MOLECULAR
METABOLISM

Parental obesity leads to metabolic changes in the F2 generation in *Drosophila*

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ABSTRACT

Objective: 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*. These studies, however, have all depended on dietary paradigms to alter parental metabolic state, which can have inconsistent heritable effects on the metabolism of offspring.
Methods: Here we use *AKHR* null alleles to induce obesity in the parental generation and then score both metabolic parameters and genome-wide transcriptional responses in *AKHR* heterozygote F1 progeny and genetically wild-type F2 progeny.
Results: Unexpectedly, we observe elevated glycogen levels and changes in gene expression in *AKHR* heterozygotes due to haploinsufficiency at this locus. We also show that genetic manipulation of parental metabolism using *AKHR* mutations results in significant physiological changes in F2 wild-type offspring of the grandpaternal/maternal lineage.
Conclusions: Our results demonstrate that genetic manipulation of parental metabolism in *Drosophila* can have an effect on the health of F2 progeny, providing a non-dietary paradigm to better understand the mechanisms behind the transgenerational inheritance of metabolic state.

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1. INTRODUCTION

Over the past several decades, the incidence of complex metabolic disease has increased at an alarming rate in developed countries. This increase is accompanied by significant social and economic costs, totaling $245 billion for diabetes in the United States in 2012 [1]. Despite this, the known genetic risk factors for diabetes account for only ∼10% of its heritability [2,3]. 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 [4,5]. 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 [6—8]. 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 [9,10]. Furthermore, 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 gestational and developmental effects [11,12].

This response appears to be conserved through evolution. Dietary conditioning of rodents in the parental generation can influence the metabolic state of their progeny. 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 [13—17].

These molecular changes are accompanied by changes in the risk of diabetes, obesity, and cardiovascular disease [15,18—20]. 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 [13,14,21].

Although promising, the time and financial cost of doing these multigenerational experiments in mammalian models have inspired a move to *Drosophila* as an ideal system in which to study the effects of parental metabolic state on offspring metabolism [22,23]. Treatment of either maternal or paternal flies with a high sugar diet leads to changes

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in glycemic levels and a propensity to develop obesity in progeny generations [24,25]. Similarly, altering parental dietary protein impacts triglyceride and glycogen levels as well as longevity and fecundity in progeny, and a parental high fat diet can result in changes in body weight and major metabolites in the F1 and F2 generations [26–28]. These types of dietary manipulations in parents, however, are subject to environmental, sex-specific, and genotypic variation, which can affect the penetrance of progeny phenotypes [24,28].

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 [29–31]. In this paradigm, a mutant is crossed to a wild-type individual to generate F1 heterozygous offspring, which are subsequently crossed to wild-type animals 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 leads to low body weight in wild-type F2 offspring, especially when passed through a grandmaternal/paternal pattern of inheritance [29]. 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 employ a genetic approach to examine the effects of obesity in the parental generation of *Drosophila* using *Adipokinetic Hormone Receptor* (*AKHR*) loss-of-function mutations. Adipokinetic hormone (AKH) is released from neuroendocrine cells of the corpora cardiaca in response to fasting and acts remotely on the fat body to promote the release of stored lipids and carbohydrates for energy production [32]. In this manner, AKH functions analogously to the fasting hormone glucagon in mammals. Consistent with the catabolic activities of its ligand, loss of *AKHR* results in reduced fat body lipid mobilization and elevated triglyceride stores [33,34]. We show that grandpaternal obesity resulting from 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 results 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. This paradigm should allow more reproducible studies of the effects of parental metabolic state on offspring health under controlled environmental and genetic conditions, facilitating the discovery of the molecular mechanisms that underlie this mode of inheritance.

2. MATERIALS AND METHODS

2.1. *Drosophila* stocks and maintenance

Drosophila melanogaster were maintained at 25 °C in an incubator with a 12 h 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% $MgSO_4 \cdot x6H_2O$, 0.05% $CaCl_2 \cdot x2H_2O$, 0.1% p-hydroxybenzoic acid methyl ester, and 0.6% propionic acid [35]. This media was prepared in the lab in large batches and stored for use over multiple generations, providing a stable nutritional environment. 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) were used to generate *AKHR* null mutants for the parental generation [33,36]. These alleles had been previously outcrossed to

the *Canton S* wild-type line that was used as a control throughout this study in order to provide a uniform genetic background. The first, third, and Y chromosomes of the mutant stocks were completely exchanged for the *Canton S* chromosomes, and the second chromosome carrying the mutations was allowed to undergo free recombination for at least three generations. *AKHR* mutants were crossed to the same *Canton S* strain to generate the F1 and F2 generations. To generate the F1 and F2 offspring, males and female virgins were crossed and maintained on egg caps, which were replaced every 24 h. 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. Most studies were performed only in males, except as noted, because the metabolism of adult *Drosophila* females is heavily dedicated to oogenesis and reproduction.

