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Jennifer Daenzer Date

Exploring the roles of UDP-galactose 4'-epimerase in *Drosophila melanogaster*

development and homeostasis

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Doctor of Philosophy

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B.S., Alma College, 2007

Advisor: Judith L. Fridovich-Keil, PhD

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Abstract

Exploring the roles of UDP-galactose 4'-epimerase in *Drosophila melanogaster* development and homeostasis

By Jennifer Marie Iris Daenzer

In both humans and *Drosophila melanogaster*, UDP-galactose 4'-epimerase (GALE) catalyzes two distinct reactions. It catalyzes the interconversion of UDP-galactose (UDP-Gal) and UDP-glucose (UDP-Glc) in the final step of the Leloir pathway of galactose metabolism, and also interconverts UDP-N-acetylgalactosamine (UDP-GalNAc) and UDP-N-acetylglucosamine (UDP-GlcNAc). All four of these UDP-sugars serve as critical substrates in the synthesis of glycoproteins and glycolipids. Loss of GALE in humans results in the spectrum disorder epimerase deficiency galactosemia and profound loss is potentially lethal.

Here we present work describing the first whole-animal model of GALE deficiency. Our studies reveal that loss of GALE is incompatible with life in *Drosophila*, and demonstrate that GALE is required throughout development. We further show that GALE is necessary and sufficient when expressed in specific tissues, and demonstrate that partial loss of GALE results in galactose-induced lethality.

Whether the outcomes observed in human patients result from loss of one GALE activity, the other, or both is unknown. To address this question we have uncoupled the activities of dGALE in our *Drosophila* model. By replacing the endogenous dGALE with either of two prokaryotic genes, *Escherichia coli galE* which efficiently interconverts only UDP-Gal/UDP-Glc, and *Plesiomonas shigelloides wbgU* which efficiently interconverts only UDP-GalNAc/UDP-GlcNAc, we were able to generate animals with only one or the other GALE activity. Using this system we demonstrate that activity with respect to both UDP-Gal and UDP-GalNAc is required for *Drosophila* survival. Furthermore, distinct roles for each activity were observed during specific windows of development and in response to dietary galactose. Combined, these data reinforce the unique and essential roles of both GALE activities in *Drosophila*, and may provide insight into the pathophysiology of human GALE deficiency.

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Chapter 1

Introduction

1.1 Galactose metabolism

The hexose sugar galactose, which is prevalent in the human diet, is essential for life. Galactose is a component of the disaccharide lactose, and is, therefore, found in high quantities in milk and dairy products. In addition, galactose is found in legumes, fruits and vegetables, to various extents [1]. Movement of galactose into the cell is facilitated by two families of transporters, GLUT (passive, facilitative transport) and SGLT (sodium-dependent active transport) [2]. Once inside the cell, galactose is metabolized via the Leloir pathway (**Figure 1.1**). This pathway is conserved from *E. coli* to humans, and allows for both the metabolism of galactose, as well as the synthesis of galactose derivatives when exogenous sources of galactose are lacking. The three enzymes of the Leloir pathway are galactokinase (GALK; EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT; EC 2.7.7.12), and uridine diphosphate (UDP) glucose 4'-epimerase (GALE; EC 5.1.3.2).

Upon entering the cell, galactose is phosphorylated by GALK into galactose-1-phosphate (gal-1P), in an ATP-dependent manner [3]. Gal-1P is then converted to glucose-1-phosphate (glc-1P) by GALT. This reaction proceeds through a double displacement mechanism, whereby uridine diphosphate glucose (UDP-Glc) is also consumed and uridine diphosphate galactose (UDP-Gal) is produced [4]. The glc-1P produced can then be converted to glucose 6-

phosphate by the enzyme phosphoglucomutase (PGM, EC 5.4.2.2) and be metabolized via the glycolytic pathway [5]. Alternatively, glc-1P can be used to form UDP-Glc by the enzyme UDP-glucose pyrophosphorylase (UGP2, EC 2.7.7.9) and used to make glycogen for short-term energy storage. The final Leloir enzyme, GALE, converts UDP-Gal to UDP-Glc, using NAD as a cofactor. This reaction regenerates UDP-Glc as well as allows the cell to maintain the proper ratio of UDP-Gal and UDP-Glc, which in humans is 1:3 [6, 7]. Since GALE is a reversible enzyme, it also allows for the production of endogenous galactose from glucose if necessary [8, 9].

While the Leloir pathway is the primary pathway for galactose metabolism, there are several alternative routes (**Figure 1.2**). Normally, these pathways metabolize galactose at low levels. However, when defects in any of the Leloir enzymes cause abnormal accumulation of galactose metabolites these pathways can become much more active.

Instead of being converted to gal-1P, galactose can take two alternative paths. The first involves reduction of galactose to galactitol by aldose reductase (EC 1.1.1.21) [10, 11]. This is a dead- end pathway, as galactitol cannot be metabolized further. Defects in the Leloir pathway cause galactitol to accumulate in tissues [12] and be excreted in the urine [13]. The tissue most often affected by galactitol buildup is the lens of the eye in which osmotic pressure can lead to

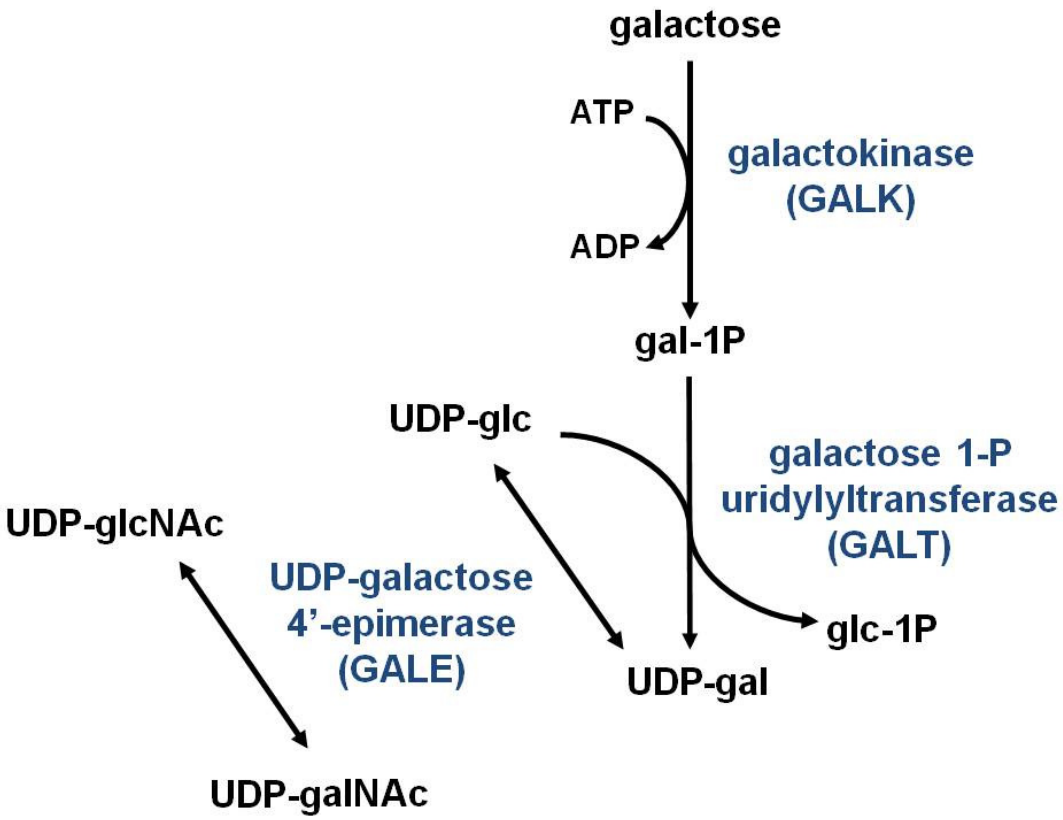

Figure 1.1: The Leloir Pathway of Galactose Metabolism. The three enzymes which constitute the Leloir pathway are shown in blue.

cataract formation. In the second pathway galactose is oxidized to galactonate. This reaction is thought to be catalyzed by a galactose dehydrogenase enzyme or by alcohol dehydrogenase [14, 15]. Galactonate can enter the pentose phosphate pathway following conversion to *D*-xylulose or be excreted in the urine [15-18].

There is also an alternate pathway for metabolizing gal-1P. Studies looking at the oxidation of galactose by patients with classic galactosemia have shown that even patients with no detectable GALT activity can metabolize galactose, albeit at a much slower rate [19, 20]. This has implicated the pyrophosphorylase pathway (catalyzed by UGP2, as reviewed above) as the potential alternative route for galactose metabolism [21]. As mentioned above, the primary role of UGP is to catalyze the formation of UDP-Glc from glc-1P in a UTP-dependent manner. However, at a much lower efficiency, human UGP can also catalyze the formation of UDP-Gal from gal-1P. Thus, the gal-1P formed by GALK can be converted to UDP-Gal by UGP, and GALE can then epimerize UDP-Gal to UDP-Glc. The ability of UGP to synthesize UDP-Gal has been observed to be approximately 1% of wild-type GALT activity [22]. In treated patients with classic galactosemia it may actually be more important for this pathway to work backwards to form UDP-Gal from glc-1p [23], since patients on galactose restriction have relatively low levels of gal-1P. Despite the presence of this alternative pathway, patients with classic galactosemia develop both acute and

long-term problems, and have very abnormal metabolite levels. This suggests that while this pathway may be helpful, it is clearly not sufficient to metabolize the levels of galactose derivatives necessary for normal cellular function [20, 24].

In addition to its role in the Leloir pathway, in humans and many other organisms GALE can interconvert a second set of substrates, UDP-N-acetyl galactosamine (UDP-GalNAc) and UDP-N-acetyl glucosamine (UDP-GlcNAc) [25-27]. Like UDP-Gal and UDP-Glc, UDP-GalNAc and UDP-GlcNAc are critical substrates in the production of glycoproteins and glycolipids. It is the role of GALE to maintain the pools of these four UDP sugars. The conversion of UDP-GlcNAc to UDP-GalNAc by GALE provides the primary source of UDP-GalNAc production, though there is some evidence that a salvage pathway exists which allows free GalNAc from glycoprotein and glycolipid turnover to be recycled back into UDP-GalNAc [28]. Proper functioning of the Leloir pathway allows galactose to be used for energy, as well as maintains proper levels of galactose derivatives, which is necessary for normal cellular function.

1.2 Galactosemia

Galactosemia is the metabolic disorder resulting from the inability to properly metabolize galactose. Deficiency of any of the three Leloir enzymes, GALK, GALT or GALE, results in a form of galactosemia. While there is some

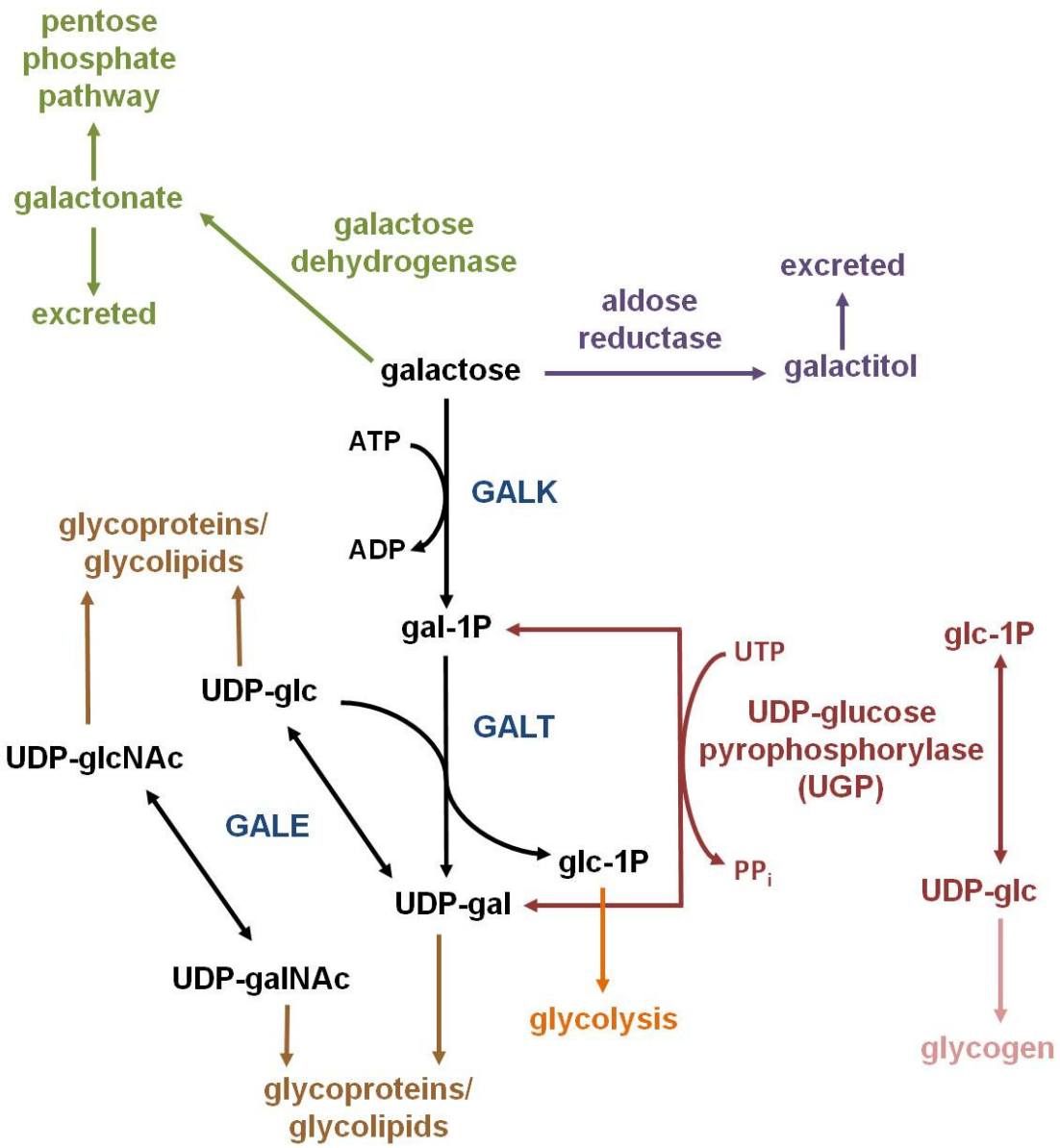

Figure 1.2 Expanded Diagram of Galactose Metabolism

overlap among the three forms of galactosemia, the biochemical and clinical outcome of each is distinct.

GALK deficiency galactosemia

First described by Gitzelmann [29], galactokinase deficiency (OMIM 230200) [30, 31], also referred to as type II galactosemia, is an autosomal recessive disorder resulting from impairment of GALK. Galactokinase deficiency is very rare (<1:100,000 live births) [32] and is the least severe form of galactosemia. When fed a milk-based diet, infants with galactokinase deficiency accumulate high levels of galactose in their cells and plasma. Galactitol, which is generated from the reduction of galactose by aldose reductase, cannot cross the cell membrane and, thus, accumulates in cells. The principal phenotypic abnormality in individuals with galactokinase deficiency is the development of cataracts due to accumulation of galactitol in the lens of the eye [31, 33]. If caught early, these cataracts typically resolve upon initiation of a galactose restricted diet. In contrast to patients with GALT and GALE deficiencies, individuals with galactokinase deficiency do not experience any known long-term complications [34, 35].

GALT deficiency galactosemia

Severe impairment of GALT results in the most common severe form of galactosemia, classic galactosemia (OMIM 230400), also known as Type I galactosemia. The incidence is typically reported as about 1/30,000 to 1/60,000 live births in the United States [34, 36]. However, a study which surveyed centers from 11 different countries reported incidences ranging from 1:14,000 to 1:80,000 live births, which may be accounted for by a combination of true differences among populations or variation in the sensitivity of the methods used for detection [37].

While infants with classic galactosemia appear normal at birth, they begin to develop symptoms rapidly upon exposure to a milk-based diet. These symptoms include, but are not limited to, poor feeding, poor weight gain, jaundice, vomiting, diarrhea, cataracts, liver enlargement, *E. coli* sepsis, and, if not treated, death. The standard of care is to initiate immediate restriction of dietary galactose upon suspected diagnosis. This typically alleviates or reverses the acute symptoms of the disease, and treated galactosemic infants appear healthy and normal. Unfortunately, although dietary galactose restriction allows for the survival of patients with galactosemia, it does not relieve the long-term problems that many patients go on to develop throughout child and adulthood [38]. Common complications include language and speech delays, cognitive

disability, primary ovarian insufficiency in >80% of females [35, 39-41], ataxic tremor, and decreased bone density [34]. In many countries, galactosemia is detected early through newborn screening, while in others infants are not diagnosed until after clinical symptoms have manifested. It has been shown that while very early detection of classic galactosemia by newborn screening does save lives, it does not appear to affect the incidence or severity of long-term complications [37, 42, 43].

GALE deficiency galactosemia

Epimerase deficiency galactosemia (OMIM 230350), also known as Type III galactosemia, results from impairment of GALE. GALE deficiency is the least well understood form of galactosemia owing, in part, to poor ascertainment of patients and lack of long-term follow-up. Identification of patients with GALE deficiency typically occurs through newborn screening or recognition of galactosemia-like symptoms. The newborn screening programs in some states include the measurement of total blood galactose. Although designed to identify patients with classic galactosemia, this test can also identify patients with GALE impairment because they too can accumulate high gal-1P in the blood. After a positive screen, GALT enzyme activity is tested and, if normal, GALE deficiency

is considered. GALE enzyme activity can then be measured to diagnose epimerase deficiency.

Epimerase-deficiency galactosemia was first reported by Gitzelmann in 1972 [44]. The patient was identified through newborn screening due to elevated gal-1P; however, no clinical symptoms were evident. After GALK and GALT deficiencies had been ruled out, samples from the patient's blood were found to be unable to convert UDP-Gal-C^{14} to UDP-Glc-C^{14}, implicating deficient GALE [44]. Upon testing various tissues for GALE activity, the patient was found to have impairment only in red and white blood cells; GALE was active in liver and cultured skin fibroblasts [45]. Several subsequent groups reported similar findings: patients with impaired GALE activity only in circulating blood cells, but no clinical complications [46-49]. The disorder in these cases was termed "benign," or "peripheral," and patients with this form of GALE deficiency typically do not receive further treatment or follow-up. Peripheral GALE deficiency is estimated to exist at frequencies ranging from 1/6,700 to <1/60,000 depending on race, with African Americans having a higher frequency than non-African populations [50].

Almost ten years later, the first case of severe GALE-deficiency was reported [23]. The child presented with symptoms reminiscent of classic galactosemia: jaundice, vomiting, weight loss, hypotonia, and galactosuria. In

addition to the red blood cells, GALE impairment was found to affect the patient's fibroblasts, and the deficiency was thus termed "generalized." Fewer than ten patients with generalized GALE deficiency have been reported in the literature [23, 51-53]. The initial acute presentation in these patients was similar to that described above. The predominant long-term outcomes observed in generalized galactosemia are developmental delay, moderate learning disabilities, sensorineural hearing impairment and growth retardation [52, 54]. Because the majority of these patients come from consanguineous families, the possibility that homozygosity at other loci could influence some of these outcomes cannot be ruled out. Of note, the two female patients who were old enough to evaluate displayed normal pubertal development [52]. This is in contrast to patients with classic galactosemia, the majority of whom experience premature ovarian insufficiency. Another patient, reported more recently in 2010, is from a nonconsangineous family [53]. He was admitted at one month of age with jaundice, lethargy, poor feeding and hepatomegaly. At fifteen months of age no long-term outcomes were reported. A galactose restricted diet with a small amount of galactose to allow for the production of essential glycoproteins and glycolipids was initiated upon diagnosis. This regimen was prescribed in some of the early cases of generalized GALE deficiency as well [23], and remains the current treatment recommendation [54].

Until recently, epimerase-deficiency was categorized into the two forms discussed above, the peripheral form, which is milder and more common, and the generalized form, which is very rare and much more severe [45, 46, 50, 55]. However, evidence of a form of the disease which did not fit into either of these two categories became apparent. Several patients were identified who appeared to have peripheral GALE deficiency, but were found to have impaired enzymatic activity in nonperipheral cells as well [56-59]. At least one of these patients, though appearing well in infancy, experienced both developmental delay and cognitive disability in childhood [56, 57]. In another study, Shin et. al. measured GALE activity in a variety of tissues from several GALE-impaired patients [58]. These patients had clinical outcomes which included cataracts and mental retardation. They found that the level of GALE activity measured from red blood cells did not always match the levels found in the lens or fibroblasts. This suggested that tissue-specific impairment of GALE could influence the clinical outcome for some patients. Similarly, a study of ten patients who had received a clinical diagnosis of peripheral epimerase deficiency demonstrated that not all of the subjects had normal levels of GALE activity in their nonperipheral tissues. Specifically, the activity values observed in patient lymphoblasts ranged from ~15% to ~64% of the control mean [60]. Furthermore, some of these patients exhibited metabolic abnormalities both *in vitro* and *in vivo*. Biochemical testing of

some of the mutations from these patients revealed enzymes with biochemical properties that were intermediate between those associated with the generalized or peripheral forms [61]. These studies suggest that GALE deficiency is not a binary condition, but point towards a disorder in which patients fall along a continuum. In addition, they have raised the question of which tissues should be tested when predicting the severity of a patient's disease.

