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Markers and Models of Outcome in Galactosemia

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Markers and Models of Outcome in Galactosemia

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ABSTRACT

Markers and Models of Outcome in Galactosemia
By Rebecca Sanders

Galactose metabolism occurs via the Leloir pathway, a highly conserved series of reactions catalyzed by three enzymes: galactokinase (GALK), galactose-1-phosphate uridylyltransferase (GALT), and UDP-galactose-4'-epimerase (GALE). When dietary galactose is insufficient, the Leloir pathway permits galactose synthesis through GALE, which interconverts UDP-galactose and UDP-glucose. In some species GALE also interconverts UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. These four UDP-sugars are essential for the biosynthesis of glycoproteins and glycolipids.

Impairment of any Leloir pathway enzyme causes galactosemia. Despite treatment with dietary galactose restriction, many galactosemics with profound GALT or GALE deficiency develop neurological complications. Additionally, 80-90% of women with severe GALT impairment develop premature ovarian insufficiency (POI). Understanding the pathophysiology of these complications has been hindered by lack of a suitable animal model. This dissertation presents a focused examination of the nature of galactosemia-associated POI, and a more general exploration of GALE deficiency using a novel *D. melanogaster* model.

Galactosemia-associated POI has been attributed to either ootoxic effects of galactose and its metabolites in the ovary, or to dysfunction of follicle-stimulating hormone (FSH) secondary to glycosylation defects. To evaluate these explanations, we tested FSH and anti-Mullerian hormone (AMH) in serum from galactosemic girls and women. FSH bioactivity, a measure of the hormone's ability to activate its receptor, was normal in our study population. However, AMH abundance, a reflection of number and quality of ovarian follicles, was strikingly low, even in girls younger than two years old. These results indicate that POI in galactosemia is not caused by ineffective FSH signaling, but by damage to the ovary in early childhood or even prenatally.

To elucidate the pathophysiology of severe GALE deficiency, we have generated the first whole animal model of GALE impairment. Our studies in *D. melanogaster* confirm that total loss of GALE is incompatible with life, and reveal that GALE is required throughout development. Furthermore, we demonstrate that GALE expression is only essential in certain tissues, and that intermediate enzyme impairment permits survival but also confers sensitivity to environmental galactose. This model of both profound and intermediate GALE-deficiency recapitulates aspects of the human disease and provides insight into the essential role of GALE in tissues.

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Chapter 1

INTRODUCTION

1.1 GALACTOSE METABOLISM

Galactose is an abundant carbon source in the diet. Together with glucose, it forms the disaccharide lactose, the primary carbohydrate in milk. As such, it is a major component of the infant diet. Free galactose is also present in fruits, vegetables, and legumes (1,2). In addition to serving as an energy source, galactose and its derivative N-acetylgalactosamine are incorporated into glycan chains that modify proteins and lipids. In the absence of sufficient dietary galactose to supply glycolipid and glycoprotein synthesis, galactose can be produced endogenously. Degradation of glycoproteins and glycolipids releases free galactose; additionally, UDP-glucose can be enzymatically converted to UDP-galactose directly.

The formation of glucose-1-phosphate from galactose occurs through the Leloir pathway (Figure 1.1), which is the chief route of galactose metabolism in the body. The Leloir pathway consists of a highly conserved series of reactions catalyzed by three enzymes. First, galactose is trapped in the cell through phosphorylation by galactokinase (GALK) in an ATP-dependent reaction (3). Next, galactose-1-phosphate (gal-1-P) and its co-substrate UDP-glucose (UDP-glc) react in a double displacement mechanism to form glucose-1-phosphate (glc-1-P) and UDP-galactose (UDP-gal) in a reaction catalyzed by galactose-1-phosphate uridylyltransferase (GALT) (4). Finally, UDP-gal is converted to

UDP-glc by UDP-galactose 4'-epimerase (GALE). This reaction, which requires NAD as a cofactor, is reversible and thereby allows for the endogenous synthesis of galactose derivatives from glucose derivatives. In some species, including humans, GALE also interconverts UDP-N-acetyl-galactosamine (UDP-galNAc) and UDP-N-acetyl-glucosamine (UDP-glcNAc) (5). These four UDP-sugars are essential substrates for glycoprotein and glycolipid production, and GALE is responsible for maintaining the proper balance of these metabolites within the cell.

