METABOLIC REGULATION AT THE INTERSECTION

OF GENES AND ENVIRONMENT

by

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ABSTRACT

The incidence of complex metabolic disease has risen to an alarming level in the last several decades. This elevated frequency has been accompanied by increased social and financial costs, with nearly $250 billion spent each year on diabetes alone. Despite this growing health crisis, only a small percentage of the heritable risk for these disorders has been identified. Possible sources of this missing heritability include gene-environment interactions and gene-gene interactions, as well as the influence of parental or grandparental metabolism. In this work, I have focused on characterizing potential sources of these effects and the impacts they may have on physiology.

The deacetylase Sir2 is a conserved metabolic regulator whose influence in normal and pathological physiology has been well documented but little understood. In characterizing *Drosophila sir2* mutants, I discovered that loss of *sir2* leads to progressive defects in carbohydrate and lipid homeostasis as well as the development of insulin resistance and glucose intolerance. I found that these functions of Sir2 are localized to the fat body and partially restore metabolic function by overexpressing the nuclear receptor dHNF4. Finally, I found that dHNF4 acetylation and stability is altered in *sir2* mutants, suggesting that this factor is a key and direct target for Sir2 in the maintenance of metabolic flexibility.

In the second part of this work, I focus on the development of both dietary and genetic paradigms to induce metabolic dysfunction in the parental generation that can

lead to heritable physiological defects in their progeny. I found that either method of altering parental metabolic state can induce heritable changes in offspring metabolism under both basal and challenge conditions for at least two generations. I also identified key sources of genetic and environmental variation that influence the degree of parental dysfunction as well as the degree of physiological responses in the progeny. These studies lay the groundwork for more careful characterization of progeny responses, the molecular pathways affected in these progeny, as well as the mechanisms by which these changes are inherited.

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CHAPTER 1

INTRODUCTION

The incidence of complex metabolic disorders has risen to an alarming level over the last several decades. Obesity rates have doubled since 1980, with nearly 2 billion overweight individuals worldwide, 600 million of whom are obese (World Health Organization, 2015). In no place is this exemplified more than in the United States, where more than 35% of adults and 17% of children under 18 are obese (Ogden et al., 2012). Furthermore, diabetes is becoming a major healthcare crisis, existing both independent of and as a complication from obesity. It is estimated that nearly 30 million adults have either type I or type II diabetes, although more than a quarter of these cases remain undiagnosed. Additionally, almost 40% of adults over 20 are prediabetic (Centers for Disease Control and Prevention, 2014). Undiagnosed and therefore untreated diabetes is often accompanied by potentially deadly complications such as high blood pressure, elevated low density lipoprotein (LDL) cholesterol, blindness, and kidney disease. Coupled with diabetic hyperglycemia, some of these risk factors can elevate the incidence of cardiovascular disease and its inherent complications (CDCP, 2014). All combined, the direct and indirect costs from diabetes in 2012 totaled $245 billion. At $176 billion, even the direct medical costs of diabetic individuals are more than twice the medical expenditures of people without diabetes (CDCP, 2014).

With the high financial and social costs of diabetes, it is imperative to find a way

to treat these disorders at early stages before complications arise. Treatment of at-risk, asymptomatic individuals may be the key to stemming this epidemic by preventing metabolic disease in the first place. Early detection requires a better understanding of the genetic and environmental risk factors that contribute to disease onset as well as the interactions between these factors.

Genetic and environmental factors influence metabolic state

Diet and exercise are the two major environmental contributors to the development of metabolic diseases. The introduction of a Western-style diet into developing countries has been linked to increases in the incidence of obesity and diabetes (Hu, 2011; Johnson et al., 2014). The all-too-common accompaniment of this type of high calorie diet with a more sedentary lifestyle appears to be the critical combination that supports the development of complex metabolic disorders (Hu, 2011; Johnson et al., 2014).

These major sources of environmental variation, however, are joined by other, more subtle influences on metabolic health, the impacts of which are less well understood. Air quality, viral infections, and smoking are just a subset of the environmental variables that can impact an individual's risk for metabolic disorders and their complications (Chen et al., 2016a; Chen et al., 2016c; Fagard and Nilsson, 2009; Izumi et al., 2015; Nilsson et al., 2009; Ross et al., 1976). Furthermore, the degree of this impact can vary from person to person based on the interaction of these environmental variables with the genotype of any given individual.

Extensive work has gone into identifying genetic risk factors for metabolic disease, which make up the heritable component of disease risk. Genome-wide

associations studies (GWAS) have explained approximately 10% of the heritability of type II diabetes by identifying risk alleles common in the population (Billings and Florez, 2010; Imamura and Maeda, 2011). Unfortunately, these alleles that contribute to metabolic health are often too rare in the population to be detected by GWAS (Sanghera and Blackett, 2012). Furthermore, metabolic disease is so complex that each individual allele may only make a small contribution to the overall risk of any given subject. As a result, a large portion of the heritability of the metabolic syndrome remains unknown (Billings and Florez, 2010; Imamura and Maeda, 2011; Sanghera and Blackett, 2012).

In the past two decades, effort has been put toward the study of gene-environment interactions as the source of this missing heritability (Sanghera and Blackett, 2012). It is hypothesized that certain risk alleles that are neutral or even beneficial under particular environmental conditions may be detrimental under an alternative set of conditions. Gene-gene interactions could also contribute to the missing heritability, wherein a particular allele of one gene could alter the phenotype of an allele in another gene from neutral to detrimental (Cordell, 2009). These interactions could take the form of physical interactions between gene products, contributions to the same cellular pathways, or influences of one gene product on the expression of another.

Metabolic regulators influence transcription by altering epigenetic state

Transcription factors play a central role in coordinating environmental and genetic influences on metabolic health. These regulators respond to changes in the environment by altering the transcription and metabolic state of the cell. Changes in the levels of particular components of metabolic pathways can alter the flux through these pathways,

increasing or decreasing the synthesis of particular metabolites and allowing the cell to adapt to its current nutritional environment.

One example of this is seen with the Foxo transcriptional activator, which is retained in the cytoplasm of hepatocytes under fed conditions by differential phosphorylation and acetylation. Upon fasting, however, Foxo is de-phosphorylated and transported into the nucleus, where it activates target genes that include components of the target of rapamycin (TOR) and gluconeogenic pathways (Alic et al., 2011; Salih and Brunet, 2008; Zhang et al., 2006).

Another example is the temporally regulated *Drosophila* Estrogen Related Receptor (ERR). Approximately halfway through embryogenesis, dERR is activated posttranscriptionally and the protein localizes to the nucleus where it contributes to the upregulation of many of the genes known to be involved in glycolysis (Tennessen et al., 2011). This primes the embryo for the rapid growth it must undergo once it hatches as a larvae, when it must increase its size nearly 200-fold before puparium formation (Church and Robertson, 1966). Larval metabolism is therefore largely dependent on aerobic glycolysis for adenosine triphosphate (ATP) production, allowing glycolytic intermediates to be used for biosynthetic purposes (Tennessen et al., 2011).

The types of metabolic regulators described above act by binding open promoter or enhancer regions and then guiding the transcriptional machinery to the promoter. Others regulators can actively alter the chromatin in these regions. Modifiers of this sort often have enzymatic activities associated with histone methylation, acetylation, or other posttranslational modifications. These modifications can either open up or restrict access to regions of chromatin in order to alter the access of transcriptional activators to DNA.

Activating marks include H3K4 methylation and acetylation, while repressive marks include H3K9 methylation and H3K27 methylation (Braunstein et al., 1993; Du et al., 2015; Greer and Shi, 2012). The degree of methylation (mono-, di-, or tri-) and interactions with concurrent histone modifications also influence the access of additional chromatin modifying enzymes, such as DNA methyltransferases, all of which determines the activation state of that chromatin region (Du et al., 2015; Greer and Shi, 2012).

Regulators with H3K4 methyltransferase activity (such as Set 1 in the Trithorax complex) or acetyltransferase activity (p300/CREBP) are associated with open chromatin and transcriptional activation, while those associated with H3K9/K27 methyltransferase activity (Su(var)3-9, EZH2 in the Polycomb complex) or deacetylase activity (sirtuins, HDACs) are associated with closed chromatin and transcriptional repression (Blander and Guarente, 2004; Krauss, 2008; Lanzuolo et al., 2012; Sadoul et al., 2008; Xu et al., 2015). These modifying enzymes directly impact transcriptional activation by altering the access of other transcriptional activators to enhancers and promoters, modifying the levels of gene products.

Sirtuins regulate global metabolism by histone deacetylation

One family of these modifying enzymes, the sirtuins, plays an important role in the regulation of metabolism at both cellular and organismal levels. The founding member of the sirtuin family, *sir2*, was originally identified in yeast as an important part of the heterochromatin-forming machinery at chromosome telomeres and mating type loci (Braunstein et al., 1993; Ivy et al., 1986; Rine and Herskowitz, 1987; Shore et al., 1984). A histone deacetylase, Sir2 contributes to the formation of constitutive heterochromatin in concert with other Sir proteins as well as the silencing of

transcriptionally active regions throughout the genome (Moynihan et al., 2005; Newman et al., 2002; Pruitt et al., 2006; Smolik, 2009; Sun et al., 2007).

The sirtuins comprise a large family of deacetylases that have been highly conserved and expanded in mammals (Blander and Guarente, 2004). The fruit fly *Drosophila melanogaster* has five sirtuins, where the gene *dsir2* is most closely related to yeast *sir2*, whereas mammals have seven sirtuins, where the gene *Sirt1* is most closely related to *sir2* (Chalkiadaki and Guarente, 2012; Frye, 2000; Guarente, 2013; Houtkooper et al., 2012). According to protein Basic Local Alignment Seach Tools (BLAST), *Drosophila* Sir2 has approximately 43% identity and 60% similarity with yeast Sir2, and 52% identity and 65% similarity with human Sirt1. The human Sirt1 protein is also highly conserved, with about 41% identity and 56% similarity to yeast Sir2 (Altschul et al., 1990). This high degree of sequence conservation suggests that sirtuins have a high degree of functional conservation as well.

The activity of sirtuins is dependent upon the electron carrier NAD^+. Deacetylation consumes NAD^+, converting it into nicotinamide and 2'-O-acetyl-ADP-ribose (Figure 1.1A) (Blander and Guarente, 2004). NAD^+ also acts an indispensable electron carrier in a number of central metabolic pathways such as glycolysis and the tri-carboxylic acid (TCA) cycle (Canto et al., 2015). In these reactions, NAD^+ is reduced to NADH as the carbons from glucose are oxidized. The electrons carried by reduced NADH are transferred to the electron transport chain in order to fuel oxidative phosphorylation and the production of ATP (Canto et al., 2015). As a result, the NAD^+ pool is itself dependent on the energetic state of the cell, as is the activity of enzymes, such as the sirtuins, that consume NAD^+.

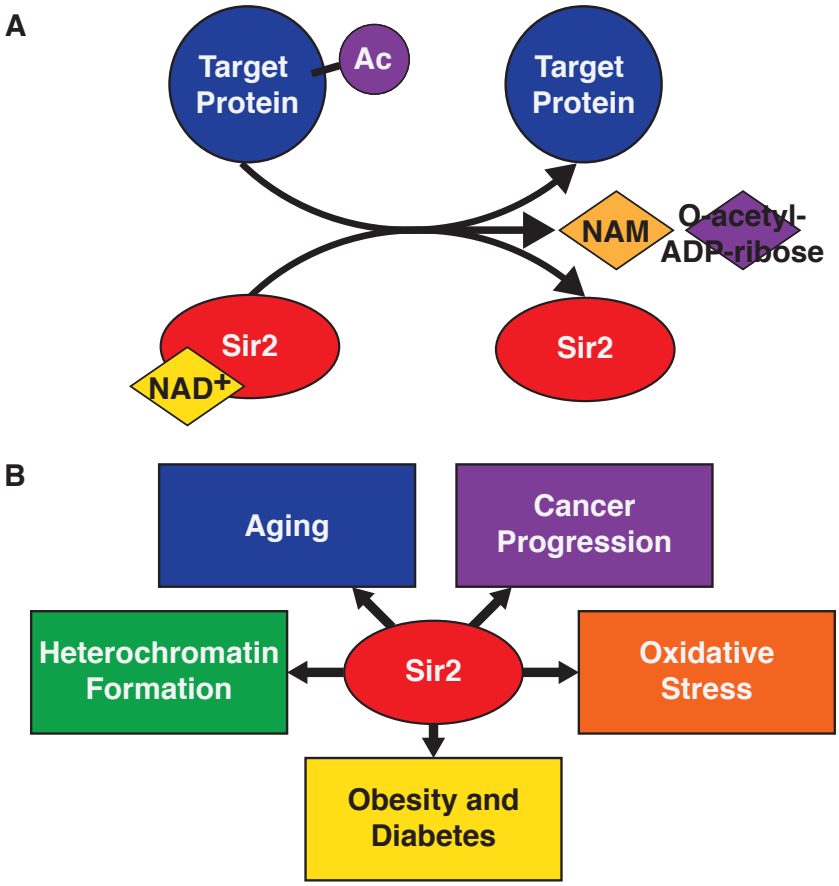

Figure 1.1 The deacetylase Sir2 regulates metabolic processes
(A) The family of sirtuin deacetylases depends upon NAD^+ to deacetylate their target proteins. During the reaction, NAD^+ is converted into nicotinamide (NAM) and 2'-O-acetyl-ADP-ribose. (B) Sirtuin proteins have been implicated in a number of complex metabolic processes. The overlapping and interacting pathways involved in the processes make it difficult to define the precise mechanism by sirtuins regulate any one process alone.

This link between the nutritional status of the cell and the activity of sirtuins supports the possibility that this connection could be used to alter the transcriptional state of the cell in response to nutritional status through changes in sirtuin activity. Consistent with this, the activity of sirtuins in multiple model systems has been associated with a number of complex processes linked to metabolic function. Sirtuins have been found to play a role in cancer progression, the response to oxidative stress, aging, and development of metabolic disorders, in addition to its known roles in the formation of

heterochromatin (Figure 1.1B) (Blander and Guarente, 2004; Boutant and Canto, 2014; Canto et al., 2015; Chalkiadaki and Guarente, 2012; Chang and Guarente, 2014; Houtkooper et al., 2012; Li, 2013; Nogueiras et al., 2012).

Importantly, SNPs in human *sirt1* have been associated with obesity, Type 1 diabetes, and autoimmune diseases, raising the possibility that the associated alleles contribute to these metabolic disorders (Biason-Lauber et al., 2013; Clark et al., 2012; van den Berg et al., 2009). One of these *sirt1* alleles has been associated with a reduction in insulin secretion from the pancreatic beta cells as well as a reduction in insulin sensitivity in peripheral muscle fibroblasts (Biason-Lauber et al., 2013). Mammalian studies in rodents lend further support to a role for sirtuins in modulating normal metabolic homeostasis. Loss of *sirt1* is embryonic lethal in some genetic backgrounds. When mutants for *sirt1* survive to adulthood, however, they develop symptoms associated with severe metabolic dysfunction (Boily et al., 2008; McBurney et al., 2003). These results have been supported by studies in *Drosophila*, although the mechanisms through which sirtuins maintain metabolic homeostasis remain unclear (Banerjee et al., 2012, 2013; Reis et al., 2010).

Some effort has been made to link the metabolic defects in *Sirt1* mutants with their role in modifying histones. For example, increasing *sirt1* expression in cultured myotubes is associated with the downregulation of the protein tyrosine phosphatase *ptp1b*, while conditions that are associated with loss of *sirt1* also lead to increased levels of Ptp1b. This phosphatase is known to act on the insulin receptor, reducing its activity and therefore reducing the insulin sensitivity of cells. The link between Sirt1 expression and its deacetylase activity with the expression of *ptp1b* suggests that Sirt1 directly

regulates *ptp1b* transcription by histone deacetylation in the regulatory regions of that gene, although this has not been directly shown (Sun et al., 2007).

Similar results were found in another study focusing on pancreatic beta cells. When *sirt1* is specifically overexpressed in pancreatic beta cells, mice have improved insulin secretion and sensitivity when they are challenged with high glucose. This over-expression is also associated with decreased expression of uncoupling protein 2 (*ucp2*). Furthermore, expression of siRNAs against *sirt1* in cell culture is associated with increased *ucp2* expression. As this protein uncouples the activity of the electron transport chain in the mitochondria from the generation of ATP, it is associated with a reduction in glucose-stimulated insulin secretion. Once again, however, this study was correlative and while it suggests that the Sirt1 impacts *ucp2* expression by directly deacetylating its regulatory sequences, this hypothesis was not tested (Moynihan et al., 2005).

Sirtuins regulate global metabolism by deacetylation of multiple transcription factors

Although histones were the first identified target for Sirt1-mediated deacetylation, this enzyme can also target a large number of additional proteins, including metabolic regulators. The transcription factors HNF4α, PPARα, PPARγ, several of the Foxo proteins, and LXR have all been identified as targets of Sirt1 in mammals (Brunet et al., 2004; Kauppinen et al., 2013; Li et al., 2007; Picard et al., 2004; Yang et al., 2009). Deacetylation of transcription factors can lead to changes in nuclear localization, cofactor binding affinity, target recognition, or stability, all of which alter the activity of these factors, thereby altering the transcriptional and thus the metabolic state of the cell.

Foxo3 is one example of altered target recognition upon deacetylation by Sirt1.

Deacetylated Foxo3 has increased affinity for targets associated with cell cycle arrest and oxidative stress resistance and decreased affinity for targets associated with cell death (Brunet et al., 2004). Other studies have suggested that acetylation of Foxo at lysines targeted by sirtuins sequesters it in the cytoplasm, preventing transcription of Foxo target proteins (Frescas et al., 2005). Deacetylation of the transcriptional coactivator PGC-1α alters its ability to bind the transcription factor HNF4α, and in this manner alters the activation of targets dependent upon this interaction (Pfluger et al., 2008). Similarly, deacetylation of LXR by Sirt1 is associated with transcriptional activation of LXR targets because of an activating ubiquitination mark on the previously acetylated lysine (Li et al., 2007).

Modulation of histone modifications by sirtuins could also indirectly regulate transcription factor target specificity. A few studies have, in turn, associated these changes with shifts in the activity of metabolic pathways (Moynihan et al., 2005; Sun et al., 2007). In these two instances, however, direct evidence of changes in histone acetylation levels was lacking. Additionally, there does not appear to be any DNA sequence that targets sirtuins to particular gene loci, preventing specific genes from being targeted as a prevalent mechanism of metabolic regulation outside of heterochromatic regions. In contrast, sirtuin-mediated modification of transcription factor activity appears to be a more likely mechanism by which these factors can modify metabolic state.

Evidence for transgenerational regulation of metabolism in humans

As discussed above, a large percentage of the risk for metabolic disease is attributable to unidentified inherited factors, some of which appears to be due to gene-environment and gene-gene interactions (Cordell, 2009; Sanghera and Blackett, 2012).

Over the course of the past few decades, another previously unexplored source of risk has been identified: the influence of parental and developmental nutritional environments on adult offspring, which can be sustained for several generations. This phenomenon has been observed in retrospective studies of human populations exposed to famine conditions in utero. The most famous and best controlled of these studies follows individuals conceived during a period of time known as the Dutch Hunger Winter (de Rooij et al., 2006; Lumey et al., 2009; Ravelli et al., 1999). Between October 1944 and May 1945, civilians in German-occupied Holland were exposed to severe caloric restriction due to strict rationing. Those exposed to the famine during fetal development, especially during the first trimester of gestation, were at a higher risk of obesity, diabetes, heart disease, and even cancer as compared to gender-matched siblings either conceived after the famine had ended or born before it began (de Rooij et al., 2006; Lumey et al., 2009; Ravelli et al., 1999). Similar observations have been made in other famine exposed populations in China and Leningrad (Li et al., 2010; Stanner et al., 1997). Furthermore, a dichotomy was found in the Chinese population, which followed individuals conceived from 1959-1961 in rural areas. Famine-exposed individuals who consumed a high calorie, western-style diet later in life were at increased risk for metabolic disease, while those who continued to consume a low calorie diet were not (Li et al., 2010).

These studies of maternal famine exposure are complicated by a combination of maternal nutritional exposure and gestational exposure during fetal development. Effects of either paternal or maternal diet for multiple generations, however, suggest that the nutritional environment of parents can indeed impact the metabolic state of offspring. The Overkalix studies followed cohorts born in northern Sweden in 1805, 1905, and 1920

(Bygren et al., 2001; Bygren et al., 2014; Kaati et al., 2002; Pembrey et al., 2014; Pembrey et al., 2006). In these cohorts, it was shown that overnutrition in the paternal grandfather at 9-12 years of age is associated with shortened lifespan and increased risk of diabetes in grandsons (Bygren et al., 2001; Kaati et al., 2002; Pembrey et al., 2006). Fluctuations in nutrient availability in paternal grandmothers during puberty are associated with shortened lifespan and increased risk of cardiovascular disease in granddaughters (Bygren et al., 2001; Bygren et al., 2014; Pembrey et al., 2006).

Common among these studies is the presence of a contrast between the parental and offspring nutritional environments, suggesting a unifying theory of transgenerational inheritance (Figure 1.2) (Lillycrop, 2011; Somer and Thummel, 2014). Exposure of an individual to an altered nutritional or metabolic environment leads to alteration in the epigenetic and transcriptional state in that individual's somatic tissues by virtue of the mechanisms discussed earlier. However, alterations are also transmitted to offspring conceived during this time through the germline, and these changes allow the offspring to be appropriately adapted to the parental environment. If instead, however, the offspring are exposed to an environment that differs from that of the parents, they are no longer adapted to that environment and are therefore susceptible to the development of metabolic disease (Lillycrop, 2011; Somer and Thummel, 2014).

Model systems for the study of transgenerational metabolism

Given the limitations of human studies, it is essential to use model systems to properly test the validity of transgenerational inheritance theories. A vast majority of these studies have focused on the use of rodent dietary models in the maternal parents. While the results have varied between studies, the general conclusions have been

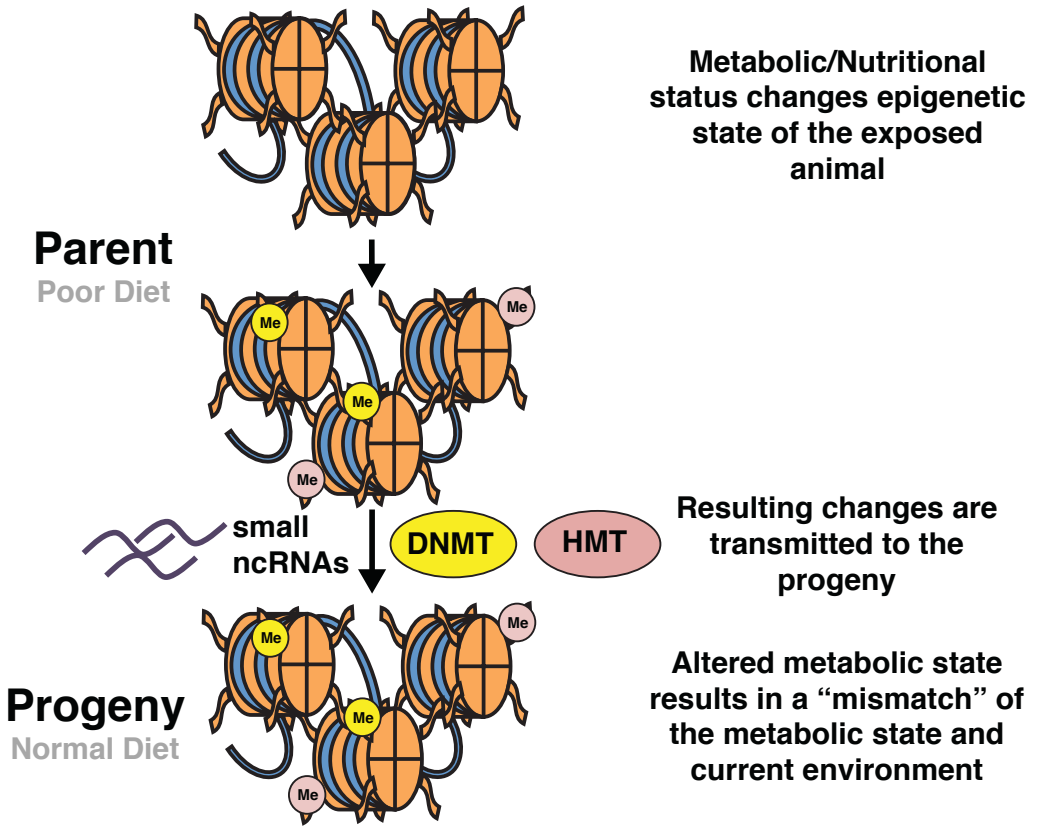

Figure 1.2. Model for the epigenetic transmission of metabolic state
A change in nutritional environment of the parental generation alters the epigenetic state of their chromatin and the transcriptional state of their cells, both in somatic tissues and in the germline. These changes can be transmitted to the progeny, setting up a metabolic state that is best adapted to the parental environment, such that any alternative environment could result in the development of disease phenotypes. DNA methylation, histone modifications, or small RNAs may be involved in the transmission of these nongenetic signals. Originally published in Somer and Thummel (2014), Figure 1.

consistent. Exposure of mothers during gestation to a low protein diet leads to an increased risk of obesity, diabetes, and cardiovascular disease accompanied by changes in hepatic gene expression, circulating and stored lipid levels, and changes in DNA methylation patterns for at least one and, in some cases, two generations (Bellinger et al., 2004; Bellinger et al., 2006; Burdge et al., 2011; Burdge et al., 2004; Duque-Guimaraes and Ozanne, 2013; Hoile et al., 2011; Jimenez-Chillaron et al., 2009; Langley-Evans, 2001; Lillycrop, 2011; Lillycrop et al., 2005; Lucas et al., 1996). Occasionally these

molecular and physiological defects have been linked to altered expression and promoter methylation of metabolic regulators such as *PPARα* and the glucocorticoid receptor (Burdge et al., 2011; Lillycrop et al., 2005). In these cases, enhancing the maternal diet with excess folate restores the expression and function of these transcription factors, suggesting a link between the methylation of genes in the progeny and the maternal diet (Lillycrop et al., 2005). Interestingly, mutation of a gene involved in folate metabolism leads to developmental defects in progeny for up to two generations after the mutant alleles are removed from the genetic background (Padmanabhan et al., 2013). However, no direct link has been made between DNA methylation in parental gametes and that in the adult progeny, suggesting that these epigenetic modifications are a secondary effect to the transmission of some other inherited factor.

While maternal paradigms such as those described above are confounded by the environmental impact of the diet on fetal development, paternal paradigms largely reduce fetal exposure to the parental condition. Most of these paradigms limit paternal exposure to the female; some go so far as to use in vitro fertilization to implant embryos of exposed and control fathers in the mothers (Carone et al., 2010; Chen et al., 2016b; Sharma et al., 2016). Importantly, the results of these studies have largely paralleled those from the maternal paradigms (Carone et al., 2010; Jimenez-Chillaron et al., 2009; Sharma et al., 2016). A paternal low protein diet is linked to increased triglycerides and reduced expression of *PPARα* in the livers of daughters. As previously reported, this reduced expression is associated with increased methylation at a potential *PPARα* enhancer region (Carone et al., 2010). Once again, however, the increase in DNA methylation at loci in the progeny genome is not correlated with detectable methylation

changes in the paternal gametes, suggesting that this is a secondary effect in the offspring and not a primary cause of transgenerational inheritance. Treatment of fathers with a high fat diet has also been associated with hyperglycemia, glucose intolerance, insulin resistance, and altered gene expression in metabolic tissues such as the pancreatic islets of offspring (Chen et al., 2016b; Fullston et al., 2013; Ng et al., 2010).

A few studies have used genetic approaches to alter parental metabolism rather than a dietary paradigm (Nelson et al., 2010; Padmanabhan et al., 2013; Yazbek et al., 2010). As described above, mutation of an enzyme in the folate metabolic pathway leads to an increased incidence of birth defects for several generations after the mutations have been outcrossed (Padmanabhan et al., 2013). Grandmaternal expression of a variant associated with low body weight and improved insulin sensitivity is associated with low body weight and improved insulin sensitivity in F2 progeny of F1 males as compared to controls, giving a grandmaternal/paternal-specific pattern of inheritance (Yazbek et al., 2010). These two studies exemplify the use of genetic paradigms to induce transgenerational effects.

Despite the progress made in rodent models, transgenerational phenotypes are still difficult to study in mammalian systems due to the time and expense of multigenerational studies that require a large number of progeny to identify subtle defects. In contrast, the fruit fly *Drosophila melanogaster* is a tractable organism for this type of study, with its short generation time, large number of progeny produced per cross, and extensive tools for studies of epigenetic mechanisms. Several groups have reported initial attempts to study this phenomenon using the fly as a model.

For two of these studies, female or male parents were exposed to a high sugar diet

known to induce hyperglycemia and insulin resistance (Buescher et al., 2013; Musselman et al., 2011; Ost et al., 2014). When the maternal parent was fed a high sugar diet, the F1 progeny were hyperglycemic during larval stages, hypoglycemic during adult stages, and obese upon challenge with the high sugar diet. Larval hyperglycemia persisted through the F2 generation (Buescher et al., 2013). When the paternal parent was fed the high sugar diet, adult F1 progeny were obese and hyperglycemic (Ost et al., 2014). The similarity of the results between maternal and paternal exposure suggests that a common mechanism may be responsible for these inherited effects, while the differences might be attributed to variations between strains and the timing of the high sugar diet exposure.

The effects of dietary protein have also been explored in *Drosophila* (Matzkin et al., 2013; Xia and de Belle, 2016). A low protein parental diet is associated with increased glycogen and reduced triglycerides in the F1 progeny, although the magnitude of these changes varies between different genetic backgrounds (Matzkin et al., 2013). Dietary protein content also appears to impact longevity and fecundity across generations. Compared with either a high or low protein-containing diet, F1 and F2 offspring descended from parents fed an intermediate protein-containing diet have longer lifespans without sacrificing fecundity. Those descended from parents fed a low protein diet, in contrast, have severely shortened lifespans and reduced fecundity as compared to offspring descended from parents fed either the high or intermediate protein-containing diets (Xia and de Belle, 2016). It therefore appears that dietary modulations of many kinds are capable of inducing intergenerational phenotypic responses.

Potential mechanisms of transgenerational inheritance

Several mechanisms have been proposed for the epigenetic inheritance of information across generations, but as of yet none has been proven. Prior to the last decade, the prevailing hypothesis focused on changes in DNA methylation in the germline (Figure 1.2). Preliminary studies identified altered patterns of DNA methylation in the progeny genome that correlated with changes in gene expression (Burdge et al., 2011; Burdge et al., 2004; Carone et al., 2010; Hoile et al., 2011; Lillycrop et al., 2005). Multiple attempts to prove this hypothesis, however, have instead demonstrated that DNA methylation is likely a secondary effect. DNA methylation patterns in parental gametes do not mirror those observed in progeny, and it has been suggested that these patterns are reset during embryogenesis (Carone et al., 2010; Shea et al., 2015). Additionally, traditional DNA methylation does not appear to occur in *Drosophila*, suggesting that a conserved mechanism must rely on another transmitted factor (Lyko et al., 2000). Direct histone modifications have also been hypothesized as a potential mechanism of inheritance, but the dynamic nature of these modifications makes this an unlikely way to transfer information across multiple generations (Hajkova et al., 2008; Hajkova et al., 2002; Lee et al., 2002). Such a mechanism would likely rely on the inclusion of modifying enzymes in the gametes to replace chromatin modifications at the correct loci (Figure 1.2) (Francis et al., 2009; Petruk et al., 2012).

Recent evidence supports a new model that involves the transmission of small noncoding RNAs through the germline. Multiple cases in *C. elegans*, *Drosophila*, and rodents support the ability of these molecules to transfer information for several generations. In both *C. elegans* and *Drosophila* there is evidence of piwi-RNAs or

piRNAs contributing to transposon and transgene silencing for several generations as well as for the transmission of RNAi to progeny in *C. elegans* (Castel and Martienssen, 2013; de Vanssay et al., 2012; Grentzinger et al., 2012; Shirayama et al., 2012). In rodents, evidence has arisen for the transmission of miRNAs and small tRNA fragments to offspring through the germline (Chen et al., 2016b; Kawano et al., 2012; Murashov et al., 2016; Sharma et al., 2016; Yuan et al., 2015). Changes in the expression of specific small tRNA fragments in response to parental diet have also been observed, and subsequently linked to hyperglycemia in the F1 progeny (Chen et al., 2016b; Sharma et al., 2016). However, while the evidence for small RNAs in transgenerational transmission of metabolic phenotypes is increasing, the data remains correlative and there is no explicit mechanism.

Thesis summary

In this dissertation, I present my studies of the epigenetic regulation of metabolic state, through the characterization of *sir2* mutants and of transgenerational effects. In Chapter 2, I describe a detailed phenotypic characterization of *sir2* mutants. This study shows that *sir2* mutants display progressive defects in carbohydrate and lipid homeostasis. They are hyperglycemic at one week of age, become insulin resistant and obese by two weeks of age, and glucose intolerant and starvation sensitive by three weeks of age. These defects are due to Sir2 functions in the fat body, regulating both insulin sensitivity and carbohydrate homeostasis. Furthermore, the insulin resistance that develops in *sir2* mutants is due to reduced stability and hyperacetylation of the nuclear receptor dHNF4. These results demonstrate that *Drosophila sir2* mutants can be used as a model for studying the mechanisms by which insulin resistance is initiated and

maintained, and to identify relevant Sir2 targets in the development of metabolic disease.

In Chapter 3, I explore dietary methods to induce transgenerational changes in metabolic state. Using an adult dietary conditioning paradigm, I show that a high protein parental diet is capable of inducing changes in stored metabolites for multiple generations. This includes changes in offspring basal metabolism as well as in their response to dietary challenge. These changes are influenced by both environmental and genetic variables. To address the impact of genetic variation on our studies, I generated a number of isogenized fly strains and showed that they respond differently to adult dietary challenge. Although this approach reduces some of the variability in our results, it was insufficient to provide reproducible significant changes in metabolites in offspring generations. It is likely that environmental variation beyond our control leads to inconsistencies that complicate the identification of molecular mechanisms for this phenomenon.

Finally, in Chapter 4, I introduce a novel genetic approach to induce parental metabolic changes and test for the effect on progeny. In this paradigm, mutation of the hormone receptor *AKHR* leads to obesity in both males and females. Although no significant changes in metabolite levels were detected in the heterozygous F1 generation due to phenotypes associated with haploinsufficiency, I was able to identify a reduction in triglyceride levels in the F2 generation. Interestingly, this defect is only observed in F2 progeny from obese grandfathers and heterozygous mothers, resulting from grandpaternal/maternal inheritance. This genetic model for altering parental metabolism provides a novel approach to study the transgenerational inheritance of metabolic phenotypes in *Drosophila*, which could allow us to identify a conserved mechanism for

this phenomenon.

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CHAPTER 2

SIR2 ACTS THROUGH HEPATOCYTE NUCLEAR FACTOR 4 TO MAINTAIN INSULIN SIGNALING AND METABOLIC HOMEOSTASIS IN *DROSOPHILA*

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RESEARCH ARTICLE

Sir2 Acts through Hepatocyte Nuclear Factor 4 to maintain insulin Signaling and Metabolic Homeostasis in *Drosophila*

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Abstract

SIRT1 is a member of the sirtuin family of NAD^+-dependent deacetylases, which couple cellular metabolism to systemic physiology. Although studies in mouse models have defined a central role for SIRT1 in maintaining metabolic health, the molecular mechanisms remain unclear. Here we show that loss of the *Drosophila* SIRT1 homolog *sir2* leads to the age-progressive onset of hyperglycemia, obesity, glucose intolerance, and insulin resistance. Tissue-specific functional studies show that Sir2 is both necessary and sufficient in the fat body (analogous to the mammalian liver) to maintain glucose homeostasis and peripheral insulin sensitivity. Transcriptional profiling of *sir2* mutants by RNA-seq revealed a major overlap with genes regulated by the nuclear receptor Hepatocyte Nuclear Factor 4 (HNF4). Consistent with this, *Drosophila* HNF4 mutants display diabetic phenotypes similar to those of *sir2* mutants, and protein levels for dHNF4 are reduced in *sir2* mutant animals. We show that Sir2 exerts these effects by deacetylating and stabilizing dHNF4 through protein interactions. Increasing *dHNF4* expression in *sir2* mutants is sufficient to rescue their insulin signaling defects, defining this nuclear receptor as an important downstream effector of Sir2 signaling. This study demonstrates that the key metabolic activities of SIRT1 have been conserved through evolution, provides a genetic model for functional studies of phenotypes related to type 2 diabetes, and establishes HNF4 as a critical downstream target by which Sir2 maintains metabolic health.

Author Summary

The sirtuins are a highly conserved family of deacetylases with targets that range from DNA-associated histones to transcription factors. The activities of these enzymes are dependent upon the energetic state of the cell as they utilize the coenzyme NAD^+, an important electron carrier in central metabolic pathways. We have found that loss of the *Drosophila* homolog of the founding member of the sirtuin family, *sir2*, leads to age-progressive metabolic disease with symptoms similar to those of type 2 diabetes. In addition, we show that the *Drosophila* HNF4 nuclear receptor is deacetylated and stabilized by Sir2,

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and that it accounts for a major part of the transcriptional program controlled by Sir2. This work provides a new genetic model of insulin resistance in *Drosophila* and establishes HNF4 as a critical downstream target in the Sir2 signaling pathway.

Introduction

The incidence of complex metabolic disorders has been on the rise for the past three decades, comprising an epidemic of ever-increasing severity. Much of this can be attributed to an increase in the prevalence of type 2 diabetes accompanied by insulin resistance, the development of which is complex and poorly understood. These trends have prompted widespread changes in public policy and a shift in biomedical research toward improving our understanding of the genetic and environmental factors that contribute to insulin resistance and its progression to a more severe disease state.

One focus for these studies has been the sirtuin family of NAD^+-dependent deacetylases, which play a central role in coupling metabolic state to systemic physiology. Sirtuin activity is dependent upon the availability of NAD^+, an important electron carrier that contributes to cellular redox balance, drives mitochondrial oxidative phosphorylation, and acts as an important enzymatic cofactor [1–4]. The founding member of the sirtuin family, yeast Sir2, was discovered based on its role in heterochromatin formation [5–7]. Subsequent studies of the mammalian Sir2 homolog, SIRT1, have defined it as a critical regulator of metabolic homeostasis, acting through multiple protein targets [1–4]. These include Foxo, the nuclear receptors PPARα and LXR, and co-activators such as PGC-1α [8–11]. The multiple downstream targets of SIRT1, combined with its dependence on NAD^+, establish it as a pivotal energy sensor that couples cellular redox state to metabolic control.

Given the complexity of SIRT1 regulation and function, it is not surprising that genetic studies of SIRT1 in mice have been complicated by environmental factors and genetic background effects, occasionally leading to contradictory results [1, 3]. This has been most evident in the context of aging, where the beneficial effects of SIRT1 remain controversial. In contrast, tissue-specific functional studies of SIRT1, combined with overexpression experiments and pharmacological activation, have established important roles for this factor in maintaining metabolic homeostasis [1, 3, 4]. These include a role in pancreatic beta-cells to promote glucose-stimulated insulin secretion (GSIS) and improve glucose tolerance, as well as activities in peripheral tissues that promote insulin sensitivity [12, 13]. SIRT1 also supports fatty acid oxidation and oxidative phosphorylation in the liver, suppresses hepatic steatosis, and acts in white adipose to suppress lipid accumulation [9]. Conversely, low-level SIRT1 overexpression promotes glucose tolerance, insulin sensitivity, and prevents fatty liver disease, highlighting the beneficial effects of SIRT1 action and supporting the proposal that SIRT1 activation could be of therapeutic value [14–16]. In spite of these advances, however, the molecular mechanisms by which SIRT1 maintains metabolic homeostasis remain unclear [2].

Studies of the *Drosophila* SIRT1 homolog, Sir2, have recently begun to provide a better understanding of its roles in systemic physiology. Null mutants for *sir2* display increased levels of stored lipid, analogous to the role of SIRT1 in suppressing obesity [17, 18]. Elevated free glucose levels were also observed in mutant adults, accompanied by starvation sensitivity [18]. Other metabolic functions for *sir2*, however, have been based on overexpression experiments and RNAi [18, 19]. One of these studies reported that glucose levels are reduced in animals with ubiquitous RNAi against *sir2*, contradicting their data from mutants [18]. The RNAi studies also resulted in only a two-fold reduction in *sir2* expression, leaving it unclear how these

results relate to gene function [18, 19]. These concerns, combined with the importance of genetic background on SIRT1 activities, led us to undertake a detailed metabolic analysis of a transheterozygous combination of *sir2* null alleles compared to genetically-matched controls. We show here that loss of *sir2* leads to the age-progressive development of obesity, hyperglycemia, glucose intolerance, and insulin resistance. Tissue-specific RNAi and genetic rescue experiments show that Sir2 function is both necessary and sufficient in the fat body to maintain insulin sensitivity. In addition, our studies show that Sir2 maintains insulin signaling through deacetylation and stabilization of the *Drosophila* ortholog of HNF4A, dHNF4. Sir2 interacts with dHNF4, dHNF4 levels are reduced in *sir2* mutants, and expressing wild-type dHNF4 restores insulin signaling in a *sir2* mutant background. Taken together, our results define dHNF4 as a key downstream target of Sir2 and provide insights into the molecular mechanisms by which Sir2 promotes insulin sensitivity and metabolic health.

