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Changes in gene expression reveal pathways to galactose sensitivity in GALT-null *Drosophila melanogaster*

By

Darwin Benjamin Hang
Master of Science

Graduate Division of Biological and Biomedical Sciences

Genetics and Molecular Biology

_____ [Advisor's signature]

Judith Fridovich-Keil, PhD.
Advisor

_____ [Member's signature]
Victor Corces, PhD.
Committee Member

_____ [Member's signature]
Dean Jones, PhD.
Committee Member

_____ [Member's signature]
William Kelly, PhD.
Committee Member

_____ [Member's signature]
Kenneth Moberg, PhD.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Changes in gene expression reveal pathways to galactose sensitivity in GALT-null *Drosophila melanogaster*

By

Darwin B. Hang

Bachelor of Science

Advisor: Judith Fridovich-Keil, Ph.D.

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Abstract

Changes in gene expression reveal pathways to galactose sensitivity in GALT-null *Drosophila melanogaster*

By Darwin Hang

Classic galactosemia is an autosomal recessive metabolic disorder with short and long term complications as a result of severe reduction in galactose-1-phosphate uridylyl transferase (GALT) activity. GALT is the second enzyme in the Leloir pathway of galactose metabolism. The mechanisms of both the short and long term outcomes are unknown. Our lab generated the first GALT-null animal model that displays acute and long term outcomes reminiscent of the human disease.

We chose to perform a gene expression based microarray experiment as an open minded approach to understand the changes that occur when GALT-null and WT larvae are exposed to galactose. GALT-null and WT L1 larvae were placed on galactose free food and then switched to either galactose free or galactose containing food. Analysis was done using Gene Ontology (GO). When both genotypes were exposed to galactose, there was enrichment for GO terms Glutathione S-Transferase (GST) and oxidation reduction processes. The number of genes involved in these processes was greater in the GALT-null larvae compared to the WT larvae, indicating that there may be differences in oxidative stress level or response in the GALT-null compared to the WT larvae after 12 hours of galactose exposure. Comparison of the gene expression profiles of the GALT-null and WT larvae raised on glucose showed increases in the amount of differentially expressed genes involved in mitochondrial, neurological, and metabolic processes.

The microarray data revealed that two GST genes showed a large increase in expression when both the GALT-null and WT larva were exposed to galactose. *GSTD6* and *GSTE7* have been shown to increase their expression as part of the *Drosophila* response to oxidative stress. Real Time PCR for these genes validated the microarray results. Both genes showed elevated expression when larvae were exposed to galactose, indicating that dietary galactose exposure induced oxidative stress. *GSTD6* in particular showed a greater increase in expression level when the GALT-null larvae were exposed to galactose than when the WT larvae were exposed to galactose. In contrast, the reduction of galactokinase (GALK, the first enzyme in the Leloir pathway) activity had no significant effect on the expression of these genes.

Changes in gene expression reveal pathways to galactose sensitivity in GALT-null *Drosophila melanogaster*

By: Darwin B. Hang

University of Minnesota, B.S. 2007

Advisor:

Judith Fridovich-Keil, Ph.D.

A Thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of

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Chapter 1

Introduction

Galactose is a monosaccharide released from the digestion of milk, and to a lesser extent, some fruits, and vegetables [1,2]. The largest source of galactose consumed in the human diet comes from the breakdown of milk lactose, a disaccharide of glucose and galactose. In humans, galactose is primarily metabolized through the Leloir pathway, which consists of three enzymes that are conserved from *E. coli* to humans[3]. The three enzymes that make up the Leloir pathway are Galactokinase (GALK), Galactose-1-phosphate uridylyl transferase (GALT), and UDP-galactose-4'-epimerase (GALE). Galactose metabolism mostly occurs in the liver. The first step in the Leloir pathway is the phosphorylation of the first carbon of galactose by GALK. Then, GALT transfers a uridyl group from UDP-glucose (UDP-glc) to galactose-1-phosphate (gal-1-p) through a ping-pong mechanism to produce UDP-galactose (UDP-gal) and glucose-1-phosphate (glc-1-p). The final step in the pathway is the isomerization of UDP-gal to UDP-glc by GALE.

Classic galactosemia (GG) is an autosomal recessive metabolic disorder that affects more than 1:60,000 newborns in the US and may result in death if left untreated[4].The short term symptoms begin shortly after the neonate is exposed to galactose and include hepatomegaly, vomiting, diarrhea, and failure to thrive[4,5]. Treatment involves the replacement of traditional formula or breast milk with a low galactose soy based formula and results in a reversal of the short term symptoms symptoms[4]. The introduction of the Beutler assay to the newborn screening panel has prevented the deaths of GG patients in the United States and many other countries, though regardless of galactose exposure, long term complications such as ataxia, seizures, speech impairment, cognitive impairment, and premature or primary ovarian insufficiency (POI) may occur[4,5]. The cause of GG is the impairment of GALT activity; however the mechanisms of the short and long term complications remain unknown[6,7].

In 1996, the Leslie lab created a GALT knock-out mouse[8]. This mouse showed a buildup of gal-1-p, which was expected without GALT activity, but was unable to replicate any

of the acute or long term complications seen in patients[8]. This resulted in the absence of a multicellular model of classic galactosemia for the next 14 years.

In 2010, our lab created a GALT-null fly line through the isolation of an imprecise P-element excision of the *GALT* gene[9]. The GALT-null flies die during development when exposed to galactose, and have movement phenotypes independent of galactose exposure[9,10]. This model can and has been used to provide potential insights into the mechanisms of both the acute a long term outcomes in classic galactosemia.

Drosophila melanogaster is a model genetic system with a genome that has been sequenced for over a decade[11,12]. The continued annotation of the *Drosophila* genome has facilitated the development of chips that contain the whole known transcriptome. These gene expression microarrays provide a powerful tool to address questions of mechanism when there is no clear direction, such as the case of our GALT-null *Drosophila* model of classic galactosemia. With one experiment, we can gain insights into both the acute and long term outcomes.

The purpose of this research was to approach the mechanism of the acute outcome by comparing the transcriptomes of GALT-null and wild-type (WT) larvae each raised on a galactose containing diet and also GALT-null and WT larvae raised on a galactose free diet. The WT larvae raised on a galactose containing diet were compared to the WT larvae raised on a galactose free diet to understand how animals with GALT activity handle the added stress of exogenous galactose exposure. The results from those comparisons showed the difference in the gene expression profiles between larvae that survive to adulthood and larvae that do not survive. The differences in transcription based on genotype were addressed by comparing the gene expression profile of GALT-null larvae raised on a galactose free diet with WT larvae raised on a galactose free diet. The results from this comparison showed expression patterns that may explain aspects of both the acute and the long term outcomes. Both larvae will survive to adulthood, but

the GALT-null larvae perform worse on the countercurrent and simple climbing assays[9,10]. Understanding the results from these comparisons will highlight pathways and processes that require a functional *GALT* gene in absence of galactose.

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Chapter 2

Gene expression analysis of GALT-null and WT larvae on glucose and galactose containing diets provides insight into pathways of the acute and long term outcomes in GALT-null *Drosophila melanogaster*

The mechanisms of both the acute and long term outcomes in our GALT-null flies are unknown. To identify possible pathways and processes that are involved in the outcome of the GALT-null flies, a gene expression analysis using the microarray platform was performed on developing GALT-null and WT larvae. The microarray platform provides an unbiased method for discovering patterns in gene expression, and has yielded powerful insights into the immune response[1], starvation response[2], and the response to stress[3]. The microarray experiment was designed to gain information on patterns of gene expression for the whole larvae when exposed to galactose and when not exposed to galactose. This provided information on possible processes and pathways that are involved in the acute (GALT-null compared to WT on galactose free food) and long term outcomes (GALT-null compared to WT on food containing galactose) seen in our GALT-null flies.

Materials and Methods

Collection of Larvae. GALT-null and WT flies were allowed to lay eggs on grape juice plates for 4 hours. 24 hours after the egg laying, newly hatched larvae (L1) were transferred from the egg laying plates to plates with food containing 500 mM glucose or 500 mM glucose + 225 mM galactose. After 12 hours on the food, the larvae were floated in 20% glucose, washed, and placed on fresh plates of food containing 555 mM glucose or 555 mM glucose + 225 mM galactose. The conditions for each genotype were 12 hours on glucose then 12 hours on glucose (glc), and 12 hours on glucose then 12 hours on galactose (switch). The switching of food every 12 hours for each condition even if the food composition was not changed to control for the changes in gene expression that may occur from the stress of being floated in sugar water. The conditions were all done in duplicate. The larvae were collected by floatation in 20% glucose and then frozen at -20 degrees C.

Extraction of RNA and generation of cDNA. The RNA from each genotype/condition combination was extracted using the RNeasy kit (Qiagen), performing the optional on-column DNase step to remove genomic DNA. The RNA was quantified by nanodrop spectrophotometry. cDNA was generated by using the High Capacity Reverse Transcription kit (Applied Biosystems). 10 ug of RNA was added to the components of the kit and random hexamers were used to generate cDNA. The cDNA was cleaned up using the Qiaquick PCR Cleanup kit (Qiagen) and quantified using the nanodrop spetrophotomer.

Expression Microarray and initial analysis. The Nimblegen (Roche) *D. melanogaster* 12 x 135K expression microarray chip was used for this experiment. This was done at the Florida State University Nimblegen microarray core. Robust Multichip Analysis normalization was also done at this facility using the Nimblescan software (Roche). Initial analysis was done using Array Star (DNA Star). Pairwise comparisons (Figure 1) were done between the GALT-null and WT larvae that had the same treatments (i.e. GALT-null swi to WT swi), and between the same genotypes with different treatments (i.e. GALT-null glc to *GALT*-null swi). With only duplicates, there was not enough statistical power to get a false discovery rate of less than 5%. I use a 2-fold change between conditions as the significance cut off, because variation between conditions was much greater than the variation between the replicates (Table 1). Gene lists were generated using a 2-fold cutoff for genes that displayed differential expression. Genes that had a 1.5 fold or greater difference between replicates were removed from these lists. A recent study has shown that using different criteria will give vastly different results, so validation by Real Time PCR is required to verify the results regardless of the parameters chosen[4]. This validation was done as part of chapter 3.

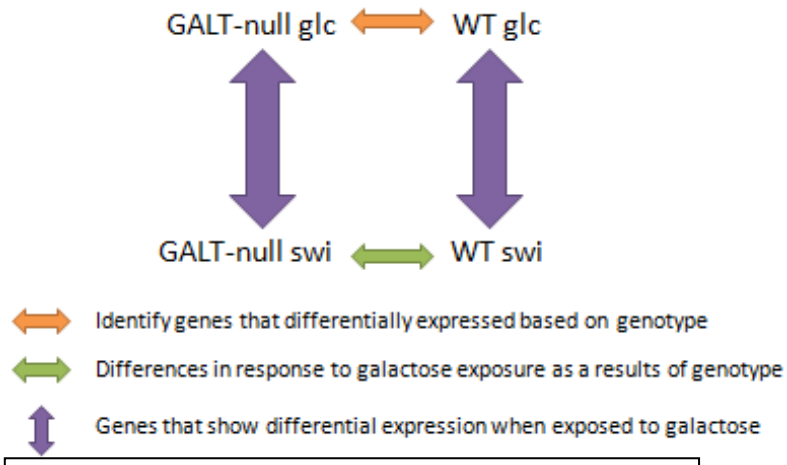

Figure 1. Analysis of transcriptomes by pairwise comparisons.

Gene Ontology and identification of human orthologs. The Gene Ontology (GO) project is an ongoing initiative to standardize the classification of genes and gene products for use in the analysis of large scale genomic data[5]. There are three ontologies that are designed to represent a different domain of molecular biology: Molecular Function (the activity of the gene or gene product at the molecular level, definitions in Supplementary Table S6), Biological Process (biological goal of the ordered molecular functions, definitions in Supplementary Table S5), and Cellular Component (the location where the processes occur, definition in Supplementary Table S7)[6]. The Database for Annotation, Visualization and Integrated Discovery (DAVID) is a publically available resource for performing gene ontology enrichment[7,8]. The gene lists were run through DAVID to identify the clusters of genes that showed differential expression. DAVID enriches the genes into 4 categories: the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and the three GO terms[9,10]. Flybase was used to find human orthologs of the genes that showed differential expression between the conditions[11].

Results and Discussion

The variation in gene expression levels between replicates. The numbers of transcripts that show at least a 2 fold difference in expression between the replicates are shown in Table 1. These represent changes that occur in either direction (increase or decrease). Besides the

comparisons between WT Glc and WT Swi, there were between 5 and 10 times more transcripts that where differentially expressed at least 2-fold between conditions than between replicates.

Condition	**2 fold difference**	**4 fold difference**	**8 fold difference**
GALT WT, Glc	111	8	0
GALT WT, Swi	161	2	0
GALT-null, Glc	227	12	1
GALT-null, Swi	31	3	0

Table 1. The variation in gene expression between the replicates.

The change in global expression between WT and GALT-null larvae on galactose free diets. The pairwise comparisons (Figure 1) showed that genotype (*GALT*-null vs. WT) had the largest effect on the transcriptome. Table 2 displays the number of transcripts that show a greater than 2 fold change (but less than 1.5 fold between replicates) in expression level between GALT-null and WT larvae that have not been exposed to galactose are compared (GALT-null Glc compared to WT Glc). This data represents the change in expression that results from the loss of the *GALT* gene. There were 1123 transcripts that showed an increase of at least 2 fold and 703 transcripts that showed at least a 2 fold decrease when the larvae do not have the GALT gene.

Fold Change	**2X↑**	**4X↑**	**8X↑**	**16X↑**	**2X↓**	**4X↓**	**8X↓**	**16X↓**
Number of transcripts	1123	104	6	0	709	70	9	1

Table 2. The total number of transcripts that show a greater than the indicated fold difference when the GALT-null larvae are compared to the WT larvae.

The KEGG pathways that are enriched (Supplementary table S1a) when the larvae do not have GALT activity include TCA cycle (9 genes), glutathione metabolism (9 genes), glycolysis/gluconeogenesis (8 genes), and the WNT signaling pathway (9 genes), drug metabolism (17 genes), metabolism of xenoiotics by cytochrome P450 (15 genes), and glutathione metabolism (11 genes). The GO terms for biological processes (Supplementary table S2a) that are enriched when there is loss of GALT include sensory perception (50 genes), cognition (53 genes), regulation of transcription (61 genes), and proteolysis (155 genes),oxidation

reduction (55 genes), and nitrogen compound biosynthesis (21 genes). The GO terms for molecular function (Supplementary Table S3a) that are enriched when there is loss of GALT includes transcription factor activity (56 genes) and odorant binding (21 genes), and carbohydrate binding (33 genes). The GO terms for cellular components (Supplementary Table S4b) enriched include integral to membrane (113 genes) and cytoskeleton (31 genes), and mitochondrial part (32 genes).

The most interesting GO terms were enriched for genes involved in glutathione metabolism, TCA cycle, cognition, and mitochondrial part. Genes involved in glutathione metabolism that were differentially expressed between the genotypes included genes with glutathione peroxidase (*CG15116*) and glutathione s-transferase activity (*GSTD8* and *GSTD11*). The *Delta* (D) subfamily of glutathione s-transferases have been shown to increase in larva when exposed to H_2O_2, an oxidant[12]. This, along with enrichment for other GO terms that have previously been associated with oxidative stress [3] indicate that there is an increase in oxidative stress in the GALT-null fly compared to the WT fly even in the absence of galactose. The enrichment of genes involved in TCA cycle and the mitochondria could mean that there is a problem with GALT-null mitochondria (genes with human orthologs, Table 6). The TCA cycle occurs in the mitochondria, so it not surprising that genes in the pathway, such as genes with isocitrate dehydrogenase activity *CG32026, CG3483*) and malate dehydrogenase activity (*CG10748, CG10749*).

An interesting gene involved in mitochondrial function, *miro*, is important for mitochondrial transport into dendrites and axons[13]. This indicates a possible energy deficiency in the neurons of GALT-null flies, at least during development. Other genes involved in neurological processes, such as *trp, eas, disco* are involved in neurological system maintenance and development. The galactose independent long term outcomes in the flies may be due to neuronal maintenance and mitochondrial dysfunction, rather than large phenotypic abnormalities.

The change in global expression in WT larvae when exposed to galactose for 12 hours. Table 4 shows the numbers of transcripts that have a greater than 2 fold change (but less than 1.5 fold between replicates) in their expression levels when the WT larvae are exposed to galactose (WT Swi to WT Glc). There were 301 transcripts that showed at least a 2 fold increase after 12 hours of continuous galactose exposure. There are 120 transcripts that show at least a 2 fold decrease in the WT larvae after 12 hours of continuous galactose exposure. This represents the least amount of changes among all the comparisons.

Fold Change	**2X↑**	**4X↑**	**8X↑**	**16X↑**	**2X↓**	**4X↓**	**8X↓**	**16X↓**
Number of transcripts	301	26	6	2	120	1	0	0

Table 3. The total number of transcripts that show a greater than the indicated fold difference when the WT larvae are exposed to galactose.

These KEGG pathways (Supplementary Table S1b) that are enriched include metabolism of xenobiotics by cytochrome P450 (16 genes), drug metabolism (16 genes), starch and sucrose metabolism (12 genes), pentose and glucuronate interconversions (9 genes), glutathione metabolism (9 genes), and galactose metabolism (4 genes). The GO terms for biological process (Supplementary Table S2b) that are enriched when WT larvae are exposed to galactose include oxidation reduction (47 genes), polysaccharide metabolic processes (18 genes), proteolysis (30 genes), superoxide metabolic process (2 genes). The GO terms for molecular function (Supplementary Table S3b) that are enriched when the WT larvae are exposed to galactose include carbohydrate binding (21 genes), monosaccharide transmembrane transporter activity (7 genes), and iron ion binding (17 genes). Supplementary Table S4b shows the DAVID results for the GO category cellular compartments. The GO terms that are enriched include ATP-binding cassette (ABC) (5 genes) and integral to membrane (40 genes).

The change in global expression in GALT-null larvae when exposed to galactose for 12 hours. Table 5 displays the total number of transcripts that show a greater than 2 fold change in expression levels when GALT-null larvae are exposed to galactose for 12 hours (GALT-null

switch compared to GALT-null glc). There were 653 transcripts that showed an increase of at least 2 fold when exposed to galactose and 598 transcripts that showed at least a 2 fold decrease when exposed to galactose.

Fold Change	**2X↑**	**4X↑**	**8X↑**	**16X↑**	**2X↓**	**4X↓**	**8X↓**	**16X↓**
Number of transcripts	653	54	5	3	598	101	10	0

Table 4. The total number of transcripts that show a greater than the indicated fold difference when the GALT-null larvae are exposed to galactose.

The KEGG pathways that are enriched (Supplementary table S1b) also include drug metabolism, metabolism of xenobiotics by cytochrome P450, and glutathione metabolism (15, 14, and 12 genes, respectively), and glycerolipid metabolism (8 genes). The GO terms for biological processes (Supplementary Table S2b) include oxidation reduction (86 genes), neuropeptide signaling pathways (17 genes), polysaccharide metabolic processes (31 genes), and nitrogen compound biosynthesis (19 genes), proteolysis (45 genes), polysaccharide metabolic processes (15 genes), and lipid biosynthetic processes (11 genes). For the genes that are enriched when the GALT-null larvae are exposed to galactose GO terms for molecular function (Supplementary Table S3c) includes carbohydrate binding (35 genes), neuropeptide hormone activity (19 genes), iron ion binding (23 genes), calcium ion binding (23 genes), and lipase activity (11 genes). GO terms for cellular components (Supplementary Table S4b) enriched includes synapse (25 genes) and integral to membrane (100 genes).

The change in global expression between WT and GALT-null larvae following 12 hours of exposure to a galactose containing diet. Table 5 displays the total number of transcripts that show a greater than 2 fold change (but less than 1.5 fold between replicates) in expression level between GALT-null and WT larvae exposed to galactose for a 12 hour period (GALT-null switch compared to WT switch). These data represent the change in expression that results when there is loss of the *GALT* gene and the larvae are exposed to galactose. There were 694 transcripts that

showed an increase of at least 2 fold and 958 transcripts that showed at least a 2 fold decrease when the larvae do not have the *GALT* gene and are exposed to galactose.

Fold Change	**2X↑**	**4X↑**	**8X↑**	**16X↑**	**2X↓**	**4X↓**	**8X↓**	**16X↓**
Number of Transcripts	925	42	1	0	694	89	12	5

Table 5. The total number of transcripts that show a greater than the indicated fold difference when the GALT-null and WT larvae are exposed to galactose.

The KEGG pathways that are enriched (Supplementary table S1c) when the larvae do not have GALT activity include glutathione metabolism (19 genes), TCA cycle (5 genes), drug metabolism (17 genes), metabolism of xenoiotics by cytochrome P450 (15 genes), and lysosome (16 genes). The GO terms for biological processes (Supplementary table S2c) that are enriched when there is loss of GALT include sensory perception (44 genes), cognition (47 genes), regulation of transcription DNA dependent (51 genes), proteolysis (107 genes), oxidation reduction (69 genes), and lipid biosynthetic processes (18 genes). The GO terms for molecular function (Supplementary table S3d) enriched when there is loss of GALT includes transcription factor activity (46 genes), carbohydrate binding (33 genes), iron ion binding (8 genes), and lipase activity (12 genes). GO terms for cellular components that are enriched (Supplementary Table S4c) include integral to membrane (91 genes), cytoskeleton (26 genes), integral to membrane (86 genes), and lysosome (6 genes).

While many of the genes that show differential expression in this comparison remain the same compared to when the larvae were on Glc, there is an increase in the number of genes involved in the glutathione metabolic processes that show differential expression when larvae of both genotypes are exposed to galactose. These include thioredoxin reductase 2 (CG11401, Table 6), and more glutathione s-transferase genes of the *delta* and *epsilon* class (such as *GSTD6* and *GSTE7*) that have been previously been shown to be increase in larva exposed to an oxidative stress inducer[12]. Thioredoxin reductase activity is thought to replace the activity of glutathione reductase in *Drosophila*, meaning that it is important in redox homeostasis [14]. The largest

difference between this comparison (both genotypes Swi) and the previous comparison (both genotypes Glc) is that there are genes involved in the oxidative stress response that are differentially expressed. Both GALT-null and WT larvae are undergoing oxidative stress when exposed to galactose, though to varying degrees. The GALT-null larvae have a stronger response to galactose exposure than the WT larvae (below and chapter 3)[15]. The larger oxidative stress response may be indicative of a higher baseline level of oxidative stress in the GALT-null compared to the WT larvae when both are on Glucose only diets (above).*Identification of Human orthologs.* Table 6 displays genes that have direct human orthologs and have roles in the processes and pathways that were enriched in the GO analysis. Besides the processes involved in oxidation reduction, the most striking result is the genes involved in mitochondrial function that show differential expression when there is loss of the *GALT* gene.