2.2. Metabolite assays

Five adult male flies were collected at the indicated ages and washed in $1 \times$ PBS. Each sample was homogenized in 100 μL $1 \times$ PBS, 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 [37]. All experiments were replicated at least three times and were consistent across all replicates unless otherwise indicated.

2.3. RNA-seq transcriptional profiling

RNA was isolated from 10 to 11 day-old F1 or F2 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. Data from F2 sequencing was further analyzed using independent filtering approaches to account for batch effects. We restricted our analysis to identify transcripts that display an approximate 10% change in expression level by using a cut-off for significance of Log2 ratio ± 0.13 and p-value < 0.05. Gene Ontology analysis was performed using DAVID [38,39]. RNA-seq data from this study can be accessed at NCBI GEO (accession numbers GSE97201 and GSE97525).

2.4. Statistical analysis

GraphPad PRISM 6 software was used to plot metabolite data and to perform statistical analysis. Pairwise 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.

3. RESULTS

3.1. Loss of *AKHR* function leads to parental obesity

We selected two loss-of-function *AKHR* alleles to induce metabolic dysfunction in the parental generation. One of these, *AKHR*[1], is a deletion mutation generated by imprecise excision of a P-element [33]. 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 [36]. 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 reporter gene encoding an eye-specific fluorescent DsRed protein, facilitating the detection of this allele [36].

We used a wild-type inbred *Canton S* line for our studies along with *AKHR^1* and *AKHR^DsRed* mutants that had been outcrossed into this *Canton S* genetic background. Metabolite measurements in transheterozygotes carrying the *AKHR^1* and *AKHR^DsRed* alleles were consistent with the results of earlier genetic studies of *AKHR* function [33,34]. Both males and females lacking *AKHR* have elevated triglycerides (Figure 1A and B), which serve as a marker for obesity in *Drosophila*, with no apparent changes in either glucose or glycogen levels (Figure 1C–F) [33,40]. Protein levels are unchanged in males and slightly reduced in females (Figure 1G and H). The disruption in female protein, however, varied among experimental replicates (p < 0.05), while this was not the case for obesity in either males or females. We conclude that *AKHR* mutants have severe, reproducible defects in triglyceride mobilization.

3.2. Genetic crosses to generate wild-type F2 offspring from *AKHR* mutant parents

The dominant eye-specific DsRed marker in our *AKHR^DsRed* mutant allowed us to follow this allele and observe the effect of parental obesity on future generations (Figure 2). Transheteroallelic *AKHR^1/AKHR^DsRed* mutant parental 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. F1 progeny were selected that carry the *AKHR^DsRed* allele, and males and females of this genotype were crossed to wild-type females and males to generate wild-type F2 offspring (Figure 2). This crossing scheme allowed us to examine physiological phenotypes in wild-type progeny descended from obese parents when compared to wild-type progeny from parents with no obesity in their genealogical history. As controls, we also set up crosses between male or female *AKHR−/+* heterozygotes and wild-type females or males to generate heterozygous *AKHR−/+* F1 offspring from non-obese parents. These offspring were derived from heterozygous parents for at least two generations such that there was no expected metabolic dysfunction in either their parents or grandparents. In addition, heterozygotes were selected from crosses in which either the mother or father carried the *AKHR* mutant allele (Figure 2).

