt1	metallothionein 1
Mme	membrane metallo endopeptidase	Mtnr1b	melatonin receptor 1B
Myh11	myosin, heavy polypeptide 11, smooth muscle	Nipal1	NIPA-like domain containing 1
Myl9	myosin, light polypeptide 9, regulatory	Nnmt	nicotinamide N-methyltransferase
Scd1	stearoyl-Coenzyme A desaturase 1	Orm1	orosomucoid 1
Slc25a30	solute carrier family 25, member 30	Orm2	orosomucoid 2
Sparcl1	SPARC-like 1	Orm3	orosomucoid 3

Tmem47	transmembrane protein 47	Saa1	serum amyloid A 1
Tpm2	tropomyosin 2, beta	Saa2	serum amyloid A 2
Tspan8	tetraspanin 8	Scara5	scavenger receptor class A, member 5
Txnip	thioredoxin interacting protein	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N
		Slc3a1	solute carrier family 3, member 1
		Slc16a5	solute carrier family 16 (monocarboxylic acid transporters), member 5
		Slc37a1	solute carrier family 37 (glycerol-3-phosphate transporter), member 1
		Usp18	ubiquitin specific peptidase 18

Gene set enrichment analysis indicates several gene sets are mTORC2 dependent in the mTORC2 experiment

To complement our Metascape analysis of short lists of genes from significantly altered due to BDE-47 exposure in mTORC2 control mice and mice lacking mTORC2 in the liver, we used GSEA to further understand subtle purturbations to gene sets within the transcriptome that may have biological meaning undetectable by analyzing only the short-list of significantly altered genes between groups. Per Broad Institute GSEA recommendations, we used an FDR q-value of 0.25 and a normalized enrichment score (NES) of less than -1.7 or greater than 1.7 as the cutoff for significance. The Hallmark, Reactome, and Kegg gene set collections were used for this analysis. By comparing the NES of each gene set between sequencing datasets for the

effect of BDE-47 exposure in mTORC2 control mice versus mTORC2 knockout mice, we were able to identify the potential relationship between mTORC2 and BDE-47 exposure in this mouse model in terms of specific genes sets and cellular pathways in the liver (Figure 30). Table 15 presents the full list of significant gene sets and their relative enrichment due to BDE-47 exposure in mTORC2 control and knockout mice. Interestingly, there were no gene sets negatively enriched in either mTORC2 control or knockout mice. However, there were a significant number of gene sets for which mTORC2 knockout abolished the effect of BDE-47 exposure. These include gene sets associated with muscle-related functions and myogenesis ("HALLMARK_MYOGENESIS," "KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC," "KEGG_CARDIAC_MUSCLE_CONTRACTION," "KEGG_DILATED_CARDIOMYOPATHY," "KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM," "REACTOME_CARDIAC_CONDUCTION," and "REACTOME_STRIATED_MUSCLE_CONTRACTION"), calcium signaling and ion transport

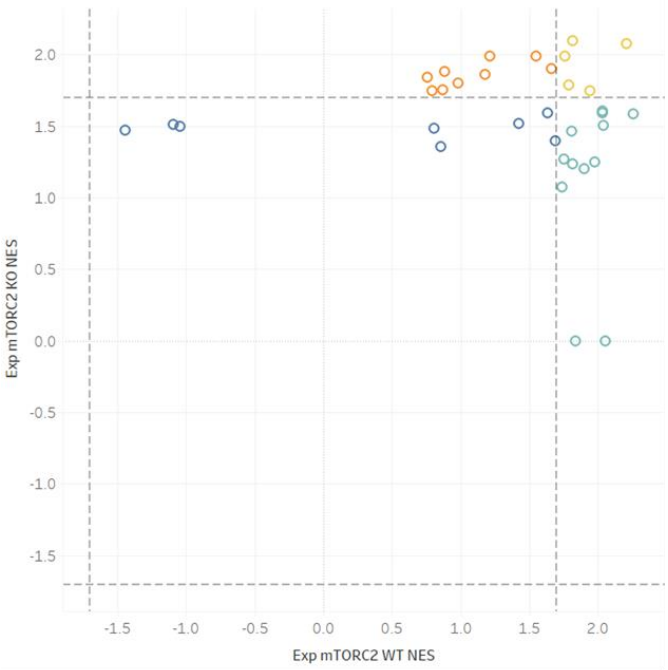

Figure 30. Comparison of Significantly Enriched GSEA Gene Sets In BDE-47 Exposed mTORC2 Control and Knockout Mice. Gene set enrichment results are presented for all gene sets with an FDR q-value of 0.25 or less for at least one of the two knockouts. Gene sets that crossed the threshold NES of 1.7 or -1.7 for one or both knockouts were considered significant. Interestingly, no gene sets were negatively enriched due to BDE-47 exposure in mTORC2 control and knockout mice. Gene sets upregulated by BDE-47 exposure in mTORC2 control mice only are shown in teal, while gene sets upregulated by BDE-47 exposure in mTORC2 KO mice only are shown in orange. Gene sets that were positively enriched due to BDE-47 exposure in both types of mice are in yellow, while gene sets that did not pass the threshold NES of 1.7 or -1.7 for either comparison are shown in blue.

("KEGG_CALCIUM_SIGNALING_PATHWAY,"
"REACTOME_ION_CHANNEL_TRANSPORT," and
"REACTOME_PHASE_0_RAPID_DEPOLARISATION"), endocytosis, actin cytoskeleton dynamics, and cellular survival via nephrin family proteins ("REACTOME_NEPHRIN_FAMILY_INTERACTIONS"), and tight junctions ("KEGG_TIGHT_JUNCTION"). There were also a number of gene sets that were upregulated via BDE-47 exposure only when mTORC2 was knocked out. These include gene sets associated with immunity ("HALLMARK_ALLOGRAFT_REJECTION,"
"HALLMARK_IL2_STAT5_SIGNALING," and
"HALLMARK_TNFA_SIGNALING_VIA_NFKB"), blood vessel formation and notch signaling ("HALLMARK_ANGIOGENESIS," and "HALLMARK_NOTCH_SIGNALING"), apoptosis and DNA damage ("HALLMARK_APOPTOSIS" and
"HALLMARK_UV_RESPONSE_DN"), KRAS signaling ("HALLMARK_KRAS_SIGNALING_UP"), and extracellular matrix function ("REACTOME_MOLECULES_ASSOCIATED_WITH_ELASTIC_FIBRES"). Lastly, some gene sets were positively enriched due to BDE-47 exposure in both mTORC2 control and knockout mice. These gene sets were
"HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION,"
"KEGG_FOCAL_ADHESION, REACTOME_ION_HOMEOSTASIS,"
"REACTOME_MUSCLE_CONTRACTION," and
"REACTOME_SMOOTH_MUSCLE_CONTRACTION."

Table 15. GSEA results for the effect of BDE-47 exposure in mTORC2 controls vs mTORC2 knockouts

Name	Exp mTORC2 WT NES	Exp mTORC2 KO NES	Result
HALLMARK_MYOGENESIS	2.258	1.583	
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	1.901	1.202	
KEGG_CALCIUM_SIGNALING_PATHWAY	1.816	1.237	
KEGG_CARDIAC_MUSCLE_CONTRACTION	2.035	1.607	
KEGG_DILATED_CARDIOMYOPATHY	2.031	1.593	
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	1.980	1.252	Upregulated due to BDE-47 exposure in mTORC2 control only
KEGG_TIGHT_JUNCTION	1.810	1.465	
REACTOME_CARDIAC_CONDUCTION	2.043	1.502	
REACTOME_ION_CHANNEL_TRANSPORT	1.737	1.072	
REACTOME_NEPHRIN_FAMILY_INTERACTIONS	1.756	1.268	
REACTOME_PHASE_0_RAPID_DEPOLARISATION	1.837	0.000	
REACTOME_STRIATED_MUSCLE_CONTRACTION	2.052	0.000	
HALLMARK_ALLOGRAFT_REJECTION	0.757	1.843	
HALLMARK_ANGIOGENESIS	1.177	1.862	
HALLMARK_APOPTOSIS	0.886	1.884	
HALLMARK_IL2_STAT5_SIGNALING	0.980	1.802	
HALLMARK_KRAS_SIGNALING_UP	1.213	1.989	Upregulated due to BDE-47 exposure in mTORC2 KO only
HALLMARK_NOTCH_SIGNALING	0.794	1.750	
HALLMARK_TNFA_SIGNALING_VIA_NFKB	0.868	1.756	
HALLMARK_UV_RESPONSE_DN	1.551	1.990	
REACTOME_MOLECULES_ASSOCIATED_WITH_ELASTIC_FIBRES	1.663	1.900	

HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.763	1.989	
KEGG_FOCAL_ADHESION	1.785	1.789	
REACTOME_ION_HOMEOSTASIS	1.943	1.747	Upregulated due to BDE-47 exposure independent of mTORC2
REACTOME_MUSCLE_CONTRACTION	2.208	2.078	
REACTOME_SMOOTH_MUSCLE_CONTRACTION	1.817	2.096	

Ingenuity Pathway Analysis reveals unique predicted upstream regulators due to BDE-47 exposure for mTORC2 control and knockout mice

To identify regulators of transcription potentially involved in mediating the statistically significant changes in gene expression observed due to BDE-47 exposure in our mTORC2 control and knockout mice, we analyzed the two short lists of differentially expressed genes related to these animals using Ingenuity Pathway Analysis (IPA) upstream regulator analysis (Table 16). We elected to only analyze our gene expression data for associated upstream transcription factors and nuclear receptors. This analysis indicated that as a result of BDE-47 exposure, 26 transcription factors were predicted to be activated upstream of our observed changes in gene expression in mTORC2 control mice (such as PPARG, EP300, RB1, MEF2C, SMARCA4, and ESR1), while 8 transcription regulators were predicted to be inhibited in these same mice (such as HDAC1, 3IRF2, and HDAC4). In BDE-47 exposed mice lacking mTORC2, only two transcription factors were predicted to be activated (SMARCA4 and STAT5B), while none were inhibited. Interestingly, it appears that mTORC2 deletion abolished the effects of BDE-47 exposure on genes regulated by most transcription factors predicted activated or inhibited due to BDE-47 exposure in control mice. The only transcription factor predicted to be activated due to BDE-47 exposure in both mTORC2 control and knockout mice was SMARCA4, while STAT5B was predicted to be activated in mTORC2 knockout mice due to BDE-47 exposure but not in mTORC2 controls.

We then took the predicted activated and inhibited transcription factors from BDE-47 exposed mTORC2 control and mTORC2 knockout mice, and used the growth function of IPA to build a network of interactions linking the transcription factors with mTORC1 and 2. The resulting network for the effect of BDE-47 exposure in mTORC2 control mice is presented in Figure 31. Interestingly, all the above transcription factors were connected to mTORC2 via mTORC1, with HIFIA being the only predicted transcription regulator directly connected to mTORC1. However, despite the fact that all of these predicted regulators were connected directly or indirectly to mTORC1, the IPA dataset of transcription regulators predicted to be affected due to BDE-47 exposure in mTORC2 knockout did not result in any network, suggesting that mTORC2 deletion may prevent the effects of early-life BDE-47 exposure on

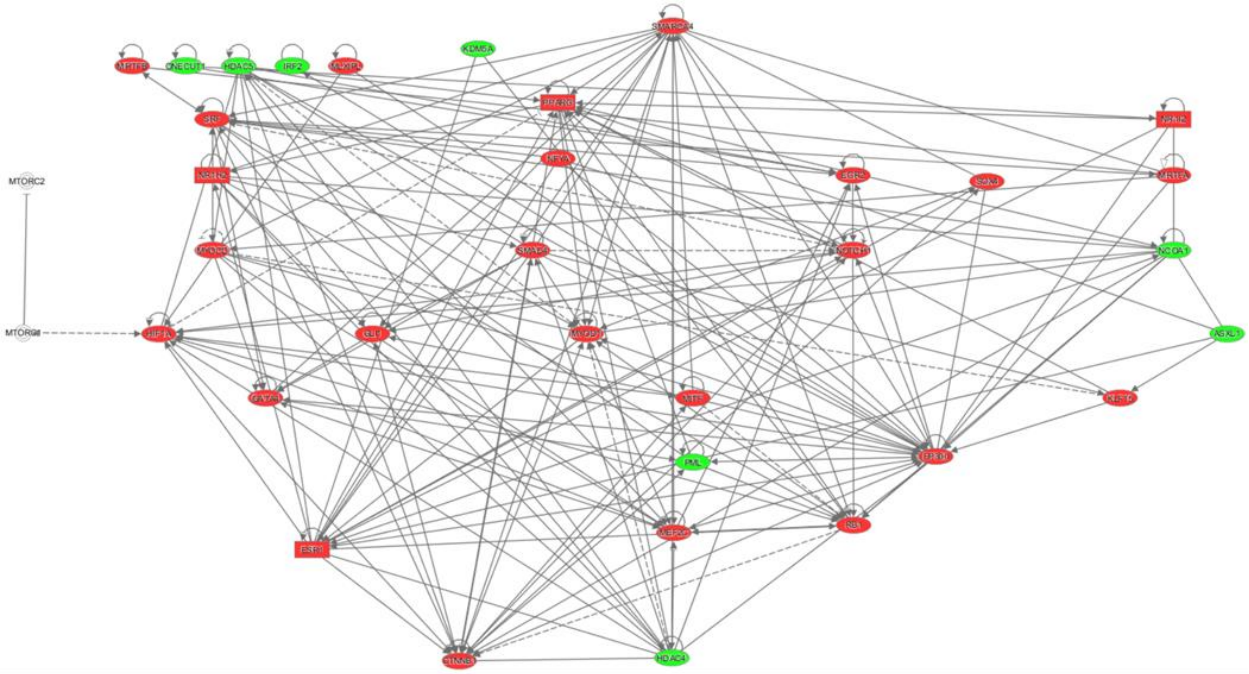

Figure 31. IPA-Generated Network of Transcription Factors Downstream of mTORC1 and 2 that are Activated and Inhibited due to BDE-47 Exposure in mTORC2 control mice. Using the predicted activated (red) and inhibited (green) transcription regulators altered due to BDE-47 exposure mTORC2 control mice in combination with IPA's growth function, we generated a predicted network of transcription factors downstream of mTORC1 and mTORC2. Interestingly, all the above transcription factors were connected to mTORC2 via mTORC1, with HIFIA being the only predicted transcription regulator directly connected to mTORC1. However, despite the fact that all of these predicted regulators were connected directly or indirectly to mTORC1, the IPA dataset of transcription regulators predicted to be affected due to BDE-47 exposure in mTORC2 knockout (STAT5B and SMARCA4 only, both activated) did not result in any network.

gene expression to a significant degree. However, in mTORC2 knockouts, SMARCA4 was still predicted to be activated, suggesting that the effect of BDE-47 exposure on this predicted transcription factor may be independent of mTORC2, unlike all other transcription factors present in Figure 31. Lastly, STAT5B was predicted to be activated in mTORC2 knockout mice only.

Table 16. IPA predicted transcription factors and nuclear receptors for BDE-47 exposed mTORC2 controls and knockouts.