Fly Annotation	Human Ortholog	Fold Change	Function*
CG7311	GPD2	2.72 increase[1]	glycerol-3-phosphate to dihydroxyacetone phosphate
CG11401	TXNRD2	3.19 increase[1]	oxidoreductase
CG10909	FBL	5.24 increase[1] 5.01 increase[3]	RNA binding; methyltransferase activity
CG34076	ND3	4.14 decrease[2]	NADH dehydrogenase (ubiquinone) activity
CG4495	MICU1	3.04 increase[2]	Calcium uptake
CG8745	AGXT2L1	3.01 increase[2]	alanine-glyoxylate transaminase activity
CG1982	SORD	7.85 increase[2]	L-iditol 2-dehydrogenase activity
CG1742	MGST1	2.48 decrease[2]	glutathione transferase activity
CG6910	MIOX	5.18 increase[2]	inositol oxygenase activity; iron ion binding
CG3879	ABCB4	6.12 increase[2]	ATPase activity, coupled; drug transmembrane transporter activity
CG31559	GRXCR1, GRXCR2	6.26 decrease[2]	protein disulfide oxidoreductase activity; electron carrier activity
CG9582	SLC25A21	4.33 increase[3]	transmembrane transporter activity; oxidative phosphorylation uncoupler activity
CG10090	TIMM17A, TIMM17B	9.21 increase[3]	P-P-bond-hydrolysis-driven protein transmembrane transporter activity; protein transporter activity
CG34132	TIMM13	4.16 decrease[3]	protein import into mitochondrial inner membrane
CG3879	ABCB4	7.46 increase[4]	ATPase activity, coupled; drug transmembrane transporter activity

Table 6. Genes that showed differential expression and have human orthologs. [1]Change in *GALT*-null compared to WT on galactose. [2]Change in *GALT*-null larvae when exposed to galactose. [3]Change in *GALT*-null compared to WT on glucose. [4]Change in WT larvae when exposed to galactose. *Functions are derived from Flybase[11].

Conclusions

Gene expression analysis by microarray has provided insights into the larval immune response[1], starvation response[2], and the response to stress[3]. The GO terms enriched in the expression analysis in this experiment, such as oxidation reduction, glutathione metabolism, iron ion binding, and proteolysis have been shown to be over-represented in adult flies exposed to paraquat[3]. Paraquat is known to induce oxidative stress, which indicates that the GALT-null larvae are undergoing increased oxidative stress compared to WT larvae since there are more genes over-represented in these categories in the GALT-null larvae exposed to galactose compared to when the WT larvae are exposed to galactose (Supplementary table S2-S4).

When exposed to galactose, the GALT-null larvae have more genes that change their expression than the WT larvae (Table 4 and Table 5).This is due to there being more of the proteins involved in the response to oxidative stress in addition to an enrichment of genes involved in the neuropeptide signaling pathways and calcium ion binding. Since the WT larvae survive on 225 mM galactose, while the *GALT*-null larvae do not, the need to increase the oxidative stress response could indicate that there is a greater amount of oxidative stress throughout the *GALT*-null larvae than in the WT under parallel conditions. Increase in oxidative stress could be a contributing factor to the acute outcome in the *GALT*-null flies raised on galactose (death during development).

This was largely due to the increase in the expression of genes that are involved in neurological system processes and regulation of transcription (Supplementary Table S2). There is at least one long term outcome in the *GALT*-null flies when raised on a galactose free diet. The *GALT*-null flies perform worse on the counter current and simple climbing assays than WT flies[16,17]. The *GALT*-null flies also perform worse on the negative geotaxis response assay. The increase in the genes that are part of the TCA pathway could indicate that the *GALT*-null

larvae have a greater need for energy, which could lead to a movement defect. *Drosophila* mutants that have impairment in mitochondrial function and also display movement phenotypes[18]. There is a decrease in genes enriched in the GO Term mitochondrial part (Supplementary Table S4).

Since the microarray analysis was performed on RNA isolated from developing larvae, it has more power to detect the causes of the acute symptoms, unless the long term outcomes are strictly caused by errors in development. One theory for the acute outcome could be that the *GALT*-null larvae are simply avoiding the food and starving to death. While experiments have already been done with colored food to ensure that the larvae are eating, this experiment showed that the expression profile does not match a starving fly[2] as much as a fly exposed to paraquat[3], indicating that oxidative stress plays an important role in the galactose induced toxicity. Chapter 3, which is a paper our lab published while I was working on the microarray data, provides more evidence that oxidative stress is involved in the galactose induced toxicity of *GALT*-null larvae. In Chapter 3, I have validated two of the GST genes (*GSTD6* and *GSTE7*) that showed differential expression when larvae of both genotypes were exposed to galactose.

The long term outcomes observed in GALT-null larvae could be at least partially due to impaired mitochondrial function, a delayed neurological development, and reduced energy sources during the early stages of development.

Supplementary Tables

Supplementary Table S1a. The KEGG pathway enrichment for the GALT-null compared to WT when both are raised on glc only food. Count = genes.

GALT-null compared to WT (both glc)		
KEGG (2 fold Up)	count	pValue
Citrate cycle (TCA cycle)	9	0.001539
Glutathione metabolism	9	0.014732
Glycolysis / Gluconeogenesis	8	0.016403
Starch and sucrose metabolism	8	0.033656
Wnt signaling pathway	9	0.035668
Galactose metabolism	5	0.060558
KEGG (2 fold down)		
Drug metabolism	17	1.48E-05
Arginine and proline metabolism	14	1.30E-04
Metabolism of xenobiotics by cytochrome P450	15	1.75E-04
Ascorbate and aldarate metabolism	10	4.79E-04
Retinol metabolism	9	0.004118
Pentose and glucuronate interconversions	9	0.00809
Glycine, serine and threonine metabolism	7	0.008624
ECM-receptor interaction	4	0.011556
Drug metabolism	11	0.011972
Glutathione metabolism	11	0.016697
Folate biosynthesis	6	0.020569
Androgen and estrogen metabolism	7	0.024674
Porphyrin and chlorophyll metabolism	8	0.042204
Neuroactive ligand-receptor interaction	7	0.042483
Cysteine and methionine metabolism	5	0.057246
Starch and sucrose metabolism	9	0.075407

Supplementary Table S1b. The KEGG pathway enrichment for the WT swi compared to glc (the WT larvae response to galactose) and also the GALT-null swi compared to glc (the GALT-null response to galactose). Count = genes.

WT Swi compared to Glc		
KEGG (2 fold up)	count	pValue
Metabolism of xenobiotics by cytochrome P450	16	2.32E-10
Drug metabolism	16	3.73E-10
Starch and sucrose metabolism	12	1.16E-06
Ascorbate and aldarate metabolism	9	4.28E-06
Pentose and glucuronate interconversions	9	1.99E-05
Androgen and estrogen metabolism	8	2.98E-05
Retinol metabolism	8	8.37E-05
Porphyrin and chlorophyll metabolism	8	3.15E-04
Drug metabolism	9	4.05E-04
Glutathione metabolism	9	5.69E-04
Limonene and pinene degradation	8	0.015735
Glycine, serine and threonine metabolism	4	0.040709
Galactose metabolism	4	0.059253
KEGG (2 fold down)		
folate biosynthesis	2	0.082678
GALT-null swi compared to glc		
KEGG (2fold Up)	count	pValue
Drug metabolism	15	4.84E-07
Metabolism of xenobiotics by cytochrome P450	14	2.20E-06
Glutathione metabolism	12	6.45E-05
Arginine and proline metabolism	10	5.21E-04
Alanine, aspartate and glutamate metabolism	7	0.001097
Pentose and glucuronate interconversions	8	0.001497
Glycerolipid metabolism	8	0.002661
Ascorbate and aldarate metabolism	7	0.002695
beta-Alanine metabolism	5	0.008379
Neuroactive ligand-receptor interaction	6	0.933126
Retinol metabolism	6	0.019411
Butanoate metabolism	5	0.022324
Non-homologous end-joining	3	0.025923
Nitrogen metabolism	4	0.070458
KEGG (2fold Down)		
Terpenoid backbone biosynthesis	6	4.27E-06
Glycerolipid metabolism	5	0.026598
Tyrosine metabolism	4	0.062187

Supplementary Table S1c. The KEGG pathway enrichment for the GALT-null compared to WT when both are exposed to galactose (swi condition). Count = genes.

GALT-null compared to WT (both swi)		
KEGG (2 fold up)	count	pValue
Glutathione metabolism	7	0.017312
Citrate cycle (TCA cycle)	5	0.057701
Fructose and mannose metabolism	4	0.083456
Glycolysis / Gluconeogenesis	5	0.094426
KEGG (2 fold down)		
Drug metabolism	17	2.23E-05
Metabolism of xenobiotics by cytochrome P450	15	2.47E-04
Terpenoid backbone biosynthesis	6	8.23E-04
Retinol metabolism	10	0.001212
Androgen and estrogen metabolism	9	0.001828
Lysosome	16	0.002394
Ascorbate and aldarate metabolism	9	0.002814
Limonene and pinene degradation	15	0.005161
Glutathione metabolism	12	0.007566
Arginine and proline metabolism	11	0.008056
Porphyrin and chlorophyll metabolism	9	0.017281
Folate biosynthesis	6	0.023335
Pentose and glucuronate interconversions	8	0.030839
Drug metabolism	10	0.038122
Starch and sucrose metabolism	10	0.038122
Glycine, serine and threonine metabolism	6	0.039023
Amino sugar and nucleotide sugar metabolism	9	0.043314
Neuroactive ligand-receptor interaction	7	0.048523
Cysteine and methionine metabolism	5	0.063096

Supplementary Table S2a. The biological process GO enrichment for the GALT-null compared to WT (both raised on glc only food). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null compared to WT (both glc)		
GOTERM (2 fold up)		
sensory perception of chemical stimulus	42	3.34E-13
sensory perception	50	1.63E-12
cognition	53	3.41E-10
sensory perception of taste	20	2.99E-09
proteolysis	74	3.45E-06
G-protein coupled receptor protein signaling pathway	40	6.61E-06
ion transport	40	6.21E-05
neurological system process	55	9.54E-05
microtubule-based movement	18	1.09E-04
glycolysis	9	7.87E-04
regulation of transcription, DNA-dependent	56	9.52E-04
cell surface receptor linked signal transduction	57	9.88E-04
carbohydrate catabolic process	14	0.001047
generation of precursor metabolites and energy	24	0.004387
transcription	41	0.007833
tricarboxylic acid cycle	7	0.009937
nitrogen compound biosynthetic process	24	0.010222
ATP biosynthetic process	11	0.015744
ATP metabolic process	11	0.018565
mitochondrial transport	8	0.03481
hexose metabolic process	10	0.061904
metal ion transport	16	0.062112
transmembrane transport	12	0.065368
regulation of transcription	61	0.0282207
GOTERM (2 fold down)	count	pValue
chitin metabolic process	28	1.59E-11
proteolysis	81	4.46E-11
aminoglycan metabolic process	30	1.34E-10
polysaccharide metabolic process	31	1.83E-10
neuropeptide signaling pathway	10	6.87E-05
oxidation reduction	55	9.83E-05
nitrogen compound biosynthetic process	21	0.018705
lipid biosynthetic process	13	0.020637
ion transport	27	0.036982
monovalent inorganic cation transport	16	0.038829
cation transport	21	0.038871

Supplementary Table S2b. The biological process GO enrichment for the WT swi compared to glc (the WT larvae response to galactose) and also the GALT-null swi compared to glc (the GALT-null response to galactose). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

WT swi compared to glc		
GOTERM (2 fold up)	count	pValue
oxidation reduction	39	3.77E-12
chitin metabolic process	18	1.01E-11
aminoglycan metabolic process	18	3.61E-10
polysaccharide metabolic process	18	1.17E-09
proteolysi	30	2.27E-05
GOTERM (2 fold down)	count	pValue
oxidation reduction	8	0.016811
superoxide metabolic process	2	0.030474
cuticle development	3	0.032108
proteolysis	8	0.037547
GALT-null swi compared to glc		
GOTERM (2 fold up)	count	pValue
chitin metabolic process	29	2.87E-14
neuropeptide signaling pathway	17	3.81E-13
aminoglycan metabolic process	30	1.58E-12
polysaccharide metabolic process	31	1.95E-12
G-protein coupled receptor protein signaling pathway	35	6.66E-07
regulation of system process	11	1.09E-06
ion transport	36	2.26E-06
cellular amino acid derivative metabolic process	13	9.55E-06
oxidation reduction	51	1.18E-05
cell-cell signaling	20	0.002027
cell surface receptor linked signal transduction	43	0.003079
nitrogen compound biosynthetic process	19	0.012664
response to oxidative stress	8	0.016003
GOTERM (2 fold down)	count	pValue
proteolysis	45	2.58E-07
chitin metabolic process	14	1.14E-05
aminoglycan metabolic process	15	2.81E-05
oxidation reduction	35	5.28E-05
polysaccharide metabolic process	15	6.45E-05
lipid biosynthetic process	11	0.001399

Supplementary Table S2c. The biological process GO enrichment for the GALT-null compared to WT (both exposed to galactose). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null compared to WT (both swi)		
GOTERM (2 fold up)	count	pValue
sensory perception	44	5.87E-13
cognition	47	4.11E-11
G-protein coupled receptor protein signaling pathway	41	2.20E-09
sensory perception of chemical stimulus	29	4.48E-08
cell surface receptor linked signal transduction	57	5.81E-07
neurological system process	51	1.15E-06
regulation of transcription, DNA-dependent	51	2.91E-05
ion transport	33	9.38E-05
microtubule-based movement	15	2.40E-04
regulation of RNA metabolic process	56	3.79E-04
regulation of transcription	11	0.001029
regulation of phosphorus metabolic process	11	0.001443
specification of segmental identity, head	10	0.001557
GO Term (2 fold down)	count	pValue
proteolysis	107	6.08E-25
chitin metabolic process	28	1.01E-11
aminoglycan metabolic process	29	4.22E-10
oxidation reduction	69	6.54E-10
polysaccharide metabolic process	29	2.52E-09
lipid biosynthetic process	18	1.03E-04

Supplementary Table S3a. The molecular function GO enrichment for the GALT-null compared to WT larvae when both were raised in glc only food. Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null compared to WT (both glc)		
GOTERM (2 fold up)		
serine-type endopeptidase activity	50	2.53E-09
taste receptor activity	19	2.10E-08
serine-type peptidase activity	51	2.31E-08
transcription factor activity	56	5.46E-07
endopeptidase activity	62	9.61E-07
peptidase activity, acting on L-amino acid peptides	71	3.75E-05
odorant binding	23	5.56E-05
specific RNA polymerase II transcription factor activity	17	8.35E-05
structural constituent of chitin-based cuticle	20	2.54E-04
protein kinase regulator activity	12	0.001073
transcription regulator activity	64	0.017684
motor activity	13	0.021776
channel activity	22	0.025165
ATPase activity, coupled	31	0.047562
GOTERM (2 fold down)	count	pValue
chitin binding	27	1.57E-10
polysaccharide binding	29	1.07E-09
carbohydrate binding	33	2.22E-07
peptidase activity	80	1.15E-09
serine-type peptidase activity	46	3.33E-08
hormone activity	14	1.30E-05
vitamin binding	17	3.12E-04
carboxylic acid binding	13	5.07E-04
metallopeptidase activity	23	5.76E-04
enzyme inhibitor activity	17	8.43E-04
cofactor binding	23	0.00208
structural molecule activity	41	0.01306

Supplementary Table S3b. The molecular function GO enrichment for the WT response to galactose exposure (swi compared to gl). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

WT swi compared to glc		
GOTERM (2 fold down)	count	pValue
structural constituent of cuticle	5	0.002488
structural molecule activity	6	0.065
GOTERM (2 fold up)		
structural constituent of peritrophic membrane	11	3.17E-11
chitin binding	18	9.91E-11
polysaccharide binding	18	3.16E-09
carbohydrate binding	21	1.46E-08
glucuronosyltransferase activity	9	7.91E-07
monosaccharide transmembrane transporter activity	7	7.95E-06
glutathione transferase activity	8	1.86E-05
iron ion binding	17	3.45E-04
peptidase activity	30	4.32E-04
ATPase activity, coupled to movement of substances	11	0.001216
vitamin binding	7	0.027431

Supplementary Table S3c. The molecular function GO enrichment for the GALT-null response to galactose exposure (swi compared to glc). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null swi compared to glc		
GOTERM (2 fold up)	count	pValue
neuropeptide hormone activity	19	7.28E-16
hormone activity	22	1.06E-14
chitin binding	29	1.14E-14
polysaccharide binding	30	6.09E-13
carbohydrate binding	35	2.30E-11
structural constituent of peritrophic membrane	12	2.36E-09
neurotransmitter receptor activity	16	8.88E-07
symporter activity	17	2.04E-06
ligand-gated ion channel activity	14	2.48E-05
ion channel activity	20	7.69E-04
hexose transmembrane transporter activity	6	0.001708
glutathione transferase activity	8	0.001713
calcium ion binding	23	0.003154
iron ion binding	23	0.007287
vitamin binding	12	0.008059
GOTERM (2 fold down)	count	pValue
structural constituent of cuticle	30	3.72E-17
serine-type endopeptidase activity	32	8.58E-09
structural molecule activity	37	2.84E-06
endopeptidase activity	37	3.26E-07
peptidase activity, acting on L-amino acid peptides	42	3.24E-05
carboxylesterase activity	14	8.14E-05
peptidase activity	42	1.18E-04
chitin binding	12	6.81E-04
lipase activity	11	0.001195
polysaccharide binding	13	0.001242

Supplementary Table S3d. The molecular function GO enrichment for the GALT-null compared to WT when both are exposure to galactose (swi condition). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null compared to WT (both swi)		
GOTERM (2 fold up)	count	pValue
transcription factor activity	46	8.56E-07
protein kinase regulator activity	11	4.68E-04
structural constituent of chitin-based cuticle	15	0.002354
specific RNA polymerase II transcription factor activity	12	0.00272
odorant binding	16	0.002766
transcription regulator activity	53	0.006701
passive transmembrane transporter activity	19	0.012491
channel activity	19	0.012491
DNA binding	58	0.033775
serine-type endopeptidase activity	23	0.050075
GOTERM (2 fold down)	count	pValue
peptidase activity	108	2.18E-22
peptidase activity, acting on L-amino acid peptides	101	1.55E-20
serine-type peptidase activity	62	1.18E-16
endopeptidase activity	72	2.27E-13
chitin binding	25	6.45E-09
exopeptidase activity	24	1.01E-07
metallopeptidase activity	31	1.26E-07
polysaccharide binding	26	1.29E-07
carbohydrate binding	33	2.22E-07
organic cation transmembrane transporter activity	12	9.65E-06
carboxypeptidase activity	12	7.26E-05
aminopeptidase activity	22	7.26E-05
tetrapyrrole binding	22	1.45E-04
heme binding	19	1.45E-04
structural constituent of cuticle	18	4.25E-04
sugar transmembrane transporter activity	14	0.001047
structural constituent of peritrophic membrane	16	0.002317
enzyme inhibitor activity	29	0.002746
iron ion binding	8	0.003165
glucuronosyltransferase activity	14	0.00434
pantetheine hydrolase activity	17	0.011172
vitamin binding	13	0.019784
lipase activity	12	0.025511

Supplementary Table S4a. The cellular component GO enrichment for the GALT-null compared to WT (both raised on glc only food). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null compared to WT (both glc)		
GOTERM (2 fold up)		
protein kinase CK2 complex	12	2.05E-09
extracellular region	56	1.93E-05
dynein complex	12	4.06E-05
chorion	8	5.68E-05
integral to membrane	113	1.87E-04
intrinsic to membrane	113	3.73E-04
microtubule associated complex	18	6.12E-04
axoneme	6	0.001101
microtubule cytoskeleton	25	0.004242
cytoskeletal part	29	0.009838
plasma membrane	62	0.026218
cytoskeleton	31	0.041035
integral to plasma membrane	19	0.062416
GOTERM (2 fold down)	count	pValue
extracellular region	78	2.69E-17
extracellular region part	20	7.33E-04
proteinaceous extracellular matrix	10	0.001643
extracellular matrix	10	0.002505
microsome	11	0.027171
membrane fraction	12	0.035415
insoluble fraction	12	0.044609
cell fraction	12	0.0525
mitochondrial part	32	0.055383

Supplementary Table S4b. The cellular component GO enrichment for the WT swi compared to glc (the WT larvae response to galactose) and also the GALT-null swi compared to glc (the GALT-null response to galactose). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

WT swi compared to glc		
GOTERM (2 fold down)	count	pValue
extracellular region	8	7.43E-04
GOTERM (2 fold up)		
extracellular region	26	1.25E-05
integral to membrane	40	0.005103
intrinsic to membrane	40	0.006855
microsome	7	0.00807
ATP-binding cassette (ABC) transporter complex	5	0.009161
extrinsic to membrane	9	0.010888
membrane fraction	7	0.018122
insoluble fraction	7	0.021367
cell fraction	7	0.024045
intrinsic to plasma membrane	9	0.045019
GALT-null swi compared to glc		
GOTERM (2 fold up)	count	pValue
extracellular region	85	1.25E-22
synapse part	23	2.29E-08
synapse	25	3.80E-08
nicotinic acetylcholine-gated receptor-channel complex	6	9.33E-05
integral to membrane	99	6.43E-04
intrinsic to membrane	100	7.39E-04
intrinsic to plasma membrane	23	0.001219
plasma membrane part	37	0.001982
integral to plasma membrane	22	0.002329
plasma membrane	56	0.022577
GOTERM (2 fold down)	count	pValue
extracellular region	30	3.60E-06
integral to membrane	48	0.001292
intrinsic to membrane	48	0.001866

Supplementary Table S4c. The cellular component GO enrichment for the GALT-null compared to WT (both exposed to galactose). Count = genes. Overlapping terms and terms that had high pValues and few genes were omitted from these tables.

GALT-null compared to WT (both swi)		
GOTERM (2 fold up)	count	pValue
protein kinase CK2 complex	11	4.22E-09
extracellular region	47	2.50E-05
intrinsic to membrane	93	3.32E-04
integral to membrane	91	5.05E-04
dynein complex	9	9.76E-04
plasma membrane	57	0.001469
axoneme	5	0.003839
microtubule cytoskeleton	20	0.010993
cytoskeletal part	24	0.012761
cytoskeleton	26	0.036131
intrinsic to plasma membrane	17	0.036879
integral to plasma membrane	16	0.060772
GOTERM (2 fold down)	count	pValue
extracellular region	62	1.41E-11
microsome	15	9.94E-05
membrane fraction	15	6.48E-04
insoluble fraction	15	9.43E-04
cell fraction	15	0.001233
integral to membrane	86	0.006399
extrinsic to membrane	16	0.008716
intrinsic to membrane	86	0.010045
lysosome	6	0.014727

Supplementary Table S5. The Definitions of the GO Terms for Biological Processes that are mentioned in the results[19].