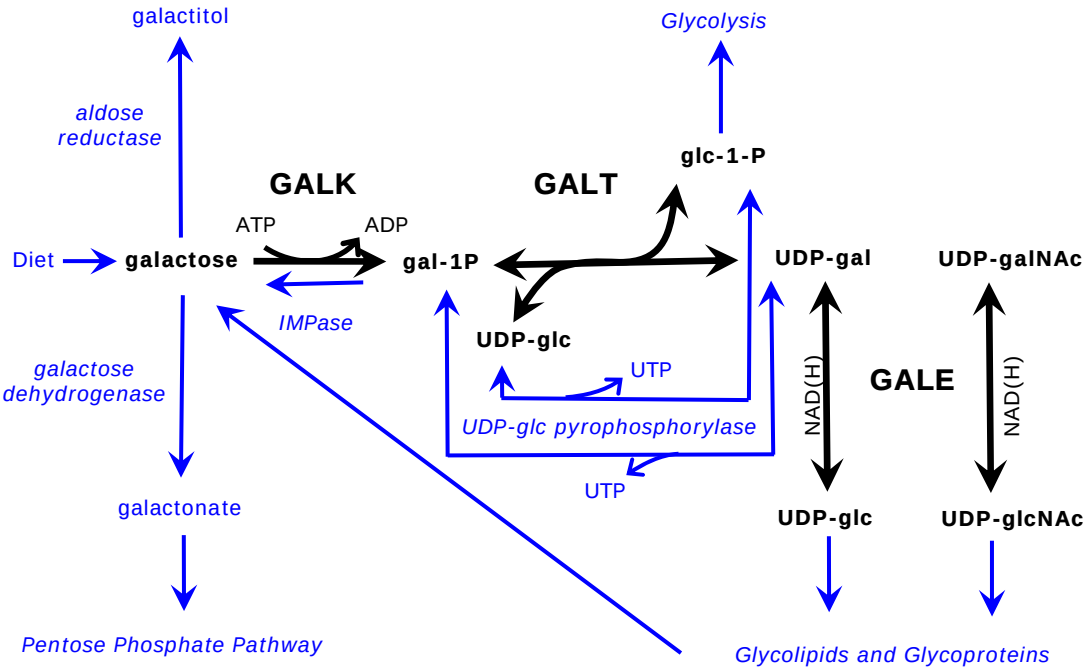

Figure 1.1 **Galactose Metabolism** The Leloir pathway is shown in black; alternative pathways of galactose metabolism are shown in blue.

If any of the Leloir enzymes are impaired, abnormal accumulation of galactose and/or its metabolites occurs. In this situation galactose is metabolized via alternate pathways, some of which can be detrimental to the cell (Figure 1.1). For example, excess accumulation of galactose can lead to the production of galactitol via aldose reductase. Galactitol cannot be metabolized further, and although it can be excreted in urine, excess production leads to a build-up in cells. In certain tissues this accumulation causes swelling from osmotic imbalance. The most frequently affected tissue is the lens of the eye, in which osmotic swelling leads to cataract formation. Additionally, buildup of galactitol in the brain can lead to pseudotumor cerebri, an elevation of intracranial pressure.

Excess galactose may also be oxidized to galactonate, either by an as yet unidentified galactose-specific dehydrogenase or by alcohol dehydrogenase (6,7). Galactonate can be cleared by the kidney or converted to D-xylulose and enter the pentose phosphate pathway (7).

Gal-1-P can also be metabolized by alternative pathways. UDP-glucose pyrophosphorylase (UGP), which normally catalyzes the formation of UDP-glucose from glc-1-P and UTP, can also form UDP-gal with gal-1-P as a substrate. This forms a functional, albeit weak, bypass of the GALT reaction, and accounts for approximately 1% of the total conversion of gal-1-P to UDP-gal (8) when the

Leloir pathway is functional. In individuals with no GALT activity, this bypass does allow for effective conversion of gal-1-P to UDP-gal (9,10). Alternately, gal-1-P can be converted back to galactose through the action of myo-inositol monophosphatase (IMPase). Of note, IMPase exhibits equal activity towards gal-1-P as towards its canonical substrates, D-1 and D-3 myo-inositol monophosphate (11).

In humans, no alternative pathway for interconversion of the UDP-sugars has been reported. Studies of galactose metabolism in the yeast *S. cerevisiae* support the idea that GALE performs a unique function. In the absence of GALE, no metabolism of galactose occurs (12); furthermore, alternative routes of gal-1-P metabolism cannot operate (13).

1.2 GALACTOSEMIA

Impairment in enzymes of the Leloir pathway causes the galactosemias, a group of autosomal recessive metabolic disorders characterized by elevated levels of galactose and its metabolites in the blood and tissues. Severity of the disease depends on the affected enzyme and the degree of impairment. The only available treatment is restriction of dietary galactose, and even with strict dietary control some galactosemics still accumulate abnormal levels of galactose metabolites.

GALK Deficiency

GALK deficiency prevents initiation of galactose metabolism by the Leloir pathway, promoting accumulation of galactose, galactitol and galactonate. First described in 1965 (14), GALK-deficiency galactosemia is rare, although incidence varies widely due to enriched carrier frequency in some communities. Studies of United States populations estimate affected frequencies between 1:50,000 and 1:1,000,000 (15), but in the European Roma Gypsy population incidence may be as high as 1:10,000 (16). The chief negative outcome in GALK-deficient individuals is bilateral neonatal cataracts (15); intermediate enzyme deficiency (such as heterozygosity for an allele conferring profound impairment) may also place individuals at higher risk for developing cataracts (17). In two cases, GALK-deficient infants have developed pseudotumor cerebri (18,19). The pathogenesis of both cataracts and elevated intracranial pressure is linked to galactitol overload. These symptoms resolve with prompt institution of a galactose-restricted diet, and with good dietary management no further complications arise.

GALT deficiency

GALT deficiency galactosemia is a disorder with marked phenotypic and allelic heterogeneity; at least 207 different mutations in the GALT gene have been reported (20). Generally, mutations that preserve some level of residual GALT

activity are associated with less severe disease and better prognosis than mutations with undetectable activity. At opposite ends of the spectrum of GALT-deficiency galactosemia are Duarte galactosemia, in which approximately 25% of wild-type activity is present, and classical galactosemia, in which less than 1% residual GALT activity is retained. Patients with varying levels of activity between 1 and 25% also exist and are said to have variant galactosemia. Because of differences in GALT activity assay sensitivities and normal ranges across locales, distinctions between classical and variant galactosemia are sometimes blurred.

