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UNIVERSITY OF CALIFORNIA,
IRVINE

Retinoid X Receptor Activation by the Endocrine Disruptor Tributyltin Promotes Adipose
Lineage Commitment and Perturbs Adipocyte Function

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Bassem Merit Shoucri

Dissertation Committee:
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Professor Thomas Schilling
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2018

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DEDICATION

To

my *Teta* and *Geddo*

in recognition of their unconditional love

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ACKNOWLEDGMENTS

First and foremost, I thank Dr. Bruce Blumberg for his invaluable mentorship. Bruce has pushed me to develop as a scientist both at the bench and as a communicator. He has taught me to be detail-oriented and compulsive, and to always be wary of “fooling yourself, because you are the easiest one to fool”. Bruce has improved my scientific writing by painstakingly editing several sizeable (and unsuccessful) fellowship applications early in my PhD. In the face of four rejections Bruce encouraged me to keep writing and, eventually, we secured two fellowships. My communication skills have greatly improved simply by watching Bruce communicate to different audiences, be they scientists, community members, or the general public through his newly published book. I am inspired by Bruce’s commitment to speaking out against the regulatory failures and business interests that have allowed for the continued production of harmful industrial chemicals. We need more scientists like him. Finally, I thank Bruce for being an encouraging and supportive mentor as I made family a priority in my life.

I also am thankful to my “second mentor”, Dr. Raquel Chamorro-García, who greatly contributed to my development as a scientist. Early in my PhD, Raquel encouraged me to take the extra step that many first-year students struggle to take. In addition to her mentorship, she became a great friend who made my time in lab more productive and gratifying. I thank Riann Egusquiza, Dr. Amanda Janesick, and Dr. Carlos Díaz-Castillo for enriching my time as a graduate student through scientific and unscientific conversations. I also thank my undergraduate students, Eric Martinez, Tim Abreo, and Victor Hung, for their hard work and companionship. Many other Blumberg Lab members have aided me over the past five years and I thank all of them.

I am grateful to my committee members, Dr. Tom Schilling, Dr. Ali Mortazavi, Dr. Maksim Plikus, and Dr. Ellis Levin. I am particularly thankful to Dr. Levin, who has taken time to mentor me along the physician-scientist career path. I also thank Dr. Toshi Shioda, our collaborator at Massachusetts General Hospital, for advising me on the analysis of genomic data.

I would like to thank my colleagues in the Department of Developmental and Cell Biology for providing a welcoming and stimulating environment for me to develop and mature as a scientist. In particular, I thank Lindsay Malter and Andrea Wiley for their incredible administrative support throughout my time here. I also thank the Medical Scientist Training Program for giving me the opportunity to develop leadership and organizational skills. In particular, I thank Dr. Al Goldin, Dr. Ed Monuki, Dr. Rob Edwards, and Dr. Steven Chessler for their mentorship, and my colleagues Edsel Abud and Michael Ghijsen for their advice and friendship throughout the program. I also thank Joanne Wu for her monumental efforts working with the faculty and student body to run our program smoothly.

I thank all of my past scientific mentors starting with Dr. Larry Feldman, who gave me my first opportunity to work in a lab at UCLA when I was 16-years-old. From UC San Diego, I thank Dr. Geert Schmid-Schönbein and Dr. Paul Mills, my Master’s thesis

advisors, as well as Dr. Kate Edwards who personally mentored me as a young (and often unfocused) scientist. From the NHLBI, I thank Dr. Alan Remaley and Dr. Kasey Vickers who opened my eyes to the world of metabolism and molecular biology.

I thank the CRC Press for permission to include portions of a book chapter in my Introduction, originally appearing in *Childhood Obesity: Causes, Consequences, and Intervention Approaches*. I also thank the Oxford University Press for permission to include Chapter 1 of my dissertation, which was originally published in *Endocrinology*.

Financial support for my research in the Blumberg Laboratory was provided by the National Institute of Environmental Health Sciences (R01ES023316). I have been supported by the UC Irvine Medical Scientist Training Program (T32GM008620), the UC Irvine School of Biological Sciences, the American Heart Association (15PRE25090030), and the Environmental Protection Agency (STAR FP917800).

Lastly, I thank all of my friends and family for encouraging me along this long career path. I especially thank my parents, not only for their love and support, but for nurturing my curiosity as a child. I am forever grateful to my wife, Taylor, who has been a rock to stand on in the seas of graduate school. Finally, I thank my baby Noura for being a bright light in this world.

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ABSTRACT OF THE DISSERTATION

Retinoid X Receptor Activation by the Endocrine Disruptor Tributyltin Promotes Adipose
Lineage Commitment and Perturbs Adipocyte Function

By

Bassem Merit Shoucri

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2018

Professor Bruce Blumberg, Chair

The obesity epidemic has reached unsustainable proportions and threatens to bankrupt the American healthcare system. While physicians continue to prescribe diet and exercise as a panacea for obesity, mounting evidence shows that the majority of diet interventions fail. Longitudinal data suggest that Americans gain more weight in the 21st century than they did three decades ago for a given level of caloric intake and exercise, implicating an environmental insult independent of energy balance that is programming obesity risk into the population.

Mounting evidence implicates early-life exposure to industrial chemicals—of which there are tens of thousands—as an overlooked contributor to the obesity epidemic. Of particular concern are a subset of at least 1,000 chemicals, termed endocrine disrupting chemicals, that have the ability to interfere with the action of endogenous hormones in the body. Our laboratory proposed the ‘obesogen hypothesis’, which asserts that there are chemicals in the environment that can program our obesity risk, particularly when we are exposed during critical windows of development.

Our lab identified and characterized the archetypal obesogen, tributyltin (TBT), which acts through two nuclear receptors, peroxisome proliferator-activated receptor γ and retinoid X receptor (RXR), to promote the development of fat tissue. Mice exposed to TBT *in utero* are born with excess fat tissue and have mesenchymal stem cells (MSCs) that favor the fat lineage over bone. *In vitro* exposure to TBT promotes the differentiation of MSCs or 3T3-L1 cells (a preadipocyte cell line) into adipocytes in a PPAR γ -dependent fashion. Incredibly, the obesogenic effects of TBT can be propagated through several generations to the F3 and F4 descendants of exposed F0 mothers.

The work described in this dissertation employs the *in vitro* MSC adipogenesis model to further explore the molecular mechanisms through which TBT alters the fate and function of developing stem cells. In **Chapter 1**, we investigate how TBT influences the earliest cell-fate decisions MSCs make during the process of lineage commitment. In **Chapter 2**, we examine the functionality of adipocytes differentiated in the presence TBT. In both studies, we found RXR to be indispensable in programming the obesogenic effects of TBT on the MSC compartment.

INTRODUCTION

The 21st Century Epidemic

The first half of the 20th century bore witness to a precipitous drop in mortality attributed to infectious disease (Centers for Disease and Prevention, 1999). This followed public health efforts to improve sanitation and implement childhood vaccination programs as well as the discovery of antibiotics. Conversely, the latter half of the 20th century witnessed an abrupt and dramatic rise in the prevalence of chronic, non-communicable disease. Among the most costly chronic diseases—both in terms of healthcare expenditure and patient suffering—are obesity, diabetes, cardiovascular disease (CVD), cancer, and arthritis. Half of all American adults suffer from a chronic disease, and 1 in 4 have two or more of these conditions (Ward et al., 2014). Critically, obesity is a key risk factor for many other chronic diseases including heart disease, hypertension, stroke, type 2 diabetes, certain cancers, and osteoarthritis. Not surprisingly, obesity is often associated with a constellation of symptoms, termed the metabolic syndrome, that include central (abdominal) adiposity, hypertension, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), and insulin resistance (Alberti et al., 2009). This places obesity at the fulcrum of a burgeoning epidemic of chronic diseases that have stretched the capacity of the American healthcare system in the 21st century.

At the population level, obesity is defined by body mass index (BMI; mass in kilograms divided by the square of height in meters, kg/m^2) a quantity that has limitations (Ahima and Lazar, 2013). Patients with a BMI of 18.5-24.9 are considered normal weight, 25-29.9 are overweight, and those ≥ 30 are obese. In 1976-1980, 15.0% of US adults were obese, but by the turn of the 21st century this number had doubled to 30.9% (Flegal et al., 2002). While there was some indication that the incessant rise in obesity prevalence had finally settled at 34.9% in 2011-2012 (Ogden et al., 2014), the latest data now show that 39.8% of American adults are

currently obese (Hales et al., 2017; Hales et al., 2018). In a recent obesity-themed issue of the Journal of the American Medical Association (JAMA), associate editor Edward Livingston noted that “Six years ago...there was optimism that progress was being made in preventing and treating obesity. As time has passed, so too has the optimism...” (Livingston, 2018; Livingston and Zylke, 2012). Among the most discouraging data cited by Livingston are the relentless rise in pediatric obesity rates. From 1999 to 2016 pediatric obesity prevalence rose from 13.9% to 18.5% (Hales et al., 2017; Hales et al., 2018). In recent years it appeared as though progress was being achieved, particularly in 2-5 year olds where obesity rates dropped to 8.4% in 2012 from a high of 13.9% in 2003 (Dietz and Economos, 2015). The latest data, however, show the 2-5 year old obesity rate back at 13.9% (Hales et al., 2017; Hales et al., 2018; Skinner et al., 2018). Current models predict that more than half of today’s youth will be obese by the time they are 35 years old (Ward et al., 2017). This unrelenting rise in adult and pediatric obesity has lead physicians, public health officials, policy makers, and scientists alike to reconsider both established and unconventional risk factors for the development of obesity.

Calories in, calories out, and much more

For decades physicians have been counseling their obese patients to improve their health through lifestyle modification. Diet and exercise are steadfastly prescribed as a panacea for obesity despite mounting evidence showing that weight loss triggers a robust physiologic response to restore body weight in the long-term (Coutinho et al., 2018; Sumithran et al., 2011). While the benefits of a healthy diet and regular exercise remain undisputed (Blair et al., 1989; Mitrou et al., 2007), their ability to specifically combat obesity has been called into question. Only 1 in 5 overweight/obese patients who set out to lose weight are able to maintain a $\geq 10\%$ reduction in body weight at 1 year (Kraschnewski et al., 2010; Wing and Hill, 2001). This problem is further aggravated by the widespread and persistent perception, particularly within

the healthcare community, that obese individuals fail to lose weight due to lack of self-discipline or willpower (Phelan et al., 2015). Weight stigmatization has, in fact, exacerbated the obesity crisis by deteriorating the mental and metabolic health of obese patients as well as stifling meaningful intervention at the public policy level (Puhl and Heuer, 2010). Dismissing the ludicrous notion that 4 out of 5 dieters lack willpower, clinicians and scientists are left asking why the vast majority of weight loss interventions fail. The answer seems to be, increasingly, that body weight is influenced by factors that act independently of caloric intake and energy expenditure, and these factors work hard to resist changes in body weight (Bray et al., 2018).

As the energy balance paradigm wanes, investigators have begun to turn their attention away from quantity and towards quality of diet. In particular, an increased intake of refined carbohydrates is thought to be responsible for the burgeoning obesity and diabetes epidemics (Ludwig, 2016; Ludwig and Friedman, 2014). Many randomized dietary interventions, however, have failed to identify any one macronutrient to best reduce weight and improve cardiometabolic risk profile (Bray et al., 2018). The POUNDS Lost Study assigned 811 overweight adults to one of four diets varying in macronutrient composition (20/15/65%, 20/25/55%, 40/15/45%, and 40/25/35% fat/protein/carbohydrate, respectively) and followed them for 2 years (Sacks et al., 2009). The authors observed no differences in weight loss between any group (high vs. low fat, protein, or carbohydrate). Importantly, only 15% of participants who completed the trial reduced their body weight by $\geq 10\%$ at 2 years. In the DIETFITS study, 609 overweight adults were assigned to one of two 'healthy' diet interventions, one low in fat and the other low in carbohydrates (Gardner et al., 2018). Again, no differences in weight loss between diets were observed at 1 year; long-term weight loss maintenance was not assessed in this study. The DIETFITS study also investigated the interaction between genetics and diet by assessing three single nucleotide polymorphisms (SNPs; FABP2, PPARG, ADRB2) thought to predict response to low-fat or low-carbohydrate diets. Furthermore, they explored the interaction between pre-

intervention insulin response and subsequent success in diet. No significant interactions were observed between genotype or insulin secretion and diet (Gardner et al., 2018), challenging the conclusions of previous studies (Chaput et al., 2008; McClain et al., 2013) and exposing the current futility in attempting to personalize diets based on simple measures such as SNPs or glycemic parameters. Taken together these studies demonstrate that dietary interventions consistently result in temporary weight loss, but they fail to identify any macronutrient that can be restricted to the benefit of the general population. Furthermore, they fail to identify factors that can predict the success or failure of a specific diet with a specific individual. More sophisticated predictive models, built on dozens of parameters and machine learning algorithms, have shown considerable promise in personalizing diets, but are many years from becoming cost-effective, routine therapy (Zeevi et al., 2015).

If not diet, what then?

Given these inconclusive results in the face of an unrelenting obesity crisis, investigators and healthcare providers have been forced to consider alternative treatments. Unfortunately, pharmacotherapies for obesity share a disappointing history, epitomized by the recent bankruptcy of Orexigen Therapeutics, which sold the anti-obesity drugs Qsymia (phentermine/topiramate) and Belviq (lorcaserin). Barriers to effective pharmacotherapy include concerns over safety and efficacy, adherence issues due to significant side effects, as well as insufficient insurance coverage (Bessesen and Van Gaal, 2018). Several pharmacologic agents have been developed for long-term treatment of obesity including pancreatic lipase inhibitors (orlistat), glucagon-like peptide-1 agonists (liraglutide), serotonin 2C agonists (lorcaserin), combination sympathomimetic and GABA receptor agonist (phentermine/topiramate), and combination opioid inhibitor and dopamine agonist (naltrexone/bupropion). All of these therapies promote weight loss to varying degrees over placebo when combined with lifestyle

modification (Dong et al., 2017). However, the most efficacious of these drugs, phentermine/topiramate and liraglutide, have substantial shortcomings. Phentermine/topiramate elevates pulse and blood pressure and causes oral clefts in offspring exposed *in utero*, while liraglutide is expensive, requires daily injection, and is associated with risk of pancreatitis and gallbladder disease (Bessesen and Van Gaal, 2018). Hence, pharmacotherapy for obesity remains unpopular with patients and physicians alike and less than 1% of eligible patients in the United States are prescribed these drugs (Zhang et al., 2016).

Yet another approach to the obesity epidemic has been to promote cardiorespiratory fitness in lieu of weight loss. This unorthodox recommendation is based on several studies showing that poor fitness bestows a greater mortality risk than high BMI (termed the “fat-but-fit” paradigm), in addition to studies revealing a paradoxical inverse relationship between BMI and mortality (termed the “obesity paradox”) (Kennedy et al., 2018). These observations are accompanied by studies documenting “metabolically healthy obesity” (MHO) where BMI is elevated, but all other metabolic parameters are normal and cardiovascular disease (CVD) risk is not elevated (Ortega et al., 2016). Since MHO individuals are somewhat fitter than their metabolically unhealthy counterparts, investigators hypothesize that exercise, not weight loss, may hold the key to CVD protection in obesity (Ortega et al., 2016). Longitudinal studies suggest that MHO is a transient condition, however, and these patients have an elevated CVD risk in the long-term (Bray et al., 2018). Hence, the focus on fitness over weight loss remains controversial.

Recent work has uncovered the critical role the circadian clock plays in regulating appetite and metabolism (Huang et al., 2011). Hormonal signals from the periphery are integrated within the suprachiasmatic nucleus of the hypothalamus, the body’s master clock. The well-documented inverse association between sleep duration and obesity, particularly during childhood, may be due to an impaired circadian regulation of the adipokine leptin (Dibner

and Gachon, 2015). Sleep quality and sleep patterns, such as “social jetlag” (delayed sleep/wake times on weekends), have also been associated with obesity, unhealthy eating behaviors, and altered metabolism (Miller et al., 2015). Furthermore, sleep quality and duration can predict successful response to diet interventions, suggesting that sleep may be a routinely overlooked factor in lifestyle interventions (Chaput and Tremblay, 2012). Chronobiologists have also explored novel fasting regimens such as intermittent fasting (e.g. every other day fasting), periodic fasting (e.g. 2 days per week fasting), and time restricted feeding (food intake limited to 9-12 hours per day) that have shown promise in improving cardiometabolic endpoints in rodents and some human trials (Longo and Panda, 2016; Rothschild et al., 2014). The ability of these interventions, however, to preserve long-term weight loss or improve glycemic control is not yet proven and further studies are underway.

An explosion of research has discovered that the trillions of microbiota that reside within our gastrointestinal tract have a profound impact on our health. Pioneering work from the Gordon lab used metagenomic analyses of intestinal microbiota to establish that obese humans and mice have a distinct biome (Turnbaugh et al., 2006). Importantly, these authors showed that transplanting the obese microbiome into lean, germ-free mice resulted in increased body fat, establishing a causal role for the gut microbiome in obesity. A subsequent study in twins concordant for leanness or obesity and their mothers found a core microbiome in the obese twins (Turnbaugh et al., 2009). The authors also found that families share much of their microbiome, in spite of significant inter-individual variation that was comparable between mono- and dizygotic twins, implying a critical role for the environment in establishing our gut flora (Turnbaugh et al., 2009). This point was strengthened by an ensuing study of twins discordant for obesity, where lean or obese human fecal microbiota was transplanted into germ-free mice (Ridaura et al., 2013). Mice that received transplants from obese co-twins gained more weight and body fat, which was associated with less efficient short-chain fatty acid fermentation and

bile acid metabolism, but increased metabolism of branched-chain amino acids. At the same time researchers were uncovering the link between obesity and the microbiome, clinicians were beginning to use fecal microbiota transplantation to treat intractable pseudomembranous colitis, the result of iatrogenic *Clostridium difficile* infection (Kassam et al., 2013). Given the success and relative safety in treating *C. difficile* with fecal transplant, investigators are beginning to explore its potential to treat obesity and metabolic disease (Hartstra et al., 2015). Furthermore, physicians are exploring the impacts that various dietary supplements, pre- and probiotics, as well as existing pharmacologic therapies (antibiotics, in particular) have on microbial abundance, diversity, and metabolism (Hartstra et al., 2015). Whether these investigations will lead to effective and affordable therapies for obesity and its comorbidities is yet to be seen.

Finally, the medical community is turning to surgical intervention to treat obesity. Bariatric surgeries, primarily, Roux-en-Y gastric bypass and sleeve gastrectomy, are increasingly common procedures that greatly diminish the size of the stomach and result in significant long-term weight loss and improved glucose control in diabetic patients (Arterburn and Gupta, 2018). Clearly, these procedures are not void of complications such as hernias, vomiting, reflux, and abdominal pain, as well as micronutrient deficiencies and alcoholism due to enhanced alcohol absorption (Bloomberg et al., 2005; King et al., 2012). In spite of beneficial effects on weight and glycemic control, the overall effect of these surgeries on all-cause mortality is minimal (Reges et al., 2018). Furthermore, a recent study of diabetics that underwent gastric bypass surgery showed that 50% of patients achieved optimal control of blood sugar, cholesterol, and blood pressure (the so-called “triple endpoint”) after 1 year, but that number had dropped to 23% at 5 years (Ikramuddin et al., 2018). This casts serious doubt on the long-term benefits of this costly and risky procedure that is becoming increasingly utilized in the fight against obesity and diabetes.

Obesity therapies in adults have produced demoralizing results for decades, be they dietary, behavioral, or pharmaceutical. Furthermore, surgical interventions, which had been considered the gold standard for obesity intervention, now appear as though they may not reverse CVD risk factors and mortality in the majority of patients long-term. This leaves the field, both clinically and scientifically, at a critical juncture from which it should continue to improve and expand upon existing treatment strategies, but also pivot toward novel and alternative therapies and interventions. Notably, current approaches are primarily aimed at treating adults already suffering from obesity and often multiple comorbidities. For decades, however, pediatricians have known that obesity begins at the moment we are born. In fact, mounting evidence now shows that obesity begin before birth, even before conception.

Obesity from cradle to grave

As highlighted previously, the pediatric obesity rate is at an all-time high of 18.5%, and the 2-5 year-old obesity rate is at an alarming 13.9% (Hales et al., 2017; Skinner et al., 2018). The vast majority of obese children become obese adults, and childhood BMI accelerates CVD progression (Berenson et al., 1998; Freedman et al., 2005). 2-5 year old obesity can predict obesity in adulthood, as can infant size and growth rate in early postnatal life (Baird et al., 2005; Freedman et al., 2005). These data suggest that environmental inputs that can alter our size or growth rate can program obesity risk from the moment we are born.

A recent study assessed growth rate in Finnish subjects for which there were decades of serial BMI data, from 6 to 49 years old (Buscot et al., 2018). Patients were divided into 4 groups based on childhood and adult BMI status: the first group contained lean children that became lean adults, the second group contained lean children who became obese adults, the third group contained obese children that escaped adult obesity, and the final group contained obese children that remained obese in adulthood. They then used a piecewise regression model to

calculate BMI growth rates as well as a change point (CP) for each group, representing the age at which BMI growth rate significantly slowed. For lean children that became lean adults, CPs were 24.1 and 17.2 years for males and females, respectively, while those that became obese adults experienced accelerated growth rates in childhood and CPs delayed to ~30 years of age in both males and females (Buscot et al., 2018). Obese children that escaped adult obesity experienced reduced growth rates in childhood as compared to those who remained obese. Remarkably, these subjects reduced their growth rates even earlier than the lean controls, at 21.4 and 16.1 years for males and females, respectively (Buscot et al., 2018). This study highlights the importance of maintaining normal size and growth in the first 6 years of life, as obese or fast-growing 6-year-olds were more likely to become obese adults. Furthermore, these data show that adolescence (for females) and early adulthood (for males) may provide a second critical window of opportunity in which to target interventions towards obese youth and, optimistically, prevent adult obesity.

Obesity begins at (and before) fertilization

Several decades ago, the British epidemiologist David Barker observed a strong geographic correlation between cardiovascular mortality rates in the 1970s and infant mortality rates 50 years prior (Barker and Osmond, 1986). Barker went on to show that this correlation was due to under-nutrition during fetal life and infancy, which (in addition to increasing the infant mortality rate) gave rise to small for gestational age (SGA) neonates that had an elevated CVD risk as adults (Barker et al., 1989a; Barker et al., 1989b; Barker et al., 1989c). These foundational studies provided convincing evidence that cardiometabolic disease could be programmed by the environment *in utero* (Barker, 2007). These studies led to the formation of a society termed the “Developmental Origins of Health and Disease” (DOHaD) dedicated to the investigation of environmental influences on the earliest windows of human development and

subsequent programming of chronic disease risk, particularly obesity and metabolic disease (Gluckman and Hanson, 2004).

Many epidemiologists went on to confirm that poor fetal growth (i.e. small for gestational age, SGA) or infant growth resulting from undernourishment increases risk of obesity and cardiometabolic disease later in life (Martyn et al., 1998; Rich-Edwards et al., 1997; Skilton et al., 2005). Barker postulated that gestational undernutrition programmed the fetus to survive in scarcity—termed the “thrifty phenotype”—but these adaptations backfire when faced with caloric excess in adulthood (Hales and Barker, 2001). In ensuing years, investigators discovered that over-nourished, large for gestational age (LGA) babies also have an elevated obesity and CVD risk (Armitage et al., 2005; Hardy et al., 2004; Wei et al., 2007). These findings have resulted in a growing list of maternal factors known to predict obesity and CVD in the offspring (Hanson and Gluckman, 2014). Most importantly, maternal pre-pregnancy obesity, excess gestational weight gain, and gestational diabetes are known to increase risk of obesity, CVD, diabetes, and asthma in the offspring (Godfrey et al., 2017).

These data have led some investigators to propose a paradigm-shifting model to better explain the obesity epidemic and account for its enormity and pace. In this model, undernourished (in the third world) or obese (in the developed world) pregnant mothers give birth to children with a developmentally encoded obesity risk, broadly termed ‘fetal programming’. As they grow, these children will struggle to maintain weight and health, since the *in utero* environment encoded some neurologic, hormonal, and/or metabolic predilection to consume and store calories. As adults, the obese daughters of obese mothers will give birth to grandchildren imbued with the same obesity risk, and a feed-forward cycle ensues.

Mechanisms behind the developmental programming of obesity

How then is obesity risk transferred to the fetus, particularly in the overfed state? Many studies have documented that maternal obesity leads to changes in hormones and metabolites such as glucose, insulin, lipids, adipokines, and inflammatory cytokines (Patel et al., 2015). These hormones either cross the placenta or alter its function to affect fetal development of tissues such as the hypothalamus, pituitary, liver, pancreatic islets, adipose tissue, and others. Maternal over-nutrition and gestational diabetes mellitus (GDM) are well-known to result in insulin resistance, fetal hyperinsulinemia, and LGA neonates (Lawlor et al., 2012). Likewise, cortisol is depressed in severe maternal obesity and is inversely correlated with increased birthweight and length of gestation (Stirrat et al., 2016). Hyperleptinemia in maternal obesity impairs parasympathetic innervation of pancreatic β cells, resulting in impaired glycemic control later in life (Croizier et al., 2016). Hence, obesity-induced alterations of maternal hormones during pregnancy can influence the development of varied fetal tissues and subsequently program obesity and/or cardiometabolic risk.

Of course, proper uterine perfusion and placental function are major determinants of fetal growth and development. Obese women have larger placentas, but lower placental efficiency (i.e. higher placenta to birth weight ratio) (Wallace et al., 2012). Furthermore, various animal models of maternal over-nutrition result in altered placental vascularity, nutrient transport, and gene expression, implicating the placenta as a central hub in the fetal programming of obesity (Burton et al., 2016). The microbiome is yet another emerging mediator of maternally programmed obesity risk. For years, maternal-fetal microbial exchange was thought to initiate with the rupture of the amniotic sac at the start of labor. The detection of bacteria in placenta, amniotic fluid, cord blood, and meconium, however, suggests that this exchange begins *in utero* (Gohir et al., 2015). Type of delivery (vaginal vs. C-section), prenatal antibiotics, type of feeding (breastmilk vs. formula), gestational age, and other factors all affected the infant microbiome (Mueller et al., 2015a; Penders et al., 2006). Importantly, both C-

section or prenatal exposure to antibiotics increase risk of obesity at 7 years of age, presumably due to an aberrant exchange of microbiota *in utero* or during delivery (Mueller et al., 2015b). Critically, obese mothers have an altered microbiome throughout the course of pregnancy (Gohir et al., 2015), implicating maternal-fetal microbial transfer in the developmental origins of obesity.

Epigenetics – an interface between the environment and the genome

As endocrine, physiologic, and microbial mechanisms of fetal programming have emerged, so too has epigenetics, which established a molecular link between the environment and the genome. Epigenetics is the study of heritable changes in phenotype that are not the result of altered DNA sequence, but rather environmentally influenced modifications of the genome. These modifications include methylation of DNA at cytosine residues 5' to guanine (CpG sites), chemical modifications of the histone proteins that package DNA into chromatin, and expression of non-coding RNAs. These epigenetic marks are thought to reflect broader changes in chromatin accessibility and architecture that have the potential to disrupt transcriptional regulation, particularly in developing cells where the chromatin state is in flux. In mammalian development, there are two major epigenetic reprogramming events during which there is a genome-wide erasure of DNA methylation marks and subsequent remethylation (Fraser and Lin, 2016; Seisenberger et al., 2013). The first reprogramming occurs in the pre-implantation embryo and the second in the developing primordial germ cells. This process plays a critical role in regulating the potency of developing cell populations from pluripotency through lineage commitment and eventual terminal differentiation (Hemberger et al., 2009).

There is ample evidence that environmental inputs during development can alter the epigenetic landscape to alter gene expression, development, and phenotype (Feil and Fraga, 2011; Jirtle and Skinner, 2007). A classic model of this phenomenon is the viable yellow agouti

(A^{vy}) allele in mice, which contains an upstream retrotransposon that dictates the coat color and health of the mice based on its methylation status. If the maternal diet is supplemented with methyl donors (folic acid or vitamin B12), the retrotransposon of the progeny is hypermethylated and the agouti gene is normally expressed, resulting in mice with a brown coat and healthier “pseudo-agouti” phenotype (Waterland and Jirtle, 2003). Conversely, A^{vy} mice prenatally exposed to the endocrine disruptor BPA have a hypomethylated retrotransposon and aberrant expression of the agouti gene, resulting in a yellow coat and poor metabolic health (Dolinoy et al., 2007).

Studies in wild-type animals have explored the effects of maternal nutrition on the epigenetic landscape and phenotype of the offspring (Seki et al., 2012). The progeny of rat dams fed a low protein diet have livers with promoter hypomethylation and increased expression of PPAR α (*Ppara*) and the glucocorticoid receptor (*Nr3c1*) (Lillicrop et al., 2005), later attributed to a reduction in DNA methyltransferase 1 (*Dnmt1*) expression (Lillicrop et al., 2007). Maternal protein restriction in mice results in fetal livers with promoter hypermethylation and underexpression of the liver X receptor alpha gene (*Lxra*) in addition to several of its target genes (van Straten et al., 2010). Maternal high-fat diet alters feeding behavior of the offspring via altered methylation and expression of genes in the dopamine and opioid pathways within areas of the brain associated with reward (Vucetic et al., 2010). Mice from dams fed a high-fat had elevated serum leptin and diminished adiponectin at 6 months (in addition to obesity and insulin resistance), which was attributed to altered gene expression in gonadal adipose tissue accompanied by increased histone 4 lysine 20 monomethylation (H4K20me1) in the leptin (*Lep*) promoter and elevated histone 3 lysine 9 dimethylation (H3K9me2) in the adiponectin (*Adipoq*) promoter (Masuyama and Hiramatsu, 2012). These animal experiments display that nutritional status of the mother has the potential to alter the epigenome in the developing fetus potentially resulting in lifelong modification of disease risk.

Interestingly, several studies of paternal over-nutrition have implicated environmentally-induced modifications of the sperm epigenome as a putative mechanism of intergenerational disease propagation. Paternal high-fat diet results in glucose intolerance and pancreatic β -cell dysfunction in female offspring, as well as hypomethylation and upregulation of a member of the JAK-STAT signaling pathway in pancreatic islets (Ng et al., 2010). Another study showed significant obesity and insulin resistance in the daughters of high-fat diet exposed fathers, as well as increased sperm reactive oxidative species, DNA damage, altered sperm microRNA expression, and diminished global DNA methylation in germ cells (Fullston et al., 2013). Insulin resistance, glucose intolerance, and an altered β -cell transcriptome, but not obesity, was reported in the male offspring of fathers fed a high-fat diet and treated with low-dose streptozotocin to induce pre-diabetes (Wei et al., 2014). Work in *Drosophila* showed that as little as two days of paternal high-sucrose diet prior to mating resulted in obese progeny (Ost et al., 2014). The authors went on to show this phenotype was passed through the male germline via modifications of histone marks passed on from sperm to developing embryos. Hence, even the paternal environment has the potential to transmit epigenetic information to the zygote and influence subsequent development. Critically, in vitro fertilization studies have confirmed the epigenetic nature of phenotypic transmission to the offspring in both paternal and maternal high-fat diet exposures (Huypens et al., 2016).

A number of human studies highlight the influence of parental dietary exposure on epigenetic programming of the offspring, primarily through examination of DNA methylation in umbilical cord blood of obese mothers (Godfrey et al., 2017). Humans exposed to famine early, but not late in gestation have a slight hypomethylation of the maternally imprinted insulin-like growth factor 2 gene (IGF2) in blood leukocytes as compared to their unexposed, same-gender siblings at 60 years of age (Heijmans et al., 2008). Interestingly, IGF2 cord blood protein levels are associated with increased birth weight and inversely correlated with IGF2 DNA methylation,

a correlation that is significantly strengthened if the mother is obese (Hoyo et al., 2012). Another study identified a single CpG residue in the retinoid X receptor alpha (RXRA) promoter whose methylation in umbilical cord predicts adiposity of the offspring at 9 years of age (Godfrey et al., 2011). An ensuing study showed that hypermethylation of 4 CpGs upstream of RXRA was inversely associated with bone mineral density of the offspring at 4 years (Harvey et al., 2014). These studies, and many others, extend the notion that the prenatal environment can interact with the epigenome to influence development in humans.

One troubling discovery within the DOHaD field has been the observation that environmentally-induced changes in phenotypic expression can propagate through multiple generations. One of the first observations documenting such a phenomenon in humans was conducted using detailed historical harvest records from the small and isolated municipality of Överkalix, in the far north of Sweden (Kaati et al., 2002). Investigators showed that food availability during the pre-pubertal period in grandmothers was associated with diminished longevity in the granddaughters, while food availability during the same period in grandfathers was associated with decreased longevity in the grandsons (Kaati et al., 2002). Another study showed that women exposed to starvation *in utero* during the Dutch Famine gave birth to neonates with excess adiposity and poor health later in life (Painter et al., 2008). Given the substantial evidence linking environmental exposures to changes in the epigenome, and the relative infrequency of DNA mutations, these transgenerational phenomena are attributed to heritable epigenetic alterations of the germline (Skinner, 2015; Wang et al., 2017). In line with this hypothesis, maternal and paternal obesity result in a number of molecular and metabolic perturbations within the gametes that manifest as transmissible epigenetic modifications (Lane et al., 2015).

Environmental chemicals and the obesity epidemic

A study setting out to examine the potential effects of the environment on obesity observed over 20,000 animals representing 12 distinct populations and 8 different species living in proximity to industrialized societies (Klimentidis et al., 2011). These animals included pets (cats and dogs), laboratory animals (mice, rats, and primates) that were fed controlled diets, and feral rats. Notably, nearly every one of these populations showed positive trends in both weight gain and odds of obesity over the past several decades. The chance of these populations all concomitantly exhibiting the same trend in obesity was calculated as approximately 1 in 10 million (Klimentidis et al., 2011). Furthermore, a recent study examining dietary and exercise data from the National Health and Nutrition Examination Survey (NHANES) from 1988 to 2006 in 14,419 adults concluded that for a given amount of caloric intake and energy expenditure, BMI was 2.3 kg/m² higher in 2006 than in 1988 (Brown et al., 2016). These studies strongly suggest that an environmental insult, independent of diet and exercise, is responsible for the parallel trend in obesity observed in humans and animals.

The use of synthetic chemicals in commerce has grown exponentially since the 1940s, numbering in the tens of thousands today (Meeker, 2012). Of particular concern is a subset of at least 1000 chemicals, termed endocrine disrupting chemicals (EDCs), which interfere with any aspect of hormone action (Diamanti-Kandarakis et al., 2009; Gore et al., 2015; Zoeller et al., 2012). The “endocrine” label can be misleading as EDCs can perturb the action of any chemical messenger, endocrine or otherwise (autocrine, paracrine, neurotransmitter). EDCs can alter hormone synthesis and transport or they can interfere with the cell signaling and receptor systems that regulate hormone response in target tissues. Since hormones play critical roles in development and metabolism, investigators suspected that EDCs might interfere with hormonal systems to promote weight gain and ultimately obesity (Heindel, 2003).

The obesogen hypothesis

In 2006, our group proposed the obesogen hypothesis, which asserts that there are EDCs in the environment that confer obesity risk upon exposed individuals, principally those exposed during critical windows of development (Grun and Blumberg, 2006). These “obesogens” promote adiposity through a variety of mechanisms that include:

- Disturbing normal fat development, thereby increasing the number of fat cells.
- Encouraging the storage of energy within fat cells, increasing fat cell size.
- Altering metabolic setpoints programmed during development.
- Interfering with neurologic and hormonal control of hedonic reward and appetite.

Obesogens are a subset of a broader group of EDCs termed ‘metabolism disrupting chemicals’ (MDCs) that can promote diseases such as type 2 diabetes, NAFLD, dyslipidemia, and metabolic syndrome (Heindel et al., 2017a).

A number of obesogens have been identified in humans and animals and the list continues to lengthen. Obesogens identified in animal studies include estrogenic chemicals [such as diethylstilbestrol (DES) (Newbold et al., 2005), genistein (Penza et al., 2006), bisphenol A (BPA) (Vom Saal et al., 2012), and nonylphenol (Hao et al., 2012b)], organotins such as tributyltin (TBT) (Grun et al., 2006); organochlorine pesticides [including polychlorinated biphenyls (PCBs) (Arsenescu et al., 2008), dichlorodiphenyltrichloroethane (DDT) (La Merrill et al., 2014), triflumizole (Li et al., 2012), and tolylfluanid (Regnier et al., 2015)], organophosphates [such as chlorpyrifos (Meggs and Brewer, 2007) and diazinon (Roegge et al., 2008)], brominated (Suvorov et al., 2009) and non-brominated flame retardants (Patisaul et al., 2013); and a number of other chemicals including nicotine (Gao et al., 2005), benzo[a]pyrene (Ortiz et al., 2013), phthalates (Schmidt et al., 2012); and perfluoroalkyl substances (PFAS) (Hines et al., 2009).

In humans, urinary phthalates are associated with waist circumference and insulin resistance in adults (Stahlhut et al., 2007) and in children and adolescents (Buser et al., 2014a; Trasande et al., 2013). Likewise, multiple studies of US (Bhandari et al., 2013; Harley et al., 2013; Trasande et al., 2012) and Chinese (Li et al., 2013) children associate urine BPA levels with obesity prevalence. Urinary levels of phenol pesticides are also correlated with obesity in children and adolescents (Buser et al., 2014b). Baseline serum PFAS levels in the POUNDS Lost study (discussed earlier) were associated with increased weight regain following dietary intervention, particularly in women (Liu et al., 2018). Finally, serum levels of several persistent organic pollutants (POPs) are associated with BMI in adults, including dichlorodiphenyldichloroethylene (DDE, a metabolite of DDT), PCBs, hexachlorobenzene (HCB), and β -hexachlorocyclohexane (β -HCH) (Tang-Peronard et al., 2011).

In addition to these environmental exposures, there is a wide range of obesogenic pharmaceuticals for which weight gain is an established side effect. These include first- and second-generation antipsychotics (Rummel-Kluge et al., 2010), selective serotonin-reuptake inhibitors (SSRIs) (Blumenthal et al., 2014), systemic glucocorticoids (Huscher et al., 2009), and the anti-diabetic thiazolidinediones (rosi-, pio-, and tro- glitazone) (Nesto et al., 2003). If clinicians have already accepted weight gain as an established side effect of these medications, then it is not unreasonable to infer that exposure to physiologically relevant doses of non-pharmaceutical xenobiotic compounds (such as EDCs) could have the same effect.

Developmental obesogen exposure

Applying the DOHaD model to the obesogen hypothesis requires monitoring of early life EDC exposure during sensitive developmental windows together with subsequent observation of obesity and metabolic disease throughout life (Heindel et al., 2017b). Given the relatively recent establishment of the DOHaD and EDC fields, such studies are limited in number and by

low sample size and lack of long-term follow up. The strongest epidemiological evidence of an environmental obesogen programming obesity risk *in utero* is maternal smoking. It is well established that the children of smoking mothers are born small-for-gestational-age, however, these children experience catch-up growth in the first year of life and eventually outpace their peers. Tens of epidemiological studies have all shown an increased overweight/obesity risk in children whose mothers smoked during pregnancy (Thayer et al., 2012).