Predicted upstream regulators affected in BDE-47 exposed mTORC2 controls			Predicted upstream regulators affected in BDE-47 exposed mTORC2 knockouts		
TF	Direction	Zscore	TF	Direction	Zscore
MRTFB	Activated	2.616	STAT5B	Activated	2.135
SRF	Activated	3.818	**SMARCA4***	**Activated**	**2.449**
MRTFA	Activated	2.221			
SOX4	Activated	2.219			
PPARG	Activated	3.108			
NOTCH1	Activated	2.145			
HIF1A	Activated	2.475			
MYOCD	Activated	2.243			
GLI1	Activated	2.236			
GATA4	Activated	2.132			
NR1H2	Activated	2.178			
EGR2	Activated	2.183			
NFYA	Activated	2			
SMAD4	Activated	2.205			
MYOD1	Activated	3.003			
EP300	Activated	2.759			
MITF	Activated	2.385			
RB1	Activated	2.536			
NR1I2	Activated	2.024			
MEF2C	Activated	3.083			
MLXIPL	Activated	2.418			
KLF15	Activated	2.125			
SMARCA4*	**Activated**	**2.744**			
CTNNB1	Activated	2.398			
ESR1	Activated	2.598			
HDAC5	Inhibited	-2.414			
IRF2	Inhibited	-2			

NCOA1	Inhibited	-2.228
ASXL1	Inhibited	-2.236
KDM5A	Inhibited	-2.646
PML	Inhibited	-2.551
ONECUT1	Inhibited	-2.219
HDAC4	Inhibited	-2.607

*SMARCA4 was the one transcription factor predicted to be activated due to BDE-47 exposure in both mTORC2 control and knockout mice

Ingenutiy pathway analysis identifies similar and dissimilar associations with diseases, biological functions, and toxicological responses at the systems level due to BDE-47 exposure in mTORC2 control and knockout mice

In addition to using IPA to understand the possible regulatory network associated with our observed gene expression changes in response to early-life BDE-47 exposure in mTORC2 control and mTORC2 knockout mice, we also utilized this platform to identify potential diseases, biological processes, and toxicological functions associated with observed changes in gene expression as a result of BDE-47 exposure in exposed mTORC2 control and knockout mice. Significantly enriched disease and biological functions in the IPA results for both datasets included organismal development, cell morphology, organ morphology, tissue morphology, cardiovascular disease, organismal injury and abnormalities, lipid metabolism, molecular transport, small molecule biochemistry, tissue development, cellular movement (the top term for BDE-47 exposed mTORC2 knockout mice), carbohydrate metabolism, metabolic disease, endocrine system disorders, endocrine system development and function, nutritional disease, connective tissue development and function, gastrointestinal disease, cellular development, and cellular growth and proliferation (Figure 32).

Diseases and biological functions enriched for BDE-47 exposed mTORC2 control mice only include skeletal and muscular system development and function (the top term for BDE-47 exposed mTORC2 control mice), neurological disease, skeletal and musclular disorders, cardiac system development and function, embryonic development, organ development, hereditary disorder, psychological disorders, cellular function and maintenance, cellular assembly and organization, hepatic system disease, protein synthesis, and energy production.

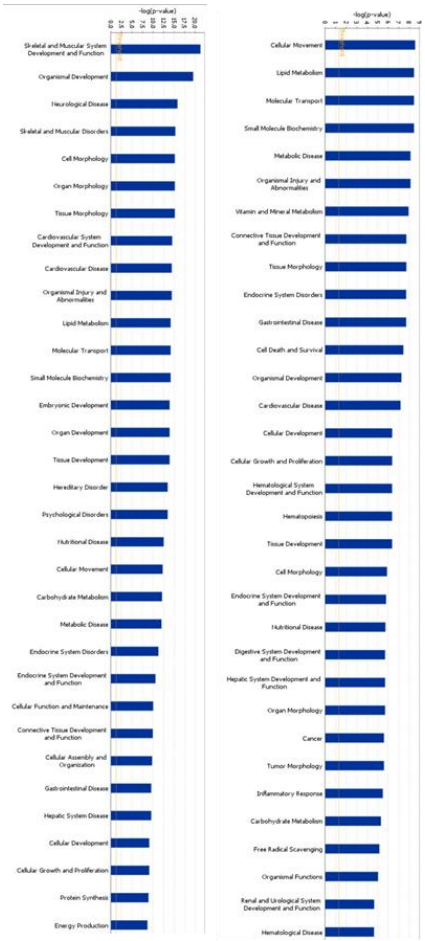

Figure 32. IPA Disease and Biological Functions for BDE-47 Exposed Control (Left) and mTORC2 Knockout (Right) mice in the mTORC2 Experiment. Many associated diseases and biological functions were common between our two sets of differentially expressed genes, such as organismal development, cell morphology, organismal injury and abnormalities, and lipid metabolism. However, there were some distinct functions for both BDE-47 exposure in mTORC2 controls (such as skeletal and muscular system development and function, organ development, hepatic system disease, and others) and BDE-47 exposure in mTORC2 knockouts (such as vitamin and mineral metabolism, cell death and survival, and hematological system development and function, and others).

For BDE-47 exposure in mTORC2 knockouts, enriched diseases and biological functions included vitamin and mineral metabolism, cell death and survival, hematological system decelopment and function, hematopoesis, digestive system development and function, hepatic system development and function, cancer, tumor morphology, inflammatory response, free radical scavenging, renal and urological system development and function, hematological disease. Interestingly, despite its being highly significant, the significance for the most enriched term in this dataset was much lower than the top term for the BDE-47 exposed mTORC2 control dataset.

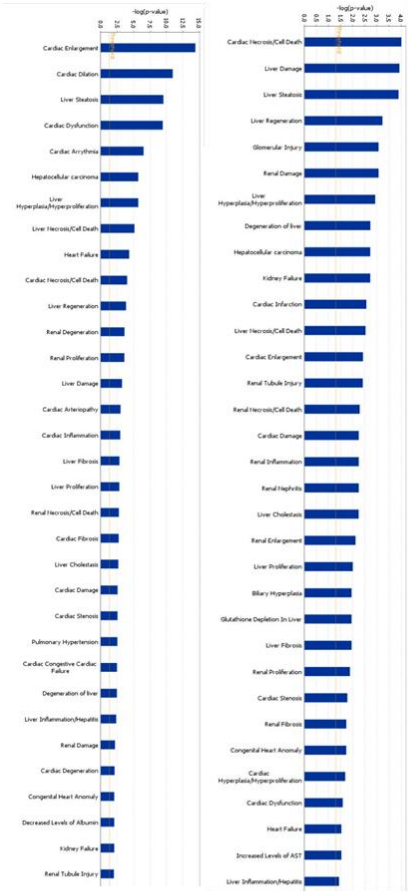

Figure 33. IPA Toxicological Functions for BDE-47 Exposed Control (Left) and mTORC2 Knockout (Right) mice in the mTORC2 Experiment. Many associated diseases and biological functions were common between our two sets of differentially expressed genes, such as cardiac enlargement, liver steatosis, liver necrosis/cell death, and cardiac dysfunction. However, there were some distinct functions for both BDE-47 exposure in mTORC2 controls (such as cardiac dealation, cardiac inflammation, cardiac fibrosis, and others) and BDE-47 exposure in mTORC2 knockouts (such as glomerular injury, biliary hyperplasia, glutathione depletion in liver, and others).

We also used IPA to predict toxicological functions associated with observed expression changes in the BDE-47 exposed mTORC2 control and knockout datasets (Figure 33). Common enriched functions due to BDE-47 exposure in both mTORC2 control and knockout mice include cardiac enlargement, liver steatosis, cardiac dysfunction, hepatocellular carcinoma, liver hyperplasia/hyperproliferation, liver necrosis/cell death, heart failure, cardiac necrosis/cell death (top term for knockouts), liver regeneration, renal proliferation, liver damage, liver fibrosis, liver proliferation, renal necrosis/cell death, liver cholestasis, cardiac damage, cardiac stenosis, degeneration of liver, congenital heart anomaly, kidney failure, liver inflammation/hepatitis, renal damage, and renal tubule injury.

Terms significantly enriched due to BDE-47 exposure in mTORC2 control mice only incude cardiac dialation, cardiac arrythmia, renal degeneration, cardiac arteriopathy, cardiac inflammation, cardiac fibrosis, pulmonary hypertension, cardiac congestive cardiac failure, cardiac degeneration, and decreased levels of albumin.

Lastly, terms enriched due to BDE-47 exposure in mTORC2 knockout mice only incude glomerular injury, cardiac infarction, renal inflammation, renal nephritis, renal enlargement, biliary hyperplasia, glutathione depletion in liver, renal fibrosis, cardiac hyperplasia/hyperproliferation, and increased levels of AST. As with disease and biological

functions, the top enriched term for mTORC2 knockouts (cardiac necrosis/cell death), while significantly enriched, was much less significant in comparison to the top enriched term for BDE-47 exposure in mTORC2 control mice (cardiac enlargement).

VII. Discussion

Understanding the differential roles of mTORC1 and mTORC2 in the regulation of hepatic gene expression

The role of the mTOR pathway in regulating hepatic gene expression is undoubtedly complex. However, our liver-specific deletions of mTORC1 and mTORC2 using a hepatocyte-specific Cre/LoxP knockout of *Raptor* or *Rictor* provide the first comparison of mTOR complex roles in hepatic gene expression regulation, and thus novel insights into mTOR biology.

The role of mTORC1 in hepatic gene expression regulation

It is interesting to note that liver-specific deletion of mTORC1 does not completely abolish phosphorylation of S6K1 at Thr389 at PND75 (Figure 3), which suggests that other molecules may also target this phosphorylation site as well. S6K1 is a key component of the most well-characterized pathway regulating protein synthesis and ribosomal biogenesis, and while the mTOR pathway is critical to normal growth and metabolism, there are likely other pathways capable of regulating S6K1 and its downstream effector functions. These pathways may play a minor role in regulating S6K1 activity, given that Thr389 phosphorylation decreases significantly following mTORC1 deletion. Given the role of S6K1 in feedback regulation of

mTORC2 activity (Zinzalla et al., 2011), it makes sense that mTORC1 deletion triggers increased mTORC2 activity based on AKT Ser473 phosphorylation (Figure 3). However, this is complicated by the fact that deletion of mTORC1 leads to positive enrichment of ribosomal and translation-related gene sets (Table 5), which suggests that there may be some compensatory mechanism or feedback loops regulating ribosome biogenesis and protein synthesis either independently of mTORC1 or in response to developmental mTORC1 knockout.

The phenomenon of increased mTORC2 activity in response to deletion of mTORC1 makes understanding the differential roles of mTORC1 and 2 in gene expression regulation complex, as genes significantly affected due to only deletion of "mTORC1" (Figure 6, Table 1) could in reality be altered due to the deletion of mTORC1, the increase in activity of mTORC2, or both. As such, it may be more appropriate to state that the genes observed to be significantly altered due to the deletion of mTORC1 in the liver are not specifically "dependent" on mTORC1 activity, but are rather altered in the directions observed due to the balance of mTOR activity shifting from mTORC1 towards mTORC2 due to mTORC1 knockout. Based on Metascape dependency analysis and GSEA, one group of genes most sensitive to mTORC1 knockout only were genes and gene sets involved in cholesterol biosynthesis (Figure 3, Table 5). Based on GSEA, these genes tended to be downregulated, which makes sense given the role of mTORC1 in activating SREBF1, one of the key regulators of metabolism of cholesterol and other lipids in the liver (Mao and Zhang, 2018). In fact, IPA predicted that SREBF1 (and SREBF2) would be "inhibited" based on the changes in gene expression observed in mTORC1 knockout (Table 6, Figure 12A). It is interesting to note, however, that mTORC1 knockout does not significantly alter triglyceride levels in the blood or liver based on our analysis, and that mTORC1 knockout

also leads to downregulation of the *Lpin1* gene, which links mTORC1 to SREBF1 by inhibiting SREBF1 until mTORC1 phosphorylates it.

Hepatocyte-specific deletion of mTORC1 also appears to have effects on innate and adaptive immunity within the liver. In particular, we say that the terms "regulation of chemokine production," "inflammatory response," "neutrophil degranulation," "platelet degranulation," and "adaptive immune system" were enriched as a result of mTORC1 knockout only in our Metascape analysis (Figure 6). GSEA also showed that gene sets associated with immunity such as "HALLMARK_ALLOGRAFT_REJECTION," "HALLMARK_INFLAMMATORY_RESPONSE," "KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION," "REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL," and more. Specific genes of interest altered due to deletion of mTORC1 that were related to the general theme of immunity include one of the key members of the classical pathway of the complement system *C1qb* (upregulated by mTORC1 KO), the chemokine *Ccl6* (upregulated by mTORC1 KO), and the rodent-specific toll-like receptor *Tlr12* (upregulated by mTORC1 KO). However, contrary to situations in which mTORC1 inhibition via rapamycin suppresses the immune system Morrisett, et al., 2002; Kasiske et al., 2008; Zimmerman et al., 2018) , it appears that genes associated with immunity in mTORC1 knockouts mice were upregulated in the liver. This difference may be due to the timing and specific methods that we used to suppress mTORC1 activity.

Deletion of mTORC1 also led to notable changes in expression of genes and gene sets associated with cell cycle and cellular senescence (Figure 6, Table 5). Metascape results indicated that mTORC1 knockout led to enrichment of ontology terms associated with cell cycle

("negative regulation of cell proliferation, negative regulation of cell cycle," Figure 6). Concordantly, GSEA showed that many gene sets associated with cell cycle progression (such as "HALLMARK_E2F_TARGETS," "REACTOME_CELL_CYCLE_CHECKPOINTS," REACTOME_MITOTIC_METAPHASE_AND_ANAPHASE, and "REACTOME_APC_C:CDC20_MEDIATED_DEGRADATION_OF_CYCLIN_B", Table 5) and actin cytoskeletal regulation ("REACTOME_RHO_GTPASE_EFFECTORS," and "REACTOME_RHO_GTPASES_ACTIVATE_FORMINS") were positively enriched due to deletion of mTORC1. Interestingly, while Metascape analysis of genes dependent on mTORC1 did not corroborate this, gene sets associated with cellular stress and senescence ("REACTOME_DNA_DAMAGE_TELOMERE_STRESS_INDUCED_SENESCENCE" and "REACTOME_THE_ROLE_OF_GTSE1_IN_G2_M_PROGRESSION_AFTER_G2_CHECKPOINT") were positively enriched as well due to mTORC1 knockout only in GSEA (Table 5). Notable specific genes altered due to mTORC1 knockout only that may contribute to these results are *Ccnd1*, *Cdk1*, *Tubb2a*, *Tubb2b* (all upregulated due to mTORC1 KO), and *Gas1* (downregulated by mTOR1 KO). The fact that mTORC1 knockout only influences these types of genes was corroborated yet again by the disease and biological functions for mTORC1 knockout produced by IPA core analysis (Figure 13).

Two other notable functions that are altered by mTORC1 knockout only include changes in genes differentially expressed in response to hypoxia (ontology term "response to hypoxia," see Figure 6) and autophagy regulation, with the most notable gene related to these two terms being the autophagy regulator *Depp1* (downregulated in mTORC1 KO) (Stepp et al., 2014).