Results

sir2 mutants develop age-progressive symptoms of diabetes

Two previously described deletion alleles of *sir2*, $sir2^{2A-7-11}$ and $sir2^{4.5}$, were used in transheteroallelic combination and compared to genetically-matched controls for all studies [20, 21]. As expected, *sir2* is not expressed in these mutants as assayed by RNA-seq or northern blot hybridization, consistent with their characterization as null alleles (S1A and S1B Fig). Unless otherwise indicated, adult male flies were used in all experiments, where the age indicated in the figures refers to the number of weeks after eclosion from the pupal case. The *sir2* mutants survive to adulthood and develop starvation sensitivity as previously reported (S1C and S1D Fig) [18, 21]. Basic metabolite measurements, however, reveal that they also display increasing metabolic dysfunction with age in the absence of significant effects on feeding rate (Figs 1 and S1E). At one week of age, *sir2* mutants have elevated levels of both free and circulating glucose as well as glycogen but no significant change in triglycerides (TAG) (Fig 1A–1C; S1F Fig). Elevated glucose and glycogen levels are still present at two weeks of age, but are also accompanied by elevated TAG, which is consistent with the increased lipid levels reported for *sir2* mutants (Fig 1D–1F) [17, 18]. In addition to this obesity, mutants at two weeks of age, but not one week, display fasting hyperglycemia, a hallmark of diabetes (Fig 1G and 1H). This is consistent with the results of metabolomic analysis of *sir2* mutants at two weeks of age, which revealed increased levels of glycolytic intermediates, including glucose-6-phosphate, dihydroxyacetone phosphate, and lactate (S2 Fig). Alternative glucose metabolites also increase significantly, such as the glucose alcohol sorbitol, which can accumulate to high levels in diabetics and may contribute to neuropathy and nephropathy [22].

An oral glucose tolerance test was used to determine if these age progressive defects in carbohydrate homeostasis can be accounted for by reduced peripheral glucose uptake. In this assay, male flies are fasted overnight and then allowed to consume 10% glucose for approximately one hour, after which they are transferred back to starvation media for either two or four hours. The kinetics with which they clear glucose from their systems is then monitored by performing glucose assays at each time point. The glucose levels in wild-type animals at both one and two weeks of age return to near fasting levels within two hours after glucose feeding (Fig 1I and 1J). Similarly, although *sir2* mutants at both two and three weeks of age are hyperglycemic after consuming glucose, they display relatively normal kinetics of subsequent glucose clearance at two weeks of age (Fig 1I). They are, however, clearly glucose intolerant by three weeks of age, as demonstrated by the continued high levels of glucose present after two hours of clearance on starvation media (Fig 1J). Taken together with our previous results, this indicates that *sir2* mutants display a progression of symptoms associated with a loss of glycemic control during early adulthood, from elevated levels of free glucose, to fasting hyperglycemia, to glucose intolerance.

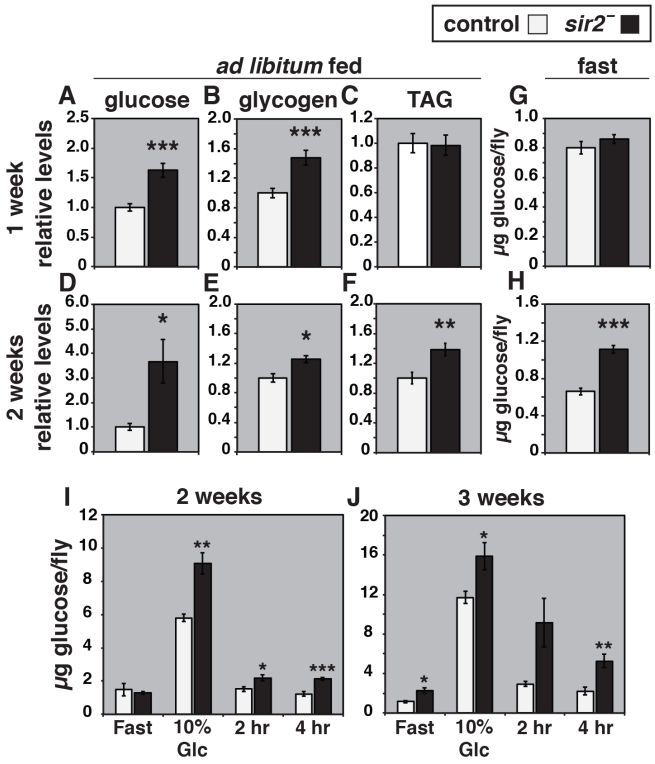

Fig 1. *sir2* mutants display age-dependent obesity and fasting hyperglycemia. Glucose (A,D), glycogen (B,E), and triglyceride (TAG) (C,F) levels were measured in control (white bars) and *sir2* mutants (black bars) at one (A-C) or two (D-F) weeks of age (n = 5–15 for each group). All values are normalized to soluble protein levels. (G-H) Glucose levels were measured in control (white bars) and *sir2* mutants (black bars) after an overnight fast, at one (G) or two weeks of age (H), and normalized to the number of animals (n = 37–43 for each group). An oral glucose tolerance test was performed on two (I) or three week (J) old controls (white bars) or *sir2* mutants (black bars). Animals were fasted overnight, fed on 10% glucose, re-fasted for either 2 or 4 hours, and free glucose levels were measured from whole animal homogenates (n = 5–10 for each group). Although *sir2* mutants do not display fasting hyperglycemia at two weeks of age in panel I, this is an exceptional result that was included because the overall profile of glucose clearance in this experiment best reflects our results from four independent replicates of this glucose tolerance test. The other three assays show fasting hyperglycemia, as depicted in panel H. Error bars are ± SEM. *p<0.05, **p<0.005, ***p<0.0005.

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Insulin signaling defects arise in *sir2* mutants due to a loss of insulin sensitivity

The development of diabetic phenotypes in *sir2* mutants with age could arise from a defect in peripheral insulin signaling. To determine if this is the case, we measured the levels of phosphorylated AKT (P-AKT), a downstream target of the insulin receptor, by western blot analysis

of extracts from control and *sir2* mutants using a fasting/refeeding paradigm. While *sir2* mutants at one week of age respond normally to feeding by increasing their P-AKT levels, this response is reduced by two weeks of age and almost completely absent by three weeks of age (Fig 2A–2C). Consistent with this result, the Foxo target *4EBP* is incompletely repressed upon feeding in *sir2* mutants as compared to controls at two weeks of age (S3A Fig).

A decrease in insulin signaling could be due to a defect in either insulin sensitivity or insulin secretion. As expected, both controls and *sir2* mutants at one week of age have increased P-AKT in response to injected insulin, consistent with the activation of insulin signaling in response to dietary glucose (Fig 2A and 2D). In contrast, while control flies at two weeks of age continue to show increasing levels of P-AKT with increasing concentrations of injected insulin, *sir2* mutants fail to respond (Fig 2E). This indicates that *sir2* mutants are insulin resistant by two weeks of age.

We also measured secreted levels of *Drosophila* insulin-like peptide 2 (DILP2) in control and *sir2* mutants to determine if reduced DILP2 secretion could contribute to their defects in insulin signaling [23]. This study revealed that circulating DILP2 increases with age in both fasting and fed controls, and increases approximately two-fold in response to feeding (Fig 2F and 2G; S3B and S3C Fig), consistent with published wild-type responses [23]. Similar responses were seen in *sir2* mutants under these conditions at both one and two weeks of age (Fig 2F and 2G; S3B and S3C Fig). Taken together, these results indicate that defects in peripheral insulin sensitivity, but not insulin secretion, can account for the reduced insulin signaling in *sir2* mutants.

Sir2 acts in the fat body to maintain insulin sensitivity and metabolic homeostasis

The GAL4/UAS system was used to determine where Sir2 is necessary and/or sufficient to regulate metabolic homeostasis using tissue-specific RNAi and rescue experiments. Ubiquitous expression of a *sir2* RNAi construct efficiently eliminates *sir2* mRNA as assayed by northern blot hybridization, indicating that this approach provides a strong loss of *sir2* function (S4A Fig). Driving the expression of this construct in the fat body, but not the muscles, intestine, insulin producing cells (IPCs), or AKH-producing cells, disrupts insulin signaling and leads to hyperglycemia (Fig 3A and 3B). Consistent with this, tissue-specific expression of a wild-type *UAS-sir2* construct in the fat body of *sir2* mutants is sufficient to restore insulin signaling in peripheral tissues, with no rescue seen upon expression of *sir2* in the muscles or IPCs (Fig 3C). In addition, expression of *sir2* in the fat body, but not the muscle or IPCs, is sufficient to rescue the obesity of mutant animals, as reported previously (S4B Fig) [18]. Moreover, GAL4-driven expression of *sir2* in the fat body of wild-type animals is sufficient to reduce TAG levels, consistent with previous reports of *SIRT1* overexpression in mice (S4C Fig) [24]. These results define a central role for Sir2 in the fat body to regulate insulin signaling and suppress obesity and hyperglycemia. Given that the fat body performs functions analogous to the mammalian liver and white adipose tissue, these results are consistent with *Sirt1* studies in mice and suggest the *Drosophila* provides a valuable model to determine the molecular mechanisms by which this sirtuin promotes a healthy metabolic state.

Sir2 regulates metabolic gene expression

As a first step to define the mechanisms by which Sir2 maintains metabolic homeostasis, we conducted RNA-seq analysis using quadruplicate RNA samples from control and *sir2* mutants at two weeks of age. A total of 400 genes were identified as differentially expressed in *sir2* mutants ($\geq$1.5-fold change, p-value <0.05), with 312 genes down-regulated and 88 genes up-

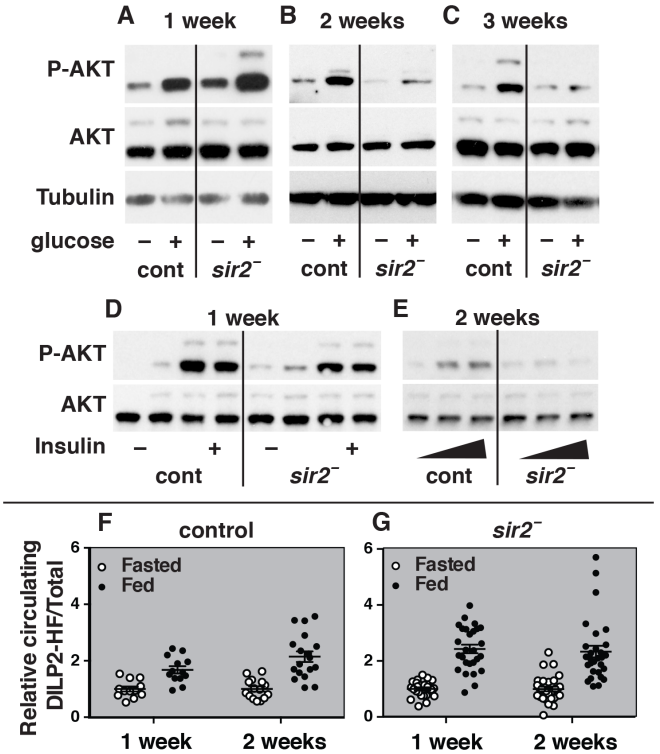

Fig 2. Loss of peripheral insulin signaling in *sir2* mutants is due to defects in insulin sensitivity. (A-C) Protein isolates from control (cont) and *sir2* mutant males (*sir2* –) were analyzed on western blots using antibodies directed against phosphorylated AKT (P-AKT), total AKT, or Tubulin as a control. Animals were collected at one, two, or three weeks of age following a fasting-refeeding paradigm (–/+ glucose). The ratio of P-AKT levels to total AKT levels in refed controls and *sir2* mutants was quantified using data from three independent experimental replicates. The fold change between these ratios in mutants and controls is as follows, representing the mean ± SEM: (A) one week of age 0.8±0.3 (NS), (B) two weeks of age 0.2±0.06 ($p = 0.005$), (C) three weeks of age 0.1±0.08 ($p = 0.008$). (D,E) Insulin tolerance tests were performed at one (D) or two weeks (E) of age by comparing P-AKT levels after injection of bovine insulin (–/+ insulin, D; increasing insulin, E) following a fast. The ratio of P-AKT levels to total AKT levels in injected controls and *sir2* mutants was quantified using data from two biological replicates for each insulin concentration. The fold change between these ratios in mutants and controls is as follows, representing the mean ± SEM. (D) One week of age (- insulin) 3.3±1.5, (+ insulin) 0.7±0.01, two-way ANOVA between genotypes across insulin concentrations (NS). (E) Two weeks of age with increasing insulin concentrations, 1.0±0.6, 0.2±0.1, 0.3±0.2, two-way ANOVA between genotypes across insulin concentrations ($p<0.05$). (F,G) The amount of circulating DILP2 in controls (open circles) or *sir2* mutants (black circles) was determined using an ELISA assay following a fasting-refeeding paradigm. Each data point represents a single biological replicate (n = 10 flies/sample, n = 12–30 samples/group). Data is normalized to the fasting state for each experiment and presented as a scatter plot with the mean ±SEM indicated. Quantification of the fold-change in secreted DILP2 levels from the fasted to fed state is: one week controls: 1.0±0.09 to 1.7±0.1, two week controls: 1.0 ±0.2 to 2.2±0.2, one week *sir2* mutants: 1.0±0.07 to 2.5±0.2, two week *sir2* mutants: 1.0±0.08 to 2.7±0.4. In both controls and *sir2* mutants, there is a significant increase in DILP2 secretion in fed versus fasted animals ($p<0.0001$), but not between one and two weeks of age, as determined by two-way ANOVA. *sir2* mutants at one week of age have a stronger induction of DILP2 secretion in response to feeding than do controls ($p = 0.008$), while at two weeks the difference is not significant as determined by two-way ANOVA.

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regulated (S1 Table). Gene ontology analysis revealed that the down-regulated genes are enriched in pathways related to the metabolic defects in *sir2* mutants, including proteolysis, lipolysis, carbohydrate metabolism, and genes involved in redox homeostasis, while many up-regulated genes are involved in *Drosophila* defense responses (S5 Fig) [25]. This could be analogous to the known role for Sirt1 in suppressing adipocyte inflammation and could contribute to the fat body-specific functions for *sir2* [26]. In addition, genes that are expressed at high levels in the intestine are enriched in the Sir2 down-regulated gene set, suggesting that this factor plays an important role in this tissue [27].

Because transcription factors are prominent targets of Sirt1 regulation, we compared our RNA-seq dataset from *sir2* mutants with similar datasets for *Drosophila* transcription factors that control metabolism and insulin signaling. A small, but significant overlap is seen with genes regulated by the LXR homolog DHR96 in adults (15% of the 136 DHR96-regulated genes; Fig 4A), consistent with the known associations between Sirt1 and LXR [11, 28]. Similarly, we saw a significant overlap between genes that are expressed at reduced levels in *sir2* mutants and genes that increase their expression in *foxo* mutants (18% of the 312 *sir2*-down-regulated genes; Fig 4B) [29]. This is consistent with the decreased insulin signaling in *sir2*

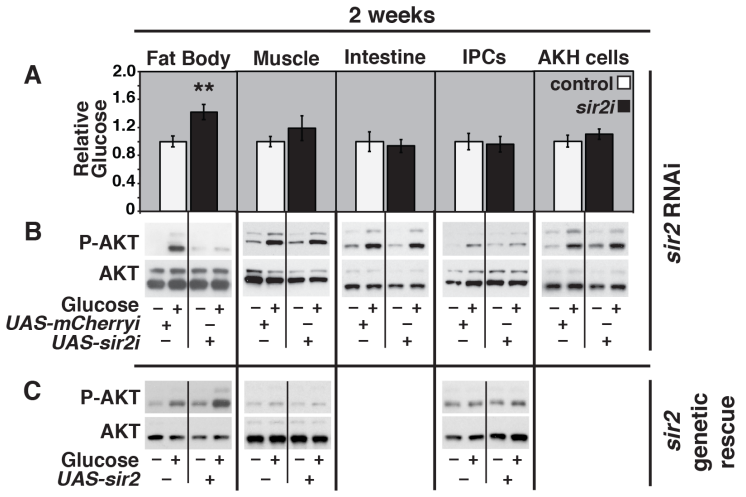

Fig 3. Sir2 acts in the fat body to regulate glucose homeostasis and insulin sensitivity. (A,B) Tissue-specific UAS-driven RNAi was directed against *mCherry* as a control (control, white bars) or *sir2* (*sir2i*, black bars) using the following GAL4 drivers: *r4-GAL4* (fat body), *mef2-GAL4* (muscle), *mex-GAL4* (intestine), *dilp2-GAL4* and *UAS-dcr2* (IPCs), or *AKH-GAL4* (AKH-producing cells). (A) Glucose levels were measured in two week old animals of each genotype and normalized to protein levels (n = 6–21 for each group, 5 males/sample). mean ± SEM is depicted. (B) Protein isolates from two week old animals of each genotype were analyzed on western blots using antibodies directed against phosphorylated AKT (P-AKT) or total AKT, following a fasting-refeeding paradigm (–/+ glucose). The ratio of P-AKT levels to total AKT levels in refed controls and *sir2* RNAi animals was quantified using data from three independent experimental replicates, except for *mef2-GAL4*, which had two replicates. The fold change between these ratios in *sir2* RNAi and control animals is as follows, representing the mean ± SEM: *r4-GAL4* 0.2±0.1 (p = 0.02), *mef2-GAL4* 1.7±0.5 (NS), *mex-GAL4* 0.9±0.2 (NS), *dilp2-GAL4>UAS-dcr2* 1.6±0.6 (NS), and *AKH-GAL4* 1.3±0.2 (NS). (C) Protein isolates were prepared from two week old *sir2* mutants carrying the following GAL4 drivers: *r4-GAL4* (fat body), *mef2-GAL4* (muscle), or *dilp2-GAL4* (IPCs), in either the absence or presence of a *UAS-sir2* rescue transgene (–/+ *UAS-sir2*). Equal amounts of protein were analyzed on western blots using antibodies directed against phosphorylated AKT (P-AKT) or total AKT, following a fasting-refeeding paradigm (–/+ glucose). The ratio of P-AKT levels to total AKT levels in refed controls (GAL4 driver/+ in $sir2^{2A\text{-}7\text{-}11/4.5}$ mutants) and rescued *sir2* mutants (GAL4 driver/+, *UAS-sir2*/+, $sir2^{2A\text{-}7\text{-}11/4.5}$) was quantified using data from two independent experimental replicates. The fold change between these ratios in *sir2* rescue and controls is as follows, representing the mean ± SEM: *r4-GAL4* 2.1±0.06 (p = 0.04), *mef2-GAL4* 0.9±0.2 (NS), *dilp2-GAL4* 0.9±0.1 (NS). **p<0.005.

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mutants as well as the known interactions between mammalian Sirt1 and Foxo [8]. Most remarkably, however, we saw a major overlap with genes regulated by the dHNF4 nuclear receptor, where more than 30% of the genes down-regulated in *sir2* mutants are also down-regulated in *dHNF4* mutants and nearly 60% of the genes up-regulated in *sir2* mutants are up-regulated in *dHNF4* mutants (Fig 4C and 4D) (W. Barry and C.S Thummel, manuscript in revision). This observation suggests that dHNF4 represents a major downstream target for Sir2 regulation in *Drosophila*.

Sir2 interacts with dHNF4 and promotes its deacetylation

The simplest explanation for the large overlap between the genes regulated by *sir2* and *dHNF4* is that dHNF4 protein levels are reduced in *sir2* mutants. This is indeed the case as assayed by western blot, with an approximately 3-fold reduction in protein levels by two weeks of age, accompanied by a 1.7-fold reduction in *dHNF4* mRNA, with more mild effects in younger flies (Fig 4E; S6A Fig). Mammalian HNF4A can be regulated by acetylation, and lysines that are targets for this modification are conserved in *Drosophila* (S6B Fig) [30]. Consistent with this, when FLAG-tagged dHNF4 is immunoprecipitated and the levels of this protein are equalized between *sir2* mutants and controls, there is a 3-fold increase in the proportion of immunoprecipitated protein that is acetylated in *sir2* mutants (Fig 4F). Sir2 protein is also present in this immunoprecipitate, indicating that these factors interact physically (Fig 4F). Taken together, these results support the model that Sir2 interacts with dHNF4 to direct its deacetylation and maintain its stability.

Expression of *dHNF4* in *sir2* mutants is sufficient to rescue insulin signaling

The reduced levels of dHNF4 protein in *sir2* mutants combined with the large overlap between the dHNF4 and Sir2-regulated gene sets suggests that Sir2 stabilizes and promotes the function of dHNF4. Consistent with this, ectopically increasing the levels of dHNF4 protein by crossing two copies of a genomic dHNF4-GFP-FLAG transgene into the *sir2* mutant background is sufficient to restore normal insulin signaling responses in these animals (Fig 4G). It is not sufficient, however, to rescue the hyperglycemia and elevated glycogen levels in *sir2* mutants (S6C and S6D Fig). We therefore conclude that some, but not all of the diabetic defects observed in *sir2* mutants are due to a reduction in dHNF4 levels.

Discussion

Here we show that *sir2* mutants display a range of metabolic defects that parallel those seen in mouse *Sirt1* mutants, including hyperglycemia, lipid accumulation, insulin resistance, and glucose intolerance [1–3]. These results suggest that the fundamental metabolic functions of Sirt1 have been conserved through evolution and that further studies in *Drosophila* can be used to provide insights into its mammalian counterpart. An additional parallel with Sirt1 is seen in our tissue-specific studies, where we show that *sir2* function is necessary and sufficient in the fat body to maintain insulin signaling and suppress hyperglycemia and obesity, analogous to the role of Sirt1 in the liver and white adipose [9, 13, 24]. These results are also consistent with published studies of insulin sensitivity in *Drosophila*, which have shown that the fat body is the critical tissue that maintains glucose and lipid homeostasis through its ability to respond properly to insulin signaling [31, 32].

Our studies also define the dHNF4 nuclear receptor as a major target for Sir2 regulation. Consistent with this, *dHNF4* mutants display a range of phenotypes that resemble those of *sir2* mutants, including hyperglycemia, obesity, and glucose intolerance [33] (W. Barry and C.S.

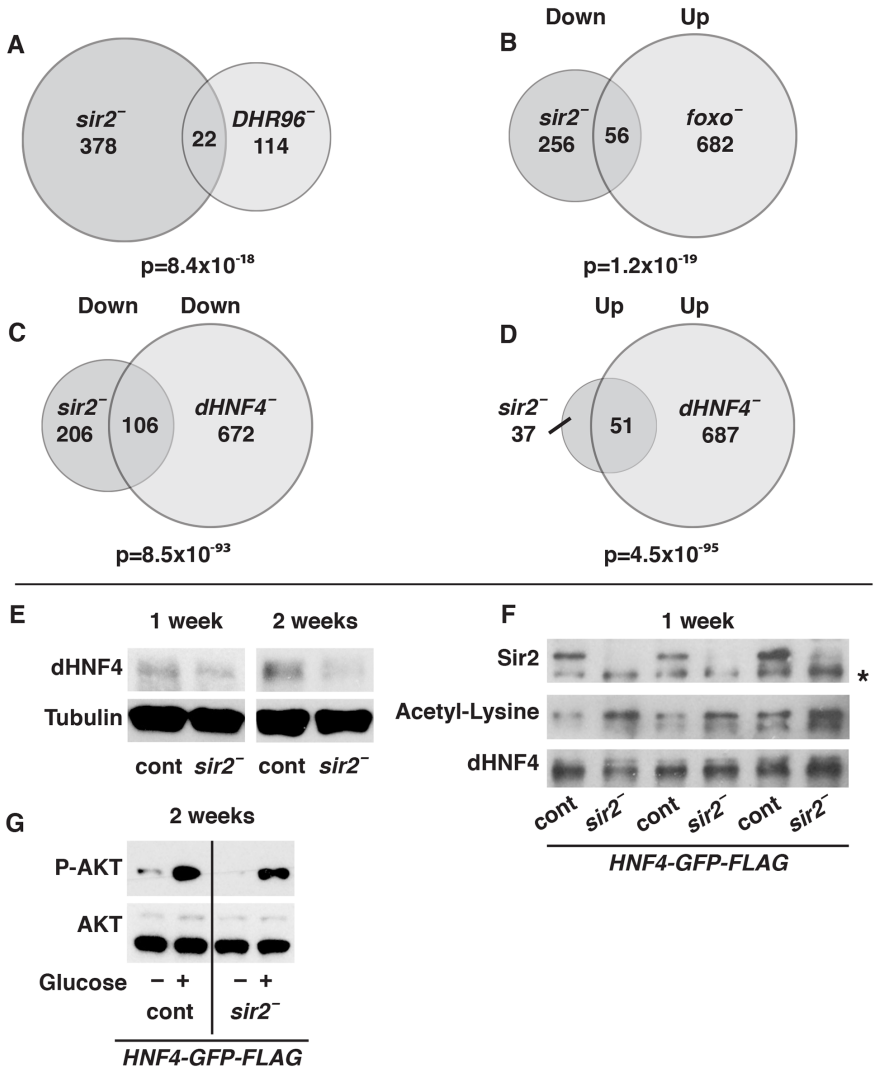

Fig 4. Sir2 acts through dHNF4 to maintain peripheral insulin signaling. Venn diagrams are shown depicting comparisons between (A) the genes regulated by *sir2* and *DHR96* mutants [28], (B) genes down-regulated in *sir2* mutants and up-regulated in *foxo* mutants [29], (C) genes down-regulated in both *sir2* and *dHNF4* mutants, and (D) genes up-regulated in both *sir2* and *dHNF4* mutants (W. Barry and C.S. Thummel, manuscript in revision). P-values were generated using a chi-square test, indicating the likelihood of the overlap by chance, given the number of genes in the *Drosophila* genome and the number of genes in each group. (E) Protein from control (cont) and *sir2* mutant (*sir2* –) adult males were isolated at either one or two weeks of age and analyzed on western blots using antibodies against dHNF4 or tubulin as a loading control. The ratio of HNF4 protein levels to tubulin levels in each sample

was quantified using data from three independent experimental replicates. The fold change between these ratios in *sir2* mutants and controls is as follows, representing the mean ± SEM: one week 0.7±0.01 (p = 0.0004), two weeks 0.3±0.03 (p = 0.002). (F) Protein extracts were prepared from controls (cont) or *sir2* mutants (*sir2*$^{-}$) at one week of age carrying two copies of a genomic dHNF4 rescue construct tagged with GFP and FLAG (2X *HNF4-GFP-FLAG*). dHNF4 protein levels were normalized by loading a larger volume of *sir2* mutant lysates than controls after anti-FLAG immunoprecipitation. Extracts were analyzed on western blots using antibodies directed against Sir2, acetyl-lysine, or dHNF4. A background band detected by the Sir2 antibodies is marked (*). The ratio of acetylated HNF4 levels to total HNF4 levels in *sir2* mutants and controls was quantified using data from four independent experimental replicates. The fold change between these ratios in controls and mutants is 3.2±0.8 (p = 0.02), representing the mean ± SEM. (G) Protein isolates were prepared from controls (cont) and *sir2* mutants (*sir2*$^{-}$) carrying two copies of the genomic dHNF4 rescue construct at two weeks of age, following a fasting-refeeding paradigm (–/+ glucose). These samples were analyzed on western blots using antibodies directed against phosphorylated AKT (P-AKT) or total AKT. The ratio of P-AKT levels to total AKT levels in refed controls and *sir2* mutants was quantified using data from four independent experimental replicates. The fold change between these ratios in controls and mutants is 2.1±1.1 (NS), representing the mean ± SEM.

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Thummel, manuscript in revision). As expected, these defects are more severe in *dHNF4* loss-of-function mutants, consistent with *sir2* mutants only resulting in a partial loss of dHNF4 protein. Sir2 interacts with dHNF4 and appears to stabilize this protein through deacetylation. This is an established mechanism for regulating protein stability, either through changes in target protein conformation that allow ubiquitin ligases to bind prior to proteasomal degradation, or through alternate pathways [34]. Further studies, however, are required to determine if this is a direct protein-protein interaction or part of a higher order complex.

Although two papers have shown that mammalian Sirt1 can control HNF4A transcriptional activity through a protein complex, only one gene was identified as a downstream target of this regulation, *PEPCK*, leaving it unclear if this activity is of functional significance [10, 30]. Our study suggests that this regulatory connection is far more extensive. The observation that one third of the genes down-regulated in *sir2* mutants are also down-regulated in *dHNF4* mutants (including *pepck*, S6A Fig), and most of the genes up-regulated in *sir2* mutants are up-regulated in *dHNF4* mutants, establishes this nuclear receptor as a major downstream target for Sir2 regulation. It will be interesting to determine if the extent of this regulatory connection has been conserved through evolution.

Despite this regulatory control, the over-expression of an HNF4 transgene was only able to partially restore the insulin signaling response and not the defects in carbohydrate homeostasis in *sir2* mutants. This lack of complete rescue is not surprising, given that the Sirt1 family targets a large number of transcription factors, histones, and enzymes, providing multiple additional pathways for metabolic regulation. Moreover, the activity or target recognition of dHNF4 may be altered when it is hyperacetylated, in which case merely over-expressing this factor would not fully restore normal function. Future studies can examine more direct targets, both previously characterized and uncharacterized, for their functions in suppressing diabetes downstream of Sir2-dependent regulation.

Finally, *sir2* mutants represent a new genetic model for studying the age-dependent onset of phenotypes related to type 2 diabetes. We show that newly-eclosed *sir2* mutant adults are relatively healthy, with elevated levels of free glucose and glycogen but otherwise normal metabolic functions. Their health, however, progressively worsens with age, with two-week-old *sir2* mutants displaying lipid accumulation, fasting hyperglycemia, and reduced insulin signaling accompanied by insulin resistance. This is followed by the onset of glucose intolerance by three weeks of age. Previous studies of type 2 diabetes in *Drosophila* have relied on dietary models using wild-type animals that are subjected to a high sugar diet [31, 32]. Although this is a valuable approach to better define the critical role of diet in diabetes onset, it is also clear that the likelihood of developing type 2 diabetes increases with age. The discovery that *sir2* mutants display this pathophysiology provides an opportunity to exploit the power of *Drosophila* genetics to better define the mechanisms that lead to the stepwise onset of metabolic dysfunction associated with diabetes.

Materials and Methods

Fly stocks and maintenance

Flies were raised at 25°C on media containing 8% yeast, 6% glucose, 3% sucrose, and 1% agar in 1XPBS for all studies, with flies maintained at 18°C for genetic rescue studies. Adult ages are indicated in the figures and text and refer to the time period after eclosion from the pupal case. Males under *ad libitum* feeding conditions were used for all experiments unless otherwise indicated. For most fasting-re-feeding paradigms, flies were transferred to 1% agar in 1XPBS for 14–18 hours and re-fed on 10% glucose, 1% agar in 1XPBS for two hours. A transheterozygous combination of the $sir2^{2A\text{-}7\text{-}11}$ and $sir2^{4.5}$ deletion alleles was used for all mutant studies [20, 21]. These alleles, all GAL4 lines (except for the *dilp2-gal4>UAS-dcr2* line), and the rescue construct, were outcrossed to a w^{1118} control strain, which was then used as a genetically-matched control for all experiments where indicated. The RNAi lines for *sir2* (#32481) and *mCherry* (#35787) were obtained from the Bloomington Stock Center. Immunoprecipitation experiments for dHNF4 were performed on lines containing a transgenic genomic construct with dHNF4 carrying GFP and FLAG tags, driven by the endogenous *dHNF4* promoter (Bloomington #38649). This transgene fully rescues *dHNF4* mutant defects and was maintained in homozygous $sir2^{2A\text{-}7\text{-}11}$ or wild-type genetic backgrounds.

Metabolite assays

Samples of five flies each were collected at one or two weeks of age and washed in 1XPBS. For triglycerides, glucose, glycogen, and protein, samples were homogenized in 120 μL of 1XPBS. For fasting glucose measurements, samples were homogenized in 100 μL trehalase buffer, and for ATP assays, samples were homogenized in 100 μL 6M guanidine HCl, 100mM Tris pH 7.8, 4mM EDTA). Assays were performed as described [35].

Western blots

Samples of ten flies were collected under the indicated conditions at one, two, or three weeks of age, and homogenized in 100 μL of RIPA buffer containing 1X protease inhibitors (Roche cOmplete Mini EDTA-free protease inhibitor tablets). For P-AKT westerns, the buffer also contained Calyculin A and okadaic acid. Equivalent amounts of protein were resolved by SDS-PAGE (10% acrylamide), transferred to PVDF membrane overnight at 4°C, and blocked with 5% BSA prior to immunoblotting. Western blots were probed with antibodies for P-AKT (1:1000, Cell Signaling #4060), pan-AKT (1:1000, Cell Signaling #4691), Tubulin (1:5000, Abcam #ab184613), dHNF4 (1:1000–1:2000, generated by L. Palanker-Musselman), Sir2 (1:50, Developmental Studies Hybridoma Bank #p4A10), and pan-acetyl-lysine (1:1000, Cell Signaling #9441). The westerns shown in the figures are representative of at least three biological replicates. Quantification was performed by measuring protein levels using ImageJ software. The values reported represent the mutant or experimental condition normalized to the control, unless otherwise specified. For P-AKT quantification, the ratio of P-AKT levels to total AKT levels was determined in refed *sir2* mutants or the experimental condition and controls using ImageJ. The data from the fasted state was not quantified for these studies because the small changes in the basal levels of P-AKT under these conditions (ranging from undetectable to low levels) results in large statistical fluctuations that are not meaningful.

RNA-seq

RNA was isolated from control and *sir2* mutants at two weeks of age using Trizol extraction (Thermo Fisher) and the Qiagen RNeasy Mini Kit. Library generation (Illumina TruSeq RNA

Sample Preparation Kit v2 with oligo dT selection) and sequencing (HiSeq 50 Cycle Single Read Sequencing v3) were performed by the High-Throughput Genomics core facility at the University of Utah. The Bioinformatics Core Facility at the University of Utah aligned this dataset to the genome, utilizing the Genome Build DM3 from April 2006. Cut-offs for significance were Log2 ratio ± 0.585 and p-value <0.05 (<0.005 in all cases but two). RNA-seq data from this study can be accessed at NCBI GEO (accession number: GSE72947).

Statistics

A standard two-tailed Student's *t*-test was used to determine significance on basic metabolite measurements (Fig 1; S1F Fig). GraphPad PRISM 6 software was used to plot data and perform statistical analysis on all other measurements. Pairwise comparison p-values were calculated using a two-tailed Student's *t*-test, and multiple comparison p-values were calculated using two-way ANOVA or Bonferroni correction. For metabolomics, p-values reflect a standard two-tailed unpaired t-test after a Welch's correction for different variances. For the starvation sensitivity experiment, the p-values reflect results from both a Log-Rank Mantel-Cox test as well as a Gehan-Breslow-Wilcoxon test. For gene-regulatory overlaps, the p-values reflect results from chi-square tests.

Cloning

The *UAS-sir2* rescue construct was generated by PCR amplification of the *sir2* coding region using primers designed to incorporate a KpnI restriction site in the forward primer (CGCG**GGTACC**CCAAATGGGTGCGAAGCTGACG) and an XbaI site in the reverse primer (CGCG**TCTAGA**GGCCCTCGGCTACGATTTCGCAG). The template for this reaction was cDNA generated from wild-type RNA using the ProtoScript M-MuLV *Taq* RT-PCR Kit (NEB). The gel-purified PCR product was digested with KpnI and XbaI and inserted into the multiple cloning site of pUAST-attB. This construct was integrated into each of three attP sites that are predicted to be silent (attP40, attP2, attP3) using standard methods (BestGene Inc.) [36]. We then combined this transgene with our $sir2^{2A\text{-}7\text{-}11}$ allele in order to study its effect in a transheterozygous *sir2* mutant background. Only the rescue line inserted on the third chromosome at attP2, however, had sufficiently low background levels of *sir2* expression to allow us to see changes in triglyceride levels and insulin signaling using tissue-specific GAL4 drivers, as shown in Figs 3C and S4B. Background expression from the *UAS-sir2* transgene in all three lines is sufficient to rescue the hyperglycemia of *sir2* mutants, preventing us from examining the tissue-specific regulation of this response in Fig 3.

Northern blots

RNA was isolated from samples containing 10–15 flies using Trizol (Thermo Fisher). Males were used for all studies except for the *Act>sir2-RNAi* experiment in S4A Fig, as only females were obtained from this cross. Northern blot transfers and hybridizations were performed as previously described [37].

Feeding rate assays

Feeding rates were measured by using radioactive media containing ~5,000 cpm/μL α-^{32}P-dCTP in 8% yeast, 6% glucose, and 3% sucrose in 1% agar. Male flies at one or two weeks of age were fasted overnight and then allowed to re-feed on the labeled media for two hours, after which they were transferred to unlabeled food for 45 minutes and sorted into samples of five

flies on ice. A scintillation counter was used to measure the radioactivity in each sample, and this value was used to determine the relative volume of media consumed.

Starvation sensitivity assays

Groups of 197–198 flies of each genotype, at two or three weeks of age, were transferred to fresh food for about 12–24 hours, and then transferred to starvation media. Lethality was monitored every four to eight hours, with surviving flies transferred to fresh starvation media at least once over the course of the experiment.

Glucose tolerance test

Flies at two or three weeks of age were fasted overnight for 15–18 hours prior to re-feeding on 10% glucose. After one hour, flies were transferred back to starvation media for either two or four hours. Samples were collected at each time point for glucose assays, which were performed as described [35].

Insulin tolerance test

Bovine insulin (Sigma) was dissolved in 1% acetic acid at 1 mg/mL before dilution to between 0.5 nM-100 nM in 1XPBS and 5% food dye. Flies were fasted overnight (one week old flies) or 4 hours (two week old flies) prior to insulin/dye injection in the thorax, until the dye was visible throughout the head and abdomen. Injections were performed for 10–15 minute intervals followed by an additional 30 minute rest period prior to collection of protein samples for western blot analysis. Concentrations at 1 week of age were 100 nM, and at 2 weeks of age were 0.5–1.0 nM [31, 38].

Assays for circulating glucose

To extract hemolymph, 30 one week old flies were punctured in the thorax between the head and wing junction using a tungsten needle and centrifuged at 9,000xg for five minutes through a Zymo-Spin IIIC filter (Zymo Research). These samples were diluted 1:100 in Trehalose buffer and heat treated at 70°C for five minutes. Final dilutions of 1:200 and 1:400 were used for glucose assays.

ELISA assay for circulating Dilp2

ELISA assays were performed as described on one and two week old flies [23]. Heterozygous control and homozygous $sir2^{2A\text{-}7\text{-}11}$ mutant lines were established that contained two copies of the transgenic *dilp2* construct carrying HA and FLAG tags, driven by the genomic *dilp2* promoter in a *dilp2* mutant background (Dilp2-HF). Ten flies were collected per sample. Undiluted circulating Dilp2-HF was measured from hemolymph samples and total Dilp2-HF levels were measured at a 1:10 dilution.

Metabolomics

Samples of fifteen adult males at two weeks of age were snap-frozen in liquid nitrogen and prepared for analysis by gas chromatography-mass spectrometry (GC/MS) as described [35]. Each experiment was performed on six independent samples, and each experiment was repeated three times. The data presented reflect the combined replicates from all three experiments, normalized within each experiment, for a total of 17–18 biological replicates per group. In one experimental replicate we failed to detect DHAP, for which there are only 12 biological replicates per group.

Immunoprecipitation

Samples were collected from control and $sir2^{2A-7-11}$ homozygous lines at one week of age containing two copies of the *dHNF4-GFP-FLAG* genomic transgene. Ten flies were homogenized in 100 μL homogenization solution consisting of RIPA buffer with protease inhibitors (Roche cOmplete Mini EDTA-free protease inhibitor tablets). Mouse anti-FLAG antibody (Sigma #F1804) was added to this homogenate at a 1:500 dilution and incubated for one hour, rotating at 4°C. A 1:1 mixture of Protein A/Protein G Dynabeads (Life Technologies) was washed with 1 mL RIPA three times before being resuspended in homogenization buffer. The equivalent of 10–20 μL of the original volume of washed beads was added to each homogenate and incubated for an additional two hours, rotating at 4°C. Immunoprecipitates were then eluted according to standard procedures in 1X sample buffer with protease inhibitors. 5–7.5 μL of the resulting elutes were loaded into a 10% SDS-PAGE gel Proteins were resolved by SDS-PAGE and analyzed on western blots as described above.