Duarte Galactosemia: Duarte galactosemia is caused by compound heterozygosity for a specific GALT allele, D2, sometimes called D, and any GALT allele that results in profound loss of GALT activity, often abbreviated G. The D2 allele carries a 4 base pair deletion in the GALT promoter region (21) that is associated with approximately 50% reduction in enzyme expression (22); therefore individuals with one D2 allele and one severe GALT mutation, referred to as a DG genotype, have only 25% residual GALT activity. This relatively moderate impairment is not associated with severe galactose toxicity and may be benign (23). Some Duarte galactosemics are treated with galactose restriction for all or part of the first year of life and some ingest a normal diet from birth; little is known about the consequences of treatment or failure to treat. However, Duarte

galactosemic infants who are not on galactose restriction do accumulate excess gal-1-P, galactonate and galactitol (24) and children with Duarte galactosemia also have elevations in blood levels of galactose and gal-1-P after galactose challenge (25). The one study comparing outcomes in treated and untreated children with Duarte galactosemia showed no significant improvements in language or cognitive development in the galactose-restricted group (26).

Classical Galactosemia: Severe GALT impairment, known as classical galactosemia, is the most common clinically significant form of the galactosemias and has an estimated incidence of approximately 1:47,000 (27). Because the primary route of conversion to UDP-gal is blocked by loss of GALT activity, classical galactosemics accumulate high levels of gal-1-P; similar to GALK deficiency, they also accumulate excess galactose, galactitol and galactonate (28).

Classical galactosemics may also have a *deficit* of UDP-gal, although there have been conflicting reports on this point (98). If UDP-gal depletion does occur, it is most likely a result of galactose-restriction and not a direct result of GALT enzyme dysfunction; non-galactosemics on lactose-restricted diets have similarly low levels of UDP-gal (29). Indeed, classical galactosemics can metabolize orally administered galactose to UDP-gal, albeit more slowly than metabolically normal controls (10). Gal-1-P to UDP-gal conversion in galactosemics is most likely through the UGP bypass pathway, since UDP-gal formation has even been

observed in an individual homozygous for a deletion removing more than 90% of the GALT coding sequence (9).

Early complications of classical galactosemia arise in the first days to weeks of life. The first symptoms may be non-specific and include vomiting, diarrhea, lethargy, and hypotonia. Symptoms can escalate to failure to thrive, jaundice, hepatosplenomegaly, bleeding diathesis and *E. coli* sepsis; affected infants may also develop cataracts or pseudotumor cerebri. Without treatment the disorder can be fatal; instigation of a galactose-restricted diet is necessary to reverse the acute, life-threatening symptoms of the disease. Even with strict dietary control classic galactosemics confront a panoply of adverse long-term outcomes: approximately 30% display cognitive deficits (30), approximately 60% have verbal dyspraxia (31), and approximately 20% develop ataxia and tremor (32). Furthermore, 80-90% of female patients experience primary or premature ovarian insufficiency (POI), which ranges from failure to develop secondary sexual characteristics to early menopause (30,32,33).

The mechanisms responsible for either the acute or chronic complications of GALT-deficiency are not known. Furthermore, there is no explanation for why some classic galactosemics develop certain complications while others do not. Although differences in residual GALT activity may partially account for differences in clinical severity, other factors must influence disease outcomes.

Phenotypic heterogeneity is present among individuals with the same degree of GALT impairment, and even among individuals with the same genotype (30,34). A likely explanation of phenotypic variability concerns the degree to which galactose metabolite levels accrue in a particular individual, and the response of that individual's body to a given metabolite.

Even assuming universal adherence to a galactose-restricted diet, other factors can produce individual variation in metabolite buildup. GALT does not act alone to metabolize galactose, but functions within a pathway alongside two other enzymes. Moreover, galactose metabolites can be acted on by enzymes of other pathways. Differences in GALK activity might directly impact the extent of gal-1-P accumulation; similarly, variation in GALE activity might mitigate or exacerbate insufficiencies of UDP-gal production. Relative levels of IMPase and UGP, as well as their relative affinities for gal-1-P as an alternative substrate, may influence how much gal-1-P is converted to galactose and how much is converted to UDP-gal. Finally, the extent of endogenous galactose production or "self-intoxication" by GALE may directly affect levels of galactose metabolites. Both classic galactosemics and metabolically normal controls synthesize gram quantities of galactose each day (35,36). In galactosemics, endogenous galactose production is age-dependent, and newborns synthesize galactose at an approximately three-fold higher rate than adults (37).

GALE Deficiency

GALE deficiency was originally described as a benign biochemical oddity affecting only circulating blood cells. In the first reported cases, patients were described as having impaired GALE activity in erythrocytes and elevated hemolysate levels of gal-1-P; these individuals did not have symptoms of galactosemia on a lactose-containing diet and demonstrated normal levels of GALE activity in other tissues (38).

The view of GALE deficiency as a clinically insignificant disorder was refuted by the discovery of individuals with severe generalized GALE deficiency. The first report of a child with profound impairment in GALE was made in 1981 (39); since then, a handful of other cases have been described (40,41). Each of these individuals, all offspring of consanguineous pairings, is homozygous for the amino acid substitution V94M. When expressed in yeast the V94M GALE mutant allele is associated with approximately 5% residual activity towards UDP-gal, and 24% residual activity towards UDP-galNAc (42); cultured fibroblasts from one patient homozygous for V94M showed residual activity of 1.8% toward UDP-gal and 11.6% toward UDP-galNAc (43).

The clinical picture of the acute phase of generalized GALE deficiency is very similar to GALT deficiency. In infancy and on a milk-based diet, affected individuals presented with vomiting, jaundice, weight loss, hypotonia, and

hepatosplenomegaly; one developed cataracts and *E. coli* sepsis. These symptoms were accompanied by biochemical abnormalities: patients had elevated levels of gal-1-P and UDP-gal (39,40,41). Later in life these individuals all suffered from learning disabilities or mental retardation, and short stature, other common complications were dysmorphic features and sensorineural deafness. Of note, the few affected females have spontaneous puberty and apparently normal ovarian function (41,44). Because of consanguinity, the possibility that some or all of the phenotypes observed in these children are caused by homozygosity for other deleterious mutations unrelated to galactose metabolism cannot be ruled out. However, in at least one of the families a sibling without GALE deficiency displays normal development (44).

