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Rebekah Felice Kushner

The Role of Galactose-1-Phosphate Uridylyltransferase in *Drosophila melanogaster* Development and Homeostasis

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The Role of Galactose-1-Phosphate Uridylyltransferase in *Drosophila melanogaster* Development and Homeostasis

By

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B.S., George Washington University, 2005

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Abstract

The Role of Galactose-1-Phosphate Uridylyltransferase in *Drosophila melanogaster* Development and Homeostasis

By Rebekah Felice Kushner

Galactose metabolism occurs primarily through the Leloir pathway, which is highly conserved from *E. coli* to humans and is catalyzed by three enzymes: **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). In humans, impairment in any one of the Leloir enzymes results in the metabolic disorder, galactosemia. The most common clinically severe form of galactosemia is **classic galactosemia**, a potentially lethal disorder resulting from profound GALT impairment. The current standard of care remains the lifelong dietary restriction of galactose, which prevents or reverses the acute and potentially lethal symptoms of the disorder, but is insufficient to prevent the long-term complications, which include cognitive, motor, speech, and female reproductive dysfunction. Decades of research have been hindered by the lack of a genetic animal model that recapitulates the human phenotype, and the underlying pathophysiology of this disorder remains poorly understood. Here we report the creation and initial characterization of a *Drosophila melanogaster* model of classic galactosemia that mimics aspects of the human disorder. Like humans, GALT-deficient flies survive under conditions of galactose restriction but die in a dose dependent manner when exposed to galactose during development. These animals also exhibit neurological complications despite dietary restriction of galactose. Both the acute and long-term phenotypes observed can be rescued by the ubiquitous transgenic expression of wild-type human *GALT*. Using this *D. melanogaster* model, we have begun to dissect the timing and extent of the galactose sensitivity in GALT-null animals, as well as the role(s) of GALT function in organismal development and homeostasis. The existence of an animal model may identify new targets for novel and effective treatments, enabling a better quality of life for patients.

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CHAPTER 1

INTRODUCTION

The primary purpose of this dissertation is to describe the creation and the initial characterization of the first genetic animal model of classic galactosemia that recapitulates the human disorder using the fruit fly, *Drosophila melanogaster*. In addition, this document contains a chapter describing the creation of an assay for hGALK activity for the application of high-throughput screening of small molecule libraries in order to identify specific inhibitors of this enzyme. These seemingly unrelated projects were attempted with the same long-term goals: to find more effective treatments for classic galactosemia and improve the quality of life for patients.

In order to introduce the reader to this field, this chapter will include information on galactose metabolism and the disorders that result when galactose metabolism is impaired (the galactosemias). The structures of the GALK and GALT genes and proteins will be examined, as they pertain, in the case of GALT, to the enzyme activity missing in classic galactosemia, and in the case of GALK, to the protein structure utilized to identify small molecule inhibitors for the hGALK protein. Next, past research that has been conducted to further the understanding of the underlying pathophysiology of classic galactosemia will be reviewed in order to highlight the gaps in our current knowledge of this disorder, which has been greatly hindered by the lack of an appropriate animal model of the disease. Finally, the use of other model systems, including yeast and mammalian cell culture will be discussed, highlighting the research these systems have made possible in this field as well and their intrinsic limitations. This chapter will conclude with an introduction to the *D. melanogaster* model system and why we have chosen this model system for the establishment of the first appropriate genetic model of classic galactosemia.

Galactose Metabolism

Galactose is an abundant sugar in milk and other dairy products as a component of the disaccharide, lactose. Both free and bound galactose is found in certain fruits, vegetables, and legumes (Cozen et al. 2002; Kim et al. 2007). Human newborns, whose diet consists entirely of breast milk, depend on galactose to provide close to half of their sugar calories. Galactose metabolism occurs primarily through the Leloir pathway, which is highly conserved from *Escherichia coli* (*E. coli*) to humans and is catalyzed by three major enzymes: **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 1.1**).

Galactose metabolism begins when a molecule of galactose enters the cell through one of the glucose transporter (GLUT) proteins and is phosphorylated by GALK to form galactose-1-phosphate (gal-1P). The next enzyme in the Leloir pathway, GALT, uses gal-1P and a molecule of UDP-glucose (UDP-glc) to form glucose-1-phosphate (glc-1P) and UDP-galactose (UDP-gal). The final enzyme in the pathway, GALE, interconverts UDP-gal and UDP-glc in a reversible and NAD+-dependent reaction (Holden et al. 2003; Leslie 2003; Ross et al. 2004; Fridovich-Keil and Walter 2008). As a product of the pathway, glc-1P is further metabolized by the enzyme phosphoglucomutase into glucose-6-phosphate (glc-6P), which enters directly into the energy producing pathway of glycolysis. Since only a single molecule of ATP is required to convert galactose into glc-1P, galactose and glucose release equal amounts of energy when metabolized (Frey 1996). Finally, UDP-gal is used as a substrate donor for the addition of galactose to

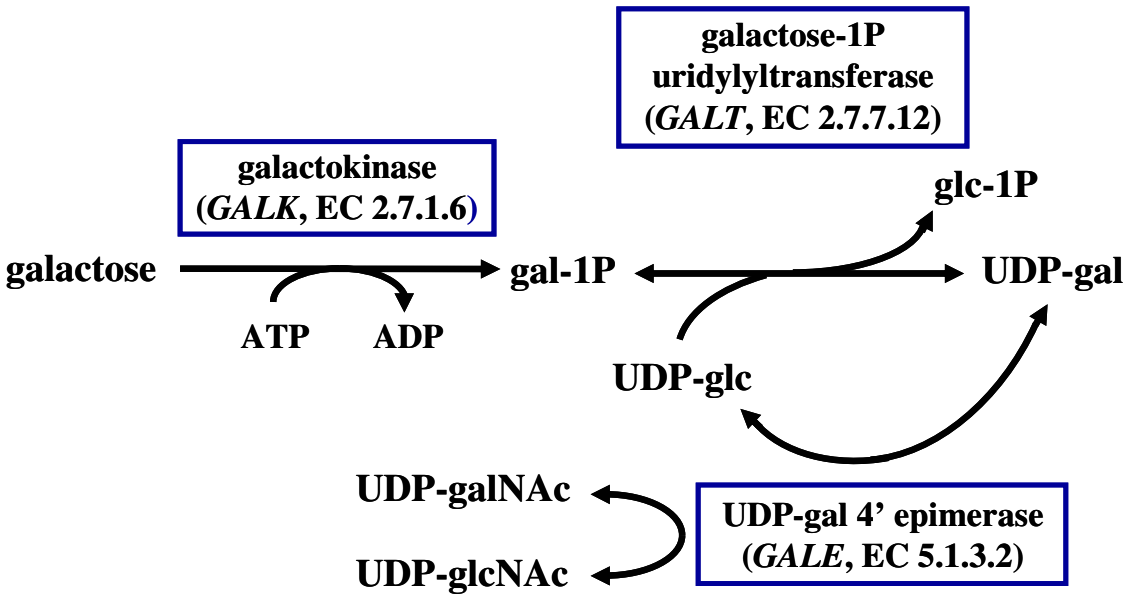

Figure 1.1

The Leloir pathway of galactose metabolism.

Free galactose is metabolized through the Leloir Pathway, which consists of three major enzymes. Galactose if first converted into galactose-1-phosphate (gal-1P) by galactokinase (GALK). Next, galactose-1-phosphate uridylyltransferase (GALT) converts gal-1P and UDP-glucose (UDP-glc) into glucose-1-phosphate (glc-1P) and UDP-galactose (UDP-gal). The final enzyme, UDP-galactose 4'-epimerase (GALE), interconverts UDP-gal and UDP-glc, and UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylglucosamine (UDP-glcNAc).

growing glycan chains (galactosylation), for the biosynthesis of glycoproteins and glycolipids (Fridovich-Keil and Walter 2008).

In humans, impairment in any one of the Leloir enzymes results in the metabolic disorder, galactosemia. As with other metabolic disorders, untreated galactosemic patients accumulate abnormal levels of the metabolites that precede and follow the impaired enzymatic step. These metabolic abnormalities may underlie or contribute to the clinical sequelae of galactosemia. Fortunately, galactose can also be shuttled into other bypass metabolic pathways in humans and other organisms (**Figure 1.2**). For example, as galactose accumulates, it can be reduced into the sugar alcohol, galactitol, by aldose reductase (AR), or oxidized into galactonate by galactose dehydrogenase (the gene for this enzyme has not yet been discovered). Additionally, levels of gal-1P can be modulated by the action of UDP-glucose pyrophosphorylase (UGP), which can convert gal-1P into UDP-gal using a molecule of UTP. It has been shown, however, that the activity of UGP on galactose derivatives is much lower than that of GALT; therefore, this enzyme cannot fully compensate for the loss of GALT activity (Leslie 2003; Fridovich-Keil and Walter 2008).

In organisms with an enzymatic impairment in the Leloir pathway, these alternative pathways of galactose metabolism may modulate phenotypic outcomes by creating or metabolizing molecules that are involved in the pathophysiology of galactosemia. In order to improve the quality of life of patients with galactosemia, these modifiers may be potential targets of novel treatments, such as small molecule inhibitors.

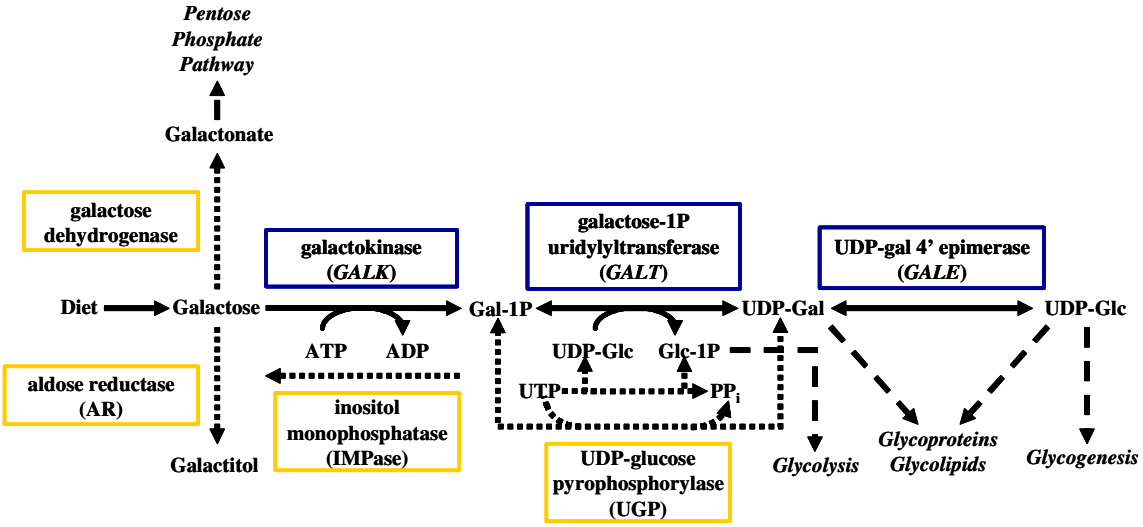

Figure 1.2

Alternative pathways of galactose metabolism.

The Leloir pathway enzymes are shown in blue. Alternative pathway enzymes are shown in yellow boxes and metabolites are linked by short-dashed arrows. Long-dashed arrows indicate where there exists overlap with other pathways, including glycosylation of proteins and lipids, glycogenesis, glycolysis, and the pentose phosphate pathway.

Galactosemia

Impairment in any one of the three Leloir enzymes in humans results in a group of metabolic disorders, generally referred to as galactosemia, but further defined based on the specific enzyme impacted. The most common, clinically severe form of galactosemia results from the profound impairment in the second enzyme of the pathway, GALT, and is referred to as **classic galactosemia**. The comparison of this group of disorders has revealed that patient outcome depends upon the enzyme activity impacted and to what extent it is impaired.

GALK deficiency galactosemia

Galactokinase deficiency (OMIM 230200) was first recognized clinically by Gitzelmann in 1967 and occurs in less than 1/100,000 live births (Gitzelmann 1967; Levy 1980; Fridovich-Keil and Walter 2008). Untreated patients accumulate high levels of galactose in their blood and tissues; without GALK activity, galactose cannot be converted to gal-1P (Bosch et al. 2002; Leslie 2003; Holden 2004; Thoden et al. 2005).

In contrast to patients with classic galactosemia, patients with galactokinase deficiency do not present with acute and potentially lethal or long-term complications. Neonatal cataracts, usually bilateral, are highly common due to the conversion of galactose into its corresponding alcohol sugar, galactitol, by AR. The lens cells of the eyes are particularly sensitive to high levels of galactitol, which cannot be transported across the cell membrane. As it accumulates within the cell, galactitol alters its osmotic potential. In the lens of the eye, this results in the formation of cataracts due to cell swelling and death (Thoden et al. 2005). Additionally, pseudotumor cerebri (elevated

intracranial pressure) has been observed in several cases of GALK deficiency (Bosch et al. 2002; Holden 2004). Cataracts and other symptoms are usually reversible upon commencement of a galactose restricted diet (Bosch et al. 2002; Tyfield and Walter 2002).

GALT deficiency galactosemia (classic galactosemia)

First described in 1908 by von Reuss and enzymatically defined by Kalckar in 1956, the most common clinically severe form of galactosemia, **classic galactosemia**, is an autosomal recessive disorder resulting from profound GALT impairment and occurring in approximately 1/60,000 live births (Kalckar 1965; Gitzelmann 1995; Ridel et al. 2005; Fridovich-Keil and Walter 2008). Other forms of GALT deficiency do exist, including a variant associated with an allele referred to as the Duarte allele, which are characterized by mutations that partially impair GALT enzyme activity (Fridovich-Keil and Walter 2008). For the purposes of the work described in this dissertation, the focus will remain on the profound impairment of GALT activity and classic galactosemia.

The diagnosis of classic galactosemia occurs within the first few weeks of life with newborn screening or the appearance of symptoms following exposure to a milk-based diet, such as cataracts, failure to thrive, diarrhea, vomiting, jaundice, *E. coli* sepsis, and death (Holton et al. 2000; Tyfield and Walter 2002; Antshel et al. 2004). In the 1930s, Mason and Turner were able to reverse the acute and potentially lethal symptoms of classic galactosemia by restricting galactose from the diets of patients (Mason and Turner 1935). In combination with the enzyme assay developed by Beutler in the 1960s, which could screen for newborns with galactosemia, early intervention was finally

possible. By the 1980s, however, it was obvious that severe long-term complications were not avoided by early detection and strict compliance with a galactose restricted diet (Ridel et al. 2005).

Today, a similar newborn screen is used from the dried blood spot collected to test for phenylketonuria (PKU) and other genetic disorders in the United States and elsewhere. The most widely accepted form of treatment remains the lifelong dietary restriction of galactose, which prevents or reverses the acute and potentially lethal symptoms, but is insufficient for the prevention of long-term complications, including cognitive, motor, speech, and female reproductive dysfunction. Approximately 30% to 50% of all patients suffer from learning disabilities and/or other complications, such as verbal dyspraxia, delayed growth, impaired motor function, ataxia (impaired coordination and balance), and decreased bone density. Concurrently, approximately 85% of female patients suffer from primary or premature ovarian insufficiency (POI) (Kaufman et al. 1988; Waggoner et al. 1990; Kaufman et al. 1994; Holton 1996; Charlwood et al. 1998; Tyfield and Walter 2002; Antshel et al. 2004; Ridel et al. 2005). Clearly, dietary restriction of galactose alone is not a sufficient treatment for the long-term management of classic galactosemia. Despite decades of research, the underlying pathophysiology of the acute and long-term complications of classic galactosemia remains unknown.

Untreated patients with classic galactosemia, like with other metabolic disorders, accumulate abnormal levels of the metabolites that precede the impaired enzymatic step; in this case, these metabolites include galactose, gal-1P, and galactitol. Patients who adhere to the dietary restriction of galactose show a dramatic improvement in these metabolic abnormalities, but blood gal-1P levels sometimes still remain above normal.

While dramatically elevated levels of gal-1P seen in untreated patients correlate with the presence of acute symptoms, there does not seem to be a reproducible correlation between the subtle levels of gal-1P in treated patients and long-term outcome severity (Kaufman et al. 1988; Waggoner et al. 1990; Ng et al. 1991; Kaufman et al. 1994; Gitzelmann 1995; Xu et al. 1995; Leslie 2003; de Jongh et al. 2008; Fridovich-Keil and Walter 2008).

One postulated mechanism for the apparent toxicity of gal-1P involves the inhibition of crucial enzymes, including glucose-6-phosphatase, glycogen phosphorylase, and UDP-galactose galactosyltransferase by high gal-1P levels *in vitro* (Gitzelmann and Bosshard 1995). UDP-galactose galactosyltransferase is a critical enzyme for the proper glycosylation of proteins and lipids because galactose is an important subterminal sugar in glycan chains; several studies have identified aberrant glycosylation patterns in classic galactosemic patients (Dobbie et al. 1990; Petry et al. 1991; Jaeken et al. 1992; Ornstein et al. 1992; Prestoz et al. 1997; Charlwood et al. 1998; Lebea and Pretorius 2005; Sturiale et al. 2005).

One rationale that has been posed to explain the differences in the outcomes of GALK deficiency galactosemia and classic galactosemia postulates that the accumulation of the product of the GALK reaction, gal-1P, contributes to the acute and long-term sequelae of classic galactosemia (Leslie 2003; Slepak et al. 2005). Many have hypothesized that the selective inhibition of GALK, and the subsequent arrest of gal-1P production, may prevent the acute and long-term complications that plague patients with classic galactosemia (Timson and Reece 2003; Bosch et al. 2004; Fridovich-Keil and

Walter 2008). However, at present, this hypothesis remains untested beyond microbial systems (Ross et al. 2004).

GALE deficiency galactosemia

The third and least well understood form of galactosemia, **epimerase deficiency** (OMIM 230350), occurs when the third enzyme of the Leloir pathway is impaired (Tyfield and Walter 2002; Openo et al. 2006). GALE deficiency is a spectrum disorder spanning from an apparently benign peripheral condition, where the enzyme impairment occurs only in circulating blood cells, to a generalized disorder, where a range of tissues are affected and patients may suffer from complications similar to those of classic galactosemia (Gitzelmann 1972; Gitzelmann and Steimann 1973; Mitchell et al. 1975; Gitzelmann et al. 1976; Sardharwalla et al. 1988; Schulpis et al. 1993; Schulpis et al. 1993; Quimby et al. 1997; Alano et al. 1998; Walter et al. 1999; Wohlers et al. 1999; Shin et al. 2000; Openo et al. 2006).

Human GALE is able to catalyze both the interconversion of UDP-gal and UDP-glc, and the interconversion of UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylglucosamine (UDP-glcNAc) (Piller et al. 1983; Schulz et al. 2004). These UDP sugars are essential for processes such as glycogenesis and glycosylation; UDP-glc is used to add molecules of glucose to a growing glycogen chain, and all four UDP sugars are necessary substrate donors for the creation of glycan chains for the glycosylation of proteins and lipids.

GALK and GALT Enzymes

The crystal structures for both GALK and GALT proteins have been elucidated, allowing for a molecular analysis of dimer interactions and the impact of specific mutations on enzyme activity. Additionally, the development of novel and more effective treatments of classic galactosemia may include small molecules that regulate enzyme activities. New technology allows for the *in silico* screening of small molecule libraries against the structure of an enzyme's active site. Such a study will be described in **Chapter III** as a method to identify small molecule inhibitors of hGALK, a predicted modifier of the patient outcome of classic galactosemia.

GALK Protein

Galactokinase (**GALK**, EC 2.7.1.6) catalyzes the first step of the Leloir pathway of galactose metabolism. A molecule of galactose enters the cell through GLUT transporter proteins and is acted upon by GALK, which converts galactose into a molecule of gal-1P, using a molecule of ATP (Leslie 2003; Timson and Reece 2003; Fridovich-Keil and Walter 2008). The phosphorylation of galactose inhibits it transport back outside the cell, as there are no transporter proteins for phosphorylated sugars. The GALK reaction allows for energy to be conserved by avoiding the repeated transport of galactose across the cell membrane, trapping the sugar in the cell for further metabolism and ATP production (Leslie 2003). GALK activity can be regulated through a feedback mechanism by its substrate, galactose, and its product, gal-1P. Therefore, in the presence of high levels of gal-1P, such as in a GALT-deficient organism, GALK activity will be inhibited by its product, leading to the accumulation of galactose in plasma and urine, and

limiting the amount of gal-1P being produced (Leslie 2003; Fridovich-Keil and Walter 2008).