Biological Process	Definition of Term
oxidation reduction	A metabolic process that results in the removal or addition of one or more electrons to or from a substance, with or without the concomitant removal or addition of a proton or protons
polysaccharide metabolic processes	The chemical reactions and pathways involving polysaccharides, a polymer of more than 20 monosaccharide residues joined by glycosidic linkages
proteolysis	This term was intentionally placed under 'protein metabolic process ; GO:0019538' rather than 'protein catabolic process ; GO:0030163' to cover all processes centered on breaking peptide bonds, including those involved in protein maturation
superoxide metabolic process	The chemical reactions and pathways involving superoxide, the superoxide anion O2- (superoxide free radical), or any compound containing this species
neuropeptide signaling pathways	The series of molecular signals generated as a consequence of a peptide neurotransmitter binding to a cell surface receptor
nitrogen compound biosynthesis	The chemical reactions and pathways resulting in the formation of organic and inorganic nitrogenous compounds
lipid biosynthetic processes	The chemical reactions and pathways resulting in the formation of lipids, compounds soluble in an organic solvent but not, or sparingly, in an aqueous solvent.
sensory perception	The series of events required for an organism to receive a sensory stimulus, convert it to a molecular signal, and recognize and characterize the signal. This is a neurological process
cognition	The operation of the mind by which an organism becomes aware of objects of thought or perception; it includes the mental activities associated with thinking, learning, and memory.
regulation of transcription	Any process that modulates the frequency, rate or extent of cellular DNA-dependent transcription
regulation of transcription DNA dependent	Any process that modulates the frequency, rate or extent of cellular DNA-dependent transcription

Supplementary Table S6. The Definitions of the GO Terms for Molecular Function that are mentioned in the results[19].

Molecular Function	Definition of Term
carbohydrate binding	Interacting selectively and non-covalently with any carbohydrate, which includes monosaccharides, oligosaccharides and polysaccharides as well as substances derived from monosaccharides by reduction of the carbonyl group (alditols), by oxidation of one or more hydroxy groups to afford the corresponding aldehydes, ketones, or carboxylic acids, or by replacement of one or more hydroxy group(s) by a hydrogen atom. Cyclitols are generally not regarded as carbohydrates
monosaccharide transmembrane transporter activity	Catalysis of the transfer of a monosaccharide from one side of a membrane to the other.
iron ion binding	Interacting selectively and non-covalently with iron (Fe) ions
neuropeptide hormone activity	The action characteristic of a neuropeptide hormone, any peptide hormone that acts in the central nervous system. A neuropeptide is any of several types of molecules found in brain tissue, composed of short chains of amino acids; they include endorphins, enkephalins, vasopressin, and others. They are often localized in axon terminals at synapses and are classified as putative neurotransmitters, although some are also hormones
calcium ion binding	Interacting selectively and non-covalently with calcium ions (Ca2+)
lipase activity	Catalysis of the hydrolysis of a lipid or phospholipid
transcription factor activity	Interacting selectively and non-covalently with a specific DNA sequence in order to modulate transcription. The transcription factor may or may not also interact selectively with a protein or macromolecular complex
odorant binding	Interacting selectively and non-covalently with an odorant, any substance capable of stimulating the sense of smell.
lipid biosynthetic processes	The chemical reactions and pathways resulting in the formation of lipids, compounds soluble in an organic solvent but not, or sparingly, in an aqueous solvent

Supplementary Table S7. The Definitions of the GO Terms for Cellular Component that are mentioned in the results[19].

Cellular Component	Definition of Term
ATP-binding cassette (ABC) Transporter Complex	A complex for the transport of metabolites into and out of the cell, typically comprised of four domains; two membrane-associated domains and two ATP-binding domains at the intracellular face of the membrane, that form a central pore through the plasma membrane. Each of the four core domains may be encoded as a separate polypeptide or the domains can be fused in any one of a number of ways into multidomain polypeptides. In Bacteria and Archaebacteria, ABC transporters also include substrate binding proteins to bind substrate external to the cytoplasm and deliver it to the transporter.
integral to membrane	Penetrating at least one phospholipid bilayer of a membrane. May also refer to the state of being buried in the bilayer with no exposure outside the bilayer. When used to describe a protein, indicates that all or part of the peptide sequence is embedded in the membrane
synapse	The junction between a nerve fiber of one neuron and another neuron or muscle fiber or glial cell; the site of interneuronal communication. As the nerve fiber approaches the synapse it enlarges into a specialized structure, the presynaptic nerve ending, which contains mitochondria and synaptic vesicles. At the tip of the nerve ending is the presynaptic membrane; facing it, and separated from it by a minute cleft (the synaptic cleft) is a specialized area of membrane on the receiving cell, known as the postsynaptic membrane. In response to the arrival of nerve impulses, the presynaptic nerve ending secretes molecules of neurotransmitters into the synaptic cleft. These diffuse across the cleft and transmit the signal to the postsynaptic membrane
cytoskeleton	Any of the various filamentous elements that form the internal framework of cells, and typically remain after treatment of the cells with mild detergent to remove membrane constituents and soluble components of the cytoplasm. The term embraces intermediate filaments, microfilaments, microtubules, the microtrabecular lattice, and other structures characterized by a polymeric filamentous nature and long-range order within the cell. The various elements of the cytoskeleton not only serve in the maintenance of cellular shape but also have roles in other cellular functions, including cellular movement, cell division, endocytosis, and movement of organelles
mitochondrial part	Any constituent part of a mitochondrion, a semiautonomous, self replicating organelle that occurs in varying numbers, shapes, and sizes in the cytoplasm of virtually all eukaryotic cells. It is notably the site of tissue respiration
lysosome	A small lytic vacuole that has cell cycle-independent morphology and is found in most animal cells and that contains a variety of hydrolases, most of which have their maximal activities in the pH range 5-6. The contained enzymes display latency if properly isolated. About 40 different lysosomal hydrolases are known and lysosomes have a great variety of morphologies and functions

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Chapter 3

Oxidative stress contributes to outcome severity in a *Drosophila melanogaster* model of classic galactosemia

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Oxidative stress contributes to outcome severity in a *Drosophila melanogaster* model of classic galactosemia

Patricia P Jumbo-Lucioni[1], Marquise L Hopson[1], Darwin Hang[2], Yongliang Liang[3], Dean P Jones[3], and Judith L. Fridovich-Keil[1*]

[1]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, [2]Graduate Program in Genetics and Molecular Biology, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA, [3]Department of Medicine, Pulmonary Division, Emory University School of Medicine, Atlanta, GA, 30322; 30322

[*]corresponding author: Judith L. Fridovich-Keil

Department of Human Genetics

Emory University School of Medicine

Rm. 325.2 Whitehead Bldg.

615 Michael St.

Atlanta, GA 30322

TEL: 404-727-3924

Email:jfridov@emory.edu

FAX: 404-727-3949

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ABSTRACT

Classic galactosemia is a genetic disorder that results from profound loss of galactose-1P-uridylyltransferase (GALT). Affected infants experience a rapid escalation of potentially lethal acute symptoms following exposure to milk. Dietary restriction of galactose prevents or resolves the acute sequelae; however, many patients experience profound long-term complications. Despite decades of research the mechanisms that underlie pathophysiology in classic galactosemia remain unclear. Recently, we developed a *Drosophila melanogaster* model of classic galactosemia and demonstrated that, like patients, GALT-null *Drosophila* succumb in development if exposed to galactose but live if maintained on a galactose-restricted diet. Prior models of experimental galactosemia have implicated a possible association between galactose exposure and oxidative stress. Here we applied our fly genetic model of galactosemia to ask whether oxidative stress contributes to the acute galactose-sensitivity of GALT-null animals. Our first approach tested the impact of pro- and anti-oxidant food supplements on the survival of GALT-null vs. control larvae. We observed a clear pattern: each of two oxidants, paraquat and DMSO, had a negative impact on the survival of mutant but not control animals exposed to galactose, and each of two anti-oxidants, vitamin C and α-mangostin, had the opposite effect. Biochemical markers also confirmed that galactose and paraquat synergistically increased oxidative stress on all cohorts tested, but interestingly, the mutant animals showed a decreased response relative to controls. Finally, we tested the expression levels of two transcripts responsive to oxidative stress, *GSTD6* and *GSTE7*, in mutant and control larvae exposed to galactose and found that both genes were induced, one by more than 40-fold. Combined, these results implicate oxidative stress and response as contributing factors in the acute galactose-sensitivity of GALT-null *Drosophila*, and by extension, suggest that reactive oxygen species may also contribute to the acute pathophysiology in classic galactosemia.

INTRODUCTION

Galactose is essential for life in metazoans. Derivatives of galactose in glycoconjugates are key elements of cell membrane structures, hormones, extracellular matrix, immunologic determinants, and structural elements of the central nervous system, among other roles (Segal, 1995). For mammalian infants, galactose is also an important source of sugar calories as it represents half of the monosaccharide liberated from the digestion of lactose. For full catabolism, however, galactose must be converted into glucose-1-phosphate (glc-1P) via the Leloir pathway (Frey, 1996; Berg JM, 2002 ; Holden et al., 2003). In humans, a deficiency of the second enzyme of the Leloir pathway, galactose-1-phosphate uridylyltransferase (GALT, E.C. 2.7.7.12), results in the autosomal recessive, potentially lethal disorder classic galactosemia (*OMIM* 230400; (Fridovich-Keil and Walter, 2008; Bennett, 2010; Bosch, 2011)).

Infants with classic galactosemia experience acute symptoms within days to weeks of beginning to nurse or drink a milk-based formula. Symptoms can escalate rapidly from vomiting and failure to thrive to cataracts, hepatomegaly, E. coli sepsis, and neonatal death (reviewed in (Fridovich-Keil and Walter, 2008)). Dietary restriction of galactose, generally implemented by switching the infant from milk to a soy-based formula, prevents or resolves the acute symptoms. Unfortunately, despite early and rigorous dietary restriction of galactose, many patients grow to experience intellectual disability, speech difficulties, locomotor impairment, and for girls and women, primary or premature ovarian insufficiency, among other complications. We, and others, have reported that these long-term complications develop regardless of how early treatment is initiated, how rigorously galactose intake is restricted, or how closely patients are followed clinically (Waggoner et al., 1990; Schweitzer-Krantz, 2003; Bosch, 2006; Fridovich-Keil, 2006; Hughes et al., 2009; Jumbo-Lucioni et al., 2012).

Despite decades of research, there is still no clear understanding of the pathophysiology that underlies either the acute or long-term complications of classic galactosemia (Tyfield and Walter, 2002; Leslie, 2003; Fridovich-Keil and Walter, 2008); however, a number of intriguing hypotheses have been put forward (reviewed in (Tyfield and Walter, 2002; Leslie, 2003; Fridovich-Keil and Walter, 2008)). These include ATP depletion via futile cycles of phosphorylation and dephosphorylation of galactose (Mayes and Miller, 1973), inhibition of key enzymes by galactose-1-phosphate (gal-1P) (Wells et al., 1969; Gitzelmann, 1995; Parthasarathy et al., 1997; Bhat, 2003), and depleted UDP-gal leading to impaired galactosylation of cerebrosides (Lebea and Pretorius, 2005).

Until recently, studies to explore factors contributing to pathophysiology in classic galactosemia have been limited by the lack of a genetic animal model that recapitulates the patient outcome. Nonetheless, numerous studies have been reported using so-called "experimental" animal models -- genetically normal animals exposed to high levels of dietary galactose -- to explore the impact of galactose on animal physiology. These experimental mouse (Wei et al., 2005; Cui et al., 2006; Long et al., 2007), and *Drosophila melanogaster* (Jordens et al., 1999; Cui et al., 2004) models have provided compelling evidence that D-galactose exposure decreases lifespan, and that this effect is galactose specific (Jordens et al., 1999). High level galactose exposure of genetically normal mice and/or dogs has also been associated with negative long-term outcomes that include neurodegeneration, and cognitive disability (Shang YZ, 2001; Shen et al., 2002), diminished immune response (Song et al., 1999; Shang YZ, 2001), and retinal degeneration (Engerman and Kern, 1984). Reports on D-galactose treated rodents (Yelinova et al., 1996; Kowluru et al., 1997) and flies (Cui et al., 2004) suggest that galactose metabolism in these systems leads to oxidative stress, and the resulting oxidative damage accounts for the life-shortening effect of the exposure. Galactose-dependent free radical generation observed in rat brain homogenates was also reversible after antioxidant administration (Tsakiris et al., 2005).

Paradoxically, despite heightened oxidative stress biomarkers, galactose-treated rodents, flies, and tissue culture cells studied also demonstrated evidence of lower than expected antioxidant enzyme activities (Cui et al., 2004; Cui et al., 2006) suggesting that the normal defenses might be compromised; no mechanism has been established. Of note, anecdotal studies have shown that galactosemic patients on poor dietary control also displayed lower total antioxidant status along with remarkably increased markers of oxidative stress (Schulpis et al., 2005; Schulpis et al., 2006).Recently, we established a *Drosophila melanogaster* genetic model of classic galactosemia that recapitulates significant aspects of the patient phenotype (Kushner et al., 2010). Like patients, GALT-null *Drosophila* succumb in development following galactose exposure but survive to adulthood under dietary galactose restriction or when rescued by expression of a wild-type human *GALT* transgene (Kushner et al., 2010). Also like patients, GALT-null flies, but not controls, accumulate significantly elevated levels of gal-1P following exposure to galactose (Kushner et al., 2010). Here we have tested whether oxidative stress contributes to the acute galactose sensitivity of GALT-null *Drosophila*. Our approach was three-fold.

First, we tested the impact of dietary oxidants and anti-oxidants on the survival rates of GALT-null and control *Drosophila* exposed to galactose in development. Second, we monitored biochemical markers of oxidative stress response, including reduced and oxidized glutathione and cysteine intermediates, in representative samples. Finally, we tested the expression levels of two genes responsive to oxidative stress, *GSTD6* and *GSTE7*, in GALT-null and control larvae after acute exposure to galactose. Our results implicate oxidative stress in the mechanism of galactose-toxicity in GALT-deficient *Drosophila*, and raise the intriguing possibility that oxidative stress might also play a role in the acute pathophysiology of classic galactosemia.

RESULTS

Oxidants paraquat and DMSO increase the acute galactose-sensitivity of GALT-null *Drosophila*

We tested the impact of two oxidants, paraquat and DMSO, on the acute galactose-sensitivity of GALT-null *Drosophila* by adding each compound at selected doses to vials of fly food either with or without galactose. Of note, all fly food also contained 555 mM glucose. To select an appropriate dose of galactose for these experiments, we first tested the relationship between galactose concentration in the food and survival of mutant and control *Drosophila* to adulthood under the conditions to be used here (see Methods). We saw a clear dose-dependent negative impact of galactose on survival of the mutant but not the control animals (Supplemental Figure 1), and selected 200 mM galactose as the "optimal" dose for further experiments because the survival impact on GALT-null animals was robust but survival rates were not so low as to prevent us from seeing a potential further negative impact from other factors (e.g. dietary oxidants).

To test the impact of paraquat and DMSO we monitored the survival rates of mutant vs. control *Drosophila* deposited in fixed numbers as first-instar larvae (L1) into replicate vials containing fly food that either did or did not include 200 mM galactose, and that also either did or did not contain specified levels of paraquat or DMSO (see Methods). Of note, only oxidant levels that had no significant impact on survival rates of control animals were pursued.

For paraquat these levels included 0, 50, 100, and 200 μM; and while these levels had no significant impact on the survival rates of control larvae regardless of the presence or absence of galactose (open bars in Figure 1, panels A and B, and Supplemental Figure 2, panels A and B), there was a marked impact on the survival rates of GALT-null larvae in the presence of galactose (shaded bars in Figure 1, panels A and B). Specifically, the three increasing levels of paraquat

decreased the survival rates of GALT-null animals to pupation in galactose-supplemented food (Figure 1, panel A) by ~24%, ~37%, and 58%, respectively, and to adulthood (Figure 1, panel B) by ~46%, ~55%, and ~73%, respectively. These differences were statistically significant, as indicated in Figure 1. Also as indicated (shaded bars in Supplemental Figure 2, panels A and B), there was no apparent impact on the survival of animals maintained on food that did not contain galactose.

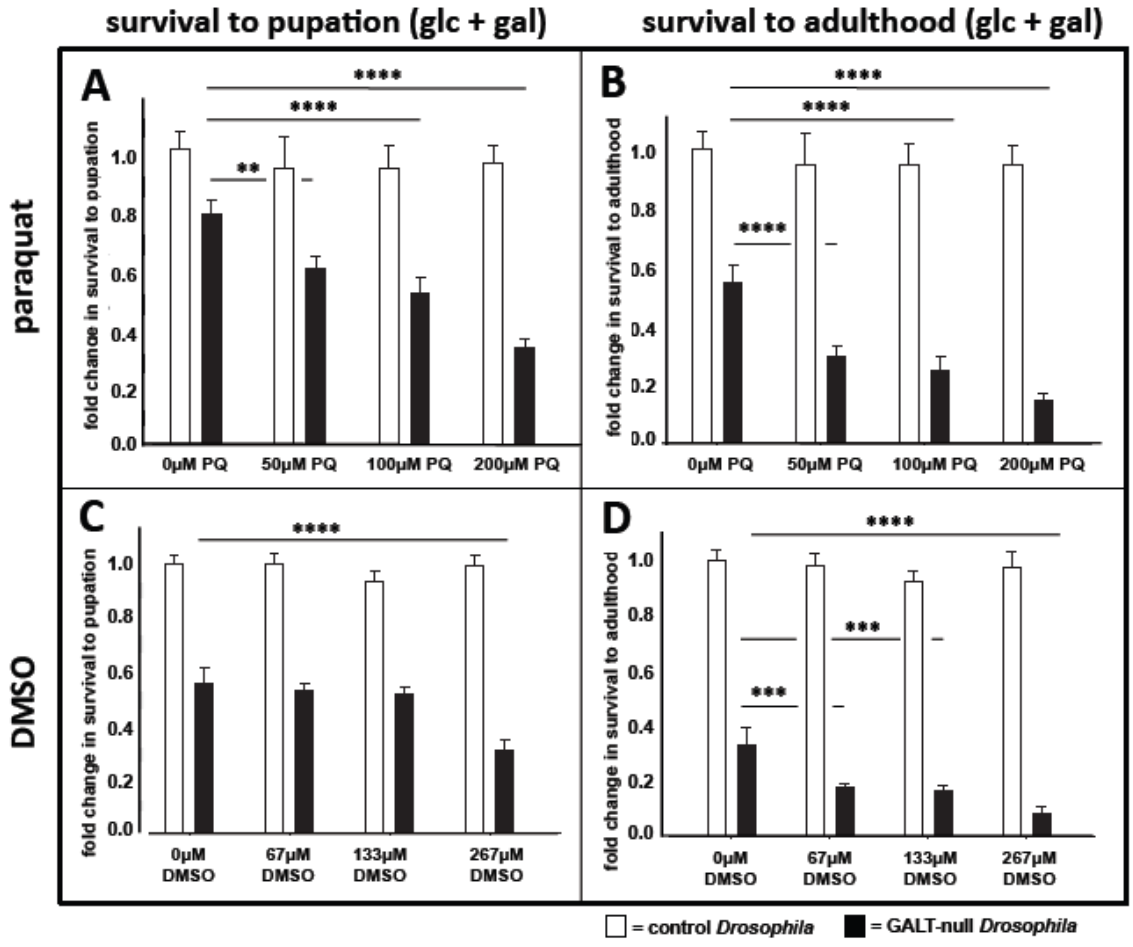


Figure 1: Effects of oxidants on survival of control and GALT-null *Drosophila* exposed to galactose

Survival of control (open bars) and GALT-null (shaded bars) *Drosophila* larvae was monitored to pupation (**A** and **C**) and to adulthood (**B** and **D**) under the conditions listed. Significant differences are denoted as: * $p<0.05$, ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Corresponding data for animals raised in the absence of galactose are presented in Supplemental Figure 2.

For DMSO, the levels tested were 0, 67, 133, and 267 μM, and as for paraquat, in the presence of galactose we saw a dose-dependent negative impact on survival of the mutant but not the control animals to adulthood (Figure 1, panels C and D). In the absence of galactose (Supplemental Figure 2, panels C and D), most vials showed unaffected survival rates although DMSO supplementation at 267 μM did decrease the survival of GALT-null *Drosophila* to pupation and eclosion by just over 10% compared to controls.

Anti-oxidants vitamin C and α-mangostin are protective against the acute galactose-sensitivity of GALT-null *Drosophila*

We also tested the impact of two anti-oxidants, vitamin C (ascorbate, (Rose and Bode, 1993; Duarte and Lunec, 2005)) and α-mangostin (Bumrungpert et al., 2010), on the survival of GALT-null and control *Drosophila* larvae deposited on fly food containing either glucose or glucose + galactose (see Methods). As with the oxidant exposures, these experiments were conducted using levels of antioxidant (20, 40, and 80 μM for vitamin C and 40, 120, and 360 μM for α-mangostin) that had no significant impact on the survival rates of control animals regardless of sugar exposure (Figure 2 and Supplemental Figure 3). Unlike controls, which demonstrated no marked response (Figure 2 and Supplemental Figure 3, open bars), we found a significant positive impact of anti-oxidant treatment on the survival rates of GALT-null larvae exposed to galactose (Figure 2, shaded bars). In brief, the addition of vitamin C at 80 μM significantly ($p=0.0022$) rescued the survival of GALT-deficient larvae to pupation (~33% increase, Figure 2 Panel A), and at both 40 and 80 μM vitamin C produced a significant ($p<0.0001$) and dose-dependent increase in the survival rates of mutant larvae to adulthood (~77% and ~127% increases, respectively, Figure 2 panel B). Similarly, all doses of α-mangostin tested significantly ($p<0.0001$) increased the survival rates of galactose-exposed mutant larvae to pupation (Figure 2 panel C), and the one

dose (40 μM) that showed the strongest impact on survival to pupation also significantly (p=0.0026) increased the survival of mutant animals to adulthood (~87% increase, Figure 2 panel D). None of the vitamin C or α-mangostin doses tested significantly impacted the survival rates of either mutant or control larvae in the absence of galactose (Supplemental Figure 3).