A number of studies have evaluated prenatal exposure to POPs, which have long half-lives and persist in the environment, despite significant regulation and outright bans on many. These include PCBs, organochlorine pesticides (DDE/DDT, chlordane, HCB), polybrominated diphenyl ethers (PBDEs), and others. A number of prospective studies have shown positive associations between POPs levels in maternal serum or umbilical cord blood and BMI of the offspring later in life (Legler et al., 2015; Tang-Peronard et al., 2011). HCB levels in umbilical cord blood are positively associated with weight, body mass index (BMI), and increased obesity risk at 6.5 years of age, independent of birth weight and maternal obesity (Smink et al., 2008). A more recent study from the Rhea cohort in Crete showed that first trimester serum HCB and DDE levels are associated with BMI, abdominal obesity, and blood pressure of the offspring at 4 years (Vafeiadi et al., 2015). Two successive pooled analyses of European birth cohorts showed an inverse association between prenatal PCB exposure and birth weight, but no effect of DDE exposure (Casas et al., 2015; Govarts et al., 2012). Another pooled analysis of European cohorts examining postnatal growth at 24 months observed increased growth associated with prenatal DDE exposure and decreased growth associated with postnatal PCB exposure (Iszatt et al., 2015). The United States Collaborative Perinatal Project (CPP) collected third trimester serum from mothers in the early 1960s, prior to regulation of POPs, and followed the offspring to 7 years of age. These higher exposure studies revealed no association between BMI and prenatal PCB, DDE, and several other POPs (Cupul-Uicab et al., 2013; Gladen et al.,

2004). The Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) Study in California published two sequential studies showing a non-significant but positive association between gestational serum DDE/DDT and odds of obesity at 7 years (Warner et al., 2013), followed by a significant association in males at 9 years (Warner et al., 2014). The Spanish Infancia y Medio Ambiente (INMA) cohort shows that DDE and PCB levels in cord blood are positively associated with gender- and dose- specific increases in obesity risk at 6.5 years (Valvi et al., 2012). In the Faroe Islands, one study showed perinatal PCB and DDE were associated with BMI change between 5 and 7 years in the female offspring of overweight, but not normal weight mothers (Tang-Peronard et al., 2014). Taken together, these studies indicate that POPs can affect intrauterine and postnatal growth to increase risk of obesity later in childhood.

The developmental origins of fat

One facet of the obesogen hypothesis that has received ample attention is the notion that EDCs can promote the excessive development of fat tissue. Adipocytes appear during the second trimester of pregnancy and proliferate through childhood and adolescence before leveling off at approximately 10% renewal per year in adulthood (Spalding et al., 2008). This phenomenon is independent of BMI, as weight gain/loss in adults is predominantly attributed to changes in cell size rather than cell number (Spalding et al., 2008). Visceral fat, which is linked to insulin resistance, may be the exception to this rule. In humans, visceral depot size is determined by cell number (Arner et al., 2013), and adult mice fed a high fat diet generate new fat cells in visceral depots (Jeffery et al., 2015; Jeffery et al., 2016). Therefore, adipogenic stimuli (such as an obesogen) during gestation and early life establish the number of fat cells in an individual, while fat mass in adults is regulated both by cell number and cell size in a depot-specific manner.

Adipocytes originate from mesenchyme, primarily from the mesoderm, via the mesenchymal stem cell (MSC or multipotent stromal cell), a multipotent cell capable of forming bone, muscle, cartilage, tendon, fat, and other tissues. MSCs and their lineage-restricted derivatives can be found in the perivascular niche of any vascularized organ, including adipose tissue (Crisan et al., 2008). Transformation of an MSC into a mature adipocyte requires initial commitment to the adipose lineage, followed by terminal differentiation into a functioning fat cell (Rosen and Spiegelman, 2014). Adipose lineage commitment requires the concerted action of multiple signaling cascades regulated by adipogenic transcription factors that induce the expression of the peroxisome proliferator-activated receptor gamma (PPAR γ), the “master regulator” of adipogenesis (Cristancho and Lazar, 2011). PPAR γ , a member of the nuclear receptor family, is a ligand-activated, DNA-binding transcription factor that dimerizes with the retinoid X receptor (RXR) to bind and regulate genomic targets that promote adipose differentiation (Tontonoz and Spiegelman, 2008). Both MSCs and mouse 3T3-L1 cells (a committed preadipocyte cell line) have become valuable *in vitro* tools for screening candidate obesogens and characterizing their mechanisms of action.

Tributyltin: a model obesogen

We and others first showed that the organotin tributyltin (TBT) binds and activates both PPAR γ and its heterodimeric partner, RXR, to promote adipogenesis and alter lipid homeostasis (Grun et al., 2006; Kanayama et al., 2005). Human and mouse MSCs as well as mouse 3T3-L1 preadipocytes exposed to environmentally relevant levels (nanomolar) of TBT, or the pharmaceutical PPAR γ agonist rosiglitazone, were shunted toward the adipocyte lineage via a PPAR γ -dependent pathway (Kirchner et al., 2010; Li et al., 2011). Mice exposed to TBT, *in utero*, show lipid accumulation in adipose depots, livers, and testis and have MSCs biased towards the adipose lineage and away from the bone lineage (Chamorro-Garcia et al., 2013;

Grun et al., 2006; Kirchner et al., 2010). Treatment of adult mice or rats with TBT results in obesity and fatty liver (Bertuloso et al., 2015; Zuo et al., 2011) as well as disrupted thyroid function (Sharan et al., 2014).

TBT was used for many years as an anti-fouling agent in paint for ship hulls until it was banned in the 80s and 90s, although it persists in marine environment to this day (Antizar-Ladislao, 2008). TBT is found in other industrial products such as wood preservatives, disinfectants, and vinyl plastics, as a byproduct of the thermal stabilizer dibutyltin (DBT). Importantly, TBT can be found in house dust (Kannan et al., 2010) and placental TBT levels are associated with growth in the first 3 months of life (Rantakokko et al., 2014). Beyond concerns over organotin exposure, there is an expanding group of obesogens to which humans are exposed that also activate PPAR γ . These include phthalates (Feige et al., 2007), triflumizole (Li et al., 2012), flavanones (Christensen et al., 2010; Saito et al., 2009), bixin (Takahashi et al., 2009), dioctyl sodium sulfosuccinate (DOSS) a component of the oil dispersant COREXIT (Temkin et al., 2015), and several flame retardants including BDE-47 (Kamstra et al., 2014), tetrabromo- and tetrachloro- bisphenol A (TBBPA, TCBPA) (Riu et al., 2011) and triphenyl phosphate (TPP) a component of the flame retardant Firemaster® 550 (FM550) (Belcher et al., 2014).

Phthalates are widely used as plasticizers and solubilizing agents and are commonly found in personal care products, medications, and medical equipment. Phthalates and their metabolites can be detected in the urine of nearly all humans (Crinnion, 2010) including infants (Sathyanarayana et al., 2008). These chemicals promote adipose differentiation of 3T3-L1 cells (Hurst and Waxman, 2003) and they stimulate adipogenesis and suppress osteogenesis in mouse MSCs (Watt and Schlezinger, 2015). Several *in vivo* studies show that prenatal phthalate exposure promotes obesity in adult mice (Hao et al., 2012a; Schmidt et al., 2012). In addition to activation of PPAR γ , phthalates may program obesity risk through their effects on

PPAR α or PPAR δ , thyroid metabolism, or gestational growth (Kim and Park, 2014). Urinary phthalates are associated with obesity and insulin resistance in children, adolescents, and adults (Buser et al., 2014a; Hatch et al., 2008; Stahlhut et al., 2007; Trasande et al., 2013). Studies of prenatal phthalate exposure that examine growth and obesity as a primary outcome are sparse, although three studies have identified a negative correlation between prenatal phthalate levels and growth in boys (de Cock et al., 2014; Maresca et al., 2015; Valvi et al., 2015).

For a half-century, brominated chemicals such as PBDEs and hexabromocyclododecane (HBCD) have been used as flame retardants in a variety of products (Shaw et al., 2014). Due to safety concerns, several PBDEs were phased out of US production in 2005, though these chemicals linger in products and migrate into house dust, a major source of human exposure (Shaw et al., 2014). BDE-47 induces adipogenesis in 3T3-L1 cells (Bastos Sales et al., 2013) in part due to a weak activation of PPAR γ (Kamstra et al., 2014). A recent screen of flame retardants and their metabolites revealed that 3-hydroxy-BDE-47 activates PPAR γ with the same potency as the pharmaceutical rosiglitazone (Fang et al., 2015). TBBPA and TCBPA have not been phased out and are still widely used. Recently, these halogenated bisphenols were identified as PPAR γ agonists that stimulate differentiation of 3T3-L1 preadipocytes (Fang et al., 2015; Riu et al., 2011). The phase-out of PBDEs increased demand for alternative flame retardants such as the organophosphate-based FM550. Perinatal FM550 exposure results in varied phenotypes in rat offspring including obesity, advanced puberty, cardiac hypertrophy, and anxiety (Patisaul et al., 2013). In a subsequent study, FM550 was shown to be a PPAR γ activator along with TPP, a triaryl phosphate that comprises 10-20% of FM550 (Belcher et al., 2014). FM550 and TPP were further shown to increase adipogenesis and inhibit osteogenic differentiation of mouse MSCs (Pillai et al., 2014).

Perfluoroalkyl substances (PFAS) are hydrophobic surfactants with hundreds of industrial applications that are found in the serum of most humans living in the United States (Kato et al., 2011). Perfluorooctanoic acid (PFOA) purportedly activates PPAR γ (Vanden Heuvel et al., 2006) though this assertion is controversial (Takacs and Abbott, 2007), and PFOA does not induce adipogenesis in 3T3-L1 preadipocytes (Bastos Sales et al., 2013). However, *in utero* exposure to low dose PFOA results in increased body weight and elevated serum insulin and leptin in post-pubertal female mice (Hines et al., 2009). These animal data were mirrored in a prospective study of 665 Danish pregnant mothers whose gestational PFOA exposure was associated with the BMI of female, but not male offspring at 20 years of age (Halldorsson et al., 2012). Another Danish cohort showed no such associations (Andersen et al., 2013). Baseline serum PFAS levels predicted weight regain in a randomized weight loss intervention (Liu et al., 2018). Women in the highest tertile of PFAS concentrations regained almost double the weight women in the lowest tertile regained.

Taken together, these results indicate a continued need to screen for industrial chemicals that can activate PPAR γ , since there is sufficient evidence in cells, animals, and humans to believe these compounds will act as obesogens, *in vivo*.

Estrogenic obesogens

Estrogens are protective against obesity and cardiovascular disease in adults, as is well demonstrated by the onset of abdominal obesity and dyslipidemia following loss of estrogen at menopause. Emerging research, however, implicates early life exposure to low-dose estrogens to be obesogenic. Prenatal exposure to the estrogenic EDCs DES, genistein, and BPA results in obese adult animals (Newbold et al., 2009), and urine BPA is associated with obesity prevalence in children (Bhandari et al., 2013; Li et al., 2013; Trasande et al., 2012). DDT and its

metabolite DDE are estrogenic and anti-androgenic, respectively, and have been implicated as obesogens in humans and animals (La Merrill et al., 2014; Valvi et al., 2012).

Diethylstilbestrol (DES) is a synthetic estrogen that was widely prescribed to pregnant women in the mid 20th century to prevent miscarriage. Though mothers were unaffected, amongst the millions of children born to DES-treated mothers there was a well-documented increase in several rare pathologies of the reproductive tract (making DES a textbook example of the DOHaD model). Data from the National Cancer Institute's DES Follow-Up Study showed a modest increase in obesity risk amongst females prenatally exposed to DES, and this risk was higher in those exposed to lower doses (Hatch et al., 2015). Mice exposed to low doses of DES prenatally become obese later in life, while high dose exposure results in decreased birth weight followed by catch-up growth and subsequent obesity (Newbold et al., 2005). Similar results were observed in mice exposed during the first 5 days of postnatal life (Newbold et al., 2009; Newbold et al., 2008). Importantly, these results were recapitulated with other estrogens (2- and 4-hydroxyestradiol), suggesting an estrogen-dependent mechanism (Newbold et al., 2005).

Of all the estrogenic EDCs, data implicating bisphenol A (BPA) as a potent obesogen are most concerning. BPA, used in polycarbonate plastics and epoxy resins, is produced in millions of tons annually and can be detected in most humans (Crinnion, 2010; Vom Saal et al., 2012). BPA is a potent activator of the estrogen receptor (ER) in the nucleus, but also at the cell membrane where it induces rapid cell signaling events (Watson et al., 2010). BPA promotes differentiation of 3T3-L1 preadipocytes (Masuno et al., 2002) and human preadipocytes via an ER-dependent mechanism (Boucher et al., 2014). Low dose prenatal BPA exposure in animals results in increased body weight in adult life (Howdeshell et al., 1999). Perinatal BPA exposure results in increased visceral fat depot size in females at weaning, as well as adipocyte hypertrophy and increased expression of adipogenic and lipogenic genes (Somm et al., 2009). Both Trasande et al and Bhandari et al have shown a correlation between urinary BPA and

obesity prevalence in US children from NHANES (Bhandari et al., 2013; Trasande et al., 2013), results echoed in a Chinese cohort (Li et al., 2013). Despite these extensive data (and data implicating BPA in numerous other pathologies) regulatory agencies do not believe levels of BPA exposure are sufficient to result in adverse outcomes, and production of the high production volume chemical continues (Abbasi, 2018).

Other obesogens and their mechanisms of action

Much attention has been paid to the ability of obesogens to act as hormone mimics that can bind nuclear receptors. Numerous EDCs have been shown to bind PPAR γ and ER, though other nuclear receptors are known to be obesogen targets. BPA, whose obesogenic effects are largely attributed to its ability to bind and activate ER, is also an activator of the steroid and xenobiotic receptor (SXR) (Sui et al., 2012), the glucocorticoid receptor (GR) (Sargis et al., 2010), and an antagonist of the androgen receptor (AR) (Teng et al., 2013). Likewise, phthalates activate all three PPAR receptors (α , δ , γ) (Hurst and Waxman, 2003; Lampen et al., 2003) as well as SXR (Hurst and Waxman, 2004). The obesogenic effects of PCB-77 were shown to be dependent on activation of the aryl hydrocarbon receptor (AhR) both *in vitro* and *in vivo* (Arsenescu et al., 2008). Tolyfluanid, a fungicide commonly used in Europe, promotes adipose differentiation of 3T3-L1 cells through activation of the glucocorticoid receptor (GR) (Neel et al., 2013), and mice fed a diet supplemented with tolyfluanid gain more weight and fat mass than controls (Regnier et al., 2015). Hence obesogens can act through several members of the nuclear receptor family, at times simultaneously, to promote obesity.

Not all obesogens are nuclear receptor ligands, and obesogens that do activate nuclear receptors may also act through alternative pathways. For example, TBT, BPA, and phthalates, all of which activate nuclear receptors, also inhibit the enzyme 11 β -hydroxysteroid dehydrogenase, a critical regulator of active/inactive intracellular glucocorticoid levels (Vitku et

al., 2014). TBT is further known to inhibit aromatase (Heidrich et al., 2001) and isocitrate dehydrogenase (Yamada et al., 2014). Prenatal exposure to nicotine results in obesity and metabolic complications (Gao et al., 2005; Somm et al., 2008), presumably through its action on nicotinic acetylcholine receptors (nAChRs), plasma membrane-associated ion channels present in the brain, hypothalamus, adrenal medulla, and other organs (Tweed et al., 2012). The pharmaceutical obesogen lithium, which has diverse mechanisms of action, promotes weight gain through increased appetite, hypothyroidism, and even a combination of thirst and improved mood that leads to consumption of high-calorie beverages (Torrent et al., 2008). Therefore, obesogens can act through varied nuclear receptor-independent mechanisms to promote weight gain.

Environmental exposures have transgenerational consequences

Of great concern is accumulating evidence linking developmental EDC exposure to disease risk not only in the offspring, but also in multiple generations of unexposed descendants. Skinner and colleagues showed that high-dose exposure of pregnant F0 rats to the fungicide vinclozolin caused reproductive abnormalities in male rats through four generations (F1-F4) (Anway et al., 2005). While the F1 fetus and F2 primordial germ cells were exposed to vinclozolin *in utero*, the F3 and F4 generations received no direct exposure and hence their phenotype is considered transgenerational. This study went on to show that the F3 and F4 phenotype was due to heritable epigenetic alterations of the male germ line (Anway et al., 2005). Similar adverse effects on male and female reproductive health have now been demonstrated in F3 or F4 descendants of rodents exposed to BPA (Salian et al., 2009), phthalates (Doyle et al., 2013; Pocar et al., 2017; Rattan et al., 2018), and atrazine (McBirney et al., 2017).

Our group first showed that developmental EDC exposure could result in a transgenerational obesity phenotype (Chamorro-Garcia et al., 2013). The F1, F2, and F3 progeny of F0 mothers exposed to environmentally relevant doses of TBT display increased adipose depot weights, hepatic steatosis, and MSCs reprogrammed to favor the adipocyte lineage (Chamorro-Garcia et al., 2013). Since that study, the list of environmental chemicals that induce a heritable, transgenerational obesity phenotype has grown to include a mixture of plastics-derived EDCs (BPA and phthalates) (Manikkam et al., 2013), a hydrocarbon mixture (jet fuel, JP-8) (Tracey et al., 2013), DDT (Skinner et al., 2013), and PCBs (Mennigen et al., 2018).

In a recent study from our group, F0 mothers were again exposed to TBT and subsequently bred through the F4 generation (Chamorro-Garcia et al., 2017). When F4 males were challenged with a moderately high-fat diet, the descendants of TBT-exposed mice gained the same amount of weight as controls, but their body fat percentage was considerably higher. Genome-wide analysis of DNA methylation in F4 adipose tissue revealed broad regions of DNA with iso-directional changes in methylation levels (termed iso-directional differentially methylated blocks or isoDMBs) in TBT animals (Chamorro-Garcia et al., 2017). Critically, isoDMBs showing TBT-induced increases in methylation were associated with AT-rich DNA sequence and lower gene expression levels, while those with reduced methylation correlated with GC-rich DNA and higher gene expression. Interestingly, the region coding for leptin (*Lep*) was within an isoDMB showing reduced methylation in mice ancestrally exposed to TBT. Accordingly, leptin mRNA and serum leptin was increased in these mice, implying a leptin-resistant, thrifty phenotype (Chamorro-Garcia et al., 2017). We have proposed a model whereby isoDMBs are a proxy for broader changes in chromatin architecture that are established in the directly-exposed F2 primordial germ cells. Importantly, unbiased clustering analysis of chromatin accessibility in sperm from F3 and F4 males shows significant separation of control and TBT samples and transgenerational similarity, in addition to overlap with isoDMBs in adipose tissue.

How exactly these developmental exposures propagate disease phenotypes to unexposed generations remains an open question. Some assert that an altered intrauterine environment is sufficient to propagate a phenotype through multiple generations, independent of epigenetic changes to the germ line (Aiken and Ozanne, 2014). This assertion is contradicted by evidence of transgenerational phenotypes following paternal exposures and studies showing phenotypes beyond the F2 generation (Lane et al., 2014). DNA methylation marks are stable through mitosis and meiosis, hence altered epigenetic reprogramming of the germ line is a key mechanism through which EDCs are proposed to cause transgenerational phenotypes (Jirtle and Skinner, 2007). DNA methylation remains the most studied epigenetic factor responsible for EDC-induced transgenerational phenomena. However, DNA hydroxymethylation, histone modifications, and a variety of noncoding RNAs have all been implicated in epigenetic inheritance (Xin et al., 2015). Looking forward, our group is turning towards higher-order readouts of chromatin access (assay for transposase-accessible chromatin using sequencing, ATAC-Seq) and chromatin architecture (chromosome conformation capture, Hi-C) to assess how low-dose environmental exposures can induce heritable alterations in the developing germline. That developmental EDC exposure may contribute to the vast and abrupt rise in global obesity through several generations raises the stakes of identifying obesogens, studying their mechanisms of action, and ultimately reducing human exposure.

RXR – a key player in the development of mesenchymal stem cells

While animal studies continue to explore the mechanisms behind the developmental and transgenerational programming of obesity by TBT, this dissertation makes use of the mesenchymal stem cell model of adipogenesis to explore the molecular consequences of TBT exposure and, hopefully, inform our ongoing *in vivo* work. The clear shortcoming of the MSC model is that it does not mimic the intricate developmental timing, complex exposure dynamics,

or diverse microenvironments these cells experience *in vivo*. Furthermore, the diversity of fat depots and their progenitors means that no one stem cell source (in our case, bone marrow) can model a highly diverse set of tissues (Jiang et al., 2014; Zhang et al., 2018; Zwick et al., 2018). With that obvious caveat, however, these cells provide several advantages over other *in vitro* systems:

1. They are an easily obtainable and expandable primary cell, making them preferable to commonly used cell lines (3T3-L1, C3H10T1/2) that have issues with reproducibility (Kassotis et al., 2017).
2. As multipotent stem cells, they allow for the study of both the lineage commitment and terminal differentiation processes, something that is not possible in committed 3T3-L1 preadipocytes.
3. They are a powerful tool to screen for industrial chemicals (of which there are tens of thousands) that may perturb adipocyte development or function, particularly if these chemicals that disrupt the commitment process, which takes place *in utero*.
4. Molecular discoveries in these cells may lead to valuable insights that shed light on how other stem cell compartments might be influenced by chemical exposures.

This last point is particularly relevant to the findings reported in this dissertation, since the role of the nuclear receptor RXR was extensively characterized in MSCs exposed to TBT. Given the transgenerational phenotypes observed in the descendants of TBT-exposed mothers and the role RXRs play in gametogenesis, this research has led us to investigate the effects of RXR ligands on the development of primordial germ cells.

RXR is a unique member of the nuclear receptor family in that it is an obligate dimer of many nuclear receptors (Evans and Mangelsdorf, 2014). It is expressed in placenta and developing embryo/fetus and is a well-described developmental regulator of many tissues (Froment, 2008; Mark et al., 2009). Given its partnership with PPAR γ , RXR and its ligands are

known to regulate adipogenesis and lipid metabolism *in vitro* and *in vivo* (Imai et al., 2001; Tontonoz et al., 1997). While TBT was established as a dual RXR/PPAR γ agonist from our earliest investigation (Grun et al., 2006), the consequences of RXR activation were largely eclipsed by PPAR γ , the master regulator of fat development and target of the thiazolidinedione class of anti-diabetic pharmaceuticals (Janesick and Blumberg, 2011). Subsequently, the effects of TBT on adipogenesis were shown to require PPAR γ (Li et al., 2011), and many other obesogens were identified as PPAR γ activators (reviewed above). Only two additional adipogenic RXR activators have been published to date (Bowers et al., 2016; Janesick et al., 2016), and the obesogenic effects of developmental exposure to RXR activators ('rexinoids') remains unexplored.

This dissertation employs the *in vitro* MSC adipogenesis model to ask two fundamental questions. In **Chapter 1**, we ask how TBT influences the earliest cell fate decisions made by MSCs. In **Chapter 2**, we ask what are the functional consequences of developmental TBT exposure on the adipocyte. In answering both questions, we found that the role of RXR was pivotal, opening the door for future studies of *in utero* rexinoid exposure.

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CHAPTER 1

Retinoid X receptor activation alters the chromatin landscape to commit mesenchymal stem cells to the adipose lineage (Endocrinology, Volume 158, Issue 10, Pages 3109-3125)

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Abstract

Developmental exposure to environmental factors has been linked to obesity risk later in life. Nuclear receptors are molecular sensors that play critical roles during development, and as such are prime candidates to explain the developmental programming of disease risk by environmental chemicals. We have previously characterized the ‘obesogen’ tributyltin (TBT), which activates the nuclear receptors Peroxisome proliferator-activated receptor γ (PPAR γ) and

Retinoid X receptor (RXR) to increase adiposity in mice exposed *in utero*. Mesenchymal stem cells (MSCs) from these mice are biased towards the adipose lineage at the expense of the osteoblast lineage, and MSCs exposed to TBT *in vitro* are shunted towards the adipose fate in a PPAR γ -dependent fashion. To address where in the adipogenic cascade TBT acts, we developed an *in vitro* 'commitment assay' that permitted us to distinguish early commitment to the adipose lineage from subsequent differentiation. TBT and RXR activators (rexinoids) had potent effects in committing MSCs to the adipose lineage, whereas the strong PPAR γ activator rosiglitazone (ROSI) was inactive. We show that activation of RXR is sufficient for adipogenic commitment and that rexinoids act through RXR to alter the transcriptome in a manner favoring adipogenic commitment. RXR activation alters expression of Enhancer of zeste 2 (EZH2) and modifies genome-wide histone 3 lysine 27 trimethylation (H3K27me3) in promoting adipose commitment and programming subsequent differentiation. These data offer novel insights into the roles of RXR and EZH2 in MSC lineage specification and shed new light on how endocrine disrupting chemicals such as TBT can reprogram stem cell fate.

Introduction

38% of American adults are obese, as are 17% of US children (Flegal et al., 2016; Ogden et al., 2016). The obese population is at risk for a number of comorbidities including cardiovascular disease, type 2 diabetes, hypertension, and many cancers. This comes at a tremendous cost to the US economy, estimated to be more than \$200 billion annually (Cawley and Meyerhoefer, 2012). Clinical management of obesity remains focused on lifestyle modification in obese and overweight adults (Hall et al., 2012; National Institutes of Health, 1998). However, substantial evidence shows that environmental factors *in utero* and in early life have a profound effect on human health in adulthood (Hanson and Gluckman, 2014). In particular, exposure to xenobiotic chemicals during early development has been implicated as

an important contributor to the obesity epidemic (Heindel et al., 2017a; Heindel et al., 2017b; Janesick and Blumberg, 2016).

Our group proposed *the obesogen hypothesis*, which holds that exposure to exogenous chemicals during development can increase risk of obesity later in life (Grun and Blumberg, 2006). Results from many laboratories support and extend this hypothesis in humans and animal models (Gore et al., 2015; Heindel et al., 2017a; Janesick and Blumberg, 2016). While the mechanisms of action for most obesogens are unclear, many obesogens are known to act through nuclear receptors to promote the development of fat tissue (Gore et al., 2015; Heindel et al., 2017a; Janesick and Blumberg, 2011).

Adipogenesis in humans and mice begins *in utero* and continues during the postnatal period (Poissonnet et al., 1983; Tang et al., 2008; Wang et al., 2013). Transformation of a mesenchymal stem cell (MSC, a.k.a. multipotent stromal cell) into a white adipocyte requires initial commitment to the adipose lineage, followed by terminal differentiation into a mature adipocyte (Cristancho and Lazar, 2011). The goal of adipose lineage commitment is to induce expression of the “master regulator” of adipogenesis, the nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ), which is both necessary and sufficient to stimulate terminal differentiation (Tontonoz and Spiegelman, 2008).

We and others demonstrated that TBT acts through the nuclear receptors PPAR γ and its heterodimeric partner retinoid X receptor (RXR) to promote adipogenesis and alter lipid homeostasis *in vitro and in vivo* (Grun et al., 2006; Kanayama et al., 2005). Mice exposed to nanomolar levels of TBT *in utero* display increased lipid accumulation in adipose depots, livers, and testis as adults, and MSCs from these animals are reprogrammed to favor the adipose lineage at the expense of the osteogenic lineage (Chamorro-Garcia et al., 2013; Grun et al., 2006; Kirchner et al., 2010). The effects of TBT are transgenerational and can be detected in the F1, F2, F3 and F4 descendants of F0 mice exposed during pregnancy (Chamorro-Garcia et

al., 2017; Chamorro-Garcia et al., 2013). In a standard *in vitro* adipogenesis assay, human and mouse MSCs or 3T3-L1 preadipocytes exposed to TBT or the PPAR γ agonist rosiglitazone (ROSI) are shunted toward the adipocyte lineage via a PPAR γ -dependent pathway (Kirchner et al., 2010; Li et al., 2011). These studies did not address precisely where in the adipogenic pathway TBT acted to both promote lineage commitment and adipogenic differentiation.

One limitation within the adipogenesis field is the widespread use of the 3T3-L1 cell line, which is already committed to the adipose lineage. Primary bone marrow-derived MSCs offer an attractive alternative to these cells since they are easy to obtain and culture, but allow for the study of lineage commitment *in vitro*. They also offer a viable platform to screen endocrine disrupting chemicals (EDCs) and study their mechanisms of action in a dish. A further limitation of current *in vitro* methods is the use of adipose induction cocktails whose components both commit and differentiate MSCs into adipocytes. Therefore, it is impossible to decipher whether a chemical of interest, such as TBT, acts during one or both of these phases of development, since chemicals are added in conjunction with the induction cocktail. One *in vitro* model that has effectively separated commitment and differentiation is the MSC-like cell line C3H10T1/2. These cells can be committed to the adipose lineage by pretreatment with Bone morphogenetic protein 4 (BMP4) or an inhibitor of DNA methylation prior to differentiation with MDI (Bowers et al., 2006; Huang et al., 2009; Tang et al., 2004). No such system has been established for primary MSCs.

Since both prenatal TBT and ROSI treatment increased the number of preadipocytes *in vivo* in F1 animals treated *in utero* (Kirchner et al., 2010), but only TBT could elicit transgenerational effects on adipogenic commitment of MSCs in F3 descendants of F0-treated animals (Chamorro-Garcia et al., 2013) we hypothesized that there was some fundamental difference in how ROSI and TBT acted during MSC commitment and/or differentiation. To test this hypothesis, we developed an *in vitro* 'commitment assay' that allowed us to distinguish

between effects on adipogenic commitment and differentiation by pretreating MSCs with candidate chemicals for 48 hours prior to differentiating them with the adipogenic cocktail. Surprisingly, a 2-day pretreatment with TBT prior to adipose induction resulted in as much lipid accumulation as the standard 2-week adipogenesis assay co-treatment. Although ROSI is a potent inducer of adipogenesis, it was unable to commit MSCs to the adipogenic lineage in our commitment assay. We infer that TBT induces adipose lineage commitment in an RXR-dependent, PPAR γ -independent manner.

RXR has long been considered indispensable for adipose differentiation due to the critical heterodimer it forms with the master regulator of adipogenesis, PPAR γ (Imai et al., 2001; Tontonoz et al., 1997). Transcriptomal analyses of MSCs revealed genome-wide changes in transcription that were induced by TBT or the RXR-selective agonist, IRX4204, but not the strong PPAR γ agonist, ROSI. Furthermore, we found that RXR activation reduced the expression of the repressive histone modifier Enhancer of zeste 2 (EZH2), resulting in a genome-wide redistribution and overall decrease of repressive histone 3 lysine 27 trimethylation (H3K27me3) marks, particularly near key adipogenic regulators. These data identify RXR as an environmental sensor that can alter epigenomic architecture to influence lineage allocation of the mesenchymal stem cell compartment during development.

Materials and Methods

Chemicals – Tributyltin, rosiglitazone (Cayman Chemical, Ann Arbor, MI), IRX4204 (aka AGN194204)(a gift from Rosh Chandraratna, IO Therapeutics, Santa Ana, CA), T0070907 (Enzo Life Sciences, Farmingdale, NY), HX531 (a gift from Claes Bavik, Acucela Inc, Seattle, WA), LG100268, 9-cis retinoic acid (Enzo Life Sciences), all-trans retinoic acid, TTNPB, WY14643, GW501516, GW3965, DZNep (ApexBio, Houston, TX), dexamethasone, insulin, isobutylmethylxanthine, ascorbic acid 2-phosphate, β -glycerolphosphate, formaldehyde (Fisher

Scientific, Waltham, MA) Nile Red, Hoechst 33342, polybrene, and puromycin were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Cell culture, chemical treatment – Bone marrow-derived multipotent mesenchymal stromal cells from the long bones of C57BL/6J mice (MSCs) were purchased at passage 6 (OriCell, Cyagen Biosciences, Santa Clara, CA) and stored at passage 8 or 9 in liquid N₂. Cells were maintained as previously described (Chamorro-Garcia et al., 2012) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf bovine serum (CBS), 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 ug/mL streptomycin. MSCs were plated at 15,000 cells/cm² in 24-well, 12-well, or 10 cm plates. For standard adipogenesis assays (see Fig 1.1A), cells were maintained in media without any treatment until confluent after 72 hours. For commitment assays (Fig 1.1A), cells were allowed to attach and acclimate for 24 hours prior to 48 hours of chemical treatment (Day -2 through Day 0). After 48 hours (Day 0) MSCs were confluent and ready for differentiation. Due to its short half-life, the PPAR γ antagonist T0070907 was dosed every 12 hrs during pretreatment (Li et al., 2011). The amount of DMSO vehicle was kept under 0.1% in all assays.

Adipose/bone differentiation and staining – At confluency, cells were differentiated in minimal essential medium alpha medium (α MEM) containing 15% fetal bovine serum (FBS), 10 mM HEPES, 2 mM L-Glutamine, 100 IU/mL penicillin, and 100 ug/mL streptomycin, supplemented with an adipose induction cocktail (1 uM dexamethasone, 5 ug/mL insulin, 500 uM isobutylmethylxanthine) or an osteogenic induction cocktail (10 nM dexamethasone, 50 uM ascorbic acid 2-phosphate, and 10 mM β -glycerophosphate). Media was changed every 3-4 days for 14 days prior to fixation in 3.7% formaldehyde. Neutral lipids were stained with Nile Red (1 ug/mL) and nucleic acid was stained with Hoechst 33342 (1 ug/mL). Total fluorescence per well was measured in a SpectraMax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) using SoftMax Pro (Molecular Devices); Nile Red relative fluorescence units

(RFU) were normalized to Hoechst RFU for each well. Cells were imaged by confocal microscopy on a Zeiss LSM 700 (Zeiss, Oberkochen, Germany) and processed using Volocity (PerkinElmer, Waltham, MA) at the UC Irvine Optical Biology Core.

Lentiviral transduction – Hairpin-pLKO plasmids (RNAi Consortium shRNA Library, TRC/Broad Institute) along with packaging and envelope plasmids were transfected into 293T packaging cells as previously described (see <http://portals.broadinstitute.org/gpp/public/>). MSCs were seeded at 20,000 cells/well in 6-well plates (2,000 cells/cm²). After 24 hrs, cells were transduced overnight in 1 mL antibiotic-free media with 8 ug/mL polybrene and 50 uL high titer lentivirus expressing vector alone (pLKO.1) or shRNA against mRNA targets (Table S5). Following transduction MSCs were allowed to recover in fresh media (with antibiotics) for 24 hrs prior to a 48 hr selection with 2 ug/uL puromycin. Successfully transduced cells were expanded and frozen for further assaying.

RNA isolation, Reverse transcription, QPCR – Cells were lysed with TriPure (Roche, Basel, Switzerland) and total RNA isolated with MiniPrep columns (Zymo Research, Irvine, CA). cDNA was generated from 1 ug total RNA using Transcriptor Reverse Transcriptase (Roche) according to the manufacturer's protocol. Quantitative real-time PCR was conducted on a Roche LightCycler 480 II (Roche) using FastStart SYBR Green Master Mix (Roche) and intron-spanning primers (Table S5). Ct values were calculated as the second derivative maximum in LightCycler software (Roche). Relative quantification and error propagation were performed using the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001).

Chromatin immunoprecipitation – ChIP was performed as previously described (Lee et al., 2006) with slight modifications. MSCs were plated and treated as described in 10 cm plates. At the end of chemical treatment (Day 0), cells were fixed at room temperature for 10 min with 1% paraformaldehyde (Ted Pella, Redding, CA) in unsupplemented DMEM, washed with ice cold PBS, then quenched for 5 min with 125 mM glycine. Fixed cells were washed, scraped

from plates, centrifuged, then resuspended in 1 mL PBS / 10^7 cells. Equal numbers of cells were spun down, flash frozen in liquid N₂, and stored at -80°C. To isolate nuclei, cell pellets were lysed at 4°C for 10 min with mild detergents (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X 100, protease inhibitors). Nuclei were spun down, washed for 10 min at room temperature (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, protease inhibitors), and finally lysed in 300 µL of nuclear lysis buffer (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine, protease inhibitors). Chromatin was sonicated in 0.5 mL thin-walled PCR tubes (BrandTech, Essex, CT) using a QSonica Q800R2 (QSonica, Newtown, CT) with the following settings: 30 min, 30 sec ON / 30 sec OFF, amplitude 40%. 1% Triton-X 100 was added to sonicated lysate prior to high-speed, cold centrifugation to remove debris. 5 µg of DNA was immunoprecipitated with pre-blocked Protein A/G Dynabeads (Thermo, Waltham, MA) complexed to 2.5 µg of antibody (anti-H3K4me3, ab8580, RRID:AB_306649, Abcam, Cambridge, UK; anti-H3K27me3, ab6002, RRID:AB_305237, Abcam). Beads were washed four times with LiCl buffer (50 mM HEPES-KOH pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate) and once with TE + 50 mM NaCl. Beads were resuspended in elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and incubated at 65°C for 30 min to release chromatin from beads. Eluate and input controls were reverse crosslinked overnight at 65°C. DNA was isolated using the Zymo CHIP DNA Clean & Concentrator kit (Zymo Research) following RNase A (0.2 mg/mL, 2 hr, 37°C) and Proteinase K (0.2 mg/mL, 2 hr, 55°C) treatment. Input DNA content was determined by Nanodrop (Thermo).

Deep sequencing – Integrity of total RNA was evaluated using Tapestation high-sensitivity RNA screen tapes (Agilent Technologies, Santa Clara, CA). Range of the RNA integrity number (RIN) was 8.0 - 8.6. Strand-specific, barcode-indexed RNA-seq deep sequencing libraries were synthesized from total RNA with ERCC spike-in controls

(ThermoFisher Scientific, Waltham, MA) using Ovation RNA-Seq Systems 1-16 for Mouse (NuGen Technologies, San Carlos, CA). Size distribution of the libraries were determined by Tapestation to be 200 – 800 bp, peaking at 300 bp. RNA-seq libraries were quantified using KAPA Illumina library quantification kit (KAPA Biosystems, Wilmington, MA), and up to 12 libraries were pooled in each run of the Illumina NextSeq500 deep sequencer (75 nt + 75 nt, paired-end) to generate fastq raw read sequence files.

Size distribution of ChIP-enriched genomic DNA fragments was evaluated using D1000 high-sensitivity DNA screen tape to be 100-300 bp, peaking at about 200 bp. ChIP-seq deep sequencing libraries were synthesized using NEBNext Ultra Library Prep Kit (New England Biolabs, Ipswich, MA). Libraries were subjected to size distribution analysis using Tapestation (125 bp – 300 bp, peaking at 200 bp) and quantified using the KAPA kit to pool up for NextSeq500 sequencing (75 nt, single) to generate fastq data.

Deep sequencing data analysis – RNA-Seq and ChIP-Seq deep sequencing reads (fastq data) were aligned to the mouse genome reference sequence GRCm38/mm10 using the STAR aligner (Dobin et al., 2013), and the resulting bam format aligned reads were subjected to QC analysis using fastQC (Babraham Institute; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) followed by extraction of uniquely mapped reads using samtools (Li et al., 2009). Depth of deep sequencing of each sample is summarized as uniquely mapped read numbers in Table S6. For RNA-seq data analysis, uniquely mapped reads were assigned to the mm10 gene model and on-exon reads were counted using Bioconductor package “Rsubread” (Liao et al., 2013). The assigned read counts were normalized using Bioconductor package “DESeq2” (Love et al., 2014). Differential expression was assessed in DESeq2 using the DESeq function with $\alpha = 0.01$. Differentially expressed genes (DEGs) were defined by Benjamini-Hochberg corrected p-values ($p\text{-adj} < 0.01$) and fold change (absolute value of $\log_2[\text{fold change}] > 0.2$). Hierarchical clustering was

performed on the most differentially expressed genes using Cluster 3.0 and visualized in Java TreeView. For GO term and pathway analysis, DEGs were converted to HUGO gene symbols, then tested for enrichment in MSigDB (Broad Institute) pathway (c2) and GO term (c5) gene sets by hypergeometric test in R. P-values were corrected for multiple testing using the Benjamini-Hochberg method.