Interestingly, unlike classic galactosemia, in which many patients have two mutations which result in complete loss of GALT activity, no patient has been identified to have complete loss of GALE activity. This is consistent with Kalckar's prediction over half a century ago that GALE would be essential for life [62]. It is also of note that some GALE mutations which have been characterized result in different levels of impairment on each of its activities. For example, the V94M substitution impairs enzyme activity to only ~5% of wild-type with respect to UDP-Gal and to ~25% of wild-type levels with respect to UDP-GalNAc [59]. The relative contributions of GALE activity with respect to each of its substrates in determining clinical outcome is unknown, and it is interesting to note that most clinical labs test only for activity with respect to UDP-Gal. A major focus of this dissertation is uncoupling these activities to better understand their individual roles in development and homeostasis.

1.3 The Pathophysiology of Galactosemia

Despite decades of study, the pathophysiology of galactosemia is poorly understood [38]. The only well-characterized disease mechanism is the increase in osmotic pressure caused by accumulation of galactitol. This accounts for the cataracts which are the primary clinical concern in patients with GALK deficiency, and also appear in patients with GALT and GALE deficiencies. Unfortunately, patients with the latter two disorders suffer from a host of other complications as well.

The mechanistic basis underlying the pathophysiologies of both the acute and long term complications of classic galactosemia and GALE deficiency are as yet unknown. Patients with classic galactosemia exhibit metabolic abnormalities including accumulation of high levels of galactose, galactitol and galactose-1-phosphate [34, 35, 38]. Patients with GALE deficiency accumulate galactose, gal-1P and UDP-Gal [23, 51, 52, 60]. Studies from yeast, combined with the observation that despite accumulating galactose and galactitol, patients with GALK deficiency have a mild disease course, suggest that neither galactose nor galactitol alone causes the long-term problems in patients with GALT or GALE deficiency [63, 64].

Gitzelmann [7] suggested that accumulated gal-1P may play a pathogenic role through its ability to inhibit the activities of other enzymes, including;

glucose-6-phosphatase [65, 66], glucose-6-phosphate dehydrogenase [67, 68], phosphoglucomutase [69, 70], liver glycogen phosphorylase [71], and UDP-glucose pyrophosphorylase [72]. Alternatively, or in addition, buildup of gal-1P might lead to futile cycles of phosphorylation/dephosphorylation, leading to a depletion of ATP [73].

Interestingly, a study in yeast demonstrated that while strains deficient in *GAL7* (yeast GALT) or *GAL10* (yeast GALE) are both negatively affected by galactose in the growth medium, the growth of GALE-null yeast is more severely affected than is that of GALT-null yeast. A double mutant with deletions of both *GAL7* and *GAL10* behaved similarly to the GALT-null strain when grown in the presence of various levels of galactose, although the level of gal-1P accumulated was similar to that seen in the GALE-null cells. Thus, the loss of GALT protected the GALE-null cells, indicating that factors besides gal-1P accumulation must be involved in mediating the galactose hyper-sensitivity of GALE-null cells [74].

Aberrant glycosylation may also be a problem in both GALT and GALE deficiencies. There is some evidence that high levels of gal-1P may inhibit UDP-galactose galactosyltransferases [75], which could interfere with the biosynthesis of galactosylated compounds [76]. In addition, the levels or ratio of UDP-Glc and UDP-Gal may be disrupted. Specifically, UDP-Gal and/or UDP-Glc may be depleted in classic galactosemia [38, 77, 78], and in GALE deficiency UDP-Gal

accumulates to abnormally high levels and UDP-Glc may be depleted [78]. Because these two sugar derivatives are key precursors for the synthesis of glycans, abnormal levels could result in improper glycosylation of proteins and lipids. In GALE deficiency, disruption of the levels or ratio of UDP-GalNAc and UDP-GlcNAc could also lead to defects in glycosylation.

The severity of outcomes in individual patients depends on multiple factors. The specific mutations each patient carries and their effect on enzyme activity seems to have a significant impact on disease outcome. For GALE deficiency, the effect of individual mutations on GALE activities in various tissues is also a major factor. In addition, natural variation in non-Leloir genes which may interact with this pathway or otherwise affect patient outcomes could influence disease severity.

1.4 UDP-galactose 4′-Epimerase

Protein structure and function

GALE is expressed in most tissues [79] and the GALE protein is highly conserved across branches of the evolutionary tree. Specifically, human GALE demonstrates 52% identity to *E. coli* galE, 54% to *S. cerevisiae* GAL10, and 60% to *D. melanogaster* GALE [80]. GALE is the most structurally characterized of the Leloir pathway enzymes. Work to understand the properties and mechanism of

GALE has been based on a number of x-ray crystallographic studies of the *E. coli* [81-86] and, later, human GALE enzymes [87].

GALE functions as a homodimer in which each 338 amino acid subunit is tightly bound to an NAD^+ or NADH moiety [81, 88, 89]. Each subunit contains an N-terminal nucleotide-binding motif and a C-terminal domain which binds the UDP-sugar substrates; the active site being located in a pocket between the two domains. The reaction mechanism proceeds via the following steps: 1) an enzymatic base from a conserved tyrosine residue abstracts the 4'-hydroxyl hydrogen of the sugar and a hydride is transferred from C-4 of the sugar to the C-4 of NAD^+, resulting in a 4'-ketopyranose intermediate and a reduced NADH bound to the enzyme; 2) the 4'ketopyranose rotates within the active site to present the opposite side of the sugar to the NADH; and finally 3) the hydride is transferred back from NADH to C-4 of the sugar, and the C-4 oxygen is reprotonated [5].

As mentioned above, in many organisms, including humans, GALE is able to catalyze the interconversion of a second set of substrates, UDP-GalNAc and UDP-GlcNAc. However, the GALE enzymes of some organisms lack this second activity, for example, those of *E. coli* and *S. cerevisiae*. Instead, some of these organisms have a separate enzyme which is capable of performing the UDP-GalNAc/UDP-GlcNAc interconversion and may or may not be able to

interconvert UDP-Gal/UDP-Glc. Examples of these include Wbgu from *P. shigelloides* [90], GNE from *E. coli* [91] and WbpP from *P. aeruginosa* [92].

The crystallization of human GALE complexed with UDP-GlcNAc elucidated the mechanism by which GALE accommodates the larger N-acetylated substrates: the side chain of Asn^{207} rotates toward the interior of the protein [25]. Further, in an attempt to explain why *E. coli* GALE is unable to interconvert UDP-GalNAc and UDP-GlcNAc, the crystal structures of the human and *E. coli* enzymes were compared. It was found that the human active site has a 15% larger volume, and predicted that the bulkier Tyr^{299} in the bacterial enzyme, which is replaced by Cys^{307} in the human protein, accounts for this difference. A subsequent study tested this hypothesis by creating a Y299C mutant form of the *E. coli* enzyme. This enzyme gained a 230-fold increase in activity with UDP-GalNAc as substrate [86]. Furthermore, our group later generated a C307Y-hGALE mutant enzyme. This enzyme had normal activity with respect to UDP-Gal, but demonstrated a complete loss of activity with respect to UDP-GalNAc, demonstrating that the more bulky residue could indeed preclude the larger substrate from the active site [93].

Molecular basis of epimerase deficiency

The human *GALE* gene maps to chromosomal position 1p36 [38]. Since human *GALE* was cloned in 1995 [94], a number of unique mutations have been identified [52, 56, 59-61, 95-97], indicating a relatively high allelic heterogeneity, though some mutations seem to occur at higher frequencies in certain populations [50, 79].

One mutation, V94M, was found in the homozygous state in five of the patients identified with generalized GALE deficiency [59]. Additional mutations which have been shown to result in severe reduction in GALE enzyme activity in yeast models include G90E, which exhibits little, if any, detectable activity, and L183P, which retains about 4% of wild-type activity [57, 59, 98]. In addition, L183P along with another mutation, N34S, which is associated with a slight decrease in GALE activity, have been shown to exhibit dominant negative effects [57]. Four mutations which have been identified in Korean patients, E165K, R239W, G302D and W336X, exhibited no detectable enzyme activity when expressed in GALE null ldlD cell lines [99], though R239W and G302D are associated with the peripheral form of GALE deficiency [97].

1.5 Model Systems for Studying Galactosemia

Yeast

Much of our current understanding of galactose metabolism and galactosemia has derived from work with the yeast, *Saccharomyces cerevisiae*. It was in yeast that the Leloir pathway was first identified [21, 100], and each human *GALT* and *GALE* have since been shown to complement deficiency of the corresponding yeast homologue [59, 101]. This has allowed for the development of an easily amenable system for studying galactose metabolism and modeling deficiencies therein.

This system has proven valuable in functionally characterizing individual mutations identified in patients with deficiency in both GALT [102, 103] and GALE [57, 59, 104-106]. Studies on GALE-null yeast strains have demonstrated a relationship between the level of impairment of GALE activity with respect to UDP-Gal and the degree of galactose-mediated growth impairment [59, 107]. Specifically, strains expressing enzymes with mutations resulting in higher activity grew better than those carrying low-activity mutations, and strains with GALE-null mutations were unable to grow.

Further studies in yeast have demonstrated that GALE-null yeast arrest growth at a 10-fold lower galactose concentration than do GALT-null yeast [64]. In addition, it was shown that there is a threshold relationship between gal-1P

accumulation and growth arrest in both GALT-null and GALE-null yeast, but that the threshold for the two strains is distinct: GALE-null strains arrested growth at lower levels of accumulated gal-1P than did GALT-null strains [74]. Further, GALT-null yeast retain at least some ability to utilize galactose from their medium whereas GALE-null cells are unable to do so [64]. This is believed to be because the UGP bypass pathway described earlier requires GALE activity, but not GALT activity.

Mammalian Systems

Though yeast is an immensely useful system for studying galactose metabolism, they do have clear limitations. GALE from yeast is unable to interconvert UDP-GalNAc and UDP-GlcNAc [93], and GalNAc is not used in their glycoproteins or glycolipids. Whereas in humans GalNAc is the obligate first sugar donor for *O*-glycosylation, yeast use mannose [108]. As a result, the study of GALE deficiency in yeast is particularly limited.

A mammalian system for studying GALE deficiency was discovered in a Chinese hamster ovary (CHO) cell line, *ldlD* [109]. This line exhibits no detectable GALE enzyme activity with respect to either UDP-Gal or UDP-GalNAc [8]. These cells have defects in both N- and O- linked glycosylation which can be corrected by supplementation with small amounts of galactose and GalNAc [109, 110].

Though the glycosylation defects were correctible, these cells were sensitive to galactose exposure, which lead to growth arrest and accumulation of gal-1P and UDP-Gal. More recently, our group found that the galactose sensitivity of the *ldlD* cell line could be rescued by supplementation with uridine, providing evidence that UTP may be depleted in these cells [6].

To extend the study of galactose metabolism into a whole-animal system, GALK and GALT knock-out mice were created [111, 112]. Disappointingly, the phenotypic outcomes in these animals did not recapitulate those of humans with GALK or GALT deficiency. Despite accumulating galactitol in its tissues, the GALK-null mouse only develops cataracts if aldose reductase is over-expressed in the lens [111]. Like humans with classic galactosemia, the GALT-null mouse model accumulates galactose, gal-1P, galactitol and galactonate. Unlike humans, however, they appear healthy and lack any known negative outcomes [112, 113]. It is not known what differences between mice and humans account for the disparity in their outcomes. Given these results, mice have not proven useful in studying the effects of GALT loss in a whole-animal system.

No GALE knock-out mouse has been reported. Given speculation that GALE may be essential for life – and the fact that no patient has ever been found to have complete loss of GALE activity - it is possible that a GALE-null mouse would be embryonic lethal.

Fruit Flies

The first whole-animal model of GALT-deficiency which recapitulated outcomes of classic galactosemia was created by our group recently in the fruit fly *Drosophila melanogaster* [114]. These flies survive well in the absence of galactose, but exhibit significant lethality when galactose is present during development. This lethality was rescued by constitutive expression of an *hGALT* transgene. In addition, the mutant flies accumulate abnormally high levels of gal-1P, particularly when exposed to galactose. Interestingly, these flies also display a long term locomotor defect. Thus, it appears that this model will prove useful in studying mechanisms and modifiers of outcome in GALT deficiency. Our group has also created a *D. melanogaster* model of GALE deficiency which is described in this dissertation.

1.6 Summary

This dissertation describes the utility of studying GALE deficiency in a *D. melanogaster* model. GALE deficiency is poorly understood, and the creation of a whole animal model has opened up many new avenues by which we can study this disorder.

Chapter 2 describes the creation and characterization of a *D. melanogaster* model of epimerase deficiency. We show that, as theorized previously, GALE is

in fact essential for life in metazoans and the minimum threshold for survival is between four and eight percent compared to wild-type GALE activity. Survival can be rescued by expression of a *hGALE* transgene. In addition, animals with low-level GALE activity were sensitive to galactose exposure during development. This whole-animal model provides a much needed system to extend our understanding of the pathophysiology of this complex disorder.

Chapter 3 provides a dissection of the roles of the two activities of GALE in development and homeostasis. By differentially expressing two prokaryotic genes encoding enzymes which catalyze only one or the other GALE activity, we were able to demonstrate that each activity of GALE is essential for *Drosophila* survival and that distinct roles are observed at specific windows of development or in response to galactose challenge. These studies lay the groundwork for elucidating the specific roles of each GALE activity in the processes underlying the pathogenesis of GALE deficiency.

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Chapter 2

UDP-galactose 4′ epimerase (GALE) is essential for development of *Drosophila melanogaster*

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2.1 ABSTRACT

UDP-galactose-4'epimerase (GALE) interconverts UDP-galactose and UDP-glucose and also UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. GALE therefore plays key roles in the Leloir pathway of galactose metabolism, in the endogenous production of galactose, and in maintaining the ratios of four key substrates for glycan biosynthesis. Partial impairment in GALE results in epimerase-deficiency galactosemia. To explore GALE's contributions to normal development and homeostasis we generated a GALE-deficient *Drosophila melanogaster*; this is the first reported whole animal model of GALE deficiency. GALE-deficient animals died in embryogenesis, and conditional loss of GALE at any stage of development through pupation was lethal. Animals with weak GALE activity were viable but sensitive to dietary galactose. Tissue-specific expression of GALE in the gut and Malpighian tubule is both necessary and sufficient for survival. These studies illuminate the timing and location of GALE requirement, and represent a first step toward understanding the pathogenesis of epimerase deficiency galactosemia.

2.2 INTRODUCTION

Galactose is a dietary source of energy, especially for mammals, and galactose derivatives are also key substrates for the biosynthesis of complex carbohydrates, glycoproteins and glycolipids. Specifically, galactose is the penultimate sugar residue in many glycans, and the galactose derivative N-acetylgalactosamine is the obligate first sugar residue in O-linked glycosylation.

In organisms from bacteria to humans, galactose is metabolized via the three enzymes of the Leloir pathway (**Figure 2.1**): galactokinase (GALK), galactose-1-phosphate uridylyltransferase (GALT), and UDP-galactose 4'-epimerase (GALE). Collectively, these enzymes are responsible for metabolizing environmental galactose and for synthesizing endogenous UDP-galactose (UDP-gal) when environmental sources of galactose are insufficient. GALE, the third and final enzyme in the pathway, regulates the supply of UDP-sugars essential for glycosylation by interconverting UDP-gal and UDP-glucose (UDP-glc) as well as UDP-N-acetyl-galactosamine (UDP-galNAc) and UDP-N-acetyl-glucosamine (UDP-glcNAc).

Impairment in any of the Leloir enzymes causes galactosemia, a family of genetically and clinically heterogeneous metabolic disorders. The specific clinical

and biochemical outcomes of the disease depend upon which enzyme is impaired, and the degree of catalytic impairment. Classical galactosemia results from profound impairment of GALT. Epimerase deficiency galactosemia, originally considered a benign condition that involved only circulating red and white blood cells in affected individual (1), is now recognized as a spectrum disorder that ranges from the ostensibly benign "peripheral" GALE deficiency through intermediate GALE deficiency to the potentially-lethal "generalized" GALE deficiency (1-8). Affected individuals show increased levels of galactose metabolites, including galactose, UDP-gal, and galactose-1-phosphate (gal-1-P) in impacted tissues, and may suffer acute and/or long-term complications, especially if exposed to dietary galactose (2-4,6,8). Unfortunately, little is known about the pathophysiology of epimerase deficiency galactosemia. Of note, unlike GALT- or GALK-deficiency, no patient completely lacking GALE activity has ever been reported; even the most severely affected patients demonstrate residual activity, at least in some tissues. More than 40 years ago Kalckar (9) noted the essential role of GALE for the biosynthesis of glycogonjugates and postulated that total absence of GALE activity would be incompatible with life.

Although there are no prior reports of a whole-animal model of GALE-deficiency, the consequence of GALE-loss has been studied in yeast and in

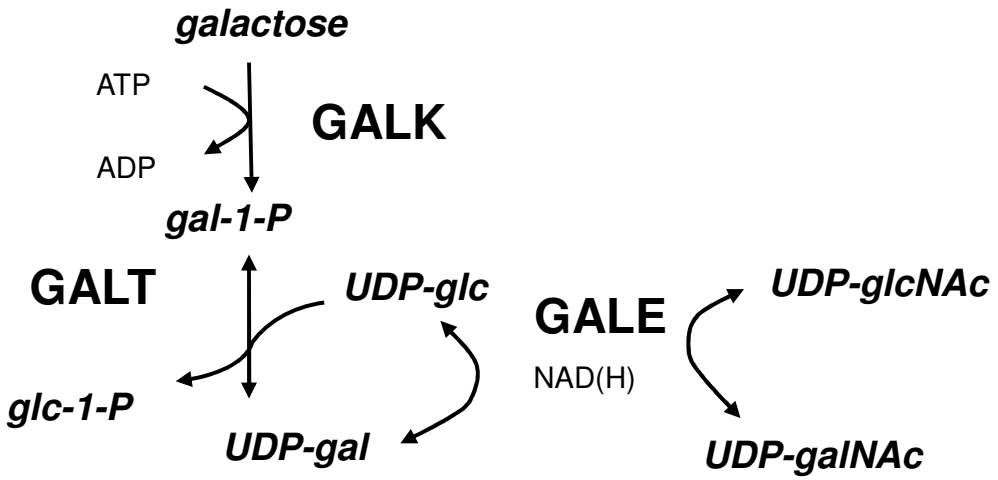

Figure 2.1 The Leloir Pathway of Galactose Metabolism. In both humans and Drosophila, GALE catalyzes UDP-galNAc/UDP-glcNAc interconversion in addition to UDP-gal/UDP-glc interconversion.

mammalian tissue-culture systems. GALE deficient yeast are viable and apparently healthy, although they arrest growth upon exposure to even trace levels of environmental galactose (10,11). GALE-deficient Chinese Hamster Ovary (CHO) cells (*ldlD*,(12)) also demonstrate galactose-sensitive growth arrest as well as defects in both N- and O-linked glycosylation (13,14). Nonetheless, the relationship between yeast or tissue culture outcomes and the pathophysiology of generalized epimerase deficiency galactosemia remains unclear and untested.

The fruit fly *Drosophila melanogaster* has been used as a powerful genetic model in laboratory studies for more than a century and recently has emerged as a facile animal model for studies of human genetic disease (15), including metabolic disease (16). Of particular relevance to galactosemia, the complexity of N-and O-linked glycans in *Drosophila* and the organismal effects of loss of specific enzymes in the glycosylation pathway have recently been reported (17). Further, we have confirmed that *D. melanogaster* encodes (*http://superfly.ucsd.edu/homophila*) and expresses functional orthologues of all three Leloir enzymes, designated *dGALK* (CG5288), *dGALT* (CG9232), and *dGALE* (CG12030) (Kushner et al, in preparation).

Here we describe a *Drosophila melanogaster* model of GALE deficiency; this is the first reported whole animal genetic model of epimerase-deficiency

galactosemia. Using this model, we confirm that *dGALE* is an essential gene in animals. *Drosophila* lacking *dGALE* die as embryos, and developing larvae from which *dGALE* is conditionally knocked down die within days of GALE-loss. Of note, tissue-specific knock-down of *dGALE* in gut primordium and Malpighian tubules is also lethal. Transgenic expression of human *GALE* (*hGALE*) restores viability, as does selective *hGALE* expression in the gut and Malpighian tubule. Notably, although *dGALE* is essential for survival at all stages of larval development, it is not required for survival of adults. Finally, animals with partial GALE impairment survive on food that lacks galactose but show reduced survival in the presence of galactose, thereby recapitulating the outcome reported for patients with generalized epimerase deficiency.