Supporting Information

S1 Fig. *sir2* null mutants are sensitive to starvation and hyperglycemic. (A) A gene model for *sir2* is shown, with the coding region in black and non-coding regions in gray. The regions deleted in the $sir2^{2A-7-11}$ and $sir2^{4.5}$ alleles are shown in green. Expression of *sir2* from controls (red) and *sir2* mutants (blue) was determined by RNA-seq analysis, with the reads assembled using an integrated genomics viewer (IGV). There is no measurable expression of the *sir2* coding region in transheterozygous mutants. (B) *sir2* transcripts are also not detectable in *sir2* mutants by northern blot hybridization, using *rp49* mRNA as a loading control. (C,D) The survival of *sir2* mutants (*sir2* –) on starvation media is similar to that of controls (cont) at two weeks of age (C), but is significantly reduced at three weeks of age (D). (E) *sir2* mutants display a normal feeding rate at both one and two weeks of age. (F) Circulating levels of glucose were measured in the hemolymph of *sir2* mutants at one week of age after 24 hours on 8% yeast 15% sugar media, demonstrating hyperglycemia.
(PDF)

S2 Fig. Glucose metabolites accumulate in *sir2* mutants. Gas chromatography-mass spectrophotometry analyses was performed on controls (red) and *sir2* mutants (blue) at two weeks of age. The results of three experimental replicates are presented with the exception of dihydroxyacetone phosphate (DHAP), which was undetectable in the third experimental replicate. Sorbitol, a sugar alcohol derived from glucose, is elevated in *sir2* mutants, as is glucose-1-phosphate, an intermediate in glycogen metabolism. Glycolytic intermediates are also elevated, including glucose-6-phosphate, DHAP, phosphoenolpyruvate, and lactate. $^{**}p<0.005$, $^{***}p<0.0005$.
(PDF)

S3 Fig. Glucose-stimulated insulin secretion is unaffected in *sir2* mutants that display reduced insulin signaling. (A) RNA was isolated from controls (*cont*) and *sir2* mutants (*sir2* –) at two weeks of age following a fasting-refeeding paradigm (–/+ glucose) and analyzed by northern blot hybridization. The reduced expression of the Foxo target gene *4EBP* in response to glucose refeeding is blunted in *sir2* mutants, indicative of reduced insulin signaling. (B,C) The non-normalized results of the ELISA assays shown in Fig 2F and 2G are depicted with the mean and ±SEM indicated. Each data point represents a single biological replicate (n = 10 flies/sample, n = 12–30 samples/group). As reported previously, circulating DILP2 levels increase during early adulthood [23]. This can be seen in both controls and *sir2* mutants between one to two weeks of age (fasted to fed). One week controls: 0.016±0.0017 to 0.027±0.0027, two weeks controls: 0.021±0.0043 to 0.042±0.006, one week *sir2* mutants: 0.0068±0.00093 to 0.016

±0.0010, two week *sir2* mutants: 0.012±0.0022 to 0.027±0.0043 (two-way ANOVA $p<0.0005$ between one and two-week-old controls, $p<0.0001$ one and two-week-old *sir2* mutants). Controls and mutants show similar fold increases in circulating DILP2 in response to feeding, although *sir2* mutants show a slightly enhanced response: one week controls, 1.9-fold, two week controls, 1.7-fold; one week mutants, 2.4-fold, two week mutants, 2.0-fold (two-way ANOVA $p<0.05$ between fasted and fed controls, $p<0.0001$ between fasted and fed *sir2* mutants).
(PDF)

S4 Fig. *sir2* RNAi results in a strong loss of gene function, and *sir2* rescue restores gene function. (A) *Act-GAL4* was used to drive ubiquitous expression of either *mCherry* (cont) or *sir2 UAS-RNAi* transgenes. Northern blot analysis of RNA isolated from females at two weeks of age following a fasting-refeeding paradigm (–/+ glucose), reveals no detectable *sir2* mRNA upon *sir2* RNAi. (B) GAL4 drivers for the fat body (*r4-GAL4*), muscle (*mef2-GAL4*), or IPCs (*dilp2-GAL4*) were used to express wild-type *UAS-sir2* in an otherwise *sir2* mutant background (black bars), with the GAL4 drivers alone in the mutant background as controls (white bars). Triglycerides were measured in extracts from these animals at two weeks of age and normalized to soluble protein levels (n = 6–15 for each group). Specific expression of wild-type *sir2* in the fat body of *sir2* mutants, but not in the muscle or IPCs, is sufficient to rescue the obese phenotype. (C) Triglycerides are reduced below those of both controls and *sir2* mutants when *UAS-sir2* is expressed in the fat body using the *r4-GAL4* driver (n = 5 for each group).
(PDF)

S5 Fig. Sir2 regulates genes involved in metabolism and innate immunity. Gene ontology categories were derived from the *sir2* RNA-seq dataset using the online program DAVID [25]. Categories are divided into "Biological Process" and "Molecular Function", for both the down-regulated and up-regulated genes. Only the top categories are listed and multiple identical categories are represented by a single entry. The down-regulated genes primarily fall into categories consisting of catabolic enzymes, represented by peptidases, mannosidases, and lipases. Up-regulated genes mainly fall in the innate immune response and stress-response categories.
(PDF)

S6 Fig. Sir2 functions are mediated by its protein target dHNF4. (A) A northern blot hybridization was performed on RNA isolated from two independent replicates of control (cont) and *sir2* mutants (*sir2* –) at 1 or 2 weeks of age, probed to detect *dHNF4*, *sir2*, and *pepck* mRNA. Levels of *dHNF4* and *pepck* mRNA are reduced in *sir2* mutants, although *pepck* is more severely affected. The ratio of *dHNF4* mRNA levels to *rp49* levels in each sample was quantified using data from three independent experimental replicates. The fold change between these ratios in *sir2* mutants and controls is as follows, representing the mean ± SEM: one week 0.7 ±0.1 (NS), two weeks 0.6±0.09 ($p = 0.05$). The ratio of *pepck* mRNA levels to *rp49* levels in each sample was quantified using data from three independent experimental replicates. The fold change between these ratios in *sir2* mutants and controls is as follows, representing the mean ± SEM: one week 0.4±0.009 ($p = 0.0002$), two weeks 0.6±0.09 ($p = 0.052$). (B) NCBI BLAST alignment of the region in the *Drosophila* (*Dm*) and human (*Hs*) HNF4 sequence shows the conserved lysine residues that are acetylated by p300/CREB in humans (highlighted in yellow). (C,D) Overexpression of *dHNF4* using two copies of the *dHNF4-GFP-FLAG* transgene in an otherwise wild-type animal (control, white bars) or *sir2* mutants (*sir2* –, black bars) has no effect on the hyperglycemia (C) or high glycogen levels (D) in mutants. Glucose and glycogen were measured at two weeks of age and are normalized to soluble protein levels (n = 6

samples per group). $^{*}p<0.05$, $^{**}p<0.005$.
(PDF)

S1 Table. List of genes from RNA-seq that display differential abundance between *sir2* mutants and matched controls, meeting a cutoff of a Log2 ratio ± 0.585 (± 1.5 fold) and p-value <0.05.
(XLSX)

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Author Contributions

Conceived and designed the experiments: RASP CST. Performed the experiments: RASP. Analyzed the data: RASP CST. Contributed reagents/materials/analysis tools: RASP. Wrote the paper: RASP CST.

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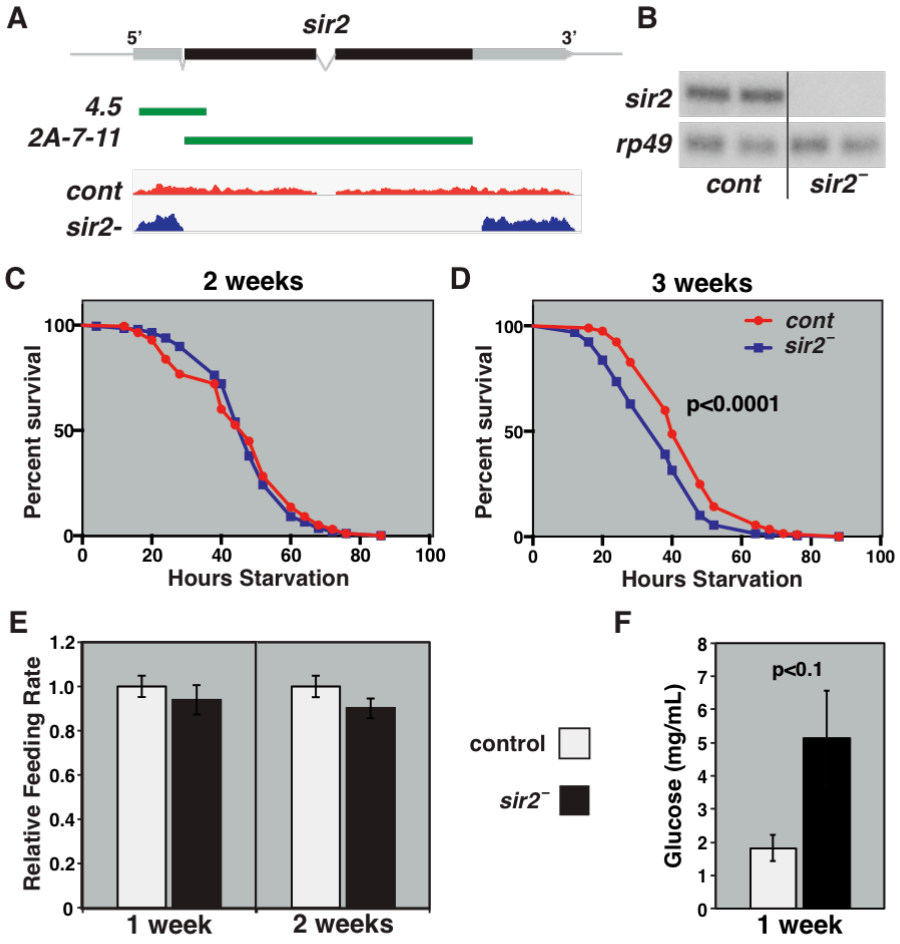
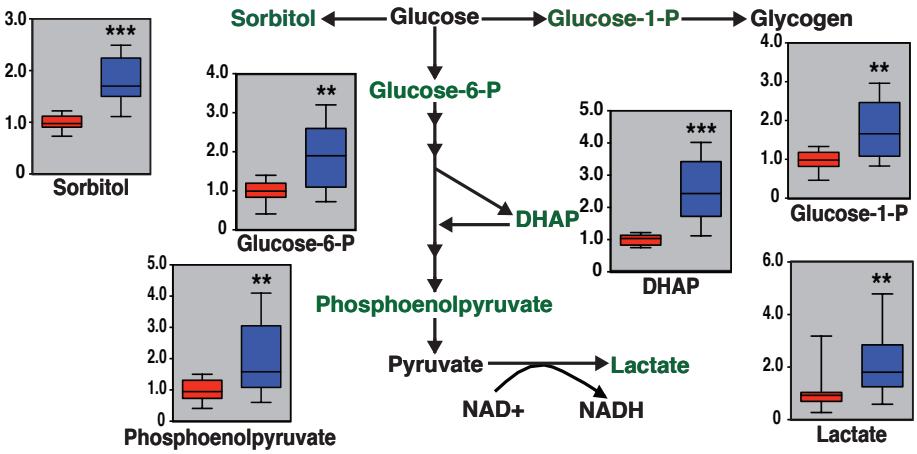
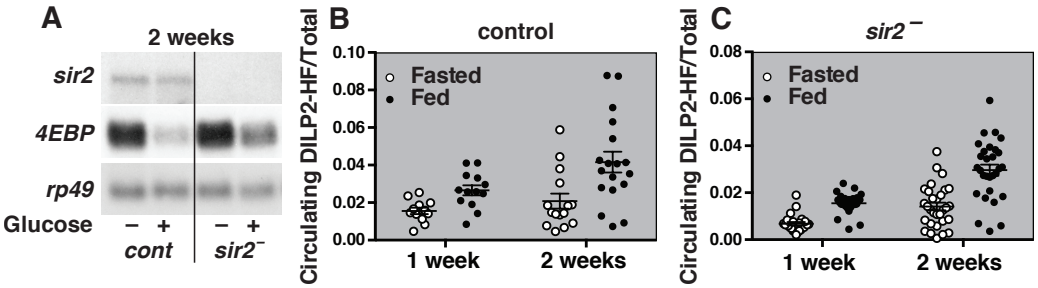


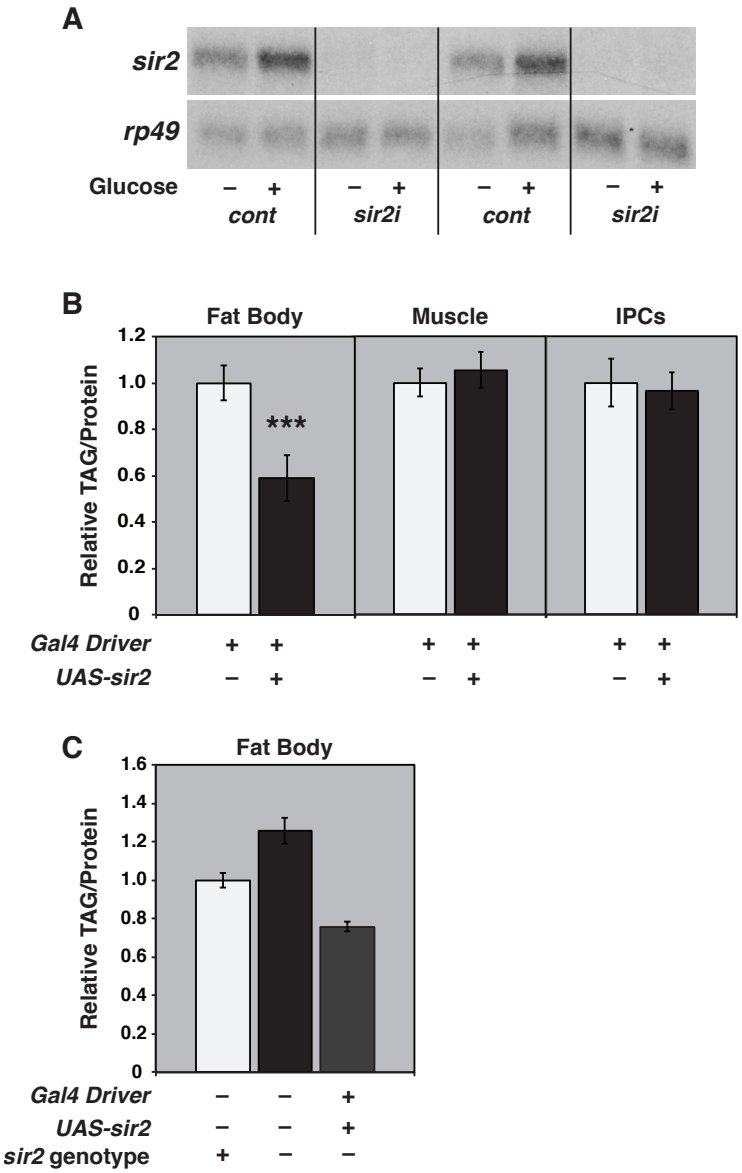
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

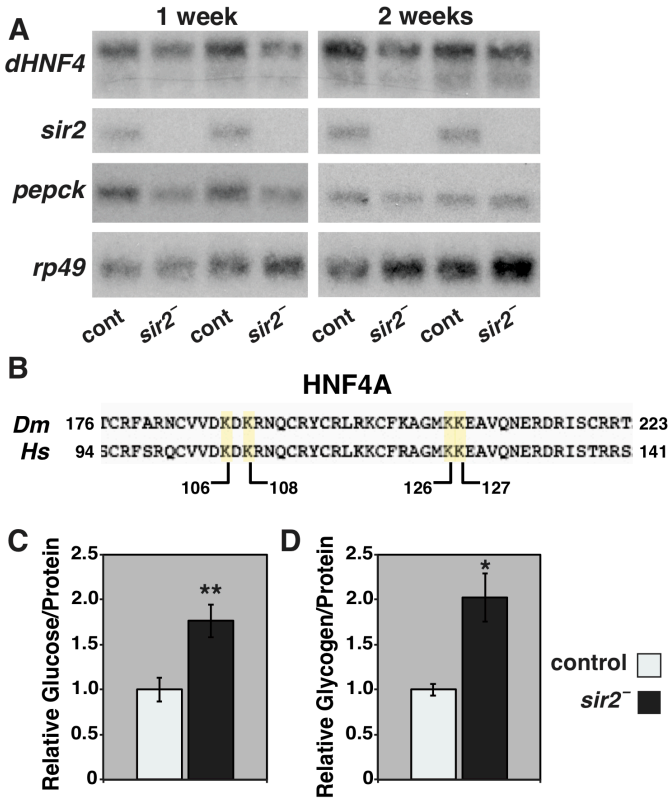


Supplemental Figure 4

DOWN	Category	# Genes	p-value	Benjamini
Biological Process	Proteolysis	47	4.80E-13	2.50E-10
	Oxidation reduction	39	1.90E-10	5.10E-08
	Defense response	16	1.60E-06	2.80E-04
	Aminoglycan metabolic process	11	5.10E-04	6.60E-02
	Xenobiotic metabolic process	4	6.00E-04	6.10E-02
	Mannose metabolic process	4	8.40E-04	7.20E-02
	Polysaccharide metabolic process	11	9.20E-04	6.80E-02
Molecular Function	Serine-type peptidase activity	32	2.30E-11	6.40E-09
	Endopeptidase activity	39	3.40E-11	3.10E-09
	Peptidase activity	47	4.90E-11	3.40E-09
	Serine-type endopeptidase activity	30	5.50E-11	3.00E-09
	Peptidase on L-amino acid peptides	45	9.50E-11	4.30E-09
	Carbohydrate binding	20	2.70E-07	1.10E-05
	Lipase activity	11	8.80E-05	3.00E-03
	Heme binding	13	2.00E-04	6.00E-03

UP	Category	# Genes	p-value	Benjamini
BP	Defense response	10	2.20E-07	2.70E-05
MF	Glutathione transferase activity	4	6.50E-04	5.20E-02

Supplemental Figure 5



Supplemental Figure 6

Supplemental Table 1. List of genes from RNA-seq that display differential abundance between sir2 mutants and matched controls*

IGB HyperLink	FlyBaseGeneID	CG	Gene	Coor D_melanogaster_Apr_2006	Strand	Total BPs	Max Abs	Max DESeq	EdgeR FDR	Lg2Rto	FC from	FDR	p-value
FBgn0033541_CG12934	FBgn0033541	CG12934	CG12934	chr2R:6536416-6537250	+	834	2.491185665	605.9547729	0	-2.49118567	0.177860042	605.9547729	2.53818E-61
FBgn0044810_TotX	FBgn0044810	CG31193	TotX	chr3R:16730637-16731240	-	603	2.339172363	506.4357605	0	-2.33917236	0.197623667	506.4357605	2.27208E-51
FBgn0044812_TotC	FBgn0044812	CG31508	TotC	chr3R:16698709-16699310	+	538	2.199834585	501.9418945	0	-2.19983459	0.217662596	501.9418945	6.39456E-51
FBgn0035666_Jon65Aii	FBgn0035666	CG6580	Jon65Aii	chr3L:6045449-6046291	+	780	2.19756937	508.6676636	0	-2.19756937	0.218004622	508.6676636	1.35904E-51
FBgn0028396_TotA	FBgn0028396	CG31509	TotA	chr3R:16696757-16697427	+	584	2.108093977	479.3804626	0	-2.10809398	0.231953259	479.3804626	1.15333E-48
FBgn0036023_CG18179	FBgn0036023	CG18179	CG18179	chr3L:9632372-9633240	+	868	2.082063437	405.4529114	0	-2.08206344	0.236176375	405.4529114	2.84911E-41
FBgn0034289_CG10910	FBgn0034289	CG10910	CG10910	chr2R:13907753-13909911	+	2010	2.065222263	405.3535767	0	-2.06522226	0.238949514	405.3535767	2.91503E-41
FBgn0002565_Lsp2	FBgn0002565	CG6806	Lsp2	chr3L:12122492-12124796	+	2304	2.005228043	405.0650635	0	-2.00522804	0.249095689	405.0650635	3.11526E-41
FBgn0042201_Nplp3	FBgn0042201	CG13061	Nplp3	chr3L:16311124-16311796	+	543	1.978522778	373.5037842	0	-1.97852278	0.253749559	373.5037842	4.46295E-38
FBgn0033521_CG12896	FBgn0033521	CG12896	CG12896	chr2R:6314384-6315418	-	846	1.925925255	377.9299622	0	-1.92592525	0.263171423	377.9299622	1.61066E-38
FBgn0002562_Lsp1alpha	FBgn0002562	CG2559	Lsp1alpha	chrX:12384415-12386933	+	2451	1.896593571	410.5925293	0	-1.89659357	0.268576769	410.5925293	8.72463E-42
FBgn0040832_CG8012	FBgn0040832	CG8012	CG8012	chr3L:8208530-8209302	+	704	1.890225649	343.1018066	0	-1.89022565	0.269764862	343.1018066	4.89575E-35
FBgn0031490_CG17264	FBgn0031490	CG17264	CG17264	chr2L:3018507-3021506	+	2734	1.86518693	281.82724	0	-1.86518693	0.274487636	281.82724	6.56562E-29
FBgn0002563_Lsp1beta	FBgn0002563	CG4178	Lsp1beta	chr2L:898646-901320	+	2606	1.837452412	354.9345703	0	-1.83745241	0.279815462	354.9345703	3.21028E-36
FBgn0040262_Ugt36Ba	FBgn0040262	CG13270	Ugt36Ba	chr2L:16794235-16795807	+	1572	1.787397146	395.09729	0	-1.78739715	0.28969423	395.09729	3.09222E-40
FBgn0003357_Jon99Ciii	FBgn0003357	CG31362	Jon99Ciii	chr3R:25747930-25748823	+	893	1.785958409	257.3994446	0	-1.78595841	0.289983273	257.3994446	1.81993E-26
FBgn0038290_CG6912	FBgn0038290	CG6912	CG6912	chr3R:10960859-10962702	-	1404	1.684785128	252.2970581	0	-1.68478513	0.311049237	252.2970581	5.89243E-26
FBgn0034229_CG4847	FBgn0034229	CG4847	CG4847	chr2R:13399027-13401364	+	1527	1.650733709	256.3705444	0	-1.65073371	0.318478148	256.3705444	2.30646E-26
FBgn0039778_Jon99Fi	FBgn0039778	CG18030	Jon99Fi	chr3R:26314686-26315625	+	939	1.640217304	256.4342346	0	-1.6402173	0.320808149	256.4342346	2.27288E-26
FBgn0038291_CG3984	FBgn0038291	CG3984	CG3984	chr3R:10962883-10964626	+	1614	1.621851325	234.5489655	0	-1.62185133	0.324918248	234.5489655	3.50835E-24
FBgn0033826_CG4734	FBgn0033826	CG4734	CG4734	chr2R:9184706-9185972	-	1011	1.582234383	222.0786591	0	-1.58223438	0.333964259	222.0786591	6.19632E-23
FBgn0033774_CG12374	FBgn0033774	CG12374	CG12374	chr2R:8649616-8651330	+	1465	1.581898212	231.1963806	0	-1.58189821	0.334042087	231.1963806	7.5921E-24
FBgn0038095_Cyp304a1	FBgn0038095	CG7241	Cyp304a1	chr3R:8789518-8791652	+	1717	1.539157271	193.6968994	0	-1.53915727	0.344086389	193.6968994	4.26884E-20
FBgn0051091_CG31091	FBgn0051091	CG31091	CG31091	chr3R:21725624-21727897	+	2152	1.528124213	234.5489655	0	-1.52812421	0.346727888	234.5489655	3.50835E-24
FBgn0036024_CG18180	FBgn0036024	CG18180	CG18180	chr3L:9634115-9635013	+	898	1.524088383	210.932663	0	-1.52408838	0.347699191	210.932663	8.0674E-22
FBgn0039472_CG17192	FBgn0039472	CG17192	CG17192	chr3R:22842896-22844009	-	1057	1.498911619	201.76651	0	-1.49891162	0.353820215	201.76651	6.65808E-21
FBgn0038914_fit	FBgn0038914	CG17820	fit	chr3R:17709639-17710176	-	537	1.494594216	227.8112335	0	-1.49459422	0.354880642	227.8112335	1.6553E-23
FBgn0259896_nimC1	FBgn0259896	CG8942	nimC1	chr2L:13974116-13976753	-	2040	1.483121753	219.3683472	0	-1.48312175	0.35771394	219.3683472	1.15655E-22
FBgn0037396_CG11459	FBgn0037396	CG11459	CG11459	chr3R:1851831-1852962	-	1131	1.480573297	221.0983734	0	-1.4805733	0.358346384	221.0983734	7.76538E-23
FBgn0030262_Vago	FBgn0030262	CG2081	Vago	chrX:10983322-10984138	+	748	1.460957885	142.0202179	0	-1.46095788	0.363251866	142.0202179	6.28027E-15
FBgn0043791_CG8147	FBgn0043791	CG8147	CG8147	chr3R:5320524-5322452	+	1867	1.439928055	239.5765839	0	-1.43992805	0.368585685	239.5765839	1.10241E-24
FBgn0051810_CG31810	FBgn0051810	CG31810	CG31810	chr2L:16849017-16852366	-	1136	1.42798686	192.1836548	0	-1.42798686	0.371649131	192.1836548	6.04832E-20
FBgn0030148_CG3106	FBgn0030148	CG3106	CG3106	chrX:9553128-9556789	-	2441	1.379285574	183.8504944	0	-1.37928557	0.384409109	183.8504944	4.12051E-19
FBgn0034317_CR14499	FBgn0034317	CG14499	CR14499	chr2R:14075226-14075794	+	568	1.362111807	160.6221313	0	-1.36211181	0.389012439	160.6221313	8.66537E-17
FBgn0040102_lectin-24Db	FBgn0040102	CG2958	lectin-24Db	chr2L:4188122-4189285	+	1163	1.358545661	193.1943817	0	-1.35854566	0.389975214	193.1943817	4.7925E-20
FBgn0004425_LysB	FBgn0004425	CG1179	LysB	chr3L:1207370-1207868	+	498	1.339680314	180.4854279	0	-1.33968031	0.395108198	180.4854279	8.94246E-19
FBgn0051266_CG31266	FBgn0051266	CG31266	CG31266	chr3R:12956975-12959441	+	2401	1.331200004	174.8326569	0	-1.3312	0.397437524	174.8326569	3.28651E-18
FBgn0036321_CG14120	FBgn0036321	CG14120	CG14120	chr3L:12878151-12882267	+	4116	1.323279262	177.3642426	0	-1.32327926	0.399625552	177.3642426	1.83475E-18
FBgn0035886_Jon66Ci	FBgn0035886	CG7118	Jon66Ci	chr3L:8384641-8385479	+	838	1.319056153	178.8871613	0	-1.31905615	0.400797064	178.8871613	1.29206E-18
FBgn0036619_Cpr72Ec	FBgn0036619	CG4784	Cpr72Ec	chr3L:16358990-16360426	-	1436	1.313179851	153.8002472	0	-1.31317985	0.402432897	153.8002472	4.16846E-16
FBgn0036948_CG7298	FBgn0036948	CG7298	CG7298	chr3L:20195169-20196786	-	1322	1.280356526	158.7246857	0	-1.28035653	0.411693756	158.7246857	1.34132E-16
FBgn0028939_nimC2	FBgn0028939	CG18146	nimC2	chr2L:13980145-13982952	+	2482	1.277209878	164.0544128	0	-1.27720988	0.412592677	164.0544128	3.9315E-17
FBgn0031472_CG2983	FBgn0031472	CG2983	CG2983	chr2L:2847631-2848997	+	1307	1.269561648	154.6938019	0	-1.26956165	0.414785783	154.6938019	3.39328E-16
FBgn0028940_Cyp28a5	FBgn0028940	CG8864	Cyp28a5	chr2L:13977302-13979573	+	1769	1.267472267	153.8002472	0	-1.26747227	0.415386931	153.8002472	4.16846E-16
FBgn0259229_CG42329	FBgn0259229	CG42329	CG42329	chr2L:1219362-1229471	+	2076	1.259700775	151.2833099	0	-1.25970078	0.41763057	151.2833099	7.44165E-16
FBgn0040260_Ugt36Bc	FBgn0040260	CG17932	Ugt36Bc	chr2L:16799028-16801585	+	1951	1.25268209	160.5906525	0	-1.25268209	0.419667286	160.5906525	8.7284E-17
FBgn0039768_CG15533	FBgn0039768	CG15533	CG15533	chr3R:26254547-26257091	+	2204	1.252268791	138.8501282	0	-1.25226879	0.419787528	138.8501282	1.30313E-14
FBgn0085465_CG34436	FBgn0085465	CG34436	CG34436	chr3R:24108301-24109236	+	819	1.251837254	129.3280945	0	-1.25183725	0.419913113	129.3280945	1.16732E-13
FBgn0033271_CG8708	FBgn0033271	CG8708	CG8708	chr2R:4046961-4048735	-	1671	1.247081399	146.8594971	0	-1.2470814	0.421299644	146.8594971	2.06087E-15
FBgn0051634_Oatp26F	FBgn0051634	CG31634	Oatp26F	chr2L:6625127-6632404	-	2425	1.244714022	164.1115875	0	-1.24471402	0.421991539	164.1115875	3.88009E-17
FBgn0026314_Ugt35b	FBgn0026314	CG6649	Ugt35b	chr3R:6994218-6995955	-	1665	1.220889449	128.4657593	0	-1.22088945	0.429018139	128.4657593	1.42372E-13
FBgn0026268_antdh	FBgn0026268	CG1386	antdh	chrX:11062047-11063091	-	965	1.218732834	141.3359528	0	-1.21873283	0.429659937	141.3359528	7.35199E-15

FBgn0004426_LysC	FBgn0004426	CG9111	LysC	chr3L:1210484-1210971	+	487	1.216709971	151.4180908	0	-1.21670997	0.430262803	151.4180908	7.21425E-16
FBgn0032066_CG9463	FBgn0032066	CG9463	CG9463	chr2L:8765358-8768708	-	3110	1.198016047	77.14193726	0	-1.19801605	0.435874271	77.14193726	1.93111E-08
FBgn0028949_CG15254	FBgn0028949	CG15254	CG15254	chr2L:15613927-15614824	-	841	1.186295986	137.6424255	0	-1.18629599	0.439429617	137.6424255	1.72091E-14
FBgn0027259_Kmn1	FBgn0027259	CG1558	Kmn1	chrX:11465399-11466475	-	1018	1.185247779	134.9971466	0	-1.18524778	0.439749006	134.9971466	3.16436E-14
FBgn0039886_CG2003	FBgn0039886	CG2003	CG2003	chr3R:27803851-27816638	+	2005	1.163382888	132.7156677	0	-1.16338289	0.44646442	132.7156677	5.35098E-14
FBgn0032266_CG18302	FBgn0032266	CG18302	CG18302	chr2L:10534972-10536315	+	1221	1.158298373	132.4556885	0	-1.15829837	0.448040678	132.4556885	5.68108E-14
FBgn0034711_CG3290	FBgn0034711	CG3290	CG3290	chr2R:18093509-18095492	-	1762	1.150375605	125.0465164	0	-1.1503756	0.450507927	125.0465164	3.12859E-13
FBgn0053926_CG33926	FBgn0053926	CG33926	CG33926	chr3L:9436363-9439480	+	780	1.142419815	98.98889923	0	-1.14241982	0.45299913	98.98889923	1.26215E-10
FBgn0039415_CG6142	FBgn0039415	CG6142	CG6142	chr3R:21960716-21962680	+	1851	1.118177533	130.4902802	0	-1.11817753	0.4606754	130.4902802	8.93248E-14
FBgn0040074_retinin	FBgn0040074	CG13057	retinin	chr3L:16325018-16325889	+	812	1.117673159	107.6719666	0	-1.11767316	0.460836483	107.6719666	1.70924E-11
FBgn0004427_LysD	FBgn0004427	CG9118	LysD	chr3L:1210724-1211196	-	472	1.112178206	125.034584	0	-1.11217821	0.462595069	125.034584	3.1372E-13
FBgn0040211_hgo	FBgn0040211	CG4779	hgo	chr2L:11136394-11138567	+	1445	1.097282648	111.5500488	0	-1.09728265	0.467396019	111.5500488	6.99834E-12
FBgn0031970_CG7227	FBgn0031970	CG7227	CG7227	chr2L:7994836-7998144	-	2264	1.093161702	116.9874268	0	-1.0931617	0.468733008	116.9874268	2.00105E-12
FBgn0030098_CG12057	FBgn0030098	CG12057	CG12057	chrX:9085705-9086582	+	877	1.078242064	71.39544678	0	-1.07824206	0.473605564	71.39544678	7.25196E-08
FBgn0032067_CG9465	FBgn0032067	CG9465	CG9465	chr2L:8769174-8772173	-	2829	1.076497674	104.0151749	0	-1.07649767	0.474178556	104.0151749	3.96719E-11
FBgn0033453_CG1667	FBgn0033453	CG1667	CG1667	chr2R:5730202-5733019	+	1566	1.075402737	95.50098419	0	-1.07540274	0.474538572	95.50098419	2.81774E-10
FBgn0036833_CG3819	FBgn0036833	CG3819	CG3819	chr3L:18913620-18915206	-	1329	1.06438899	104.4587784	0	-1.06438899	0.478175132	104.4587784	3.58197E-11
FBgn0052199_CG32199	FBgn0052199	CG32199	CG32199	chr3L:18604999-18607103	+	1691	1.061729193	109.3707809	0	-1.06172919	0.479057524	109.3707809	1.1559E-11
FBgn0034052_CG8299	FBgn0034052	CG8299	CG8299	chr2R:11828205-11828988	-	783	1.060340166	104.4587784	0	-1.06034017	0.479518983	104.4587784	3.58197E-11
FBgn0031925_Cyp4d21	FBgn0031925	CG6730	Cyp4d21	chr2L:7605599-7607703	+	1823	1.055058718	107.0802612	0	-1.05505872	0.481277633	107.0802612	1.95873E-11
FBgn0038918_CG6690	FBgn0038918	CG6690	CG6690	chr3R:17737537-17739313	+	1776	1.053726077	106.7432861	0	-1.05372608	0.481722402	106.7432861	2.11676E-11
FBgn0030331_CG15221	FBgn0030331	CG15221	CG15221	chrX:11618372-11630650	+	2400	1.043711305	105.7779312	0	-1.0437113	0.485078014	105.7779312	2.64367E-11
FBgn0032536_Ance-3	FBgn0032536	CG17988	Ance-3	chr2L:13914724-13955757	+	2995	1.03850472	129.8636017	0	-1.03850472	0.486831789	129.8636017	1.03191E-13
FBgn0039203_CG13618	FBgn0039203	CG13618	CG13618	chr3R:20350521-20353261	+	1021	1.028724909	117.8227692	0	-1.02872491	0.490143159	117.8227692	1.65091E-12
FBgn0038660_CG14291	FBgn0038660	CG14291	CG14291	chr3R:14736772-14738641	-	1869	1.027870059	109.625824	0	-1.02787006	0.490433672	109.625824	1.08998E-11
FBgn0035933_CG13309	FBgn0035933	CG13309	CG13309	chr3L:8803510-8804368	+	787	1.026771069	96.52690125	0	-1.02677107	0.490807409	96.52690125	2.2249E-10
FBgn0259950_CG42460	FBgn0259950	CG42460	CG42460	chr2L:3387897-3388496	-	537	1.021081686	104.0411148	0	-1.02108169	0.492746768	104.0411148	3.94356E-11
FBgn0031389_CG4259	FBgn0031389	CG4259	CG4259	chr2L:2128214-2129211	-	935	0.996433258	96.51486206	0	-0.99643326	0.501237668	96.51486206	2.23107E-10
FBgn0051041_CG31041	FBgn0051041	CG31041	CG31041	chr3R:25415789-25416616	-	690	0.987115383	101.5753098	0	-0.98711538	0.504485468	101.5753098	6.95775E-11
FBgn0036996_CG5932	FBgn0036996	CG5932	CG5932	chr3L:20453798-20455374	+	1461	0.984843552	54.73109436	0	-0.98484355	0.505280514	54.73109436	3.36427E-06
FBgn0035673_CG6602	FBgn0035673	CG6602	CG6602	chr3L:6065243-6066639	+	1396	0.982366621	88.49137878	0	-0.98236662	0.506148763	88.49137878	1.41534E-09
FBgn0034468_Obp56a	FBgn0034468	CG11797	Obp56a	chr2R:15585227-15586016	+	550	0.970967352	84.32910156	0	-0.97096735	0.510163874	84.32910156	3.69054E-09
FBgn0031273_CG2839	FBgn0031273	CG2839	CG2839	chr2L:625662-628143	+	2481	0.96732384	111.3503799	0	-0.96732384	0.511453917	111.3503799	7.3276E-12
FBgn0053282_CG33282	FBgn0053282	CG33282	CG33282	chr2L:3327935-3329625	+	1383	0.967047989	86.11668396	0	-0.96704799	0.511551718	86.11668396	2.4453E-09
FBgn0039470_CG6296	FBgn0039470	CG6296	CG6296	chr3R:22828957-22831191	-	2127	0.961502314	109.2414246	0	-0.96150231	0.513521892	109.2414246	1.19085E-11
FBgn0027578_CG14526	FBgn0027578	CG14526	CG14526	chr3R:24709414-24711737	+	2323	0.959546685	92.90088654	0	-0.95954669	0.514218463	92.90088654	5.12757E-10
FBgn0052368_CG32368	FBgn0052368	CG32368	CG32368	chr3L:7908535-7908954	+	419	0.95279938	65.76847839	0	-0.95279938	0.516629031	65.76847839	2.64943E-07
FBgn0031559_CG3513	FBgn0031559	CG3513	CG3513	chr2L:3692698-3693182	-	423	0.952117741	78.87372589	0	-0.95211774	0.516873183	78.87372589	1.29607E-08
FBgn0052483_CG32483	FBgn0052483	CG32483	CG32483	chr3L:688522-690081	-	1382	0.951913059	89.66381836	0	-0.95191306	0.51694652	89.66381836	1.08048E-09
FBgn0030827_CG18258	FBgn0030827	CG18258	CG18258	chrX:17106360-17108010	+	1476	0.949385345	83.96373749	0	-0.94938534	0.517853044	83.96373749	4.01445E-09
FBgn0031261_nAcRbeta-21C	FBgn0031261	CG11822	nAcRbeta-21C	chr2L:545147-547096	+	1634	0.948699355	118.1137161	0	-0.94869936	0.518099338	118.1137161	1.54393E-12
FBgn0038733_CG11407	FBgn0038733	CG11407	CG11407	chr3R:15606973-15608868	+	1784	0.945176482	82.25688171	0	-0.94517648	0.519366015	82.25688171	5.94719E-09
FBgn0053109_CG33109	FBgn0053109	CG33109	CG33109	chr3R:10046272-10047301	-	1029	0.945140123	76.4164505	0	-0.94514012	0.519379104	76.4164505	2.28221E-08
FBgn0033443_CG1698	FBgn0033443	CG1698	CG1698	chr2R:5680847-5685512	+	4090	0.936742425	91.53044891	0	-0.93674242	0.522411143	91.53044891	7.03E-10
FBgn0250841_CG17242	FBgn0250841	CG17242	CG17242	chr2L:2268298-2269119	-	821	0.936051071	71.39544678	0	-0.93605107	0.522661548	71.39544678	7.25196E-08
FBgn0038398_sxe2	FBgn0038398	CG4979	sxe2	chr3R:11911226-11912951	+	1590	0.935572147	71.39544678	0	-0.93557215	0.522835082	71.39544678	7.25196E-08
FBgn0243514_eater	FBgn0243514	CG6124	eater	chr3R:22921588-22925401	-	3241	0.934553266	95.77642822	0	-0.93455327	0.523204456	95.77642822	2.64458E-10
FBgn0033867_Cpr50Ca	FBgn0033867	CG13338	Cpr50Ca	chr2R:9657419-9675310	+	3430	0.929915786	76.92827606	0	-0.92991579	0.52488898	76.92827606	2.02849E-08
FBgn0038482_CG4053	FBgn0038482	CG4053	CG4053	chr3R:12957149-12957881	-	732	0.925573289	81.04771423	0	-0.92557329	0.52647127	81.04771423	7.85649E-09
FBgn0034553_CG9993	FBgn0034553	CG9993	CG9993	chr2R:16737650-16739626	-	1798	0.923963964	79.38973999	0	-0.92396396	0.527058876	79.38973999	1.15087E-08
FBgn0011556_zetaTry	FBgn0011556	CG12387	zetaTry	chr2R:7226690-7227633	-	943	0.916778684	65.46170044	0	-0.91677868	0.529690418	65.46170044	2.84335E-07
FBgn0038236_Cyp313a1	FBgn0038236	CG3360	Cyp313a1	chr3R:10346792-10348696	+	1511	0.916253984	77.2197876	0	-0.91625398	0.529883098	77.2197876	1.8968E-08
FBgn0000477_DNaseII	FBgn0000477	CG7780	DNaseII	chr3R:13845098-13846883	+	1226	0.915816605	73.34700012	0	-0.91581661	0.530043766	73.34700012	4.62701E-08
FBgn0053290_CG33290	FBgn0053290	CG33290	CG33290	chr3L:21535458-21536112	+	654	0.905557632	66.48740387	0	-0.90555763	0.533826329	66.48740387	2.24522E-07
FBgn0032669_CG15155	FBgn0032669	CG15155	CG15155	chr2L:18154863-18155944	+	898	0.905284345	79.20513916	0	-0.90528435	0.53392746	79.20513916	1.20084E-08