For ChIP-seq data, from the bam file data of uniquely mapped reads, gDNA areas enriched with the H3K4me3 or H3K27me3 histone marks were detected using SICER (Xu et al., 2014) with the following arguments: W=200, G=600, FDR cutoff = 0.001. The output BED files of SICER were then subjected to detection of differential enrichment of the histone marks using Bioconductor package “DiffBind” with the default parameters of function dba.analyze (Ross-Innes et al., 2012). Data were visualized using Bioconductor package “ChIPseeker” (Yu et al., 2015). Genomic DNA areas showing significant differential enrichment of the histone marks were annotated to nearby genes using GREAT (Genomic Regions Enrichment of Annotations Tool) (McLean et al., 2010) with 5 kb upstream, 1 kb downstream, 1 Mb distal search parameters. GO term and pathway analyses were conducted on annotated genes as described above.

All sequencing data is available on the Gene Expression Omnibus (GSE99565).

Statistical analysis – Data visualization and statistical analyses were conducted in Prism 7 (GraphPad Software, La Jolla, CA), excluding much of the genomic analyses which was completed in R and other software packages as noted above. 4-6 biological replicates were used for all experiments. Errors were propagated, when appropriate, according to standard methods (Bevington and Robinson, 2003). A p-value < 0.05 was considered statistically significant for all assays, also excluding genomic analyses where stricter thresholds were applied for large numbers of comparisons (see above).

Results

TBT pretreatment commits MSCs to the adipose lineage

In a standard MSC adipogenesis assay, cells are seeded and allowed to proliferate until confluency (Day 0), and then differentiated for 14 days in the presence of adipogenic cocktail (MDI, see Methods) and chemicals of interest (Fig 1.1A) (Chamorro-Garcia et al., 2012). To assess if TBT can commit MSCs to the adipose lineage, cells were pretreated with chemical ligands for 48 hours (Day -2 through Day 0), then induced to differentiate with MDI in the absence of ligands beginning from Day 0 (Fig 1.1A). Ligand doses were determined using our previous studies in reporter assays, standard MSC adipogenesis assays, and 3T3-L1 adipogenesis assays (Chamorro-Garcia et al., 2012; Grun et al., 2006; Janesick et al., 2016; Kirchner et al., 2010; Li et al., 2011). We ran the standard and commitment assays in parallel, then assessed lipid accumulation at Day 14. Remarkably, MSCs pretreated with TBT or the RXR-selective agonist IRX4204 (4204) (Vuligonda et al., 2001) for only 48 hours accumulated as much lipid as those exposed for 2 weeks in a standard assay (Fig 1.1B). Cells pretreated with the PPAR γ agonist ROSI did not accumulate more lipid in the commitment assay than vehicle controls, despite its strong effect in the standard assay (Fig 1.1B). Lipids were imaged by fluorescence microscopy to confirm an increased number of lipid-containing cells in MSCs pretreated with TBT or 4204 (Fig 1.1C). Gene expression analysis conducted at the end of the commitment assay (Day 14) confirmed up-regulation of adipose lineage markers such as *Fatty acid binding protein 4 (Fabp4)*, *Fat-specific protein 27 (Fsp27)*, and *Lipoprotein lipase (Lpl)* in cells pretreated with TBT or 4204, but not ROSI (Fig S1.1A).

Gene expression was assessed at Day 0, following pretreatment but prior to induction of differentiation (Fig 1.1D-E). TBT or 4204 pretreatment up-regulated the adipose commitment marker *Zinc finger protein 423 (Zfp423)*, as well as the early differentiation markers *Peroxisome proliferator-activated receptor gamma 2 (Pparg2)* and *CCAAT/enhancer-binding protein alpha*

(*Cebpa*) (Fig 1.1D). Importantly, ROSI pretreatment up-regulated the PPAR γ target gene *Fabp4*. We infer that PPAR γ protein is present but that its activation is unable to elicit adipogenic commitment in MSCs (Fig 1.1E). The RXR target gene, *ATP-binding cassette transporter 1* (*Abca1*) was strongly up-regulated by TBT and 4204, but not ROSI (Fig 1.1E). Taken together, these data led us to hypothesize that TBT and 4204 commit MSCs to the adipose lineage via an RXR-dependent but PPAR γ -independent mechanism.

The adipose and osteogenic lineages are thought to be mutually exclusive (Kang et al., 2009). Therefore, we conducted the same commitment assay, but instead differentiated pretreated MSCs with an osteogenic cocktail. At Day 0, TBT did not alter expression of the osteogenic commitment marker *Runt-related transcription factor 2* (*Runx2*) or the myogenic *Myogenic differentiation1* (*Myod1*), although there was a slight down-regulation of the osteo/chondrogenic marker *SRY-box 9* (*Sox9*) (Fig S1.1B). Interestingly, pretreatment with ROSI, 4204, or TBT did not inhibit osteogenesis as assessed by gene expression of bone lineage markers at Day 14 (Fig S1.1C). However, markers of the adipose lineage were persistently up-regulated in MSCs pretreated with TBT or 4204 after two weeks of osteogenic induction (Fig S1.1D). These data suggest that MSCs pretreated with TBT or 4204 are specified (which is reversible), but not determined (which is irreversible) to become adipocytes.

TBT-induced lineage commitment is RXR-dependent and PPAR γ -independent

We used pharmacologic inhibitors of PPAR γ and RXR in the commitment assay to test our hypothesis that TBT acts through RXR during lineage commitment. Inhibition of PPAR γ with T0070907 during pretreatment did not diminish the effect of TBT or 4204, whereas the RXR antagonist HX531 attenuated lipid accumulation (Fig 1.2A). Expression of *Fabp4* was significantly inhibited by antagonizing either RXR or PPAR γ at Day 0, although T0070907 was less effective against TBT or 4204 than against ROSI (Fig 1.2B). *Abca1* expression was significantly inhibited by HX531, but unaffected by T0070907 treatment (Fig 1.2B). Analysis of

early adipogenic marker gene expression revealed RXR-dependent changes in expression, particularly of the key adipogenic gene *Pparg2* (Fig 1.2C). These data support an RXR-dependent, PPAR γ -independent mechanism of adipose lineage commitment by TBT and RXR activators (rexinoids) such as IRX4204.

To confirm the results of pharmacologic inhibition, we used lentivirus-delivered shRNAs to knock down expression of *Pparg* and *Rxra* in mouse MSCs. Compared to vector-transduced controls, MSCs transduced with lentiviruses expressing shRNAs against *Pparg* and *Rxra* efficiently knocked down their mRNA targets at Day 0 (Fig 1.3A). Although the *Pparg* shRNA was designed to knock down both *Pparg1* and *Pparg2*, *Pparg1* was more efficiently inhibited at Day 0 (Fig 1.3A). Literature suggests that *Pparg1* is the predominant isoform in undifferentiated MSCs, whereas *Pparg2* expression is induced early in adipogenesis (Aprile et al., 2014). As expected, cells transduced with shRNA targeting *Pparg* or *Rxra* accumulated minimal lipid, irrespective of ligand treatment, since PPAR γ and RXR are essential for adipose differentiation (Fig 1.3B). At Day 0, *Fabp4* expression was greatly diminished by knockdowns of either PPAR γ or RXR α . In samples treated with TBT or 4204, *Abca1* expression was slightly reduced in sh*Pparg* MSCs, but strongly inhibited in sh*Rxra* cells (Fig 1.3C). Interestingly, knockdown of PPAR γ increased basal *Abca1* expression in MSCs treated with DMSO or ROSI, possibly due to increased availability of RXR protein. Expression of the early adipogenic marker genes *Zfp423* and *Cebpa* was diminished by sh*Rxra*, but not sh*Pparg*, suggesting RXR-dependent regulation (Fig 1.3D). As seen with *Abca1*, basal expression of these genes increased in sh*Pparg* MSCs suggesting positive regulation by RXR. Taken together, these studies support an RXR-dependent transcriptional regulation of early adipogenic markers by TBT and rexinoids.

RXRs can homodimerize or form heterodimers with several other nuclear receptor partners (Evans and Mangelsdorf, 2014). Therefore, we exposed MSCs to a panel of RXR agonists or agonists of RXR heterodimeric partners to test whether the action of RXR

heterodimers or homodimers was required for adipogenic commitment. Pretreatment with TBT, 4204, or another synthetic rexinoid LG100268 (LG268) increased subsequent adipose differentiation (Fig 1.4A). These results were not mirrored by the endogenous RXR activator 9-cis retinoic acid (9cRA) (Fig 1.4A), likely due to its ability to also activate the retinoic acid receptor (RAR) which inhibits adipogenic differentiation. RAR activators are known to be pro-osteogenic and anti-adipogenic (Lee et al., 2011; Marchildon et al., 2010; Schwarz et al., 1997). As expected, pretreatment with all-*trans* retinoic acid (atRA) or the synthetic RAR agonist TTNPB inhibited adipose differentiation (Fig 1.4A).

'Permissive' heterodimers can activate their gene targets in the presence of either an RXR ligand, or their own cognate ligands (Evans and Mangelsdorf, 2014). Pretreatment with agonists of the permissive RXR partners PPAR α , PPAR δ , PPAR γ , or Liver X receptor (LXR) all failed to commit MSCs to an adipose fate (Fig 1.4B). FXR was not tested because subsequent RNA-seq analysis revealed that FXR expression is not detected in MSCs (not shown). These data demonstrate that activation of individual permissive heterodimers is insufficient to induce adipose commitment. Treating MSCs with combinations of permissive dimer agonists, however, revealed that targeting PPAR δ and PPAR γ simultaneously reproduced rexinoid-induced commitment, albeit with less potency (Fig 1.4C). Interestingly, the effect of PPAR δ /PPAR γ agonist combination is attenuated by the further addition of PPAR α or LXR agonists (Fig 1.4C). Furthermore, simultaneous activation of LXR with PPAR α , PPAR δ , or a combination of PPAR α and PPAR γ were all mildly adipogenic (Fig 1.4C). Taken together, these data show that high-dose activation of several combinations of permissive RXR partners can recapitulate the effect of nanomolar RXR activation. Furthermore, liganded RXR may preferentially act at PPAR δ and PPAR γ target genes in committing MSCs to the adipose fate.

RNA-Seq analysis confirms the RXR-dependent nature of adipogenic commitment.

To better understand what genes might be responsible for adipose lineage commitment, we performed a transcriptomal analysis of MSCs pretreated with TBT (50 nM), 4204 (100 nM), ROSI (100 nM), or vehicle control (0.05% DMSO) (Day 0 samples from Fig 1.1). Unsupervised hierarchical clustering revealed two distinct clusters: one of TBT and 4204 replicates and the other of ROSI and DMSO replicates (Fig 1.5A). Principal component analysis (PCA) confirmed these results (Fig 1.5B). These data support the existence of a distinct pro-adipogenic transcriptional program activated by TBT and 4204 via RXR. In contrast, activation of PPAR γ by ROSI resulted in minimal overall change in the MSC transcriptome, consistent with the inability of ROSI to elicit adipogenic commitment (Fig 1.5C). Analysis of differential gene expression between treatments strengthened these conclusions, showing that TBT and 4204 alter the expression of many of the same transcripts, while ROSI altered the expression of few genes (Fig 1.5C, S2A, Table S1). Among the top transcripts up-regulated by TBT and 4204 are many well characterized gene targets of permissive RXR partner receptors, including LXR [*Abca1*, *ATP-binding cassette subfamily G member 1 (Abcg1)*] and all three PPARs [*Perilipin 4 (Plin4)*, *Fabp4*, *Pyruvate dehydrogenase kinase 4 (Pdk4)*, *Angiopoietin-like 4 (Angptl4)*, *Lipoprotein lipase (Lpl)*, *Fibroblast growth factor 21 (Fgf21)*] (Fig 1.5D). QPCR confirmed significant changes in gene expression of novel up and down-regulated transcripts (Fig S1.2B).

Extensive analyses of our transcriptome data were performed by comparing TBT-induced expression changes with previous studies of preadipocyte markers *in vivo* (Fig S1.3) (Gupta et al., 2012; Tang et al., 2008), as well as conducting gene ontology (GO) and pathway analyses (Fig S1.4, Table S2). Interestingly, 10 gene sets among the top pathways up-regulated by TBT had a corresponding, oppositely-regulated gene set within the top down-regulated pathways (red numbers, Fig S1.4C-D). For example, *berenjeno_transformed_by_rhoa_dn* (Fig S1.4C) and *berenjeno_transformed_by_rhoa_up* (Fig S1.4D) are gene sets from a study of

NIH/3T3 fibroblasts (an uncommitted cell line) transformed with a constitutively active *Ras* homolog family member A (*RhoA*) (Berenjeno et al., 2007). TBT-induced expression is oppositely correlated with the results in these cells, which is consistent with existing literature showing that *RhoA* is an anti-adipogenic, pro-osteogenic regulator of MSC lineage commitment (McBeath et al., 2004).

RXR activation alters genome-wide H3K27me3 in committing MSCs to the adipose lineage

TBT and 4204-induced changes in gene expression are similar to what was observed with a knockdown of the repressive histone modifier *Enhancer of zeste 2* (*Ezh2*) in PC3 cells (*nuytten_ezh2_targets_up* and *nuytten_ezh2_targets_dn*; Fig S1.4C-D, number 3) (Nuytten et al., 2008). This suggested to us that RXR activation inhibits EZH2 expression thereby de-repressing critical adipogenic genes. EZH2 is the catalytic member of the Polycomb repressive complex 2 (PRC2) which deposits repressive H3K27me3 (histone 3, lysine 27 trimethylation) marks on chromatin. We queried our RNA-Seq data set for all histone modifying enzymes whose expression was altered by TBT and noted altered expression of several genes known to modify H3K27 including *Ezh1*, *Ezh2*, the H3K27me3 demethylase *Kdm6b* (*Lysine demethylase 6b*), as well as several acetyltransferases (Fig S1.5A). This led us to hypothesize that TBT acts through RXR to remodel the repressive chromatin landscape and promote adipose lineage commitment. Pretreatment of MSCs with the EZH2 inhibitor 3-deazaneplanocin (DZNep) mildly increased subsequent lipid accumulation (Fig S1.5B). QPCR of MSCs at Day 0 revealed that DZNep up-regulated important adipogenic genes including *Ebf1*, *Ebf2*, *Pparg1*, and *Pparg2* (Fig S1.5C-D). The RXR antagonist HX531 inhibited up-regulation of pro-adipogenic transcripts by DZNep, particularly genes that are previously characterized (*Pparg*, *Fabp4*) or suspected (*Ebf2*, *Irx3*) to be direct targets of RXR (Fig S1.5C-E). Other transcripts up-regulated by DZNep were only mildly repressed by HX531 (*Ebf1*, *Foxo1*, *Klf4*), indicating that these may be indirect targets of RXR regulated via EZH2 (Fig S1.5D-E). Notably, *Zfp423* was down-regulated by DZNep

treatment, suggesting that not all adipogenic targets of RXR are regulated through EZH2 (Fig S1.5D).

We performed ChIP-Seq analyses of H3K27me3 and H3K4me3 on Day 0 MSCs to identify potential genome-wide consequences of RXR activation on chromatin state. Thousands of sharp H3K4me3 peaks were detected, primarily in promoters and gene bodies (Fig S1.6A-C). Broad H3K27me3 peaks, or 'islands', were found near genes and at distal intergenic sites in equal proportion (Fig S1.6A-C). Thousands fewer H3K27me3 islands were detected in TBT-treated MSCs as compared to DMSO (Fig S1.6A), and TBT reduced the presence of islands near promoters (Fig. S6B, D). Differential binding analysis of peaks/islands showed a distinct, genome-wide change in H3K27me3 upon RXR activation as assessed by correlation clustering (Fig 1.6B) and principal component analysis (Fig 1.6C).

TBT-induced chromatin alterations were more striking for H3K27me3 than H3K4me3 (Fig 1.6A-B). To quantitate the effect of TBT on genome-wide H3K27me3, we assessed the probability density of normalized read counts within differential islands (Fig 1.6D). Overall, TBT significantly reduced H3K27me3 within differential islands, primarily due to an enrichment of weak H3K27me3 signal ($< 5.5 \log_2$ normalized reads) and confirmed to be significant by Wilcoxon-Mann-Whitney test (Fig 1.6D). These data agree with TBT-induced expression changes of *Ezh2* and *Kdm6b* (Fig S1.5A).

Activation of RXR resulted in thousands of differential islands with altered ChIP signal (Fig S1.7A, Table S3). Given the overall decrease in H3K27me3 upon TBT treatment, we were particularly interested in regions with decreased signal, since these sites are susceptible to histone marks that control enhancer activity (Calo and Wysocka, 2013; Ferrari et al., 2014). Distribution of differential islands mirrored genome-wide patterns (Fig S1.7B, Fig S1.6B), although decreased islands were overrepresented in distal intergenic regions, suggesting a role at distal enhancers (Fig S1.7B). Differential H3K27me3 islands were also found within the

promoters/gene bodies of differentially expressed genes (DEGs) from our RNA-Seq data, many of which have established roles in chromatin modification, cell lineage specification, and adipogenesis (Fig S1.7C). Since most H3K27me3 islands map to distal sites, we annotated all H3K27me3 differential islands to 4701 neighboring genes (<1 Mb) (Fig S1.7A). We focused on up-regulated DEGs annotated to decreased differential islands and found many regulators of adipogenesis, including, *Pparg* and *Cebpa* (Fig S1.7A). GO term analysis of all genes annotated to decreased H3K27me3 islands uncovered several terms concerning development (Fig 1.7A, Table S4). Genes driving these enrichments included known transcriptional regulators of adipogenesis (*Pparg*, *Cebpa*, *Klf4*, *Foxo1*, and *Irx3*), several of which were up-regulated DEGs at Day 0 (Fig 1.7A). Pathway analysis revealed an enrichment of genes from preexisting studies of PRC2 in varied biological systems (Fig 1.7B, Table S4). These gene sets included transcripts that control early adipogenic signals (Fig 1.7B), many of which were upregulated by the EZH2 inhibitor DZNep (Fig S1.5E). Taken together, these data indicate that liganded RXR decreases genome-wide H3K27me3 in proximity to genes that regulate adipose commitment.

Discussion

Adipogenesis in humans and mice begins *in utero* and continues during the postnatal period (Poissonnet et al., 1983; Tang et al., 2008; Wang et al., 2013). Studies in genetic mouse models suggest two distinct adipose progenitor populations *in vivo*: one that develops the fat organ and another of mural origin that maintains adipose turnover in adults from the perivascular niche (Hong et al., 2015; Jiang et al., 2014; Wang et al., 2015). Both of these progenitors are committed *in utero* (Jiang et al., 2014). Therefore, the prenatal period represents a critical window during which environmental insults can perturb adipose lineage commitment resulting in potential lasting effects on adiposity in the adult.

Tributyltin is a potent obesogen in rodents exposed prenatally or as adults via activation of the nuclear receptors PPAR γ and RXR (Bertuloso et al., 2015; Grun et al., 2006; Kirchner et al., 2010; Zuo et al., 2011). The obesogenic effects of prenatal TBT exposure are propagated transgenerationally to unexposed generations, presumably via epigenetic modifications of the germline (Chamorro-Garcia and Blumberg, 2014; Chamorro-Garcia et al., 2017; Chamorro-Garcia et al., 2013). Undifferentiated MSCs from mice prenatally exposed to TBT have a pro-adipogenic, anti-osteogenic gene expression profile (Chamorro-Garcia et al., 2013; Kirchner et al., 2010), which led us to investigate how TBT influences early cell fate decisions in MSCs.

Current understanding of adipose lineage commitment lags behind that of terminal differentiation (Rosen and Spiegelman, 2014). This is in part due to the popularity of the 3T3-L1 cell line (which is a committed pre-adipocyte line) and an emphasis on the master regulator PPAR γ , despite that induction of PPAR γ 2 expression occurs after lineage commitment (Cristancho and Lazar, 2011; Rosen and Spiegelman, 2014). *In vitro* models of commitment have been limited because the components of adipose induction cocktails induce both commitment and differentiation of MSCs, making it difficult to decipher when each phase begins and ends. To circumvent these issues, we developed a commitment assay using primary bone marrow-derived MSCs and used it to show that a 2-day pretreatment with TBT or the rexinoid IRX4204 was as potent inducing adipogenesis as a 14-day co-treatment with induction cocktail (the standard assay) (Fig 1.1). We went on to show that adipogenic commitment is RXR-dependent (Fig 1.2 & 3), revealing for the first time that RXR activation is sufficient for the adipogenic commitment of MSCs. Furthermore, we show that TBT reduces and redistributes genome-wide H3K27me3 in programming adipose lineage bias (Fig 1.6 & 7). Hence, we have provided an *in vitro* model of an environmental exposure altering the epigenome of a stem cell compartment to perturb subsequent development. Studying this phenomenon mechanistically *in*

vitro may shed light on how *in utero* exposures can program disease risk in adulthood (Hanson and Gluckman, 2014).

RXR is a unique member of the nuclear receptor superfamily because it heterodimerizes with many other receptors (Evans and Mangelsdorf, 2014). We therefore investigated whether TBT acts as a homodimer or in concert with permissive RXR heterodimers in committing MSCs to an adipose fate. Chemical inhibition of PPAR γ in MSCs only partially blocks induction of its canonical target gene, *Fabp4*, by rexinoids (Fig 1.2B). We previously showed, however, that the PPAR γ /RXR heterodimer remains permissive in the presence of a PPAR γ antagonist (Li et al., 2011). Lentiviral knockdown of PPAR γ ablates induction of *Fabp4* by RXR agonists (Fig 1.3C), suggesting that liganded RXR must act as a PPAR γ /RXR heterodimer at *Fabp4* and likely other loci. Interestingly, *Pparg* knockdown slightly attenuated rexinoid-induced upregulation of *Abca1* and *Zfp423* (Fig 1.3C-D). Thus, while these transcripts are predominantly regulated by other permissive partners or RXR homodimers, they obtain some activation from PPAR γ /RXR heterodimers in the presence of RXR activators. We also noted several discrepancies between chemical inhibition of RXR with HX531 (Fig 1.2) and lentiviral knockdown of RXR α (Fig 1.3). For example, HX531 strongly inhibited *Pparg2*, but not *Zfp423* expression (Fig 1.2C); the opposite trends were observed in lentiviral knockdown of RXR α (Fig 1.3A, 3D). These differences may be due to compensation by RXR β when RXR α is knocked down (RXR γ is not expressed in MSCs), or to RXR-mediated recruitment of corepressors that can take place during chemical inhibition, but not when the receptor is knocked down.

Pretreatment of MSCs with individual permissive heterodimer agonists failed to commit MSCs to an adipose fate (Fig 1.4B). However, combining PPAR δ and PPAR γ agonists at micromolar doses successfully reproduced the effect of nanomolar rexinoid pretreatment, more effectively than other combinations of PPAR and LXR agonists (Fig 1.4C). In 3T3-L1 cells, permissive PPAR δ /RXR heterodimers occupy key adipogenic sites early in differentiation, days

before PPAR γ /RXR is detected (Nielsen et al., 2008). Hence, RXR may preferentially bind PPAR δ and PPAR γ gene targets in committing MSCs to the adipose fate. The ability of RXR to recruit and target multiple partners from the nuclear receptor pool likely contributes to the unique ability of rexinoids to alter MSC fate.

Transcriptomal profiling of MSCs exposed to TBT identified a distinct RXR-dependent transcriptional program (Fig 1.5, S2A, Table S1). Gene ontology (GO) analysis of transcripts up-regulated by TBT revealed genes enriched for terms associated with extracellular matrix (ECM) proteins, which are known to play a role in MSC commitment (Cristancho and Lazar, 2011), and lipid metabolism genes, which include many well-known regulators of adipogenesis and adipocyte function [(*Pparg*, *Cebpa*, *Sterol regulatory element-binding protein 1 (Srebf1)*, *Cluster of differentiation 36 (Cd36)*, *Lipase E (Lipe*, a.k.a. Hormone-sensitive lipase)] (Fig S1.4A, Table S2). Transcripts down-regulated by TBT and 4204 were enriched for cell cycle-related terms, indicating that these cells are exiting the cell cycle as they become committed preadipocytes (Fig S1.4B, Table S2).

We compared TBT-induced expression changes with previously published datasets characterizing the transcriptome of committed preadipocytes *in vivo* (Fig S1.3). Graff and colleagues used genetic reporter mice to show that adipose expansion in the early postnatal period arises from PPAR γ -expressing progenitors that are committed prenatally and reside, in part, within the perivascular niche (Tang et al., 2008). Not surprisingly, PPAR γ + SVF and TBT or rexinoid-treated MSCs both show up-regulation of PPAR γ target genes, such as *Angptl4*, *Nr1h3* (LXR α), *Cebpa*, and *Pyruvate carboxylase (Pcx)* (Fig S1.3C). This suggests that the ability of liganded RXR to activate PPAR γ target genes is central to RXR-induced adipose commitment of MSCs. Spiegelman and colleagues identified *Zfp423* as a regulator of adipose commitment upstream of PPAR γ (Gupta et al., 2010), and subsequently used reporter mice to show that *Zfp423* marks committed preadipocytes *in vivo*, also within the perivascular niche

(Gupta et al., 2012). Two of the most enriched genes in Zfp423+ SVF are *Ebf1* and *Ebf2*, both of which are up-regulated by TBT and 4204 (Fig S1.3B). *Ebf1* or *Ebf2* overexpression in uncommitted NIH 3T3 fibroblasts promotes adipogenesis upstream of PPAR γ , whereas knockdown of either gene inhibits adipogenesis (Jimenez et al., 2007). Therefore, we infer that *Ebf1* and *Ebf2* are critical regulators of RXR-induced lineage commitment of MSCs, acting upstream of PPAR γ . Genes significantly up-regulated in both *in vivo* studies and in our TBT-treated MSCs included the master regulator of adipose differentiation *Pparg* and the pericyte/preadipocyte marker *Platelet derived growth factor receptor β* (*Pdgfrb*) (Fig S1.3A) (Vishvanath et al., 2016). Down-regulated transcripts included two of the top 5 genes repressed by TBT, *Angiopoietin-like 7* (*Angptl7*) and *Argininosuccinate synthase 1* (*Ass1*) (Fig S1.3A), whose functions in adipogenesis are unknown. Taken together, these data show that RXR activation induces gene expression changes in MSCs that define the preadipocyte *in vivo*.

Previous work in bone marrow-derived macrophages showed that RXR ligands do not alter the genomic distribution of RXR, but rather increase recruitment of transcriptional activators and histone modifiers to pre-established RXR binding sites (Daniel et al., 2014). Our pathway analysis of TBT-regulated genes suggested an RXR-dependent inhibition of the repressive histone modifier EZH2 (Fig S1.4C-D). Chemical inhibition of EZH2 increased expression of several adipogenic regulators such as *Ebf1*, *Ebf2*, *Pparg1*, and *Pparg2*, but not others such as *Zfp423* and *Cebpa* (Fig S1.5C-D). These conflicting changes in gene expression may explain the mild overall effect of DZNep on lipid accumulation (Fig S1.5B), and suggests that not all adipogenic targets of RXR are regulated through EZH2. Notably, RXR activation also diminishes expression of the H3K9me3 methyltransferase *Suppression of variegation 3-9 homolog 2* (*Suv39h2*). H3K9me3 has previously been shown to regulate expression of *Pparg* and *Cebpa* at their promoters in 3T3-L1 cells (Matsumura et al., 2015).

EZH2 was previously implicated as a positive regulator of adipogenesis and an inhibitor of osteogenesis through regulation of H3K27me3 at the promoters of WNT genes (Hemming et al., 2014; Wang et al., 2010; Wei et al., 2011; Yi et al., 2016). In contrast to our work, these studies were carried out either in preadipocytes or in MSCs following treatment with adipogenic or osteogenic induction cocktails and did not assess the genome-wide chromatin state in undifferentiated MSCs, where EZH2 appears to have a distinct role in lineage allocation. Our analysis of H3K27me3 revealed a striking, RXR-induced redistribution and reduction of this repressive mark (Fig 1.6B-D, Fig S1.6D, Table S3). Pathway analysis of genes in proximity to decreased H3K27me3 islands revealed an enrichment of gene sets from previous studies of PRC2, suggesting that liganded RXR alters H3K27me3 near bona fide targets of PRC2 (Fig 1.7B). These genes included *Insulin growth factor 1 (Igf1)*, *Iriquois homeobox 3 (Irx3)*, *Kruppel-like factor 4 (Klf4)*, and *Lysyl oxidase (Lox)* all of which have been implicated as positive regulators of early adipogenesis (Birsoy et al., 2008; Claussnitzer et al., 2015; Hu et al., 2015; Huang et al., 2009). Importantly, both *Irx3* and *Klf4* were up-regulated by the EZH2 inhibitor DZNep (Fig S1.5E). Future studies will attempt to decipher which genes are direct targets of RXR and which are regulated through EZH2. In addition, we aim to confirm RXR-dependent changes in H3K27me3 near critical adipogenic regulators (*Pparg*, *Ebf1*, *Ebf2*, *Irx3*, *Klf4*) by ChIP-Seq in the presence and absence of 4204 and HX531.

In human and mouse embryonic stem (ES) cells, H3K27me3 marks poised distal enhancers and has a well-studied reciprocal relationship with H3K27Ac, a mark of active enhancers (Calo and Wysocka, 2013). A recent study of chromatin dynamics during osteogenic differentiation confirmed the presence of these marks at distal enhancers in mouse bone marrow-derived MSCs, and described their importance during osteogenesis (Wu et al., 2017). Furthermore, the EZH2-mediated divalent H3K27me2 mark has now been implicated in distal enhancer regulation and lineage specification of ES cells (Ferrari et al., 2014; Juan et al., 2016).

Hence, RXR-regulated demethylation of H3K27 may leave differential sites susceptible to modifications that interfere with enhancer activity in undifferentiated MSCs. In macrophages, RXR is known to influence gene expression through long-range enhancers (Daniel et al., 2014). Therefore, RXR-initiated genome-wide changes in H3K27me3 may reflect a broader restructuring of the nuclear architecture that influences subsequent development (Chamorro-Garcia et al., 2017; Guelen et al., 2008). Further studies into how EDCs can reorganize the chromatin of stem cell compartments should provide critical evidence in understanding how developmental exposures program disease risk later in life and propagate that risk to future generations.

Conservative estimates of the cost of endocrine disruption in the US and Europe are in the hundreds of billions of dollars annually, with a significant portion attributed to increases in obesity and diabetes (Attina et al., 2016; Legler et al., 2015; Trasande et al., 2015). Many xenobiotic chemicals are known to promote adipogenesis through nuclear receptors such as PPAR γ and Estrogen receptor (ER) (Heindel et al., 2017a; Heindel et al., 2017b). Few chemicals other than TBT have been definitively identified as RXR activators, with the fungicide fludioxonil (Janesick et al., 2016) and the nonionic surfactant Span 80 (Bowers et al., 2016) being two notable exceptions. Considering that our data reveal a novel role for RXR in MSC lineage specification, a concerted effort to determine whether there are other, bona fide chemical activators of RXR that can act as obesogens may be worthwhile. The ability of rexinoids or dual RXR-PPAR γ activators such as TBT, but not strong PPAR γ activators such as ROSI, to elicit transgenerational effects on fat accumulation (Chamorro-Garcia et al., 2013) underscores the potential importance of this effort. Within the EDC field, 3T3-L1 cells remain the most popular tool for screening and characterizing obesogens *in vitro*, despite their inability to model lineage commitment and long-standing issues with reproducibility across laboratories and vendors (Bastos Sales et al., 2013; Kassotis et al., 2017; Pereira-Fernandes et al., 2014; Taxvig

et al., 2012). Our commitment assay offers a new approach to identify chemicals that can affect lineage specification upstream of PPAR γ in a primary, multipotent stem cell model. Moreover, this approach offered new insights into the biology of MSC lineage commitment that would not have been apparent in a standard MSC assay.

RXR is known for its roles in adipogenic (Tontonoz et al., 1997) and myogenic (AISudais et al., 2016) terminal differentiation, in myogenic commitment of ES cells (Le May et al., 2011), and for its dramatic effects on the epigenome of macrophages (Daniel et al., 2014). The role of RXR in the lineage commitment of MSCs is novel, and we found that RXR activation in MSCs produces genome-wide reduction and redistribution of H3K27me3 marks to promote adipose lineage commitment. These data identify RXR as an important interface between the environment and the epigenome that can influence the developmental programming of obesity. RXR is widely expressed *in utero* and has multiple roles in the development of tissues such as the eye, brain, heart, and, importantly, the placenta and gametes (Froment, 2008; Mark et al., 2009). This raises concerns that RXR activators may disrupt the development of multiple organ systems, and raises the stakes for effectively identifying which chemicals in production can activate RXR *in vitro* and *in vivo*. In particular, the investigation of developmental RXR disruption in the placenta, gametes, and the soma that support gametogenesis may prove critical in understanding how EDCs such as tributyltin can propagate disease risk over multiple generations.

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FIGURE 1.1

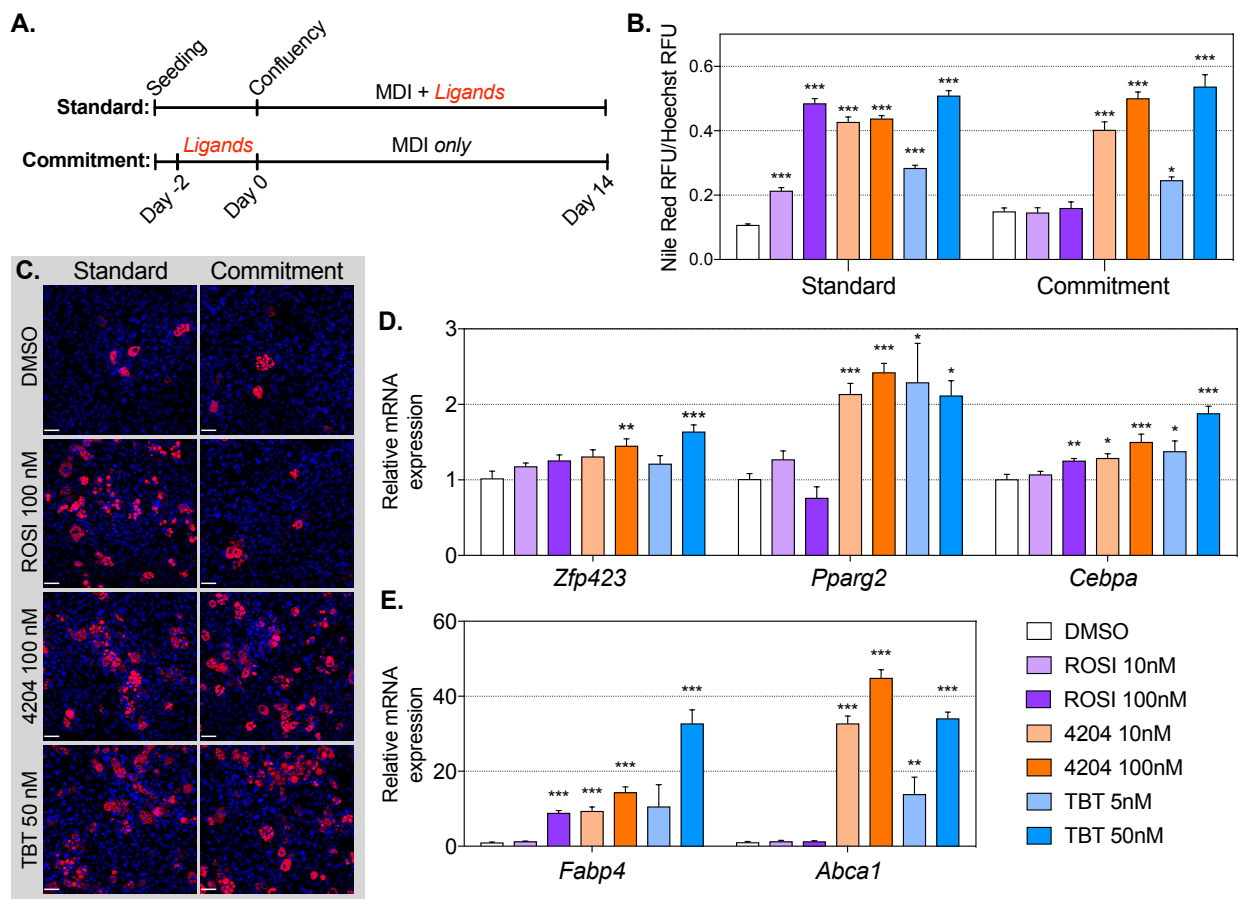


Figure 1.1. MSCs pretreated with TBT and RXR agonist, but not PPAR γ agonist, accumulate more lipid during subsequent adipose differentiation. Mouse bone marrow-derived MSCs were pretreated with vehicle control (0.05% DMSO), ROSI (10, 100 nM), 4204 (10, 100 nM), or TBT (5, 50 nM) for 48 hrs prior to induction with an adipose induction cocktail (MDI) for 2 weeks. (A) This commitment assay was run in parallel with a standard 2-week adipogenesis assay whereby chemical ligands were added in conjunction with MDI. (B) After 14 days of adipose induction, MSCs were fixed and then stained with Nile Red and Hoechst 33342 for lipid and nucleic acid content, respectively. Nile Red fluorescence (RFU) was normalized to Hoechst. (C) Cells were imaged by confocal fluorescence microscopy. Scale bar = 55 μ m. (D) Expression of early adipose lineage markers was assessed at Day 0 by QPCR. (E) Expression of canonical PPAR γ and RXR targets was also analyzed by QPCR at Day 0. Data are represented as mean $\pm$ SEM. 1-way ANOVA, Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 1.2