A general theme that emerged as a result of mTORC1 deletion is that in comparison to mTORC1 inhibition later in life via rapamycin, deletion of mTORC1 before birth appears to

affect expression of mTORC1-regulated genes and gene sets in the opposite manner including genes and gene sets related to ribosomal biogenesis and protein translation, cell cycle, immunity, and potentially autophagy. Interestingly, lipid metabolism was the major exception. Why this is the case remains to be understood, but it likely has to do with both the developmental timepoint at which mTORC1 activity is suppressed using both methods, as well as the method used (exon deletion vs a chemical inhibitor). To our knowledge, this is the first experiment to characterize mTORC1 gene expression regulation in the liver using a hepatocyte-specific deletion of Raptor. As such, the results here provide an interesting and important distinction to make for future experiments characterizing mTORC1 activity, as the timing and method of mTORC1 activity perturbations lead to very different, potentially conflicting results, and may be indicative of a complex developmental program regulating the transcriptome for which mTORC1 plays a critical role in directing the trajectory.

The role of mTORC2 in hepatic gene expression regulation

In contrast to how mTORC1 activity does not fully abolish Thr398 phosphorylation of S6K1, hepatocyte-specific deletion of mTORC2 completely abolishes AKT Ser473 phosphorylation at PND75 (Figure 3). Additionally, mTORC2 knockout, which should in theory lead to decreased mTORC1 activity given that the mTORC2 substrate AKT activates mTORC1 via PRAS40 phosphorylation, leads to an increase in activity of mTORC1 (Figure 3). The question of why decreased phosphorylation of AKT is associated with increased mTORC1 activity, even though AKT canonically activates mTORC1 (Laplante and Sabatini, 2009) remains to be answered. However, the fact that mTORC1 activity is increased in mTORC2 knockouts is consistent with the upregulation of ribosome genes observed due to mTORC2 knockout in GSEA (Table 5). However, as with our observation of how mTORC1 knockout also

upregulates ribosome biogenesis genes, it is entirely possible that an unknown compensatory mechanism is involved.

Like our observations with mTORC1, the fact that mTORC1 activity increases in response to mTORC2 knockout makes interpreting the results of genes that are "dependent on mTORC2 only" (Figure 7, Table 2) more complex. In this case, it appears that the balance of both mTOR complexes is completely shifted towards mTORC1 due to mTORC2 knockout. As such, it may be more appropriate to state that the genes observed to be significantly altered due to mTORC2 knockout in the liver are not specifically "dependent" on mTORC2 activity, but are rather altered in the directions observed due to the balance of mTOR activity shifting from mTORC2 towards mTORC1 due to mTORC2 knockout.

Metascape analysis revealed that genes significantly altered due to mTORC2 knockout only (Figure 7) were associated with lipid metabolism and transport (the term "lipid transport" was not enriched for mTORC1 knockout), as well as innate immunity (associated ontology terms: "neutrophil degranulation," "regulation of symbiosis, encompassing a mutualism through parasitism," and "regulation of reactive oxygen species metabolic process"). The fact that the top enriched ontology term associated with this group of genes was "transmembrane receptor protein tyrosine kinase signaling pathway" is likely due to the fact that the above genes are regulated by mTORC2 (given that mTORC2 receives many signals from receptor protein tyrosine kinases such as the insulin receptor). However, it is interesting to note that mTORC1 knockout did not yield enrichment of this same ontology term in Metascape. Specific genes of interest in this groups include *Fabp5*, *Igfbp2*, and *Nlrp12* (all upregulated due to mTORC2 KO). While these genes of interest are all upregulated, it is important to note that the majority of genes altered by mTORC2 knockout were downregulated (Table 2).

Notably, GSEA only produced two genes sets that passed our threshold for significant enrichment due to mTORC2 knockout only (both positively): "HALLMARK_MYC_TARGETS_V2" and "HALLMARK_OXIDATIVE_PHOSPHORYLATION." These suggest that developmental knockout of mTORC2, in addition to affecting the specific subsets of genes observed in Metascape, may upregulate gene sets involved in cellular proliferation and oxidative metabolism. Interestingly, one study found that mTORC2 acting via AKT was essential for c-Myc-driven oncogenesis of hepatocellular carcinoma (Xu et al., 2019), indicating that mTORC2 is linked to c-Myc in some manner. Additionally, c-Myc itself stimulates glucose catabolism and oxidative phosphorylation via pyruvate dehydrogenase kinase 1 (PDK1), and AKT stimulates similar functions via activation of hexokinase 2 (HK2) (Méndez-Lucas et al., 2017; Luo et al., 2018). However, our observations with mTORC2 in the context of our liver-specific developmental knockout follow the trend that developmental knockout of mTOR complexes actually leads to long-lasting upregulation of the functions that are canonically activated by the mTOR pathway, in this case specifically gene sets regulated by mTORC2. Additionally, IPA core analysis also confirmed that mTORC2 knockout affects liver lipid and carbohydrate metabolism (Figure 13), and suggested that mTORC2 knockout (but not mTORC1 knockout) was associated with changes in gene expression observed in glutathione depletion (Figure 14).

The transcriptome regulatory roles of mTORC1 and mTORC2: similar and opposing roles in developmental regulation of key functions

One of the most interesting findings of our Metascape analysis of genes regulated in opposing directions by deletion of mTORC1 or mTORC2 (Figure 8, Table 3) was that genes associated with mTOR signaling itself, (based on the ontology terms "negative regulation of

cellular response to growth factor stimulus," "positive regulation of protein kinase activity," "regulation of wound healing," and "fluid shear stress and atherosclerosis," see Figure 8) are altered in opposing directions due to mTORC1 and mTORC2 deletion, alongside genes associated with xenobiotic metabolism and temperature homeostasis. At least for the mTOR signaling-related ontology terms in Figure 8, it is interesting to think that the shift in the balance of mTOR complex activity may be leading to opposing effects on growth factor-stimulated and canonically "mTOR-controlled" genes. While it is possible that these genes are sensitive to the balance of mTOR complex activity, it is also plausible that these genes are sensitive to only the activity of a single complex. As an example, a gene may be downregulated by mTORC1 knockout, and upregulated due to mTORC2 knockout as a result of increased activity of mTORC1.

To our knowledge, the fact that deletions of mTORC1 and mTORC2 have opposing effects in liver xenobiotic metabolism, or simply that the mTOR pathway may play a role in xenobiotic metabolism in the first place, has not been demonstrated elsewhere. It is quite plausible that the mTOR pathway, which integrates so many different internal and external environmental stimuli and regulates growth and metabolism, accordingly, would be sensitive to environmental xenobiotics and coordinate their metabolism. In fact, another line of research from our lab analyzing over 600,000 chemical-gene interactions covering over 2000 chemicals and the human, mouse, and rat genomes suggests that the mTOR pathway is one of the most sensitive to external chemical stimuli of all kinds, including nutrients, environmental toxins, and pharmaceuticals (paper in progress). However, one interesting nuance is that in GSEA, the term "HALLMARK_XENOBIOTIC_METABOLISM" was only significantly (negatively) enriched

due to mTORC1 knockout, suggesting that mTORC1 knockout may lead to subtle perturbations of gene sets involved in xenobiotic metabolism that are not altered in mTORC2 knockout.

Cytochrome P450 monooxygenases play a significant role in xenobiotic metabolism (McDonnel and Dang 2013), and unsurprisingly genes within this gene set included three cytochrome P450 genes: *Cyp2b10*, *Cyp26a1*, and *Cyp46a1*. All three were upregulated by mTORC1 knockout and downregulated by mTORC2 knockout. Another gene that fell into this category was the glutathione-S-transferase gene *Gsta1*, which also plays a major role in xenobiotic metabolism (Table 3) (Allocati et al., 2018). However, it appears that the regulatory relationship of mTORC1 and 2 to cytochrome P450 monooxygenases and glutathione-S-transferases in the liver is not simple, as several genes within these two families were altered by mTORC1 deletion only (*Cyp4f14*, *Cyp7b1*, *Cyp8b1*, *Cyp27a1*, *Cyp51*, and *Gstp1*, all downregulated, see Table 1), or mTORC2 deletion only (*Cyp4a32* and *Gstt2* were upregulated, while *Cyp26b1* was downregulated, see Table 2), as well as altered in similar directions in both knockouts (Cyp2a4, Cyp2b9, and Cyp4a14 were upregulated in both knockouts). One possible reason for the complex relationship of mTOR-controlled xenobiotic metabolism genes is that xenobiotic metabolism in the liver must balance two different requirements: the ability to sense environmental stimuli such as xenobiotics in order to metabolize toxins when they challenge the cell (possibly via mTOR given the role of mTOR in sensing the chemical environment and its aforementioned sensitivity to xenobiotics) (paper in progress), and also maintain a stable level of xenobiotic processing in the face of rapidly fluctuating mTOR activity. As such, the cytochrome P450 monooxygenase system may have developed the observed regulatory complexity and redundancy to stay stable at the systems level when the liver is challenged by environmental xenobiotics. However, this is highly speculative and requires further testing. Lastly, cytochrome

P450 monooxygenases also play a significant role in lipid metabolism in the liver, providing a potential link between xenobiotic and lipid metabolism that may provide one possible explanation regarding why environmental xenobiotics such as BDE-47 can trigger dyslipidemia in the liver.

One other gene that fell into the category of genes that were upregulated by mTORC1 knockout and downregulated by mTORC2 knockout was the antigen, fatty acid translocase, and scavenger receptor gene *Cd36*. This follows the trend of developmental knockouts causing long-lasting gene expression to be regulated in the direction opposite of what might be expected. In this case, deletion of mTORC1 triggers increased *Cd36* expression, contrary to the general understanding that increased mTORC1 activity stimulates *Cd36* (Laplante and Sabatini, 2013). Meanwhile, mTORC2 knockout (and increased mTORC1 activity as a result) led to downregulation of *Cd36*. One gene set was observed to fall into this category as well: "REACTOME_INTERLEUKIN_4_AND_INTERLEUKIN_13_SIGNALING," which is known to regulate *Cd36* and *Dusp1* (another gene that fell into this category, Table 3) (Bhattacharjee et al., 2013). Notable genes downregulated in response to mTORC1 knockout and upregulated in response to mTORC2 knockout included the smooth muscle actin *Acta2*, the transcriptional regulator *Egr1*, and *Mir671*, a miRNA whose expression, as mentioned previously, varies significantly between the alcoholic and non-alcoholic forms of fatty liver disease (Estep et al., 2010).

A subset of genes was also altered in similar directions due to deletion of mTORC1 or mTORC2 (Figure 9, Table 4). The most notable biological function associated with these genes based on Metascape analysis was lipid metabolism, but another interesting biological function regulated similarly due to mTORC1 and mTORC2 deletion was "response to stillbenoid" (Figure

9). Notably, the mTOR pathway is responsive to the stilbene resveratrol (Park et al., 2016). Two notable genes were upregulated due to knockout of both mTOR complexes in addition to the set of aforementioned cytochrome P450 monooxygenases: the leptin receptor *Lepr*, and *Myc* (which is interesting to note given that genes associated with "*Myc* targets" were upregulated in mTORC2 knockout only, see Table 5). Both knockouts led to downregulation of *Srebf1*, despite the fact that gene sets associated with *Srebf1* and cholesterol biosynthesis were downregulated in mTORC1 knockout only. Interestingly, knockout of mTORC1 and 2 stimulated up-regulation of the glucokinase gene *Gcn*, the key enzyme that initiates glycolysis, as well as the aquaporin gene *Aqp8*. *Aqp8* localization within hepatocytes is partially controlled by PI3K signaling, is upregulated during bile production, and is dysregulated in liver cholestasis (Lehmann et al., 2008).

Unlike the apparent dependency of SREBF1-regulated genes on mTORC1 only, it appears that genes associated with PPAR signaling, a central node where lipid metabolism and inflammation intersect (Wahli and Michalik, 2012)., are altered in similar directions due to both mTORC1 and mTORC2 knockout, alongside several genes associated with "monocarboxylic acid metabolic process," as well as lipid and cholesterol synthesis (Figure 9, Table 4). Canonically, PPAR signaling is also downstream of mTORC1 (Laplante and Sabatini, 2013), so it remains to be understood why both developmental knockout of mTORC1 and mTORC2 influence the activity of these kinds of genes.

As observed and discussed previously, developmental mTORC1 and 2 knockouts both trigger upregulation of gene sets associated with ribosomal biogenesis and protein translation. While this may, as mentioned previously, be indicative of some compensatory mechanism that is stimulated due to altered activity of both mTOR complexes, it is also possible that

developmental knockout during the prenatal period leads to permanent reprogramming of gene expression through an epigenetic mechanism. Canonically “mTOR-controlled” gene sets related ribosomal biogenesis, cell cycle, oxidative phosphorylation, and cell proliferation, but not cholesterol synthesis, were upregulated due to knockout of mTORC1 and/or 2 (Table 5), indicating that compensatory mechanisms that respond to mTOR complex deletion may exist for many but not all functions regulated by the mTOR pathway.

Based on dependency analysis, there were 112 genes altered due to both mTORC1 and mTORC2 knockouts in total (Figure 10), while 175 genes are dependent on mTORC1 knockout only and only 50 are dependent on mTORC2 knockout only. As such, we can conclude that perturbations of mTORC1 activity beginning in the prenatal stage and lasting to PND75 have a broader effect on the liver transcriptome than mTORC2 perturbations. This is consistent with the fact that as of now, mTORC1 appears to have a larger number of functional roles in the cell than mTORC2 (Liu and Sabatini, 2020). However, it is important to note that these effects overlap to a degree, most notably with genes associated with ribosome biogenesis, protein synthesis (Table 5), and lipid metabolism via PPAR signaling and other mechanisms (Figure 9).

IPA also indicated that changes in gene expression due to both mTOR knockouts were strongly associated with changes diseases and biological functions such as in lipid metabolism, metabolic disease, organismal injury, and more (Figure 13). Additionally, some of the top toxiological functions associated with IPA results in both mTORC1 and mTORC2 knockout mice were liver steatosis, hepatocellular carcinoma, and liver hyperplasia/hyperproliferation (for these and more, see Figure 14). Upstream analysis for both mTORC1 and 2 revealed that one transcription factor was predicted to be inhibited: STAT5B (Table 6). This suggests that the highest common signaling. node upstream of genes altered due to mTORC1 and 2 knockout is

STAT5B, which is plausible given that growth-hormone stimulated STAT5B activity triggers the production of the insulin-like growth factor IGF1 in the liver (Baik, Yu, and Hennighausen, 2012), and the receptor for IGF1, *Igf1r*, is upstream of both mTORC1 and 2. Comparing pathways generated using the predicted upstream regulator results for both mTORC1 and 2 knockouts and IPA growth function (Figure 12) suggests that the regulatory network affected by knockout of both complexes intersects at both SREBF1, CEBPA, CEBPB, and RORA. However, the remaining regulators in each network were unique, suggesting that mTORC1 and 2 have both overlapping and differential roles in regulating the transcriptome by unknown mechanisms. One confusing observation is that mTORC1 knockout results generated a larger network than results for mTORC2 knockout, even though mTORC2 is canonically upstream of mTORC1. The specific mechanisms behind this observation remain to be understood, but it is clear that the roles of mTORC1 and mTORC2 in gene expression regulation and the response of hepatocytes to deletion of either complex are complicated.

