Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Emily Lu Ryan Date

Mediators of outcome in a *Drosophila* model and patients with classic galactosemia

By
Emily Lu Ryan
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Biochemistry, Cell and Developmental Biology

Judith L. Fridovich-Keil, Ph.D. Advisor	Guy M. Benian, M.D. Committee Member
Joseph Cubells, M.D. Ph.D. Committee Member	William Kelly, Ph.D. Committee Member
Kenneth Moberg, Ph.D. Committee Member	Subhabrata Sanyal, Ph.D. Committee Member

Accepted:

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

_____ Date

Mediators of outcome in a *Drosophila* model and patients with classic galactosemia

By

Emily Lu Ryan

B.S., University of California, San Diego 2000

Advisor: Judith L. Fridovich-Keil, Ph.D.

An abstract of
A dissertation 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
Doctor of Philosophy
in
Graduate Division of Biological and Biomedical Science
Biochemistry, Cell and Developmental Biology
2012

ABSTRACT

Mediators of outcome in a *Drosophila* model and patients with classic galactosemia

By: Emily Lu Ryan

Classic galactosemia is a rare autosomal recessive disorder caused by the loss of function of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme in the Leloir pathway of galactose metabolism. Most cases in the US are diagnosed through newborn screening and dietary galactose is restricted before the disease can progress to life threatening symptoms like severe *E. coli* sepsis. Unfortunately, even with dietary restriction most individuals with classic galactosemia will go on to experience quality of life complications, including cognitive disability and movement disorders, among others. Patient studies suggest these long-term complications are independent of dietary galactose exposure and may be more common in individuals with certain GALT mutations.

A GALT knockout mouse was generated almost 20 years ago but these animals did not recapitulate any of the symptoms seen in the human disease, including dietary galactose toxicity. The work detailed here establishes the first two phenotypes found in a *Drosophila melanogaster* model of classic galactosemia. *GALT* null *Drosophila* exhibits both a dietary galactose dependent larval lethality and an adult climbing defect.

We investigated the effects of dietary galactose exposure and low levels of GALT activity on these phenotypes. The timing, duration, and level of dietary galactose exposure modified the larval lethality; however dietary galactose exposure at low levels had no effect on the adult movement defect even though the animals accumulated high levels of galactose-1phosphate, the metabolite upstream of GALT. Both phenotypes were rescued by transgenic expression of human GALT.

We also explored the scholastic and behavioral consequences of GALT deficiency in school age children. In our study of such children with classic galactosemia we saw a higher than expected incidence of behavior problems. Cryptic GALT activity explained some of the variation in our population. Combined, these results help further our understanding of the complications that result from loss of GALT in fruit flies and in humans.

Mediators of outcome in a *Drosophila* model and patients with classic galactosemia

By

Emily Lu Ryan

B.S., University of California, San Diego 2000

Advisor: Judith L. Fridovich-Keil, Ph.D.

A dissertation 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
Doctor of Philosophy
in
Graduate Division of Biological and Biomedical Science
Biochemistry, Cell and Developmental Biology
2012

Table of Contents Page

List of Figures Page

List of Tables Page

CHAPTER 1

INTRODUCTION

1.1 CLASSIC GALACTOSEMIA

Classic galactosemia is a rare autosomal recessive disorder of galactose metabolism with a prevalence of 1/40,000 to 1/60,000 in the United States (1). The gene disrupted in classic galactosemia, galactose-1-phosphate uridylyltransferase or *GALT*, is located on the short arm of chromosome 9 and in cases of classic galactosemia both alleles no longer encode sufficient enzymatic function (2, 3). Of the over 200 causative mutations that have been reported in *GALT* most are missense mutations (4). World-wide prevalence and severity of classic galactosemia varies based upon population with very few reported cases among Asians (5, 6). In populations of European ancestry, prevalence varies between Northern European and Eastern European populations due to the varying prevalence of two common mutations, a point mutation in exon 6 c.563A>G p.Q188R or a different point mutation in exon 9 c.865C.T p.K285N (7-13). The mutation, c.404C>T p.S135L, is more prevalent in groups with African ancestry and may lead to less severe symptoms (14, 15). All three of these mutations occur in pan-ethnic groups along with a 5.5 kilobase deletion, common in those of Ashkenazi decent, with varying frequencies (12, 16-18). These four mutations are common but represent a subset of the many mutations that have been studied in order to understand the disease, classic galactosemia.

Classic galactosemia presents early in life; infants appear normal at birth but with the initiation of a milk-based diet symptoms begin to arise (1). Symptoms can progress from poor feeding and failure-to-thrive to diarrhea, vomiting, lethargy and hypotonia and, if left untreated, to hepatomegaly and cirrhosis which eventually could lead to severe *E. coli* sepsis and death (19). Standard treatment is lifelong dietary restriction of galactose either via an elemental or soy-based formula (19). Infants on a galactose

restricted diet often develop long-term complications (20, 21). Due to the severity and preventability of these acute symptoms newborn screening for classic galactosemia was initiated and today most infants with classic galactosemia in the US are treated after a positive screen result (22, 23). Newborn screening occurs in almost all developed countries, including the United States, via dried blood spot analysis (24, 25). In almost all instances, newborn screening for classic galactosemia is based upon the assay that Ernest Beutler and colleagues developed in the 1960's which takes advantage of the fluorescent properties of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the enzyme glucose-6-phosphate dehydrogenase in conjunction with measuring total galactose in the dried blood spot (24, 26). Prior to newborn screening it was estimated that approximately half of all infants born with classic galactosemia did not survive to diagnosis. Now after 20 to 30 years of newborn screening most infants with classic galactosemia do not experience the full-range of acute symptoms but the long-term quality of life complications are still prevalent (22). A large percentage of classic galactosemics have at least one long-term complication: 40-60% experience development delay or have IQs below normal, up to 90% have speech difficulties, and 80 to 90% of females with classic galactosemia experience premature ovarian insufficiency (20, 21, 27-31). The underlying mechanism of these complications is not understood, though it is theorized to be independent of galactose exposure (32).

Minimizing galactose exposure is a main focus of clinical management of classic galactosemia; galactose restriction relieves the early life-threatening symptoms but it is unclear whether it has an effect on long-term outcome(22, 33, 34).Galactose is found in food in one of the following forms: as the disaccharide lactose (paired with glucose), or

as a component of complex carbohydrates like raffinose, or free in various fruits and vegetables (35-37). Galactose restricted diet excludes milk and all dairy products along with blueberries, grapes, most tomatoes, fresh peas, and some melons from the diet (25, 35-37). Galactose can also be produced endogenously and studies suggest that this endogenous production is age-dependent, decreasing into adulthood (38, 39). Therefore it is difficult to determine if cryptic dietary galactose exposure is toxic independent of endogenous production. Galactose, whether ingested or produced internally, is metabolized via the conserved Leloir pathway of galactose metabolism. Disruption of this pathway causes galactosemia.

1.2 GALACTOSE METABOLISM

Classic galactosemia, the most common clinically severe galactosemia, is due to loss of galactose-1-phosphate uridylyltransferase (GALT) function. GALT is the second enzyme in the Leloir pathway of galactose metabolism (Figure 1.1). Galactose is an epimer of glucose, the difference being the placement of the hydroxyl group on the fourth carbon. Since galactose and glucose are closely related, when galactose concentrations are elevated various enzymes that normally react with glucose, or its metabolites, can instead react with galactose (Figure 1.1) (40). These enzymes may play important roles in the pathophysiology of classic galactosemia.

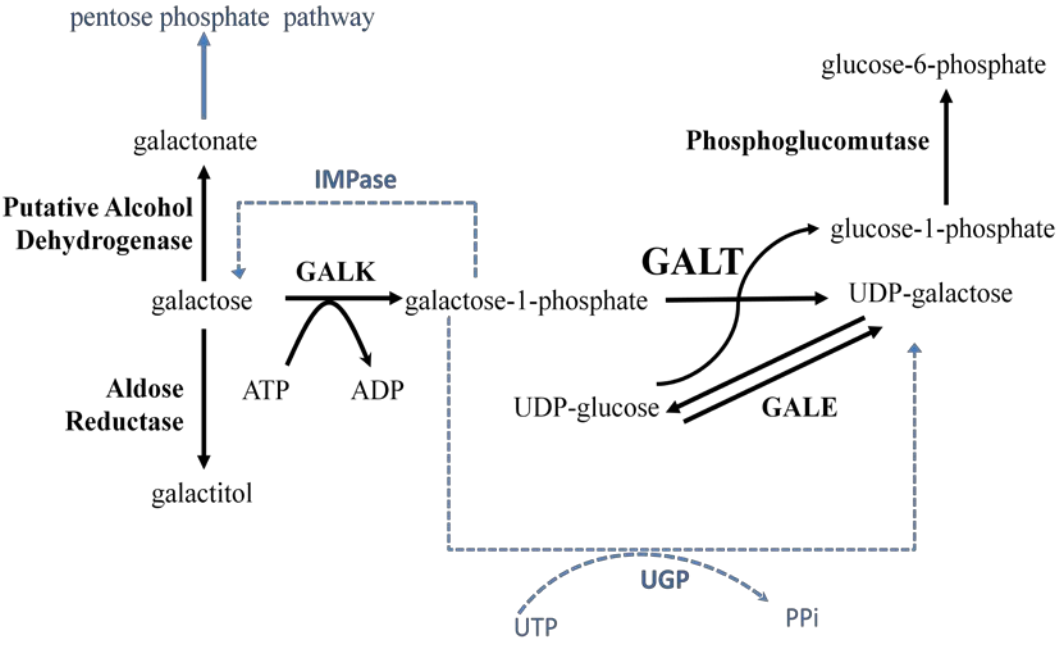

Figure 1.1: Galactose metabolism. When GALT activity is absent or impaired metabolites build up and other non-Leloir enzymes act on galactose and galactose-1-phophate.

Leloir pathway

The highly conserved Leloir pathway of galactose metabolism consists of three enzymes, galactokinase (GALK), galactose-1-phosphate uridylyltransferase (GALT) and uridine diphosphate (UDP)-4' epimerase (GALE) (Figure 1.1). Galactose enters the cell and is phosphorylated by GALK, a member of the GHMP super family, releasing a large amount of energy with the dephosphorylation of adenine triphosphate (ATP) to adenine diphosphate (ADP) (41). GALT transfers the uridine monophosphate (UMP) moiety from UDP-glucose to a histidine residue, releases glucose-1-phosphate and then adds the UMP to galactose-1-phosphate forming UDP-galactose (42). Finally GALE interconverts UDP-galactose to UDP-glucose, favoring UDP-glucose (43). These three enzymes and their reactions have been studied extensively to uncover the galactose specificity that has been conserved from *E. coli* to humans; for instance, GALK specifically phosphorylates galactose and not glucose, fucose, or arabinose (44, 45). All three of these enzymes form dimers, though through different mechanisms. GALK forms disulfide bonds between monomers while GALT and GALE dimers are not covalently linked but both enzyme dimers are stable through purification (44-48). GALE, in higher organisms, also functions to control concentrations of UDP-N-acetyl-glucosamine and UDP-N-acetyl-galactosamine as well as UDP-galactose and UDP-glucose, essential in N- and O- link glycosylation (43). Because these substrates are essential in many cellular processes, the Leloir pathway has an impact beyond solely turning galactose into glucose.

A key role of the Leloir pathway is to convert galactose into glucose-1-phosphate that can contribute to glycolysis after phosphoglucomutase converts it to glucose-6-phosphate (Figure 1.1). Glucose-1-phosophate is not the only pathway product that is

used in vital cellular activities, UDP-glucose and UDP-galactose are also essential. As the building block of glycogen, UDP-glucose is needed in the liver and skeletal muscle, and other organs, for energy storage (49). Both UDP-galactose and UDP-glucose are used in glycosylation of proteins and lipids and one of GALE's essential roles may be to modulate the relative concentrations of the UDP-sugars (50). Glycosylation dependent signaling during development is well established; a classical example is the role of fringe, an N-acetylglucosaminyltransferase, in the Notch dependent dorsal/ventral patterning of the fruit fly wing (51). A possible theory behind the long-term complications seen in patients with classic galactosemia is that they are caused by subtle glycosylation mistakes during development, though the evidence in support of this is mixed.

Common GALT Mutations

Severe GALT impairment, leading to classic galactosemia, is caused by over 200 known mutations (4). Most causative mutations are point mutations within the coding region resulting in either an amino acid change or a truncation; there is a common large deletion of 5.5 kilobases (16).Many studies have focused on two common mutations (c.563A>G p.Q188R and c.404C>T p.S135L) and their effect on GALT activity. These mutations have been well investigated through modeling and crystallization studies, as well as expression and activity studies in the yeast, *Saccharomyces cerevisiae,* and in the patient population at large. On a structural level the change from glutamine (Q) to arginine (R) at position 188 is thought to destabilize the active site making it more difficult for the histidine at position 186 to attack the oxygen-phosphate bond and remove glucose-1-phosphate from the UMP-moiety (52, 53). The exact mechanism of this destabilization is unclear; the crystal structure of the *E. coli* sequence and the computer

models of the human sequence disagree regarding the exact alteration hydrogen bonding patterns with the Q188R substitution (42). In the case of the serine (S) to leucine (L) mutation at position 135, there is little known about how this amino acid substitution affects the protein structure therefore most of the information regarding this mutation has come from expression studies (42).

Much of our understanding of these substitutions comes from expression studies where either mutant or wild-type human GALT protein is expressed in place of the endogenous enzyme; most of these studies used the yeast, *Saccharomyces cerevisiae* but the bacteria, *Escherichia coli* has also been useful. Yeast expressing only human Q188R GALT had a similar phenotype to yeast not expressing GALT. Neither had detectable GALT activity, nor did they grow in the presence of galactose and both accumulated galactose-1-phosphate (54, 55). Conversely, yeast expressing mutant S135L GALT retained a low level of activity (about 3% of wild-type human GALT) and grew in the presence of galactose (56). These yeast accumulated galactose-1-phosphate but less than half of the amount accumulated by yeast expressing human Q188R GALT (55). These data support the conclusion that the Q188R substitution disrupts GALT activity. It also clarifies the effect the S135L substitution has on enzyme activity. Using this system other substitutions have been classified as having a cryptic level of GALT activity, similar to S135L. The structural analyses of GALT showed that it forms dimers; the yeast expression system is ideal to explore this behavior (44). From yeast expressing both wild-type hGALT and either S135L GALT or Q188R GALT dimers were purified (57). When further investigating the enzymatic activity of these dimers most heterodimers, i.e. S135L/wild-type, had approximately 50% of the activity wild-type homodimers (57). But

Q188R/wild-type heterodimers had reduced activity, suggesting that the Q188R allele may act in a dominant negative fashion in a heterodimer (57, 58). This observation is relevant to the patient population because most patients are compound heterozygotes and Q188R is the most common allele in classic galactosemia. These data regarding dimerization and specifically the behavior of Q188R hGALT in the dimer show us a patient's endogenous GALT activity is a composite of many different factors.

Patients with classic galactosemia must have two causative mutations in *GALT* and combined with the heterogeneity at the *GALT* locus it is difficult to infer the endogenous impact of different GALT mutations. Since Q188R is an extremely common mutation, some studies have compared individuals who are homozygous for Q188R with those who carry only one copy of the allele. In these studies, individuals who are Q188R homozygotes may be at a higher risk for intellectual disability and speech disorders but due to the heterogeneity of the population these results are less than conclusive (21, 28, 59). It is unclear what the effect of the S135L mutation has on the long-term outcome of classic galactosemics. Very few individuals who are homozygous of the S135L have been studied. Those that have been studied are more capable of metabolizing galactose and have lower levels of galactose-1-phosphate than those who are Q188R homozygotes (14, 15). Regardless, it seems that the mutation Q188R severely impacts GALT activity and dimer formation and may increase the risk of some long-term complication of classic galactosemia, while S135L may lead to a less severe phenotype due to the low-level residual enzyme activity remaining.

Alternative pathways of galactose metabolism

Galactose is metabolized via the Leloir pathway, but in instances where the pathway is disrupted enzymes involved in metabolizing other hexose sugars can act on metabolites of galactose to provide a bypass around the crippled enzyme. When GALT is absent or severely crippled galactose-1-phosphate and galactose build up inside cells, in the circulating blood stream, and in urine (60). In the absence of GALT activity UDP-glucose pyrophosphorylase (UGP) will convert galactose-1-phosphate into UDP-galactose, even though UGP has a greater affinity for glucose-1-phosphate. UGP's activity toward galactose-1-phosphate may decrease the circulating concentrations in patients with classic galactosemia (Figure 1.1) (61, 62). Other enzymes involved in the metabolism of sugars, aldose reductase and alcohol dehydrogenase, can convert galactose to galactitol or galactonate, respectively, and decrease circulating galactose (Figure 1.1). In individuals with a working the Leloir pathway, galactitol is present only after a bolus dose of galactose and quickly decreases to an undetectable concentration; in contrast to individuals with classic galactosemia where galactitol may be elevated even in individuals on dietary restriction (63, 64). Galactitol is excreted at high concentrations in the urine of classic galactosemics for it cannot be reduced further in humans (64). Galactonate, the oxidized form of galactose, is produced from galactose by either an unknown galactose-specific dehydrogenase or a more promiscuous alcohol dehydrogenase. In individuals with classic galactosemia, galactonate accumulates in the blood and tissues and will enter the pentose phosphate pathway for further metabolism (1, 65-67). Metabolites such as galactose-1phosphate, galactitol and galactonate, are used to monitor dietary adherence in patients with classic galactosemia. They may also be

elevated in the two previously described genetic models of classic galactosemia: the yeast, *Saccharomyces cerevisiae* and the mouse, displaying the pathway conservation of not only the Leloir pathway but also these alternative pathways of galactose metabolism (54, 60, 63, 67, 68).

1.3 GENETIC MODELS OF CLASSIC GALACTOSEMIA

Yeast

The budding yeast, *Saccharomyces cerevisiae*, has a fully conserved Leloir pathway and has been used extensively in studies of galactose metabolism for decades (54, 55, 61, 69-72). Initially discovered in the 1960s, yeast lacking GALT or GALE activity, due to loss of the genes *GAL7*and*GAL10*, respectively, were galactose sensitive and accumulated galactose-1-phosphate (69). Yeast's role as a model system for classic galactosemia began in 1993 when the galactose sensitive *GAL7* null yeast was rescued via expression of human GALT (54). The utility of this system became the strongest molecular tool to understanding the severity of different point mutations in human GALT; each amino acid substitution could be tested to determine its impact on protein conformation and enzymatic activity (55, 58, 70, 72, 73). The Q188R substitution was determined to not only interfere with the enzymatic reaction but also act in a dominate negative fashion on the wild-type subunit in a heterodimer (58). The S135L substitution was found to have residual GALT activity and yeast expressing the S135L.hGALT were no longer galactose sensitive (55). Many mutant forms of human GALT have been expressed in the yeast to determine: the impact to enzymatic activity, the ability of yeast to grow in galactose only media when expressing the mutant GALT, and the concentration of galactose-1-phosphate that accumulates under these conditions (55, 70, 73, 74). Not all mutations in GALT are functionally equivalent and some may cripple the enzymatic function in this model system while others retain cryptic levels of activity.

The yeast model of classic galactosemia has also been instrumental in understanding the metabolic consequences of GALT impairment. In yeast the capacity for removal and addition of different enzymes has been essential for testing enzymes other than GALT for their ability to relieve the galactose dependent phenotypes. For instance, in the absence of GALT, yeast also lacking GALK were not galactose sensitive and did not accumulate galactose or galactose-1-phosphate internally (71). Some non-Leloir enzymes have also rescued the galactose dependent phenotypes when expressed in GALT null yeast. Human UGP, an enzyme that normally converts glucose-1-phosphate and UTP to UDP-glucose, was expressed in GALT null yeast and shown to rescue galactose sensitivity and theoretically lower galactose-1-phosphate levels (Figure 1.1) (61). The enzyme, inositol mono-phosphatase (IMPase) normally dephosphorylates inositol but when human IMPase was expressed in GALT null yeast it rescued the galactose sensitive phenotype (75, 76). Therefore it is possible that human IMPase can also dephosphorylate galactose-1-phophate to galactose decreasing the amount of galactose-1-phosphate inside the cell. And finally a yeast specific aldose reductase, *GRE3*, was over-expressed in a GALT null strain relieving the galactose sensitivity, decreasing galactose-1phophate levels and increasing galactitol levels (77). With the observations of these three enzymes relieving the galactose growth inhibition, is it accumulation of galactose-1-phosphate that causes the GALT null galactose sensitivity (71)? In the experiments with GALK and aldose reductase we know the loss of GALK and the over-expression of aldose reductase decrease galactose-1phoshate levels; in the instances of UGP and IMPase over-expression it is inferred (61, 71, 76, 77). The addition of exogenous or the removal of endogenous enzymes in this model system has offered the

galactosemia community a wealth of understanding how the galactose sensitive phenotype relates to metabolic accumulation.

The strengths of the yeast model of classic galactosemia are rooted in the ability to isolate factors that may contribute to classic galactosemia, whether it is the specific GALT mutation, the effects of galactose, and the various enzymes that may elevate the galactose specific phenotype. Along with these strengths, there are limitations with this model system. The major limitation is that *Saccharomyces cerevisiae* is uni-cellular and thereby offers little information regarding the effect of absence of GALT activity on developmentand in the nervous system. The insights gained from yeast reveal there may be an imbalance in sugar metabolites and high concentrations of galactose, galactose-1phosphate, and other metabolites may affect downstream pathways (i.e. glycosylations). What is needed to explore the mechanisms of the long-term complications is a multi-cellular animal model that shows galactose sensitivity like the yeast system.

Mouse

In an effort to make a multi-cellular model of classic galactosemia, Dr. Nancy Leslie and her group created the first GALT knockout mouse by disrupting the exons which encode the catalytic site (78). Like patients, GALT null mice were viable, lacked GALT enzymatic activity, and accumulated large amounts of galactose-1-phosphate when consuming milk; but unlike the patients they did not become ill when exposed to dietary galactose (78). Even when mouse chow was supplemented with galactose after weaning these animals still showed no toxic effect (68, 79-81). Further investigation of the GALT null mouse demonstrated that galactose-1-phosphate accumulates in many

tissues, including liver, kidney, brain, heart, and muscle, while it is barely detectable in control animals given the same bolus does of galactose (82). With knowledge of alternative pathways of galactose metabolism gained from the yeast system, the enzyme UGP was investigated as a possible reason for the lack of galactose-dependent toxicity in the mouse. Murine UGP is present in these mice and can account for a portion of converted galactose-1phophate to UDP-galactose, but enzyme levels are not sufficient to prevent the accumulation of galactose-1-phosphate (62). The GALT null mouse's ability to tolerate high amounts of galactose is puzzling and theories abound. One possibility lies in the fact mice do not rely on aldose reductase in the same manner humans do (83). Though present in mice, aldose reductase activity is reduced and mice do not accumulate galactitol to the same levels as neonates with classic galactosemia (83). Whether galactitol is the source of galactose-dependent toxicity is still under debate and is discussed in the mechanism section at the end of this chapter.

The knowledge gained in the yeast model of classic galactosemia strengthened our understanding of the metabolic consequences in a cell that lacks GALT activity. But we cannot begin to address the long-term outcomes of patients in a model that does not develop into a multi-cellular organism. In this context, the mouse phenotype seen is disheartening. These animals obviously lack GALT activity, metabolically, but have none of the acute or long-term outcome complications of the patients. With patient complications like premature ovarian failure and cognitive deficits, a multi-cellular animal model which mimics some of the patient complications is essential to ask questions about pathophysiology and timing during development. Without such a model,

researchers have searched for non-invasive ways to ask these questions in patients with classic galactosemia with limited success.

1.4 INCIDENCE AND PATHO-PHYSIOLOGY OF THE LONG-TERM CONSEQUENCES OF CLASSIC GALACTOSEMIA

Classic galactosemia is a rare disease and most studies report fewer than 50 individuals. One early study collected extensive outcome information from over 300 patients in the US and Europe, by surveying their medical professionals (20). They found a significant delay in growth among children but adults reached normal height; 81% of the females had experienced premature or primary ovarian failure (POI); 56% of individuals over three years of age reported problems with speech; and 45% of individuals over six years of age were classified as developmentally delayed (20). They also found speech, motor function, and growth covaried with IQ. With these high incidences of complications in classic galactosemia, it is clear that GALT deficiency affects multiple tissues and that in the absence of GALT activity development is perturbed. The underlying mechanisms and modifiers of these complications are not known; it is possible that there is more than one mechanism by which these complications arise.

Bone and Growth

Among the many cohort studies of classic galactosemia, bone health and growth have been studied both in children and adults. Children with classic galactosemia have below average heights, weights, and bone mineral densities (BMD) for their respective age and gender (84, 85). Conversely adults with classic galactosemia reach normal heights and weights but, like the children, have low bone mineral densities (86). Women with classic galactosemia have BMD one to two standard deviations below average,

significantly lower than men with classic galactosemia. In the general population women with low BMD are also deficient in estradiol but in women with classic galactosemia had estradiol levels within the normal range (86). Because low BMD and POI are prevalent in women with classic galactosemia, an active area of research is to understand if these two long-term complications, bone health and fertility problems are linked.

The significantly lower bone mineral density in the population with classic galactosemia could be due to diet and a dependence on supplemental calcium to correct for dietary lactose-restriction. In fact, one study found the addition of vitamins D and K to calcium supplementation increased bone density among pubertal children and increased body fat and lean tissue mass in all children (87). In an effort to determine if the lower than expected bone mineral density was due to a decrease in bone mineralization or an increase in bone metabolism, biomarkers of each process were tested in patient plasma and serum samples (84-86). Evidence of abnormal bone metabolism, modeling and turnover was evident in prepubertal, pubertal, and adult individuals with classic galactosemia (84-86). Regardless of calcium supplementation, growth and bone health in children and adults with classic galactosemia is perturbed leading to low bone mineral density throughout life; the underlying reason remains a mystery.

Ovarian Insufficiency

Early in the study of classic galactosemia, it was noted that a large proportion of females did not proceed through puberty unassisted or failed to maintain sufficient ovarian function afterward (88). The severity of impairment presents a broad range; most girls with classic galactosemia start puberty without assistance but many need exogenous

hormones to finish and most will experience premature menopause (89-91). Further studies have reported 60 to 90% of females with classic galactosemia will be diagnosed with premature ovarian insufficiency (POI) (20, 21, 30, 90, 91). Follicle stimulating hormone (FSH) is often abnormally elevated in young women with classic galactosemia (30, 90-92). FSH requires glycosylation for bio-activation and early studies suggested that FSH in classic galactosemic woman may not be fully glycosylated or active (93, 94). More recent studies however have shown that FSH from women with classic galactosemia is biologically active and does not appear to have any glycosylation abnormalities (30, 95). In girls and woman anti-Müllerian hormone (AMH) is produced by developing follicles within the ovary and is an accurate marker for ovarian reserve in most age groups (96). AMH levels in girls with classic galactosemia are consistently low in all age groups and do not rise upon exogenous FSH stimulation (30, 97). The mechanism of ovarian insufficiency in girls and women with classic galactosemia is not known but currently it is believed the ovaries are affected early in life, possibly in infancy or earlier (30). GALT genotype may modify the severity of POI; one study determined that Q188R homozygotes were at a 16-fold higher risk for POI than were those who either had one or no copies of the Q188R allele (90). While little is known about the mechanism for POI among classic galactosemic woman, hormone levels are aberrant before puberty and most women with classic galactosemia will experience premature menopause.

Speech and Language

A large portion of the population with classic galactosemia has speech difficulties though the cause, severity, and the specific difficulty may vary significantly. In a study of

over 300 people with classic galactosemia 56% over the age of 3 had been diagnosed with a speech difficulty and most were classified as delayed with vocabulary and articulation problems (20). In this group and others, many individuals with speech difficulties also had IQ's below 85 (20, 28). Recently, work has focused on trying to understand the pathology of the more prevalent speech problems found in the classic galactosemic population (98-100). Adults with classic galactosemia have difficulty repeating pseudo-words, rather than real words, and the most common speech error is elision, the omission of a sound from a word, like in a contraction (98). Most children with classic galactosemia have a diagnosis of a motor speech disorder (MSD); a rare type of MSD, childhood apraxia of speech (CAS), is more common (24%) in children with classic galactosemia than in other populations of MSD (100). CAS is considered a neurodevelopmental disorder with either idiopathic or genetic cause. Children with classic galactosemia and MSD have difficulty sustaining vowel vocalization and have poor voice quality (99). Preliminary work looking at language processing suggests that brain activity while speaking is delayed in adults with classic galactosemia (101). The difficulties with speech do not affect every individual with classic galactosemia and in some populations those homozygous for the Q188R allele are more severely affected than those who are compound heterozygotes with only one Q188R allele (28). The mechanism behind the different speech problems may include muscular problems with the larynx, may be neuromuscular in nature, like CAS, or may be more of a brain disorder as the language processing work suggest.

Neurologic

Neurological complications from classic galactosemia include intellectual disability (ID) and, less often, tremors and ataxia (20, 21, 27). Most adults and children with classic galactosemia have an intelligence quotient (IQ) below average, though some have an IQ in the normal range, 80-120 (20, 21, 102, 103). Initial studies suggested a population-wide decline in IQ as a function of age. Currently, the decrease in IQ scores between 6 to 9 year olds and 10 to 16 year olds is thought to be test specific (20, 102, 103). Tests for older children and adults rely more heavily on processing speed than do the tests for younger children; it is thought processing speed might be affected in individuals with classic galactosemia and ID (101, 102). Though more rare than other complications of classic galactosemia, problems of coordination and gait along with tremors and ataxia have been noted in 18% of the population (20). A recent study of 33 adults with classic galactosemia demonstrated a higher rate of tremor (46%), and ataxia (15%), and one individual had adult onset seizures (21).

The underlying pathology behind the neurologic complications is not understood but some patients with classic galactosemia have abnormal brain morphology (27). MRI results from one study of 40 patients found 93% had abnormal white matter signal and 20% had visible white matter lesions throughout their brains, but these abnormalities did not correlate with cognition (27). A smaller study of families with multiple affected children saw abnormal deep cerebral white matter as the most common MRI abnormality which was attributed to a delay in or absent myelination (32). It is possible that disruption of GALT activity may lead to incorrect synthesis of glycolipids critical to the formation of myelin (104). A small study of 5 adults with classic galactosemia and tremors reported

aberrant glucose metabolism in the cerebellum, anterior cingulate gyrus, and basal ganglia in comparison to healthy controls (105). These changes in glucose metabolism could either be symptomatic of or the underlying cause of the tremors. Studies using families with multiple affected individuals have shown that early dietary galactose exposure does not influence the severity of neurological complications of classic galactosemia (27, 32, 102). The true mechanism of these neurological complications is unclear.

Behavior and Quality of Life

Many adults with classic galactosemia have long-term complications that impact their quality of life (21, 102, 106, 107). There is a high lifetime incidence of depression and anxiety among adults with classic galactosemia, well above the incidence in the general population (21). German adults with classic galactosemia asked about their quality of life scored lower than the general German population in the categories of "positive mood", "social well-being" and "social functioning" (107). Many individuals with classic galactosemia not only have IQs below normal but there is also a high incidence of impaired executive functioning (21, 102, 106, 107). A significant proportion of adults with classic galactosemia still live with family members and are not employed, possibly due to a high incidence of intellectual disability, impaired executive functioning and adaptive behaviors (21, 107). Most adults with classic galactosemia complete high school and some attend university; many who are employed in long-term work are in a skilled manual labor position (21, 107). While there are individuals with classic galactosemia who complete university, live independently, and raise families, these are the minority (21, 106, 107). These quality of life measures correlate with ID, in most

populations, but both have a high incidence in classic galactosemia therefore it is unclear whether they are related or separate complications of the disease.

Mechanism and Modifiers

Not every individual with classic galactosemia is affected by the long term complications described above; therefore, much work has been done to identify risk factors for these different complications. Dietary galactose exposure does not increase risk or severity of long-term complications (20, 32, 102). Individuals homozygous for the Q188R allele may be at increased risk for more severe outcomes (20, 28, 32, 59, 90, 102). While these factors have been studied extensively, most of the variation in outcome is unexplained (108). There must be other factors (environmental or genetic) that can modify the severity of these many long-term complications. Understanding the mechanism behind these long-term complications would help us discover these modifiers.