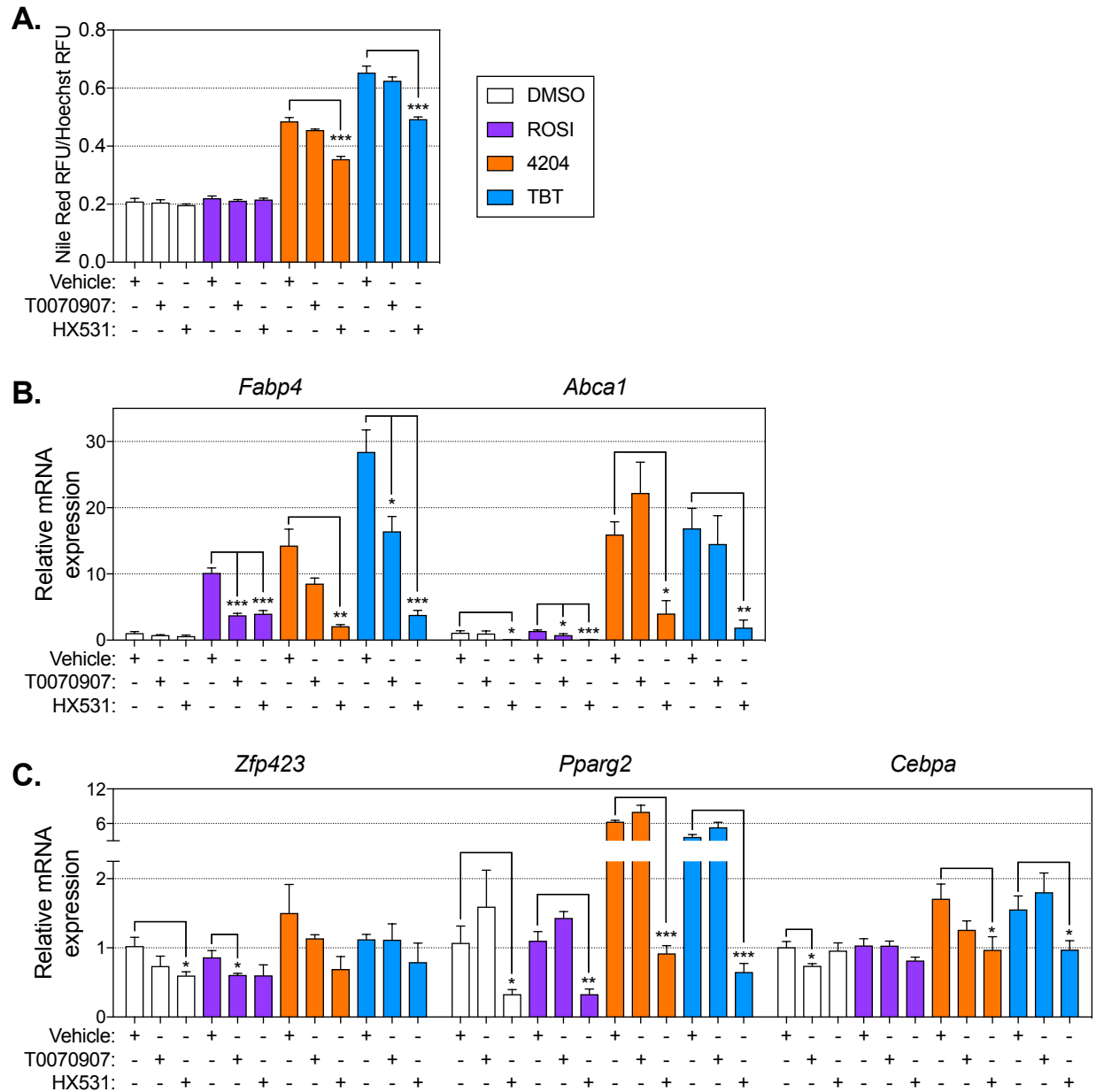


Figure 1.2. Pharmacologic inhibition of RXR, but not PPAR γ , abrogates the effect of TBT pretreatment. MSCs were pretreated for 48 hours with vehicle control (DMSO), ROSI (100 nM), 4204 (100 nM), or TBT (50 nM) in the presence of the PPAR γ antagonist T0070907 (100 nM) or the RXR antagonist HX531 (10 μ M). (A) Following pretreatment with, cells were induced to differentiate with MDI for 14 days and analyzed for lipid accumulation. (B, C) RNA was collected following pretreatment (Day 0) for analysis of gene expression by QPCR. Data are represented as mean $\pm$ SEM. Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 1.3

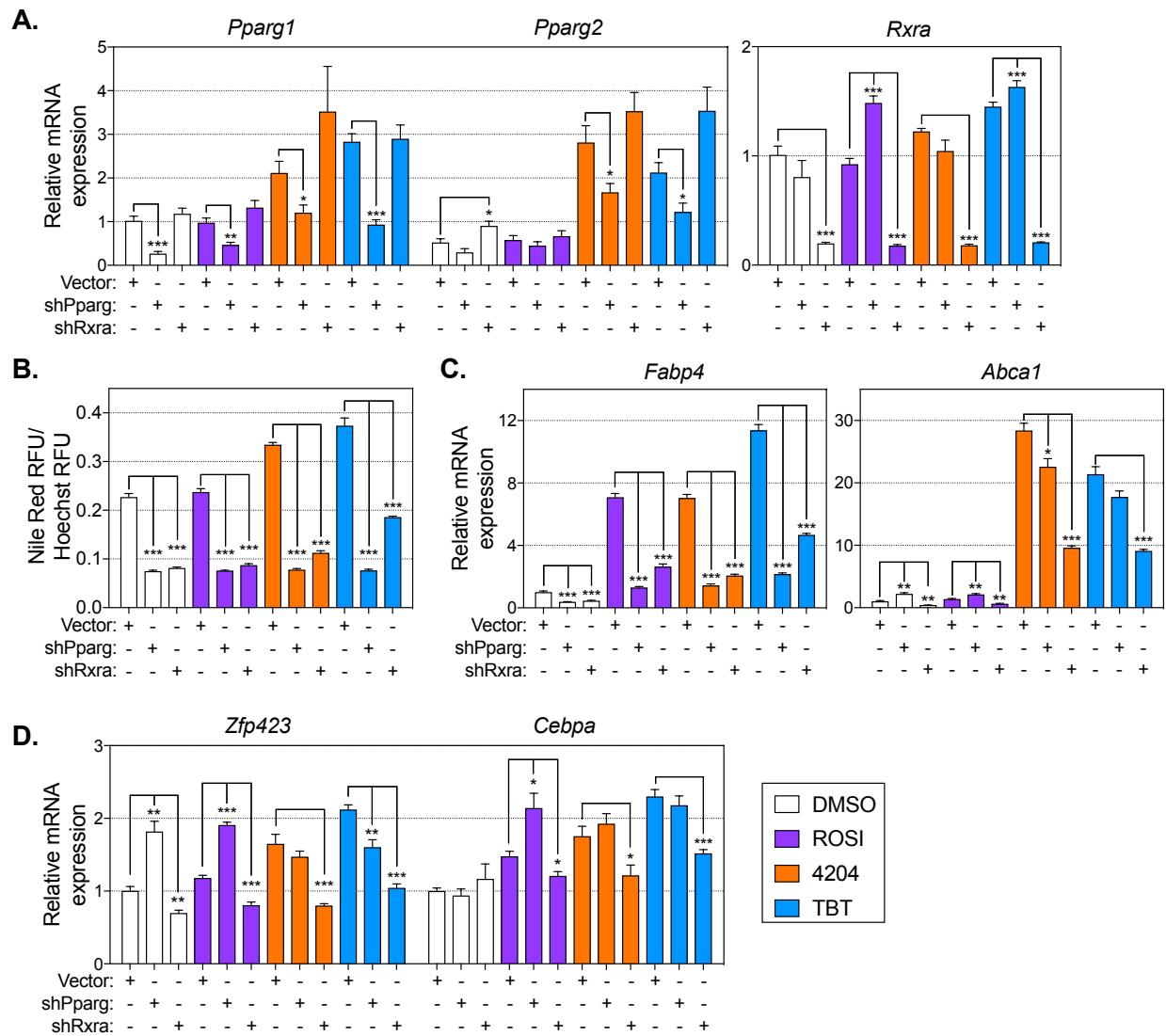


Figure 1.3. Lentiviral knockdown of RXRα diminishes expression of adipogenic transcriptional targets of TBT and 4204. MSCs were transduced with lentivirus carrying the pLKO.1 vector with and without shRNA against *Pparg* or *Rxra* transcript. Stable lines were treated with vehicle control (DMSO), ROSI (100 nM), 4204 (100 nM), or TBT (50 nM) for 48 hours and then differentiated into adipocytes with an adipogenic induction cocktail (MDI). (A) QPCR of *Pparg* and *Rxra* transcripts at Day 0. (B) Lipid accumulation after 2 weeks of adipose induction. (C, D) QPCR analysis of adipogenic gene expression at Day 0. Data are represented as mean $\pm$ SEM. Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[illegible]

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FIGURE 1.5

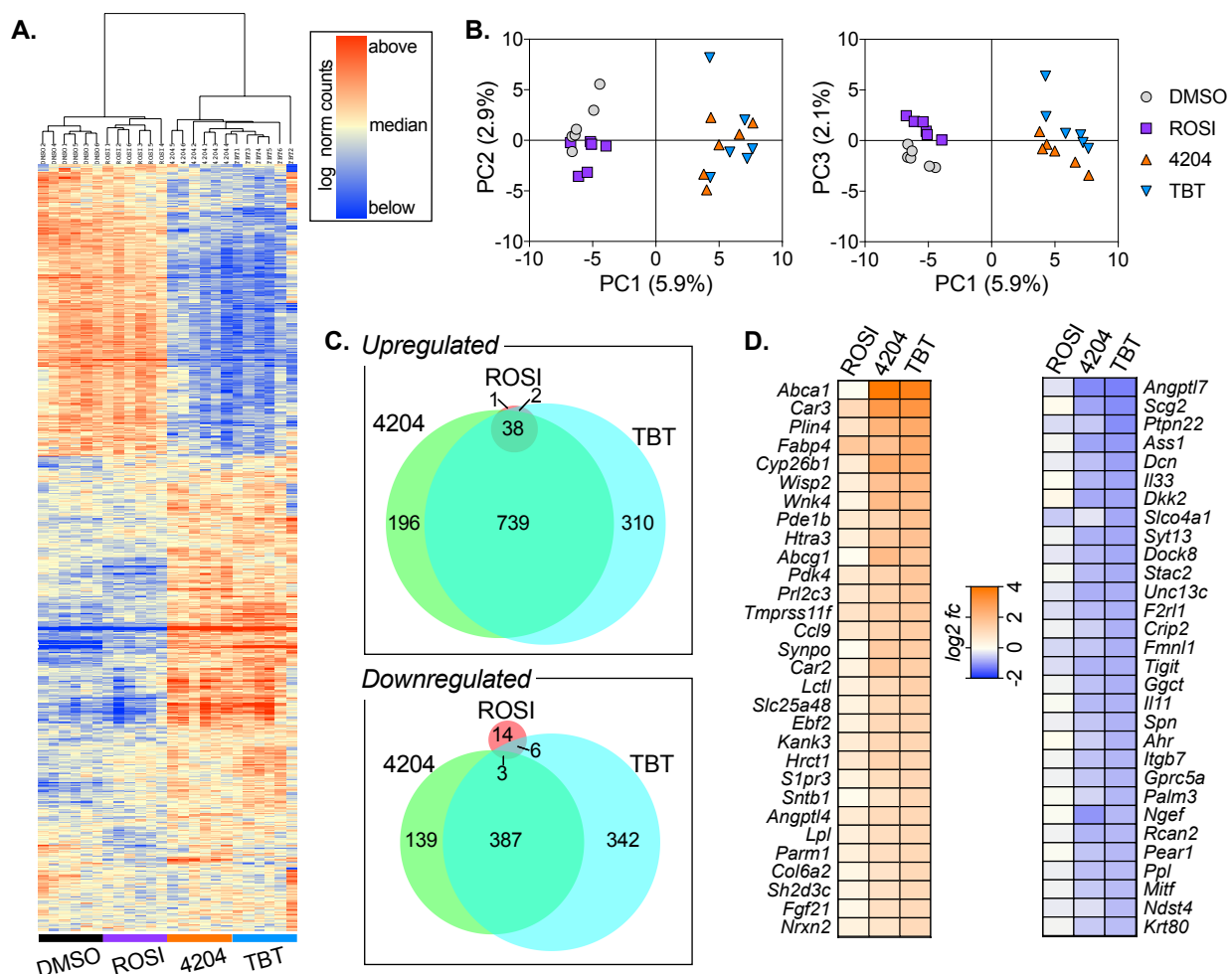


Figure 1.5. Transcriptomal profiling of MSCs reveals a distinct RXR-dependent transcriptional program in cells treated with 4204 or TBT. RNA from MSCs pretreated for 48 hours with vehicle control, ROSI (100 nM), 4204 (100 nM), or TBT (50 nM) was reverse transcribed, subjected to library preparation, and sequenced. Reads were aligned to the mouse genome (mm10, STAR aligner) and transcript abundance was analyzed (DESeq2, Bioconductor). (A) Unbiased hierarchical cluster analysis of normalized counts (regularized logarithm transformation, DESeq2). (B) Differential expression relative to vehicle control (DMSO) was analyzed for each chemical treatment; filtered gene lists (Benjamini-Hochberg adjusted p-value <0.01) were queried for overlap of up-regulated (log₂ fold change >0.2) and down-regulated (log₂ fold change <-0.2) genes. (C) Ranked list of the top 30 genes up-regulated (left) and down-regulated (right) by TBT with corresponding expression change for 4204 and ROSI. (D) QPCR validation of novel targets of TBT in undifferentiated MSCs. Data are represented as mean ± SEM. 1-way ANOVA, Dunnett's test, * p < 0.05, ** p < 0.01, *** p < 0.001.

FIGURE 1.6

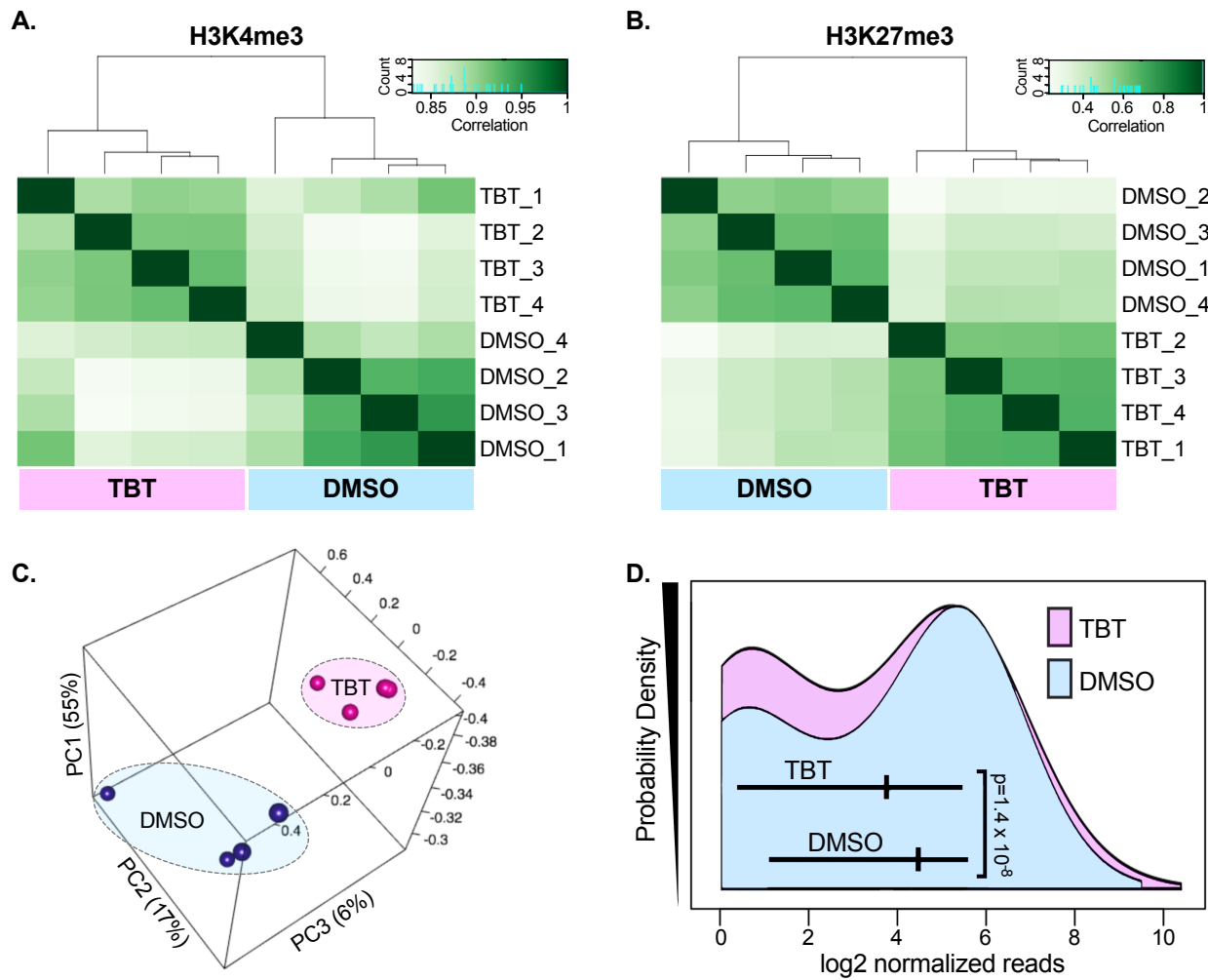


Figure 1.6. RXR activation in undifferentiated MSCs results in genome-wide alterations of H3K27me3. Chromatin from MSCs pretreated with vehicle control (DMSO) or TBT (50 nM) was immunoprecipitated for the histone marks H3K4me3 and H3K27me3 and subjected to library preparation and sequencing. Reads were aligned to the mouse genome (mm10), peaks/islands were called in SICER, and differential binding was assessed using DiffBind (R/Bioconductor). (A-B) Correlation clustering of affinity scores within differential islands for (A) H3K4me3 and (B) H3K27me3. (C) Principal component analysis of H3K27me3 affinity scores within differential islands for each sample replicate. (D) Violin plots of normalized reads within H3K27me3 differential islands are shown for DMSO and TBT. p-value was calculated by two-sided Wilcoxon-Mann-Whitney test.

FIGURE 1.7

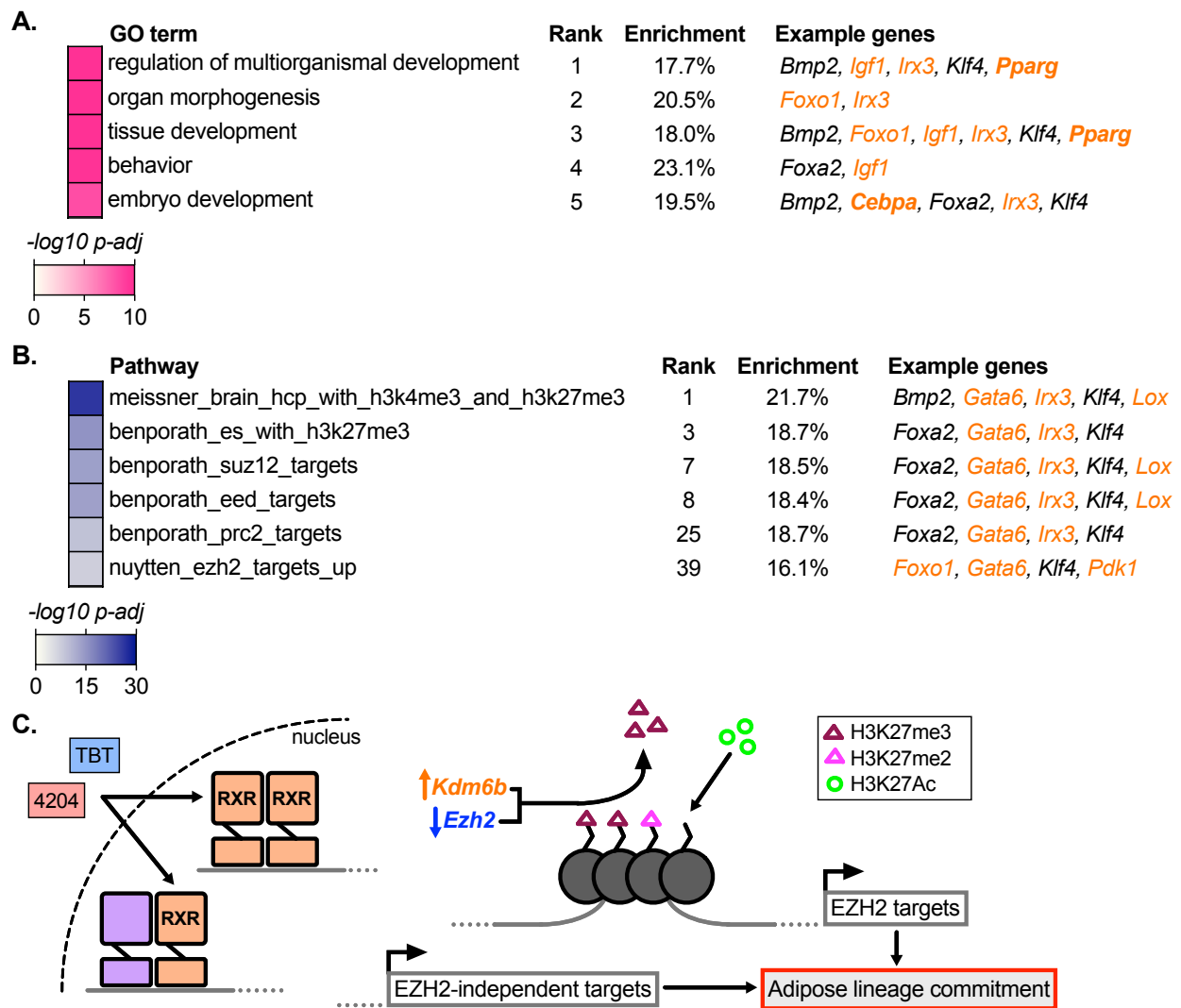
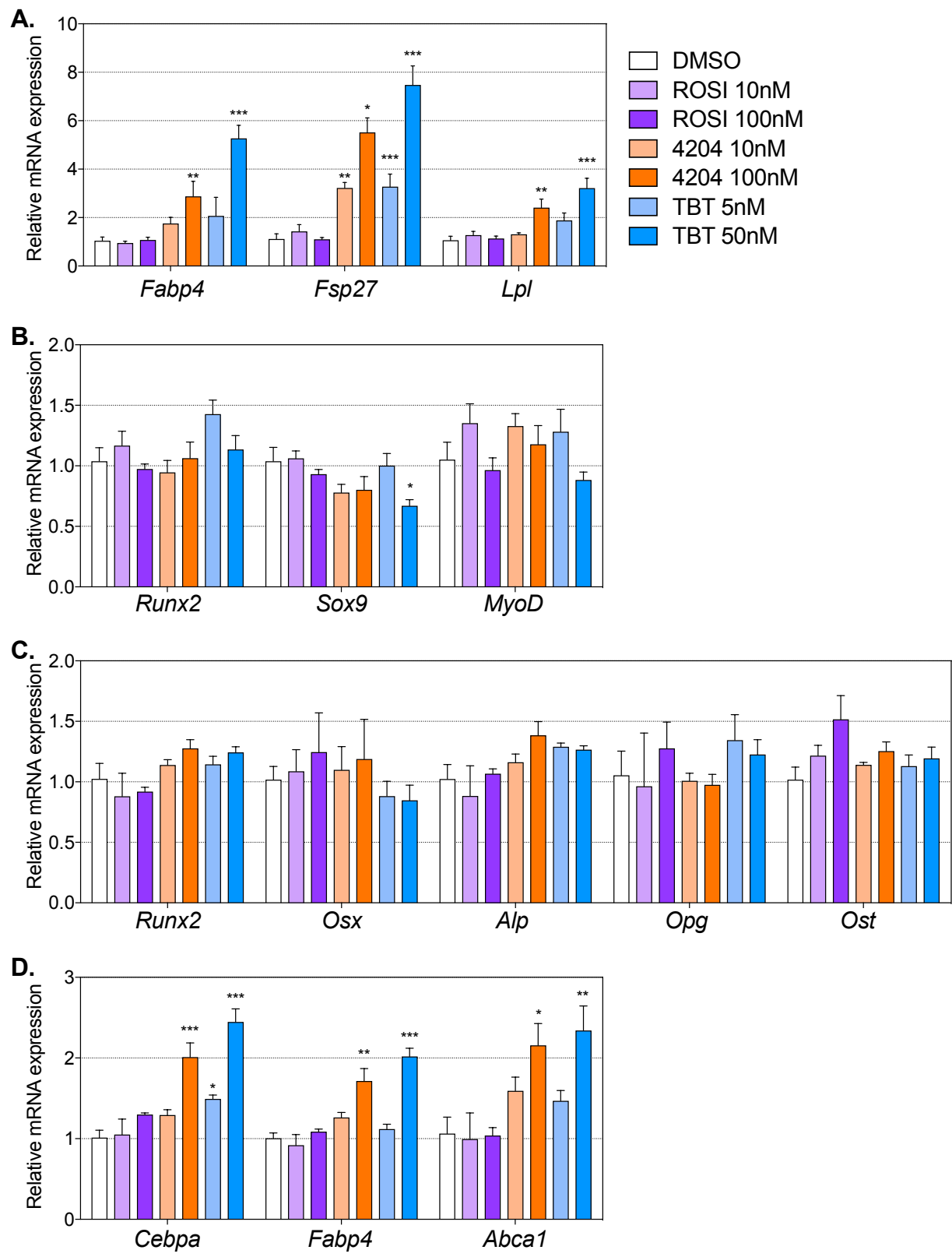


Figure 1.7. TBT alters H3K27me3 near transcriptional regulators that control adipogenesis. H3K27me3 differential islands were annotated to neighboring genes (< 1Mb) using GREAT. (A-B) Differential islands decreased by TBT treatment were annotated to 2803 genes. These genes were subjected to (A) GO term and (B) pathway analysis and ranked by Benjamini-Hochberg adjusted p-values. The fraction of annotated genes found in each GO term or pathway is displayed as a percentage. Examples of annotated genes are shown; genes that are up-regulated DEGs in our RNA-seq data set are shown in orange. (B) Pathways enriched in genes proximal to differential islands decreased upon TBT treatment that are from previous studies of the PRC2 complex. (C) Model of RXR-induced adipose lineage commitment of MSCs.

Figure S1.1. TBT does not attenuate osteogenic differentiation, but results in persistent expression of adipose lineage markers (Related to Figure 1.1). (A) Mouse MSCs were pretreated with vehicle control (DMSO), ROSI (10, 100 nM), 4204 (10, 100 nM), or TBT (5, 50 nM) and then differentiated for 2 weeks with adipose induction cocktail. RNA from mouse MSCs subjected to a commitment assay was collected at Day 14 and analyzed for markers of the adipose differentiation. (B-D) MSCs were pretreated for 48 hours with DMSO, ROSI (10, 100 nM), 4204 (10, 100 nM), or TBT (5, 50 nM) and then differentiated towards the bone lineage with an osteogenic induction cocktail. RNA was collected at Day 0 (B) and Day 14 (C-D) for gene expression analysis by QPCR. (B) RNA from Day 0 was analyzed for expression of genes involved in MSC lineage commitment. (C-D) Gene expression of osteogenic (C) and adipogenic (D) gene markers at Day 14. Data are represented as mean $\pm$ SEM. 1-way ANOVA, Dunnett's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SUPPLEMENTAL FIGURE S1.1



SUPPLEMENTAL FIGURE S1.2

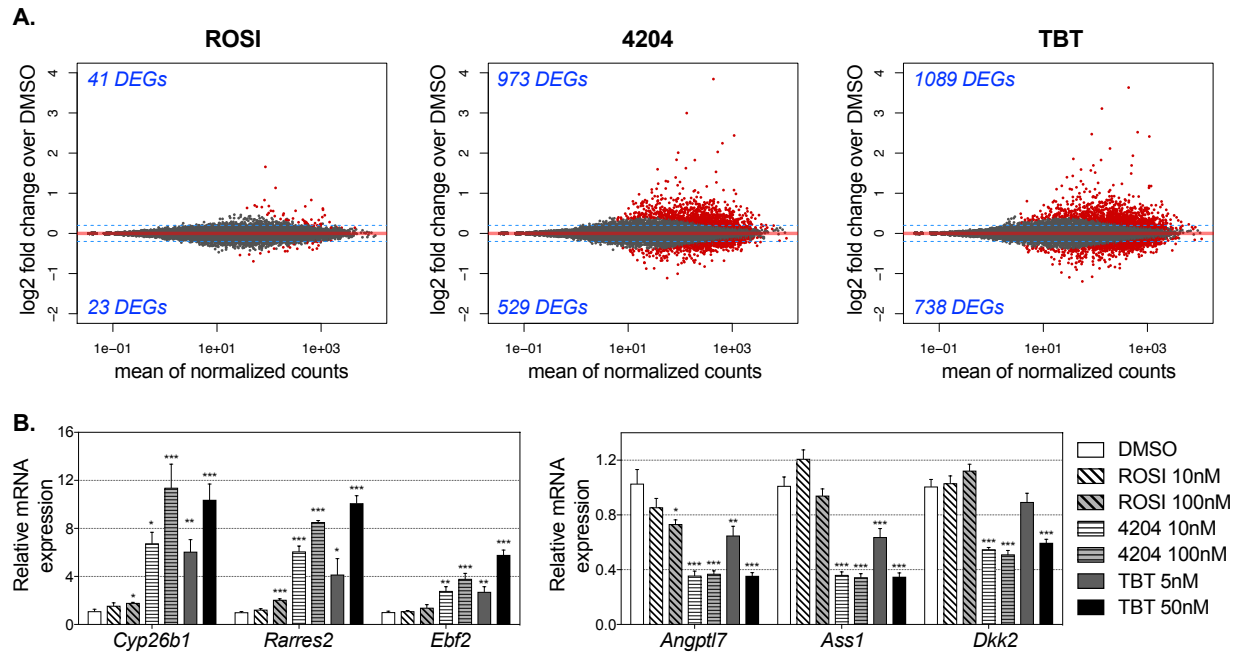


Figure S1.2. Genome-wide expression changes in undifferentiated MSCs exposed to TBT, 4204, ROSI, or DMSO control (Related to Figure 1.5). (A) MA-plots of expression changes for each treatment over vehicle control. The total number of up- and down-regulated differentially expressed genes (DEGs) (adjusted p-value <0.01, red dots; absolute value[log₂ fold change] >0.2, blue dotted lines) are indicated in blue text. (B) RNA from Day 0 pretreated MSCs was analyzed for expression of novel RXR target genes identified in RNA-Seq data. Data are represented as mean ± SEM. 1-way ANOVA, Dunnett's test, * P < 0.05, ** P < 0.01, *** P < 0.001.

SUPPLEMENTAL FIGURE S1.3

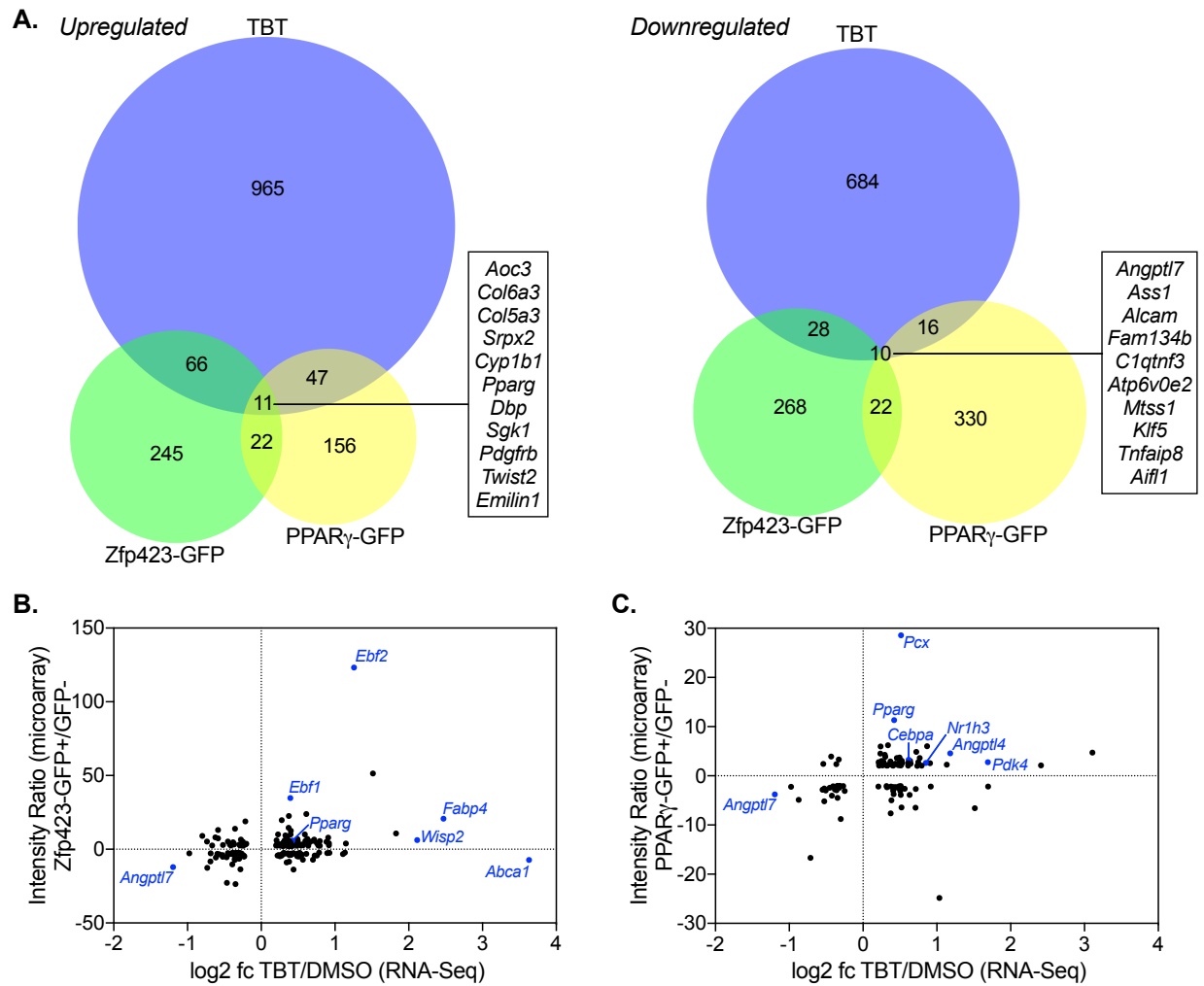


Figure S1.3. TBT targets genes that define the preadipocyte *in vivo* (Related to Figure 1.5). Genes differentially expressed in TBT-exposed MSCs were compared with previous microarray studies of adipose progenitors *in vivo*. (A) Genes up and downregulated by TBT were compared with the expression profile of Zfp423+ and PPAR γ + gonadal adipose tissue SVF. (B, C) Comparison of genes differentially expressed in TBT and (B) Zfp423+ or (C) PPAR γ + SVF.

Figure S1.4. Gene ontology and pathway analysis of TBT-exposed MSCs (Related to Figure 1.5). GO term and pathway analysis was conducted using the Molecular Signaling Database (MSigDB, Broad Institute) curated gene sets on genes altered by TBT, 4204, and ROSI. Differentially expressed genes were analyzed for enrichment in GO terms and pathways by hypergeometric test; enriched gene sets were ranked by adjusted p-value (Benjamini-Hochberg). (A, B) Top GO terms enriched in genes (A) upregulated and (B) downregulated by TBT, with corresponding 4204 and ROSI. (C, D) Top MSigDB pathways enriched in genes (C) upregulated and (D) downregulated by TBT, with corresponding 4204 and ROSI enrichment. 10 pathways with corresponding, oppositely regulated gene sets are numbered in red.