Overall, it is interesting to observe that the two mTOR complexes have distinct and overlapping roles in transcription regulation, and that liver-specific knockout of one mTOR complex leads to heightened activity of the other complex (Figure 3). Our western blotting and sequencing experiments provide additional evidence that the two mTOR complexes engage in coordinated crosstalk to maintain optimal activity in the cell. Taken in the context of the fact that chronic mTORC1 inhibition using rapamycin leads to inhibition of mTORC2 activity, it appears that regulation of mTORC1 activity via a chemical intervention and by preventing the complex from forming in the first place have very different effects on mTORC2 activity. Understanding why this is the case may eventually lead to the development of more effective cancer therapies and more effective immunosuppressants. It would be interesting to determine whether siRNA-

mediated knockdown of *Raptor* and *Rictor* results in the same effect as either rapamycin or our observations using the Cre/LoxP system. Additionally, suppressing activity of each complex separately via different methods at different timepoints and characterizing the resulting activity of the opposing complex may yield insights into the complex developmental roles of mTORC1 and 2 and how their interactions change across the lifespan. Additionally, our experiments yielded some results similar to the work done by the Gruppuso lab (Lamming et al, 2014; Boylan et al, 2015), with the most notable examples being observed upregulation of ribosomal and oxidative phosphorylation gene sets in response to mTORC2 knockout, but did not corroborate these experiments in their entirety (for example, genes associated with the 26S proteasome were not enriched in GSEA due to mTORC2 knockout in our experiments). Interestingly, the fasting-refeeding protocol with rapamycin used to probe mTORC1 function triggered upregulation of ribosomal gene sets (Boylan et al, 2015) similar to the ones we observed in mTORC1 knockout mice (Table 5). While the Gruppuso group could only find a few individual genes significantly altered by mTORC2 knockout and no genes significantly altered by mTORC1 knockout (Lamming et al, 2014; Boylan et al, 2015), we observed statistically significant changes in expression of hundreds of genes in response to mTOR complex knockout, a testament to the power of revisiting this type of experiment using next-generation sequencing.

The role of mTORC1 and 2 in mediating the effects of early-life BDE-47 exposure on the liver transcriptome

With an understanding of the differential roles of mTORC1 and 2 in developmental regulation of the transcriptome, we looked to see whether the effects of early-life BDE-47 exposure on the liver transcriptome were dependent on mTORC1. To our knowledge, this is the

first experiment to investigate the role of mTORC1 in mediating the effects of an environmental xenobiotic (BDE-47)

Perinatal BDE-47 exposure exhibits mTORC1-dependent and independent effects on the liver transcriptome

Perinatal BDE-47 exposure did not alter notably the activity of either mTORC1 or 2 in mTORC1 control mice at PND75 (Figure 15). However, in mTORC1 knockout mice, it appears that BDE-47 exposure may lessen the effects of mTORC1 knockout observed in vehicle-exposed mTORC1 knockout by increasing phosphorylation of S6K1 at Thr389 and decreasing AKT phosphorylation at Ser473 to a small degree (Figure 15). This result, if it is accurate, suggests that BDE-47 exposure may subtly trigger S6K1 phosphorylation independently of mTORC1, thus maintaining the negative feedback loop that suppresses mTORC2 activity to a degree. Given the nature of our western blots, additional work must be done to ascertain the validity of this claim.

Despite the fact that BDE-47 exposure did not alter the activity of mTORC1 or 2 at PND75 in mTORC1 control mice, there were a number of genes for which perinatal BDE-47 exposure significantly altered expression at PND75 and for which the effect of BDE-47 exposure was abolished when mTORC1 was deleted from the liver (Figure 18, Table 7). Our Metascape analysis of these genes (Figure 18) indicated that these genes were primarily involved in innate immunity (ontology terms: “recycling pathway of L1,” “prolactin signaling,” and “negative regulation of cytokine production”), lipid metabolism (ontology terms: “metabolism of lipids” and “fat cell differentiation”), cell cycle regulation (ontology terms: “positive regulation of mitotic cell cycle” and “prolactin signaling”), cell adhesion and motility (ontology terms:

"recycling pathway of L1" and "tight junction") ion transport (ontology terms: "anion transport," "cellular metal ion homeostasis," and "tight junction"). The term "regulation of carbohydrate metabolic process" was also present as an enriched. Interestingly, it appears that while the results are not entirely overlapping, these general biological themes are regulated by mTORC1 and 2. The one exception to this at first glance was the ontology term "biomineral tissue development." However, further investigation into this ontology term showed that the genes triggering its enrichment were *Ercc2* (upregulated due to BDE-47 exposure, involved in DNA damage repair), as well as *Gas6* and *Smpd3* (downregulated due to BDE-47 exposure, involved in cell proliferation and cell cycle regulation).

Additional specific genes of note for which mTORC1 knockout abolished the effect of BDE-47 exposure include *Ccnd1*, *Cyp17a1*, *Fabp2*, *Gcn*, Rbp1, and *Lpin1* (All upregulated due to BDE-47 exposure in mTORC1 control mice, see Table 7). Interestingly, *Ccnd1* and *Lpin1* expression were demonstrated to be altered in our mTORC1 knockout mice (Table 1), while *Gcn* was affected due to deletion of mTORC1 or mTORC2 (Table 4). Notable genes downregulated due to BDE-47 exposure in mTORC1 control animals only include the autophagy regulator *Depp1*, the aquaporin *aqp8*, the cytochrome P450 *Cyp4a14*, *Gas6*, the myosin light chain *Myl9*, the phosphate transporter *Slc17a8*, and the tubulin genes *Tubb2a* and *Tubb2b* (Table 7). *Depp1*, *Slc17a8*, *Tubb2a* and *Tubb2b* were demonstrated to be altered in our mTORC1 knockout mice (Table 1), while *Aqp8* and *Cyp4a14* were altered due to both mTORC1 and mTORC2 deletions (Table 4).

Interestingly, while there were changes in gene expression of many individual genes, GSEA did not produce any gene sets significantly enriched due to BDE-47 exposure in mTORC1 control mice. This suggests that perturbations to the genome due to perinatal BDE-47

exposure in these mice are relatively focused in nature, rather than effects being subtly spread across large sets of genes with functional significance as well. Additionally, upstream analysis for IPA only predicted alterations in activity of a single transcription factor: SMARCA4 (also known as BRG1, a key component of the SWI/SNF chromatin remodeling complex). The transcription factor was inhibited, suggesting that due to perinatal BDE-47 exposure, chromatin may be more compact than normal in mTORC1 control mice at regions controlled by SMARCA4. However, IPA associated many different diseases and biological functions with gene expression changes in BDE-47 exposed mTORC1 control mice only (as compared to BDE-47 exposed mTORC1 knockouts as well) such as cell and tissue morphology, inflammatory response, cell death and survival, and metabolic disease. In terms of predicted toxicological functions, IPA predicted that changes in gene expression observed due to perinatal BDE-47 exposure in mTORC1 control mice were associated with negative hepatic, cardiac, and renal health outcomes such as cell death and organ degeneration, inflammation, general dysfunction of these three types of organs. Overall, there were many notable permanent changes in gene expression due to BDE-47 exposure in the mTORC1 wild type mice that did not occur in BDE-47 exposed mTORC1 knockout mice, and these chances were associated with many different biological functions and health outcomes related to cell growth and death, inflammation, and metabolic disease.

The specific ontology terms enriched for Metascape analysis of genes altered due to BDE-47 exposure in mTORC1 knockout mice only were entirely unique from those enriched due to BDE-47 exposure in mTORC1 control mice, though some similarities in terms of general biological functions were present (Figure 19). The most notable changes were related to the acute phase of the innate immune response and lipid biosynthesis, though other terms related to

innate immunity and lipid metabolism were present as well. Other notable enriched terms distinct from BDE-47 exposed mTORC1 control mice were ontology terms associated with connective tissue development, temperature homeostasis, adenylate cyclase-modulating GPCR signaling, and regulation of the ERK1/2 cascade.

Notable genes altered due to BDE-47 exposure in mTORC1 knockout mice only within these ontology terms included the circadian rhythm-controlled *Noct*, IGF-binding *Igfbp5*, scavenger receptor *Marco* (all upregulated). Downregulated genes in this category included *Rnase4*, *Cxcl1* (altered in mTORC2 knockout, see Table 2), *Cyp7b1* (altered in mTORC1 knockout, see Table 1), *Cyp39a1*, the fatty acid elongase *Elovl6*, *Orm1*, *Orm2*, and *Orm3*, and the thyroid-hormone responsive lipid metabolism/lipogenesis regulator *Thrsp*. While only a few genes are described here, most significant genes altered due to BDE-47 exposure in knockouts appear to be downregulated (Table 8).

In contrast to the lack of enriched gene sets due to BDE-47 exposure in mTORC1 control mice, GSEA indicated that many different gene sets were enriched due to BDE-47 exposure in mTORC1 knockouts (Table 11). The overwhelming majority of gene sets were positively enriched. Many of these gene sets were related to immunity such as ("HALLMARK_ALLOGRAFT_REJECTION," and "HALLMARK_INFLAMMATORY_RESPONSE," both of which were upregulated due to mTORC1 knockout alone as well (Table 5). However, other immune-related gene sets not previously seen on mice with a liver-specific mTORC1 or 2 deletion appeared when mTORC1 knockout mice were exposed to BDE-47 as well, such as "KEGG_CHEMOKINE_SIGNALING_PATHWAY," "KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION,"

"KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY,"
"KEGG_PRIMARY_IMMUNODEFICIENCY,"
"KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION,"
"REACTOME_COSTIMULATION_BY_THE_CD28_FAMILY,"
"REACTOME_INFLAMMASOMES," "REACTOME_INTERLEUKIN_10_SIGNALING," and "REACTOME_SCAVENGING_BY_CLASS_A_RECEPTORS" (Table 11). It should be noted that Kupffer cells in the liver express scavenger receptors at high levels (Murphy and Weaver, 2017), such as CD36 (Kawanishi et al., 2018), which appears to be regulated in opposing directions by developmental mTORC1 and 2 knockouts (Table 3). All this suggests that perinatal BDE-47 exposure when mTORC1 is absent in hepatocytes triggers sustained dysregulation of the innate and adaptive immune system, potentially triggering the recruitment and increased activity of natural killer cells, T cells, and Kupffer cells via cytokines and other methods.

Additionally, mTORC1 knockout mice also exhibited positive enrichment of two gene sets associated with the mitotic spindle checkpoint: "HALLMARK_MITOTIC_SPINDLE," and "REACTOME_MITOTIC_SPINDLE_CHECKPOINT" (Table 11), the latter of which was demonstrated to be upregulated in vehicle-exposed mTORC1 knockout mice relative to vehicle-exposed mTORC1 control mice (Table 5), which may suggest increased progression through this phase of the cell cycle. The last notable category of gene sets positively enriched due to BDE-47 exposure in mTORC1 knockout mice were gene sets related to the extracellular matrix, the epithelial-mesenchymal-transition, and cell motility. These included gene sets such as "HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION,"
"REACTOME_COLLAGEN_FORMATION,"

“REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX,” “REACTOME_ECM_PROTEOGLYCANS,” “REACTOME_ELASTIC_FIBRE_FORMATION,” “REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS,” KEGG_DILATED_CARDIOMYOPATHY,” “REACTOME_SYNDECAN_INTERACTIONS,” and “REACTOME_SIGNALING_BY_RHO_GTPASES” (Table 11). Interestingly, Rho GTPases have been described as acting directly downstream of mTORC2 to control actin cytoskeleton dynamics (Jacinto et al, 2004. All this suggests that BDE-47 exposure in mTORC1 knockout mice leads to increased production of the extracellular matrix, potentially via mTORC2 in hepatocytes and/or activity of myofibroblasts (Iwaisako, Brenner, and Kisseleva, 2016). Interestingly, mTORC2 signaling has been demonstrated to play a role in development of the fibrotic matrix in fibrotic lung diseases (Chang et al., 2014). All the above is indicative of liver damage due to BDE-47 when mTORC1 is absent in mTORC1 mice, but when mTORC1 is present in these mice, the effects of BDE-47 are lessened at the level of gene sets. The last upregulated individual gene set was “REACTOME_SIGNALING_BY_PDGF.” PDGF (platelet-derived growth factor) is known to act via mTOR signaling, and previous research shows that activated mTOR signaling (most likely via mTORC1) suppresses expression of the receptor for PDGF, PDGFR (Zhang et al., 2007). It is quite possible that mTORC1 knockout induces to abnormal upregulation of PDGFR, which in turn triggers hyperactivation of mTORC2 signaling when stimulated by BDE-47.

Interestingly, the only three negatively enriched gene sets due to BDE-47 exposure in mTORC1 knockout mice were related to SREBF-controlled cholesterol metabolism: “REACTOME_METABOLISM_OF_STEROIDS,”

"REACTOME_CHOLESTEROL_BIOSYNTHESIS," and "REACTOME_REGULATION_OF_CHOLESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF (Table 11). Notably, the last two of these gene sets were also downregulated due to mTORC1 knockout only (Table 5), suggesting that perinatal exposure to BDE-47 when mTORC1 is absent induces further suppression of cholesterol synthesis through some yet unknown mechanism.

While GSEA suggested that the effect of mTORC1 knockout at the gene set level was exacerbated due to BDE-47 exposure, IPA upstream analysis, based on significantly differentially expressed genes rather than gene sets, predicted that activity of single transcription factor, the estrogen receptor ESR1, was altered (in this case, ESR1 was predicted to be inhibited). In breast cancers, ESR1 has been demonstrated to be downstream of both the mTORC1 and ERK1/2 pathways, indicating that in mTORC1 knockout mice, BDE-47 exposure may be acting via ERK1/2 (Yamamoto-Ibusuki, Arnedos, and André, 2015) and other mechanisms to suppress ESR1 activity relative to vehicle-exposed mTORC1 knockout mice. However, this is only speculative. IPA also indicated that in mice lacking mTORC1, many of the top predicted diseases and biological functions were associated with development and function of specific systems, such as skeletal and muscular system development and function, digestive system development and function, embryonic development, hair and skin development and function, organ development, tissue development, and cellular development. Why this is the case remains to be understood, but it may have to do with changes in expression of transcription factors due to BDE-47 exposure in mTORC1 knockout mice. The term connective tissue disorders was also associated with our gene expression data, corroborating GSEA results related to the extracellular matrix. Interestingly, IPA predicted notably less associated toxicological functions for BDE-47 exposed mice lacking mTORC1 in the liver relative to BDE-47 exposed

mTORC1 control mice. The toxicological terms predicted to be affected due to BDE-47 exposure in mTORC1 knockout mice only (as compared to BDE-47 exposed mTORC1 control mice as well) included liver cirrhosis, liver cholestasis, liver proliferation, and increased levels of ALT, consistent with GSEA results.

There were only 10 genes that were altered in opposing directions due to BDE-47 exposure in mTORC1 control and knockout mice (Table 9). The one that stuck out on this list was the alpha smooth muscle actin *Acta2*, which was downregulated due to BDE-47 exposure in mTORC1 control mice and upregulated due to BDE-47 exposure in mTORC1 knockouts. Interestingly, *Acta2* was also downregulated due to mTORC1 knockout and upregulated due to mTORC1 knockout (Table 3). Another intriguing gene, in this case upregulated due to BDE-47 exposure in mTORC1 controls and downregulated in mTORC1 knockouts, was the circadian rhythm-associated transcription regulator *Ciart*.