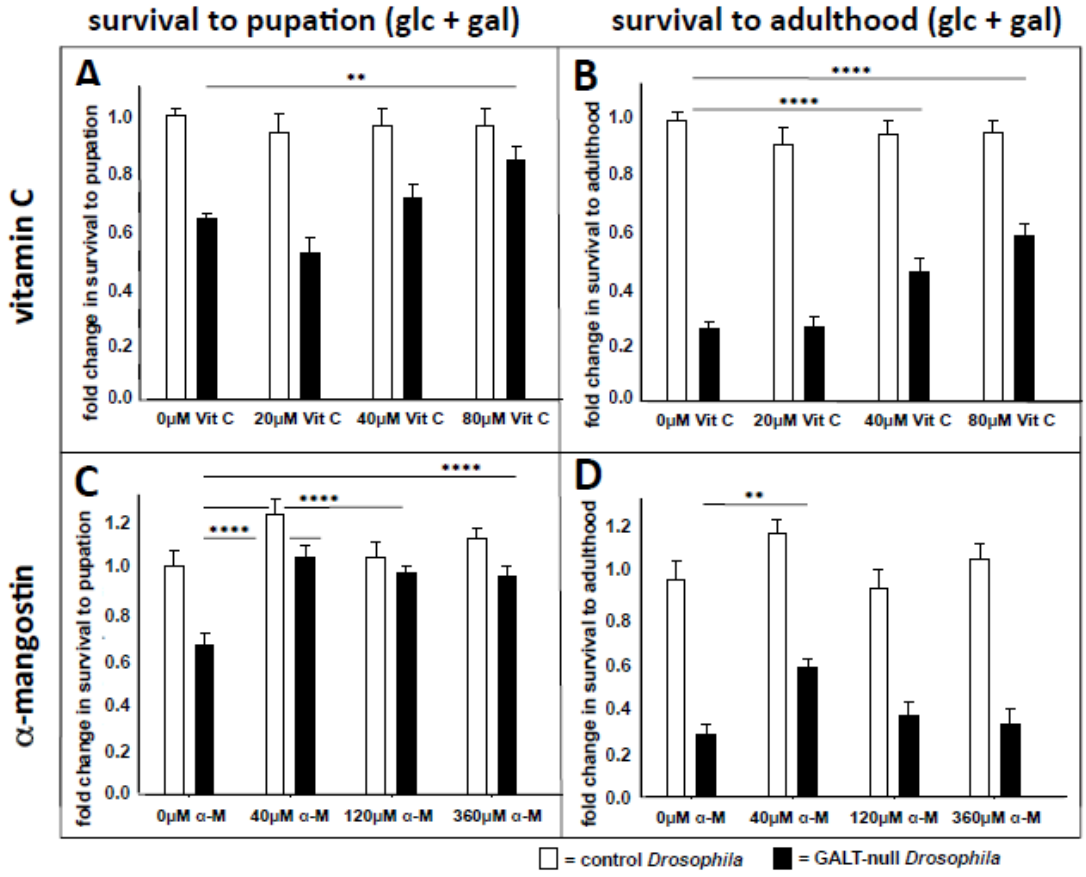


Figure 2: Effects of anti-oxidants on survival of control and GALT-null *Drosophila* exposed to galactose

Survival of control (open bars) and GALT-null (shaded bars) *Drosophila* larvae was monitored to pupation (**A** and **C**) and to adulthood (**B** and **D**) under the conditions listed. Significant differences are denoted as: * p<0.05, **p<0.01; ***p<0.001; ****p<0.0001. Corresponding data for animals raised in the absence of galactose are presented in Supplemental Figure 3.

Gal-1P accumulation in GALT-null *Drosophila* is unaffected by oxidant or anti-oxidant exposures

Accumulation of gal-1P is a common marker of impaired Leloir function in patients and model systems, and all factors reported to date that relieve the lethal or growth-inhibitory effects of galactose exposure in the face of impaired GALT function have done so apparently by lowering the accumulation of gal-1P (Douglas and Hawthorne, 1964; Mehta et al., 1999; Kabir et al., 2000; Lai and Elsas, 2000; Ross et al., 2004). We therefore sought to test whether oxidants or anti-oxidants might also impact galactose sensitivity by modulating the accumulation of gal-1P in our GALT-null *Drosophila*. Toward this end, we extracted and quantified gal-1P from control and mutant third-instar (L3) larvae that had been exposed to food containing glucose or glucose + galactose, with or without 100 μM paraquat or 80 μM vitamin C (see Methods).

As expected, when raised on food lacking galactose (Figure 3, panel A) control animals (open bars) accumulated only trace levels of gal-1P and GALT-null animals (solid bars) accumulated levels that were notably higher, likely reflecting the endogenous biosynthesis of galactose (Berry et al., 1995). In the presence of dietary galactose, GALT-null larvae accumulated levels of gal-1P that were more that 30-fold higher than those seen in their GALT-normal counterparts (Figure 3, panel B). What was most striking, however, was that the mutant larvae demonstrated the same extremely high levels of gal-1P regardless of the presence or absence of either vitamin C or paraquat (Figure 3 panel B). In short, the marked impacts of paraquat and vitamin C on survival of galactose-exposed GALT-null *Drosophila* were not explained by changes in the levels of gal-1P that accumulated in those animals.

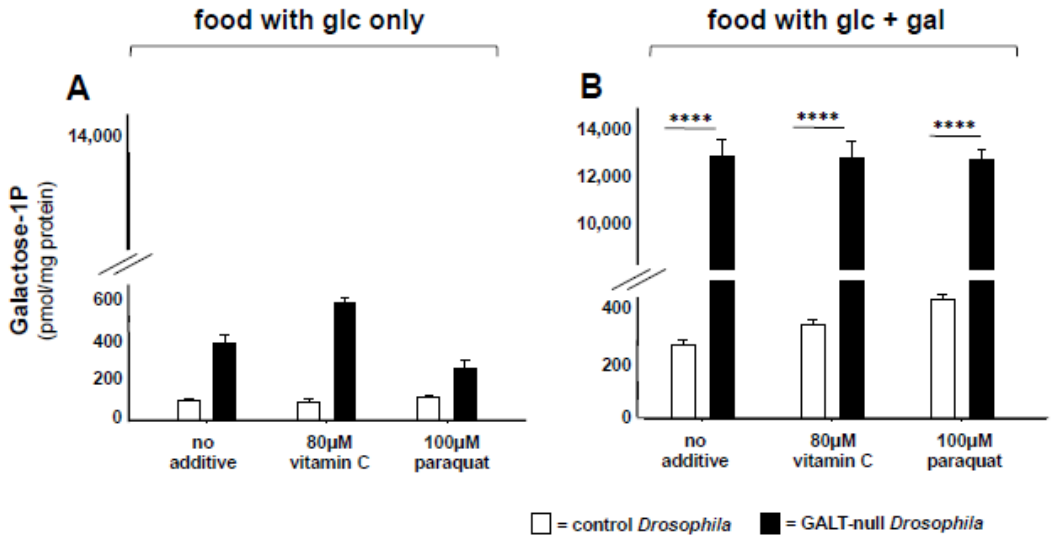


Figure 3: Impact of vitamin C and paraquat on the accumulation of gal-1P in control and GALT-null *Drosophila* maintained in the absence and presence of galactose

Gal-1P was extracted from control (open bars) and GALT-null (shaded bars) larvae maintained under the conditions listed. (**A**) Gal-1P values in animals maintained on food lacking galactose, and (**B**) gal-1P values in animals maintained on food including galactose. Significant differences are denoted as: * p<0.05, **p<0.01; ***p<0.001; ****p<0.0001.

Impact of paraquat and vitamin C on oxidized and reduced glutathione and cysteine levels in GALT-null *Drosophila* exposed to galactose

As a biochemical approach to explore the impact of galactose exposure on oxidative stress in control and GALT-null *Drosophila* we monitored the levels of reduced and oxidized glutathione (GSH and GSSG, respectively) and cysteine (Cys and CySS, respectively) in control and mutant larvae exposed to galactose. We also used the ratios of these reduced and oxidized moieties to estimate intracellular and extracellular redox potentials (E_h), respectively. As before, parallel cohorts of mutant and control larvae were exposed to food containing either glucose or glucose + galactose supplemented either with no additive, with 100 μM paraquat, or with 80 μM vitamin C, as described in Methods.

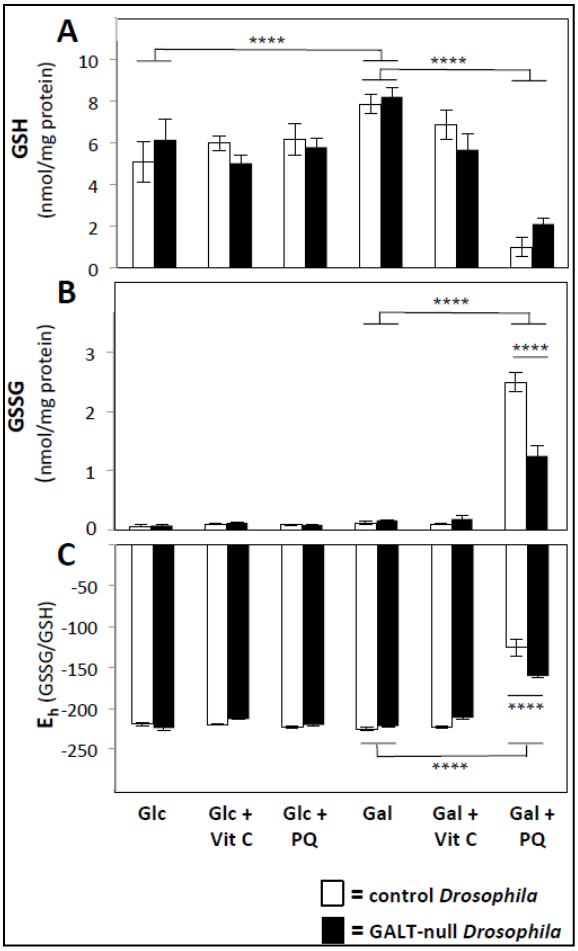


Figure 4: Impact of vitamin C (Vit C) and paraquat (PQ) on the levels of reduced and oxidized glutathione in control and GALT-null *Drosophila* maintained on glucose vs. glucose + galactose food

Samples tested were from control animals (open bars) or GALT-null animals (shaded bars). (**A**) Levels of reduced glutathione (GSH), (**B**) levels of oxidized glutathione (GSSG), (**C**) intracellular redox state estimated from the GSH and GSSG levels. Significant differences are denoted as:

* $p<0.05$, **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Galactose supplementation alone produced a small but significant ($p<0.0001$) increase in GSH levels in both mutant and control animals (Figure 4 panel A); this increase reverted to near normal levels in the presence of vitamin C (Figure 4 panel A). Interestingly, paraquat exposure of both genotypes in the presence of galactose dramatically decreased GSH levels (Figure 4 panel A). The converse was true for oxidized glutathione (GSSG); paraquat exposure in the presence of galactose caused a marked increase ($p<0.0001$) in GSSG levels in both mutant and control animals, but the magnitude of the increase for mutants was only about half that seen for controls (Figure 4 panel B). Multivariate analysis of variance (MANOVA) also revealed a significant ($p<0.0001$) genotype by diet by treatment interaction for intracellular redox state (E_h). Specifically, paraquat exposure in the presence of galactose caused a significant increase in

intracellular E_h for both mutant and control animals ($p<0.0001$), but the magnitude of the change was diminished for mutants relative to controls (Figure 4 panel C). **Notably, i**n the absence of galactose (first three sets of bars in each panel) we saw no significant impact of either vitamin C or paraquat on GSH, GSSG, or intracellular redox state (E_h) in mutants or controls (Figure 4 panels A, B, C).

MANOVA also revealed a significant ($p<0.0001$) interaction between GALT genotype, diet, and exposure to vitamin C or paraquat for cysteine (Cys, $p<0.05$), cystine (CySS, $p<0.02$), and Cys-GSH (a disulfide intermediate of glutathione metabolism, Figure 5). Specifically, galactose exposure alone triggered a small but significant ($p=0.0004$) increase in Cys level in control but not mutant animals (Figure 5 panel A), and this increase was largely prevented by vitamin C. We saw no significant difference in Cys levels between mutant and control animals raised in the absence of galactose (Figure 5 panel A, first three sets of bars). In the presence of galactose, however, there was a >5-fold decrease in Cys levels observed in both mutant and control larvae exposed to paraquat as compared to all other conditions ($p<0.0001$, Figure 5 panel A). This change was accompanied by a commensurate rise in CySS in both mutant and control animals ($p<0.0001$, Figure 5 panel B), but as with GSSG, the magnitude of the increase for mutants was less than that seen for controls ($p<0.0001$, Figure 5 panel B). Extracellular redox potential (E_h), calculated from the levels of Cys and CySS, was also affected by diet and treatment ($p<0.0001$), with both mutant and control larvae showing a significant ~2-fold increase when maintained on food supplemented with both galactose and paraquat (Figure 5 panel C). Strikingly, the disulfide Cys-GSH also increased more than 10-fold in both mutant and control animals exposed to the combination of paraquat and galactose ($p<0.0001$) and again the levels in mutants were significantly lower than those seen in controls ($p<0.0001$; Figure 5 panel D).

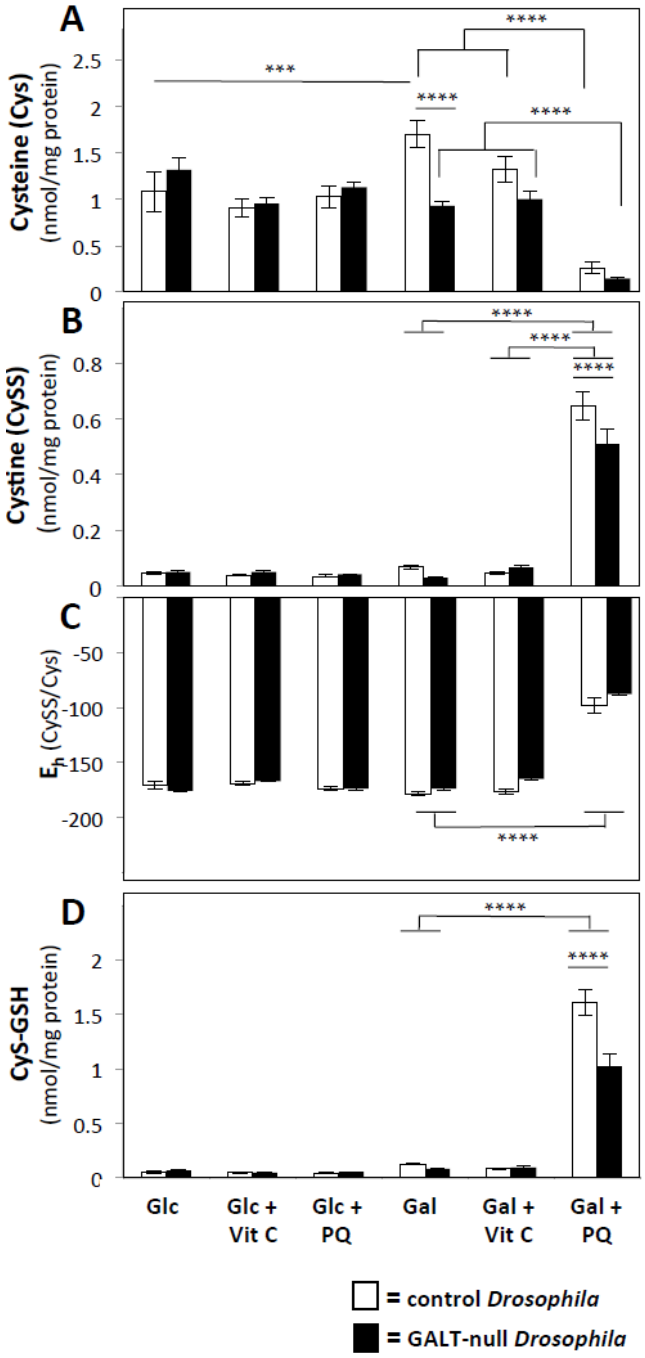


Figure 5: Impact of vitamin C (Vit C) and paraquat (PQ) on the levels of reduced and oxidized cysteine in control and GALT-null *Drosophila* maintained on glucose vs. glucose + galactose food

Samples tested were from control animals (open bars) or GALT-null animals (shaded bars). (**A**) Levels of reduced cysteine (Cys), (**B**) levels of oxidized cystine (CySS), (**C**) extracellular redox state estimated from the Cys and CySS levels, and (**D**) CyS-GSH levels. Significant differences are denoted as: * $p<0.05$, **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Finally, we compared the levels of total glutathione (GSH+GSSG) and total cysteine (Cys+CySS) in lysates prepared from mutant and control larvae exposed to food containing either glucose or glucose + galactose with no additive, with 80 µM vitamin C, or with 100 µM paraquat. Galactose exposure alone slightly increased the level of total glutathione in both mutants and

controls ($p= 0.0003$); this increase was prevented by vitamin C (Figure 6 panel A). Total glutathione was decreased by about a factor of two in both mutants and controls exposed to galactose + paraquat as compared with galactose alone; this difference was highly significant ($p<0.0001$, Figure 6 panel A). In contrast, total cysteine revealed a differential impact of galactose + paraquat on mutants and controls. Specifically, galactose exposure alone resulted in a 60% increase in the total cysteine level in controls ($p=0.0005$) but not mutants; total cysteine tended to decrease in these animals in the presence of vitamin C (Figure 6 panel B), but the effect was not statistically significant. Similarly, galactose + paraquat exposure resulted in a 33% increase in total cysteine relative to galactose alone for controls, and a 61% increase relative to galactose alone for mutants (Figure 6 panel B). Despite this difference, control animals exhibited significantly higher total cysteine levels compared to mutants when exposed to galactose + paraquat ($p=0.0003$; Figure 6, panel B). There were no significant changes in total glutathione or cysteine for either mutant or control animals in the absence of galactose (Figure 6, first three sets of bars in both panels).

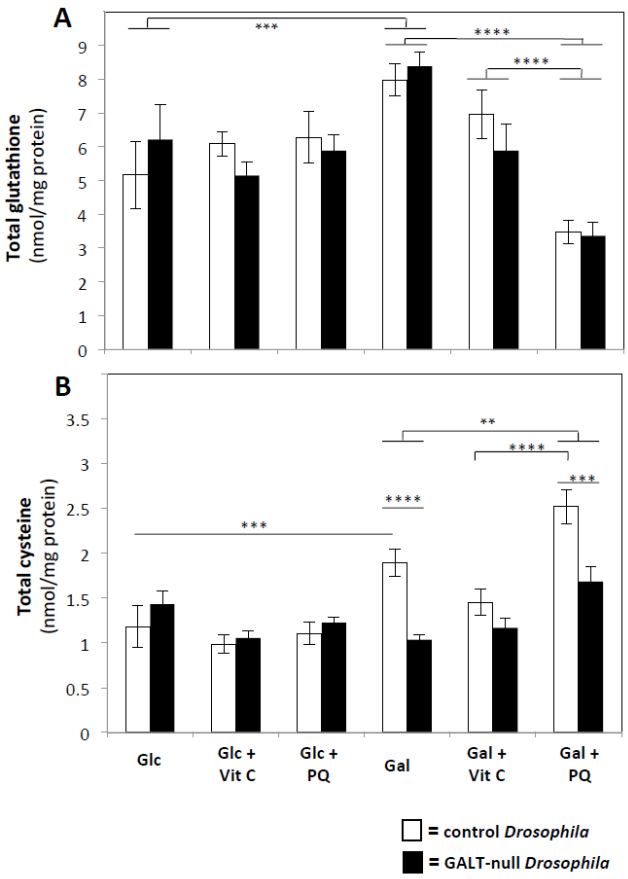


Figure 6: Impact of vitamin C (Vit C) and paraquat (PQ) on the levels of total glutathione and total cysteine in control and GALT-null *Drosophila* maintained on glucose vs. glucose + galactose food

Samples tested were from control animals (open bars) or GALT-null animals (shaded bars). (**A**) Total glutathione (GSH + GSSG) and (**B**) total cysteine (Cys + CySS + CyS-GSH). Significant differences are denoted as: * $p<0.05$, ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Drosophila **larvae exposed to galactose show a striking induction of genes responsive to oxidative stress**

As a final approach to test whether galactose exposure causes oxidative stress in *Drosophila*, we used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to monitor the expression levels of two genes, *GSTD6* and *GSTE7*, both involved in glutathione metabolism and known to function in oxidative stress response (Alias and Clark, 2007; Li et al., 2008), and also a housekeeping gene (*ACT5C*) that encodes actin. We quantified the levels of all three transcripts in cohorts of mutant and control larvae maintained under glucose vs. glucose + galactose conditions (see Methods). Of note, all of the larvae were between 48 and 52 hours old at the time of harvest, and the period of galactose exposure was limited to the final 12 hours of life so that even the

GALT-null larvae were still very much alive at the time of harvest. Both mutant and control larvae exposed to galactose demonstrated dramatic increases ($p<0.0001$) in the expression levels of both *GST* genes relative to *ACT5C* (Table 1). Specifically, *GSTE7* was induced by galactose exposure >5-fold in controls and >8-fold in GALT-null larvae, and *GSTD6* was induced by galactose exposure >40-fold in controls and >80-fold in GALT-null larvae (Table 1).

	expression level relative to *ACT5C*	
	GSTD6	***GSTE7***
control larvae (no galactose exposure)	0.344 ± 0.052	0.591 ± 0.114
control larvae (12 hrs galactose exposure)	14.794 ± 0.456 (>40-fold)	3.122 ± 0.195 (>5-fold)
GALT-null larvae (no galactose exposure)	0.354 ± 0.058	0.456 ± 0.131
GALT-null larvae (12 hrs galactose exposure)	29.467 ± 1.189 (>80-fold)	3.885 ± 1.004 (>8-fold)

Table 1: Impact of 12 hours of galactose exposure on the expression levels of genes in GALT-null and control *Drosophila* larvae.

GSTD6 and *GSTE7* each encode proteins (glutathione S-transferase) responsive to oxidative stress; *ACT5C* encodes a housekeeping protein (actin). Values presented are mean ± SEM (n=3). The fold change in expression level for each gene attributable to the galactose exposure is indicated in parenthesis in each relevant table cell; each of these changes was statistically significant at $p<0.0001$.

DISCUSSION

The underlying basis of pathophysiology in classic galactosemia has remained a mystery for more than fifty years; the work described here brings us one important step closer to unraveling that mystery. Using a *Drosophila melanogaster* genetic model of classic galactosemia we have asked whether oxidative stress and response contribute to the mechanism of acute galactose-toxicity in GALT-deficiency; our results provide compelling evidence that the answer is yes.

The hypothesis tested here was based on a preponderance of evidence amassed over decades from studies of genetically wild-type animals exposed to high levels of galactose in what have been called "experimental" models of galactosemia (Yelinova et al., 1996; Jordens et al., 1999;

Ho et al., 2003; Wei et al., 2005; Cui et al., 2006; Long et al., 2007). Collectively, these studies demonstrated that exposure to high levels of galactose result in accelerated aging and decreased lifespan as a result of oxidative stress and damage. This result was specific to galactose, and paradoxically, while galactose exposure caused oxidative stress, it also compromised antioxidant defenses, leaving the organism especially vulnerable to damage (Cui et al., 2004). Here we used genetically modified GALT-null animals exposed to biologically relevant levels of dietary galactose (e.g. 25% of the monosaccharide present) to test the potential role(s) of oxidative stress response in galactose-sensitivity. Our approach was three-fold.