2.3 MATERIALS AND METHODS

Drosophila Stocks

Except as noted, crosses were maintained on standard cornmeal-molasses-agar medium supplemented with yeast at 25°C and 75% humidity.

Unless otherwise specified, stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University. For experiments involving a wild-type control, the isogenic strain w^{1118} (FBst0005905) was used.

Stocks used for complementation testing in the excision screen were *PBac{WH}CG12030*^{f00624} (FBst1016354), Harvard Exelixis Collection, *Df(3L)emc-E12/TM6B, Tb*^{1} *ca*^{1} (FBst0002577), and *w*^{1118}*; Df(3L)ED4196, P{3'.RS5+3.3'}ED4196/TM2* (FBst0008050).

For viability assays, GALE alleles y and *PBac{WH}CG12030*^{f00624} were balanced using *w*^{1118}*; Dr*^{Mio}*/TM3, P{GAL4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb*^{1} *Ser*^{1} (FBst0006663). The following stocks were used to generate germline clone mutants: *w***; P{FRT(w*^{hs}*)}2A* (FBst0001997), *P{hsFLP}1, y*^{1} *w*^{1118}*; Dr*^{Mio}*/TM3, ry** *Sb*^{1} (FBst0000007), *w***; P{ovoD1-18}3L P{FRT(w*^{hs}*)}2A/st*^{1} *βTub85D*^{D} *ss*^{1} *e*^{s}*/TM3, Sb*^{1} (FBst0002139).

Stocks used to determine timing, tissue-specific requirements and sufficiency of GALE were the UAS-RNAi stock *12030R-2*, (NM_138200.2), National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan; *w*; P{tubP-GAL80*^{ts}*}20; TM2/TM6B, Tb1*(FBst0007019), *y*^{1} *w***; P{Act5C-GAL4}25FO1/CyO, y*$^{+}$ (FBst0004414), *w*^{1118}*; P{drm-GAL4.7.1}1.1/TM3, Sb*^{1} (FBst0007098), *P{GawB}elav*^{C155} (FBst0000458), *P{GawB}c754, w*^{1118} (FBst0006984),

w^{1118}; *P{Sgs3-GAL4.PD}TP1* (FBst0006870), w^{1118}; *P{GMR-GAL4.w-}2/CyO* (FBst0009146) and y^1 w^{1118}; *P{ey1x-GAL4.Exel}3* (FBst0008227).

Generation and Characterization of Excision Alleles

Excision alleles were generated by mobilizing a P-element insertion in *CG12030, P{EPgy2}CG12030*^{EY22205} (FBst0022544) through exposure to a transposase source, *H{PDelta2-3}HoP8, y*^{1}*w***;;D/TM3,Sb,* (gift of Dr. Subhabrata Sanyal, Emory University). 278 excisions were identified by loss of the *white* marker gene and tested for complementation to chromosomal deficiencies *Df(3L)emc-E12* and *Df(3L)4196* and to the lethal *PBac* insertion *PBac{WH}CG12030*^{f00624}. Homozygous lethal excisions which failed to complement these three stocks were selected as potential strong loss of function alleles. Homozygous viable excisions which demonstrated failure to complement were selected as potential hypomorphs and further characterized for environmental galactose sensitivity and GALE enzyme activity. Homozygous viable excisions that did not fail to complement were retained as probable precise excisions. The strong loss of function excision *dGALE*^{y}, the hypomorph *dGALE*^{h}, and the precise excision *dGALE*^{d} were further characterized. The breakpoints for excisions *dGALE*^{h} and *dGALE*^{y} were determined by PCR amplification and

sequencing using the following primers: RSexcF1195'GTCAGCCTGCTGCTAGC ACTTG, RSexc1055'AGTGCTATCGTGTGCTAACC, RSexcR7205'GAATGGTAT CCAGTTGGCAC, and RSexc17675'CTACCTTGTCAGACTTGCAC. These primers were also used to confirm that *CG12030* was not disrupted in precise excision $dGALE^{d}$. For $dGALE^{d}$ and $dGALE^{h}$, genomic DNA was extracted from homozygous adults. Because $dGALE^{y}/dGALE^{y}$ was not viable, genomic DNA was obtained from $dGALE^{y}/+$ heterozygotes. The amplicons were separated by electrophoresis on a 1.5% agarose gel and the $dGALE^{y}$ amplicon was excised and purified (QIAquick Gel Extraction Kit, Qiagen) prior to sequencing.

Enzymatic Assays

General assay conditions: Crude lysate was prepared from a pool of 10 adult males in 100 μL lysis buffer (1 Complete Mini protease inhibitor cocktail pellet, EDTA-free (Roche) dissolved in 10mL of 100 mM glycine, pH 8.7). Samples were ground on ice for 15 seconds using a teflon micropestle and handheld micropestle motor (Kimble-Kontes) and centrifuged at 16,100 rcf for 5 min at 4° C. The resulting supernatant was passed over a Micro Bio-Spin P-6 Chromatography Column (Bio-Rad) to remove endogenous metabolites and diluted in lysis buffer to within the linear range of the assay. 30 μL of diluted

protein and 20 μL of a premix of substrates and cofactors were combined to start each reaction. Time from crude lysate preparation to initiation of the assay was no more than 35 minutes. Reaction mixtures were incubated at 25° C for 30 min and then quenched by the addition of 450 μL of ice-cold HPLC grade water (Fisher). Particulates were removed from the samples by centrifugation at 4000 rcf for 4 min at 4º C in .22 μm Costar Spin-X centrifuge tube filters (Corning); reactants and products were quantified by HPLC as described previously (11) and specific activity reported as pmol product formed per μg protein per minute of reaction. Protein concentration was determined using the Bio-Rad DC protein assay according to the manufacturer's protocol, with BSA as a standard.

GALK assay conditions: Activity was calculated from the conversion of galactose to gal-1-P. Initial reaction mixture concentrations were 2 mM $MgCl_2$, 40 mM Tris pH 8.0, 40μM dithiothreitol, 4 mM galactose, and 4 mM ATP. Lysates were diluted 1:10.

GALT assay conditions: Activity was calculated from the conversion of gal-1-P to UDP-gal. Initial reaction mixture concentrations were 125 mM glycylglycine pH 8.7, .8 mM UDP-glc and (when relevant) 1.6 mM gal-1-P. To account for conversion of UDP-glc to UDP-gal by GALE, assays were performed both with

and without gal-1-P. UDP-gal formed in the no gal-1-P reaction was subtracted from UDP-gal formed in the gal-1-P reaction, and the net UDP-gal used to calculate specific activity. Lysates were diluted 1:10.

GALE UDP-gal assay conditions: Activity was calculated from the conversion of UDP-gal to UDP-glc. Initial reaction mixture concentrations were 100 mM glycine pH 8.7, .8 mM UDP-gal and .5 mM NAD. Lysates were diluted 1:60 except for those prepared from $dGALE^{h}$ / $dGALE^{h}$, which were diluted 1:30, and those prepared from animals overexpressing *hGALE* transgenes. $Act5C{>}hGALE^{33B}$ was diluted 1:120; $Act5C{>}hGALE^{32A}$ was diluted 1:200, and $Act5C{>}hGALE^{40B2}$ was diluted 1:600.

GALE UDP-galNAc assay conditions: Activity was calculated from the conversion of UDP-galNAc to UDP-glcNAc. Initial reaction mixture concentrations were 100 mM glycine pH 8.7, 1.6 mM UDP-galNAc and .5 mM NAD. Lysates were diluted 1:2 or 1:4. Volumes for this assay were reduced; 7.5 µL of soluble protein lysate was added to 5 µL of premix and the reactions were quenched with 112.5 µL of ice-cold HPLC water.

Viability Assays

Zygotic mutants were generated by crossing *dGALE*^{y} and *PBac{WH}CG12030*^{f00624} heterozygotes. Females carrying *PBac{WH}CG12030*^{f00624} germ line clone embryos were generated by the FLPase-Dominant Female Sterile (FLP-DFS) technique (18), and crossed to either heterozygous *dGALE*^{y} males or wild-type males. As a control, females carrying wild-type germ line clone embryos were also generated and crossed to wild-type males. Virgin females and males of the appropriate genotypes were crossed and placed into egg-laying cages. For all assays in which embryos need to be genotyped for presence or absence of a third chromosome balancer, *TM3, P{w*$^{+mC}$*GAL4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb*^{1} *Ser*^{1} was used. Embryos were collected on grape juice agar plates (10% w/vol dextrose, 3% w/vol agar in organic, unfiltered Concord grape juice) spread with yeast paste. Embryos were dechorinated in 50% bleach for 3 minutes and rinsed with ddH_2O before sorting. Trans-heterozygote embryos, identified as GFP negative, were placed under halocarbon oil on grape juice plates spread with yeast paste overnight. Any embryos that did not hatch as L1's by the following morning (~36 hours after egg-laying) were scored as dead embryos. Surviving L1's were followed and scored for survival every 24 hours. Control crosses that did not need to be genotyped were dechorinated and placed

on grape juice agar plates for an equivalent period of time to approximate sorting.

Generation of hGALE transgenic animals

UAS-hGALE was constructed using standard cloning procedures. Plasmid mm22.hGALE was cut with EcoR1and Sal1 to release the *hGALE* insert. The *hGALE* sequence was then cloned into the expression vector pP{UAST} (19) using EcoR1 and Xho1 sites located in the multiple cloning site. Transgenic lines were created by standard techniques using the helper plasmid pP{wc Δ2,3} (20). Transformants were selected by presence of the *white* gene within pUAST and their insertions mapped by standard methods. We confirmed that the transgenic lines were expressing hGALE by enzymatic assay as described above.

Timing of GALE requirement

So that animals could be scored for the presence or absence of a *Gal4* driver during development, *P{Act5C-GAL4}25FO1* was balanced over *T(2;3)TSTL, Tb, Hu* (FBab0026935). The resulting stock was crossed to animals homozygous for both *P{tubP-GAL80^{ts}}10* and *12030R-2*. The *GAL80(ts)* allele we used allowed

for maximal repression of *GAL4* at 18°C with progressive de-repression at increasing temperatures (21). Crosses were maintained at 18° to allow negative regulation of the GAL4-*UAS* system by GAL80(ts) and tapped at 24 hour intervals to fresh vials. When adults from the first vial eclosed, all vials were shifted to 29° to relieve repression of the GAL4-UAS system by GAL80(ts). In this manner, we created cohorts of flies in which GALE knockdown occurred at 24 hour intervals in development. Vials were examined daily and scored for the presence of non-tubby larvae and pupae. Adults which eclosed from the vials were scored for the presence of humeral.

Galactose Sensitivity

For assays of galactose sensitivity and metabolite accumulation, crosses were maintained on cornmeal-agar-yeast extract medium containing 555 mM dextrose. In some vials, galactose or mannose was added to this media at a final concentration of 111 mM. To minimize metabolism of sugars in the food by microbes, vials were not supplemented with yeast. $dGALE^{h}$ / $dGALE^{h}$ or $dGALE^{d}$ / $dGALE^{d}$ virgin females were crossed to $dGALE^{h}$ or $dGALE^{d}$ heterozygote males balanced over *TM6B, Tb, Hu*. Offspring were scored for presence or absence of humeral. The proportions of unbalanced offspring were compared in $dGALE^{d}$

and *dGALE*^{h} crosses raised on a given type of food. Based on the results of an F-test of variance equality, a two-tailed, nonhomoscedastic Student's t-test was used to determine for each type of food whether differences in proportion of unbalanced offspring in *dGALE*^{h} crosses as compared to *dGALE*^{d} were statistically significant.

2.4 RESULTS

Generation and characterization of mutations in dGALE (CG12030)

To assess the requirement for GALE in *D. melanogaster*, we examined the consequence of mutations in *dGALE* (*CG12030).* A homozygous lethal piggyBac insertion within the second intron, *PBac{WH}CG12030*^{f00624}, was identified as part of the Exelixis collection at Harvard (22). Soluble protein lysates from adults heterozygous for this allele demonstrated ~50% GALE enzymatic activity compared to age- and gender-matched wild-type animals (Table 2.1), indicating that this insertion creates a strong loss-of-function allele and suggesting that profound loss of GALE is lethal.

The Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org/) gene disruption project also identified a P element insertion within the 5'UTR of

Genotype	Enzyme Specific Activity (pmol product formed/ug protein/min)			
	GALK	GALT	GALE (UDP-gal)	GALE (UDP-galNAc)
w1118	19.91 ± 1.61	24.31 ± 2.03	81.70 ± 2.80	-----
dGALE^{d}	26.79 ± 7.47	29.64 ± 2.50	91.65 ± 6.58	1.93 ± 0.11
dGALE^{f00624}	16.50 ± 1.39	25.99 ± 6.06	39.24 ± 4.18	0.46 ± 0.06
dGALE^{y}	15.44 ± 4.68	25.02 ± 2.57	42.95 ± 4.01	0.78 ± 0.12
dGALE^{h}	14.28 ± 0.51	24.06 ± 0.63	6.62 ± 0.48	0.26 ± 0.01
hGALE^{32A}	14.28 ± 0.52	-----❖	580.56 ± 16.77	-----
hGALE^{33B}	13.75 ± 1.41	9.78 ± 1.36❖	275.66 ± 32.62	-----
hGALE^{40B2}	14.28 ± 0.54	-----❖	2016.94 ± 23.30	-----

Table 2.1 Leloir Pathway Enzyme Activities for wild-type, dGALE mutants, and flies expressing hGALE. Lethal *dGALE* alleles were assayed as heterozygotes over wild-type. Expression of *hGALE* transgenes was driven with *Act5C-GAL4* in *dGALE^{f00624}/ dGALE^{y} trans* heterozygotes. Values for cells marked ----- are not reported. ❖: Overexpression of *hGALE* in these stocks prevented accurate quantification of *dGALT* activity, due to high background levels of UDP-gal.

dGALE; this insertion (*P{EPgy2}CG12030*^{EY22205} , **Figure 2.2**) does not affect viability. By mobilizing the P element we generated 278 excisions, as defined by loss of the *white* marker. In this study we have focused on two imprecise excision alleles identified from that cohort: *dGALE*^{y}, which is homozygous lethal, and *dGALE*^{h}, which is homozygous viable. As a control, we also studied a precise excision allele, *dGALE*^{d}, identified from the same cohort.

As with the lethal transposon insertion *PBac{WH}CG12030*^{f00624}, *dGALE*^{y} heterozygotes showed ~50% wild-type levels of GALE activity. Animals homozygous for the precise excision *dGALE*^{d} allele have wild-type levels of GALE activity, and *dGALE*^{h} homozygotes show ~8% of wild-type GALE activity (Table 2.1). Both *dGALE*^{h} and *dGALE*^{y} fail to complement chromosomal deficiencies that remove *CG12030*, *Df(3L)emc-E12* and *Df(3L)4196*. Similarly, *dGALE*^{h} and *dGALE*^{y} are lethal in *trans* to each other, and both imprecise excisions are also lethal in *trans* to *PBac{WH}CG12030*^{f00624}.

Molecular characterization of *dGALE*^{y} revealed a 1339bp deletion from *dGALE* that included the first coding exon (**Figure 2.2**). Molecular characterization of *dGALE*^{h} revealed a complex partial excision that left ~1500bp of P-element sequence behind with no other visible perturbation of *CG12030* . This creation of an allele from partial deletion of a P insertion is not without

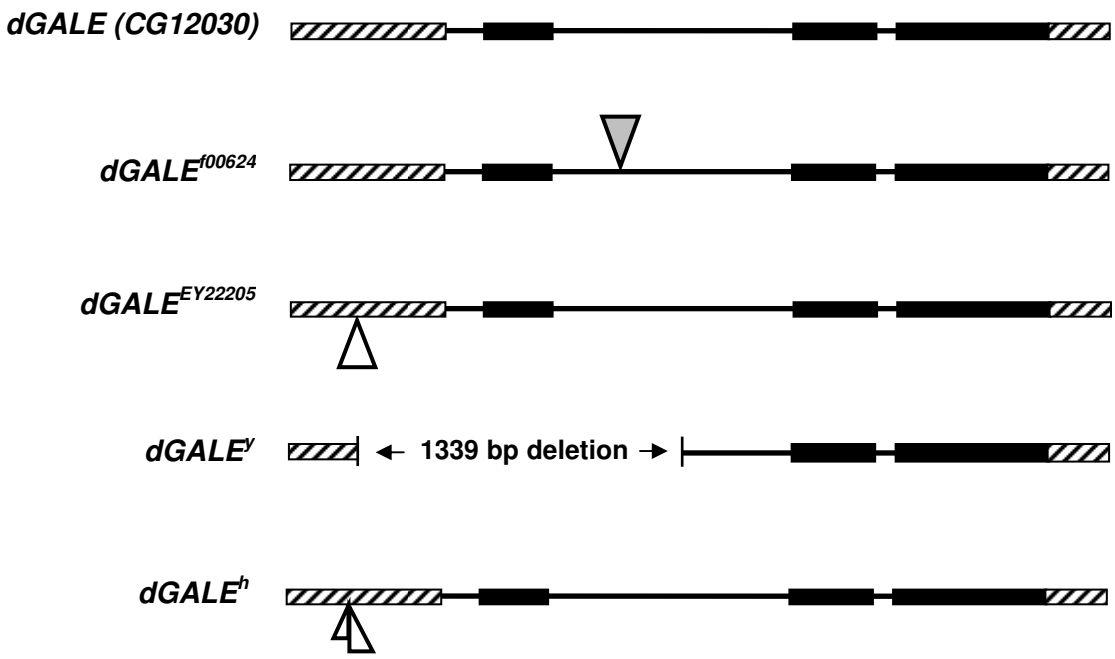

Figure 2.2 Schematic of *dGALE* and Alleles. Introns are shown as thin lines and exons as thick lines, with coding regions in solid black and non-coding regions cross-hatched. Transposon insertion sites are denoted by triangles.

precedent (23). Enzyme activities for animals heterozygous or homozygous (where viable) for each of these genotypes, in concert with complementation and sequencing data, indicate that *PBac{WH}CG12030^{f00624}* and *dGALE^{y}* are strong loss-of-function alleles and that *dGALE^{h}* is a hypomorphic allele of *dGALE.*

Loss of dGALE (CG12030) is embryonic lethal in Drosophila

Trans heterozygotes of *PBac{WH}CG12030^{f00624}* and *dGALE^{y}* show increased death during embryogenesis relative to wild-type animals, and larvae that hatch do not survive past the second instar (L2) stage of development (**Figure 2.3**). Suspecting that this prolonged and somewhat variable period of death was due to the variable persistence of maternally loaded *dGALE* mRNA and protein in individual animals, we created germline clone mutants of *PBac{WH}CG12030^{f00624}*, as described in Methods. With the maternal *dGALE* component removed, *PBac{WH}CG12030^{f00624}, P{FRT}^{2A}/dGALE^{y}* trans-heterozygotes died uniformly in late embryogenesis, confirming our suspicion about maternal loading and implicating an absolute requirement for GALE at this stage of development (**Figure 2.3**). Interestingly, *dGALE* germline clone mutant embryos that carry a wild-type paternal *dGALE* allele, *PBac{WH}CG12030^{f00624}*, *P{FRT}^{2A}/+*, are viable and eclose as healthy, fertile adults that appear morphologically normal.