The various long-term complications of classic galactosemia: low bone density, POI, speech difficulties, tremors, ID, and decreased quality of life, may share a common mechanism, or may be caused by different, but related defects. There are published reports of aberrant protein glycosylation in serum and urine of patients with classic galactosemia (109-111).The loss of GALT activity could impact the production of glycolipids and glycosylated proteins through a subcellular imbalance of UDP-gal and UDP-glc concentrations. One theory is aberrant UDP-gal and UDP-glc concentrations during development leads to neurologic complications via a drop in the locally available UDP-gal leading to inhibition of galactoceramide synthesis and affecting myelination

(104). FSH requires proper glycosylation for bioactivity, therefore it was theorized that POI in classic galactosemia was due to the ovaries not receiving the FSH signal due to improper glycosylation. Current studies of serum from girls with classic galactosemia and POI demonstrate FSH is active and properly glycosylated (30, 97). Recent studies also suggest that plasma glycosylation abnormalities in adults and older children with classic galactosemia do not correlate with long-term complications, (110). There may still be differences in glycosylation patterns prior to galactose restriction, but it is unclear whether these are due the toxic effect of galactose, or are specific to diet, or the source of clinical symptoms (110). While aberrant glycosylation during development is still a possible cause of long-term complications, it appears that ongoing abnormal glycosylation in individuals with classic galactosemia is minimal and may be not causative.

A different mechanism for these long-term complications may include the loss of GALT activity leading to metabolic imbalances that interfere with normal enzymatic function and disregulate some cellular processes. Along those lines the results from a brain-MRI study of an 8 day old infant with classic galactosemia revealed increased levels of galactitol and decreased levels of myo-inositol in different parts of the brain when compared to scans of an unaffected 9 day old infant (112). Since IMPase can convert galactose-1-phosphate to galactose in yeast, high concentrations of galactose-1-phosphate might compete with inositol monophosphate for IMPase active sites (75, 76). Inhibition of the dephosphorylation of inositol monophosphate could disrupt of myo-inositol synthesis in the brain and decrease the level of myelin produced during brain development (113). Since the phosphorylation/dephosphorylation of phosphatidylinositol

is essential in various cellular signaling pathways it is conceivable that the disruption of IMPase could alter other tissues as well as brain.

A third possibility for the mechanism of long-term complications in classic galactosemia comes from work where rodents and *Drosophila* are given large amounts of galactose (114, 115). Wild strains of fruit flies when kept on food containing galactose, as the sole monosaccharide, do not live as long as those kept on food containing glucose (114). The fruit flies exposed to galactose also show increased levels of reactive oxygen species, a sign of aging (114). Similarly, mice who received daily subcutaneous injections of galactose showed an increase of oxidative stress biomarkers and neurodegeneration when compared to mice injected with saline (115). While these models were developed to study the process of aging, it becomes apparent that large doses of galactose increase oxidative stress and are galactose specific (114, 115). If giving galactose to these animals increases oxidative stress, it is possible that the loss of GALT activity could have a similar affect. If oxidative stress was the mechanism behind these outcome complications it would suggest that tissues more susceptible to oxidative stress, like neurons, would be more affected by the loss of GALT activity. In order to determine the true mechanisms at work in these long-term complications, an animal model that recapitulates at least some of these phenotypes is needed.

1.5 SUMMARY

The underlying cause of classic galactosemia, severe impairment of GALT, has long been known but the pathophysiology behind the complications and disease severity are far from understood. As with any rare disease, the limited numbers of affected individuals available for study impedes the ability to study how environmental exposures and background genetics can affect the severity of symptoms in patients. Some environmental exposures, like dietary galactose, have been assessed in different groups with mixed results (20, 32, 102). Work in the yeast model has shown that not all GALT mutations are functionally equivalent; some still retain significant cryptic levels of activity (55). In order to rigorously test these environmental exposures along with different GALT genotypes and background genetic variation an animal model is essential. Unfortunately, the phenotypic results from the mouse model have not answered these questions and instead left more questions needing answers. It is a puzzle why in the presence of a galactose diet GALT null mice do not display any toxic symptoms while still accumulating galactose-1-phosphate in serum and tissue (68). We need a multi-cellular animal model that recapitulates some, if not all, of the patient's phenotype to further understand how the complications of classic galactosemia arise and why they vary in severity. The goal of this dissertation is to establish a fruit fly model of classic galactosemia and to address some unanswered questions about potential modifiers of classic galactosemia using this model organism and a retrospective patient cohort study.

The work detailed in chapters Two and Three was undertaken with the desire to understand the modifiers underlying outcome severity in GALT deficiency. The aim of these chapters is to develop a *Drosophila melanogaster* model of classic galactosemia,

assess its usefulness for modeling patient phenotype, and test some patient specific modifiers. Chapter Two establishes the model and begins to assess the acute phenotype in the presence of a galactose supplemented diet. The *GALT* null animals fail to develop in the presence of high concentrations of galactose but survive with either the removal of galactose from the diet or the addition of the human enzyme. Chapter two also presents evidence for movement phenotype in *GALT* null animals. Chapter Three delves into the movement phenotype, demonstrating it is a climbing defect, and explores a number of modifiers relevant to the patient experience, dietary exposure to galactose, cryptic GALT activity and age. It also examines the possibility of gross morphological differences that arise in the absence of GALT activity. These results show that low level galactose exposure during development does not exacerbate the movement deficit, that cryptic GALT activity can improve ability to climb and that aged GALT null animals are less able than younger animals to climb repeatedly. These conclusions regarding the affects of GALT activity and galactose exposure during development and homeostasis on this movement phenotype in the adult fly suggests that some of these modifiers may also affect the severity of complications for patients with classic galactosemia.

Work in the *Drosophila* model of classic galactosemia allows us to manipulate one factor at a time and assess its contribution to the severity of the phenotype in question. However, there are limits to the model and some of the complications in classic galactosemia may not have analogous phenotypes in the fruit fly. Previously, reports in the literature document patients with classic galactosemia having behavioral differences from the general population, therefore we investigated what kinds of behavioral differences exist in children of school age with classic galactosemia at home or in school

(21). As described in chapter Four, we assessed their behavior and social interaction via parent and teacher surveys and their scholastic achievement via school records. We found our study volunteers have more problems with social skills and internalizing problems, than expected, and that those enrolled in special education or those whose academic achievement was below grade level are more likely also to have these behavior problems. We assessed cryptic GALT activity in our study volunteers and it modified the severity or presence of some of these behavior problems, specifically social interactions. These results showed that, in children with classic galactosemia, behavior and achievement were not independent and may impact quality of life on a broader scale.

This dissertation addresses factors that modify the severity and type of complications of the disease classic galactosemia using results from studies involving a fruit fly model and a retrospective cohort study of school age children with the disease. This work advances what we know about classic galactosemia in a number of ways. First it establishes the first multi-cellular animal model of the disease that demonstrates aspects of the patient phenotype, both in regard to galactose-dependent toxicity during developmental and long-term galactose-independent phenotypes. Second using the fly model, it explores a series of patient-relevant modifiers to see if they influence the impaired movement phenotype of GALT null flies. Finally, it expands our understanding of the behavior problems experienced by school-age children with classic galactosemia. With these results we can begin to see that cryptic levels of GALT activity, in the GALT null fly or in patients, may improve outcome and decrease severity of symptoms. With the establishment of the fly model of classic galactosemia it may be possible to begin to

explore the mechanism for some of the complications seen in the patient population, hopefully one day leading to better intervention and treatment of classic galactosemia.

1.6 REFERENCES

1. Fridovich-Keil JL & Walter JH (2008) Galactosemia. *The Online Metabolic & Molecular Bases of Inherited Disease*, eds Valle D, Beaudet A, Vogelstein B, Kinzler K, Antonarakis S, & Ballabio A (McGraw Hill), p http://www.ommbid.com/.
2. Isselbacher KJ, Anderson EP, Kurahashi K, & Kalckar HM (1956) Congenital galactosemia, a single enzymatic block in galactose metabolism. *Science* 123(3198):635-636.
3. Kalckar HM, Anderson EP, & Isselbacher KJ (1956) Galactosemia, a congenital defect in a nucleotide transferase. *Biochim Biophys Acta* 20(1):262-268.
4. Tyfield L, *et al.* (1999) Classical galactosemia and mutations at the galactose-1-phosphate uridyl transferase (GALT) gene. *Hum Mutat* 13(6):417-430.
5. Hirokawa H, *et al.* (1999) Molecular basis for phenotypic heterogeneity in galactosaemia: prediction of clinical phenotype from genotype in Japanese patients. *Eur J Hum Genet* 7(7):757-764.
6. Lee BH, *et al.* (2011) Low prevalence of classical galactosemia in Korean population. *J Hum Genet* 56(1):94-96.
7. Bosch AM, *et al.* (2005) Identification of novel mutations in classical galactosemia. *Hum Mutat* 25(5):502.
8. Gort L, *et al.* (2006) Mutational spectrum of classical galactosaemia in Spain and Portugal. *J Inherit Metab Dis* 29(6):739-742.

9. Lukac-Bajalo J, Kuzelicki NK, Zitnik IP, Mencej S, & Battelino T (2007) Higher frequency of the galactose-1-phosphate uridyl transferase gene K285N mutation in the Slovenian population. *Clin Biochem* 40(5-6):414-415.

10. Lukac-Bajalo J, *et al.* (2002) Frequencies of Q188R and N314D mutations and IVS5-24g>A intron variation in the galactose-1-phosphate uridyl transferase (GALT) gene in the Slovenian population. *Clin Chem Lab Med* 40(11):1109-1113.

11. Murphy M, *et al.* (1999) Genetic basis of transferase-deficient galactosaemia in Ireland and the population history of the Irish Travellers. *Eur J Hum Genet* 7(5):549-554.

12. Suzuki M, West C, & Beutler E (2001) Large-scale molecular screening for galactosemia alleles in a pan-ethnic population. *Hum Genet* 109(2):210-215.

13. Velazquez-Aragon J, *et al.* (2008) Low allelic heterogeneity in a sample of Mexican patients with classical galactosaemia. *J Inherit Metab Dis* 31 Suppl 2:S333-337.

14. Lai K, *et al.* (1996) A prevalent mutation for galactosemia among black Americans. *J Pediatr* 128(1):89-95.

15. Manga N, Jenkins T, Jackson H, Whittaker DA, & Lane AB (1999) The molecular basis of transferase galactosaemia in South African negroids. *J Inherit Metab Dis* 22(1):37-42.

16. Goldstein N, *et al.* (2011) The GALT rush: high carrier frequency of an unusual deletion mutation of the GALT gene in the Ashkenazi population. *Mol Genet Metab* 102(2):157-160.

17. Ng WG, *et al.* (1994) Biochemical and molecular studies of 132 patients with galactosemia. *Hum Genet* 94(4):359-363.

18. Yang YP, Corley N, & Garcia-Heras J (2002) Molecular analysis in newborns from Texas affected with galactosemia. *Hum Mutat* 19(1):82-83.

19. Isselbacher KJ (1957) Clinical and biochemical observations in galactosemia. *Am J Clin Nutr* 5(5):527-532.

20. Waggoner DD, Buist NR, & Donnell GN (1990) Long-term prognosis in galactosaemia: results of a survey of 350 cases. *J Inherit Metab Dis* 13(6):802-818.

21. Waisbren SE, *et al.* (2011) The adult galactosemic phenotype. *J Inherit Metab Dis*.

22. Berry GT (2012) Galactosemia: when is it a newborn screening emergency? *Mol Genet Metab* 106(1):7-11.

23. Fernhoff PM, *et al.* (1982) Coordinated system for comprehensive newborn metabolic screening. *South Med J* 75(5):529-532.

24. Beutler E (1991) Galactosemia: screening and diagnosis. *Clin Biochem* 24(4):293-300.

25. Jumbo-Lucioni PP, *et al.* (2012) Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. *J Inherit Metab Dis*.

26. Beutler E & Baluda MC (1966) Improved method for measuring galactose-I-phosphate uridyl transferase activity of erythrocytes. *Clin Chim Acta* 13(3):369-379.

27. Kaufman FR, McBride-Chang C, Manis FR, Wolff JA, & Nelson MD (1995) Cognitive functioning, neurologic status and brain imaging in classical galactosemia. *Eur J Pediatr* 154(7 Suppl 2):S2-5.

28. Potter NL, Lazarus JA, Johnson JM, Steiner RD, & Shriberg LD (2008) Correlates of language impairment in children with galactosaemia. *J Inherit Metab Dis* 31(4):524-532.

29. Robertson A, Singh RH, Guerrero NV, Hundley M, & Elsas LJ (2000) Outcomes analysis of verbal dyspraxia in classic galactosemia. *Genet Med* 2(2):142-148.

30. Sanders RD, *et al.* (2009) Biomarkers of ovarian function in girls and women with classic galactosemia. *Fertil Steril* 92(1):344-351.

31. Schweitzer S, Shin Y, Jakobs C, & Brodehl J (1993) Long-term outcome in 134 patients with galactosaemia. *Eur J Pediatr* 152(1):36-43.

32. Hughes J, *et al.* (2009) Outcomes of siblings with classical galactosemia. *J Pediatr* 154(5):721-726.

33. Bosch AM (2011) Classic galactosemia: dietary dilemmas. *J Inherit Metab Dis* 34(2):257-260.

34. Forges T, Monnier-Barbarino P, Leheup B, & Jouvet P (2006) Pathophysiology of impaired ovarian function in galactosaemia. *Hum Reprod Update* 12(5):573-584.

35. Gropper SS, Weese JO, West PA, & Gross KC (2000) Free galactose content of fresh fruits and strained fruit and vegetable baby foods: more foods to consider for the galactose-restricted diet. *J Am Diet Assoc* 100(5):573-575.

36. Kim HO, Hartnett C, & Scaman CH (2007) Free galactose content in selected fresh fruits and vegetables and soy beverages. *J Agric Food Chem* 55(20):8133-8137.

37. Scaman CH, Jim VJ, & Hartnett C (2004) Free galactose concentrations in fresh and stored apples (Malus domestica) and processed apple products. *J Agric Food Chem* 52(3):511-517.

38. Berry GT, *et al.* (2004) The rate of de novo galactose synthesis in patients with galactose-1-phosphate uridyltransferase deficiency. *Mol Genet Metab* 81(1):22-30.

39. Schadewaldt P, Kamalanathan L, Hammen HW, & Wendel U (2004) Age dependence of endogenous galactose formation in Q188R homozygous galactosemic patients. *Mol Genet Metab* 81(1):31-44.

40. Fridovich-Keil JL (2006) Galactosemia: the good, the bad, and the unknown. *J Cell Physiol* 209(3):701-705.

41. Holden HM, Thoden JB, Timson DJ, & Reece RJ (2004) Galactokinase: structure, function and role in type II galactosemia. *Cell Mol Life Sci* 61(19-20):2471-2484.

42. McCorvie TJ & Timson DJ (2011) Structural and molecular biology of type I galactosemia: disease-associated mutations. *IUBMB Life* 63(11):949-954.

43. Timson DJ (2006) The structural and molecular biology of type III galactosemia. *IUBMB Life* 58(2):83-89.

44. Holden HM, Rayment I, & Thoden JB (2003) Structure and function of enzymes of the Leloir pathway for galactose metabolism. *J Biol Chem* 278(45):43885-43888.

45. Thoden JB, Timson DJ, Reece RJ, & Holden HM (2005) Molecular structure of human galactokinase: implications for type II galactosemia. *J Biol Chem* 280(10):9662-9670.

46. Thoden JB, Wohlers TM, Fridovich-Keil JL, & Holden HM (2001) Human UDP-galactose 4-epimerase. Accommodation of UDP-N-acetylglucosamine within the active site. *J Biol Chem* 276(18):15131-15136.

47. Wedekind JE, Frey PA, & Rayment I (1995) Three-dimensional structure of galactose-1-phosphate uridylyltransferase from Escherichia coli at 1.8 A resolution. *Biochemistry* 34(35):11049-11061.

48. Elsevier JP, Wells L, Quimby BB, & Fridovich-Keil JL (1996) Heterodimer formation and activity in the human enzyme galactose-1-phosphate uridylyltransferase. *Proc Natl Acad Sci U S A* 93(14):7166-7171.

49. Roach PJ, Depaoli-Roach AA, Hurley TD, & Tagliabracci VS (2012) Glycogen and its metabolism: some new developments and old themes. *Biochem J* 441(3):763-787.

50. Daenzer JM, Sanders RD, Hang D, & Fridovich-Keil JL (2012) UDP-Galactose 4'-Epimerase Activities toward UDP-Gal and UDP-GalNAc Play Different Roles in the Development of Drosophila melanogaster. *PLoS Genet* 8(5):e1002721.

51. Moloney DJ, *et al.* (2000) Fringe is a glycosyltransferase that modifies Notch. *Nature* 406(6794):369-375.

52. Geeganage S & Frey PA (1998) Transient kinetics of formation and reaction of the uridylyl-enzyme form of galactose-1-P uridylyltransferase and its Q168R-

variant: insight into the molecular basis of galactosemia. *Biochemistry* 37(41):14500-14507.

53. Marabotti A & Facchiano AM (2005) Homology modeling studies on human galactose-1-phosphate uridylyltransferase and on its galactosemia-related mutant Q188R provide an explanation of molecular effects of the mutation on homo- and heterodimers. *J Med Chem* 48(3):773-779.

54. Fridovich-Keil JL & Jinks-Robertson S (1993) A yeast expression system for human galactose-1-phosphate uridylyltransferase. *Proc Natl Acad Sci U S A* 90(2):398-402.

55. Riehman K, Crews C, & Fridovich-Keil JL (2001) Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase. *J Biol Chem* 276(14):10634-10640.

56. Wells L & Fridovich-Keil JL (1997) Biochemical characterization of the S135L allele of galactose-1-phosphate uridylyltransferase associated with galactosaemia. *J Inherit Metab Dis* 20(5):633-642.

57. Christacos NC & Fridovich-Keil JL (2002) Impact of patient mutations on heterodimer formation and function in human galactose-1-P uridylyltransferase. *Mol Genet Metab* 76(4):319-326.

58. Elsevier JP & Fridovich-Keil JL (1996) The Q188R mutation in human galactose-1-phosphate uridylyltransferase acts as a partial dominant negative. *J Biol Chem* 271(50):32002-32007.

59. Shield JP, *et al.* (2000) The relationship of genotype to cognitive outcome in galactosaemia. *Arch Dis Child* 83(3):248-250.

60. Ning C & Segal S (2000) Plasma galactose and galactitol concentration in patients with galactose-1-phosphate uridyltransferase deficiency galactosemia: determination by gas chromatography/mass spectrometry. *Metabolism* 49(11):1460-1466.

61. Lai K & Elsas LJ (2000) Overexpression of human UDP-glucose pyrophosphorylase rescues galactose-1-phosphate uridyltransferase-deficient yeast. *Biochem Biophys Res Commun* 271(2):392-400.

62. Leslie N, Yager C, Reynolds R, & Segal S (2005) UDP-galactose pyrophosphorylase in mice with galactose-1-phosphate uridyltransferase deficiency. *Mol Genet Metab* 85(1):21-27.

63. Palmieri M, *et al.* (1999) Urine and plasma galactitol in patients with galactose-1-phosphate uridyltransferase deficiency galactosemia. *Metabolism* 48(10):1294-1302.

64. Yager C, Wehrli S, & Segal S (2006) Urinary galactitol and galactonate quantified by isotope-dilution gas chromatography-mass spectrometry. *Clin Chim Acta* 366(1-2):216-224.

65. Cuatrecasas P & Segal S (1966) Mammalian galactose dehydrogenase. I. Identification and purification in rat liver. *J Biol Chem* 241(24):5904-5909.

66. Cuatrecasas P & Segal S (1966) Galactose conversion to D-xylulose: an alternate route of galactose metabolism. *Science* 153(3735):549-551.

67. Yager CT, Chen J, Reynolds R, & Segal S (2003) Galactitol and galactonate in red blood cells of galactosemic patients. *Mol Genet Metab* 80(3):283-289.

68. Yager C, Ning C, Reynolds R, Leslie N, & Segal S (2004) Galactitol and galactonate accumulation in heart and skeletal muscle of mice with deficiency of galactose-1-phosphate uridyltransferase. *Mol Genet Metab* 81(2):105-111.

69. Douglas HC & Hawthorne DC (1964) Enzymatic Expression and Genetic Linkage of Genes Controlling Galactose Utilization in Saccharomyces. *Genetics* 49:837-844.

70. Chhay JS, Openo KK, Eaton JS, Gentile M, & Fridovich-Keil JL (2008) A yeast model reveals biochemical severity associated with each of three variant alleles of galactose-1P uridylyltransferase segregating in a single family. *J Inherit Metab Dis* 31(1):97-107.

71. Ross KL, Davis CN, & Fridovich-Keil JL (2004) Differential roles of the Leloir pathway enzymes and metabolites in defining galactose sensitivity in yeast. *Mol Genet Metab* 83(1-2):103-116.

72. Quimby BB, Wells L, Wilkinson KD, & Fridovich-Keil JL (1996) Functional requirements of the active site position 185 in the human enzyme galactose-1-phosphate uridylyltransferase. *J Biol Chem* 271(43):26835-26842.

73. Fridovich-Keil JL, Quimby BB, Wells L, Mazur LA, & Elsevier JP (1995) Characterization of the N314D allele of human galactose-1-phosphate uridylyltransferase using a yeast expression system. *Biochem Mol Med* 56(2):121-130.

74. Henderson JM, Wells L, & Fridovich-Keil JL (2000) Covalent heterogeneity of the human enzyme galactose-1-phosphate uridylyltransferase. *J Biol Chem* 275(39):30088-30091.

75. Bhat PJ (2003) Galactose-1-phosphate is a regulator of inositol monophosphatase: a fact or a fiction? *Medical hypotheses* 60(1):123-128.

76. Mehta DV, Kabir A, & Bhat PJ (1999) Expression of human inositol monophosphatase suppresses galactose toxicity in Saccharomyces cerevisiae: possible implications in galactosemia. *Biochim Biophys Acta* 1454(3):217-226.

77. Masuda CA, *et al.* (2008) Overexpression of the aldose reductase GRE3 suppresses lithium-induced galactose toxicity in Saccharomyces cerevisiae. *FEMS Yeast Res* 8(8):1245-1253.

78. Leslie ND, Yager KL, McNamara PD, & Segal S (1996) A mouse model of galactose-1-phosphate uridyl transferase deficiency. *Biochem Mol Med* 59(1):7-12.

79. Lai K, *et al.* (2008) ARHI: A new target of galactose toxicity in Classic Galactosemia. *Bioscience hypotheses* 1(5):263-271.

80. Ning C, *et al.* (2001) Galactose metabolism in mice with galactose-1-phosphate uridyltransferase deficiency: sucklings and 7-week-old animals fed a high-galactose diet. *Mol Genet Metab* 72(4):306-315.

81. Ning C, *et al.* (2000) Galactose metabolism by the mouse with galactose-1-phosphate uridyltransferase deficiency. *Pediatr Res* 48(2):211-217.

82. Wehrli S, Reynolds R, & Segal S (2007) Metabolic fate of administered [13C]galactose in tissues of galactose-1-phosphate uridyl transferase deficient mice determined by nuclear magnetic resonance. *Mol Genet Metab* 90(1):42-48.

83. Leslie ND (2003) Insights into the pathogenesis of galactosemia. *Annu Rev Nutr* 23:59-80.

84. Gajewska J, *et al.* (2008) Serum markers of bone turnover in children and adolescents with classic galactosemia. *Adv Med Sci* 53(2):214-220.

85. Panis B, *et al.* (2004) Bone metabolism in galactosemia. *Bone* 35(4):982-987.

86. Batey LA, *et al.* (2012) Skeletal health in adult patients with classic galactosemia. *Osteoporos Int*.

87. Panis B, *et al.* (2006) Effect of calcium, vitamins K1 and D3 on bone in galactosemia. *Bone* 39(5):1123-1129.

88. Kaufman FR, *et al.* (1981) Hypergonadotropic hypogonadism in female patients with galactosemia. *N Engl J Med* 304(17):994-998.

89. Gubbels CS, Land JA, & Rubio-Gozalbo ME (2008) Fertility and impact of pregnancies on the mother and child in classic galactosemia. *Obstet Gynecol Surv* 63(5):334-343.

90. Guerrero NV, *et al.* (2000) Risk factors for premature ovarian failure in females with galactosemia. *J Pediatr* 137(6):833-841.

91. Rubio-Gozalbo ME, *et al.* (2010) Gonadal function in male and female patients with classic galactosemia. *Hum Reprod Update* 16(2):177-188.

92. Kaufman FR, Donnell GN, Roe TF, & Kogut MD (1986) Gonadal function in patients with galactosaemia. *J Inherit Metab Dis* 9(2):140-146.

93. Menezo YJ, Lescaille M, Nicollet B, & Servy EJ (2004) Pregnancy and delivery after stimulation with rFSH of a galatosemia patient suffering hypergonadotropic hypogonadism: case report. *J Assist Reprod Genet* 21(3):89-90.

94. Prestoz LL, Couto AS, Shin YS, & Petry KG (1997) Altered follicle stimulating hormone isoforms in female galactosaemia patients. *Eur J Pediatr* 156(2):116-120.

95. Gubbels CS, *et al.* (2011) FSH isoform pattern in classic galactosemia. *J Inherit Metab Dis* 34(2):387-390.

96. Hagen CP, *et al.* (2010) Serum levels of anti-Mullerian hormone as a marker of ovarian function in 926 healthy females from birth to adulthood and in 172 Turner syndrome patients. *J Clin Endocrinol Metab* 95(11):5003-5010.

97. Gubbels CS, *et al.* (2012) Primary ovarian insufficiency in classic galactosemia: role of FSH dysfunction and timing of the lesion. *J Inherit Metab Dis*.

98. Hoffmann B, Wendel U, & Schweitzer-Krantz S (2011) Cross-sectional analysis of speech and cognitive performance in 32 patients with classic galactosemia. *J Inherit Metab Dis* 34(2):421-427.

99. Potter NL (2011) Voice disorders in children with classic galactosemia. *J Inherit Metab Dis* 34(2):377-385.

100. Shriberg LD, Potter NL, & Strand EA (2011) Prevalence and phenotype of childhood apraxia of speech in youth with galactosemia. *J Speech Lang Hear Res* 54(2):487-519.

101. Timmers I, van den Hurk J, Di Salle F, Rubio-Gozalbo ME, & Jansma BM (2011) Language production and working memory in classic galactosemia from a

cognitive neuroscience perspective: future research directions. *J Inherit Metab Dis* 34(2):367-376.

102. Antshel KM, Epstein IO, & Waisbren SE (2004) Cognitive strengths and weaknesses in children and adolescents homozygous for the galactosemia Q188R mutation: a descriptive study. *Neuropsychology* 18(4):658-664.

103. Ridel KR, Leslie ND, & Gilbert DL (2005) An updated review of the long-term neurological effects of galactosemia. *Pediatr Neurol* 33(3):153-161.

104. Lebea PJ & Pretorius PJ (2005) The molecular relationship between deficient UDP-galactose uridyl transferase (GALT) and ceramide galactosyltransferase (CGT) enzyme function: a possible cause for poor long-term prognosis in classic galactosemia. *Medical hypotheses* 65(6):1051-1057.

105. Dubroff JG, *et al.* (2008) FDG-PET findings in patients with galactosaemia. *J Inherit Metab Dis* 31(4):533-539.

106. Doyle CM, Channon S, Orlowska D, & Lee PJ (2010) The neuropsychological profile of galactosaemia. *J Inherit Metab Dis* 33(5):603-609.

107. Hoffmann B, Dragano N, & Schweitzer-Krantz S (2012) Living situation, occupation and health-related quality of life in adult patients with classic galactosemia. *J Inherit Metab Dis*.

108. Panis B, *et al.* (2006) Untreated classical galactosemia patient with mild phenotype. *Mol Genet Metab* 89(3):277-279.

109. Coss KP, *et al.* (2012) IgG N-glycans as potential biomarkers for determining galactose tolerance in Classical Galactosaemia. *Mol Genet Metab* 105(2):212-220.

110. Liu Y, *et al.* (2012) N- and O-linked glycosylation of total plasma glycoproteins in galactosemia. *Mol Genet Metab*.

111. Staubach S, Schadewaldt P, Wendel U, Nohroudi K, & Hanisch FG (2012) Differential glycomics of epithelial membrane glycoproteins from urinary exovesicles reveals shifts toward complex-type N-glycosylation in classical galactosemia. *Journal of proteome research* 11(2):906-916.

112. Berry GT, *et al.* (2001) In vivo evidence of brain galactitol accumulation in an infant with galactosemia and encephalopathy. *J Pediatr* 138(2):260-262.

113. Berry GT (2011) Is prenatal myo-inositol deficiency a mechanism of CNS injury in galactosemia? *J Inherit Metab Dis* 34(2):345-355.

114. Cui X, *et al.* (2004) D-galactose-caused life shortening in Drosophila melanogaster and Musca domestica is associated with oxidative stress. *Biogerontology* 5(5):317-325.

115. Cui X, *et al.* (2006) Chronic systemic D-galactose exposure induces memory loss, neurodegeneration, and oxidative damage in mice: protective effects of R-alpha-lipoic acid. *J Neurosci Res* 84(3):647-654.

Chapter 2

A *DROSOPHILA MELANOGASTER* MODEL OF CLASSIC GALACTOSEMIA

This chapter contains work published as: Rebekah F Kushner*, Emily L Ryan*, Jennifer MI Sefton, Rebecca D Sanders, Patricia Jumbo Lucioni, Kenneth H Moberg & Judith L Fridovich-Keil *Dis Model Mech*. 2010 Sep-Oct;3(9-10):618-27. This chapter is the authors' version, including changes resulting from the peer-review process. Changes resulting from the publishing process are not reflected in this work.

* Authors contributed equally to this work.

2.1 ABSTRACT

Classic galactosemia is a potentially lethal disorder that results from profound impairment of galactose-1P uridylyltransferase (GALT). Despite decades of research, the underlying pathophysiology of classic galactosemia remains unclear, in part due to the lack of an appropriate animal model. Here we report the establishment of a *Drosophila melanogaster* model of classic galactosemia; this is the first whole animal genetic model to mimic aspects of the patient phenotype. Analogous to humans, GALT-deficient *D. melanogaster* survive under conditions of galactose restriction, but accumulate elevated levels of galactose-1 phosphate and succumb during larval development following galactose exposure. As in patients, the potentially lethal damage is reversible if dietary galactose restriction is initiated early in life. GALT-deficient *Drosophila* also exhibit locomotor complications despite dietary galactose restriction, and both the acute and long-term complications can be rescued by transgenic expression of human *GALT*. Using this new *Drosophila* model, we have begun to dissect the timing, extent, and mechanism(s) of galactose sensitivity in the absence of GALT activity.

2.2 INTRODUCTION

Galactose and its derivatives serve in higher eukaryotes as key constituents of glycoproteins, glycolipids, and complex carbohydrates. As a component of lactose, galactose also provides close to half of the sugar calories in mammalian milk. Galactose is metabolized in mammals and other species by the enzymes of the Leloir Pathway: galactokinase (GALK, EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12), and UDP-galactose 4' epimerase (GALE, EC 5.1.3.2) (Figure 2.1) (1). In humans, profound impairment of GALT results in the potentially lethal disorder classic galactosemia [OMIM #230400, reviewed in (2)].

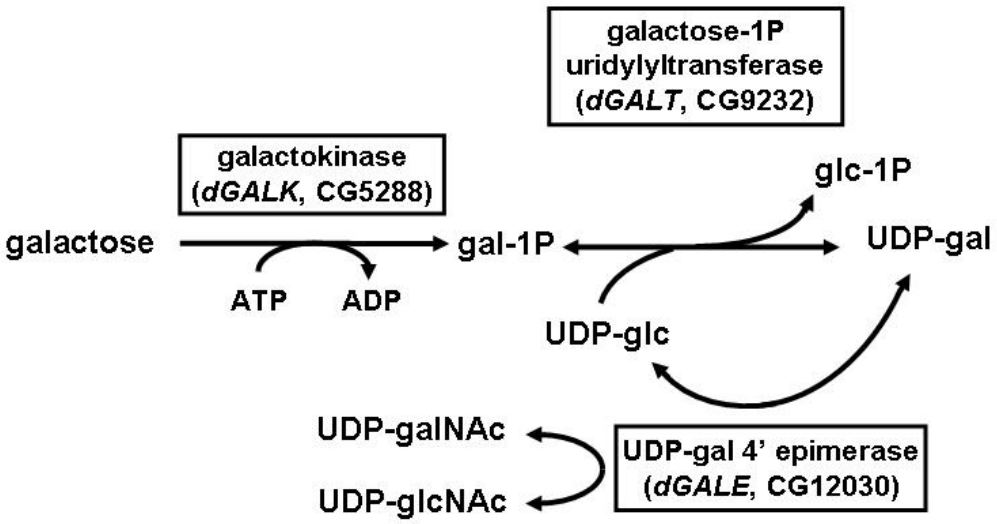

Figure 2.1: The Leloir pathway of galactose metabolism. Classic galactosemia results from profound impairment of the second enzyme in the Leloir pathway, galactose-1P uridylyltransferase (GALT). GALT catalyzes the synthesis of glucose-1P (glc-1P) and UDP-galactose (UDP-gal) from UDP-glucose (UDP-glc) and galactose-1P (gal-1P).