First, we quantified the impact of two oxidants and two anti-oxidants on survival of control and GALT-null (mutant) *Drosophila* raised from an early larval stage on food containing either glucose as the only sugar or glucose + galactose. The oxidants we used were DMSO and paraquat; the antioxidants were vitamin C and α-mangostin. DMSO, commonly used as a solvent and in cryogenic preservation of mammalian cells, is also a potent oxidant that leads to the formation of superoxide anion when exposed to air (oxygen) and hydroxide (OH^-) (Hyland and Auclair, 1981). Paraquat (1,1'dimethyl-4-4'-bipyridynium dichloride) has long been used as a pesticide; its toxicity derives from the generation of superoxide anions, and the oxidation of the NADPH pool with the subsequent disruption of biochemical processes requiring NADPH (Bonilla et al., 2006). Vitamin C (ascorbate) is regarded as a potent antioxidant, capable of scavenging a wide array of reactive oxygen and nitrogen radicals, and particularly protective of DNA and low-density lipoproteins (Rose and Bode, 1993; Duarte and Lunec, 2005). Vitamin C also has the ability to recycle other cellular antioxidant defenses, such as glutathione, from their respective free radical forms (Duarte and Lunec, 2005). Finally, α-mangostin is one of the major xanthones found in the tropical fruit mangosteen (Bumrungpert et al., 2010; Larson et al., 2010). Potent antioxidant (Williams et al., 1995; Jung et al., 2006; Martinez et al., 2011) and anti-inflammatory potentials (Udani et al., 2009; Bumrungpert et al., 2010) have been ascribed to this

compound. Interestingly, α-mangostin is able to scavenge singlet oxygen, superoxide and peroxynitrite anions, but not hydroxyl radicals or hydrogen peroxide under in vitro conditions (Pedraza-Chaverri et al., 2009). Other studies (Williams et al., 1995) suggest that α-mangostin enhances the initial free radical scavenging potential and prolongs the early resistance to oxidative stress until all antioxidants are exhausted.

From our oxidant and antioxidant experiments we observed a clear pattern: both oxidants exacerbated the galactose sensitivity of GALT-null but not the control *Drosophila*, and conversely, both antioxidants had a protective effect. Of note, α-mangostin's ability to enable galactose-challenged GALT-null larvae to survive to pupation (Figure 2 panel C) but not always to adulthood (Figure 2 panel D) may reflect the reported strong initial but not sustained antioxidant impact of this compound (Williams et al., 1995).

Next, we quantified biochemical markers of oxidative stress response in mutant and control larvae maintained on glucose-only or glucose + galactose food and exposed either to no additive, to vitamin C, or to paraquat. The markers tested included oxidized and reduced glutathione and cysteine, and again a pattern was clear: galactose and paraquat synergized to create heightened markers of oxidative stress in both mutant and control animals. However, there were also notable quantitative differences evident in the responses of mutants and controls to oxidative stress, namely, in many instances mutants showed a significantly diminished response relative to controls. The implications for mechanism are discussed below.

Finally, we used quantitative RT-PCR to monitor the expression levels of two genes known to function in response to oxidative stress, *GSTD6* and *GSTE7* (Alias and Clark, 2007; Li et al., 2008). We tested RNA levels in both mutant and control larvae maintained in either the presence and absence of galactose for a short window of time (12 hours). The levels of both *GST* genes were induced dramatically and differentially by galactose exposure in both control and mutant

animals; for *GSTE7* the induction was >5-fold for controls and >8-fold for mutants, and for *GSTD6* the induction was >40 fold for controls and >80-fold for mutants. Combined, these data provide compelling evidence that galactose exposure leads to oxidative stress in both GALT-null and control *Drosophila*, but that GALT-null larvae respond differently in some way that leaves them unusually vulnerable to the stress.

Implications for mechanism: The data presented here raise two important and distinct points with regard to mechanism. First, galactose exposure both causes oxidative stress and sensitizes to other sources of oxidative stress. Second, while GALT-null and control *Drosophila* clearly both experience oxidative stress as a result of galactose exposure, the impacts of that stress may differ -- qualitatively and quantitatively. For example, the survival rates of galactose-exposed mutant animals are dramatically affected by the presence of other oxidants and antioxidants; the survival rates of control animals are not. In isolation, these data could suggest that galactose exposure causes higher oxidative stress in mutants than in controls, but combined with the biochemical data presented here, the difference may be in how the animals respond to oxidative stress rather than in the level of stress itself.

The observation that both oxidants and antioxidants impact the survival of GALT-null *Drosophila* exposed to galactose without substantially impacting the levels of gal-1P is also important because it implies either that the oxidant and antioxidant modifiers each act downstream of gal-1P in the ostensible pathway of galactose toxicity, or alternatively, that each acts independently of it. Either way, these data run counter to the common assumption that gal-1P accumulation is central to the negative outcomes associated with classic galactosemia (Pesce and Bodourian, 1982; Gitzelmann and Steinmann, 1984; Gitzelmann, 1995). Of note, the oxidant and antioxidant modifiers of acute outcome described here represent the first modifiers of

outcome in GALT-deficiency that do not appear to work by either preventing the synthesis of gal-1P or promoting its catabolism (Wierenga et al., 2008; Boxer et al., 2010; Tang et al., 2011). It is important to note that the data presented here do not address the question of whether oxidative stress may also contribute to long-term, apparently galactose-independent outcomes in GALT-deficient flies; that question will be a focus of future attention.

Why might galactose exposure promote oxidative stress in GALT-deficient Drosophila? The results presented here confirm that galactose exposure leads to oxidative stress, and demonstrate that GALT-null animals show heightened sensitivity to that stress. But why? A number of possibilities exist. For example, the production of ATP via metabolism of galactose is by definition a more indirect process than is the production of ATP via glycolysis of glucose, because to be fully metabolized galactose must first be "converted" into glc-1P by the Leloir pathway. This reality might lead cells to rely more heavily on mitochondrial oxidative phosphorylation to produce energy (Aguer et al., 2011). Indeed, studies from yeast demonstrate that "Leloir competent" yeast consume substantially more oxygen when cultured in medium containing galactose as the carbon source than when cultured in medium containing glucose as the carbon source (De Deken, 1966). Further, as has been suggested (Obrosova et al., 1997), accumulated gal-1P might inhibit key glycolytic enzymes such as phosphoglucomutase (Gitzelmann, 1995), and futile cycles of phosphorylation and dephosphorylation of galactose (Mayes and Miller, 1973) might further deplete ATP stocks, putting increased energy strain on cells. Again, this could stress mitochondrial function, which could potentially both lead to and sensitize to oxidative stress. That paraquat and vitamin C both alter survival rates of galactose-exposed GALT-null Drosophila without altering their gal-1P levels suggests that the gal-1P level might not be what is important here; this favors the dynamic phosphorylation/ dephosphorylation of galactose hypothesis, though it does not rule out the possibility that gal-1P might also inhibit

key enzymes, exacerbating the problem. Future studies, with *Drosophila* and other model systems, will be required to distinguish between the possibilities to explain the mechanism(s) behind the observations reported here.

Implications for patients: The studies presented here were performed using control and GALT-null *Drosophila* and therefore the results may or may not translate to the human condition. That said, anecdotal studies have shown that galactosemic patients on poor dietary control display increased markers of oxidative stress yet lower total antioxidant status (Schulpis et al., 2005; Schulpis et al., 2006). Considering that antioxidant supplements (e.g. vitamin C) and supplements designed to improve mitochondrial function (e.g. creatine) are apparently well-tolerated, at least in healthy people, it is tempting to speculate whether such supplements might prove beneficial for patients with classic galactosemia.

METHODS

Drosophila stocks and maintenance

We used two excision alleles of *Drosophila melanogaster GALT*, $dGALT^{\Delta AP2}$ and $dGALT^{C2}$, generated by mobilizing an existing SUPor-P insertion in the 5'-UTR of the *CG9232* locus (KG00049) as previously detailed (Kushner et al., 2010). The $dGALT^{\Delta AP2}$ allele carries a 1647bp deletion that removes almost the entire *dGALT* gene; flies homozygous for this allele demonstrate no detectable GALT activity. In contrast, the $dGALT^{C2}$ allele carries a precise excision of the P element and flies homozygous for this allele demonstrate wild-type GALT activity. Both stocks have been characterized and we have reported previously that flies homozygous for $dGALT^{\Delta AP2}$

mimic aspects of classic galactosemia including a significant galactose-dependent decrease in survival and considerable accumulation of a metabolic intermediate, gal-1P (Kushner et al., 2010).

For this study, fly stocks were maintained at 25°C on molasses-based food that contained 43.5 g/l cornmeal, 17.5 g/l yeast extract, 8.75 g/l agar, 54.7 ml/l molasses, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol). For experiments that measured galactose sensitivity, animals were reared under non-overcrowding conditions on a diet that consisted of 5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 555 mM glucose (Fisher Scientific Co., Pittsburgh, PA), 10 ml/l propionic acid, 14 ml/l tegosept mold inhibitor (10% w/v in ethanol), and the indicated amount of D(+)-galactose (Sigma-Aldrich Corp, St. Louis, MO) measured from a 20% w/v galactose stock solution.

Survival assays

To test the impact of varying dietary exposures on survival of developing *Drosophila* we established the following protocol. First, both $dGALT^{\Delta AP2}$ and $dGALT^{C2}$ were raised under non-overcrowding conditions in parallel on foods containing either glucose-only (555 mM) or glucose (555 mM) + galactose. To control for larval density, parents of the desired genotypes were allowed to mate and deposit embryos for 24 hours on grape juice/agar medium to generate embryo collections. Twenty-four hours later, cohorts of 20 first-instar larvae were collected under the microscope and transferred to replicate 12x55 mm polystyrene vials each containing 2 ml of the appropriate fly food. Each vial was plugged with cotton and maintained under conditions of controlled temperature (25°C) and humidity (60%), and monitored for ~19 days. Over the course of this time, the numbers of pupa and adults were recorded. Ten to 20 replicate vials were monitored for each genotype and condition. Initial studies testing the impact of galactose at 0

mM, 150 mM, 175 mM, 200 mM and 225 mM revealed that 200 mM galactose was the preferred amount (Supplemental Figure 1) as explained in Results.

This same protocol was applied to test the impacts of oxidants and antioxidants on GALT-null and control animals. The additives tested included dimethyl sulfoxide (DMSO, Sigma-Aldrich Corp, St. Louis, MO), paraquat (methyl viologen dichloride hydrate; Sigma-Aldrich Corp, St. Louis, MO), vitamin C (Fisher Scientific Co., Pittsburgh, PA) and α-mangostin (Gaia Chemical Corp., Gaylordsville, CT). These supplements were selected based on their established or predicted roles as oxidants (paraquat and DMSO) or antioxidants (α-mangostin and vitamin C). Different stock solutions of each additive were prepared by dissolving each compound into the appropriate solvent (water for vitamin C and paraquat, DMSO for α-mangostin) so the same volumes of solvent and additive were added to each batch of food. We were careful to avoid exposing supplements to high temperatures or excessive light, as recommended by the manufacturers (e.g. (Naidu, 2003)). For food containing α-mangostin, a comparable amount of DMSO was added to the control food to counter the impact of DMSO alone. Doses for vitamin C and paraquat were selected based on earlier reports in *Drosophila* (Bahadorani et al., 2008; Rzezniczak et al., 2011), while for α-mangostin, which had not been previously studied in flies, we tested a broader range: 40, 120, and 360 μM. Doses for DMSO were selected based on the observed survival rates of animals exposed to DMSO as compared to animals raised in food containing no DMSO.

Of note, survival rates for specific genotypes and food exposures were highly reproducible within experiments, and relative survival rates were also reproducible between experiments, but absolute survival rates sometimes differed between experiments, presumably reflecting the impact of varying cryptic environmental factors such as moisture content of the food. All comparisons described here involved mutant and control cohorts tested side by side in the same experiment, and with experiments replicated by each of two different experimenters.

Metabolite extraction and measurement

Newly-eclosed adult flies were allowed to lay eggs for 5 to 7 days in vials containing 10 ml of glucose-only (555 mM) or glucose (555 mM) + galactose (200 mM) food with and without vitamin C (80 µM) or paraquat (100 µM). Doses of vitamin C and paraquat were selected based on their impact on survival of mutant animals. Cohorts of 20 third instar wandering larvae were collected from appropriate vials. Each cohort was placed into 125 µl of ice-cold high performance liquid chromatography (HPLC) grade water and ground for 15 sec using a Teflon micropestle and handheld motor (Kimble Chase Life Science and Research Products LLC, Vineland, NJ). A sample was taken from each lysate for protein quantification (using the BioRad DC Assay with BSA as a standard). Metabolites were extracted from the remaining lysate as previously described (Ross et al., 2004; Openo et al., 2006). The aqueous phase was dried under vacuum with no heat (Eppendorf Vacufuge). All samples were diluted with HPLC grade water to normalize for protein concentration and then centrifuged through 0.22 µm Costar Spin-X centrifuge tube filters (Corning Inc, Lowell, MA) at 4000 X g for 4 minutes to remove any particulates. The soluble phase from each sample was then transferred to a glass HPLC vial. Metabolites were separated and quantified using a Dionex ICS-2500 Ion Chromatograph fitted with a CarboPac PA10 4x250 mm analytical column as previously described (Ross et al., 2004). At least 5 replicates were tested for each genotype-diet combination.

Measuring oxidized and reduced glutathione and cysteine

Newly-eclosed *dGALT*$^{\Delta AP2}$ and *dGALT*^{C2} flies were allowed to lay eggs for 5 to 7 days in vials containing 10 ml of glucose-only (555 mM) or glucose (555 mM) + galactose (200 mM) fly food with and without vitamin C (80µM) or paraquat (100µM). Doses of vitamin C and paraquat were

selected based on their impact on survival of mutant animals. Cohorts of 30 third instar wandering larvae (~50 mg of fresh tissue) were collected in Eppendorf tubes containing 500 µl ice-cold 50 g/L perchloric acid solution containing 0.2 M boric acid and 10 µM γ-Glu-Glu and placed on ice. Larvae were homogenized for 15 sec using a Teflon micropestle and handheld motor (Kimble Chase Life Science and Research Products LLC, Vineland, NJ), and the homogenate was centrifuged at 14,000 X g for 2 minutes. Aliquots of 300µl of the supernatant were transferred to fresh tubes for further analysis. The remaining supernatant fluid was discarded and the protein pellet was resuspended in 200 µl of 1N NaOH. Ten microliters of this suspension were aliquoted to measure the amount of acid-insoluble protein using the BioRad DC Assay with bovine serum albumin (BSA) as a standard. Samples were stored at -80°C until they were derivatized with 60ul of 7.4mg/ml sodium iodoacetic acid; pH was adjusted to 8.8-9.2 with 1M KOH saturated $K_3B_4O_7$ and 300µl of 20 mg/ml dansyl chloride, followed by incubation in the dark at room temperature for 16-24 hours. Analysis by HPLC with fluorescence detection was performed as previously described (Jones et al., 1998; Miller et al., 2002). Concentrations of thiols and disulfides were determined by integration relative to an internal standard (Jones et al., 2000). Redox potential (E_h) was calculated from the cellular GSH and GSSG concentrations by the Nernst equation as described (Kirlin et al., 1999). Whole-body total cysteine and glutathione levels were calculated by adding all cysteine and glutathione intermediates, respectively.

Statistical analyses

Experiments to determine the relationship between galactose exposure and survival of GALT-null Drosophila were carried out in at least ten replicate vials; two-way ANOVA with genotype and diet as independent variables was used to determine significant differences in survival to adulthood for genotypes $dGALT^{\Delta AP2}$ and $dGALT^{C2}$ raised on food containing 555 mM

glucose plus 0, 150, 175, 200, or 225 mM galactose. Survival rate was calculated as the proportion of animals that survived to adulthood. For each additive tested, we analyzed survival for each diet (i.e. glucose-only and glucose + galactose) separately. For this purpose, we used two-way ANCOVA to compare significant differences in survival to pupation and adulthood for both $dGALT^{\Delta AP2}$ and $dGALT^{C2}$ animals with genotype and treatment as independent variables and with experimenter as covariate. Experiments were performed by two experimenters each loading comparable numbers of replicate vials per treatment group. Survival rate for each replicate was calculated as the fold-change relative to the average survival of control animals raised under control conditions (i.e. no additive). Two-way ANOVA with genotype and treatment group as independent variables was used to compare differences in metabolite accumulation for each diet separately. The interaction of genotype and treatment was tested for each dependent variable. We used multivariate analysis of variance (MANOVA) to determine the significance of differences in the levels of oxidative stress biomarkers for the different "genotype by diet by treatment" groups. In all cases, post-hoc tests were performed on the least-square means to determine differences between groups. The criterion for statistical significance was $p<0.05$ but *p*-values were adjusted for multiple comparisons as applicable. All statistical analyses were performed using SAS (Version 9.2; SAS Institute Cary, NC, USA).

Quantitative RT-PCR

GALT-null and control larvae were prepared for harvest as follows. First, stocks of control and GALT-null flies were allowed to deposit embryos on grape juice agar plates at 25°C for 4 hours, after which the adults were removed and the plates were maintained at 25°C for another 24 hours. Early stage larvae (L1s) from each plate were then picked and transferred to fresh plates

containing fly food with 555 mM glucose. After 12 hours, the larvae were collected by floatation in a 20% glucose solution, rinsed with phosphate buffered saline (PBS), and transferred again, either to a fresh plate with glucose fly food, or to a fresh plate with fly food containing 555 mM glucose + 225mM galactose. After 12 hours on the new food, the larvae were again collected by floatation and stored at -20°C until the RNA was extracted using the RNeasy Mini Kit (Qiagen) as recommended by the manufacturer with DNase digestion performed on the column. The resulting RNA was quantified using a nanodrop 1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit with random hexamers as primers (Applied Biosystem), followed by RNase digestion. The cDNA was then purified using a Qiaquick PCR Purification Kit (Qiagen) and quantified using the nanodrop.

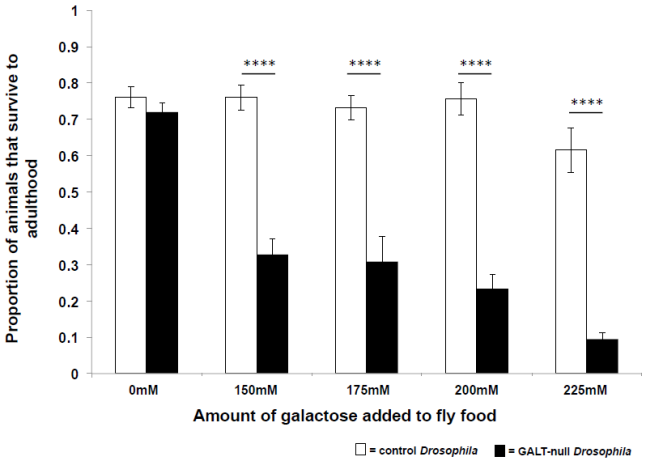
The targets for real time PCR amplification were *Actin5C*, *GSTD6*, and *GSTE7*. The primers for *Actin5C* amplification were actin 5C F (5' GCC CAT CTA CGA GGG TTA TGC 3') and actin 5C R (5' CAA ATC GCG ACC AGC CAG3'), which defined an amplicon of 66bp (Guenin et al., 2010). The primers for *GSTD6* were *dGSTD6* F1 (5’ TCC CCA GAA GCA AGC GCT GA 3’) and *dGSTD6* R1 (5’ GGG TTT GCC CGT CCG AAG CA 3’) which defined an amplicon of 106bp. Finally, the primers for *GSTE7* were *dGSTE7* F1 (5’ ACC TTG GCT GCC CTG GAG GT 3’) and *dGSTE7* R1 (5’ CGT CCT CCA ACG TGG GCA CC 3’) which defined an amplicon of 121bp.

Prior to use each of the primers was verified for specificity using BLAST (NCBI) to look for unintended matches in the *Drosophila melanogaster* genome sequence. Primer sets were also confirmed by the appearance of a single band of the anticipated size following traditional PCR amplification off a cDNA template followed by gel electrophoresis and staining.

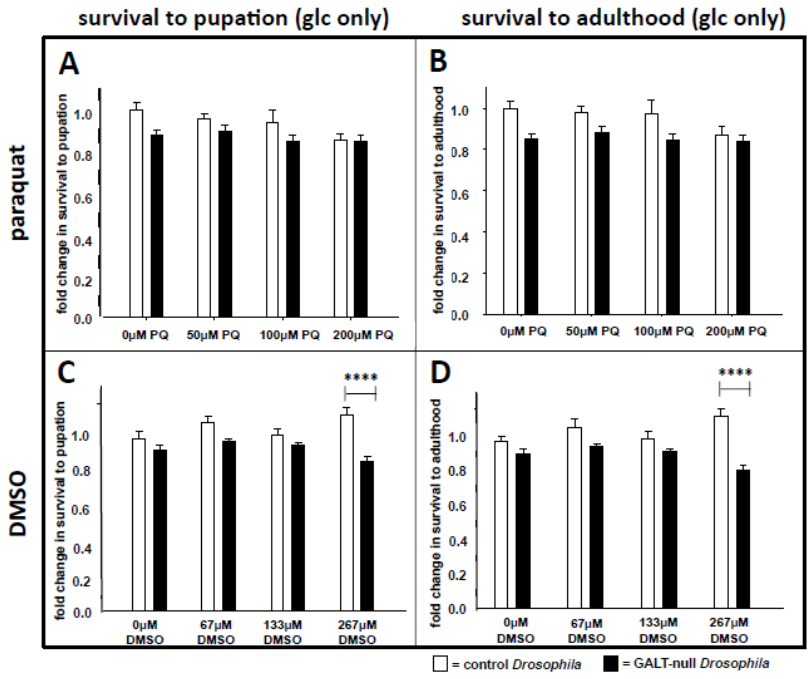
The real time PCR was performed using the Lightcycler® 480 SYBR green I Master Kit (Roche) in 20 μL reactions. The reactions were set up in 96 well plates covered with optical tape

(Genesee Scientific). The amplification was performed on a CFX96™ Real Time System (Bio-Rad). The cycling conditions for the real time PCR were: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 92°C for 10s, 55°C for 20s, and 68°C for 10s. This was followed by a melting curve analysis from 65°C to 95°C at 0.5°C increments to confirm the amplification of single products. Statistical significance was determined by two-way ANOVA with genotype and diet as independent variables.

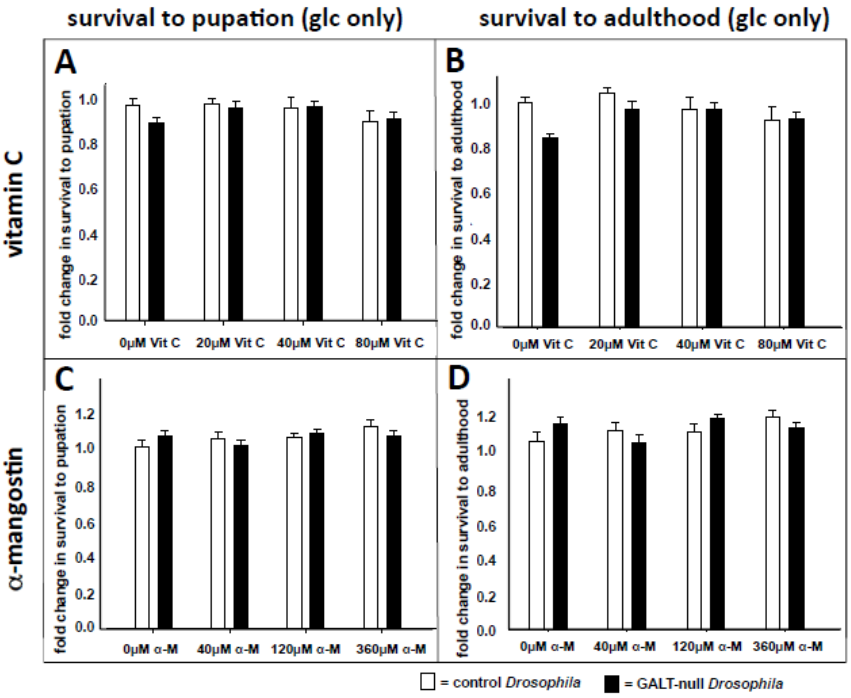
Supplementary Data



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

Acknowledgments

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Author contributions

PP Jumbo-Lucioni and M Hopson performed or contributed to all experiments except those involving qRT-PCR (Table 1); Y Liang and DP Jones quantified the levels of oxidized and reduced glutathione and cysteine in samples provided by PPJ-L and MH and interpreted those data; D Hang performed and interpreted the experiments involving qRT-PCR. JL Fridovich-Keil and PP Jumbo-Lucioni conceived of and directed the project, and all authors contributed to writing and editing the manuscript.