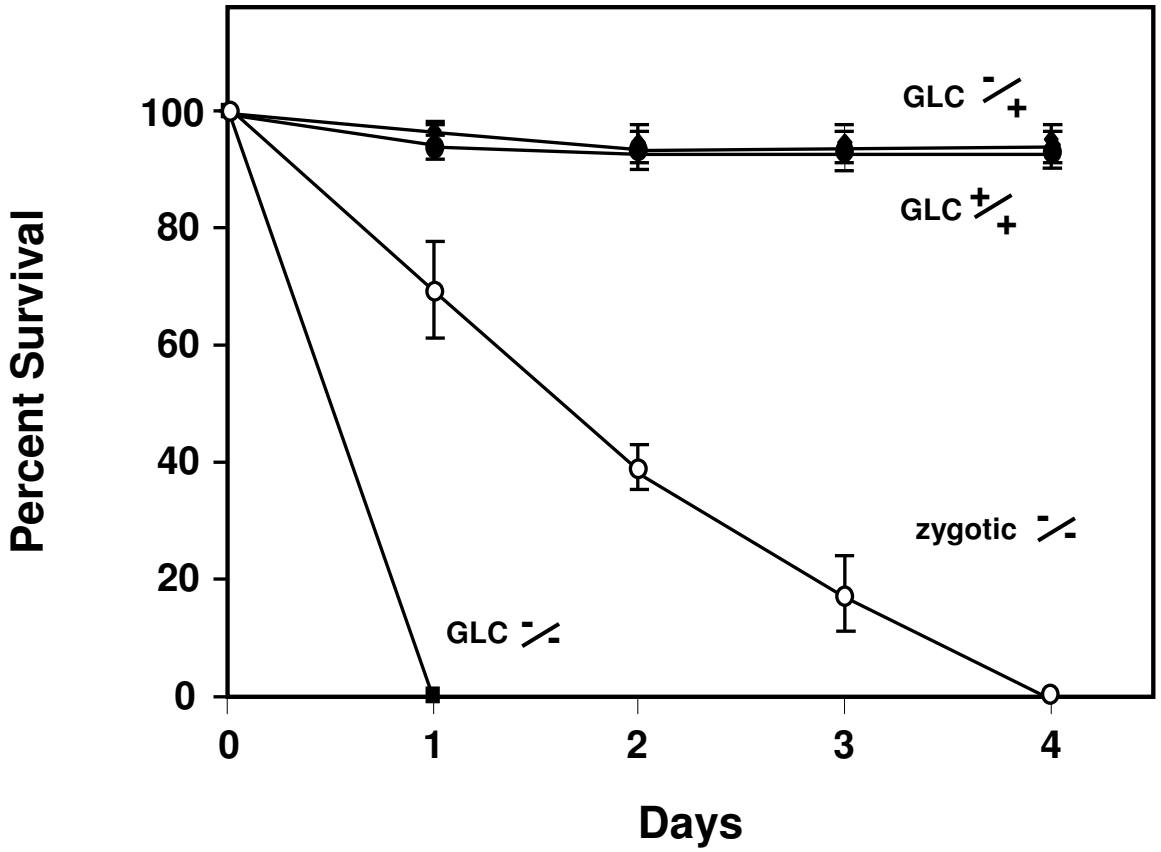

Figure 2.3 Viability of *dGALE* Mutants. Some zygotic $dGALE^{f00624}/dGALE^{y}$ (open circles, denoted -/-) mutants die as embryos while others survive through L1 or L2, while all $dGALE^{f00624}/dGALE^{y}$ germ line clone mutants (filled squares, denoted GLC -/-) die as embryos. Survival of germ line clone $dGALE^{f00624}$/+ heterozygotes (filled triangles, denoted GLC -/+) was not distinguishable from germ line clone wild-type homozygotes (filled circles, denoted GLC +/+).

Furthermore, these *dGALE* heterozygotes did not differ from their wild-type *dGALE* homozygous (*P{FRT}^{2A} /+)* counterparts in survival rates (**Figure 2.3**).

As a final test of the requirement for *dGALE* function in developing *Drosophila* we used flies carrying the inducible UAS-RNAi *dGALE* knock-down construct *12030-R2*. Strong ubiquitous expression of this construct by *Act5C-GAL4* phenocopied the strong loss of function alleles *PBac{WH}CG12030^{f00624}* and *dGALE^{y}*; no viable *Act5C-GAL4>UAS-RNAi^{dGALE}* offspring were observed out of 894 animals scored.

Human GALE rescues viability of dGALE-deficient Drosophila

To confirm that the homozygous lethality of *PBac{WH}CG12030^{f00624}* and *dGALE^{y}* alleles resulted from loss of GALE activity, and not some cryptic off-target effects of the disruption or intragenic deletion, we created compound heterozygotes which carried these two alleles in *trans* and also ubiquitously expressed a human *GALE (hGALE*) transgene. Three separate insertions were tested and yielded the following ratios of rescued animals: *UAS-hGALE^{32A}* (observed 174/916; expected 131/916), *UAS-hGALE^{33B}* (observed 294/973; expected 195/973), and *UAS-hGALE^{40B2}* (observed 418/1540; expected 308/1540). In every case our observed proportion of rescued animals was higher than the expected

Mendelian ratio; this result is not surprising given the reduced fitness of animals carrying balancer chromosomes. Animals of the genotype *Act5C-GAL4*/*UAS-hGALE; PBac{WH}CG12030*^{f00624}/ *dGALE*^{y} were viable, fertile, and morphologically normal.

Transgenic expression of *hGALE* similarly rescued the lethality of *dGALE* knockdown animals, confirming that the lethality was a specific effect of GALE-loss and not an off-target RNAi effect. Strong, ubiquitous expression of both *UAS-hGALE*^{40B2} and *UAS-RNAi*^{dGALE} by *Act5C-GAL4* resulted in 124 viable progeny out of 274 total, no progeny expressing only *RNAi*^{dGALE} were observed. Rescued animals appeared morphologically normal and were fertile.

As expected, transgenic expression of *hGALE* also reconstituted GALE enzymatic activity (Table 2.1). Indeed, the detected levels of GALE activity in these transgenic animals were increased as much as 25-fold relative to wild-type, yet no negative phenotypic consequences of *hGALE* over-expression were apparent.

GALE activity in the gut and Malpighian tubules is both necessary and sufficient for Drosophila development

To determine which organ system(s) *require* GALE function, we used tissue-specific knock-down of *dGALE*. We tested the impact of *UAS-RNAi*^{GALE} expression, and therefore *dGALE* knockdown, in a total of five different tissues or tissue combinations, including: salivary gland (using *Sgs3-GAL4* (L. Cherbas, FlyBase communication, FBrf0155387)), neurons (using *elaV-GAL4* (24) and *pros-GAL4*) larval brain and fat body (using *P{GawB}c754* (25)), eye (using *gmr-GAL4* (26) and *eyeless-GAL4* drivers (A. Parks, FlyBase communication, FBrf0178842)), and embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules, and small intestine (using a *drm-GAL4* driver, (J. Lengyel, FlyBase communication, FBrf0159889)). Of these five, only *dGALE* knockdown in embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules, and small intestine, driven by the *drm-GAL4*, resulted in a clear phenotype; 100% of these animals died at 28°C (0 knock-down/ 128 total scored).

To specify *Drosophila* tissues in which GALE function might be *sufficient* for viability, we selectively expressed the *hGALE* transgene in an otherwise *dGALE*-deficient background using tissue-specific *GAL4* drivers. We observed that *drm-GAL4* driven *hGALE* was sufficient to rescue *PBac{WH}CG12030*^{f00624}/

dGALE^{y} trans heterozygotes (15 rescued /484 total offspring scored). In contrast, hGALE expressed in the nervous system *elaV-GAL4* or in the larval brain and fat body by *P{GawB}c754,* was insufficient to rescue or delay lethality in *PBac{WH}CG12030*^{f00624}*/ dGALE*^{y} trans-heterozygotes (0 rescued / >400 offspring scored for each condition).

dGALE function is required throughout Drosophila development

By employing a temperature-sensitive allele of *GAL80* that negatively regulates *GAL4*-dependent transgene expression at 18° but not at 29°, we established conditional knockdown of *dGALE* and defined the developmental window of GALE requirement in *Drosophila*. As described in Methods, we generated a staggered series of cohorts, each of which experienced loss of *dGALE* beginning one day later in development. As controls, some crosses were maintained at 18°C for the duration of the experiment; functional *GAL80(ts)* suppressed *dGALE* knockdown and these animals survived. Other crosses were maintained at 29°C for the duration of the experiment; *GAL80(ts)* in these animals did not suppress *dGALE* knockdown and they died early in development.

By staging the *dGALE* knockdown and recording the outcomes of resulting animals we determined that GALE is required at stages of fly development from embryogenesis through pupation (**Figure 2.4**). Crosses shifted during pupation, as pharate adults, or after eclosion produced viable flies, though *dGALE* knockdown prior to eclosion had some negative effects on lifespan and fertility (**Figure 2.4**). Although females shifted in early pupal stages laid few or no embryos, their ovaries and egg chambers appeared morphologically normal (data not shown).

Partial impairment in dGALE results in sensitivity to dietary galactose

To determine the impact of dietary galactose exposure on *Drosophila* expressing minimal *dGALE* activity we compared the survival rates of progeny from crosses between homozygotes for the hypomorphic excision allele $dGALE^{h}$ or the precise excision allele $dGALE^{d}$, and balanced flies heterozygous for each of these *dGALE* alleles. All crosses were set up in parallel on each of three different foods: food containing 555 mM glucose as the sole sugar, food containing both 555 mM glucose and 111 mM galactose, and food containing both 555 mM glucose and 111 mM mannose. On glucose-only food both crosses yielded approximately 50/50 ratios of balanced and unbalanced flies, demonstrating that

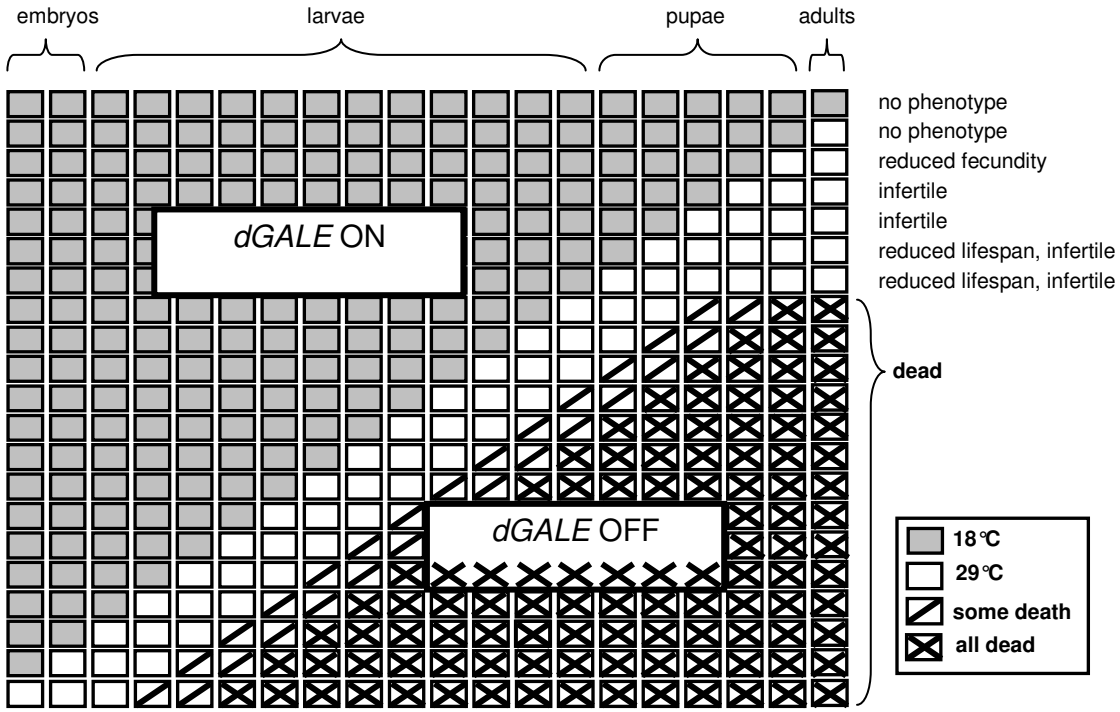

Figure 2.4 ***dGALE*** **Is Required Throughout Development.** Each row represents a cohort of animals in which GALE knockdown was initiated on a particular day in development. Shaded boxes represent days in which dGALE knockdown was prevented by GAL80(ts) at 18°; open boxes represent days after animals were shifted to 29°, when dGALE knockdown is no longer negatively regulated by GAL80(ts). Slashed and crossed boxes denote the time period at which dGALE knockdown was lethal in each cohort.

both *dGALE*^{h} and *dGALE*^{d} homozygotes are viable and fertile in the absence of galactose exposure. On food containing both glucose and galactose, however, the proportion of unbalanced offspring was reduced to ~25% for *dGALE*^{h}, but remained at 50% for *dGALE*^{d}; this difference was highly statistically significant ($p<.000001$). This reduction in *dGALE*^{h} / *dGALE*^{h} viability was not seen in crosses raised on food containing 555 mM dextrose with 111 mM mannose (**Figure 2.5**), indicating that the impact was galactose-specific.

2.5 DISCUSSION

We report here the development and application of a first whole-animal genetic model of GALE deficiency. Using this fly model system we have confirmed what Kalckar first postulated over 40 years ago--that complete loss of GALE is incompatible with life (9). We have further addressed a number of important questions whose answers begin to define the role(s) of GALE in normal *Drosophila* development and homeostasis. These questions include: When in development is *dGALE* required, in what tissues is *dGALE* required, and how much *dGALE* function is required? Finally, in an effort to model epimerase deficiency galactosemia we have begun to explore the impact of partial GALE impairment on organismal viability and galactose sensitivity.

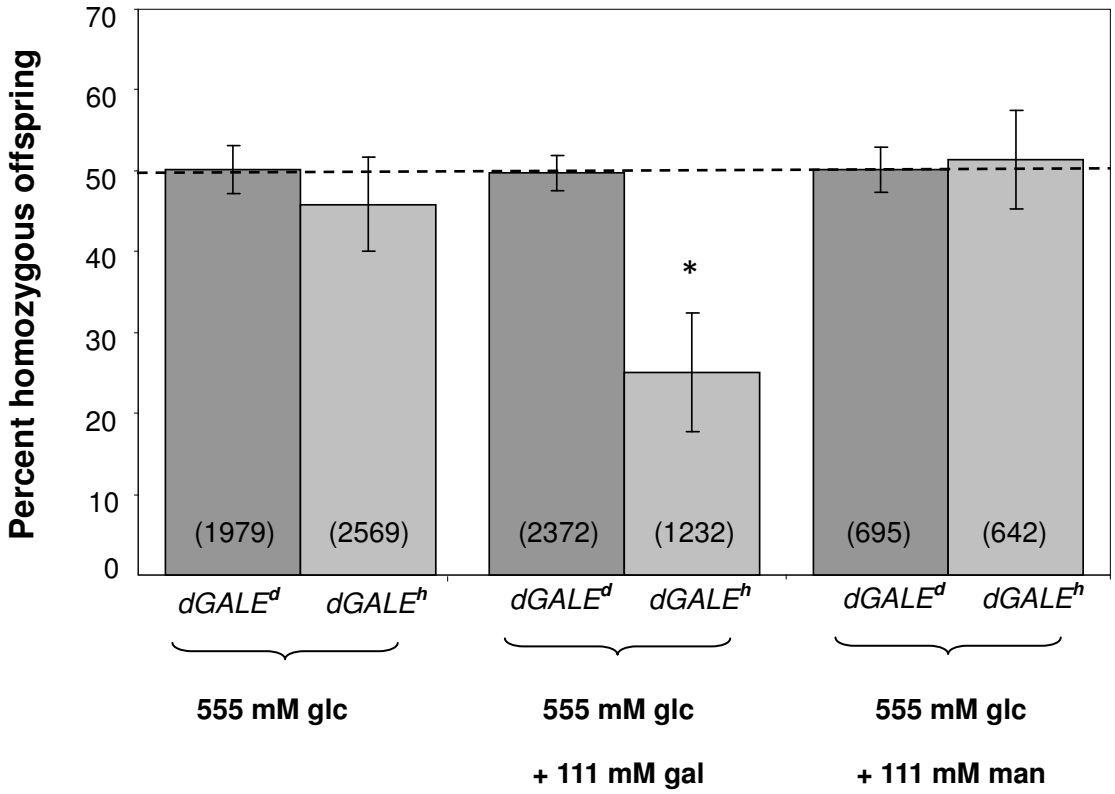

Figure 2.5 Hypomorphic *dGALE* Mutants Are Sensitive To Dietary Galactose. The percentage of homozygous offspring which eclosed as adults is shown for both the precise excision $dGALE^{d}$ (dark columns) and the hypomorph $dGALE^{h}$ (light columns) on food containing dextrose, dextrose and galactose, or dextrose and mannose. The number at the base of each column represents the total number of animals scored for each condition. The expected proportion of 50% homozygotes is represented by a dashed line. On food containing galactose, $dGALE^{h}$ produced significantly fewer homozygotes than $dGALE^{d}$.

When is dGALE required?

The results of our conditional knockdown of *dGALE* experiments demonstrated that epimerase is required for organismal viability continuously from embryonic development through mid-pupation. GALE was also required in late pupation for normal fertility and lifespan, however, the observed failure of these animals to deposit embryos could have reflected reproductive, behavioral, or neuromuscular defects. Of note, the *GAL80(ts)* system allowed us to modulate the expression of an RNAi cassette at will, but the actual timing of *dGALE*-loss was a function of the abundance and halflife of any *dGALE* message and/or protein pre-existing at the time of shutdown. The span of time from temperature shift to death, four to five days in most instances (**Figure 2.3**), therefore represents a likely over-estimate of the length of time an animal at that stage of development can survive in the absence of GALE. Surprisingly, *dGALE* was not required in early embryonic development, prior to the onset of zygotic transcription, as demonstrated by the paternal rescue of germ line clones (**Figure 2.3**). Finally, *dGALE* was not required for adult viability, although we cannot rule out that in the absence of *dGALE* adults may have experienced some subtle negative outcome(s).

Where is dGALE required?

We addressed the question of tissue-specific requirement for epimerase function from the perspectives of both necessity and sufficiency using conditional knockdown and conditional add-back of *dGALE*, respectively. In short, we found that *dGALE* expression in the embryonic proventriculus, midgut, Malpighian tubules, and small intestine (using a *drm-GAL4* driver) was both necessary and sufficient for survival, and that none of the other tissues tested demonstrated either necessity or sufficiency. These results underscore the importance of galactose synthesis through GALE. It is not completely unexpected that expression of *dGALE* (or *hGALE*) in the midgut and tubule could rescue $dGALE^{y}$ */f00624* trans-heterozygotes, given that normal *dGALE* expression is enriched in those tissues (27,28). Of course, despite the absence of demonstrated *dGALE* necessity or sufficiency in the other tissues tested we cannot rule out the possibility that dGALE may be necessary and/or sufficient in combinations of those tissues, as experimental design required that they were tested individually. We also cannot rule out the impact of potential quantitative rather than qualitative differences in *dGALE* expression afforded by the different drivers tested.

It is interesting to note that the midgut and tubule express specific UDP-galNAc transferases involved in mucin-type O-linked glycosylation reactions (29), and are enriched in glycan production during embryogenesis (30). It has been suggested that during tubulogenesis in these tissues, as well as in the trachea, glycan expression is required for proper establishment of apical-luminal polarity (30,31). Since *dGALE* supplies the cell with UDP-galNAc, the obligate first sugar donor in all mucin-type glycosylation, it stands to reason that its requirement in the gut and tubule may be related to glycan expression in these tissues. By extension, human *GALE* may play a similar role in development and maintenance of cell polarity; the highest level of human GALE tissue expression is in the bronchial epithelium, a site with strong apicobasal polarity (32).

How much GALE is required?

Our results address the question of how much epimerase function is required for viability in *Drosophila* from the perspectives of defining both a lower limit and an upper limit. The galactose sensitivity of $dGALE^{h}$ homozygotes, that express about 8% of wild-type GALE levels, and the lethality of $dGALE^{h}$ and either $dGALE^{y}$ or *f00624* compound heterozygotes, that would express about 4% residual GALE activity, implies that the minimum level of GALE activity

sufficient for survival is somewhere between 4 and 8%. These data also provide evidence that metabolite accumulation may be detrimental to individuals with low level residual GALE activity. These results are fully consistent with studies of individuals with generalized epimerase deficiency galactosemia; all of these cases reported to date are homozygotes for the V94M mutation (8); which reduces GALE catalysis to 5% wild-type levels with regard to UDP-gal, and about 24% with regard to UDP-galNAc (33).

We addressed the question of a possible upper limit of *dGALE* expression using our human *GALE* transgene that was expressed at up to almost 25-times the wild-type level, and yet resulted in no apparent negative outcomes. This result is not surprising, given that epimerase catalyzes a reversible reaction. Once levels of enzyme are sufficient to manage the substrate pool, excess enzyme should not perturb the equilibrium ratio of substrates to products achieved.

Why is dGALE required?

GALE has a dual role in the cell, assisting in the metabolism of dietary or endogenously-produced galactose as part of the Leloir pathway and maintaining supplies of UDP-gal/UDP-glc and UDP-galNAc/UDP-glcNAc, essential

substrates for glycosylation reactions. One key question surrounds the relative importance of these roles, whether GALE is required to prevent the accumulation of metabolites from dietary galactose, whether GALE is required to produce adequate galactose for glycan production, or both. The observations from our germ line clone mutants, that animals lacking GALE die during embryogenesis *before any exposure to dietary galactose could occur*, reinforces the notion that GALE deficiency is a problem not only of galactose metabolism, but of galactose synthesis. These studies are a first step toward understanding the full extent of GALE's function in the whole animal, and offer many opportunities for further exploration.