Classic galactosemia is an autosomal recessive disorder that affects ~1/60,000 live births. Following exposure to milk, affected infants experience a rapid clinical

demise with symptoms that escalate from vomiting, diarrhea, and failure-to-thrive to jaundice, *Escherichia coli* sepsis, and neonatal death. Untreated infants with classic galactosemia also accumulate dramatically elevated levels of galactose-1-phosphate (gal-1P) in their cells and tissues. While careful dietary galactose restriction prevents or reverses the acute and potentially lethal symptoms of classic galactosemia, and also largely resolves the abnormal accumulation of gal-1P, significant long-term complications are common; these include cognitive impairment, speech difficulties, neuromuscular problems, and premature ovarian insufficiency, among others [reviewed in (2)].

Despite decades of study, the underlying bases of the pathophysiology of both the acute and long-term complications of classic galactosemia remain unclear [reviewed in (3), (4, 5)]. One of the principal factors limiting research progress has been the lack of an animal model that recapitulates either the acute or the long-term complications of the disorder. Studies conducted by ourselves and others using yeast and mammalian tissue-culture models have provided biochemical insights into the consequences of GALT activity loss (e.g. (6-16)), but the absence of an appropriate multicellular model has precluded studies into the timing and impact of GALT deficiency on organismal development or on intact tissues and organ systems. Of note, more than a decade ago Leslie and colleagues (17) reported a *GALT* knockout mouse that demonstrated complete loss of GALT activity and that accumulated gal-1P; however, these mice unexpectedly remained healthy and fertile despite dietary exposure to large quantities of galactose (17-21). This outcome disparity between GALT-deficient humans and mice remains unexplained.

As an alternative to mice, we turned to the fruit fly *Drosophila melanogaster*. Fruit flies have facilitated genetic experiments for more than a century, and in recent years have emerged as an extremely powerful system to model human genetic diseases (22), including complex metabolic disorders, such as diabetes and obesity (23-25). Sequence alignments show that >75% of recognized human disease genes, among them all three Leloir pathway genes (http://superfly.ucsd.edu/homophila/), have related sequences in the *D. melanogaster* genome (22).

Here we report the establishment and first application of a *D. melanogaster* model of classic galactosemia. Like human patients, and unlike mice, GALT-deficient *Drosophila* survive if maintained on food that contains only glucose (glc) but die as larvae if exposed to food that contains both glucose and galactose. This galactose-dependent lethality is dose-dependent, sugar-specific, and can be rescued by expression of a human *GALT* transgene. GALT-deficient animals are also rescued from death by initiation of a galactose-restricted diet early in development. Of note, larval animals transferred from a glucose diet to a glucose-plus-galactose diet die within days of the transfer, but adult flies do not, arguing for stage-specific or dose-dependent consequences to impaired galactose metabolism. As expected, metabolite studies of GALT-deficient larvae and adults exposed to galactose show abnormal accumulation of gal-1P. Finally, GALT-deficient flies raised and maintained exclusively on a galactose-restricted diet demonstrate a clear deficit in the normal negative geotaxic response seen in controls (26, 27). As with the acute galactose-dependent phenotype, this galactose-independent muscular or neuromuscular deficit is rescued by expression of a human GALT transgene. These data confirm that GALT-deficient *D. melanogaster* mimic aspects of both the

acute and long-term outcomes of classic galactosemia, paving the way for future studies to explore the genetic and environmental factors that underlie and modify these phenotypes.

2.3 METHODS

Fly stocks and maintenance

Details about stocks of *D. melanogaster* used in this study are provided in Table 2.1. 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, 10mLs 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, 10mL/L propionic acid, and 14.4 mL/L tegosept mold inhibitor (10% w/v in ethanol) (28) supplemented with galactose or mannose, as indicated. For some experiments liquid food coloring was also added to facilitate confirmation that larvae were eating.

Creation and molecular characterization of the $dGALT^{\Delta 1AP2}$, $dGALT^{\Delta 1V2}$, and $dGALT^{C2}$ alleles

Excision alleles of *dGALT* were generated by mobilizing an existing SUP or-P insertion in the 5' UTR of the *CG9232* locus (*KG00049*) via transient expression of the Δ*2-3* transposase enzyme in the male germ line, according to standard methods (29). Flies carrying excision alleles were identified by loss of the associated mini-*w*+ marker (white eyes) and homozygous stocks derived from those flies were sorted according to the level of GALT enzymatic activity detected in soluble lysates. Of >50 excision stocks tested, five demonstrated a profound loss of GALT enzymatic activity; those stocks were designated as imprecise excision candidates, while stocks demonstrating wild-type GALT activity were designated as precise excision candidates.

Fly stock or allele name	Comments
w^{1118}	Wild-type *D. melanogaster* (Bloomington Stock #3605)
P{SUP or-P}CG9232^{KG00049}	P-element insertion stock used for excision scheme (Bloomington Stock #14339)
$y^{1}w^{*}$;$ry^{506}Sb^{1}$P{Δ2-3}99B/TM6B	Transposase *stock*, Bloomington # 3664
cup^{01355}	Female-sterile *P{PZ}* insertion within the untranslated region of the first exon of the *cup* gene (Bloomington Stock #12218)
Df(2L)Exel7027	Chromosome 2 Df that removes sequence including the entire *dGALT* gene (Bloomington Stock #7801)
dGALT^{C2}	Precise excision allele of *P{SUPor-P}CG9232*^{KG00049}
dGALT$^{\Delta 1AP2}$ and *dGALT*$^{\Delta 1V2}$	Imprecise excisions of *P{SUPor-P}CG9232*^{KG00049} that produce a ~1.6kb deletion in *dGALT*
hGALT^{10A11}	*UAS-hGALT* insertion allele (leaky), chr III
hGALT^{9B12}, *hGALT*^{10A12}, *and hGALT*^{10B22}	*UAS-hGALT* insertion alleles, chr III

Table 2.1: *D. melanogaster* stocks and alleles used in this study

Given the genomic location of *dGALT* within the second intron of *cup*, we further tested all excision alleles of interest for their ability to complement a strong mutant allele of *cup* (*cup*^{01355}). Of note, the *KG00049* P-element insertion allele was itself homozygous

viable and female fertile, and also complemented the *cup*^{01355} allele. Similarly, female flies carrying the *cup*^{01355} allele *in trans* to either *dGALT*$^{\Delta 1AP2}$, *dGALT*$^{\Delta 1V2}$, or *dGALT*^{C2} remained viable and fertile, confirming that *cup* remained functional in each.

To characterize the *dGALT*$^{\Delta 1AP2}$, *dGALT*$^{\Delta 1V2}$, and *dGALT*^{C2} alleles at the molecular level we amplified the relevant genomic sequences using primers that annealed within the flanking exons of *cup*. Primer sequences were 5'-GCTGACTGCTGATCTCGCCGTTGT-3' and 5'-CCAAGGAGAGCTTTGTGATGCCT-3'. Control PCR amplification of the corresponding region from *w*^{1118} flies revealed the anticipated 4kb amplicon. The *dGALT*^{C2} stock also produced a 4 kb amplicon, while both the *dGALT*$^{\Delta 1AP2}$ and *dGALT*$^{\Delta 1V2}$ stocks produced ~2.4 kb amplicons[1]. Direct sequencing of the amplicons from all three excision templates revealed that the presumed precise excision allele was indeed precise (Figure 2C), and that each of the presumed imprecise excision alleles carried the same 1647 bp deletion removing virtually the entire coding region of the *dGALT* gene, with a small remnant of the P-element sequence left behind (Figure 2.2A, B).

Transgenic lines

A *UAS-hGALT* transgene was generated by subcloning the wild-type human *GALT* coding sequence, as an *Eco*R1-*Sal*1 fragment, into *pUAST* (30) using the *Eco*R1

[1] The published lengths of the amplicons are incorrect. The correct lengths of the amplicons are 3kb for the *dGALT*^{C2} stock and 1.4kb for the *dGALT*$^{\Delta 1AP2}$ and *dGALT*$^{\Delta 1V2}$ stocks.

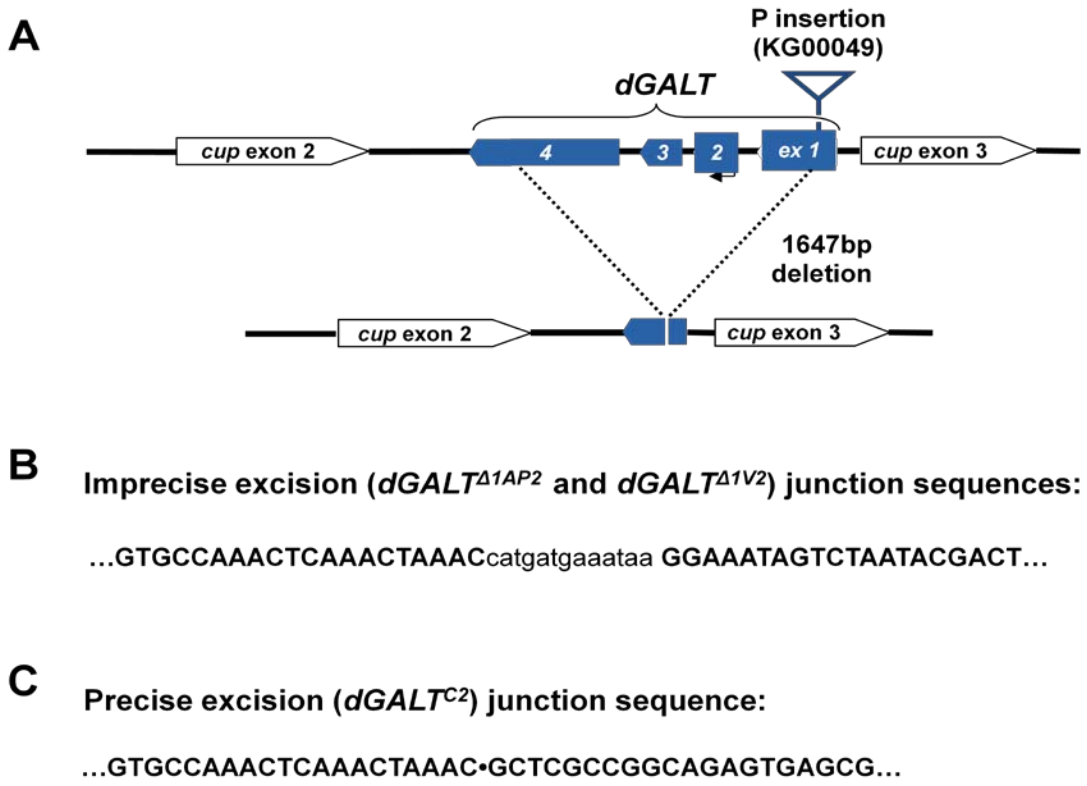

Figure 2.2 Creation of an imprecise excision allele of *dGALT*. (A) The *dGALT* gene (CG9232) exists entirely within the second intron of the gene cup. As described in the Methods, the *KG00049* P-element insertion in the first (noncoding) exon of dGALT was mobilized by transient exposure to transposase, and excision alleles were selected and characterized. (B) An imprecise excision identified in both $dGALT^{\Delta 1AP2}$ and $dGALT^{\Delta 1V2}$ alleles that removed 1647 bp of *dGALT* sequence, including almost the entire coding region, and that left behind 13 bp of P-element sequence (lower case font). (C) Most excisions were precise (e.g. $dGALT^{C2}$), restoring the wild-type *dGALT* sequence.

and Xho1 sites in the pUAST polylinker region. The resulting plasmid was confirmed by sequence analysis. UAS-hGALT stocks were generated using standard transgenic techniques by the Fly Core of the Massachusetts General Hospital, Charlestown, MA. Candidate insertion lines were mapped and balanced. Three homozygous viable

insertions (dGALT9B12, dGALT10A11, dGALT10A12, and dGALT10B22) on chromosome III were used in this study.

GALK, GALT, and GALE enzyme assays

Lysates were prepared and assays ($n \geq 3$) were performed (for Table 2.2) as described in (31).

STOCK (comments)	**GALK activity[1]**	**GALT activity[1]**	**GALE activity[1]**
w^{1118}/w^{1118} (wild-type control)	19.91 ± 1.61	24.31 ± 2.03	81.70 ± 2.80
$CG9232^{KG00049}$ / $CG9232^{KG00049}$ (starting P-element insertion in *dGALT*)	11.56 ± 2.06	22.50 ± 3.60	65.77 ± 10.21
$dGALT^{C2}/dGALT^{C2}$ (precise excision)	20.87 ± 3.62	29.17 ± 1.43	73.48 ± 17.36
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ (imprecise excision)	42.78 ± 4.74	-0.07 ± 0.02	87.38 ± 10.47
$dGALT^{\Delta 1V2}/dGALT^{\Delta 1V2}$ (imprecise excision)	38.29 ± 3.33	0.02 ± 0.29	80.90 ± 7.86
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10A11}$/ $UAS\text{-}hGALT^{10A11}$ (low expression transgenic rescue)	12.22 ± 2.46	1.01 ± 0.06	81.18 ± 15.15
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10A11}/\beta tub\text{-}GAL4$ (high expression transgenic rescue)	18.70 ± 8.65	172.41 ± 60.80	98.00 ± 1.65
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{9A12}/\beta tub\text{-}GAL4$ (high expression transgenic rescue)	14.71 ± 3.54	651.93 ± 21.49	66.45 ± 3.97
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10B22}/\beta tub\text{-}GAL4$ (high expression transgenic rescue)	15.47 ± 2.08	260.63 ± 35.86	74.22 ± 12.28

Table 2.2: Leloir enzyme activities in *D. melanogaster*. Enzyme activities were measured on lysates prepared from cohorts of 10 male flies raised on molasses food and harvested 8-48 h after eclosion. Values presented are average ± S.E.M. (n ≥ 3 sets of duplicate samples).[1]Enzyme activity was expressed as pmol/ µg/ min.

Measuring gal-1P in larvae and adults

Cohorts of newly hatched *dGALT*$^{\Delta 1AP2}$ homozygous and *dGALT*^{C2} homozygous larvae and newly eclosed adults were transferred to cages or vials containing either 555 mM glucose-only or 555 mM glucose-plus-222 mM galactose food. After 4 days, pools of 10 adult flies or ≥20 larvae were anesthetized with CO_2, suspended in 125 µL of ice-cold HPLC grade water and ground on ice for 15 s using a teflon micropestle and handheld micropestle motor (Kimble Chase Life Science and Research Products LLC, Vineland NJ). Ten µL of each lysate was saved for protein quantification (using the BioRad DC assay with BSA as a standard). Intracellular metabolites were extracted from the remainder as described previously (13, 32). The extracted samples were then dried under vacuum with no heat (Eppendorf Vacufuge) until no liquid remained visible. Dried metabolite pellets were rehydrated with HPLC-grade water in volumes normalized for protein concentration and centrifuged through 0.22µm Costar Spin-X centrifuge tube filters (Corning Inc, Lowell, MA) at 4000xg for four minutes to remove any insoluble matter. The soluble phase of each sample was transferred to a glass HPLC vial and metabolites were separated and quantified using a Dionex HPLC as described previously (13). For each sample 20 µL were injected into a 25 µL injection loop.

Time of death experiments

At least 100 each of embryos homozygous for the *dGALT*$^{\Delta 1AP2}$ (imprecise excision) or *dGALT*^{C2} (precise excision) alleles were harvested from egg-laying plates at <24 hrs old and deposited individually into the wells of 96-well plates pre-loaded to about ¾ full with either 555 mM glucose-only or 555 mM glucose-plus-111 mM or 222 mM galactose fly food. Once loaded, plates were tightly covered with a clear plastic wrap

which was pricked with a needle to introduce a small hole over each well to permit an exchange of air. All animals were monitored and scored for viability daily under a dissecting microscope until they either died or eclosed as adults.

Window of galactose sensitivity

Embryos homozygous for the *dGALT*$^{\Delta 1AP2}$ (imprecise excision) and *dGALT*^{C2} (precise excision) alleles were deposited on plates containing either 555 mM glucose food or 555 mM glucose-plus-222 mM galactose food. Every 24 hours, starting with 1st instar larvae (L1s), cohorts of >20 developing animals were transferred from glucose food individually into the wells of 96-well plates pre-loaded with 555 mM glucose-plus-222 mM galactose food, and similarly larvae were transferred from 555 mM glucose-plus-222 mM galactose food into the wells of 96-well plates pre-loaded with 555 mM glucose-only food. To control for a potential impact of the transfer process itself, cohorts of larvae also were transferred from glucose-only food to wells containing glucose-only food, and from glucose-plus-galactose food to wells containing glucose-plus-galactose food. This transfer process was continued for the first six days of development after which all animals were allowed to continue developing without further transfer.

2.4 RESULTS

D. melanogaster metabolize galactose via the Leloir Pathway

To explore the role of galactose metabolism in *D. melanogaster*, we first confirmed that flies encode and express orthologs of human *GALK*, *GALT*, and *GALE* (Figure 2.1). The *D. melanogaster* genes whose predicted protein products show the greatest amino acid sequence homology to the human Leloir enzymes are *CG5288* (on chr. III, designated here as *dGALK*), *CG9232* (on chr. II, designated here as *dGALT*), and *CG12030* (on chr. III, designated here as *dGALE*) (33) (http://superfly.ucsd.edu/homophila/). Both *dGALT* and *dGALE* show strong conservation with their corresponding human orthologs, having 57% sequence identity with 72% sequence similarity, and 60% sequence identity with 76% sequence similarity, respectively. *dGALK* demonstrates slightly lower conservation, with 27% amino acid sequence identity and 44% sequence similarity.

Publicly available *in situ* RNA hybridization data from the Berkeley Drosophila Genome Project (BDGP; http://www.fruitfly.org/) confirm that all three fly Leloir genes are expressed during embryogenesis. Assays of lysates prepared from wild-type (w^{1118}) *D. melanogaster* adults also reveal the presence of all three enzyme activities (Table 2.1**).** Finally, studies of animals carrying disruptions or deletions of each of the three fly genes (Table 2.2; (Sanders, 2010 #2563), and unpublished data) demonstrate loss of the corresponding enzymatic activity, thereby confirming a functional connection between each gene sequence and the enzymatic activity attributed to its predicted protein product.

The *dGALT* gene is approximately 2 kb in length and includes four exons, the first of which is noncoding (Figure 2.2); the predicted mRNA is ~1.5 kb. The fly *dGALE* and

dGALK genes will be described in detail elsewhere (31), and Jumbo-Lucioni et al, in preparation). Of note, the *dGALT* gene is located entirely within the second intron of another gene, *cup* (34). *cup* is required for ovarian function and egg production in flies; female flies deficient in *cup* are infertile and fail to lay embryos (34) [reviewed in (35)].

Creation of a *dGALT*-deficient allele within a functional allele of *cup*

We created an impaired allele of *dGALT* by imprecise excision of an existing P-element insertion, *KG00049*, in the 5' untranslated region of the *CG9232* gene (Figure 2.2, panel A). This $CG9232^{KG00049}$ allele fully complemented a strong *cup* allele (cup^{01355}), suggesting that it has little if any effect on *cup* expression; female $cup^{01355}/dGALT^{KG00049}$ compound heterozygotes crossed to w^{1118} males produced viable embryos that developed into phenotypically normal flies. The $dGALT^{KG00049}$ allele also failed to impair *dGALT* expression or function, as indicated by the wild-type level of GALT activity detected in lysates from homozygotes (Table 2.2).

Excision of the P-element was achieved by transient exposure to the *Δ2-3* transposase in the germ line of male flies (29), as described in Methods. From among the resulting excision alleles we identified two, designated $dGALT^{\Delta 1AP2}$ and $dGALT^{\Delta 1V2}$, each carrying the same 1647 bp deletion removing almost the entire *dGALT* gene (Figure 2.2, panel B). Flies carrying a precise excision of the *KG00049* P-element insertion, designated $dGALT^{C2}$, were also identified and characterized (Figure 2.2, panel C). As expected, $dGALT^{\Delta 1AP2}$ and $dGALT^{\Delta 1V2}$ homozygotes demonstrated a complete lack of detectable GALT activity, while $dGALT^{C2}$ homozygotes demonstrated wild-type GALT activity (Table 2.2). Finally, all three excision alleles demonstrated normal GALE activity (Table 2.2) and complemented the cup^{01355} allele, indicating that *cup* remained

functional on these chromosomes. Of note, there was a small apparent increase in GALK activity when GALT activity was deficient (Table 2.2). The basis for this apparent increase remains unknown but may reflect either a true compensatory increase in GALK expression or activity, or alternatively an artifact of the assay, perhaps resulting from increased stability of the GALK reaction product, gal-1P, when GALT is not present to metabolize it.

Dietary galactose is lethal to *dGALT*-deficient flies

To test the impact of galactose exposure on *dGALT*-deficient *Drosophila*, we assessed the survival of F1 progeny from crosses of homozygous $dGALT^{\Delta 1AP2}$ or $dGALT^{\Delta 1V2}$ virgin females to heterozygous $dGALT^{\Delta 1AP2}/CyO$ or $dGALT^{\Delta 1V2}/CyO$ males. Crosses were performed on fly food containing 555 mM glucose or 555 mM glucose plus 222 mM galactose (Figure 2.3 and Table 2.3). Surviving adult progeny were genotyped by the presence or absence of the balancer chromosome (*CyO*), which conferred a dominant curly wing phenotype. Viability of the *dGALT*-deficient progeny resulting from each cross was quantified in terms of the percentage of surviving F1 animals that were unbalanced (exhibiting straight wings).

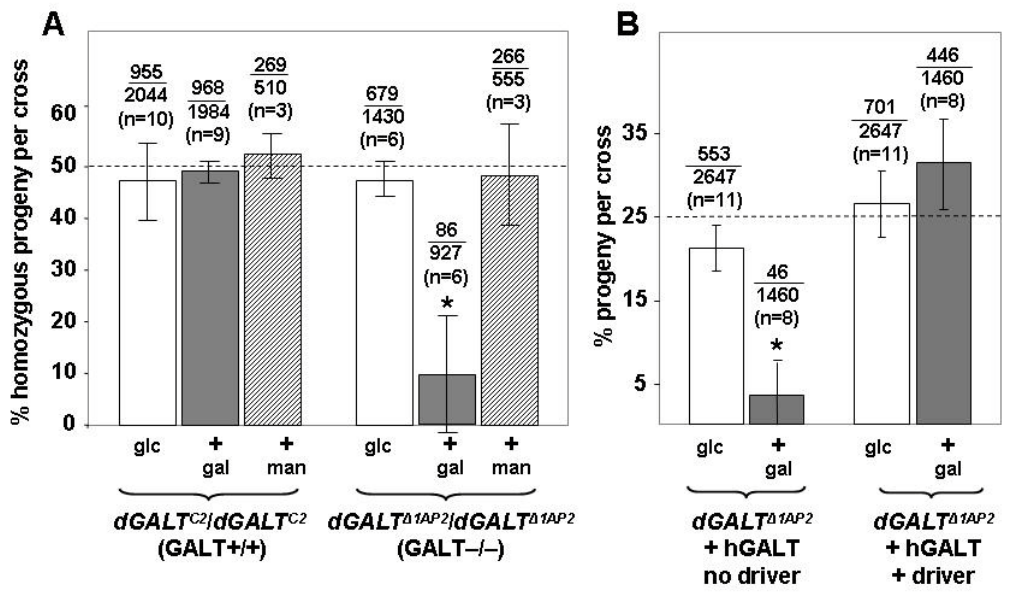

Figure 2.3: Loss of *dGALT* results in galactose sensitivity of *D. melanogaster*.

(A) Percent viable *dGALT*-deficient progeny resulting from crosses of *dGALT* −/− virgin females with *dGALT* +/− males on the food sources indicated (glc = 555 mM glucose food; + gal = 555 mM glucose-plus-222 mM galactose food; + man = 555 mM glucose-plus-222 mM mannose). Fractions over the bars indicate the combined numbers of homozygous adult flies over the total numbers of progeny counted in each category; n = number of crosses from which those progeny were derived. Each cross produced at least 75 viable F1 adults. Flies carrying a precise excision ($dGALT^{C2}$) rather than an imprecise excision ($dGALT^{\Delta 1AP2}$) allele of *dGALT* served as the positive control. Asterisk (*) indicates statistical significance, $p<0.0001$ for a two-tailed t-test at the 95% confidence interval. **(B)** Expression of a human *GALT* transgene rescued viability of $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ *D. melanogaster* exposed to galactose. Note these data were generated using *hGALT* transgene alleles *9A12, 10A12,* and *10B22* that were minimally, if at all, leaky. Asterisk (*) indicates statistical significance, $p<0.0001$ for a two-tailed t-test at the 95% confidence interval.

Experimental cross	Genotype	Anticipated Mendelian proportion of F1 with this genotype	Observed proportion of viable F1 with this genotype in glc food	Observed proportion of viable F1 with this genotype in glc+gal food
$dGALT^{C2}/dGALT^{C2}$ x $dGALT^{C2}/CyO$	$dGALT^{C2}/dGALT^{C2}$ (*dGALT* control)	0.5	0.466 ± 0.003	0.454± 0.003
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ x $dGALT^{\Delta 1AP2}/CyO$	$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ (*dGALT*-null)	0.5	0.403± 0.005	0.062± 0.008
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10A11}/UAS\text{-}hGALT^{10A11}$ x $dGALT^{\Delta 1AP2}/CyO$; $UAS\text{-}hGALT^{10A11}/UAS\text{-}hGALT^{10A11}$	$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10A11}/UAS\text{-}hGALT^{10A11}$ (*dGALT*-null with a leaky *hGALT* transgene)	0.5	0.443± 0.006	0.160 ± 0.015*

Table 2.3: Partial rescue of viability by low-level expression of *hGALT* in *D. melanogaster*. Expression of about 4% wild-type levels of human GALT partially rescues the viability phenotype in *dGALT*-null *Drosophila* raised on glucose-plus-galactose food (*t-test, $p<0.05$). Glc=glucose; gal=galactose. Values represent the average of replicates ± SEM.

The result was striking; on food that contained glucose as the sole sugar, unbalanced *dGALT*-deficient flies accounted for close to the expected 50% of viable offspring, but on food supplemented with 222 mM galactose, *dGALT*-deficient flies accounted, on average, for <10% of viable offspring (Figure 2.3A and Table 2.3). When the galactose concentration was raised to 278 mM, the number of viable *dGALT*-deficient animals plummeted nearly to zero (data not shown). This same result was also obtained using the $dGALT^{\Delta 1V2}$ allele, as well as using $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1V2}$ trans-heterozygotes.

As a control for galactose specificity of the apparent food sensitivity, we repeated each cross on food containing 555 mM glucose plus 222 mM mannose rather than galactose; all *dGALT*-deficient crosses yielded close to 50% unbalanced offspring (Figure 2.3A). We also performed crosses using flies homozygous for the precise excision allele ($dGALT^{C2}$); these crosses also yielded close to 50% unbalanced offspring regardless of the sugar composition of the food (Figure 2.3A).

Finally, to test whether $dGALT^{\Delta 1AP2}$ behaved as a genetic null allele, we crossed homozygous $dGALT^{\Delta 1AP2}$ virgin females to males heterozygous for a large genomic deletion that removes the entire *dGALT* locus (*Df(2L)Exel7027)* and assessed the effect of this $dGALT^{\Delta 1AP2}/Df(2L)Exel7027$ genotype on galactose sensitivity. The outcomes of these crosses (data not shown) were comparable to the outcomes of crosses using $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ or $dGALT^{\Delta 1V2}/dGALT^{\Delta 1V2}$ flies, indicating that $dGALT^{\Delta 1AP2}$ and $dGALT^{\Delta 1V2}$ behave as genetic nulls.

Transgenic expression of human *GALT* rescues viability of *dGALT*-deficient *D. melanogaster* exposed to galactose

To confirm that the galactose sensitivity of *dGALT*-deficient *Drosophila* resulted

from loss of GALT activity, we attempted transgenic rescue using a *UAS*-driven human *GALT* transgene (*UAS-hGALT*). The rationale for using a human rather than a fly *GALT* transgene was to test whether *dGALT* and *hGALT* are true functional orthologs. Male flies homozygous for $dGALT^{\Delta 1AP2}$ and heterozygous for a *β-tubulin-GAL4* driver were crossed to virgin females heterozygous for $dGALT^{\Delta 1AP2}$ and homozygous for either of three *UAS-hGALT* transgenes, $UAS\text{-}hGALT^{9A12}$, $UAS\text{-}hGALT^{10A12}$, or $UAS\text{-}hGALT^{10B22}$. These crosses were conducted in parallel on foods containing 555 mM glucose or 555 mM glucose plus 222 mM galactose. As illustrated in Figure 2.3B and Table 2.2, the presence of an *hGALT* transgene together with the *β-tubulin-GAL4* driver resulted in over-expression of *hGALT* activity and fully rescued viability of $dGALT^{\Delta 1AP2}$ homozygous *Drosophila* exposed to galactose. The transgenic rescue data further demonstrated that over-expression of *hGALT* appears benign, although we have not ruled out subtle effects.

To test the ability of low level expression of human *GALT* to rescue viability of *dGALT*-null *Drosophila* we generated animals homozygous for $dGALT^{\Delta 1AP2}$ and also homozygous for a leaky *hGALT* transgene, $UAS\text{-}hGALT^{10A11}$. These animals expressed just over 4% wild-type levels of GALT activity despite the absence of any *GAL4* driver (Table 2.2), and even this low level was sufficient for partial rescue of the viability phenotype on glucose-plus-galactose food (Table 2.3).

Timing and dose-dependent lethality of GALT-deficient *D. melanogaster* exposed to galactose

To identify the stage of development at which GALT-deficient *D. melanogaster* succumb to galactose toxicity, we followed the fates of cohorts of >100 individual progeny derived from crosses of $dGALT^{\Delta 1AP2}$ homozygous virgin females and males. In

brief, <12-h-old embryos harvested from egg-laying plates were transferred to the wells of 96-well plates preloaded with each of three types of fly food: food containing 555 mM glucose, food containing 555 mM glucose plus 111 mM galactose, and food containing 555 mM glucose plus 222 mM galactose. Each day the numbers of live and dead animals were recorded; the results (Figure 2.4) revealed four important points:

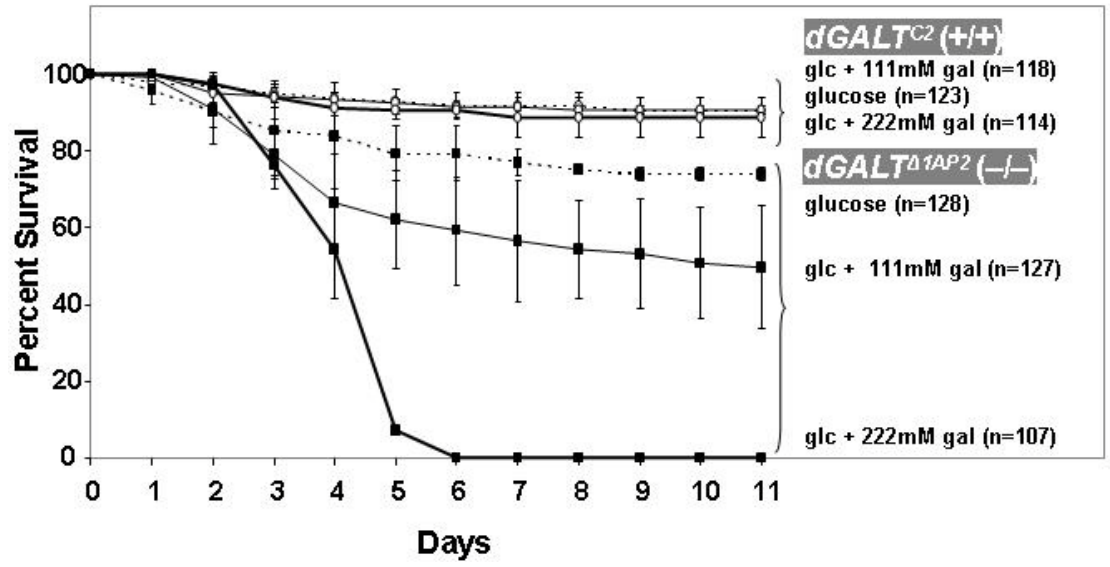

Figure 2.4: Timing of death in *dGALT*-deficient *Drosophila* exposed to galactose. Age of animals, in days, is plotted on the x-axis; percent of animals remaining viable in each cohort is plotted on the y-axis. Animals homozygous for the *dGALT*^{C2} precise excision allele served as a positive control; these animals remained predominantly viable throughout the 11-day course of the experiment, regardless of the food content. In contrast, animals homozygous for the *dGALT*$^{\Delta 1AP2}$ imprecise excision allele remained predominantly viable in the absence of galactose, but died in increasing numbers during larval development when exposed to increasing levels of dietary galactose.