There were several genes that were affected in similar directions due to BDE-47 exposure in mTORC1 control and knockout mice. Metascape analysis yielded three enriched ontology terms that were related to lipid metabolism: “steroid metabolic process,” “regulation of steroid metabolic process,” and “monocarboxylic metabolic process” indicating that BDE-47 exposure may affect some steroid and lipid metabolism genes independently of mTORC1 (Figure 20). Based on other enriched Metascape terms, BDE-47 exposure may affect immunity (“inflammatory response” and “lymphocyte differentiation”), as well as glucose metabolism (“response to glucose”) independent of mTORC1 as well (Figure 20). Interestingly, the ontology term “transmembrane receptor protein serine/threonine kinase signaling pathway” was enriched as well. Our Metascape results for the effect of mTORC2 knockout on the liver transcriptome produced the enriched ontology term “transmembrane receptor protein tyrosine kinase signaling

pathway" (Figure 7), a highly similar ontology term to "transmembrane receptor protein serine/threonine kinase signaling pathway." This may be evidence that there are some genes affected by BDE-47 exposure independent of mTORC1 that are affected via mTORC2. Some notable genes within these ontology terms that were upregulated in response to early-life BDE-47 exposure in both mTORC1 control and knockout mice include the fatty acid transporter *Mfsd2a* (which was downregulated due to mTORC2 knockout, see Table 2). BDE-47 exposure downregulated many more genes independent of mTORC1, including cytochrome P450 monooxygenases *Cyp26a1* (upregulated by mTORC1 knockout, downregulated by mTORC2 knockout, see Table 3) and *Cyp26b1* (downregulated by mTORC2 knockout, see Table 2), the transcription factor *Egr1* (downregulated by mTORC1 knockout, upregulated by mTORC2 knockout), the insulin-like growth factor *Igf2*, and the hepatic acute-phase proteins *Saa1* and *Saa2* (both upregulated by mTORC1 and mTORC2 knockouts). For a full list of genes affected by BDE-47 exposure independently of mTORC1, please refer to Table 10.

It is interesting to note that there were no gene sets significantly enriched due to BDE-47 exposure in both mTORC1 control and knockout mice, and the one transcription factor predicted to be altered due to BDE-47 exposure was distinct for each genotype. However, many disease and biological functions predicted to be influenced due to BDE-47 exposure in both genotypes were still produced by IPA related to connective tissue development and function, organ injury, morphology, and abnormalities, hepatic system development and function, lipid metabolism, immunological disease, cell-to-cell signaling and interaction, cell growth and proliferation, and cancer. Common predicted toxicological functions included liver hyperplasia/hyperproliferation, liver damage, and increased levels of AST.

Visual inspection of the individual genes affected due to perinatal BDE-47 exposure in the mTORC1 experiment suggest that, consistent with our hypothesis, BDE-47 may act via mTORC1 to reprogram the liver transcriptome (Figure 17). This is based on the fact that the majority of genes lie in the cross-shape formed by dependency analysis cutoffs in the graph illustrating the dependency of BDE-47 exposure effects on mTORC1 (Figure 17), which suggests that most genes are significantly affected by BDE-47 exposure only in the presence or absence of mTORC1. For both genotypes, perinatal BDE-47 exposure triggered long-lasting permanent changes in the liver transcriptome, but the nature of these changes were somewhat different for both, with many of the genes altered by BDE-47 exposure independent of mTORC1 potentially being altered via mTORC2 based on the results of our analysis of mTORC2 knockout data. Knockout of mTORC1 may remove multiple inhibitory mechanisms that attenuate the signals that mTORC2 receives. If this is the case, and BDE-47 induces mTORC2 signaling via multiple mechanisms, then many of the positively enriched gene sets due to BDE-47 exposure in mTORC1 knockout mice may be due to increased mTORC2 activity that triggers subtle but biologically meaningful permanent alterations to the liver transcriptome. This is corroborated by the fact that exposure to BDE-47 triggers acute increases in mTORC1 and 2 activity in HepG2 cells (Khalil et al., 2017), and that developmental knockout of mTORC1 increases mTORC2 activity (Figure 3). This may be one explanation for why, at the gene set level, exposure to BDE-47 exacerbates some of the effects of mTORC1 knockout. Regardless of upstream mechanisms, it appears that mTORC1 plays a key role in mediating the long-lasting effects of perinatal BDE-47 exposure, and its presence or absence directs the effects of BDE-47 exposure to different regions of the transcriptome that are largely distinct in terms of their individual genes affected but somewhat overlapping in terms of biological function at the systems level.

The role of mTORC2 in mediating the effects of early-life BDE-47 exposure on the liver transcriptome

Our understanding of the differential roles of mTORC1 and 2 in developmental regulation of the transcriptome gave us a more informed perspective when investigating the role of mTORC2 in mediating the effects of early-life BDE-47 exposure as well. To our knowledge, this is the first experiment to investigate the role of mTORC2 in mediating the effects of an environmental xenobiotic (BDE-47).

Perinatal BDE-47 exposure exhibits mTORC2-dependent and independent effects on the liver transcriptome

Perinatal BDE-47 exposure did not alter notably the activity of either mTORC1 or 2 in mTORC2 control mice at PND75 in the experiment used to investigate the role of mTORC2 in mediating the effects of BDE-47 exposure (Figure 24). However, in mTORC2 knockout mice, it appears that BDE-47 exposure may exacerbate the effects of mTORC2 knockout observed in vehicle-exposed mTORC2 knockout by subtly increasing phosphorylation of S6K1 at Thr389. This result, if it is accurate, suggests that BDE-47 exposure may subtly trigger S6K1 phosphorylation independently of mTORC1 or by inducing activity of mTORC1 when mTORC2 is deleted. However, given the nature of our western blots, additional work must be done to ascertain if this is true or not.

Even though BDE-47 exposure did not alter the activity of mTORC1 or 2 at PND75 in mTORC2 control mice, BDE-47 exposure appeared to exert many mTORC2-dependent effects in the mTORC2 experiment. Unlike in the mTORC1 experiment, BDE-47 exposure triggered a shift in the blood-liver lipid balance towards the blood by significantly increasing serum

triglycerides (Figure 25). However, knockout of mTORC2 completely abolished this effect of BDE-47 exposure in this experiment (Figure 25). It is interesting to note that when comparing the effects of hepatic mTORC1 knockout vs mTORC2 knockout, genes significantly affected by mTORC2 knockout only were associated with “lipid transport” in our Metascape analysis (Figure 7). However, this could also be due to subtle differences in genetic background between mice used in both experiments, as will be discussed later.

There were several genes for which perinatal BDE-47 exposure significantly altered expression at PND75 and for which the effect of BDE-47 exposure was abolished when mTORC2 was deleted from the liver (Figure 28). Our Metascape analysis of these genes (Figure 28) indicated that the biological roles of these genes included glucose and pyruvate metabolism (“pyruvate metabolism” and “glucose homeostasis), muscle-associated processes (“muscle organ development,” “muscle adaptation,” and “striated muscle contraction”), innate immunity (“positive regulation of inflammatory response,” “acute-phase response,” and “cellular response to interleukin-1”), and lipid metabolism (“lipid homeostasis”). Additionally, terms were present related to hormone and IGF signaling (“response to hormone,” and “regulation of insulin-like growth factor receptor signaling pathway”), organismal homeostasis (“multicellular organismal homeostasis”), viral entry (“regulation of viral entry into host cell”), membrane permeability (“regulation of membrane permeability”), liver development (“liver development”), and organic ion transport (“organic anion transport”).

Notable genes within this category include the aquaporin *Aqp8*, the cytochrome P450 enzyme Cyp2c23, *Fabp2* and *Fabp4*, *Igfbp5*, *Jun*, the skeletal muscle myosin *Mylpf*, and *Tubb2a* and *Tubb2b* (Table 12). Notable genes downregulated due to BDE-47 exposure in mTORC2 control mice only include *Rbp1* the RNase *Rnase4*, *Ciart*, *Cyp39a1*, the insulin-like growth

factor binding protein *Igfp2*, the interleukin 1 receptor *Il1r1*, and the ubiquitin-specific peptidase *Usp2* (Table 12). See Table 12 for a full list of genes for which the effect of BDE-47 exposure is mTORC2-dependent.

Unlike the effect of BDE-47 exposure in mTORC1 control mice in the mTORC1 experiment, BDE-47 exposure in mTORC2 control mice triggered the positive enrichment of many "muscle" related gene sets such as "HALLMARK_MYOGENESIS," "KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC," "KEGG_CARDIAC_MUSCLE_CONTRACTION," "KEGG_DILATED_CARDIOMYOPATHY," and "KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM." Calcium signaling and ion transport ("KEGG_CALCIUM_SIGNALING_PATHWAY," "REACTOME_ION_CHANNEL_TRANSPORT," and "REACTOME_PHASE_0_RAPID_DEPOLARISATION") were also present, alongside gene sets associated with endocytosis, actin cytoskeleton dynamics, and cellular survival via nephrin family proteins ("REACTOME_NEPHRIN_FAMILY_INTERACTIONS"), and tight junctions ("KEGG_TIGHT_JUNCTION"). While these results are difficult to interpret, one possibility is that the effects of BDE-47 exposure in these mice are significant enough to trigger differentiation of hepatic stellate cells into myofibroblasts subsequent alterations to the extracellular matrix in response to liver damage (Hinz et al., 2007). It should be noted that mTORC2 deletion in the liver abolished the enrichment of these gene sets, suggesting that these gene sets are mTORC2 dependent.

BDE-47 exposure in mTORC2 control mice yielded a large network of predicted upstream regulators via IPA upstream analysis (Figure 31, Table 16). Interestingly, none of these

transcription factors could be linked to mTORC2 using the grow function or the connect function (which links different proteins based on a database of predicted or experimentally confirmed interactions) for pathway building in IPA and were instead linked to mTORC2 indirectly via mTORC1 (Figure 31). However, mTORC2 knockout abolished all effects of BDE-47 exposure in upstream analysis except for the effect of BDE-47 exposure on SMARCA4, which was predicted to be activated in both BDE-47 exposed mTORC2 control and knockout mice (Table 16). Given that mTORC1 knockout, but not mTORC2 knockout, abolishes the effect of BDE-47 exposure on predicted activity of SMARCA4, it is likely that the genes predicted to be regulated by SMARCA4 are influenced by BDE-47 exposure via mTORC1 independently of mTORC2. Interestingly, BDE-47 exposure in mTORC2 knockout mice also triggered the predicted activation of STAT5B (Table 16), the only transcription regulator predicted to be downregulated due to BDE-47 exposure in both vehicle-exposed mTORC1 and mTORC2 knockout mice (Table 6). Another interesting result of this generated network was that every other transcription regulator was predicted to be linked to mTORC1 (and thus mTORC2) indirectly via HIF1A. Interestingly, previous work suggests that xenobiotic metabolism and the hypoxia response intersect at the aryl hydrocarbon receptor nuclear translocator, which is required for normal function of both the hypoxic response regulated by HIF1A and the regulation of cytochrome P450 monooxygenases by the aryl hydrocarbon receptor (Vorrink and Domann, 2014). It is possible that the mTOR pathway, when challenged by environmental xenobiotics, triggers the activation of the hypoxic response to allow for oxygen to be diverted for use in xenobiotic metabolism, at least in the liver. IPA also predicted that some disease and biological functions would be affected in BDE-47 exposed mTORC2 control mice but not BDE-47 exposed mTORC2 knockout mice such as skeletal and muscular system development and function,

skeletal and muscular disorders, cardiac system development and function, cellular assembly and organization, hepatic system disease, protein synthesis, and energy production (Figure 32). Additionally, IPA predicted that affected toxicological functions due to BDE-47 exposure in mTORC2 control mice only included many different "cardiac" functions such as cardiac dilation, cardiac arrythmia, cardiac arteriopathy, cardiac inflammation, and cardiac fibrosis (Figure 33). All these results corroborate our results from Metascape and GSEA. Overall, there were many notable permanent changes in gene expression due to BDE-47 exposure in the mTORC2 control mice that did not occur in BDE-47 exposed mTORC2 knockout mice, and these changes were associated with many different biological functions and health outcomes related to glucose metabolism, hepatic stellate cell functioning, immunity, lipid metabolism, and more.

There were only three enriched ontology terms enriched for genes altered due to BDE-47 exposure in mTORC2 knockout mice only: "heart development" (*Id1*, *Slc9a1*, *Dusp6*, *Dipk2a*), "positive regulation of apoptotic process" (*G0s2*, *Slc9a1*, *Dusp6*, *Klf11*), and "regulation of growth" (*Slc9a1*, *Dusp6*, *Osgin1*). Notably, not all genes contributed to enrichment. There were also only 14 genes altered due to BDE-47 exposure in mTORC2 knockout (Table 13). Notable upregulated genes in this gene group include *Slc9a1* (Downregulated in vehicle-exposed mTORC1 knockout only, see Table A1), *Dusp6* (upregulated in vehicle-exposed mTORC1 knockout only, see Table A1), *Gs02*, and *Osgin1*. Notable genes downregulated due to BDE-47 exposure in this gene group include *Chpf2* and *Mir671* (downregulated by mTORC1 KO, upregulated by mTORC2 KO, see Table 3).

Interestingly, some gene sets were also upregulated due to BDE-47 exposure in mTORC2 knockout mice that were distinct from those in mTORC2 control mice. These include sets associated with immunity with immunity ("HALLMARK_ALLOGRAFT_REJECTION,"

"HALLMARK_IL2_STAT5_SIGNALING," and "HALLMARK_TNFA_SIGNALING_VIA_NFKB"), blood vessel formation and notch signaling ("HALLMARK_ANGIOGENESIS," and "HALLMARK_NOTCH_SIGNALING"), apoptosis and DNA damage ("HALLMARK_APOPTOSIS" and "HALLMARK_UV_RESPONSE_DN"),

KRAS signaling ("HALLMARK_KRAS_SIGNALING_UP"), and extracellular matrix function ("REACTOME_MOLECULES_ASSOCIATED_WITH_ELASTIC_FIBRES") (Table 15). It is interesting to not that mTORC1 knockout also induces positive enrichment of the "HALLMARK_ALLOGRAFT_REJECTION" gene set, and IPA predicted the activation of NOTCH1 due to BDE-47 exposure in mTORC2 control mice (which mTORC2 knockout abolished) despite the fact that the gene set "HALLMARK_NOTCH_SIGNALING" is positively enriched due to BDE-47 exposure in mTORC2 knockout mice. These results suggest that the mechanisms surrounding the role of mTORC1, mTORC2, and BDE-47 are highly complex.

As mentioned previously, knockout of mTORC2 in BDE-47 exposed animals abolished almost all the predicted effects on specific transcription factors in IPA, except for SMARCA4, and triggered the predicted activation of STAT5B (Table 16). IPA also predicted that affected diseases and biological functions in these mice only (as compared to BDE-47 exposed mTORC2 control mice as well) included vitamin and mineral metabolism, cell death and survival, hematological hepatic system development and function, cancer, inflammatory response, and free radical scavenging (Figure 32), while predicted toxicological functions included renal inflammation, renal nephritis, renal enlargement, bilary hyperplasia, glutathione depletion in liver, renal fibrosis, cardiac hyperplasia/hyperproliferation, and increased levels of AST (Figure 33). Overall, it appears that while the majority of effects of BDE-47 exposure at the level of

significant genes and gene sets are abolished due to mTORC2 knockout, mTORC2 knockout permits the effect of BDE-47 exposure on a small number of genes and induces subtle pertutbations in specifc gene sets.