SUPPLEMENTAL FIGURE S1.4

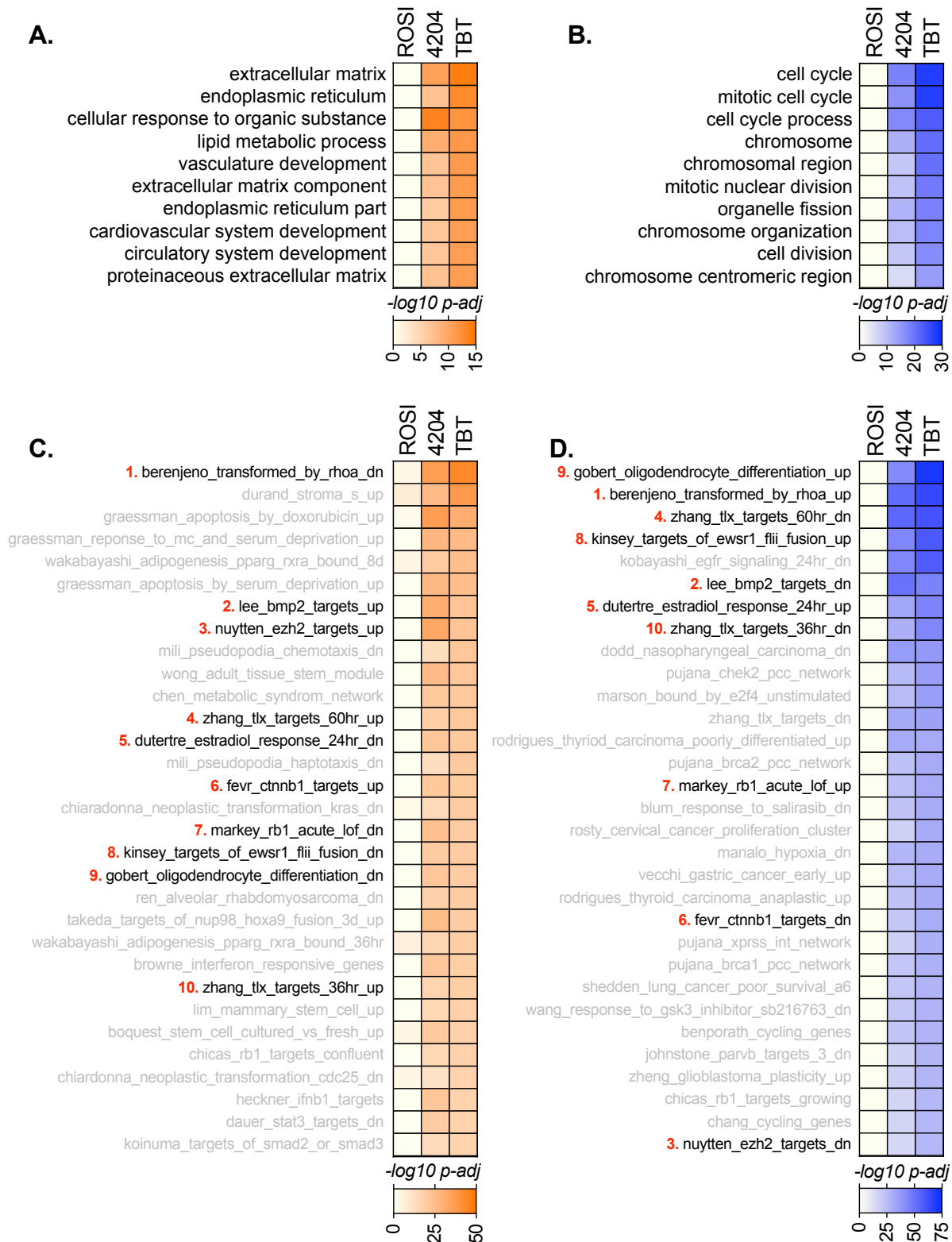


Figure S1.5. TBT acts through RXR to repress EZH2 and other histone modifying enzymes (Related to Figure 1.6). (A) All histone modifiers identified as DEGs by RNA-Seq. (B) MSCs were pretreated with vehicle control or TBT (50 nM) with and without the EZH2 inhibitor DZNep (1 μ M), then differentiated into adipocytes and stained for lipid accumulation. Data are represented as mean $\pm$ SEM. Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C-E) Day 0 gene expression following chemical pretreatment with vehicle control or TBT (50 nM), with or without the RXR inhibitor HX531 (10 μ M) and/or DZNep (1 μ M). Expression of (C-D) adipogenic markers and (E) genes in proximity to decreased H3K27me3 differential islands was assessed. For each DMSO and TBT group, 1-way ANOVA, Tukey's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SUPPLEMENTAL FIGURE S1.5

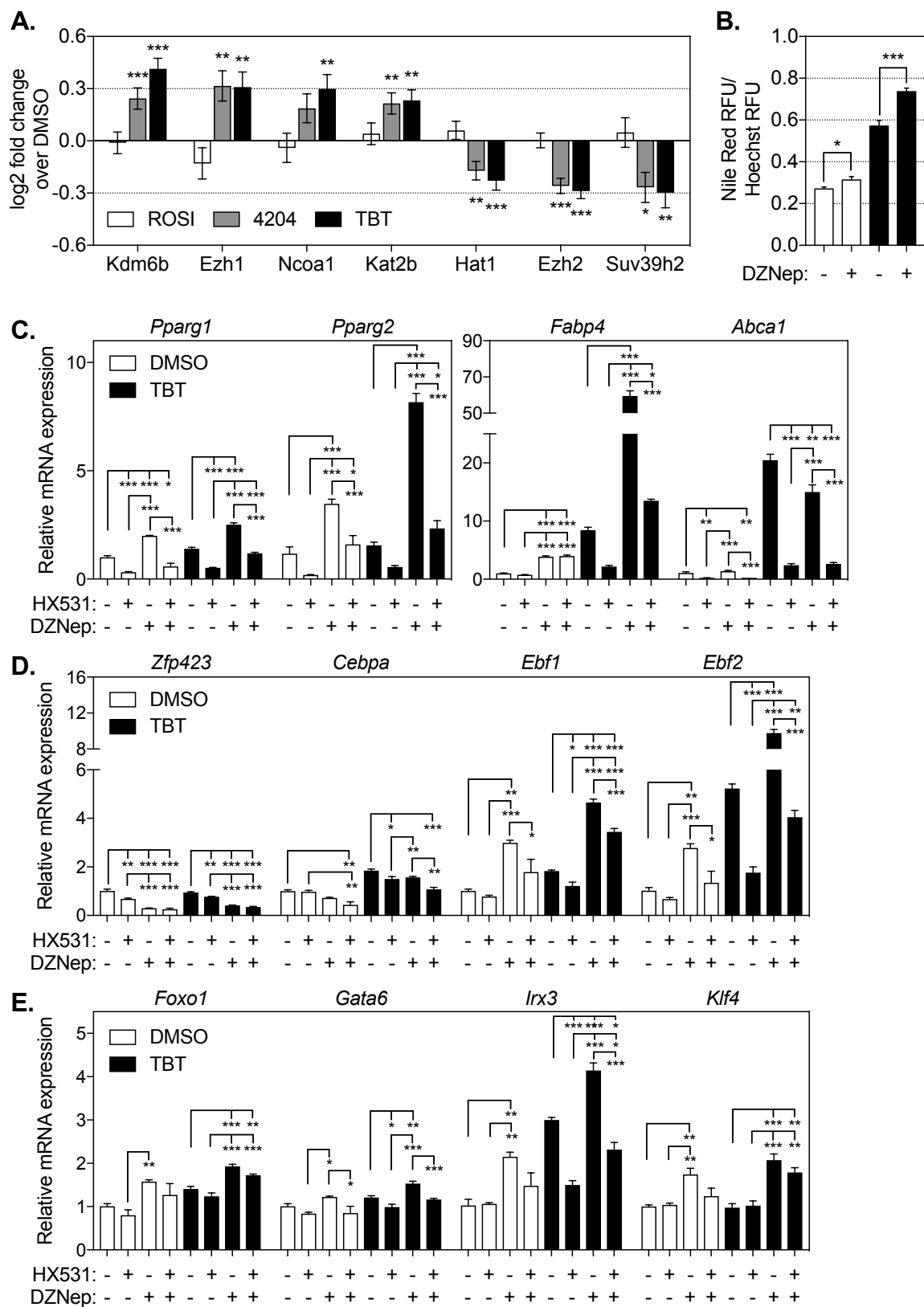
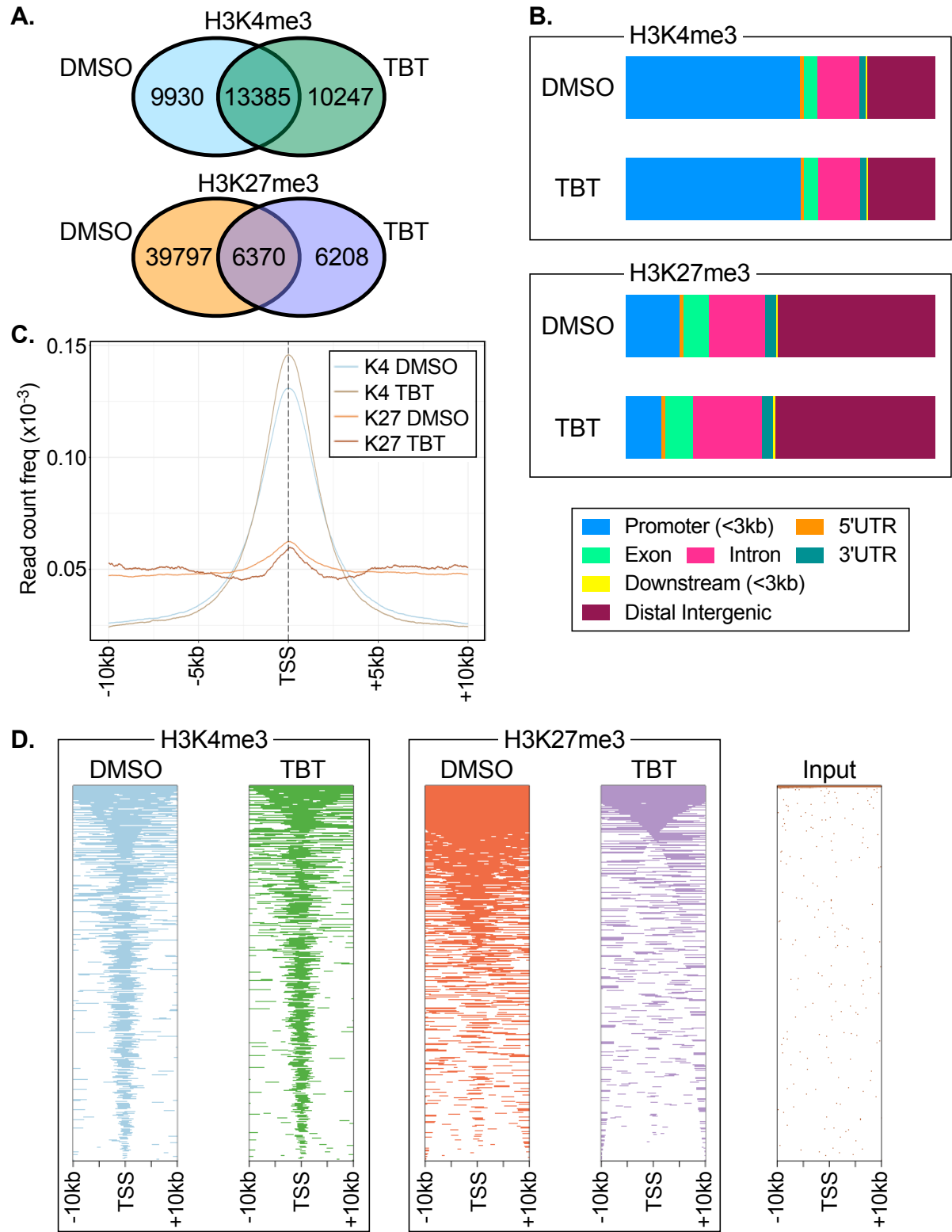


Figure S1.6. TBT the genome-wide distribution of H3K27me3 islands (Related to Figure 1.6). Chromatin from MSCs pretreated with vehicle control (DMSO) or TBT (50 nM) was immunoprecipitated for the histone marks H3K4me3 and H3K27me3 and subjected to library preparation and sequencing. Reads were aligned to the mouse genome (mm10), peaks/islands were called in SICER, and data was visualized in ChipSeeker (R/Bioconductor). (A) Overlaps of H3K4me3 and H3K27me3 peaks/islands from both treatment groups. (B) Genomic distribution, displayed as a percentage, of all H3K4me3 peaks and H3K27me3 islands. (C) Read count frequency of each histone mark and treatment 10 kb proximal and distal to the TSS. (D) Heatmap of ChIP signal for each histone mark, treatment, and input control around the TSS.

SUPPLEMENTAL FIGURE S1.6



SUPPLEMENTAL FIGURE S1.7

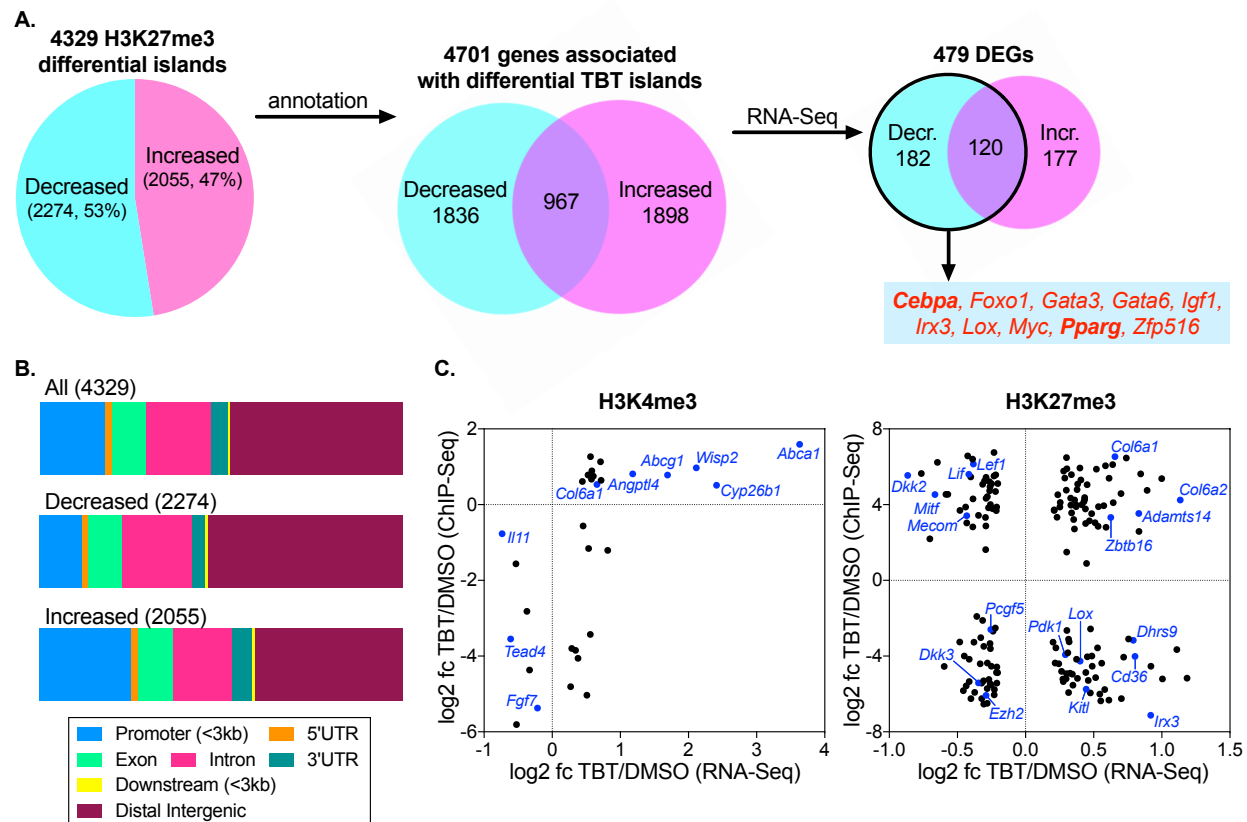


Figure S1.7. H3K27me3 differential islands are near differentially expressed genes that play critical roles in adipogenesis (Related to Figure 1.7). (A) All 4329 H3K27me3 differential islands were annotated to 4701 neighboring genes (< 1Mb) using GREAT. 479 of these genes were differentially expressed; up-regulated DEGs annotated by at least one decreased differential island are indicated in red. (B) Genome-wide distribution of all H3K27me3 differential islands, decreased islands, and increased islands. (C) DEGs with differential peaks/islands within promoters or gene bodies were identified. Gene-level correspondence between RNA expression and differential H3K4me3 (left panel) or H3K27me3 (right panel) ChIP signal.

SUPPLEMENTAL TABLE 1.1

Gene	Forward primer (5' > 3')	Reverse primer (5' > 3')
<i>36B4</i>	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGGCAGCAGTGGT
<i>Abca1</i>	GCTTGTTGGCCTCAGTTAAGG	GTAGCTCAGGCGTACAGAGAT
<i>Alp</i>	GGATAACGAGATGCCACCAG	TGAGATCCAGGCCATCTAGC
<i>Angptl7</i>	AACAACCAGATTGACATCATGC	CTCGTCAGGAGGAAGCTTGT
<i>Ass1</i>	CTTGCATAGCTCGCAGACAG	GGTGCCAGTGAATAGCAGGT
<i>Cebpa</i>	ACAAGAACAGCAACGAGTACC	GGTCATTGTCACTGGTCAACT
<i>Cyp26b1</i>	CAATTCCATTGGCGACATCCAC	CTCCAGGCTCGAAGTGTATCC
<i>Dkk2</i>	TGAACCAAGGACTGGCTTTC	GTGGGGACTGTGGCAGTATC
<i>Ebf1</i>	GGATACGGACAGAACAGGATTTC	ACATGATCTCGTGTGTGAGCAA
<i>Ebf2</i>	GGGATTCAAGATACGCTAGGAAG	GGAGGTTGCTTTTCAAATGGG
<i>Fabp4</i>	GTCACCATCCGGTCAGAGAG	TCGACTTTCCATCCCACTTC
<i>Foxo1</i>	TTCAAGGATAAGGGCGACAG	CCACCAAGAACTCTTTCCAG
<i>Fsp27</i>	CTGGAGGAAGATGGCACAAT	GGGCCACATCGATCTTCTTA
<i>Gata6</i>	GTCTCTACAGCAAGATGAATGG	CATAAGGTAGTGGTTGTGGT
<i>Igf1</i>	AGGAGACTGGAGATGTACTG	CTTGTTTCCTGCACTTCCTC
<i>Irx3</i>	TGGAACAGATCGCTGTAGTG	GAGCTGATAAGACCAGAGCA
<i>Klf4</i>	AGGGAAGACTCCGAAGAAGC	CGCATCCAGGATAGCAGAAT
<i>Lox</i>	CAGAGGAGAGTGGCTGAAGG	CCAGGACTCAATCCCTGTGT
<i>Lpl</i>	ACAACCAGGCCTTCGAGATT	TCAGGCCAGCTGAAGTAGGA
<i>Myod1</i>	TACAGTGGCGACTCAGATGC	ACACAGCCGCACTCTTCC
<i>Opg</i>	TGTTCCGGAACAGAGAAGC	CTCTCGGCATTCACTTTGGT
<i>Ost</i>	TGCTTGTGACGAGCTATCAG	GAGGACAGGGAGGATCAAGT
<i>Osx</i>	TCCTCGGTTCTCTCCATCTG	GGACGTGGAGCCATAGTGAGC
<i>Pparg1</i>	GTCACGTTCTGACAGGACTGTGTGAC	GGGTCAGCTCTTGTGAATGGAATG
<i>Pparg2</i>	TGGGTGAACTCTGGGAGATTTC	AATGCGAGTGGTCTTCCATC
<i>Rarres2</i>	AAGCTCCAGCAGACCAACTG	AATTTTACCCTTGGGGTCCA
<i>Runx2</i>	GCCTCCGCTGTTATGAAAAA	GGACCGTCCACTGTCACTTT
<i>Rxra</i>	AGCCATTGTCCTGTTCAACC	GCTCAGGGTACTTGTGTTTGC
<i>Sox9</i>	GAGAAGAGACCCTTCGTGGA	CGTTCTTCACCGACTTCCTC
<i>Zfp423</i>	TGGACCACCGTGATCTTACA	TCGTTCTCGAACGTCATCTG
shRNA	Clone ID	Target sequence (5' > 3')
<i>shPparg</i>	TRCN0000001658	GCCCTTTACCACAGTTGATTT
<i>shRxra</i>	TRCN0000222392	CCTGTAGAGAAGATTCTGGAA

SUPPLEMENTAL TABLE 1.2

ChIP	Sample	Uniquely mapped reads
Input	DMSO	121,055,082
Input	TBT	135,392,656
H3K4me3	DMSO_1	56,766,566
H3K4me3	DMSO_2	43,538,943
H3K4me3	DMSO_3	49,151,790
H3K4me3	DMSO_4	31,415,894
H3K4me3	TBT_1	58,054,687
H3K4me3	TBT_2	29,932,720
H3K4me3	TBT_3	33,332,713
H3K4me3	TBT_4	26,919,115
H3K27me3	DMSO_1	135,290,659
H3K27me3	DMSO_2	88,564,520
H3K27me3	DMSO_3	99,793,202
H3K27me3	DMSO_4	87,806,882
H3K27me3	TBT_1	138,898,096
H3K27me3	TBT_2	97,443,766
H3K27me3	TBT_3	91,340,354
H3K27me3	TBT_4	95,071,628

CHAPTER 2

Retinoid X receptor activation during adipogenesis programs a dysfunctional adipocyte

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Abstract

Early life exposure to endocrine disrupting chemicals (EDCs) is an emerging risk factor for the development of obesity and diabetes later in life. We previously showed that prenatal exposure to the EDC tributyltin (TBT) results in increased adiposity in the offspring. These effects linger into adulthood and are propagated through successive generations. TBT activates two nuclear receptors, the peroxisome proliferator-activated receptor γ (PPAR γ) and its heterodimeric partner retinoid X receptor (RXR), that promote adipogenesis *in vivo* and *in vitro*.

We recently employed a mesenchymal stem cell (MSC) model to show that TBT promotes adipose lineage commitment by activating RXR, not PPAR γ . This led us to consider the functional consequences of PPAR γ versus RXR activation in developing adipocytes. We used a transcriptomal approach to characterize genome-wide differences in MSCs differentiated with the PPAR γ agonist rosiglitazone (ROSI) or TBT. Pathway analysis suggested functional deficits in TBT-treated cells. We then compared adipocytes differentiated with ROSI, TBT, or a pure RXR agonist IRX4204 (4204). Our data show that RXR activators ('rexinoids', 4204 and TBT) attenuate glucose uptake, blunt expression of the anti-diabetic hormone adiponectin, and fail to down-regulate pro-inflammatory and pro-fibrotic transcripts as does ROSI. Finally, 4204 and TBT treatment results in an inability to induce markers of adipocyte browning, in part due to sustained interferon signaling. Taken together, these data implicate rexinoids in the development of dysfunctional white adipose tissue that could potentially exacerbate obesity and/or diabetes risk *in vivo*. These data warrant further screening and characterization of EDCs that activate RXR.

Introduction

The economic burden of obesity (Cawley and Meyerhoefer, 2012) and diabetes (ADA, 2013) in the United States is colossal, with each pathology independently estimated to cost upwards of \$200 billion annually. Moreover, rates of obesity and diabetes are on the rise globally, even in developing countries where prevalence has been historically low (Ng et al., 2014; WHO, 2016). Obesity is by far the strongest predictor of type 2 diabetes (T2D) (2016), which raises the stakes for identifying key drivers of the obesity epidemic beyond caloric intake and energy expenditure. Twin studies show that obesity is highly heritable (40-70%), yet genetic variants identified in large-scale genome-wide association studies (GWAS) only account for a small fraction (< 3%) of the variance in body mass index (BMI) (Herrera et al., 2011; Locke et

al., 2015). This has led investigators to consider how environmental factors interact with the genome to contribute to the obesity and diabetes epidemics. In particular, researchers now understand that environmental factors *in utero* and in early life can program disease risk in adulthood (Hanson and Gluckman, 2014; Herrera et al., 2011), collectively referred to as the Developmental Origins of Health and Disease (DOHaD) paradigm (Gluckman and Hanson, 2004). Maternal obesity, maternal weight gain, and gestational diabetes are all linked to the risk of obesity in the offspring later in life. Likewise, *in utero* exposure to hyperglycemia via maternal type 1, type 2, or gestational diabetes predicts diabetes risk in the offspring (reviewed in Hanson and Gluckman, 2014). Hence, researchers are examining a number of environmental factors, both maternal and paternal, that can influence metabolic disease risk in the offspring.

Emerging evidence links exposure to industrial chemicals with a number of non-communicable diseases, including obesity and diabetes (Gore et al., 2015; Heindel et al., 2017a; Heindel et al., 2017b; Meeker, 2012). Of the tens of thousands of chemicals used in industry, at least 1000 of them are considered endocrine disrupting chemicals (EDCs) that can interfere with any aspect of hormone action (Zoeller et al., 2012). These chemicals can target endocrine and metabolic organs including the hypothalamus, pituitary, thyroid, liver, pancreas, and, of course, adipose tissue (Heindel et al., 2017a). The ‘obesogen hypothesis’ postulated that developmental exposure to EDCs might promote obesity and metabolic disease later in life by reprogramming metabolic set points, promoting adipose hyperplasia and hypertrophy, or dysregulating hypothalamic and cortical circuits that control appetite, satiety, and reward (Grun and Blumberg, 2006; Grun et al., 2006). In the ensuing decade, numerous ‘obesogens’ have been identified in humans and animals and further characterized *in vitro* and *in vivo* (Heindel et al., 2017a).

Although the mechanisms of action of many obesogens remain elusive, many EDCs act through nuclear receptors such as peroxisome proliferator-activated receptor gamma (PPAR γ),

estrogen receptor (ER), and glucocorticoid receptor (GR) to stimulate adipogenesis (Heindel et al., 2015; Janesick and Blumberg, 2011; Mimoto et al., 2017). To study these chemicals mechanistically, many laboratories have employed the murine 3T3-L1 preadipocyte cell line as well as multipotent mesenchymal stromal cells (MSCs, a.k.a. mesenchymal stem cells) from bone marrow, adipose tissue, and other organs (Bateman et al., 2016; Pereira-Fernandes et al., 2013). We first showed that the fungicide tributyltin (TBT) activates PPAR γ and its heterodimeric partner retinoid X receptor (RXR) to promote adipogenesis in 3T3-L1 cells and MSCs (Grun et al., 2006; Kirchner et al., 2010; Li et al., 2011), results corroborated by independent studies in several laboratories (Baker et al., 2015; Kanayama et al., 2005; Pereira-Fernandes et al., 2013; Regnier et al., 2015). Mice exposed to TBT *in utero* have increased fat mass and excess ectopic fat (Grun et al., 2006; Kirchner et al., 2010), characteristics that persist into adulthood and across several generations (Chamorro-Garcia et al., 2017; Chamorro-Garcia et al., 2013). We recently showed that TBT promotes adipose lineage commitment of undifferentiated MSCs through activation of RXR, but not PPAR γ (Shoucri, 2017). This led us to question the implications of RXR versus PPAR γ activation on the function of the induced adipocytes, especially since pharmaceutical PPAR γ agonists are considered to improve adipocyte health (Sugii et al., 2009) (in spite of important harmful side-effects).

Adipocytes perform many functions essential to maintaining metabolic health. Principally, they are tasked with taking up glucose from the bloodstream in response to post-prandial insulin (Saltiel and Kahn, 2001). In obesity, disrupted insulin signaling through the protein kinase B (Akt/PKB) pathway can lead to insulin resistance and T2D; if unmanaged, the disease will eventually progress to pancreatic β -cell failure requiring exogenous insulin (Kahn and Flier, 2000). Adipose tissue is also an endocrine organ, releasing several 'adipokines' that can improve or impair metabolic health (Ouchi et al., 2011). Chief among these is adiponectin, which inversely correlates with T2D risk and acts, in part, by suppressing gluconeogenesis and

stimulating β -oxidation in the liver (Li et al., 2009b). Importantly, PPAR γ agonists increase serum adiponectin levels through direct binding to and activation of its promoter in adipocytes (Maeda et al., 2001). Two hallmarks of metabolically unhealthy obesity are inflammation and fibrosis (Kusminski et al., 2016; Sun et al., 2013). Both are believed to be initiated by hypoxic signaling in hypertrophic adipocytes which induces the expression of fibrotic (collagens, collagen crosslinking enzymes) and inflammatory (interleukin-6, IL-6; tumor necrosis factor α , TNF α ; macrophage migration inhibitory factor, MIF) genes. The resultant increase in extracellular matrix (ECM) and cytokines stimulate adipocyte necrosis and recruit pro-inflammatory immune cells, such as M1-type macrophages, and a positive feedback loop ensues (Sun et al., 2013). Hence, functional adipocytes are characterized by 1) insulin sensitivity, 2) a healthy adipokine profile, and 3) small, normoxic cells that promote an anti-inflammatory and anti-fibrotic milieu (Kusminski et al., 2016; Sun et al., 2013).

One final dimension of adipocyte function that has advanced greatly in recent years is the discovery that thermogenic brown adipose tissue (BAT) persists in adults, and white adipose tissue (WAT) possesses the capability to 'brown' into 'beige/brite' fat (Kajimura and Saito, 2014; Nam and Cooper, 2015). These thermogenic adipocytes are characterized by their abundant mitochondria, which are able to uncouple cellular respiration from ATP synthesis and dissipate their proton gradient to generate heat. This is achieved by Uncoupling Protein 1 (UCP1), whose activity is stimulated by lipolysed free fatty acids (FFAs), but inhibited by purine nucleotides, such as ATP. Both cold exposure and dietary intake can stimulate lipolysis through β -adrenergic signaling, resulting in sufficient FFAs to promote thermogenesis. BAT has great therapeutic potential as a treatment for obesity and diabetes since it readily converts blood glucose and fats into heat (Poher et al., 2015). Interestingly, perinatal exposure to the insecticide dichlorodiphenyltrichloroethane (DDT) results in cold intolerance, reduced core temperature, and diminished energy expenditure in adult females (La Merrill et al., 2014). This was driven by

diminished BAT expression of *Peroxisome proliferator-activated receptor γ coactivator 1 α* (*Ppargc1a*, aka. PGC-1 α , a transcriptional master regulator of BAT genes) and *iodothyronine deiodinase 2* (*Dio2*, which converts thyroxine, T₄, to the more potent and thermogenic triiodothyronine, T₃). Further studies are needed to clarify how DDT and other EDCs might disrupt adipose development to interfere with non-shivering thermogenesis in adulthood. Several reports in the literature show that brown/beige-like adipocytes can be differentiated from MSCs using genetic and pharmacologic approaches (Huang et al., 2011; Morganstein et al., 2010; Wang et al., 2016). Hence, these cells may serve as a platform to screen for EDCs that interfere with BAT function and to study the effects of these EDCs mechanistically.

RXR is a unique member of the nuclear receptor family in that it forms heterodimers with multiple other nuclear receptors (Evans and Mangelsdorf, 2014). These partners are categorized either as 'permissive,' where the heterodimer can be activated either by activating RXR with a rexinoid or the partner receptor with its cognate ligand, or as 'non-permissive,' in which RXR is unable to be activated and the heterodimer activated only by the partner receptor ligand. Given the multitude of permissive partners expressed in adipose tissue (e.g. liver X receptor, LXR; bile acid receptor, FXR; PPAR α ; PPAR δ ; PPAR γ), rexinoids would be predicted to activate numerous, potentially interacting pathways in these cells. Interestingly, 3T3-L1 cells differentiated in the presence of the PPAR γ agonist troglitazone or TBT were shown to have divergent expression of adipogenic transcripts and proteins and an altered glucose metabolism (Regnier et al., 2015). Hence, we hypothesized that RXR activation in differentiating MSCs would result in a functionally distinct adipocyte compared with activating PPAR γ . We employed the MSC model to differentiate adipocytes in the presence of a PPAR γ agonist (ROSI), an RXR agonist (4204), or the dual PPAR γ /RXR activator TBT, and subsequently assessed the gene expression profiles and function of these cells. Despite comparable levels of lipid accumulation, MSCs differentiated in the presence of TBT had a strongly divergent transcriptome from ROSI-

treated cells that suggested deficits in adipocyte function. Further analyses revealed that when compared to cells treated with ROSI, rexinoids reduced glucose uptake, ablated the secretion of adiponectin, sustained the expression of fibrotic and inflammatory genes, and repressed adipose browning. These data point to a deleterious role for rexinoids and RXR disrupting chemicals on adipose tissue development and function.

Materials and Methods

Chemicals – All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated: dimethyl sulfoxide (Fisher Scientific, Waltham, MA), tributyltin, rosiglitazone (Cayman Chemical, Ann Arbor, MI), IRX4204 (a.k.a. AGN194204; a gift from Rosh Chandraratna, IO Therapeutics, Santa Ana, CA), T0070907 (Enzo Life Sciences, Farmingdale, NY), HX531 (a gift from Claes Bavik, Acucela Inc, Seattle, WA), dexamethasone, human recombinant insulin, isobutylmethylxanthine, triiodothyronine, mouse recombinant interferon α (PBL Assay Science, Piscataway, NJ), tofacitinib (Selleck Chemicals, Houston, TX), 2-deoxy-D-glucose, 2-deoxy-D-[^3H]-glucose (American Radiolabeled Chemicals, Saint Louis, MO), Nile Red, Hoechst 33342, and Collagenase D.

Mesenchymal stem cell culture and differentiation – Mouse bone marrow-derived mesenchymal stem cells (MSCs; OriCell, Cyagen Biosciences, Santa Clara, CA) from female C57BL/6 mice were stored at passage 9 in liquid N₂. Cells were maintained and expanded at subconfluence as previously described (Janesick et al., 2016; Shoucri, 2017) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum (CBS, Sigma Aldrich), 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 ug/mL streptomycin. To differentiate MSCs into adipocytes cells were plated at 15,000 cells/cm² and allowed to proliferate for 72 hours, at which point they were confluent and ready for adipose induction. Media was then switched to Minimal essential medium alpha medium (α MEM)

supplemented with 15% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), 10 mM HEPES, 2 mM L-Glutamine, 100 IU/mL penicillin, 100 ug/mL streptomycin, and an adipose induction cocktail (MDI) containing 1 uM dexamethasone, 5 ug/mL insulin, and 0.5 mM isobutylmethylxanthine; 10 nM T₃ was added for brown adipose differentiation. Ligands were added in conjunction with differentiation media. Media was changed every 3-4 days for 14 days at which point differentiated MSCs were ready for further analysis. For antagonist assays, the PPAR γ antagonist T0070907 (1 μ M) or the RXR antagonist HX531 (10 μ M) were added during the last 7 days of differentiation. Due to its short half-life, T0070907 was re-dosed every 12 hours (Li et al., 2011). To assess the role of the interferon signaling, 1000 U/mL IFN α or 5 μ M tofacitinib were added during the last 7 days of differentiation. All cell culture experiments were carried out at 37°C with 5% CO₂ unless otherwise indicated.

Stromal vascular fraction isolation and differentiation – 9 female C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and housed in a temperature controlled room (25-27°C) with a 12 hour light/dark cycle and unlimited access to food and water. All procedures were approved by the University of California, Irvine Institutional Animal Care and Use Committee. At 9 weeks of age, mice were euthanized and stromal vascular fraction (SVF) was harvested from inguinal fat pads as previously described (Shao et al., 2016). Inguinal fat pads were dissected, minced, pooled, and subsequently digested in a collagenase solution (100 mM HEPES, 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 mM CaCl₂, 1.5% BSA, and 1 mg/mL collagenase D) at 37°C for 2 hours. Digested adipose tissue was passed through a 100 μ M filter and the SVF was separated from adipocytes by centrifugation (5 minutes, 600 x g). SVF was resuspended in α MEM 15%FBS (see above), plated at high density in collagen-coated plates, and allowed to attach overnight. Media was changed daily to remove dead and non-adherent cells until adherent cells (preadipocytes, MSCs, fibroblasts) reached confluency. SVF was induced to differentiate in α MEM 15%FBS, MDI, T₃, and ligands (DMSO, ROSI, 4204,

TBT) as described above. After 48 hours, the induction cocktail was switched to insulin (5 $\mu\text{g/mL}$), T_3 , and ligands and differentiated for 4 more days.

Assessment of lipid accumulation – MSCs were fixed in 3.7% buffered formaldehyde; neutral lipids were stained with Nile Red (1 $\mu\text{g/mL}$) and nucleic acid was stained with Hoechst 33342 (1 $\mu\text{g/mL}$). Relative fluorescence units (RFUs) were measured in a SpectraMax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) using SoftMax Pro (Molecular Devices). Lipid accumulation was calculated as Nile Red RFU normalized to Hoechst RFU.

Insulin challenge, glucose uptake – To assess insulin response, MSCs were differentiated for 12 days, then maintained in media (αMEM 15% FBS) without MDI or ligands for the final 2 days of differentiation (Day 12-14). Cells were washed with Krebs-Ringer-HEPES buffer (KRH) containing 0.1% BSA and subsequently serum-starved overnight in the same buffer supplemented with 5.5 mM glucose. MSCs were then washed with glucose-free KRH 0.1% BSA prior to insulin challenge in the same buffer. For protein and gene expression analysis, cells were collected at baseline or after 15 minutes (protein) or 4 hours (RNA) of 100 nM insulin treatment. Glucose uptake was measured as previously described (Yamamoto et al., 2015). In brief, cells were treated with insulin (100 nM) or vehicle control (DMSO) in glucose-free KRH 0.1% BSA for 30 min. Wells were then spiked with 0.5 μCi 2-deoxy-D- ^3H -glucose (^3H -2-DG) in 6.5 mM unlabeled 2-deoxy-D-glucose (2-DG). After 5 min cells were thoroughly washed in ice cold KRH 0.1% BSA and subsequently lysed in 0.05 N NaOH. Lysates were transferred into scintillation vials and radioactivity was measured in a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Brea, CA).

Measurement of secreted adiponectin – Differentiated MSCs (14 days) were maintained for 72 hours in differentiation media lacking MDI or ligands (αMEM 15% FBS). Mouse adiponectin protein levels in conditioned media were measured by enzyme-linked

immunosorbent assay (ELISA) according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA).

Lipolysis assay – Differentiated MSCs (14 days) were washed and subsequently incubated in the presence or absence of the β -adrenergic agonist, isoproterenol (100 nM) for 4 hours. Free glycerol in the media was measured by a colorimetric method according to the manufacturer's protocol (Abcam, Cambridge, United Kingdom). All assay buffers and chemicals were supplied by Abcam. Cells were fixed and stained with Nile Red to quantify lipid accumulation as described above. Free glycerol was normalized to total lipid (Nile Red RFU) for each well.

Mitochondrial stress test – Mitochondrial function was evaluated using a Seahorse XF24 Analyzer (Agilent Technologies, Santa Clara, CA). MSCs were plated in 24-well seahorse plates at 3,000 cells/well ($\sim 11,000$ cells/cm²) in 100 μ L of DMEM 10% CBS. Media volume was brought up to 200 μ L the following day and this volume was used for the remainder of the assay. Cells were allowed to grow to confluence and differentiated as described above in the presence of T₃. The assay was carried out on 2 plates with 5 replicates from each treatment group on each plate (n=10 total per group). After 2 weeks of differentiation, cells were washed and then incubated in buffer-free Seahorse XF media supplemented with 5.55 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine (identical to concentrations in α MEM) for 1 hour at 37°C without CO₂. Plates were then loaded into the Seahorse XF analyzer and subjected to a Mito Stress Test. Cells were sequentially exposed to a Complex V inhibitor (1.5 μ M oligomycin), a mitochondrial uncoupler [1 μ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone or FCCP], and a combination of Complex I (1 μ M rotenone) and Complex III (1 μ M antimycin A) inhibitors. Oxygen consumption rate (OCR, pmol/min) is measured repeatedly at baseline and following exposure to each compound. At the end of the assay, cells were immediately fixed and stained with Hoechst 33342 as described above; OCR measurements were normalized to

Hoechst RFU for each well. ATP production, maximal respiration, and spare respiratory capacity were extrapolated from changes in normalized OCR in response to compounds.

Immunoblotting – To isolate protein, cells were washed twice in ice cold PBS and then lysed in RIPA buffer containing protease (Research Products International, Mount Prospect, IL) and phosphatase (ThermoFisher Scientific, Waltham, MA) inhibitors. Lysates were shaken at 4°C for 1 hour and then sheared with a 27-gauge needle prior to cold centrifugation. Supernatants were collected and protein concentrations were measured using the DC protein assay (BioRad, Hercules, CA). 20 µg of SDS denatured protein was run on 4-20% tris-glycine polyacrylamide gels (BioRad) and transferred to a PVDF membrane (Millipore, Burlington, MA) by semi-dry transfer (BioRad). Blots were blocked, washed, and incubated with primary antibodies according to product specifications (Phospho-Akt Ser473, rabbit polyclonal, #9271, Cell Signaling Technology, Danvers, MA; Pan-Akt, mouse monoclonal, #2920, Cell Signaling Technology; GAPDH, sc-25778, Santa Cruz Biotechnology, Dallas, TX). Following incubation with HRP-conjugated secondary antibody (GE Healthcare Life Sciences, Marlborough, MA), blots were incubated with chemiluminescent substrate (ThermoFisher) and visualized in a ChemiDoc imaging system (BioRad). Blots were stripped in acidic glycine (pH = 2.2, 0.1% SDS, 1% Tween20) and reprobed as needed.

RNA/DNA isolation, Reverse transcription, QPCR – Cells were lysed in Trizol (ThermoFisher Scientific, Waltham, MA) and RNA was isolated by phenol:chloroform extraction. RNA concentrations were measured on a NanoDrop spectrophotometer (ThermoFisher) and RNA quality was confirmed by gel electrophoresis. cDNA was generated from 1 µg total RNA using Transcriptor Reverse Transcriptase (Roche, Basel, Switzerland) according to the manufacturer's protocol. For DNA isolation, cells were collected in lysis buffer (10 mM Tris-HCl pH 8.0, 400 mM NaCl, 2 mM EDTA, 1% SDS) and subsequently treated with Proteinase K (0.25 mg/mL, 2 hours, 55°C). DNA was isolated by phenol:chloroform:isoamyl alcohol extraction and

concentrations were measured on a NanoDrop. Quantitative real-time PCR was conducted as previously described (Janesick et al., 2016; Shoucui, 2017) using intron-spanning primers (Table S2.1).

Deep sequencing – RNA for sequencing was isolated using a miRNeasy Micro Kit (Qiagen, Hilden, Germany). Intact RNA integrity (RIN > 9.0) of total RNA samples was confirmed using the Agilent RNA 6000 Nano Kit for Bioanalyzer (Agilent Technologies, Santa Clara, CA). After ribosomal RNA removal using the RiboMinus Eukaryote System v2 kit (Life Technologies), non-directional RNA-seq deep sequencing libraries for the ABI/SOLiD platform were constructed using the SOLiD Total RNA-seq Kit and EZ Bead (Life Technologies). Deep sequencing was performed using the SOLiD 5500XL deep sequencer (50 nt, single non-directional reads; Life Technologies), and the XSQ-format raw data were converted to the csfasta/QV.qual format using the XSQ tool Linux script provided by Life Technologies.