3.3. *AKHR* heterozygotes display elevated levels of glycogen

We first examined metabolite levels in the F1 generation of progeny from all five genetic crosses (Figure 2) to determine if any physiological phenotypes are dependent on the parental metabolic state. Interestingly, glycogen levels are significantly increased in all F1 *AKHR* heterozygotes descended from either obese or non-obese parents (Figure 3D, Supplementary Figure 1). Triglyceride levels are not changed between wild-type and either heterozygous controls or heterozygotes descended from an obese parent (Figure 3A). The same is true for glucose (Figure 3B) and protein (Figure 3C) levels. Our studies thus demonstrate that heterozygosity at the *AKHR* locus results in a reproducible effect on stored glycogen levels, something that has not been reported in past work [33,34].
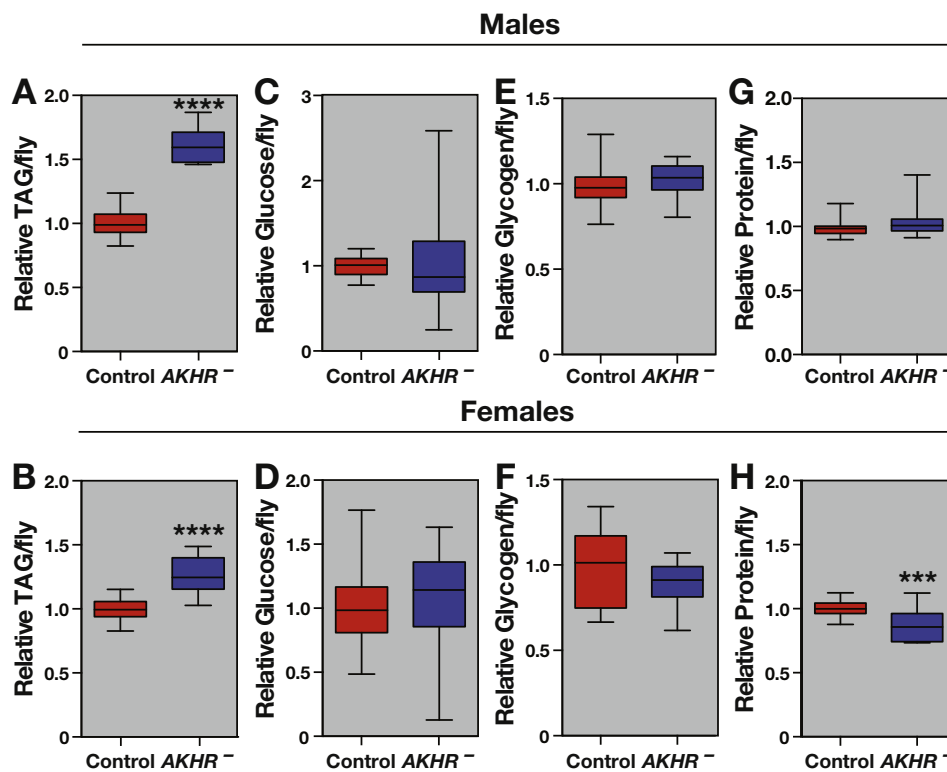

Figure 1: AKHR mutants are obese compared to genetically matched controls. Metabolites were measured from *AKHR^DsRed/AKHR^1* mutant males and females (*AKHR−*) and genetically matched controls (n = 16). Triglycerides in males (A) and females (B) are substantially elevated relative to controls, while glycogen (C, D) and glucose levels (E, F) are unchanged. Protein is unchanged in males (G) but slightly reduced in females (H). 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.