FBgn0001128_Gpdh	FBgn0001128	CG9042	Gpdh	chr2L:5943681-5949092	+	2913	0.904717922	74.77774811	0	**-0.90471792**	0.534137129	74.77774811	3.32832E-08
FBgn0052282_dro4	FBgn0052282	CG32282	dro4	chr3L:3315636-3315945	+	309	0.904699922	70.56919098	0	**-0.90469992**	0.534143794	70.56919098	8.77164E-08
FBgn0085195_CG34166	FBgn0085195	CG34166	CG34166	chr2L:14743252-14743859	+	533	0.896599948	73.46181488	0	**-0.89659995**	0.537151164	73.46181488	4.50628E-08
FBgn0028542_nimB4	FBgn0028542	CG33115	nimB4	chr2L:13968279-13969973	-	1475	0.890149713	75.383255	0	**-0.89014971**	0.539558124	75.383255	2.89517E-08
FBgn0036831_CG6839	FBgn0036831	CG6839	CG6839	chr3L:18910680-18912361	+	1327	0.890119016	73.40615845	0	**-0.89011902**	0.539569604	73.40615845	4.5644E-08
FBgn0011283_Pbprp5	FBgn0011283	CG6641	Pbprp5	chr2L:7496784-7497360	-	576	0.889808536	72.93947601	0	**-0.88980854**	0.539685737	72.93947601	5.08221E-08
FBgn0054005_CG34005	FBgn0054005	CG34005	CG34005	chr2R:13935616-13936417	+	693	0.880963445	71.95018005	0	**-0.88096344**	0.543004687	71.95018005	6.38237E-08
FBgn0033027_TpnC4	FBgn0033027	CG12408	TpnC4	chr2R:1390941-1395371	-	849	0.879566014	71.97568512	0	**-0.87956601**	0.54353091	71.97568512	6.345E-08
FBgn0002939_ninaD	FBgn0002939	CG31783	ninaD	chr2L:18081629-18083608	+	1786	0.879103303	57.18892288	0	**-0.8791033**	0.543705262	57.18892288	1.91033E-06
FBgn0036362_CG10725	FBgn0036362	CG10725	CG10725	chr3L:13431055-13432109	+	874	0.874769747	68.7076416	0	**-0.87476975**	0.545340895	68.7076416	1.34659E-07
FBgn0039777_Jon99Fii	FBgn0039777	CG2229	Jon99Fii	chr3R:26312734-26313648	-	914	0.874013126	73.71974182	0	**-0.87401313**	0.545626974	73.71974182	4.24645E-08
FBgn0067311_CheB38b	FBgn0067311	CG33321	CheB38b	chr2L:20816548-20817262	-	594	0.870666623	69.36270905	0	**-0.87066662**	0.54689409	69.36270905	1.15805E-07
FBgn0051446_CG31446	FBgn0051446	CG31446	CG31446	chr3R:11518810-11519920	+	870	0.870225132	72.73542023	0	**-0.87022513**	0.547061475	72.73542023	5.3267E-08
FBgn0038214_CG9616	FBgn0038214	CG9616	CG9616	chr3R:10070525-10071193	-	550	0.867015898	69.42029572	0	**-0.8670159**	0.548279752	69.42029572	1.1428E-07
FBgn0035434_dro5	FBgn0035434	CG10812	dro5	chr3L:3316808-3317144	+	336	0.866655588	60.03764725	0	**-0.86665559**	0.548416701	60.03764725	9.91369E-07
FBgn0050289_CG30289	FBgn0050289	CG30289	CG30289	chr2R:17407959-17409338	-	1107	0.863750398	67.57527161	0	**-0.8637504**	0.549522174	67.57527161	1.74772E-07
FBgn0034276_CG6385	FBgn0034276	CG6385	CG6385	chr2R:13641051-13643923	-	2754	0.861352146	77.31217957	0	**-0.86135215**	0.550436427	77.31217957	1.85687E-08
FBgn0032069_CG9468	FBgn0032069	CG9468	CG9468	chr2L:8777659-8781251	-	3160	0.858777642	28.68061447	0	**-0.85877764**	0.551419563	28.68061447	0.001354998
FBgn0033367_CG8193	FBgn0033367	CG8193	CG8193	chr2R:4929764-4932213	-	2280	0.857548952	68.81678009	0	**-0.85754895**	0.551889387	68.81678009	1.31317E-07
FBgn0085420_CG34391	FBgn0085420	CG34391	CG34391	chr3L:5424731-5433143	+	1776	0.849556386	65.56064606	0	**-0.84955639**	0.554955353	65.56064606	2.7793E-07
FBgn0051313_CG31313	FBgn0051313	CG31313	CG31313	chr3R:10392301-10393148	-	631	0.849449515	65.30342102	0	**-0.84944952**	0.554996464	65.30342102	2.94889E-07
FBgn0039611_CG14528	FBgn0039611	CG14528	CG14528	chr3R:24701344-24703529	+	2185	0.847614467	62.77233124	0	**-0.84761447**	0.555702846	62.77233124	5.28162E-07
FBgn0030828_CG5162	FBgn0030828	CG5162	CG5162	chrX:17109334-17111249	+	1386	0.845253587	67.74649048	0	**-0.84525359**	0.556612963	67.74649048	1.68016E-07
FBgn0036550_CG17026	FBgn0036550	CG17026	CG17026	chr3L:15968481-15969528	+	990	0.84186691	66.37082672	0	**-0.84186691**	0.557921127	66.37082672	2.30631E-07
FBgn0037975_CG3397	FBgn0037975	CG3397	CG3397	chr3R:7808819-7810263	-	1187	0.841273665	67.23045349	0	**-0.84127367**	0.558150595	67.23045349	1.89215E-07
FBgn0062928_pncr009_3L	FBgn0062928	CR33940	pncr009_3L	chr3L:19479845-19481488	-	961	0.838473439	61.58298874	0	**-0.83847344**	0.559235	61.58298874	6.94546E-07
FBgn0039685_Obp99b	FBgn0039685	CG7592	Obp99b	chr3R:25541972-25542532	-	560	0.837808251	53.99556732	0	**-0.83780825**	0.559492908	53.99556732	3.98514E-06
FBgn0052212_CG32212	FBgn0052212	CG32212	CG32212	chr3L:19481053-19481448	+	336	0.835855782	60.67612839	0	**-0.83585578**	0.560250609	60.67612839	8.55829E-07
FBgn0034437_CG10051	FBgn0034437	CG10051	CG10051	chr2R:15251149-15254799	+	2764	0.834272861	70.10697174	0	**-0.83427286**	0.560865652	70.10697174	9.7567E-08
FBgn0030270_CG15199	FBgn0030270	CG15199	CG15199	chrX:11034503-11035868	-	667	0.834271729	63.48817825	0	**-0.83427173**	0.560866092	63.48817825	4.47901E-07
FBgn0085356_CG34327	FBgn0085356	CG34327	CG34327	chrX:17117155-17117848	-	693	0.834097147	60.80177307	0	**-0.83409715**	0.560933967	60.80177307	8.31424E-07
FBgn0033980_Cyp6a20	FBgn0033980	CG10245	Cyp6a20	chr2R:10769624-10771551	+	1799	0.833497703	63.71591187	0	**-0.8334977**	0.561167085	63.71591187	4.25019E-07
FBgn0022700_Cht4	FBgn0022700	CG3986	Cht4	chr2R:16952884-16954592	-	1472	0.831578732	65.92649841	0	**-0.83157873**	0.561914007	65.92649841	2.55476E-07
FBgn0038865_CG10824	FBgn0038865	CG10824	CG10824	chr3R:16984959-16986815	-	1856	0.830061018	63.64976883	0	**-0.83006102**	0.562505451	63.64976883	4.31542E-07
FBgn0031694_Cyp4ac2	FBgn0031694	CG17970	Cyp4ac2	chr2L:5266391-5268455	+	1709	0.829509616	64.11354828	0	**-0.82950962**	0.562720483	64.11354828	3.87833E-07
FBgn0001114_Glt	FBgn0001114	CG9280	Glt	chr2L:8672042-8676811	+	3474	0.829120219	66.48228455	0	**-0.82912022**	0.562872387	66.48228455	2.24787E-07
FBgn0030590_CG9518	FBgn0030590	CG9518	CG9518	chrX:14757021-14765207	-	3672	0.826018274	60.69790649	0	**-0.82601827**	0.564083924	60.69790649	8.51548E-07
FBgn0032839_CG10659	FBgn0032839	CG10659	CG10659	chr2L:20025112-20025781	-	669	0.81995976	53.83994293	0	**-0.81995976**	0.566457742	53.83994293	4.13053E-06
FBgn0036320_CG10943	FBgn0036320	CG10943	CG10943	chr3L:12854891-12855818	-	927	0.818407476	70.93554688	0	**-0.81840748**	0.567067557	70.93554688	8.06205E-08
FBgn0037562_CG11671	FBgn0037562	CG11671	CG11671	chr3R:4113660-4114340	+	623	0.81788528	67.52224731	0	**-0.81788528**	0.567272849	67.52224731	1.76919E-07
FBgn0033958_CG12858	FBgn0033958	CG12858	CG12858	chr2R:10627870-10632834	-	3409	0.816757321	59.91179276	0	**-0.81675732**	0.56771654	59.91179276	1.02052E-06
FBgn0034076_Jhedup	FBgn0034076	CG8424	Jhedup	chr2R:12004615-12006654	+	1756	0.814508915	61.99991226	0	**-0.81450891**	0.568602003	61.99991226	6.3097E-07
FBgn0040106_lectin-21Cb	FBgn0040106	CG13686	lectin-21Cb	chr2L:620432-621540	-	774	0.814241111	77.74076843	0	**-0.81424111**	0.56870756	77.74076843	1.68238E-08
FBgn0032286_CG7300	FBgn0032286	CG7300	CG7300	chr2L:10695208-10698024	-	2816	0.812558889	62.8878479	0	**-0.81255889**	0.569371076	62.8878479	5.14298E-07
FBgn0034010_CG8157	FBgn0034010	CG8157	CG8157	chr2R:11213337-11213888	-	491	0.811412334	60.57084656	0	**-0.81141233**	0.569823753	60.57084656	8.7683E-07
FBgn0034582_Cht9	FBgn0034582	CG10531	Cht9	chr2R:16955486-16956813	-	1193	0.799338698	54.82371521	0	**-0.7993387**	0.574612508	54.82371521	3.29328E-06
FBgn0038845_CG10827	FBgn0038845	CG10827	CG10827	chr3R:16830881-16832709	-	1671	0.79738301	61.53202438	0	**-0.79738301**	0.575391969	61.53202438	7.02745E-07
FBgn0054033_CG34033	FBgn0054033	CG34033	CG34033	chr2R:5708067-5708691	+	566	0.796114862	60.05387497	0	**-0.79611486**	0.575897969	60.05387497	9.87671E-07
FBgn0033408_CG8800	FBgn0033408	CG8800	CG8800	chr2R:5282833-5283602	-	651	0.792444527	55.52608109	0	**-0.79244453**	0.577364966	55.52608109	2.80151E-06
FBgn0000299_Cg25C	FBgn0000299	CG4145	Cg25C	chr2L:5029614-5037114	+	5992	0.791682363	56.96994781	0	**-0.79168236**	0.577670064	56.96994781	2.00912E-06
FBgn0038481_CG17475	FBgn0038481	CG17475	CG17475	chr3R:12955136-12956141	-	1005	0.79065007	58.4499321	0	**-0.79065007**	0.578083552	58.4499321	1.42892E-06
FBgn0000533_ea	FBgn0000533	CG4920	ea	chr3R:11154451-11156135	+	1434	0.789096177	53.59971237	0	**-0.78909618**	0.578706528	53.59971237	4.36545E-06
FBgn0032638_CG6639	FBgn0032638	CG6639	CG6639	chr2L:17454262-17455917	-	1581	0.782385886	68.91329193	0	**-0.78238589**	0.581404488	68.91329193	1.28431E-07
FBgn0053173_CG33173	FBgn0053173	CG33173	CG33173	chrX:15667203-15674095	+	5504	0.782050848	53.99556732	0	**-0.78205085**	0.581539524	53.99556732	3.98514E-06
FBgn0031629_CG3244	FBgn0031629	CG3244	CG3244	chr2L:4796583-4801923	-	1295	0.779838145	57.76351929	0	**-0.77983814**	0.582432132	57.76351929	1.67359E-06

FBgn0034166_CG6472	FBgn0034166	CG6472	CG6472	chr2R:12767777-12772005	+	1185	0.779387116	49.16386795	0	**-0.77938712**	0.582614246	49.16386795	1.21231E-05
FBgn0034783_CG9825	FBgn0034783	CG9825	CG9825	chr2R:18724885-18726814	+	1541	0.778582573	59.97364807	0	**-0.77858257**	0.582939241	59.97364807	1.00609E-06
FBgn0032068_CG9466	FBgn0032068	CG9466	CG9466	chr2L:8772947-8776261	-	3070	0.777826369	24.44740486	0	**-0.77782637**	0.583244875	24.44740486	0.003591365
FBgn0033769_CG8768	FBgn0033769	CG8768	CG8768	chr2R:8578812-8580193	-	1208	0.774892271	53.11039352	0	**-0.77489227**	0.584432264	53.11039352	4.88608E-06
FBgn0034784_CG9826	FBgn0034784	CG9826	CG9826	chr2R:18728357-18729988	+	1569	0.774801016	51.89162064	0	**-0.77480102**	0.584469232	51.89162064	6.46901E-06
FBgn0041711_yellow-e	FBgn0041711	CG9792	yellow-e	chr3R:9235263-9241006	-	1843	0.773439765	48.49638367	0	**-0.77343976**	0.585020966	48.49638367	1.41371E-05
FBgn0031489_CG17224	FBgn0031489	CG17224	CG17224	chr2L:3016590-3018304	-	1423	0.771544755	45.30586243	0	**-0.77154475**	0.585789909	45.30586243	2.94723E-05
FBgn0031701_TotM	FBgn0031701	CG14027	TotM	chr2L:5329856-5330466	+	554	0.768813312	81.04771423	0	**-0.76881331**	0.58690003	81.04771423	7.85649E-09
FBgn0030737_CG9914	FBgn0030737	CG9914	CG9914	chrX:16200944-16202416	-	1161	0.768745303	52.81612396	0	**-0.7687453**	0.586927698	52.81612396	5.22863E-06
FBgn0250815_Jon65Aiv	FBgn0250815	CG6467	Jon65Aiv	chr3L:6032256-6033248	+	915	0.76648587	51.87677002	0	**-0.76648587**	0.587847617	51.87677002	6.49117E-06
FBgn0014865_Mtk	FBgn0014865	CG8175	Mtk	chr2R:11296350-11296621	+	271	0.761907518	66.77789307	0	**-0.76190752**	0.589716098	66.77789307	2.09996E-07
FBgn0040099_lectin-28C	FBgn0040099	CG7106	lectin-28C	chr2L:7857089-7857931	+	842	0.760580659	46.30659866	0	**-0.76058066**	0.590258714	46.30659866	2.34067E-05
FBgn0051265_CG31265	FBgn0051265	CG31265	CG31265	chr3R:12953945-12954842	+	897	0.758543551	54.07886505	0	**-0.75854355**	0.591092758	54.07886505	3.90943E-06
FBgn0037228_CG1092	FBgn0037228	CG1092	CG1092	chr3R:92675-94166	+	1491	0.754663229	52.15791702	0	**-0.75466323**	0.592684721	52.15791702	6.08427E-06
FBgn0033789_CG13324	FBgn0033789	CG13324	CG13324	chr2R:8936646-8937123	-	477	0.753686249	54.73092651	0	**-0.75368625**	0.593086217	54.73092651	3.3644E-06
FBgn0259951_Sfp24Ba	FBgn0259951	CG42461	Sfp24Ba	chr2L:3668015-3668551	-	483	0.749959171	50.25251389	0	**-0.74995917**	0.594620385	50.25251389	9.43515E-06
FBgn0016075_vkg	FBgn0016075	CG16858	vkg	chr2L:5012143-5027412	-	6343	0.749751985	49.3323288	0	**-0.74975199**	0.594705785	49.3323288	1.16618E-05
FBgn0050083_CG30083	FBgn0050083	CG30083	CG30083	chr2R:11599101-11600149	-	868	0.749426484	50.48039246	0	**-0.74942648**	0.594839978	50.48039246	8.95284E-06
FBgn0030108_Gr8a	FBgn0030108	CG15371	Gr8a	chrX:9121169-9122864	+	1513	0.74899441	51.72303391	0	**-0.74899441**	0.595018154	51.72303391	6.72507E-06
FBgn0033809_CG4630	FBgn0033809	CG4630	CG4630	chr2R:9103158-9106752	-	2126	0.743646383	51.38430786	0	**-0.74364638**	0.597227961	51.38430786	7.27058E-06
FBgn0051104_CG31104	FBgn0051104	CG31104	CG31104	chr3R:21111194-21112696	+	1382	0.741925776	33.46109009	0	**-0.74192578**	0.597940661	33.46109009	0.000450704
FBgn0030332_CG9360	FBgn0030332	CG9360	CG9360	chrX:11657504-11658621	-	1035	0.740362167	47.7388649	0	**-0.74036217**	0.598589066	47.7388649	1.68311E-05
FBgn0031653_Jon25Biii	FBgn0031653	CG8871	Jon25Biii	chr2L:4949946-4950763	-	817	0.740338385	40.27270889	0	**-0.74033839**	0.598598934	40.27270889	9.39137E-05
FBgn0036836_CG11619	FBgn0036836	CG11619	CG11619	chr3L:18979158-18981258	-	2100	0.738587439	48.75450516	0	**-0.73858744**	0.599325873	48.75450516	1.33214E-05
FBgn0261362_proPO-A1	FBgn0261362	CG42639	proPO-A1	chr2R:13774717-13777477	-	2344	0.738519788	50.55744171	0	**-0.73851979**	0.599353977	50.55744171	8.7954E-06
FBgn0011554_etaTry	FBgn0011554	CG12386	etaTry	chr2R:7228017-7228928	-	911	0.736695945	56.44908524	0	**-0.73669595**	0.600112154	56.44908524	2.26512E-06
FBgn0053983_obst-H	FBgn0053983	CG33983	obst-H	chr3L:15521364-15522343	-	915	0.735724568	60.03764725	0	**-0.73572457**	0.60051635	60.03764725	9.91369E-07
FBgn0035665_Jon65Aiii	FBgn0035665	CG6483	Jon65Aiii	chr3L:6043873-6044800	+	868	0.735331297	47.77249908	0	**-0.7353313**	0.60068007	47.77249908	1.67013E-05
FBgn0031726_Cyp6a16Psi	FBgn0031726	CG7249	Cyp6a16Psi	chr2L:5621601-5623760	-	1529	0.732524753	38.93075562	0	**-0.73252475**	0.60184974	38.93075562	0.000127916
FBgn0038732_CG11391	FBgn0038732	CG11391	CG11391	chr3R:15604487-15606438	+	1773	0.731240451	45.1533432	0	**-0.73124045**	0.602385751	45.1533432	3.05257E-05
FBgn0036992_CG11796	FBgn0036992	CG11796	CG11796	chr3L:20431265-20432968	-	1514	0.731087565	48.22304153	0	**-0.73108757**	0.60244959	48.22304153	1.50555E-05
FBgn0005633_fln	FBgn0005633	CG7445	fln	chr3L:19982650-19984279	-	987	0.729717314	49.86195374	0	**-0.72971731**	0.60302206	49.86195374	1.0323E-05
FBgn0024289_Sodh-1	FBgn0024289	CG1982	Sodh-1	chr3R:2878056-2880264	+	1388	0.729613185	47.9500618	0	**-0.72961318**	0.603065586	47.9500618	1.60322E-05
FBgn0003358_Jon99Ci	FBgn0003358	CG31039	Jon99Ci	chr3R:25750947-25751907	-	903	0.72943455	38.57003784	0	**-0.72943455**	0.603140262	38.57003784	0.000138994
FBgn0051089_CG31089	FBgn0051089	CG31089	CG31089	chr3R:21728418-21729812	+	1266	0.726763785	51.51879501	0	**-0.72676378**	0.60425785	51.51879501	7.04889E-06
FBgn0011828_Pxn	FBgn0011828	CG12002	Pxn	chr3L:2601449-2630072	-	6053	0.723542511	47.55329514	0	**-0.72354251**	0.605608554	47.55329514	1.75659E-05
FBgn0033312_CG8642	FBgn0033312	CG8642	CG8642	chr2R:4499398-4501416	-	1044	0.720008373	51.95011139	0	**-0.72000837**	0.607093919	51.95011139	6.38247E-06
FBgn0032405_CG14946	FBgn0032405	CG14946	CG14946	chr2L:12063522-12066772	-	2521	0.719266057	48.38692856	0	**-0.71926606**	0.60740637	48.38692856	1.4498E-05
FBgn0035476_CG12766	FBgn0035476	CG12766	CG12766	chr3L:3938623-3940084	-	1034	0.718410194	47.14804077	0	**-0.71841019**	0.607766814	47.14804077	1.92839E-05
FBgn0036778_Cyp312a1	FBgn0036778	CG5137	Cyp312a1	chr3L:18123199-18125333	-	1767	0.716673791	58.24914551	0	**-0.71667379**	0.608498752	58.24914551	1.49653E-06
FBgn0035280_Cpr62Bb	FBgn0035280	CG13935	Cpr62Bb	chr3L:1832635-1835166	-	994	0.716573596	46.34235001	0	**-0.7165736**	0.608541014	46.34235001	2.32148E-05
FBgn0030311_CG11699	FBgn0030311	CG11699	CG11699	chrX:11464656-11465445	+	639	0.716177583	44.38174438	0	**-0.71617758**	0.608708078	44.38174438	3.64607E-05
FBgn0039612_CG14523	FBgn0039612	CG14523	CG14523	chr3R:24703735-24705963	-	2228	0.714852989	51.56359482	0	**-0.71485299**	0.609267213	51.56359482	6.97655E-06
FBgn0033763_CG8646	FBgn0033763	CG8646	CG8646	chr2R:8543460-8546846	+	1761	0.712617636	44.38174438	0	**-0.71261764**	0.610211961	44.38174438	3.64607E-05
FBgn0050043_CG30043	FBgn0050043	CG30043	CG30043	chr2R:8246957-8250670	+	3065	0.711295724	43.01633072	0	**-0.71129572**	0.610771342	43.01633072	4.99306E-05
FBgn0031693_Cyp4ac1	FBgn0031693	CG14032	Cyp4ac1	chr2L:5264000-5265899	+	1666	0.710297763	47.90266418	0	**-0.71029776**	0.611193979	47.90266418	1.62082E-05
FBgn0250836_CG8628	FBgn0250836	CG8628	CG8628	chr3L:7123973-7124605	+	436	0.709876478	43.50517654	0	**-0.70987648**	0.611372482	43.50517654	4.46151E-05
FBgn0037553_CG18249	FBgn0037553	CG18249	CG18249	chr3R:4045862-4047910	+	2048	0.709574819	48.58502197	0	**-0.70957482**	0.61150033	48.58502197	1.38515E-05
FBgn0033540_StIP	FBgn0033540	CG11887	StIP	chr2R:6533229-6536144	+	2553	0.707346916	42.16955185	0	**-0.70734692**	0.612445377	42.16955185	6.06799E-05
FBgn0032706_Irk3	FBgn0032706	CG10369	Irk3	chr2L:18711070-18713763	-	1994	0.703576207	40.32255554	0	**-0.70357621**	0.614048193	40.32255554	9.2842E-05
FBgn0033978_Cyp6a23	FBgn0033978	CG10242	Cyp6a23	chr2R:10763337-10765159	+	1757	0.701786399	44.77055359	0	**-0.7017864**	0.614810454	44.77055359	3.33384E-05
FBgn0032002_CG8353	FBgn0032002	CG8353	CG8353	chr2L:8214304-8215239	+	751	0.699335396	41.26245117	0	**-0.6993354**	0.615855847	41.26245117	7.47747E-05
FBgn0259967_Sfp53D	FBgn0259967	CG42477	Sfp53D	chr2R:12632054-12632609	-	555	0.698752344	46.22535706	0	**-0.69875234**	0.61610479	46.22535706	2.38487E-05
FBgn0013686_mt_lrRNA	FBgn0013686	CR34094	mt_lrRNA	chrM:12733-14058	-	1325	0.694636762	61.69473267	0	**-0.69463676**	0.617864863	61.69473267	6.76903E-07
FBgn0040261_Ugt36Bb	FBgn0040261	CG13271	Ugt36Bb	chr2L:16796594-16798273	+	1620	0.693942964	45.84912872	0	**-0.69394296**	0.618162069	45.84912872	2.60068E-05

FBgn0034154_CG5267	FBgn0034154	CG5267	CG5267	chr2R:12642605-12643328	+	723	0.693773866	43.69057465	0	**-0.69377387**	0.618234528	43.69057465	4.27506E-05
FBgn0085358_CG34329	FBgn0085358	CG34329	CG34329	chrX:18944505-18945010	+	505	0.693428099	35.15674591	0	**-0.6934281**	0.618382716	35.15674591	0.000305018
FBgn0038716_CG7342	FBgn0038716	CG7342	CG7342	chr3R:15429832-15432200	+	1823	0.691609859	47.13195801	0	**-0.69160986**	0.61916256	47.13195801	1.93555E-05
FBgn0010044_GstD8	FBgn0010044	CG4421	GstD8	chr3R:8205744-8206537	+	793	0.691520154	39.70695114	0	**-0.69152015**	0.61920106	39.70695114	0.000106981
FBgn0033857_CG13335	FBgn0033857	CG13335	CG13335	chr2R:9458888-9461734	-	2782	0.691242874	40.82149887	0	**-0.69124287**	0.619320079	40.82149887	8.27656E-05
FBgn0040091_Ugt58Fa	FBgn0040091	CG4414	Ugt58Fa	chr2R:18529400-18531420	-	1752	0.689831018	47.85686111	0	**-0.68983102**	0.619926457	47.85686111	1.638E-05
FBgn0051087_CG31087	FBgn0051087	CG31087	CG31087	chr3R:21136748-21139555	-	1445	0.688758969	47.74721146	0	**-0.68875897**	0.620387288	47.74721146	1.67988E-05
FBgn0033501_CG12911	FBgn0033501	CG12911	CG12911	chr2R:6144193-6148781	+	1636	0.686865211	41.70524597	0	**-0.68686521**	0.621202176	41.70524597	6.75267E-05
FBgn0034187_CG6967	FBgn0034187	CG6967	CG6967	chr2R:12950281-12954051	+	3472	0.684923768	50.7770462	0	**-0.68492377**	0.622038694	50.7770462	8.36172E-06
FBgn0013680_mt_ND2	FBgn0013680	CG34063	mt_ND2	chrM:239-1265	+	1026	0.683237135	63.68108749	0	**-0.68323714**	0.622766335	63.68108749	4.28441E-07
FBgn0038719_CG16727	FBgn0038719	CG16727	CG16727	chr3R:15439102-15441506	+	2014	0.683192968	42.16793823	0	**-0.68319297**	0.622785401	42.16793823	6.07024E-05
FBgn0051380_CG31380	FBgn0051380	CG31380	CG31380	chr3R:21613718-21615177	-	1335	0.68298167	41.16876602	0	**-0.68298167**	0.622876622	41.16876602	7.64053E-05
FBgn0002856_Acp26Ab	FBgn0002856	CG9024	Acp26Ab	chr2L:5892297-5892862	-	504	0.682685852	43.69204712	0	**-0.68268585**	0.623004353	43.69204712	4.27361E-05
FBgn0031926_CG6739	FBgn0031926	CG6739	CG6739	chr2L:7608389-7616528	+	2707	0.682161033	40.81496048	0	**-0.68216103**	0.623231028	40.81496048	8.28903E-05
FBgn0011280_Pbprp2	FBgn0011280	CG1668	Pbprp2	chrX:20290639-20292009	+	1104	0.681736887	38.48994827	0	**-0.68173689**	0.623414283	38.48994827	0.000141581
FBgn0038986_CG5278	FBgn0038986	CG5278	CG5278	chr3R:18384582-18388293	-	1202	0.680657029	37.09020996	0	**-0.68065703**	0.623881083	37.09020996	0.000195424
FBgn0031520_CG8837	FBgn0031520	CG8837	CG8837	chr2L:3320254-3323141	+	1825	0.680536985	36.47890091	0	**-0.68053699**	0.623932997	36.47890091	0.000224962
FBgn0036939_CG7365	FBgn0036939	CG7365	CG7365	chr3L:20117395-20120465	-	1656	0.678112566	40.87987518	0	**-0.67811257**	0.624982385	40.87987518	8.16606E-05
FBgn0034660_lox2	FBgn0034660	CG4402	lox2	chr2R:17677120-17679160	-	1811	0.677809656	41.04854584	0	**-0.67780966**	0.625113621	41.04854584	7.85499E-05
FBgn0036877_CG9452	FBgn0036877	CG9452	CG9452	chr3L:19494244-19496961	-	1508	0.676176071	39.54067993	0	**-0.67617607**	0.625821847	39.54067993	0.000111156
FBgn0261059_Sfp78E	FBgn0261059	CG42607	Sfp78E	chr3L:21676197-21677142	-	778	0.6754179	40.81496048	0	**-0.6754179**	0.626150818	40.81496048	8.28903E-05
FBgn0036750_CG6034	FBgn0036750	CG6034	CG6034	chr3L:17738472-17739912	-	1440	0.675055206	46.61749649	0	**-0.67505521**	0.626308253	46.61749649	2.17897E-05
FBgn0085285_CG34256	FBgn0085285	CG34256	CG34256	chr3L:18981835-18982915	-	762	0.671286285	41.70524597	0	**-0.67128628**	0.62794657	41.70524597	6.75267E-05
FBgn0033742_CG8550	FBgn0033742	CG8550	CG8550	chr2R:8359419-8361321	+	1902	0.670977294	38.90427017	0	**-0.67097729**	0.628081075	38.90427017	0.000128698
FBgn0039666_CG11501	FBgn0039666	CG11501	CG11501	chr3R:25319573-25319987	+	414	0.670065761	69.43541718	0	**-0.67006576**	0.628478039	69.43541718	1.13883E-07
FBgn0032472_CG9928	FBgn0032472	CG9928	CG9928	chr2L:13142480-13142902	-	422	0.6693362	39.50466156	0	**-0.6693362**	0.628795937	39.50466156	0.000112081
FBgn0053296_CG33296	FBgn0053296	CG33296	CG33296	chr2L:7714068-7716645	-	1878	0.66709584	38.80708694	0	**-0.66709584**	0.629773152	38.80708694	0.000131611
FBgn0037498_CG10029	FBgn0037498	CG10029	CG10029	chr3R:3141445-3142929	-	1380	0.666860461	41.96142578	0	**-0.66686046**	0.629875909	41.96142578	6.36586E-05
FBgn0030981_CG14191	FBgn0030981	CG14191	CG14191	chrX:18975699-18976606	+	792	0.666527867	42.44948196	0	**-0.66652787**	0.630021135	42.44948196	5.68921E-05
FBgn0024291_Sir2	FBgn0024291	CG5216	Sir2	chr2L:13165550-13169551	+	3822	0.663832664	39.32303619	0	**-0.66383266**	0.631199223	39.32303619	0.000116868
FBgn0054034_CG34034	FBgn0054034	CG34034	CG34034	chr3R:17563218-17564832	-	847	0.662555635	40.3934021	0	**-0.66255563**	0.631758189	40.3934021	9.13397E-05
FBgn0036972_CG6434	FBgn0036972	CG6434	CG6434	chr3L:20315476-20316309	-	659	0.661959767	37.48912048	0	**-0.66195977**	0.632019174	37.48912048	0.000178274
FBgn0036951_CG7017	FBgn0036951	CG7017	CG7017	chr3L:20203537-20205262	-	1454	0.65979898	27.34539223	0	**-0.65979898**	0.632966486	27.34539223	0.001842726
FBgn0035232_CG12099	FBgn0035232	CG12099	CG12099	chr3L:1555362-1558454	-	2905	0.659337759	34.54815292	0	**-0.65933776**	0.633168874	34.54815292	0.000350901
FBgn0036727_CG7589	FBgn0036727	CG7589	CG7589	chr3L:17463525-17467617	+	1950	0.658911467	39.80805969	0	**-0.65891147**	0.633355993	39.80805969	0.000104519
FBgn0038701_CG18493	FBgn0038701	CG18493	CG18493	chr3R:15215993-15217854	+	1564	0.657310188	41.05121613	0	**-0.65731019**	0.634059359	41.05121613	7.85016E-05
FBgn0036969_CG6663	FBgn0036969	CG6663	CG6663	chr3L:20314116-20315420	+	1113	0.655966103	32.51223755	0	**-0.6559661**	0.634650354	32.51223755	0.000560759
FBgn0005592_btl	FBgn0005592	CG32134	btl	chr3L:14068871-14075985	+	3624	0.653815567	40.9065361	0	**-0.65381557**	0.635597093	40.9065361	8.11608E-05
FBgn0037807_CG6293	FBgn0037807	CG6293	CG6293	chr3R:6133983-6137317	-	2127	0.651299655	40.60920334	0	**-0.65129966**	0.636706477	40.60920334	8.6912E-05
FBgn0033203_CG2070	FBgn0033203	CG2070	CG2070	chr2R:3549516-3550764	+	1075	0.65014261	33.67996597	0	**-0.65014261**	0.637217322	33.67996597	0.000428552
FBgn0034160_CG5550	FBgn0034160	CG5550	CG5550	chr2R:12704095-12705086	+	928	0.649903238	38.35747528	0	**-0.64990324**	0.637323058	38.35747528	0.000145966
FBgn0036975_CG5618	FBgn0036975	CG5618	CG5618	chr3L:20347051-20349794	+	1968	0.649856746	35.11803818	0	**-0.64985675**	0.637343596	35.11803818	0.000307749
FBgn0031942_CG7203	FBgn0031942	CG7203	CG7203	chr2L:7752167-7753156	-	702	0.649247229	32.6360054	0	**-0.64924723**	0.637612921	32.6360054	0.000545004
FBgn0037678_CG16749	FBgn0037678	CG16749	CG16749	chr3R:5156606-5157617	-	883	0.649065077	35.03314972	0	**-0.64906508**	0.63769343	35.03314972	0.000313823
FBgn0052447_CG32447	FBgn0052447	CG32447	CG32447	chr3L:21685901-21699447	-	3116	0.647221386	37.82257843	0	**-0.64722139**	0.638508891	37.82257843	0.000165098
FBgn0036208_CG10361	FBgn0036208	CG10361	CG10361	chr3L:11792043-11793956	-	1430	0.646652758	37.89844131	0	**-0.64665276**	0.638760604	37.89844131	0.000162239
FBgn0050042_Cpr49Ab	FBgn0050042	CG30042	Cpr49Ab	chr2R:8265961-8266901	+	835	0.64576602	37.78203583	0	**-0.64576602**	0.639153333	37.78203583	0.000166647
FBgn0053533_lectin-37Db	FBgn0053533	CG33533	lectin-37Db	chr2L:19419074-19419767	+	562	0.645267427	39.22560883	0	**-0.64526743**	0.639374261	39.22560883	0.00011952
FBgn0034709_CG3074	FBgn0034709	CG3074	CG3074	chr2R:18077325-18090178	-	2170	0.643200874	29.82616234	0	**-0.64320087**	0.640290773	29.82616234	0.00104084
FBgn0043575_PGRP-SC2	FBgn0043575	CG14745	PGRP-SC2	chr2R:4604471-4605026	+	555	0.642103851	45.74254608	0	**-0.64210385**	0.640777835	45.74254608	2.6653E-05
FBgn0051267_CG31267	FBgn0051267	CG31267	CG31267	chr3R:12959918-12961005	+	921	0.639095783	34.30060196	0	**-0.63909578**	0.642115272	34.30060196	0.000371484
FBgn0043578_PGRP-SB1	FBgn0043578	CG9681	PGRP-SB1	chr3L:16720398-16721118	-	652	0.636855423	45.88137817	0	**-0.63685542**	0.643113187	45.88137817	2.58144E-05
FBgn0054035_CG34035	FBgn0054035	CG34035	CG34035	chr3R:13238809-13240253	+	639	0.636056185	35.90251541	0	**-0.63605618**	0.643469564	35.90251541	0.000256891
FBgn0037288_CG14661	FBgn0037288	CG14661	CG14661	chr3R:779227-780974	-	1083	0.635538578	32.57567215	0	**-0.63553858**	0.643700468	32.57567215	0.000552628
FBgn0053105_p24-2	FBgn0053105	CG33105	p24-2	chr3R:5517624-5518516	-	830	0.634959519	36.81855774	0	**-0.63495952**	0.643958884	36.81855774	0.000208039