Once these patients were reported, the view of GALE-deficiency shifted to a binary model: in exceedingly rare cases, patients could be affected by a severe, generalized form of the disorder caused by homozygosity for the V94M allele, all other cases were classified as a peripheral, benign form of galactosemia.

Reports of individuals with presumedly peripheral GALE deficiency who nevertheless displayed poor outcomes have challenged the prevailing tendency to dichotomize GALE deficiency as either benign/peripheral or severe/generalized disease. One child who was assumed to have peripheral disease because he was clinically well as a newborn subsequently developed

gross motor and language delay on a diet with no galactose restriction (45). He was later determined to have approximately 15% residual GALE activity in non-peripheral cells (46). In another case, a newborn with ostensibly peripheral disease developed vomiting, diarrhea, jaundice and hypoglycemia that resolved upon initiation of galactose restriction, erythrocyte GALE was measured in the low-normal range (47). Supposedly peripheral GALE deficiency has also been implicated as a potential cause of unclassified myelodysplasia in one child; the authors speculate that impaired GALE activity in her hematopoietic cells led to inadequate glycosylation of hematopoietic hormones or their receptors, which in turn caused a defect in platelet and white cell production (48). These case reports indicate that peripheral deficiency may be neither isolated to circulating blood cells nor benign.

Comparisons within small groups of individuals with presumedly peripheral disease have highlighted the inadequacy of erythrocyte or fibroblast activity studies in distinguishing benign and clinically relevant disease across a spectrum of GALE deficiency. In a group of patients with juvenile cataracts, GALE from red blood cells or fibroblasts was impaired to varying degrees, but was uniformly severely deficient in the lens of the eye. Some of these individuals also had hearing impairment and cognitive or speech delay (49). Studies of tissues and transformed lymphoblasts from GALE-deficient individuals

provided further evidence for a clinical spectrum of disease. Lymphoblast cell lines (a non-peripheral tissue) derived from patients with ostensibly peripheral deficiency had varying levels of GALE impairment; the level of residual activity in these cells was inversely correlated with accumulation of galactose metabolites in culture. Many of the individuals from whom these cells were derived also had abnormally elevated erythrocyte gal-1-P measurements. These results did not correlate with the amount of erythrocyte GALE activity reported for each individual (50).

The broad range of outcomes now recognized to form the GALE-deficiency disease spectrum should not be surprising. Degree of enzyme impairment, relative activity towards each substrate, and variability in residual activity across tissues provides a fertile ground for clinical heterogeneity.

The example of peripheral deficiency demonstrates that measures from an isolated tissue or a single-cell cannot be extrapolated to describe deficiencies and their potential impact within the entire body; however, it is important to note that while low, GALE activity is detectable in even the most severely affected individuals. This finding contrasts sharply with observations of classical galactosemia, in which most patients with severe disease have undetectable GALT activity. Whether or not the cell is able to metabolize galactose for energy, galactose is an essential component of glycoproteins and glycolipids that are

critical for proper cellular function. GALE is responsible for *de novo* production of galactose in the absence of sufficient dietary intake and no alternate pathway exists; therefore, it has been speculated that total loss of GALE would be incompatible with life (51).

1.3 EXPERIMENTAL MODELS OF GALACTOSEMIA

Yeast

The pathways of galactose metabolism were first elucidated in yeast (52,53) and have since contributed greatly to our understanding of the biochemical consequences of Leloir enzyme impairment. In the baker's yeast *S. cerevisiae*, the inability of strains lacking GALK, GALT or GALE to metabolize galactose was first described 45 years ago (54). Since then, yeast have been instrumental in defining the relationship between enzyme impairment and metabolite accumulation. GALT and GALE null yeast exhibit galactose-sensitive growth impairment (12,54) that correlates directly with accumulation of gal-1-P; GALE-null yeast also accumulate high UDP-gal relative to UDP-glc (12,55). Interestingly, GALE-impaired yeast are 10-fold more sensitive to environmental galactose (12) than their GALT-null counterparts, and require a higher threshold of residual GALE activity to grow normally in galactose supplemented media (56). Yeast have also allowed us to explore possible modifiers of these biochemical phenotypes; a genetic screen of GALT-null yeast first identified loss

of GALK and over-expression of IMPase and UGP as potential suppressors of galactose sensitivity (13,54).

Moreover, expression of human GALT or GALE can complement deletions of the respective yeast homologs (46,57), allowing for studies of patient alleles in an easily manipulable system. Yeast human enzyme expression studies have permitted the functional characterization of novel patient alleles associated with both GALT (58,59) and GALE deficiency (42,46,60,61). This ability to "humanize" yeast has also made it possible to model the *in vivo* impact of combinations of known patient mutations. Because of the marked allelic heterogeneity of galactosemia, affected individuals are often compound heterozygotes. Co-expression of two different GALT alleles in a single strain of yeast has allowed for better understanding of the interaction between mutant alleles, particularly their impact on enzyme dimer formation and function (62,63,64).