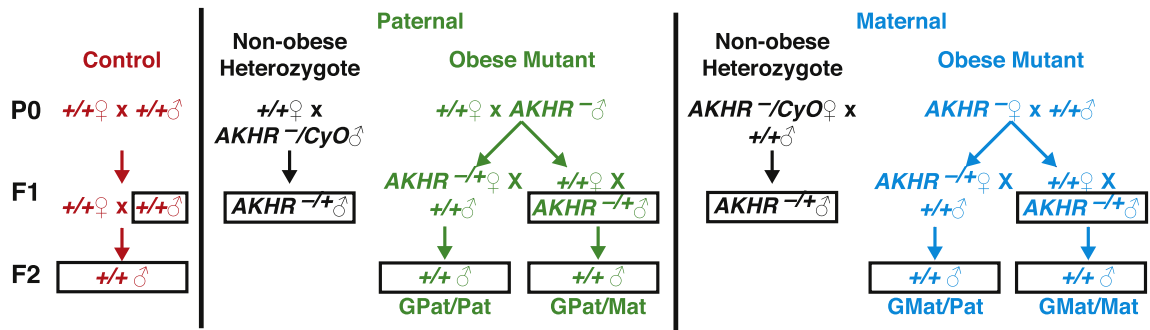**Figure 2: Crossing scheme to generate F1 and F2 progeny from obese individuals.** *AKHR^DsRed^/AKHR^1^* mutant males or females (*AKHR−*) were crossed to wild-type females or males to generate heterozygous *AKHR^DsRed^/+* F1 male and female progeny (*AKHR−/+*). These were subsequently crossed to wild-type 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−/+*). Wild-type control crosses (+/+) were maintained in both the F1 and F2 generations. Descendants from the wild-type control line are colored in red, from the heterozygote control lines in black, from the paternal obese line in green, and from the maternal obese line in cyan. Metabolites were measured in both F1 and F2 males (black boxes).
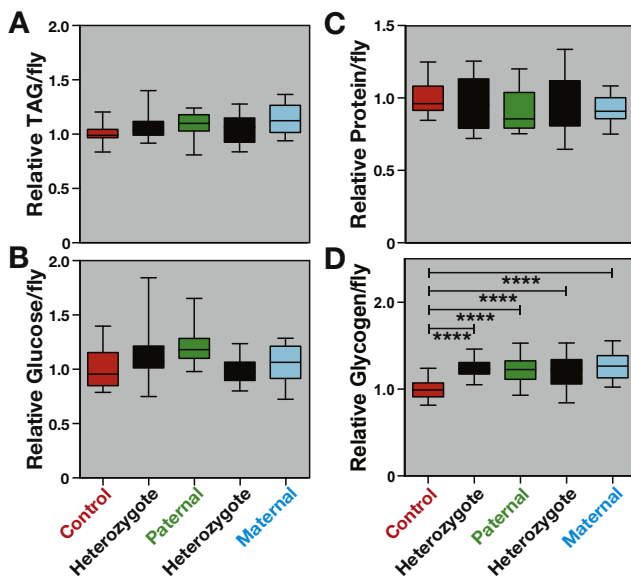

Figure 3: *AKHR* heterozygotes have elevated glycogen levels. Metabolites were measured in F1 progeny from parents of the following genotypes: wild-type (red), *AKHR−/+* heterozygotes (black), *AKHR* mutant fathers (green), and *AKHR* mutant mothers (cyan). Results from F1 progeny (n = 20/each) were 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 when compared to heterozygote controls or when compared to wild-type controls. Glycogen levels (D) are elevated in all heterozygous progeny when 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. Brackets are present to indicate the two samples being compared for the p-value presented. Only comparisons resulting in a significant difference that is consistent between the different experimental lines and controls are indicated. ****$p < 0.0001$.

3.4. Transcriptional profiling of F1 progeny

The lack of an effect 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 read-out of epigenetic state, we hypothesized that changes in mRNA levels might provide a better assessment of phenotypes in F1 offspring. We performed RNA-seq analysis on three biological replicates of F1 progeny

from wild-type controls, *AKHR−/+* heterozygous controls descended from non-obese 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. RNA was extracted from 60 adult male flies for each replicate at approximately 10−11 days of age. Because we expected that the transcriptional differences caused by parental obesity might be subtle, transcripts were selected that changed more than 9.5% in expression level, with a significance of $p < 0.05$.

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 (Supplementary Table 1; Figure 4). There are 143 mis-regulated genes (94 down and 49 up) in heterozygotes descended from non-obese parents compared to wild-type controls. Gene Ontology (GO) analysis of these gene sets revealed that the down-regulated genes affect reproduction, while the up-regulated genes act in carbohydrate metabolic pathways, consistent with the increased stored carbohydrates in *AKHR−/+* heterozygotes (Supplementary Tables 2 and 3).

There are 38 genes that are uniquely down-regulated in *AKHR−/+* heterozygotes derived from obese fathers compared to wild-type controls (Figure 4A, Supplementary Tables 4 and 5). These include genes involved in sperm function and mating behavior, including seminal fluid proteins. These could indicate differences in the fecundity and germline function in F1 descendants of obese fathers, which may contribute to the lineage-specific F2 phenotypes described below. One gene is uniquely down-regulated in the maternal lineage: *CR43494*. This presumptive non-coding RNA, however, has no known function [41]. Five genes are expressed at reduced levels in both paternal and maternal obese lineages (Figure 4A, Supplementary Table 4). Interestingly, these genes include three gut-specific predicted serine proteases, suggesting a possible effect on dietary nutrient uptake (Supplementary Table 4).