FBgn0034294_Muc55B	FBgn0034294	CG5765	Muc55B	chr2R:13937730-13939610	-	1593	0.634917676	39.33638	0	-0.63491768	0.643977561	39.33638	0.00011651		
FBgn0032834_CG13965	FBgn0032834	CG13965	CG13965	chr2L:19961836-19962298	-	402	0.634766817	31.68521118	0	-0.63476682	0.644044903	31.68521118	0.000678389		
FBgn0035090_CG2736	FBgn0035090	CG2736	CG2736	chr2R:20860943-20862894	-	1766	0.63123256	34.94607162	0	-0.63123256	0.645624593	34.94607162	0.000320179		
FBgn0086711_mol	FBgn0086711	CG4482	mol	chr2L:14975746-14997556	-	3009	0.630545795	34.6198349	0	-0.63054579	0.645932003	34.6198349	0.000345157		
FBgn0036752_Adgf-A	FBgn0036752	CG5992	Adgf-A	chr3L:17744817-17757253	-	2596	0.63032788	39.33638	0	-0.63032788	0.646029576	39.33638	0.00011651		
FBgn0050151_CG30151	FBgn0050151	CG30151	CG30151	chr2R:16416008-16416969	-	961	0.630227327	35.15674591	0	-0.63022733	0.646074604	35.15674591	0.000305018		
FBgn0032082_CG18088	FBgn0032082	CG18088	CG18088	chr2L:8967536-8970264	+	2263	0.630056739	34.52384949	0	-0.63005674	0.646151003	34.52384949	0.00035287		
FBgn0004108_Nrt	FBgn0004108	CG9704	Nrt	chr3L:16759173-16770678	+	4390	0.629628718	35.51385117	0	-0.62962872	0.646342732	35.51385117	0.000280941		
FBgn0260746_Ect3	FBgn0260746	CG3132	Ect3	chr3R:7825028-7829239	+	2208	0.627761543	32.51223755	0	-0.62776154	0.647179788	32.51223755	0.000560759		
FBgn0259957_CG42467	FBgn0259957	CG42467	CG42467	chr2L:3701306-3701604	+	249	0.627344847	35.44692993	0	-0.62734485	0.647366741	35.44692993	0.000285303		
FBgn0033388_CG8046	FBgn0033388	CG8046	CG8046	chr2R:5049541-5052455	-	2308	0.627323329	34.72802734	0	-0.62732333	0.647376396	34.72802734	0.000336664		
FBgn0039473_CG17191	FBgn0039473	CG17191	CG17191	chr3R:22844329-22845473	-	1090	0.625852287	56.1021843	0	-0.62585229	0.64803683	56.1021843	2.45347E-06		
FBgn0028738_ETH	FBgn0028738	CG18105	ETH	chr2R:20682177-20682981	-	746	0.625750363	33.95674133	0	-0.62575036	0.648082614	33.95674133	0.000402092		
FBgn0085200_CG34171	FBgn0085200	CG34171	CG34171	chr2L:18378625-18379619	+	858	0.623434603	36.21242142	0	-0.6234346	0.649123727	36.21242142	0.000239198		
FBgn0040060_yip7	FBgn0040060	CG6457	yip7	chr3L:6028329-6029279	+	894	0.622882903	32.51223755	0	-0.6228829	0.649372006	32.51223755	0.000560759		
FBgn0047205_CR32385	FBgn0047205	CR32385	CR32385	chr3L:6969786-6972326	+	2211	0.622698247	30.47714233	0	-0.62269825	0.649455127	30.47714233	0.000895954		
FBgn0031800_CG9497	FBgn0031800	CG9497	CG9497	chr2L:6346541-6348151	-	1386	0.620432794	32.55652237	0	-0.62043279	0.650475762	32.55652237	0.00055507		
FBgn0033733_CG8834	FBgn0033733	CG8834	CG8834	chr2R:8307315-8309988	-	1869	0.61885798	36.47890091	0	-0.61885798	0.651186195	36.47890091	0.000224962		
FBgn0037936_CG6908	FBgn0037936	CG6908	CG6908	chr3R:7514098-7515745	-	1415	0.61775589	33.81573486	0	-0.61775589	0.651683833	33.81573486	0.000415362		
FBgn0052284_CG32284	FBgn0052284	CG32284	CG32284	chr3L:3186663-3187245	+	457	0.616998196	39.97199631	0	-0.6169982	0.652026183	39.97199631	0.000100647		
FBgn0034296_CG10912	FBgn0034296	CG10912	CG10912	chr2R:13943567-13944564	-	905	0.615125656	33.88800049	0	-0.61512566	0.652873027	33.88800049	0.000408507		
FBgn0039326_CG10562	FBgn0039326	CG10562	CG10562	chr3R:21146773-21148473	-	1522	0.614394605	33.39171219	0	-0.61439461	0.653203939	33.39171219	0.000457961		
FBgn0032144_CG17633	FBgn0032144	CG17633	CG17633	chr2L:9769066-9770415	+	1349	0.612184048	22.23976326	0	-0.61218405	0.654205572	22.23976326	0.005970678		
FBgn0039323_CG10559	FBgn0039323	CG10559	CG10559	chr3R:21140390-21141826	-	1248	0.610370219	33.27112961	0	-0.61037022	0.655028589	33.27112961	0.000470855		
FBgn0030776_CG4653	FBgn0030776	CG4653	CG4653	chrX:16598120-16599188	+	765	0.608005881	35.69763565	0	-0.60800588	0.656102953	35.69763565	0.0002693		
FBgn0035484_CG11594	FBgn0035484	CG11594	CG11594	chr3L:4022969-4026009	-	2861	0.603887737	34.55995178	0	-0.60388774	0.657978461	34.55995178	0.000349949		
FBgn0032285_CG17108	FBgn0032285	CG17108	CG17108	chr2L:10692939-10694103	-	1164	0.601350307	30.16062546	0	-0.60135031	0.65913674	30.16062546	0.00096369		
FBgn0034942_CG13566	FBgn0034942	CG13566	CG13566	chr2R:19849177-19850034	-	624	0.600716949	26.5952282	0	-0.60071695	0.659426172	26.5952282	0.002190167		
FBgn0031907_CG5171	FBgn0031907	CG5171	CG5171	chr2L:7395831-7404526	+	1535	0.600085795	31.32741928	0	-0.60008579	0.659714722	31.32741928	0.000736645		
FBgn0038207_CG14356	FBgn0038207	CG14356	CG14356	chr3R:9956399-9957791	-	739	0.599981487	27.81986237	0	-0.59998149	0.659762422	27.81986237	0.001652014		
FBgn0019940_Rh6	FBgn0019940	CG5192	Rh6	chr3R:11308879-11310421	+	1474	0.599803686	27.37844086	0	-0.59980369	0.659843737	27.37844086	0.001828757		
FBgn0039609_CG14529	FBgn0039609	CG14529	CG14529	chr3R:24698626-24700753	+	2127	0.598185182	34.24668121	0	-0.59818518	0.660584406	34.24668121	0.000376125		
FBgn0033302_Cyp6a14	FBgn0033302	CG8687	Cyp6a14	chr2R:4451797-4453481	-	1627	0.598050594	27.40278435	0	-0.59805059	0.660646034	27.40278435	0.001818535		
FBgn0030334_Karl	FBgn0030334	CG4139	Karl	chrX:11682878-11686287	+	1417	0.595384896	32.70236588	0	-0.5953849	0.661867852	32.70236588	0.000536739		
FBgn0036068_CG6640	FBgn0036068	CG6640	CG6640	chr3L:10088846-10093981	-	2888	0.593503535	19.52510643	0	-0.59350353	0.662731531	19.52510643	0.011155508		
FBgn0085342_CG34313	FBgn0085342	CG34313	CG34313	chr2L:16879516-16886323	+	2243	0.591353238	29.55621719	0	-0.59135324	0.66372005	29.55621719	0.001107588		
FBgn0032618_CG31743	FBgn0032618	CG31743	CG31743	chr2L:16879516-16886323	+	2243	0.591353238	29.55621719	0	-0.59135324	0.66372005	29.55621719	0.001107588		
FBgn0038752_CG4462	FBgn0038752	CG4462	CG4462	chr3R:15747465-15749660	-	1896	0.590847552	28.99768829	0	-0.59084755	0.663952735	28.99768829	0.001259596		
FBgn0034756_Cyp6d2	FBgn0034756	CG4373	Cyp6d2	chr2R:18540077-18542032	-	1756	0.58652401	34.5811882	0	-0.58652401	0.665945487	34.5811882	0.000348242		
FBgn0040001_CG17374	FBgn0040001	CG17374	CG17374	chr3LHet:1135557-1145369	-	7100	0.585628748	30.87971306	0	-0.58562875	0.666358866	30.87971306	0.000816636		
FBgn0035453_CG10357	FBgn0035453	CG10357	CG10357	chr3L:3615381-3616682	+	1111	0.585063756	33.39171219	0	0.585063756	1.50010528	33.39171219	0.000457961		
FBgn0033464_CG1441	FBgn0033464	CG1441	CG1441	chr2R:5786522-5790788	-	1862	0.585196495	31.72974205	0	0.585196495	1.500243309	31.72974205	0.000671469		
FBgn0035023_itp	FBgn0035023	CG13586	itp	chr2R:20427494-20446273	+	4207	0.588579535	31.32846832	0	0.588579535	1.503765424	31.32846832	0.000736467		
FBgn0028407_Drep-3	FBgn0028407	CG8364	Drep-3	chr2R:7940448-7950374	+	2208	0.595180631	30.30714226	0	0.595180631	1.510661714	30.30714226	0.000931721		
FBgn0052512_CG32512	FBgn0052512	CG32512	CG32512	chrX:20933410-20938549	-	2197	0.598717868	31.72974205	0	0.598717868	1.514370139	31.72974205	0.000671469		
FBgn0035607_CG4835	FBgn0035607	CG4835	CG4835	chr3L:5472949-5476763	+	3675	0.59877485	24.98650551	0	0.59877485	1.514429953	24.98650551	0.003172119		
FBgn0030904_upd2	FBgn0030904	CG5988	upd2	chrX:18135380-18137038	-	1113	0.602122843	28.69023705	0	0.602122843	1.517948498	28.69023705	0.001351999		
FBgn0033188_CG1600	FBgn0033188	CG1600	CG1600	chr2R:3413622-3420487	-	2280	0.604378402	39.84326172	0	0.604378402	1.520323567	39.84326172	0.000103675		
FBgn0039131_CG12268	FBgn0039131	CG12268	CG12268	chr3R:19769349-19774141	-	1770	0.60799849	32.50553131	0	0.60799849	1.524143235	32.50553131	0.000561626		
FBgn0034329_IM1	FBgn0034329	CG18108	IM1	chr2R:14271456-14271883	+	358	0.615513921	31.72974205	0	0.615513921	1.53210367	31.72974205	0.000671469		
FBgn0053960_CG33960	FBgn0053960	CG33960	CG33960	chr2R:12274052-12318273	+	4155	0.617728889	31.68453407	0	0.617728889	1.534457714	31.68453407	0.000678495		
FBgn0003495_spz	FBgn0003495	CG6134	spz	chr3R:22890791-22895790	-	2282	0.627616704	38.10078049	0	0.627616704	1.545010565	38.10078049	0.000154854		
FBgn0033928_Arc2	FBgn0033928	CG13941	Arc2	chr2R:10249448-10250330	+	882	0.632899106	29.93289948	0	0.632899106	1.550677963	29.93289948	0.00101557		
FBgn0259098_CG42246	FBgn0259098	CG42246	CG42246	chrX:7305299-7305939	-	578	0.653190255	37.41325378	0	0.653190255	1.572641962	37.41325378	0.000181416		
FBgn0033926_Arc1	FBgn0033926	CG12505	Arc1	chr2R:10245427-10247799	-	2372	0.662656903	29.69349289	0	0.662656903	1.582995225	29.69349289	0.001073126		

FBgn0029738_CG4068	FBgn0029738	CG4068	CG4068	chrX:4820693-4832077	-	3839	0.663899601	41.22670364	0	**0.663899601**	1.58435936	41.22670364	7.53928E-05		
FBgn0035544_CG15021	FBgn0035544	CG15021	CG15021	chr3L:4436720-4438320	+	1545	0.683696866	42.11875916	0	**0.683696866**	1.606250458	42.11875916	6.13937E-05		
FBgn0034126_CG4398	FBgn0034126	CG4398	CG4398	chr2R:12319677-12320682	+	887	0.687191963	42.35001755	0	**0.687191963**	1.610146505	42.35001755	5.82101E-05		
FBgn0038074_CG6188	FBgn0038074	CG6188	CG6188	chr3R:8500330-8501806	+	989	0.692652583	40.87987518	0	**0.692652583**	1.616252479	40.87987518	8.16606E-05		
FBgn0259711_CG42365	FBgn0259711	CG42365	CG42365	chr2R:17554251-17555099	+	848	0.694446445	39.15617752	0	**0.694446445**	1.618263394	39.15617752	0.000121446		
FBgn0063494_GstE6	FBgn0063494	CG17530	GstE6	chr2R:14293517-14294266	+	749	0.694953859	39.96063232	0	**0.694953859**	1.618832658	39.96063232	0.000100911		
FBgn0033731_Cpr49Ah	FBgn0033731	CG8515	Cpr49Ah	chr2R:8301566-8302358	+	573	0.703395665	56.68758774	0	**0.703395665**	1.628332885	56.68758774	2.14408E-06		
FBgn0035845_CG13675	FBgn0035845	CG13675	CG13675	chr3L:8153414-8157552	-	1399	0.708165765	43.01633072	0	**0.708165765**	1.633725686	43.01633072	4.99306E-05		
FBgn0035409_CG14963	FBgn0035409	CG14963	CG14963	chr3L:3171613-3172545	-	932	0.709760666	43.22269058	0	**0.709760666**	1.63553277	43.22269058	4.76136E-05		
FBgn0033696_Cyp6g2	FBgn0033696	CG8859	Cyp6g2	chr2R:8075842-8077882	-	1989	0.713146746	50.18395233	0	**0.713146746**	1.639375959	50.18395233	9.58528E-06		
FBgn0032598_ChLD3	FBgn0032598	CG17905	ChLD3	chr2L:16738851-16742614	+	2267	0.714519501	46.23756409	0	**0.714519501**	1.640936602	46.23756409	2.37817E-05		
FBgn0039925_Kif3C	FBgn0039925	CG17461	Kif3C	chr4:1168091-1189865	+	4376	0.714587152	40.94236755	0	**0.714587152**	1.641013551	40.94236755	8.0494E-05		
FBgn0005660_Ets21C	FBgn0005660	CG2914	Ets21C	chr2L:547413-552454	-	2215	0.728065133	49.92996597	0	**0.728065133**	1.656416103	49.92996597	1.01626E-05		
FBgn0051226_CG31226	FBgn0051226	CG31226	CG31226	chr3R:14677024-14677526	-	397	0.732221782	43.36656189	0	**0.732221782**	1.6611954	43.36656189	4.60621E-05		
FBgn0040653_IM4	FBgn0040653	CG15231	IM4	chr2R:16756340-16756826	-	427	0.732766569	50.09978485	0	**0.732766569**	1.661822815	50.09978485	9.77286E-06		
FBgn0035144_CG17181	FBgn0035144	CG17181	CG17181	chr3L:582823-589272	-	2823	0.750435352	61.58298874	0	**0.750435352**	1.68230041	61.58298874	6.94546E-07		
FBgn0085264_CG34235	FBgn0085264	CG34235	CG34235	chr2R:9097313-9099680	-	1699	0.753148496	48.14463806	0	**0.753148496**	1.685467135	48.14463806	1.53298E-05		
FBgn0050058_CG30058	FBgn0050058	CG30058	CG30058	chr2R:9097313-9099680	-	1699	0.753148496	48.14463806	0	**0.753148496**	1.685467135	48.14463806	1.53298E-05		
FBgn0085341_CG34312	FBgn0085341	CG34312	CG34312	chr2R:9097313-9099680	-	1699	0.753148496	48.14463806	0	**0.753148496**	1.685467135	48.14463806	1.53298E-05		
FBgn0042206_GstD10	FBgn0042206	CG18548	GstD10	chr3R:8190495-8191440	-	837	0.753200591	69.46659088	0	**0.753200591**	1.685527997	69.46659088	1.13068E-07		
FBgn0037126_CG14567	FBgn0037126	CG14567	CG14567	chr3L:21730861-21731434	-	573	0.757774174	57.2524147	0	**0.757774174**	1.69087988	57.2524147	1.8826E-06		
FBgn0033438_Mmp2	FBgn0033438	CG1794	Mmp2	chr2R:5498643-5571356	-	4149	0.766392708	57.2524147	0	**0.766392708**	1.70101128	57.2524147	1.8826E-06		
FBgn0038525_CG14329	FBgn0038525	CG14329	CG14329	chr3R:13435083-13436255	+	1100	0.767341495	49.47335434	0	**0.767341495**	1.702130316	49.47335434	1.12892E-05		
FBgn0029831_CG5966	FBgn0029831	CG5966	CG5966	chrX:5882844-5886677	+	2602	0.772948563	76.21277618	0	**0.772948563**	1.708758558	76.21277618	2.39179E-08		
FBgn0025693_CG11163	FBgn0025693	CG11163	CG11163	chr2R:1662278-1670360	-	2460	0.790486574	56.64445114	0	**0.790486574**	1.729657722	56.64445114	2.16548E-06		
FBgn0053319_CR33319	FBgn0053319	CR33319	CR33319	chr2L:20928823-20929525	+	593	0.797964454	56.24847794	0	**0.797964454**	1.738646281	56.24847794	2.3722E-06		
FBgn0035439_CG14961	FBgn0035439	CG14961	CG14961	chr3L:3330141-3332942	+	2745	0.800153017	79.61412048	0	**0.800153017**	1.741285803	79.61412048	1.09292E-08		
FBgn0040736_IM3	FBgn0040736	CG16844	IM3	chr2R:14275624-14275995	+	300	0.803055227	59.72331619	0	**0.803055227**	1.744792201	59.72331619	1.06578E-06		
FBgn0001225_Hsp26	FBgn0001225	CG4183	Hsp26	chr3L:9369517-9370527	-	1010	0.809127748	79.05286407	0	**0.809127748**	1.752151773	79.05286407	1.24369E-08		
FBgn0001223_Hsp22	FBgn0001223	CG4460	Hsp22	chr3L:9366030-9368070	+	1906	0.816953123	66.55504608	0	**0.816953123**	1.761681505	66.55504608	2.21052E-07		
FBgn0001228_Hsp67Bb	FBgn0001228	CG4456	Hsp67Bb	chr3L:9366030-9368070	+	1906	0.816953123	66.55504608	0	**0.816953123**	1.761681505	66.55504608	2.21052E-07		
FBgn0259708_CG42362	FBgn0259708	CG42362	CG42362	chr2R:17551937-17553613	+	1623	0.821015894	56.32909012	0	**0.821015894**	1.766649566	56.32909012	2.32858E-06		
FBgn0259709_CG42363	FBgn0259709	CG42363	CG42363	chr2R:17551937-17553613	+	1623	0.821015894	56.32909012	0	**0.821015894**	1.766649566	56.32909012	2.32858E-06		
FBgn0053110_CG33110	FBgn0053110	CG33110	CG33110	chr3R:18395910-18406120	+	2010	0.821952701	67.97875977	0	**0.821952701**	1.767797103	67.97875977	1.59266E-07		
FBgn0010381_Drs	FBgn0010381	CG10810	Drs	chr3L:3369568-3369944	+	376	0.82347858	69.43541718	0	**0.82347858**	1.769667818	69.43541718	1.13883E-07		
FBgn0038160_CG9759	FBgn0038160	CG9759	CG9759	chr3R:9434474-9434968	-	494	0.827426374	82.25688171	0	**0.827426374**	1.774516973	82.25688171	5.94719E-09		
FBgn0033355_CG13748	FBgn0033355	CG13748	CG13748	chr2R:4827919-4828641	+	722	0.829433501	68.6137085	0	**0.829433501**	1.77698746	68.6137085	1.37603E-07		
FBgn0067905_IM14	FBgn0067905	CG33990	IM14	chr2R:16757929-16758134	+	141	0.835850894	67.86061859	0	**0.835850894**	1.784909459	67.86061859	1.63658E-07		
FBgn0085483_CG34454	FBgn0085483	CG34454	CG34454	chr3L:231736-232302	-	499	0.844677329	74.92918396	0	**0.844677329**	1.795863041	74.92918396	3.21426E-08		
FBgn0038611_CG14309	FBgn0038611	CG14309	CG14309	chr3R:14206850-14210723	-	3721	0.873231232	75.46025085	0	**0.873231232**	1.83176094	75.46025085	2.8443E-08		
FBgn0013772_Cyp6a8	FBgn0013772	CG10248	Cyp6a8	chr2R:10774672-10776514	-	1783	0.884602189	70.92247772	0	**0.884602189**	1.846255462	70.92247772	8.08634E-08		
FBgn0027073_CG4302	FBgn0027073	CG4302	CG4302	chr2R:17100384-17102485	-	1762	0.894615531	74.98602295	0	**0.894615531**	1.859114377	74.98602295	3.17247E-08		
FBgn0039684_Obp99d	FBgn0039684	CG15505	Obp99d	chr3R:25540260-25540674	+	414	0.901110947	88.10211945	0	**0.901110947**	1.8675035	88.10211945	1.54806E-09		
FBgn0039670_CG7567	FBgn0039670	CG7567	CG7567	chr3R:25412957-25413822	-	767	0.904607952	78.83452606	0	**0.904607952**	1.872035705	78.83452606	1.30782E-08		
FBgn0001224_Hsp23	FBgn0001224	CG4463	Hsp23	chr3L:9374983-9375867	+	884	0.918300927	110.9287109	0	**0.918300927**	1.889888246	110.9287109	8.07475E-12		
FBgn0035412_CG14957	FBgn0035412	CG14957	CG14957	chr3L:3187562-3188106	+	423	0.927136123	92.19454193	0	**0.927136123**	1.901497607	92.19454193	6.03317E-10		
FBgn0040734_CG15065	FBgn0040734	CG15065	CG15065	chr2R:14277462-14277752	+	224	0.939682961	79.7280426	0	**0.939682961**	1.918106679	79.7280426	1.06462E-08		
FBgn0004187_snRNA_U1_95Cc	FBgn0004187	CR31185	snRNA_U1_95	chr3R:19652055-19652219	+	164	0.943053722	88.21485138	0	**0.943053722**	1.922593448	88.21485138	1.50839E-09		
FBgn0025583_IM2	FBgn0025583	CG18106	IM2	chr2R:14274101-14274535	+	372	0.971523106	90.17692566	0	**0.971523106**	1.960909707	90.17692566	9.6008E-10		
FBgn0034470_Obp56d	FBgn0034470	CG11218	Obp56d	chr2R:15590634-15591300	-	596	0.978462696	98.00818634	0	**0.978462696**	1.970364713	98.00818634	1.58191E-10		
FBgn0039452_CG14245	FBgn0039452	CG14245	CG14245	chr3R:22554264-22554929	+	665	0.980756581	97.0276947	0	**0.980756581**	1.973500084	97.0276947	1.98258E-10		
FBgn0031430_CG3528	FBgn0031430	CG3528	CG3528	chr2L:2517195-2518037	-	789	1.001010537	91.53044891	0	**1.001010537**	2.001401393	91.53044891	7.03E-10		
FBgn0033830_CG10814	FBgn0033830	CG10814	CG10814	chr2R:9263475-9265219	-	1445	1.020808697	139.5068665	0	**1.020808697**	2.02905602	139.5068665	1.12025E-14		
FBgn0014019_Rh5	FBgn0014019	CG5279	Rh5	chr2L:12008375-12009846	+	1363	1.024037838	100.7226181	0	**1.024037838**	2.033602682	100.7226181	8.46717E-11		
FBgn0034328_IM23	FBgn0034328	CG15066	IM23	chr2R:14270208-14270737	-	465	1.031030774	93.63851929	0	**1.031030774**	2.043483754	93.63851929	4.32661E-10		

FBgn0038610_CG7675	FBgn0038610	CG7675	CG7675	chr3R:14203841-14206897	-	1273	1.154345751	137.9318542	0	**1.154345751**	2.225833612	137.9318542	1.60996E-14
FBgn0051815_CG31815	FBgn0051815	CG31815	CG31815	chr2L:16605944-16607277	+	1333	1.198985934	135.4275818	0	**1.198985934**	2.295782445	135.4275818	2.86577E-14
FBgn0034335_GstE1	FBgn0034335	CG5164	GstE1	chr2R:14285894-14286729	+	835	1.232788563	151.5424652	0	**1.232788563**	2.350208192	151.5424652	7.01057E-16
FBgn0031277_CG13947	FBgn0031277	CG13947	CG13947	chr2L:779203-779563	+	360	1.250569224	151.7890778	0	**1.250569224**	2.379352833	151.7890778	6.62357E-16
FBgn0010385_Def	FBgn0010385	CG1385	Def	chr2R:5941682-5942081	-	399	1.295354486	173.0514526	0	**1.295354486**	2.454372946	173.0514526	4.95284E-18
FBgn0010038_GstD2	FBgn0010038	CG4181	GstD2	chr3R:8197692-8198426	+	734	1.393217683	218.0830994	0	**1.393217683**	2.626638545	218.0830994	1.55486E-22
FBgn0005626_ple	FBgn0005626	CG10118	ple	chr3L:6706454-6712624	-	3806	1.458168268	235.1699829	0	**1.458168268**	2.747592913	235.1699829	3.0409E-24
FBgn0033207_CG12826	FBgn0033207	CG12826	CG12826	chr2R:3558054-3558507	-	393	1.516606927	291.3965454	0	**1.516606927**	2.861173386	291.3965454	7.25012E-30
FBgn0028853_CG15263	FBgn0028853	CG15263	CG15263	chr2L:15285026-15285905	+	879	1.553354502	267.8150024	0	**1.553354502**	2.93498779	267.8150024	1.65386E-27
FBgn0000047_Act88F	FBgn0000047	CG5178	Act88F	chr3R:11265709-11267895	+	1572	1.592026711	341.3789978	0	**1.592026711**	3.014725634	341.3789978	7.27948E-35
FBgn0085256_CG34227	FBgn0085256	CG34227	CG34227	chr2R:7135630-7136117	+	487	1.609744668	272.1794739	0	**1.609744668**	3.051978223	272.1794739	6.05414E-28
FBgn0037724_Fst	FBgn0037724	CG9434	Fst	chr3R:5470850-5471874	-	1024	1.857282281	347.6725159	0	**1.857282281**	3.623244795	347.6725159	1.70902E-35
FBgn0036659_CG9701	FBgn0036659	CG9701	CG9701	chr3L:16726673-16732471	+	1984	1.911488414	420.2203979	0	**1.911488414**	3.761970182	420.2203979	9.50518E-43
FBgn0033593_CG9080	FBgn0033593	CG9080	CG9080	chr2R:7129249-7129700	-	451	1.965174794	405.8503113	0	**1.965174794**	3.904600088	405.8503113	2.59997E-41
FBgn0260011_nimC4	FBgn0260011	CG16876	nimC4	chr2L:14047583-14048863	+	1280	2.076827765	489.7662048	0	**2.076827765**	4.21878558	489.7662048	1.05531E-49
FBgn0039759_CG9733	FBgn0039759	CG9733	CG9733	chr3R:26069697-26072425	-	1469	2.155241966	503.1905212	0	**2.155241966**	4.4544335	503.1905212	4.79676E-51
FBgn0010357_betaTry	FBgn0010357	CG18211	betaTry	chr2R:7233843-7234641	+	798	3.366718531	985.6813354	0	**3.366718531**	10.31533329	985.6813354	2.7031E-99
FBgn0010425_epsilonTry	FBgn0010425	CG18681	epsilonTry	chr2R:7232733-7233567	-	834	4.300736427	1710.463623	0	**4.300736427**	19.70836823	1710.463623	8.9875E-172

***Genes listed meet a fold change cutoff of a Log2 ratio ± 0.585 (± 1.5 fold) and p-value <0.05.**

CHAPTER 3

EFFECTS OF PARENTAL DIET ON THE METABOLIC STATE OF OFFSPRING

Summary

While the incidence of complex metabolic disorders such as obesity and diabetes has increased in the last several decades, a majority of the heritable risk is as yet unexplained. Research over the course of the last decade has suggested that some of this missing heritability can be explained by the metabolic state of parents and even grandparents through several generations. *Drosophila* models of this phenomenon have indicated that inheritance of metabolic phenotypes is conserved across evolution, and likely the mechanism of this inheritance is as well. Here I present three separate but related dietary methods to manipulate parental metabolic state. I show that alteration in parental metabolism does indeed impact progeny physiology for up to two generations. I also demonstrate that genetic variation can contribute to how parental metabolism is affected by dietary manipulation and subsequently the effects of dietary manipulation on progeny metabolism. Using isogenic lines, I demonstrate reproducible reductions in F1 triglycerides and glycogen in response to a high protein parental diet as well as alterations in responses to F1 dietary challenge. Unfortunately, the control response to high protein diet was not reproducible in the F2 generation, suggesting that environmental variables can also impact the progeny metabolic response to dietary conditioning. As a result, I am

unable to induce a reliable transgenerational physiological response to parental diet, preventing us from identifying the molecular mechanisms behind this phenomenon.

Introduction

Over the last several decades, the incidence of obesity, diabetes, and related disorders has increased exponentially (CDCP, 2014; Ogden et al., 2012). Although the prevalence of high calorie diets contributes to this upsurge, complex metabolic disorders also have a high degree of heritability (Barron et al., 2016; Elks et al., 2012). Some of the heritable risk can be attributed to changes in gene sequence, but much of that risk cannot be explained by canonical Mendelian inheritance. Over the last decade, evidence has arisen suggesting that the diet and metabolic state of parents, grandparents, and even great-grandparents can influence the health of their offspring. This heritable information, in combination with genetic and environmental contributions, can predispose an individual to resistance or sensitivity to a range of metabolic disorders, including diabetes and obesity. Identifying the mechanism(s) by which parental metabolic health influences the development of metabolic syndrome could lead to new methods for the identification of at-risk patients as well as new preventative therapies.

One explanation for the missing heritability could be that an individual's metabolism is influenced by the metabolic state of his/her ancestors. This model is supported by a number of retrospective studies in humans that have tracked the metabolic phenotypes of patients who were exposed to famine in utero (de Rooij et al., 2006; Li et al., 2010; Lumey et al., 2009; Ravelli et al., 1999; Stanner et al., 1997). The best known of these studies involves a period in Holland known as the Dutch hunger winter. This famine occurred from 1944-1945 during World War II when a German blockade led to

food shortages and severe rationing. Individuals exposed while in utero had a significantly higher chance than controls to be obese, diabetic, and develop heart disease or cancer (de Rooij et al., 2006; Lumey et al., 2009; Ravelli et al., 1999). These effects were greatest when exposure took place during the first trimester (Ravelli et al., 1999). The controls were age-matched siblings either born prior to the famine or conceived after May 1945, making this one of the best-controlled studies of its kind (de Rooij et al., 2006; Lumey et al., 2009; Ravelli et al., 1999). Similar findings have been reported in other famine-exposed populations in China and Leningrad, especially when individuals had access to a calorically rich diet during adulthood (Li et al., 2010; Stanner et al., 1997). A few studies have also provided evidence for paternal influence on progeny metabolism over several generations, indicating that changes in the fetal environment during development are not sufficient to explain this phenomenon (Bygren et al., 2001; Bygren et al., 2014; Kaati et al., 2002; Pembrey et al., 2006).

Importantly, evidence supporting the transgenerational inheritance of metabolic state is not limited to humans but has also been studied in mammals through both maternal and paternal lineages. Dietary manipulation of either male or female rodents has been shown to alter the risk of obesity, hyperglycemia, and heart disease in their progeny, in many cases for at least two generations (Aerts and Van Assche, 2006; Bellinger et al., 2004; Bellinger et al., 2006; Burdge et al., 2011; Burdge et al., 2004; Bygren et al., 2014; Carone et al., 2010; Chen et al., 2016; Dalgaard et al., 2016; Dunn and Bale, 2011; Duque-Guimaraes and Ozanne, 2013; Jimenez-Chillaron et al., 2009; Kirchner et al., 2013; Langley-Evans, 2001; Lillycrop, 2011; Lillycrop et al., 2005; Lucas et al., 1996; Ng et al., 2010; Sharma et al., 2016). Furthermore, these changes in metabolic state have

been correlated with molecular changes in the liver, as well as changes in circulating lipid levels, gene expression, and in some cases alterations in chromatin modifications such as DNA methylation (Burdge et al., 2011; Burdge et al., 2004; Carone et al., 2010; Chen et al., 2016; Lucas et al., 1996; Ng et al., 2010). Similar results have been found in multiple model systems including *Drosophila melanogaster* (Buescher et al., 2013; Matzkin et al., 2013; Ost et al., 2014). In spite of these correlations in a wide range of organisms, however, no clear molecular mechanism has emerged to explain this phenomenon.

The results of these studies in both model systems and humans support the "mismatch" theory of transgenerational inheritance of metabolic state (Lillycrop, 2011; Somer and Thummel, 2014). According to this theory, exposure to a poor or stressful nutritional environment leads to alterations in the transcriptional and epigenetic state that better adapts the organism to its environment. Since these adaptations can also occur in the germline, alterations in the epigenetic state of the gametes lead to changes in the transcriptional and the metabolic state of progeny conceived during this period. Theoretically, this would allow the progeny to be best adapted to continued exposure to the parental environment. If, however, the offspring environment does not match the parental environment, the epigenetic state of these organisms could lead to potential metabolic dysfunction (Lillycrop, 2011; Somer and Thummel, 2014).

Hypotheses to explain the nongenetic transmission of phenotypes across generations have ranged from direct transmission of DNA or histone methylation marks, to retention of chromatin-modifying enzymes, to the transmission of small RNAs in the germline that guide more direct epigenetic modifications during development (Francis et al., 2009; Kirchner et al., 2013; Lillycrop et al., 2005; Petruk et al., 2012). A number of

studies have provided correlative evidence for the direct transmission of chromatin marks; however, recent studies have suggested that chromatin modifications might be secondary effects (Carone et al., 2010; Hajkova et al., 2008; Hajkova et al., 2002; Lee et al., 2002). More evidence supports a model wherein small RNAs are transmitted to the embryo through the gametes, with evidence for the participation of piRNAs, miRNAs, and tRNA fragments in nematodes, flies, and mice (Ashe et al., 2012; Castel and Martienssen, 2013; Chen et al., 2016; de Vanssay et al., 2012, 2013a; de Vanssay et al., 2013b; Grentzinger et al., 2012; Kawano et al., 2012; Murashov et al., 2016; Pembrey et al., 2014; Sharma et al., 2016; Shirayama et al., 2012; Sienski et al., 2012; Yuan et al., 2015). The mechanisms by which small RNAs might be inducing changes in epigenetic, transcriptional, and metabolic state, however, remain unclear.

The use of mammalian systems in these types of studies is time consuming, expensive, and difficult to control due to the multigenerational nature of the phenomenon and the need for large sample sizes to detect the often subtle defects. For this reason, a shift has been made to the use of *Drosophila* as an appropriate model system (Buescher et al., 2013; Ost et al., 2014). *Drosophila* have short lifespans, are easy and inexpensive to maintain, and can produce hundreds of offspring from a single cross. In addition, many of the tools needed to test the epigenetic mechanisms that might be involved in transmitting metabolic state are available in *Drosophila*. Moreover, previous work has shown that exposure of male or female flies to a high sugar diet can alter the metabolic state and gene expression in progeny through at least the F1 generation (Buescher et al., 2013; Ost et al., 2014). It thus appears that transgenerational metabolic inheritance is a phenomenon that is conserved in *Drosophila*. As with other systems, however, no clear

molecular mechanism has been tested in this organism.

In this study, I attempt to develop a dietary model for the transgenerational inheritance of metabolic state in *Drosophila*. I show that exposing parents to a high protein diet can indeed impact the metabolic state of offspring. Unlike previous studies, however, I observed inconsistent transmission of phenotypes to subsequent generations. This variability makes the use of a dietary paradigm incompatible with the objective to track the molecular changes in progeny and in parental gametes, or to identify important mechanistic pathways. I distinguished both genetic and environmental variables as contributing to these inconsistencies in the response of offspring to parental dietary conditioning.

Materials and methods

Fly stocks and maintenance

Wild-type flies from a *Canton S* inbred line were used for studies in a nonisogenic background. For isogenic studies, lines *Canton S 9* and *Canton S 2* were derived as described in the main text. Parental flies were raised at room temperature on media containing approximately 1% agar, 1.8% yeast, 6.1% corn meal, 1.3% corn syrup, 8.2% malt, 0.1% p-hydroxybenzoic acid methyl ester, and 0.75% propionic acid (to suppress mold). Once transferred to one of the control diets, the parental generation and all subsequent generations were maintained at 25°C in a circadian incubator and transferred to fresh media every two to four days. Flies were aged at a ratio of one male to one female, with anywhere from 40-60 flies maintained per vial. The first control diet is a modified version of the Semidefined diet, containing 1% agar, 8% yeast, 4% corn meal, 2% peptone, 3% sucrose, 6% dextrose, 0.05% $MgSO_4x6H_2$), 0.05% $CaCl_2x2H_2O$, 0.1%

p-Hydroxy-benzoic acid methyl ester, and 0.6% propionic acid (Backhaus et al., 1984). The conditioning diet for this study contains 1% agar, 10% sucrose, and 10% yeast extract, where the agar and yeast extract have been depleted of lipids using chloroform extraction (Sieber and Thummel, 2009). The second control diet contains 1% agar, 8% yeast, 3% sucrose, and 6% dextrose, while the conditioning diet contains 1% agar, 16% yeast, 3% sucrose, and 6% dextrose. The nutrient composition and caloric content of the control and conditioning diets are summarized in Table 3.1.

Chloroform-mediated lipid extraction

Yeast extract or agar was mixed with chloroform in a 1:5 ratio, allowed to stir overnight, and filtered through Whatman paper. The remaining solids were resuspended in the same volume of chloroform and allowed to stir for three to four hours, and filtered again through Whatman paper. The resulting lipid-extracted medium was allowed to dry for at least five days and used to make fly media (Sieber and Thummel, 2009).

Metabolite assays

Samples of five male flies were collected at the indicated adult ages and washed in 1X PBS. Each sample was homogenized in 120 µL 1XPBS or 1XPBST (0.3%

Table 3.1: Diets used for parental metabolic conditioning

	Control Semi-defined	Low Nutrient Diet Conditioning	Control Yeast	High Yeast Conditioning
Dietary Composition	8% Yeast 2% Peptone 4% Corn meal 6% Dextrose 3% Sucrose 1% Agar	10% Yeast extract 10% Sucrose 1% Agar	8% Yeast 6% Dextrose 3% Sucrose 1% Agar	16% Yeast 6% Dextrose 3% Sucrose 1% Agar
% kcal from Carbohydrate	73.5	79.2	79.1	69.6
% kcal from Protein	20.7	20.6	16.5	24.1
% kcal from Fat	5.9	0.2	4.4	6.3
Total kcal/L	953	830	740	1015

TritonX100), after which 10 μL was reserved for protein quantification and the remainder of the lysate was heat-treated for 10 min at 70°C. Metabolic assays were then performed as described (Tennessen et al., 2014).

Northern Blots

RNA was isolated from samples of approximately 16 male flies using Trizol (Thermo Fisher). Northern blot transfer and hybridization was performed as previously described (Karim and Thummel, 1991).

Statistics

GraphPad PRISM 6 software was used to plot metabolite data and to perform statistical analyses. Simple comparisons were performed using a standard Student's T-test with Welch's correction for unequal variances. In the challenge experiments, two-way ANOVA was performed to compare the effects of parental diet and progeny diet on metabolite levels, while Tukey's multiple comparisons test was used to determine the statistical significance of differences between the individual groups. To determine the effect of experimental replicates on variation between the groups, a two-way ANOVA was performed comparing the effects of dietary variation and experimental replicate on metabolite levels.

Results

Dietary manipulation in the parental generation induces metabolic changes in progeny.

I employed a dietary-switch conditioning paradigm to simulate a temporary alteration in the parental nutritional environment (Figure 3.1). In this paradigm, recently

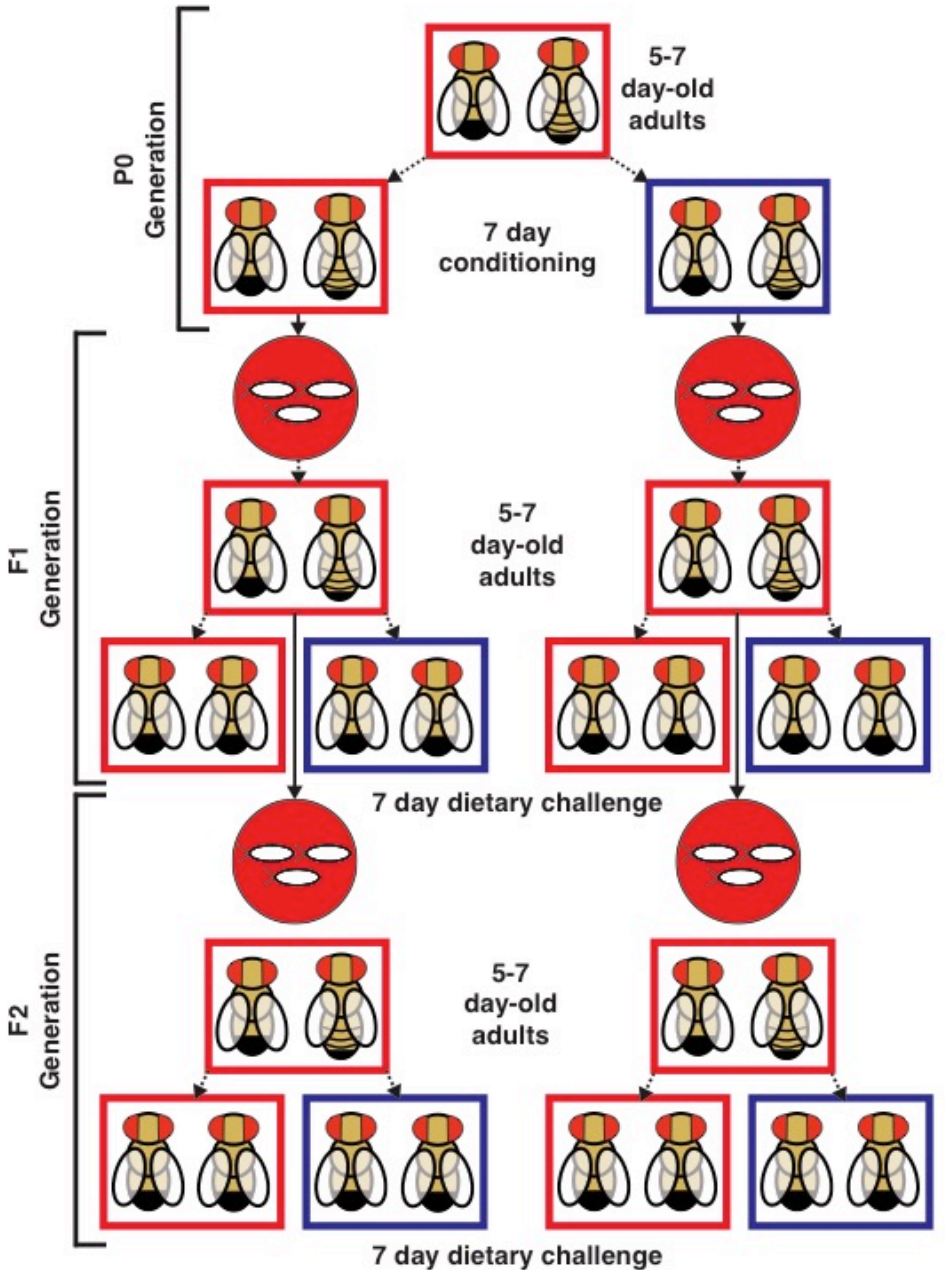


Figure 3.1. Paradigm for dietary manipulation in the parental generation
Both male and female parental flies (P0 generation) were equilibrated on a control diet (red) until they had aged approximately 5-7 days. These were then transferred to either fresh control media or a conditioning diet (blue) on which they were conditioned for seven days. At that point they were returned to the control media for egg lay for 12-100 hours while embryo density is monitored. These F1 embryos mature to adulthood on the control media. After eclosion, the F1 progeny were aged on control media for 5-7 days before they were collected for metabolites, mated to produce the F2 generation, or subjected to dietary challenge for seven days. The F2 and F3 generations were raised in the same way. Mature adults were collected for basal metabolites, mated to produce the next generation, or subjected to the dietary challenge. Solid lines indicate mating to produce the next generation. Dashed lines represent aging or transfer of the same flies.

eclosed adult males and females were aged together for five to seven days on a control diet to equilibrate their metabolic state to that media. The mature flies were then transferred to either the control diet or the associated conditioning diet. This conditioning diet differed from the control diet in both nutritional composition (carbohydrates, fat, and protein) and caloric content (Table 3.1). After seven days, all flies were transferred back to the original control media and allowed to mate. The parental flies were then removed after an appropriate amount of time that resulted in a consistent density of embryos between the two conditions. Embryo density was estimated by eye and confirmed by quantifying the number of pupae. Any vials exceeding a 150-200% difference in F1 density between the two groups were discarded.