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Chapter 3

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

This chapter contains work published as: Jennifer M.I. Sefton, Rebecca D. Sanders, Darwin Hang, and Judith L. Fridovich-Keil

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3.1 Abstract

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

a galactose challenge. By extension, these data also suggest that both activities might play distinct and essential roles in humans.

3.2 Author Summary

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

3.3 Introduction

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

The reactions of the Leloir pathway are catalyzed by the sequential activities of three enzymes: (1) galactokinase (GALK) which phosphorylates alpha-D-galactose to form galactose-1-phosphate (gal-1P), (2) galactose-1-phosphate uridylyltransferase (GALT), which transfers uridine monophosphate (UMP) from uridine diphosphoglucose (UDP-glc) to gal-1P, forming UDP-galactose (UDP-gal) and releasing glucose-1-phosphate (glc-1P), which can proceed to phosphoglucomutase and the glycolytic pathway, and (3) UDP-galactose 4'-epimerase (GALE) which interconverts UDP-gal and UDP-glc [1]. In addition to a role in the Leloir pathway, metazoan GALE enzymes also interconvert UDP-N-acetylgalactosamine (UDP-glcNAc) and UDP-N-acetylglucosamine (UDP-glcNAc) (**Figure 3.1**). Because it catalyzes reversible reactions, GALE therefore not only contributes to the catabolism of dietary

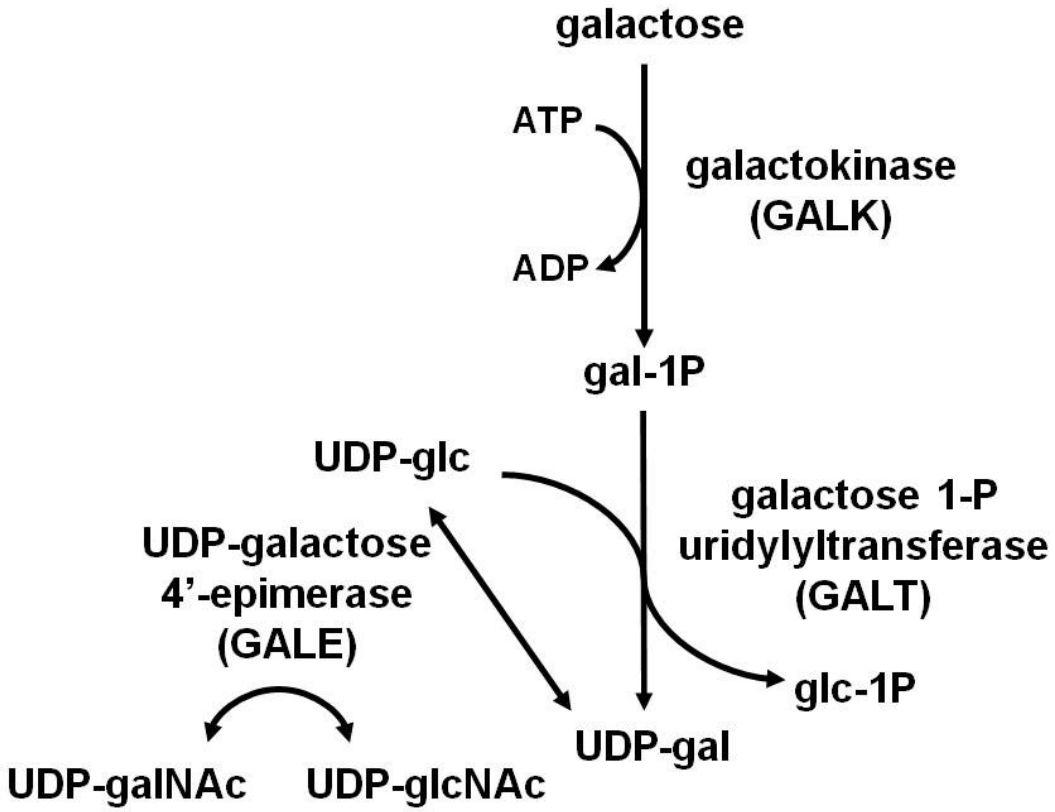

Figure 3.1 The Leloir pathway of galactose metabolism. UDP-galactose 4′-epimerase, the third enzyme in the pathway, also interconverts UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylglucosamine (UDP-glcNAc) in humans, *Drosophila*, and other metazoans tested.

galactose, but also enables the endogenous biosynthesis of both UDP-gal and UDP-galNAc [2,3] when exogenous sources are limited.

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

To address this question, we applied a *Drosophila melanogaster* model of *GALE* deficiency [10]. Using this model, we have previously established that *GALE* is essential in *Drosophila*; animals completely lacking endogenous *dGALE* succumb as embryos, and conditional loss of *dGALE* in larvae results in death within two to four days of knockdown. Finally, partial loss of *dGALE* leads to galactose sensitivity in larvae, and transgenic expression of human *GALE* (*hGALE*) rescues each of these negative outcomes [7].

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

of *dGALE* in *Drosophila* development and homeostasis, and by extension, suggest that *hGALE* may play similarly complex and essential roles in humans.

3.4 Results

The Drosophila GALE enzyme efficiently interconverts both UDPgal/UDPglc and UDPgalNAc/UDPglcNAc

Human and other mammalian GALE enzymes efficiently interconvert both UDPgal/UDPglc and UDPgalNAc/UDPglcNAc (e.g. [13-15]). Previously, we reported that *Drosophila* GALE interconverts the first of these substrate pairs (UDPgal/UDPglc) [10], but did not address whether dGALE could also interconvert the second. Here we demonstrate that dGALE from wild-type adults flies efficiently interconverts both substrate sets (left most bar, **Figure 3.2**). Of note, while purified human GALE [15] and dGALE each interconvert both UDPgal/UDPglc and UDPgalNAc/UDPglcNAc, the apparent specific activity of both human and fly enzymes toward UDPgal is significantly higher than toward UDPgalNAc.

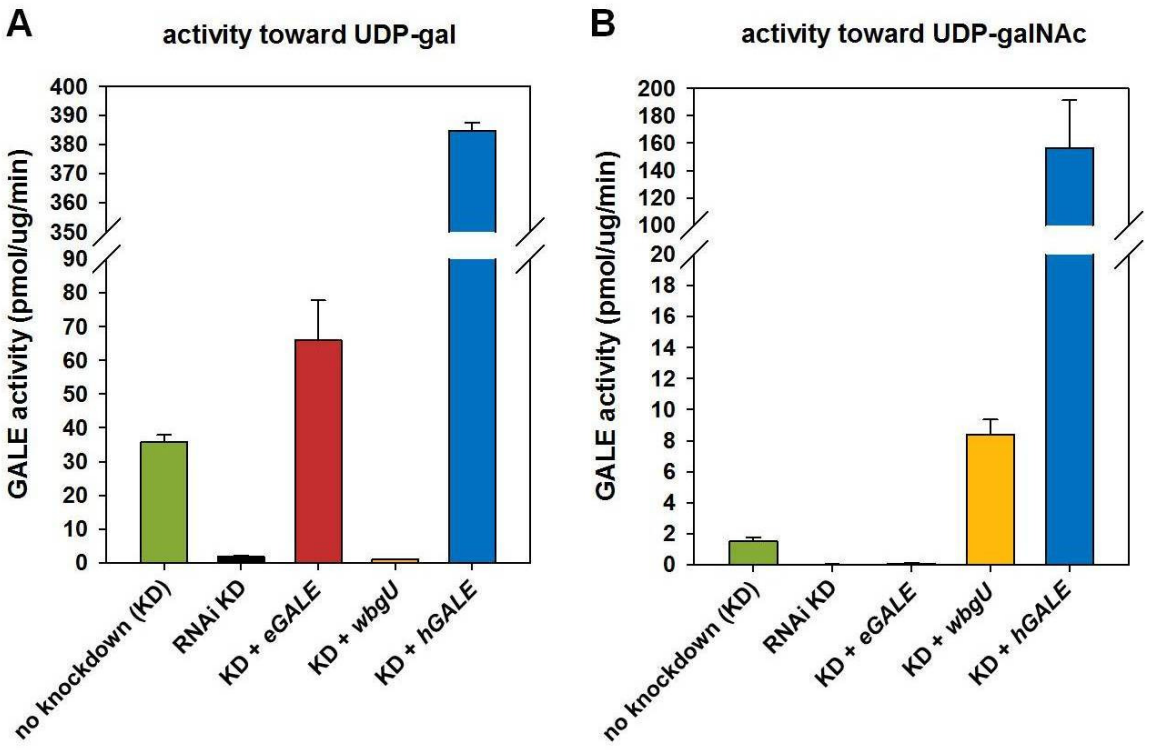

Figure 3.2 Enzyme activities of flies expressing different *GALE* transgenes. Assays for all genotypes were performed on flies with *dGALE* knockdown (KD) driven by the *Act5C-GAL4* driver with the exception of flies labeled "no knockdown"; those flies carried the same *UAS-RNAi*^{dGALE} and *GAL80*^{ts} alleles, but were balanced over TSTL, and thus lacked the driver. In addition to RNA^{i} knockdown of *dGALE*, *Act5C-GAL4* also drives expression of the specified transgenes in these animals. Panel A: GALE activity using UDP-gal as substrate. Panel B: GALE activity using UDP-galNAc as substrate.

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

To generate flies with epimerase activity toward only UDPgal/UDPglc or only UDPgalNAc/UDPglcNAc, we created transgenic lines expressing *eGALE* (*UAS-eGALE*) or *wbgU* (*UAS-wbgU*), respectively, each in a conditionally *dGALE*-impaired background. Each of these prokaryotic *GALE* genes has been demonstrated previously to encode epimerase activity toward only one of the two sets of epimer pairs (e.g. [11,12]). To minimize background, activities of the encoded eGALE and WbgU enzymes toward UDP-gal and UDP-galNAc were assayed in flies knocked down for endogenous *dGALE*; results for the transgenes that demonstrated activities closest to those seen in wild-type *Drosophila, eGALE^{62A} and wbgU^{19A},* are presented in **Figure 3.2**. As expected, lysates from *dGALE* knockdown flies expressing the *eGALE* transgene demonstrated strong activity toward UDP-gal, but not UDP-galNAc, and lysates from *dGALE* knockdown flies expressing the *wbgU* transgene demonstrated strong activity toward UDP-galNAc, but not UDP-gal. As a control we also tested lysates from *dGALE* knockdown flies expressing a human *GALE* transgene; as expected, those samples demonstrated very strong activity toward both substrates.

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

Previously, we created and characterized two *dGALE*-deficient alleles, $dGALE^{f00624.4}$ and $dGALE^{\Delta y}$, which allowed us to demonstrate that *GALE* is essential for survival in *Drosophila* [10]. To examine the requirement for the two different epimerase activities separately, we set up crosses which allowed for the expression of *eGALE* or *wbgU,* individually or in combination, driven by *Act5C-GAL4* in an otherwise *dGALE*-deficient background ($dGALE^{f00624.4}/dGALE^{\Delta y}$). Table 3.1 shows the observed to expected ratios of surviving transgenic offspring that eclosed from these crosses. As presented in Table 3.1, neither *eGALE* alone nor *wbgU* alone was sufficient to rescue survival of the *dGALE*-deficient animals; however, expression of both *eGALE* and *wbgU*, in combination, was sufficient. These results demonstrate that GALE activities toward both UDPgal and UDPgalNAc are essential for survival of *D. melanogaster*. To rule out the possibility that rescue with *eGALE* plus *wbgU* in combination occurred not because both GALE activities are essential, but rather because neither individual transgene expressed sufficient enzyme, we also tested additional *eGALE* and *wbgU* transgenes that individually demonstrated higher levels of expression; none was sufficient to rescue (data not shown). Of note, there also was no

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

Table 3.1 Crosses to test rescue of *wbgU* and *eGALE* transgenes individually and in combination.

apparent over-expression phenotype; for example, animals expressing either *eGALE* or *wbgU* in *addition* to endogenous *dGALE*, and animals dramatically over-expressing human GALE, remained viable, fertile, and appeared morphologically normal (data not shown).

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

Previously, we described an approach that achieves conditional knockdown of *dGALE* in *Drosophila* using a *UAS-RNAi*^{dGALE} transgene (12030-R2, National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan) in combination with a temperature sensitive allele of yeast *GAL80* (*GAL80*^{ts}) ([10] and **Figure 3.3A**). Using this system, we found that *dGALE* is required from embryogenesis through pupation, and that loss of *dGALE* during pupation leads to defects in fecundity and perhaps also a shortened life span [10].

Here we have expanded the *GAL80*^{ts}conditional *dGALE* knockdown system to include different *GAL4*-dependent *GALE* transgenes and have applied this expanded system to test the ability of each transgene, or pair of transgenes, to compensate for the loss of endogenous *dGALE*. By using age-synchronized

cohorts of animals and shifting from the permissive (18°C) to the restrictive temperature (28-29°C) at different times we also were able to test the ability of each *GALE* transgene, or pair of transgenes, to sustain survival and fecundity at different stages of development. At 18°C these animals expressed endogenous *dGALE*, but not their transgenes, and at 28-29°C these animals expressed their transgenes but not *dGALE* (**Figure 3.3A**). Specifically, we tested *Drosophila* that carried no *GALE* transgene, an *eGALE* transgene, a *wbgU* transgene, both *eGALE* and *wbgU* transgenes, or an *hGALE* transgene.

As expected from prior results ([10] and Table 3.1), animals expressing no *GALE* transgene succumbed when shifted to the restrictive temperature as larvae, while animals expressing either human *GALE* or both *eGALE* plus *wbgU* remained viable and fertile (**Figure 3.3C**). Surprisingly, expression of either *eGALE* or *wbgU* alone was also sufficient to rescue survival, albeit to a lesser extent. The fact that the individual prokaryotic transgenes were sufficient to rescue *dGALE* knockdown animals, but not animals genetically null for *dGALE* (Table 3.1), suggests that trace residual *dGALE* expression in the knockdown animals lowered the threshold of transgene function required for rescue.

Of note, while *dGALE* knockdown animals encoding either *eGALE* or *wbgU* remained viable following a shift to the restrictive temperature in early to

mid-development (**Figure 3.3C**), these survivors were not entirely healthy. Specifically, these animals demonstrated either partial or complete loss of fecundity as adults. To test whether the degree of *dGALE* knockdown was comparable between males and females, and therefore would not be a confounding factor, we performed GALE and GALT enzyme assays on newly eclosed male and female adults that carried no GALE transgene and that had been switched to the restrictive temperature as pupa,; the degree of GALE knockdown in both genders was profound and comparable (**Figure 3.3B**), and as expected, GALT activity was apparently unaffected (**Figure 3.3B**). Of note, male and female flies also switched to the restrictive temperature as pupa but harvested three days after eclosion showed the same results (data not shown).

To examine fecundity, we collected and sequestered newly eclosed virgin female and male flies from each surviving cohort, crossed them to an equal number of wild-type flies of the opposite sex, and counted the numbers of viable offspring resulting from each cross. Crosses resulting in large numbers of viable offspring (>50) were scored as "normal fecundity". Crosses resulting in fewer than 10 viable offspring were scored as "reduced fecundity," and crosses resulting in no viable offspring were scored as "loss of fecundity" (**Figure 3.3C**). For example, when *dGALE* knockdown was initiated during early to mid-stage

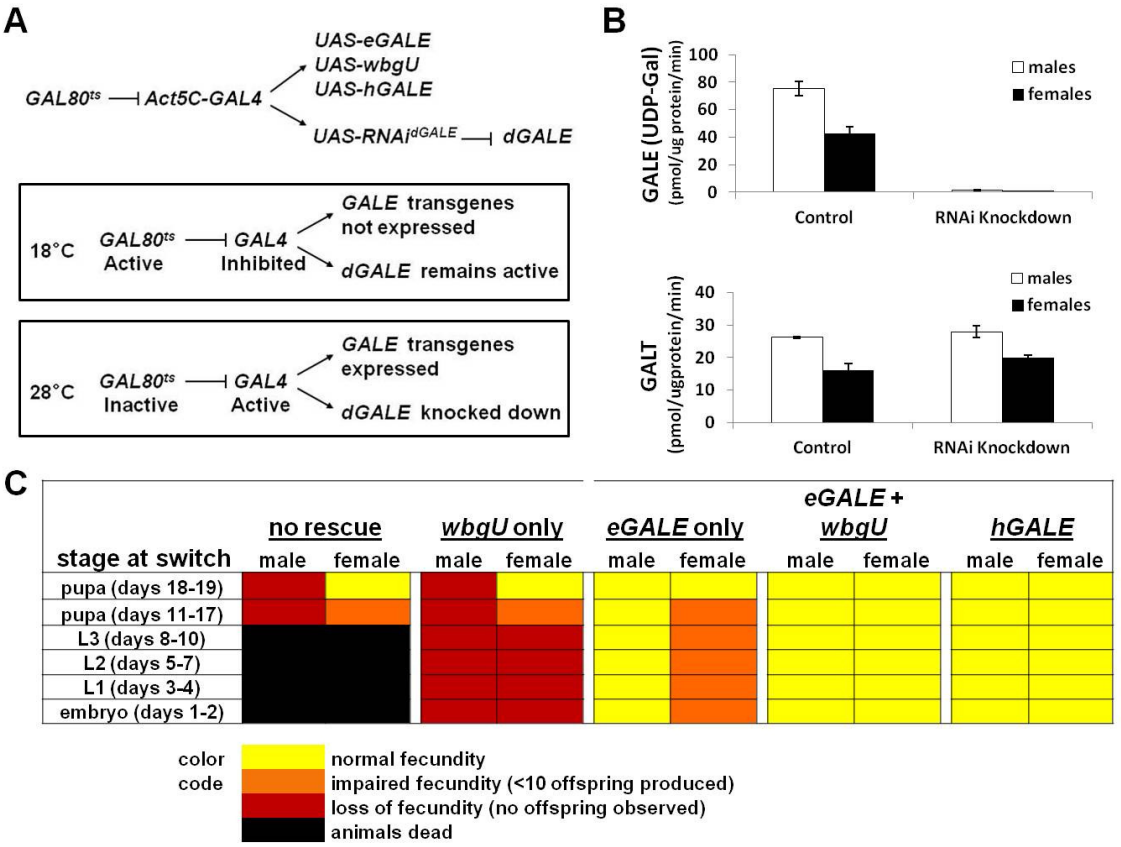

Figure 3.3 Differentially impaired fecundity of flies lacking different GALE activities. (A) Diagram of the method used to achieve expression of different *GALE* transgenes in the background of *dGALE* knockdown animals. The timing of knockdown and concurrent transgene expression was controlled by switching flies from the permissive temperature (18°C) to the restrictive temperature (28–29°C), as indicated. (B) Knockdown efficiency in male and female animals switched to the restrictive temperature as early to mid-stage pupa and harvested for biochemical analysis as newly eclosed adults or three days after eclosion. Of note, GALT activity was completely normal in all samples tested and apparently unaffected by the *dGALE* knockdown (data not shown). (C) Each box represents the outcome of flies switched from 18°C to 28°C at the stage indicated in the column on the left. The number of days the flies developed at 18°C to reach each stage is shown in parentheses.

pupal development, animals of both sexes displayed diminished fecundity. Expression of*eGALE* alone, but not *wbgU* alone, rescued the male defect, whereas expression of both prokaryotic transgenes in combination, or *hGALE* alone, was required to rescue the female defect. These results indicate that GALE activity toward UDP-gal is both necessary and sufficient for male fecundity, but that GALE activities toward both UDP-gal and UDP-galNAc are required for female fecundity.

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

We have previously demonstrated that *Drosophila* expressing a hypomorphic allele of *dGALE* are viable but sensitive to galactose exposure [10]. To assess the roles of the two *GALE* activities in coping with environmental galactose, we collected adult flies in which *dGALE* knockdown coupled with *hGALE*, *eGALE*, *wbgU*, or *eGALE* plus *wbgU* transgene expression was initiated using the $GAL80^{ts}$ system during late larval or early-to-mid-pupal development. These animals were allowed to develop on a standard molasses-based food, and were then transferred as newly eclosed adults to food containing either 555 mM glucose as the sole sugar, or 555 mM glucose plus 175 mM galactose.

We assessed the lifespan of each cohort of animals on both foods; as a control, knockdown animals expressing no *GALE* transgene were also monitored (**Figure 3.4**). In the absence of galactose, all cohorts showed similar longevity profiles, although females (**Figure 3.4**, panel C) showed greater variability than males (**Figure 3.5**, panel A). In the presence of galactose, however, both males and females expressing either no *GALE* transgene, or only the *wbgU* transgene, demonstrated a dramatic reduction in life span ($p<0.0001$, **Figure 3.4**, panels B and D). Females expressing *eGALE* alone exhibited a slight decrease in life span that was independent of diet. Animals expressing *hGALE* or *eGALE* + *wbgU* had lifespans comparable to control animals expressing endogenous *dGALE*, regardless of diet. These data implicate loss of UDP-gal activity as responsible for the galactose-dependent early demise of adult *dGALE*-impaired *Drosophila*.