There are nine genes that are uniquely up-regulated in *AKHR−/+* heterozygotes descended from obese fathers compared to wild-type controls (Figure 4B, Supplementary Tables 4 and 5). These genes include *Larval serum protein 1α* (*Lsp1α*), which is thought to provide a source of amino acids and energy during metamorphosis, as well as *MtnB*, which is involved in metal ion homeostasis. Two genes are uniquely up-regulated in the maternal lineage: *lectin-28C* and *CG5966*
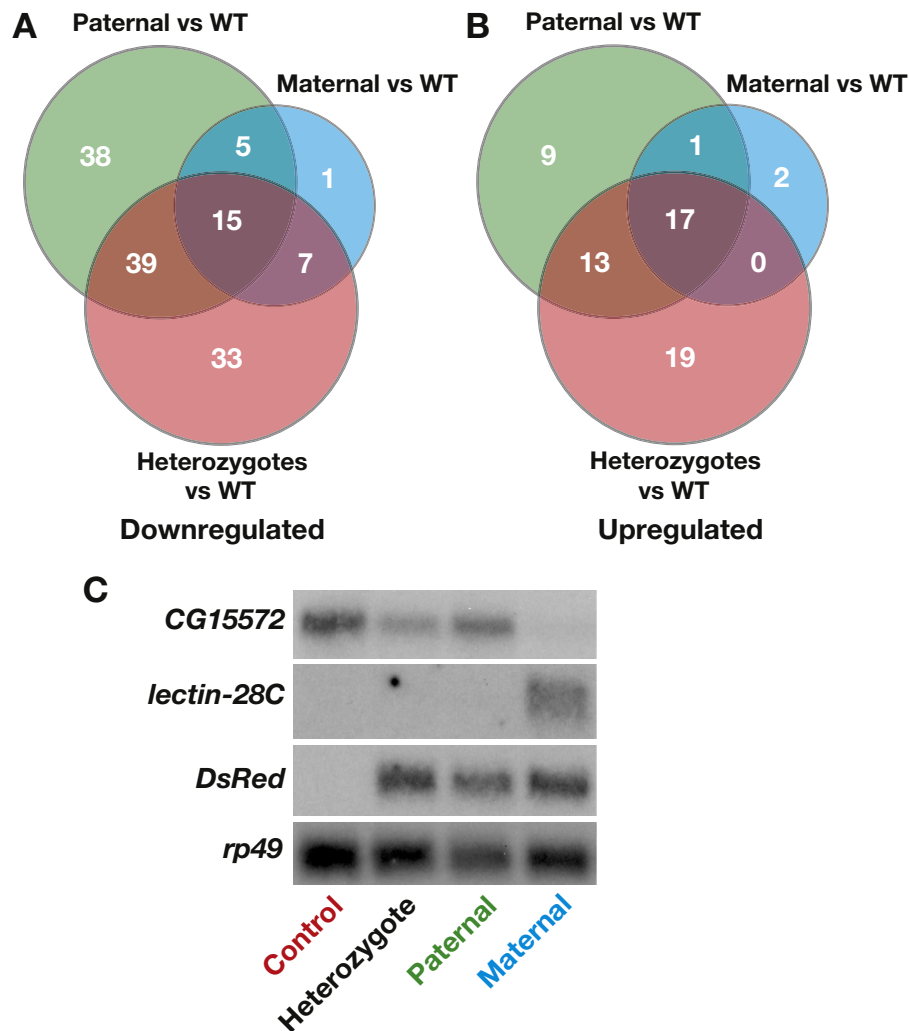
Figure 4: Transcriptional profiling of F1 offspring. Venn diagrams are presented that depict overlaps in genes that are either down-regulated (A) or up-regulated (B) in *AKHR−/+* heterozygotes and F1 flies descended from obese paternal or maternal parents. (C) RNA was isolated from F1 controls, *AKHR−/+* heterozygotes, or F1 flies descended from obese paternal or maternal parents and analyzed for *CG15572* or *lectin-28C* expression by northern blot hybridization, as described [48]. Probes to detect *DsRed* and *rp49* mRNA were used as controls. The changes in *CG15572* and *lectin-28C* expression parallel those seen by RNA-seq.

(Figure 4B, Supplementary Tables 4 and 6). Interestingly, *lectin-28C* encodes a predicted galactose-binding protein and *CG5966* encodes a presumptive triglyceride lipase that is highly expressed in the fat body [42,43]. The mis-regulation of these genes could be indicative of metabolic changes in the F1 that are buffered by compensating pathways. Finally, only one up-regulated gene, *Gpdh*, is shared between the paternal and maternal obese lineages (Figure 4B, Supplementary Table 4). This gene encodes glycerol-3-phosphate dehydrogenase, which plays an important role in lipid biosynthesis as well as in coupling NADH production during glycolysis with mitochondrial oxidative phosphorylation through the glycerol-phosphate shuttle. The mis-regulation of this gene could thus contribute to the F2 metabolic defects described below.