The human galactokinase (hGALK) protein is composed of 392 residues and is encoded by the *GALK1* gene, which is located on the human chromosome 17p24 and is composed of 8 exons spanning 7.3 kb (Fridovich-Keil and Walter 2008). GALK is a member of the GHMP superfamily of proteins based on amino acid sequence similarity (GHMP stands for the members of this family: *g*alactokinase, *h*omoserine kinase, *m*evalonate kinase, and *p*hosphomevalonate kinase) (Holden et al. 2003; Holden 2004). GHMP kinases contain three major conserved motifs, including a motif involved in the binding of ATP, and the conserved sequence Pro-X-X-X-Gly-Leu-X-Ser-Ser-Ala (where X refers to any amino acid) (Holden 2004).

Thoden and colleagues have determined the structure of hGALK in the presence of α-D-galactose and the nonhydrolyzable ATP analog, Mg-AMPPNP, using x-ray crystallography (Thoden et al. 2005). Studies have demonstrated that hGALK acts through an ordered reaction mechanism, in which MgATP binds the active site first, followed by the binding of galactose (Holden et al. 2003; Timson and Reece 2003). A ball-and-stick model of the potential interactions between hGALK and its substrates has been designed by Thoden and colleagues. The kinetics of this reaction have been determined, where the K_m for α-D-galactose is 970±220 μM and the K_m for ATP is 34±4 μM; the K_{cat} for human galactokinase is 8.7±0.5 s^{-1} (Timson and Reece 2003). Size-exclusion chromatography suggests that hGALK is a 42 kDa monomer, but the structural data suggest that hGALK is a homodimer (Timson and Reece 2003; Thoden et al. 2005). Additional experiments need to be conducted in order to clear up these inconsistent data.

The existence of the crystal structure of this enzyme is essential for the discovery of specific, small molecule inhibitors. It has been widely hypothesized that the selective inhibition of the hGALK protein *in vivo* may modulate the sequelae of classic galactosemia (Bosch et al. 2002; Timson and Reece 2003; Bosch 2006). Fortunately, the structure of hGALK is markedly different from that of human glucokinase, which is involved in important cellular processes including carbohydrate metabolism, presenting the possibility of the selective inhibition of hGALK.

Kinases have been implicated in roles in cancer and some forms of diabetes (specifically, glucokinase in the later). Research has focused on the identification of small molecule inhibitors of these enzymes in order to provide new and effective treatments for these diseases (Fabian et al. 2005; Printz RL 2005). **Chapter III** will describe the creation of a high-throughput assay to screen for small molecule inhibitors of hGALK. The goal of this study is to identify novel, supplemental treatments for classic galactosemia.

GALT Protein

Galactose-1-phosphate uridylyltransferase (**GALT**, EC 2.7.7.12) catalyzes the second step of the Leloir pathway of galactose metabolism. After GALK converts galactose into gal-1P, GALT uses the molecule of gal-1P and one of UDP-glc to create molecules of glc-1P and UDP-gal via a ping-pong (double displacement) reaction mechanism. During this reaction, the uridylyl (UMP) group is transferred from UDP-glc to gal-1P through an intermediate UMP-GALT step, where the UMP group covalently binds GALT at residue His^{166}. Glc-1P is released and the UMP-GALT intermediate

reacts with gal-1P, producing UDP-gal and the regenerated GALT enzyme (Frey 1996; Geeganage and Frey 2002; Marabotti and Facchiano 2005).

The human galactose-1-phosphate uridiylyltransferase (hGALT) protein is composed of 379 amino acids and is encoded by the *GALT* gene on the human chromosome 9p13, which is composed of 11 exons spanning 4.3 kb (Leslie et al. 1992; Tyfield et al. 1999; Bosch et al. 2005; Ridel et al. 2005). The translated product is a 44 kDa polypeptide, but the active hGALT protein is a homodimer of 88 kDa, which requires two Zn/Fe ions for stable dimeric interactions (Wang et al. 1998; Tyfield et al. 1999; Fridovich-Keil and Walter 2008).

GALT is a member of the histidine triad (HIT) family of enzymes, which contain the HisϕHisϕHis$\phi\phi$ motif (where ϕ refers to a hydrophobic amino acid) (Holden et al. 2003; Leslie 2003; Marabotti and Facchiano 2005). The GALT active site, encoded by exon 6, includes a HPH sequence, which is conserved from *E. coli* to humans (Quimby et al. 1996; Fridovich-Keil and Walter 2008). In 1995, the structure of the *E. coli* GALT enzyme was elucidated in the presence of UDP-glc using x-ray crystallography (Wedekind et al. 1995; Holden et al. 2003). Additionally, Marabotti and colleagues have created theoretical three-dimensional models of the human GALT enzyme to study the effects of mutations on structure and dimeric interactions (Marabotti and Facchiano 2005).

More than 200 GALT mutations have been identified and cataloged in online databases (www.arup.utah.edu/database/galactosemia/GALT_welcome.php) (Tyfield 2000; Bosch et al. 2005; Ridel et al. 2005; Fridovich-Keil and Walter 2008). Due to the large number of known GALT mutations, it is possible that genomic heterogeneity is a

contributing factor in the range of phenotypic outcomes observed in patients with classic galactosemia (Quimby et al. 1996). The most common mutation linked to classic galactosemia in European and Caucasian populations is the Q188R mutation, where an adenine (A) to guanine (G) transition in exon 6 leads to the glutamine (Q) to arginine (R) conversion in the translated protein (Tyfield 2000; Ridel et al. 2005). The Q188R mutation has been demonstrated to have no detectable GALT activity in studies with the yeast and mammalian cell culture model systems (Fridovich-Keil et al. 1995). It has been hypothesized by Holden and colleagues that this mutation leads to the stabilization of the uridylyl-GALT intermediate, inhibiting its reaction with gal-1P (Holden et al. 2003).

Many of the remaining mutations have not yet been characterized by their effect on enzyme activity. Characterizing human mutations becomes more complicated due to the fact that hGALT exists as a dimer and that many classic galactosemic patients are compound heterozygotes. Therefore, the effect of mutations on subunit interactions and the activity of heterodimers becomes important in understanding the relationship between genotype and outcome (Elsevier et al. 1996; Marabotti and Facchiano 2005).

Pathophysiology of classic galactosemia

Despite the fact that galactosemia has been studied for decades, the underlying pathophysiology of the disease is poorly understood (Holton et al. 2000; Tyfield and Walter 2002; Leslie 2003; Fridovich-Keil and Walter 2008). It is known that patients with classic galactosemia accumulate abnormally high levels of the metabolites preceding the impaired enzymatic step (galactose, galactitol, gal-1P, etc.) and have aberrant protein

and lipid glycosylation patterns (Leslie 2003; Fridovich-Keil and Walter 2008). The adherence of patients to a galactose restricted diet markedly improves these metabolic and biochemical phenotypes, but gal-1P levels remain above normal and some patients continue to show mildly aberrant glycosylation patterns. Therefore, it is apparent that the current treatment is not sufficient for the reversal of these phenotypes, or a high quality of life for classic galactosemic patients, who suffer from severe long-term complications despite early and continuous treatment (Waggoner et al. 1990).

Metabolites

The relationship between the accumulation of metabolites and the tissue and organ damage that underlies the acute and long-term complications of classic galactosemia is unclear. Classic galactosemic patients accumulate high levels of galactose, gal-1P, and galactitol in their blood and tissues. Galactonate is also produced in high quantities, but is excreted in urine and does not build up in tissues (Holton et al. 2000; Tyfield and Walter 2002).

In utero studies have shown that at 10 weeks of gestation, a classic galactosemic fetus displayed elevated levels of galactitol in the amniotic fluid. At 20 weeks, two fetuses studied showed elevated levels of gal-1P in the blood and high levels of galactose, gal-1P, and galactitol in their livers. These levels were similar to those seen in newborn infants succumbing to the disease (Ng et al. 1977; Allen et al. 1980; Holton et al. 2000). Additionally, high gal-1P levels have been found in the umbilical cord blood of classic galactosemic babies of mothers who adhered to a galactose restricted diet during pregnancy. The restriction of dietary galactose during pregnancy does not correlate with

an improvement in prognosis or outcome in patients (Waggoner et al. 1990; Gitzelmann 1995; Holton 1996). These data suggest that metabolite toxicity and subsequent damage to tissues and organs may begin *in utero*. Additionally, the removal of galactose from the diet is not sufficient to avoid this damage.

Classic galactosemic patients who adhere to a galactose restricted diet, which prevents or reverses the acute and potentially lethal symptoms of the disease early in life, show marked improvement in metabolic abnormalities. Galactitol and gal-1P levels, however, sometimes remain higher than normal and several studies have correlated high gal-1P levels with the severity of the acute complications in classic galactosemia (Kaufman et al. 1988; Waggoner et al. 1990; Ng et al. 1991; Kaufman et al. 1994; Gitzelmann and Bosshard 1995; Xu et al. 1995; Tyfield and Walter 2002; de Jongh et al. 2008; Fridovich-Keil and Walter 2008). It is not apparent whether the residual elevation of gal-1P in treated patients is due to the fact that it is impossible to completely remove galactose from the human diet or due to the endogenous production of galactose via the Leloir pathway (Berry et al. 1995; Berry et al. 2004).

Gal-1P

One longstanding hypothesis in this field is that the cellular accumulation of gal-1P contributes to the acute and long-term complications of classic galactosemia (Gitzelmann and Bosshard 1995; Holton 1996; de Jongh et al. 2008; Fridovich-Keil and Walter 2008). Evidence supporting this hypothesis comes from studies in the yeast and mammalian cell culture model systems, and observations in human patients.

GALT-deficient yeast and human cells growth arrest or die and accumulate abnormal levels of gal-1P when exposed to galactose. The galactose sensitivity phenotype is reversed by the prevention of the synthesis or the further metabolism of gal-1P (Douglas and Hawthorne 1964; Pourci et al. 1990; Parthasarathy et al. 1997; Lai and Elsas 2000; Lai et al. 2003; Ross et al. 2004; Slepak et al. 2005). As mentioned previously, patients with classic galactosemia suffer from severe acute and long-term complications, while patients with GALK deficiency galactosemia are healthy, apart from treatable cataracts (Bosch et al. 2002; Tyfield and Walter 2002). An obvious difference between these two disorders is that patients with classic galactosemia accumulate abnormally high levels of gal-1P. While blood gal-1P levels decrease when dietary galactose restriction is commenced (>5 mM gal-1P in untreated patients, ~0.1 mM gal-1P in treated patients), levels remain above normal. Additionally, continuously high levels of gal-1P may indicate that a patient is not adhering to the galactose restricted diet, although elevated endogenous production may also contribute (Kaufman et al. 1988; Waggoner et al. 1990; Ng et al. 1991; Kaufman et al. 1994; Gitzelmann 1995; Xu et al. 1995; Tyfield and Walter 2002; de Jongh et al. 2008).

It has been hypothesized that high levels of gal-1P may be involved in the sequelae of classic galactosemia, perhaps by the direct inhibition of several crucial enzymes, including enzymes essential for the proper glycosylation of proteins and lipids (Gitzelmann 1995; Bosch 2006; de Jongh et al. 2008). Several studies have demonstrated that patients with classic galactosemia show aberrant patterns of glycosylation in proteins and lipids, even with galactose restriction. Abnormal glycosylation patterns may be a contributing factor in the acute and long-term complications of classic galactosemia, as

glycoproteins and glycolipids play integral roles in several cellular processes (Dobbie et al. 1990; Petry et al. 1991; Jaeken et al. 1992; Ornstein et al. 1992; Prestoz et al. 1997; Charlwood et al. 1998; Lebea and Pretorius 2005; Sturiale et al. 2005).

Galactitol

The role of galactitol in neonatal cataracts has been confirmed through studies with GALK deficient mice (Ai et al. 2000). Free galactose is reduced to galactitol via the aldose reductase (AR) enzyme. In humans, the production of galactitol is not reversible and the alcohol cannot easily diffuse across cell membranes, changing the osmotic potential of the cell (Leslie 2003). In the lens of the eye, this leads to cellular swelling and death, and the formation of cataracts (Holden et al. 2003; Thoden et al. 2005). Fortunately, these cataracts are treatable with a galactose restricted diet (Tyfield and Walter 2002; Bosch 2006; de Jongh et al. 2008). While its role in this symptom of galactosemia is well understood, it is still unknown whether galactitol contributes (alone or in combination with other galactose metabolites) to other acute and long-term complications of classic galactosemia. It is known that galactitol accumulates in the blood, urine, liver, and brain of classic galactosemics, and that in these patients, cataracts are a sign of a failure to adhere to the galactose restricted diet (Beigi et al. 1993; de Jongh et al. 2008).

UDP-gal

In addition to elevated levels of gal-1P, there is speculation about whether patients with classic galactosemia have diminished levels of UDP-gal, the product of the

GALT reaction. This finding, however, is controversial and studies are contradictory due, in part, to the use of different methodologies, including enzymatic, radioactive, and chromatography-based methods. In some studies, the low levels of UDP-gal still lie within the range of normal values (Ng et al. 1989; Berry et al. 1992; Keevill et al. 1993; Schweitzer et al. 1993; Keevill et al. 1994; Gibson et al. 1995; Segal 1995; Holton 1996; Riehman et al. 2001; Tyfield and Walter 2002; Lai et al. 2003). Therefore, it is unclear whether a UDP-gal deficiency exists in patients with classic galactosemia. If a deficiency in UDP-gal does exists, it has been hypothesized that it may have a role in the aberrant glycosylation patterns in patients, as UDP-gal is an integral sugar donor involved in the biosynthesis of glycoproteins and glycolipids (Petry et al. 1991; Bhat 2003; Lebea and Pretorius 2005; Slepak et al. 2005; Fridovich-Keil and Walter 2008).

As long as GALE is active in the cell, however, UDP-gal can be created from UDP-glc. Therefore, GALE should keep cellular UDP-gal levels from being affected, even in the absence of GALT activity. Additionally, the existence of such a deficiency does not indicate whether it is significant enough to have an effect on glycosylation or other cellular processes (Holton 1996; Fridovich-Keil and Walter 2008). Therefore, it is still unknown and controversial whether a UDP-gal deficiency exists in patients with classic galactosemia, and if it does, whether it has any impact on pathophysiology.

Endogenous production of galactose

GALE catalyzes a reversible reaction and, in times of need, can produce galactose, an important sugar involved in the biosynthesis of the glycan chains of glycoproteins and glycolipids. Therefore, while the diets of classic galactosemics can be

restricted, this treatment cannot control for the endogenous production of galactose in patients, referred to as self-intoxication (Gitzelmann and Steinmann 1984).

Berry and colleagues demonstrated that patients with classic galactosemia produce substantial amounts of endogenous galactose. The endogenous production of galactose may contribute to the fact that levels of metabolites such as gal-1P and galactitol remain elevated in patients despite adherence to a galactose restricted diet (Berry et al. 1995). Adult patients can produce 0.55 mg/kg (3.1 μmol/kg) body weight of galactose per hour endogenously; for example, a 70 kg adult classic galactosemic can produce more than 900 mg of galactose a day. Normal intake for a patient adhering to the galactose restriction is less than 50 mg of galactose a day (Berry et al. 1993; Bosch 2006). The rate of endogenous production of galactose is affected by the intake of dietary galactose and decreases with age, but remains higher in adults with classic galactosemia compared to unaffected individuals (Berry et al. 1995; Leslie 2003; Berry et al. 2004; Bosch et al. 2004; Schadewaldt et al. 2004).

Inhibition of enzymes

In 1995, Gitzelmann reviewed studies that found that enzymes that normally metabolize glc-1P may be targets for inhibition by gal-1P when this metabolite accumulates at high levels in the cell (Gitzelmann 1995). Additionally, those enzymes that use UDP-gal as a substrate in the biosynthesis of glycans that will later be attached to glycoproteins or glycolipids may be targets as well. *In vitro* studies have demonstrated that high levels of gal-1P inhibit enzymes involved in sugar metabolism and

glycosylation, including glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, phosphoglucomutase, glycogen phosphorylase, UDP-glucose pyrophosphorylase, and UDP-galactose galactosyltransferase (Gitzelmann 1995; Lai et al. 2003; Lebea and Pretorius 2005; de Jongh et al. 2008).

The inhibition of UDP-galactose galactosyltransferase by gal-1P may be involved in the aberrant glycosylation patterns detected in patients with classic galactosemia (Dobbie et al. 1990; Petry et al. 1991; Jaeken et al. 1992; Ornstein et al. 1992; Prestoz et al. 1997; Charlwood et al. 1998; Lai et al. 2003; Lebea and Pretorius 2005; Sturiale et al. 2005; Bosch 2006). This inhibition may be worsened by the unconfirmed deficiency of UDP-gal, the substrate of UDP-galactose galactosyltransferase and other proteins, in patients (Lai et al. 2003; Bosch 2006).

Bhat hypothesized that gal-1P may also regulate inositol monophosphatase (IMPase), an enzyme involved in inositol-mediated signal transduction in the brain (Bhat 2003). Studies conducted in the yeast model system identified gal-1P as a substrate of IMPase. Overexpression of human IMPase reverses the abnormal accumulation of gal-1P in GALT-null yeast (Mehta et al. 1999). Additionally, Parthasarathy and colleagues demonstrated that human brain IMPase can efficiently hydrolyzes gal-1P (Parthasarathy et al. 1997).

Whether gal-1P regulates IMPase activity has not been determined experimentally. The fact that IMPase is able to act on gal-1P with a equal affinity for its inositol monophosphate substrates implies that high gal-1P levels may competitively inhibit IMPase from its normal functions (Parthasarathy et al. 1997; Bhat 2003). If IMPase activity is modulated by gal-1P, inositol-mediated signaling in the brain may be

affected in patients with classic galactosemia, who have elevated gal-1P levels in their blood and tissues (Holton et al. 2000). In support of this hypothesis, autopsies of two classic galactosemic neonates found that brain inositol and phosphatidylinositol levels were 80% lower than unaffected control samples (Wells et al. 1965). As mentioned previously, patients with classic galactosemia suffer from cognitive impairments. These data suggest that proper brain development may be inhibited by elevated levels of gal-1P and its interaction with IMPase.

Glycosylation

Over the years, several studies have detected aberrant glycosylation patterns of proteins and lipids in classic galactosemic patients (Haberland et al. 1971; Petry et al. 1991; Charlwood et al. 1998; Lebea and Pretorius 2005; Sturiale et al. 2005). As mentioned previously, elevated levels of metabolites, such as gal-1P, have been shown to inhibit important enzymes for sugar metabolism and glycosylation, including UDP-galactose galactosyltransferase, which adds galactose, an essential subterminal sugar, to growing glycan chains (Dobbie et al. 1990; Petry et al. 1991; Ornstein et al. 1992; Jaeken et al. 1996; Prestoz et al. 1997; Charlwood et al. 1998). Glycosylated proteins and lipids are integral for many cellular processes, including cell-cell recognition, cell cycle regulation, signal transduction, growth, and differentiation. Therefore, aberrant glycosylation patterns may underlie or contribute to the acute and long-term sequelae of classic galactosemia, including the cognitive and motor impairments, and premature ovarian insufficiency (POI).

Studies in fibroblasts derived from patients with classic galactosemia detected a deficiency in glycoproteins containing galactose (Dobbie et al. 1990; Ornstein et al. 1992). Both Charlwood and colleagues and Sturiale and colleagues found aberrantly galactosylated serum proteins in untreated classic galactosemic patients; a small subset of these patients showed signs of abnormal glycosylation even after a galactose restricted diet was commenced (Holton 1996; Stibler et al. 1997; Charlwood et al. 1998; Sturiale et al. 2005). Furthermore, Jaeken and colleagues found abnormal electrophoretic isoforms of plasma transferrins, most likely due to their lack of galactosyl side chains, in classic galactosemic infants prior to the commencement of a galactose restricted diet (Jaeken et al. 1992).

In 1971, Haberland and colleagues described the abnormal glycoprotein pattern in the brain of a classic galactosemic (Haberland et al. 1971; Witting et al. 1972). Two decades later, Petry found in the brain of a newborn classic galactosemic a deficiency of glycolipids containing either galactose or galNAc, and a build-up of precursors molecules (Petry et al. 1991).