CHO cells

Although yeast have been instrumental in exploring functional impairment of mutant alleles and the metabolic consequences of enzyme loss, they are not a good model for the effects of Leloir pathway impairment on glycan synthesis. This particularly limits the application of yeast to the study of GALE disease pathogenesis. Unlike human GALE, yeast GALE does not interconvert

UDP-galNAC and UDP-glcNAc (65) and they do not use galNAc in the synthesis of glycoproteins and glycolipids (66). Discovery of a Chinese hamster ovary (CHO) cell line, *ldlD*, that is completely lacking in GALE activity toward either UDP-gal or UDP-galNAc (67) has aided our understanding of the role of GALE in glycosylation reactions. This cell line is the only reported mammalian model of total GALE deficiency.

ldlD cells have defects in both N- and O- linked glycosylation; these defects can be rescued with low-level supplementation of galactose and galNAc (43,67). However, increasing levels of galactose exposure lead to growth arrest and accumulation of both gal-1-P and UDP-gal. This galactose sensitivity can be rescued by either uridine supplementation--suggesting that metabolite accumulation creates a UTP sink--or by expression of human GALE (68).

Mice

Though yeast and CHO cells have contributed to our understanding of biochemical and growth phenotypes associated with galactosemia, they cannot be used to understand the impact of enzyme impairment in complex tissues or in developmental processes. In the hope of better understanding the pleiotropic effects of galactosemia, GALK and GALT knock-out mice have been created (69,70). Unfortunately, neither model fully recapitulates the human disease phenotypes observed in GALK or GALT deficiency.

The GALK-null mouse accumulates galactitol in liver, brain and kidney, especially after galactose exposure, but does not develop cataracts without concomitant over-expression of aldose reductase in the lens (69). The GALT-null mouse model is even more disappointing. Despite accumulation of galactose, galactonate, galactitol and gal-1-P, the mice are healthy, fertile, and have no neurological deficits (70,71). Consequently, the knock-out mouse has been of little use in examining the effects of GALT impairment in organ systems. This model may be most useful in one day explicating the biological differences between mice and humans that permit GALT-deficient mice to escape the negative outcomes experienced by classic galactosemics.

No GALE knock-out mouse--nor any other whole animal model of GALE-deficiency--has been reported in the literature. In light of GALE's unique cellular role in maintenance of the UDP-sugar pools and the speculation that complete absence of GALE would be incompatible with life, it is possible that a GALE knock-out mouse could not be created.

1.4 THE PATHOPHYSIOLOGY OF GALACTOSEMIA

The lack of a suitable animal model has hampered our understanding of galactosemia pathophysiology. Only GALK deficiency, which has limited symptoms and a mild clinical course, is fully understood. As discussed above,

the only adverse effects of GALK deficiency are cataracts, and quite rarely, pseudotumor cerebri; the cause of both symptoms is easily explained as osmotic swelling from galactitol accumulation. These symptoms can also be present in other forms of galactosemia, but both GALT and GALE-deficiency are accompanied by a host of additional symptoms of unknown pathophysiology. The remainder of this section focuses on the pathophysiology of GALT and GALE deficiency galactosemia.

Although the underlying mechanisms of disease pathogenesis in GALT- or GALE-deficiency galactosemia are not well understood, observations from galactosemic patients and from various experimental models have given rise to several possible hypotheses. Proposed mechanisms of disease pathology fall into three broad categories. First, accumulated galactose metabolites constitute a "sink" for precious resources, sequestering molecules that are essential for proper cellular function. Second, at high levels galactose metabolites may competitively inhibit enzymes that catalyze essential reactions. And third, that in the presence of excess gal-1-P and altered levels of the UDP-sugars, proteins and lipids are aberrantly glycosylated to their functional detriment.

Depletion of ATP and UTP

Given the comparatively mild phenotypes observed in GALK-deficiency, in which galactose, galactitol and galactonate all accumulate but gal-1-P does

not, it seems likely that elevated gal-1-P levels are directly tied to the pathogenesis of GALT and GALE deficiency. Although no definitive causal link between gal-1-P and galactose toxicity has been established, a number of hypotheses have been put forth. Inability to efficiently metabolize gal-1-P and its subsequent buildup may initiate a futile phosphorylation/dephosphorylation cycle which depletes the cell of ATP (72); this mechanism might be especially detrimental to tissues with high metabolic demand, such as the brain and nervous system.

In both GALE-deficient patients and in the GALE-null CHO cell line *ldlD*, galactose exposure produces abnormally high levels of UDP-gal relative to UDP-glc. Human GALE normally maintains a UDP-glc:UDP-gal ratio of 3:1 (68,73); this inability to interconvert the UDP sugars can deplete the cell of UTP. Studies of *ldlD* cells have demonstrated that uridine supplementation restores UTP and UDP-glc levels. This treatment relieves galactose induced growth arrest without any reduction of elevated gal-1-P or UDP-gal levels (68), suggesting that the primary mode of galactose toxicity, at least for CHO cells, is UTP depletion.

Enzyme Inhibition

As an alternate substrate for IMPase, gal-1-P may indirectly deplete the cell of inositol by competitively inhibiting the formation of inositol from inositol monophosphate. Since IMPase has equal activity toward gal-1-P and its

traditional substrates, excessive amounts of gal-1-P may deprive the brain and other tissues of inositol (11,13), thereby disrupting inositol-mediated signaling cascades. Inositol depletion has been reported in the brains of infants with classical galactosemia in conjunction with elevated galactitol (74,75); by promoting osmotic swelling, galactitol accumulation may further dilute inositol concentrations that are already low.

Elevated galactitol and gal-1-P levels may also act synergistically to inhibit enzymes. In *S. cerevisiae*, the presence of both metabolites has an inhibitory effect on phosphoglucomutase (76), which converts glc-1-P to glc-6-P, allowing it to enter glycolysis. Both metabolites also inhibit the Na^+-K^+-ATPase in rat brain; inactivation of this ion transporter causes edema and cell death (77).