Several transcripts were selected for validation by northern blot hybridization based on their statistically significant differences in expression between the heterozygous controls and the maternal or paternal F1 lineages. The testes specific gene *CG15572* is up-regulated in the paternal lineage when compared to heterozygous progeny of non-obese parents ($\sim$1.2-fold increased, p $= 10^{-73}$),

while it is down-regulated in the maternal lineage ($\sim$1.4-fold decreased, p $= 10^{-143}$). When comparing *CG15572* levels between heterozygous progeny of non-obese parents with wild-type controls, however, it is still significantly down-regulated ($\sim$1.2-fold decreased, p $= 10^{-67}$). This pattern of expression is confirmed by northern blot analysis (Figure 4C). Only one other gene, the predicted galactose-binding *lectin-28C*, is significantly up-regulated in the maternal line compared to heterozygous progeny of non-obese parents ($\sim$1.4-fold increased, p $= 10^{-156}$). This was confirmed by northern blot analysis (Figure 4C). While the transcriptional differences between the heterozygous progeny of obese and non-obese parents are limited, the few we have identified could be indicative of broader defects that cannot be detected above the more dramatic effects of heterozygosity at the *AKHR* locus.

3.5. Grandpaternal/maternal inheritance of metabolic dysfunction in the F2 generation

We measured stored metabolites in wild-type F2 offspring in order to determine if parental obesity might lead to reproducible changes in

their physiology (Figure 5). Glycogen, glucose, and protein levels are unchanged between controls and all F2 progeny descended from an obese parent (Figure 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 5D). This reduction is significant whether the comparison is made between flies of grandpaternal/maternal lineage and controls (p < 0.01) or the grandpaternal/paternal lineage (p < 0.01). Furthermore, there is no significant variation between experimental replicates in triglyceride levels between flies of the grandpaternal/maternal lineage and controls (Supplementary Figure 2).

3.6. Transcriptional profiling of F2 progeny

As described above for the F1 offspring, the effects of parental obesity might have a greater impact on the transcriptome of wild-type F2 offspring than it does on stored metabolite levels. We therefore performed RNA-seq analysis on F2 offspring that are genetically identical to the wild-type controls at the *AKHR* locus. Because we observed changes in triglycerides only in F2 offspring descended through a grandpaternal/maternal lineage, we only analyzed samples with a history of grandpaternal obesity. RNA was again extracted from three biological replicates of 60 adult male flies at approximately 10−11 days of age. Analysis was performed comparing wild-type controls, F2 offspring of the grandpaternal/paternal lineage, and F2 offspring of the grandpaternal/maternal lineage. Because changes might be even subtler in the F2 generation than in the F1 generation, the batches in
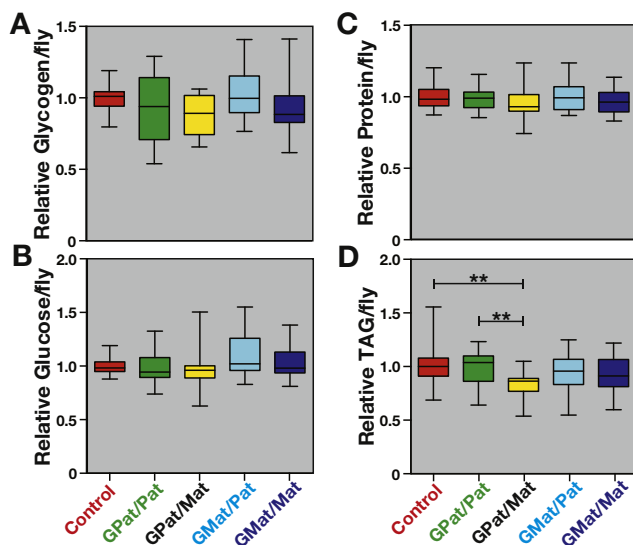
Table 1 — Gene altered in expression in grandpaternal/maternal F2 progeny

Flybase gene ID	Associated gene name	LgR2rt	Fold change	P-value	Function
Grandpaternal−maternal vs control					
FBgn0044812	*totC*	0.251	1.19	1.51E-06	Stress response
FBgn0028396	*totA*	0.232	1.17	1.95E-06	Stress response
FBgn0033246	*ACC*	0.180	1.13	8.02E-03	Fatty acid biosynthesis
FBgn0001168	*h*	0.181	1.13	8.53E-03	Hairy (Hes family of suppressors)
Grandpaternal−maternal vs grandpaternal−paternal					
FBgn0037724	*fst*	0.262	1.20	1.68E-06	Cold/Stress response

which samples were collected were taken into account for the analysis. Once again, we expected that the transcriptional changes might be small so we selected transcripts that changed more than 9.5% with a significance of p < 0.05.