First, *dGALT* wild-type animals homozygous for the precise excision allele

(*dGALT*^{C2}) remained viable and developed normally throughout the 11-day course of the experiment, regardless of the presence or absence of galactose in their food. By day 11, close to 90% of these animals had eclosed as viable adults, demonstrating that the 96-well plate format was not itself a major cause of delayed development or death.

Second, even in the absence of dietary galactose, animals homozygous for the imprecise excision allele (*dGALT*$^{\Delta 1AP2}$) were slightly less robust than their precise excision counterparts. By day 11 only 74% of these homozygotes had eclosed as viable adults, compared with 90% of controls. Of note, this difference did not appear as pronounced in standard culture vials, suggesting that the 96-well plate format may have disproportionately stressed the *dGALT*-deficient animals, although this possibility remains unexplained.

Third, in the presence of increasing concentrations of galactose, we saw a decreasing percentage of *dGALT*-deficient animals that survived to adulthood. Specifically, on food containing 111 mM galactose, only 50% of the animals eclosed as viable adults, while on food containing 222 mM galactose, none of the *dGALT*-deficient animals eclosed as viable adults.

Finally, the timing of death of the *dGALT*-deficient *Drosophila* exposed to galactose occurred during mid-to-late larval stages. Essentially, all the mutant embryos visibly hatched into viable first instar (L1) larvae and began to eat food, as evidenced by the presence of coloring from the food in their gut, but none of these larvae survived to pupation. It is difficult to distinguish the precise stage of lethality, because the dying animals were stunted relative to controls (Figure 2.5). Whether this small organismal size reflected a true developmental delay or simply a growth defect remains unclear.

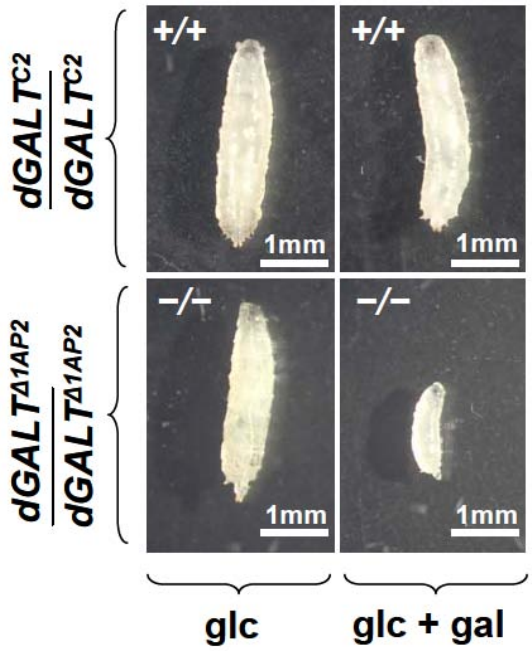

Figure 2.5: **Digital images of living *dGALTC2/dGALTC2* precise excision (+/+) and *dGALTΔ1AP2/dGALTΔ1AP2* imprecise excision (−/−) homozygotes**, each photographed at 25X on day five of development; larvae were collected from food containing only 555 mM glucose (glc)or 555 mM glucose plus 222 mM galactose (glc+gal), as indicated.

Window of galactose-sensitivity of GALT-deficient *D. melanogaster*

To define the stage of development at which GALT-deficient *D. melanogaster* are most sensitive to galactose, we performed dietary "cross-over" experiments (Figure 4) in which cohorts of $dGALT^{\Delta 1AP2}$ homozygotes (GALT-deficient) were transferred as embryos or larvae from food containing only 555 mM glucose to food containing 555 mM glucose plus 222 mM galactose, or from food containing 555 mM glucose plus 222 mM galactose to food containing only 555 mM glucose. As a control for the transfer process, animals were also transferred each day from glucose food to glucose food, and from glucose-plus-galactose food to glucose-plus-galactose food. The transfers were performed for six days, which covered the span of larval development (L1 to L3), after which all animals were allowed to complete development without further interference.

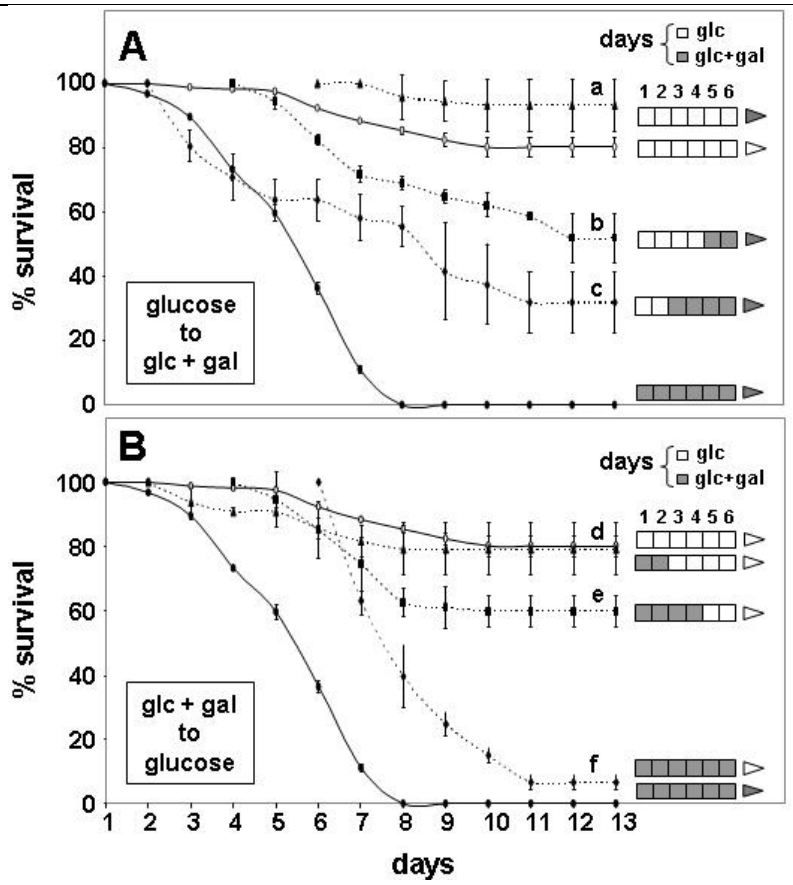

Figure 2.6: Window of galactose sensitivity of *dGALT*$^{\Delta 1AP2}$ imprecise excision homozygotes. Day of development is plotted on the x-axis; percent survival in each cohort is plotted on the y-axis. *dGALT*-deficient embryos and larvae were transferred daily in cohorts of >20 animals each from 555 mM glucose-only food or from 222 mM galactose-supplemented food to 555 mM glucose-only food **(**dashed lines in **B)**, for six days, after which the animals in each cohort were allowed to complete development without further interference. To the right of each plotted curve is a key illustrating the food exposure of the corresponding cohort of animals; each open box represents one day of development on glucose food; each shaded box represents one day of development on food supplemented with 222 mM galactose. The shading of the arrow to the right of each key indicates whether subsequent development occurred on glucose (open arrow) or glucose-plus-galactose (shaded arrow) food. Of note, only

live animals were transferred, which led to an apparent but artificial upward shift in the survival curves for some cohorts of animals, as explained in Results. As a control, animals were also transferred from glucose-only to glucose-only food (upper solid line in each panel), and from galactose-supplemented food to galactose-supplemented food (lower solid line in each panel). In both experiments animals demonstrated a reversible response to galactose exposure during early larval development.

The results (Figure 2.6) clearly showed that GALT-deficient animals that started developing on glucose-only food nonetheless succumbed if they were transferred to glucose-plus-galactose food early in larval development (e.g., day two, which corresponded to late L1 or early L2 stage). Transfer on subsequent days (e.g., day four, which corresponded to late L2 or early L3 stage) resulted in diminished, albeit detectable, loss of viability. Significantly, GALT-deficient animals exposed to galactose late in larval development (e.g., day six, which corresponded to L3 stage) or as adults demonstrated no significant loss of viability relative to their galactose-restricted counterparts.

Similarly, GALT-deficient animals that started life on food supplemented with galactose were rescued from death by transfer to glucose-only food, as long as the transfer occurred within the first two days of development. Transfer on later days conferred only limited survival benefit. Of note, only live animals were transferred between foods, which led to an apparent but artificial upward shift in the survival curves of animals transferred from galactose-supplemented food to glucose-only food (Figure 2.6B), and also to a slight upward shift in the survival curves of animals transferred between foods on later days of development (e.g. day 6, Figure 2.6A and B). While

cohorts of animals were transferred on each of the first six days of these experiments, for simplicity, only the results from transfers on the even-numbered days are presented in Figure 2.6; however, the cohorts transferred on odd-numbered days showed survival profiles fully consistent with these patterns.

Finally, the vast majority of animals transferred from glucose-only food to fresh glucose-only food developed into viable adults, whereas all GALT-deficient animals transferred from galactose-supplemented food to fresh galactose-supplemented food died as larvae, demonstrating that the physical transfer process itself did not noticeably alter the outcomes observed.

GALT-deficient *Drosophila* accumulate gal-1P when exposed to galactose

To test whether GALT-deficient *Drosophila* exposed to dietary galactose accumulate abnormal levels of gal-1P we exposed developing larvae and newly eclosed adults to food containing either 555 mM glucose alone or 555mM glucose-plus-222 mM galactose for four days. Extracts of these animals were analyzed as described in Methods. In the absence of dietary galactose $dGALT^{\Delta 1AP2}$ homozygous larvae and adults demonstrated 0.75 ± 0.13 (n=3) and 0.22 ± 0.09 (n=3) pmol gal-1P/µg protein, respectively, and in the presence of dietary galactose, these animals accumulated 11.82 or 12.09 (n=2) and 7.95 ± 4.25 (n=3) pmol gal-1P/µg protein, respectively. Parallel samples from $dGALT^{C2}$ homozygous larvae and adults demonstrated only 0.37 ± 0.09 and 0.10 ± 0.04 (n=3, glucose-only food) and 0.85 ± 0.12 and 0.30 ± 0.10 pmol gal-1P/µg protein (n=3, glucose -plus-galactose food), respectively.

Galactose-restricted GALT-deficient flies demonstrate an impaired negative geotaxic response

To address whether GALT-deficient *D. melanogaster* might, like their human counterparts, exhibit long-term complications despite lifelong dietary restriction of galactose, we assessed the negative geotaxic response of $dGALT^{\Delta 1AP2}$ homozygous, $dGALT^{\Delta 1V2}$ homozygous, and $dGALT^{C2}$ homozygous flies. All of these animals were raised on standard molasses food, which contains negligible if any galactose, and tested using a previously described “countercurrent” apparatus (26, 27) that quantifies negative geotaxic response. Normal flies exhibit a strong negative geotaxic response and a quick response to stimulus, meaning they begin to move shortly after being tapped to the bottom of a tube, and they prefer to run "up" the sides of a tube rather than stand still or run "down."

The apparatus and procedure we applied offered cohorts of approximately 60 two-to-three-day-old male flies each six sequential opportunities to walk or run "up" the sides of a tube in a well-lighted room within a specified period of time (15 seconds). Flies that moved to the top of inverted tube one within the first 15-second period were tapped to the bottom of tube two, and the next round was initiated. At the end of six sequential rounds, the "fastest" flies would be found in tube six, and the "slowest" flies would be found in tube one.

Control flies subjected to this "countercurrent test" presented a final distribution in which ~60% of the flies were in tube six, with the remainder distributed almost evenly between the other five tubes (Figure 2.7A); this result is fully consistent with the original

report of this behavior (26). In striking contrast, both GALT-deficient stocks demonstrated an almost random distribution of flies among the six tubes with no enrichment in tube six (Figure 2.7B, C). This differential distribution profile was highly statistically significant ($p<0.01$) and was rescued by expression of the human *GALT* transgene (Figure 2.7D), thereby demonstrating that this phenotype resulted from the absence of GALT activity.

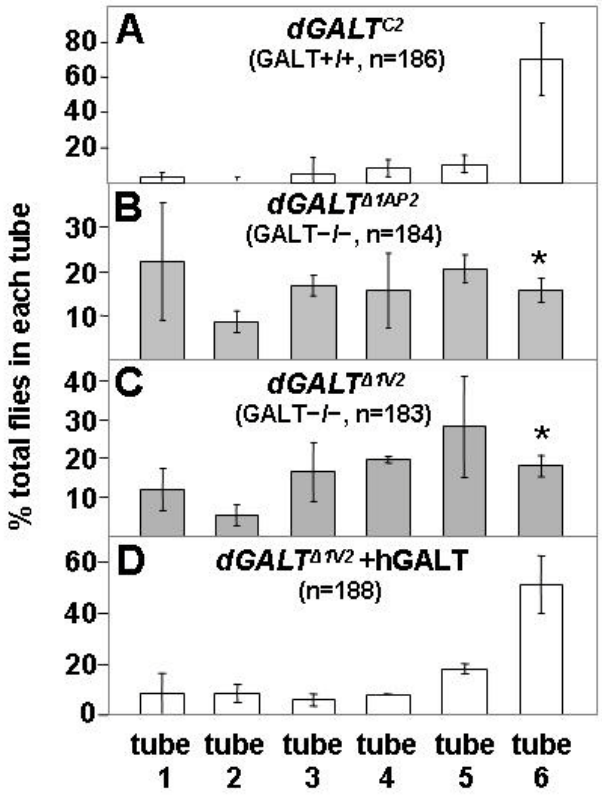

Figure 2.7: *dGALT*-deficient flies demonstrate an impaired negative geotaxic response despite dietary restriction of galactose Cohorts of 60 male two-to-three-day-old flies of the indicated genotypes were subjected to "countercurrent" analysis as a measure of negative geotaxic response. All flies began the experiment in the bottom of tube one. Those flies that walked to the top of tube one in 15 seconds were tapped to the bottom of tube two, and so forth. After six rounds of tapping and transfer, the fastest flies could be found in tube six, and the slowest flies could be found in tube one. Values plotted represent averages ± standard deviation (n = 3). Flies homozygous for the $dGALT^{\Delta 1AP2}$ (**panel B**) and $dGALT^{\Delta 1V2}$ (**panel C**) imprecise excision alleles demonstrated a statistically significant impairment of negative geotaxic response relative to flies homozygous for the $dGALT^{C2}$ precise excision allele (**panel A**) (*, $p<0.01$ in a one-way ANOVA with 95% confidence intervals); this impairment was rescued by expression of a human *GALT* transgene (**panel D**).

2.5 DISCUSSION

Here we report the generation and initial characterization of a *D. melanogaster* model of classic galactosemia. This is the first whole animal genetic model of *GALT* deficiency to mimic any of the clinical manifestations of classic galactosemia and as such represents a major step forward for the field.

Galactose sensitivity of GALT-deficient flies:

Our data establish two main points about galactose sensitivity. First, loss of *dGALT* in developing *D. melanogaster* results in a galactose-dependent lethality, and this outcome is rescued by transgenic expression of human *GALT*. While obvious in its simplicity, this result is also profound, because it largely puts to rest a nagging concern that has plagued the field since Leslie and colleagues first reported that loss of *GALT* in mice is apparently benign (17). In short, the lack of a phenotype in *GALT*-deficient mice raised the unlikely but disturbing possibility that the clinical outcomes of patients with classic galactosemia might result from some cryptic impact of their mutations, rather than from the detected loss of GALT enzymatic activity. With the work presented here, the causal relationship between loss of GALT activity and galactosemia-related phenotypes in a multicellular organism is confirmed.

Our data presented here further establish that even trace expression (~4%) of human GALT can at least partially rescue the galactose-sensitivity of *dGALT*-deficient *Drosophila*. This result is especially notable in light of patient data; humans homozygous for the *S135L-hGALT* allele, associated with about 5% residual GALT activity, tend to show a relatively mild clinical course (14). This result is also notable in contrast to GALE. The most severely affected patients with GALE-deficiency galactosemia express

at least 5% residual GALE activity [reviewed in (2)], and *Drosophila* expressing ~4% residual GALE activity (from a hypomorphic *dGALE* allele) are inviable (31). The results observed in flies therefore parallel what is seen in patients, namely that marginal levels of GALT activity are sufficient for life, while comparably marginal levels of GALE are not.

Second, the galactose-dependent mortality observed in *dGALT*-deficient *D. melanogaster* is both dose- and time-dependent. Exposure to a lower level of galactose or exposure to galactose later in development resulted in less severe outcomes. These data implicate a potential threshold, or series of thresholds, of galactose sensitivity in GALT-deficient *Drosophila.* Alternatively, or in addition, there may be a finite developmental window of sensitivity. As we did not quantify the exact amount of food (and therefore galactose) consumed by each animal on each day, we cannot distinguish between these possible options. The possibility therefore remains that younger animals appeared more sensitive to galactose because they consumed proportionally larger quantities of food, and therefore likely accumulated proportionally larger quantities of galactose than their older counterparts.

Consistent with this conclusion, we observed that the numbers of *dGALT*-deficient flies that eclosed as viable adults when exposed to 222 mM galactose fell, on average, below 10%; the precise proportion varied from experiment to experiment, even for crosses involving flies of the same genotype. Raising the level of galactose to 278 mM was sufficient to bring the number of *dGALT*-deficient survivors virtually to zero, but at lower concentrations of galactose, subtle factors, such as the number of flies placed in a vial, or whether the animals were allowed to develop in vials or in the wells of a 96-well plate, seemed to impact the ability of some *dGALT*-deficient animals to escape early

death. Future experiments will address the nature and extent of these cryptic modifying factors.

Also evident here was our finding that, if galactose exposure was early but transient, the potentially lethal “damage” was reversible. This observation is not surprising given decades of clinical experience reported from human populations under surveillance by newborn screening, where affected infants are routinely rescued from acute disease by rapid initiation of dietary galactose restriction [reviewed in (2)]. The fly data are nonetheless compelling, because they suggest that future experiments using this model system may reveal with greater precision both the nature of the damage and the mechanism by which it is reversed or overcome when galactose is removed from the diet.

Despite the significance of these findings, it is important not to overinterpret the fly data. For example, although exposure of *Drosophila* adults or late-stage larvae to galactose was not lethal, we do not know that it was completely benign. It would be premature, therefore, to conclude that *dGALT*-deficient flies, or classic galactosemia patients, do not require lifelong dietary restriction of galactose. Nevertheless, there are intriguing anecdotal reports from the literature (36, 37) that describe a similar finding in patients; namely, that older children and adults with classic galactosemia appear able to consume dietary galactose with no acute negative clinical consequences.

Long-term complications:

Although we have just begun to test our *dGALT*-deficient flies for long-term phenotypes, abnormalities clearly exist (Figure 2.7) and these can be rescued by expression of a human *GALT* transgene. The galactose-independent phenotype we report here, impaired negative geotaxic response, is a complex trait that could reflect any of a

number of underlying muscular or neuromuscular abnormalities. While many patients with classic galactosemia do experience apparent neuromuscular complications, the relationship between those patient outcomes and the fly phenotype reported here remains to be explored. Future studies will address the extent and nature of other potential abnormalities in *dGALT*-deficient flies, as well as the genetic and environmental factors that underlie them.

Metabolic abnormalities in GALT-impaired *D. melanogaster*:

That GALT-deficient *Drosophila* exposed to galactose accumulate elevated levels of gal-1P is fully consistent with metabolic abnormalities reported in patients [reviewed in (2)]. What is striking, however, is that the levels of gal-1P accumulated from four days of galactose exposure in larvae and adults is fairly comparable (11.82 or 12.09 (n=2) and 7.95 ± 4.25 (n=3) pmol gal-1P/μg protein, respectively), yet the larvae are progressing rapidly toward death, while the adults are not. There are many possible explanations for this apparent disparity, but we must also consider that gal-1P itself may not be the primary cause of symptoms. Rather, the symptoms may result from stage-specific ways in which the body responds to an elevated gal-1P level. This observation, in essence, parallels the lesson of the *GALT* knockout mouse, which also accumulated high levels of gal-1P in response to dietary galactose exposure, and which also did not die as a result (17). Hence, mice do not succumb in response to galactose exposure regardless of age, whereas flies only succumb to galactose-exposure within an apparent developmental window of sensitivity. Clearly, if we are to understand the underlying pathophysiology of galactosemia we must find out what occurs downstream of gal-1P accumulation in developing animals. With a fly genetic model that recapitulates aspects of the patient

phenotype, at long last we have tools to begin the work.

Acknowledgments

We are grateful to Kerry Garza and members of the Moberg and Sanyal Labs at Emory University for contributions and helpful discussions throughout the course of this project, to Jewels Chhay for contributions to the creation of the *UAS-hGALT* construct, and to Doug Rennie for embryo injection of the *hGALT* transgene. We are also grateful to Cheryl Strauss for helpful comments on the manuscript. This work was supported by National Institutes of Health grant DK046403 (to JLFK and KHM).

Author contributions

RFK generated the *dGALT*-excision alleles and designed and performed the majority of experiments presented in Figures 2.2-2.6; ELR generated some of the data for Figure 2.3 and all of the data for Figure 2.7; RDS and JMIS generated most of the data presented in Table 2.2; PJL generated some of the data for Tables 2.2 and 2.3; KHM provided general oversight for experiments involving fly genetic manipulation; JLFK conceived the project and provided oversight for much of its completion. All authors contributed to writing the final manuscript.

2.6 REFERENCES

1. Holden HM, Rayment I, & Thoden JB (2003) Structure and function of enzymes of the Leloir pathway for galactose metabolism. *J Biol Chem* 278(45):43885-43888.
2. Fridovich-Keil JL & Walter JH (2008) Galactosemia. *The Online Metabolic & Molecular Bases of Inherited Disease*, eds Valle D, Beaudet A, Vogelstein B, Kinzler K, Antonarakis S, & Ballabio A (McGraw Hill), p http://www.ommbid.com/.
3. Chhay JS, Openo KK, Eaton JS, Gentile M, & Fridovich-Keil JL (2008) A yeast model reveals biochemical severity associated with each of three variant alleles of galactose-1P uridylyltransferase segregating in a single family. *J Inherit Metab Dis* 31(1):97-107.
4. Tyfield L & Walter J (2002) Galactosemia. *The Metabolic and Molecular Bases of Inherited Disease*, eds Scriver C, Beaudet A, Sly W, Valle D, Childs B, Kinzler K, & Vogelstein B (McGraw-Hill, New York).
5. Leslie ND (2003) Insights into the pathogenesis of galactosemia. *Annu Rev Nutr* 23:59-80.
6. Christacos NC & Fridovich-Keil JL (2002) Impact of patient mutations on heterodimer formation and function in human galactose-1-P uridylyltransferase. *Mol Genet Metab* 76(4):319-326.
7. Elsevier JP & Fridovich-Keil JL (1996) The Q188R mutation in human galactose-1-phosphate uridylyltransferase acts as a partial dominant negative. *J Biol Chem* 271(50):32002-32007.
8. Elsevier JP, Wells L, Quimby BB, & Fridovich-Keil JL (1996) Heterodimer formation and activity in the human enzyme galactose-1-phosphate uridylyltransferase. *Proc Natl Acad Sci U S A* 93(14):7166-7171.
9. Fridovich-Keil JL & Jinks-Robertson S (1993) A yeast expression system for human galactose-1-phosphate uridylyltransferase. *Proc Natl Acad Sci U S A* 90(2):398-402.

10. Fridovich-Keil JL, *et al.* (1995) Identification and functional analysis of three distinct mutations in the human galactose-1-phosphate uridyltransferase gene associated with galactosemia in a single family. *Am J Hum Genet* 56(3):640-646.

11. Fridovich-Keil JL, Quimby BB, Wells L, Mazur LA, & Elsevier JP (1995) Characterization of the N314D allele of human galactose-1-phosphate uridylyltransferase using a yeast expression system. *Biochem Mol Med* 56(2):121-130.

12. Riehman K, Crews C, & Fridovich-Keil JL (2001) Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase. *J Biol Chem* 276(14):10634-10640.

13. Ross KL, Davis CN, & Fridovich-Keil JL (2004) Differential roles of the Leloir pathway enzymes and metabolites in defining galactose sensitivity in yeast. *Mol Genet Metab* 83(1-2):103-116.

14. Lai K, *et al.* (1996) A prevalent mutation for galactosemia among black Americans. *J Pediatr* 128(1):89-95.

15. Lai K & Elsas LJ (2000) Overexpression of human UDP-glucose pyrophosphorylase rescues galactose-1-phosphate uridyltransferase-deficient yeast. *Biochem Biophys Res Commun* 271(2):392-400.

16. Lai K, Langley SD, Khwaja FW, Schmitt EW, & Elsas LJ (2003) GALT deficiency causes UDP-hexose deficit in human galactosemic cells. *Glycobiology* 13(4):285-294.

17. Leslie ND, Yager KL, McNamara PD, & Segal S (1996) A mouse model of galactose-1-phosphate uridyl transferase deficiency. *Biochem Mol Med* 59(1):7-12.

18. Leslie ND & Bai S (2001) Functional analysis of the mouse galactose-1-phosphate uridyl transferase (GALT)promoter. *Mol Genet Metab* 72(1):31-38.

19. Leslie N, Yager C, Reynolds R, & Segal S (2005) UDP-galactose pyrophosphorylase in mice with galactose-1-phosphate uridyltransferase deficiency. *Mol Genet Metab* 85(1):21-27.

20. Ning C, *et al.* (2000) Galactose metabolism by the mouse with galactose-1-phosphate uridyltransferase deficiency. *Pediatr Res* 48(2):211-217.

21. Ning C, *et al.* (2001) Galactose metabolism in mice with galactose-1-phosphate uridyltransferase deficiency: sucklings and 7-week-old animals fed a high-galactose diet. *Mol Genet Metab* 72(4):306-315.

22. Bier E (2005) Drosophila, the golden bug, emerges as a tool for human genetics. *Nat Rev Genet* 6(1):9-23.

23. Lasko P (2002) Diabetic flies? Using Drosophila melanogaster to understand the causes of monogenic and genetically complex diseases. *Clin Genet* 62(5):358-367.

24. Bharucha KN (2009) The epicurean fly: using Drosophila melanogaster to study metabolism. *Pediatr Res* 65(2):132-137.

25. Zhang H, *et al.* (2009) Deletion of Drosophila insulin-like peptides causes growth defects and metabolic abnormalities. *Proc Natl Acad Sci U S A* 106(46):19617-19622.

26. Benzer S (1967) BEHAVIORAL MUTANTS OF Drosophila ISOLATED BY COUNTERCURRENT DISTRIBUTION. *Proc Natl Acad Sci U S A* 58(3):1112-1119.

27. Akai S (1979) Genetic variation in walking ability of Drosophila melanogaster. *Jpn J. Genet.* 54:317-324.

28. Honjo K & Furukubo-Tokunaga K (2005) Induction of cAMP response element-binding protein-dependent medium-term memory by appetitive gustatory reinforcement in Drosophila larvae. *J Neurosci* 25(35):7905-7913.

29. Ryder E & Russell S (2003) Transposable elements as tools for genomics and genetics in Drosophila. *Brief Funct Genomic Proteomic* 2(1):57-71.

30. Brand AH & Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118(2):401-415.

31. Sanders RD, Sefton JM, Moberg KH, & Fridovich-Keil JL (2010) UDP-galactose 4' epimerase (GALE) is essential for development of Drosophila melanogaster. *Dis Model Mech* 3(9-10):628-638.

32. Openo KK, *et al.* (2006) Epimerase-deficiency galactosemia is not a binary condition. *Am J Hum Genet* 78(1):89-102.

33. Chien S, Reiter LT, Bier E, & Gribskov M (2002) Homophila: human disease gene cognates in Drosophila. *Nucleic Acids Res* 30(1):149-151.

34. Keyes LN & Spradling AC (1997) The Drosophila gene fs(2)cup interacts with otu to define a cytoplasmic pathway required for the structure and function of germ-line chromosomes. *Development* 124(7):1419-1431.

35. Piccioni F, Zappavigna V, & Verrotti AC (2005) A cup full of functions. *RNA Biol* 2(4):125-128.

36. Panis B, *et al.* (2006) Untreated classical galactosemia patient with mild phenotype. *Mol Genet Metab* 89(3):277-279.

37. Bosch AM, Bakker HD, Wenniger-Prick LJ, Wanders RJ, & Wijburg FA (2004) High tolerance for oral galactose in classical galactosaemia: dietary implications. *Arch Dis Child* 89(11):1034-1036.

Chapter 3

Mediators of a long-term movement abnormality in a *Drosophila melanogaster* model of classic galactosemia

This chapter contains work published as: Emily L Ryan, Brian DuBoff, Mel B Feany, and Judith L Fridovich-Keil *Dis Model Mech*. 2012 Aug 10. [Epub ahead of print]. This chapter is the authors' version, including changes resulting from the peer-review process. Changes resulting from the publishing process are not reflected in this work.

3.1 ABSTRACT

Despite neonatal diagnosis and life-long dietary restriction of galactose, many patients with classic galactosemia grow to experience significant long-term complications. Among the more common are speech, cognitive, behavioral, ovarian, and neurological/ movement difficulties. Despite decades of research, the pathophysiology of these long-term complications remains obscure, hindering prognosis and attempts at improved intervention. As a first step to overcome this roadblock we have begun to explore long-term outcomes in our previously reported GALT-null *Drosophila melanogaster* model of classic galactosemia. Here we describe the first of these studies. Using a countercurrent device, a simple climbing assay, and a startle response test to characterize and quantify an apparent movement abnormality, we explored the impact of cryptic GALT expression on phenotype, tested the role of sub-lethal galactose exposure and galactose-1-phosphate (gal-1P) accumulation, tested the impact of age, and searched for potential anatomical defects in brain and muscle. We found that about 2.5% residual GALT activity was sufficient to reduce outcome severity. Surprisingly, sub-lethal galactose exposure and gal-1P accumulation during development showed no effect on adult phenotype. Finally, despite the apparent neurological or neuromuscular nature of the complication we found no clear morphological differences between mutants and controls in brain or muscle, suggesting that the defect is subtle and/or may be physiologic rather than structural. Combined, our results confirm that, like human patients, GALT-null *Drosophila* experience significant long-term complications that occur independent of galactose exposure, and serve as a proof of principle demonstrating utility of the GALT-

null *Drosophila* model as a tool to explore genetic and environmental modifiers of long-term outcome in GALT deficiency.

3.2 INTRODUCTION

Classic galactosemia (OMIM 230400) is an autosomal recessive disorder that results from profound impairment of galactose-1-P uridylyltransferase (GALT, EC 2.7.7.12), the middle enzyme in the Leloir pathway of galactose metabolism (reviewed in (1)). In most western populations, classic galactosemia occurs with a frequency of at least 1/60,000 live births; the rate is substantially higher in some groups. Infants with classic galactosemia generally appear normal at birth but present with escalating symptoms within days of exposure to dietary galactose, which, as a constituent monosaccharide of lactose, is abundant in breast milk and milk-based formulae. Acute symptoms range from cataracts, failure to thrive, vomiting, and diarrhea to hepatomegaly, bleeding abnormalities, and *E. coli* sepsis, which can be lethal. Absent intervention, infants with classic galactosemia often succumb in the neonatal period (reviewed in (1)).

The advent of population newborn screening for GALT deficiency in the 1960s (e.g. (2, 3)) and its rapid implementation in the decades that followed enabled early, sometimes even pre-symptomatic identification of affected infants. Switched to a galactose-restricted diet – generally a soy or elemental formula -- these infants appeared to thrive, leading to early predictions that neonatal diagnosis coupled with rigorous life-long dietary restriction of galactose would enable patients with classic galactosemia to escape the negative consequences of their disease (reviewed in (1)).