After the F1 progeny from either control or conditioned parents eclosed, they were aged to maturity for five to seven days, after which they were collected for either metabolite measurements or RNA isolation. In addition, some F1 progeny were subjected to an adult dietary challenge. This challenge was performed in a manner similar to the adult conditioning, wherein half of each F1 group was maintained on the control media while the other half was transferred to the conditioning media for seven days. At the end of this challenge, samples were collected for metabolite measurements.

The F2 generation was obtained from vials in which the F1 generation had aged for five-seven days post-eclosion. Pupal density was estimated rather than directly quantified, since the F1 progeny groups were maintained on the same control diet and displayed high fecundity. F2 progeny were treated in the same way as F1 progeny, with newly-eclosed F2 progeny aged for five to seven days, whereupon they were either collected for metabolite measurements or subjected to a seven day dietary challenge.

Samples were also collected at the end of the dietary challenge.

It is generally accepted that a phenotype passing through the maternal line must appear in the F3 before it is considered transgenerational, while a phenotype passing through the paternal line must appear in the F2 (Skinner, 2011). This is because the F1 progeny are exposed to an altered developmental environment in addition to any maternal effects. Even the F2 generation is potentially exposed to an altered developmental environment, as the germline of the F1 is specified during embryogenesis (Skinner, 2011). Therefore, for my studies, I set out to identify a phenotype that appears through at least two, and ideally three generations.

Parental low protein diet induces metabolic changes in the progeny

Several diets were tested with the paradigm described above, two of which are described in Table 3.1. The first of these utilized a modified version of the rich Bloomington Semidefined medium as the control diet, and a low nutrient diet consisting of sucrose and yeast extract that was depleted of lipids (Table 3.1) (Backhaus et al., 1984; Sieber and Thummel, 2009). F1 offspring of parents fed the low nutrient diet have, on average, higher triglyceride levels compared to controls (Figure 3.2A). The results of 14 independent experimental replicates, including more than 100 biological replicates per condition, were compiled to generate this overall 20% increase in triglycerides. I observed, however, a great deal of variation between these experimental replicates. Indicative of this, a two-way ANOVA analysis comparing the influence of experimental replicates versus the effect of parental diet on relative triglyceride levels revealed that the variation among replicates was almost as significant ($p < 0.001$) as were the effects seen in response to altered parental diet ($p = 0.0001$).

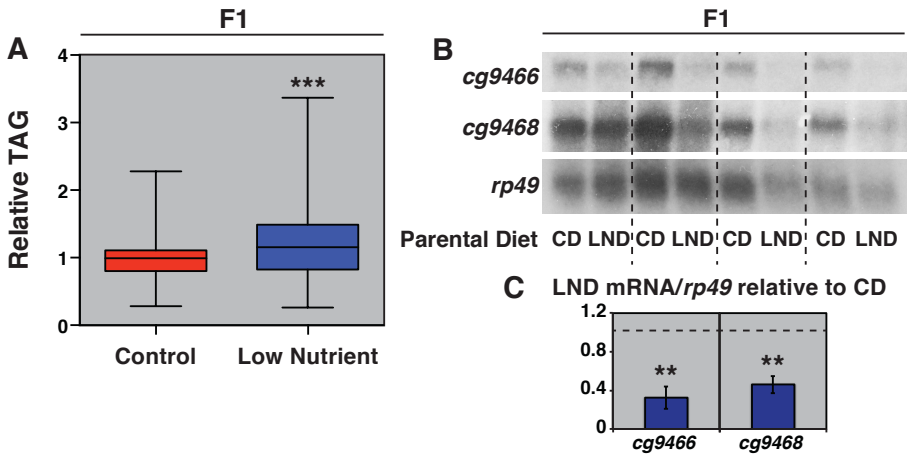


Figure 3.2. Parental low protein diet in inbred fly strains induces measurable but inconsistent changes in physiology
Triglyceride (TAG) levels were measured in F1 progeny descended from parents fed either a control or a low nutrient diet. Offspring of parents fed a low nutrient diet (LND) (n = 116) have increased triglyceride levels as compared to controls (CD) (n = 113) over 14 experimental replicates (A). Northern blot hybridization was used to detect transcriptional differences between F1 offspring from parents subjected to either the control diet (CD) or the low nutrient diet (LND) (B). Levels of *rp49* mRNA was used as a control for loading and transfer. Transcript levels of the putative α-mannosidases *cg9466* and *cg9468* were reduced 60-70% in F1 male offspring of parents fed the low nutrient diet as compared to controls (n = 4, error bars ±SD) (C). Triglyceride levels are internally normalized to soluble protein. Basic comparisons were analyzed using a student's t-test with Welch's correction for unequal variance, while batch effects were analyzed using two-way ANOVA. **p < 0.01, ***p < 0.001.

In contrast, I obtained more consistent results by monitoring the levels of select transcripts using northern blot analysis of RNA isolated from F1 offspring (Figure 3.2B). Two genes encoding presumptive α-mannosidases, *CG9466* and *CG9468*, are downregulated in F1 progeny of parents fed the low nutrient diet as compared to controls (n = 4/group) (Figure 3.2C) (Sieber and Thummel, 2009). These transcriptional changes are observed even when metabolite levels are unchanged, suggesting that the physiological outcomes of robust transcriptional changes might be buffered to prevent broad metabolic defects. An appropriate dietary challenge, however, might elicit measurable physiological responses that are undetectable under control conditions.

Parental high protein diet induces metabolic changes in the progeny

It is possible that the inconsistent physiological responses to the initial conditioning paradigm might be due to the components that make up the two diets (Table 3.1). In particular, the efficiency of chloroform extraction could vary between batches, leading to changes in the lipid content of the low nutrient diet. It was also difficult to obtain sufficient numbers of density-controlled F1 progeny because the low nutrient diet tends to reduce fecundity. Accordingly, in the second paradigm, I used control and conditioning diets that are more comparable in composition and richer in calories. The control diet was a simple 8% yeast, 6% glucose, 3% sucrose composition, while the conditioning diet contained twice as much yeast (16%), shifting the balance of nutrients toward a higher protein/sugar ratio (Table 3.1). The F1 offspring of parents fed this high yeast/protein diet have decreased triglycerides and elevated glycogen relative to controls (Figure 3.3A,B). These results were compiled from eight experimental replicates including 32-33 biological replicates per condition. No variation was detected in triglyceride levels among these replicates as determined by two-way ANOVA. The variation in glycogen levels due to experimental replicate, however, was more significant ($p < 0.005$) than the effects of the parental diet ($p < 0.05$).

Unexpectedly, examination of stored energy levels in F2 progeny revealed phenotypes that mirror those observed in the F1 generation. Triglyceride levels are decreased in F2 offspring descended from parents fed the high yeast/protein diet as compared to controls over eight independent replicates, although the reduction is only half of that seen in the F1 generation (Figure 3.3C). This change was observed in the absence of any significant variation among experimental replicates as determined by two-

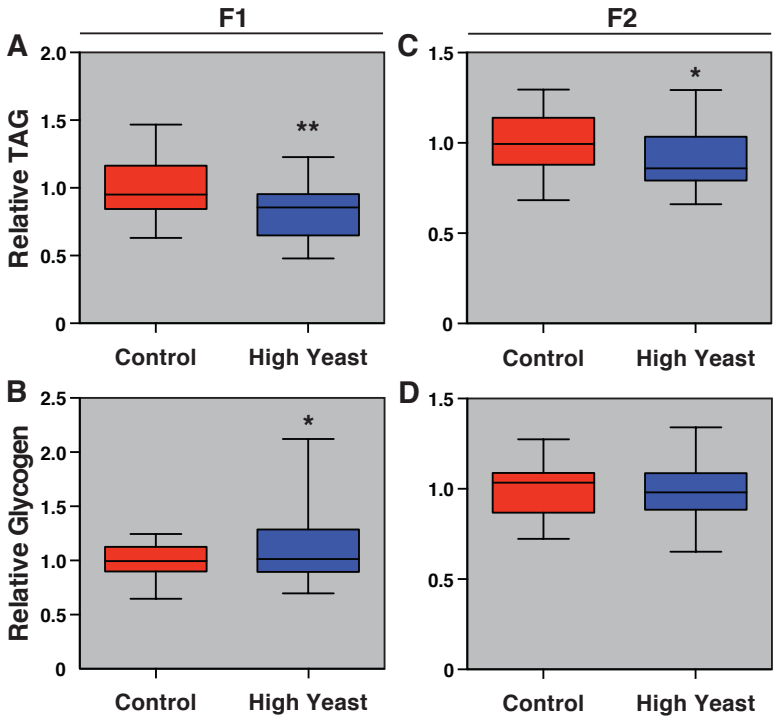


Figure 3.3. Parental high protein diet in inbred fly strains induces measurable but inconsistent changes in physiology

Triglyceride (TAG) and glycogen levels were measured in F1 and F2 progeny descended from parents fed either a control or high yeast/protein diet. Offspring of parents fed a high yeast/high protein diet (n = 33) have reduced triglycerides as compared to controls (n = 32) over eight experimental replicates (A), while glycogen levels are significantly increased (B). Offspring of parents fed the high yeast/protein diet (n = 37) as compared to controls (n = 38) have reduced triglycerides (C) over eight experimental replicates, while glycogen levels are unchanged (D). All metabolite levels are internally normalized to soluble protein. Basic comparisons were analyzed using a student's t-test with Welch's correction for unequal variance, while batch effects were analyzed using two-way ANOVA. *$p < 0.05$, **$p < 0.01$.

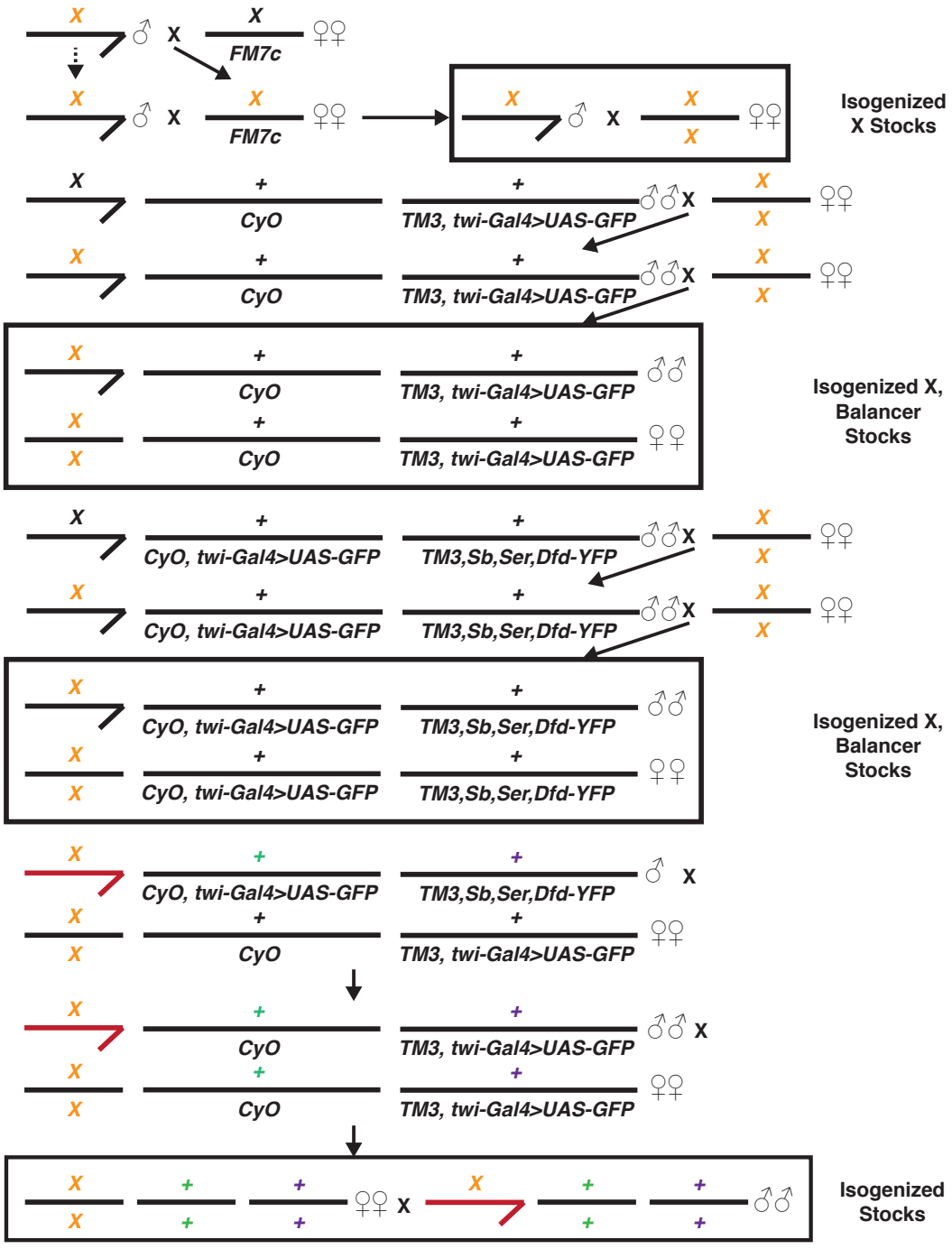
way ANOVA. In contrast, glycogen levels are not significantly affected (Figure 3.3D). This high yeast/protein parental conditioning thus appears to generate more reproducible results in offspring than does the lipid-depleted diet.

Parental high protein diet in isogenized lines reduces variation in the progeny response

It is possible that genetic variation within the inbred *Canton S* line could contribute to experimental variation in the dietary conditioning paradigm. It has been well documented in both flies and mammals that genetic variation within what are otherwise classified as "wild-type" backgrounds can lead to wildly different responses to environmental or genetic manipulation (Churchill et al., 2004; Mackay et al., 2012). To date, no studies have addressed the impact of genetic variation on transgenerational inheritance in any organism, including *Drosophila*. Accordingly, I generated a number of isogenized lines from two laboratory strains commonly used as wild-type controls: a *Canton S* line, and a w^{1118} line. These isogenized lines, in turn, were used for the subsequent studies of transgenerational inheritance with the goal of providing more reproducible responses in the F1 and F2 generations.

The isogenized lines were established using a modified version of the genetic crosses described in Ryder et al. (2004). In this manner, I generated 22 viable lines that are isogenic across the X, Y, second, and third chromosomes from an initial 40 single males each from the *Canton S* and w^{1118} stocks (Figure 3.4) (Ryder et al., 2004). These lines are homozygous at each gene locus with the exception of any located on the small fourth chromosome. By the conclusion of the isogenization process, nine *Canton S*-derived lines were established, along with 13 w^{1118}-derived lines.

Figure 3.4. Isogenization of fly lines
Crossing scheme modified from Ryder et al. (2004). The X, second, and third chromosomes were homozygoused across the entirety of the chromosomes. 80 individual males (40 from a *Canton S* background, 40 from a w^{1118} background) were chosen for the initial crosses to an X-balancer line (*FM7c*). These same individual males were then crossed to daughters carrying the male X and the *FM7c* balancer to generate male and female progeny all carrying the same X. These were then crossed to lines balanced at the second and third chromosomes. The two second chromosome balancers are differentially marked, as are the two third chromosome balancers. I was thus able to isolate single males from one double-balanced line to be crossed to females from the second double-balanced line. By selecting male and female progeny carrying the maternal balancers, I ensured that the other second and third chromosomes descended from the single male parent. Males and females carrying these maternal balancers were mated, and any progeny from this cross that lack these balancers have identical X, Y, second, and third chromosomes. The resulting stock is therefore homozygous at all gene loci across these chromosomes. Of the 80 starting lines, 22 (9 from a *Canton S* background, 13 from a w^{1118} background) were maintainable after the isogenization process.


X
X
FM7c
Isogenized X Stocks
CyO
TM3, twi-Gal4>UAS-GFP
Isogenized X, Balancer Stocks
CyO, twi-Gal4>UAS-GFP
TM3,Sb,Ser,Dfd-YFP
Isogenized X, Balancer Stocks
Isogenized Stocks

Interestingly, the preliminary characterization of these lines suggests that individual stocks derived from the same inbred population can have different responses to dietary manipulation. For example, two lines derived from the *Canton S* strain (*Canton S 2* and *Canton S 9*) were subjected to a dietary challenge on either a low sugar diet (8% yeast, 2% dextrose, 1% sucrose, red boxes) or a high sugar diet (8% yeast, 12% dextrose, 6% sucrose, blue boxes), after which triglycerides were measured. While *Canton S 2* flies have increased triglycerides upon exposure to the high sugar diet, *Canton S 9* flies do not display any apparent response (Figure 3.5). These results suggest that the use of isogenized parental lines may increase the consistency of parental phenotypes, and therefore improve the likelihood of transmitting a reproducible phenotype to the progeny. I chose to perform the analysis of transgenerationally inherited metabolic phenotypes using the *Canton S 9* (*Cs9*) line because it has a strong response to the previously

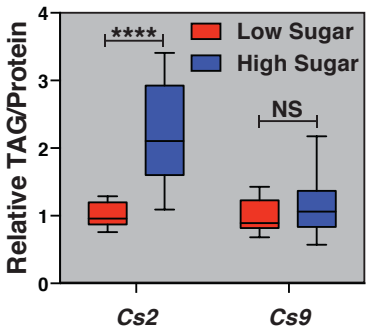


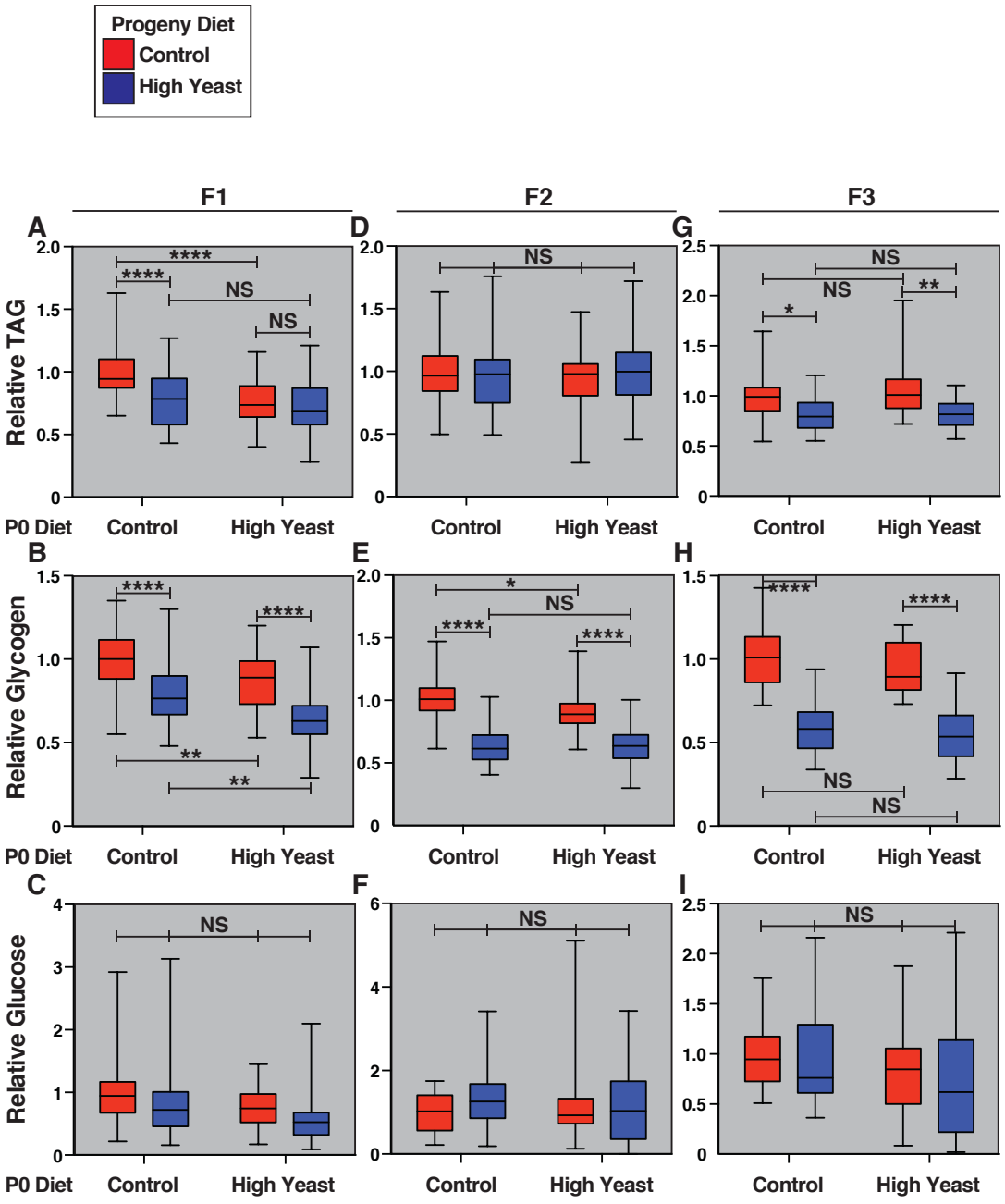
Figure 3.5. Isogenic lines derived from the same wild-type stock respond differently to a high sugar diet
Triglyceride levels are reported relative to levels on the low sugar diet and are internally normalized to soluble protein levels. Adult flies of either the *Canton S 2* (*Cs 2*) or the *Canton S 9* (*Cs 9*) lines were fed a low sugar diet containing 8% yeast, 2% dextrose, and 1% sucrose until approximately one week of age. At this point the flies were either transferred to a high sugar diet containing 8% yeast, 12% dextrose, and 6% sucrose or maintained on the control diet for an additional seven days. In response to challenge on the high sugar diet, *Cs 2* flies have elevated triglycerides while *Cs9* flies display no change (n = 9-11/group). Triglyceride levels are internally normalized to soluble protein. Comparisons were analyzed using two-way ANOVA across lines and diets, with individual comparisons analyzed using Sidak's multiple comparisons test. Batch effects were analyzed using two-way ANOVA. ****$p < 0.0001$.

described high yeast/protein diet and is robust enough to enable the collection of large numbers of offspring in the F1 and F2 generations (Figure 3.4).

For the studies using *Cs9*, I utilized the control-high yeast/protein paradigm described above and compared the results to those observed previously for the nonisogenized populations (Table 3.1, Figure 3.3). I also included an adult dietary challenge, wherein mature progeny were either maintained on the control media on which they had been raised or transferred to the high yeast/protein media for seven days (Figure 3.1). The resultant 12-14 day-old adults were then collected for metabolite measurements.

The F1 data presented in Figure 3.6A-C represents the compiled results of four independent experimental replicates, with 35-36 biological replicates per group. F1 offspring of parents fed the high yeast/protein diet have significantly reduced triglyceride and glycogen levels when compared to controls (Figure 3.6A,B, red boxes). Furthermore, while triglycerides are reduced in controls challenged with the high yeast/protein diet, they are unchanged in F1 offspring of parents fed the high yeast/protein diet when subjected to the dietary challenge (Figure 3.6A, blue boxes). There is some variation among the four experimental replicates ($p < 0.05$), but these effects are mild when compared to the combined effects of P0 and F1 diet ($p < 0.0001$). In contrast, there is no effect of parental diet on glycogen levels in the response to dietary challenge. Both F1 progeny of parents fed either the high yeast/protein diet and controls have reduced glycogen levels upon dietary challenge (Figure 3.6B, blue boxes). Once again, variation among experimental replicates ($p < 0.01$) is minor when compared to the combined effects of the P0 and F1 diets ($p < 0.0001$). Glucose levels are unchanged across dietary conditions (Figure 3.6C).

Figure 3.6. Parental high protein diet in isogenized fly strains induces measurable but inconsistent changes in physiology in response to dietary challenge
Metabolite levels were measured in F1, F2, and F3 progeny descended from parents fed either a control or high yeast/protein diet. Measurements were taken at approximately two weeks of adult age after maturation through one week of adulthood on control media followed by a one week challenge on either the control (red) or high yeast/protein (blue) media. F1 offspring of parents fed the high yeast/protein diet (n = 36) have reduced triglycerides (A) and glycogen (B) as compared to controls (n = 36) over four experimental replicates. Both triglyceride (A) and glycogen (B) levels are reduced when controls are challenged with the high yeast/protein diet (n = 36). In F1 offspring of parents fed the high yeast/protein diet, triglyceride levels are unchanged (A) while glycogen levels are reduced (B) when they are challenged with the high yeast/protein diet (n = 35). Glucose levels are unaffected by either parental or progeny diet (C). F2 metabolites were compiled from four experimental replicates (n = 36/each). F2 offspring descended from parents fed the high yeast/protein diet (n = 36) have unaffected triglycerides (D) and reduced glycogen (E) as compared to controls (n = 36) over four experimental replicates. Both F2 offspring descended from parents fed the high yeast/protein diet (n = 36) and controls (n = 36) have unchanged triglyceride levels (D) and reduced glycogen levels (E) when they are challenged with the high yeast/protein diet. Glucose levels are unaffected by either parental or progeny diet (F). F3 metabolites were compiled from three experimental replicates (n = 23/each). F3 offspring descended from parents fed the high yeast/protein diet (n = 23) have similar triglyceride (G) and glycogen levels (H) as compared to controls (n = 23). Both F3 offspring descended from parents fed the high yeast/protein diet (n = 23) and controls (n = 23) have reduced triglyceride (G) and glycogen levels (H) when they are challenged with the high yeast/protein diet. Glucose levels are unaffected by either parental or progeny diet (I). All metabolite levels are internally normalized to soluble protein. Challenge comparisons were analyzed using two-way ANOVA across parental and progeny diets, with individual comparisons analyzed using Tukey's multiple comparisons test. Effects of experimental replicates were analyzed using two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.


Progeny Diet
Control
High Yeast
F1
F2
F3
A
B
C
D
E
F
G
H
I
Relative TAG
Relative Glycogen
Relative Glucose
P0 Diet
Control
High Yeast
****
NS
**
*

While the F1 results were promising, striking irregularities were observed in the F2 generation. The data displayed in Figure 3.6D-F is compiled across four experimental replicates, with 36 biological replicates per group. F2 offspring descended from parents fed the high yeast/protein diet have no change in triglycerides as compared to controls (Figure 3.6D, red boxes). Of greater concern, the expected change in triglyceride levels by exposure of F2 offspring to dietary challenge was not observed (Figure 3.6D, blue boxes). This loss of a triglyceride response makes it difficult to interpret the effects of dietary challenge on F2 progeny of conditioned parents. The control glycogen response, on the other hand, was well conserved across generations. There is a slight reduction in glycogen ($p < 0.05$) in F2 offspring descended from parents fed the high yeast/protein diet as compared to controls (Figure 3.6E, red boxes). There is, however, a larger reduction in glycogen levels in response to dietary challenge in both F2 progeny descended from parents fed the high yeast/protein diet and controls (Figure 3.6E, blue boxes). There is no significant variation among experimental replicates that contributes to the variation in glycogen measurements. Glucose levels are once again unchanged across dietary conditions (Figure 3.6F).

In the F3 generation, I observed a return of the normal response to dietary challenge but an absence of any measurable difference between the parental dietary groups. The data displayed in Figure 3.6G-I is compiled across three experimental replicates, with 23 biological replicates per group. There is no difference in triglyceride or glycogen levels between F3 progeny descended from parents fed the high yeast/protein diets and controls (Figure 3.6G,H, red boxes). Additionally, descendants of both parental dietary groups have significantly reduced triglyceride and glycogen levels upon dietary

challenge (Figure 3.6G,H, blue boxes). There is mild variation in triglyceride levels among the experimental replicates ($p < 0.05$), but these effects are minor when compared to the combined effects of P0 and F3 diet ($p < 0.0001$). This is also true for glycogen levels, although the effect of variation among the experimental replicates is slightly stronger ($p < 0.005$). As in the previous two generations, glucose is unchanged across dietary conditions (Figure 3.6I).

Discussion

This multigenerational study demonstrates an effect of parental diet on basal metabolic state as well as a response to dietary challenge in the F1 generation. Unfortunately, the lack of a response to dietary challenge in the F2 generation makes it difficult to interpret effects of parental diet on triglyceride levels in this generation. In addition, by the F3 generation, physiological effects induced by the parental diet are undetectable. Without a reliable response beyond the F1 generation, we cannot designate these responses as transgenerational. In summary, my data suggest that even in the absence of genetic variation, dietary manipulations of parental metabolic state are subject to variations in the environment that can lead to inconsistent progeny responses.

Variation in genetic background can lead to different progeny responses to parental dietary conditioning

My work suggests that while a strong and consistent dietary manipulation in the parental generation can induce changes in the physiology of their progeny, there is a lack of consistency in this response across experimental replicates. While some of this variation can be attributed to irregularities in the composition of the diets from one batch

to another, it is unlikely that this accounts for all of the observed experimental variation (Table 3.1).

One possibility that I address in my studies is the contribution of natural genetic variation within the in-bred fly strains. The subtle effects of standing genetic variation have already been reported in population studies across many model organisms (Churchill et al., 2004; Mackay et al., 2012). While the wild-type fly stocks used in the initial studies are already highly inbred, there is still some degree of heterozygosity, made clear by the number of inviable genotypes that emerged when the chromosomes were homozygosed. Approximately half of the forty isogenized lines initiated from the *Canton S* stock were lethal once the X chromosome was homozygosed. The same was true for those lines that originated from single males of the w^{1118} stock. In this way, the isogenization of these healthy inbred lines demonstrates the high degree of genetic variation within each stock.

These isogenized lines also demonstrate differential metabolic responses to dietary challenge. Two lines isolated from the inbred *Canton S* line have different responses to a high sugar diet. Flies of the *Canton S 2* (*Cs2*) line become obese on this diet, while flies of the *Cs9* line are unaffected (Figure 3.5). Given this result, one would expect that the progeny of these two lines would have different physiological responses to parental diet. Indeed, this may contribute to some of the discrepancies observed in the glycogen response of offspring descended from isogenized or nonisogenized parents fed the high yeast/protein diet (Figures 3.3, 3.6). While there is a trend toward an increase in glycogen in F1 progeny of nonisogenized parents fed the high yeast/protein diet, there is a reproducible reduction in glycogen in the progeny of the isogenized *Cs9* line parents

fed the high yeast/protein diet (Figures 3.3B,3.6B,E). Genetic variation in the inbred line may thus partially explain the variation between experimental replicates in this study ($p < 0.001$) (Figure 3.3B,D). More complete characterization of all 22 isogenized lines may clarify how they differ metabolically, while genome sequencing might uncover potential genetic causes of these differences.

Responses to dietary challenge in descendants can be influenced by parental diet

Although genetic variation contributes to the consistency of offspring responses to parental conditioning, these studies demonstrate that other factors make it difficult to obtain reproducible results even under conditions of strict genetic control. For example, in the F1 generation there is a striking triglyceride phenotype that reveals not only differences in basal metabolic state on a control media, but also differences in the response to dietary challenge (Figure 3.6A). While glycogen levels are altered in the F1 progeny, the impact of parental diet is independent from that of the F1 dietary challenge (Figure 3.6B). The differences in regulation of each stored metabolite suggests a tantalizing hypothesis, wherein different cellular processes are autonomously affected by the parental metabolic state in order to adapt to alterations in energy supply and demand. Not all metabolites are affected equally, reflecting the complexity of the network involved in the regulation of whole animal physiology.

Unfortunately, results from the F2 generation cloud even these broad interpretations. The loss of a triglyceride response to dietary challenge prevents the interpretation of results in the F2 generation. Additionally, while there is technically a significant reduction in glycogen levels in F2 progeny descended from high

yeast/protein-fed flies when compared to controls, more rigorous analyses suggest that this 10% reduction may not be statistically significant. Two-way ANOVA comparing the effect of parental and F2 diets indicates that there is a significant impact of F2 diet ($p < 0.0001$) but not of parental diet on metabolite levels. I therefore do not have a reliable physiological read-out to monitor the effects of parental diet on F2 progeny metabolism. Furthermore, even though the proper response to dietary challenge returns in the F3 generation, there is no longer any significant difference in metabolite levels between controls and F3 progeny descended from conditioned parents. My inability to reliably recapitulate the response to dietary challenge in controls indicates that the high yeast/protein diet described in Table 3.1 may not induce a strong enough metabolic response for us to reliably monitor subtle changes in progeny metabolism. Similar issues were encountered when only male or female parents were exposed to the high yeast/protein diet. F1 progeny of parents who were both fed the control diet did not reliably display the expected triglyceride response to dietary challenge (data not shown). Without any distinguishing physiological phenotypes in the F2 or F3 generations, I cannot interpret potential transgenerational effects of dietary manipulation in the parental generation.

There are a number of environmental factors that could be contributing to this variability in my results. While the diets were kept as consistent as possible from experiment to experiment, they were still made in small batches that might be susceptible to small changes in composition. Additional variables such as temperature, humidity, and the light-dark cycle could also contribute to inter-experimental variation. While I attempted to control these as tightly as possible by maintaining experimental replicates in

the same incubator, there could still be differences that might subtly alter parental or progeny metabolic state. to a high enough degree that the progeny responses are subsequently altered or lost. Other studies have proposed similar variables in studies of transgenerational inheritance of metabolic state (Ost et al., 2014).

In conclusion, I have shown that dietary manipulation in the parental generation can influence metabolism in at least the first generation of progeny and, perhaps, in the second generation as well. Using isogenic stocks can reduce variability in experimental results, but inconsistencies due to environmental fluctuations make it difficult to use a dietary paradigm as a reliable approach to induce transgenerationally inherited metabolic changes.

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CHAPTER 4

EFFECTS OF PARENTAL OBESITY ON THE METABOLIC STATE OF OFFSPRING

Summary

A significant portion of the heritable risk for complex metabolic disorders cannot be attributed to classic Mendelian genetic factors. At least some of this missing heritability is thought to be due to the epigenetic influence of parental and grandparental metabolic state on offspring health. Previous work suggests that this transgenerational phenomenon is evolutionarily conserved in *Drosophila*, but dietary models have proven to be inconsistent in generating stable heritable effects on offspring metabolism. We show here that genetic manipulation of parental metabolism using *AKHR* mutations results in significant physiological changes in F2 wild-type offspring. F2 offspring descended from obese grandfathers and heterozygous mothers (a grandpaternal/maternal line of inheritance) display reduced triglyceride levels when compared to siblings from all other genetic combinations. We also observe an unexpected phenotype in *AKHR* heterozygotes due to haploinsufficiency at this locus, with elevated glycogen levels and changes in gene expression. Taken together, our results demonstrate that genetic manipulation of adult metabolism can be used to induce transgenerational metabolic phenotypes in *Drosophila*, providing an alternative approach to better understand the mechanisms behind this mode of inheritance.

Introduction

Over the past several decades, the incidence of complex metabolic disease in developed countries has increased at an alarming rate. This increase is accompanied by significant social and economic costs, totaling $245 billion for diabetes in the United States in 2012 (CDCP, 2014). Despite this, the known genetic risk factors for diabetes account for only ~10% of its heritability (Billings and Florez, 2010; Imamura and Maeda, 2011). Some of this missing heritability can be explained by gene-environment and gene-gene interactions, but the majority of the heritable risk is still unidentified (Cordell, 2009; Sanghera and Blackett, 2012). Over the last decade, however, it has become clear that at least some of this risk can be attributed to the epigenetic inheritance of the parental and even grandparental metabolic state.

Studies in famine-exposed human populations have suggested that parental and developmental caloric restriction can lead to metabolic dysfunction in adult progeny. The best controlled of these studies followed individuals conceived during a period from October 1944 through May 1945 in German-occupied Holland, when civilians were subjected to severe rationing. Compared to sibling controls born before October 1944 or conceived after May 1945, individuals exposed to famine during the first trimester were at a higher risk for diabetes, obesity, cardiovascular disease, and certain types of cancer (de Rooij et al., 2006; Lumey et al., 2009; Ravelli et al., 1999). Similar results were found in famine-exposed populations from China or Leningrad, especially when exposed individuals had access to a rich, western-style diet later in life (Li et al., 2010; Stanner et al., 1997). Furthermore, it is clear that paternal and multigenerational exposure to caloric restriction can impact the metabolism of adult children and grandchildren, suggesting that

these phenotypes are due to more than developmental defects (Bygren et al., 2001; Bygren et al., 2014; Kaati et al., 2002; Pembrey et al., 2014; Pembrey et al., 2006).

This response appears to be conserved through evolution, with effects on offspring metabolism in rodents exposed to dietary conditions that induce metabolic dysfunction in the parental generation. Both maternal and paternal low protein or high fat diets alter circulating and liver lipid levels, DNA methylation, and gene expression patterns through at least one and often two generations (Bellinger et al., 2004; Bellinger et al., 2006; Burdge et al., 2004; Carone et al., 2010; Chen et al., 2016; Dunn and Bale, 2011; Duque-Guimaraes and Ozanne, 2013; Hoile et al., 2011; Jimenez-Chillaron et al., 2009; Langley-Evans, 2001; Lillycrop, 2011; Lillycrop et al., 2005; Lucas et al., 1996; Ng et al., 2010; Sharma et al., 2016). These molecular changes are accompanied by changes in the risk of diabetes, obesity, and cardiovascular disease (Jimenez-Chillaron et al., 2009; Langley-Evans, 2001). Recent evidence has linked these molecular and physiological phenotypes to changes in the expression of small RNAs in the germline, suggesting that metabolic changes in offspring might be due to the effects of these small RNAs on gene expression (Chen et al., 2016; Kawano et al., 2012; Murashov et al., 2016; Sharma et al., 2016; Yuan et al., 2015).

Although promising, the time and financial costs of doing these types of multigenerational experiments in mammalian models have inspired a move to *Drosophila* as an ideal system in which to study this phenomenon. Treatment of either maternal or paternal flies with a high sugar diet leads to changes in glycemic levels and a propensity to develop obesity in progeny generations (Buescher et al., 2013; Ost et al., 2014). Similarly, altering parental dietary protein impacts triglyceride and glycogen levels as

well as longevity and fecundity in progeny (Matzkin et al., 2013; Xia and de Belle, 2016). These types of dietary manipulations in parents, however, are subject to a large degree of environmental variation, which can affect the penetrance of progeny phenotypes (Ost et al., 2014).

An alternative approach to induce metabolic changes in the parental generation is through genetic mutation, which has been used successfully for transgenerational studies in rodent models (Nelson et al., 2010; Padmanabhan et al., 2013; Yazbek et al., 2010). In this paradigm, a parental mutant is crossed to a wild-type individual to generate the subsequent F1 heterozygous offspring, which are subsequently crossed to another wild-type individual to generate genetically wild-type F2 offspring. In one example of this, the presence of one allele at the *Obrq2A* quantitative trait locus (QTL) in the parental generation is associated with low body weight and insulin sensitivity. Interestingly, the presence of this allele in grandparents is associated with low body weight in wild-type F2 offspring, especially when passed through a grandmaternal/paternal pattern of inheritance (Yazbek et al., 2010). This and other findings demonstrate that genetic perturbations in metabolic state of parents can effectively impact the physiology of progeny in rodents.

In this study, we developed a novel genetic paradigm to examine the effects of obesity in the parental generation of *Drosophila* using *AKHR* loss-of-function mutations. Grandpaternal obesity caused by a loss of *AKHR* is associated with low triglyceride levels in wild-type F2 offspring of heterozygous F1 mothers. In addition, we observed an unexpected effect of *AKHR* mutant heterozygosity in the F1 generation that resulted in elevated levels of stored glycogen and effects on gene expression. Our results indicate that genetic manipulation of parental metabolism in *Drosophila* provides an effective

approach to induce transgenerational changes in metabolic state through the F2 generation. More detailed characterization could allow the use of this system to identify molecular mechanisms behind this mode of inheritance.