Deep sequencing data analysis – The csfasta/QV.qual sequences were aligned to the NCBI37/mm9 mouse genome reference sequence to obtain the bam format aligned read data using the NovoAlignCS color- space aligner (Novocraft Technologies, Selangor, Malaysia; www.novocraft.com/documentation/novoaligncs-3/) with the following command line: “novoalignCS -d Mouse.mm9.Exons.index -f csfastafile.csfasta -H -k -oSAM -t 110 -v 0 0 70 [>][^:]*) -F CSFASTAnQV -r All 10 -oSAM.” The bam-format data of mapped reads were examined using the fastQC deep-sequencing reads quality control tool (Simon Andrews, Babraham Institute; www.bioinformatics.babraham.ac.uk/projects/fastqc/) to exclude poor-quality reads from the study. Quality control-passed mapped reads were subjected to extraction of uniquely mapped reads using samtools (Li et al., 2009a). Uniquely mapped reads were assigned to the mm9 gene model and on-exon reads were counted using the Bioconductor package “Rsubread” (Liao et al., 2013). The assigned read counts were normalized using Bioconductor package “DESeq2” (Love et al., 2014) using the regularized logarithm (rlog)

transformation. Prior to clustering analysis, normalized counts were filtered based on variance (standard deviation > 0.2) and expression level (minimum 4 replicates > 4 rlog normalized counts). Hierarchical and k-means clustering were performed on the 4,770 genes that met the indicated criteria in Cluster 3.0; dendrograms were visualized in Java TreeView. For GO term and pathway analysis, DEGs were converted to HUGO gene symbols, then tested for enrichment in MSigDB (Broad Institute) pathway (C2) gene sets by hypergeometric test in R. p-values were corrected for multiple testing using the Benjamini-Hochberg method. Differential expression was assessed in DESeq2 using the DESeq function with $\alpha = 0.01$. Differentially expressed genes (DEGs) were defined by Benjamini-Hochberg corrected p-values ($p\text{-adj} < 0.01$) and fold change (absolute value of $\log_2[\text{fold change}] > \log_2[1.2]$).

All sequencing data is available on the Gene Expression Omnibus (pending).

Statistical analysis – Data visualization and statistical analyses were conducted in Prism 7 (GraphPad Software, La Jolla, CA), excluding RNA-seq analysis which was completed in R and other software packages as noted above. Four to six biological replicates were used for all experiments except the Seahorse assay, which used ten replicates. The primary endpoint of our study was to compare the function of terminally differentiated adipocytes with similar levels of lipid accumulation (ROSI, 4204, and TBT groups). Vehicle controls (DMSO) are presented, but were excluded from the majority of statistical tests presented in Figures 2.2-2.6 and S2.5, since these cells form far less adipocytes than ligand-treated MSCs (Fig 2.2A). However, since comparisons to vehicle control are useful to evaluate differentiation, we have included these data as a supplement (Table S2.2). Standard propagation of error was used where appropriate (Bevington and Robinson, 2003). A p-value ≤ 0.05 was considered statistically significant for all assays other than RNA-Seq analysis (see above).

Results

Differentiation of MSCs with ROSI and TBT forms adipocytes with distinct transcriptomes

Mouse bone marrow MSCs were differentiated into adipocytes using a standard adipogenic cocktail (insulin, dexamethasone, and isobutylmethylxanthine; a.k.a. MDI) (Scott et al., 2011). Cells were additionally treated with the PPAR γ agonist rosiglitazone (ROSI), the endocrine disruptor tributyltin (TBT), or vehicle control (0.1% DMSO). Doses of ROSI (500 nM) and TBT (50 nM) were chosen to achieve similar levels of lipid accumulation based on previously published reporter assays and adipogenesis assays (Chamorro-Garcia et al., 2012; Grun et al., 2006; Janesick et al., 2016; Kirchner et al., 2010; Li et al., 2012; Li et al., 2011). After 2 weeks of differentiation, mature adipocytes were assessed for lipid accumulation and genome-wide transcription (Fig S1A). ROSI and TBT treatment resulted in comparable levels of lipid accumulation over DMSO control (Fig S1B). The transcriptomes of differentiated MSCs were analyzed by hierarchical clustering (Fig S1C) and principal component analysis (Fig S1D) of normalized transcript counts. Despite achieving similar levels of lipid accumulation, TBT and ROSI transcriptomes were distinct from one another (Fig S1C-F). In fact, TBT-differentiated MSCs clustered closer to vehicle controls than to ROSI samples (Fig S1C). Genes differentially expressed by TBT and ROSI samples as compared to DMSO controls were largely distinct from one another (Fig S1E, Table S2.3) and the overall correlation between TBT and ROSI expression changes over DMSO was modest ($R^2 = 0.3426$, Fig S1F).

To better assess differences in gene expression patterns between TBT and ROSI-differentiated MSCs, we performed k-means clustering on 4,770 transcripts with the highest variance and meaningful expression levels (Fig 2.1A, Table S2.4, see Methods). These transcripts were binned into six clusters which were subsequently evaluated for enrichment in the Molecular Signaling Database's curated gene sets (C2 collection, MSigDB, Broad Institute, Cambridge, MA). From this analysis we focused on enrichments in the Kyoto Encyclopedia of

Genes and Genomes (KEGG, Kyoto University, Kyoto, Japan), Pathway Interaction Database (PID, National Cancer Institute, Bethesda, MD), and BioCarta (Allele Biotechnology, San Diego, CA) gene sets (Fig 2.1B, Table S2.5). Broadly, these clusters can be grouped into three categories. In the first group, TBT expression is distinct from both DMSO and ROSI (Clusters 1 and 5, Fig 2.1A). In the second group, ROSI expression is distinct from both DMSO and TBT (Clusters 3 and 6, Fig 2.1A). While these clusters are the smallest in number (n=153 and 287, respectively), they represent a failure by TBT to target a subset of genes effectively altered by ROSI. The final group contains genes congruently altered by ROSI and TBT treatments over DMSO controls (Clusters 2 and 4, Fig 2.1A), likely representing adipocyte differentiation induced by either ligand.

Pathway analysis reveals that Cluster 1 is enriched for the pro-fibrotic transforming growth factor β (TGF- β) signaling pathway, suggesting that TBT may push developing MSCs towards a myofibroblast-like phenotype. Clusters 2 and 3 are enriched for cell adhesion and extracellular matrix (ECM) gene sets (Fig 2.1B). Genes driving these enrichments include transcripts coding for collagens (*Col1a1*, *Col1a2*, *Col3a1*, *Col5a1*, *Col6a1*), integrins (*Itga1*, *Itga3*, *Itgb3*), thrombospondins (*Thbs1*, *Thbs2*), and fibronectin (*Fn1*) (Fig 2.1C). Remarkably, Cluster 3 is highly enriched for inflammatory pathways, particularly interferon (IFN) signaling (Fig 2.1B). These data reveal that TBT is unable to repress fibrotic and inflammatory transcripts that are effectively down-regulated by ROSI. Furthermore, TBT activates the TGF- β pathway, which is known to promote adipose tissue fibrosis and dysfunction.

Genes from Clusters 4-6 are highly enriched for metabolic pathways, particularly cellular respiration and lipid metabolism (Fig 2.1B). Enrichment for cellular respiration terms was primarily found in Clusters 4 and 5, driven by genes coding members of the electron transport chain (ETC, Fig S2A). The unique repression of nuclear-encoded ETC genes (Cluster 5) implies respiratory deficits in TBT-differentiated cells. Lipid metabolism enrichment was principally seen

in Cluster 6, driven by classic adipose markers such as *Acyl-CoA oxidase 1 (Acox1)*, *Cell death-inducing DFFA-like effector C (Cidec, a.k.a. Fsp27)*, *Fatty acid binding protein 4 (Fabp4)*, and *Sterol regulatory element binding transcription factor 1 (Srebf1)* (Fig 2.1C). The inability of TBT to effectively activate these genes suggests an inability to activate PPAR γ targets as effectively as ROSI, despite comparable lipid accumulation. Taken together, these data demonstrate that energy metabolism is disrupted in TBT-treated MSCs.

Treatment with a selective RXR agonist mirrors TBT-induced changes in gene expression

Since TBT is a dual PPAR γ /RXR activator, we hypothesized that differences in the ROSI and TBT transcriptomes might be RXR-dependent. To explore this, MSCs were differentiated in the presence of ROSI, TBT, or the selective RXR agonist IRX4204 (4204) (Vuligonda et al., 2001). After 2 weeks of differentiation, all three treatment groups accumulated similar levels of intracellular lipid compared to vehicle control (Fig 2.2A) although 4204 produced slightly less lipid accumulation at the dose used here (100 nM).

We investigated gene expression in 4204 samples to compare with ROSI and TBT treatments. This began with the canonical PPAR γ and RXR target genes, *Fabp4* and *ATP-binding cassette transporter 1 (Abca1)*, respectively (Fig 2.2B). As expected expression of the PPAR γ target *Fabp4* was highest in ROSI-treated samples, while expression of the RXR target, *Abca1* was highest in 4204-treated samples; TBT-induced expression levels fell between ROSI and 4204 (Fig 2.2B). The transcriptional regulators of adipogenesis *Zinc finger protein 423 (Zfp423)* and *CCAAT/enhancer binding protein α (Cebpa)* have higher expression in ROSI samples, while *Pparg2* expression is highest with 4204 treatment (Fig 2.2C), consistent with the ability of rexinoids to commit cells to the adipogenic lineage (Shoucri, 2017). For all three of these genes, TBT-induced expression levels resemble 4204 more so than ROSI (Fig 2.2C). Genes critical for adipocyte function such as *Acox1* and *Cidec* are preferentially targeted by the

PPAR γ agonist, ROSI, whereas *Fatty acid synthase (Fasn)* is better induced by rexinoids. The lipases *Lipase E (Lipe)*, a.k.a. Hormone-sensitive lipase or HSL) and *Lipoprotein lipase (Lpl)* are expressed similarly between ligand treatment groups (Fig 2.2D). These data show a clear rift in gene expression between MSCs differentiated with PPAR γ and RXR agonists, and suggest that the differences observed in ROSI and TBT transcriptomes might be driven by RXR.

Rexinoid-differentiated MSCs have impaired glucose uptake, but intact insulin signaling

Chief among the functions of a mature adipocyte is the ability to take up glucose in response to insulin. Transcript levels of the insulin-dependent glucose transporter *Solute carrier family 2 member 4 (Slc2a4)*, a.k.a. GLUT4) are attenuated in 4204 and TBT samples when compared to ROSI (Fig 2.3A). To test whether altered expression was reflected in glucose uptake, MSCs were differentiated with MDI and ligands as previously described (Fig S1A) for 12 days, then maintained in basal media for 2 days and finally starved overnight in a serum-free buffer. In this way, cells were starved first of the insulin in MDI and, subsequently, of insulinomimetic compounds in serum (Fig 2.3B). Following serum starvation, cells were challenged with insulin and assessed for their ability to take up glucose (Fig 2.3C), for signaling through the insulin pathway (Fig 2.3D), and altered expression of insulin-response genes (Fig 2.3E). Both basal and insulin-stimulated glucose uptake were highest in ROSI-differentiated MSCs compared to 4204 and TBT, as assessed by uptake of radiolabeled 2-deoxyglucose (Fig 2.3C). Akt/PKB signaling was assessed by detecting phosphorylation of Akt at Serine 473 (Fig 2.3D). No discernable differences were seen between samples, suggesting that insulin signaling was intact across treatments (Fig 2.3D, Fig S4).

Finally, the transcriptional response to insulin was assayed after 4 hours of insulin treatment. Basal *Slc2a4* expression remained high in ROSI-differentiated MSCs, even after removal of MDI, ligands, and serum (Fig 2.3E). Insulin treatment strongly repressed *Slc2a4* in

ROSI samples, mildly so in TBT treatment, but not in 4204-differentiated cells (Fig 2.3E).

Glycerol kinase (Gyk), an insulin target expressed in brown but not white adipose tissue, was induced by insulin in all treatments (Fig 2.3E). Its basal expression, however, was highest in ROSI-differentiated samples (Fig 2.3E). The gluconeogenic *Phosphoenolpyruvate carboxykinase 1 (Pck1*, a.k.a. PEPCK) was induced in ROSI-treated MSCs, while expression remained low in 4204 and TBT samples (Fig 2.3E). Hence, the transcriptional response to insulin is altered in rexinoid-differentiated adipocytes, as is observed in human obesity (Ryden et al., 2016).

RXR agonists blunt the expression and secretion of adiponectin compared to ROSI-treated cells

A major role of adipose tissue in health and disease is the secretion of adipokines, some of which are insulin-sensitizing and anti-inflammatory while others promote insulin resistance and inflammation (Ouchi et al., 2011). Adiponectin belongs to the former group, acting on the liver, skeletal muscle, and pancreas to ameliorate glycemic control. In analyzing our RNA-Seq data, we noted that *Adiponectin (Adipoq)* was one of the most differentially expressed genes between ROSI and TBT (Fig 2.4A, Table S2.3). In agreement with previous work in human and mouse MSCs (Kirchner et al., 2010), we see minimal to modest induction of *Adipoq* in ROSI treatment over MDI plus vehicle controls. Diminished expression of *Adipoq* by TBT (and 4204) relative to ROSI was confirmed by QPCR (Fig 2.4B). Notably, *Adipoq* transcript was repressed by 4204 (60%) and TBT (52%) as compared to vehicle control (DMSO), although this only reached statistical significance in the 4204 group (Table S2.2). To assess whether these differences resulted in diminished secretion of adiponectin protein, we measured levels of adiponectin in cell media. ROSI and control cells secreted substantial levels of adiponectin over 3 days, but the adipokine was nearly undetectable in 4204 and TBT-conditioned media (Fig 2.4C, Table S2.2). In fact, diminished protein secretion by rexinoids relative to ROSI was

greater than their effect on mRNA expression (92% vs. 78%, respectively), suggesting that RXR activators may also inhibit adiponectin export. Therefore, we assayed transcript levels of *Endoplasmic reticulum oxidoreductase 1-like (Ero1l)* which regulates the secretion of adiponectin from adipocytes (Qiang et al., 2007). Indeed, expression of this gene in rexinoid treatments was less than half of what was detected in ROSI samples (Fig 2.4D), suggesting that RXR activators not only produce less adiponectin mRNA, but may either fail to induce or interfere with protein export compared to the ROSI group.

Rexinoids promote or fail to silence fibrotic and inflammatory genes that are down-regulated by ROSI

As in the liver, adipose fibrosis is promoted by TGF- β signaling, which recruits wound-healing myofibroblasts and elevates expression of pro-fibrotic ECM genes such as collagens, fibronectin, and *Connective tissue growth factor (Ctgf)* (Biernacka et al., 2011). In analyzing our RNA-seq data, we noted an enrichment of ECM terms in Clusters 1-3, (where expression is higher in TBT than ROSI groups). A comprehensive list of ECM genes expressed in adipose tissue or adipose progenitors (Mariman and Wang, 2010) revealed 59 genes within our k-means cluster, 45 of which (76%) were found in Clusters 1-3 (Fig S2B). TGF- β signaling was chiefly enriched in the TBT-induced Cluster 1, driven by genes such as *Activin A receptor type 2A (Acvr2a)*, *Bone morphogenetic protein receptor type 1a and 2 (Bmpr1a, Bmpr2)*, *Inhibin A (Inhba)*, and *Rho associated coiled-coil containing protein kinase 2 (Rock2)*. QPCR confirmed down-regulation of fibrosis-associated genes by ROSI, but not 4204 or TBT (Fig 2.5A). This included the myofibroblast marker *Actin, α 2, smooth muscle, aorta (Acta2)*, a.k.a. α SMA), the TGF- β marker *Inhba*, as well as several ECM genes such as *Col1a1*, *Col3a1*, and *Ctgf*, while the pro-inflammatory glycoprotein *Tenascin C (Tnc)* was induced by rexinoids (Fig 2.5A, Table S2.2).

RNA-seq analysis also revealed an enrichment of inflammatory signaling pathways in Cluster 3 (Fig 2.1B). While expression of classic markers of adipose inflammation such as IL-6, TNF α , and MIF were not altered by TBT, levels of mRNAs encoding the macrophage recruiting *C-C motif chemokine ligand 5, 7, and 9* (*Ccl5*, *Ccl7*, *Ccl9*) were all expressed at higher levels than ROSI either due to incomplete repression (*Ccl5*, *Ccl7*) or an RXR-mediated induction (*Ccl9*) (Fig 2.5B, Table S2.2). Genes involved in interferon (IFN) signaling were among the most up-regulated transcripts in TBT-differentiated MSCs when compared to ROSI (Fig S3A-B). QPCR confirmed high levels of *Interferon regulatory factor 7* and *9* (*Irf7*, *Irf9*) as well as the interferon-stimulated genes (ISGs) *Signal transducer and activator of transcription 1* and *2* (*Stat1*, *Stat2*), *Interferon induced protein with tetratricopeptide repeats 2* (*Ifit2*), and *2'-5' oligoadenylate synthetase 1a* (*Oas1a*) in MSCs differentiated in the presence of rexinoids (Fig 2.5C). Hence, rexinoid treatment promotes a pro-inflammatory, pro-fibrotic gene expression profile in differentiated MSCs either by inducing (Cluster 1) or failing to silence these genes (Clusters 2 and 3) during adipogenesis.

Rexinoid-differentiated MSCs fail to induce the expression of BAT markers and respire less oxygen in the uncoupled state

Analysis of Clusters 4 and 5 revealed a pronounced enrichment of genes coding subunits of the ETC (Complex I-IV and ATP Synthase). Of the 75 ETC genes within our k-means cluster, 72 of them (96%) were in Clusters 4-6 (Fig S2A). Along with mitochondrial uncoupling and lipid catabolism, elevated respiration is a key feature that distinguishes thermogenic brown or beige adipose tissue from white adipose tissue. Recent literature shows that bone marrow-derived MSCs can be differentiated into brown/beige-like adipocytes *in vitro* (Wang et al., 2016). Therefore, we hypothesized that rexinoids were inhibiting a brown/beige adipocyte phenotype in our MSCs. To test this hypothesis, MSCs were differentiated with MDI

and ligands (as in Fig S1A) in the presence or absence of thyroid hormone (triiodothyronine, T₃) to encourage browning (Wang et al., 2016). At the end of differentiation MSCs were assessed for expression of brown/beige markers including *Uncoupling protein 1 (Ucp1)*, *Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3 (Elovl3)*, *Cell death-inducing DFFA-like effector A (Cidea)*, *Cytochrome C oxidase subunit VIIIb (Cox8b)*, *PR/SET domain 16 (Prdm16)*, *Peroxisome proliferator-activated receptor α (Ppara)*, and *Peroxisome proliferator-activated receptor γ coactivator 1 α and 1 β (Ppargc1a, Ppargc1b a.k.a. PGC-1 α , PGC-1 β)* (Fig 2.6A). T₃ effectively induced *Ucp1* (4.3-fold in ROSI samples, Fig 2.6A), although several other BAT markers (*Elovl3*, *Cidea*, and *Ppara*) were attenuated by T₃ treatment. Importantly, 4204- and TBT-treated samples failed to up-regulate the majority of BAT markers over vehicle controls, revealing an inability to activate the brown/beige transcriptional program (Fig 2.6A).

We also assessed expression of *Patatin like phospholipase domain containing 2 (Pnpla2, a.k.a. adipose triglyceride lipase or ATGL)*, since intracellular lipolysis is thought to be critical for BAT thermogenesis (Cannon and Nedergaard, 2004). Interestingly, T₃ increased expression of ATGL, and this increase was blocked in the ROSI and TBT groups, but not 4204 (Fig 2.6A). We then assessed lipolysis in our differentiated cells, as measured by free media glycerol normalized to lipid accumulation, both under basal conditions and upon β -adrenergic stimulation with isoproterenol (Fig 2.6B). In concordance with the ATGL expression data, 4204-treated cells lipolysed the most fat, while TBT treatment resulted in slightly more lipolysis than the ROSI group (Fig 2.6B). We infer from these data that thermogenesis in 4204 and TBT-differentiated adipocytes would not be limited by their ability to lipolyse intracellular fat. Consistent with this idea, recent *in vivo* work suggests that circulating, rather than intracellular free fatty acids drive BAT thermogenesis (Schreiber et al., 2017).

Since BAT is, in part, defined by an abundance of mitochondria, we assessed whether rexinoid treatment reduced mitochondrial number by quantifying relative abundance of

mitochondrial DNA. Compared to ROSI-differentiated MSCs, 4204 and TBT treated cells had an approximately 20% reduction in mitochondrial DNA, although this only reached statistical significance in the 4204 group (Fig 2.6C). We then subjected differentiated MSCs to a Seahorse Mito Stress Test (see Methods) to assess mitochondrial function. Basal oxygen consumption rate (OCR) was significantly higher in the TBT group as compared to 4204, suggesting an RXR-independent regulation of basal respiration by TBT (Fig 2.6D). We also found that ATP production in the ROSI and TBT groups was higher than in 4204-treated cells. However, when respiration was uncoupled from ATP synthesis ROSI-differentiated cells were the best equipped to respire oxygen (Fig 2.6D). Hence, under basal conditions, TBT-differentiated MSCs maintain functional mitochondria that respire oxygen and produce ATP at the same levels seen in the ROSI group. Under stressed conditions (uncoupling) the rexinoid groups are unable to respire at the rate achieved by ROSI again suggesting adipocyte dysfunction.

Multiple studies have now shown that long-term cold exposure induces the proliferation of beige adipocytes in inguinal fat depots from vascular progenitors (Berry et al., 2017; Vishvanath et al., 2016). Hence, we isolated the stromal vascular fraction (SVF) from inguinal fat depots and differentiated them in the presence of MDI, T_3 , and ligands. Interestingly, rexinoids were not as effective as ROSI in inducing adipogenesis in SVF (Fig S5A), likely due to the fact that the majority of non-hematopoietic cells in SVF are committed preadipocytes (Rodeheffer et al., 2008; Shoucri, 2017). On average, DMSO, 4204, and TBT-treated SVF reached 47%, 66% and 75% of the lipid accumulation achieved in ROSI samples, respectively (Fig S5A). In contrast, differences in gene expression of PPAR γ target genes (*Fabp4*, GLUT4, *Adipoq*) were far more dramatic than the lipid accumulation data would suggest (Fig S5A-B). This suggests that ROSI induces expression of its target genes to levels beyond their maximal effect on adipose turnover, and sheds light on how rexinoid-treated MSCs achieve the same level of lipid accumulation in spite of lower expression of PPAR γ targets. The most striking

difference in gene expression was seen in BAT markers, particularly *Ucp1*, whose expression was 1000- and 100-fold lower than ROSI in 4204 and TBT groups, respectively (Fig S5D). Interestingly, we do not see repression of adiponectin by rexinoids relative to the DMSO control as was observed in MSCs (Fig S5C, Table S2.2), implying that this may occur during the lineage commitment process where RXR is known to play an important role (Shoucri, 2017). We additionally observe activation, not repression of IFN genes by ROSI (and rexinoids to a lesser extent) (Fig S5E), again suggesting regulation during the commitment process or a depot-specific difference between the bone marrow and inguinal compartments.

Chemical antagonists reveal PPAR γ - and RXR-dependent differences in ROSI- versus rexinoid-differentiated MSCs

To evaluate the PPAR γ /RXR-dependence of our observed phenotypes, we differentiated MSCs into adipocytes for 7 days with MDI and ligands as before, then added in chemical antagonists of PPAR γ (T0070907 a.k.a. T007) and RXR (HX531) for the final 7 days of treatment (Fig S6A). In this way, we did not completely block the formation of adipocytes by inhibiting PPAR γ /RXR early in adipogenesis (Day 0-7), but could still assess the functions of these receptors in committed preadipocytes and in mature adipocytes. As expected, *Fabp4* was strongly induced by ROSI and this induction was blunted by T007 and, to a lesser extent, HX531 (Fig 2.7A). *Fabp4* induction was lower in both rexinoid treatments, however, in TBT-treated cells its expression was elevated by HX531 (Fig 2.7A, left). *Abca1* also behaved as predicted, showing induction by both rexinoid treatments that was inhibited by HX531, but not T007 (Fig 2.7A, center). Interestingly, ROSI induction of the PPAR γ target *Adipoq* was more effectively blocked by HX531 than T007, while RXR agonists 4204 and TBT strongly repressed *Adipoq* expression (Fig 2.7A, right). Similar to what was observed with *Fabp4*, HX531 could only rescue *Adipoq* expression levels in TBT-treated samples. The interferon-stimulated genes

(ISGs) *Irf7*, *Oas1a*, and *Stat2* all showed expression patterns that suggested RXR-dependence, with HX531 inhibiting ISG expression in TBT-treated samples (Fig 2.7B). The BAT markers *Elovl3*, *Cidea*, and *Ppara* all showed a PPAR γ -dependent expression pattern reminiscent of *Fabp4* or *Adipoq* (Fig 2.7C). BAT gene expression in 4204 samples was nearly unchanged by T007 or HX531 treatment, while, once again, expression in TBT samples was elevated in the presence of HX531.

Finally, we asked whether repression of BAT genes in rexinoid-treated cells might be due to the sustained expression of ISGs, as was reported in a recent study by Seale and colleagues (Kissig et al., 2017). To test this, we differentiated MSCs for one week in the presence of MDI and ligands, and then modulated IFN signaling by adding either mouse recombinant IFN α to ROSI-treated cells or the Janus kinase (JAK) inhibitor Tofacitinib (TCN) to 4204 and TBT-treated cells. TCN was previously shown to induce browning in human iPS-derived adipocytes through JAK1/3 inhibition and, ultimately, by suppressing the expression of ISGs (Moisan et al., 2015). IFN α treatment was able to reactivate the expression of ISGs in ROSI samples, while TCN strongly inhibited their expression in 4204/TBT samples (Fig 2.7D). Interestingly, IFN α had no effect on the expression of BAT markers in ROSI-differentiated MSCs (Fig 2.7E, S6C). On the other hand, TCN significantly increased the expression of PGC-1 α in 4204 and TBT-differentiated cells, and slightly elevated the expression of *Ppara* and *Elovl3* (Fig 2.7E, S6C). These data are consistent with the possibility that persistent IFN signaling in rexinoid-treated samples suppresses the critical BAT regulator PGC-1 α . Taken together, our data show that rexinoids fail to silence inflammatory signals in developing adipocytes that can interfere with the ability of these cells to acquire a brown/beige phenotype.

Discussion

Here we demonstrated that while rexinoids are potent inducers of lipid accumulation in differentiating MSCs, the resulting cells are dysfunctional when compared to MSCs differentiated with the thiazolidinedione rosiglitazone. MSCs differentiated into adipocytes were assessed for four basic functions: 1) sensitivity to insulin and subsequent glucose uptake, 2) adipokine secretion, 3) expression of pro-fibrotic and pro-inflammatory markers, and 4) the acquisition of a 'brown/beige' fat phenotype. In all of these functions, rexinoids programmed poorly functioning adipocytes that displayed diminished glucose uptake (Fig 2.3), ablated adiponectin secretion (Fig 2.4), persistent pro-inflammatory and pro-fibrotic gene expression (Fig 2.5), and a limited ability to respire oxygen in response to mitochondrial uncoupling (Fig 2.6). These data shed light on a key question raised by our previous publications and within the EDC field: if pharmacologic PPAR γ agonists improve adipocyte function then why do environmental PPAR γ activators such as TBT not produce a corresponding effect? For the prototypical obesogen TBT, the culprit appears to be its ability to activate the promiscuous nuclear receptor heterodimer RXR. Of course, *developmental* exposure (during gestation or early life) to any PPAR γ agonist bears the risk of increasing total adipocyte number, a strong predictor of fat mass in adults (Janesick and Blumberg, 2012; Spalding et al., 2008). However, our data highlight the potential for RXR activators to promote both adipose hyperplasia (Shoucri, 2017) and adipocyte dysfunction.

We recently showed that TBT exposure of MSCs prior to differentiation promotes adipose lineage commitment in an RXR-dependent manner (Shoucri, 2017). Transcriptomal analyses of undifferentiated MSCs revealed a distinct RXR-dependent gene expression profile in TBT-treated cells, while ROSI exposure had little effect, likely due to minimal PPAR γ 2 isoform expression in MSCs prior to adipose induction (Aprile et al., 2014; Shoucri, 2017). This led us to consider the potential role of RXR as a target of TBT in mature adipocytes. In our current study,

MSCs differentiated in the presence of ROSI have a significantly different transcriptome from that of TBT-treated cells (Fig 2.1, S2.1). Unlike our previous work in undifferentiated cells (Shoucri, 2017), ROSI treatment altered the expression of numerous genes (Fig S1C-F) some of which were congruently regulated by TBT (Fig S1E-F). This result is expected, since PPAR γ 2 is highly expressed in mature adipocytes and because TBT can activate PPAR γ gene targets via either half of the RXR-PPAR γ heterodimer. The consequence of RXR promiscuity, however, is that rexinoids will act not only on targets of RXR-PPAR γ , but also upon gene targets of other permissive RXR partners such as liver X receptor (LXR), farnesoid X receptor (FXR), PPAR α and PPAR δ , as well as targets of RXR homodimers (Evans and Mangelsdorf, 2014). Hence, it is not surprising that the overlap between ROSI and TBT-induced expression changes is relatively poor (Fig S1E-F). Subsequent pathway analysis revealed that transcriptomal differences between ROSI and TBT suggest functional deficits and inappropriate development of TBT-treated MSCs into adipocytes (Fig 2.1).

Given gene expression data implying that TBT fails to activate PPAR γ targets to levels achieved by ROSI, how then do these cells achieve the same levels of lipid accumulation? Notably, several lipogenic genes show higher levels of expression in TBT-treated samples including *Pparg2* (Fig 2.2C), *Fasn* (Fig 2.2D), *ATP citrate lyase (Acly)*, and *Elovl6* (Fig 2.1C). ROSI treatment, however, highly induces the expression of many lipogenic genes found in Cluster 6 (e.g. *Srebf1* and *Dgat2*, Fig 2.1C). What is more likely is that rexinoids induce the expression of enough adipogenic genes to a level sufficient for meaningful adipose differentiation, while ROSI promotes high levels of these transcripts such that they achieve their maximal adipogenic effect. Despite significant lipogenesis, rexinoids fail to silence genes that may belong to alternate mesenchymal lineages or preadipocytes, which may explain their dysfunction. Hence, these rexinoid-treated cells could also be viewed as immature or

incompletely differentiated (i.e., dysfunctional) adipocytes, despite their ability to store and mobilize fat.

MSCs and 3T3-L1 cells have become common platforms to screen EDCs for obesogenic potential (Janesick et al., 2016; Kirchner et al., 2010; Pereira-Fernandes et al., 2013; Pereira-Fernandes et al., 2014; Watt and Schlezinger, 2015). Typically, the endpoints of these adipogenesis assays are intracellular lipid accumulation and gene expression of adipose lineage markers. These endpoints are then used to infer accelerated or inhibited commitment (MSCs only) and/or terminal differentiation (MSCs or 3T3-L1 cells). More recently, investigators have begun assessing the effects of EDCs on mature adipocyte function, primarily insulin sensitivity and adipokine secretion (Heindel et al., 2017a; Mimoto et al., 2017; Nadal et al., 2017; Regnier and Sargis, 2014). *In vitro* studies of adipocyte function using 3T3-L1 cells are nearly universal, with few exceptions utilizing primary adipose tissue explants or differentiated MSCs (Hugo et al., 2008; Steinmetz et al., 1997; Valentino et al., 2013). This is despite literature showing that MSCs maintain lipolytic and endocrine functions when differentiated into adipocytes (Dicker et al., 2005; Ryden et al., 2003).

Sargis and colleagues investigated functional differences in 3T3-L1 cells differentiated with TBT or the thiazolidinedione troglitazone (Regnier et al., 2015). These authors observed lower levels of adiponectin and CEBP α mRNA and intracellular protein in TBT-treated cells, as well as lower GLUT4 expression, but no differences in glucose uptake. They speculated that the observed differences may be RXR-dependent. Here, we have used the primary MSC model to show that rexinoids fail to induce GLUT4 expression to levels achieved by ROSI, and therefore attenuate glucose uptake under basal and insulin-stimulated conditions (Fig 2.3). Notably, we observed intact insulin signaling, suggesting that diminished glucose uptake is indeed due to lower expression of GLUT4.

There are multiple sources of ECM in adipose tissue fibrosis, although M1 macrophages and preadipocytes are major contributors. Recent work shows that TGF- β and PDGFR α signaling in preadipocytes induces a myofibroblast-like phenotype that results in ECM deposition and adipose fibrosis (Marcelin et al., 2017). ROSI-treated MSCs effectively down-regulate the myofibroblast marker *Acta2* and the TGF- β marker *Inhba* as well as several genes coding for ECM proteins, whereas rexinoid treatment sustains their expression at preadipocyte (vehicle control) levels (Fig 2.1, 2.5, S2.2B). Unlike obese adipose tissue, fibrotic gene induction in our MSCs is not a product of hypoxia. Rather, it may reflect a developmental mis-programming by RXR whereby markers of other mesenchymal lineages (muscle, bone, cartilage, etc.) are either inappropriately activated or left unsilenced. This notion is supported by the fact that RXR is known to promote myogenic differentiation (AlSudais et al., 2016) and its heterodimeric partner retinoic acid receptor (RAR) is known to promote osteogenesis (Geng et al., 2013; Wan et al., 2006). While RAR is considered to be a ‘non-permissive’ RXR partner, previous studies report induction of RAR gene targets by rexinoids (Daniel et al., 2014). This might occur through RXR homodimers or in concert with retinoids present in serum, since the RXR-RAR heterodimer is permissive when RAR is liganded (Altucci et al., 2007). Notably, we observe markers of the myogenic (*Myosin heavy chain 11*, *Myh11*) and osteogenic (*Carbonic anhydrase II*, *Car2*, *Cytochrome P450 family 26 subfamily B member 1*, *Cyp26b1*) lineages among the top genes up-regulated by TBT in our RNA-seq data (Table S2.3). Furthermore, many ECM genes are known to be expressed in preadipocytes, but are down-regulated during terminal differentiation (Mariman and Wang, 2010). Given the diminished ability of rexinoids to induce PPAR γ targets (Cluster 6, Fig 2.1B), it is likely that many of these transcripts reflect a failure by rexinoids to properly differentiate these cells into fully functional, mature adipocytes. Since adipose fibrosis is maintained through a positive feedback loop (Sun et al., 2013),

aberrant rexinoid signaling in preadipocytes during adipogenesis could potentially initiate an inflammatory/fibrotic response *in vivo*.

In addition to a pro-fibrotic transcripts, rexinoids also sustained the expression of pro-inflammatory genes in MSCs. The macrophage chemoattractants CCL5, CCL7, and CCL9 were highly expressed in MSCs treated with RXR activators when compared to ROSI (Fig 2.5B). Importantly, these chemokines are up-regulated in human obesity (Huber et al., 2008), with the exception of CCL9 which has no human ortholog, but is up-regulated in mouse models of obesity (Jiao et al., 2009). Remarkably, rexinoids failed to repress many genes involved in interferon signaling (Fig 2.1, 2.5, S2.3). The role of IFN signaling in adipose tissue is complex, as IFNs and its effectors play roles in adipogenesis and in mature adipocyte function (Eguchi et al., 2008; Richard and Stephens, 2014). Importantly, IFN γ acts through STAT1 to induce insulin resistance and inhibit adipogenesis in human preadipocytes (McGillicuddy et al., 2009) and IFN γ -null mice on a high-fat diet have improved insulin sensitivity, smaller adipocytes, and an M2-shift in adipose tissue macrophages (O'Rourke et al., 2012). While the source of interferons in obese adipose tissue is primarily T cells and natural killer cells, here we report the induction of IFN signaling within MSCs differentiated in the presence of RXR activators. Notably, IFN genes are strongly repressed by ROSI treatment, but remain elevated in vehicle and TBT samples (Fig 2.1A-B, Cluster 3, Fig 2.5B). Hence, the enrichment of IFN genes in rexinoid-treated samples probably reflects a failure to down-regulate these transcripts via PPAR γ , rather than an RXR-mediated induction. At the same time, chemical inhibition of RXR consistently repressed ISGs (Fig 2.7B), suggesting some role for RXR in maintaining basal expression of these genes. Where in the IFN cascade PPAR γ and RXR might act is unclear, however, recruiting or ousting IRFs to and from the genome is a plausible mechanism.

Research into the molecular regulation of brown and beige adipose tissue has exploded in the past decade (Kajimura and Saito, 2014), in part due to its potential as a pharmacologic

target for the treatment of obesity and diabetes (Poher et al., 2015). Our data show that MSCs differentiated in the presence of rexinoids are unable to induce the expression of transcriptional and functional markers of brown and beige fat (Fig 2.6A) despite uninhibited adipose turnover and T₃ supplementation in the media. Interestingly, ATGL expression and lipolysis were induced by T₃ in the 4204 group, but this was blunted by ROSI and TBT treatment (Fig 2.6A-B), exposing a rare example of functional similarity between ROSI and TBT-differentiated adipocytes. The other instance of congruence between ROSI and TBT can be seen in the basal respiration and ATP production rates measured in the Mito Stress Test (Fig 2.6D), revealing that TBT-differentiated adipocytes are as functional ROSI cells under basal conditions. Only when respiration is uncoupled from ATP synthesis (FCCP treatment) are the deficits of rexinoid-treated mitochondria apparent (Fig 2.6D). Whether similar deficits would be seen in response to sympathetic activation (by isoproterenol or β_3 agonist) was not tested. Higher respiration in ROSI cells is likely due to higher expression of PGC-1 α , a master regulator of mitochondrial biogenesis, and a subsequent increase in mitochondrial abundance (Fig 2.6A, 6C). Our results in MSCs were mirrored in SVF differentiated into adipocytes, where rexinoid treatment resulted in slightly less adipose turnover, but a >100-fold decrease in *Ucp1* expression compared with ROSI (Fig S5D).

Interestingly, recent work has highlighted the importance of IFN signaling in brown and beige adipose tissue. For example, IRF3 was shown to repress white adipose browning (Kumari et al., 2016) while IRF4 is induced upon cold exposure and promotes thermogenesis (Kong et al., 2014). A recent high-throughput screen for potential browning agents using human pluripotent stem cell-derived adipocytes identified two JAK inhibitors (one of which was TCN) that were shown to act via repression of IFN signaling (Moisan et al., 2015). Importantly, these pharmaceuticals increased mitochondrial content and basal lipolysis in these cells. Seale and colleagues showed that the BAT transcriptional regulator PRDM16 represses type I IFN

signaling in adipocytes to promote thermogenesis (Kissig et al., 2017). PRDM16 was shown to negatively regulate ISGs in brown preadipocytes including *Irf7*, *Ifi44*, *Oas2*, *Oas3*, *Stat1*, and *Stat2*, all genes that are highly up-regulated in MDI-induced MSCs treated with vehicle or TBT compared to ROSI (Fig S3). Importantly, a downstream inhibitor of IFN signaling (TCN) was able to rescue expression of PGC-1 α in 4204/TBT samples (Fig 2.7E). Taken together, these data implicate persistent IFN signaling in rexinoid-treated MSCs as a repressor of adipose browning/beiging.