Unfortunately, the escape was short-lived. By the 1970s and 1980s, first anecdotal reports, and then large retrospective studies demonstrated that despite early diagnosis and rigorous dietary intervention, patients with classic galactosemia remained at strikingly increased risk for an unusual constellation of long-term complications, including a speech

disorder, cognitive disability, and neurological or neuromuscular complications in close to half of all patients, and primary or premature ovarian insufficiency in more than 80% of girls and women (e.g. (4-9)). Other complications were also noted. Attempts to pinpoint the underlying causes of these disparate complications have been disappointing, hindered in part by the fact that classic galactosemia is a rare disorder so that most studies have been conducted with relatively small numbers of patients, and in part by the failure of a knock-out mouse model to mimic patient outcomes (10).

Recently, we reported the development and initial characterization of a *Drosophila melanogaster* model of classic galactosemia (11). These GALT-null animals recapitulate the fundamental acute patient phenotype in that they die in development if exposed to food containing substantial galactose (in addition to glucose and other nutrients), but live if maintained on a galactose-restricted diet. These animals are also rescued by expression of a wild-type human GALT transgene early in development. Finally, GALT-null *Drosophila* demonstrate a movement disorder that is evident when they attempt to traverse a countercurrent device; as in patients, this movement abnormality occurs despite life-long dietary restriction of galactose (11).

In the work described here we have first characterized and then used this movement abnormality to test four fundamental questions about long-term outcome in GALT-deficient animals: 1-does cryptic GALT activity impact outcome severity? 2-does dietary exposure to sub-lethal galactose in development exacerbate the phenotype and is there a relationship between gal-1P level and outcome severity? 3-does age impact severity of the phenotype, and finally 4-are there any structural defects evident in brain or muscle of adult GALT-null *Drosophila* that might account for the movement

abnormality? Our results demonstrate that cryptic residual GALT activity *does* make a difference; about 2.5% and 6% normal GALT activity were each sufficient to significantly improve outcome in our flies. Strikingly, exposure of GALT-null larvae to sub-lethal levels of dietary galactose, which markedly increased their gal-1P levels, did *not* exacerbate the adult phenotype, although increasing age did have this effect. Finally, microscopic inspection of brain and muscle structures in GALT-null and control flies revealed no evident defects, implying that the abnormality is either subtle or may be physiologic rather than anatomic. These results further our understanding of the etiology of long-term outcome in GALT-null *Drosophila*, and by extension, may have implications for our understanding of long-term complications in classic galactosemia.

3.3 METHODS

Fly stocks and maintenance

All stocks were maintained at 25ºC on molasses-based food that contained 44.4 g/l corn meal, 19.2 g/l yeast extract, 6 g/l agar, 52.5 ml/l molasses, 3 ml/l propionic acid, and 13.8 ml/l methyl paraben (tegosept, 10% w/v in ethanol). For experiments designed to test the impact of dietary galactose exposure, animals were fed a glucose-based food [5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l glucose 10 ml/l proprionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol)] with supplemental galactose added, as indicated. The *dGALT* alleles $dGALT^{\Delta AP2}$ and $dGALT^{C2}$ and *UAS-hGALT* transgenes $UAS\text{-}hGALT^{10A11}$ and $UAS\text{-}hGALT^{10B22}$ used here have been described previously (11). All other alleles or stocks, including the P-element insertion stock y^1w^{67c23}; P{SUPor-P}$GALT^{KG00049}cup^{KG00049}$ (FBst0014339) from which the excisions were made, w^{1118}; Df(2L)Exel7027/ Cyo (FBst0007801), y^1w^*; P{Act5C-GAL4}25FO1/Cyo,y^+ (FBst0004414) were obtained from the Bloomington Drosophila Stock Center at Indiana University. To generate animals carrying *Actin5c-GAL4* in the $dGALT^{\Delta AP2}$ background the two alleles were recombined onto the same second chromosome. Therefore, in experiments with *hGALT* transgene rescue the following genotypes were used: $dGALT^{\Delta AP2}$ *Actin5c-GAL4/* $dGALT^{\Delta AP2}$; +/+ and $dGALT^{\Delta AP2}$ *Actin5c-GAL4/* $dGALT^{\Delta AP2}$; $UAS\text{-}hGALT^{10B22}$ /+.

Countercurrent analysis of a movement abnormality in flies

For each experiment, a cohort of approximately 60 male flies, each less than 24 hours post eclosion, was collected and aged for 36 to 48 hours on molasses food. On the day of testing flies were added to the first tube in the lower rack and given 15 seconds per

round to climb into the corresponding inverted tube in the upper rack. After each round flies in the inverted tubes were shifted into juxtaposition with the next tubes in the lower rack and tapped down. After completing all 9 rounds flies were removed from all tube positions and counted. The proportion of flies in the final tube (chamber 10) was calculated. Every experimental day, cohorts of *dGALT*$^{\Delta AP2}$ and *dGALT*^{C2} homozygotes were analyzed in parallel with all the other experimental cohorts. Details of the data analysis are described in the "Statistics and regression analysis" section below.

GALT enzyme activity assays

Lysates from 10 to 20 male flies each less than 24 hours post-eclosion were prepared and analyzed as previously described (12). GALT activity values less than 0.05 pmol/μg protein/min were indistinguishable from zero and were reported as not detectable.

Dietary galactose exposure

Cohorts of newly eclosed *dGALT*$^{\Delta AP2}$ and *dGALT*^{C2} adults were allowed to lay embryos for 24 to 48 hours in vials containing food with either 555 mM glucose as the sole sugar, or 555 mM glucose plus 50 mM galactose; at the end of this time period the adults were removed and the embryos were allowed to develop and eclose. Cohorts of approximately 60 adult male flies, each less than 24 hours post-eclosion, were then collected from among the F1 and placed for 36-48 hours in vials containing food with 555 mM glucose as the sole sugar. These cohorts were tested in the countercurrent apparatus in parallel with their counterparts who had developed on food containing molasses. For animals of each genotype there was no statistical difference between the results obtained with any of these different cohorts.

Galactose metabolites in larvae and adults

Cohorts of newly eclosed male and female *dGALT*^{C2} or *dGALT*$^{\Delta AP2}$ animals were allowed to lay embryos for 24 to 48 hours in vials containing food with either 555mM glucose as the sole sugar, or 555 mM glucose spiked with 50 mM galactose. Cohorts of larvae from these vials (approximately 100 µL packed volume) were collected after 7 days and washed in phosphate buffered saline prior to analysis. Animals remaining in the vials were allowed to pupate and eclose. Some of the newly eclosed males, in cohorts of 10, were collected directly for analysis, while others were transferred to vials containing food with 555mM glucose as the sole sugar, where they were allowed to remain for two days prior to harvest for analysis. Finally, each cohort of larvae or adult flies to be analyzed for metabolites was resuspended in 125 µL of ice-cold HPLC-grade water and then homogenized, processed, and analyzed as previously described (11).

Histological analysis

Male flies aged for 1 to 14 days post-eclosion were fixed in 4% paraformaldehyde and processed for paraffin embedding. Serial 4 µm sections were taken through the entire head (for analysis of the brain) or thorax (for indirect flight muscle analysis). Slides were processed through xylene, ethanol, and into water. Standard hematoxylin and eosin staining was performed to evaluate overall anatomy and tissue integrity. Antigen retrieval by boiling in sodium citrate, pH 6.0, was used before immunostaining. Slides were blocked in phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 5% milk. Immunostaining was performed using the following mouse monoclonal primary antibodies: anti-synapsin, Developmental Studies Hybridoma Bank; anti-vGlut, Feany laboratory; anti-futsch, Developmental Studies Hybridoma Bank; anti-ATP synthase,

MitoSciences. For immunofluorescence, an Alexa Fluor 488-conjugated anti-mouse secondary antibody was used. For immunohistochemistry, biotin-conjugated anti-mouse secondary antibody and avidin-biotin-peroxidase complex (Vectastain) staining was performed. Histochemical detection was performed by developing with diaminobenzidine (DAB).

Statistics and regression analysis

Data were analyzed with JMPSAS software version 8.0. Countercurrent data illustrated in Figure 3.1 were modeled using a one-way ANOVA with a variable to control for day-to-day variation and test for differences in experimental conditions. Data illustrated in Figure 3.4 panel A were analyzed using a hierarchically well-formulated linear regression to control for day-to-day variation and to test the interaction term (diet*genotype). In all instances, the data presented in the figures are the averages and standard errors from these analyses. Multiple comparisons were corrected using the Bonferroni correction ($\alpha=0.05/n$). Mean and standard error of the mean for enzyme activity (Table 3.1 and Figure 3.3) and gal-1P levels (Figure 3.4 panel B) were calculated from the individual values.

3.4 RESULTS

GALT-deficient flies demonstrate a movement abnormality despite lifelong dietary restriction of galactose

We have reported previously that GALT deficient flies demonstrate a movement abnormality despite lifelong dietary restriction of galactose (11); this phenotype was previously quantified using a classic six-chambered countercurrent device first introduced more than 40 years ago (13, 14). To confirm and expand upon this observation we repeated the analysis using a custom-made 10-chambered countercurrent device (see Methods); experiments were conducted using cohorts of approximately 60 male flies between the ages of 36 to 72 hours post-eclosion.

Using this expanded countercurrent device we confirmed our previous result, demonstrating that GALT-null animals, homozygous for the $dGALT^{\Delta AP2}$ deletion allele (11), were less likely than controls ($dGALT^{C2}$ homozygotes (11)) to reach the final chamber of the apparatus in a fixed period of time (Figure 3.1). Specifically, under the conditions of the assay the average proportion of $dGALT^{\Delta AP2}$ homozygotes to reach the final chamber was only 0.21± 0.01, while the corresponding proportion for control animals was 0.75 ± 0.01. This difference was highly significant ($p<0.0001$).

As a further control, we tested flies homozygous for the P-element insertion allele, $dGALT^{KG00049}$; this is the ancestral allele from which both $dGALT^{\Delta AP2}$ and $dGALT^{C2}$ were generated by imprecise and precise excision, respectively, of the P-insertion (11). Animals homozygous for the $dGALT^{KG00049}$ allele demonstrate essentially normal GALT activity (11) and, as expected, progressed to the final chamber of the

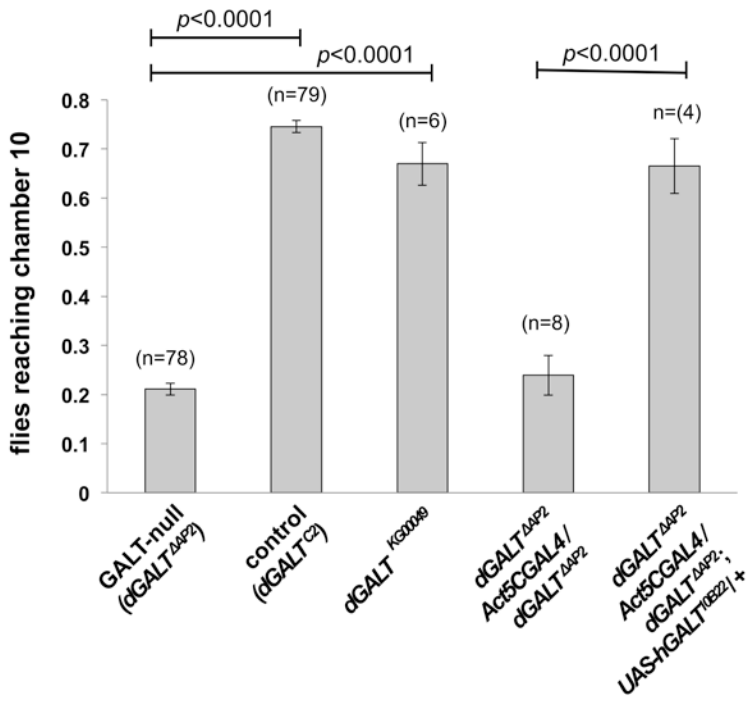

Figure 3.1: A movement abnormality in GALT-null *D. melanogaster*. The proportion of flies from each indicated cohort to reach the tenth (final) chamber of a countercurrent device is graphed. All values were modeled in a one-way ANOVA with a variable to control for day-to-day variations in testing. *dGALT*^{KG00049} carries a P-element insertion just upstream of the *dGALT* gene that does not interfere with GALT expression or activity. *dGALT*$^{\Delta AP2}$ is a nearly full deletion of the *dGALT* gene achieved by imprecise excision of the P-element from *dGALT*^{KG00049} (11). *dGALT*^{C2} is a wild-type allele generated by precise excision of the same P-element (11). In flies with endogenous GALT enzyme activity (*dGALT*^{C2} and *dGALT*^{KG00049}) more than 60% of each cohort reached the final chamber. Flies lacking GALT activity (*dGALT*$^{\Delta AP2}$ and *dGALT*$^{\Delta AP2}$ with *Actin5c-GAL4*) had difficulty with this behavior and on average only about 20% reached the final chamber. Expression of a human GALT transgene rescued the phenotype. Significant differences discussed in the text are indicated. n= number of cohorts tested.

countercurrent device in proportions comparable to those of the positive control (0.67 ± 0.04 vs. 0.75 ± 0.01, respectively).

We also tested flies that were compound heterozygotes for *dGALT*$^{\Delta AP2}$ and *Df(2L)Exel7027*, a deficiency that removes the entire *dGALT* gene (11). As expected, these animals progressed through the countercurrent device indistinguishably from *dGALT*$^{\Delta AP2}$ homozygotes (data not shown). Finally, we crossed both the *dGALT*$^{\Delta AP2}$ and *dGALT*^{C2} alleles into an Oregon-R background, crossed the resulting animals again to achieve homozygosity at the *dGALT* locus, and then tested the homozygotes using the countercurrent device; as in the w^{1118} background, the *dGALT*$^{\Delta AP2}$ Oregon-R homozygotes were significantly less proficient at progressing to the final chamber than were their *dGALT*^{C2} Oregon-R counterparts (data not shown).

To further test the connection between loss of GALT enzyme activity and the movement abnormality in *dGALT*$^{\Delta AP2}$ homozygotes we restored GALT activity in these animals using a human GALT transgene driven by the *GAL4/UAS* system. To achieve high level, nearly ubiquitous transgene expression we used the *Actin5cGAL4* driver. *Drosophila* homozygous for the *dGALT*$^{\Delta AP2}$ allele and carrying only the *Actin5cGAL4* driver without the *UAS-hGALT*^{10B22} human GALT transgene demonstrated a countercurrent result similar to that described above for *dGALT*$^{\Delta AP2}$ homozygotes (Figure 3.1). In contrast, *dGALT*$^{\Delta AP2}$ homozygotes carrying both the *Actin5cGAL4* driver and the *UAS-hGALT*^{10B22} *human GALT* transgene showed a dramatically improved outcome (Figure 3.1); this change was highly significant ($p<0.0001$).

GALT-null *Drosophila* demonstrate a defect in climbing but not startle response

Progression through a ten-tubed countercurrent device requires repeated climbing and also repeated startle response in the form of rapid recovery from being tapped to the bottom of a tube. The "countercurrent defect" evident in GALT-null *Drosophila* as compared with controls might therefore have reflected a defect in one ability, or the other, or both. To distinguish between these possibilities we subjected GALT-null and control flies to a "simple climbing" assay and also to a "tap recovery" assay. All animals tested in these assays were males ages 36 to 48 hours post-eclosion. To test climbing, cohorts of 9 to 11 flies were tapped to the bottom of a clear graduated cylinder and then allowed to climb; the number of flies that reached above a predetermined height by 20 seconds were counted and compared to the total number of flies in that cohort.

As with the countercurrent device, GALT null animals had difficulty with the climbing assay. The average proportion of $dGALT^{\Delta AP2}$ homozygotes that climbed above the predetermined height in 20 seconds was 0.256 ±0.068 while the corresponding number for $dGALT^{C2}$ homozygotes was 0.673 ±0.062 (Figure 3.2 panel A). Animals carrying the *Actin5cGAL4* driver without a *UAS-hGALT*^{10B22} transgene in the $dGALT^{\Delta AP2}$ background performed similarly to $dGALT^{\Delta AP2}$ homozygotes, with 0.263±0.058 of each cohort climbing above the mark in 20 seconds (Figure 3.2 panel A). Finally, expression of human GALT in the $dGALT^{\Delta AP2}$ background rescued this phenotype, with 0.519±0.049 of each rescued cohort climbing above the mark in 20 seconds (Figure 3.2 panel A).

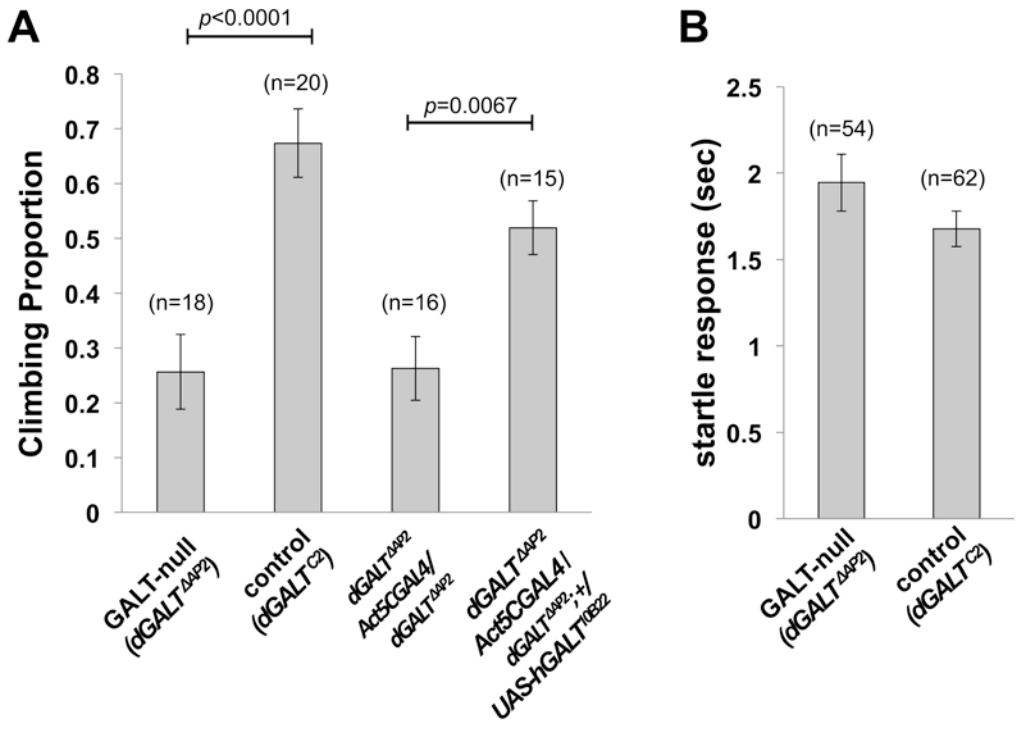

Figure 3.2: *GALT*-null *D. melanogaster* are defective in climbing but not startle response. (A) The proportion of flies in each indicated cohort that reached above a designated mark in 20 seconds is graphed. All values were modeled in a one-way ANOVA and mean and standard error are plotted. **(B)** The average number of seconds it took all of the flies in each cohort to regain a standing posture after vigorous agitation for 10 seconds. n= number of cohorts tested.

To test startle response we modified from a previously published assay (15). In brief, cohorts of 3-5 animals were subjected to vortex agitation in flat-bottom 25 mm diameter plastic vials at a fixed speed for 10 seconds and then observed. Under these conditions flies with a normal startle response take less than 15 seconds to stand upright (15). Repeated cohorts of both GALT-null and control animals subjected to this assay

were able to right themselves within one to three seconds (Figure 3.2 panel B); we saw no apparent startle response defect in any of the cohorts tested.

Relevant genotype	**GALT activity ± SEM (n)** [1] % wild-type
dGALT^{C2} homozygote	30.77 ± 2.29 (12) 100%
dGALT$^{\Delta AP2}$ homozygote	Not Detected (10) 0%
dGALT$^{\Delta AP2}$; *UAS-hGALT*^{10A11}/ +	0.7037 ± 0.2950 (5) 2.3%
dGALT$^{\Delta AP2}$; *UAS-hGALT*^{10A11} *homozygote*	1.995 ± 0.429 (4) 6.5%
dGALT$^{\Delta AP2}$ / *dGALT*^{C2}	25.39 ± 3.88 (3) 82.5%
dGALT$^{\Delta AP2}$ *Act5cGAL4* / *dGALT*$^{\Delta AP2}$*;* *UAS-hGALT*^{10B22}/ +	395.7 ± 85.5 (4) 1286%
dGALT^{C2} */Act5cGAL4* ; *UAS-hGALT*^{10B22}/ +	618.97 ± 259.57 (5) 2017%

Table 3.1: GALT enzyme activity levels detected in adult flies. Activities were measured in lysates prepared from adult flies of the indicated genotypes as described in Methods. .[1]Enzyme activity was expressed as pmol/ µg/ min.

Relationship between GALT activity level and severity of the movement abnormality

To test whether trace GALT activity might modify long-term outcome severity we explored the relationship between GALT activity and the movement abnormality revealed by the countercurrent device. The lowest levels of GALT activity were achieved using a "leaky" human GALT transgene, *UAS-hGALT*^{10A11}, that expressed low levels of GALT despite the absence of a *GAL4* driver. In a *dGALT*$^{\Delta AP2}$ (endogenous GALT-null) background, animals carrying one allele of *UAS-hGALT*^{10A11} demonstrated about 2.5% wild-type GALT activity; animals carrying two alleles demonstrated just over 6% (Table 3.1). Intermediate GALT activity was achieved using animals heterozygous for one allele of the GALT deletion, *dGALT*$^{\Delta AP2}$, and one control allele, *dGALT*^{C2}; these animals demonstrated about 82% wild-type GALT activity (Table 3.1). Finally, GALT over-expression was achieved using a *UAS*-human *GALT* transgene coupled with a strong, ubiquitous driver (*Act5cGAL4*) in either an endogenous GALT-null (*dGALT*$^{\Delta AP2}$) or a control (*dGALT*^{C2}) background; both genotypes exhibited a dramatic excess of GALT activity (Table 3.1).

Phenotypic analyses of animals representing these different genotypes revealed a very steep relationship between GALT activity and outcome at the lowest levels of GALT activity. Animals expressing as little as about 2.5% or 6.5% wild-type GALT activity demonstrated significant phenotypic rescue ($p<0.0001$, Figure 3.3). This relationship leveled off asymptotically as GALT activity approached or exceeded the carrier level so that there was no marked difference in outcome between GALT heterozygotes, wild-type animals, and animals expressing >10-fold excess GALT activity (Figure 3.3). Indeed, flies expressing a human *GALT* transgene on top of an intact

endogenous *dGALT* gene demonstrated close to 20-fold excess GALT activity (Table 3.1), and yet even these animals exhibited "normal" progression through the countercurrent device (0.79 +/- 0.05 reached the tenth chamber, data not illustrated in

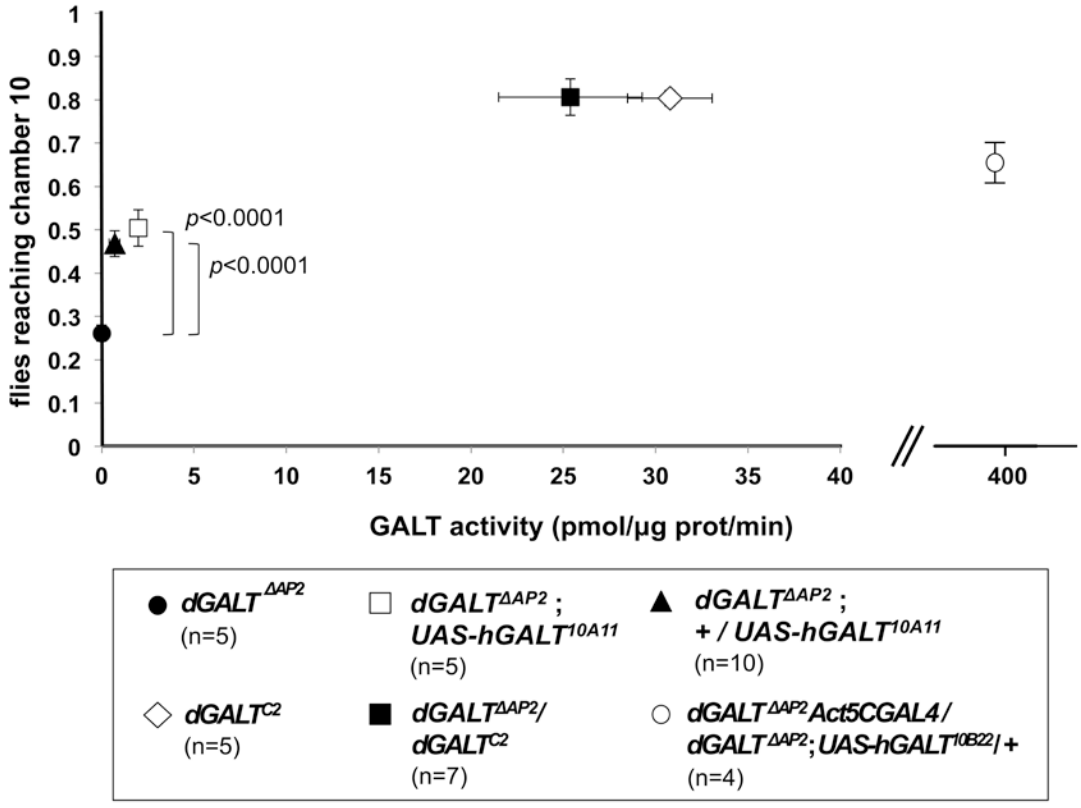

Figure 3.3: Relationship between GALT activity and a movement abnormality in *D. melanogaster*. The ability of animals of each indicated genotype to reach the tenth chamber of a countercurrent device is plotted as a function of their adult GALT enzymatic activity. As GALT activity increased from null toward wild-type levels an animal's ability to reach the tenth chamber improved. There was no apparent impact from GALT over-expression. All values were modeled in a one-way ANOVA with a variable to control for day-to-day variations in testing. All values graphed are mean and standard error.

Figure 3.3). These results demonstrate the importance of even trace GALT activity on long-term outcome, and also confirm that in flies as in humans, long-term outcome in galactosemia, like acute outcome, is recessive.

Impact of sub-lethal dietary galactose exposure in development and gal-1P accumulation on severity of the movement abnormality

Previously, we have demonstrated that GALT-null Drosophila raised in the absence of dietary galactose exhibit a movement abnormality as adults (11), but that observation left open the question of whether dietary exposure to low, sub-lethal levels of galactose during development might exacerbate the phenotype. This is an important question considering the clinical parallels. To address this question we tested the outcome severity of both GALT-null (dGALTΔAP2 homozygotes) and control (dGALTC2 homozygotes) flies reared on food containing either glucose as the sole monosaccharide or both glucose and a small amount of galactose. The level of galactose used in these experiments (50 mM) represents <10% of the monosaccharide in the fly food, and we have previously demonstrated that this level of galactose causes no survival loss in GALT-null larvae (data not shown). All animals to be tested in the countercurrent device were switched to glucose-only food upon eclosion so that dietary galactose exposure was limited to the larval period.

Countercurrent analyses of all four categories of flies – those with and without GALT, and with and without larval exposure to dietary galactose – reconfirmed that GALT-null flies have a movement abnormality revealed by the countercurrent device, but also that early galactose exposure has no apparent impact on that phenotype (Figure 4 panel A).

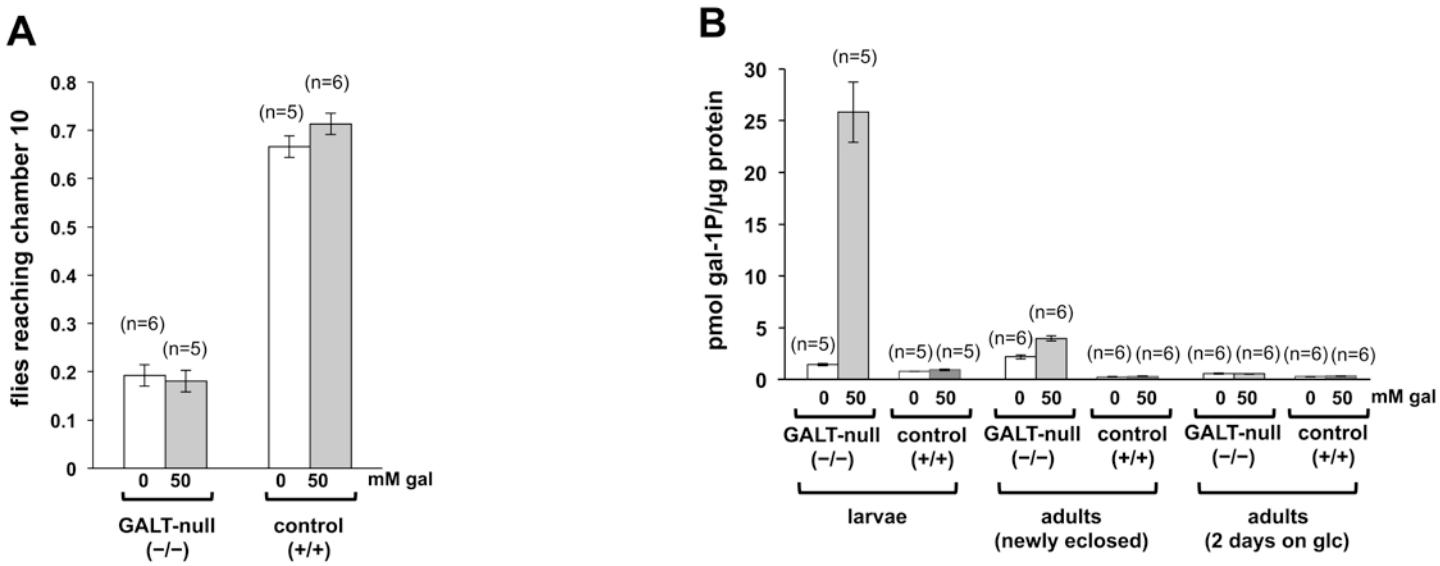

Figure 3.4: Low-level galactose exposure during development has no impact on a movement abnormality in adult *GALT*-null *D. melanogaster*. **(A)** GALT-null and control animals were tested in the countercurrent device after being raised on a diet of food containing 555 mM glucose as the sole monosaccharide (open bars), or 555 mM glucose supplemented with 50 mM galactose (shaded bars). **(B)** Gal-1P accumulation in GALT-null and control larvae, newly eclosed adults, or adults transferred following eclosion to glucose-only food for two days prior to analysis. As described in **(A)**, some animals were exposed during development to food containing glucose as the sole monosaccharide (open bars), while others were exposed to food containing glucose spiked with 50 mM galactose (shaded bars).Late-stage larvae and adult animals were collected and analyzed as described in Methods. Gal-1P levels were standardized to protein concentration.

To test whether exposure to galactose at this low level has *any* impact on the GALT-null *Drosophila,* we characterized the gal-1P levels in late stage larvae, both GALT-null and control, each harvested after seven days of life on food containing either the standard level of glucose (555 mM), or that level of glucose supplemented with 50 mM galactose. Our results (Figure 3.4 panel B) confirmed that GALT-null larvae exposed to 50 mM galactose accumulate very high levels of gal-1P, while control larvae do not.

Finally, to ask whether the movement abnormality observed in GALT-null adult flies might reflect a continued presence of high gal-1P in these animals we also measured gal-1P in newly eclosed adults and in adults transferred to glucose-only food for 48 hours before analysis. As illustrated in Figure 3.4 panel B, gal-1P levels remained marginally elevated in newly eclosed GALT-null flies exposed to galactose during development, but after 48 hours on food lacking galactose this gal-1P had fallen essentially to baseline. These data confirm that by the time adult GALT-null flies were tested in the countercurrent device they no longer harbored high levels of gal-1P, regardless of whether or not they had been exposed to galactose as larvae.

Impact of age on the movement abnormality in GALT-null *Drosophila*

Climbing behavior in normal adult *Drosophila melanogaster* slows as a function of age, largely due to decreased climbing speed (16). To test the impact of age on the movement phenotype of GALT-null *Drosophila* we collected newly eclosed mutant and control males and allowed them to age, maintained at 25°C under non-overcrowding conditions, for an additional 7 or 14 days before subjecting the different cohorts to the countercurrent assay. The results were striking (Figure 3.5). As expected, the control

animals demonstrated a slow, progressive loss of ability to navigate the countercurrent device, such that at 2 days of age >70% of the animals reached the 10th tube, at 9 days of age only about 50% reached the 10th tube, and at 16 days of age fewer than 30% reached the 10th tube. In contrast, the GALT-null animals demonstrated a loss of ability that was both accelerated and profound, such that at 2 days of age about 20% reached the 10th tube, and at 9 or 16 days of age <5% reached the 10th tube. In terms of raw numbers of flies that lost the ability to reach the 10th tube between 2 and 9 days of age, the GALT-null and control flies showed similar losses. However, in relative terms the losses were markedly different; the control animals suffered less than a 30% loss, while the mutants suffered a >4-fold loss. In short, aging the animals by one or two weeks prior to testing greatly widened the outcome gap between mutants and controls.