Petry has hypothesized that the cognitive sequelae of classic galactosemia may be related to improper galactosylation of myelin in patient brains (Petry et al. 1991; Antshel et al. 2004). Myelin is necessary for the rapid conduction of neuronal signals; the abnormal production of myelin may lead to a decrease in nerve conduction speed, which may be causal in the cognitive, speech, and motor impairments of classic galactosemics (Antshel et al. 2004; Ridel et al. 2005).

Neuroimaging studies have demonstrated that the brains of classic galactosemics have abnormal white matter, which is composed mainly of myelinated axons (Koch et al.

1992; Nelson et al. 1992; Kaufman et al. 1995; Wang et al. 2001). This finding, which could be due to abnormal myelination, may be due to the improper biosynthesis of glycoproteins and glycolipids necessary for normal myelin production, including galactosylceramide and sulphogalactosylceramide (Lebea and Pretorius 2005). The cognitive, motor, and speech sequelae of classic galactosemia may be caused by the lack of proper myelination and neuronal tissue damage. Neuronal development and myelination occur both *in utero* and after birth; therefore, the removal of galactose from the diet of newly born patients may not entirely ameliorate the long-term complications of classic galactosemia.

Prestoz and colleagues found abnormal isoforms of follicle stimulating hormone (FSH) in the sera of several women with classic galactosemia and POI (Prestoz et al. 1997). As mentioned previously, approximately 85% of female patients with classic galactosemia suffer from POI; abnormally glycosylated FSH, and other glycoproteins necessary for proper ovary development and health, may contribute to this long-term complication of classic galactosemia.

Two metabolic abnormalities may be involved in the aberrant glycosylation patterns in classic galactosemics: a deficiency of UDP-gal (decreased availability as a substrate for galactosyltransferases) and elevated levels of gal-1P (inhibitor of galactosyltransferases). As discussed previously, there is a great deal of controversy around the biological significance of a UDP-gal deficiency, if such a deficiency does in fact exist. On the other hand, a great deal of *in vitro* data implicates gal-1P as an inhibitor of enzymes involved in glycosylation, such as UDP-galactose

galactosyltransferase (Gitzelmann 1995; Holton 1996; Bhat 2003; Lai et al. 2003; Lebea and Pretorius 2005; de Jongh et al. 2008).

Untreated classic galactosemia is considered a secondary carbohydrate-deficient glycoprotein syndrome (CDG) (also known as congenital disorders of glycosylation syndrome), which is characterized by aberrant glycosylation patterns, including a deficiency of galactosylation (Sturiale et al. 2005). The organ systems affected include the central nervous system (CNS), the peripheral nervous system (PNS), and the liver. Patients with CDG show delays in mental and motor skills, cognitive impairments, ataxia, underdevelopment (hypoplasia) of certain areas of the brain, and hypergonadotropic hypogonadism (abnormal development of the ovaries or testes) (Stibler et al. 1997). The myriad similarities between CDG and classic galactosemia lend more weight to the hypothesis that aberrant glycosylation in classic galactosemics may be involved in the acute and long-term complications of this disorder. A great deal may be learned from the comparison of classic galactosemia and CDG disorders.

Model Systems for the Study of Galactosemia

The underlying pathophysiology of classic galactosemia is not well understood. A major hindrance in the field is the lack of a genetic animal model system of classic galactosemia that recapitulates the human disorder. So far, the yeast, *Saccharomyces cerevisiae*, mammalian cell culture, and mice have been utilized to study galactose metabolism and GALT deficiency *in vivo*.

Saccharomyces cerevisiae

A model of classic galactosemia was established in *Saccharomyces cerevisiae* (baker's yeast) in 1993 by Dr. Judith Fridovich-Keil, the principal investigator of this laboratory (Fridovich-Keil and Jinks-Robertson 1993). The *S. cerevisiae* model has been utilized for studies describing the impact of patient mutations on GALT enzymatic activity and the discovery of modifiers of galactose sensitivity of GALT-null yeast (Fridovich-Keil and Jinks-Robertson 1993; Fridovich-Keil 1995; Fridovich-Keil et al. 1995; Elsevier and Fridovich-Keil 1996; Quimby et al. 1996; Wells and Fridovich-Keil 1997; Crews et al. 2000; Riehman et al. 2001; Ross et al. 2004).

S. cerevisiae have an active Leloir pathway, consisting of the genes *GAL1* (*GALK*), *GAL7* (*GALT*), and *GAL10* (*GALE*). GALT-null yeast are growth impaired in the presence of 0.2% galactose, even when an alternative carbon sources (glycerol and ethanol) are present in the medium (Douglas and Hawthorne 1964; Ross et al. 2004; Slepak et al. 2005) Wild-type hGALT protein is sufficient to complement the galactose-induced growth arrest phenotype, making the yeast model an excellent system for the study of patient hGALT mutations (Fridovich-Keil et al. 1995). The most common hGALT mutant, Q188R, shows no detectable activity in yeast, which mimics results from human lymphoblasts (Fridovich-Keil and Jinks-Robertson 1993; Fridovich-Keil et al. 1995). Additionally, the ability to study the correlation between genotype and biochemical phenotype has been possible; Riehman and colleagues expressed 16 patient hGALT mutations, which conferred activity levels ranging from null to wild-type levels of activity, in GALT-null yeast. Metabolite levels and growth were measured in the

presence of galactose, and were shown correlate inversely with the activity level encoded by the GALT allele (Riehman et al. 2001).

The yeast model has been amenable to the search for modifiers of the growth arrest phenotype. Studies have come to the same conclusion: the galactose-induced growth arrest phenotype of GALT-null yeast is rescued by preventing either the synthesis of gal-1P (loss of GALK activity) or by increasing the metabolism of gal-1P through other pathways (overexpression of UGP or inositol monophosphatase (IMPase), both of which can hydrolyze gal-1P) (Douglas and Hawthorne 1964; Parthasarathy et al. 1997; Mehta et al. 1999; Kabir et al. 2000; Lai and Elsas 2000; Riehman et al. 2001; Bhat 2003; Ross et al. 2004).

In this laboratory, Ross and colleagues studied the growth and metabolic phenotypes of yeast impaired for one or a combination of two of the Leloir enzymes. GALT-null yeast growth arrest and accumulate intracellular gal-1P levels, which reach a threshold level of 10 nmol/mg, in the presence of galactose and an alternative carbon source (glycerol and ethanol) (Ross et al. 2004). These phenotypes are rescued by the secondary deletion of *GALK*. These data, and the fact that patients with GALK deficiency galactosemia do not experience the acute or long-term complications of classic galactosemia, support the hypothesis that GALK and the metabolite gal-1P are involved in the galactose sensitivity in GALT-null yeast (Segal 1995; Lai and Elsas 2000; Ross et al. 2004). As mentioned previously, patients with classic galactosemia have elevated levels of blood gal-1P despite adherence to a galactose restricted diet and several studies have correlated high gal-1P levels with the acute and potentially lethal complications of

this disease (Kaufman et al. 1988; Ng et al. 1991; Kaufman et al. 1994; Xu et al. 1995; Tyfield and Walter 2002; Leslie 2003; Fridovich-Keil and Walter 2008).

The yeast model system increased the field's understanding of the biochemistry of GALT and the effect of specific mutations on structure and function. This model has been used for the screening of genetic modifiers of the galactose-induced growth phenotype. As to be expected, there are several limitations on the use of this model organism in the study of galactosemia: *S. cerevisiae* is a single-celled organism, eliminating the potential to study the effects of GALT deficiency in different tissues and organ systems or the requirement of GALT in developmental processes. Additionally, *S. cerevisiae* has a more simplified glycosylation pathway compared to higher eukaryotes.

Mammalian cell culture

The utilization of cell culture has allowed the field to study galactosemia in a mammalian context. Cell lines derived from patients with classic galactosemia can be used in experiments where they are exposed to galactose in the controlled environment of cell culture. The resulting biochemical and metabolic phenotypes can then be analyzed, such as the role of GALK and gal-1P as mediators of galactose sensitivity (Pourci et al. 1990; Lai et al. 2003).

Fibroblasts derived from human patients with classic galactosemia undergo growth arrest and accumulate intracellular gal-1P when exposed to galactose (Pourci et al. 1990; Lai et al. 2003). The overexpression of human UGP rescues these galactose sensitivity phenotypes (Lai et al. 2003). These findings parallel those from the yeast model system.

The intrinsic limitations of this model system prevent the study of GALT deficiency in developmental processes, the pathophysiology of the acute and long-term complications of classic galactosemia, and in the affected tissues and organ systems.

Mus musculus

A *GALT* knockout mouse was created by Leslie and colleagues in 1996 in order to establish the first mammalian model of classic galactosemia. The knockout region consisted of exons 6 to 8 of the *GALT* gene, which contain the coding region for the active site of the enzyme. These mice showed no detectable GALT activity but, unfortunately, the mice did not recapitulate the human phenotype—acute or long-term (Leslie et al. 1996). The knockout mice did accumulate high levels of galactose and gal-1P in their blood and tissues, including the liver, kidneys and brain, but remained healthy and fertile, unlike their human counterparts (Ning et al. 2000; Leslie and Bai 2001; Bosch et al. 2004; Leslie et al. 2005). When fed a diet of 40% galactose, the mice continued to accumulate high levels of galactose and gal-1P in their blood and tissues, but they were able to tolerate the high galactose diet and continued to show no acute or long-term phenotypes (Ning et al. 2001).

This model has been useful in the establishment of a new hypothesis concerning the underlying pathophysiology of classic galactosemia: the accumulation of both gal-1P and galactitol, not gal-1P alone, may be necessary for the acute toxicity and long-term complications of classic galactosemia (Bosch 2006; de Jongh et al. 2008). Ai and colleagues created a mouse deficient for GALK, which on a 40% galactose diet accumulated galactose and galactitol. While galactose and gal-1P levels were

comparable to those observed in humans, galactitol levels were low, most likely because mice express lower levels of aldose reductase (AR), ubiquitously and in the lens of the eye (Ai et al. 2000; Bosch et al. 2004). Therefore, it was not surprising when the mice did not form cataracts like their human counterparts. When the human AR (hAR) gene was transgenically expressed in the lens of the eye, however, GALK deficient mice developed cataracts, even on a normal diet (Ai et al. 2000). While galactitol cannot be solely responsible for the negative outcomes in GALT deficiency, it has been hypothesized that in high levels and in combination with high levels of gal-1P, or another galactose metabolite, galactitol may be responsible for the acute toxicity and long-term complications of classic galactosemia (Bosch 2006; de Jongh et al. 2008). In the absence of an animal model that recapitulates the human phenotypes, however, this is not a testable hypothesis.

These findings suggest that, while yeast and humans seem to share the same sensitivity to galactose, mice and humans are different in this aspect of biology. This may be due to differences in the efficiencies of mouse Leloir enzymes and alternative pathways, the differing sensitivity of mouse and human tissues to galactose and its metabolites, and unpredictable factors (Leslie 2003). Currently, further experimentation is necessary to explain the disparities in the phenotypes of GALT-impaired mice and humans.

Drosophila melanogaster

Despite decades of research, the underlying pathophysiology of the acute and long-term complications of classic galactosemia remains unknown (Holton et al. 2000;

Tyfield and Walter 2002; Leslie 2003). In order to further the understanding of classic galactosemia, the field is in desperate need of an animal model system that recapitulates the human phenotype. The existence of an animal model would allow us to analyze the effects of GALT deficiency on complex processes such as development, glycosylation, tissue and organ system function, and the role of diet and other genetic factors in the outcome of this disorder. A greater knowledge of the pathophysiology of classic galactosemia will lead to more effective treatments for patients. This laboratory has established the first animal model of classic galactosemia in the fruit fly, *Drosophila melanogaster*.

First studied by Thomas Hunt Morgan more than 100 years ago, *D. melanogaster* is a well-established genetic model organism for the study of biology, genetics, and development. *D. melanogaster* been widely used to study metabolism and has become a useful model for disease as well, including diabetes and obesity (Celotto et al. 2006; Baker and Thummel 2007; Schlegel and Stainier 2007; Bharucha 2009). *D. melanogaster* have the same basic metabolic pathways as vertebrates and many similar metabolic tissues, which are necessary for the regulation of energy homeostasis, the balance between metabolism and catabolism, and the storage and uptake of nutrients. Additionally, about 70% of human disease-causing genes have a *D. melanogaster* orthologs, many of which show corresponding phenotypes when mutated (Baker and Thummel 2007).

This laboratory was the first to show that the Leloir pathway is conserved and active in *D. melanogaster*. We have shown that all three enzymes are expressed as active proteins using our established, HPLC-based enzyme assays. Additionally, we were able

to control for this organism's environment and diet, making *D. melanogaster* an excellent organism in which to further the study of galactose metabolism and its role in normal fly development and homeostasis. This dissertation will describe studies with the *D. melanogaster* model system to define acute phenotypes and biochemical abnormalities of GALT deficient flies, the developmental period during which flies are most sensitive to dietary galactose, the role of dietary galactose as a modifier of the phenotypes tested, and the developmental period of galactose sensitivity. Finally, whether these phenotypes parallel those seen in patients will be discussed.

Significance

Classic galactosemia is a potentially fatal disorder if untreated early in life. Patients who adhere to treatment, which predominantly only consists of the dietary restriction of galactose, still suffer from the severe long-term sequelae of the disease. Despite decades of research in this field, the biochemical bases of the pathophysiology of the acute and long-term complications of classic galactosemia remain unknown. The existence of an animal model of classic galactosemia would allow researchers to analyze the effects of GALT deficiency on complex processes, including development, glycosylation, and tissue and organ function. A greater knowledge of the fundamental pathophysiology of this disease will allow for the creation of new, more effective treatments, perhaps including a specific, small molecule inhibitor of hGALK, which may be used in conjunction with a galactose restricted diet. Additionally, a better

understanding will be gained of the role of diet and genetic factors in the phenotypic outcome of classic galactosemia.

The focus of this dissertation is the creation and initial characterization of the first genetic animal model of classic galactosemia and the search for a small molecule inhibitor of hGALK, both of which are necessary in order to provide novel treatments and a better quality of life for patients with classic galactosemia.

CHAPTER II

Modified from
A *Drosophila melanogaster* model of classic galactosemia

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Introduction

Galactose and its derivatives are essential in higher eukaryotes because these molecules serve as key constituents of glycoproteins, glycolipids, and complex carbohydrates. Galactose is also of particular importance to mammalian infants because, as a component of lactose, it accounts for close to half of the sugar calories in milk.

Galactose is metabolized in mammals and other species by the three 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**) (Holden et al. 2003). In humans, profound impairment of GALT results in the potentially lethal metabolic disorder, classic galactosemia (OMIM #230400) (Fridovich-Keil and Walter 2008).

Classic galactosemia is an autosomal recessive disorder that affects 1/60,000 live births in many human populations. 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 within days to weeks. Untreated infants with classic galactosemia also accumulate dramatically elevated levels of galactose (gal), galactose-1-phosphate (gal-1P), and galactitol in their cells and tissues. While the careful restriction of dietary galactose prevents or reverses the acute and potentially lethal symptoms of classic galactosemia and also largely resolves the abnormal accumulation of metabolites, significant long-term complications persist; these include cognitive impairment, speech difficulties, neurological problems, and premature ovarian insufficiency, among others (Fridovich-Keil and Walter 2008).

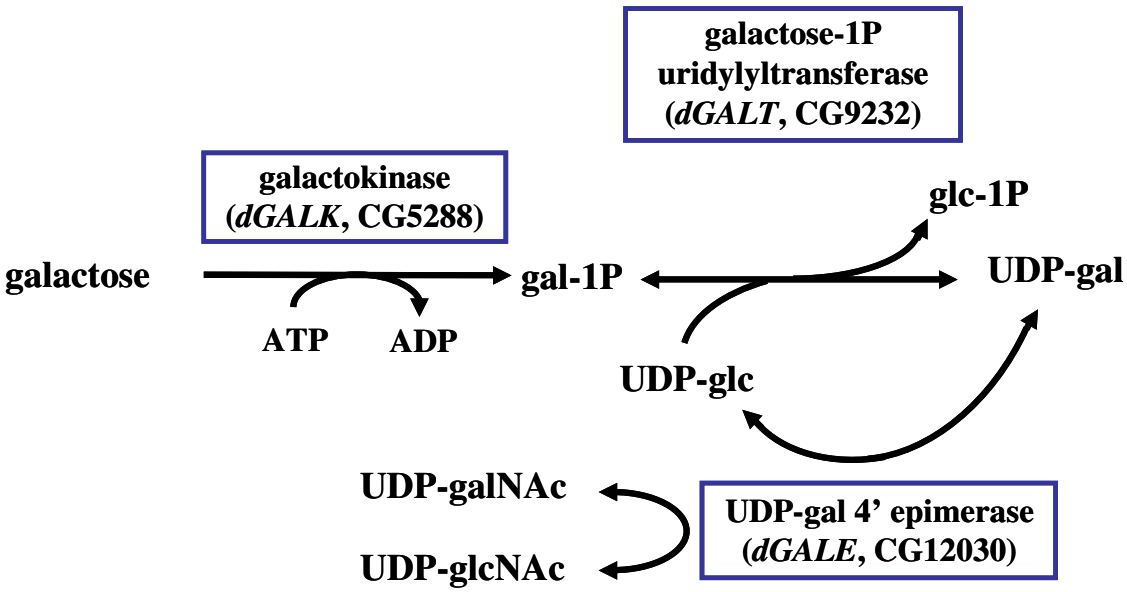


Figure 2.1

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 glc-1P and UDP-gal from UDP-glc and gal-1P.

Despite decades of study, the underlying bases of the pathophysiology of both the acute and long-term complications of classic galactosemia remain unclear (Tyfield and Walter 2002; Leslie 2003; Fridovich-Keil and Walter 2008). One of the principle factors limiting research progress has been the lack of an appropriate animal model system that recapitulates either the acute or long-term complications seen in patients. Studies conducted by ourselves and others using microbial and mammalian tissue-culture models have provided biochemical insights into the consequences of GALT deficiency, but the absence of a multicellular model has precluded studies into the timing and impact of GALT deficiency on development or on intact tissues and organ systems (Fridovich-Keil and Jinks-Robertson 1993; Fridovich-Keil 1995; Fridovich-Keil et al. 1995; Elsevier and Fridovich-Keil 1996; Elsevier et al. 1996; Lai et al. 1996; Lai and Elsas 2000; Riehman et al. 2001; Christacos NC 2002; Lai et al. 2003; Ross et al. 2004). Of note, more than a decade ago, Leslie and colleagues reported a *GALT* knockout mouse that demonstrated complete loss of GALT activity and that accumulated Leloir pathway metabolic intermediates; however, these mice unexpectedly remained healthy and fertile, despite dietary exposure to large quantities of galactose (Leslie et al. 1996; Ning et al. 2000; Leslie and Bai 2001; Ning et al. 2001; Leslie et al. 2005). This outcome disparity between GALT-deficient humans and mice remains unexplained.

As an alternative to mice, we turned to the fruit fly, *Drosophila melanogaster* as a model to understand the genetic and metabolic bases of classic galactosemia. 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 , including complex metabolic disorders, such as diabetes and obesity (Lasko 2002; Bier 2005;

Bharucha 2009). Indeed, sequence alignments show that more than 70% of recognized human disease genes, among them all three Leloir pathway genes, have related sequences in the *D. melanogaster* genome (Bier 2005).

Here we report the establishment and first application of a *D. melanogaster* model of classic galactosemia. Like human patients, but unlike mice, GALT-deficient *D. melanogaster* survive if maintained on food that contains only glucose (glc), but these animals 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 galactose enriched diet die within days of the transfer, but adult flies do not, arguing for stage-specific consequences to impaired galactose metabolism. Finally, GALT-deficient flies raised and maintained exclusively on a galactose restricted diet nonetheless demonstrate a clear deficit in the normal negative geotaxic response seen in controls (Benzer 1967; Akai 1979). As with the acute galactose-dependent phenotype, this galactose-independent neurological or neuromuscular deficit is rescued by expression of a human *GALT* transgene. These data confirm that GALT-deficient *D. melanogaster* mimic aspects of both acute and long-term patient outcomes, paving the way for future studies to explore the genetic and environmental factors that underlie and modify these phenotypes.

Materials and Methods

Fly stocks and maintenance

Stocks of *D. melanogaster* used in these studies are listed in **Table 2.2**. Unless otherwise noted, stocks were maintained at 25°C and 5% humidity 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, and 14.4 mL/L tegasept mold inhibitor (10% w/v in ethanol). For experiments in which the levels and types of sugars 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, 14.4 mL/L tegasept mold inhibitor (10% w/v in ethanol) (Honjo and Furukubo-Tokunaga 2005). To this basic recipe, galactose or mannose was added, as indicated (e.g. final concentration 111 mM or 222 mM galactose or 222 mM mannose). Of note, for some experiments (e.g. the 96-well plate survival experiments) one to two mL of liquid food coloring was added per liter of food to facilitate confirmation that the larvae were eating normally.