Aberrant Glycosylation of Proteins and Lipids

High gal-1-P may also interfere with galactosyltransferases, perturbing normal glycosylation reactions essential to protein processing (73). Alterations in levels of the UDP-sugars are implicated in this aspect of disease pathogenesis as well. Cultured human GALT-deficient fibroblasts are not only UDP-gal depleted (78,79), but also produce hypogalactosylated proteins when exposed to galactose (80). Since UDP-gal is a key sugar donor for lipid and protein glycosylation, its depletion in classical galactosemia may account for the aberrant glycosylation observed in the disease. In human galactosemic samples, glycosylation defects

include reduced incorporation of galactosyl and galactosamine residues and loss of terminal galactose-linked sialic acids. These defects are observed in a variety of sample types: glycoproteins and glycolipids from galactosemic brain tissue (81,82), serum transferrin (83,84,85) the serum lysosomal enzymes β-hexosaminidase and α-fucosidase (86), and in circulating isoforms of the glycoprotein follicle-stimulating hormone (87).

Although galactosylation has not been extensively studied in GALE-impaired humans, glycoprotein and glycolipid synthesis are most likely deranged in these individuals as well. Similar to classical galactosemics, they have elevated levels of gal-1-P; moreover, their GALE impairment results in a compromised ability to regulate UDP-sugar pools. Studies in *ldlD* cells support this assumption; this cell line was first identified by its non-functional ldl receptor, a defect that was traced back to a deficiency in N- and O-linked glycosylation due to loss of GALE (43,67).

Damage to Specific Tissues

The two most common adverse outcomes in galactosemia are neurological complications, which are present for both GALT and GALE deficiency, and POI, which is only reported in classical galactosemia.

Imaging studies have shown diffuse white matter abnormalities in the brain and nervous tissue of galactosemics; one study found that greater than 90%

of the individuals studied had changes in white matter (88). This common finding may be due to defective myelin production secondary to reduced glycoprotein and galactosphingolipid synthesis (89). Autopsies in two severely affected individuals have also shown necrotic foci and neuronal loss (81,90) which may be due to the various cellular insults from metabolite accumulation described above.

In females with classic galactosemia, POI is the most common complication. Classic galactosemics produce some galactose-deficient follicle-stimulating hormone (87); it has been speculated that these less acidic isoforms may be unable to efficiently activate the FSH receptor in the ovary (87,91). An alternative explanation is that POI arises within the ovary itself, resulting from galactose toxicity to the ovary or developing oocytes (92,93), and/or from reduced glycoprotein or glycolipid content (94).

Discouragingly, early initiation of galactose restriction has not reduced the incidence of neurological and reproductive complications in patients with classical galactosemia (30,31,33,34). This finding suggests that the mechanisms of long-term complication are developmental in nature, and are initiated in the prenatal or perinatal period, before galactose restriction is initiated. The Leloir enzymes are present in the fetal liver as early as 10 weeks gestation (95), allowing ample opportunity for endogenous galactose production and self-intoxication *in*

utero. Galactose, galactitol and gal-1-P all accumulate in galactosemic fetuses (95), most likely from *de novo* galactose synthesis; even with maternal abstinence from milk ingestion during pregnancy, gal-1-P is present in cord blood (96) and galactitol is measurable in amniotic fluid (97) of galactosemic infants.

1.5 SUMMARY

The specific effects of impaired galactose metabolism in tissues and their consequences in the body as a whole are not well understood. Poor outcomes in galactosemia are most commonly associated with its theorized affects on the nervous and reproductive systems; obtaining samples of these tissues from individuals with the disease is clearly not possible, and there is no animal model that fully parallels the human phenotypes. Progress toward understanding the cellular mechanisms by which organ damage occurs in galactosemia has been hindered by lack of a suitable animal model, and by inadequate studies of humans affected by the disease. These lacunae further prohibit the formulation of effective diagnosis, treatment and prevention methods relating to acute and chronic complications of the disease. In particular, the prenatal and perinatal effects of galactose exposure and their contribution to disease complications are not well examined. This dissertation addresses issues related to adverse outcomes in both GALT and GALE deficiency galactosemia.

Chapter 2 is a focused examination of the possible causes of galactosemia-related POI, which is exclusively associated with GALT deficiency. POI is the most common long-term complication in women with classic galactosemia. The predominance of POI in the galactosemic population has been attributed to either ootoxic effects of galactose and its metabolites in the ovary, or to dysfunction of FSH secondary to glycosylation defects. Without knowledge of the underlying cause of ovarian insufficiency in these women, effective treatment or prevention strategies cannot be developed.

Measurements of FSH and anti-Mullerian hormone (AMH) in serum from girls and women with classical galactosemia and metabolically normal age-matched controls were used to examine the causes of galactosemia-associated POI. The bioactivity of FSH, the ability to bind and activate its receptor, did not significantly differ between galactosemics and controls. AMH levels, which depend upon the number and maturation status of follicles within the ovary, was abnormally low in >90% of galactosemic girls and women in this study, including girls less than 2 years old.

These results provide evidence that severe GALT deficiency has direct negative consequences within the ovary, and discount the hypothesis that ineffective FSH signaling is responsible for POI. Moreover, the early age at which diminished follicular function could be observed in galactosemic girls

suggests that damage to the ovary occurs early in life, perhaps even before birth. This finding supports the hypothesis that the long-term complications of galactosemia have their root in early development.