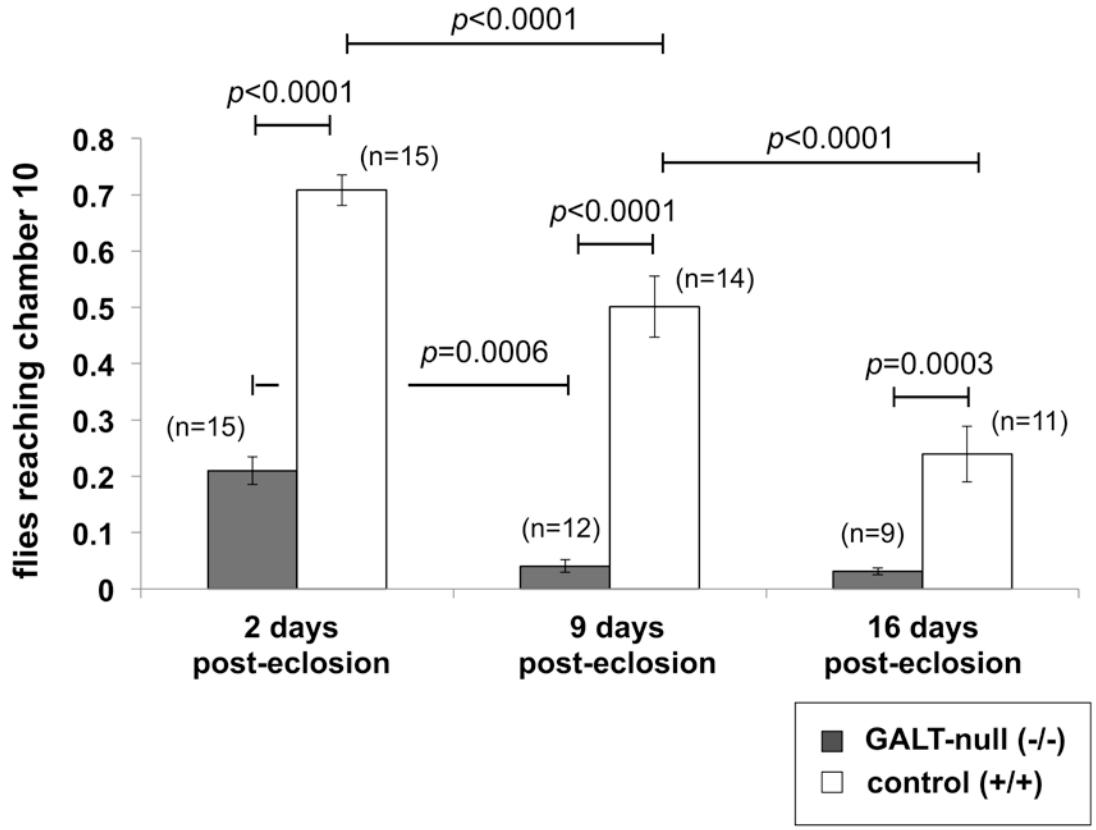

Figure 3.5: Impact of age on movement in adult *GALT*-null *D. melanogaster*. GALT-null (shaded bars) and control flies (open bars) were tested using the countercurrent device at ages 2 days, 9 days, and 16 days post-eclosion. While the control flies demonstrated a gradual, progressive decline with age the GALT-null flies demonstrated a decline that was notably accelerated and profound.

Microscopy reveals no clear anatomical defects in adult GALT-null fly brain or muscle

Considering the nature of the movement abnormality we hypothesized that GALT-null flies might have an anatomical defect visible in brain or muscle, and so performed the following histological studies. Sections from paraffin-embedded adult male animals, 24 to 48 hours post-eclosion, were stained with hematoxylin and eosin to

visualize overall anatomy and tissue integrity. Histological examination of the brain demonstrated normal configuration of major brain structures (Figure 3.6, panel A, top). The cortex, which contains cell bodies of neurons and glia, was well preserved in GALT-null animals (Figure 3.6, panel A, top, arrows) as was the neuropil (Figure 3.6, panel A, top, asterisks). Vacuoles (Figure 3.6, panel A, top, arrowheads), which often accompany neurodegeneration in *Drosophila*, were modest in size and number and were present in both mutants and controls with equivalent frequencies. Histological examination of indirect flight muscle similarly revealed overall normal structure with no clear indication of malformation or degeneration (Figure 3.6 panel A, bottom).

To probe brain structures further we performed immunostaining for well-characterized markers of mitochondria (ATP synthase, Figure 3.6 panel B), synapses (synapsin (SYN) and the vesicular glutamate transporter (VGLUT)), and axons (futsch) (Figure 3.6, panel C). No clear abnormalities were evident in the GALT-null animals. Finally, we repeated these studies on control and GALT-null flies that had been aged for one or two weeks following eclosion; again no clear differences were detected (data not shown)

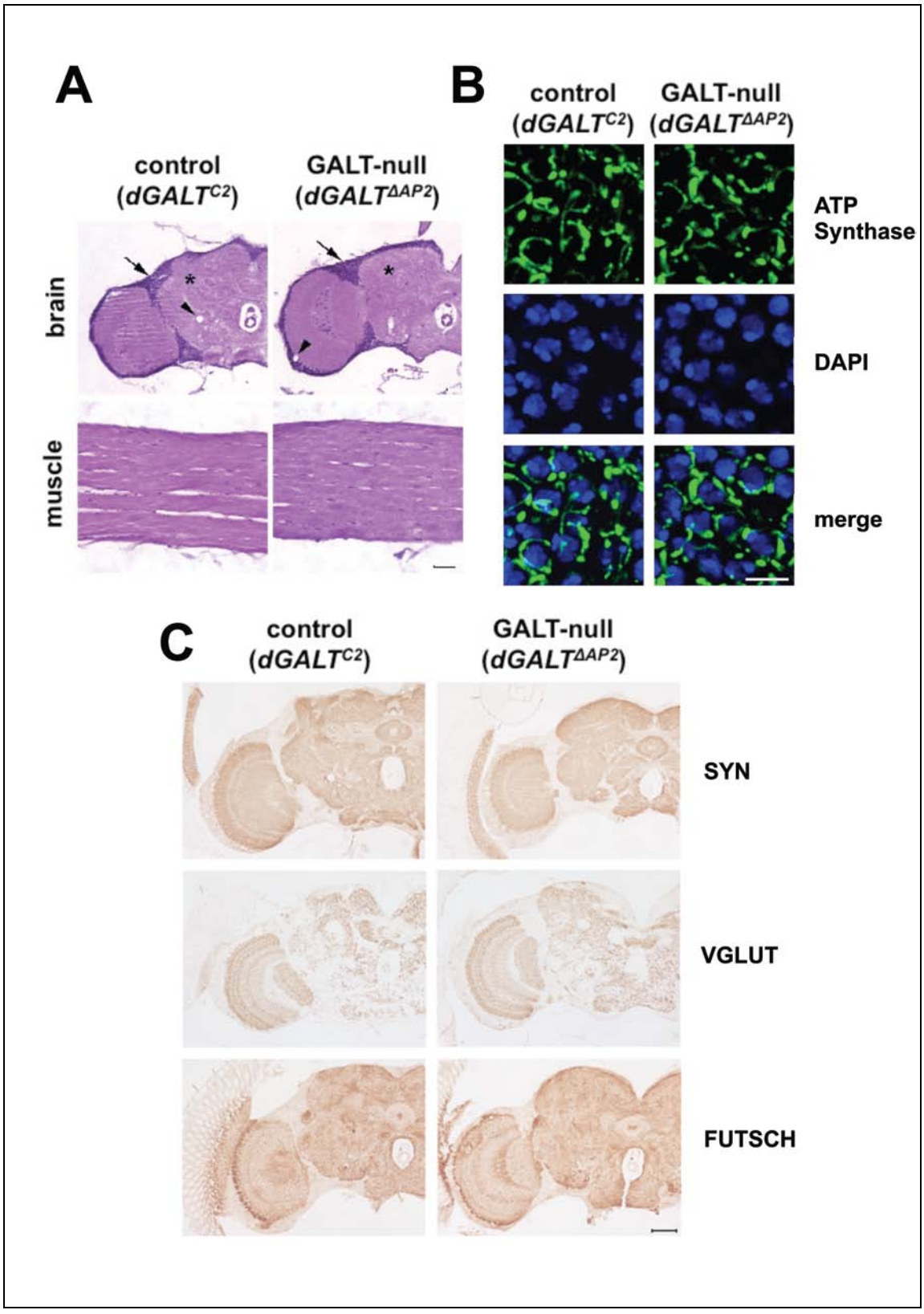
A
control
(dGALT^C2)
GALT-null
(dGALT^ΔAP2)
brain
muscle
B
control
(dGALT^C2)
GALT-null
(dGALT^ΔAP2)
ATP
Synthase
DAPI
merge
C
control
(dGALT^C2)
GALT-null
(dGALT^ΔAP2)
SYN
VGLUT
FUTSCH

Figure 3.6: GALT-null *D. melanogaster* show no apparent morphological defects in brain or muscle. (A) Representative images of adult brain (upper panels) and muscle tissue (lower panels) from GALT-null and control animals are presented. Arrows in the brain images point to cortex, arrowheads point to vacuoles, and the asterisk (*) indicates normal neuropil. There were no apparent differences in gross anatomical structures between the GALT-null and control animals in either tissue. Scale bar is 20 microns. **(B)** Representative immunofluorescence stains for mitochondria using an antibody directed to ATP synthase reveals no clear abnormalities of mitochondrial structure or number in GALT-null neurons. A DAPI counterstain was used to stain nuclei. Scale bar is 5 microns. **(C)** Representative immunohistochemical stains for synapsin (SYN), the vesicular glutamate transporter (VGLUT) and the microtubule binding protein futsch revealed no clear defects in GALT-null animals. Scale bar is 40 microns.

3.5 DISCUSSION

Effective newborn screening coupled with prompt and rigorous dietary restriction of galactose prevents or resolves the acute and potentially lethal sequelae of classic galactosemia but does little, if anything, to prevent the long-term complications of the disease. Our goal is to understand the fundamental bases of long-term complications in galactosemia in hopes that this knowledge will lead to improved options for prognosis and intervention. In the work reported here we have applied a *Drosophila melanogaster* model of classic galactosemia to begin defining the genetic and environmental factors that modify long-term outcome in GALT deficiency. Of note, *Drosophila* is the only animal model reported to date that recapitulates aspects of either the acute or long-term complications of classic galactosemia (11).

Previously we reported that GALT-null *Drosophila* adults exhibit a movement defect despite being raised on food with no added galactose (11). Here we have extended from that result in five important ways.

First, we tested whether the “countercurrent” abnormality reported earlier reflects a defect in climbing or in startle response. This is an important question because of implications for mechanism. The answer was climbing, not startle response.

Second, we asked whether trace levels of GALT activity might impact severity of the movement defect. The answer was “yes;” about 2.5% wild-type GALT activity was sufficient to rescue most of the movement defect. This is an important result that parallels the clinical experience.

Third, we tested whether low-level galactose exposure in development, and the elevated gal-1P values that result, would impact severity of the movement defect in

GALT-null adult flies. In our earlier report we demonstrated that the countercurrent defect occurred despite complete dietary galactose restriction, but we did not test whether cryptic galactose exposure might make the phenotype more severe (11). Here we demonstrate that exposure to 50 mM galactose, which causes no significant increase in mortality despite a >20-fold increase in gal-1P accumulation, does *not* exacerbate the movement defect. This result challenges the idea that accumulated gal-1P leads to long-term complications in GALT deficiency.

Fourth, we tested the impact of age on the movement phenotype and noted a marked difference between controls and GALT-null animals; where the decline with age for controls was gradual and progressive, for GALT-null flies it was rapid and profound. This result suggests that physiological changes associated with aging overlap with the pathways that underlie the climbing defect in GALT-null flies.

Finally, careful light microscopic studies of brain and muscle in *GALT*-null and control flies collected at three different ages revealed no clear morphological differences. While this result cannot rule out the existence of morphological defects that are subtle or tissue-specific, or evident only at a specific developmental stage, at face value it strengthens the argument that the defect in the mutants might be physiological rather than anatomical. Simply put, our current understanding is that a primary defect in biochemistry (GALT-deficiency) leads to physiological changes that are either localized or systemic, and that these physiological changes ultimately lead to impaired neuronal or neuromuscular function and a movement abnormality in the mutant flies.

Combined these data add substantially to our knowledge of long-term outcomes in GALT-null *Drosophila,* better characterizing the nature of the movement defect and also using it as a tool to begin exploring mechanism. These results further establish the utility of the fly model system for studies of long-term outcome in galactosemia, setting the stage for future work to define other outcomes and the genetic and/or environmental factors that underlie and modify those outcomes.

Acknowledgments

We are grateful to members of the Fridovich-Keil, Moberg, and Sanyal laboratories at Emory University for many helpful discussions.

Funding

This work was supported in part by National Institutes of Health grant DK046403 (to JLFK); ER was supported in part by NIH Training Grants T32 MH087977, TL1 RR025010 and T32 GM008367.

Author contributions

EL Ryan performed all experiments except those illustrated in Figure 3.6; MB Feany and B DuBoff performed the microscopy illustrated in Figure 3.6. JL Fridovich-Keil conceived of and directed the project. All authors contributed to writing and editing the manuscript.

3.6 REFERENCES

1. Fridovich-Keil JL & Walter JH (2008) Galactosemia. *The Online Metabolic & Molecular Bases of Inherited Disease*, eds Valle D, Beaudet A, Vogelstein B, Kinzler K, Antonarakis S, & Ballabio A (McGraw Hill), p http://www.ommbid.com/.
2. Beutler E, Baluda MC, Sturgeon P, & Day R (1965) A New Genetic Abnormality Resulting in Galactose-1-Phosphate Uridyltransferase Deficiency. *Lancet* 1(7381):353-354.
3. Mellman WJ & Tedesco TA (1965) An improved assay of erythrocyte and leukocyte galactose-1-phosphate uridyl transferase: stabilization of the enzyme by a thiol protective reagent. *J Lab Clin Med* 66(6):980-986.
4. Komrower GM (1983) Clouds over galactosemia. *Lancet* 1(8317):190.
5. Gitzelmann R & Steinmann B (1984) Galactosemia: how does long-term treatment change the outcome? *Enzyme* 32(1):37-46.
6. Kaufman FR, Xu YK, Ng WG, & Donnell GN (1988) Correlation of ovarian function with galactose-1-phosphate uridyl transferase levels in galactosemia. *J Pediatr* 112(5):754-756.
7. Segal S (1989) Disorders of Galactose Metabolism. *the Metabolic Basis of Inherited Disease*, eds Scriver D, Beaudet A, Sly W, & Valle D), 6th Ed, pp 453-480.
8. Waggoner DD, Buist NR, & Donnell GN (1990) Long-term prognosis in galactosaemia: results of a survey of 350 cases. *J Inherit Metab Dis* 13(6):802-818.

9. Waisbren S, *et al.* (2012) The adult galactosemic phenotype. *J Inherit Metab Dis* 35(2):279-286.

10. Leslie ND, Yager KL, McNamara PD, & Segal S (1996) A mouse model of galactose-1-phosphate uridyl transferase deficiency. *Biochem Mol Med* 59(1):7-12.

11. Kushner RF, *et al.* (2010) A Drosophila melanogaster model of classic galactosemia. *Dis Model Mech* 3(9-10):618-627.

12. Sanders RD, Sefton JM, Moberg KH, & Fridovich-Keil JL (2010) UDP-galactose 4' epimerase (GALE) is essential for development of Drosophila melanogaster. *Dis Model Mech* 3(9-10):628-638.

13. Benzer S (1967) BEHAVIORAL MUTANTS OF Drosophila ISOLATED BY COUNTERCURRENT DISTRIBUTION. *Proc Natl Acad Sci U S A* 58(3):1112-1119.

14. Akai S (1979) Genetic variation in walking ability of Drosophila melanogaster. *Jpn J. Genet.* 54:317-324.

15. Ganetzky B & Wu CF (1982) Indirect Suppression Involving Behavioral Mutants with Altered Nerve Excitability in DROSOPHILA MELANOGASTER. *Genetics* 100(4):597-614.

16. Rhodenizera D, Martina I, Bhandaria P, Pletcherb S, & Grotewiel M (2008) Genetic and environmental factors impact age-related impairment of negative geotaxis in Drosophila by altering age-dependent climbing speed. *Experimental Gerontology* 43(8):739–748.

.

Chapter 4

CRYPTIC RESIDUAL GALT ACTIVITY IS A MODIFIER OF OUTCOME IN SCHOOL AGE CHILDREN WITH CLASSIC GALACTOSEMIA

Emily L. Ryan, Mary Ellen Lynch, Elles Taddeo, Tyler J. Gleason, Michael P. Epstein, and Judith L. Fridovich-Keil

4.1 ABSTRACT

Classic galactosemia is a potentially lethal disorder that results from profound deficiency of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme in the Leloir pathway of galactose metabolism. Although early diagnosis and rigorous dietary restriction of galactose prevent or resolve the potentially lethal acute symptoms, patients are at markedly increased risk of long term complications including significant cognitive, speech, and behavioral difficulties, among other problems. The mechanisms that underlie these long term complications remain unclear, as do the factors that modify their severity. Here we explored the scholastic and behavioral outcomes experienced by a cohort of 54 school age children with classic galactosemia. Data collected included survey responses from parents and teachers, school records including standardized test scores, and GALT genotype data used to estimate predicted residual GALT activity based on a yeast expression system. As expected, many but not all of the children in our study demonstrated speech, scholastic, and behavioral difficulties. Perhaps most striking, we found that predicted cryptic residual GALT activity, often below the threshold of detection of clinical assays, appeared to modify behavioral outcome severity. These data raise the intriguing possibility that cryptic GALT activity might also influence the severity of other long term complications in classic galactosemia.

4.2 INTRODUCTION

Classic galactosemia (OMIM 230400) is a potentially lethal genetic disorder that impacts more than 1/60,000 newborns and results from profound deficiency of galactose-1P uridylyltransferase (GALT, E.C. 2.7.7.12), the middle enzyme in the Leloir pathway of galactose metabolism (reviewed in (1)). In many countries, infants with classic galactosemia are identified by population newborn screening, which enables early diagnosis and life-saving intervention in the form of dietary restriction of galactose. Unfortunately, despite even pre-symptomatic diagnosis and rigorous dietary modification, many affected infants grow to experience significant long-term complications (e.g. (2), reviewed in (1)). Cognitive, speech, and behavioral disabilities are among the most prevalent long term complications in classic galactosemia, impacting between 30% and 80% of all children and adults (2-11).

Cognitive outcomes:

Measures of cognitive development and function, such as Intelligence Quotient (IQ) for children and adults with classic galactosemia range from high normal in some, to low normal in many, to significantly impaired in some. Waggoner and colleagues reported that of the 177 children in their study ≥6 years old, 45% were considered developmentally delayed (11). Studies of teens and adults also report evidence of long term cognitive disability: Hoffmann and colleagues (12) found a mean IQ of 76.2 ± 14.8 in their cohort of 32 patients in Germany, and an independent study of 20 volunteers in Britain found a mean IQ in the mid to high 80s (4); mean IQ in the general population is 100. Deficits in executive, adaptive, and visuoperceptual function for patients with classic galactosemia have also been reported (4, 13), as have deficits in working memory (3). Of

note, although some early studies suggested that cognitive disability in galactosemia worsens with age (8, 11, 14, 15), more recent studies dispute that conclusion (3, 4, 16).

A number of prior studies have also identified brain structural differences in patients with classic galactosemia. For example, white matter abnormalities were reported from brain magnetic resonance imaging (MRI) studies of pediatric and adult patients on galactose restricted diets (6, 8, 17), and positron emission tomography (PET) scans of patients revealed metabolic differences in regions of the brain associated with both motor and cognitive function (18). Whether these structural or metabolic differences underlie the functional deficits reported, or are a result of the same cause, but not themselves causal, remains unclear.

One question about cognitive outcome in classic galactosemia that has been addressed thoroughly is whether exposure to high levels of dietary galactose in infancy causes the developmental delay or long term cognitive disability experienced later in life; the answer is no. This conclusion was reached both from studies of outcome among unrelated children diagnosed at different ages (11) and also from comparisons of outcome severity among affected siblings (6, 11). The sibling comparisons reported by Waggoner (11) and Hughes (6) from studies conducted almost 20 years apart, and on different continents, are particularly meaningful because, by definition, the sibling sets were matched for GALT genotype and, since they were raised by the same parents in the same household, were also matched for many other significant environmental influences. Both studies found that older siblings, who were exposed to milk and became clinically ill as infants before dietary galactose-restriction, fared no worse, long term, than did their younger siblings who never consumed milk; some of these were even born to mothers

who abstained from milk during pregnancy. This apparent independence of long term outcome from milk exposure in infancy is also consistent with the apparent independence of long term outcome severity from hemolysate gal-1P values, either at diagnosis, or later in childhood (11).

Another proposed modifier of cognitive outcome in galactosemia that has been addressed by multiple studies, sometimes with contradictory results, is GALT genotype (10, 13, 19). Most studies have examined whether homozygosity for the common Q188R allele correlates with outcome severity. Some studies have supported this viewpoint (10) while others have not (13). The relatively small sizes of the cohorts studied, combined with differences in the identity and prevalence of the non-Q188R alleles in the cohorts, may explain the apparent disparity. Of note, even sib-pairs who have the same GALT genotype may be discordant for cognitive or behavioral outcomes (6, 11), reinforcing the idea that unknown modifiers, some likely genetic and others perhaps environmental, contribute to the severity of these outcomes.

Speech and language outcomes:

Speech/language disorders have been reported in close to 50% of all galactosemic patients (11, 20-25) and are disproportionately common among galactosemic children with borderline to low cognitive development (24). Even galactosemic children with typical cognitive function are at increased risk for speech/language difficulties (24), although these children more often demonstrate an expressive language disorder, whereas galactosemic children with borderline or low cognitive development are more likely to demonstrate a mixed receptive expressive language disorder (24). Of note, children with galactosemia who demonstrate a speech disorder are four to six times more likely than

non-galactosemic children who demonstrate a speech disorder also to demonstrate language impairment (24).

Behavioral outcomes:

Behavioral or social disturbances that negatively impact quality of life are also frequent among both children and adults with classic galactosemia (3, 7, 11, 13). For younger and school-age children behavior problems may include difficulty sitting still, difficulty paying attention, difficulty getting along with others, and/or a tendency to be victims of bullying (26). Among adults, some of these same challenges hold, and in addition Waisbren and colleagues (13) found that 39% of the adults in their study had experienced depression and more than half reported heightened anxiety. Whether these behaviors or psycho-social difficulties experienced by children and adults with classic galactosemia are secondary to cognitive or other complications, or occur as an independent outcome of GALT-deficiency, remains unclear.

The goal of the study reported here was to characterize the nature of scholastic and behavioral outcomes experienced by school age children with classic galactosemia and to explore the impact of cryptic GALT activity as a candidate modifier of this outcome. We therefore recruited 54 volunteers with classic galactosemia ranging in age from 4 to 18 years and gathered scholastic and behavioral outcome information about those volunteers using established survey instruments completed by parents and teachers, and school records including standardized test results. We also gathered GALT genotype data and from that predicted the GALT activity level in most volunteers. The results of this study demonstrated that children with classic galactosemia exhibit a variety of behavioral difficulties noted by both parents and teachers; many of the concerning

behaviors were classified as indicative of internalizing rather than externalizing problems. Finally, we observed a statistically-significant association between predicted GALT activity and social skills, and also noted trends between predicted GALT activity and other outcomes that, while not statistically significant, were intriguing and merit follow up using larger samples with improved power to detect significant associations. These results both confirm prior reports and extend from prior conclusions to implicate cryptic residual GALT activity as a potential modifier of long term outcome in classic galactosemia.

4.2 METHODS

Study volunteers

Fifty-four study volunteers (25 boys and 29 girls) between the ages of 4 and 18 years old were recruited by self-referral through the Galactosemia Foundation (www.galactosemia.org) or by referral from a health care professional who treats patients with galactosemia. Our recruitment strategy did not allow us to calculate a response rate as we do not know how many eligible families saw our advertisements or heard about the study. All volunteers had been diagnosed previously with classic galactosemia, either by a clinical red blood cell GALT assay or by a recognized *GALT* genotype. This study was reviewed and approved as part of Protocol eIRB00024933 (formerly protocol 619-99, PI: JL Fridovich-Keil) by the Emory University Institutional Review Board. Informed consent, assent (where appropriate), and authorization to contact teachers and access school records were obtained for each volunteer.

Behavioral Assessments

We asked the parents of each study volunteer to provide the names of one or two teachers or other adults with whom their galactosemic child interacted on a regular basis in a scholastic setting (e.g. a classroom teacher, an instructional aide, or a special education tutor). Following written authorization to contact these individuals we sent hard copy surveys, together with an explanatory cover letter and a large self-addressed, stamped return envelope, to the parents and to the designated teachers. Parents received the age-appropriate child behavior checklist (CBCL for ages 1½ to 5 years, or for ages 6-18 years) from the Achenbach System of Empirically Based Assessment (ASEBA) and also the Social Skills improvement System (SSIS) parent form (27-29). Each teacher

received an age-appropriate (as above) Teacher Report Form (TRF from ASEBA) and also the SSIS teacher form. For the 54 children enrolled in this study we received completed parental surveys for 53, and we also received a total of 62 completed teacher surveys for 45 children; the data set includes two teacher observations for 17 of the children. Survey respondents each received a small gift card ($20) in recognition of their time spent taking the surveys.

Scholastic outcome measures

We attempted to collect school records for each study volunteer. Some of these records, such as standardized test scores, report cards, and IEP records, were provided by parents from their files; others came from teachers or other school officials following written authorization by the parents. We were not able to obtain all records for every volunteer. For example, essentially all of our youngest study volunteers were missing standardized test scores because most schools do not begin standardized testing until second or third grade. Nonetheless, the information we were able to gather was substantial, and where necessary we dealt with the problem of missing information in statistical analyses by using permutations to predict probabilities rather than a normal distribution to set significance (see below).

Survey respondents for each study volunteer were asked to send a copy of relevant state or national standardized test results from the current or prior year. We also asked whether the child was currently receiving speech therapy or any other form of special educational support in the classroom. Respondents for students who received special classroom assistance were asked to send any available files that might explain the reason why the child was receiving special assistance, the level of assistance provided,

and any progress in the classroom (i.e. Individual Educational Plans, IEP). From this information we scored each study volunteer as either achieving at their grade level, or not achieving at their grade level. Those students who did not take standardized tests were omitted from this analysis unless other clear documentation was received indicating that the child was excused from standardized testing because their achievement status was so low as to preclude testing. Of the 54 volunteers enrolled in our study, we received sufficient information for 33 to designate each as "achieves as grade level" or "achieves below grade level," and also to understand what form of special classroom support, if any, they received.

GALT genotype analysis and predicted GALT activity level

We collected medical history information for each study volunteer, including the information upon which the diagnosis of classic galactosemia was originally based. Some volunteers had documentation of *GALT* genotype analysis performed as part of the diagnostic work-up. For volunteers whose *GALT* genotype information was either missing or incomplete (e.g. one or both alleles were designated as "unknown") we determined *GALT* genotype by sequence analysis of DNA obtained from a blood or saliva sample, as described elsewhere (Gleason et al, in preparation).

All missense and nonsense mutations in the *GALT* open reading frame were assessed for residual GALT activity using our previously described yeast expression system for the human enzyme (30, 31). Volunteers with one or more *GALT* alleles that carried only non-coding sequence variants were excluded from analysis. Finally, a single "predicted GALT activity" value was calculated for each volunteer by averaging the predicted activity levels of the two *GALT* alleles identified in that individual.

Statistical analyses

To examine the relationship between the outcomes of interest and GALT activity, we applied appropriate regression procedures that adjusted for the effects of age, gender, speech therapy, and special education status. Of these four covariates, only speech therapy was significantly associated with the various outcomes ($p<0.05$) so all other covariates were dropped to maintain parsimony. For dichotomous outcomes, such as achievement in math and/or language arts, we used logistic regression for inference. For the other continuous outcomes (internalizing problems, externalizing problems, adaptive functioning, total competence, problem behaviors, social skills), we used linear regression when analyzing data from parents and used generalized-estimating-equation (GEE) models when analyzing data from teachers (to account for the correlation in observations that arises when the same student has reports from multiple teachers). For each test performed, we assumed a one-sided alternative hypothesis based on prior belief of expected trends based on independent literature. To account for multiple testing, we used permutation-based inference to establish significance. For analyses of behavior and social outcomes based on teacher evaluations, we conducted permutations in a manner that preserved within-subject correlation of outcomes due to multiple teacher observations. All analyses were performed using the R programming language.

4.3 RESULTS

This section describes characteristics of our study population and presents data collected regarding the academic achievement and behavioral outcomes of these children. Due to the limited size of our study cohort we were only able to apply statistical analyses to a small subset of the questions addressed.

Characteristics of the study population

To explore the scholastic and behavioral outcomes of school age children with classic galactosemia we recruited 54 study volunteers, ages 4 to 18 years, from across the United States and Canada, and collected school records and survey responses (explained below) from parents and up to two teachers for each child. Most participants were from Caucasian families, although a few were of mixed heritage, and most were of middle or upper-middle class socioeconomic status. Most families self-referred from a family support group for galactosemia, or were referred by a health care specialist who sees patients with galactosemia.

The children were well distributed with regard to gender (25 males and 29 females) and age, though older teens were slightly under-represented (Table 4.1). Most volunteers (38/54) were enrolled in public school, though most of the youngest children (9/12) were enrolled in private school, and four children were homeschooled (Table 4.1). Of the 54 volunteers in this study, we were able to definitively classify the level of educational support for 33, and of those, 17 received some degree of formalized special education. The level of support received by different children in this group varied widely, however. Only one child spent the entire school day in a special education classroom. Five additional children received all-day support, but that extra support was provided

either full-time by aides in a regular education classroom, or by aides part-time in a regular education classroom and part-time in a resource classroom. For the remaining 11 students who received special educational support, the services received were part-time and only in specific subjects. Some of these students remained in the regular classroom with an aide, while others relocated to a special classroom for less than 30% of the school day.

Finally, we classified each volunteer with regard to whether or not they received speech therapy at the time of the study. Other studies have indicated that a large percentage of school age children with classic galactosemia receive speech therapy (24); our population was no exception, especially in the younger age group (Table 4.1).

Outcomes explored in this study

In this section we present results obtained from a review of school records and parent and teacher survey responses for each of the study volunteers. These data are presented in a descriptive format because the limited sample size restricted the number of hypotheses we could test in this dataset without incurring an overwhelming multiple-testing burden.

	Overall	M:F ratio	4-6 yr olds	7-10 yr olds	11-14 yr olds	15-18 yr olds
Study total:	54	25:29	12	14	19	9
School Environment						
Public:	**70.4%** (38/54)	17:21	**16.7%** (2/12)	**71.4%** (10/14)	**89.5%** (17/19)	**100%** (9/9)
Private:	**22.2%** (12/54)	7:5	**75.0%** (9/12)	**21.4%** (3/14)	**0.0%** (0/19)	**0.0%** (0/9)
Other:	**7.4%** (4/54)	1:3	**8.3%** (1/12)	**7.1%** (1/14)	**10.5%** (2/19)	**0.0%** (0/9)
Achievement[1]						
Math:	**47.2%** (17/36)	8:9	NA	**36.4%** (4/11)	**52.9%** (9/17)	**50.0%** (4/8)
Language Arts:	**52.8%** (19/36)	7:12	NA	**63.6%** (7/11)	**47.1%** (8/17)	**50.0%** (4/8)
Special Educational Services						
Special Education Classes[2]:	**51.5%** (17/33)	5:12	**20.0%** (1/5)	**50.0%** (4/8)	**71.4%** (10/14)	**33.3%** (2/6)
Speech Therapy[3]:	**47.1%** (24/51)	10:14	**70.0%** (7/10)	**42.9%** (6/14)	**55.6%** (10/18)	**11.1%** (1/9)

Table 4.1. Demographics of the 54 children with classic galactosemia who participated in this study with regard to age, gender, school environment, scholastic achievement, and enrollment in special education classes or speech therapy: [1]Achievement is presented as the percentile (and fraction) of volunteers in that group who met established achievement standards in either mathematics or language arts/English subjects. [2]Percentile (and fraction) of volunteers in that group who were enrolled in special education classes at the time surveyed. [3]Percentile (and fraction) of volunteers in that group who were receiving speech therapy at the time surveyed.