Creation and molecular characterization of the dGALT$^{\Delta IAP2}$, dGALT$^{\Delta IV2}$, and dGALT^{C2} alleles

To create an intragenic deletion allele of *dGALT*, we mobilized an existing SUPor-P insertion in the 5' UTR of the *CG9232* locus (*KG00049*; Bloomington Stock #14339); excision was achieved by transient expression of the Δ*2-3* transposase enzyme in the male germ line, according to standard methods (Ryder and Russell 2003). 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 adult flies using a biochemical

Fly stock or allele name	Comments
w^{1118}	Wild-type *D. melanogaster* (Bloomington Stock #3605)
$P\{SUPor\text{-}P\}CG9232^{KG00049}$	P-element insertion stock used for excision scheme (Bloomington Stock #14339)
$y^1w^*;ry^{506}Sb^1P\{\Delta 2\text{-}3\}99B/TM6B$	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 $P\{SUPor\text{-}P\}CG9232^{KG00049}$
$dGALT^{\Delta 1AP2}$	Imprecise excision of $P\{SUPor\text{-}P\}CG9232^{KG00049}$ that produced a ~1.6kb deletion of the *dGALT* coding region
$dGALT^{\Delta 1V2}$	Imprecise excision of $P\{SUPor\text{-}P\}CG9232^{KG00049}$ that produced a ~1.6kb deletion of the *dGALT* coding region
$hGALT^{10A11}$	*UAS-hGALT* insertion allele, chr III, homozygous viable
$hGALT^{9B12}$	*UAS-hGALT* insertion allele, chr III, homozygous viable
$hGALT^{10B22}$	*UAS-hGALT* insertion allele, chr III, homozygous viable

Table 2.1

***D. melanogaster* stocks and alleles used in this study.**

The stocks used in these experiments are listed with their genotypes and a brief description.

assay (described below). Of >50 excision stocks tested, five demonstrated a profound loss of GALT enzymatic activity; those stocks were designated as candidates to harbor imprecise excisions of the P-element, while stocks demonstrating wild-type GALT activity were designated as candidates to harbor precise excisions of the P-element.

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}, Bloomington Stock #12218). Of note, the P-element insertion allele from which the *dGALT*$^{\Delta 1AP2}$, *dGALT*$^{\Delta 1V2}$, and *dGALT*^{C2} excision alleles were derived was itself homozygous viable and female fertile, and also complemented the *cup*^{01355} allele (data not shown). *cup* expression and function were, therefore, tolerant of the insertion of a ~11.5kb P-element in the *cup* second intron. Similarly, female flies carrying the *cup*^{01355} allele *in trans* to either *dGALT*$^{\Delta 1AP2}$, *dGALT*$^{\Delta 1V2}$, or *dGALT*^{C2} alleles 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* (exons two and three). 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} precise excision stock also produced a 4 kb amplicon, while both the *dGALT*$^{\Delta 1AP2}$ and *dGALT*$^{\Delta 1V2}$ imprecise excision stocks produced an amplicon that was only ~2.4 kb. Direct sequencing of the amplicons from all three excision templates revealed that the presumed precise excision allele was indeed precise (**Figure 2.2C**), and

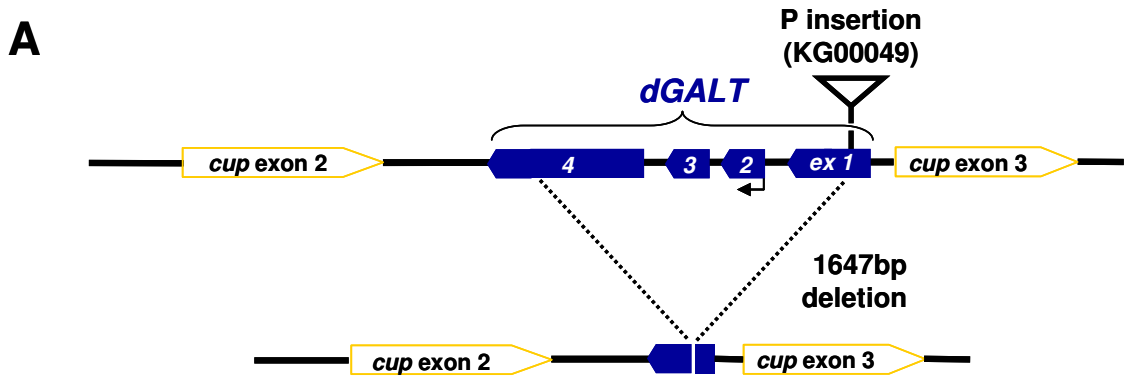
that each of the presumed imprecise excision alleles carried the same 1647 bp deletion removing virtually the entire coding region of the *dGALT* gene, with a small remnant of the P-element sequence left behind (**Figure 2.2A, B**).

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* (Brand and Perrimon 1993) using the *Eco*R1 and *Xho*1 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 ($dGALT^{9B12}$, $dGALT^{10A11}$, and $dGALT^{10B22}$) on chromosome III were used in this study.

Initial GALT biochemical screens looking for impaired dGALT alleles

Ten adult flies homozygous for each *dGALT* candidate excision allele were anesthetized with CO_2 and added to 100 µL glycine buffer and Complete Mini protease inhibitor cocktail pellet, EDTA-free (Roche). Samples were ground on ice for 15 s and then centrifuged at 16,100 rcf for five min at 4°C. Each resulting supernatant was passed over a Micro Bio-Spin Chromatography Column (Bio-Rad) to remove endogenous metabolites and then 30 µL of undiluted protein extract were added to 20 µL of assay cocktail (312.5 uL 1 M glycylglycine, pH 8.7, 20 µL 100 mM UDP-glc, 400 µL 10 mM gal1P, 267.5 µL water) as described previously (Ross et al. 2004). As a negative control,



B **Imprecise excision (*dGALT*$^{\Delta 1AP2}$ and *dGALT*$^{\Delta 1V2}$) junction sequences:**

...GTGCCAAACTCAAACTAAACcatgatgaaataa**GGAAATAGTCTAATACGACT...**

C **Precise excision (*dGALT*^{C2}) junction sequence:**

...GTGCCAAACTCAAACTAAAC•GCTCGCCGGCAGAGTGAGCG...

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 **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.

assays were also run in the absence of gal-1P. Each reaction was incubated for 30 min at 25ºC, then placed on ice and diluted 10-fold with ice-cold HPLC grade water (Fisher) to stop the reaction. Particulates were removed from the samples by centrifugation at 4000 rcf for four min at 4°C through 0.22 µm nylon filters (Costar 8169); reactants and products were separated and quantified by HPLC as described previously (Ross et al. 2004). Protein concentration was determined using the Bio-Rad DC protein assay with bovine serum albumin (BSA) as a standard.

GALK, GALT, and GALE enzyme assays

Lysates: 10 adult male flies, eight to 48 h old, of the appropriate genotype were anesthetized with CO_2 and added to 100 µL lysis buffer and Complete Mini protease inhibitor cocktail pellet, EDTA-free (Roche). Samples were ground on ice for 15 s using a teflon micropestle and handheld micropestle motor (Kimble-Kontes) and then centrifuged at 16,100 rcf for 5 min at 4°C. Each resulting supernatant was passed over a Micro Bio-Spin P-6 Chromatography Column (Bio-Rad) to remove endogenous metabolites and then diluted in lysis buffer to an appropriate concentration to achieve a linear range in the assay. 30 µL of diluted protein extract were added to 20 µL of a cocktail of substrates and cofactors to start each reaction (details below). Approximate time from grinding to initiation of the assay was 25 to 35 minutes. Reaction mixtures were incubated at 25°C for 30 min, then placed on ice and diluted 10-fold with ice-cold HPLC grade water (Fisher) to stop the reactions. Particulates were removed from the samples by centrifugation at 4000 rcf for 4 min at 4°C through 0.22 µm nylon filters (Costar 8169); reactants and products were quantified by HPLC as described previously

and specific activity reported as pmol product formed per µg protein per minute of reaction time (Ross et al. 2004). Protein concentration was determined using the Bio-Rad DC protein assay according to the manufacturer's protocol using BSA as a standard.

GALK assay conditions: Lysates were diluted 1:10. Initial concentration of the reaction mixture was 2 mM $MgCl_2$, 40 mM Tris pH 8.0, 40 µM dithiothreitol, 4 mM galactose, and 4 mM ATP.

GALT assay conditions: Lysates were diluted 1:10, except for lysates from flies homozygous for alleles $dGALT^{\Delta 1V2}$ and $dGALT^{\Delta 1AP2}$, which were not diluted. To account for background conversion of UDP-glc to UDP-gal by GALE in the lysates, assays were performed both with and without added gal-1P. UDP-gal formed in the absence of gal-1P for each lysate was subtracted from the UDP-gal formed in the presence of gal-1P, and the net amount of UDP-gal was used to calculate specific activity. Initial concentration of the reaction mixture was 125 mM glycylglycine pH 8.7, 0.8 mM UDP-glc and (when relevant) 1.6 mM gal-1P.

GALE assay conditions: Lysates were diluted 1:60. Initial concentration of the reaction mixture was 80 mM glycine pH 8.7, 0.8 mM UDP-gal and 0.5 mM NAD.

Measuring gal-1P in larvae and adults

Cohorts of $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ (GALT-deficient) and $dGALT^{C2}/dGALT^{C2}$ (control) newly hatched larvae and newly eclosed adults were transferred to cages

containing either 555 mM glucose-only or 555 mM glucose plus 222 mM galactose food. After 4 days, pools of ten adult flies or ≥20 larvae were anesthetized, 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-Kontes). 10 µL of the 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(Ross et al. 2004; Openo et al. 2006). 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 concentrations and centrifuged through .22 µm Costar Spin-X centrifuge tube filters (Corning) 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(Ross et al. 2004). For all samples, 20 µL were injected into a 25 µL injection loop.

Time of death experiments

Embryos homozygous for the $dGALT^{\Delta 1AP2}$ (imprecise excision) and $dGALT^{C2}$ (precise excision) alleles were harvested from egg-laying plates and deposited individually into the wells of 96-well plates pre-loaded with either 555 mM glucose or 555 mM glucose and 111 mM or 222 mM galactose fly food. All animals were monitored and scored for viability daily 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 and 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 preloaded with 555 mM glucose and 222 mM galactose food, and similarly larvae were transferred from 555 mM glucose and 222 mM galactose food into the wells of 96-well plates preloaded with 555 mM glucose food. To control for a potential impact of the transfer process itself, cohorts of larvae also were transferred from glucose food to wells containing glucose food, and from glucose and galactose food to wells containing glucose and 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.

Microscopy of larvae

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 and 222 mM galactose food. Each day larvae were collected from the plates and imaged at 25X using a Zeiss Stemi SV6 dissection microscope equipped with a Leica DFC 500 digital camera.

Results

D. melanogaster metabolize galactose via the Leloir Pathway

To explore the role of galactose metabolism in *D. melanogaster* development, we first confirmed that flies encode and express homologs of human *GALK*, *GALT*, and *GALE* (**Figure 2.1**). The *D. melanogaster* genes with the greatest predicted amino acid sequence homology to the human Leloir enzymes were *CG5288* (on chromosome III, designated here as *dGALK*), *CG9232* (on chromosome II, designated here as *dGALT*), and *CG12030* (on chromosome III, designated here as *dGALE*) (Chien et al. 2002) (http://superfly.ucsd.edu/homophila/). Both *dGALT* and *dGALE* showed strong conservation with their human orthologs, having 57% sequence identity with 72% sequence similarity, and 60% sequence identity with 76% sequence similarity, respectively. *dGALK* demonstrated slightly lower conservation, with 27% sequence identity and 44% sequence similarity.

Publicly available *in situ* RNA hybridization data from the Berkeley Drosophila Genome Project (BDGP; http://www.fruitfly.org/) further confirmed that all three fly genes are expressed during embryogenesis. Assays of lysates prepared from *D. melanogaster* adults also revealed 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 2.1**; R. Sanders *et al*., in preparation and unpublished data) demonstrated loss of the corresponding enzymatic activities, thereby confirming a functional connection between each gene sequence and the enzymatic activity attributed to its encoded protein product.

STOCK	GALK activity (pmol/ µg/ min)	GALT activity (pmol/ µg/ min)	GALE activity (pmol/ µg/ min)
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	39.13 ± 4.66	73.48 ± 17.36
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ (imprecise excision)	42.78 ± 4.74	-0.23 ± 0.10	87.38 ± 10.47
$dGALT^{\Delta 1V2}/dGALT^{\Delta 1V2}$ (imprecise excision)	38.29 ± 3.33	0.02 ± 0.29	80.90 ± 7.86
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10A11}/\beta tub\text{-}GAL4$	18.70 ± 8.65	172.41 ± 60.80	98.00 ± 1.65
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{9A12}/\beta tub\text{-}GAL4$	14.71 ± 3.54	651.93 ± 21.49	66.45 ± 3.97
$dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10B22}/\beta tub\text{-}GAL4$	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 harvested 8 to 48 h after eclosure on molasses food. Values presented are average ± S.E.M. ($n \geq 3$ sets of duplicate samples).

The *dGALT* gene is located within cytological interval 26F on the left arm of chromosome II. The gene is approximately 2 kb in length and includes four exons, the first of which is noncoding; the predicted mRNA is ~1.5 kb. The fly *dGALK* and *dGALE* genes will be described in detail elsewhere. Of note, the *dGALT* gene is located entirely within the second intron of another gene, *cup* (Keyes and Spradling 1997). *cup* is required for ovarian function and egg production in flies; female flies deficient in *cup* are infertile and fail to lay embryos (Keyes and Spradling 1997; Piccioni et al. 2005).

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* (Bloomington Stock #14339) in the 5' UTR of the *CG9232* gene (**Figure 2.2A**). 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 adults. 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 flies homozygous for this allele (**Table 2.1**).

Excision of the P-element was achieved by transient exposure to the *Δ2-3* transposase in the germ line of male flies, as described in **Methods** (Ryder and Russell 2003). White-eyed (F1) progeny were backcrossed and bred to homozygosity, and lysates prepared from stocks were tested for loss of GALT enzymatic activity. We identified five GALT-deficient stocks, each of which had no detectable GALT activity and the same 1647 bp deletion removing almost the entire *dGALT* gene (**Figure 2.2B**).

Two of these alleles, designated *dGALT*$^{\Delta 1AP2}$ and *dGALT*$^{\Delta 1V2}$, were chosen for further study.

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, these animals demonstrated wild-type levels of GALT activity (**Table 2.1**). Also as expected, all three P-element excision alleles, *dGALT*$^{\Delta 1AP2}$, *dGALT*$^{\Delta 1V2}$, and *dGALT*^{C2}, demonstrated normal or even slightly elevated levels of GALK and GALE activity (**Table 2.1**). Finally, all three *dGALT* excision alleles also complemented the *cup*^{01355} allele (data not shown), indicating that *cup* function was essentially unperturbed.

Dietary galactose is lethal to dGALT-deficient flies

To test the impact of galactose exposure on the viability of *dGALT*-deficient *D. melanogaster*, 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 as the sole sugar, or 555 mM glucose supplemented with 222 mM galactose (**Figure 2.3**). Surviving adult progeny were counted and 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).

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

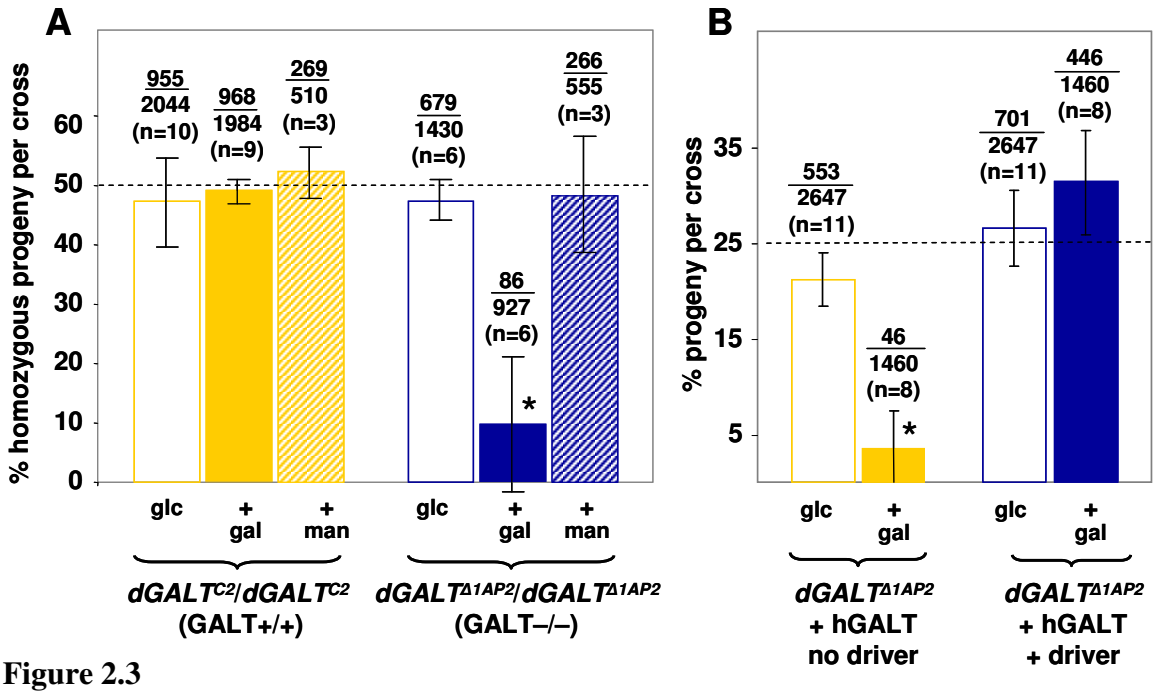


Figure 2.3

Loss of *dGALT* results in galactose sensitivity in *D. melanogaster*.

(A) Percent viable *dGALT*-null 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. Asterisk (*) indicates statistical significance ($p<0.0001$ for a two-tailed t-test at the 95% confidence interval).

offspring, but on food supplemented with 222 mM of galactose, *dGALT*-deficient flies accounted for only 0 to ~20% of viable offspring (**Figure 2.3A).** 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 $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1V2}$ transheterozygotes (data not shown).

As a control for galactose specificity of the apparent food sensitivity, we repeated each cross on food containing 555 mM glucose supplemented with 222 mM mannose, rather than galactose. All crosses yielded close to 50% unbalanced offspring, despite the presence or absence of the mannose (**Figure 2.3A**). We also performed crosses using flies homozygous for the precise excision allele ($dGALT^{C2}$); as expected, these crosses 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 outcome of these crosses (data not shown) was 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 the galactose sensitivity of dGALT-deficient D. melanogaster

To confirm that the galactose sensitivity of *dGALT*-null *D. melanogaster* resulted from loss of GALT activity and not from some unrecognized off-target effect of our excision, 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 minimize the possibility that the transgene might restore some theoretical fly-specific *dGALT* function in addition to its GALT catalytic activity, in short, to test whether *dGALT* and *hGALT* are true functional orthologs. Flies homozygous for either of the two imprecise *dGALT* excision alleles ($dGALT^{\Delta 1AP2}$ or $dGALT^{\Delta 1V2}$) and also homozygous for any one of three *UAS-hGALT* transgenes tested, $UAS\text{-}hGALT^{10A11}$, $UAS\text{-}hGALT^{9A12}$, or $UAS\text{-}hGALT^{10B22}$ (e.g., $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; $UAS\text{-}hGALT^{10A11}/UAS\text{-}hGALT^{10A11}$), were crossed to flies homozygous for the same imprecise *dGALT* excision allele and heterozygous for the *β-tubulin-GAL4* driver (e.g., $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$; *β-tubulin-GAL4*/*CyO*). These crosses were set up in parallel on foods containing 555 mM glucose or 555 mM glucose plus 222 mM galactose. As illustrated in **Figure 2.3B**, the presence of an *hGALT* transgene together with the *β-tubulin-GAL4* driver fully rescued viability of $dGALT^{\Delta 1AP2}$ homozygous larvae exposed to galactose.