Chapter 3 is a more general exploration of adverse outcomes associated with GALE deficiency, using the fruit fly *Drosophila melanogaster* as a model. Observations from individuals with intermediate and severe impairment raise many questions about both GALE deficiency disease pathogenesis and the enzyme's role in healthy tissue: How much GALE activity is enough? In what tissues is adequate GALE function most critical? What cellular processes require GALE? Is activity towards one substrate more important than the other, and if so, in what contexts?

A first step toward answering these questions has been the creation and characterization of the first whole animal model of GALE deficiency, detailed in Chapter 3. Work described here addresses the consequences of GALE loss and the timing and location of requirement for GALE. Complete loss of GALE is, as previously theorized, incompatible with life, and moderate impairment renders the animals sensitive to environmental galactose. The minimal threshold of GALE activity required for survival is between four and eight percent of wild-type activity levels. Animals lacking endogenous GALE can be rescued by

expression of a human transgene, and expression in the gut and Malpighian tubule is necessary and sufficient for survival.

This novel model of GALE deficiency confirms the essential role of GALE during development and in specific tissues, and lays the groundwork for further exploration of the role of GALE in both physiological and pathological processes.

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Chapter 2

BIOMARKERS OF OVARIAN FUNCTION IN GIRLS AND WOMEN WITH CLASSIC GALACTOSEMIA

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2.1 ABSTRACT

Capsule: Ovarian insufficiency in classic galactosemia is demonstrated by decreased AMH levels and is evident by early childhood; the cause appears to be direct ovarian toxicity rather than abnormal FSH function.

Study objective: To determine whether premature ovarian insufficiency (POI) associated with classic galactosemia results from a true deficiency of ovarian function or from aberrant follicle stimulating hormone (FSH).

Design: Cross-sectional study

Setting: University research laboratory

Patients or other participants: Study subjects included 35 girls and women with galactosemia and 43 control girls and women between the ages of <1 yrs to 51 yrs.

Interventions: Blood sampling and medical and reproductive histories were obtained.

Main outcome measures: We determined FSH and anti-Müllerian hormone (AMH) levels in subjects with and without classic galactosemia. FSH bioactivity was measured in a subset of girls and women with and without galactosemia who were not on hormone therapy.

Results: FSH levels were significantly higher and AMH levels were significantly lower in our galactosemic cases relative to controls. FSH bioactivity did not significantly differ between cases and controls.

Conclusions: Close to 90% of girls and women with classic galactosemia have a profound absence of ovarian function, a deficit which is evident shortly after birth, if not before. These patients have no evidence of abnormally functioning FSH. AMH levels can be assessed prior to menarche or after initiation of hormone therapy and may supplement FSH as a useful blood biomarker of ovarian function for patients with classic galactosemia.

Key Words: galactosemia, ovarian insufficiency, AMH, FSH, FSH bioactivity

2.2 INTRODUCTION

Classic galactosemia is an autosomal recessive disorder which results from profound impairment of galactose-1-phosphate uridylyltransferase (GALT) (reviewed in (1)), the second enzyme in the Leloir pathway of galactose metabolism (Figure 2.1). Classic galactosemia affects ~1:47,000 newborns (2). In the first weeks of life, untreated infants develop vomiting, jaundice, hepatosplenomegaly and *E. coli* sepsis. These life-threatening symptoms can be reversed or prevented by dietary restriction of galactose (reviewed in (1)).

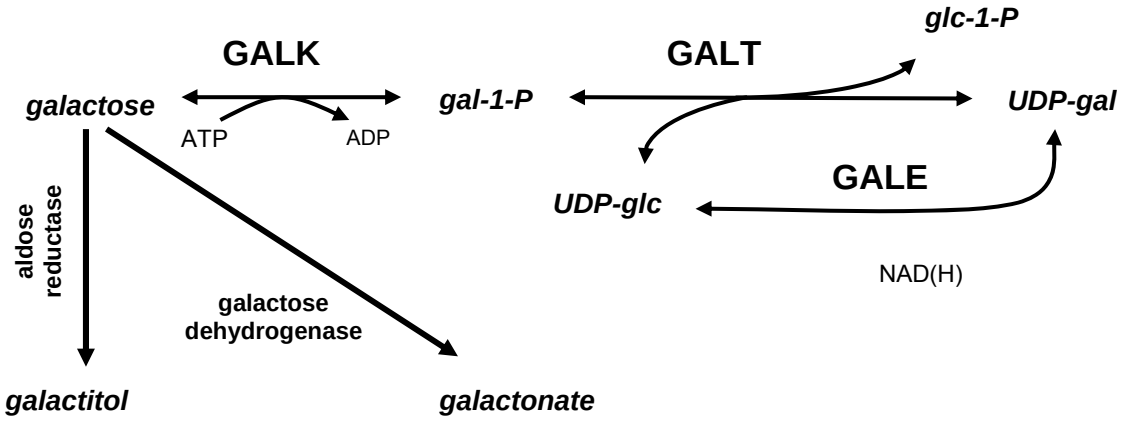

Figure 2.1 **The Leloir pathway of normal galactose metabolism**. Classic galactosemia is caused by profound impairment of galactose-1-phosphate uridylyltransferase (GALT) leading to an increased accumulation of galactose, gal-1-P, galactitol and galactonate, and perhaps a depletion of UDP-gal and/or UDP-glc

However, despite neonatal diagnosis and careful dietary management, many patients experience serious long-term complications which can include cognitive, motor, and speech delays. Further, >80% of girls and women with classic

galactosemia experience premature ovarian insufficiency (POI (3)) which can range in severity from primary complete gonadal failure without the development of secondary sex characteristics to premature menopause (4-6). Girls with this disorder may require hormone therapy to complete puberty, and quality-of-life studies indicate that infertility is a significant cause of concern for galactosemic girls and their parents (7).