Scholastic achievement:

Of the 54 volunteers in our study, we were able to gather records detailing scholastic achievement in math and language arts for 36; of the remaining 18 volunteers, 12 were under the age of 6 and therefore too young to have been formally assessed, and six were missing relevant records. Of the 36 students for whom appropriate records were available, in most cases achievement status, designated as “meets grade level” or “does not meet grade level” was based on scores from state or national standardized tests in math and/or language arts. Of note, some volunteers enrolled in special education classes took the same standardized tests as their “regular education” peers but were allowed extra time, verbal instruction, or other accommodations. In these instances we used the available standardized test scores to calculate achievement status. In some cases the scholastic files contained a phrase to the effect that the child was “exempted from standardized testing because achievement is well below grade level.” In these instances, where it was clearly stated that achievement was below grade level, we classified the student accordingly without standardized test scores. Volunteers for whom achievement status was unclear were excluded from this part of the study.

Close to half the volunteers in our study, for whom achievement status was documented, were not achieving at grade level in either math or language arts at the time of our survey (Table 4.1). Of note, not all volunteers receiving special educational support were classified as achieving below grade level; three of the 17 students receiving special services were achieving at or above their respective grade level in math, and four of the 17 were achieving at or above their respective grade level in language arts.

Behavioral outcomes:

To measure behavioral outcomes in our study volunteers we used two different survey instruments: the Social Skills Improvement System (SSIS) and the Achenbach System of Empirically Based Assessment (ASEBA) (27-29). These two surveys were selected because each has been used extensively in the general population, providing established reference values, and also in populations with known disabilities (27-29). Both survey sets assessed similar behaviors, but they grouped these behaviors differently. Therefore, we used both surveys to capture the full spectrum of behavioral outcomes in our study volunteers. We also sought to distinguish problem behaviors that might exhibit only in one setting (e.g. at home) from those that exhibited consistently in different settings (e.g. at home and at school); we therefore requested survey responses for our study volunteers both from parents and teachers.

The SSIS surveys assessed both social skills (communication, cooperation, and engagement) and problematic behaviors (including bullying, hyperactivity/inattention, internalizing and externalizing behaviors) (32, 33). SSIS survey scores were transformed to a standard scale for each age and gender group. Of note, the scores were also standardized to control for age and gender differences in the reference population, and were compared to established cut-offs limits distinguishing a “clinical range”, or affected score, from an unaffected score.

The ASEBA surveys also yielded scores, standardized for age and gender, for problematic behaviors grouped into variables by category (34-37). Neither competence nor adaptive function variables were assessed on the preschool forms and therefore we do not have those scores for the youngest volunteers in this study. Problematic behaviors

assessed via the ASEBA surveys were categorized as either *Internalizing* or *Externalizing*. The ASEBA splits problematic behaviors into these two groupings because the appearance of one, the other, or both is important for distinguishing the type of problem present (38). *Internalizing Problems*, defined as problems "within the self," such as anxiety, depression, somatic complaints, and withdrawal from social contact, may be present independently from *Externalizing Problems*, defined as specific problems stemming from conflicts with other people and with their expectations, such as bullying, oppositional or aggressive behavior, or difficulty making and keeping friends (28, 29).

Tables 4.2 to 4.4 provide details of the patterns of the observed behavioral data in our sample. Table 4.2 provides the mean scores in the entire sample, as well as scores stratified by the child's gender. Table 4.3 provides the mean scores stratified by either special-education or speech-therapy status. Finally, Table 4.4 provides mean scores stratified by math and language-arts achievement. Within each Table, we also provide the mean scores for the reference population.

Respondent		*Internalizing Problems* (ASEBA)	*Externalizing Problems* (ASEBA)	*Problem Behaviors* (SSIS)	*Total Competence* or *Adaptive Functioning* (ASEBA)	*Social Skills* (SSIS)
Reference Population (survey-specific)						
mean value		50.0-50.4	50.0-50.6	100	49.9-51.8**	100
clinical range (CR)		>63	>63	>115	<37	<85
% ref group in CR		<10%	<10%	16%	<10%	16%
Children with classic galactosemia (boys and girls combined)						
Parent	**mean ± SEM**	55.0 ± 1.55	48.2 ±1.34	102.7 ± 2.16	44.2 ± 1.74	93.7 ± 1.77
	% in CR (N)	25% (52)	8% (52)	22% (51)	28% (40)	24% (51)
Teacher*	**mean ± SEM**	53.0 ± 1.25	50.5 ± 1.08	102.0 ± 1.57	48.4 ± 1.27	94.3 ± 1.74
	% in CR (N)	16% (62)	6% (62)	16% (61)	7% (44)	20% (59)
Children with classic galactosemia (boys only)						
Parent	**mean ± SEM**	54.6 ± 2.14	49.8 ± 1.70	104.1 ± 3.43	42.2 ± 2.84	96.0 ± 3.14
	% in CR (N)	22% (23)	4% (23)	26% (23)	41% (17)	22% (23)
Teacher*	**mean ± SEM**	52.5 ± 1.68	49.8 ± 1.57	100.5 ± 2.39	51.3 ± 1.86	97.4 ± 2.53
	% in CR (N)	11% (27)	4% (27)	15% (26)	7% (15)	13% (24)
Children with classic galactosemia (girls only)						
Parent	**mean ± SEM**	55.4 ± 2.23	46.9 ± 1.97	101.6 ± 2.77	45.7 ± 2.18	91.8 ± 1.91
	% in CR (N)	28% (29)	10% (29)	18% (28)	17% (23)	25% (28)
Teacher*	**mean ± SEM**	53.5 ± 1.81	51.1 ± 1.50	103.0 ± 2.09	46.9 ± 1.62	92.2 ± 2.33
	% in CR (N)	20% (35)	9% (35)	17% (35)	7% (29)	26% (35)

Table 4.2. Behavioral outcomes for boys and girls: The mean score ± standard error (SEM), percentage of responses in the clinical range, and number of responses from parents and teachers for each of the five score categories is presented. Note that the sample size was insufficient to make statistical comparisons across genders meaningful. *Total Competence* scores are from the ASEBA parent surveys while the *Adaptive Functioning* scores are from the ASEBA teacher surveys. The reference population mean values, percentage in the clinical range, and clinical range cut-offs were derived from the reference population for each survey (27, 49, 50). *Means for teacher-rated variables are based on more than one observation for some volunteers (see Methods). **The range given is the combined range for both *Total Competence* and *Adaptive Functioning*; the range for *Total Competence* alone went only to 50.1.

Respondent		*Internalizing Problems* (ASEBA)	*Externalizing Problems* (ASEBA)	*Problem Behaviors* (SSIS)	*Total Competence or Adaptive Functioning* (ASEBA)	*Social Skills* (SSIS)
Reference Population (survey-specific)						
	mean value	50.0-50.4	50.0-50.6	100	49.9-51.8**	100
	clinical range (CR)	>63	>63	>115	<37	<85
	% ref group in CR	<10%	<10%	16%	<10%	16%
Children with classic galactosemia enrolled in special education						
Parent	**mean ± SEM**	60.2 ± 3.04	52.4 ± 2.69	108.6 ± 4.34	39.6 ± 2.21	86.3 ± 3.14
	% in CR (N)	47% (17)	18% (17)	38% (16)	36% (14)	50% (16)
Teacher*	**mean ± SEM**	56.9 ±2.46	52.6 ±1.54	105.3 ± 2.42	44.7 ± 1.40	88.5 ± 1.94
	% in CR (N)	30% (20)	5% (20)	20% (20)	12% (17)	30% (20)
Children with classic galactosemia not enrolled in special education						
Parent	**mean ± SEM**	51.3 ± 2.86	44.3 ± 2.19	97.3 ± 2.82	56.3 ± 2.38	99.6 ± 2.45
	% in CR (N)	13% (16)	6% (16)	6% (16)	0% (12)	6% (16)
Teacher*	**mean ± SEM**	48.1 ± 1.67	47.6 ± 1.62	95.9 ±1.75	55.3 ± 2.08	101.0 ± 2.74
	% in CR (N)	0% (23)	0% (23)	0% (23)	0% (15)	14% (21)
Children with classic galactosemia currently receiving speech therapy						
Parent	**mean ± SEM**	57.0 ± 2.51	50.1 ± 2.07	109.2 ± 3.28	38.3 ± 2.34	89.0 ± 2.63
	% in CR (N)	30% (23)	9% (23)	35% (23)	44% (16)	39% (23)
Teacher*	**mean ± SEM**	56.8 ± 1.65	54.5 ± 1.55	106.5 ± 2.01	43.2 ±1.44	87.2 ± 2.22
	% in CR (N)	21% (28)	11% (28)	21% (28)	6% (17)	36% (28)
Children with classic galactosemia not currently receiving speech therapy						
Parent	**mean ± SEM**	53.5 ± 2.07	46.9 ± 1.84	98.3 ± 2.57	48.7 ± 2.13	96.7 ± 2.02
	% in CR (N)	22% (27)	7% (27)	13% (26)	13% (23)	12% (26)
Teacher*	**mean ± SEM**	50.1 ± 1.72	47.2 ±1.32	98.3 ± 2.20	51.6 ±1.57	100.0 ±1.98
	% in CR (N)	12% (33)	3% (33)	13% (32)	7% (27)	7% (30)

Table 4.3. Behavioral Outcome, Special Education and Speech Therapy: The mean score ± standard error (SEM), percentage of responses in the clinical range, and number of responses from parents and teachers for each of the five scores is reported. Note that the sample size was insufficient to make statistical comparisons across the special education or speech therapy categories meaningful. *Total Competence* scores were derived from ASEBA parent surveys while the *Adaptive Functioning* scores were derived from ASEBA teacher surveys. The reference population mean values, percentage in the clinical range, and clinical range cut-offs come from the reference population for each survey (27, 49, 50). Our study population was stratified by whether the child was currently enrolled in or receiving special education or speech therapy. *Means for teacher-rated variables are based on more than one observation for some volunteers (see Methods). **The range given is the combined range for both *Total Competence* and *Adaptive Functioning*; the range for *Total Competence* alone went only to 50.1.

Respondent		*Internalizing Problems* (ASEBA)	*Externalizing Problems* (ASEBA)	*Problem Behaviors* (SSIS)	*Total Competence* or *Adaptive Functioning* (ASEBA)	*Social Skills* (SSIS)
Reference Population (survey-specific)						
	mean value	50.0-50.4	50.0-50.6	100	49.9-51.8**	100
	clinical range (CR)	>63	>63	>115	<37	<85
	% ref group in CR	<10%	<10%	16%	<10%	16%
Children with classic galactosemia achieving at grade level in math						
Parent	**mean ± SEM**	51.7 ± 2.31	42.6 ± 1.33	93.8 ± 2.48	50.0 ± 2.82	97.2 ± 2.48
	% in CR (N)	18% (17)	0% (17)	0% (17)	19% (16)	12% (17)
Teacher*	**mean ± SEM**	50.0 ± 1.99	45.4 ± 1.44	93.5 ± 1.51	53.8 ± 1.91	103.9 ± 2.71
	% in CR(N)	5% (20)	0% (20)	0% (19)	0% (19)	6% (17)
Children with classic galactosemia achieving below grade level in math						
Parent	**mean ± SEM**	58.0 ± 2.47	50.6 ± 2.42	107.6 ± 3.60	41.2 ± 1.89	87.6 ± 2.99
	% in CR (N)	32% (19)	11% (19)	28% (18)	22% (18)	44% (18)
Teacher*	**mean ± SEM**	53.5 ± 2.34	50.2 ± 1.54	102.8 ±2.33	45.1 ± 1.24	91.4 ± 1.91
	% in CR(N)	18% (22)	5% (22)	14% (22)	10% (21)	23% (22)
Children with classic galactosemia achieving at grade level in language arts						
Parent	**mean ± SEM**	51.9 ± 2.39	41.9 ±1.27	94.5 ± 2.74	48.4 ± 2.50	97.5 ± 2.61
	% in CR (N)	16% (19)	0% (19)	5% (19)	16% (19)	16% (19)
Teacher*	**mean ± SEM**	48.7 ± 1.61	44.9 ±1.05	92.9 ± 0.10	52.0 ± 1.73	100.2 ±2.66
	% in CR(N)	0% (23)	0% (23)	0% (22)	0% (21)	10% (20)
Children with classic galactosemia achieving below grade level in language arts						
Parent	**mean ± SEM**	58.5 ± 2.40	52.3 ± 2.38	108.5 ± 3.56	41.5 ± 2.31	86.1 ± 2.73
	% in CR (N)	35% (17)	12% (17)	25% (16)	27% (15)	44% (16)
Teacher*	**mean ± SEM**	55.6 ±2.63	51.5 ± 1.80	104.9 ± 2.56	46.2 ± 1.76	93.3 ± 2.45
	% in CR(N)	26% (19)	5% (19)	16% (19)	11% (19)	21% (19)

Table 4.4. Behavioral Outcomes and Scholastic Achievement: The mean score ± standard error (SEM), percentage of responses in the clinical range, and number of responses from parents and teachers for each of the five scores is reported. Note that the sample size was insufficient to make statistical comparisons across achievement categories meaningful. *Total Competence* scores were derived from ASEBA parent surveys while the *Adaptive Functioning* scores were derived from ASEBA teacher surveys. The reference population mean values, percentage in the clinical range, and clinical range cut-offs come from the reference population for each survey (27, 49, 50). Our study population was stratified by whether or not the child was meeting grade level achievement standards in math and language arts. *Means for teacher-rated variables are based on more than one observation for some volunteers (see Methods). **The range given is the combined range for both *Total Competence* and *Adaptive Functioning*; the range for *Total Competence* alone went only to 50.1.

Social skills and adaptive behavior:

We measured behavior in social and adaptive areas for our study volunteers using three different variables: *Adaptive Functioning* (ASEBA surveys), *Total Competence* (ASEBA surveys), and *Social Skills* (SSIS surveys). *Adaptive Functioning* was a composite of teacher's observations of the student's ability to work, behave, learn and interact with peers in the classroom. *Total Competence* was assessed from responses on the ASEBA parent survey concerning the number of activities, clubs, and sports the study volunteer was involved in, whether they were as capable at activities as their peers, and how they interacted with adults, peers and family. *Social Skills* were assessed from both the SSIS parent and teacher surveys from questions addressing the study volunteer's ability to communicate, follow directions, and interact with their peers and adults. A clinical score in *Social Skills* could reflect either an inability to perform a given behavior or an inability to understand when a given behavior was appropriate for a specific situation. In Tables 4.2 to 4.4, we present the average values of these measures for our study volunteers, and for the reference population. Like the behavioral outcome data described above, we provide these data stratified by gender (Table 4.2), special-education/speech-therapy status (Table 4.3), and math/language arts achievement status (Table 4.4).

Cryptic GALT activity may be a predictor of scholastic and behavioral outcomes.

Previous studies have looked at GALT genotype as a candidate modifier of long-term outcomes in classic galactosemia with mixed results (3, 10, 24); we addressed the question from a slightly different angle, asking not whether GALT genotype itself correlates with scholastic outcome, but whether the residual GALT activity predicted by

GALT genotype correlates with outcome.

Our study volunteers demonstrated a variety of GALT genotypes: 33 were homozygotes for the common Q188R allele, associated with no residual activity, 17 were compound heterozygotes for Q188R and another allele, and 4 carried only non-Q188R alleles. Residual GALT activity for each volunteer was estimated from the arithmetic mean of the predicted activities for each allele present in the genotype. To quantify the level of cryptic GALT activity associated with each allele we applied our previously described null background yeast expression system for human GALT (39), averaging the activities predicted for each allele in any given patient. Of note, this system allows over-expression of any *hGALT* open reading frame in a null-background strain of yeast, so that even very low levels of residual GALT activity, well below the threshold of detection by clinical assays of hemolysate, are detectable (see Methods and Gleason et al, in preparation). However, alleles that involve non-coding changes cannot be accurately modeled. We therefore excluded from this portion of the study any patients whose GALT genotype included an allele with only non-coding sequence changes; of the 54 volunteers in this study, only one had to be excluded for this reason.

For the purpose of our analyses we stratified volunteers into two groups: those with <1% predicted residual GALT activity, and those with ≥1% predicted residual GALT activity (Table 4.5). Of the 29 volunteers with <1% predicted GALT activity for whom academic achievement status was known, only 11 were achieving at grade level in math, and only 13 were achieving at grade level in language arts; most of the children were achieving below grade level in one or both subjects (Table 4.5). In contrast, of the 6 volunteers with predicted GALT activity ≥1% for whom academic achievement status

Predicted GALT activity (as a % of wild-type hGALT)	<1%	≥1%
all study volunteers (n=53)	47	6
Special Educational Support		
Enrolled in special education (n=17)	15	2
Not enrolled in special education (n=15)	12	3
Math Achievement[1]		
At grade level in Math (n=16)	11	5
Below grade level in Math (n=19)	18	1
Language Arts Achievement[2]		
At grade level in Language Arts (n=18)	13	5
Below grade level in Language Arts (n=17)	16	1

Table 4.5. Achievement and predicted GALT activity: Each study volunteer was classified by the level of residual GALT activity predicted for their *GALT* genotype based on yeast expression data (see Methods). One volunteer was omitted from this analysis because one of their mutations was intronic and so could not be assessed using the yeast system. Scholastic achievement shows a clear tendency with predicted residual GALT activity but due to the small number of volunteers with ≥1% predicted GALT activity this tendency is not statistically significant. [1]Math achievement and GALT activity p-value=0.436. [2]Language Arts achievement and GALT activity p-value=0.312.

was known, all but one were achieving at grade level in math and/or language arts (Table 4.5). A similar finding, though not as striking, was seen when the volunteers were stratified with regard to special education status rather than scholastic achievement; of those with <1% GALT activity more than half (15/27) were enrolled in special education, while of the volunteers with ≥1% GALT activity more than half (3/5) were not enrolled

in special education. While these differences were certainly consistent, none reached statistical significance in our model, likely due to the limited number of total volunteers in the study, and the even more limited number of volunteers with ≥1% predicted residual GALT activity (see Methods).

We also explored whether residual GALT activity might correlate with behavioral outcomes in our study volunteers (Figures 4.1 and 4.2). We found that the fraction of volunteers with scores for problematic behaviors in the clinical range was higher for volunteers with <1% predicted GALT activity than for volunteers with ≥1% predicted GALT activity (shaded areas, Figure 4.1). These findings were evident in the social and adaptive behavior scores as well (Figure 4.2). For example, the *Social Skills* scores from both the parent and teacher surveys regarding study volunteers with <1% predicted GALT activity showed a large percentage of scores in the clinical range (28% and 21%, respectively), while for study volunteers with ≥1% predicted GALT activity there were no scores in the clinical range (Figure 4.2). Due to small sample size, most of these findings were not statistically significant; however, the difference in teacher responses concerning *Social Skills* for study volunteers with <1% predicted GALT activity vs. ≥1% predicted GALT activity was statistically significant in our model after an appropriate adjustment for multiple testing ($p=0.042$) (Figure 4.2). That this difference was statistically significant despite the limited sample size, and despite the correction for multiple testing, is striking. This relationship demonstrates that cryptic residual GALT activity may be a modifying factor, at least for social skills if not for other long term outcomes, among school aged children with classic galactosemia.

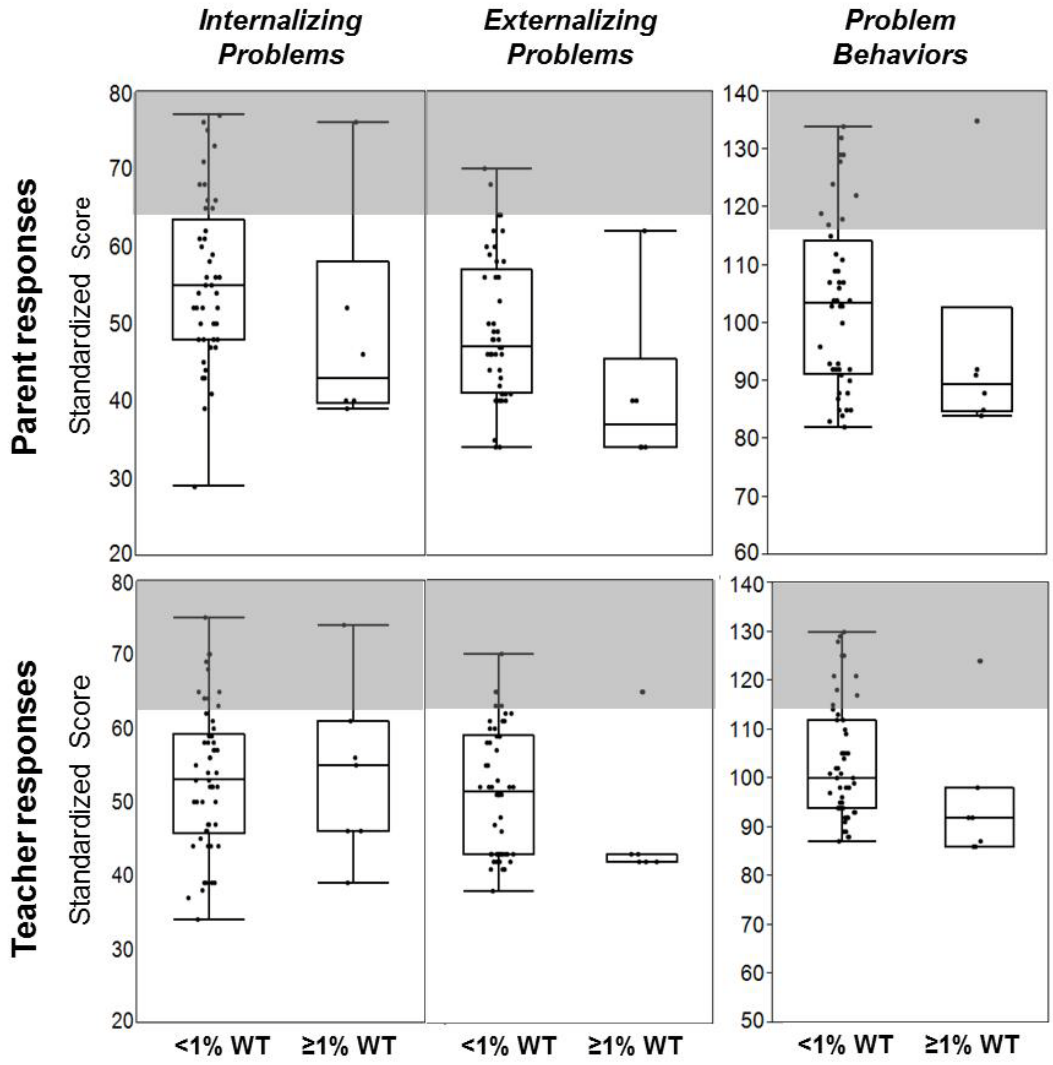
Internalizing Problems
Externalizing Problems
Problem Behaviors
Parent responses
Teacher responses
Standardized Score
<1% WT
≥1% WT

Figure 4.1: *Problematic Behavior* scores are displayed as a function of residual GALT activity. For each data set illustrated, box and whisker plots indicate the median (center line in each box), limits of the 75th and 25th percentiles (top and bottom of each box), and 95th percentile confidence limits (top and bottom whiskers). Note that for some distributions the 95% percentile limits coincided with the 75th and/or 25th percentile limits so no whiskers are visible. Predicted residual GALT activity for each volunteer was estimated for that volunteer's *GALT* genotype based on the level of residual activity associated with the relevant *GALT* alleles using a null-background yeast expression system for the human enzyme. Volunteers with non-coding mutations that could not be assessed using this system were excluded from analysis. Both parent and teacher surveys indicated that, on average, volunteers predicted to have <1% wild-type residual GALT activity demonstrated more problem behaviors, and were more likely to have scores in the clinical range, than volunteers predicted to have ≥1% wild-type GALT activity. However, likely reflecting the small sample size, none of these apparent differences reached statistical significance.

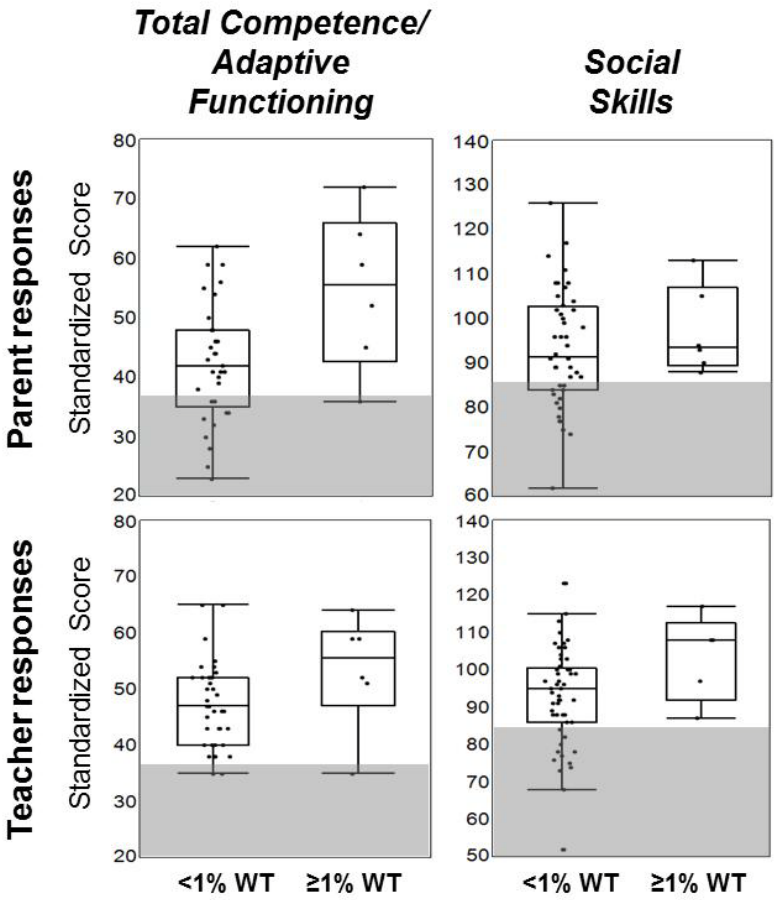
Total Competence/ Adaptive Functioning
Social Skills
Parent responses
Teacher responses
Standardized Score
<1% WT
≥1% WT

Figure 4.2: *Social Interaction* scores are displayed as a function of predicted residual GALT activity. For each data set illustrated, box and whisker plots indicate the median (center line in each box), limits of the 75th and 25th percentiles (top and bottom of each box), and 95th percentile confidence limits (top and bottom whiskers). Predicted residual GALT activity for each volunteer was estimated for that volunteer's *GALT* genotype based on the level of residual GALT activity associated with the relevant *GALT* alleles using a null-background yeast expression system for the human enzyme. Volunteers with non-coding mutations that could not be assessed using this system were excluded from analysis. Both parent and teacher surveys indicated that, on average, volunteers predicted to have ≥1% wild-type GALT activity demonstrated better *Total Competence* and *Adaptive Functioning*, and stronger *Social Skills* than volunteers predicted to have <1% wild-type GALT activity. Only the difference in scores from the teacher responses for *Social Skills* was statistically significant (p=0.042).

4.5 DISCUSSION

The goal of this study was to characterize the nature of the scholastic and behavioral outcomes experienced by a group of 54 school age children with classic galactosemia and to ask whether cryptic GALT activity predicted for these children might influence parameters of long term outcome in this population. Our results both confirm earlier findings and extend them.

Scholastic achievement and behavioral outcomes:

We used many different approaches to assess scholastic achievement and behavioral outcomes in our study volunteers, gathering information from parent and teacher surveys, standardized test scores, and school records. Our goal was to provide as comprehensive as possible a phenotypic description of scholastic and behavioral outcomes in school-aged children with classic galactosemia, recognizing that some of these outcomes might be highly correlated. Previous research, outside the field of galactosemia, has shown strong correlations between working memory, cognitive ability, social interactions and behavior (40). Our results suggest that at least some of these relationships might also hold for children with classic galactosemia, though further studies with a substantially larger cohort will be required to confirm or refute this hypothesis in a statistically meaningful way.

Like other groups of patients with classic galactosemia who have been studied (e.g. (3, 4, 8, 9, 11, 21, 22, 26)), a large percentage of the children in our cohort struggled academically, many required speech therapy, and many exhibited problem behaviors. A small number of children demonstrated academic achievement at grade level for math but not language arts, or for language arts but not math, but most either achieved at grade

level, or below grade level, for both subjects. This apparent concordance implies that the underlying deficit may be broad and that the achievement gap may not be simply a complication secondary to difficulties with speech.

Results from Table 4.2 appear to suggest that many of the volunteers in our cohort demonstrated *internalizing* rather than *externalizing* problem behaviors. This was observed both in the parents' and teachers' evaluation forms, which would imply these behaviors likely were not simply responsive to a specific environment (e.g. home vs. school). As above, studies in a larger cohort will be required to test the significance of this possible relationship. However, Antshel and colleagues (3) also noted *internalizing* problems in their study cohort of 25 children and adolescents with classic galactosemia, but all of their behavioral outcome data derived from parent surveys; they did not also survey teachers. Given the cognitive and speech challenges faced by many children with classic galactosemia, if the *internalizing* behaviors observed were simply responsive to contextual feelings of frustration or isolation one might have anticipated that they would be even more pronounced at school than at home, but that is not what we observed. For almost every behavioral parameter measured the parent and teacher survey responses were similar.

Cryptic GALT activity as a candidate prognostic indicator:

Patients with classic galactosemia display a striking allelic heterogeneity at *GALT*, leading to the logical supposition that these allelic differences might underlie the differences in outcome severity observed between patients. Indeed, a number of prior studies have addressed the hypothesis of genotype-phenotype correlation with sometimes contradictory results (10, 11, 13, 14, 19). Here we have addressed this question from a

slightly different angle, asking not whether *GALT* genotype itself predicts outcome severity, but rather whether the cryptic residual GALT activity associated with some alleles might impact outcome severity. This is a logical extension of early reports demonstrating that patients who carry an *S135L GALT* mutation, which is common in patients of African ancestry and associated with residual GALT activity in both clinical studies and model systems (41-46), tend to experience milder long term outcomes. Work in a *Drosophila melanogaster* (fruit fly) model of GALT deficiency also suggests that even very low levels of residual GALT activity can modify long term outcome (47, 48).

To address the role of cryptic GALT activity as a candidate modifier of scholastic or behavioral outcomes in our study population we first assessed the *GALT* genotype of each volunteer, and then applied our previously-described yeast model system (31, 39) to predict the level of residual GALT function associated with each missense or nonsense mutation. Finally, we averaged the activity levels predicted for each of the alleles in a given patient to derive a total predicted GALT activity value for that patient. The strength of this approach derives from the sensitivity of the null-background yeast model system to detect even trace amounts of GALT function. Indeed, most of the patients predicted to have some residual GALT function by the yeast model demonstrated no detectable GALT activity by standard hemolysate clinical assays (Gleason et al, in preparation).

Of the 53 volunteers in our study whose GALT function we could assess using the yeast system (one was excluded from study due to a non-coding mutation), only 6 had ≥1% predicted residual GALT function. This low prevalence of patients with ≥1% predicted residual GALT activity in our cohort may reflect the predominantly northern European heritage of most of our volunteers, and the high prevalence of the null-activity

Q188R allele that goes with that demography. Even with this limitation, however, we observed a relationship between predicted residual GALT activity and *Social Skills,* as reported on the teacher surveys that was statistically significant even after an appropriate adjustment for multiple testing. That said, even among volunteers with <1% predicted residual GALT activity we observed a range of scholastic and behavioral outcomes, implicating the existence of other genetic and/or environmental modifiers outside the *GALT* locus.

Limitations and implications:

The major limitation of this work was the small sample size; a common problem among studies of patients with rare conditions. This problem is compounded with regard to testing the impact of predicted residual GALT activity on the outcomes measured because such a small fraction of patients with classic galactosemia in the US have evidence of residual GALT activity. This study is further limited by the absence of a matched control group, although we attempted to minimize that problem by using very well established survey instruments that have reference ranges already defined from prior studies.