We found significantly altered transcript levels at five loci in the grandpaternal/maternal lineage compared with either the wild-type controls or with matched F2 offspring descended from the grandpaternal/paternal lineage (Table 1). This is consistent with the changes in triglyceride levels detected only in F2 progeny descended from a grandpaternal/maternal lineage. The genes *ACC* (~1.13-fold increased, p = 0.008), *hairy* (~1.13-fold increased, p = 0.009), *totA* (~1.17-fold increased, p = 10^{-6}), and *totC* (~1.19-fold increased, p = 10^{-6}) are all reproducibly and significantly elevated in F2 descendants of the grandpaternal/maternal lineage when compared to controls, while the gene *fst* (~1.2-fold increased, p = 10^{-6}) is significantly elevated in F2 descendants of the grandpaternal/maternal lineage when compared to those of the grandpaternal/paternal lineage. No significant changes were observed when the transcriptomes of the grandpaternal/paternal and control lineages were compared.

4. DISCUSSION

4.1. Genetic models induce reproducible changes in parental metabolic state

By using *AKHR* mutants to induce obesity, we were able to generate a reliable and severe metabolic defect in the parental generation. Male triglycerides are significantly increased in *AKHR* mutants when compared to controls. This degree of obesity is difficult to induce using standard dietary methods. The *AKHR* model therefore provides a more severe and consistent alteration in the metabolic state of the parental generation under constant nutritional conditions. This consistency likely contributed to the significant changes in metabolite levels seen in wild-type F2 offspring.

4.2. Heterozygosity at the *AKHR* locus produces unexpected metabolic phenotypes

Previous genetic studies of *AKHR* have assumed that the existing loss-of-function mutations behave as classic recessive alleles and thus should not display haploinsufficient phenotypes [33,34]. Here we show that not only is there a reproducible 20−30% increase in glycogen levels in flies lacking one copy of the *AKHR* locus but also that these changes are accompanied by a transcriptional response. Our RNA-seq study identified 143 genes that change their expression when compared to wild-type levels, all with p-values that are less than 10^{-14}. This demonstrates that the loss of one copy of *AKHR* leads to significant transcriptional and physiological effects. Moreover, the

Figure 5: F2 progeny descended from obese paternal grandparents and heterozygous mothers are lean. Metabolite levels were measured in wild-type controls (red) and F2 descendants of obese parents of the following lineages: grandpaternal/paternal (GPat/Pat, green), grandpaternal/maternal (GPat/Mat, yellow), grandmaternal/paternal (GMat/Pat, cyan), and grandmaternal/maternal (GMat/Mat, blue). Whole body extracts were prepared at approximately 10 days of adult age and represent combined data from 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 when compared to controls (D). There was no effect of experimental variation across replicates pooled in this dataset when comparing control and grandpaternal/maternal samples. Comparisons between inheritance lines were performed using one-way ANOVA and Sidak's multiple comparisons test. Batch effects were determined using two-way ANOVA. Brackets are present to indicate the two samples being compared for the p-value presented. Only comparisons resulting in a significant difference are indicated. **p < 0.01.

increased glycogen we report is consistent with the well-known role of AKH in mobilizing glycogen stores in response to nutrient deprivation [34]. Our results suggest that the genetic dose of *AKHR* is important for maintaining carbohydrate homeostasis in *Drosophila* under normal feeding conditions.

The presence of defects due to haploinsufficiency may also impact our ability to reliably detect metabolic and transcriptional changes in the F1 progeny of obese parents. The changes we would expect to see from inherited metabolic phenotypes are subtle and therefore likely to be weaker than genotypic effects caused by haploinsufficiency. This is a general concern in any genetic transgenerational paradigm and bears consideration when developing such a system. In addition, heterozygosity in the F1 generation could potentially complicate our interpretation of the F2 phenotypes. It is possible that the elevated glycogen associated with *AKHR* heterozygosity in the F1 generation could contribute to the phenotypes we report in F2 offspring. Future studies will be necessary to clarify the effects of altered metabolism in F1 *AKHR*−/+ animals on the F2 generation. These efforts could also include the use of alternative approaches to genetically alter metabolism in the parental generation using mutations that have no demonstrable haploinsufficient phenotype.