Materials and methods

Fly stocks and maintenance

Flies were maintained at 25°C in an incubator with a 12 hr light/dark cycle and transferred every two to four days to fresh media containing 1% agar, 8% yeast, 2% yeast extract, 2% peptone, 3% sucrose, 6% dextrose, 0.05% $MgSO_4x6H_2O$, 0.05% $CaCl_2x2H_2O$, 0.1% p-Hydroxy-benzoic acid methyl ester, and 0.6% propionic acid (Backhaus et al., 1984). Adult males at 10 days of age under *ad libitum* feeding conditions were used for all experiments unless otherwise indicated. A transheteroallelic combination of the $AKHR^{DsRed}$ (Bloomington 140835) and $AKHR^{1}$ alleles (a generous gift from R. Kühnlein) was used to generate *AKHR* null mutants for the parental generation (Gronke et al., 2007; Schuldiner et al., 2008). These alleles were previously outcrossed to a *Canton S* wild-type genetic background. *AKHR* mutants were crossed to the same *Canton S* strain to generate the F1 and F2 generations. *Canton S* flies were also used as controls for all experiments.

To generate the F1 and F2 offspring, males and female virgins were crossed and maintained on egg caps, which were replaced every 24 hr. The first two egg collections were discarded, after which embryos were collected from egg caps and transferred to fresh vials at a density of approximately 50-100 embryos/vial. Embryos were collected on two consecutive days from each cross.

Metabolite assays

Five adult male flies were collected at the indicated ages and washed in 1X PBS. Each sample was homogenized in 100 µL 1XPBS, after which 10 µL was reserved for a protein assay and the remainder of the lysate was heat-treated for 10 min at 70°C. Protein, glucose, glycogen, and triglyceride assays were performed as described (Tennessen et al., 2014).

RNA-seq transcriptional profiling

RNA was isolated from 10-11 day-old F1 male progeny using Trizol extraction (Thermo Fisher) and the Qiagen RNeasy Mini Kit. Library generation (Illumina TruSeq RNA Sample Preparation Kit v2 with oligo dT selection) and sequencing (HiSeq 50 Cycle Single Read Sequencing v3) were performed by the High-Throughput Genomics core facility at the University of Utah. The Bioinformatics Core Facility at the University of Utah aligned this dataset to the genome, utilizing the Genome Build DM3 from April 2006. Cut-offs for significance were Log2 ratio ± 0.13 and p-value <0.05.

Statistical analysis

GraphPad PRISM 6 software was used to plot metabolite data and to perform statistical analysis as appropriate. Simple comparisons in the P0 generation were performed using a standard Student's T-test with Welch's correction for unequal variances. In the F1 and F2 generations, one-way ANOVA was performed to compare the effects of parental or grandparental obesity on metabolite levels, with multiple comparisons tests to determine the statistical significance of differences between the individual groups by Sidak's multiple comparisons test. A two-way ANOVA was used to determine the effect of experimental replicates on variation between the groups in

comparison with the effect of parental/grandparental obesity.

Results

Loss of *AKHR* function leads to parental obesity

We used loss of the hormone receptor *AKHR* as a model of robust metabolic dysfunction in the parental generation to induce physiological defects in their progeny. AKHR is the receptor for the adipokinetic hormone (AKH). AKH is released from the neuroendocrine cells of the corpora cardiaca in response to fasting and activates enzymes in the fat body to catabolize stored lipids and carbohydrates for energy production (Kim and Rulifson, 2004). In this manner, AKH functions analogously to the fasting hormone glucagon in mammals. Consistent with the catabolic activities of AKH, the loss of *AKHR* results in reduced lipid mobilization and elevated triglyceride stores (Bharucha et al., 2008; Gronke et al., 2007). We selected two loss-of-function *AKHR* alleles for our study. One of these, $AKHR^{1}$, is a deletion allele generated by imprecise excision of a P-element inserted into the locus (Gronke et al., 2007). The other allele, $AKHR^{DsRed}$, carries a *piggyBac* insertion in the second intron of *AKHR* along with a splice acceptor and stop codon, resulting in early termination of AKHR translation (Schuldiner et al., 2008). Based on the location of the *piggyBac* insertion, this is predicted to be a null allele for the *AKHR* locus. The transposon also carries a gene encoding the fluorescent DsRed protein under the control of the synthetic *3XP3* promoter, which drives gene expression in the eye (Schuldiner et al., 2008). As a result, individuals carrying the $AKHR^{DsRed}$ allele can be identified by eye-specific DsRed expression.

We used a wild-type *Canton S* line for our studies along with $AKHR^{1}$ and $AKHR^{DsRed}$ mutants that had been outcrossed into the *Canton S* genetic background.

Metabolite measurements in transheterozygotes carrying the *AKHR*^{1} and *AKHR*^{DsRed} alleles were consistent with the results of earlier studies (Bharucha et al., 2008; Gronke et al., 2007). Both males and females lacking *AKHR* are obese (Figure 4.1A,B), with no apparent defects in either glucose or glycogen levels (Figure 4.1C-F). Protein levels are unchanged in males and slightly reduced in females (Figure 4.1G,H). The disruption in female protein, however, varied among experimental replicates ($p < 0.05$), while this was

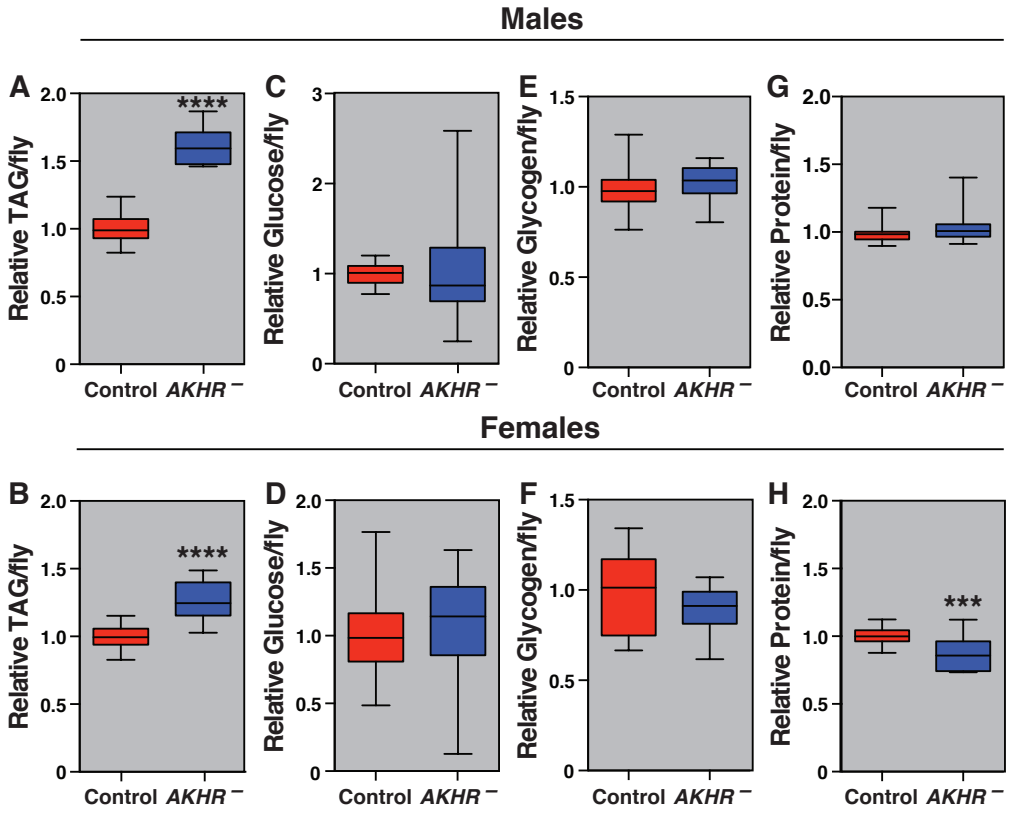

Figure 4.1. *AKHR* mutants are obese compared to genetically matched controls
Metabolites were measured from *AKHR*^{DsRed}/*AKHR*^{1} transheterozygous mutant males and females (*AKHR*$^{-}$) and genetically matched controls (n = 16). Triglycerides in males (A) and females (B) are substantially elevated relative to controls while glycogen (C,D) and glucose levels (E,F) are unchanged. Protein is unchanged in males (G) but slightly reduced in females (H). There was no effect of experimental variation across replicates pooled in this dataset for triglyceride levels in either males or females, while there was an effect of experimental variation for protein levels in females ($p < 0.05$). Basic comparisons were analyzed using a Student's t-test with Welch's correction for unequal variance, while batch effects were determined using two-way ANOVA. ***$p < 0.001$, ****$p < 0.0001$.

not the case for obesity in either males or females. We conclude that *AKHR* mutants have severe, reproducible defects in triglyceride homeostasis.

Paradigm to generate wild-type offspring from *AKHR* mutant parents

The dominant eye-specific DsRed marker in our $AKHR^{DsRed}$ allele allowed us to outcross this mutation and observe the effect of parental obesity on future generations (Figure 4.2). Transheteroallelic $AKHR^{1}/AKHR^{DsRed}$ mutant males and females were crossed to wild-type *Canton S* females and males to generate the F1 offspring, all of which were heterozygous for one of the two *AKHR* mutant alleles. As controls, we also set up crosses between male or female $AKHR^{-}$/+ heterozygotes with wild-type females or males to generate heterozygous $AKHR^{-}$/+ F1 offspring. These offspring were descended from heterozygous parents for at least two generations, such that there was no obesity in

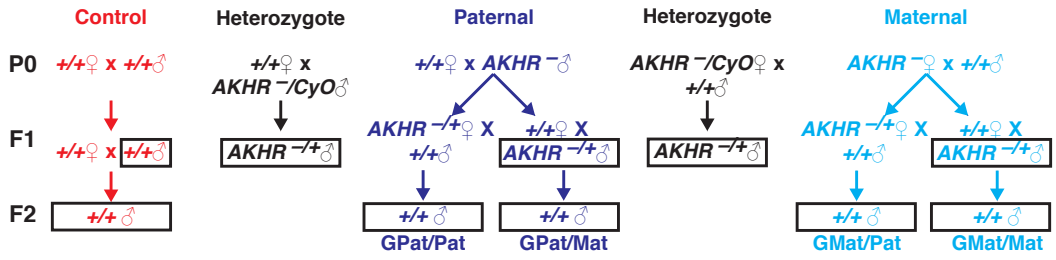

Figure 4.2. Crossing scheme to generate F1 and F2 progeny from obese individuals. $AKHR^{DsRed}/AKHR^{1}$ transheterozygous mutant males or females ($AKHR^{-}$) were crossed to wild-type females or males to generate heterozygous $AKHR^{DsRed}$/+ F1 male and female progeny ($AKHR^{-}$/+). These were subsequently crossed to wild-type females or males to generate genetically wild-type F2 progeny (+/+). As controls for the F1 generation, $AKHR^{DsRed}/CyO$ heterozygote males or females ($AKHR^{-}/CyO$) were crossed to wild-type females or males to generate heterozygous $AKHR^{DsRed}$/+ F1 male and female progeny ($AKHR^{-}$/+). In both the F1 and F2 generations, wild-type control crosses (+/+) were maintained and measured. Descendants from the wild-type control line are colored in red, from the heterozygote control line in black, from the paternal obese line in blue, and from the maternal obese line in cyan. Metabolites were measured in both F1 and F2 males (black boxes).

either their parents or grandparents.

Half of the F1 progeny of obese *AKHR* mutant parents carried the $AKHR^{DsRed}$ allele and half carried the unmarked $AKHR^{1}$ allele. We therefore selected F1 progeny carrying the $AKHR^{DsRed}$ allele and crossed males and females of this genotype to wild-type females and males to generate the F2 offspring (Figure 4.2). From the F2 offspring we selected flies lacking the $AKHR^{DsRed}$ allele, effectively isolating those that are wild-type at the *AKHR* locus (Figure 4.2). In the F2 generation we could therefore identify physiological defects in wild-type progeny descended from obese parents as compared to wild type progeny with no genetically induced obesity in their genealogical history.

AKHR heterozygotes display elevated levels of glycogen

We first examined metabolite levels in the F1 generation to determine if any physiological defects are independent of heterozygosity for *AKHR* and dependent on the parental metabolic state. Triglyceride levels are not changed between wild-type and either heterozygous controls or heterozygotes descended from an obese parent (Figure 4.3A). The same is true for glucose (Figure 4.3B) and protein (Figure 4.3C) levels. Interestingly, glycogen levels are significantly increased in all F1 *AKHR* heterozygotes descended from either obese or nonobese parents (Figure 4.3D, Figure 4.4). Our studies thus demonstrate that heterozygosity for *AKHR* results in a reproducible effect on stored glycogen levels, something that has not been reported in past work (Bharucha et al., 2008; Gronke et al., 2007). These results have been consistent across three or more independent biological replicates.

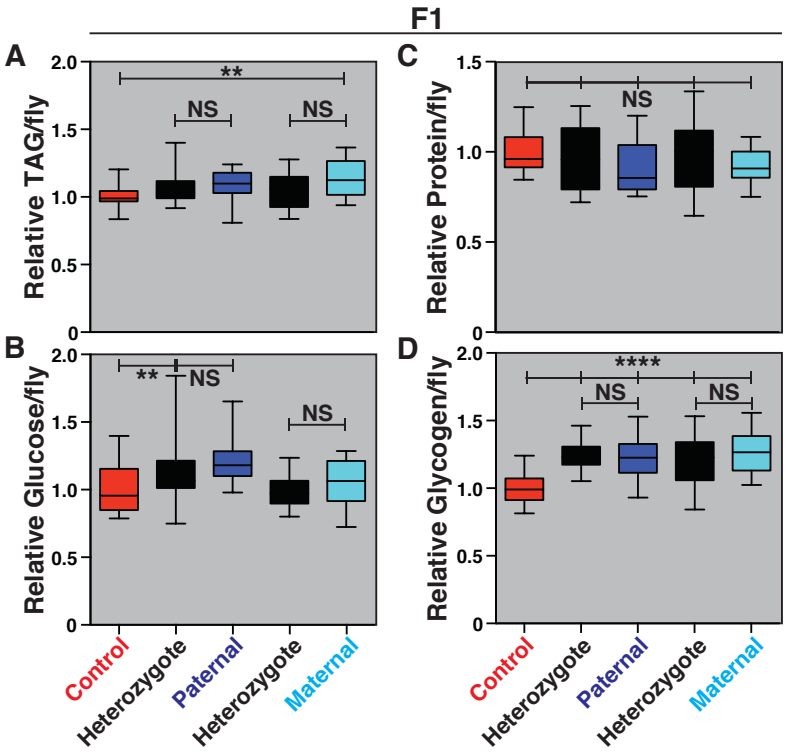

Figure 4.3 Haploinsufficiency masks F1 metabolic phenotypes
Metabolites were measured in F1 progeny from wild-type (red), heterozygous (black), obese paternal (blue), and obese maternal parents (cyan). Results from F1 progeny (n = 20/each) were pooled across two experimental replicates. F1 progeny from obese maternal or paternal parents have no physiological defects that are independent of genotype. Triglyceride (A), glucose (B), and protein (C) levels are largely unaffected in F1 heterozygous progeny descended from obese parents as compared to heterozygote controls or as compared to wild-type controls. Glycogen levels (D) are elevated in all heterozygous progeny as compared to controls. Comparisons among inheritance lines were performed using one-way ANOVA and Sidak's multiple comparisons test. Batch effects were determined using two-way ANOVA. **$p < 0.01$, ****$p < 0.0001$.

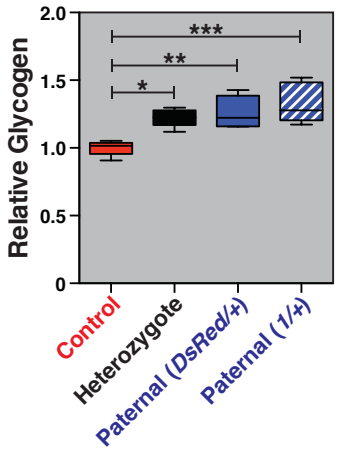

Figure 4.4. Defects in F1 heterozygotes are independent of the *AKHR* allele. Triglyceride levels in F1 progeny from wild-type (red), heterozygous (black), or obese paternal parents (blue and blue striped). Heterozygote controls are $AKHR^{DsRed}/+$, while heterozygotes descended from obese parents are either $AKHR^{DsRed}/+$ (blue) or $AKHR^{1}/+$ (blue striped). Measurements are taken at approximately 10 days of adult age (n = 5/each). All *AKHR* heterozygotes have significantly elevated glycogen as compared to controls. Comparisons among inheritance lines were performed using one-way ANOVA and Sidak's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Grandpaternal/maternal inheritance of metabolic dysfunction in the F2 generation

We also measured basic stored metabolites in the F2 generation in order to determine if parental obesity might lead to a reproducible change in the physiology of wild-type F2 offspring (Figure 4.2). Glycogen, glucose, and protein levels are unchanged between controls and all F2 progeny descended from an obese parent (Figure 4.5A-C). Importantly, however, triglyceride levels are significantly lower in F2 progeny descended from obese grandfathers and heterozygous mothers, or with grandpaternal/maternal inheritance (Figure 4.5D). This reduction is significant whether the comparison is made between flies of grandpaternal/maternal lineage and controls or the grandpaternal/paternal lineage. Furthermore, there is no significant variation between experimental replicates in triglyceride levels between flies of the grandpaternal/maternal

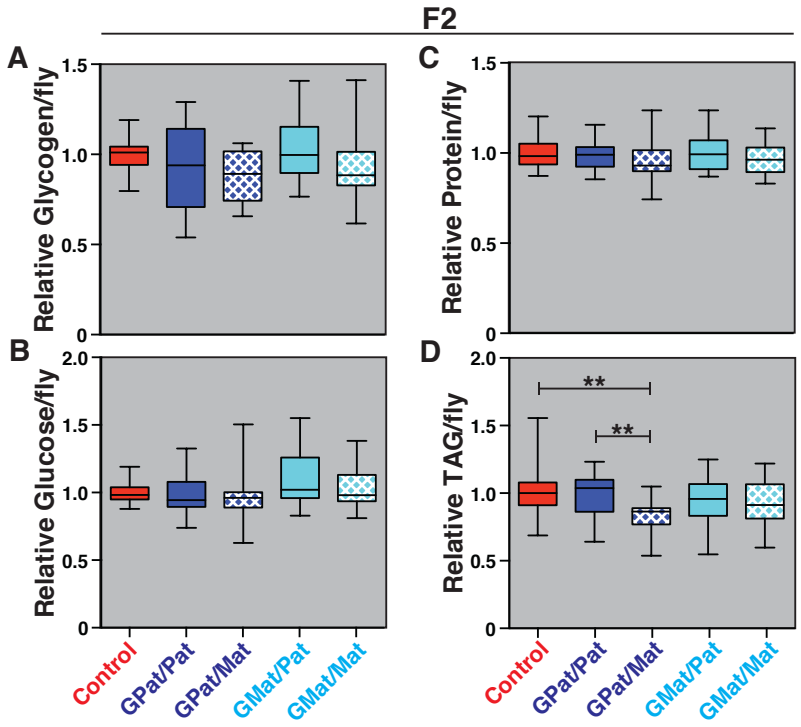

Figure 4.5. F2 progeny descended from obese grandpaternal and heterozygous maternal parents are lean.
Metabolite levels in wild-type controls (red) and F2 descendants of obese parents of the following lineages: grandpaternal/paternal (GPat/Pat, blue), grandpaternal/maternal (GPat/Mat, blue/white), grandmaternal/paternal (GMat/Pat, cyan), and grandmaternal/maternal (GMat/Mat, cyan/white). Measurements were taken at approximately 10 days of adult age and represent three experimental replicates (n = 25/each). There is no significant difference between controls and any of the F2 descendants of obese parents in glycogen (A), glucose (B), or protein (C) levels. Descendants of the grandpaternal/maternal inheritance line have reduced triglycerides as compared to controls, but all other F2 progeny are not significantly altered (D). There was no effect of experimental variation across replicates pooled in this dataset when comparing control and grandpaternal/maternal samples. Comparisons among inheritance lines were performed using one-way ANOVA and Sidak's multiple comparisons test. Batch effects were determined using two-way ANOVA. **$p < 0.01$.

lineage and controls. These results are consistent across three experimental replicates.

Transcriptional profiling of F1 progeny

The lack of an effect of parental obesity on stored metabolites in the F1 generation may be due to the multiple physiological pathways that maintain energy homeostasis. Because transcription is a more direct product of epigenetic state, we hypothesized that changes in mRNA levels may not be subject to the same buffering effects that prevented us from identifying physiological defects in the F1. We therefore performed RNA-seq analysis on F1 offspring to identify possible transcriptional changes in the progeny of obese parents compared with both wild-type and heterozygous controls. RNA was extracted from 60 adult male flies at approximately 10-11 days of age, when they had reached maturity and metabolic homeostasis. Three biological replicates were collected from wild-type controls, $AKHR^{-}$/+ heterozygous controls descended from nonobese parents, $AKHR^{-}$/+ heterozygotes descended from obese paternal parents, and $AKHR^{-}$/+ heterozygotes descended from obese maternal parents. Approximately half of the heterozygote controls were descended from paternal parents carrying the $AKHR^{DsRed}$ allele, while the other half were descended from maternal parents carrying the $AKHR^{DsRed}$ allele. Because we expected that the transcriptional differences caused by parental obesity would be subtle, we selected transcripts that changed more than 9.5% ($p < 0.05$).

Consistent with the reproducible effects of *AKHR* heterozygosity on glycogen levels in F1 offspring, we detected a number of genes that are significantly affected by the loss of one copy of this receptor (Table 4.1; Figure 4.3A). There are 143 misregulated genes (94 down and 49 up) in heterozygotes descended from nonobese parents compared to wild-type controls, 137 misregulated genes (97 down and 40 up) in

Table 4.1: Heterozygous controls versus wild-type controls

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0031910	CG15818	-0.814	0.569	299.1	7.49E-300
FBgn0264352	CR43808	-0.507	0.704	95.9	1.38E-96
FBgn0263321	CG43402	-0.505	0.705	118.4	4.36E-119
FBgn0031430	CG3528	-0.496	0.709	95.9	1.38E-96
FBgn0031791	CG9486	-0.461	0.727	80.6	2.43E-81
FBgn0261055	Sfp26Ad	-0.447	0.733	137.1	8.14E-138
FBgn0064237	Idgf5	-0.420	0.747	91.2	5.88E-92
FBgn0263320	CG43401	-0.419	0.748	64.3	5.23E-65
FBgn0262616	CR43142	-0.403	0.756	55.4	3.85E-56
FBgn0019928	Ser8	-0.389	0.764	60.5	3.04E-61
FBgn0031918	CG6055	-0.376	0.771	47.6	2.34E-48
FBgn0032601	yellow-b	-0.359	0.779	42.0	8.99E-43
FBgn0039084	CG10175	-0.353	0.783	65.3	5.26E-66
FBgn0038449	CG17562	-0.346	0.787	50.6	2.51E-51
FBgn0040733	CG15068	-0.340	0.790	80.9	1.21E-81
FBgn0031538	CG3246	-0.324	0.799	79.3	4.51E-80
FBgn0029702	CG15572	-0.319	0.802	66.3	5.37E-67
FBgn0034318	CG14500	-0.311	0.806	33.8	1.45E-34
FBgn0011722	Tig	-0.310	0.806	54.4	4.04E-55
FBgn0031275	GABA-B-R3	-0.304	0.810	34.5	3.25E-35
FBgn0259949	Sfp23F	-0.291	0.817	25.3	4.86E-26
FBgn0030098	CG12057	-0.286	0.820	22.8	1.58E-23
FBgn0031857	CG11321	-0.286	0.820	48.8	1.51E-49
FBgn0033164	CG11112	-0.285	0.821	26.5	2.84E-27
FBgn0036024	CG18180	-0.283	0.822	35.8	1.62E-36
FBgn0032367	CG6555	-0.278	0.825	53.6	2.34E-54
FBgn0046212	CG15841	-0.278	0.825	53.6	2.34E-54
FBgn0027552	CG10863	-0.277	0.825	76.0	9.83E-77
FBgn0262476	CG43066	-0.274	0.827	29.3	4.93E-30
FBgn0263597	Acp98AB	-0.270	0.829	51.1	8.62E-52
FBgn0028583	lcs	-0.267	0.831	38.5	2.99E-39
FBgn0261061	Sfp96F	-0.265	0.832	24.3	5.17E-25
FBgn0011669	Mst57Db	-0.264	0.833	42.0	8.99E-43
FBgn0038930	CG5778	-0.262	0.834	41.5	3.09E-42
FBgn0033593	Listericin	-0.256	0.838	23.2	5.66E-24
FBgn0039094	CG10184	-0.254	0.838	35.7	2.18E-36
FBgn0051935	CG31935	-0.254	0.838	26.0	1.11E-26

Table 4.1 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0020385	pug	-0.253	0.839	52.6	2.34E-53
FBgn0028987	Spn2	-0.253	0.839	35.8	1.62E-36
FBgn0036756	cln3	-0.250	0.841	28.0	9.63E-29
FBgn0262233	mir-2494	-0.250	0.841	15.0	9.45E-16
FBgn0031906	CG5160	-0.243	0.845	15.5	2.94E-16
FBgn0032122	CG31883	-0.241	0.846	28.8	1.47E-29
FBgn0046113	GluRIIC	-0.240	0.847	15.3	5.61E-16
FBgn0033603	Cpr47Ef	-0.238	0.848	14.1	7.23E-15
FBgn0263322	CG43403	-0.237	0.848	18.1	8.44E-19
FBgn0260396	CG42521	-0.237	0.849	15.1	8.03E-16
FBgn0031561	CG16712	-0.234	0.850	49.8	1.63E-50
FBgn0259971	CG42481	-0.231	0.852	35.8	1.62E-36
FBgn0003249	Rh3	-0.231	0.852	23.1	8.47E-24
FBgn0026755	Ugt37b1	-0.230	0.853	14.4	3.75E-15
FBgn0262961	CG43272	-0.230	0.853	22.0	8.92E-23
FBgn0031560	CG16713	-0.229	0.853	19.0	9.64E-20
FBgn0040107	lectin-21Ca	-0.228	0.854	14.1	7.19E-15
FBgn0031689	Cyp28d1	-0.226	0.855	34.5	3.25E-35
FBgn0032868	CG17472	-0.224	0.856	24.6	2.48E-25
FBgn0020908	Scp1	-0.220	0.858	27.9	1.26E-28
FBgn0259958	Sfp24F	-0.215	0.862	15.2	5.79E-16
FBgn0259969	Sfp65A	-0.213	0.863	18.9	1.36E-19
FBgn0262899	CG43254	-0.210	0.865	16.9	1.25E-17
FBgn0031515	CG9664	-0.210	0.865	13.1	8.00E-14
FBgn0039031	CG17244	-0.209	0.865	16.5	3.32E-17
FBgn0054043	CG34043	-0.208	0.866	14.2	5.70E-15
FBgn0003274	RpLP2	-0.208	0.866	19.9	1.26E-20
FBgn0053123	CG33123	-0.205	0.867	14.4	3.75E-15
FBgn0011670	Mst57Dc	-0.204	0.868	24.3	5.17E-25
FBgn0015010	Ag5r	-0.203	0.869	26.5	2.84E-27
FBgn0031305	Iris	-0.203	0.869	22.2	6.35E-23
FBgn0020509	Acp62F	-0.200	0.871	18.6	2.44E-19
FBgn0004242	Syt1	-0.199	0.871	21.2	5.77E-22
FBgn0259966	Sfp51E	-0.198	0.872	15.0	9.45E-16
FBgn0259968	Sfp60F	-0.198	0.872	20.0	1.01E-20
FBgn0016675	Lectin-galC1	-0.198	0.872	14.4	3.75E-15
FBgn0014427	CG11899	-0.197	0.872	14.2	6.23E-15

Table 4.1 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0259964	Sfp33A3	-0.196	0.873	15.3	4.79E-16
FBgn0011559	Acp36DE	-0.192	0.876	34.5	3.25E-35
FBgn0028986	Spn3	-0.189	0.877	19.9	1.25E-20
FBgn0015521	RpS21	-0.187	0.878	26.4	4.04E-27
FBgn0002863	Acp95EF	-0.180	0.883	20.4	3.88E-21
FBgn0263657	CR43648	-0.179	0.883	14.3	4.87E-15
FBgn0032350	CG6287	-0.178	0.884	13.9	1.18E-14
FBgn0020513	ade5	-0.178	0.884	15.0	9.45E-16
FBgn0086691	UK114	-0.177	0.885	13.1	8.00E-14
FBgn0263984	CG43733	-0.175	0.886	27.6	2.24E-28
FBgn0050395	CG30395	-0.174	0.886	17.6	2.36E-18
FBgn0000120	Arr1	-0.174	0.886	25.8	1.46E-26
FBgn0031176	CG1678	-0.173	0.887	23.4	4.18E-24
FBgn0040007	RpL38	-0.172	0.887	16.2	6.94E-17
FBgn0028433	Ggamma30A	-0.172	0.888	26.5	3.42E-27
FBgn0034200	CG11395	-0.167	0.890	14.6	2.40E-15
FBgn0031294	IA-2	-0.160	0.895	14.3	4.93E-15
FBgn0002940	ninaE	-0.157	0.897	22.9	1.20E-23
FBgn0038236	Cyp313a1	-0.155	0.898	13.7	2.11E-14
FBgn0031453	CG9894	-0.151	0.900	14.3	4.92E-15
FBgn0031545	CG3213	0.154	1.113	19.2	5.72E-20
FBgn0015600	toc	0.158	1.116	15.1	8.03E-16
FBgn0037329	CG12162	0.159	1.117	14.7	2.15E-15
FBgn0000182	BicC	0.169	1.125	14.0	9.61E-15
FBgn0035988	CG3982	0.172	1.126	18.6	2.26E-19
FBgn0038598	CG7131	0.173	1.128	17.6	2.43E-18
FBgn0035724	CG10064	0.175	1.129	14.3	4.87E-15
FBgn0037064	CG9389	0.177	1.130	24.4	3.79E-25
FBgn0034435	CG9975	0.179	1.132	21.8	1.50E-22
FBgn0035491	Dpy-30L2	0.183	1.135	14.2	5.70E-15
FBgn0032424	CG17010	0.184	1.136	16.8	1.53E-17
FBgn0033330	CG8746	0.184	1.136	16.1	7.23E-17
FBgn0031418	CG3609	0.190	1.141	25.8	1.46E-26
FBgn0031853	TTLL3B	0.195	1.144	22.2	5.63E-23
FBgn0031343	CG18131	0.197	1.146	15.0	9.45E-16
FBgn0014906	Hydr2	0.201	1.149	24.9	1.17E-25
FBgn0034840	CG3124	0.201	1.150	26.5	2.91E-27

Table 4.1 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0027524	CG3909	0.214	1.160	17.3	4.81E-18
FBgn0259111	Ndae1	0.218	1.163	26.5	2.87E-27
FBgn0001089	Gal	0.222	1.166	18.1	7.80E-19
FBgn0033246	ACC	0.222	1.167	20.4	3.88E-21
FBgn0031208	CG11023	0.226	1.170	14.2	5.70E-15
FBgn0040502	CG8343	0.231	1.173	20.4	3.88E-21
FBgn0032434	CG5421	0.239	1.180	13.7	2.00E-14
FBgn0031389	CG4259	0.245	1.185	14.2	5.80E-15
FBgn0031856	CG11322	0.246	1.186	15.8	1.44E-16
FBgn0030326	CG2444	0.246	1.186	15.1	8.03E-16
FBgn0261805	CG42751	0.247	1.187	22.8	1.58E-23
FBgn0032402	CG14945	0.253	1.192	27.5	3.43E-28
FBgn0051950	CG31950	0.255	1.194	18.2	6.01E-19
FBgn0031710	CG7371	0.259	1.196	19.9	1.25E-20
FBgn0032965	CG11629	0.273	1.208	23.6	2.32E-24
FBgn0032900	CG14401	0.280	1.214	25.2	5.67E-26
FBgn0262097	CG42850	0.295	1.226	39.2	6.72E-40
FBgn0031520	CG8837	0.300	1.231	33.1	7.82E-34
FBgn0044810	TotX	0.314	1.244	34.9	1.27E-35
FBgn0031869	CG18304	0.321	1.249	48.0	9.20E-49
FBgn0053282	CG33282	0.341	1.266	36.7	1.99E-37
FBgn0040723	CG5011	0.345	1.270	49.8	1.63E-50
FBgn0020545	kraken	0.348	1.273	80.7	1.90E-81
FBgn0264369	CR43821	0.353	1.277	104.3	5.62E-105
FBgn0031472	CG2983	0.374	1.296	47.1	7.26E-48
FBgn0031323	CG5139	0.383	1.304	75.6	2.35E-76
FBgn0031345	CG18132	0.401	1.320	64.9	1.19E-65
FBgn0031360	CG31937	0.443	1.359	144.8	1.72E-145
FBgn0259229	CG42329	0.541	1.455	118.4	3.98E-119
FBgn0031925	Cyp4d21	0.613	1.530	235.2	6.35E-236
FBgn0031805	CG9505	0.697	1.621	199.8	1.60E-200
FBgn0262944	CR43263	1.081	2.115	594.3	0.00E+01

heterozygotes descended from obese fathers compared to wild-type controls, and 48 misregulated genes (28 down and 20 up) in heterozygotes descended from obese mothers compared to wild-type controls (Tables 4.1-4.3). 15 of the downregulated genes and 17 of the upregulated genes are shared between all three heterozygous lineages (Figure 4.6A,B).

33 genes are uniquely downregulated in heterozygotes descended from nonobese parents compared to wild-type controls (Figure 4.6A). These are enriched for genes involved in translation and ribosome structure (Table 4.4) (Huang da et al., 2009a, b). In addition, although the category is not significantly enriched, there are also two accessory gland proteins (*acp62F* and *acp95EF*) that are downregulated in heterozygotes descended from nonobese parents. These seminal fluid proteins are highly expressed in the accessory gland and are likely involved in male fertility (Mueller et al., 2008; Mueller et al., 2005). Potentially this could be indicative of downstream impacts of metabolic homeostasis on fecundity.

39 downregulated genes are shared between heterozygotes descended from nonobese parents and those descended from obese fathers, while five are shared only between the paternal and maternal lineages (Figure 4.6A). This means that approximately 55% of genes downregulated in heterozygotes descended from obese fathers are also downregulated in heterozygotes descended from nonobese fathers. The genes that are uniquely downregulated in the paternal lineage (38) are enriched for genes involved in sperm function and mating behavior (Table 4.5) (Huang da et al., 2009a, b). Many of these are known seminal fluid proteins and could indicate subtle differences in the fecundity and germline function in F1 descendants of obese fathers, which may

Table 4.2: Maternal versus wild-type controls

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0029702	CG15572	-0.846	0.556	382.3	0.00E+01
FBgn0031910	CG15818	-0.825	0.565	257.3	5.23E-258
FBgn0031791	CG9486	-0.435	0.740	55.7	1.85E-56
FBgn0262616	CR43142	-0.406	0.755	44.7	1.78E-45
FBgn0264352	CR43808	-0.402	0.757	43.1	7.23E-44
FBgn0031741	CG11034	-0.374	0.772	41.0	9.37E-42
FBgn0038449	CG17562	-0.372	0.773	40.2	6.34E-41
FBgn0034318	CG14500	-0.368	0.775	41.7	2.04E-42
FBgn0263505	CR43494	-0.363	0.778	36.8	1.42E-37
FBgn0263321	CG43402	-0.355	0.782	40.1	7.55E-41
FBgn0031857	CG11321	-0.332	0.795	45.5	2.99E-46
FBgn0031653	Jon25Biii	-0.329	0.796	30.3	5.18E-31
FBgn0261055	Sfp26Ad	-0.326	0.798	42.8	1.77E-43
FBgn0263322	CG43403	-0.317	0.803	28.9	1.36E-29
FBgn0262233	mir-2494	-0.307	0.809	19.2	5.74E-20
FBgn0031275	GABA-B-R3	-0.305	0.809	23.3	4.79E-24
FBgn0011722	Tig	-0.304	0.810	28.8	1.47E-29
FBgn0262476	CG43066	-0.295	0.815	23.3	4.79E-24
FBgn0040726	dpr	-0.293	0.816	16.9	1.32E-17
FBgn0020906	Jon25Bi	-0.291	0.817	21.7	1.82E-22
FBgn0013684	mt:ND5	-0.280	0.824	14.0	1.01E-14
FBgn0263320	CG43401	-0.276	0.826	14.1	7.67E-15
FBgn0031430	CG3528	-0.273	0.828	14.0	1.01E-14
FBgn0019928	Ser8	-0.271	0.829	14.1	7.67E-15
FBgn0064237	Idgf5	-0.266	0.832	18.2	6.37E-19
FBgn0020908	Scp1	-0.265	0.832	32.8	1.57E-33
FBgn0038930	CG5778	-0.229	0.853	16.1	8.76E-17
FBgn0031538	CG3246	-0.219	0.859	15.2	6.66E-16
FBgn0029831	CG5966	0.272	1.207	19.7	2.16E-20
FBgn0262097	CG42850	0.272	1.207	22.2	6.99E-23
FBgn0032965	CG11629	0.278	1.212	15.9	1.25E-16
FBgn0031323	CG5139	0.282	1.215	18.7	2.16E-19
FBgn0031869	CG18304	0.304	1.235	30.3	5.18E-31
FBgn0031389	CG4259	0.313	1.242	20.6	2.26E-21
FBgn0031472	CG2983	0.317	1.246	22.2	6.99E-23
FBgn0001128	Gpdh	0.324	1.252	32.1	7.63E-33
FBgn0264369	CR43821	0.338	1.264	80.5	3.49E-81

Table 4.2 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0053282	CG33282	0.348	1.273	29.8	1.58E-30
FBgn0031360	CG31937	0.369	1.291	59.5	2.92E-60
FBgn0031345	CG18132	0.375	1.297	40.6	2.68E-41
FBgn0020545	kraken	0.380	1.301	60.0	1.00E-60
FBgn0259229	CG42329	0.436	1.353	57.6	2.49E-58
FBgn0031925	Cyp4d21	0.451	1.367	66.2	5.63E-67
FBgn0044810	TotX	0.453	1.369	72.8	1.69E-73
FBgn0040099	lectin-28C	0.624	1.541	154.6	2.70E-155
FBgn0032402	CG14945	0.624	1.542	131.4	4.19E-132
FBgn0031805	CG9505	0.716	1.642	180.3	4.70E-181
FBgn0262944	CR43263	0.891	1.854	381.3	0.00E+01

Table 4.3: Paternal versus wild-type controls

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0031910	CG15818	-0.821	0.566	296.8	1.59E-297
FBgn0264352	CR43808	-0.525	0.695	103.9	1.37E-104
FBgn0031430	CG3528	-0.468	0.723	84.0	1.06E-84
FBgn0263321	CG43402	-0.459	0.727	89.8	1.77E-90
FBgn0031791	CG9486	-0.446	0.734	72.9	1.29E-73
FBgn0261055	Sfp26Ad	-0.429	0.743	148.4	4.37E-149
FBgn0262616	CR43142	-0.423	0.746	61.9	1.14E-62
FBgn0031741	CG11034	-0.395	0.761	65.9	1.29E-66
FBgn0020906	Jon25Bi	-0.394	0.761	55.8	1.71E-56
FBgn0064237	Idgf5	-0.392	0.762	60.0	1.07E-60
FBgn0032601	yellow-b	-0.381	0.768	47.9	1.26E-48
FBgn0031654	Jon25Bii	-0.381	0.768	51.7	1.89E-52
FBgn0004414	msopa	-0.377	0.770	67.3	5.14E-68
FBgn0263320	CG43401	-0.362	0.778	44.2	6.24E-45
FBgn0031538	CG3246	-0.349	0.785	89.1	8.44E-90
FBgn0263597	Acp98AB	-0.329	0.796	57.2	6.18E-58
FBgn0019928	Ser8	-0.325	0.798	37.7	2.12E-38
FBgn0031918	CG6055	-0.312	0.805	28.5	2.91E-29
FBgn0262621	CG43145	-0.303	0.811	27.7	2.05E-28
FBgn0261061	Sfp96F	-0.299	0.813	34.0	9.35E-35
FBgn0262099	CG42852	-0.292	0.817	57.5	3.03E-58
FBgn0028987	Spn2	-0.292	0.817	47.7	2.15E-48
FBgn0011722	Tig	-0.283	0.822	40.8	1.53E-41
FBgn0040733	CG15068	-0.282	0.822	39.6	2.30E-40
FBgn0259970	Sfp70A4	-0.281	0.823	37.7	2.12E-38
FBgn0038467	CG3590	-0.279	0.824	31.6	2.69E-32
FBgn0259949	Sfp23F	-0.276	0.826	21.2	6.11E-22
FBgn0036756	cln3	-0.273	0.827	38.1	7.57E-39
FBgn0039084	CG10175	-0.272	0.828	37.0	1.07E-37
FBgn0031857	CG11321	-0.266	0.831	39.6	2.30E-40
FBgn0053307	CG33307	-0.263	0.834	28.3	4.64E-29
FBgn0027552	CG10863	-0.262	0.834	62.2	6.11E-63
FBgn0262961	CG43272	-0.262	0.834	29.2	6.53E-30
FBgn0031275	GABA-B-R3	-0.261	0.834	21.4	4.15E-22
FBgn0259959	Sfp26Ac	-0.260	0.835	16.4	3.99E-17
FBgn0040726	dpr	-0.258	0.836	16.1	7.31E-17
FBgn0013684	mt:ND5	-0.258	0.837	16.1	7.31E-17