The implications of this work are two-fold. First, our results provide a more comprehensive overview of the types of scholastic and behavioral outcomes experienced by school age children with classic galactosemia than has been reported previously. Recognizing the nature of these challenges empowers families and schools to provide the needed support structure to enable each child to reach his or her fullest potential. Second, our results demonstrate compelling, if limited evidence that predicted cryptic GALT activity may be a modifier of long term outcome severity in patients with classic

galactosemia. The outcomes we have measured here are scholastic and behavioral, but these results raise suspicion that other outcomes might also be modified by the presence of even trace levels of residual GALT activity. Studies to test this hypothesis are currently underway.

Acknowledgments

First and foremost, we are grateful to the many study volunteers and their families and teachers who participated in this project; without these amazing people this project could never have been completed. We are also grateful to our colleagues in the Departments of Human Genetics and Psychiatry and Behavioral Sciences at Emory for their many helpful discussions. This work was supported in part by funds from the National Institutes of Health grant R01 DK059904 (to JLFK); ELR was also supported in part by NIH Training Grants T32 MH087977, TL1 RR025010 and T32 GM008367.

Author contributions

This project was a true collaboration on many levels. JLFK and ELR initiated the project, recruited the study volunteers, gathered the survey and school records data, assembled the Tables and Figures, and wrote the majority of the manuscript; MEL and ET reviewed the school records and provided valuable insight and interpretation; TJG conducted all of the experiments related to patient GALT genotyping and GALT functional analysis using the yeast system; MPE performed the statistical analyses; all co-authors contributed to writing and editing the final manuscript.

4.6 REFERENCES

1. Fridovich-Keil JL & Walter JH (2008) Galactosemia. *The Online Metabolic & Molecular Bases of Inherited Disease,* eds Valle D, Beaudet A, Vogelstein B, Kinzler K, Antonarakis S, & Ballabio A (McGraw Hill), p http://www.ommbid.com/.
2. Coss K, *et al.* (2012) Classical Galactosaemia in Ireland: incidence, complications and outcomes of treatment.. *J Inherit Metab Dis.* 2012 Jul 3. [Epub ahead of print].
3. Antshel K, Epstein I, & Waisbren S (2004) Cognitive strengths and weaknesses in children and adolescents homozygous for the galactosemia Q188R mutation: a descriptive study. *Neuropsychology* 18(4):658-664.
4. Doyle C, Channon S, Orlowska D, & Lee P (2010) The neuropsychological profile of galactosaemia. *J Inherit Metab Dis* 33(5):603-609.
5. Gitzelmann R & Steinmann B (1984) Galactosemia: how does long-term treatment change the outcome? *Enzyme* 32(1):37-46.
6. Hughes J, *et al.* (2009) Outcomes of siblings with classical galactosemia. *J Pediatr* 154(5):721-726.
7. Jumbo-Lucioni P, *et al.* (2012) Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. *J Inherit Metab Dis* In press.
8. Kaufman FR, McBride-Chang C, Manis FR, Wolff JA, & Nelson MD (1995) Cognitive functioning, neurologic status and brain imaging in classical galactosemia. (Translated from eng) *Eur J Pediatr* 154(7 Suppl 2):S2-5 (in eng).

9. Schweitzer S, Shin Y, Jakobs C, & Brodehl J (1993) Long-term outcome in 134 patients with galactosemia. *Eur J Pediatr* 152:36-43.

10. Shield JP, *et al.* (2000) The relationship of genotype to cognitive outcome in galactosaemia. *Arch. Dis. Child* 83:248-250.

11. Waggoner DD, Buist NR, & Donnell GN (1990) Long-term prognosis in galactosaemia: results of a survey of 350 cases. (Translated from eng) *Journal of inherited metabolic disease* 13(6):802-818 (in eng).

12. Hoffmann B, Wendel U, & Schweitzer-Krantz S (2011) Cross-sectional analysis of speech and cognitive performance in 32 patients with classic galactosemia. *J Inherit Metab Dis* 34(2):421-427.

13. Waisbren S, *et al.* (2012) The adult galactosemic phenotype. *J Inherit Metab Dis* 35(2):279-286.

14. Kaufman FR, *et al.* (1994) Correlation of cognitive, neurologic, and ovarian outcome with the Q188R mutation of the galactose-1-phosphate uridyltransferase gene. (Translated from eng) *J Pediatr* 125(2):225-227 (in eng).

15. Schweitzer S, Shin Y, Jakobs C, & Brodehl J (1993) Long-term outcome in 134 patients with galactosaemia. (Translated from eng) *European journal of pediatrics* 152(1):36-43 (in eng).

16. Schadewaldt P, *et al.* (2010) Longitudinal assessment of intellectual achievement in patients with classical galactosemia. *Pediatrics* 125(2):e374-381.

17. Nelson MD, Jr., Wolff JA, Cross CA, Donnell GN, & Kaufman FR (1992) Galactosemia: evaluation with MR imaging. (Translated from eng) *Radiology* 184(1):255-261 (in eng).

18. Dubroff JG, *et al.* (2008) FDG-PET findings in patients with galactosaemia. (Translated from eng) *J Inherit Metab Dis* 31(4):533-539 (in eng).

19. Tyfield LA (2000) Galactosaemia and allelic variation at the galactose-1-phosphate uridyltransferase gene: a complex relationship between genotype and phenotype. (Translated from eng) *Eur J Pediatr* 159 Suppl 3:S204-207 (in eng).

20. Bosch AM (2006) Classical galactosaemia revisited. (Translated from eng) *J Inherit Metab Dis* 29(4):516-525 (in eng).

21. Hughes J, *et al.* (2009) Outcomes of Siblings with Classical Galactosemia. *The Journal of Pediatrics* 154(5):721-726.

22. Nelson CD, Waggoner DD, Donnell GN, Tuerck JM, & Buist NR (1991) Verbal dyspraxia in treated galactosemia. (Translated from eng) *Pediatrics* 88(2):346-350 (in eng).

23. Potter N (2011) Voice disorders in children with classic galactosemia. *J Inherit Metab Dis* 34(2):377-385.

24. Potter N, Lazarus J, Johnson J, Steiner R, & Shriberg L (2008) Correlates of language impairment in children with galactosaemia. *J Inherit Metab Dis* 31(4):524-532.

25. Shriberg L, Potter N, & Strand E (2011) Prevalence and phenotype of childhood apraxia of speech in youth with galactosemia. *J Speech Lang Hear Res* 54(2):487-519.

26. Lambert C & Boneh A (2004) The impact of galactosaemia on quality of life--a pilot study. (Translated from eng) *J Inherit Metab Dis* 27(5):601-608 (in eng).

27. Gresham FM & Elliott SN (2008) *SSIS (Social Skills Improvement System) Rating Scales* (NCS Pearson, Inc,, Minneapolis, MN).

28. Achenbach TM & Rescorla L (2001) *Manual for the ASEBA school-age forms & profiles : an integrated system of multi-informant assessment* (ASEBA, Burlington, VT) pp xii, 238 p.

29. Achenbach TM & Rescorla L (2000) *Manual for the ASEBA preschool forms & profiles : an integrated system of multi-informant assessment* (ASEBA, Burlington, VT) pp xi, 178 p.

30. Chhay J, Openo K, Eaton J, Gentile M, & Fridovich-Keil J (2008) A yeast model reveals biochemical severity associated with each of three variant alleles of galactose-1P uridylyltransferase segregating in a single family. *J Inherit Metab Dis* 31:97-107.

31. Fridovich-Keil JL & Jinks-Robertson S (1993) A yeast expression system for human galactose-1-phosphate uridylyltransferase. *Proc. Natl. Acad. Sci. USA* 90:398-402.

32. Gresham FM, Elliott SN, & Kettler RJ (2010) Base rates of social skills acquisition/performance deficits, strengths, and problem behaviors: an analysis of the Social Skills Improvement System--Rating Scales. (Translated from eng) *Psychol Assess* 22(4):809-815 (in eng).

33. Gresham FM, Elliott SN, Cook CR, Vance MJ, & Kettler R (2010) Cross-informant agreement for ratings for social skill and problem behavior ratings: an investigation of the Social Skills Improvement System-Rating Scales. (Translated from eng) *Psychol Assess* 22(1):157-166 (in eng).

34. Achenbach TM & Dumenci L (2001) Advances in empirically based assessment: revised cross-informant syndromes and new DSM-oriented scales for the CBCL, YSR, and TRF: comment on Lengua, Sadowksi, Friedrich, and Fischer (2001). (Translated from eng) *J Consult Clin Psychol* 69(4):699-702 (in eng).

35. Achenbach TM & Ruffle TM (2000) The Child Behavior Checklist and related forms for assessing behavioral/emotional problems and competencies. (Translated from eng) *Pediatr Rev* 21(8):265-271 (in eng).

36. Achenbach TM, Verhulst FC, Baron GD, & Althaus M (1987) A comparison of syndromes derived from the Child Behavior Checklist for American and Dutch boys aged 6-11 and 12-16. (Translated from eng) *J Child Psychol Psychiatry* 28(3):437-453 (in eng).

37. Verhulst FC, Achenbach TM, Althaus M, & Akkerhuis GW (1988) A comparison of syndromes derived from the child behavior checklist for American and Dutch girls aged 6-11 and 12-16. (Translated from eng) *J Child Psychol Psychiatry* 29(6):879-895 (in eng).

38. Achenbach TM (1966) The classification of children's psychiatric symptoms: a factor-analytic study. (Translated from eng) *Psychol Monogr* 80(7):1-37 (in eng).

39. Riehman K, Crews C, & Fridovich-Keil JL (2001) Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase. *J. Biol. Chem.* 276(14):10634-10640.

40. Vuontela V, *et al.* (2012) Working Memory, Attention, Inhibition, and Their Relation to Adaptive Functioning and Behavioral/Emotional Symptoms in

School-Aged Children. (Translated from Eng) *Child Psychiatry Hum Dev* (in Eng).

41. Henderson H, Leisegang F, Brown R, & Eley B (2002) The clinical and molecular spectrum of galactosemia in patients from the Cape Town region of South Africa. *BMC Pediatr* 2:7.

42. Lai K & Elsas LJ (2001) Structure-Function Analyses of a Common Mutation in Blacks with Transferase-Deficiency Galactosemia. *Mol Genet Metab* 74:264-272.

43. Reichardt JKV, Levy HL, & Woo SL (1992) Molecular characterization of two galactosemia mutations and one polymorphism: implications for structure-function analysis of human galactose-1-phosphate uridyltransferase. *Biochemistry* 31:5430-5433.

44. Crushell E, Chukwu J, Mayne P, Blatny J, & Treacy E (2009) Negative screening tests in classical galactosaemia caused by S135L homozygosity. *J Inherit Metab Dis.* 32(3):412-415.

45. Landt M, *et al.* (1997) Black children deficient in galactose 1-phosphate uridyltransferase: Correlation of activity and immunoreactive protein in erythrocytes and leukocytes. *The Journal of Pediatrics* 130(6):972-980.

46. Wells L & Fridovich-Keil JL (1997) Biochemical characterization of the S135L allele of galactose-1-phosphate uridylyltransferase associated with galactosemia. *J. Inher. Metab. Dis.* 20(5):633-642.

47. Kushner RF, *et al.* (2010) A Drosophila melanogaster model of classic galactosemia. (Translated from eng) *Disease models & mechanisms* 3(9-10):618-627 (in eng).

48. Ryan E, Duboff B, Feany M, & Fridovich-Keil J (2012) Mediators of a long-term movement abnormality in a Drosophila melanogaster model of classic galactosemia. *Dis Model Mech* 2012 Jun 26. [Epub ahead of print].

49. Achenbach T & Rescorla L (2000) Manual for ASEBA Preschool Forms & Profiles (University of Vermont, Research Center for Children, Youth, & Families, Burlington, VT).

50. Achenbach T & Rescorla L (2001) Manual for ASEBA School-Age Forms & Profiles. (University of Vermont, Research Center for Children, Youth, & Families, Burlington, VT).

CHAPTER 5

CONCLUSION

5.1 SUMMARY

This dissertation explores potential modifiers of outcome in classic galactosemia using a model system, *GALT* deficient *Drosophila melanogaster* and a cohort of patients with the disease. Initially in this dissertation we began with creating and validating a fruit fly model of classic galactosemia. Unlike the mouse model of classic galactosemia and like the patients, the fruit fly succumbs early in development on a diet enriched with galactose and accumulates galactose-1-phosphate under these conditions. We then examined the movement defect in adult *GALT* deficient fruit flies in chapter three and determined that galactose exposure had no affect on this phenotype although small amounts of GALT activity could improve the phenotype, age made the *GALT* deficient flies worse. We found no morphological defect in the adult *GALT* deficient fly. Therefore the acute patient phenotype, galactose dependent toxicity, is recapitulated in the fly, the *GALT* deficient *Drosophila* also have a movement phenotype that may be similar to some of the neurological complications reported for patients.

In Chapter Four we looked at the relationship between scholastic achievement and behavior outcomes in school age children with classic galactosemia. In our study cohort we detected a high incidence of internalizing behavior problems and difficulties with social interactions. In our study a select number of volunteers were predicted to have cryptic GALT activity, based on genotype and a yeast model system. Individuals with ≥1% predicted residual GALT activity were more likely to be achieving at grade level, had lower problem behavior scores, and higher social competence scores than those whose predicted GALT activity was <1%. The small size of our study cohort makes it difficult to determine many broad scope conclusions beyond confirming the observation

that individuals with classic galactosemia are at higher risk for intellectual disability and social and behavioral problems.

5.2 MODELING CLASSIC GALACTOSEMIA IN *DROSOPHILA MELANOGASTER*

Summary

The work discussed in Chapter Two establishes that *Drosophila melanogaster* have a functional Leloir pathway via genetic homology, gene disruption studies, and enzymatic activity. The *GALT* deficient allele is an imprecise excision that removed 1.6 kb of the *dGALT* and disrupted enzymatic activity of GALT but left *cup* function intact, the gene in which *dGALT* exists in one of its introns (Figure 2.2 and Table 2.2). Animals homozygous for the *GALT* deficiency failed to develop on food supplemented with high amounts of galactose (222 mM) while their heterozygous counterparts developed (Figure 2.3). Expression of the human transgene in the GALT deficient background rescued survival on food supplemented with galactose to the expected Mendelian ratio. *GALT* deficient larvae and adults accumulated galactose-1-phosphate when exposed to high amounts of dietary galactose. Combined, these results demonstrated that the fruit fly model of classic galactosemia recapitulates portions of the human disease phenotype.

The movement phenotype introduced in Chapter Two is explored more fully in Chapter Three. *GALT* deficient animals could not progress through the countercurrent apparatus at the same rate as control flies and this was due to a defect in climbing ability, not startle response (Figure 3.2). Transgenic expression of human GALT, via the *GAL4/UAS* system rescued this phenotype completely (Figure 3.3). A transgenic insertion of human GALT that showed a low level of expression independent of the *GAL4/UAS* driver modified the climbing phenotype (Figure 3.3). Animals carrying one copy of this

"leaky" transgene had about 2% of GALT activity seen in the control animals, *dGALT*^{C2}, and this low level of activity yielded an intermediate climbing phenotype. In Chapter Two, we showed *GALT* null animals carrying two copies of this leaky transgene had an intermediate survival rate when exposed to high levels of dietary galactose during development (Table 2.3). Combined, these results demonstrate that flies with a small level of GALT activity are less severely affected than flies that are completely *GALT* deficient.

It is important to note that not all larvae exposed to dietary galactose fail to develop. It was only when larvae were exposed throughout development and at high concentration that dietary galactose was lethal to nearly all *GALT* null animals. At lower concentrations of dietary galactose most *GALT* null animals continued through development in spite of high levels of galactose-1-phosphate they accumulated as larvae (Figures 2.4 and 3.4). Results from the diet switching experiments showed an intermediate level of lethality under a number of conditions (Figure 2.6). About half of *GALT* null animals switched to a galactose restricted diet after exposure to high galactose concentrations for 2 to 4 days early in larval development survived. Animals that were switched to a diet supplemented with galactose after 2 or more days of development on glucose only food showed a increase in survival as a function of the length of time before switching to the high galactose food (Figure 2.6). Therefore lethality in *GALT* null fruit flies exposed to dietary galactose occurred only if dietary galactose was persistently present at elevated levels. Independent of galactose exposure, there was lethality seen among *GALT* null animals (Table 2.3 and Figures 2.4 and 2.6). Within the population of *GALT* null flies there was variation of galactose-dependent lethality, a few animals died

even when not exposed to galactose; most did not survive exposure to high-levels of dietary galactose throughout development. This variability is also seen in patients where two patients with the same genotype may have drastically different dietary restrictions. If we understood the mechanism behind this variation in the fruit fly we might be able to improve the prognosis of different patients with classic galactosemia (1).

Dietary galactose exposure modified survival and therefore, in Chapter Three, we explored its ability to modify the movement phenotype. *GALT* null animals exposed to low levels of dietary galactose perform similarly in the climbing apparatus as *GALT* null animals that developed without dietary galactose exposure; neither group was proficient. These findings showed that this specific movement phenotype was not exacerbated by dietary galactose, an interesting finding given questions regarding outcome severity in patients with classic galactosemia and cryptic dietary exposure to galactose (2).

Finally, we looked at age as a candidate modifier of the movement phenotype for two reasons: 1-fruit fly climbing behavior has been shown to decline as a function of age and 2-climbing behavior and age are both impacted by reactive oxygen species (3). *GALT* null animals were already less capable than control animals but after an additional one or two weeks of age these animals were incapable of routinely completing the countercurrent apparatus while most control animals still completed the task (Figure 3.5). Two day old *GALT* null animals climbed in a similar fashion to 16 day old control animals; implying that *GALT* null animals started out prematurely affected.

In an effort to determine the origins of the movement defect in the *GALT* null animals, a collaborator performed anatomical studies of the brains and indirect flight

muscle on control and *GALT* null adults. We hoped to discover a defect in one tissue or both that would elucidate the *GALT* null movement phenotype. We saw no difference or defect in either tissue; we also examined older animals to no avail. We speculate that the cause of the movement defect in the *GALT* null animals is a subtle physiological effect from the loss of GALT function; possibly a transitory imbalance during development that leads to slight damage to some cell-type or tissues in the individual animal. If we understood what non-Leloir modifiers affected the movement phenotype, we could begin to understand the defect at an anatomical level.

Implications and Future Directions

These chapters establish a *Drosophila* model of classic galactosemia that recapitulates both the lethality after exposure to dietary galactose during development and the movement disorder in adult flies that appears to be independent of dietary galactose exposure. These chapters give examples of how the phenotypes seen in *GALT* null fruit flies behave similarly to phenotypes in people with classic galactosemia. *GALT* null flies have a movement defect; the entire population is not affected but the population as a whole is significantly slower than controls. The movement defect is not worsened by exposure to low levels of dietary galactose. Similarly, patients with classic galactosemia on average have IQs below normal, but there are individuals with IQs in the normal range (4). Studies of siblings with classic galactosemia showed that dietary galactose exposure, as measured as the number of days the infant was given a milk-based diet, does not correlate with IQ (2). We see a similar pattern with cryptic GALT activity modifying both the movement phenotype in GALT null fruit flies and IQ in patients (5). With these similarities, it may be possible to explore the mechanisms behind the phenotypes seen in

the *GALT* null fruit fly and one day to improve prognosis and treatment of individuals with classic galactosemia.

We were also able to draw correlates between the *GALT* null fruit fly carrying a transgenic insertion of *hGALT* cDNA that conferred *GAL4* independent enzymatic activity and classic galactosemic alleles that retain a cryptic level of GALT activity. We used our leaky transgene in the *GALT* null background to show that minimal GALT activity (approximately 6% of control) was sufficient to increase survival during development on a diet high in galactose (Table 2.2). These animals, when raised in a galactose-restricted manner, also climb better than those without GALT activity (Figure 3.3). These flies are similar to patients homozygous for the S135L.hGALT allele in their level of enzymatic activity and phenotype. Individuals homozygous for the S135L allele are more capable of metabolizing galactose and may be less affected with long-term outcome complications (6-8). It appears that a significant modifier of outcome is cryptic levels of GALT activity in both the fruit fly and patients with classic galactosemia.

In our study of the GALT null fruit fly, dietary galactose exposure is lethal if persistent and elevated throughout development, but if the dietary galactose is at lower concentrations and/or during shorter times during development it is not lethal. A few days of exposure to high levels of dietary galactose early in larval development may not be lethal to significant numbers of flies (Figure 2.3 and 2.4) . Low level dietary galactose exposure throughout development did not exacerbate the movement defect. Is the dietary galactose dependent lethality specific to a time in development? Or an overall galactose load? Or both? Published reports of adult wild-type flies exposed to galactose as their sole sugar source showed a decreased in lifespan and an increase of reactive oxygen

species (9). In our lab, others have investigated the affect of high dietary galactose exposure (while glucose is present) on the lifespan of our control or *GALT* null animals. When glucose is supplemented with high galactose concentrations, neither the controls nor *GALT* null animals had consistent decreases in lifespan, leaving the role of galactose exposure in adult flies unanswered (data not shown). Since it is unclear what role dietary galactose exposure plays in the long-term complications of patients with classic galactosemia, the fly model is an excellent tool to parse out when and how much galactose is tolerated. For instance, in humans we know in newborns with the disease, galactose exposure can be lethal but in adults with classic galactosemia galactose exposure may be tolerated (10). Trying to understand the timing and impact of galactose exposure in the fruit fly may eventually inform patient treatment and diet options.

We do not understand the understanding mechanism of GALT loss leading to a movement defect in the adult fruit fly; there was no gross anatomical defect present. Therefore, the movement defect must be caused by a subtle imbalance in an organelle, cell-type or tissue at some point during development. While the various parts of neurons appear normal it is possible that glucose metabolism, oxidative stress response or cellular phosphatidylinositol signaling is substandard because of GALT impairment and galactose-1-phosphate accumulations. Since heads are easily removed from adult fruit flies, we could measure metabolites in the brains of *GALT* null and control fruit flies to investigate if galactose-1-phosphate accumulates in the adult fly head in spite dietary galactose restriction. Another possibly would be to test the glucose metabolism, oxidative stress response and cellular phosphatidylinositol signaling in a *GALT* null S2 cell-line. Screening pathway modifiers would be efficient in the cell-line; candidates would then be

tested in the whole fly for their ability to modify the adult movement defect. These experiments would help us understand if the modifying pathways in the yeast model are relevant in a multi-cellular model of classic galactosemia

With the establishment of this multi-cellular model of classic galactosemia, work can begin to understand the pathophysiology of the various symptoms. Work exploring the mechanism behind the galactose dependent lethality has already begun (11). Oxidative stress plays a significant role in the toxicity associated with galactose exposure in the *GALT* null fly. Work ongoing in the lab is exploring oxidative stress and its role in the movement phenotype as well. If oxidative stress is a mechanism behind the tissue specific complications of classic galactosemia, this could open new avenues for patient care.

There may be other consequences of GALT impairment in the fruit fly that we have yet to investigate. We hope to determine if the *GALT* null fly has impaired fertility; girls and women with classic galactosemia face an 80 to 90% rate of POI (12). The genetic location of *GALT* in the *Drosophila* genome, in an intron of *cup*, complicates any investigation of female fertility impairment in the *GALT* null fly. The gene *cup* is expressed in female ovaries and is essential in setting up the location and repression of a number of RNAs required for patterning during embryogenesis. The *GALT* null alleles complemented a strong *cup* allele and the GALT null excision is contained inside the intron of *cup*. Therefore it is possible that females with the *GALT* null allele have normally functioning *cup* and therefore could be examined for problems with fertility. The information gathered from the fruit fly model of classic galactosemia has begun to inform our understanding on how the symptoms in patients relate to their genotype and

dietary galactose exposure. Potentially one day our understanding of the mechanisms at work in the *GALT* null phenotypes can inform better treatment for people with classic galactosemia. The *GALT* null fruit fly still has much to reveal to us about the pathophysiology of classic galactosemia.

5.3 SCHOLASTIC AND BEHAVIORAL CONSEQUENCES IN CLASSIC GALACTOSEMIA

Summary

Classic galactosemia is a disease with many complications that impact a patient's quality of life; these complications may co-vary to some extent in the population (13, 14). Before we undertook the work summarized in Chapter Four there were reports of behavioral consequences as well as the cognitive problems in children and adults with classic galactosemia (15-17). Similar reports, in the general population, have shown that developmental delay and problems with executive functioning co-vary with behavioral problems as well (18). We focused on school age children because they are the most at risk for developing behavior problems and could benefit the most from a potential intervention. Classic galactosemia is rare; therefore our study volunteers were limited in number and come from throughout the US and Canada. We used parent and teacher specific surveys to assess behavior. (19-23). We found our study volunteers had a higher than expected rate of behavior problems (Table 4.2). Specifically our study volunteers had a higher incidence of internalizing problems, including anxiety and depression, and problems with social interaction, like the ability to communicate and follow directions. We assessed our study volunteer's scholastic ability as well and found that it and their enrollment in special education co-varied to some extent with these behaviors.

We looked at GALT activity and its ability to modify symptom severity in our study volunteers. Most studies prior have looked at genotype (e.g. Q188R homozygotes v. Q188R/other) to determine if cryptic GALT activity could play a role in modifying the

severity of a complication. Because of the heterogeneity of GALT genotypes in our population we harnessed information gained from the yeast model system previously developed in the lab (24). Most recently, work done by Tyler Gleason (manuscript in preparation) has shown some classic galactosemia alleles have low levels of cryptic GALT activity when they are expressed in yeast. Using these results, we calculated our study volunteers cryptic GALT activity based on the activity of each allele, in comparison to wild-type hGALT expressed in yeast. Only six study volunteers (out of 54) were predicted to have residual GALT activity ≥1% and therefore we lost statistical power due to our small sample size. Even with the limits of our study, we saw definite trends among those with predicted GALT activity. Individuals with cryptic levels of GALT activity were more likely to achieve at grade level in math and language arts and had less likely to have behavior problems (Figures 4.1 and 4.2 and Table 4.5). In the instance of the teacher reported social skills we saw a statistically significant difference between the group who had ≥1% predicted residual GALT activity and the group that had ≤1%. The link between GALT activity, or genotype, and severity of phenotype has been shown in other studies of classic galactosemia establishing one modifier of the long-term complications of classic galactosemia (5, 14, 25). Cryptic GALT activity is not the only modifier; there were still some study volunteers with no predicted residual GALT activity doing well in school and reporting no behavioral problems (Figures 4.1 and 4.2 and Table 4.5). This study establishes that there is a behavioral phenotype in school age children with classic galactosemia.

Implications and Future Directions

The complications of classic galactosemia may not be independent of each other. In our study we found behavior and scholastic ability highly correlated; others have seen a correlation between speech and IQ (14). With many complications being common among individuals with classic galactosemia it is difficult to determine if a delay in language is associated with a delay in development, or if an inability to interact in social settings is caused by a low IQ. In the general population these quality of life complications appear together and independently; in classic galactosemia is it co-morbidity or just happenstance? There is no clear answer. What we know from studies in the general population is different interventions are needed for individuals with more than one complication, i.e. those with a low IQ and difficulty with social interactions need different types of support than those with only one of these complications. To that end this study has helped us understand that school age children with classic galactosemia are at higher risks for internalizing problems and more likely to have problems with social interactions as well as difficulty in school. Therefore it is not sufficient to treat just one problem. This initial study will inform a colleague, Dr. Elles Taddeo, and from this she hopes to formulate a parent based intervention toolkit based on her previous work with autism spectrum disorder and children with fetal alcohol spectrum disorder (26). These interventions could lead to improvement of behavior and scholastic achievement in children with classic galactosemia and improve their quality of life.

As the population of individuals with classic galactosemia age it has become obvious that the level of independent function is different for each individual. Most finish high school and begin skilled labor jobs but may not be able to live independently; a few

graduate college and live completely “normal” independent adult lives (13, 15). Many complications common in classic galactosemia can impact quality of life and this has become a new avenue of research in classic galactosemia. As this field expands it will become increasing important to identify the behavioral and psychosocial issues for individuals with classic galactosemia to determine the best treatment. The goal of this study was to begin to assess the behavioral and scholastic consequences in children with classic galactosemia

5.4 REFERENCES

1. Segal S (2006) Of mice and men: galactosemia. *Mol Genet Metab* 89(4):401-402.
2. Hughes J, *et al.* (2009) Outcomes of siblings with classical galactosemia. *J Pediatr* 154(5):721-726.
3. Rhodenizer D, Martin I, Bhandari P, Pletcher SD, & Grotewiel M (2008) Genetic and environmental factors impact age-related impairment of negative geotaxis in Drosophila by altering age-dependent climbing speed. *Exp Gerontol* 43(8):739-748.
4. Doyle CM, Channon S, Orlowska D, & Lee PJ (2010) The neuropsychological profile of galactosaemia. *J Inherit Metab Dis* 33(5):603-609.
5. Shield JP, *et al.* (2000) The relationship of genotype to cognitive outcome in galactosaemia. *Arch Dis Child* 83(3):248-250.
6. Wells L & Fridovich-Keil JL (1997) Biochemical characterization of the S135L allele of galactose-1-phosphate uridylyltransferase associated with galactosaemia. *J Inherit Metab Dis* 20(5):633-642.
7. McCorvie TJ & Timson DJ (2011) Structural and molecular biology of type I galactosemia: disease-associated mutations. *IUBMB Life* 63(11):949-954.
8. Lai K, *et al.* (1996) A prevalent mutation for galactosemia among black Americans. *J Pediatr* 128(1):89-95.
9. Cui X, *et al.* (2004) D-galactose-caused life shortening in Drosophila melanogaster and Musca domestica is associated with oxidative stress. *Biogerontology* 5(5):317-325.

10. Krabbi K, Uudelepp ML, Joost K, Zordania R, & Ounap K (2011) Long-term complications in Estonian galactosemia patients with a less strict lactose-free diet and metabolic control. *Mol Genet Metab* 103(3):249-253.

11. Jumbo-Lucioni PP*, et al.* (2012) Oxidative stress contributes to outcome severity in a Drosophila melanogaster model of classic galactosemia. *Dis Model Mech* .

12. Sanders RD*, et al.* (2009) Biomarkers of ovarian function in girls and women with classic galactosemia. *Fertil Steril* 92(1):344-351.

13. Hoffmann B, Dragano N, & Schweitzer-Krantz S (2012) Living situation, occupation and health-related quality of life in adult patients with classic galactosemia. *J Inherit Metab Dis* .

14. Potter NL, Lazarus JA, Johnson JM, Steiner RD, & Shriberg LD (2008) Correlates of language impairment in children with galactosaemia. *J Inherit Metab Dis* 31(4):524-532.

15. Waisbren SE*, et al.* (2011) The adult galactosemic phenotype. *J Inherit Metab Dis* .

16. Lambert C & Boneh A (2004) The impact of galactosaemia on quality of life--a pilot study. *J Inherit Metab Dis* 27(5):601-608.

17. Antshel KM, Epstein IO, & Waisbren SE (2004) Cognitive strengths and weaknesses in children and adolescents homozygous for the galactosemia Q188R mutation: a descriptive study. *Neuropsychology* 18(4):658-664.

18. Vuontela V*, et al.* (2012) Working Memory, Attention, Inhibition, and Their Relation to Adaptive Functioning and Behavioral/Emotional Symptoms in School-Aged Children. *Child psychiatry and human development* .

19. Gresham FM, Elliott SN, & Kettler RJ (2010) Base rates of social skills acquisition/performance deficits, strengths, and problem behaviors: an analysis of the Social Skills Improvement System--Rating Scales. *Psychol Assess* 22(4):809-815.

20. Gresham FM & Elliott SN (2008) *SSIS (Social Skills Improvement System) Rating Scales* (NCS Pearson, Inc,, Minneapolis, MN).

21. Achenbach TM & Ruffle TM (2000) The Child Behavior Checklist and related forms for assessing behavioral/emotional problems and competencies. *Pediatr Rev* 21(8):265-271.

22. Achenbach TM & Rescorla L (2001) *Manual for the ASEBA school-age forms & profiles : an integrated system of multi-informant assessment* (ASEBA, Burlington, VT) pp xii, 238 p.

23. Achenbach TM & Rescorla L (2000) *Manual for the ASEBA preschool forms & profiles : an integrated system of multi-informant assessment* (ASEBA, Burlington, VT) pp xi, 178 p.

24. Riehman K, Crews C, & Fridovich-Keil JL (2001) Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase. *J Biol Chem* 276(14):10634-10640.

25. Guerrero NV, *et al.* (2000) Risk factors for premature ovarian failure in females with galactosemia. *J Pediatr* 137(6):833-841.