Timing and dose-dependent lethality of dGALT-null D. melanogaster exposed to galactose

To identify the stage of development at which GALT-null *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-hour-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 as the sole sugar, food containing 555 mM glucose supplemented with 111 mM galactose, or food containing 555 mM glucose supplemented with 222 mM galactose. Each day the wells were inspected, and the numbers of live and dead animals were recorded. The results (**Figure 2.4**) revealed four important points:

First, *dGALT* wild-type animals homozygous for the precise excision allele ($dGALT^{C2}/dGALT^{C2}$) remained viable and developed normally throughout the 11-day 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 the $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ animals had eclosed as viable adults, compared with 90% of controls. Of note, this difference was not routinely observed in standard culture vials, suggesting that the 96-well plate format may have disproportionately stressed the *dGALT*-null animals relative to their wild-type counterparts.

Third, in the presence of increasing concentrations of galactose, we saw a decreasing percentage of *dGALT*-null 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, there were no adult *dGALT*-null survivors.

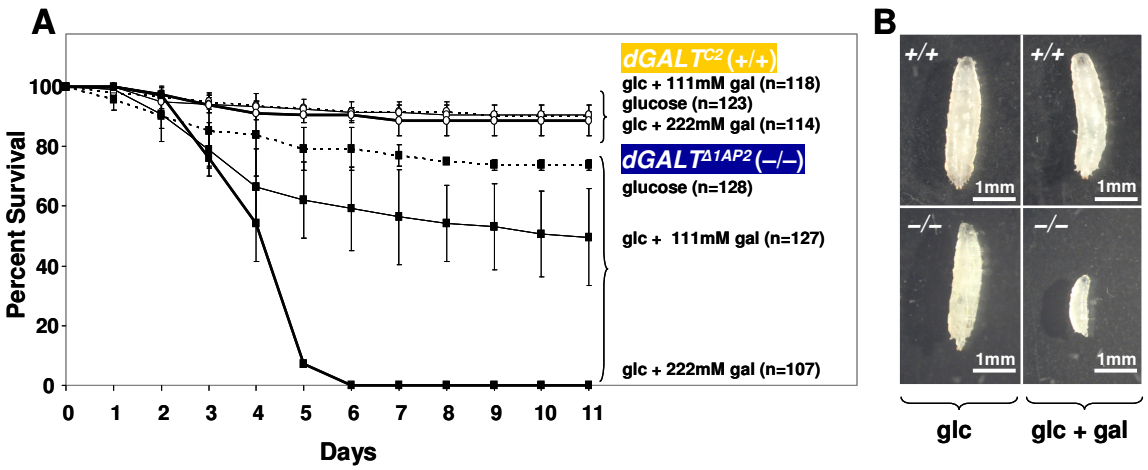


Figure 2.4

Timing of death in *dGALT*-null *D. melanogaster* exposed to galactose.

(A) 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. **(B)** Digital images of living $dGALT^{C2}/dGALT^{C2}$ precise excision (+/+) and $dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ imprecise excision (–/–) homozygotes, each photographed on day five of development; larvae were collected from vials of food containing only 555 mM glucose (glc) or 555 mM glucose plus 222 mM galactose (glc+gal), as indicated.

Finally, the timing of death of the *dGALT*-null *D. melanogaster* 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 (data not shown), but none of these larvae survived to pupation. It is difficult to distinguish further the precise stage of lethality, because the dying animals were quite stunted relative to control animals (**Figure 2.4B**); whether this small organismal size reflected a true developmental delay or simply a growth defect remains unclear.

Window of galactose sensitivity of dGALT-null D. melanogaster

To define the stage of development at which *dGALT*-null *D. melanogaster* are most sensitive to galactose exposure, we performed dietary "crossover" experiments (**Figure 2.5**) in which cohorts of $dGALT^{\Delta IAP2}$ homozygous animals were transferred as embryos or larvae from food containing only 555 mM glucose to food containing both 555 mM glucose and 222 mM galactose, or from food containing both 555 mM glucose and 222 mM galactose to food containing only 555 mM glucose. As a control for the transfer process, animals were also transferred each day from 555 mM glucose food to fresh 555 mM glucose food, or from 555 mM glucose and 222 mM galactose food to fresh 555 mM glucose and 222 mM galactose food. The transfers were continued 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.

The results (**Figure 2.5**) clearly showed *dGALT*-null animals that started developing on glucose food nonetheless succumbed if they were transferred to glucose

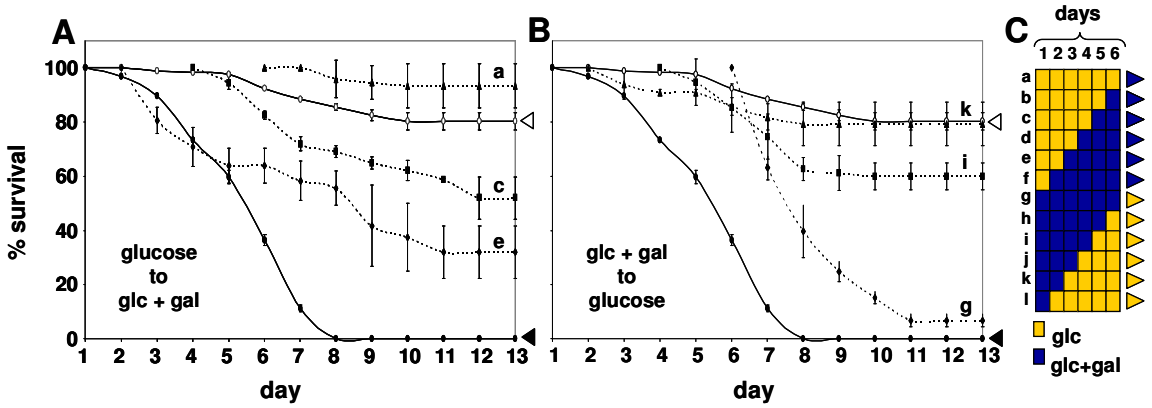


Figure 2.5

Window of galactose sensitivity of *dGALT*$^{\Delta 1AP2}$/*dGALT*$^{\Delta 1AP2}$ imprecise excision homozygotes.

dGALT-null embryos and larvae were transferred in cohorts of >20 animals each from 555 mM glucose food to 222 mM galactose enriched food (dashed lines in **A**), or from 222 mM galactose enriched food to 555 mM glucose food (dashed lines in **B**), for six days, after which each cohort was allowed to complete development without further interference. 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 food to glucose food (solid line indicated with an open triangle), and from galactose enriched food to galactose enriched food (solid line indicated with a black triangle). In both experiments animals demonstrated a reversible response to galactose exposure during early larval development. **(C)** The diagram illustrates the food switching regimen; each row represents one cohort of animals; lower case letters correspond to data curves plotted in **A** and **B**. Each gold box represents one day of development on glucose food; each blue box represents one day of development on food supplemented with 222 mM galactose.

and galactose food early in larval development (e.g., on day two of larval development, which corresponded to late L1 or early L2 stage); transfer on subsequent days of development (e.g., day four, which corresponded to late L2 or early L3 stage) resulted in diminished, albeit detectable, loss of viability. Significantly, *dGALT*-null animals exposed to galactose in late larval development (e.g., day six, which corresponded to L3 stage) or as adults (data not shown) demonstrated no significant loss of viability relative to their galactose restricted counterparts.

Similarly, *dGALT*-null animals that started developing on food supplemented with galactose were rescued from death by transfer to glucose 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 enriched food to glucose food (**Figure 2.5B**), and also to a slight upward shift in the survival curve of animals transferred from glucose food to fresh glucose food on day six of development (**Figure 2.5A**). 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 (two, four, and six) are presented in **Figure 2.5**; however, the cohorts transferred on odd-numbered days showed survival profiles fully consistent with these patterns (data not shown).

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

dGALT-deficient Drosophila accumulate gal-1P when exposed to galactose

To test whether *dGALT*-deficient *Drosophila* exposed to dietary galactose accumulate abnormal levels of gal-1P developing larvae and newly eclosed adults were exposed for four days to food containing either 555 mM glucose alone or 555mM glucose supplemented with 222 mM galactose. Extracts of these animals were prepared and analyzed as described in Methods. As presented in **Table 2.3**, in the presence of dietary galactose, *dGALT*-deficient ($dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$) larvae and adults accumulated abnormally high levels of gal-1P; the control animals ($dGALT^{C2}/dGALT^{C2}$) did not.

Galactose restricted dGALT-null flies demonstrate an impaired negative geotaxic response

To address whether *dGALT*-null *D. melanogaster* might, like their human counterparts, exhibit long-term complications despite lifelong dietary restriction of galactose, we assessed the negative geotaxic response of two stocks of *dGALT*-null flies ($dGALT^{\Delta 1AP2}/dGALT^{\Delta 1AP2}$ and $dGALT^{\Delta 1V2}/dGALT^{\Delta 1V2}$), as well as control flies ($dGALT^{C2}/dGALT^{C2}$). All of these flies were raised on standard molasses food and tested using a previously described countercurrent apparatus that quantifies phototaxic and negative geotaxic response, and also time to recovery (Benzer 1967; Akai 1979). Normal flies exhibit a strong negative geotaxic response and a quick response to stimulus, meaning they prefer to run up the sides of a tube rather than stand still or run down, and they recover quickly after being tapped to the bottom of a tube.

The apparatus and procedure we applied offered cohorts of approximately 60 two

Genotype	Gal-1P (pmol/ µg protein)	
	555mM glc food	555mM glc + 222 mM gal food
dGALT^{C2}/dGALT^{C2}		
larvae	**0.37 ± 0.09**	**0.85 ± 0.12**
adults	**0.10 ± 0.04**	**0.30 ± 0.10**
dGALT$^{\Delta 1AP2}$/dGALT$^{\Delta 1AP2}$		
larvae	**0.75 ± 0.13**	**11.82, 12.09***
adults	**0.22 ± 0.09**	**7.95 ± 4.25**

Table 2.3

Accumulation of Gal-1P in larvae and flies exposed to galactose.

Metabolites were extracted from cohorts of wild-type and mutant larvae and adults maintained for four days on food with or without galactose supplementation, as described in **Methods**. Values presented are average ± SD (n = 3). *Individual values are presented when n=2.

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.

Cohorts of control flies subjected to this countercurrent test ended up distributed among the six tubes, as presented in **Figure 2.6A**. Close to 60% of the flies were in tube six, with the remainder distributed in small numbers between the other five tubes; this result is fully consistent with the original report of this behavior more than 40 years ago (Benzer 1967). In striking contrast, both *dGALT*-null stocks demonstrated an almost random distribution of flies among the six tubes; there was no enrichment of flies in tube six relative to the other tubes (**Figure 2.6B, C**). This differential distribution profile was highly statistically significant ($p<0.01$) and was rescued by expression of the human *GALT* transgene (**Figure 2.6D**), thereby demonstrating that it resulted from the absence of GALT activity.

Discussion

Here we report the generation and initial characterization of a *D. melanogaster* model of classic galactosemia. This is the first genetic animal 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.

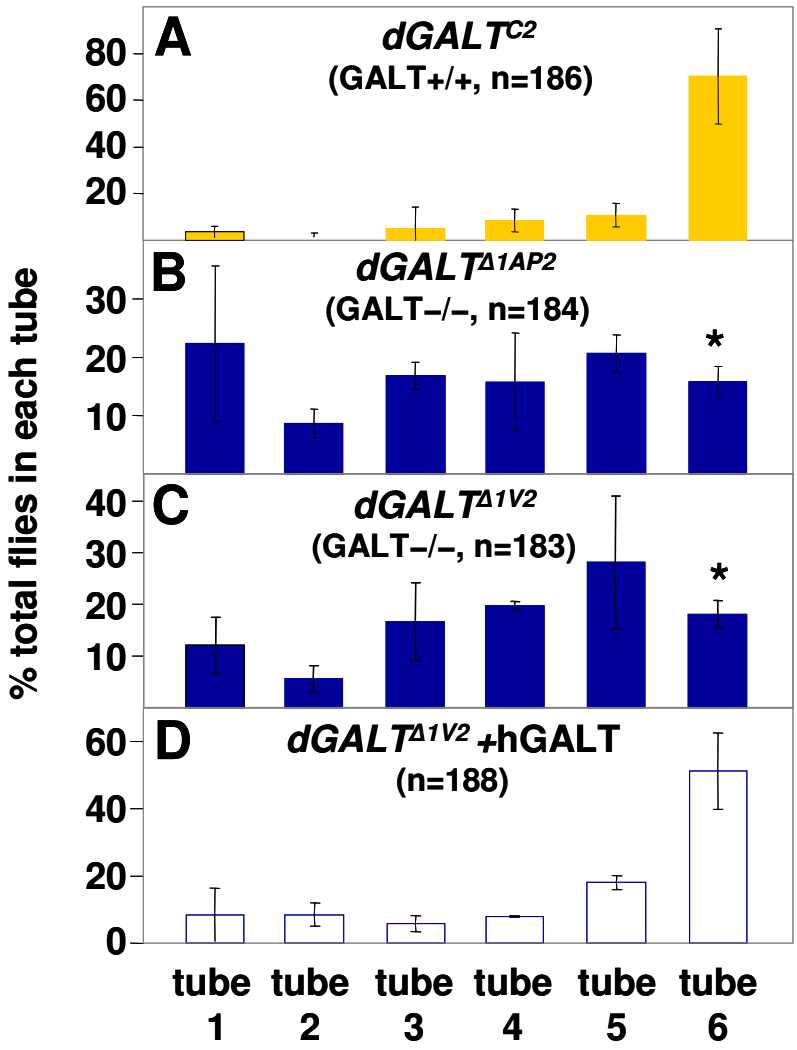


Figure 2.6

***dGALT*-null 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 ± s.d (n = 3). Flies homozygous for the $dGALT^{\Delta 1AP2}$ (**B**) and $dGALT^{\Delta 1V2}$ (**C**) imprecise excision alleles demonstrated a statistically significant impairment of negative geotaxic response relative to flies homozygous for the $dGALT^{C2}$ precise excision allele (**A**) (*, $p<0.01$ in a one-way ANOVA with 95% confidence intervals); this impairment was rescued by expression of a human *GALT* transgene (**D**).

Galactose sensitivity of dGALT-null 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 for the most part it 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; namely, the lack of a phenotype in *GALT*-null mice raised the unlikely but disturbing possibility that the clinical outcomes of patients with classic galactosemia might result from some cryptic and mysterious impact of their mutations, rather than from the detected loss of GALT enzymatic activity (Leslie et al. 1996). With the work presented here, the causal relationship between loss of GALT activity and galactose-induced phenotypes in a multicellular animal is confirmed.

Secondly, the galactose-dependent mortality observed in *dGALT*-null *D. melanogaster* is both dose and time dependent. Exposure to a lower level of galactose (e.g., 111 mM), or exposure to galactose later in development (e.g., L3 stage) resulted in less severe outcomes than exposure to a higher level of galactose (e.g., 222 mM) or exposure earlier in organismal development. These data implicate a potential threshold, or series of thresholds, of galactose sensitivity in GALT-null *D. melanogaster*, and/or 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 yet distinguish between these possibilities.

Consistent with this conclusion, we observed that the numbers of *dGALT*-null flies that eclosed as viable adults when exposed to 222 mM galactose hovered between

0% and 20%; the precise number varied from experiment to experiment, even for crosses involving flies of the same *dGALT* genotype. Raising the level of galactose from 222 mM to 278 mM was sufficient to bring the number of *dGALT*-null survivors virtually to zero, but at the lower concentration of galactose, other factors also seemed to play a role. 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, likely influenced the overall stress level in the animals and may have impacted the ability of some *dGALT*-null 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 as a result of newborn screening, where affected infants are routinely spared from potentially lethal acute symptoms by rapid initiation of a galactose restricted diet (Fridovich-Keil and Walter 2008). The fly result is nonetheless compelling, since it suggests 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, however, we must be careful not to over interpret the data. For example, although exposure of *D. melanogaster* adults or late-stage larvae to galactose was not lethal, we do not know whether it was completely benign. The possibility remains that exposure of *dGALT*-null animals to sub-lethal levels of galactose might have negatively impacted some aspects of development or

homeostasis we did not test. It would therefore be premature to conclude from the data presented here that *dGALT*-null flies, or classic galactosemia patients, do not require lifelong dietary galactose-restriction. Nevertheless, there are intriguing anecdotal reports from the literature that describe a similar finding in patients; namely, that older children and adults with classic galactosemia appear to be able to consume dietary galactose with no acute negative clinical consequences (Bosch et al. 2004; Panis et al. 2006).

Long-term complications

Although we have just begun to test our *dGALT*-null flies for galactose-independent phenotypes, abnormalities clearly exist (**Figure 2.6**) and they 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 neurological or neuromuscular abnormalities. While many patients with classic galactosemia do experience neurological and/or neuromuscular complications, the relationship between those patient outcomes and the fly phenotype reported here remains to be explored. Future studies will also address the extent and nature of other potential abnormalities in the *dGALT*-null flies, as well as the genetic and environmental factors that underlie them.

Metabolic abnormalities in dGALT-impaired D. melanogaster

That *dGALT*-deficient *D. melanogaster* exposed to galactose accumulate elevated levels of gal-1P is fully consistent with metabolic abnormalities reported in patients (reviewed in (Fridovich-Keil and Walter 2008)). 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 (**Table 2.3**) yet the larvae are progressing toward death, but the adults are not. While there are many possible explanations for apparent disparity, 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(Leslie et al. 1996). Hence, mice do not succumb in response to galactose exposure regardless of age, whereas flies only succumb to galactose-exposure within a 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

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CHAPTER III

THE CREATION OF A SIMPLE, QUANTITATIVE ASSAY FOR THE IDENTIFICATION OF HUMAN GALACTOKINASE INHIBITORS

Introduction

The Leloir pathway is conserved from *E. coli* to humans and is responsible for the majority of galactose metabolism via three major enzymes: **galactokinase** (**GALK**), **galactose-1-phosphate uridylyltransferase** (**GALT**), and **UDP-galactose 4'-epimerase** (**GALE**) (**Figure 1.1**) (Holton et al. 2000; Leslie 2003). In humans, impairment in any one of the Leloir enzymes results in the metabolic disorder, galactosemia.

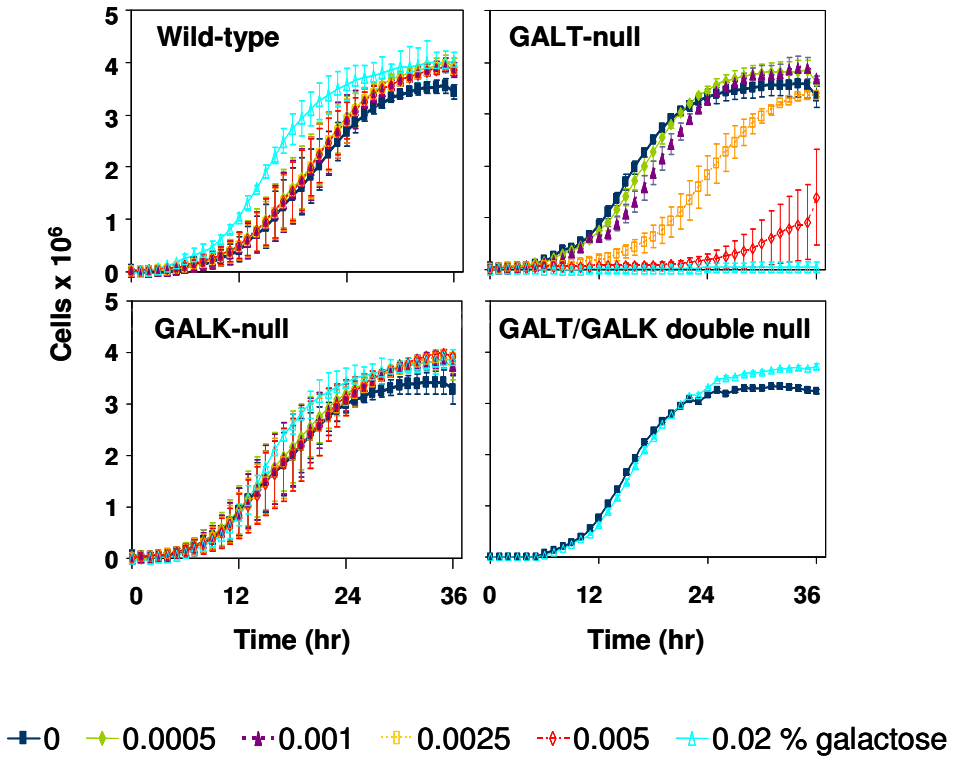
Impairment in the second enzyme, GALT, results in the most common clinically sever form of galactosemia, named classic galactosemia. It has been well established through observations in human patients and research in yeast and mammalian cell model systems that the first enzyme of the Leloir pathway, GALK, is an important modifier of the acute galactose sensitivity resulting from the loss of GALT activity. Humans with GALK deficiency demonstrate none of the acute and potentially lethal or long-term complications of classic galactosemia. The most common symptom of GALK deficiency is neonatal cataracts; fortunately, upon commencement of dietary restriction galactose, these cataracts usually resolve (Gitzelmann 1967; Levy 1980; Holden 2004).