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Chapter 4

The expression levels of *GSTD6* and *GSTE7* are not significantly altered by the reduction of GALK activity.

In classic galactosemia, the severe impairment of GALT activity results in elevated gal-1-p levels, which may play a role in the pathology of the disease[1]. It has been proposed that reducing GALK activity (the first enzyme in the Leloir pathway) could have a positive impact on the long term outcome in patients with classic galactosemia through the reduction of gal-1-p levels[2]. However, *GALT* knock-out mice show elevated levels of gal-1-p compared to WT mice, but fail to replicate any of the acute and long term outcomes seen in humans[3]. In our published study from the previous chapter (chapter 3), *GALT*-null flies that were fed antioxidants on a galactose containing diet showed an improved acute outcome independent of gal-1-p levels[4]. Results from that study also indicate that galactose exposure exerts oxidative stress on both *GALT*-null and WT *Drosophila melanogaster*[5].

Expression microarray analysis (Chapter 2) showed that many Glutathione S-Transferase genes were increased when both WT and *GALT*-null drosophila were exposed to a galactose containing diet. Two of the genes that showed the largest increase in expression in response to galactose exposure (regardless of genotype) were *GSTD6* and *GSTE7*. GSTs of the Delta and Epsilon class, including *GSTD6* and *GSTE7*, are more highly expressed in the larval midgut in response to oxidative stress from H2O2 exposure than other GSTs[6]. *GSTD6* and *GSTE7* do not increase their expression in response to drug exposure (phenorbital or atrazine), indicating that their role is more specific to the oxidative stress response[7].

We generated a GALK deficient fly was generated by imprecise P-element excision. This fly line will be used to observe the effect that the loss of GALK activity has on the oxidative stress response in the *GALT*-null background. The imprecise excision was mapped through PCR, followed by gel electrophoresis and sequencing (Figures 1 and 2). A GALK RNAi knock-down allele was crossed into the *GALT*-null background to test the effect that reduction in GALK activity has on the expression of GSTD6 and GSTE7 (Table 1, Figures 3 and 4), which are involved in the oxidative stress response when the larvae are exposed to galactose. While there

was reduced GALK activity in this line, the GALK-RNAi allele did not prevent the buildup of galactose-1-phosphate in GALT-null flies.

Experimental Methods

Stocks. The fly stocks used for the experiment were *GALT*-null [8], the precise excision of the *dGALT* P-element (WT)[8], and a *Btub-GAL4* allele crossed onto a *UAS-GALKRNAi* chromosome. The fly stocks were kept on molasses food at 25 degrees C.

Mapping the dGALK imprecise excision. The breakpoints of the P-element excision were mapped through PCR of the *dGALK* excision flies and WT flies. The sequence of the forward primer used was 5'-TCT TGA AAT CCG CGC CAG CTG -3' and the sequence of the reverse primer was 5'-AGC CAA CTC TGC CCA AAT AGG C-3'. These primer sequences were put through the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) to ensure that they were specific for the target sequence. The cycling conditions were: 1 cycle of 94 degrees C for 2 minutes (initial denaturation), followed by 32 cycles of 94 degrees for 30 seconds (denaturation), 58 degrees C for 30 seconds (annealing), and 72 degrees C for 4 minutes (extension). The junction amplicons were run on a 1% agarose gel to ensure and estimate the product size. The junction amplicons were then submitted to Eurofins MWG Operon (Huntsville, AL) for sequencing.

Larval collection, RNA Extraction, and cDNA generation. Larval collection was done as previously described[5]. One difference was that the flies were stored at -80 degrees C instead of -20 degrees C as the flies in this experiment were set up for long term storage.

Real Time PCR and analysis. The real time PCR was done as described previously[5]. The results were organized in Excel and analysis was done in R using a Two-way ANOVA with genotype and diet as independent variables[9].

Results and Discussion

Mapping the dGALK imprecise excision. The P-element that was mobilized was located in the 5' UTR of fly gene *CG5068* (Figure 1). *CG5068* and *GALK* are transcribed in opposite directions. The result of the imprecise excision was that a major portion of the 5' UTR of *CG5068* was removed along with the 5'UTR and most of the first 3 exons of *GALK* (Supplementary Figure 1). This size of the imprecise P-element excision is 2,991bp. This was confirmed through PCR product analysis of the imprecise excision and subsequent sequencing of the junction amplicon.

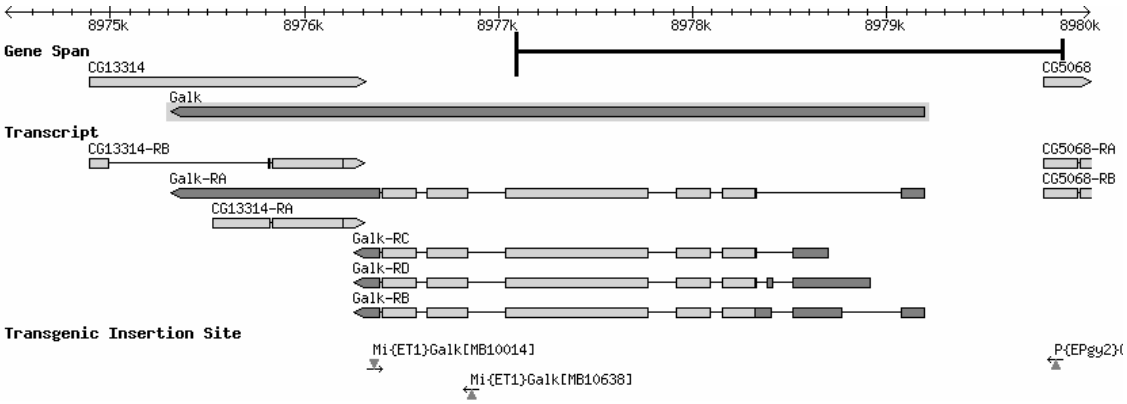

Figure 1. The approximate location of the imprecise p-element excision (located between the bracket). The fragment removed was from 8980895 – 8977904.

CG5068 is predicted to have protein phosphatase methylesterase activity based on sequence analysis done and uploaded to Flybase[10].

Real Time PCR to detect the expression of GSTD6 and GSTE7. There was a significant interaction between diet and genotype on the relative expression of *GSTD6* in the larvae that were WT for *GALK*. There was a significantly larger increase in the expression of *GSTD6* in *GALT-/-* flies compared to WT flies when both flies were exposed to galactose. Diet but not genotype had a significant effect on the relative expression of *GSTE7* in larvae that are WT for *GALK*. *GALT -/-* and *GALT+/+* (WT) flies both showed increases in the expression of *GSTE7* that were statistically similar. These results are similar to what was seen in a previous experiment[5]. In

larvae that carry the GALK-RNAi construct, there was no interaction between diet and genotype on the relative expression of *GSTD6* and *GSTE7*, as only diet had a significant effect on the relative expression of these genes. However, there was still an increase in the expression of GSTD6 and GSTE7 when the *GALK-RNAi* allele was expressed in the GALT-null and WT backgrounds exposed to galactose that was comparable to the increase in expression seen in flies without the *GALK-RNAi* allele (Figures 3 and 4).

GALK WT	**GSTD6 Expression (relative to Actin5c)**	**GSTE7 Expression (relative to Actin5c)**
GALT +/+, glc to glc	0.662492 ± 0.013001	0.919668 ± 0.02505
GALT -/-, glc to glc	0.452807 ± 0.066172	0.885618 ± 0.040665
GALT +/+, glc to glc + gal	16.19909 ± 0.48474	3.035815 ± 0.100769
GALT -/-, glc to glc + gal	22.8511 ± 1.034269	4.526921 ± 0.612593
GALK RNAi	**GSTD6 Expression (relative to Actin5c)**	**GSTE7 Expression (relative to Actin5c)**
GALT +/+, glc to glc	0.360468 ± 0.028484	0.711845 ± 0.034217
GALT -/-, glc to glc	0.481658 ± 0.100437	1.158046 ± 0.132049
GALT +/+, glc to glc + gal	21.70399 ± 3.898693	2.833344 ± 0.429226
GALT -/-, glc to glc + gal	18.56056 ± 3.537623	2.622105 ± 0.353272

Table 1. The relative expression ratios of *GSTD6* and *GSTE7* for each genotype and treatment condition. GALT +/+ = wild type larvae, GALT -/- = GALT-null larvae, glc = glucose food (12 hour exposure), glc + gal = glucose + galactose containing food (12 hours exposure).

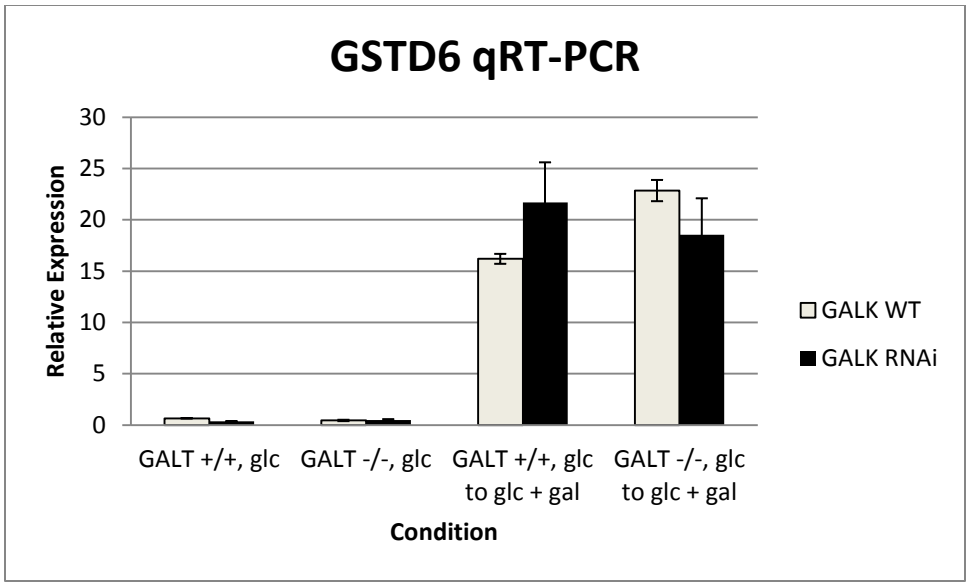

Figure 2. The relative expression levels of *GSTD6* in the *GALK-RNAi* flies compared to the wild type (WT) flies. GALT +/+ = wild type larvae, GALT -/- = GALT-null larvae, glc = glucose food (12 hour exposure), glc + gal = glucose + galactose containing food (12 hour exposure).

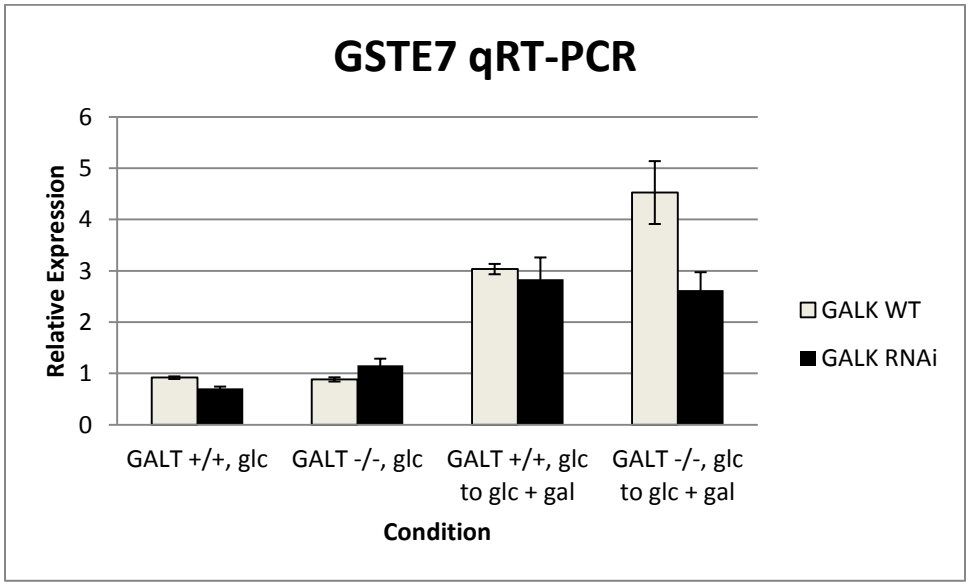

Figure 3. The relative expression levels of *GSTE7* in the *GALK-RNAi* flies compared to the wild type (WT) flies. GALT +/+ = wild type larvae, GALT -/- = GALT-null larvae, glc = glucose food (12 hour exposure), glc + gal = glucose + galactose containing food (12 hour exposure).

While this experiment showed that the reduction of GALK activity does not have a significant effect on the expression of two oxidative stress markers, *GSTD6* and *GSTE7*, it remains to be seen whether the buildup of gal-1-p, the byproduct of GALK activity and a biochemical marker of classic galactosemia, contributes to oxidative stress. A qRT-PCR measuring *GSTD6* and *GSTE7* expression using the imprecise excision that has removed most of the GALK 5' end (Figure 1) would be able to address this question.

Supplementary Figure.

8980985 CTCCACAACATCGATGACGGTTATTCCAATCAAATTGGGCACCAGAGCCATGTGGGCAAA 8980926

↓

8980925 GTGGACGGCGATGGCGCCGCCCATCGAGTGGCCAACCACAAAAAGCTGCGGCACTTCTTC 8980866

8980865 AGGATAGAGCTTCAGGATTAAGTCACCGATGTCCCTGTTTAGAATATTTTAAACATTATT 8980806

8980805 ATAGATTATAGATTAGATTATTATGGTTTTTAGCTTCAACCGTGAATAAAGTAACTATAA 8980746

8980745 AAAACTAAATAATATTTACAAATGAGTGATCGTTCAAATAGATCGACCTACTTTAAGTGC 8980686

8980685 AGTGCGCTATTTTTATAAATAAAGAGATTATGCAAGTTTAGCCTAATCGTTCATAAAGCT 8980626

8980625 AAGAGTTAATGCGAGAAGTTTTACTCGACAAAATCTTAAAAATATTGGTTTTTAATGTAA 8980566

8980565 GCAGTAGAAAAATTTGCATAACCATCTCTAAAACATATTTTGAGAACAATATTTTTTTGG 8980506

8980505 ATTCAACTCACTTAGCCAACGTATCAGCGGAGAGATCGTCCTCGTCGTCCACCTTGCTGT 8980446

8980445 CCCCATGACCTCGCATATCGATGCACAGGCACTGGCAGTGGATCATGCTGGTCACCTCAG 8980386

8980385 ACTTAAATGGATATATGTACATGTTAAGACTATTTAACAGAATATATCGCATTCACTCAC 8980326

8980325 ACAAAAGTGCGCCCAGGTGAGGGCAGAGTAGCCACCGCCGTGGAGCAGAAGGAGAACTGG 8980266

8980265 ACCCGGCTTCTCCGGTTGCTTGGTGCGATAGATGCGGAAGGTGCGTTGCTCATCCACCGT 8980206

8980205 GACGTCCTCCTTCTCCGCAAAGAATTCGTTCCACATGCCCGGCTTGTAGTCGCGAATGCG 8980146

8980145 CGACTTCTTGAACGAGTCCCTGTAATTAGCCAAATTGGGTAGAGATTGCGTACTCCCCAC 8980086

8980085 TCCAGTCGCAGAATACCCTGCACTTACGCTCTGCCAATGCGCCCGCCAGGAATTGTGGGT 8980026

8980025 GGCAGCTTGCCCTTCAACATCGTGCGCTGTAAACTCGACATTGATCCGATTGTCTTCGCT 8979966

8979965 GTGGGGTCAACAGGTGGTGCTCCTACGGTGCAGGTGCAAGTGGTTATTCACACAGTCGTT 8979906

8979905 GCTCGCAATTATCCGTTTTCGAAAACAAAAAAATAGTTCCAATTCAACGAAGAAGTATTG 8979846

8979845 CAGTGTGACCGTAATCGAGAAAACCGTAACTGACGCCAGCTGGCAGCGCTGGTTCAAGAG 8979786

8979785 CTGCACTTGGCAATGCCCTTGTCTGGCAACGCCTAACCGCAACAGTCAACATTTCAAATT 8979726

8979725 CCAAAACGGATATTTTAGGCTTTTGCAATCAAAGACGAAAATCCGTACCATACTGAAACA 8979666

8979665 ACAACAGTTGTATATTTTAATGTTTGTACATTTTAATCAATTAAGTATTTTAAAAGTTTA 8979606

8979605 TCTACGAAAAAATTGATGAGCTCCCCGAAACACTTGTACCTTATCAGCTTTGTAAATACT 8979546

8979545 TCACATTAAT----GGCAAAATAATAAGCAAGGTAATAACCTTTCGCAAAGTGGATGTCA 8979490

8979489 AAACCACTTTTTAAAGCAGACAAAATTTGGAAACCAGTTTTTTAGAATGCTAACAAGAAA 8979430

8979429 GAATTCATATGCTATGAAGATGTTTGACCCATTTAACGGTAAAGACAGCTAGTTATAAAC 8979370

8979369 TAACAAAGCTCTCAACAAGTTCCTACTGATACACACAGTGCAACATCGCTAGCTGGGAAA 8979310

8979309 CTGGTGTTCTATAAGGTCTCCGAGCATTTATATTTCAGTAATGCTACTGATAAGGAAGTA 8979250

8979249 TCACTTTATTGTTCATAGTATTCCGGACTAAGCAAAAACGGAAACGGAACGGGCCGGATT 8979190

8979189 GCTCAGTTGCTTGATGGAACGCGACGTGCTCACACCGAAAACTAGAACTCCTCTCTGATA 8979130

8979129 AGACGGCACCAAGTTAATTTGACCTCAAACAGAGAAAGATAAGGCATTGTTGGGTATGTA 8979070

8979069 TCGGCAAAAGTGTAAAAATCTTGACAAAATCCTTTATATCTGTGTTTATTCTGGTATGTA 8979010

8979009 AACAAAAGGTAAGCTCAATACAGGTTAAGTTTATGGTTTCGAGATAAGATAAATGTCATG 8978950

8978949 TTTGTCATTAATGGTCTATTTAGATTATGCGGCAGTAAGTTTAAGCGGTAGTACTTTTGA 8978890

8978889 AAAACAATTACCAGCTAAGAACTTTATTGCCAAATAATTTACTATTAGTTGGGACTAGAC 8978830

8978829 ATAAACATCAATTTCGCGGATAAATATTCAAAATAAAATCTTTATCCATTGCGCATTTCA 8978770

8978769 AGGGTTAATGCTTCTGCAAGCCTGGCAACTCCTAAATAACAGCTGTTGGCTATAGTCCTC 8978710

8978709 TCGCTCCCGCACACCCTTCCTTTTCATTTACCGGCAGAAATTGCACTTTTGTACTTGTTT 8978650

8978649 TTTGCCCAATTAAGCGGTCAAATTGGCGTCAATCTAATGTTTTAGTGACTTTGATGCCGT 8978590

8978589 TTCCCCAACTGATAACCGCAATGTTAGTACATCACTTTGTGTGCTAATAGCTATTTGGAC 8978530

8978529 ATATTGATAAGGTGAGATGGGTTGATAAGCTAATTTTTGCACAACCACAGGTGCAATTGT 8978470

8978469 TTTTCCCTTTGCCACGGCTATTTGTTTACGTATGTAAATTTGTTTTTGCCCATAAATTTA 8978410

8978409 GATTAACCAGTTGCGACACGTTTACGGTTCGTCGACTGCGAAAATAATGGGCATTATTAA 8978350

8978349 AGTTTCACCCGCCTGCAGGTGAACATGACTGCCAACGGGAACGGAACTGCTCAGGATTTG 8978290

8978289 GGTGCCGGCATTGAAATCGTGGCCCCACAGAAGCTCCAGCCGGAATCCAAGGTTTACGGG 8978230

8978229 CGCGTGCAGCAATTGACCGCATTTTTCAAACAACAATTCGGAGCGAATCCGGACTTTATC 8978170

8978169 GTTCGAGTGCCAGGAAGGTGAGCAGTTCCACTCGTGGTGCACATCTATAGTCCCTTATCG 8978110

8978109 ATGATATCTCCTCCTTCTCCTAGGGTCAACATCATTGGCGAGCATGTGGACTACTGCGGC 8978050

8978049 TATTCGGTGCTGCCCATGGCTGTAAGCCAGAGCATTTTCCTTGCAGTGGCCAAGAATCCT 8977990

8977989 AGTGATAGCCAGCTTCAACTCAGAAATCTCGAGGAAGCGAAGTTTACGGGCTACGATGCC 8977930

↓

8977929 GATCTCAAAACGCTCAGGTGGGTGAATCAGAAACATGATAATTACCGACCTTAAGCACTA 8977870

8977869 GAAAATTTTCCATTTAATTTGTTATAATAACAATGTCACCTTGAAGGCGGTATCTAAACG 8977810

8977809 AATTTAGAAATGATAAGTCCAAATATTTTTTTGCTATTTTAGAATCGAGCTTCCAAAGAG 8977750

8977749 TGGTGGACCCGCTTGGTATAACTACTTCTTGTGCGGCATCAAAGGAATACAGGAGAGTCT 8977690

8977689 GGGCAGCCAGTGGAAGCCCATTGGCATGCGCATTGCTGTGGACGGTAATGTGCCCTTGGC 8977630

8977629 GGCAGGACTCTCCAGTTCGAGTGCCATGGTCAGCTCCGCTGTGTTGGCCACCGCACACGT 8977570

8977569 CCAGGGCAAGAAACTAGATCGCAGGGAGCTGGCT 8977536

Supplementary Figure 1. The sequence that has been removed due to imprecise excision of the P-element in the 5'UTR of *CG5068*. The dark gray highlighted sequence is from *CG5068* and the light gray highlighted sequence is from *dGALK*. The location of the excised sequence are bases 8980895 to 8977904 (denoted by the arrows).

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Chapter 5

Conclusions

The mechanisms of the acute and long term outcomes in classic galactosemia are currently unknown[1,2]. In this thesis, gene expression analysis has been used to provide insights into the galactose sensitivity of GALT-null *Drosophila melanogaster*. This was done through the comparison of global changes in gene expression through microarray technology followed by the more sensitive real time PCR to validate the results (Chapters 2 and 3). Finally, it was shown that a reduction in GALK activity does not have a significant effect on galactose induced oxidative stress in the fruit fly (Chapter 4).