PPARs are 'permissive' partners of RXR, hence, rexinoid treatment should induce the expression of PPAR γ targets (Evans and Mangelsdorf, 2014). To some extent, this is achieved in our experiments, since rexinoid-differentiated cells accumulate lipid and express many genes congruently with ROSI-treated MSCs (Fig S1). However, we observe a vastly divergent transcriptome between ROSI and rexinoid-treated samples (Fig 2.1, S2.1). Our chemical antagonist assay sheds light on the complex mechanisms through which the effects of these ligands ultimately diverge (Fig 2.7A-C). *Fabp4*, for example, is effectively induced by ROSI, 4204, and TBT over vehicle, but this induction is much stronger in ROSI (Fig 2.7A). Lower expression levels in rexinoid treatments might be due to dispersion of RXR among many permissive partners thereby reducing the availability of RXR protein to partner with PPAR γ . Interestingly, chemical inhibition of RXR in TBT-treated cells increases expression of *Fabp4* and many other PPAR γ targets (*Adipoq*, *Elovl3*, *Cidea*, *Ppara*) (Fig 2.7A, C). This may be due to a 'redirecting' of TBT towards its other nuclear receptor target, PPAR γ . Alternatively, inhibition of RXR in the presence of rexinoids might increase RXR availability, and subsequent partnering with PPAR γ is only unmasked in the presence of PPAR γ agonist (i.e. TBT, but not 4204). We also note that *Adipoq* mRNA levels are depressed by rexinoids relative to vehicle control, and this depression is only relieved by HX531 in TBT, but not 4204 samples (Fig 2.7A). We hypothesize that RXR, likely as a PPAR γ /RXR heterodimer, positively regulates the *Adipoq*

promoter even in the absence of exogenous ligand (represented by the vehicle control group). Hence, ROSI treatment activates *Adipoq*, while the rexinoid-induced dispersal of RXR throughout the genome results in repression. Similar to *Fabp4*, this repression can be relieved by HX531 only in the presence of a PPAR γ agonist, TBT (Fig 2.7A). Taken together, we infer from these results that rexinoids are uniquely poised to disrupt normal adipocyte development and function due to the promiscuous nature of RXR. Notably, LXR has been implicated as a negative regulator of adiponectin (Zheng et al., 2014) and adipose browning (Korach-Andre et al., 2011), making this permissive RXR partner suspect in mediating some of the adverse effects of rexinoids during adipogenesis.

Work in animals suggest that RXR agonists are, in fact, insulin-sensitizing and glucose-lowering agents, in spite of persistent hypertriglyceridemia and repression of the thyroid axis (Li et al., 2005; Macchia et al., 2002; Ogilvie et al., 2004; Pinaire and Reifel-Miller, 2007). Notably, every published animal study conducted on this class of drug used genetic models of obesity and/or diabetes in rodent models (ob/ob mouse, db/db mouse, or Zucker fatty rats). It is possible that rexinoids have unique effects in animals with these genetic backgrounds, and may in fact worsen glycemic control in wild type animals or in dietary models of obesity. Diabetic patients taking the rexinoid bexarotene, indicated for cutaneous T-cell lymphoma, are warned that bexarotene might enhance the effect of insulin and to be wary of hypoglycemic crises (Wong, 2001). We find no evidence in the literature of such a case and presume that the warning was issued based on rodent data. We do find, however, a documented case of a pediatric patient developing insulin-dependent diabetes that persisted after discontinuation of bexarotene (Mehta et al., 2012). Our data demonstrate that these pharmaceutical rexinoids stifle adipose browning, which, in combination with central repression of the thyroid axis, could result in deficits in non-shivering thermogenesis and energy expenditure that are worthy of further investigation. A recent paper identified bexarotene in a small molecule screen of

browning agents in a muscle cell line (Nie et al., 2017), and subsequently showed increased browning and beiging in BAT and WAT, respectively, of adult mice fed a high-fat diet. The authors proposed that RXRs are master regulators of brown fat development and activation, more so than PPAR γ . Importantly, doses of bexarotene used in this study (10 μ M *in vitro*, 50 mg/kg/day *in vivo*) exceed the window of RXR selectivity for this drug (Gottardis et al., 1996) and likely activate RAR, which is a well-documented positive regulator of thermogenesis (Alvarez et al., 1995; Kiefer et al., 2012; Wang et al., 2017).

In conclusion, we have shown that RXR activation during the differentiation of MSCs promotes the formation of a dysfunctional and mis-programmed adipocyte *in vitro*. This comes on the heels of our recent work showing that rexinoids promote adipose lineage commitment in undifferentiated MSCs (Shoucri, 2017). Taken together, these data support a model in which RXR activators have the potential to increase the allocation of stem cells to the fat lineage in addition to programming a poorly functioning adipocyte. Given the focus within the endocrine disruption field on PPAR γ activators, it would be prudent not to ignore EDCs that activate RXR, as they may prove more deleterious. Few of these chemicals have been identified to date (Bowers et al., 2016; Janesick et al., 2016), and none, other than TBT, have been characterized *in vivo* or studied in human populations. Finally, we have shown for the first time that MSCs may provide a useful platform to screen and study EDCs that repress adipose browning. Given the high cost of endocrine disruption in the US and Europe (Attina et al., 2016; Legler et al., 2015; Trasande et al., 2015), the need to identify obesogens and characterize their mechanisms of action becomes increasingly critical.

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Figure 2.1. Differentiation of MSCs with ROSI and TBT forms adipocytes with distinct transcriptomes. MSCs were differentiated for 2 weeks in the presence of ROSI (500 nM), TBT (50 nM), or vehicle control (0.1% DMSO) and an adipose induction cocktail (MDI). After two weeks, RNA was isolated and prepared for deep sequencing. Reads were aligned to the mouse genome (mm9), counted (Rsubread, R/Bioconductor), normalized (DESeq2, R/Bioconductor), and clustered using the k-means method (Cluster 3.0) as described in Materials and Methods. (A) Median-adjusted normalized counts for all genes within each of the 6 clusters are presented as Tukey box plots. (B) Pathway analysis of genes within each k-means cluster was performed using the MSigDB C2 gene sets. Results from KEGG (blue text), PID (green text), and Biocarta (brown text) gene sets are displayed. Pathways were ranked by Benjamini-Hochberg adjusted p-value (p-adj). The number of genes from a given cluster within a particular gene set divided by the total number of genes in the set, as a percentage, is indicated by the size of the circle. Pathways not relevant to adipocyte biology are presented in gray, but are listed in Table S4. (C) Heatmap of median-adjusted normalized counts for ten representative genes from each of the 6 clusters are presented for each sample replicate.

FIGURE 2.1

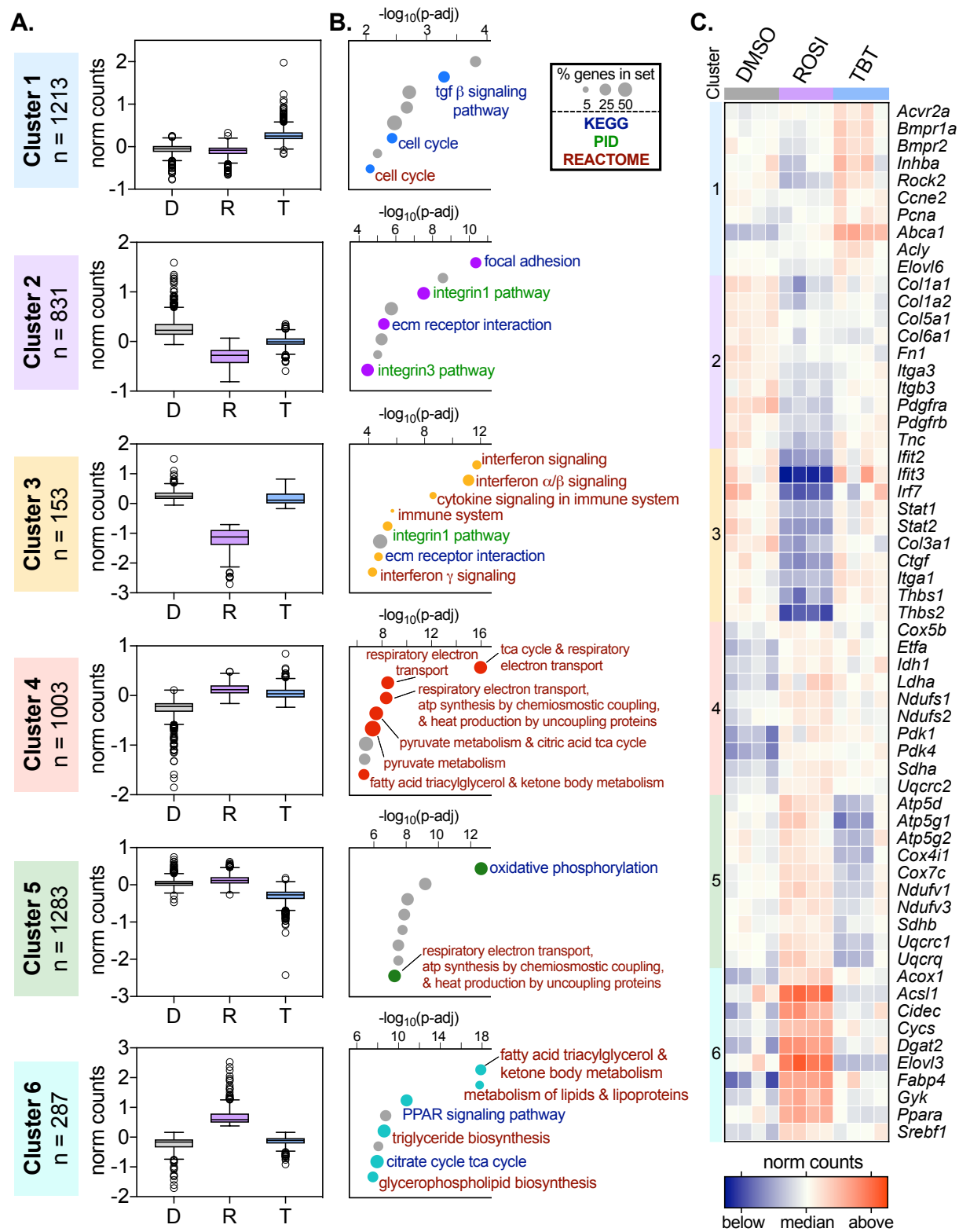


FIGURE 2.2

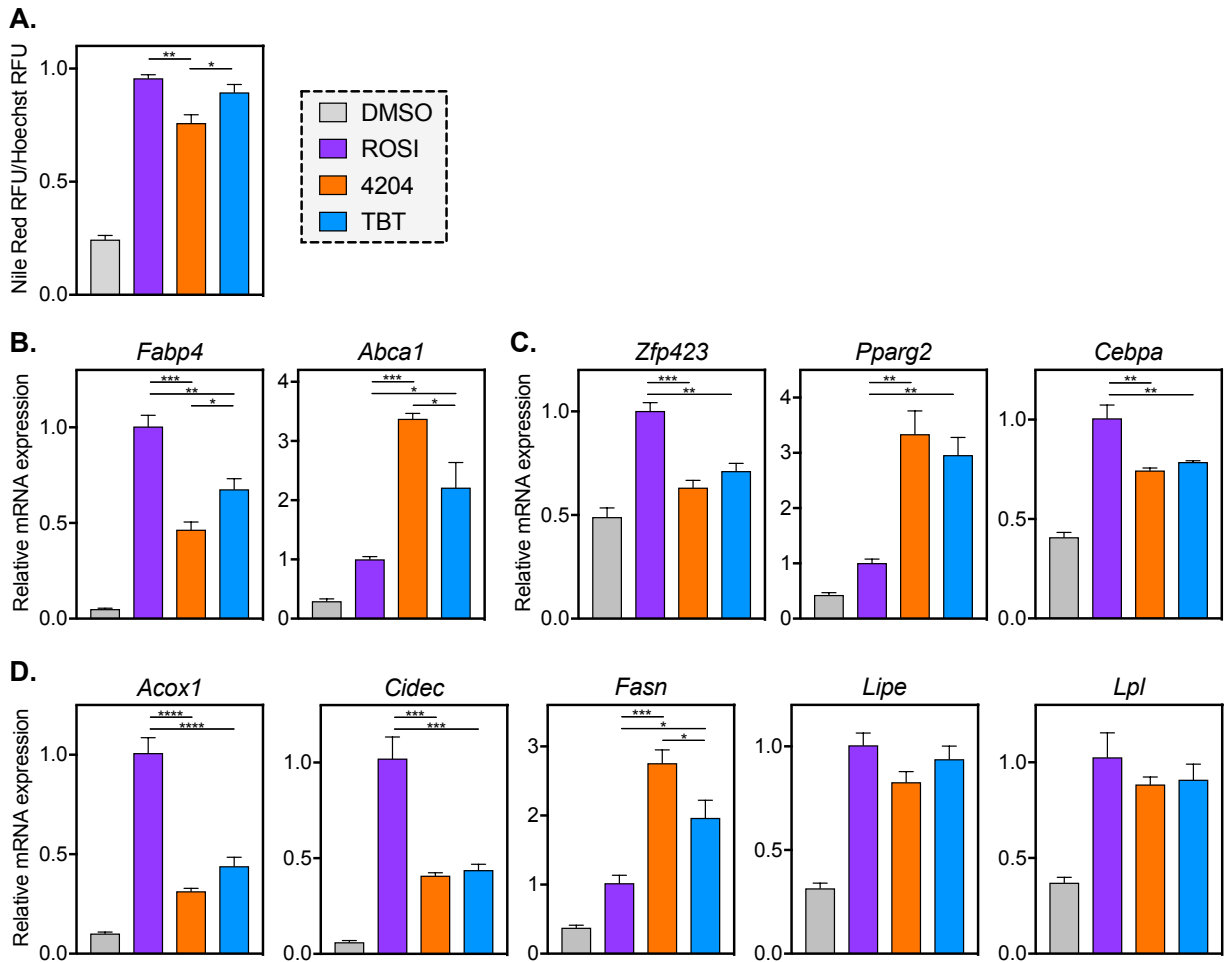


Figure 2.2. Treatment with a selective RXR agonist mirrors TBT-induced changes in gene expression. MSCs were differentiated into adipocytes with MDI and ROSI (500 nM), 4204 (100 nM), TBT (50 nM), or vehicle control (0.1% DMSO) for 2 weeks. Cells were fixed and stained to assess lipid accumulation (A) or RNA was extracted for analysis of gene expression (B-D). (A) Fixed MSCs were stained for neutral lipids (Nile Red) and nuclei (Hoechst 33342). Nile Red fluorescence was normalized to Hoechst for each well. QPCR analysis of (B) canonical PPAR γ and RXR targets, (C) early adipose lineage markers, and (D) late markers of adipocyte function. For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data are represented as the mean $\pm$ s.e.m.

Figure 2.3. Reginoid-differentiated MSCs have impaired glucose uptake, but intact insulin signaling. (A) QPCR analysis of *Slc2a4* (GLUT4) from material described in Figure 2. For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$. (B) For insulin challenges, MSCs were differentiated for 12 days in the presence of MDI and ligands, then tapered off MDI, ligands, and serum as described in the Materials and Methods. Cells were starved overnight in serum-free buffer prior to insulin challenge and measurement of (C) glucose uptake, (D) Akt/PKB signaling, or (E) transcriptional response. (C) Glucose uptake was measure using radiolabeled 2-DG as described in the Materials and Methods. For ROSI, 4204, and TBT samples: 2-way ANOVA, Tukey's multiple comparisons test between treatment groups: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (D) Protein lysates were prepared from samples at baseline or following insulin treatment (100 nM) for 15 minutes as described in the Materials and Methods. Samples were immunoblotted for phosphorylation of AKT at serine 473 (pAKT S473), panAKT, and GAPDH (loading control). (E) RNA was collected at baseline or after 4 hours of insulin treatment (100 nM). Gene expression of insulin-responsive transcripts was assessed by QPCR. For ROSI, 4204, and TBT samples: 2-way ANOVA, Tukey's multiple comparisons test between treatment groups: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data are represented as the mean $\pm$ s.e.m.

FIGURE 2.3

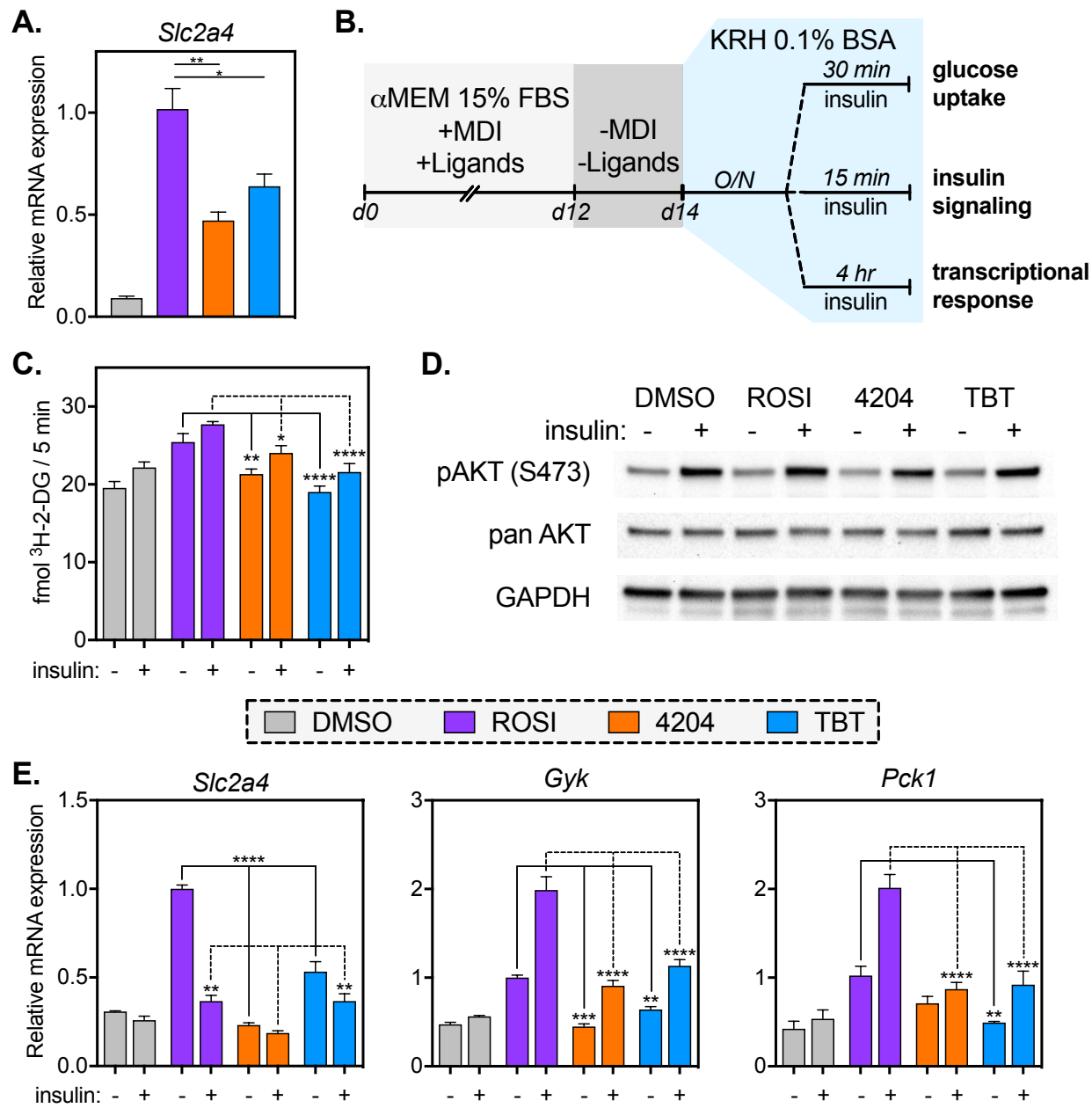


FIGURE 2.4

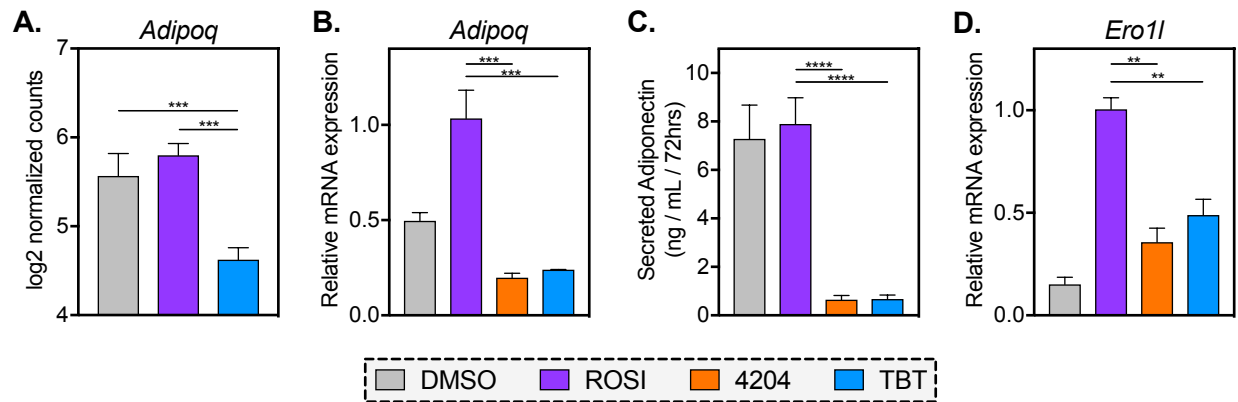


Figure 2.4. RXR agonists blunt the expression and secretion of adiponectin compared to ROSI-treated cells. (A) Normalized read counts of *Adipoq* transcript were analyzed as described in Materials and Methods. (B) QPCR analysis of *Adipoq* expression in differentiated MSCs treated with ROSI (500 nM), 4204 (100 nM), TBT (50 nM), or vehicle control (0.1% DMSO). (C) MSCs were differentiated with MDI and ligands for 2 weeks, washed, and allowed to incubate in media for 72 hours. Adiponectin protein in conditioned media was measured by ELISA. (D) QPCR analysis of *Ero1l* in MSCs differentiated as described in (B). For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: ** p < 0.01, *** p < 0.001, **** p < 0.0001. All data are represented as the mean $\pm$ s.e.m.

FIGURE 2.5

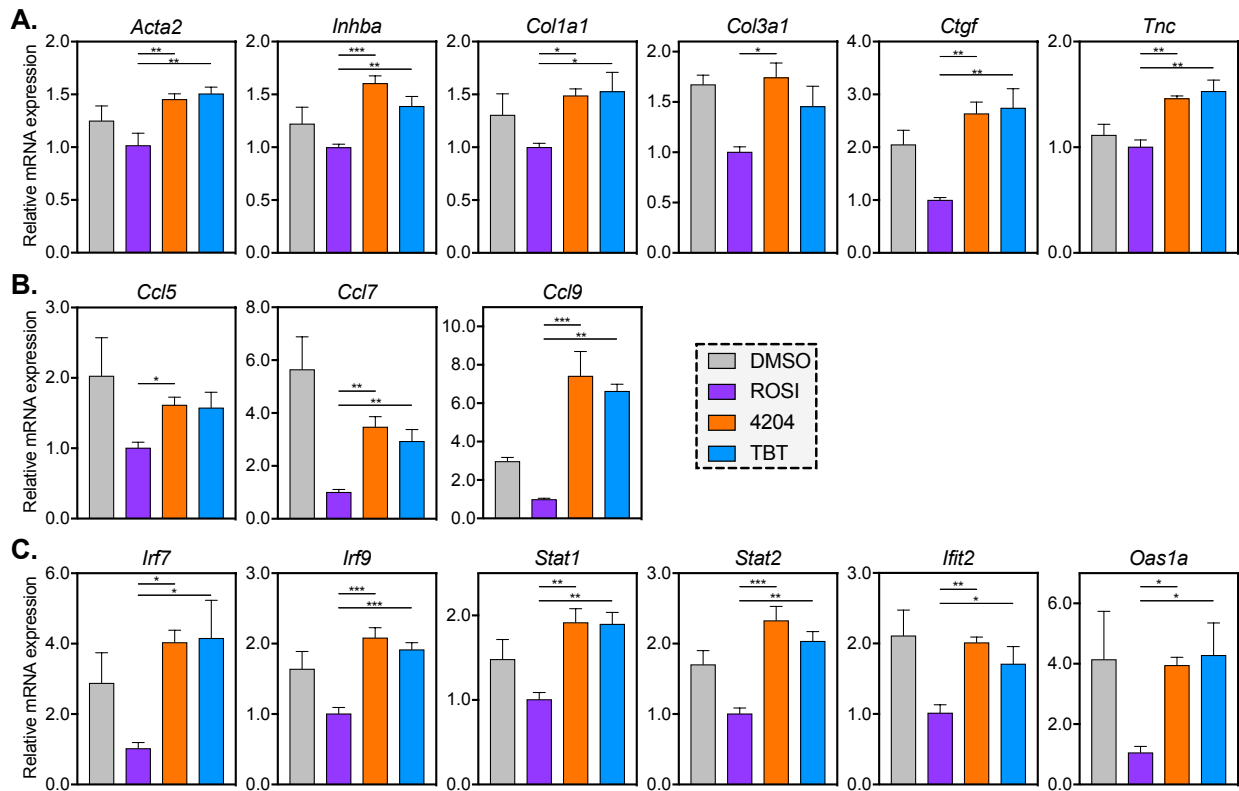


Figure 2.5. Reginoids promote or fail to silence the expression of fibrotic and inflammatory genes that are down-regulated by ROSI. RNA was collected from MSCs differentiated with MDI and ROSI (500 nM), 4204 (100 nM), TBT (50 nM), or vehicle control (0.1% DMSO). QPCR analysis of gene expression was conducted for (A) genes involved in adipose tissue fibrosis, (B) chemokines, and (C) IFN genes. For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All data are represented as the mean $\pm$ s.e.m.

Figure 2.6. Reginoid-differentiated MSCs fail to induce the expression of BAT markers and respire less oxygen in the uncoupled state.

MSCs were differentiated into adipocytes with MDI and ligands (500 nM ROSI, 100 nM 4204, 50 nM TBT, 0.1% DMSO) in the presence or absence of T₃. (A) RNA was collected at the end of differentiation and analyzed for expression of BAT markers. For ROSI, 4204, and TBT samples: 2-way ANOVA, Tukey's multiple comparisons test: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. (B)

Differentiated MSCs were exposed to the β -adrenergic agonist isoproterenol (100 nM) or vehicle control (0.1% DMSO) for 4 hours. Lipolysis was measured by assaying free glycerol in the media and normalizing to lipid stain as described in the Materials and Methods. For ROSI, 4204, and TBT samples: 2-way ANOVA, Tukey's multiple comparisons test between treatment groups: * p < 0.05, ** p < 0.01, **** p < 0.0001. (C) DNA was isolated as described in the Materials and Methods. Mitochondrial content was assessed by QPCR as the ratio of mitochondrial DNA (*mt-Co1*) to nuclear DNA (*Ndufv1*). For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: * p < 0.05. (D) MSCs were differentiated into adipocytes in the presence of MDI, T₃, and ligands as described in the Materials and Methods. After 2 weeks, MSCs were starved of buffer and oxygen for 1 hour prior to analysis of mitochondrial function by Mito Stress Test (see Methods). Oxygen consumption rate (OCR) was normalized to cell number (Hoechst 33342 RFU) for each well. Parameters of mitochondrial function were extrapolated from normalized OCRs. For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: * p < 0.05, ** p < 0.01, *** p < 0.001. All data are represented as the mean $\pm$ s.e.m.

FIGURE 2.6

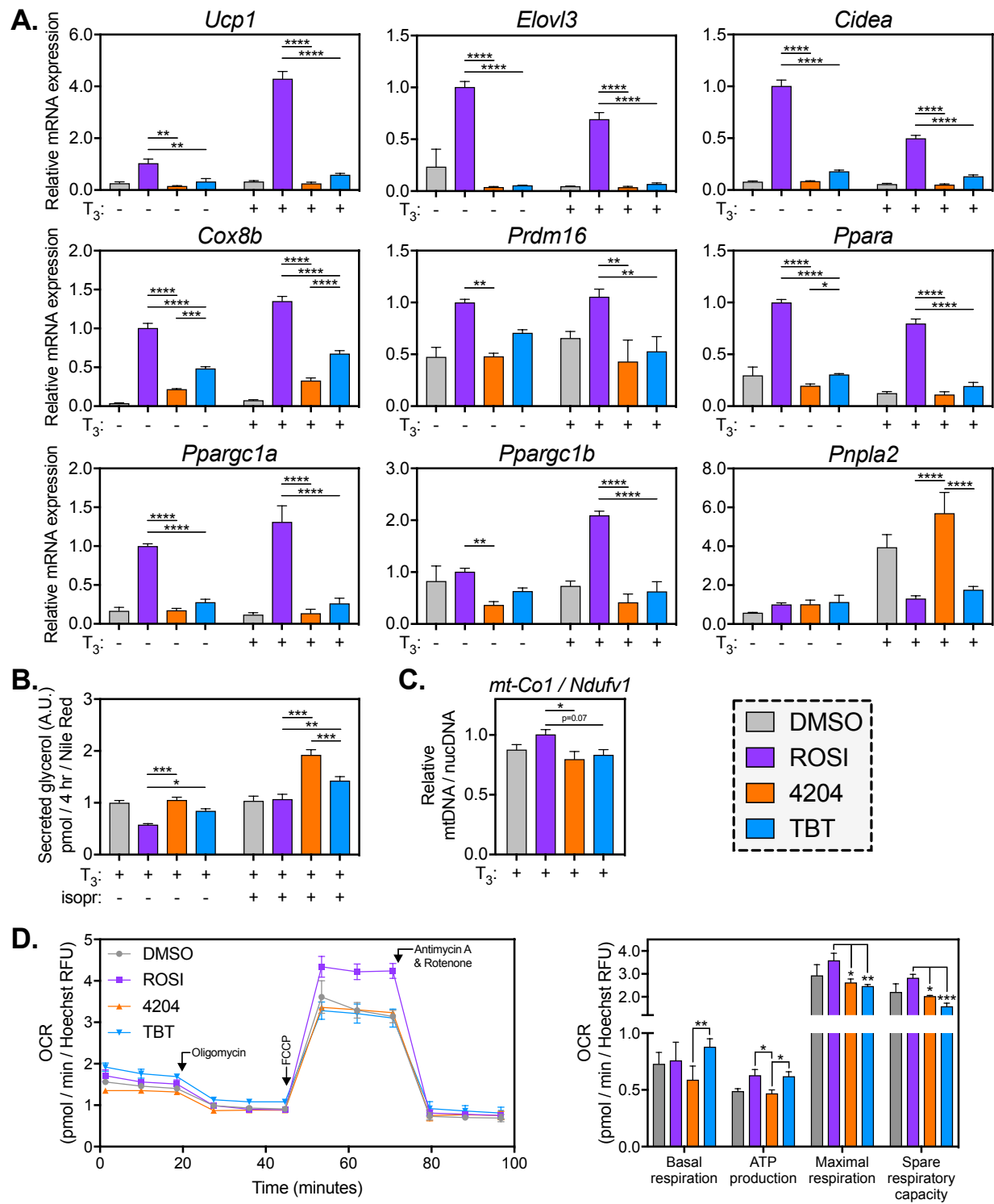


Figure 2.7. Chemical antagonists reveal PPAR γ - and RXR-dependent differences in ROSI- versus rexinoid-differentiated MSCs. (A-C) MSCs were differentiated into adipocytes in the presence of MDI and ligands (500 nM ROSI, 100 nM 4204, 50 nM TBT, 0.1% DMSO). On day 7 of differentiation, chemical antagonists of PPAR γ (T0070907, 1 μ M) or RXR (HX531, 10 μ M) were added to the culture medium through the end of differentiation (see Fig S6A). RNA was collected and analyzed by QPCR for gene expression of (A) classic PPAR γ and RXR targets, (B) IFN genes, or (C) BAT markers. 2-way ANOVA, Tukey's multiple comparisons within each treatment group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (D-E) MSCs were differentiated for 7 days in the presence of MDI and ligands as in panel (A-C). On day 7, mouse recombinant IFN α (1000 U/mL) was added to ROSI samples and the JAK inhibitor TCN (5 μ M) was added to 4204 and TBT samples (see Fig S6B). Gene expression of (D) IFN genes and (E) BAT markers was analyzed by QPCR. Unpaired t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data are represented as the mean $\pm$ s.e.m.

FIGURE 2.7

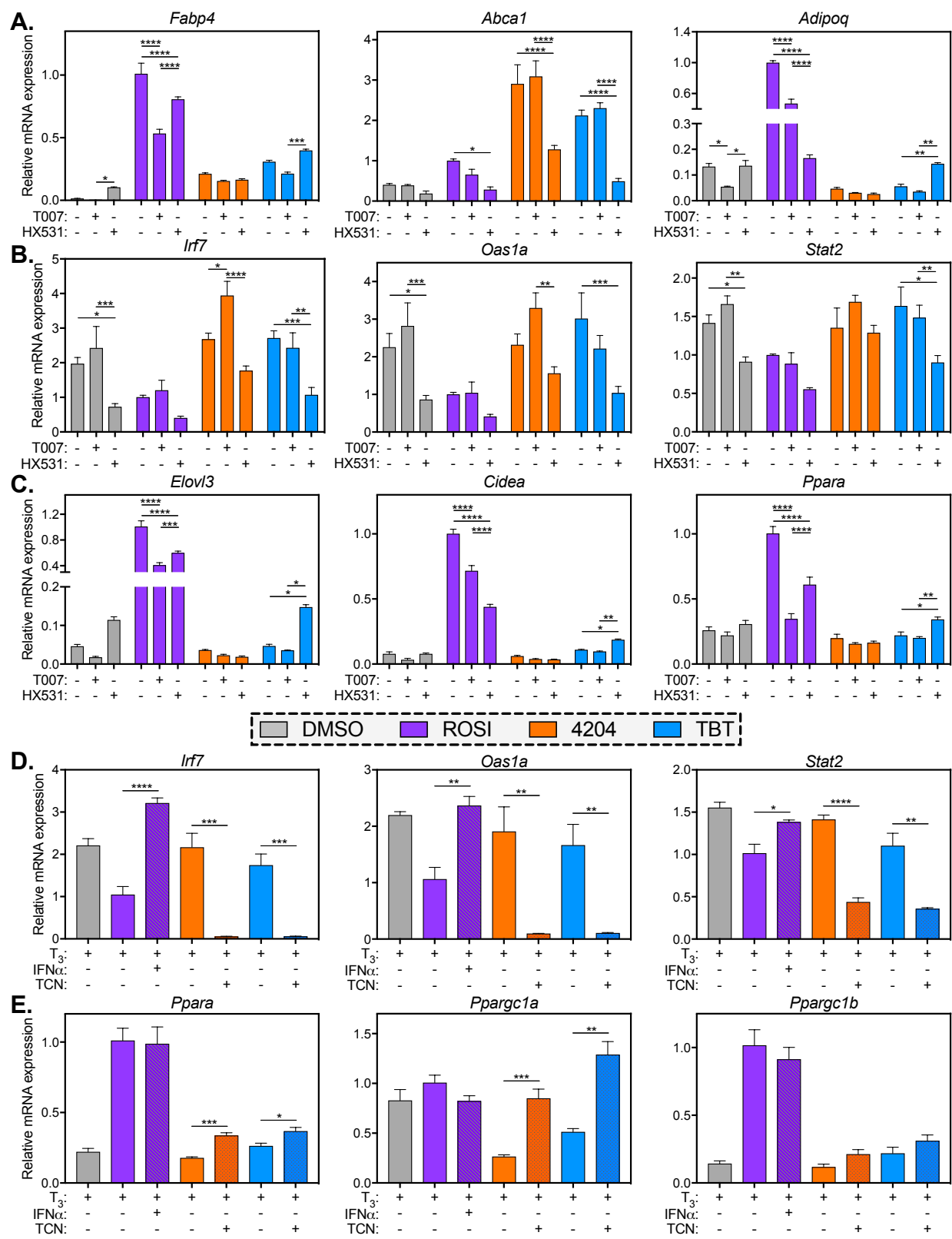
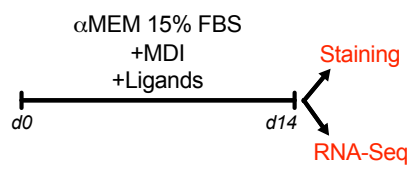


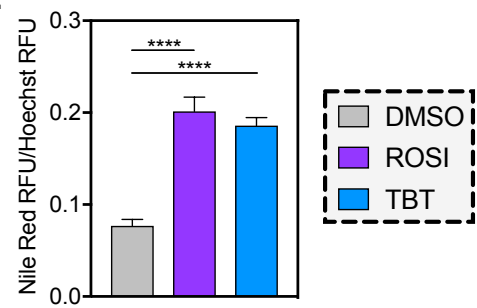
Figure S2.1. TBT and ROSI-differentiated cells accumulate similar amounts of lipid, but have distinct transcriptomes (Related to Figure 2.1). Mouse MSCs were differentiated with an adipogenic cocktail (MDI) and vehicle control (0.1% DMSO), ROSI (500 nM), or TBT (50 nM) for 2 weeks. (A) At day 14, MSCs were either fixed for analysis of lipid accumulation or RNA was collected for deep sequencing. (B) Lipid accumulation was determined by normalizing Nile Red relative fluorescence units (RFU) to Hoechst 33342 RFU. 1-way ANOVA, Tukey's multiple comparisons test: **** $p < 0.001$. Data are represented as the mean $\pm$ s.e.m. (C-F) Libraries for sequencing were prepared from isolated RNA, sequenced, aligned, counted, quantified, and further analyzed using a bioinformatics pipeline detailed in the Materials and Methods. (C) Normalized counts from 4770 genes with the highest variance above a specified threshold (see Methods) from each replicate were subjected to unbiased hierarchical clustering analysis. (D) Normalized counts from all genes were analyzed using principal component (PC) analysis. (E) Overlap of up- and down-regulated differentially expressed genes (see Methods) in ROSI and TBT replicates as compared to vehicle control (DMSO). (F) Correlation of ROSI and TBT fold-change-over-DMSO values for all genes.

SUPPLEMENTAL FIGURE 2.1

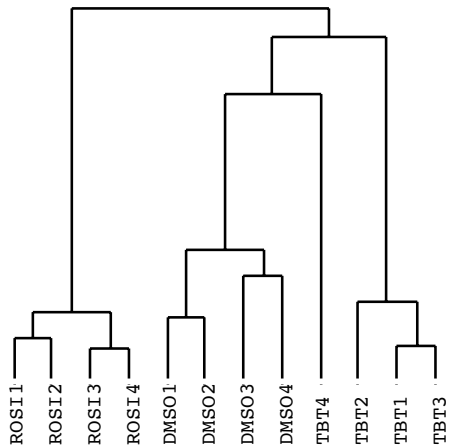
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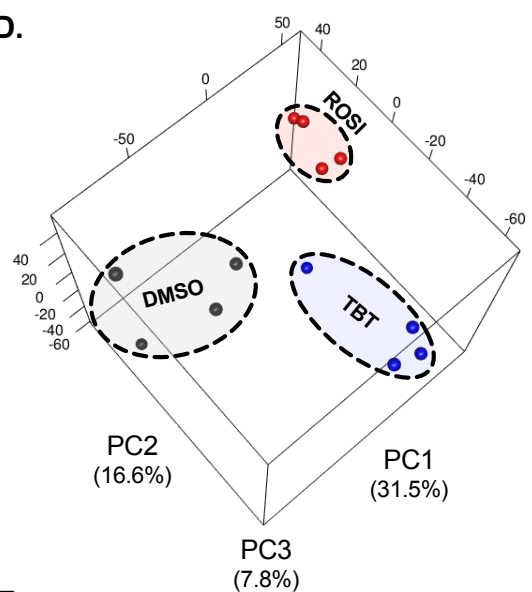
B.



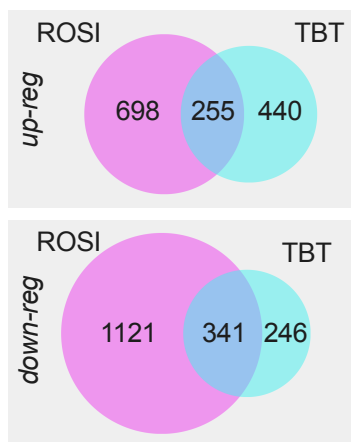
C.



D.



E.



F.