In both GALK and GALT deficiency, patients accumulate galactose, leading to the formation of cataracts via the enzyme aldose reductase (AR), which converts galactose to galactitol (Bosch et al. 2002; Holden 2004; Thoden et al. 2005). This area of the pathophysiology of galactosemia is well understood and, as mentioned previously, cataracts are treatable with the galactose restricted diet. The most obvious difference in the pathophysiology of these two disorders is patients with classic galactosemia accumulate abnormal levels of galactose-1-phsosphate (gal-1P), the product of GALK, implicating this metabolite as a contributing factor in the acute and long-term complications of this metabolic disorder (Holton et al. 2000; Leslie 2003; Slepak et al.

2005). The pathophysiology of the severe acute and long-term complications of classic galactosemia and the relationship between these sequelae and other metabolite abnormalities, including gal-1P levels, are unknown. It has been widely hypothesized that the selective inhibition of GALK, and the subsequent prevention of the gal-1P production, may prevent or at least minimize the sequelae of classic galactosemia.

Untreated patients with classic galactosemia accumulate abnormal levels of gal-1P and several studies have shown a correlation between elevated gal-1P levels and severity of phenotypic outcome. Additionally, patients demonstrate aberrant glycosylation patterns, which may be related to the gal-1P inhibition of the enzymes involved in galactosylation (Haberland et al. 1971; Dobbie et al. 1990; Petry et al. 1991; Jaeken et al. 1992; Ornstein et al. 1992; Prestoz et al. 1997; Charlwood et al. 1998; Sturiale et al. 2005).

Studies in the yeast and mammalian cell culture model systems of GALT deficiency have looked at the roles of GALK and gal-1P in galactose sensitivity. GALT-null yeast growth arrest and accumulate high levels of gal-1P in the presence of galactose, even when alternative carbon sources (glycerol and ethanol) are present (**Figure 3.1**) (Douglas and Hawthorne 1964; Riehman et al. 2001; Ross et al. 2004; Slepak et al. 2005). The secondary deletion of *GALK* is able to prevent both the metabolic abnormality and the growth arrest phenotype (Mehta et al. 1999; Kabir et al. 2000; Lai and Elsas 2000; Ross et al. 2004). Additionally, studies have found that mediators of the galactose-induced sensitivity are active in either the prevention of gal-1P production (GALK) or in the further metabolism of gal-1P (UDP-glucose


Wild-type
GALT-null
GALK-null
GALT/GALK double null
Cells x 10^6
Time (hr)
Time (hr)
0
0.0005
0.001
0.0025
0.005
0.02 % galactose

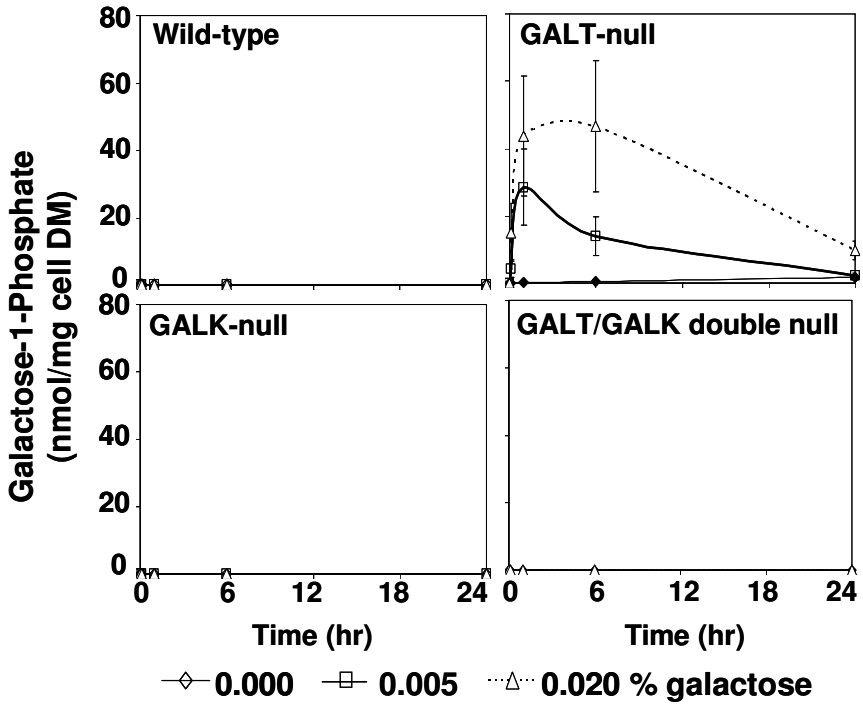

Wild-type
GALT-null
GALK-null
GALT/GALK double null
Galactose-1-Phosphate (nmol/mg cell DM)
Time (hr)
Time (hr)
0.000
0.005
0.020 % galactose

Figure 3.1

The secondary deletion of *GALK* prevents galactose sensitivity in *GALT*-null yeast

The secondary deletion of *GALK* in *GALT*-null yeast prevents galactose-induced growth arrest and the abnormal accumulation of gal-1P. Yeast were exposed to media containing galactose and an alternative carbon source (2% glycerol and 2% ethanol medium) [Ross 2004].

pyrophosphorylase (UGP) and inositol monophosphatase (IMPase)) (Douglas and Hawthorne 1964; Parthasarathy et al. 1997; Mehta et al. 1999; Kabir et al. 2000; Lai and Elsas 2000; Riehman et al. 2001; Bhat 2003; Ross et al. 2004). Studies in mammalian cell culture mirror these findings; cell lines derived from classic galactosemics are growth impaired and accumulate abnormal levels of gal-1P when galactose is present in their medium (Pourci et al. 1990; Lai et al. 2003). The overexpression of human UGP improves the growth and metabolic phenotypes of the GALT-impaired cells (Lai et al. 2003).

Together, these data suggest the inhibition of GALK by a small molecule inhibitor may have a large impact on gal-1P accumulation and patient outcome. The short-term goal of this project was to develop a quantitative assay for human galactokinase (hGALK) activity in a 384-well microplate format in order to screen for small molecule inhibitors of this enzyme. The long-term goal of this project was to develop an alternative or supplemental treatment for classic galactosemic patients. Additionally, an inhibitor of hGALK would be an invaluable laboratory tool for the further understanding of galactosemia.

Materials and Methods

This assay used three purified enzymes:

1) human galactokinase (hGALK, E.C. 2.7.1.6)

   His6 tagged hGALK was recombinantly expressed in *E. coli* and purified using nickel-affinity chromatography by Drs. Jim Thoden and Hazel Holden and was

stored at -85°C at a concentration of 8 mg/mL in freezing buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% glycerol) in small aliquots. When the protein was needed for an assay, it was thawed on ice and diluted to 300 nM in buffer (40 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 100 μM DTT, 10% glycerol), and then further diluted to 10 nM in the reaction master mixture.

2) pyruvate kinase (PK, E.C. 2.7.1.40)

700 U/mL (Sigma Chemicals, Inc. #P0294)

3) lactate dehydrogenase (LDH, E.C. 1.1.1.27)

1000 U/mL (Sigma Chemicals, Inc. #P0294)

This assay uses the following reagents:

α-D-galactose (Sigma-Aldrich #G0750)

ATP (Roche Pharmaceuticals #11140-965)

Tris-HCl buffer (prepared with components from Fisher Scientific and HPLC -grade water)

$MgCl_2$ (Fisher Scientific #BP214-500)

glycerol (US Biologicals #G8145)

phosphoenolpyruvate (PEP, Aldrich #860077)

NADH (Calbiochem #481913)

This assay uses the following microplates:

black 384-well, flat and clear bottom microplates (Corning #3711)

The 384-well microplates are first loaded with 40 μL "reaction master mixture," which contains all components of the assay except galactose, to each well, followed by

either 5 µL of DMSO (negative control) or a candidate inhibitor. The mixture was allowed to incubate for 10 minutes to allow inhibitors and their potential targets time to interact (Copeland 2003). Next, to begin the reaction, 5 µL of 2.5 mM galactose was added with gentle mixing. The final concentration of the components of the assay are 10 nM hGALK, 0.55 U/L pyruvate kinase, 0.74 U/L lactate dehydrogenase, 680 µM ATP, 200 µM PEP, 200 µM NADH, and 250 µM galactose in buffer (40 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 100 µM DTT, 10% glycerol). As an additional negative control, some wells received reaction master mixture that does not contain hGALK.

With the addition of galactose, the microplate was placed in an absorbance reader, where absorbance of each well is measured at 340 nm every five minutes until t=50 minutes after the addition of galactose. In order to account for the fact that the absorbance of the reaction at 340 nm can be affected by small changes in the concentration of NADH and hGALK in the reaction master mixture, and by the intrinsic spectral properties of the candidate inhibitors being screened, the change in absorbance at 340 nm (Abs_{340}) was monitored over time as a slope, which accurately reflects the rate of the hGALK reaction.

Results

A simple, quantitative assay for hGALK activity

In order to identify small molecule inhibitors of hGALK, we have first developed a simple and quantitative assay for hGALK activity using a well-established strategy for

detecting the production of ADP using the enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) (**Figure 3.2**). The assay is initiated by the addition of α-D-galactose, which is converted into gal-1P by hGALK, and a molecule of ATP. The resulting molecule of ADP is consumed by PK to create a molecule of pyruvate, using a molecule of phosphoenolpyruvate (PEP). Pyruvate is then converted into lactate by LDH with the oxidation of NADH into NAD+. This final reaction is detected by the loss of absorbance at 340 nm. The entire reaction occurs at room temperature (approximately 25^{o}C) and in a total volume of 50 µL in a 384-well microplate. The use of a coupled enzyme assay allows for the easy detection of hGALK activity via spectrophotometry. Such an assay can be easily applied to a high-throughput screening format once optimized. The identification of small molecules that inhibit hGALK activity will occur via a decrease in the rate of NADH consumption.

To account for the fact that the absorbance of the reaction at 340 nm can be affected by small changes in the concentration of NADH and hGALK in the reaction master mixture, and by the intrinsic spectral properties of the candidate inhibitors being screened, we will be measuring the change in absorbance at 340 nm (Abs_{340}) over time as a calculated slope, which accurately reflects the rate of the hGALK reaction.

In order for the assay to accurately measure small changes in hGALK activity, both galactose and hGALK must be limiting in the reaction, while all other components of the reaction must be in excess. hGALK is limiting at 10 nM in the assay, which is sufficient to produce a strong, reproducible activity, but low enough to allow the detection of inhibitors at low concentrations or of low potency (**Figure 3.3**). Galactose is

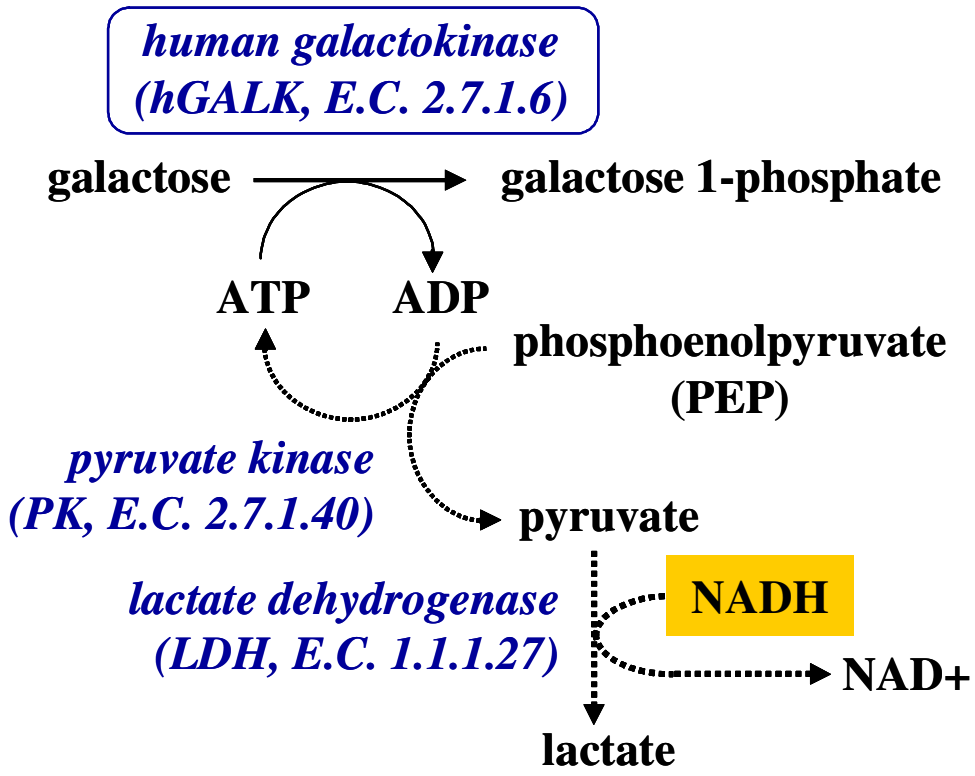
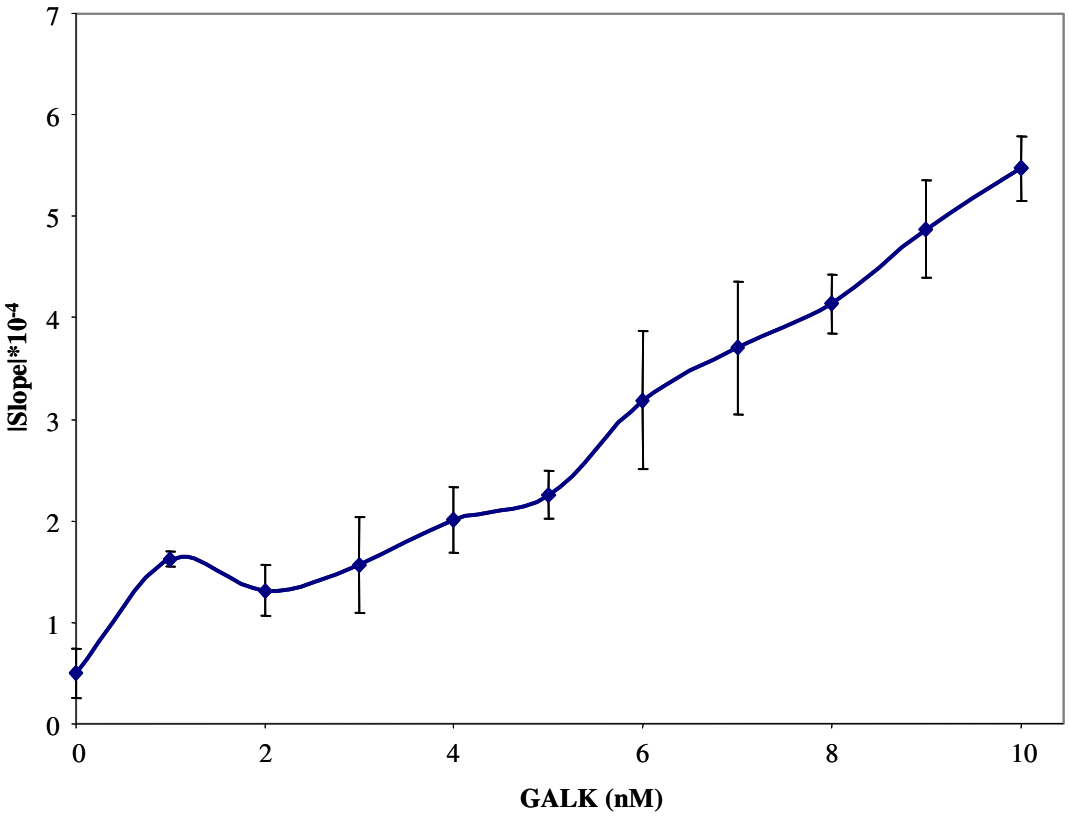


Figure 3.2

A simple, coupled enzyme assay for hGALK activity

The assay is based on a well-established strategy of detecting the production of ADP via a coupled enzyme reaction. The assay starts with the addition of α-D-galactose. hGALK converts a molecule of α-D-galactose into a molecule of gal-1P, using a molecule of ATP. The resulting molecule of ADP is converted into pyruvate by PK, using a molecule of PEP. Pyruvate is converted into lactate by LDH. This final reaction uses a molecule of NADH, which is oxidized into NAD+. The NADH absorbs strongly at 340 nm and the hGALK assay is monitored by the loss of absorbance at this wavelength. The reaction occurs at room temperature (approximately 25^{o}C) and in a total volume of 50 µL in a 384-well plate.


|Slope|*10-4
0
1
2
3
4
5
6
7
0
2
4
6
8
10
GALK (nM)

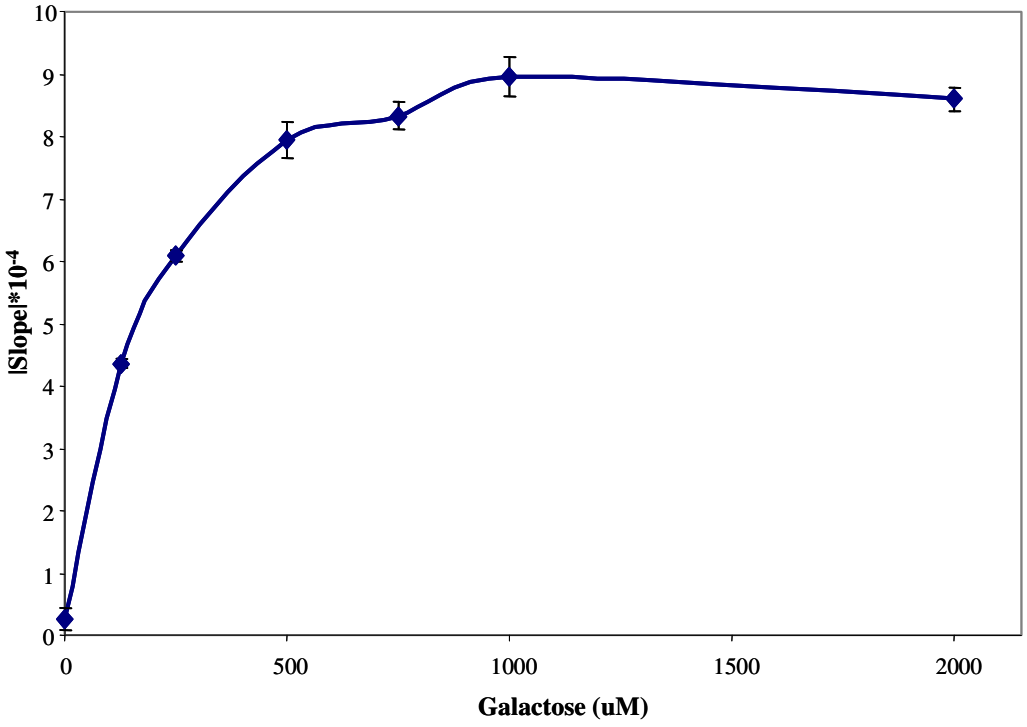

|Slope|*10-4
0
1
2
3
4
5
6
7
8
9
10
0
500
1000
1500
2000
Galactose (uM)

Figure 3.3

hGALK and galactose are limiting in the hGALK assay

In order for the assay to accurately measure small changes in hGALK activity, hGALK and galactose must be limiting in the reaction, while the coupling enzymes and other reaction components are kept in excess. A titration of hGALK was conducted from 1 nM to 10 nM. hGALK is limiting at 10 nM in the assay, producing a strong and reproducible signal. Additionally, this experiment demonstrated that the assay is able to reproducibly detect small changes in hGALK activity. This will be useful in identifying weak inhibitors.