There were only five genes regulated in opposing directions in mTORC2 control and knockout mice due to BDE-47 exposure. The one gene upregulated due to BDE-47 exposure in mTORC2 control mice and downregulated in mTORC2 knockouts was the cytochrome P450 monooxygenase *Cyp8b1*. The four genes downregulated due to BDE-47 exposure in mTORC2 controls but upregulated due to BDE-47 exposure in mTORC2 knockouts were *Cyp26b1* (downregulated by mTORC2 knockout, see Table 2), *Angptl8*, the monooxygenase *Moxd1* (downregulated by both mTORC1 and 2 knockouts, see Table 4), and the major urinary protein *Mup5*. It is very clear that for the vast majority of genes altered due to BDE-47 exposure in the mTORC2 experiment, the effect of BDE-47 exposure is either abolished due to mTORC2 deletion, permitted due to mTORC2 deletion, or acts independent of mTORC2.

Interestingly, there were a large number of genes for which the effect of BDE-47 exposure acted independently of mTORC2 in the mTORC2 experiment (Figure 29, Table 14). Metascape analysis of genes for which the effects of BDE-47 exposure are independent of mTORC2 (Figure 29) are primarily associated with innate immunity (“acute-phase response,” “regulation of response to wounding,” and “platelet degranulation”), lipid metabolism (“steroid metabolic process,” and “PPAR signaling pathway”), and ion transport (“organic anion transport,” “cellular metal ion homeostasis,” and “cellular transition metal ion homeostasis”). Interestingly, knockout of mTORC1 also permitted the effect of BDE-47 exposure on genes involved in the acute phase response (Figure 19), while mTORC1 status altered the ion homeostasis-related genes affected by BDE-47 exposure (Figures 19 and 20). Additionally,

expression of genes altered by PPAR signaling appears to be influenced by both mTOR knockouts as well (Figure 20). The term "response to stillbenoid" was also enriched. As discussed previously, this term was affected by deletion of both mTORC1 and mTORC2, suggesting that BDE-47 exposure affects genes involved in the response to stillbenoids such as resveratrol (Park et al., 2016) independently of mTORC2 and are potentially mTORC1-dependent. The effect of BDE-47 exposure on the term "positive regulation of cold-induced thermogenesis," may be to be influenced by mTOR status as well, given that some thermogenesis-related ontology terms appear as sub-terms for the intilogy term "multicellular organismal homeostasis" for which BDE-47 exposure enriched due to mTORC1 knockout in the mTORC1 experiment (Figure 19). The term "vascular smooth muscle contraction" was also enriched (Figure 29) Notable genes upregulated by BDE-47 exposure independently of mTORC2 include *Depp1* (downregulated by mTORC1 knockout, see Table 1), *Acta2* (downregulated by mTORC1 knockout, upregulated by mTORC2 knockout, see Table 3), *Cd24a* (downregulated by mTORC1 knockout, see Table 1), *Cd36* (upregulated by mTORC1 knockout, downregulated by mTORC2 knockout, see Table 3), *Egr1*, and *Myl9*. Notable genes downregulated due to BDE-47 exposure include *Noct*, *msfd2a*, *Mt1* (upregulated by mTORC1 knockout, downregulated by mTORC2 knockout, see Table 3), *Orm1*, *2*, and *3*, *Saa1*, and *Saa2*. For a list of all genes in this category, refer to Table 14).

Interestingly, there were four gene sets upregulated due to BDE-47 exposure independently of mTORC2 (see 31 and Table 15):
"HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION,"
"KEGG_FOCAL_ADHESION, REACTOME_ION_HOMEOSTASIS,"
"REACTOME_MUSCLE_CONTRACTION," and

"REACTOME_SMOOTH_MUSCLE_CONTRACTION." These indicate that in the mTORC2 mice, some subtle perturbations to gene sets related to "muscle" structures and remodeling of the extracellular matrix may take place due to BDE-47 exposure independently of mTORC2. In IPA, SMARCA4 was the only transcription factor that was predicted to be affected by BDE-47 exposure independently of mTORC2, suggesting that this transcription factor may be involved in regulation of these gene sets and genes involved in the predicted ontology term "vascular smooth muscle contraction" from Metascape. Interestingly, this chromatin remodeler has been shown to be a biomarker for the activation of hepatic stellate cells and the progression of liver fibrosis (Li et al., 2018). In this case, it is likely that BDE-47 exposure is triggering SMARCA4 to open chromatin at SMARCA4-controlled regions, potentially resulting in fibrosis due to the activation of hepatic stellate cells. IPA also predicted many different diseases and biological functions to be commonly affected due to BDE-47 exposure in both mTORC2 controls and knockouts, related to terms such as cell morphology, organismal injury and abnormalities, lipid metabolism, molecular transport, small molecule biochemistry, carbohydrate metabolism, metabolic disease, endocrine system disorders, connective tissue development and function, and cellular growth and proliferation, and more (Figure 32). Common predicted toxicological functions included liver steatosis, cardiac dysfunction, hepatocellular carcinoma, liver hyperplasia/hyperproliferation, liver necrosis/cell death, heart failure, cardiac necrosis/cell death, liver regeneration, renal proliferation, liver damage, liver fibrosis, liver proliferation, renal necrosis/cell death, liver cholestasis, cardiac stenosis, and more (Figure 33).

Visual inspection of the individual genes affected due to perinatal BDE-47 exposure in the mTORC1 experiment suggest that consistent with our hypothesis, BDE-47 may act via mTORC2 to reprogram the liver transcriptome (Figure 27). This is based on the fact that the

most dense cluster of genes lies within the cross-shape formed by dependency analysis cutoffs such that effects of BDE-47 exposure on these genes appear to be mTORC2-dependent (blue and orange gene groups, Figure 27). Interestingly, there were very few genes affected by BDE-47 exposure in mTORC2 knockouts only (Figure 27, yellow and red), almost no genes altered in opposing directions due to BDE-47 exposure in mTORC2 controls and knockouts (Figure 27, purple and green), were many genes affected by BDE-47 exposure independently of mTORC2 (Figure 27, pink and teal). All this information suggests that while there are many long-lasting effects of perinatal BDE-47 exposure that are dependent on mTORC2, there are many that act independently of mTORC2, at least in this model. Given the fact that many genes altered independently of mTORC2 due to BDE-47 exposure are also altered due to mTORC1 knockout it may be that the effects of BDE-47 exposure on many of these genes are dependent on mTORC1 rather than mTORC2. This may include activation of hepatic stellate cells in response to BDE-47 exposure to promote liver fibrosis. Based on analysis of individual genes and gene sets altered due to BDE-47 exposure in mTORC2 knockout mice only, as well as western blotting data (Figure 24) it is possible that BDE-47 exposure in mTORC2 knockout mice triggers a subtle increase in mTORC1 activity, but we cannot be certain. Since many genes that appear to be mTORC1-dependent are altered independently of mTORC2, it is also possible that BDE-47 modulated mTORC1 through other mechanisms beyond mTORC2. Regardless of upstream mechanisms, it appears that mTORC2 regulates the long-lasting effects of perinatal BDE-47 exposure for a large subset of genes, and whether genes will be affected by BDE-47 exposure or not. However, for another subset of genes, mTORC2 does not play a role in mediating the effects of BDE-47 exposure.

Comparative effects of mTORC1 and 2 in mediating effects of BDE-47 exposure

Overall, it appears that our hypothesis was correct. Based on dependency analysis of BDE-47 exposure in mTORC1 in the mTORC1 experiment (Figure 5), the effect of BDE-47 exposure for approximately 80 percent of genes (104 out of 131 genes significantly affected by BDE-47 exposure in the dependency analysis for mTORC1) were influenced by mTORC1 status (mTORC1 either present or absent). In the mTORC2 experiment, the effect of BDE-47 exposure on 65 percent of genes (118 out of 182 genes significantly affected by BDE-47 exposure in the dependency analysis for the mTORC2 experiment) were influenced by mTORC2 status. This result supports the general idea that while both mTORC1 and mTORC2 play a significant role in mediating the effects of BDE-47 exposure, mTORC1 is generally "closer" to the mechanisms that play a direct role in permanently reprogramming and maintaining altered expression of the largest number of genes (which are likely epigenetic) in comparison to mTORC2, and may act to stimulate the activation or inhibition of transcription factors such as HIF1A and SMARCA4. This notion that mTORC1 is "closer" is corroborated by the fact that the vast majority of genes altered due to BDE-47 exposure in the mTORC1 experiment are altered either in the presence (Figure 18) or absence (Figure 19) of mTORC1, as compared to genes altered in opposing directions in both or independently of mTORC1, and is also supported by the fact that predicted upstream regulators in the IPA experiment are linked to mTORC2 via mTORC1 (Figure 31). However, mTORC2 still plays a critical role in mediating effects of BDE-47 exposure, as evidenced by the fact that almost all upstream regulators within Figure 31 were no longer predicted to be activated or inhibited when mTORC2 was knocked out (with the exception of SMARCA4, which may be mTORC1-dependent only). Canonically, mTORC2 is upstream of mTORC1, and this makes some sense in the context of these BDE-47 exposure results.

However, the effect of BDE-47 exposure does not likely act through mTORC2 and then mTORC1 for every single gene. In fact, for 74 genes, the effect of BDE-47 exposure was dependent on the status of mTORC1 only (from the mTORC1 experiment), the effect of BDE-47 exposure for 88 genes was dependent on mTORC2 status only (from the mTORC2 experiment), and for 30 genes the effect of BDE-47 exposure was dependent on the status of both complexes in their respective experiments notable genes falling onto this last category (influenced by both complexes) included *Aqp8*, *Tubb2a*, *Tubb2b*, *Fabp2*, *Rbp1*, *Usp2*, *Rnase4*, *Mir671*, *Igfbp5*, and *Ciart*. There were also a number of genes that were affected by BDE-47 exposure independently of mTORC1 status only (22 genes) in the mTORC1 experiment, and a number of genes that were affected by BDE-47 exposure independently of mTORC2 (59 genes) in the mTORC2 experiment. However, it is interesting to note that some genes (but not all) that were affected by BDE-47 exposure independently of mTORC1 status in the mTORC1 experiment were influenced by mTORC2 status in the mTORC2 experiment in the mTORC2 experiment (for example, *Nrep*, *Me1*, *Onecut1*, and *Serpina12*). Likewise, some but not all genes altered independently of mTORC2 status due to BDE-47 exposure in the mTORC2 experiment were influenced by mTORC1 status in the mTORC1 experiment (Such as *Depp1*, *Acta2*, *Cd24a*, *Mt1*, *Myl9*, and *Noct*). There were also 5 genes for which the effect of BDE-47 exposure was independent of mTOR complex status in both experiments: *Asns*, *Mfsd2a*, *Egr1*, *Saa1*, and *Saa2*. These five genes are the only genes that we can say with certainty are affected by BDE-47 exposure independently of both mTOR complexes. For a summary of all genes involved in this comparison, please refer to Table A2 in the appendix.

Evidence for a gene-environment interaction between early-life BDE-47 exposure and genetic background mediated by the mTOR pathway

When comparing the effects of BDE-47 exposure in the "control" mice in the mTORC1 and mTORC2 experiments, it becomes very clear that BDE-47 exposure triggers different effects in both types of mice. For example, BDE-47 exposure triggers the mTORC2-dependent upregulation of many "muscle" associated genes and gene sets in the mTORC2 experiment relative to BDE-47 exposure in the mTORC1 experiment, and BDE-47 exposure alters the blood liver lipid balance (in an mTORC2-dependent manner) in the mTORC2 experiment only, with no significant effects on this balance in the mTORC1 experiment (Figure 25). Additionally, there were some genes altered due to BDE-47 exposure independently of mTORC1 that were not altered due to BDE-47 exposure in the mTORC2 experiment and vice versa, three genes that were altered due to BDE-47 exposure independently of both mTORC1 and 2 (*Asns*, *Mfsd2a*, and *Egr1*) and a number of genes that were altered due to BDE-47 exposure in opposite directions in mTORC1 and mTORC2 controls (such as *Aqp8*, *Tubb2a*, *Tubb2b*, *Rbp1*, and *Usp2*). IPA also predicted that in mTORC1 control mice, SMARCA4 would be inhibited due to BDE-47 exposure, while it predicted that SMARCA4 would be activated due to BDE-47 exposure in mTORC2 control mice. Interestingly, there were a few genes that were altered due to BDE-47 exposure in one direction in one type of knockout model in one experiment, and altered due to BDE-47 exposure in the opposite direction of the control model in the other experiment (such as *Rnase4*), and genes altered due to BDE-47 exposure in opposite directions in mTORC1 and 2 knockout mice (such as *Mir671*). All this evidence indicates that while mTORC1 and 2 do mediate most of the effects of BDE-47 exposure, some effect is inducing differential expression patterns in wild type mice in the mTORC1 experiment relative to the mTORC2 experiment.

These observed effects are, in many cases, reminiscent of the dose-response curve observed in previous experiments, where low doses of BDE-47 regulated expression of genes in one direction, while high doses regulated these same genes in the opposite directions (Khalil et al., 2018). There some genetic differences in the mice tested in the mTORC1 experiment in comparison to the mTORC2 experiment due to the different genotypes of available mice with floxed *Raptor* and floxed *Rictor* used in each breeding scheme to generate the mice used for our experiment (even though these mice were back-crossed to C57BL/6 mice for multiple generations even before being used in our breeding scheme to generate mTORC1 and 2 controls and knockouts). This may be a limitation of our experiment, but it allowed us to conclude that mTORC1 and 2 mediate the effects of a gene-environment interaction between BDE-47 and different genetic backgrounds that determines both the types of genes whose expression are altered as a result of BDE-47 exposure, as well as the directions in which these genes are altered. In many cases, it appears that at the same dose, some genes are altered in opposing directions in mTORC1 and 2 controls. Given this along with all of the other above information, it appears that different genetic backgrounds in our mice lead to a "shift" in the dose-response curve for each gene in the mOTRC1 mice relative to

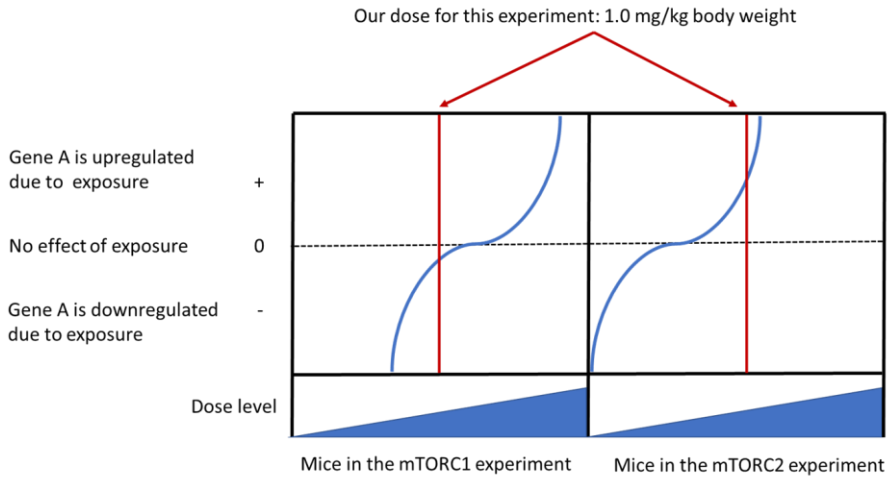

Figure 34. A Visual Representation of the Gene-Environment Interaction that Modulates Expression of a Hypothetical Gene in the mTORC1 and mTORC2 experiments. Given that in previous experiments, BDE-47 exposure to low doses of BDE-47 would regulate individual genes in directions opposite to that of high doses, it is possible that the different genetic backgrounds of mice in the mTORC1 and 2 experiments shifts the "expression response curve" for the same gene in both experiments, resulting in the same dose triggering upregulation of a gene (in this case, a hypothetical gene A) in one experiment and downregulation of this same gene in the other experiment. It is important to note that in this model, this dose may trigger nonsignificant or changes in gene expression, or none at all, depending on the expression response curve of each individual gene.

the mTORC2 mice. This concept is illustrated in Figure 34. Given that the curve is shifted differently for each gene, and that each gene likely responds to different doses of BDE-47 differently, the level of complexity of mTORC1- and 2-dependent changes in hepatic gene expression triggered by early-life BDE-47 exposure makes sense. It is important to note that in this model, this dose may trigger nonsignificant or changes in gene expression, or none at all, depending on the expression response curve of each individual gene. This may explain why we saw no significant changes in the blood-liver lipid balance in the mTORC1 experiment, despite observing them in the mTORC2 experiment. This work is compelling evidence for a gene-environment interaction between early-life exposure to BDE-47 and different genetic backgrounds mediated by the mTOR pathway that induces long-lasting changes in hepatic gene expression (likely due to epigenetic effects). It is also possible that same effect is triggered not by the genotype of the mice tested, but the genotype of the dams by which they were nursed. If somewhat different genetics result in different levels of BDE-47 and its metabolites in the milk of dams in the mTORC1 experiment relative to the mTORC2 experiment, it could be that the animals tested were actually just receiving different doses of BDE-47. However, this explanation is still consistent with the concept that genetics and environment intersect to alter liver gene expression via the mTOR pathway in early life; in this case the difference is that it is the genetics of the mother that modulates the effect of the environment on their offspring. We did not measure the concentration of BDE-47 in the milk of nursing dams in this experiment, so we cannot be certain. However, it is also possible that both explanations are not mutually exclusive.