The results from the Jumbo, *et al*. paper showed that oxidative stress is a modifier of acute outcome in the GALT-null fly[3]. The Gene Ontology (GO) results from the gene expression microarray experiment bolster the idea that the increase of oxidative stress plays a significant role in the acute outcome, as the GO patterns of *GALT-/-* flies match previous data that observed the response to paraquat, a known inducer of oxidative stress (Chapter 2)[4]. However, this may be part of the long term stress response to galactose exposure. A shorter time of galactose exposure (2-6 hours) could reveal the immediate response to galactose.

The mechanism(s) of the long term outcomes are more difficult to approach. We do not know whether the movement defects seen in our GALT-null model are a result of aberrant developmental processes or an inability to maintain homeostasis. In the gene expression microarray, the differences between *GALT-/-* and *GALT+/+* larvae at 48-52 hours post egg laying tissue include the expression of mitochondrial genes, neurological system process genes, transcriptional regulation genes, and TCA pathway genes. These could be all be the result of a lack of energy (ATP, etc.) or the differential gene expression of each GO term could be caused by a different mechanism. Performing tissue specific (brain, fat body, midgut, etc.) rather than global transcriptome analysis may provide a more sensitive look into the developmental factors that influence the long term outcomes. This is difficult for L1 larvae, due to the small size and the amount of RNA needed for each microarray experiment.

Based on our results and a previous data, *GSTD6* and *GSTE7* expression can be used as markers for oxidative stress in future studies with *Drosophila melanogaster[3,5]*. Both genes show increased expression when *GALT*-/- and *GALT*+/+ larvae are exposed to galactose, though *GSTD6* increases a significantly greater amount in the *GALT*-/- flies exposed to galactose containing food, indicating that GSTD6 may play a larger role in the *Drosophila* response to galactose induced oxidative stress[3]. There are other Glutathione S-Transferase (GST) genes that had a reduction in expression when the larvae were exposed to galactose. The restoration of repressed GST expression has been shown to restore aspects of other fly models that have shown evidence of oxidative stress[6,7]. Restoring the activity of one or some of these GSTs could possibly increase the galactose tolerance in the *GALT-/-* flies.

References

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Appendix

Publication that I was an author on while a student in the Fridovich-Keil lab:

UDP-galactose 4'-epimerase activities toward UDP-Gal and UDP-GalNAc play different roles in the development of Drosophila melanogaster. Daenzer JM, Sanders RD, Hang D, Fridovich-Keil JL. PLoS Genet. 2012;8(5):e1002721. Epub 2012 May 24. (PMID:22654673)

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UDP-galactose 4'-epimerase activities toward UDP-gal and UDP-galNAc play different roles in the development of *Drosophila melanogaster*

Jennifer M. I. Daenzer[1], Rebecca D. Sanders[2], Darwin Hang[1], and Judith L. Fridovich-Keil[3*]

[1]Graduate Program in Genetics and Molecular Biology, Emory University, Atlanta, GA; [2]Graduate Program in Biochemistry, Cell, and Developmental Biology, Emory University, Atlanta, GA; [3]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, 30322

[*]corresponding author: Judith L. Fridovich-Keil

Department of Human Genetics

Emory University School of Medicine

Rm. 325.2 Whitehead Bldg.

615 Michael St.

Atlanta, GA 30322

TEL: 404-727-3924

Email:jfridov@emory.edu

FAX: 404-727-3949

ABSTRACT

In both humans and *Drosophila melanogaster*, UDP-galactose 4'-epimerase (GALE) catalyzes two distinct reactions, interconverting UDP-galactose (UDP-gal) and UDP-glucose (UDP-glc) in the final step of the Leloir pathway of galactose metabolism, and also interconverting UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylglucosamine (UDP-glcNAc). All four of these UDP-sugars serve as vital substrates for glycosylation in metazoans. Partial loss of GALE in humans results in the spectrum disorder epimerase deficiency galactosemia; partial loss of GALE in *Drosophila melanogaster* also results in galactose-sensitivity, and complete loss in *Drosophila* is embryonic lethal. However, whether these outcomes in both humans and flies result from loss of one GALE activity, the other, or both, has remained unknown. To address this question, we uncoupled the two activities in a *Drosophila* model, effectively replacing the endogenous *dGALE* with prokaryotic transgenes, one of which (*E. coli GALE)* efficiently interconverts only UDP-gal/UDP-glc, and the other of which (*P. shigelloides wbgU)* efficiently interconverts only UDP-galNAc/UDP-glcNAc. Our results demonstrate that both UDP-gal and UDP-galNAc activities of *dGALE* are required for *Drosophila* survival, although distinct roles for each activity can be seen in specific windows of developmental time, or in response to a galactose challenge. By extension, these data also suggest that both activities might play distinct and essential roles in humans.

AUTHOR SUMMARY

In this manuscript we apply a fruit fly model to explore the relative contributions of each of two different activities attributed to a single enzyme – UDP-galactose 4'-epimerase (GALE); partial impairment of human GALE results in the potentially severe metabolic disorder epimerase deficiency galactosemia. One GALE activity involves interconverting UDP-galactose and UDP-glucose in the Leloir pathway of galactose metabolism; the other activity involves interconverting UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. We have previously demonstrated that complete loss of GALE is embryonic lethal in fruit flies, but it was unclear which GALE activity loss was responsible for the outcome. Using genetically modified fruit flies we were able to remove or give back each GALE activity individually at different times in development and observe the consequences. Our results demonstrate that both GALE activities are essential, although they play different roles at different times in development. These results provide insight into the normal functions of GALE and also have implications for diagnosis and intervention in epimerase deficiency galactosemia.

INTRODUCTION

Galactose is an essential component of glycoproteins and glycolipids in metazoans, and as a constituent monosaccharide of the milk sugar, lactose, also serves as a key nutrient for mammalian infants. Galactose is also found in notable quantities in some fruits, vegetables, and legumes. Galactose is both synthesized and catabolized in all species via the Leloir pathway, which is highly conserved across branches of the evolutionary tree [1].

The reactions of the Leloir pathway are catalyzed by the sequential activities of three enzymes: (1) galactokinase (GALK) which phosphorylates alpha-D-galactose to form galactose-1-phosphate (gal-1P), (2) galactose-1-phosphate uridylyltransferase (GALT), which transfers uridine monophosphate (UMP) from uridine diphosphoglucose (UDP-glc) to gal-1P, forming UDP-galactose (UDP-gal) and releasing glucose-1-phosphate (glc-1P), which can proceed to phosphoglucomutase and the glycolytic pathway, and (3) UDP-galactose 4'-epimerase (GALE) which interconverts UDP-gal and UDP-glc [1]. In addition to a role in the Leloir pathway, metazoan GALE enzymes also interconvert UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylglucosamine (UDP-glcNAc) (Figure 1). Because it catalyzes reversible reactions, GALE therefore not only contributes to the catabolism of dietary galactose, but also enables the endogenous biosynthesis of both UDP-gal and UDP-galNAc [2,3] when exogenous sources are limited.

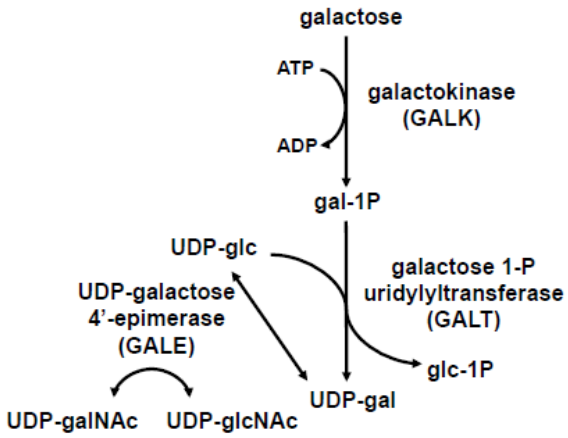

Figure 1: the Leloir pathway of galactose metabolism. UDP-galactose 4'-epimerase, the third enzyme in the pathway, also interconverts UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylglucosamine (UDP-glcNAc) in humans, *Drosophila*, and

Deficiency in any of the three Leloir enzymes in humans results in a form of the metabolic disorder galactosemia, although the symptoms and clinical severity differ according to which enzyme is impaired and the extent of the impairment. Profound loss of hGALE results in generalized epimerase-deficiency galactosemia, an autosomal recessive and potentially severe disorder. To date, however, no patient has been reported with complete loss of GALE, and even the most severely affected demonstrate at least 5% residual enzyme activity [4]. Previous studies have indicated that different patient mutations impair hGALE to different extents [5-9]. Further, while some mutations impair both GALE activities similarly, others do not. For example, the *hGALE* allele V94M, which leads to severe epimerase-deficiency galactosemia in the homozygous state, encodes an enzyme that retains ~5% residual activity toward UDP-gal but ~25% residual activity toward UDP-galNAc [8,9]. Disparities such as this have raised the question of whether the pathophysiology of epimerase deficiency galactosemia results from the loss of GALE activity toward UDP-gal/UDP-glc, or toward UDP-galNAc/UDP-glcNAc, or both.

To address this question, we applied a *Drosophila melanogaster* model of *GALE* deficiency [10]. Using this model, we have previously established that *GALE* is essential in *Drosophila*; animals completely lacking endogenous *dGALE* succumb as embryos, and conditional loss of *dGALE* in larvae results in death within two to four days of knockdown. Finally, partial loss of

dGALE leads to galactose sensitivity in larvae, and transgenic expression of human *GALE* (*hGALE*) rescues each of these negative outcomes [7].

Here we have applied our transgenic *Drosophila* model to uncouple and examine the individual roles of GALE separately. Toward that end, we generated flies that lacked endogenous *dGALE* and expressed either of two prokaryotic transgenes, one encoding *E. coli* GALE (*eGALE)* which exhibits an approximately 8,000-fold substrate preference for UDP-gal/UDP-glc over UDP-galNAc/UDP-glcNAc [11], and the other encoding *P. shigelloides wbgU*, which exhibits an approximately 2,000-fold substrate preference for UDP-galNAc/UDP-glcNAc over UDP-gal/UDP-glc [12]. By expressing these prokaryotic transgenes individually or in combination in *dGALE*-deficient *Drosophila* we determined that both GALE activities are required for survival of embryos and larvae. We also found that restoration of one activity or the other in later development rescued some phenotypes. Combined, these results provide insight into the varied roles of *dGALE* in *Drosophila* development and homeostasis, and by extension, suggest that *hGALE* may play similarly complex and essential roles in humans.

Results

The Drosophila GALE enzyme efficiently interconverts both UDP-gal/UDP-glc and UDP-galNAc/UDP-glcNAc

Human and other mammalian GALE enzymes efficiently interconvert both UDP-gal/UDP-glc and UDP-galNAc/UDP-glcNAc (e.g. [13-15]). Previously, we reported that *Drosophila* GALE interconverts the first of these substrate pairs (UDP-gal/UDP-glc) [10], but did not address whether dGALE could also interconvert the second. Here we demonstrate that dGALE from wild-type adult flies efficiently interconverts both substrate sets (left most bar, Figure 2). Of note, while purified human GALE [15] and dGALE each interconvert both UDP-gal/UDP-glc and

UDP-galNAc/UDP-glcNAc, the apparent specific activity of both human and fly enzymes toward UDP-gal is significantly higher than toward UDP-galNAc.

eGALE* and *wbgU* transgenes enable the expression of individual GALE activities in *Drosophila

To generate flies with epimerase activity toward only UDP-gal/UDP-glc or only UDP-galNAc/UDP-glcNAc, we created transgenic lines expressing *eGALE* (*UAS-eGALE*) or *wbgU* (*UAS-wbgU*), respectively, each in a conditionally *dGALE*-impaired background. Each of these prokaryotic *GALE* genes has been demonstrated previously to encode epimerase activity toward only one of the two sets of epimer pairs (e.g. [11,12]). To minimize background, activities of the encoded eGALE and WbgU enzymes toward UDP-gal and UDP-galNAc were assayed in flies knocked down for endogenous *dGALE*; results for the transgenes that demonstrated activities closest to those seen in wild-type *Drosophila*, $eGALE^{62A}$ *and* $wbgU^{19A}$, are presented in Figure 2. As expected, lysates from *dGALE* knockdown flies expressing the *eGALE* transgene demonstrated strong activity toward UDP-gal, but not UDP-galNAc, and lysates from *dGALE* knockdown flies expressing the *wbgU* transgene demonstrated strong activity toward UDP-galNAc, but not UDP-gal. As a control we also tested lysates from *dGALE* knockdown flies expressing a human *GALE* transgene; as expected, those samples demonstrated very strong activity toward both substrates.

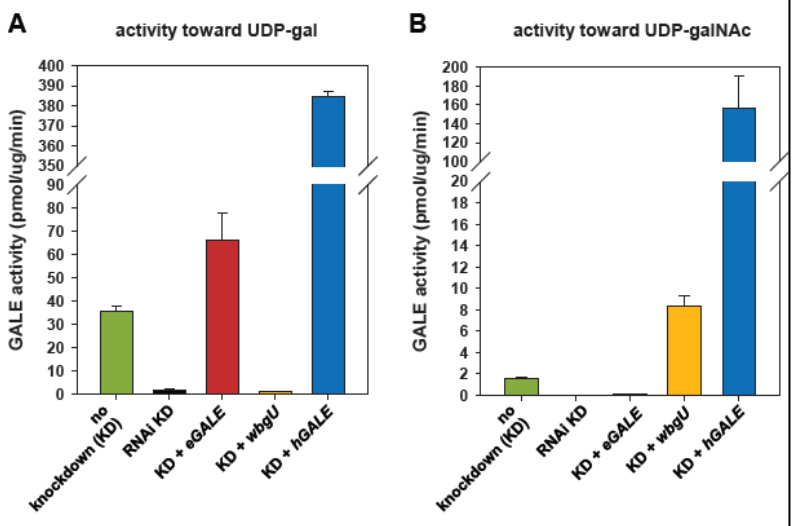

Figure 2: Enzyme activities of flies expressing different *GALE* transgenes. Assays for all genotypes were performed on flies with *dGALE* knockdown (KD) driven by the *Act5C-GAL4* driver with the exception of flies labeled "no knockdown"; those flies carried the same *UAS-RNAi*^{dGALE} and *GAL80*^{ts} alleles, but were balanced over TSTL, and thus lacked the driver. In addition to RNA^{i} knockdown of *dGALE*, *Act5C-GAL4* also drives expression of the specified transgenes in these animals. Panel A: GALE

Both *eGALE* and *wbgU* transgene activities are required, in combination, to rescue viability of *dGALE*-deficient *Drosophila*

Previously, we created and characterized two *dGALE*-deficient alleles, *dGALE*$^{f00624.4}$ and *dGALE*$^{\Delta y}$, which allowed us to demonstrate that *GALE* is essential for survival in *Drosophila* [10]. To examine the requirement for the two different epimerase activities separately, we set up crosses which allowed for the expression of *eGALE* or *wbgU*, individually or in combination, driven by *Act5C-GAL4* in an otherwise *dGALE*-deficient background (*dGALE*$^{f00624.4}$/*dGALE*$^{\Delta y}$). Table 1 shows the observed to expected ratios of surviving transgenic offspring that eclosed from these crosses. As presented in Table 1, neither *eGALE* alone nor *wbgU* alone was sufficient to rescue survival of the *dGALE*-deficient animals; however, expression of both *eGALE* and *wbgU*, in combination, was sufficient. These results demonstrate that GALE activities toward both UDP-gal and UDP-galNAc are essential for survival of *D. melanogaster*. To rule out the possibility that rescue with *eGALE* plus *wbgU* in combination occurred not because both GALE activities are essential but rather because neither individual transgene expressed sufficient enzyme, we also tested additional *eGALE* and *wbgU* transgenes that individually demonstrated higher levels of

expression; none was sufficient to rescue (data not shown). Of note, there also was no apparent over-expression phenotype; for example, animals expressing either *eGALE* or *wbgU* in *addition* to endogenous *dGALE*, and animals dramatically over-expressing human GALE, remained viable, fertile, and appeared morphologically normal (data not shown).

experimental cross	**genotype**	**Expected mendelian proportion of F1 with this genotype**	**Observed proportion of viable F1 with this genotype**
Actin5C-GAL4/CyO ; dGALE^{y}/TM6B x *Actin5C-GAL4/CyO ; UAS-wbgU, dGALE$^{f00624.4}$/TM6B*	*Actin5C-GAL4/CyO ; UAS-wbgU, dGALE$^{f00624.4}$/dGALE^{y}*	0.333	0.000 ± 0.000
Actin5C-GAL4/CyO ; dGALE^{y} /TM6B x *UAS-eGALE/CyO ; dGALE$^{f00624.4}$/TM6B*	*Actin5C-GAL4/UAS-eGALE; dGALE$^{f00624.4}$/dGALE^{y}*	0.143	0.000 ± 0.000
UAS-eGALE/CyO ; dGALE^{y}//TM6B x *Actin5C-GAL4/CyO ; UAS-wbgU,dGALE$^{f00624.4}$/TM6B*	*Actin5C-GAL4/UAS-eGALE; UAS-wbgU,dGALE$^{f00624.4}$/dGALE^{y}*	0.143	0.176 ± 0.018

Table 1: Crosses to test rescue of *wbgU* and *eGALE* transgenes individually and in combination

Different requirements for GALE activities at different stages of *Drosophila* development

Previously, we described an approach that achieves conditional knockdown of *dGALE* in *Drosophila* using a *UAS-RNAi^{dGALE}* transgene (12030-R2, National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan) in combination with a temperature sensitive allele of yeast *GAL80* (*GAL80^{ts}*) ([10] and Figure 3A). Using this system, we found that *dGALE* is required from embryogenesis through pupation, and that loss of *dGALE* during pupation leads to defects in fecundity and perhaps also a shortened life span [10].

Here we have expanded the *GAL80*^{ts}conditional *dGALE* knockdown system to include different *GAL4*-dependent *GALE* transgenes and have applied this expanded system to test the ability of each transgene, or pair of transgenes, to compensate for the loss of endogenous *dGALE*. By using age-synchronized cohorts of animals and shifting from the permissive (18°C) to the restrictive temperature (28-29°C) at different times we also were able to test the ability of each *GALE* transgene, or pair of transgenes, to sustain survival and fecundity at different stages of development. At 18°C these animals expressed endogenous *dGALE*, but not their transgenes, and at 28-29°C these animals expressed their transgenes but not *dGALE* (Figure 3A). Specifically, we tested *Drosophila* that carried no *GALE* transgene, an *eGALE* transgene, a *wbgU* transgene, both *eGALE* and *wbgU* transgenes, or an *hGALE* transgene.

As expected from prior results ([10] and Table 1), animals expressing no *GALE* transgene succumbed when shifted to the restrictive temperature as larvae, while animals expressing either human *GALE* or both *eGALE* plus *wbgU* remained viable and fertile (Figure 3C). Surprisingly, expression of either *eGALE* or *wbgU* alone was also sufficient to rescue survival, albeit to a lesser extent. The fact that the individual prokaryotic transgenes were sufficient to rescue *dGALE* knockdown animals, but not animals genetically null for *dGALE* (Table 1), suggests that trace residual *dGALE* expression in the knockdown animals lowered the threshold of transgene function required for rescue.

Of note, while *dGALE* knockdown animals encoding either *eGALE* or *wbgU* remained viable following a shift to the restrictive temperature in early to mid-development (Figure 3C), these survivors were not entirely healthy. Specifically, these animals demonstrated either partial or complete loss of fecundity as adults. To test whether the degree of *dGALE* knockdown was comparable between males and females, and therefore not a confounding factor in differential outcome, we performed GALE and GALT enzyme assays on newly eclosed and three day old male and female knockdown adults that carried no GALE transgene and that had been switched

to the restrictive temperature as early to mid-stage pupa. The degree of GALE knockdown in both males and females was profound and comparable (Figure 3B). As expected, the level of GALE activity was even lower in the older animals, presumably because any GALE synthesized prior to the temperature switch had three additional days to decay. Also as expected, GALT activity was normal and apparently unaffected by the *dGALE* knockdown in all samples tested (data not shown).

To examine fecundity, we collected and sequestered newly eclosed virgin female and male flies from each surviving cohort, crossed them to an equal number of wild-type flies of the opposite sex, and counted the numbers of viable offspring resulting from each cross. Crosses resulting in large numbers of viable offspring (>50) were scored as "normal fecundity". Crosses resulting in fewer than 10 viable offspring were scored as "reduced fecundity," and crosses resulting in no viable offspring were scored as "loss of fecundity" (Figure 3C). For example, when *dGALE* knockdown was initiated during early to mid-stage pupal development, animals of both sexes displayed diminished fecundity. Expression of *eGALE* alone, but not *wbgU* alone, rescued the male defect, whereas expression of both prokaryotic transgenes in combination, or *hGALE* alone, was required to rescue the female defect. These results indicate that GALE activity toward UDP-gal is both necessary and sufficient for male fecundity, but that GALE activities toward both UDP-gal and UDP-galNAc are required for female fecundity.

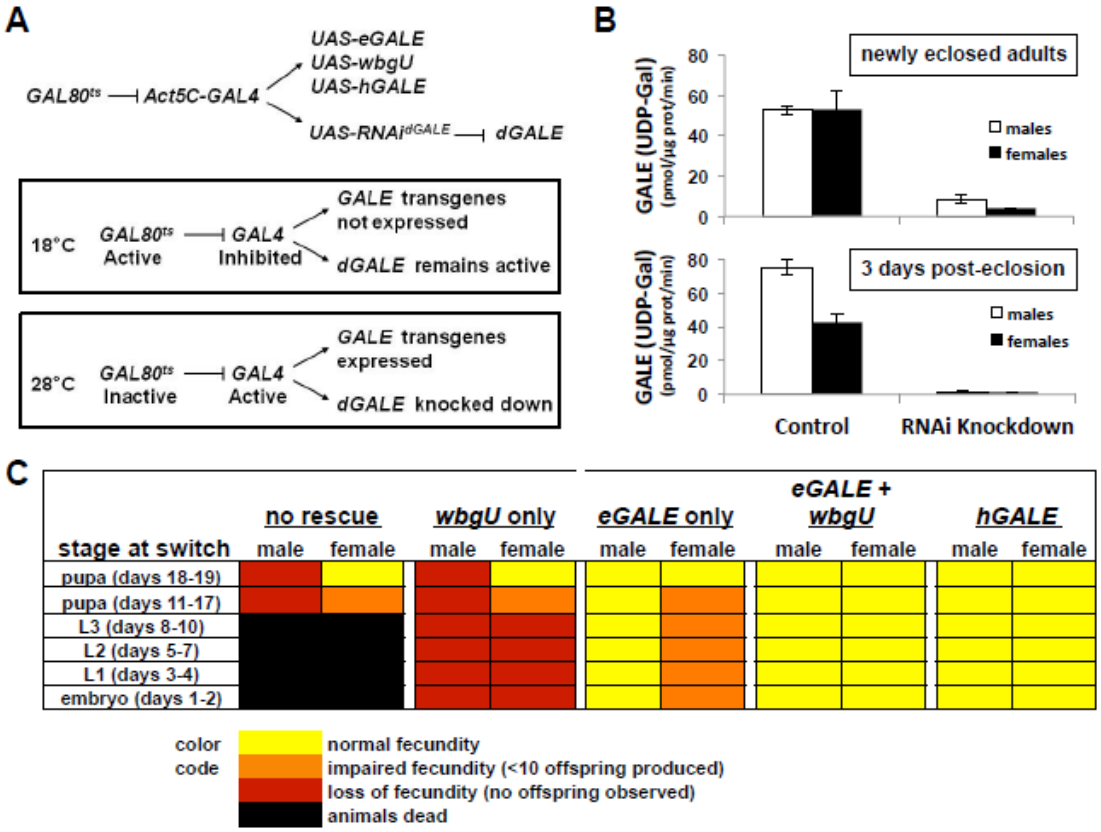

Figure 3: Differentially impaired fecundity of flies lacking different GALE activities. (A) Diagram of the method used to achieve expression of different *GALE* transgenes in the background of *dGALE* knockdown animals. The timing of knockdown and concurrent transgene expression was controlled by switching flies from the permissive temperature (18°C) to the restrictive temperature (28-29°C), as indicated. (**B**) Knockdown efficiency in male and female animals switched to the restrictive temperature as early to mid-stage pupa and harvested for biochemical analysis as newly eclosed adults or three days after eclosion. Of note, GALT activity was completely normal in all samples tested and apparently unaffected by the *dGALE* knockdown (data not shown). **(C)** Each box represents the outcome of flies switched from 18°C to 28°C at the stage indicated in the column on the left. The number of days the flies developed at 18°C to reach each stage is shown in parentheses.