Galactose was titrated in the reaction from 125 μM to 2000 μM (2 mM) and was found to be limiting at 250 μM, which is about ¼ K_m for hGALK. This concentration will allow for the detection of weak and competitive inhibitors.

limiting in the reaction at 250 μM, which is ~¼ K_m for hGALK (**Figure 3.3**). Again, this concentration allows for the detection of weak inhibitors and competitive inhibitors, which would be difficult to identify if substrate levels were increased. In order to maximize the sensitivity of our assay, ATP, the PK and LDH enzymes, and the other reaction reagents, are held in great excess in the reaction. ATP, the second substrate of hGALK, is held at ~20 times K_m, allowing for the activity of hGALK to be at a maximum, while making possible the identification of competitive inhibitors that mimic galactose, but not ATP. These types of inhibitors will be more specific inhibitors of hGALK, as few enzymes selectively interact with free galactose and many enzymes use ATP as a substrate. Finally, the enzymes PK and LDH and their substrates, PEP and NADH, are kept in excess in order to, again, increase the activity and sensitivity of the assay, and avoid identifying inhibitors of these enzymes.

The enzymatic reaction is reproducible, linear, and stable at room temperature

Once the reaction is initiated with the addition of galactose, the rate remains linear for at least 90 minutes (**Figure 3.4**). For the purposes of the screen, we will be using incubation times of 15-30 minutes, which is still adequate for excellent S:B, S:N, and Z" values (see next section). Additionally, the assay is able to reproducibly detect small changes in hGALK activity, allowing for even weak inhibitors to be identified (**Figure 3.3**).

In order to test the stability of the reaction, we maintained the reaction master mixture in the absence of galactose at room temperature (25°C) for 36 hours. At several time points, the reaction was started with the addition of galactose and monitored. Very

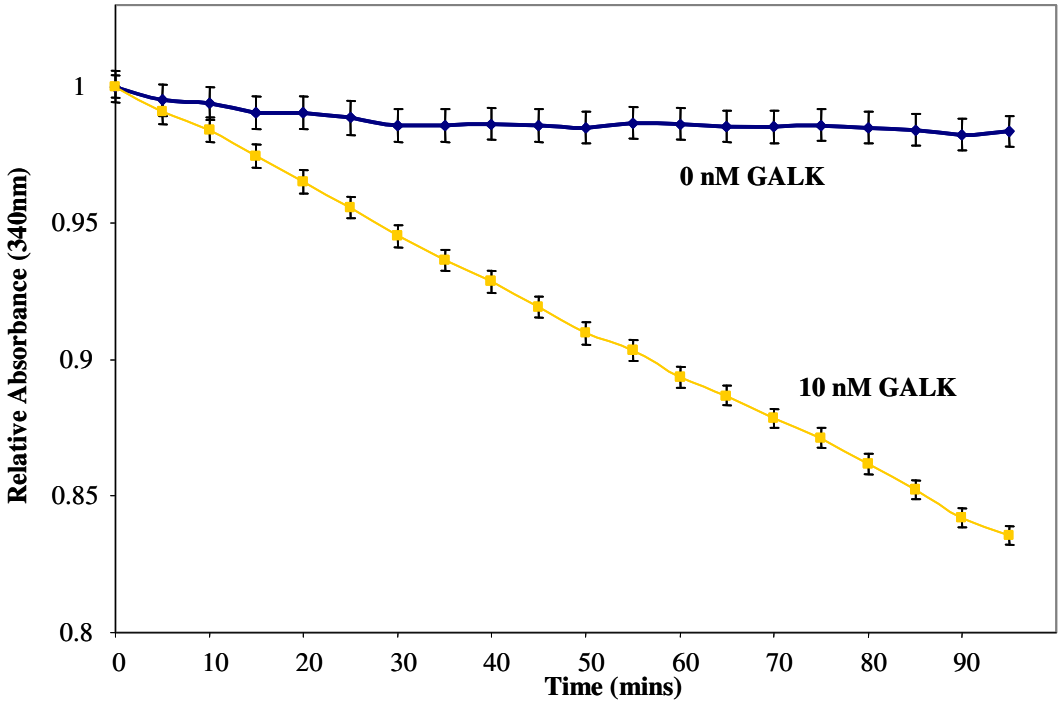


Figure 3.4

hGALK assay is linear for at least 90 minutes

The reaction remains linear for at least 90 minutes after the addition of α-D-galactose. For the purposes of the screen, incubations times of 15 to 30 minutes will be used in order to minimize the length of time necessary to screen large libraries.

little activity was lost over the first 24 hours (**Figure 3.5**). The ability for the reaction to remain very active at room temperature allows for easy facilitation of the robotic high-throughput screening instrumentation necessary for the screening of large libraries.

Finally, in order to account for the fact that many small molecule libraries use DMSO as a solvent, we tested the impact of DMSO on hGALK activity by performing a dilution series up to 20% (**Figure 3.6**). About 50% sensitivity is lost at a DMSO concentration of 20%, which still allows adequate levels of sensitivity for the proposed screens. Usually, inhibitors are tested so that the final concentration of DMSO in the reaction is 10%.

Assay parameters: signal-to-background, signal-to-noise, and Z

As a measure of the sensitivity, reproducibility, and overall quality of the hGALK assay, we calculated the signal-to-background (S:B), signal-to-noise (S:N), and Z' parameters. These parameters are measures of the ability of an assay to be applied to high-throughput screening. The equations used to calculate S:B, S:N, and Z' are as follows:

S:B = mean slope (GALK)/mean slope (no GALK)

$S:N = (\mu_{GALK} - \mu_{no\ GALK})/(SD_{GALK}^2 + SD_{no\ GALK}^2)^{0.5}$

$Z' = 1 - (3SD_{GALK} + 3SD_{noGALK})/(\mu_{GALK} - \mu_{no\ GALK})$

where SD_{GALK} and $SD_{no\ GALK}$ are the standard deviations for enzyme assays conducted in the presence and absence of hGALK, respectively, and $\mu_{GALK} - \mu_{no\ GALK}$ is the difference in mean signals (slopes) for these two data sets. In order for an assay to be considered ready for high-throughput screening, S:B and S:N must be greater than 10 and Z' must be

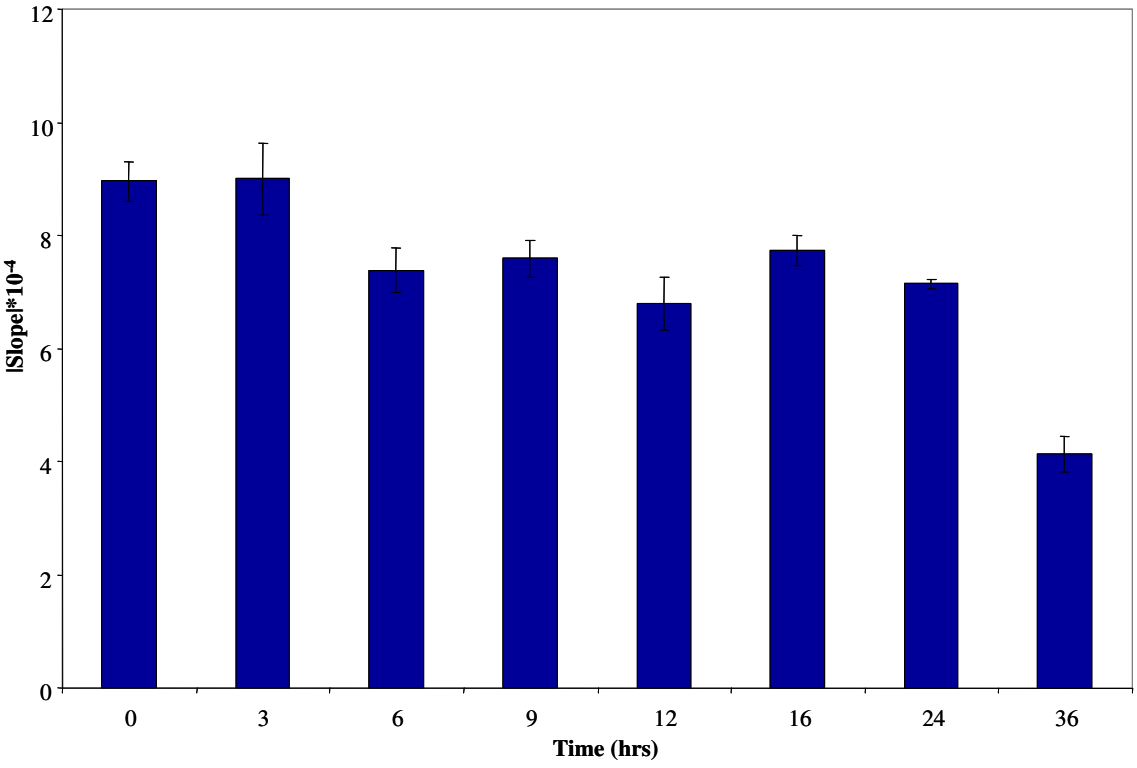


Figure 3.5

Stability of the reaction components at room temperature

The stability of the hGALK assay was tested at room temperature (approximately 25°C). The reaction master mix was maintained at room temperature for 36 hours, during which the reaction was initiated with the addition of α-D-galactose and monitored at several time points. Minimal activity was lost over the first 24 hours, making the reaction easy to apply to the robotic high-throughput screening instrumentation used for the screening of large libraries.

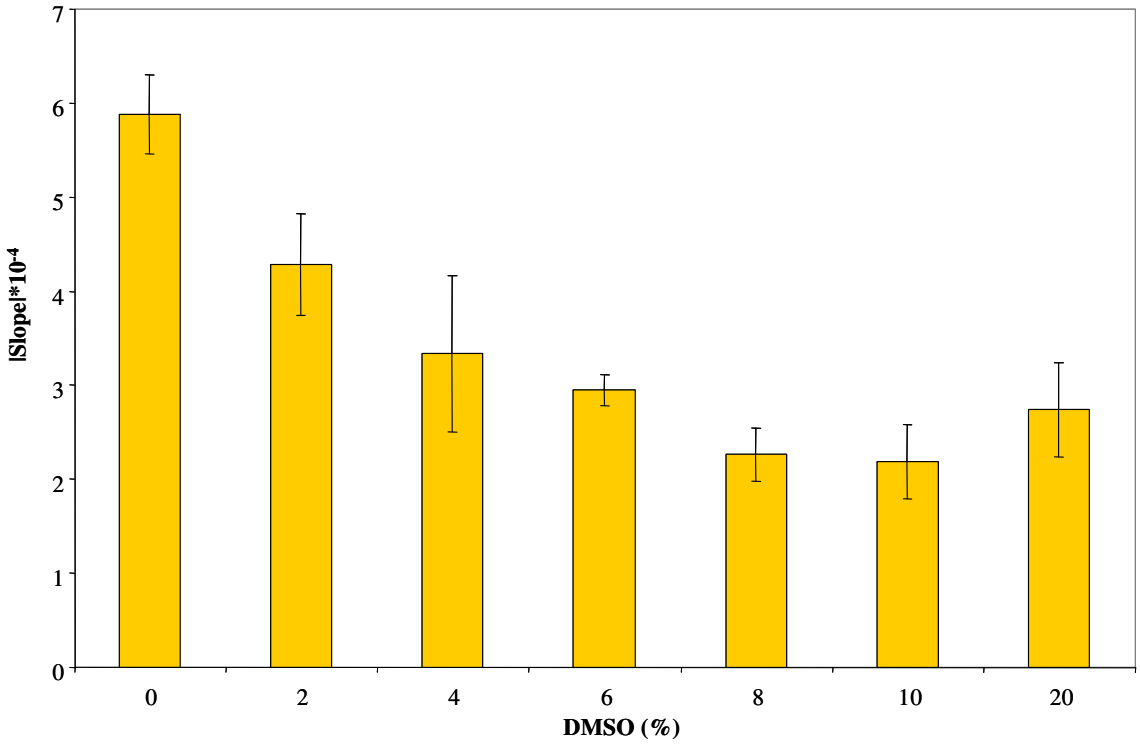


Figure 3.6

The impact of DMSO on the hGALK assay

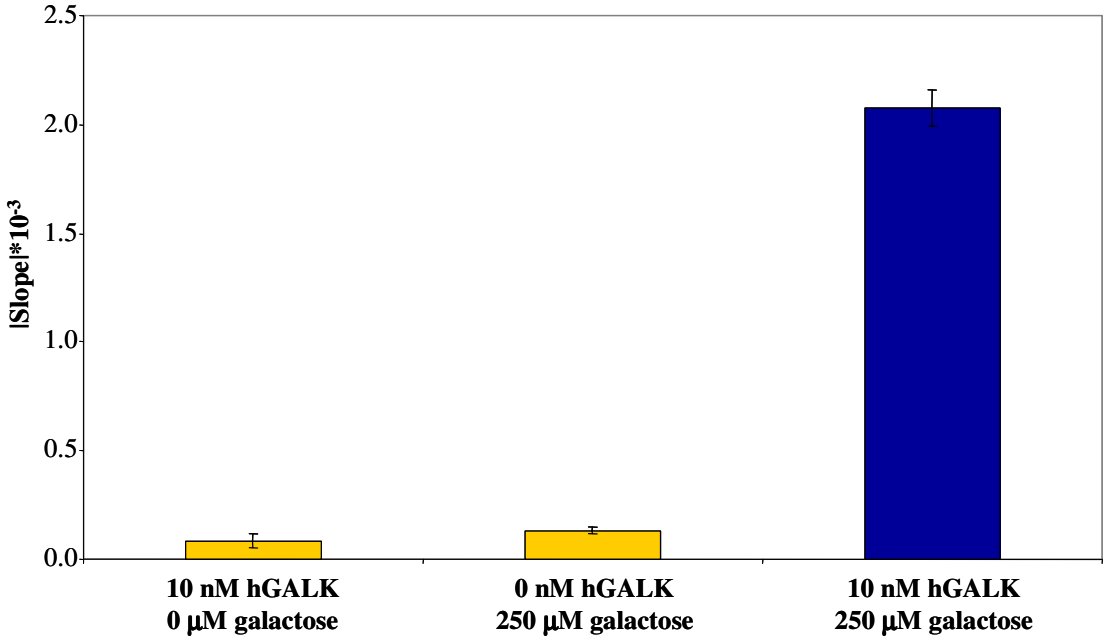
Many inhibitor libraries are maintained in DMSO. In order to account for this, a titration of DMSO from 2% to 20% was tested for the impact on hGALK activity. In most cases, inhibitors are tested where the final concentrations of DMSO is 10% in the reaction. About 50% of hGALK activity, and therefore sensitivity, is lost at this concentration of DMSO.

greater than 0.5 (Zhang et al. 1999). All three parameters of the assay meet criteria when 0 μM galactose is used as a negative control, indicating that the assay can be applied to a high-throughput screen with minor further optimization (**Figure 3.7 and Table 3.1**)

In silico screen

To run an initial assessment of our assay's ability to detect inhibition, we tested six predicted hGALK inhibitors: one previously shown to inhibit GALK and five predicted via an *in silico* screen conducted by our collaborators (**Figure 3.8**). These data demonstrate that the assay is sensitive enough to detect as little as 10% inhibition.

1-deoxygalactose has been reported to competitively inhibit GALK with a K_I of 4.1 mM (Reutter and Bauer 1985). Structurally, 1-deoxygalactose is similar enough to α-D-galactose to bind the active site of hGALK; since the molecule lacks the C1 hydroxyl group, hGALK cannot react with 1-deoxygalactose to produce gal-1P. The additional five compounds assayed were identified in collaboration with Drs. Chuck Parkos and Eric Severson as predicted inhibitors. Dr. Severson took a virtual picture of the hGALK x-ray crystal structure and identified the active site and the binding site for galactose and ATP. Using the corresponding coordinates, he used a software program to estimate the binding affinity of the 100,000 compounds of the NCI library at the active site of hGALK. More than 1200 compounds were identified and predicted to bind the hGALK active site with high affinity via calculations of predicted binding energy using the Xscore and DrugScore algorithms. The five NSC compounds tested here were in the top 1.3% of predicted binding affinity. The best predicted inhibitors bind to the active site to occupy both the galactose and ATP binding sites. Libraries containing compounds



Parameter	0 µM galactose as negative	0 nM GALK as negative
S:B	12.2	9.6
S:N	21.7	22.3
Z'	0.82	0.84

Figure 3.7 and Table 3.1

S:B, S:N, and Z' parameters for the hGALK assay meet criteria for high-throughput screening

Negative and positive control hGALK assays performed in 384-well format with manual pipetting and monitored using an absorbance plate reader (n=128). S:B, S:N, and Z'

parameters were calculated from these data. For an assay to be considered highly sensitive, reproducible, and robust assay for the use in high-throughput screens, S:B and S:N values must be greater than 10 and Z' must be greater than 0.5. All three parameters of the assay meet criteria for high-throughput screening when 0 μM galactose is the negative control.

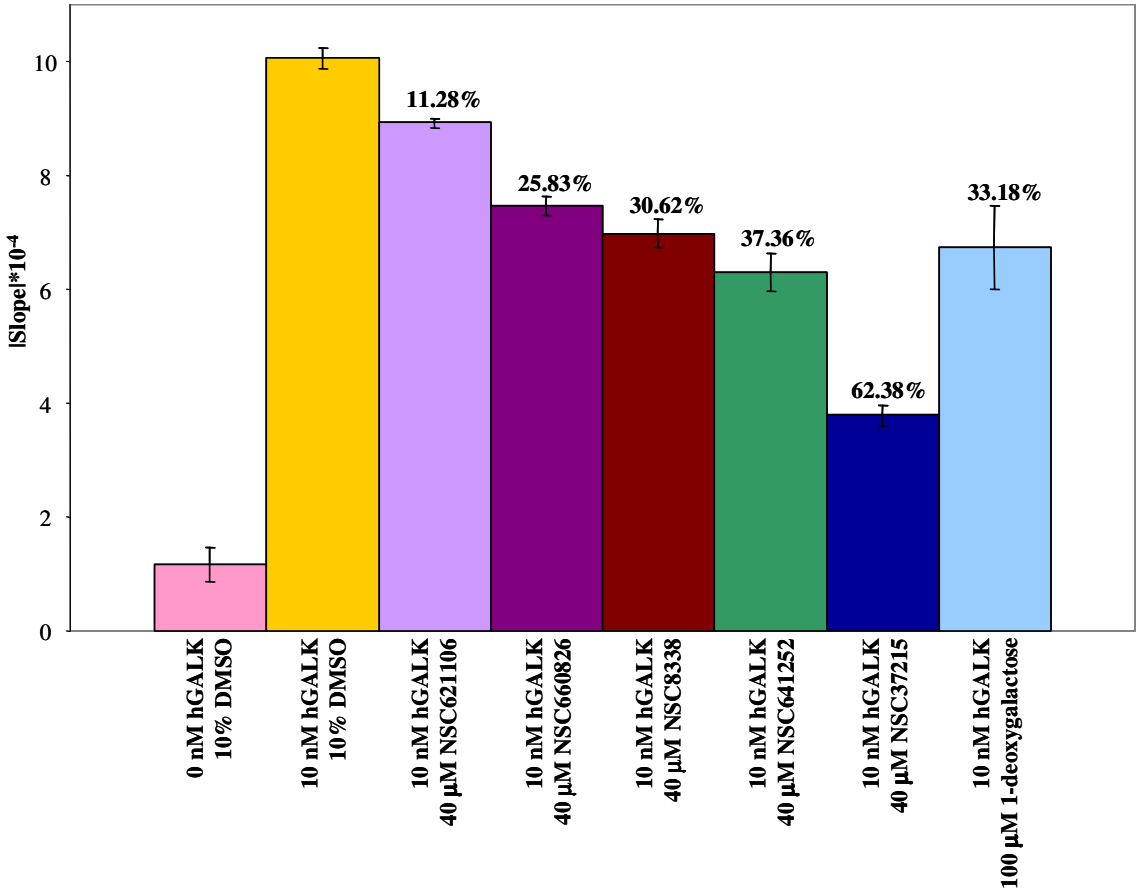


Figure 3.8

Inhibition of hGALK by six predicted small molecule inhibitors

Inhibition of hGALK by 1-deoxygalactose and five NCI compounds predicted to bind and inhibit hGALK (n=8 for negative and positive controls, n=4 for inhibition assays). Above each bar is the level of inhibition as a percentage. Each level of inhibition detected was statistically significant from the positive control. The final concentration of DMSO in each reaction was 10%.

similar in structure to this class of compounds may lead to the identification of potent and specific inhibitors of hGALK.

While these compounds were predicted to be competitive inhibitors, other classes of inhibitors can influence enzyme activity allosterically. It is difficult to predict where such an inhibitor will bind. Therefore, large-scale screening of structurally diverse molecules will still be necessary in parallel with the directed screens.

Future plans for the assay

Currently, our hGALK assay is sensitive and robust. Following further optimization to maximize the S:B, S:N, and Z' parameters, we plan to screen small libraries to confirm ability of the assay to be applied to a high-throughput screens of large libraries.