A theoretical model illustrating the relationship between BDE-47 and mTOR-controlled gene expression reprogramming in early life

Based on the all the above results and interpretations, we came up with a theoretical model for how BDE-47 exposure modifies mTORC1 and 2-dependent gene expression via mTOR in early life to permanently reprogram downstream gene expression. It appears that most effects of gene expression first act via mTORC2 to stimulate mTORC2 and mTORC1 activity, though some effects of BDE-47 exposure may directly activate mTORC1. Activated mTORC2 stimulates mTORC1 activity via AKT, and activated mTORC1 suppresses mTORC2 activity via S6K1, leading to a balance in mTORC1 and 2 activity, though both may be hyperactivated in response to BDE-47 exposure. Low doses trigger up- and downregulation of genes regulated by both mTORC1 and 2, while high doses trigger the regulation of these genes in opposing directions. Based on dependency analysis and IPA, it is likely that the majority of genes affected by BDE-47 exposure are directly downstream of mTORC1, but are in fact dependent on both complexes given that the majority of signal from BDE-47 likely signals the mTOR pathway via a cascade upstream of mTORC2. Additionally, BDE-47 exposure in mTORC1 knockout mice relative to

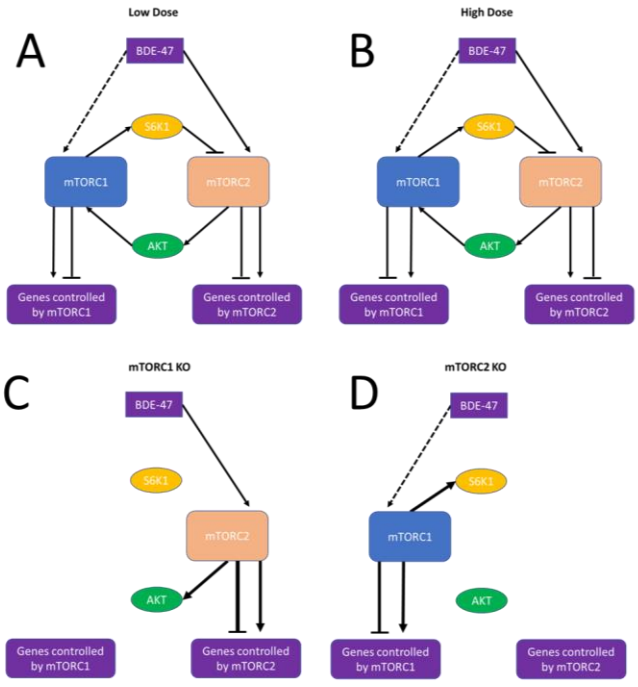

Figure 35. A Theoretical Model of Modulation of Gene Expression by BDE-47 via mTORC1 and 2. A. Early-life BDE-47 exposure at low doses triggers the coordinated activation of both mTOR complexes, resulting in long-term upregulation and downregulation of specific gene groups downstream of each complex. **B.** High doses trigger the opposite effect on mTOR-regulated gene groups. **C.** BDE-47 Exposure in mice lacking mTORC1 triggers hyperactivation of mTORC2 and exacerbates the long-term effect of BDE-47 on genes downstream of mTORC2 relative to unexposed mTORC1 knockout mice. **D.** BDE-47 Exposure in mice lacking mTORC2 triggers hyperactivation of mTORC2 and exacerbates the long-term effect of BDE-47 on genes downstream of mTORC1 relative to unexposed mTORC2 knockout mice. However, given that the majority of signal from BDE-47 acts via mTORC2, the effects of BDE-47 exposure in **D** are less significant than in **C**.

vehicle-exposed mTORC1 knockout mice hyperactivates mTORC2 beyond the level of activation induced by mTORC1 knockout while leaving the majority of mTORC1-controlled genes unaffected, which results in exacerbated long-term effects of BDE-47 exposure on genes activated and inhibited by mTORC2. Likewise, BDE-47 exposure in mTORC2 knockout mice relative to vehicle-exposed mTORC2 knockout mice triggers the hyperactivation of mTORC1 and exacerbates the activation of mTORC1 beyond the level of activation induced by mTORC2 knockout only while leaving the majority of mTORC2-regulated genes unaffected, which results in exacerbated effects of BDE-47 exposure on genes activated and inhibited by mTORC1. However, given that the majority of signal from BDE-47 transmits via mTORC2 and then mTORC1, the exacerbation of mTORC1 activity in mTORC2 knockout is less than the exacerbation of mTORC2 activity due to BDE-47 exposure in mTORC1 knockout mice. After acute BDE-47 exposure in early life, mTOR activity settles back to normal (at least in control animals) by PND75, but effects of BDE-47 exposure are already permanently ingrained in the epigenome or other potential gene expression regulation mechanisms downstream of the mTOR pathway, resulting in lifelong alterations in liver gene expression as a result of early-life BDE-47 exposure. See Figure 35 for a visual representation of this model.

Limitations of these studies

While this series of experiments shed light on many novel findings, it is important to note yet again that due to the coronavirus pandemic, we were unable to obtain western blots of total S6K1 or AKT, which render it difficult to fully interpret our western blot data. This work will be done as soon as the opportunity presents itself to obtain this necessary control for our experiments, and our conclusions will be updated accordingly. Additionally, while the mice used for the mTORC1 and 2 experiments are genetically similar, they are not identical, which may

prevent us from making some direct comparisons but also provided the opportunity to understand why animals with subtle genetic (or potentially epigenetic) differences respond to early-life BDE-47 exposure in different ways. Given that we only conducted BDE-47 exposure and sequencing analyses at set timepoints, our experiments also only provide a "snapshot" into what is likely notably different developmental and long-lasting changes in the liver transcriptome over time. Additionally, in biological reality, many genes are not "dependent" or "not dependent" but rather vary on a spectrum in terms of how significantly different modifications such as the genetic deletions used here influence their expression. As such, while our experiments illustrate many statistically significant findings, these may not illustrate the full biological reality of the dependency of BDE-47-induced effects on the mTOR pathway. Our systems biology approach, while it highlights novel mTOR biology such as the role of mTOR in xenobiotic metabolism, also relies on manually curated ontology terms and gene sets, and thus may be susceptible to database effects. This may be one potential reason we saw so many cardiac striated muscle terms in a location that typically lacks these types of muscle. Sample size may also play a role, particularly in the mTORC1 experiment, where sample sizes were smaller as a result of our breeding scheme which produced only a small number of mice with a respective genotype and BDE-47 exposure status for some of the groups within the mTORC1 experiment. Given all the above, different portions or the entirety of this series of experiments should be validated by independent parties in the name of scientific accuracy. However, given previous studies cited in the introduction, and the concordance between both the mTORC1 and 2 experiments, we have confidence in our results.

VIII. Conclusions

Overall, this series of experiments demonstrates that mTORC1 and 2 have significant partially overlapping roles in developmental programming of hepatic gene expression, regulating ribosomal biogenesis, lipid metabolism, immunity, and more. Given the recent advances in sequencing technology and bioinformatic analysis, this is the most comprehensive analysis of the developmental roles of mTORC1 and 2 in regulating hepatic gene expression. Additionally, mTORC1 and 2 both mediate a complex gene-environment interaction between perinatal exposure to BDE-47 and different genetic backgrounds that induces permanent changes in the hepatic transcriptome and influences biological functions controlled by mTOR such as lipid metabolism, glucose metabolism, immunity, metabolism, and cellular growth and replication. This study also indicates that BDE-47 exposure truly affects a few genes independent of mTOR, providing an opportunity to delve into potential mTOR-independent mechanisms that mediate the effect of BDE-47 exposure in the future. However, it appears that our hypothesis overall was very on-target, given that most genes affected by BDE-47 exposure are dependent on mTOR in some manner.

It is interesting to note that contrary to previous experiments (Khalil et al., 2018) BDE-47 exposure did not lead to altered expression of ribosomal gene sets. This may be due to the dose and BDE-47 exposure period used in this experiment, which was a different combination than used in previous experiments, which in this case was 1.0 mg/kg body weight per day for the first 21 days of postnatal life, instead of 0.2 mg/kg postnatally as done in previous experiments (Khalil et al., 2018). Given our proposed model of chemical-gene interaction for BDE-47, it is possible that either this perinatal dose lies in the middle of the BDE-47 exposure curve (Figure

34) for mice used in Khalil et al. (2018) and for this series of experiments, or genetic differences in the mice used here shift the expression response curve relative to the mice used in Khalil et al. (2018) such that this dose has no effect on the mice used in this series of experiments. However, it is very likely that BDE-47 acts via the mTOR pathway to trigger programming of ribosomal genes, as developmental knockout of either mTORC1 or 2 triggered upregulation of ribosomal gene sets in exactly the same way as seen for 0.2 mg/kg body weight per day of perinatal BDE-47 exposure in Khalil et al (2018). It is interesting to note that developmental knockout of mTORC1 and 2 triggers the upregulation, rather than downregulation, or ribosomal gene sets as would be expected for a pathway that canonically controls expression of ribosomal genes. This is evidence for compensatory or alternative mechanisms for ribosome biogenesis and protein synthesis, and BDE-47 may act to influence ribosomal gene expression through both the mTOR pathway and these mechanisms, though this is only a theory.

One of the major themes that arose from this study is the fact that, in addition to sensing amino acid and energy availability, oxygen levels, growth factors, DNA damage, inflammation, and more, mTOR may also be responsive to environmental xenobiotics. This may be due to the possibility that a major responsibility of the mTOR pathway is to coordinate the metabolism of environmental xenobiotics and other growth and metabolism-related processes to compensate for chemical challenges. It makes sense that mTOR, as a key sensor of internal and external environmental stimuli, would sense challenges by environmental chemicals and program growth and metabolism accordingly, and in the case of perinatal BDE-47 exposure, mTOR may be receiving a chemical signal that causes it to “misread” the external environment and trigger the maladaptive permanent programming of liver gene expression that may make individuals more vulnerable to metabolic disease later in life as a result. A significant example of how the mTOR

signaling axis may influence metabolic disease susceptibility may be the study of children affected by the Dutch Hunger Winter, where children prenatally affected by the famine from 1944 to 1945 had significant demethylation of the *IGF2* gene (Heijmans et al., 2008) which is indirectly both upstream and downstream of mTOR (Ge and Chen, 2012). These children were also more susceptible to obesity later in life (Schulz, 2010). Essentially, it may not be that BDE-47 "dysregulates" gene expression, but rather triggers the liver to preemptively "adapt" to what it anticipates the lifelong external environment will be like in a similar manner to how nutrient deprivation affected children developing prenatally during the Dutch Hunger Winter. However, in the case of environmental toxins, as compared to early-life nutrient deprivation, it may also be that the mTOR pathway is truly just vulnerable to dysregulation by toxins. This will need to be investigated in future studies. Regardless of the biological reason, mTOR appears to be sensitive to BDE-47 exposure and, as mentioned previously, is one of the most sensitive to external chemical stimuli of all kinds, be it nutrients, pharmaceuticals, or environmental toxins across different doses and tissue types based on another line of research from our lab analyzing over 600,000 chemical-gene interactions covering over 2000 chemicals and the human, mouse, and rat genomes (paper in progress). This series of studies described in this thesis provide experimental evidence corroborating this observation.

Given our observations about how BDE-47 exposure permanently reprograms the liver transcriptome, many mechanistic questions remain. What is the downstream mechanism that sustains permanent changes in the liver transcriptome once BDE-47 acts via mTORC1 and 2 and mTOR pathway activity returns to normal? These mechanisms are likely epigenetic but remain to be characterized. Given that we observe a potential gene-environment interaction in the case of mTOR-mediated effects of BDE-47, what are the genetic differences that result in this effect?

Are there any epigenetic differences that contribute as well? Future work sequencing and comparing the genomes and epigenomes of mice used in the mTORC1 and 2 experiments may shed light on this. Future work into understanding the genetic components of the gene-environment interaction associated with early-life BDE-47 exposure and changes to the liver transcriptome could also have translational value, as this line of research could potentially lead to the identification of alleles that make subsets of the heterogeneous human population more or less sensitive to metabolic diseases and other kinds of disease as a result of BDE-47 exposure to different doses of BDE-47, if indeed there are any. Also, if there is a critical period early in life for which the liver is highly sensitive to reprogramming by BDE-47, can this critical period be reopened?

This work suggests that environmental xenobiotics like BDE-47 may reprogram the liver transcriptome early in life and contribute to susceptibility to different kinds of metabolic diseases such as NAFLD, diabetes, obesity, and even potentially cancer alter on in life. Interestingly, PBDEs were first produced in the 1970s (Cdc.gov, 2017). The prevalence of metabolic diseases in the general population are now increasing (Wisocky and Paul, 2017; Saklayen, 2018) potentially corroborating the theory that early-life exposure to PBDEs beginning in the 1970s may predispose individuals to metabolic disease later on, especially given that the prevalence of metabolic disease also increases with age (Saklayen, 2018). However, the prevalence of metabolic diseases is rising even in young children (Saklayen, 2018), which may be a side effect of the increasing concentration of PBDEs (and likely other toxicants) in human tissues (Dodson et al., 2012). As mentioned previously, increased CD36 expression (which was upregulated due to BDE-47 exposure in the mTORC2 experiment (see Table 14) and whose expression is both dependent on mTORC1 and 2 (see Table 3)) was found to be strongly associated in aging with

healthy patients, but not in patients with NAFLD, for which expression of CD36 was already high throughout the life course, with the first measurement based on liver biopsies taking place at 20 years of age (Sheedfar et al., 2014). Thus, our data may be consistent with the hypothesis that early-life BDE-47 exposure may permanently reprogram the liver transcriptome to advance age- and metabolic-disease associated changes in the liver transcriptome in genetically vulnerable populations, making them more susceptible to metabolic disease. Additional evidence for this is that certain levels of BDE-47 exposure trigger the dysregulation of ribosomal biogenesis genes, as well as dyslipidemia (Khalil et al., 2018). Dyslipidemia is correlated with aging (Shanmugasundaram, Rough, and Alpert, 2010), and changes in ribosomal gene expression have been theorized to be involved in either active or compensatory mechanisms in response aging as well (Turi et al., 2019). Lastly, the mTOR pathway is one of the few pathways known to regulate lifespan (Saxton and Sabatini, 2017, Papadopoli et al., 2019). Taken together, it is possible that early-life exposure to BDE-47 may contribute to the prevalence of metabolic- and age-associated disease via mTOR (Lim et al., 2008; Zhang et al., 2018). It is quite possible that perturbations to the mTOR pathway and downstream effectors, early in life and otherwise, may lead to a change in the rate or trajectory of cellular aging at many different levels of organization, such as the transcriptomic or epigenomic levels, to influence complex disease outcomes. Additionally, given all the above, caution should be taken when using mTOR inhibitors as cancer therapies or to prevent organ rejection, especially in genetically vulnerable or younger populations.