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

As one approach to explore the pathophysiology underlying the different galactose-dependent outcomes observed in *Drosophila* deficient in GALE activity toward UDP-gal or UDP-galNAc we measured the levels of gal-1P, UDP-gal, and UDP-galNAc in lysates prepared from galactose-exposed third instar larvae

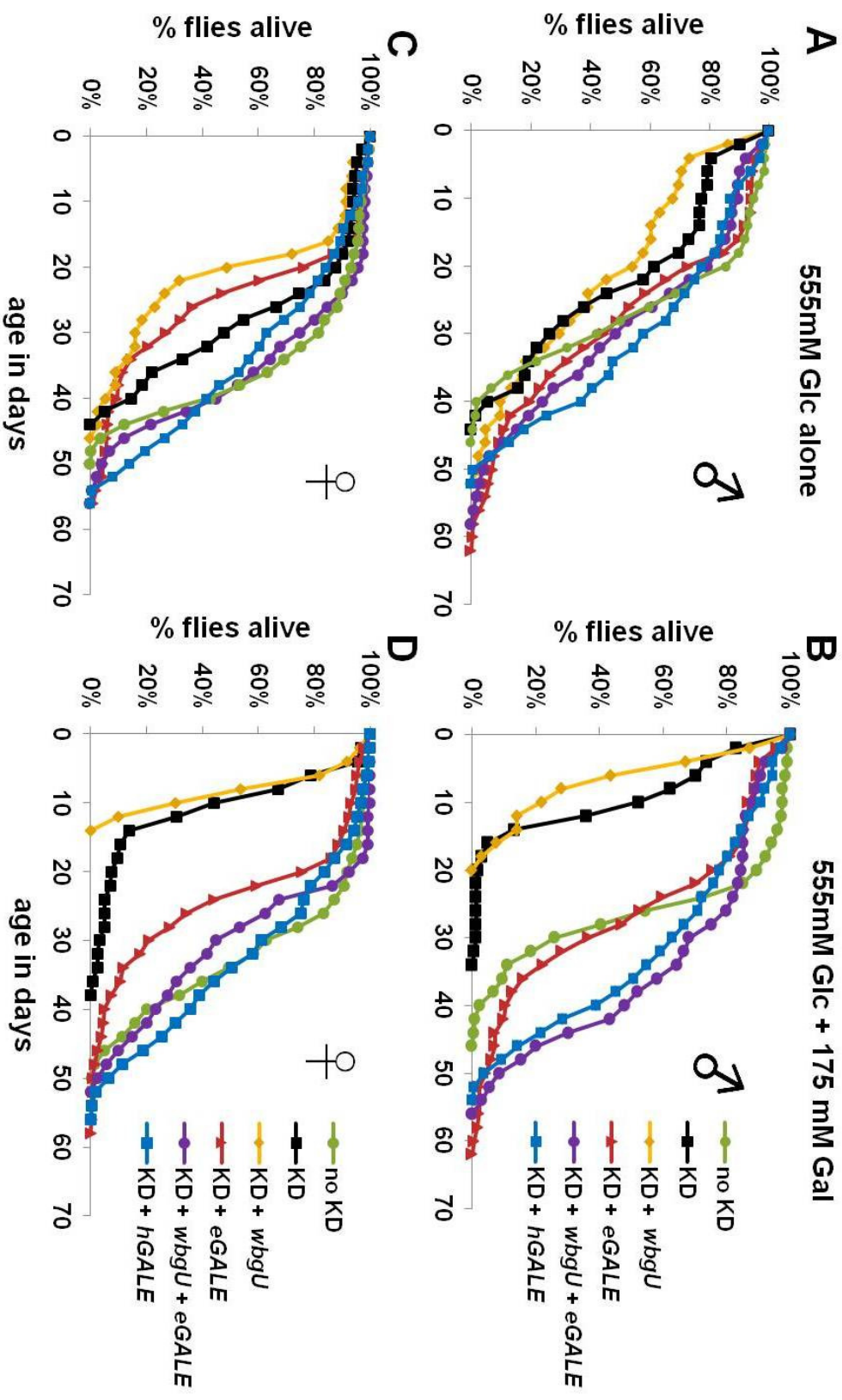
A
555mM Glc alone
% flies alive
B
555mM Glc + 175 mM Gal
% flies alive
C
% flies alive
age in days
D
% flies alive
age in days
no KD
KD
KD + wbgU
KD + eGALE
KD + wbgU + eGALE
KD + hGALE

Figure 3.4 Flies lacking GALE activity toward UDP-gal/UDP-glc have a shortened life span when exposed to galactose as adults. The life spans of male (A and B) and female (C and D) flies reared on molasses food and then tapped as newly eclosed adults to food containing either 555 mM glucose only (A and C), or 555 mM glucose plus 175 mM galactose (B and D), is illustrated. As indicated by the key, these cohorts of flies included controls expressing endogenous *dGALE* as well as animals that expressed endogenous *dGALE* early in development but then were subjected late in development to *dGALE* knockdown coupled with induced expression of either no *GALE* transgene, or *wbgU*, *eGALE*, *hGALE*, or both *wbgU* and *eGALE* in combination. Based on Log rank and Wilcoxon tests for significance, the life spans of knockdown animals expressing either no transgene or expressing only *wbgU* were significantly decreased on food containing galactose compared with food containing only glucose ($p<0.0001$).

expressing different *GALE* transgenes. As illustrated in **Figure 3.5**, galactose exposed animals deficient in both GALE activities (bars marked "KD" for knockdown) accumulated abnormally high levels of gal-1P (**Figure 3.5A and 3.5D**) and UDP-gal (**Figure 3.5B and 3.5E**). Animals deficient only in GALE activity toward UDP-gal (bars marked "*wbgU*" in **Figure 3.5**) also demonstrated elevated gal-1P (**Figure 3.5A and 3.5D**) and UDP-gal (**Figure 3.5B and 3.5E**). In contrast, galactose exposed larvae deficient only in GALE activity toward UDP-galNAc (bars marked "*eGALE*" in **Figure 3.5C and 3.5F**) demonstrated no extraordinary metabolic abnormalities, although, as expected, the absolute level of UDP-galNAc was diminished in these animals independent of diet relative to the "no knockdown" control (**Figure 3.5C**). Also as expected, animals expressing either *hGALE* or both *eGALE* plus *wbgU* demonstrated no clear metabolic abnormalities (**Figure 3.5**).

3.5 Discussion

UDP-galactose 4'-epimerase (GALE) is an essential enzyme in *Drosophila* [10] and in humans [16], but until now the relative contributions of the two distinct GALE activities to development and galactose-tolerance has remained

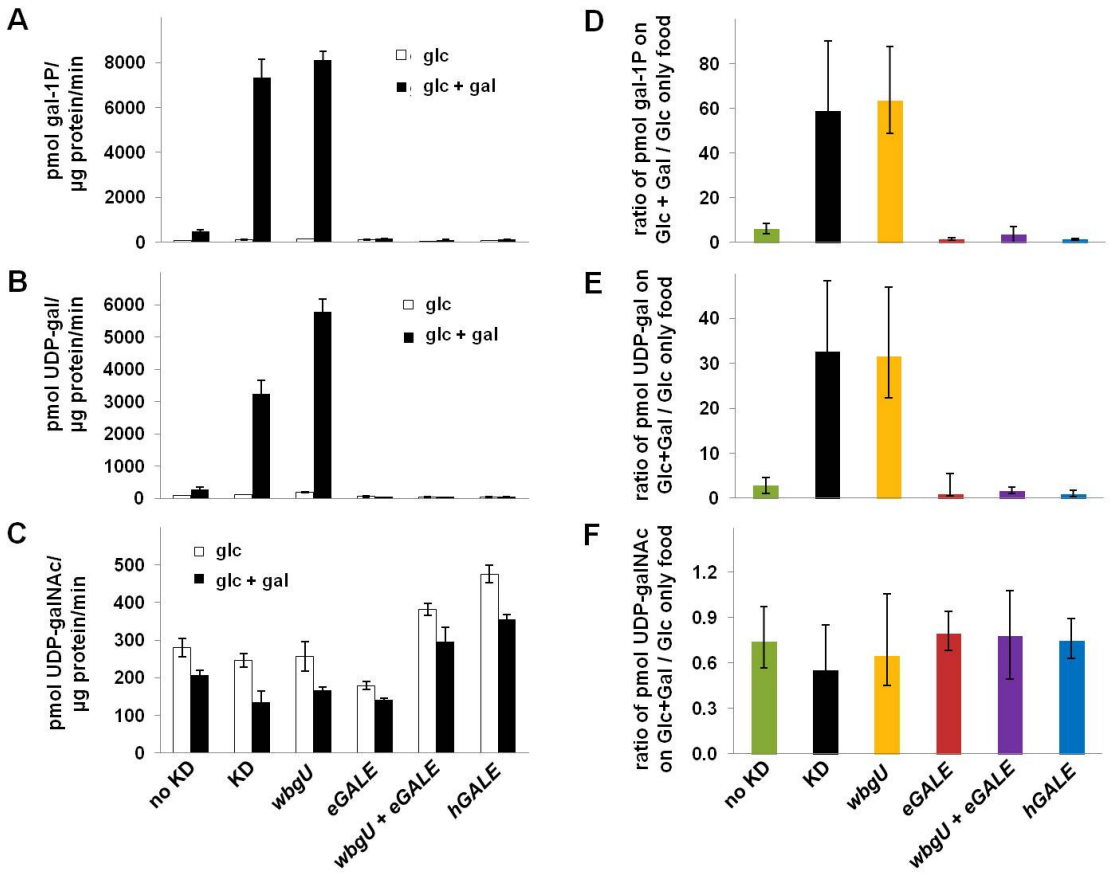

Figure 3.5 Metabolite profiles of *Drosophila* exposed to galactose.

Metabolites were extracted from cohorts of larvae raised on food containing either 555 mM glucose or 555 mM glucose+175 mM galactose. Animals were shifted from the permissive temperature (18°C) to the restrictive temperature (28°C) as first instar larvae and allowed to develop for four days before harvest. Accumulated metabolite values for gal-1P (A), UDP-gal (B), and UDP-galNAc (C) are shown on food containing glucose and glucose+galactose. To demonstrate the impact of diet on metabolite levels, values for gal-1P (D), UDP-gal (E), and UDP-galNAc (F) are shown as ratios of the amount of each metabolite accumulated by animals on food containing galactose over that accumulated by animals of the same genotype on food containing only glucose. Error bars show the 95% confidence interval for each ratio.

unclear. Understanding these roles has important implications regarding mechanism of galactose sensitivity, and may be applicable to diagnosis and prognosis in humans with epimerase-deficiency galactosemia. Our experiments described here exploit the genetic and biochemical facility of *Drosophila melanogaster* to test the consequences of losing each of the two GALE activities individually at different stages of development, or under different conditions of galactose exposure.

Our results demonstrate that developing animals require at least some GALE activity toward both epimer pairs, even in the absence of dietary galactose. Complete loss of either activity in embryos is lethal (Table 1). In animals with trace dGALE activity left by knockdown rather than genetic deletion or disruption, however, transgenic expression of either GALE activity alone is sufficient for rescue (**Figure 3.4B**). Further, in animals that expressed both GALE activities as larvae, knockdown of both activities during pupation is not lethal.

However, knockdown of either GALE activity in early development, or knockdown of both activities in later development has consequences. For example, loss of activity toward UDP-gal in larvae results in impaired fecundity of both males and females, while loss of activity toward UDP-galNAc in larvae

results in impaired fecundity of females but not males. Individual loss of one activity or the other later in development also results in differential sensitivity to galactose. Specifically, both male and female flies deficient in GALE activity toward UDP-gal exhibit a markedly reduced lifespan when exposed to galactose; this effect is not seen in wild-type flies or in flies uniquely deficient in GALE activity toward UDP-galNAc.

These results support two important conclusions. First, the essential role of GALE in development and homeostasis of *Drosophila* extends beyond the Leloir pathway. Whether GALE activity toward UDP-galNAc is essential because of its presumed role in establishing and maintaining substrate pools for glycosylation, or for some other reason, remains unknown. Prior studies in GALE-deficient mammalian cells [17] showed that uridine supplementation could rescue growth and some metabolic abnormalities caused by galactose exposure, raising the possibility that depleted pools of uridine or uridine-derivatives might also be contributing factors. In the current study it is also unclear whether animals subjected to knockdown of one or both GALE activities later in development demonstrate a less severe outcome than those knocked down earlier in development because the products of GALE function, namely UDP-gal, UDP-glc, UDP-galNAc, and UDP-glcNAc, are less essential later in

development, or rather because these UDP sugars have already accumulated to sufficient levels and can be recycled for use. Similarly, the differential sensitivities of male and female fecundity to loss of GALE activity later in development may reflect fundamental differences in male and female development, or alternatively may reflect differential sensitivity to loss; for example, eggs may require a more substantial pool of specific UDP-sugar substrates than sperm to give rise to a viable embryo.

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

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

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

reproductive issues in epimerase-deficiency galactosemia patients might be warranted.

3.6 Materials and Methods

Drosophila stocks and maintenance

The Drosophila stocks used in this study are listed in Table 3.2. Stocks were maintained at 25°C on a molasses-based food that contained 43.5 g/l cornmeal, 17.5 g/l yeast extract, 8.75 g/l agar, 54.7 ml/l molasses, 10 mls propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol). For experiments in which the levels and types of sugar were to be varied, we used a glucose-based food [5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l glucose, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol)] [21] supplemented with galactose, as indicated.

Generation of transgenic Lines

UAS-eGALE and *UAS-wbgU* transgenes were generated by subcloning the *eGALE* and *wbgU* coding sequences, respectively, as *Eco*RI/*Xho*I fragments, into

pUAST [22] using the *Eco*RI and *Xho*I sites in the *pUAST* polylinker region. The resulting plasmids were confirmed by sequence analysis. *UAS-eGALE* stocks were generated using standard transgenic techniques following injection of the transgene into embryos by the fly core of the Massachusetts General Hospital, Charlestown, MA. *UAS-wbgU* stocks were generated using standard transgenic techniques following injection of the transgene into embryos by Genetic Services, Inc., Cambridge, MA. Transformants were selected by the presence of the white gene within *pUAST*. Expression of functional *eGALE* or *wbgU* was confirmed by enzymatic assay of lysates from transformants.

GALK, _GALT_ and _GALE_ (UDP-gal) enzyme assays

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

GALE assay conditions for activity toward UDP-galNAc

Activity was calculated from the conversion of UDP-galNAc to UDP-glcNAc. The initial reaction mixture concentrations were: 100mM glycine pH 8.7, 1.6mMUDPgalNAc and 0.5 mM NAD. Enzyme assays were performed as

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

Table 3.2 *D. melanogaster* stocks and alleles used in this study.

described in Sanders et al. [10] except for the following changes: To start each reaction, 7.5 μl of diluted protein and 5 μl of a cocktail of substrates and cofactors were combined. Reaction mixtures were incubated at 25ºC for 30 minutes and then quenched by the addition of 125 μl of ice-cold high-performance liquid chromatography (HPLC)-grade water (Fisher). Lysates were diluted 1:4, except for those prepared from animals with RNAi knockdown, which were undiluted, and those prepared from animals overexpressing *hGALE* or *wbgU* transgenes, which were diluted to a greater extent. Lysates from *Act5C>hGALE*^{22C} animals were diluted 1:60. Lysates from *Act5C>wbgU*^{19A} animals were diluted 1:20.

Determining requirement for *GALE* activities in development and homeostasis

Generation of animals in which *GALE* knockdown was initiated at 24-hour intervals throughout development was achieved as described previously [10]. A stock homozygous for both *P{tubP-GAL80*^{ts}*}10* and *12030R-2* was used in all crosses. These flies were then crossed to the appropriate genotypes to obtain offspring expressing various transgenes; for: no transgene, *P{Act5C-GAL4}25FO1 ; + / T(2;3)TSTL, Tb, Hu*; *eGALE only*, *P{Act5C-GAL4}25FO1, UAS-eGALE*^{62A}*/ CyO*; *wbgU* only, *P{Act5C-GAL4}25FO1 / CyO; P{Act5C-GAL4}25FO1 / CyO ; UAS-wbgU*^{19A}/TM6B; *eGALE* plus *wbgU*, *P{Act5C-GAL4}25FO1, UAS-eGALE*^{62A}*/ CyO* ;

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

Measurement of life span

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

Measuring metabolite accumulation in GALE-deficient larvae

Cohorts of newly hatched larvae raised at 18ºC were transferred to vials of food containing either 555mM glucose only or 555mM glucose plus 175mM

galactose. The larvae were maintained at 18ºC for one additional day, then transferred to 28º-29ºC and allowed to develop for another four days prior to harvest. Metabolites were extracted and quantified as described previously [10], and were separated and quantified using a Dionex HPLC, as described previously [23] with the following changes: UDP-gal, UDP-glc, UDP-galNAc, and UDP-glcNAc were separated using a high salt isocratic procedure with a flow rate of 0.5 mL/min and buffer concentrations of 45% A and 55% B (0–61 min), followed by washing with a linear increase of B to 95% (61-80 min). For all samples, 20 μl were injected into a 25 μl injection loop.

Acknowledgments

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Chapter 4

Conclusions and Future Directions

4.1 Summary

The work presented in this dissertation highlights the essential roles of GALE in *Drosophila* development and homeostasis. The discovery that both UDP-Gal and UDP-GalNAc activities of GALE are essential and play unique roles emphasizes the complexity of GALE loss and underscores the importance of studying this enzyme from multiple perspectives. The models we have created provide a much needed resource for furthering our understanding of the disease mechanisms underlying GALE deficiency.

In creating and characterizing the first model of GALE deficiency, we were able to answer several important questions. We confirmed that GALE is essential for life in *Drosophila* which, by extension, may explain why all human patients identified to date maintain residual GALE activity. By combining our loss of function alleles with our hypomorphic allele, which retains approximately 4% of wild-type activity, we established a threshold of requirement for GALE activity in flies of between 4% and 8% compared to wild-type GALE.

By creating mutants lacking a maternal contribution of GALE mRNA or protein, but including a paternal GALE allele, we determined that GALE is not required during early embryogenesis, but becomes essential after the initiation of zygotic transcription later in embryogenesis. Furthermore, we demonstrated that GALE is required throughout development: loss during early stages is lethal,

while loss in late development results in live, though not completely normal, adult flies.

Flies with low level GALE activity appeared healthy and fertile. However, when these flies developed on a diet containing galactose they exhibited a galactose-induced lethality. With ~8% residual activity, these flies may be a good model of generalized epimerase deficiency galactosemia.

To look at tissue-specific requirement of GALE we selectively knocked down dGALE in a variety of tissues. Only knockdown in the proventriculus, midgut and Malpighian tubules was lethal. In addition, expression of hGALE in these tissues was sufficient to rescue lethality. This result makes sense in that these tissues play roles in metabolism, and GALE expression is enriched in these tissues during development [1-3].

We then took the next step in characterizing the roles of GALE by uncoupling its two activities. By differentially expressing each GALE activity in genetic loss of function alleles, we demonstrated that activities toward both UDP-gal and UDP-galNAc are required for *Drosophila* survival even in the absence of galactose. Furthermore, in animals with approximately 5% dGALE residual activity resulting from RNA^{i} knockdown, either activity was sufficient to rescue the lethality observed during larval stages of development.

Using conditional RNAi knockdown of dGALE at each stage of development, we showed that each activity plays a distinct role in fecundity. Specifically, activity with regard to UDP-gal was required for fecundity in both males and females, and UDP-galNAc activity was required for normal female, but not male, fecundity. In males that lost UDP-gal activity at any stage prior to the pharate adult stage, no offspring were produced when mated to wild-type females. When female flies lost UDP-gal activity at any stage through larval development no offspring were observed. However, when this activity was lost in early pupal development this phenotype was less severe, with some, though still relatively few, offspring produced, and when this activity was lost during mid to late pupal development fecundity appeared normal. Loss of UDP-galNAc activity resulted in a milder phenotype. Females that lost activity at any point through late pupal development exhibited compromised fecundity, but not a complete loss of fecundity. Male fecundity did not appear to be affected by loss of UDP-galNAc activity. All of these flies were observed mating in individual pairings with wild-type flies of the opposite gender, thus defects in courtship or mating do not seem to account for this phenotype (Jennifer Daenzer, unpublished data).

To explore the requirement for each GALE activity in animals challenged with galactose, we took flies that experienced dGALE knockdown during late

larval or pupal development and, after these eclosed as adults, measured their lifespan in the presence or absence of galactose. Flies lacking either activity or flies lacking only UDP-gal activity demonstrated a dramatic reduction in lifespan when exposed to galactose, indicating that activity toward UDP-gal, but not UDP-galNAc, is required for successful metabolism of exogenous galactose. Furthermore, even in the absence of galactose flies with only one or the other activity appeared less healthy, showing some decrease in lifespan.