Table 4.3 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0020908	Scp1	-0.253	0.839	47.9	1.26E-48
FBgn0053516	dpr3	-0.249	0.841	21.4	4.32E-22
FBgn0031653	Jon25Biii	-0.247	0.842	14.7	1.95E-15
FBgn0030098	CG12057	-0.245	0.844	14.2	6.86E-15
FBgn0050151	CG30151	-0.244	0.844	21.4	4.15E-22
FBgn0067905	IM14	-0.241	0.846	20.9	1.23E-21
FBgn0054043	CG34043	-0.241	0.846	20.4	4.39E-21
FBgn0032122	CG31883	-0.239	0.847	33.9	1.35E-34
FBgn0031689	Cyp28d1	-0.239	0.847	44.2	6.24E-45
FBgn0026755	Ugt37b1	-0.237	0.849	16.1	7.31E-17
FBgn0259971	CG42481	-0.233	0.851	39.3	5.39E-40
FBgn0032367	CG6555	-0.233	0.851	47.4	4.44E-48
FBgn0046212	CG15841	-0.233	0.851	47.4	4.44E-48
FBgn0011669	Mst57Db	-0.232	0.851	44.2	6.24E-45
FBgn0031561	CG16712	-0.232	0.852	53.9	1.25E-54
FBgn0039474	CG6283	-0.232	0.852	16.2	6.37E-17
FBgn0036795	CG18233	-0.229	0.853	15.2	6.31E-16
FBgn0039094	CG10184	-0.227	0.855	25.6	2.68E-26
FBgn0263762	CG43679	-0.227	0.855	19.2	6.61E-20
FBgn0032868	CG17472	-0.226	0.855	24.3	5.62E-25
FBgn0015584	Acp53Ea	-0.225	0.856	26.1	8.03E-27
FBgn0011670	Mst57Dc	-0.222	0.857	33.7	1.84E-34
FBgn0015583	Acp29AB	-0.221	0.858	15.4	4.09E-16
FBgn0036796	CG18231	-0.217	0.860	22.0	9.66E-23
FBgn0031176	CG1678	-0.215	0.862	34.2	6.26E-35
FBgn0040098	lectin-29Ca	-0.214	0.862	25.1	7.50E-26
FBgn0015010	Ag5r	-0.212	0.863	27.3	4.87E-28
FBgn0051659	CG31659	-0.211	0.864	14.8	1.44E-15
FBgn0036024	CG18180	-0.210	0.864	23.6	2.53E-24
FBgn0031558	CG16704	-0.208	0.866	15.4	3.58E-16
FBgn0263024	CG43319	-0.205	0.868	24.2	6.18E-25
FBgn0028583	lcs	-0.203	0.869	16.1	7.31E-17
FBgn0003249	Rh3	-0.203	0.869	15.3	4.83E-16
FBgn0015586	Acp76A	-0.202	0.869	20.0	9.25E-21
FBgn0033247	Nup44A	-0.201	0.870	13.1	8.09E-14
FBgn0259965	Sfp35C	-0.200	0.870	14.3	4.91E-15
FBgn0020385	pug	-0.200	0.871	34.7	2.20E-35

Table 4.3 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0052203	Spn75F	-0.199	0.871	16.8	1.62E-17
FBgn0047334	BG642312	-0.198	0.871	29.1	7.71E-30
FBgn0011559	Acp36DE	-0.198	0.872	37.7	2.12E-38
FBgn0262899	CG43254	-0.197	0.873	14.2	6.86E-15
FBgn0016675	Lectin-galC1	-0.196	0.873	13.9	1.15E-14
FBgn0051077	CG31077	-0.195	0.874	20.2	6.54E-21
FBgn0050395	CG30395	-0.194	0.874	25.8	1.54E-26
FBgn0040097	lectin-30A	-0.193	0.875	17.1	8.70E-18
FBgn0036232	CG14125	-0.192	0.876	15.1	8.32E-16
FBgn0031359	CG18317	-0.185	0.880	18.2	6.56E-19
FBgn0034153	Acp53C14b	-0.184	0.880	19.3	4.57E-20
FBgn0028986	Spn3	-0.178	0.884	18.2	6.56E-19
FBgn0003034	Acp70A	-0.175	0.886	18.5	3.11E-19
FBgn0043825	CG18284	-0.171	0.888	18.4	3.88E-19
FBgn0034152	Acp53C14a	-0.169	0.890	13.4	3.94E-14
FBgn0083938	BG642163	-0.168	0.890	16.1	7.31E-17
FBgn0031453	CG9894	-0.160	0.895	16.8	1.74E-17
FBgn0039342	CG5107	-0.157	0.897	13.6	2.66E-14
FBgn0051872	CG31872	-0.154	0.899	16.0	9.71E-17
FBgn0263984	CG43733	-0.152	0.900	16.1	7.31E-17
FBgn0028433	Ggamma30A	-0.149	0.902	15.6	2.43E-16
FBgn0002940	ninaE	-0.139	0.908	15.4	4.09E-16
FBgn0032505	CG16826	-0.132	0.912	14.7	2.10E-15
FBgn0038598	CG7131	0.164	1.120	15.6	2.43E-16
FBgn0015600	toc	0.169	1.124	16.1	7.31E-17
FBgn0031853	TTLL3B	0.176	1.129	13.7	1.90E-14
FBgn0031418	CG3609	0.183	1.135	20.8	1.75E-21
FBgn0014906	Hydr2	0.185	1.137	18.5	3.53E-19
FBgn0036924	hale	0.197	1.146	15.5	2.82E-16
FBgn0031785	CG13991	0.208	1.155	16.6	2.81E-17
FBgn0001089	Gal	0.210	1.157	14.1	7.30E-15
FBgn0038722	Nup58	0.211	1.158	15.4	4.09E-16
FBgn0259111	Ndae1	0.217	1.162	25.1	7.50E-26
FBgn0031292	CG15824	0.220	1.165	17.2	5.99E-18
FBgn0033246	ACC	0.222	1.166	18.5	3.11E-19
FBgn0040723	CG5011	0.234	1.176	16.1	7.31E-17
FBgn0002562	Lsp1alpha	0.234	1.176	15.6	2.35E-16

Table 4.3 continued

FlyBase Gene ID	Gene Name	LgR2rt	Fold Change	AdjP	P-value
FBgn0031490	CG17264	0.237	1.179	16.1	7.31E-17
FBgn0263080	CG43348	0.237	1.179	17.2	5.99E-18
FBgn0040502	CG8343	0.249	1.188	21.0	1.02E-21
FBgn0002869	MtnB	0.252	1.191	15.6	2.43E-16
FBgn0031695	Cyp4ac3	0.253	1.192	16.2	6.57E-17
FBgn0262097	CG42850	0.267	1.204	34.9	1.31E-35
FBgn0031710	CG7371	0.268	1.204	21.3	5.41E-22
FBgn0031389	CG4259	0.270	1.206	18.4	3.88E-19
FBgn0264369	CR43821	0.285	1.218	74.3	5.14E-75
FBgn0032900	CG14401	0.288	1.221	27.8	1.77E-28
FBgn0031520	CG8837	0.310	1.240	36.5	3.54E-37
FBgn0032402	CG14945	0.313	1.243	44.2	6.24E-45
FBgn0031472	CG2983	0.321	1.249	31.6	2.69E-32
FBgn0001128	Gpdh	0.363	1.287	87.6	2.59E-88
FBgn0053282	CG33282	0.367	1.290	44.4	4.07E-45
FBgn0031345	CG18132	0.374	1.296	52.6	2.30E-53
FBgn0031869	CG18304	0.375	1.297	73.1	8.78E-74
FBgn0031360	CG31937	0.384	1.305	102.1	8.44E-103
FBgn0031323	CG5139	0.387	1.308	73.2	6.71E-74
FBgn0020545	kraken	0.390	1.310	89.1	7.54E-90
FBgn0044810	TotX	0.407	1.326	69.1	7.89E-70
FBgn0032965	CG11629	0.410	1.329	60.6	2.79E-61
FBgn0031925	Cyp4d21	0.618	1.535	231.9	1.14E-232
FBgn0259229	CG42329	0.643	1.561	185.3	4.59E-186
FBgn0031805	CG9505	0.718	1.645	212.4	3.85E-213
FBgn0262944	CR43263	1.115	2.166	620.1	0.00E+01

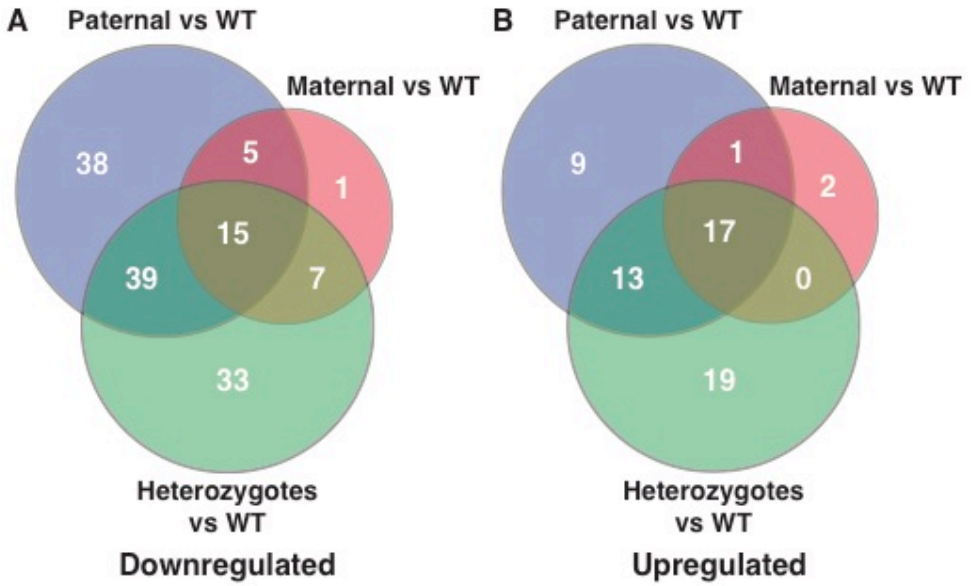

Figure 4.6. Overlap between heterozygous control, maternal, and paternal down and upregulated genes compared to wild-type controls.
RNA-seq from F1 offspring. Control heterozygotes descended from nonobese parents, paternal F1 progeny, and maternal F1 progeny were compared independently to wild-type controls. Overlaps in genes downregulated in heterozygotes and in F1 flies descended from obese paternal or maternal parents (A). Overlaps in genes upregulated in heterozygotes and in F1 flies descended from obese paternal and maternal parents (B).

Table 4.4: Uniquely downregulated genes in heterozygotes compared to wild-type

Category	Genes	p-value	Benjamini
L-serine biosynthetic process	Cg6287, cg11899	8.50E-03	0.074
Nitrogen compound biosynthetic process	Cg6287, cg11900, Ade5	0.087	0.91
Translation	cg33123, RPL38, RPLP2, RPS21	0.096	0.89
Structural constituent of the Ribosome	RPL38, RPLP2, RPS21	0.093	1
Structural molecule activity	RPL38, RPLP2, RPS21, Cpr47EF	0.093	0.99

Table 4.5: Uniquely downregulated genes in paternal line compared to wild-type

Category	Genes	p-value	Benjamini
Mating	Acp70A, Acp29AB, Acp53Ea	8.40E-03	0.32
Behavior	Acp70A, Acp29AB, Acp53Ea, Acp76A	0.03	0.43
Carbohydrate Binding	Acp29AB, Cg14125, Cg17011, Cg17799, Cg31077	6.10E-04	0.014
Hormone Activity	Acp70A, Acp29AB, Acp53Ea	5.60E-03	0.049
Serine-type-endopeptidase inhibitor activity	Acp76A, Cg16704, Cg32203	8.30E-03	0.061
Lipase activity	cg18284, Cg31872, Cg6283	1.60E-02	0.077
Peptidyl-proline dioxygenase activity	Cg18231, Cg18233	5.60E-02	0.2

contribute to the lineage-specific F2 phenotypes observed.

Seven downregulated genes are shared between the maternal lineage and heterozygous descendants of nonobese parents (Figure 4.6A). Approximately 79% of genes downregulated in heterozygotes descended from obese mothers are also downregulated in heterozygotes descended from nonobese fathers, leaving only one gene uniquely downregulated in the maternal lineage: *CR43494*. This presumptive noncoding RNA has no known function (FlyBase Genome, 2011).

There are 19 genes that are uniquely upregulated in heterozygotes descended from nonobese parents when compared to wild-type controls (Figure 4.6B). No gene ontology categories are statistically enriched from this gene set, but one of these upregulated genes is *bicC*. BicC contains a putative RNA-binding domain and may be involved with oogenesis (Mahowald, 2001; Schupbach and Wieschaus, 1991). This could potentially be indicative of downstream impacts of metabolic homeostasis on the female germline and embryonic development.

Thirteen genes are shared between heterozygotes descended from nonobese parents and those descended from obese fathers, while none are shared only between the paternal and maternal lineages (Figure 4.6B). This means that approximately 75% of genes upregulated in heterozygotes descended from obese fathers are also upregulated in heterozygotes descended from nonobese fathers. No gene ontology categories are statistically enriched from this gene set, but we can identify yet another protein potentially involved in fertility. *CG13991* is a gene of unknown function expressed in the testes and packaged into sperm (Dorus et al., 2006; FlyBase Genome, 2011). As with the downregulated ACP proteins from the paternal lineage, this could potentially be

indicative of downstream impacts on germline function and embryonic development.

One upregulated gene is shared only between the maternal lineage and heterozygous descendants of nonobese parents (Figure 4.6B). This means that 90% of genes upregulated in heterozygotes descended from obese mothers are also upregulated in heterozygotes descended from nonobese fathers, thus leaving only two genes uniquely upregulated in the maternal lineage: *lectin-28C* and *CG5966*. Interestingly, *lectin-28C* encodes a predicted galactose-binding protein, and *CG5966* encodes a presumptive triglyceride lipase that is highly expressed in the fat body (Curators, 2008; Theopold et al., 1999). The misregulation of these two genes could be indicative of metabolic changes in the F1 that are buffered by compensating pathways.

When heterozygotes descended from nonobese parents are directly compared with F1 heterozygotes descended from either obese mothers or fathers, however, there are few transcriptional differences. The testes specific gene *CG15572* is upregulated in the paternal lineage as compared to heterozygous progeny of nonobese parents (~1.2-fold increased, $p = 10^{-73}$), while it is downregulated in the maternal lineage (~1.4-fold decreased, $p = 10^{-143}$). When comparing *CG15572* levels between heterozygous progeny of nonobese parents with wild-type controls, however, it is still significantly downregulated (~1.2-fold decreased, $p = 10^{-67}$). The differences in *CG15572* expression among the three heterozygous lineages are therefore likely due to variations in the response to haploinsufficiency. Only one other gene, the predicted galactose-binding *lectin-28C*, is significantly upregulated in the maternal line compared to heterozygous progeny of nonobese parents (~1.4-fold decreased, $p = 10^{-156}$). While the differences between the descendants of obese and nonobese parents are limited, the few we have

identified could be indicative of a larger effect that is buffered by haploinsufficiency at the *AKHR* locus. Indeed, the differences elicited when we directly compare each heterozygous group to the wild-type controls indicate that conditions of nutritional stress might elicit a stronger signature.

Discussion

Heterozygosity at the *AKHR* locus produces an unexpected metabolic phenotype

Previous genetic studies have not addressed the possibility of haploinsufficiency at the *AKHR* locus (Bharucha et al., 2008; Gronke et al., 2007). We show here that not only is there a reproducible 20-30% increase in glycogen levels in *AKHR* heterozygotes as compared to wild-type controls, but that these changes are accompanied by a transcriptional response. Our RNA-seq study identified 143 genes that change their expression from 1.1-2.1-fold when compared to wild-type levels, all with p-values that are less than 10^{-14}. This demonstrates that loss of one copy of *AKHR* leads to reproducible transcriptional and physiological effects. Indeed, the increased glycogen we report is consistent with the well-known role of AKH in mobilizing glycogen stores in response to nutrient deprivation (Bharucha et al., 2008). Our results suggest that the genetic dose of *AKHR* is important for maintaining normal carbohydrate homeostasis in *Drosophila* under *ad libitum* feeding conditions.

The presence of defects due to haploinsufficiency has the added effect of potentially masking our ability to reliably detect metabolic and transcriptional changes in the F1 progeny of obese parents. The changes we would expect to detect from transgenerational metabolic phenotypes are subtle, and therefore likely to be weaker than

any genotypic effects. This is a general concern in any genetic transgenerational paradigm and bears consideration when developing such a system. Future work could account for this genotypic effect by using alternative tools to genetically alter metabolism in the parental generation. For example, RNAi directed against *AKHR* could be used to deplete this receptor in the parental generation. These flies could be crossed to wild-type controls, as we have done in the paradigm used for this study (Figure 4.2). The F1 progeny would have two intact copies of *AKHR*, preventing haploinsufficiency from masking transgenerational phenotypes. An additional benefit is the ability to specifically deplete *AKHR* from particular metabolic tissues or even the reproductive organs and observe the effects of this depletion on progeny phenotypes.

Genetic models induce reproducible changes in parental metabolic state

By using a loss of *AKHR* function to induce obesity, we were able to generate a reliable and severe metabolic defect in the parental generation. Male triglycerides are increased approximately two-fold in *AKHR* mutants as compared to controls. This degree of obesity is difficult to induce using standard dietary methods. Previous studies from our lab using adult dietary conditioning have, at best, resulted in a ~1.4-fold change in triglycerides using a high protein diet (described in Chapter 3). The genetic obesity of the *AKHR* model therefore provides a more severe and consistent alteration in the metabolic state of the parental generation. This consistency is likely what allowed us to observe changes in metabolite levels in wild-type F2 offspring using this genetic conditioning paradigm.

Inheritance of metabolic phenotypes in a lineage specific pattern

One of the most interesting results from our studies is the specific inheritance pattern required to induce a physiological effect in the genotypically wild-type F2 generation. Only F2 progeny descended from obese grandfathers through heterozygous mothers have reduced triglycerides as compared to controls. This specific pattern is reminiscent of genetic rodent models, in which phenotypes are often only seen in one gender or another, or through one parental gender or another (Yazbek et al., 2010). It is unclear why the phenotypes we have observed are only inherited in an alternating parental gender pattern. Identifying the mechanism by which metabolic effects induce the transmission of physiological effects to the next generation might help explain this pattern of inheritance.

Altered F2 physiology fluctuates with environmental variation

It is important to note that this F2 phenotype is subject to the same kind of experimental variation as observed in our dietary conditioning paradigms (Chapter 3). All of the F2 results presented here were derived from three independent biological replicates, conducted over a period of two months (Figure 4.5). As mentioned above, this reproducibility was not seen in any of our dietary studies and likely reflects the effectiveness of the *AKHR* mutant-induced obese state in the parental generation. Despite these successes, however, the phenotypes reported for the F2 generation do not appear to be completely penetrant. When a fourth replicate was performed two to three months later, triglyceride levels were unchanged in the F2 lineages (data not shown).

This is not completely unexpected since environmental conditions likely changed during the intervening period of time. Additionally, previous studies have reported

variable penetrance in the F2 generation, if any phenotype is detected at all (Buescher et al., 2013; Ost et al., 2014; Xia and de Belle, 2016). Even with this lack of penetrance, however, we may be able to detect reproducible transcriptional changes in the F2 progeny, which are genetically wild-type and therefore are free of the genotypic complications of the F1 heterozygotes. This is currently being attempted in our lab. If we are able to detect some subtle changes in F2 transcription, we may be able to determine an appropriate environmental or dietary challenge to which we could subject the F2 progeny and elicit a distinct physiological response. Detailed characterization of this nature could allow the use of this genetic system to identify pathways involved in the physiological responses reported in progeny and the molecular mechanisms behind this mode of inheritance.

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CHAPTER 5

CONCLUSIONS

The transcriptional state of the cell is a major determinant of its metabolic properties; gene expression dynamically governs the metabolic pathways that are active and the responses to dietary changes by defining the proteins and enzymes that are expressed at any given time. The cellular and organismal nutritional environment provides an additional level of control that can alter the activity of transcription factors or chromatin modifiers, both of which contribute to the repression or activation of genes encoding key metabolic enzymes (Giudici et al., 2016; King-Jones and Thummel, 2005; Kirchner et al., 2013). If the activity of these regulators is reduced or disrupted, the cell will no longer be able to adapt to environmental changes. This loss of metabolic flexibility can be a key predecessor of metabolic disease, contributing to both diabetes and obesity (Boutant and Canto, 2014; Kirchner et al., 2013).

In Chapter 2, I presented the progressive hyperglycemia and insulin resistance that can occur upon loss of the metabolic regulator *sir2*, which is due, in part, to its effects on dHNF4 acetylation and stability. In Chapters 3 and 4, I described two different approaches, dietary and genetic, to alter metabolic state in the parental generation in order to examine the heritable effects on progeny metabolism. While I showed that both could induce metabolic defects in progeny for at least two generations, I found that these changes are often not fully penetrant and can be influenced by subtle environmental and

genetic variation. This prevented the identification of molecular mechanisms regulating this transgenerational inheritance of metabolic state. Taken together, my studies address the ways in which the activity of transcription factors and chromatin modifiers can be regulated and, moreover, how their misregulation can contribute to the development of complex metabolic disorders like diabetes and obesity.

Sir2 is required for the maintenance of metabolic homeostasis

It has long been known that the activity of sirtuins has a major impact on metabolism (Blander and Guarente, 2004; Boutant and Canto, 2014; Canto et al., 2015; Chalkiadaki and Guarente, 2012; Chang and Guarente, 2014; Houtkooper et al., 2012; Nogueiras et al., 2012). Loss of *Sirt1* in mice is associated with defects ranging from insulin resistance, to obesity, to inflammatory disease (Chalkiadaki and Guarente, 2012; Kauppinen et al., 2013; Sun et al., 2007). Furthermore, SNPs in human *SIRT1* have been associated with type I diabetes, obesity, and autoimmunity, indicating that its central metabolic functions are conserved through evolution (Biason-Lauber et al., 2013; Clark et al., 2012; van den Berg et al., 2009). The dependence of sirtuin activity on the electron carrier NAD^+ offers some explanation as to how sirtuins are able to respond to changes in the nutritional environment, but does not account for how downstream pathways are modified by this altered activity (Blander and Guarente, 2004; Canto et al., 2015). In Chapter 2, I identified progressive metabolic defects in *Drosophila sir2* mutants that mimic the onset of type II diabetes. Young *sir2* mutants are hyperglycemic but display few other physiological defects (Figures 2.1A, 2.2A,D,F,G). By the time they are two weeks of age, however, *sir2* mutants have more pronounced defects in carbohydrate homeostasis and are obese and insulin resistant (Figures 2.1D,H, 2.2B,E). These defects

worsen with increasing age until the flies eventually develop glucose intolerance, a hallmark of diabetes (Figure 2.1J).

I associated these defects with a loss of *sir2* function specifically in the fat body, a tissue that is known to have functions analogous to liver and adipose tissues in mammals (Figure 2.3). This was not completely unexpected, as previous studies have localized *Drosophila* Sir2 function to the fat body (Banerjee et al., 2013; Reis et al., 2010). This published work, however, focused primarily on lipid metabolism, which my studies suggest is likely to be a secondary effect as obesity arises later than hyperglycemia in *sir2* mutants (Figure 2.1C,F). My use of tissue-specific RNAi coupled with rescue supports an clear role for Sir2 in regulating systemic metabolism through its activity in the fat body (Figure 2.3).

It is interesting that loss of *sir2* specifically in the fat body can have such a strong effect on whole fly insulin sensitivity (Figure 2.3B). Previous work suggests that the fat body is a key tissue for the regulation of insulin signaling (Musselman et al., 2013). Not only is this tissue highly insulin responsive in a tissue autonomous manner, but there is evidence that signals originating from the fat body can alter the metabolism and insulin responsiveness of other tissues as well (Geminard et al., 2009; Musselman et al., 2013; Nassel et al., 2013; Rajan and Perrimon, 2012). It is therefore unclear whether the insulin signaling defects demonstrated when *sir2* is lost in the fat body are due only to insulin signaling defects in the fat body or if they are due to changes in insulin sensitivity in other tissues as well. It is possible that Sir2 activity is important for the production of secreted signals from the fat body that alter the physiology of other, distantly located tissues. Future studies could address this question by isolating specific tissues from flies

lacking *sir2* only in the fat body and determining the insulin sensitivity and other metabolic parameters in these tissues.

Regulation of dHNF4 is reliant on Sir2-dependent deacetylation

The activities of a number of important metabolic transcriptional regulators have been shown to be modified by direct deacetylation by Sir2 and its orthologues (Brunet et al., 2004; Li et al., 2007; Pfluger et al., 2008; Picard et al., 2004; Rodgers et al., 2005; Yang et al., 2009). This is a major mechanism by which sirtuins regulate metabolic state, and appears to allow for the appropriate up or downregulation of specific target genes that encode the proteins and enzymes involved in specific biochemical pathways. I would therefore expect that if the activity of one of these transcription factors was altered by the loss of *sir2*, then I would see a significant enrichment for the targets of that factor in the genes with altered expression in *sir2* mutants. This is what was observed for dHNF4, a nuclear receptor that has been linked with a monogenic form of diabetes in humans and with severe diabetic defects in *Drosophila* (Barry and Thummel, in press) (Navas et al., 1999; Yang et al., 2009). This is consistent with a role for Sir2 in modifying, but not abolishing, the activity of dHNF4; I would expect to see less dramatic diabetic defects in *sir2* mutants as compared to *dHNF4* mutants, which is indeed the case.

I did not, however, expect the remarkable reduction in dHNF4 protein stability observed in the absence of *sir2*. This reduction is measurable even before the onset of severe metabolic dysfunction, as is hyperacetylation of dHNF4 in the absence of *sir2* (Figure 2.4E,F). Although uncommon, acetylation-dependent protein degradation has been previously reported (Asano et al., 2007; Caron et al., 2005; Jeong et al., 2002; Palermo et al., 2012; Sadoul et al., 2008; Shimazu et al., 2006). There is even a case

where Sirt1-dependent deacetylation is required for protein stability (Shimazu et al., 2006). Such degradation is sometimes due to the exposure of lysine residues that are targets for ubiquitination upon acetylation of a different lysine residue. These proteins are then targeted for destruction by the proteasome (Jeong et al., 2002; Palermo et al., 2012). In other cases, the mechanism of protein degradation is unknown (Caron et al., 2005; Sadoul et al., 2008).

Finally, the regulation of dHNF4 stability by Sir2-dependent deacetylation accounts for only a part of the broad range of phenotypes observed in *sir2* mutants. As shown in Figure 2.4, only a subset of the genes misregulated in *dHNF4* mutants are also misregulated in *sir2* mutants. Additionally, previous work has suggested that loss of *Sirt1* in mammals alters the ability of HNF4α to bind the promoter of at least one key target gene (Yang et al., 2009). My studies demonstrate that ectopically increasing dHNF4 levels in *sir2* mutants does not fully restore metabolic homeostasis (Figure 2.4G). I therefore expect that hyperacetylation of dHNF4 not only impacts its stability, but its activity as well. Preliminary studies in the lab suggest that dHNF4 localization is unaffected by the absence of *sir2*, leading me to hypothesize that target recognition may be altered instead (data not shown). This could be due either to changes in binding affinity of dHNF4 to enhancers and promoters or to changes in its binding affinity to transcriptional cofactors. There is evidence for both of these modes of regulation in the literature, and there is the additional possibility that both could be happening simultaneously (Pfluger et al., 2008; Yang et al., 2009). Future work will focus on identifying the mechanisms by which dHNF4 activity is altered in the absence of Sir2, including the mechanisms by which protein stability is disrupted.

Parental metabolic dysfunction impacts progeny physiology

Evidence from a number of different model systems, as well as from humans, suggests that parental metabolism has a profound effect on the metabolic state of their progeny, sometimes for several generations (Kirchner et al., 2013; Somer and Thummel, 2014). In Chapters 3 and 4, I demonstrate the ability of a wide variety of dietary and genetic approaches to model this phenomenon. High levels of dietary protein leads to reduced triglycerides in progeny, as does genetically induced parental obesity (Figures 3.6A, 4.5D). In both paradigms there is evidence of the "mismatch" theory for the transgenerational inheritance of metabolic state (Lillycrop, 2011; Somer and Thummel, 2014). In the dietary paradigm, I see that adult challenge with a high protein diet does not further reduce triglycerides in F1 offspring of parents conditioned on that same diet (Figure 3.6A). This suggests that the metabolic state of these offspring is primed to survive on the high protein diet without losing fat mass, unlike control offspring. In the genetic paradigm, grandpaternal obesity leads to lower triglycerides in F2 offspring of heterozygous mothers. This lean phenotype suggests that the progeny are predisposed to store less fat in response to obesity from two generations previous. The results in these diverse models therefore support the ability of the parental metabolic state to alter progeny physiology in order to better adapt them to the parental environment. Challenge of these offspring with nutritional or environmental stresses may uncover even more ways in which they are adapted to parental conditions.

Environmental and genetic factors influence progeny responses to parental metabolism

Despite the success of the paradigms described in Chapters 3 and 4 to induce metabolic changes in progeny generations, most of my efforts resulted in variable effects on progeny metabolism. This lack of reproducibility has thus far prevented the identification of a molecular mechanism through which these changes are transmitted. Even when the progeny phenotypic changes are relatively robust, they are rarely fully penetrant. It is likely that much of this variability can be accounted for by environmental and genetic variables other than those that were purposefully altered (Ost et al., 2014). I successfully accounted for several of these in the dietary studies described in Chapter 3 by shifting from the use of complex diets with a number of variable components to simple diets that differed in only one component between the control and conditioning media (Table 3.1). Additionally, I generated a number of isogenic *Drosophila* lines from inbred fly stocks to control for basal genetic variation that might affect the results (Figure 3.4). Both of these changes improved the reproducibility of my results, but did not completely alleviate inconsistencies between experimental replicates.

The obesity induced by the loss of *AKHR* is stronger and more consistent than the defects induced in parents using dietary methods (Figures 3.6, 4.1). Even this genetic model, however, failed to induce F2 phenotypes that could be reproduced over an extended period of time. Furthermore, few changes were identified in the transcriptional response of the heterozygous F1 progeny of *AKHR* mutant parents as compared to genotypically matched controls (Figure 4.6, Tables 4.1-4.5).

While disappointing, this is not wholly unexpected. Previous studies have raised

the possibility that environmental and genetic variables can influence the effects of transgenerational inheritance. Fluctuations in intestinal microbiota, temperature, humidity, light cycles, and even small changes in the dietary media can alter the physiological responses of both parents and progeny to dietary and genetic manipulation of the parental metabolic state (Ost et al., 2014). More sensitive methods to monitor physiological and transcriptional changes in progeny could be used to identify the molecular pathways affected by altered metabolism in the parental generation. Similarly, appropriate dietary challenges or environmental stresses may unmask physiological defects in progeny descended from affected parents. Additionally, tissue-specific studies might reveal changes that are restricted to particular organs or systems within the fly. Finally, a better understanding of the mechanism by which these defects are transmitted to the progeny might allow the more specific alteration of progeny responses and identification of the pathways involved in these responses. Efforts along these lines could allow future studies using the models described in Chapters 3 and 4 to identify the conserved pathways and mechanisms responsible for transgenerational inheritance of metabolic phenotypes.

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APPENDIX

FLUID RETENTION IN *SIR2* MUTANT ADULTS

During the course of my studies of *sir2* mutants outlined in Chapter 2, I noticed that these animals develop visible fluid retention that correlates with the development of insulin signaling defects. Here I describe my attempts to study this phenotype and quantify the amount of fluid retained in mutant animals.

Methods

Hemolymph extraction

Hemolymph was collected from control or *sir2* mutant males that were sorted based on the degree of abdomen distention they displayed. Flies were anesthetized on CO_2 and fixed to the surface of a fly pad on their sides using double-sided tape. The attached flies were punctured in the thorax just above the wing joint and gently pressed on the abdomen to force the hemolymph fluid out through the hole in the thorax. This fluid was collected with one microliter capillary tubes. The proportion of the capillary tube filled with hemolymph divided by the total length (1 μL) was calculated to determine the volume collected. Hemolymph was pooled from ten male flies in three separate biological replicates for both the wild-type flies and the phenotypically wild-type *sir2* mutants, such that the calculated hemolymph volume per fly represents the average of thirty flies for each of these groups. Due to volume limits in the capillary

tubes, I collected hemolymph from ten single *sir2* mutant males with visible bloating, such that the calculated hemolymph volume per fly represents the average of ten flies.

Results

Mature males lacking *sir2* function tend to have visibly distended abdomens when compared to genetically and age-matched controls (Figure A.1A). This defect is noticeable at a low frequency in young adults between 5-10 days of age and increases in prevalence with increasing age, correlating with the time frame during which *sir2*

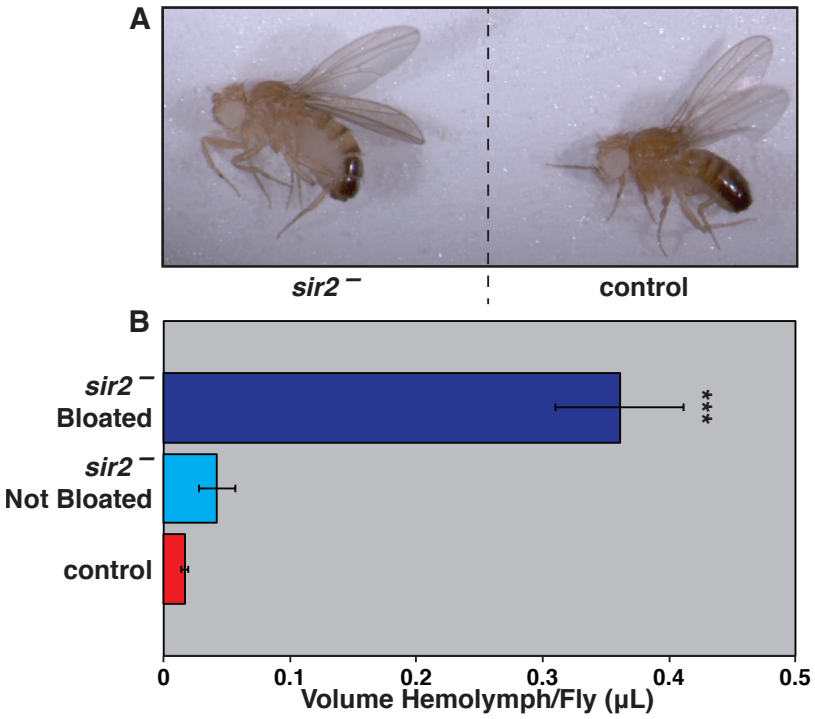

Figure A.1. Loss of *sir2* leads to aberrant accumulation of fluid in the abdomen
(A) Representative male flies are depicted at 15-19 days of age (*sir2* $^{-}$ on the left and control on the right). Mutant flies have a visibly expanded abdomen when compared to controls. **(B)** Fluid was extracted from 16-20 day old control flies and both bloated and unbloated *sir2* mutant flies using a thoracic puncture and quantified using volumetric capillaries. Mutants for *sir2* were sorted based on the presence (blue, n = 10 single flies) or absence (cyan, n = 3x groups of 10 flies) of visible expansion of the abdomen. Even *sir2* mutants that are not visibly bloated retain excess fluid when compared to controls (red, n = 3x groups of 10 flies). Error bars are ±SEM. ***p < 0.0005.

mutants shift from insulin sensitivity to insulin resistance (see Chapter 2). Distention of this magnitude has previously been associated with excess fluid or hemolymph retention (Denholm et al., 2013; Liu et al., 2015). Using capillary tubes, I quantified the amount of hemolymph present in control and *sir2* mutant males between two and three weeks of age (Figure A.1B). The *sir2* mutants were divided into two categories: those with visible abdomen expansion (Figure A.1A), and those with a wild-type appearance. Visibly bloated *sir2* mutants distended abdomens display hemolymph levels that are more than twenty-fold greater than those found in control flies. Unexpectedly, there is also an approximate two to three-fold increase in fluid volume in *sir2* mutant flies that do not display visible signs of fluid retention, although this was not significant. These results suggest that *sir2* mutants may have defects in fluid retention even before it is phenotypically obvious.

Conclusions

Complexity in metabolic diseases, especially diabetes, is not limited to the multiple genetic and environmental factors that influence the development of this disorder. Multiple organ systems are impacted both autonomously and nonautonomously under conditions that lead to the disruption of metabolic homeostasis (CDCP, 2014). Malfunctioning signals or functions in one tissue or set of tissues can impact the signals and functions of other, apparently unrelated tissues. As a result, an important area of research in any diabetic model is to determine how upstream dysfunction influences downstream processes autonomously within the original tissue as well as nonautonomously within other tissues. One example of this kind of downstream dysfunction can be seen in the mammalian kidney. In cases of uncontrolled or untreated

type I and type II diabetes, the kidneys can be damaged due to high levels of circulating glucose and glucose products, development of high blood pressure, and activation of both obesity-dependent and independent inflammatory pathways, (CDCP, 2014; Toth-Manikowski and Atta, 2015). These organs that ordinarily filter waste from circulation are overworked under these circumstances, and as a result eventually shut down.

In *Drosophila*, the Malpighian tubules function as a kidney analog, filtering waste from the circulating hemolymph (Beyenbach et al., 2010; Dow et al., 1994; Ugur et al., 2016). Disruption of Malpighian tubule function can lead to defects in toxin accumulation and tissue failure that are similar to those observed in cases of human kidney failure (Beyenbach et al., 2010; Dow et al., 1994). There are multiple cases where disruption of proper signaling to or within the Malpighian tubules leads to the retention of fluid, occasionally to a visible degree (Denholm et al., 2013; Liu et al., 2015). This type of "bloating" phenotype is made obvious by stretching of the cuticle as the abdominal cavity expands with fluid. This can be confirmed by using standard methods of hemolymph extraction to quantify the amount of fluid retained in the cuticle (Denholm et al., 2013; Liu et al., 2015). My studies demonstrate that the loss of *sir2* leads to this type of fluid retention defect, where aged mutant males have visible abdomen distention accompanied by quantifiable increases in circulating fluid.

Previous studies have demonstrated a role for insulin signaling in the regulation of osmotic homeostasis by altering hormonal signaling from leucokinin (LK) (Liu et al., 2015). When LK secretion from neurons in the abdomen is suppressed, there is evidence of abdominal expansion, fluid retention, and resistance to desiccation. Furthermore, overexpression of the *Drosophila* insulin receptor (*dInR*) in the LK-expressing cells leads

to increased LK secretion, while depletion of *dInR* mediated by RNAi in these cells leads to a reduction in LK secretion (Liu et al., 2015). This indicates that systemic disruption of insulin signaling could alter the secretion of this or other hormones that normally regulate osmotic homeostasis by regulating function in the Malpighian tubule nonautonomously.

In contrast, modulation of the expression of the leucokinin receptor (LKR) within the Malpighian tubules can also disrupt osmotic homeostasis (Denholm et al., 2013). Disruption of the transcription factor *teashirt* (*tsh*), known to be required for the expression of *LKR*, leads to a similar fluid retention phenotype as described above (Denholm et al., 2013). If loss of *sir2* in the Malpighian tubules alters the activity of *tsh* or in some other way inhibits the expression, localization, activity, or downstream signaling of LKR, this could also lead to disruption of osmotic homeostasis by altering Malpighian tubule function autonomously.

Not addressed in the initial studies reported here is the issue of tissue autonomy. It could be that the loss of *sir2* in the Malpighian tubules directly influences their ability to properly regulate fluid balance. The tissue-specific studies of *sir2* reported in Chapter 2, however, did not include a *Gal4*-driver specific to the Malpighian tubules, leaving it unclear how it might function in this tissue. Interestingly, there is some bloating when RNAi against *sir2* is driven specifically in the fat body, suggesting that its function in this tissue contributes to fluid balance. This, however, is only observed in much older flies and has not been quantified, so the significance of this observation remains unclear. As a result, more detailed tissue-specific functional studies of *sir2* are required in order to define its roles in maintaining proper fluid retention in the animal.

In conclusion, there is evidence for multisystemic dysfunction in *sir2* mutants that

contributes to the development of diabetic phenotypes. While the source of this dysfunction has not yet been identified, it is clear that loss of *sir2* has downstream impacts on not just insulin signaling and metabolic homeostasis, but also on osmotic homeostasis. The identification of the cause of this defect will be a fascinating avenue of future study.

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