BDE-47 is merely one of many environmental chemicals that modulate the mTOR pathway, with perfluorooctanoic acid being another known mTOR modulator (Yan et al., 2015). It remains to be seen how the summative effects of exposure to BDE-47 and other many other ubiquitous environmental mTOR modulators influence health and disease throughout life. We

are only beginning to understand how mTOR complex activity influences development, health, and disease, but a more holistic understanding of the role of environmental chemical exposures in modulating the mTOR pathway in different tissues across the lifespan may provide critical insight into for the current epidemic of metabolic diseases, cancers, and many other kinds of diseases. In addition, it may reveal potential new avenues for targeted and preventative therapies for these age-associated diseases, and maybe unveil a complex molecular program for aging itself.

Understanding the effects of mTOR modulation via early-life chemical exposure in tissues such as the liver, which may be highly exposed to environmental chemicals due to processing portal vein blood containing the components of breast milk and other ingested substances things in early life (Carneiro et al., 2019), may have implications for identifying whether mTOR may mediate both adaptive and maladaptive effects of the internal and external chemical environment on organisms. This understanding may also influence our current understanding of mTOR inhibitors in the clinic and future generations of mTOR-targeting therapies. As of now, what is clear is that mTOR is a likely mediator of gene-environment interactions in health and disease, at least in terms of metabolic disease susceptibility triggered by BDE-47. However, more mechanistic studies into the realm of mTOR as mediator of the long-term effects of environmental chemical exposure are sorely needed.

IX. References

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X. Appendix

Table A1. Genes dependent on mTORC1 only.

Genes upregulated by mTORC1 KO		Genes downregulated by mTORC1 KO	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
Fyb2	FYN binding protein 2	Tedc2	tubulin epsilon and delta complex 2
2010003K11Rik	RIKEN cDNA 2010003K11 gene	Depp1	DEPP1 autophagy regulator
2410006H16Rik	RIKEN cDNA 2410006H16 gene	Aacs	acetoacetyl-CoA synthetase
4931408D14Rik	RIKEN cDNA 4931408D14 gene	Abcb10	ATP-binding cassette, sub-family B (MDR/TAP), member 10
Acot9	acyl-CoA thioesterase 9	Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
Adam11	a disintegrin and metallopeptidase domain 11	Acss2	acyl-CoA synthetase short-chain family member 2
AI413582	expressed sequence AI413582	Adcy9	adenylate cyclase 9
Ajuba	ajuba LIM protein	Adra1b	adrenergic receptor, alpha 1b
Anxa5	annexin A5	Aldoc	aldolase C, fructose-bisphosphate
Apoa4	apolipoprotein A-IV	Apom	apolipoprotein M
Arhgap19	Rho GTPase activating protein 19	Asl	argininosuccinate lyase
None (Gstp3)	None (BC021614), glutathione S-transferase pi 3	Asns	asparagine synthetase
Birc5	baculoviral IAP repeat-containing 5	Car5a	carbonic anhydrase 5a, mitochondrial
Blnk	B cell linker	Cbs	cystathionine beta-synthase

Btg2	B cell translocation gene 2, anti-proliferative	Cd24a	CD24a antigen
C1qb	complement component 1, q subcomponent, beta polypeptide	Ces4a	carboxylesterase 4A
Capg	capping protein (actin filament), gelsolin-like	Clcn2	chloride channel, voltage-sensitive 2
Car2	carbonic anhydrase 2	Cyp4f14	cytochrome P450, family 4, subfamily f, polypeptide 14
Ccl6	chemokine (C-C motif) ligand 6	Cyp7b1	cytochrome P450, family 7, subfamily b, polypeptide 1
Ccnd1	cyclin D1	Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1
Mir3962	microRNA 3962	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1
Cd5l	CD5 antigen-like	Cyp51	cytochrome P450, family 51
Cd9	CD9 antigen	Dhcr7	7-dehydrocholesterol reductase
Cd63	CD63 antigen	Dio1	deiodinase, iodothyronine, type I
Cd83	CD83 antigen	Elovl3	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3
Cd84	CD84 antigen	Fbxo31	F-box protein 31
Cd300ld	CD300 molecule like family member d	Fdps	farnesyl diphosphate synthetase
Cdca3	cell division cycle associated 3	Fetub	fetuin beta
Cdk1	cyclin-dependent kinase 1	Gas1	growth arrest specific 1
Cdkn2c	cyclin dependent kinase inhibitor 2C	Gldc	glycine decarboxylase
Chka	choline kinase alpha	Got1	glutamic-oxaloacetic transaminase 1, soluble

Clec7a	C-type lectin domain family 7, member a	Gstp1	glutathione S-transferase, pi 1
Clec12a	C-type lectin domain family 12, member a	Hexim1	hexamethylene bis-acetamide inducible 1
Cotl1	coactosin-like 1 (Dictyostelium)	Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
Ctss	cathepsin S	Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7
Cybb	cytochrome b-245, beta polypeptide	Hspa1a	heat shock protein 1A
Dancr	differentiation antagonizing non-protein coding RNA	Hspa1b	heat shock protein 1B
Snora26	small nucleolar RNA, H/ACA box 26	Hspb6	heat shock protein, alpha-crystallin-related, B6
Dusp6	dual specificity phosphatase 6	Idi1	isopentenyl-diphosphate delta isomerase
Endod1	endonuclease domain containing 1	Il6ra	interleukin 6 receptor, alpha
Fam81a	family with sequence similarity 81, member A	Itpr2	inositol 1,4,5-triphosphate receptor 2
Folr2	folate receptor 2 (fetal)	Ldhd	lactate dehydrogenase D
Gdf15	growth differentiation factor 15	Ldlr	low density lipoprotein receptor
Haus8	4HAUS augmin-like complex, subunit 8	Lpin1	lipin 1
Hexa	hexosaminidase A	Lss	lanosterol synthase
Idh2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	Mir5101	microRNA 5101

Igsf11	immunoglobulin superfamily, member 11	Syne2	spectrin repeat containing, nuclear envelope 2
Klf10	Kruppel-like factor 10	Slc9a1	solute carrier family 9 (sodium/hydrogen exchanger), member 1
Lair1	leukocyte-associated Ig-like receptor 1	Slc9a1	solute carrier family 9 (sodium/hydrogen exchanger), member 1
Large1	LARGE xylosyl- and glucuronyltransferase 1	Msmo1	methylsterol monoxygenase 1
Lgals1	lectin, galactose binding, soluble 1	Mthfd1	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase
Lgals3	lectin, galactose binding, soluble 3	Mvd	mevalonate (diphospho) decarboxylase
Lilr4b	leukocyte immunoglobulin-like receptor, subfamily B, member 4B	Npr2	natriuretic peptide receptor 2
Lpl	lipoprotein lipase	Nsdhl	NAD(P) dependent steroid dehydrogenase-like
Ly6a	lymphocyte antigen 6 complex, locus A	Nxpe2	neurexophilin and PC-esterase domain family, member 2
Ly6d	lymphocyte antigen 6 complex, locus D	Pcx	pyruvate carboxylase
Lyz1	lysozyme 1	Plekhb1	pleckstrin homology domain containing, family B (evectins) member 1
Lyz2	lysozyme 2	Pmvk	phosphomevalonate kinase
Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	Rdh11	retinol dehydrogenase 11

Neat1	nuclear paraspeckle assembly transcript 1 (non-protein coding)	Rptor	regulatory associated protein of MTOR, complex 1
Nupr1	nuclear protein transcription regulator 1	Scap	SREBF chaperone
Pcolce2	procollagen C-endopeptidase enhancer 2	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	Serpina6	serine (or cysteine) peptidase inhibitor, clade A, member 6
Plek	pleckstrin	Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1
Plscr1	phospholipid scramblase 1	Serpinf2	serine (or cysteine) peptidase inhibitor, clade F, member 2
Rab11fip4	RAB11 family interacting protein 4 (class II)	Shmt2	serine hydroxymethyltransferase 2 (mitochondrial)
Rab34	RAB34, member RAS oncogene family	Slc13a2	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2
Rarb	retinoic acid receptor, beta	Slc17a8	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8
Rbp1	retinol binding protein 1, cellular	Slc22a18	solute carrier family 22 (organic cation transporter), member 18
Renbp	renin binding protein	Slc37a4	solute carrier family 37 (glucose-6-phosphate transporter), member 4
Rgs5	regulator of G-protein signaling 5	Slc39a4	solute carrier family 39 (zinc transporter), member 4
Rhoc	ras homolog family member C	Slco2a1	solute carrier organic anion transporter family, member 2a1
Rragd	Ras-related GTP binding D	Sort1	sortilin 1

Sgms1	sphingomyelin synthase 1	Sult5a1	sulfotransferase family 5A, member 1
Slc16a5	solute carrier family 16 (monocarboxylic acid transporters), member 5	Sytl1	synaptotagmin-like 1
Slc51b	solute carrier family 51, beta subunit	Tecr	trans-2,3-enoyl-CoA reductase
Socs3	suppressor of cytokine signaling 3	Tfr2	transferrin receptor 2
Stmn1	stathmin 1	Tlcd2	TLC domain containing 2
Synpo	synaptopodin	Tm7sf2	transmembrane 7 superfamily member 2
Tff3	trefoil factor 3, intestinal	Vps51	VPS51 GARP complex subunit
Tlr12	toll-like receptor 12	Tmem19	transmembrane protein 19
Tmem86a	transmembrane protein 86A	Tpm2	tropomyosin 2, beta
Tmem98	transmembrane protein 98	Zfpm1	zinc finger protein, multitype 1
Trem2	triggering receptor expressed on myeloid cells 2		
Tuba8	tubulin, alpha 8		
Tubb2a	tubulin, beta 2A class IIA		
Tubb2b	tubulin, beta 2B class IIB		
Tyrobp	TYRO protein tyrosine kinase binding protein		
Uap1l1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1		

Vsig4	V-set and immunoglobulin domain containing 4
Tceal9	transcription elongation factor A like 9
Wfdc17	WAP four-disulfide core domain 17

Table A2. Status dependency overlap results for BDE-47 exposure in the mTORC1 and 2 experiments.

Dependent on mTORC1 only	Dependent on mTORC2 only	Dependent on both mTORC1 and 2	Independent of mTORC1	Independent of mTORC2	Independent of both mTORC1 and 2
2900076A07Rik	Bcl3	Acacb	Mir369	Apcs	Asns
Mir1839	Cd5l	Aqp8	Mir410	Apoa4	Mfsd2a
Depp1	Cebpb	Atp11a	Mir412	Noct	Egr1
Cd24a	Dnajc12	Slc17a8	Mirg	Cxcl1	Saa1
Clic5	Enho	Tubb2a	Mir8104	Cyb561	Saa2
Cyp4a14	Gfra1	Tubb2b	Ppm1h	Cyp2c38	
Ddhd1	Gse1	Hectd2os	Bcl6	Cyp4a14	
Mir5131	Hipk3	1810053B23Rik	Nat8f5	Cyp7a1	
Ezr	Mir1902	Clec2h	Cyp26a1	Cyp17a1	
Gask1a	Hpx	Fabp2	Cyp26b1	Fgl1	
Gas6	Ifitm2	Fam81a	Dancr	Fkbp5	
Hspb1	Igfbp2	Rbp1	Snora26	Hp	
Krt19	Il1r1	Tff3	Ehf	Itih3	
Lama5	Isyna1	Tsku	Fst	Lcn2	
Myl9	Kcnk5	Usp2	Nckap1	Lepr	
Ppp1r10	Lbp	Ang	Mir675	Lrg1	

Prom1	Ly6e	Rnase4	Igf2	Mmd2
Smpd3	Mafb	Arrdc3	Me1	Mt1
Tspan8	Ppl	Atxn1	Nrep	Mtnr1b
Abcd2	Saa3	Cyp39a1	Onecut1	Nipal1
Ccnd1	Sh3bp2	Elovl6	Serpina12	Nnmt
Mir3962	Slc13a3	Gbp11	Slc41a2	Orm1
Cyp17a1	Sult5a1	Rgs16		Orm2
Ercc2	Tmprss2	Saa4		Orm3
Mir343	Tsc22d3	Sucnr1		Scara5
Fkbp5	Acaca	Chpf2		Serpina3n
Gck	Aldoa	Mir671		Slc3a1
Lpin1	Asb2	Igfbp5		Slc16a5
Mt1	Btbd19	Marco		Slc37a1
Mtnr1b	Car3	Ciart		Usp18
Nr4a1	Cldn1			Depp1
Rabggtb	Cryab			Acta2
Snord45c	Cyp2c23			Calcrl
Scara5	Des			Cav1
Slc13a5	Dpt			Cd24a
Zbtb16	Eno3			Cd34
Adgrf1	Fabp4			Cd36
C730036E19Rik	Fam13a			Cox7a1
Cxcl1	Fbxo32			Cpe
Cyp7b1	Fhl1			Cyp2c55
Dclk3	Gsn			Dct
Dct	Gys1			Dsg1c

Fabp5	Hspb6	Gdf15
Fam47e	Inmt	Gm4956
Fgl1	Jun	Zfp982
Fos	Ldhb	Igfbp6
Foxq1	Lpl	Junb
Gm4956	Me1	Krt19
Lin7a	Mgp	Maff
Mme	Mir331	Mme
Msmo1	Vezt	Myh11
Mup9	Mir5625	Myl9
Orm1	Slc35f6	Scd1
Orm2	Mylpf	Slc25a30
Orm3	Mup5	Sparcl1
Phlda1	Nexn	Tmem47
Rmrp	Nrep	Tpm2
Slc3a1	Onecut1	Tspan8
Tfrc	Oxct1	Txnip
Thrsp	Pdk4	
Noct	Pfkm	
Col1a1	Pkm	
Ccn2	Ptp4a3	
Gpnmb	Rilpl1	
Slc25a24	Serpina4-ps1	
Acta2	Serpina12	
Cfap69	Slc25a4	

Erdr1	St5
Fam83b	Timp2
Gnai1	Tmem38a
Ly6d	Tnnt1
Mir678	Tpm1
Prmt2	Atp6v0d2
Pls1	Mir192
	Dipk2a
	Dusp6
	G0s2
	Glce
	Mir5133
	Id1
	Klf11
	Marcks
	Slc9a1
	Osgin1
	Cyp8b1
	Cyp26b1
	Angptl8
	Moxd1