Finally, we showed that, like humans with GALE deficiency, these flies accumulate abnormally high levels of both gal-1P and UDP-gal. We did not see any abnormal accumulation of UDP-galNAc.

4.2 Significance and Future Directions

This work has a significant impact towards furthering our understanding of GALE deficiency. Establishing the roles of GALE has important implications with regard to understanding the varied roles of galactose in normal development and cellular function, as well as uncovering the underlying disease mechanisms. In addition, this work may have implications for patients with GALE deficiency both in terms of diagnosis and prognosis.

Implications for Mechanism

The metabolic profiles we measured in flies in both the presence and absence of galactose give some insight into the potential mechanisms behind the outcomes we observed. For example, animals lacking GALE activity toward UDP-gal accumulate abnormally high levels of both gal-1P and UDP-gal in the presence of galactose. Both males and females of this genotype displayed compromised fecundity, and either gal-1P or UDP-gal could contribute to this outcome. In addition, these animals have a significantly reduced lifespan when exposed to galactose as adults. Interestingly, unpublished data from our lab showed that GALT impaired flies did not display the dramatic reduction in lifespan when exposed to galactose as adults that we observed in GALE impaired flies lacking activity toward UDP-gal (Patty Jumbo-Luccioni and Marquise Hopson, unpublished data). We have previously shown that GALT impaired flies accumulate very high levels of gal-1P when exposed to galactose [4], thus gal-1P alone does not appear to be responsible for the lifespan defect in GALE deficient flies.

Animals lacking UDP-galNAc activity display lethality very early in development and compromised fecundity in females that lose activity later in development. These flies do not accumulate abnormal levels of galactose metabolites and, therefore, gal-1P and UDP-gal probably do not play a role in

these outcomes. These data challenge the common hypothesis that gal-1P accumulation underlies the pathogenesis of both GALT and GALE deficiencies and demonstrates that there must be other mechanisms resulting in at least some of these outcomes.

It is interesting that despite displaying negative outcomes, flies lacking activity with respect to UDP-galNAc/UDP-glcNAc did not have any marked abnormality in their levels of UDP-galNAc, nor any other metabolite measured. Given that epimerization of UDP-glcNAc by GALE is the primary pathway for UDP-galNAc synthesis, we hypothesized that these flies would have diminished levels of UDP-galNAc. However, this was not the case, at least in the samples we measured. There are a number of possible explanations for this finding. These flies lost dGALE activity during larval development, and therefore, had UDP-galNAc activity for the first few days of development. There is evidence in mammals that a secondary pathway exists which allows free GalNAc from glycoprotein and glycolipid turnover to be converted back to UDP-galNAc [5]. To our knowledge, this pathway has not been demonstrated in *Drosophila*, but it is plausible that our flies synthesized sufficient levels of UDP-galNAc very early in development, which was incorporated into glycoproteins and glycolipids. After initiation of the conditional knockdown, this salvage pathway might have allowed the flies to recycle their GalNAc and produce normal levels of UDP-

galNAc. To test whether this is the case, we could measure the metabolite levels in flies that lose dGALE activity even earlier in development or in flies carrying our genetic loss of function alleles. As to why these flies go on to develop phenotypic consequences, at least with regard to female fecundity, there could be tissue specific or subtle differences in metabolite levels that we were unable to detect with our assay. It might be interesting to test the metabolite levels in ovary versus the whole animal.

Given the critical roles of UDP-gal, UDP-glc, UDP-galNAc, and UDP-glcNAc as substrates in the synthesis of glycoproteins and glycolipids, it seems likely that our flies would have glycosylation defects. Interestingly, some phenotypes we have reported have been observed in studies of glycosylation defects. For example, O-linked glycans play an important role in sperm-egg binding. The zona pelucida surrounding the egg is composed of three glycoproteins, and sperm use a glycosylphosphatidylinositol (GPI)-anchored surface hyaluronidase to penetrate the extracellular matrix [6]. Our group has recently tested O- and N- linked glycosylation of plasma proteins from patients with classic galactosemia and found abnormalities in untreated infants compared with controls [7]. We hope to be able to examine possible glycosylation defects in our GALE-deficient flies or patient samples in the near future.

Implications for Patients

Our work demonstrates that both the UDP-gal and UDP-galNAc activities of GALE are essential for survival and health of *Drosophila* and, by extension, possibly also of people. Most clinical labs currently test for activity toward only UDP-gal, meaning that rare patients with loss of only UDP-galNAc activity would be missed. In addition, knowing the levels of both activities might be beneficial in predicting patient prognosis, though more information regarding the contributions of each activity toward outcome is needed.

Our lifespan data reveal that flies lacking activity with respect to UDP-gal have a substantial decrease in lifespan when exposed to galactose as adults. This result demonstrates that GALE activity is not only essential for development, but also for homeostasis. A big question in the field is whether or not it is essential for patients with GALT or GALE deficiencies to remain on a galactose restricted diet for life. These data suggest that for GALE deficiency, it may be important to maintain galactose restriction, and studies on the correlation between diet and long-term outcome in patients may be warranted.

Issues related to puberty and fertility have not been reported in patients with GALE deficiency. However, primary ovarian insufficiency (POI) affects >80% of girls and women with classic galactosemia [8-10]. Our studies revealed

compromised fecundity in both male and female flies with GALE impairment. This finding suggests that long-term studies of reproductive issues in patients with GALE-deficiency may be warranted. In addition, studies of flies might offer insight into the mechanism underlying ovarian insufficiency in classic galactosemia.

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Appendix

Development of an Assay to Permit High-throughput Screening for Modulators of UGP Activity

Jennifer M.I. Daenzer and Judith L. Fridovich-Keil

Automated screens were conducted by Clinton Maddox at the Emory Chemical Biology Discovery Center

A.1 Introduction

Classic galactosemia is a potentially lethal disorder resulting from profound impairment of the enzyme galactose-1-phosphate uridylyltransferase (GALT). GALT is the second enzyme in the Leloir pathway of galactose metabolism **(Figure A.1)**, and is responsible for converting galactose-1-phosphate (gal-1P) to uridine diphosphate galactose (UDP-gal), consuming uridine diphosphate glucose (UDP-glc) and producing glucose-1-phosphate (glc-1P) in the process. Loss of GALT prevents normal metabolism of galactose, and patients accumulate abnormally high levels of galactose metabolites including galactose, galactitol, and gal-1P [1-3]. Classic galactosemia affects between 1/30,000 and 1/60,000 live births in the United States [1, 4]. Because the milk sugar lactose is a disaccharide containing one molecule each of glucose and galactose, infants are exposed to a high level of galactose from birth. Accordingly, symptoms of classic galactosemia develop within days to weeks of birth. These symptoms include poor feeding, poor weight gain, jaundice, hepatomegaly, *E. coli* sepsis, and can include death if left untreated. In all states in the United States and many other countries classic galactosemia has been added to the newborn screening panel, and newly identified patients are immediately placed on dietary galactose

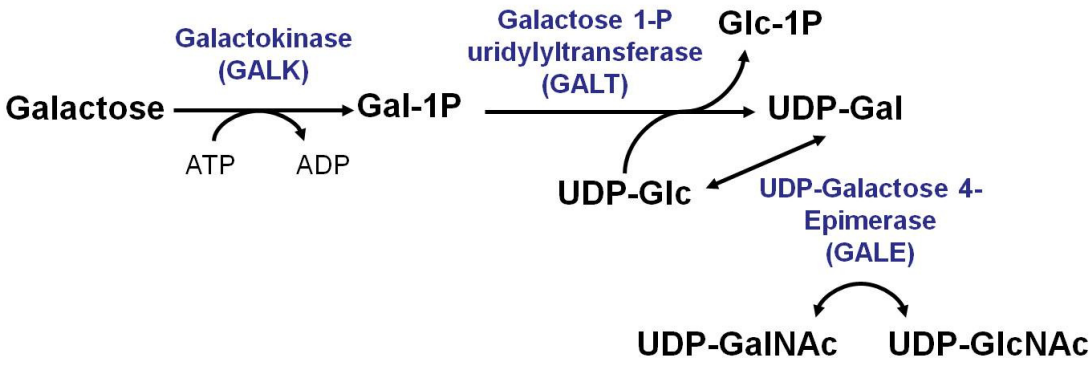


Figure A.1 The Leloir Pathway of Galactose Metabolism. The Leloir pathway is highly conserved from bacteria to humans and provides the primary route for galactose metabolism.

restriction [5]; this reverses the acute symptoms and has lead to a dramatic decrease in neonatal mortality. Unfortunately, despite dietary intervention, many patients go on to develop negative long-term outcomes including language and speech delays, cognitive disability, primary ovarian insufficiency in >80% of females, ataxic tremor, and decreased bone density [2, 6-10]. There is currently no effective therapy to prevent the long-term outcomes patients with classic galactosemia experience.

It is known that patients with classic galactosemia who have no detectable GALT activity can metabolize galactose at a very slow rate [11]. This indicates that there is an alternative route for galactose metabolism, which is believed to involve UDP-glucose pyrophosphorylase (UGP) [12]. The primary role of UGP is to catalyze the formation of UDP-glc from glc-1P in a UTP-dependent manner. This is a key step in glycogenesis, as UDP-glc is then used to synthesize glycogen. However, at a much lower efficiency, human UGP can also catalyze the formation of UDP-gal from gal-1P. **Figure A.2** depicts the bypass route that UGP could form around a deficient GALT enzyme. Studies in yeast have shown that over-expression of UGP rescues the galactose induced growth arrest in GALT deficient yeast [13], supporting the idea that this bypass route could be a therapeutic target. We believe that expanding this natural bypass route would

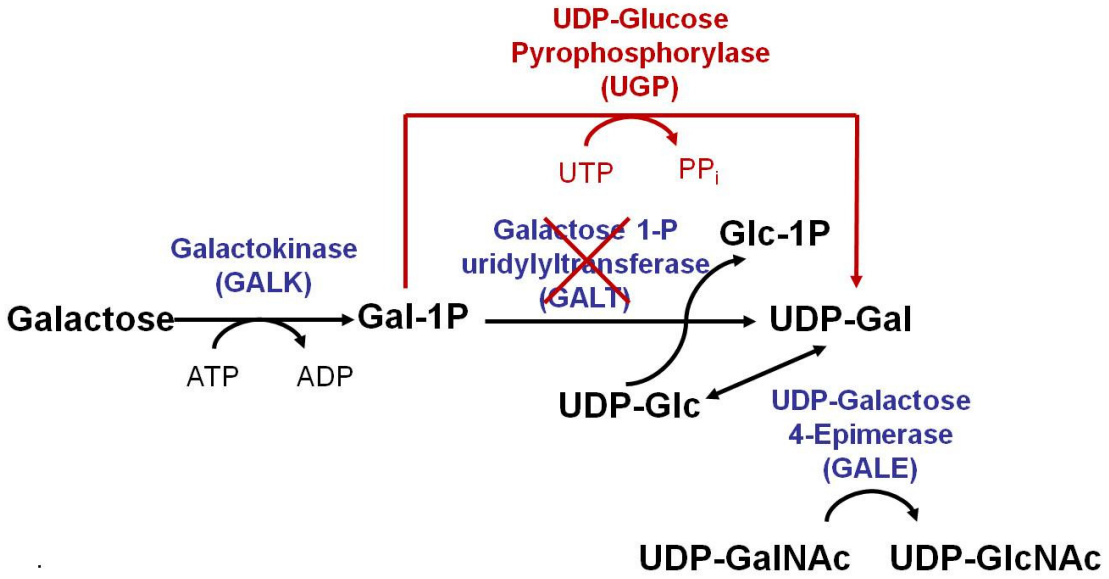


Figure A.2 UGP Forms a Bypass Pathway Around a Deficient GALT Enzyme. The enzymes of the Leloir pathway are shown in blue. The UGP bypass pathway around a deficient GALT enzyme is highlighted in red.

help restore galactose metabolism to GALT deficient cells and/or individuals. Our overall goal is to identify a small molecule which alters the substrate selectivity of UGP, allowing it to work more efficiently with gal-1P without severely affecting activity toward glc-1P.

Utilizing a compound which modulates UGP activity toward gal-1P is an attractive therapeutic strategy for several reasons. UGP is an abundant enzyme which is widely expressed. This is an advantage since even a molecule which confers a modest improvement in UGP function toward gal-1P could decrease or eliminate accumulation of galactose metabolites. In addition, UGP modulation is not cell-autonomous. That is, since this compound would be helping cells to actually metabolize galactose, treatment of cells in one tissue, liver for example, could decrease blood galactose levels, thereby protecting other tissues as well.

Here we describe the development of an assay which permits high-throughput screening for small molecule modulators of UGP activity. Our results indicate that our assay is scalable and robust, and validate its use for high-throughput screening of therapeutic compounds.

A.2 Materials and Methods

Luciferase-coupled UGP assay

Purified hUGP used in all assays was purified by our collaborators James Thoden and Hazel Holden at the University of Wisconsin-Madison.

For the initial development of the luciferase-coupled assay, 100 μL reactions were performed in black 96-well plates (Corning). A mixture containing all substrates and enzymes except for hUGP was added to wells of the plate, and hUGP was added separately to initiate the reactions. The plate was immediately placed in a plate reader (Tecan Ultra Evolution) at room temperature, and luminescence was measured every minute. Data from the plate reader was recorded and analyzed using Magellan V5.00 software. We optimized the assay to ensure that all coupling enzymes and substrates were in excess. Initial reaction concentrations were 60 mM gal-1P (Sigma), 2 mM UTP (Sigma), 60.0 μM APS (Biolog), 0.15 units ATP sulfurylase (NEB), 11.2 mM $MgCl_2$, 56 mM Tris HCl pH 8.0, and 2.1 mM DTT, and 0.32 nL reconstituted ATP Assay Mix (Sigma, FLAAM) diluted in 50 μL ATP Assay Mix Dilution Buffer (Sigma, FLAAB) per 100 μL reaction. 0.026 μg of hUGP was added to each well.

The data were validated by calculating two variables: signal-to-background (S:B) ratio and Z′ factor. S:B ratio is the mean of the signal divided by the mean of the background, and Z′ is calculated using the following equation: $1-[(3\sigma_s + 3\sigma_b) / (\mu_s - \mu_b)]$, where σ is the SD of signal (σ_s) or background (σ_b) and μ is the mean [14, 15].

To calculate the K_M of hUGP, concentrations of gal-1P were varied by factors of two from 0.986 to 63.1 mM. Concentrations of glc-1P were varied by factors of two from 8 to 2096 μM. hUGP was used at a concentration of 0.1 μg per reaction. The initial reaction rates were calculated, and the program SigmaPlot was used to calculate the K_M values.

High-throughput Screening

To optimize the assay for high-throughput screening we miniaturized to 384-well plates for the LOPAC screen and 1536-well plates for the 30K library. We decreased the gal-1P concentration from 60 mM to 3 mM in order to decrease the cost of the assay. This did not negatively affect the robustness of the assay. The plates were set up so that the test wells contained compounds plus the UGP assay with gal-1P, and the control wells did not contain compounds, only the UGP assay with glc-1P as the positive control. To allow the compounds we were screening to have time to interact with UGP before the reaction was initialized,

we changed the order reagents were added. Everything except for gal-1P and UTP was added to the wells with the compounds. The plates were allowed to sit at room temperature for approximately 30 minutes, and finally, gal-1P and UTP were added to initiate the reactions. The compounds were diluted in DMSO for screening. The reactions were incubated at room temperature, and endpoint readings were taken at 10 minutes.

High-throughput screening of both the Library of Pharmacologically Active Compounds (LOPAC) and the 30,000 compound ChemDiv library was performed at the Emory Chemical Biology Discovery Center. To validate the performance of the high throughput screening assay, we calculated the coefficient of variation in addition to Z′. The coefficient of variation is defined as the ratio between the SD of the sample and the mean of the sample. This was multiplied by 100 and presented as a percentage.

Direct UGP assay

For direct assays to measure UGP activity 30 μl of diluted protein and 20 μl of a mixture of substrates were combined to start each reaction. Initial reaction concentrations were 1 mM UTP, 1mM gal-1P, 11.2 mM $MgCl_2$, 56 mM Tris HCl pH 8.0, and 2.1 mM DTT. For assays measuring activity toward gal-1P, 5 μg of UGP was added, and for assays measuring activity toward glc-1P, 2.5 ng/μL UGP

was added. Reaction mixtures were incubated at 37°C for 20 minutes and then quenched by the addition of 700 μl of ice-cold high-performance liquid chromatography (HPLC)-grade water (Fisher). Particulates were removed from the samples by centrifugation at 4000 x ***g*** for 4 minutes at 4°C through 0.22-μm Costar Spin-X centrifuge tube filters (Corning). Reactants and products were quantified by HPLC, as described previously (Ross et al., 2004), and the specific activity reported as pmol of product formed per μg protein per minute of reaction time.

For assays testing the effects of DMSO, concentrations ranging from 4% to 0.0156% in 1:2 dilutions were used. In assays comparing the effect of DMSO on UGP activity toward gal-1P versus glc-1P, 4% DMSO was used.

A.3 Results

Development of a coupled UGP assay

Our goal was to develop an assay which could feasibly be used for high throughput screening. As described in Materials and Methods and depicted in **Figure A.3**, we used a luciferase-coupled approach. Briefly, the pyrophosphate