4.3. Inheritance of metabolic phenotypes in a lineage specific pattern

Earlier studies that used a dietary paradigm to induce parental obesity found that this led to significantly elevated glycogen and triglyceride levels in F1 offspring [24,25]. Although we did not observe any metabolic defects in the F1 generation using our genetic paradigm, this could be due to the effects of *AKHR* heterozygosity, as described above, or a fundamental difference in the nature of parental obesity induced by either a dietary or genetic approach. However, by carrying our study out to the F2 generation, we made the interesting observation that a specific inheritance pattern is required to induce a physiological effect in the genotypically wild-type F2 offspring. Only F2 progeny descended from obese grandfathers through heterozygous mothers have reduced triglycerides when compared to controls. This grandpaternal/maternal pattern of inheritance is reminiscent of genetic models in rodents and humans, in which phenotypes often are seen only in one sex in the offspring or through a specific lineage [11,12,29]. It is unclear why the phenotypes we have observed are only inherited in an alternating parental gender pattern. Future studies including both male and female progeny could clarify these inheritance patterns. Furthermore, identifying the mechanism by which metabolic effects induce the transmission of physiological effects to the next generation might help explain this pattern of inheritance.

As a first step toward addressing the mechanisms that underlie transgenerational inheritance, we performed RNA-seq transcriptional profiling on both F1 and F2 offspring. Most notably, we observed only a handful of genes that displayed significant changes in gene expression in F2 descendants of the grandpaternal/maternal lineage. Remarkably, these genes include two of the *Tot* genes, *totA* and *totC*, out of a family of only seven such genes. Although not directly involved in metabolism, these genes, along with *fst*, contribute to stress responses and may indicate a compensatory response to the physiological state of F2 offspring [44,45]. Interestingly, we also identified a significant change in the expression of *ACC*, which encodes a conserved acetyl-CoA carboxylase that acts as the rate-limiting step in fatty acid synthesis [46]. Although the low triglyceride levels observed are the opposite of what would be expected from elevated ACC activity, it is possible that this transcriptional change is a compensatory response to the reduction in stored lipid levels. The *hairy* (*h*) locus, on the other hand,

encodes a well-characterized transcriptional repressor that is required for embryonic development and has recently been associated with the proper expression of key genes in the glycolytic pathway and tricarboxylic acid cycle [47]. Taken together, these changes in gene expression focus attention on stress responses and specific metabolic pathways, providing directions for future studies that could address how these transcriptional changes might contribute to the metabolic defects observed in F2 offspring.

4.4. Altered F2 physiology is sensitive to environmental effects

It is important to note that the reduced triglycerides observed in F2 offspring from obese grandfathers and heterozygous mothers is subject to the same kind of experimental variation as observed in dietary conditioning paradigms. The metabolic assays for the F2 presented here were derived from three independent biological replicates conducted over a period of two months. However, when a fourth replicate was performed several months later, triglyceride levels were unchanged in the F2 lineages. We were also unable to detect transcriptional changes seen in our RNA-seq by northern blot hybridization studies using RNA samples isolated from animals several months later. This variability is not unexpected because the effects reported here in F2 offspring are relatively small and the environmental conditions likely changed during the intervening period of time. Additionally, we and others have noted that transgenerational metabolic studies using dietary paradigms often result in variable offspring phenotypes [24,28]. The success of this study likely reflects the effectiveness of the obese phenotype of *AKHR* parents in the absence of the subtle variations inherent in the use of dietary paradigms to alter parental metabolism. Many other genes that play central roles in metabolism have mutant alleles that carry a dominant marker. For example, a *dsRed*-marked presumptive null allele for *bmm*, which encodes the rate-limiting enzyme in triglyceride mobilization, is available from the Bloomington stock center [40]. Future experiments using this or other mutant alleles hold the promise of providing robust changes in parental metabolism that can facilitate more detailed functional analysis of the mechanisms underlying the transgenerational inheritance of metabolic state.

CONFLICTS OF INTEREST

There are no known conflicts of interest associated with the research outlined in this publication.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.03.012.

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Original Article

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