Galactose exposure of transgenic flies with late-onset *dGALE* knockdown reveals differential roles of GALE activities toward UDP-gal and UDP-galNAc

We have previously demonstrated that *Drosophila* expressing a hypomorphic allele of *dGALE* are viable but sensitive to galactose exposure [10]. To assess the roles of the two *GALE* activities in coping with environmental galactose, we collected adult flies in which *dGALE* knockdown

coupled with *hGALE*, *eGALE*, *wbgU*, or *eGALE* plus *wbgU* transgene expression was initiated using the *GAL80*^{ts} system during late larval or early-to-mid-pupal development. These animals were allowed to develop on a standard molasses-based food, and were then transferred as newly eclosed adults to food containing either 555 mM glucose as the sole sugar, or 555 mM glucose plus 175 mM galactose.

We assessed the lifespan of each cohort of animals on both foods; as a control, knockdown animals expressing no *GALE* transgene were also monitored (Figure 4). In the absence of galactose, all cohorts showed similar longevity profiles, although females (Figure 4, panel C) showed greater variability than males (Figure 4, panel A). In the presence of galactose, however, both males and females expressing either no *GALE* transgene, or only the *wbgU* transgene, demonstrated a dramatic reduction in life span ($p<0.0001$, Figure 4, panels B and D). Females expressing *eGALE* alone exhibited a slight decrease in life span that was independent of diet. Animals expressing *hGALE* or *eGALE* + *wbgU* had lifespans comparable to control animals expressing endogenous *dGALE*, regardless of diet. These data implicate loss of UDP-gal activity as responsible for the galactose-dependent early demise of adult *dGALE*-impaired *Drosophila*.

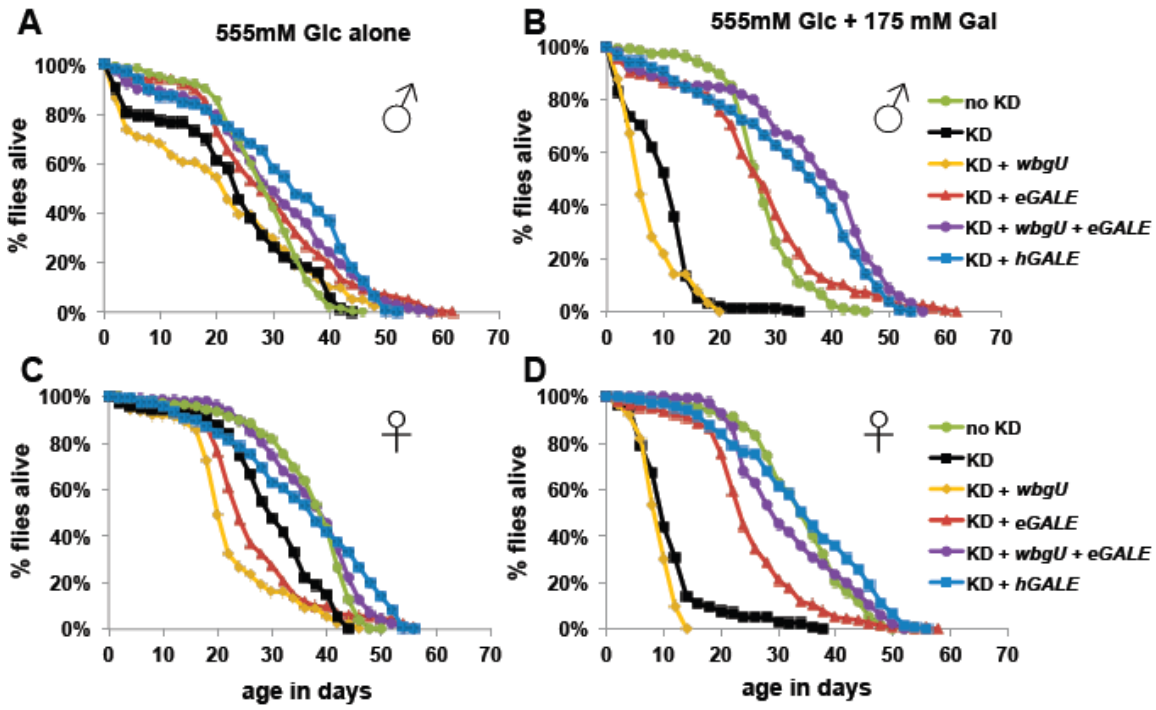

Figure 4: Flies lacking GALE activity toward UDP-gal/UDP-glc have a shortened life span when exposed to galactose as adults. The life spans of male **(A** and **B)** and female **(C** and **D)** flies reared on molasses food and then tapped as newly eclosed adults to food containing either 555 mM glucose only **(A** and **C)**, or 555 mM glucose plus 175 mM galactose **(B** and **D)**, is illustrated. As indicated by the key, these cohorts of flies included controls expressing endogenous *dGALE* as well as animals that expressed endogenous *dGALE* early in development but then were subjected late in development to *dGALE* knockdown coupled with induced expression of either no *GALE* transgene, or *wbgU*, *eGALE*, *hGALE*, or both *wbgU* and *eGALE* in combination. Based on Log rank and Wilcoxon tests for significance, the life spans of knockdown animals expressing either no transgene or expressing only *wbgU* were significantly decreased on food containing galactose compared with food containing only glucose ($p<0.0001$).

Differentially *GALE*-impaired flies exposed to galactose demonstrate different metabolic abnormalities

As one approach to explore the pathophysiology underlying the different galactose-dependent outcomes observed in *Drosophila* deficient in GALE activity toward UDP-gal or UDP-galNAc we measured the levels of gal-1P, UDP-gal, and UDP-galNAc in lysates prepared from galactose-exposed third instar larvae expressing different *GALE* transgenes. As illustrated in Figure 5, galactose exposed animals deficient in both GALE activities (bars marked "KD" for knockdown) accumulated abnormally high levels of gal-1P (panels A and D) and UDP-gal (panels B and E).

Animals deficient only in GALE activity toward UDP-gal (bars marked "*wbgU*" in Figure 5) also demonstrated elevated gal-1P (panels A and D) and UDP-gal (panels B and E). In contrast, galactose exposed larvae deficient only in GALE activity toward UDP-galNAc (bars marked "*eGALE*" in Figure 5, panels C and F) demonstrated no extraordinary metabolic abnormalities, although, as expected, the absolute level of UDP-galNAc was diminished in these animals independent of diet relative to the "no knockdown" control (Figure 5, panel C). Also as expected, animals expressing either *hGALE* or both *eGALE* plus *wbgU* demonstrated no clear metabolic abnormalities (Figure 5).

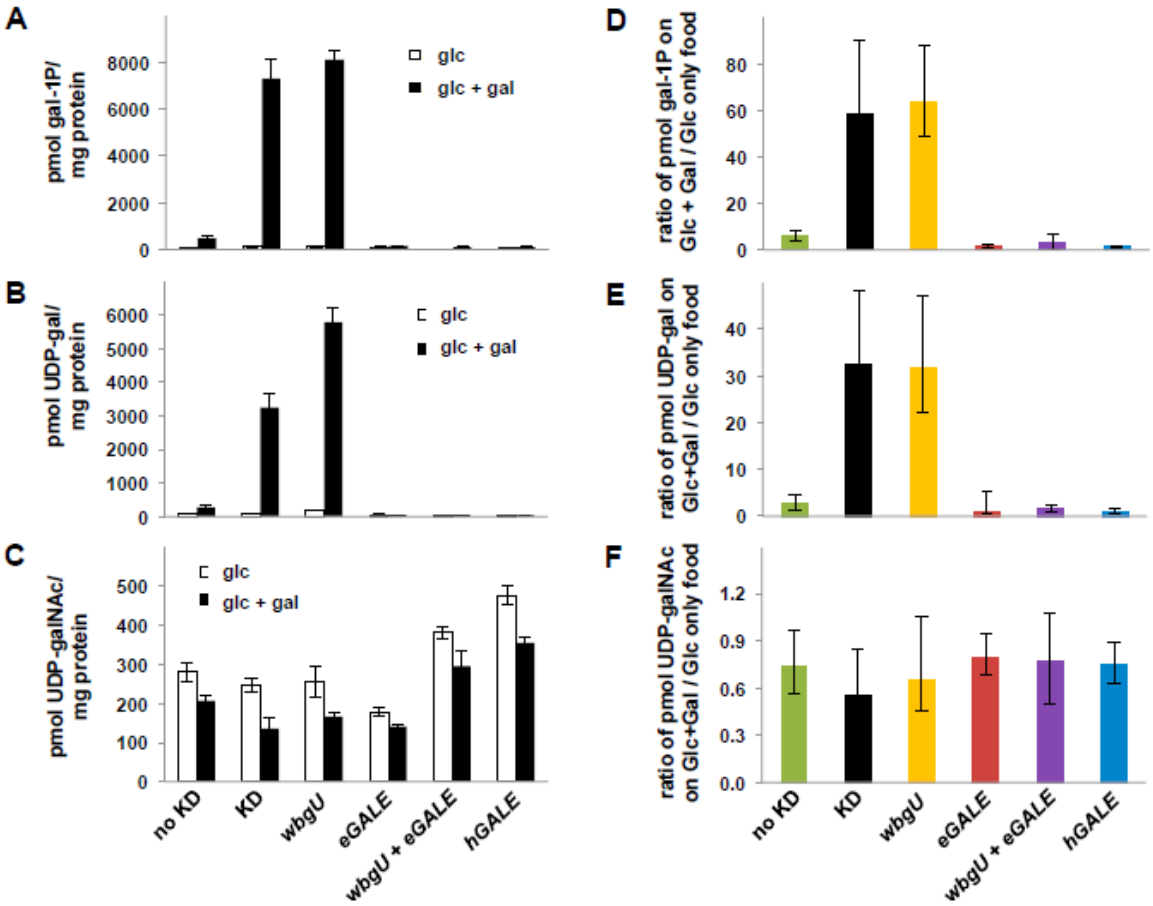

Figure 5: Metabolite profiles of *Drosophila* exposed to galactose. Metabolites were extracted from cohorts of larvae raised on food containing either 555 mM glucose or 555 mM glucose + 175 mM galactose. Animals were shifted from the permissive temperature (18°C) to the restrictive temperature (28°C) as first instar larvae and allowed to develop for four days before harvest. Accumulated metabolite values for gal-1P **(A)**, UDP-gal **(B)**, and UDP-galNAc **(C)** are shown on food containing glucose and glucose + galactose. To demonstrate the impact of diet on metabolite levels, values for gal-1P **(D)**, UDP-gal **(E)**, and UDP-galNAc **(F)** are shown as ratios of the amount of each metabolite accumulated by

animals on food containing galactose over that accumulated by animals of the same genotype on food containing only glucose. Error bars show the 95% confidence interval for each ratio.

Discussion

UDP-galactose 4'-epimerase (GALE) is an essential enzyme in *Drosophila* [10] and in humans [16], but until now the relative contributions of the two distinct GALE activities to development and galactose-tolerance has remained unclear. Understanding these roles has important implications regarding mechanism of galactose sensitivity, and may be applicable to diagnosis and prognosis in humans with epimerase-deficiency galactosemia. Our experiments described here exploit the genetic and biochemical facility of *Drosophila melanogaster* to test the consequences of losing each of the two GALE activities individually at different stages of development, or under different conditions of galactose exposure.

Our results demonstrate that developing animals require at least some GALE activity toward both epimer pairs, even in the absence of dietary galactose. Complete loss of either activity in embryos is lethal (Table 1). In animals with trace dGALE activity left by knockdown rather than genetic deletion or disruption, however, transgenic expression of either GALE activity alone is sufficient for rescue (Figure 3C). Further, in animals that expressed both GALE activities as larvae, knockdown of both activities during pupation is not lethal.

However, knockdown of either GALE activity in early development, or knockdown of both activities in later development has consequences. For example, loss of activity toward UDP-gal in larvae results in impaired fecundity of both males and females, while loss of activity toward UDP-galNAc in larvae results in impaired fecundity of females but not males. Individual loss of one activity or the other later in development also results in differential sensitivity to galactose. Specifically, both male and female flies deficient in GALE activity toward UDP-gal exhibit a

markedly reduced lifespan when exposed to galactose; this effect is not seen in wild-type flies or in flies uniquely deficient in GALE activity toward UDP-galNAc.

These results support two important conclusions. First, the essential role of GALE in development and homeostasis of *Drosophila* extends beyond the Leloir pathway. Whether GALE activity toward UDP-galNAc is essential because of its presumed role in establishing and maintaining substrate pools for glycosylation, or for some other reason, remains unknown. Prior studies in GALE-deficient mammalian cells [17] showed that uridine supplementation could rescue growth and some metabolic abnormalities caused by galactose exposure, raising the possibility that depleted pools of uridine or uridine-derivatives might also be contributing factors. In the current study it is also unclear whether animals subjected to knockdown of one or both GALE activities later in development demonstrate a less severe outcome than those knocked down earlier in development because the products of GALE function, namely UDP-gal, UDP-glc, UDP-galNAc, and UDP-glcNAc, are less essential later in development, or rather because these UDP sugars have already accumulated to sufficient levels and can be recycled for use. Similarly, the differential sensitivities of male and female fecundity to loss of GALE activity later in development may reflect fundamental differences in male and female development, or alternatively may reflect differential sensitivity to loss; for example, eggs may require a more substantial pool of specific UDP-sugar substrates than sperm to give rise to a viable embryo.

Implications for mechanism: The disparate metabolic profiles observed in GALE-impaired flies exposed to galactose provide a window of insight into potential mechanisms behind the outcomes observed. For example, gal-1P accumulates to abnormal levels in animals missing GALE activity toward UDP-gal but not UDP-galNAc, and only those animals demonstrate substantially reduced lifespan when exposed to galactose as adults. This metabolic result is expected, since only GALE activity toward UDP-gal should impact the Leloir pathway, and this outcome result implies that gal-1P might contribute to the early demise of these animals.

However, the gal-1P result also implies that the negative outcomes observed in *Drosophila* deficient in GALE activity toward UDP-galNAc, e.g. compromised survival in embryos and compromised fecundity in adult females, do not result from gal-1P accumulation. This is an important point because it challenges the common supposition that gal-1P underlies pathophysiology in both classic and epimerase deficiency galactosemias. Clearly there must be another basis for the negative outcomes observed in these animals. It is also interesting to note that while loss of GALE activity toward UDP-galNAc in developing animals has phenotypic consequences, at least for female fecundity, it does not appear to negatively impact the "global" level of UDP-galNAc in animals exposed to galactose. The explanation for this apparent disparity might involve subtle or tissue-specific differences below the threshold of detection of our experimental approach.

Implications for patients: The implications of this work for patients with epimerase deficiency galactosemia are two-fold. First, these results demonstrate that both GALE activities are essential for health of flies, and possibly also people. To our knowledge clinical laboratories that test patient samples for GALE activity only test activity toward UDP-gal. While this practice is certainly understandable, given that mutations may impact the two GALE activities differently [18-20], the results presented here raise the possibility that rare patients with GALE deficiency limited to UDP-galNAc activity could be missed. Second, given the impact of GALE-loss on both male and female fecundity in flies, these results suggest that long-term studies of both male and female reproductive issues in epimerase-deficiency galactosemia patients might be warranted.

Materials and Methods

Drosophila stocks and maintenance

The Drosophila stocks used in this study are listed in Table S1. Stocks were maintained at 25°C on a molasses-based food that contained 43.5 g/l cornmeal, 17.5 g/l yeast extract, 8.75 g/l agar, 54.7 ml/l molasses, 10 mls propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol). For experiments in which the levels and types of sugar were to be varied, we used a glucose-based food [5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l glucose, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol)] [21] supplemented with galactose, as indicated.

Generation of transgenic Lines

UAS-eGALE and *UAS-wbgU* transgenes were generated by subcloning the *eGALE* and *wbgU* coding sequences, respectively, as *Eco*RI/*Xho*I fragments, into *pUAST* [22] using the *Eco*RI and *Xho*I sites in the *pUAST* polylinker region. The *wbgU* sequence was amplified from a plasmid generously provided by Peng George Wang (Ohio State University). Resulting plasmids were confirmed by sequence analysis. *UAS-eGALE* stocks were generated using standard transgenic techniques following injection of the transgene into embryos by the fly core of the Massachusetts General Hospital, Charlestown, MA. *UAS-wbgU* stocks were generated using standard transgenic techniques following injection of the transgene into embryos by Genetic Services, Inc., Cambridge, MA. Transformants were selected by the presence of the white gene within *pUAST*. Expression of functional *eGALE* or *wbgU* was confirmed by enzymatic assay of lysates from transformants.

***GALK, GALT* and *GALE* (UDP-gal) enzyme assays**

Lysates were prepared and assays for GALK, GALT and GALE with UDP-gal as the substrate were performed (n≥3) as described previously [10].

GALE assay conditions for activity toward UDP-galNAc

Activity was calculated from the conversion of UDP-galNAc to UDP-glcNAc. The initial reaction mixture concentrations were: 100 mM glycine pH 8.7, 1.6 mM UDP-galNAc and 0.5 mM NAD. Enzyme assays were performed as described in Sanders et al. [10] except for the following changes: To start each reaction, 7.5 µl of diluted protein and 5 µl of a cocktail of substrates and cofactors were combined. Reaction mixtures were incubated at 25ºC for 30 minutes and then quenched by the addition of 112.5 µl of ice-cold high-performance liquid chromatography (HPLC)-grade water (Fisher). Lysates were diluted 1:4, except for those prepared from animals with RNAi knockdown, which were undiluted, and those prepared from animals overexpressing *hGALE* or *wbgU* transgenes, which were diluted to a greater extent. Lysates from *Act5C>hGALE*^{22C} animals were diluted 1:60. Lysates from *Act5C>wbgU*^{19A} animals were diluted 1:20.

Determining requirement for *GALE* activities in development and homeostasis

Generation of animals in which *GALE* knockdown was initiated at 24-hour intervals throughout development was achieved as described previously [10]. A stock homozygous for both *P{tubP-GAL80*^{ts}*}10* and *12030R-2* was used in all crosses. These flies were then crossed to the appropriate genotypes to obtain offspring expressing various transgenes; for: no transgene, *P{Act5C-GAL4}25FO1 ; + / T(2;3)TSTL, Tb, Hu; eGALE only*, *P{Act5C-GAL4}25FO1, UAS-*

eGALE^{62A}*/ CyO*; *wbgU* only, *P{Act5C-GAL4}25FO1 / CyO; P{Act5C-GAL4}25FO1 / CyO ; UAS-wbgU*^{19A}/TM6B; *eGALE* plus *wbgU*, *P{Act5C-GAL4}25FO1*, *UAS-eGALE*^{62A}*/ CyO* ; *UAS-wbgU*^{19A}/ TM6B; *hGALE, P{Act5C-GAL4}25FO1 / CyO ; UAS-hGALE*^{22C} */ TM6B*. Adult flies eclosing from the vials were scored for the presence or absence of humeral and/or curly, as appropriate for each cross.

Measurement of life span

Animals in which *dGALE* knockdown with concurrent transgene expression was achieved throughout development were obtained as described above. These animals were maintained on standard molasses medium until eclosion. Within 24 hours after eclosion, approximately 20 virgin male or female flies were placed in fresh vials of food containing 555 mM glucose only or 555 mM glucose plus 175 mM galactose. Flies were transferred to fresh food every 2-3 days, and the number of dead flies in each vial was recorded every other day. Log rank and Wilcoxon tests were used for statistical analysis using the program JMP (http://www.jmp.com/).

Measuring metabolite accumulation in GALE-deficient larvae

Cohorts of newly hatched larvae raised at 18ºC were transferred to vials of food containing either 555 mM glucose only or 555 mM glucose plus 175 mM galactose. The larvae were maintained at 18ºC for one additional day, then transferred to 28º-29ºC and allowed to develop for another four days prior to harvest. Metabolites were extracted and quantified as described previously [10], and were separated and quantified using a Dionex HPLC, as described previously [23] with the following changes: UDP-gal and UDP-galNAc were separated using a high salt isocratic procedure with a flow rate of 0.5 mL/min and buffer concentrations of 45% A

and 55% B (0–61 min), followed by washing with a linear increase of B to 95% (61-80 min). For all samples, 20 µl were injected into a 25 µl injection loop. Ratios of the level of each metabolite on food containing galactose over the level on food containing glucose only were calculated, and 95% confidence intervals were determined using Fieller's theorem.

Table S1: *D. melanogaster* stocks and alleles used in this study.

Fly stock or allele name	Comments
w^{1118}	Wild-type *D. melanogaster* (FBst0005905)
$dGALE^{y}$	Imprecise excision of $P\{EPgy2\}CG12030^{EY22205}$ (FBst0022544)
$dGALE^{f00624.4}$	P-element insertion $PBac\{WH\}CG12030^{f00624}$ in second intron of *dGALE* (FBst1016354), Harvard Exelixis Collection
12030R-2	UAS-RNAi to *dGALE* (National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan NM_138200.2)
w; P{tubP-GAL80ts}20;TM2/TM6B, Tb1*	Temperature sensitive allele of *GAL80* (FBst0007019)
y1 w; P{Act5C-GAL4}25FO1/CyO, y+*	*Actin5C-GAL4* driver used for rescue of *dGALE* loss and for *dGALE* knockdown (FBst0004414)
$eGALE^{62A}$	*UAS-eGALE* insertion allele, chr II, homozygous lethal
$wbgU^{19A}$	*UAS-wbgU* insertion allele, chr III, homozygous lethal
$hGALE^{22C}$	*UAS-hGALE* insertion allele, chr III, homozygous viable

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