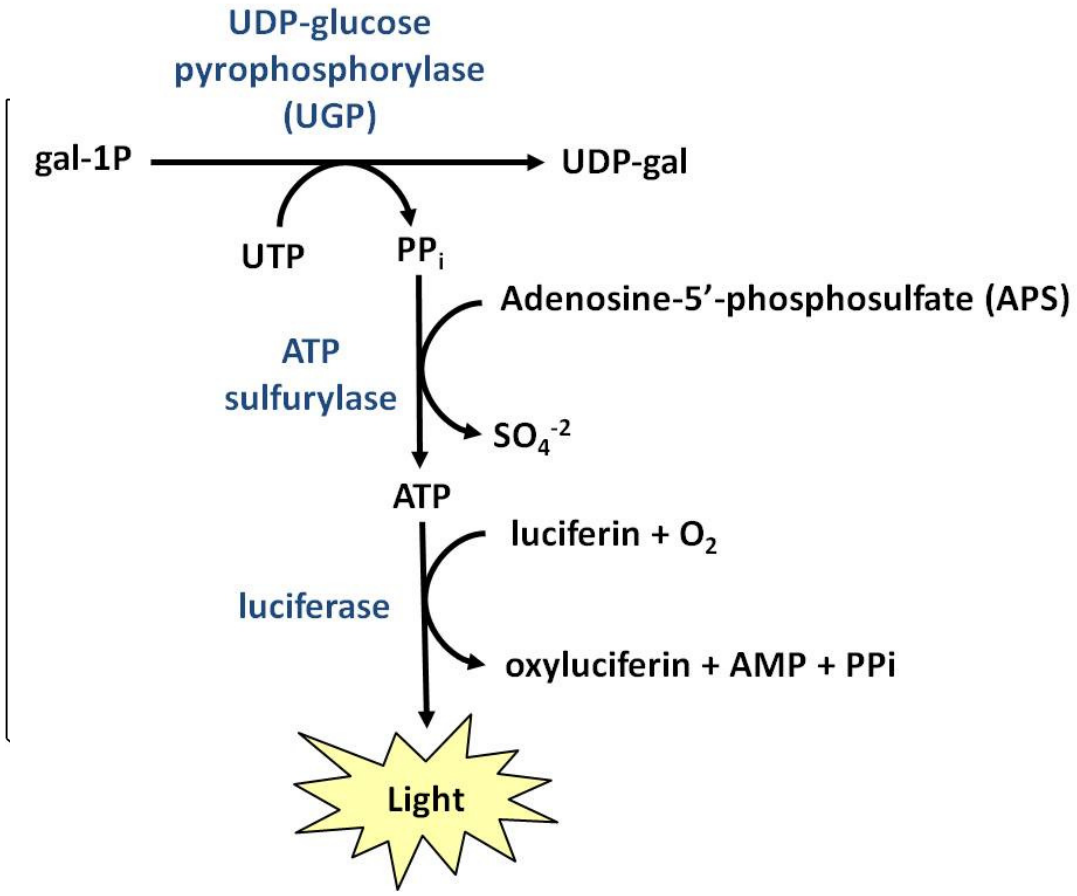


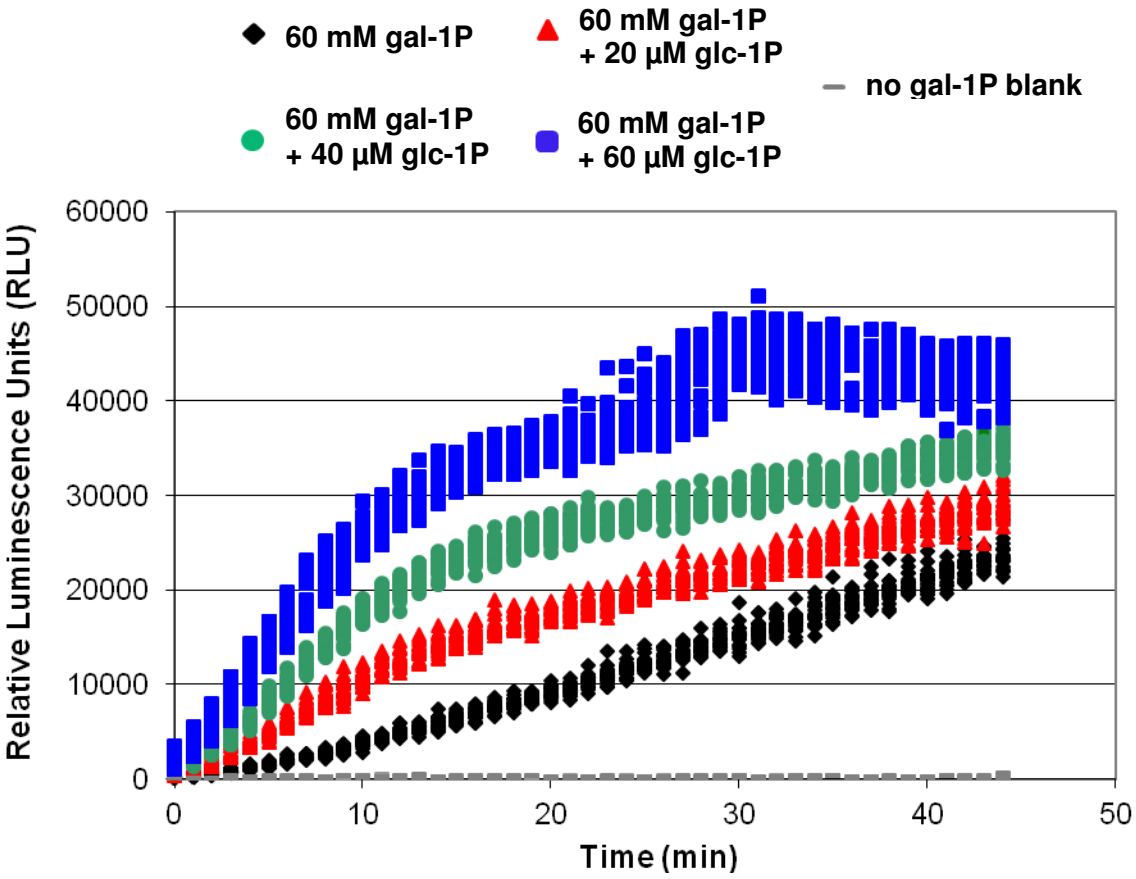
Figure A.3 Schematic of Coupled UGP Assay. We modeled our assay on pyrosequencing, which uses the sequential reactions of ATP sulfurylase and luciferase to convert pyrophosphate (PP_i), which is a reaction product of UGP, into light. This assay provides a convenient readout in the form of luminescence which facilitates high throughput screening.

(PP_i) produced from the UGP reaction is converted to adenosine triphosphate (ATP) by an enzyme called ATP-sulfurylase, which uses adenosine 5'-phosphosulfate (APS) as a co-substrate. ATP and luciferin are then utilized by luciferase to produce light, which we measured in units of relative luminescence units (RLU). This assay has several benefits. The reaction is very sensitive, reacting quickly to an increase in PP_i concentration. Furthermore, luminescence is an easily measurable readout, and is used in many assays for high-throughput screening.

Figure A.4 shows results generated from the assay with gal-1P and gal-1P spiked with a low concentration of glc-1P to emulate what we would expect to see if UGP activity toward gal-1P was improved. We saw a sizable window in which we could detect improvement of UGP activity. Also shown are the signal to background (S:B) ratio and Z' factors calculated at the various concentrations of glc-1P. We calculated the Z' factor of this assay to be 0.784 and the signal to background (S:B) ratio to be 7.41, indicating that the assay was robust and reproducible.

Calculation of K_M

Using our optimized assay, we were able to determine the K_M of hUGP2 for both gal-1P and glc-1P to be 15.31 mM and 152.9 μM respectively (**Figure A.5**



[glc-1P] (µM)	S:B	Z'
20	2.82	0.50
40	4.61	0.74
60	6.83	0.77

Figure A.4 Performance of the Coupled UGP Assay. Luminescence was read every minute for 45 minutes. Assays contained: black diamond - 60mM gal-1P only; red triangles – 60 mM gal-1P and 20 µM glc-1P; green circles – 60 mM gal-1P and 40 µM glc-1P; blue squares – 60 mM gal-1P and 60 µM glc-1P. n=23 for all conditions. Grey dashes (n=3) contained no gal-1P or glc-1P and demonstrate the background level of luminescence. The chart shows the signal to background (S:B) and Z' factors for assays with 60 mM gal-1P only compared to assays with 60 mM gal-1P + 20, 40, or 60 µM glc-1P.

and Figure A.6). As expected, with an approximately 100-fold difference, the K_M with respect to glc-1P was significantly lower than that toward gal-1P.

HTS Screening

Our pilot screen of the Library of Pharmacologically Active Compounds (LOPAC) was done as described in Materials and Methods. This library contains 1,280 pharmacologically active compounds. To determine the impact of the compounds on hUGP activity, fold induction was calculated by comparing the luminescence from each individual well to the mean value for the plate containing a given well. Because we do not have a true positive control in the form of a compound which is known to modulate or enhance UGP activity, we performed assays with 100 μM glc-1P instead of gal-1P to simulate a positive control. The assay was validated using the Z′ factor and coefficient of variation (described in Materials and Methods). The Z′ factors for each plate ranged from 0.74 to 0.83, all > 0.5, which is generally accepted as excellent for high throughput screens [15]. The coefficient of variation for each plate was approximately 3%. Though none of the compounds screened significantly increased UGP activity, these data validated the assay for use in a high throughput screening strategy.

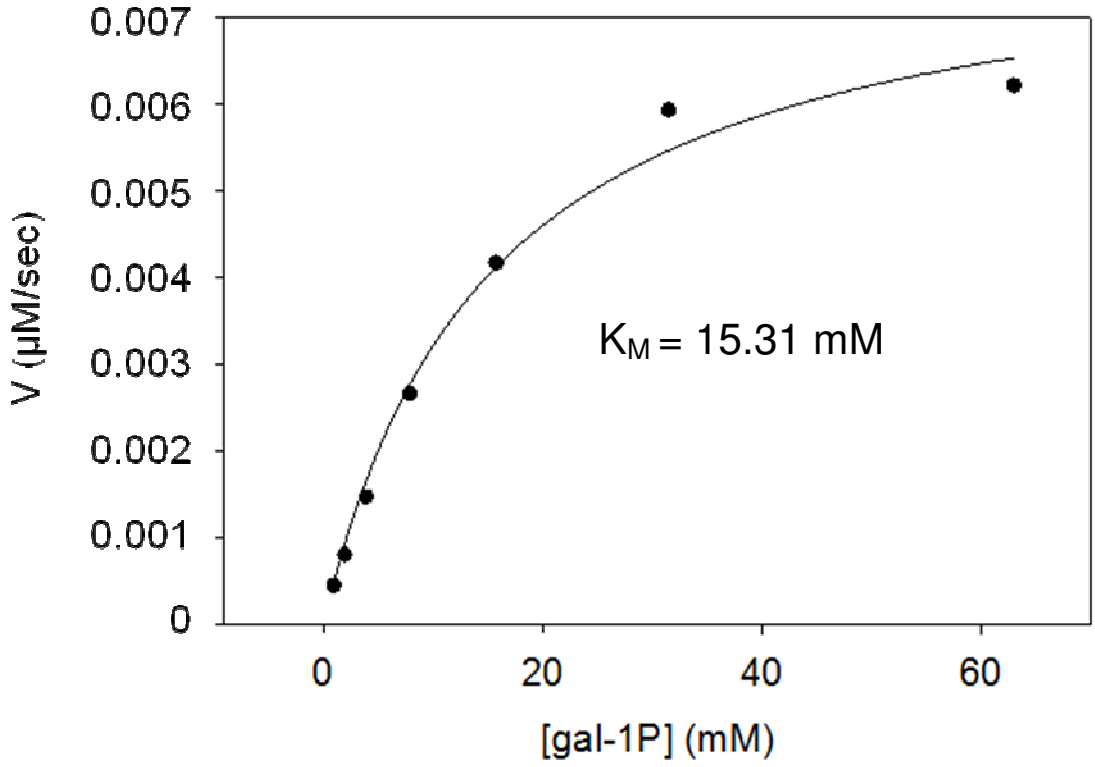


Figure A.5 hUGP K_M Determination Toward Gal-1P. Rates were calculated at gal-1P concentrations of 0.986, 1.97, 3.94, 7.89, 15.8, 31.6, and 63.1 mM. K_M was calculated using the program SigmaPlot.

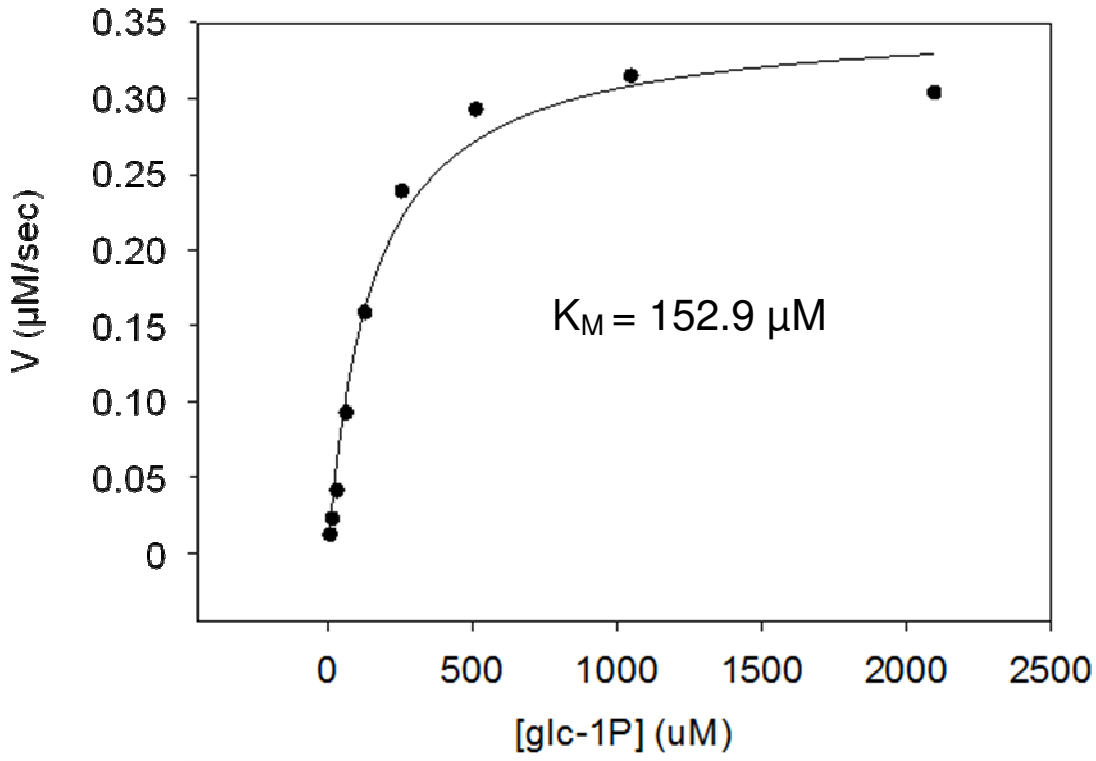


Figure A.6 hUGP K_M Determination Toward Glc-1P. Rates were calculated at glc-1P concentrations of 8, 16, 32, 64, 128, 256, 512, 1048, and 2096 µM. K_M was calculated using the program SigmaPlot.

We next performed a larger screen of the ChemDiv library, which contains approximately 30,000 compounds. Again, the Z' factors were very good, ranging from 0.64 to 0.81. The 132 compounds which gave the highest level of modulation were chosen for follow-up. These compounds imparted a 1.3 to 2.7 fold induction of UGP activity. Dose response curves were performed with compound concentrations ranging from 40 μM to 0.3125 μM in 1:2 dilutions. 11 compounds which displayed a dose response were tested in the direct assay as described in Materials and Methods. This was necessary to show that the compound was affecting UGP activity and not the activity of either of the coupling enzymes. Disappointingly, none of these compounds gave a significant improvement in UGP activity.

In testing the compounds in the direct assay, we serendipitously discovered that DMSO has an interesting effect on UGP activity. When low concentrations of DMSO were added to the coupled assay to ensure that it would not affect the assay during screening, we saw a very slight decrease in signal. However, in the direct assay, we found that DMSO increased UGP activity with respect to gal-1P **(Figure A.7 and Figure A.8A)**. In addition, preliminary data shows that DMSO appears to have the opposite effect on activity toward glc-1P, dramatically decreasing activity **(Figure A.8B)**.

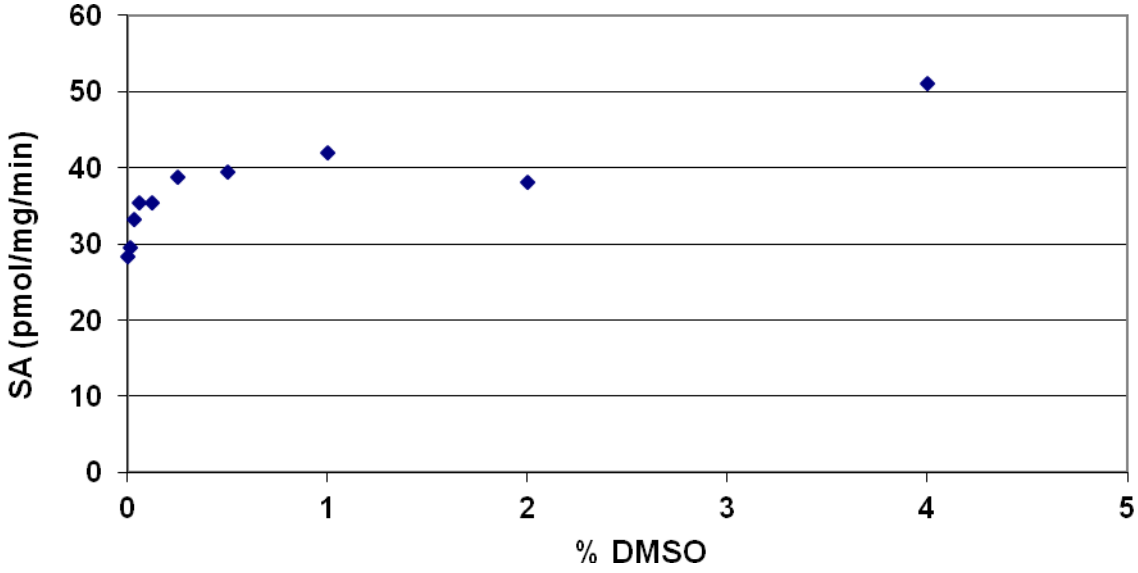


Figure A.7 DMSO increases hUGP Activity Toward Gal-1P. The effects of DMSO concentrations ranging from 4% to 0.0156% in 1:2 dilutions are shown.

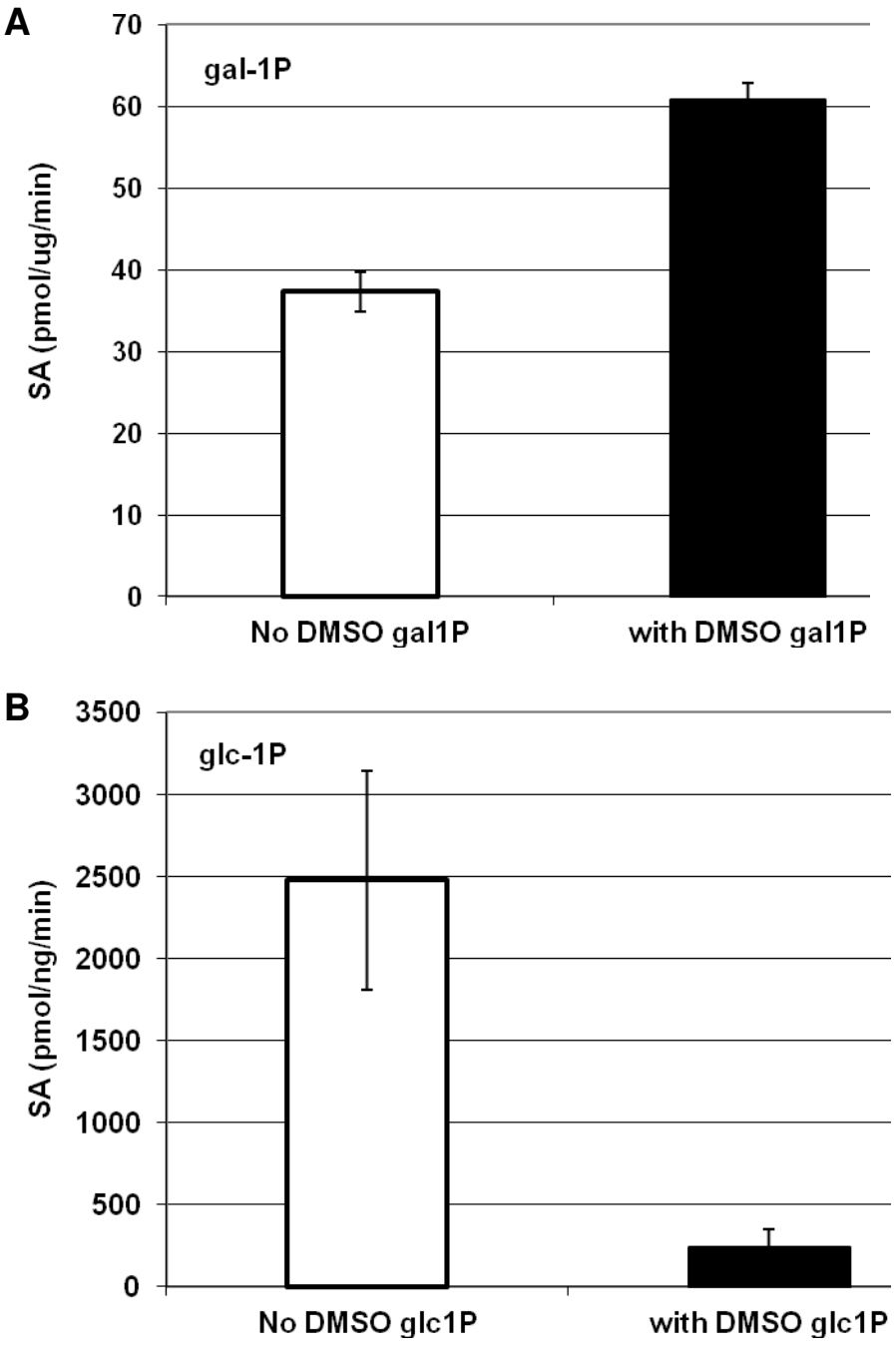


Figure A.8 DMSO Displays Opposite Effects on UGP Activity Toward Gal-1P versus Glc-1P. Preliminary data shows that DMSO increases UGP activity with respect to gal-1P (A), but dramatically decreases activity with respect to glc-1P (B). n=3 for all conditions.

A.4 Discussion

Here we describe the development of a scalable, robust assay for detecting modulators of UGP activity. While it is disappointing that we were unable to confirm any hits as positive modulators of UGP, we were able to successfully validate the assay in two pilot screens. It is not incredibly surprising that we were unable to find a modulator of UGP activity given that only 30,000 compounds were screened. It is more likely for a small molecule to interact with an enzyme in such a way that it inhibits its activity than increases activity or modulates substrate selectivity. Therefore, we believe it would be necessary to screen much larger libraries in order to find such a molecule.

We found that although DMSO had little impact on our coupled assay, it actually increased UGP activity toward gal-1P in our direct assay. We believe the explanation may be that DMSO exhibits a slightly negative effect on one or both coupling enzymes, balancing out the modest increase in activity it provides to UGP. It is interesting that UGP activity with respect to glc-1P was significantly decreased in the presence of DMSO. This result is to some extent a proof of principle that it is possible to differentially modulate UGP substrate selectivity. We are interested in determining whether this effect is caused by mild denaturation of UGP, and could test other known denaturants to investigate this.

Finally, it is of note that the crystal structure of human UGP was recently solved without a bound substrate [16]. In order to determine the structural basis for UGP substrate selectivity, it would be useful to have the structures when bound to both gal-1P and glc-1P. Comparing the active site conformations could facilitate the development of an effective therapeutic compound.

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