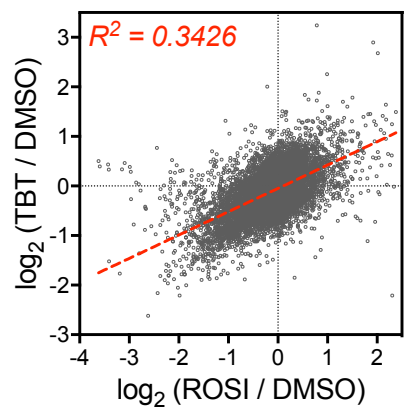
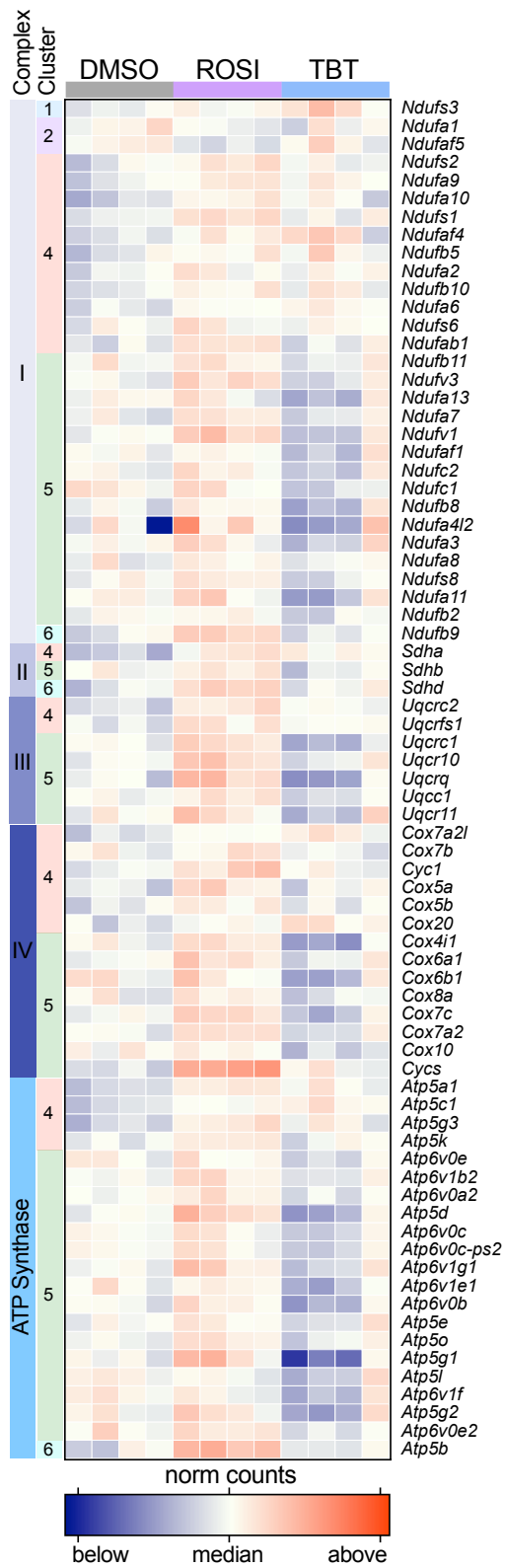


Figure S2.2. Clustering bias of genes involved in cellular respiration and adipose extracellular matrix (Related to Figure 2.1). Heatmaps of median-adjusted normalized counts (from Cluster 3.0 / Java TreeView) are presented for (A) nuclear-encoded genes in the electron transport chain (ETC) and (B) extracellular matrix genes known to play a role in adipose tissue (ref 58). (A) Genes are organized by ETC complex; k-means cluster number (see Fig 1A) is also indicated. (B) Genes are organized by k-means cluster number (see Fig 1A).

SUPPLEMENTAL FIGURE 2.2

A.



B.

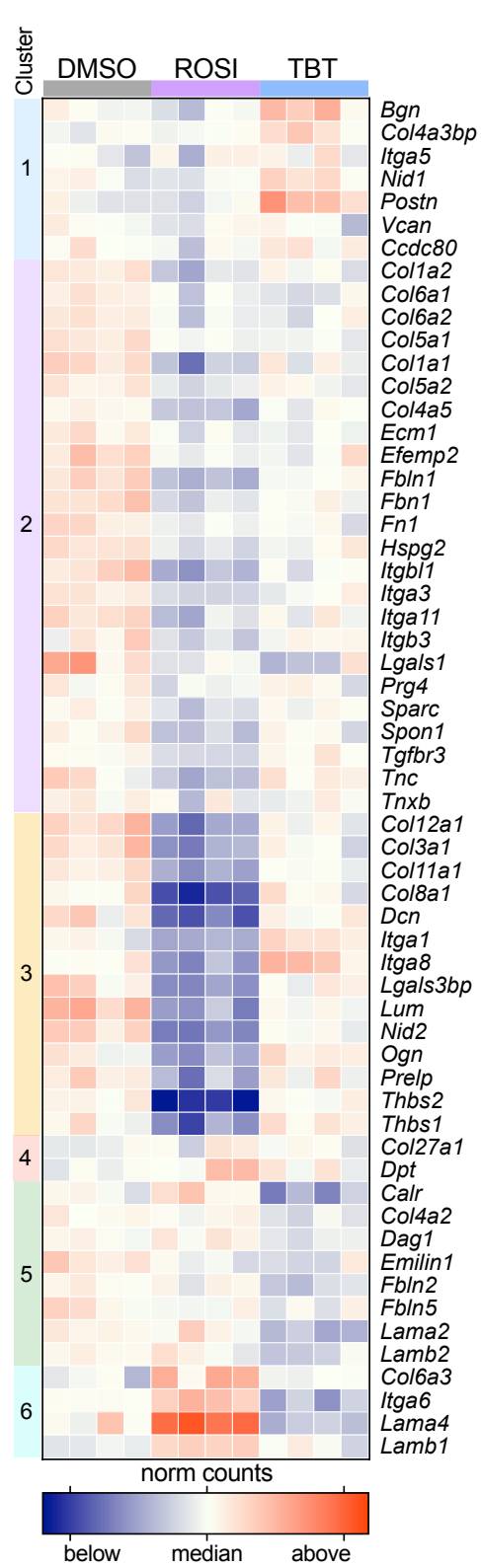
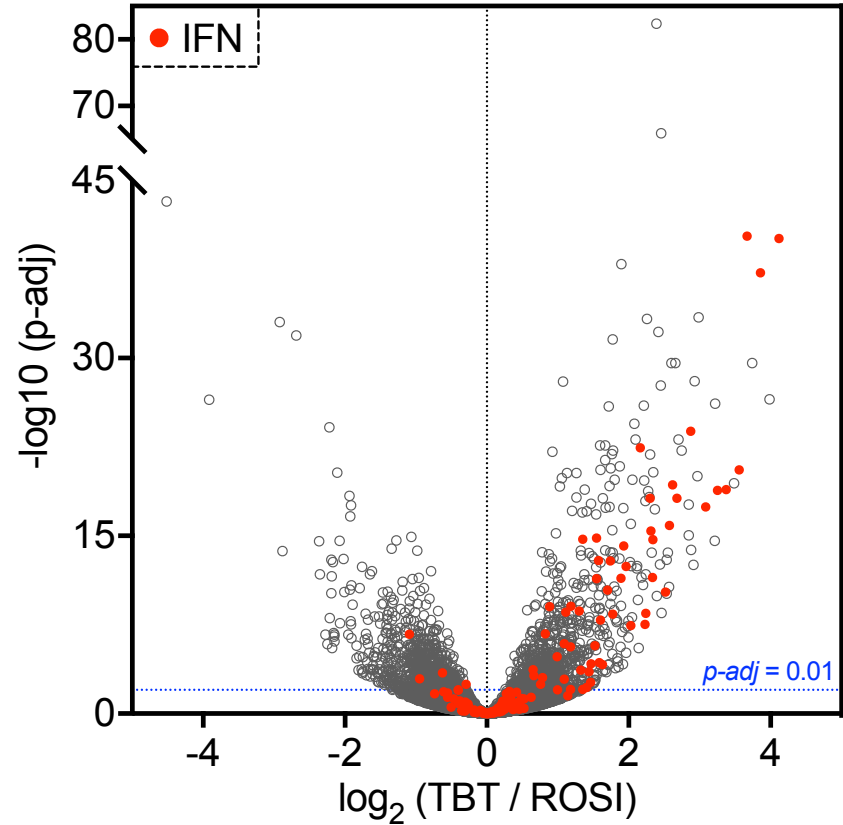


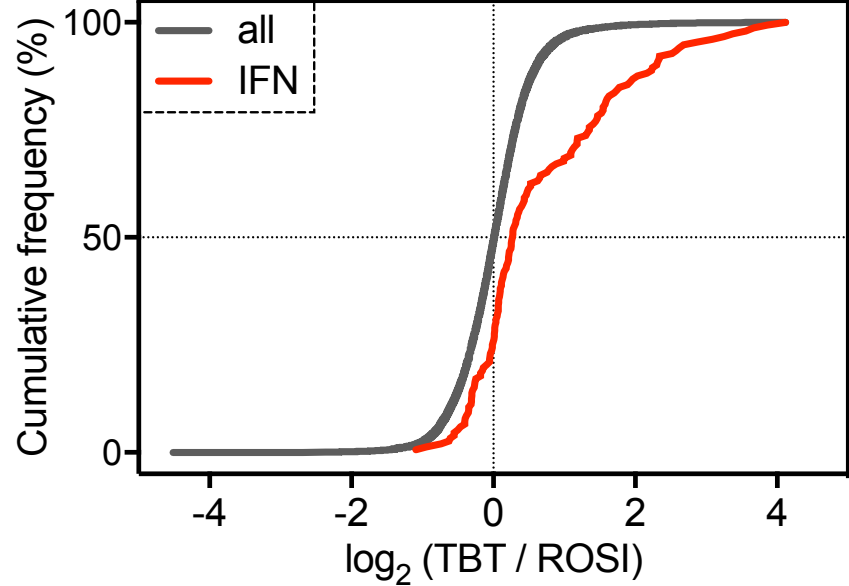
Figure S2.3. MSCs differentiated in the presence of TBT have highly enriched expression of genes involved in interferon signaling as compared to ROSI (Related to Figure 2.1). (A) Volcano plot of differential gene expression between TBT- and ROSI-treated MSCs. A manually curated list of genes either directly involved in interferon (IFN) signaling or well-characterized interferon-stimulated genes (ISGs) is highlighted in red (n = 152 genes). (B) Cumulative frequency graph of $\log_2(\text{fold-change})$ of TBT versus ROSI samples.

SUPPLEMENTAL FIGURE 2.3

A.



B.



SUPPLEMENTAL FIGURE 2.4

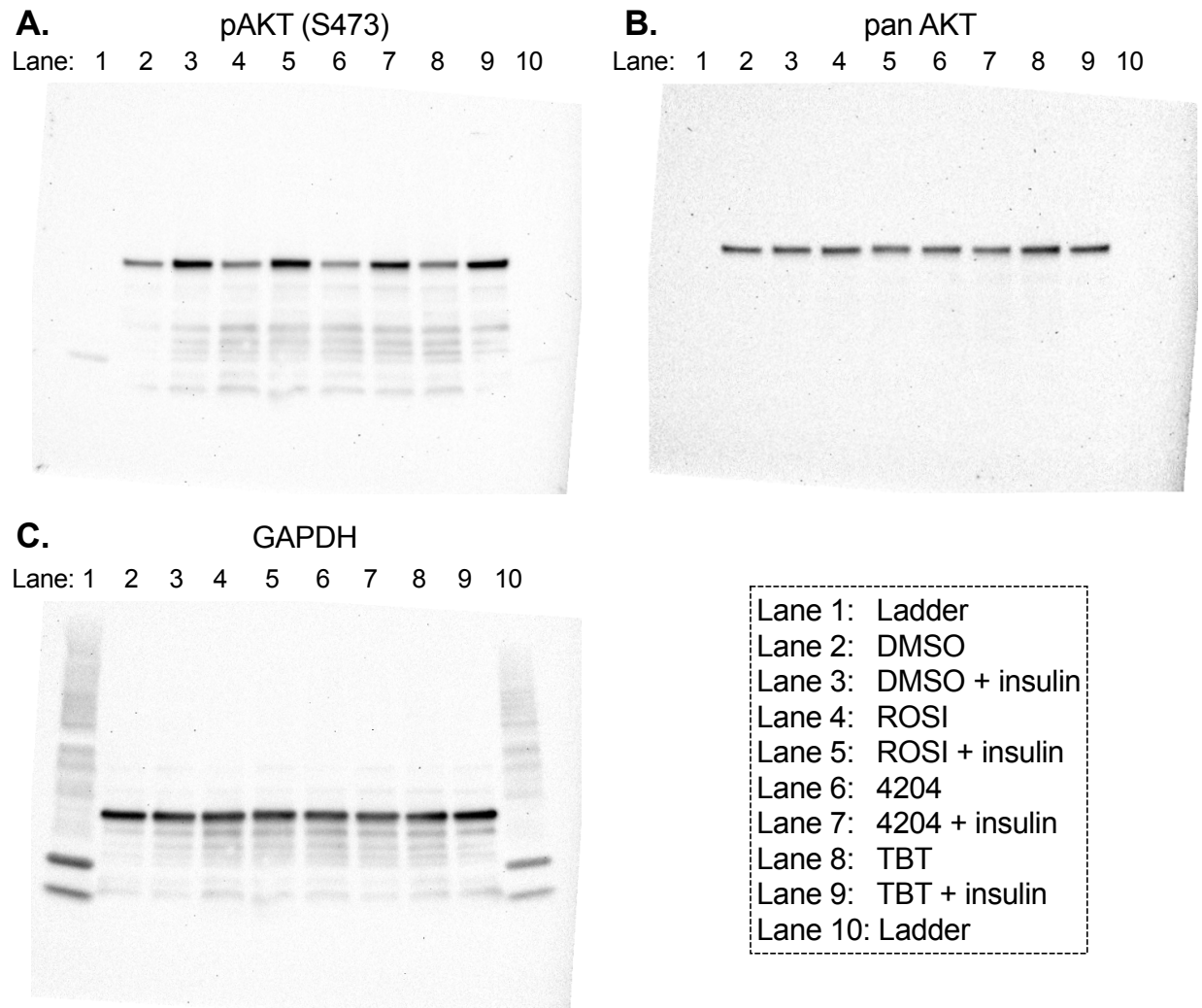


Figure S2.4. Full-screen images of western blots analysis of insulin signaling proteins (Related to Figure 2.3D). Western blots were prepared and imaged as described in the Materials and Methods. Raw blot images of (A) phospho-AKT, (B) pan-AKT, and (C) GAPDH. All images are from the same blot that was stripped and reprobed twice (see Methods) in the order presented (A → B → C).

SUPPLEMENTAL FIGURE 2.5

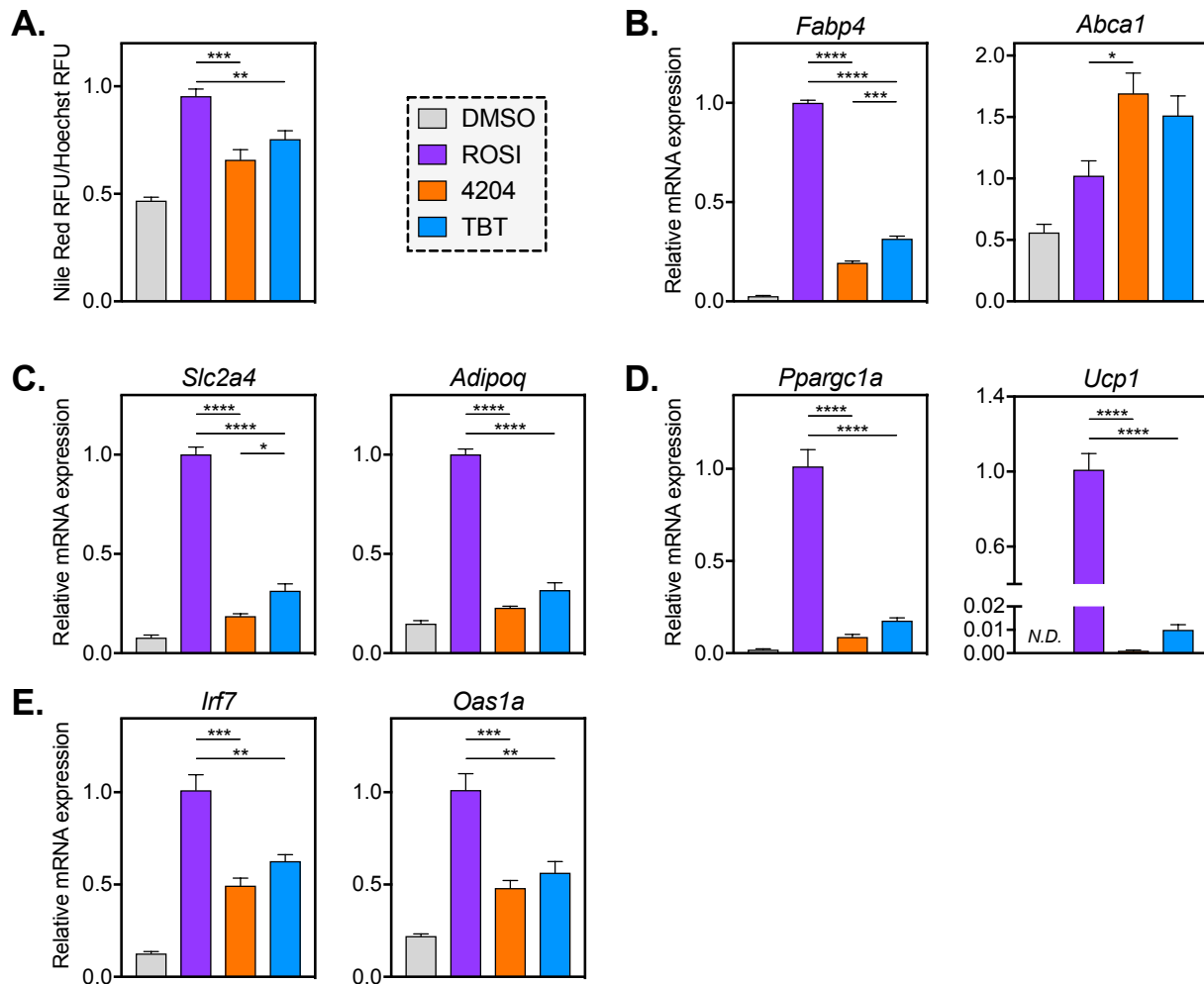


Figure S2.5. Reginoid treatment is less adipogenic than ROSI in stromal vascular fraction, but still reveal striking differences in gene expression of BAT markers (Related to Figure 2.6). SVF was isolated from the inguinal fat depots of female mice and differentiated into adipocytes as described in the Materials and Methods in the presence of T₃ and ROSI (500 nM), 4204 (100 nM), TBT (50 nM), or vehicle control (0.1% DMSO). After 6 days of differentiation, cells were either (A) fixed and stained to assess lipid accumulation or (B-E) lysed for RNA extraction and subsequent analysis of gene expression. (A) Fixed MSCs were stained for neutral lipids (Nile Red) and nuclei (Hoechst 33342). Nile Red fluorescence was normalized to Hoechst for each well. QPCR analysis of (B) canonical PPAR γ and RXR targets, (C) markers of adipocyte function, (D) BAT markers, and (E) IFN genes. For ROSI, 4204, and TBT samples: 1-way ANOVA, Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data are represented as the mean $\pm$ s.e.m. *N.D.* - not detected. s

SUPPLEMENTAL FIGURE 2.6

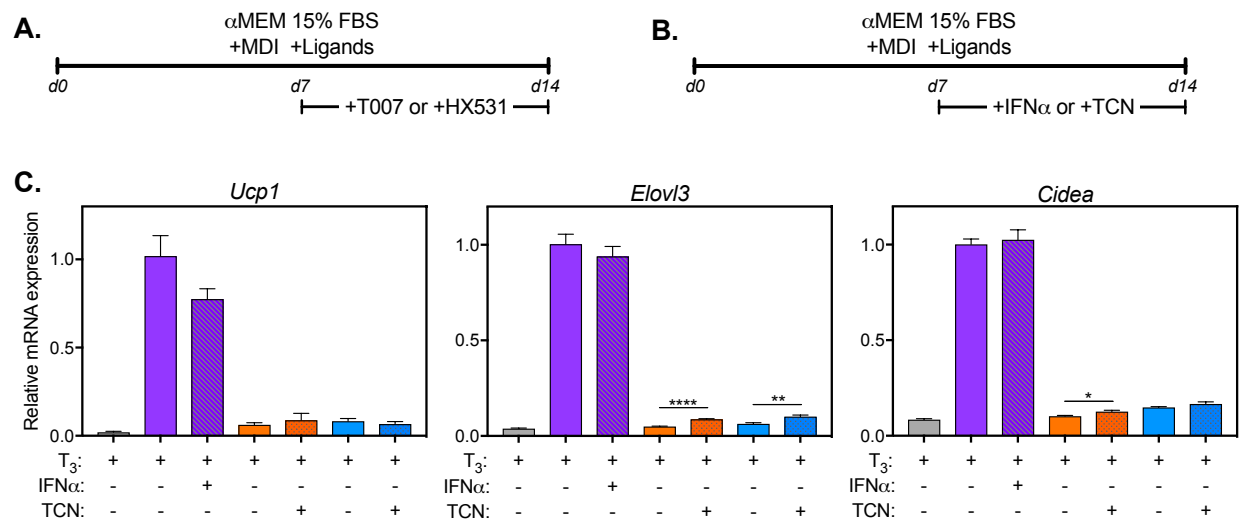


Figure S2.6. Mechanistic assays to assess the role of PPAR γ /RXR or IFN signaling in rexinoid-differentiated MSCs (Related to Figure 2.7). (A-B) Assay formats for mechanistic studies of differentiating MSCs. MSCs were differentiated into adipocytes in the presence of MDI and ligands (500 nM ROSI, 100 nM 4204, 50 nM TBT, 0.1% DMSO) for 7 days. (A) On day 7 of differentiation, chemical antagonists of PPAR γ (T0070907, 1 μ M) or RXR (HX531, 10 μ M) were added to the culture medium through the end of differentiation. (B) For studies of IFN signaling, mouse recombinant IFN α (1000 U/mL) was added to ROSI samples and the JAK inhibitor TCN (5 μ M) was added to 4204/TBT samples. (C) Gene expression of BAT markers in cells treated with modulators of IFN signaling (see panel B). Unpaired t-test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. All data are represented as the mean $\pm$ s.e.m.

SUPPLEMENTAL TABLE 2.1

Gene	Forward primer (5' > 3')	Reverse primer (5' > 3')
<i>36B4</i>	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGCAGCAGTGGT
<i>Abca1</i>	GCTTGTTGGCCTCAGTTAAGG	GTAGCTCAGGCGTACAGAGAT
<i>Acox1</i>	TCGCAGACCCTGAAGAAATC	GCCCTGATTCAGCAAGGTAG
<i>Acta2</i>	GCACCACTCTTTCTATAACGA	GTCTCAAACATAATCTGGGTC
<i>Adipoq</i>	AACTTGTGCAGGTTGGATGG	GCCCTTCAGCTCCTGTCATT
<i>Ccl5</i>	GAGTATTTCTACACCAGCAGCA	GTATTCTTGAACCCACTTCTTCTC
<i>Ccl7</i>	AAGAGGAATCTCAAGAGCTACAG	GTATGCTATAGCCTCCTCGAC
<i>Ccl9</i>	TTCATACTGCCCTCTCCTTCC	TTTCAATTTCAAGCCCTTGCTG
<i>Cebpa</i>	ACAAGAACAGCAACGAGTACC	GGTCATTGTCACTGGTCAACT
<i>Cidea</i>	GAGTTTCAAACCATGACCGA	GCCAGTTGTGATGACTAAGAC
<i>Cidec</i>	CTGGAGGAAGATGGCACAAT	GGGCCACATCGATCTTCTTA
<i>Col1a1</i>	AGGTATGCTTGATCTGTATCTG	TTGGTGATACGTATTCTTCCG
<i>Col1a3</i>	TTCTTCTCACCTTCTTCATCC	CCAGACATCTCTAGACTCATAGG
<i>Cox8b</i>	AAGTTCACAGTGGTTCCCAA	AACGACTATGGCTGAGATCC
<i>Ctgf</i>	AATGACAATACCTTCTGCAGAC	CTCAAACCTGACAGGCTTGG
<i>Elovl3</i>	GTCTATCTGTTGCTCATCGT	CATAAACTTCCACATCCTCAG
<i>Ero1l</i>	TGCGAAATTGACGATATACAGTCC	CATAGATGACACTCCATATCCTCC
<i>Fabp4</i>	GTCACCATCCGGTCAGAGAG	TCGACTTTCCATCCCCTTC
<i>Fasn</i>	GTGTCCAAGAAGTGCAGCAA	GGAGCGCAGGATAGACTCAC
<i>Gyk</i>	AATCCGCTGGCTAAGAGACAACT	CCTGAAAATGCTGGAACGAAGTA
<i>Ifit2</i>	TGAGTACAACGAGTAAGGAG	CACTGTTCTGAAACTCATCC
<i>Inhba</i>	GAACGGGTATGTGGAGATAGAG	CTTGGAATCTCAAAGTGCAG
<i>Irf7</i>	ATTTTCGGTCGTAGGGATCTG	GGCAGGTTAACTCCACTAGG
<i>Irf9</i>	CTTCAAGACCACCTACTTCTG	ATGAGATTCTCTTGGCTATGG
<i>Lipe</i>	ACGCTACACAAAGGCTGCTT	TCGTTGCGTTTGTAGTGCTC
<i>Lpl</i>	ACAACCAGGCCTTCGAGATT	TCAGGCCAGCTGAAGTAGGA
<i>mt-Co1</i>	TGCTAGCCGCAGGCATTAC	GGGTGCCCAAAGAATCAGAAC
<i>Ndufv1</i>	CTTCCCCACTGGCCTCAAG	CCAAAACCCAGTGATCCAGC
<i>Oas1a</i>	CAAGCTGAAGAGTCTCATCC	ATCCATTCCCTTGTTCCCAG
<i>Pck1</i>	CTGGCACCTCAGTGAAGACA	TCGATGCCTTCCAGTAAAC
<i>Pnpla2</i>	CACTCACATCTACGGAGCCT	CTTGGACACCTCAATAATGTTGG
<i>Ppara</i>	TTTCCCTGTTTGTGGCTGCTA	CCCTCCTGCAACTTCTCAATG
<i>Pparg2</i>	TGGGTGAAACTCTGGGAGATTC	AATGCGAGTGGTCTTCCATC
<i>Ppargc1a</i>	GGAGCTGGATGGCTTGGGACAT	TTTCGAGGCTCATTGTTGTACTGGT
<i>Ppargc1b</i>	TTGGCAAGAAGAGCTTTGAG	CATTGGCTTGTATGGAGGTG
<i>Prdm16</i>	GAGGTGTCATCCCAGGAGAG	CACGGATGTACTTGAGCCAG
<i>Slc2a4</i>	AGCTGGTGTGGTCAATACGG	AGAAGGCCACAAAGCCAAAT
<i>Stat1</i>	TCCCGTACAGATGTCCATGAT	TGACTTTACTGTCCAGCTCCTTC
<i>Stat2</i>	CACGACCACCTGAAGGATCT	TTCACTGAAGCGCAGTAGGA
<i>Tnc</i>	TTTACCACAGACCTCGATTCC	TACCATCCACAGATTCATAGACC
<i>Ucp1</i>	CAAAGTCCGCCTTCAGATCCAA	CCACACCTCCAGTCATTAAGCC
<i>Zfp423</i>	TGGACCACCGTGATCTTACA	TCGTTCTCGAACGTCATCTG

CONCLUSION OF THE DISSERTATION

The Endocrine Disruptor Tributyltin Acts Through Retinoid X Receptor to Promote Adipose Lineage Commitment and Perturb Mature Adipocyte Function

This dissertation investigated the roles of RXR in mediating the obesogenic and metabolism-disrupting effects of TBT. In **Chapter 1**, we showed that TBT acts through RXR to commit undifferentiated MSCs to the adipose lineage, in part through reductions in the repressive H3K27me3 histone mark near critical adipogenic genes (Shoucri, 2017). In **Chapter 2**, we showed that activation of RXR during adipogenesis forms a dysfunctional adipocyte that struggles to perform basic adipocyte functions. These studies have set the stage for future research *in vitro* and *in vivo* to further characterize the molecular and physiologic mechanisms through which developmental exposure to rexinoids—environmental or pharmacologic—program obesity and metabolic disease.

Future studies of MSC adipose lineage commitment by rexinoids

Genomic analyses of MSCs exposed to TBT in the commitment assay are ongoing. Presently, we are broadening our analysis of MSCs following pretreatment (Day 0) to include DNA methylation, ATAC-Seq, and Hi-C. By integrating these data with the RNA-Seq and H3K27me3 data presented in **Chapter 1**, we hope to understand how broader changes in chromosome structure are related to epigenetic marks (e.g. DNA methylation and H3K27me3) and transcription. Hi-C and promoter-capture Hi-C analysis of 3T3-L1 cells suggests that overall chromatin architecture is stable during adipogenesis, but there are extensive modifications of chromatin interactions between promoters and *cis*-regulatory elements (Siersbaek et al., 2017).

These authors found that promoter interaction was associated with the recruitment of coactivators, corepressors, and several transcription factors known to play roles in early 3T3-L1 cell differentiation, including RXR (Siersbaek et al., 2017). Hence, we are anxious to investigate how TBT or rexinoids alter chromatin structure during adipogenesis in our commitment assay model. It is possible that there may not be significant changes in nuclear organization following pretreatment (Day 0), rather, changes in structure may only become apparent during subsequent differentiation. Hence, we would eventually like to extend our genomic analyses to include Days 1, 3, 7, and 14. This would shed light on what TBT-induced alterations at Day 0 persist as these cells differentiate into mature adipocytes. Furthermore, performing ChIP-Seq of PPAR γ and RXR would clarify what changes in chromatin structure, architecture, and gene expression are nuclear receptor-dependent. Given that RXR also regulates gametogenesis (Froment, 2008; Mark et al., 2009), future studies will take a similar approach to investigating if RXR mediates the transgenerational effects of TBT through epigenetic alterations of developing primordial germ cells.

Given our *in vitro* data showing that TBT commits MSCs to the adipose fate, we would like to translate our findings into *in vivo* models of adipogenesis. Several reporter lines have been created to study adipogenesis and adipocyte turnover in mice (Hudak et al., 2014; Jiang et al., 2014; Tang et al., 2008; Wang et al., 2013). The majority of these mouse models are designed for use in adult mice, where the adipose depot is maintained by a pool of progenitors that reside in the vasculature (Tang et al., 2008). Importantly, these adult progenitors are distinct from developmental progenitors that generate the depot during the perinatal period (Jiang et al., 2014). Both progenitor pools, however, are committed *in utero* (Jiang et al., 2014). Hence, to study the role of RXR and its ligands on adipose development, it is necessary to study both progenitor pools. Sul and colleagues were able to employ *Preadipocyte factor 1* (*Pref-1* a.k.a. *Dlk1*) reporter mice to visualize adipose progenitors as early as E10.5 (Hudak et

al., 2014). This reporter mouse could be used to visually track developmental progenitors in embryos prenatally exposed to ROSI, 4204, or TBT. The AdipoTrak reporter mice developed by Graff and colleagues could serve as a useful tool to assess how prenatal exposure to different ligands alters the vascular ‘adult’ progenitor pool later in life (Jiang et al., 2014; Tang et al., 2011; Tang et al., 2008). Either of these reporter mice can be subsequently crossed with heterozygous *Rxra*^{+/-} mice to assess the RXR-dependence of any observed phenotypes. Finally, reporter mice allow for cell sorting, which would facilitate the sort of genomic analyses discussed above to be employed in an *in vivo* model.

Future studies of developmental rexinoid exposure and adipocyte dysfunction

Rexinoid exposure during adipogenesis was shown to result in functional deficits in developed adipocytes (see **Chapter 2**). Glucose uptake, adipokine release, intracellular lipolysis, and mitochondrial consumption (Seahorse Mito Stress Test) were all assessed in differentiated MSCs. While intracellular lipolysis was measured in the absence and presence of a pan- β adrenergic receptor agonist isoproterenol (**Figure 2.6B**), we did not evaluate mitochondrial consumption following sympathetic activation. Treatment with a mitochondrial uncoupler (FCCP) displayed that rexinoid-differentiated MSCs were not able to respire oxygen at the level ROSI-differentiated cells could achieve (**Figure 2.6D**). However, treatment with isoproterenol or a β_3 -selective agonist would better model sympathetic activation of non-shivering thermogenesis in adipocytes. This can be easily achieved on the Seahorse platform by injecting a β agonist following measurement of basal respiration and prior to the standard three Mito Stress Test injections (oligomycin, FCCP, rotenone/antimycin A). In this way, the ability of MSCs to uncouple respiration from ATP synthesis in response to sympathetic input can be quantified.

We hypothesized in the discussion of **Chapter 2** that the adipose dysfunction induced by rexinoids is a result of RXR promiscuity, which aberrantly disperses RXR and/or nuclear receptor cofactors throughout the genome (**Figure i**). Moving forward, we are testing this hypothesis by performing ChIP of RXR, PPAR γ , and LXR in MSCs differentiated in the presence of ROSI, 4204, TBT, or vehicle control. We are

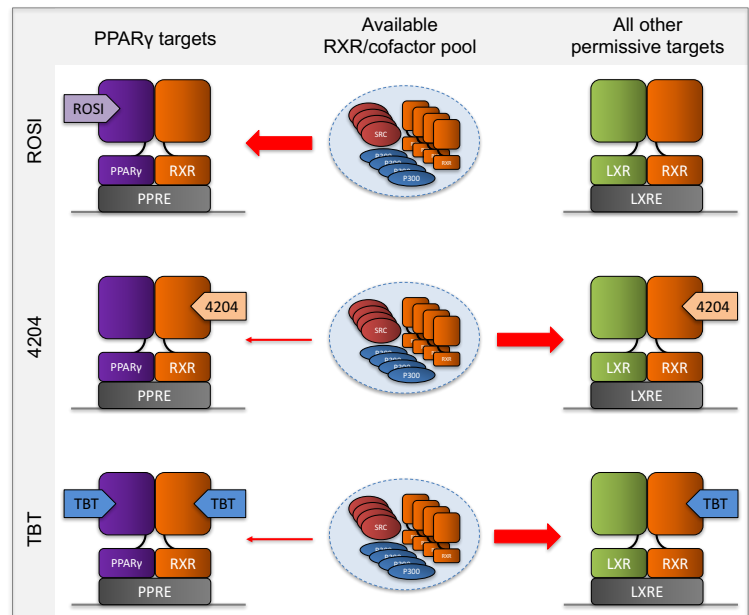


Figure i. Model for rexinoid-induced disruption of adipose development and function. Selective PPAR γ agonists (ROSI) effectively recruit available RXR and cofactors to PPAR γ target genes, whereas rexinoids (4204 and TBT) disperse these limited proteins to permissive RXR partners throughout the genome.

beginning this process by quantifying (by QPCR) nuclear receptor binding at known regulatory elements of canonical target genes (e.g. *Fabp4* and *Abca1*). If our hypothesis is correct, rexinoids (4204 and TBT) should reduce PPAR γ /RXR occupancy at PPAR γ target genes (*Fabp4*) and increase occupancy at targets of other permissive heterodimers, such as LXR (*Abca1*). Alternatively, if nuclear receptor occupancy remains relatively unchanged by ligand treatments, then perhaps cofactor recruitment (e.g. p300 or SRC-1) is shunted away from PPAR γ targets by rexinoids. In addition to our current hypothesis, there is data implicating LXR as a putative mediator of the adverse effects rexinoids have on thermogenesis (Korach-Andre et al., 2011) and adipokine secretion (Zheng et al., 2014). Hence, in parallel with our ChIP study, chemical modulators of LXR can be employed to test what role this permissive heterodimer plays in the mis-programming of rexinoid-treated adipocytes. To start, we can test whether the 'healthy' effects of ROSI are blocked by an LXR agonist (GW3965) and if the adverse effects of 4204 and TBT are rescued by an LXR antagonist (GSK2033). These data could further inform

our ChIP study by revealing important transcriptional targets of LXR that are engaged by rexinoids to program adipose dysfunction.

As mentioned in **Chapter 2**, a recent study implicates RXRs as master regulators of brown and beige fat development and activation (Nie et al., 2017). This investigation began with a small molecule screen that identified the RXR agonist bexarotene as a browning agent in the C2C12 myoblast cell line, since brown fat (unlike white or beige/brite fat) is derived from the myogenic lineage (Rosen and Spiegelman, 2014). Notably, the doses of bexarotene (10 μ M *in vitro*, 50 mg/kg/day *in vivo*) used are non-selective, as they also activate RAR (Gottardis et al., 1996), which is known to promote browning (Alvarez et al., 1995; Kiefer et al., 2012; Wang et al., 2017). In a pilot experiment, we found that 100 nM bexarotene produced similar morphology and lipid accumulation as is seen with the same doses of 4204 and LG268, while high-dose bexarotene (10 μ M) strongly inhibited adipogenesis, potentially through activation of RAR. Moving forward, we would like to see if we can rescue adipogenesis in high-dose bexarotene treatment with an RAR antagonist (AGN193109). If we can prove RAR-dependence *in vitro*, then we might set out to investigate whether the thermogenic effects of bexarotene *in vivo* rely on RAR activation.

Does perinatal RXR exposure program obesity and/or metabolic disease in adulthood?

Finally, we wish to integrate our findings that rexinoids promote both adipose lineage commitment and adipose dysfunction by exposing mothers to environmental (TBT, fludioxonil) and pharmaceutical (4204, LG268) rexinoids throughout pregnancy and lactation. Adipose depots are specified as early as E14.5 (inguinal) and as late as P15 (mesenteric) (Jiang et al., 2014), hence exposure from conception through lactation should influence the commitment of all depots. As discussed above, both developmental and adult adipose progenitors are specified *in utero* (Jiang et al., 2014), so rexinoid exposure is hypothesized to influence adipose function

across the lifespan. Following *in utero* exposure, committed and uncommitted progenitor cells and adipocytes can be isolated from the stromal vascular fraction of different adipose depots (Rodeheffer et al., 2008). These can be analyzed for relative abundance and gene expression by QPCR or RNA-Seq.

One pillar of the DOHaD paradigm is that disease-associated exposures *in utero*—the “first hit”—often must be followed by a “second hit” later in life in order to manifest as phenotypic expression of disease (Boone-Heinonen et al., 2015). This is often seen in the study of EDCs, where exposure-dependent phenotypes are only revealed upon dietary challenge in adulthood (Heindel et al., 2017). For this reason, we would like to challenge mice exposed to rexinoids perinatally with a Western diet (high fat, high sugar) in adulthood, tracking body composition and metabolic parameters throughout the experiment, both before and after the diet challenge. Given data showing that high-fat diet promotes adipogenesis within visceral adipose depots (Jeffery et al., 2015; Jeffery et al., 2016), we would hypothesize that mice exposed to rexinoids perinatally would produce a greater number of less functional adipocytes in response to the diet. Finally, following the diet challenge, we would like to further challenge a subset of mice by exposing them to the cold. One week of cold exposure (4-6°C) will result in a proliferation of beige adipocytes into inguinal adipose depots (Berry et al., 2017; Jiang et al., 2017; Lim et al., 2012). Given our *in vitro* data, we would predict that early-life rexinoid exposure would interfere with the ability of progenitor cells to form functional beige adipocytes.

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