We plan to first screen 1000 small molecules of the 100,000 NCI library predicted from the *in silico* study to bind to the active site of hGALK. Next, we will screen the Spectrum Collection from MicroSource Discovery Systems, Inc., a library of 2000 biologically active and structurally diverse small molecules, many of which are FDA-approved drugs, and for which we also have *in silico* predictions of hGALK binding affinity. Finally, we will screen the 100,000 molecules of the ChemDiv library, which contains structurally diverse Lipinski "rule of five" compounds. We are in the process of applying to use the NIH Molecular Libraries Screening Center Network (MLSCN) to screen additional libraries. The screening of large libraries in addition to our directed screens will allow us to identify promising inhibitors of structures and binding sites to hGALK that we could not predict. Putative hGALK inhibitors identified through any of

these screens will be applied to further securitization with secondary assays to confirm specificity, potency, and bioactivity.

Discussion

The experiments reported here describe the work to create a simple and sensitive assay for hGALK activity to be used in high-throughput screening for the identification of inhibitors. A longstanding hypothesis in the galactosemia field is that the inhibition of hGALK, and the presumed resulting significant decrease in gal-1P production, will prevent or limit the sequelae of classic galactosemia (Bosch et al. 2002; Timson and Reece 2003; Bosch 2006). This hypothesis is supported, in concept, by studies performed in the yeast and mammalian cell culture model systems, and observations in human patients. A specific and potent inhibitor of hGALK would be a valuable tool for the further understanding of the pathophysiology of classic galactosemia. Additionally, such a molecule may serve as a powerful supplemental treatment for this metabolic disorder.

Before the assay could be further optimized and applied to the high-throughput screening of libraries of potential small molecule inhibitors, another laboratory published a similar study. Wierenga and colleagues have created a simple, robust microplate assay for measurement of GALK activity with a calculated Z' value of 0.91. A library of 50,000 structurally diverse compounds was screened, from which 150 promising compounds were identified to inhibit hGALK *in vitro* by more than 86.5% at an average concentration of 33.3 μM. In order to develop a pharmaceutical treatment for classic

galactosemia, this group has chosen to further characterize 34 of the 150 compounds identified (Wierenga et al. 2008).

Studies from this laboratory by Mumma and colleagues showed, using a doxycycline-regulated allele of GALK in GALT-null yeast, that almost complete knockdown of GALK activity is necessary to prevent the galactose-induce growth arrest phenotype and cellular accumulation of gal-1P (Mumma et al. 2007). With further characterization and structural analysis, Wierenga and colleagues may discover or manufacture a compound that inhibits more than 86.5% of hGALK activity. The more completely a molecule can inhibit hGALK activity, however, may determine the effectiveness of this supplemental treatment. Identifying molecules such as specific, suicide inhibitors may be most successful in the complete, or near complete, inhibition of hGALK *in vivo*.

Acknowledgements

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CHAPTER IV

Concluding Remarks and Future Directions

This dissertation reports the creation and initial characterization of the first genetic animal model of classic galactosemia that recapitulates aspects of the human disease using the model system, *Drosophila melanogaster*. The prior lack of an appropriate model system has hindered the advancement of research in classic galactosemia. Prior studies characterizing the biochemical and enzymatic aspects of the disease have been made possible using yeast and mammalian cell culture model systems, but none of these studies could be conducted on an organismal level. With the *D. melanogaster* model, questions concerning complex processes, such as development, homeostasis, tissue and organ system function, and the role of diet and genetic modifiers in the phenotypic outcome of a GALT-deficient animal can be addressed for the first time. A greater knowledge of the underlying pathophysiology of the acute and long-term sequelae in an animal model will provide specific hypotheses that can be tested in patients, or using patient samples. Ultimately, this work may contribute to the goal of providing more effective treatments and a better quality of life to patients with classic galactosemia

Our laboratory is the first to show that the Leloir pathway is functionally conserved in *D. melanogaster*. We have identified the fly orthologs of all three Leloir enzymes, and this dissertation describes the creation of an allele of the fly GALT (dGALT) gene using imprecise P-element excision mutagenesis. GALT deficiency in *D. melanogaster* results in acute phenotypes similar to those seen in human patients, unlike the mouse model of classic galactosemia. This model is a first step to understanding what damage occurs in patients with classic galactosemia, leading to the acute and long-

term complications, when and where this damage occurs, and what (*i.e.*, toxic metabolite levels, aberrant glycosylation) is causing the damage.

When are flies most sensitive to dietary galactose?

Early in life, patients with classic galactosemia are very sensitive to dietary galactose. If classic galactosemia remains undiagnosed or untreated, newborns will eventually succumb to the disease within the first days to weeks of postnatal life. The ingestion of a milk-based diet, which is high in galactose, results in the severe acute complications in newborn classic galactosemics, including failure to thrive, diarrhea, vomiting, jaundice, *E. coli*, sepsis, and death (Holton et al. 2000; Tyfield and Walter 2002; Antshel et al. 2004). These acute and potentially lethal complications are prevented or reversed upon the commencement of a galactose restricted diet if the dietary restriction is initiation quickly. The long-term sequelae of classic galactosemia, including cognitive, motor, speech and reproductive impairments, are not avoided by this treatment (Ridel et al. 2005).

In utero studies have determined that gal-1P and galactitol accumulate at very high levels in the developing fetus, the amniotic fluid, and the umbilical cord blood (Ng et al. 1977; Allen et al. 1980; Holton et al. 2000). These metabolic abnormalities are not resolved if the mother adheres to a galactose restricted diet during pregnancy, nor does it correlate with improved prognosis or outcome in patients (Waggoner et al. 1990; Gitzelmann 1995; Holton 1996). These studies suggest that damage to tissues and organs, perhaps due to metabolite toxicity, may occur very early in development.

Additionally, it is again clear that the removal of galactose from the diet is not sufficient to avoid this damage. Later in life, however, the impact of dietary galactose may not be as severe. Several case studies have identified patients who go untreated later in life and continue to exhibit only from mild phenotypes (Lee et al. 2003; Schadewaldt et al. 2004; Panis et al. 2006).

Using the *Drosophila melanogaster* model, we found that GALT-null flies are most sensitive to dietary galactose early in development. When developing in the presence of a high galactose diet, *D. melanogaster* die during mid to late larval development and viability decreases in an apparently dose-dependent manner. Additionally, we tested the stage of development during which GALT-null animals were most sensitive to dietary galactose and found that flies are most sensitive to galactose early in life. Adults fed a high galactose diet did not show an overt decrease in viability. These findings are similar to what has been observed in humans, as mentioned above.

Where is the damage occurring?

Patients with classic galactosemia suffer from acute and long-term complications that involve different tissues, including the nervous system, liver, and ovaries, among others. The abnormal accumulation of metabolites in certain tissues may contribute to the sequelae of this disorder. *In utero* studies have detected elevated levels of galactose, gal-1P, and galactitol in the liver of 20 week old fetuses (Ng et al. 1977; Allen et al. 1980; Holton et al. 2000). Additionally, studies from the *GALT* knockout mouse have

detected high levels of galactose and gal-1P in the animals' liver, kidneys, and brain (Ning et al. 2000; Leslie and Bai 2001; Bosch et al. 2004; Leslie et al. 2005).

Two distinct strategies can be used to determine which fly tissues are most sensitive to galactose exposure and are involved in the lethality phenotype. One approach is tissue-specific transgenic expression of wild-type hGALT protein in a GALT-null animal, which may reveal determine which tissues require GALT function to avoid toxicity caused by dietary galactose exposure. Additionally, were a profound RNAi GALT knockdown transgene available, the tissue-specific knockdown of dGALT expression might identify sensitive tissues that are sufficient for the lethality phenotype. Candidate tissues include the nervous system, the digestive system, and the ovaries.

In order to test whether certain metabolites abnormally accumulate in certain tissues, metabolite extraction from specific fly tissues should be performed. Preliminary studies have detected an abnormal accumulation of gal-1P specifically in the heads of GALT-null *D. melanogaster* fed a high galactose diet (data not shown). As described above, GALT-null flies show abnormal negative geotaxis response, which may be due to defects in motor or neurological function. The abnormal accumulation of gal-1P in the head of GALT-null flies may be a contributing factor in this phenotype.

How is the damage occurring?

Patients with classic galactosemia who do not adhere to the galactose restricted diet accumulate abnormal levels of the metabolites that precede the impaired enzymatic step in metabolism, including galactose, gal-1P, and galactitol. When treatment is

commenced, patients show a dramatic improvement in these metabolic abnormalities, but gal-1P levels remain above normal (Fridovich-Keil and Walter 2008). Gal-1P has been implicated, from experiments done in yeast and mammalian cell culture model systems and observations in human patients, as being involved in the pathophysiology of the acute and long-term complications of classic galactosemia (Leslie 2003; Slepak et al. 2005; Fridovich-Keil and Walter 2008). It is unclear what is causing the residual elevation of gal-1P in treated classic galactosemics; it may be due to the fact that completely removing galactose from the human diet is impossible, or from the continuous, endogenous production of galactose.

Elevated levels of metabolites, such as gal-1P, have been shown to inhibit important enzymes for sugar metabolism and glycosylation, including UDP-galactose galactosyltransferase (Gitzelmann 1995). Additionally, several studies has detected aberrant protein and lipid glycosylation in classic galactosemia patients (Haberland et al. 1971; Dobbie et al. 1990; Petry et al. 1991; Ornstein et al. 1992; Jaeken et al. 1996; Prestoz et al. 1997; Charlwood et al. 1998; Lebea and Pretorius 2005; Sturiale et al. 2005). Two metabolic abnormalities (a deficiency of UDP-gal and elevated levels of gal-1P) may be involved in the aberrant glycosylation patterns of classic galactosemic patients. As discussed previously, there is a great deal of controversy around the existence and biological significance of a UDP-gal deficiency in classic galactosemics. The fly model is a powerful tool for determining whether there are metabolic abnormalities, including a UDP-gal deficiency, in GALT-null animals.

Preliminary studies with our collaborators at the University of Georgia have detected aberrant glycosylation patterns in GALT-null larvae being fed a high galactose

diet (data not shown). Glycosylated proteins and lipids are crucial for many cellular processes, and the abnormal glycosylation patterns seen in both humans and flies may be contributing to the GALT-deficient phenotypes. For the first time, experiments can be conducted in a GALT-null animal to dissect the role of abnormal metabolite levels and aberrant glycosylation patterns in the acute and long-term complications.

Is galactitol necessary for a GALT-deficient phenotype?

One obvious and well studied difference between mice and humans is that mice express much lower levels of the AR enzyme compared to humans. The *GALK* knockout mouse created by Ai and colleagues does not suffer from galactose-induced cataracts unless hAR is transgenically expressed in the lens of the eye (Ai et al. 2000). It has recently been hypothesized that the accumulation of both gal-1P and galactitol may be necessary for the acute toxicity and long-term complications of classic galactosemia (Bosch 2006; de Jongh et al. 2008). Therefore, it is believed that the lack of a phenotype in the *GALT* knockout mouse may be due to low levels of AR expression, and the resulting absence of galactitol accumulation (de Jongh et al. 2008). The fact that the galactose-dependent cataracts form from high galactitol levels is evidence that this molecule can have adverse effects on cellular health. Galactitol, however, has only currently been implicated in this aspect of the pathophysiology of classic galactosemia. Studies in other genetic models and observations in human patients have not implicated this metabolite in the other complications of classic galactosemia at this point.

The mouse studies demonstrate that the human and mouse AR expression patterns are different, potentially including levels, tissue patterning, and timing differences in expression. The role of galactitol in the complications of classic galactosemia could be tested by the ubiquitous expression of hAR in the *GALT* knockout mouse, producing an animal that accumulates abnormal levels of both gal-1P and galactitol in its tissues and blood. If the combination of galactitol and gal-1P is necessary for the galactose toxicity phenotype, *GALT* knockout mice expressing hAR would demonstrate such a phenotype. Such an experiment has yet to be reported in the literature, due in part, to the fact that a ubiquitous expressing hAR transgenic mouse does not exist.

Why do flies have a phenotype but mice don't?

A *GALT* knockout mouse was created by Leslie and colleagues in 1996 as the first attempt to create a mammalian model of classic galactosemia. Unfortunately, while these mice had no detectable GALT activity, they did not recapitulate the human phenotype, even when fed a 40% galactose diet (Leslie et al. 1996; Ning et al. 2000; Leslie and Bai 2001; Ning et al. 2001). *GALT* knockout mice did accumulate high levels of galactose and gal-1P in their blood and tissues, but were, somehow, able to tolerate high levels of galactose in their diets, remaining healthy and fertile, unlike their human counterparts.

These findings suggest that, while GALT-deficient yeast, flies, and humans are sensitive to dietary galactose, mice seem to be different in this aspect of biology. Differences in the expression levels of the Leloir enzymes and enzymes active in the alternative pathways of galactose metabolism, differing sensitivities of tissues to

galactose and other metabolites, and other unpredictable factors may account for the lack of an apparent phenotype in *GALT* knockout mice. Deciphering the differences between the fly and mouse models may lead to a further understanding of the pathophysiology of classic galactosemia.

In addition to AR expression levels being different between humans and mice, mice may also have alternate expression patterns for other enzymes involved in galactose metabolism, such as IMPase and UGP. These differences may allow the *GALT* knockout mice to overcome the galactose toxicity, perhaps by the further metabolism of gal-1P. Additionally, mouse enzymes, such as UDP-galactose galactosyltransferase, may be more resistant to the gal-1P inhibition due to slight structural differences between orthologs, allowing these animals to be more resistant to high levels of this metabolite. Whether *GALT* knockout mice have aberrant glycosylation patterns when fed a galactose enriched diet has not yet been determined; if abnormal protein and lipid glycosylation is involved in the acute and long-term complications of classic galactosemia, the glycosylation patterns in these mice may be normal despite high levels of galactose in their diets.

While work still needs to be done to answer these questions in the mouse, the fly model is an excellent resource for testing the roles of galactitol and glycosylation in the acute and long-term complications of classic galactosemia. We hypothesize that GALT-null flies will accumulate abnormal levels of both galactitol and gal-1P when fed a galactose enriched diet. Using strong alleles of GALK and AR, RNAi knockdown technology, or specific small molecule inhibitors, the production of one of both of these molecules can be decreased in a GALT-null background, allowing for the analysis of the roles of these metabolites in the galactose-induced lethality phenotype. Additionally, we

have, in collaboration with Dr. Lance Wells at the University of Georgia Complex Carbohydrate Research Center (CCRC), been able to study the glycosylation patterns of proteins in GALT-null larvae. Preliminary data suggests that, like patients, GALT-null flies have aberrant glycosylation patterns when consuming a high galactose diet.

Can the fly be used as a model for studies of patient GALT alleles?

Just as wild-type hGALT activity is sufficient to complement the galactose-induced growth arrest in the yeast model system, the ubiquitous transgenic expression of wild-type *hGALT* in GALT-null flies complemented the galactose-induced lethality phenotype (Fridovich-Keil et al. 1995). Finally, we have shown in an animal model that the absence of GALT enzymatic activity is the cause of the galactose sensitivity phenotypes. Before an animal model existed, it had been speculated that the acute and long-term complications of classic galactosemia may be due to more than the loss of GALT enzymatic activity. Now that an animal model of classic galactosemia exists, the link between the loss of GALT activity and galactose sensitivity has been verified.

The imprecise P-element excision allele created by this laboratory removes the majority of the dGALT gene, resulting in the loss of detectable GALT enzyme activity. The GALT-null fly allows for the first characterization of human mutant GALT alleles in a multicellular model, including studies concerning the correlation between genotype and phenotype of the acute and long-term complications of classic galactosemia. This includes the study of variant alleles of GALT deficiency, such at the Duarte allele, and the impact of the loss of partial GALT activity on the development and homeostasis of an

organism. Since variant alleles and partial loss of GALT activity is much more common than classic galactosemia, studies involving partial loss of function alleles of GALT may influence a relatively large pool of patients in addition to classic galactosemics.

Can findings from the fly model be applied to humans?

Based on observations and studies from human patients, the damage that contributes to the long-term complications may occur very early in human development, perhaps even *in utero* or before diagnosis and the commencement of treatment. Additionally, factors such as poor dietary management and the endogenous production of galactose may contribute as well. If damage is occurring early, the subsequent removal of galactose from the diets of patients may not prevent the long-term sequelae of this disease, which is consistent with current clinical observations. Novel and more effective treatments to be used in combination with dietary galactose restriction may lead to a better prognosis and quality of life for classic galactosemics.

Studies that identify the timing in development and the tissues which are most sensitive and damaged during galactose exposure may lead to more effective treatments in human patients by identifying the most effective timing for the treatments and the tissues that needed to be targeted. Additionally, we have described an experiment that measures the geotaxic response of GALT-null flies in order to detect an impairment in motor and neurological function. Further experimentation using the fly model is being conducted to test the possibility of other long-term complications, such as fertility and

cognitive impairments. Finally, the fly model of classic galactosemia may be a building block for the further understanding of other human metabolic diseases.

What are genetic modifiers of the GALT-null phenotype?

As with the yeast model system, *D. melanogaster* is a powerful genetic model for the search for modifiers of the GALT-deficient phenotype. In yeast, a screen for genetic modifiers found that mutations in genes that led to the prevention of the synthesis of gal-1P (loss of GALK activity) or the increase in its further metabolism by other pathways (overexpression of UGP or IMPase) prevented the galactose-induced growth arrest phenotype (Douglas and Hawthorne 1964; Parthasarathy et al. 1997; Mehta et al. 1999; Kabir et al. 2000; Lai and Elsas 2000; Riehman et al. 2001; Bhat 2003; Ross et al. 2004). In the mammalian system, overexpression of UGP was also found to rescue galactose-induced sensitivity phenotypes (Lai et al. 2003).

From the current knowledge of galactose metabolism and studies in the yeast and mammalian cell culture model systems, we have identified several candidate modifiers, including GALK, UGP, AR, and IMPase. Currently in this laboratory, experiments using the fly model are taking place to genetically test the impact of the loss of GALK activity and the overexpression of UGP on the GALT-null phenotype.

Drosophila melanogaster is a powerful tool for unbiased forward genetic screens. Genetic screens in flies have uncovered pathways active in signal transduction, development, growth, and differentiation (St Johnston 2002; Brumby et al. 2004; Luschnig et al. 2004). While there are promising candidate modifiers (see above) of the

GALT deficiency phenotype, identified from studies in the yeast and mammalian cell culture model systems and observations from human patients, there may be genetic modifiers that cannot be predicted from what is currently known about galactose metabolism. An unbiased screen may reveal unknown pathways involved in the pathophysiology of classic galactosemia, identifying new targets for novel and more effective treatments.

How can we take advantage of this model to develop new treatments?

Classic galactosemia is a potentially fatal disorder early in life if it goes untreated. Patients who adhere to treatment, which currently consists of the dietary restriction of galactose, still suffer from severe long–term complications of the disorder. Despite decades of research in the field, the biochemical bases of the pathophysiology of the acute and long-term sequelae of classic galactosemia remain unknown. The existence of a genetic animal model of classic galactosemia now enables researchers to analyze the effects of GALT deficiency on complex processes, such as development and tissue and organ function. A greater knowledge of the fundamental pathophysiology of this disease will allow for the development of new, more effective treatments, such as specific, small molecule inhibitors of candidate modifiers such as hGALK. These treatments may be used in conjunction with the dietary restriction of galactose in order to prevent the long-term complications of this disease.

A standing hypothesis in the field is that the selective inhibition of hGALK, leading to the prevention of gal-1P production, may prevent the acute and long-term complications of classic galactosemia (Timson and Reece 2003; Bosch *et al.* 2004). While this laboratory and others are searching for a small molecule inhibitor of hGALK, using *in vitro*, high-throughput screening, the effects of such an inhibitor on an organism needs to be tested before it can be applied to human patients. In the case of *Drosophila melanogaster*, unbiased and high-throughput screening of small molecule libraries can be performed using the animal model instead of using *in vitro* assays. Currently, flies have been used for drug discovery for several disorders, including chronic pain, addiction, and Parkinson's disease (Manev and Dimitrijevic 2005; Nichols 2006; Whitworth et al. 2006). Directly testing compounds on a living system can determine the target of the small molecule, how well it permeates live tissues, the prevention of phenotypes, and whether the compound itself confers toxicity. The long-term goal of this research is to provide novel and more effective treatments to our patients in order to greatly improve their quality of life.

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