The developmental timing and underlying cause of galactosemia-associated POI remain unknown. The presumed etiology involves abnormal accumulation of galactose metabolites both pre- and postnatally, although the target and pathway of toxicity remain unclear (reviewed in (1)). Anecdotal studies of patients have offered some insight, although the small numbers of patients studied and the recognized variability of outcome among different patients confound sweeping conclusions. For example, Levy and colleagues (8) reported finding apparently normal folliculogenesis and abundant oocytes in an ovary from a 5 day old infant who died from complications of galactosemia. In contrast, Kaufman and colleagues (9) reported patients between the ages of 9-29 years who demonstrated decreased or completely absent ovarian tissue with pelvic ultrasonography. Similarly, Fraser and colleagues (10) reported that ovarian biopsies from two sisters with galactosemia demonstrated true ovarian failure in one sister and resistant ovary syndrome, defined by the presence of

unresponsive primordial follicles, in the other. These authors hypothesized that resistant ovary syndrome might precede true ovarian failure in patients with galactosemia. Another ostensible case of resistant ovary syndrome was reported by Twigg and colleagues (11), who described a patient treated with hormone replacement early in puberty; this young woman later experienced a series of menstrual cycles even after hormone replacement was terminated.

Studies in animal models also support a link between galactose metabolism and ovarian toxicity. For example, adult female rats fed a high galactose diet have diminished follicular development (12), and galactose exposure *in utero* both inhibits germ cell migration to the developing ovary (13) and reduces oocyte pools (14). In contrast, a GALT knock-out mouse (15) has no apparent reproductive compromise, even when challenged with a high galactose diet (16). The basis for this apparent galactose-*sensitivity* of human and rat ovaries, in contrast to the apparent galactose-*insensitivity* of mouse ovaries, remains unclear.

Aside from its role in energy metabolism, the Leloir pathway is also responsible for regulating the supply of UDP sugars necessary for post-translational glycosylation of proteins and lipids; not surprisingly, patients with classic galactosemia exhibit glycosylation defects (reviewed in (1)). Glycosylation defects reported from studies of patient samples include reduced

incorporation of galactosyl and galactosamine residues and loss of terminal galactose-linked sialic acid. These defects have been observed in a variety of sample types, including glycoproteins and glycolipids from post-mortem galactosemic brain tissue (17, 18), serum transferrin (19, 20), and the serum lysosomal enzymes β-hexosaminidase and α-fucosidase (21).

Circulating follicle stimulating hormone (FSH) is also partially abnormally glycosylated in women with classic galactosemia (22). The glycosylation state of FSH is directly linked to its bioactivity *in vivo*, as well as to its rate of clearance, potency, and receptor binding and activation; hypoglycosylated isoforms of FSH may even act as antagonists (23). This observation raises the possibility that POI in women with classic galactosemia may arise from aberrant FSH function rather than from direct ovarian incompetence. In 2004, a case report described a woman with galactosemia-associated POI who successfully conceived after treatment with recombinant FSH (24). While intriguing, however, this result is difficult to generalize; recombinant FSH may have succeeded in this case by superovulating residual follicles rather than by replacing the patient's aberrant endogenous FSH.

Traditionally, studies of serum FSH levels have been used to predict ovarian insufficiency in galactosemic girls (reviewed in (1)), but this practice does not provide direct information about follicular function. Elevated FSH

levels can indicate either depressed gonadal function or intrinsic hormone/receptor dysfunction. In addition, GnRH pulsatility may not yet be mature in pre-pubertal girls, further confounding interpretation of FSH levels.

In contrast, anti-Müllerian hormone (AMH), which plays an important role in folliculogenesis (25), is produced by the granulosa cells of preantral and small antral follicles of the ovary and levels are relatively stable across the menstrual cycle (26-28). AMH levels are also largely unaffected by oral contraceptives (29) or gonadotropin treatment (30), making AMH a particularly useful marker for patients on hormone therapy, as are many pubertal or post-pubertal girls and women with classic galactosemia.

To distinguish between true gonadal insufficiency and aberrant FSH function in galactosemia we measured serum and/or plasma levels of AMH and FSH in 35 girls and women with classic galactosemia and in 43 age-matched controls; we also assessed FSH bioactivity in a subset of these study subjects. As predicted from the frequency of POI among galactosemic patients, only 2 of the 35 patients tested demonstrated normal levels of AMH; one additional patient demonstrated near-normal AMH. Further, while many patients demonstrated abnormally elevated levels of FSH, the bioactivity of that FSH remained normal. This result is consistent with an earlier report by Kaufman and colleagues (9) who applied an *in vivo* mouse method to test the function of urinary

gonadotropins from 18 girls and women with galactosemia; no abnormality was detected in those samples. Combined, these results strongly favor the "direct ovarian toxicity" hypothesis of ovarian insufficiency in galactosemia and suggest that studies of AMH should supplement studies of FSH as measures of ovarian function in girls and women with classic galactosemia.

2.3 MATERIALS AND METHODS

Subjects

Girls and women between the ages of <1 to 46 years with classic galactosemia confirmed by biochemical and/or mutational analyses were enrolled in this study. Participation was solicited at the 2006 Parents of Galactosemic Children (PGC) Conference in Philadelphia, PA, by announcements posted online on the PGC website (www.galactosemia.org) and a discussion forum devoted to galactosemia (www.galactosemics.org), and through referral of patients from metabolic disorders clinics.

Adult controls between the ages of 18 and 51 years who were not pregnant and had no self-reported history of impaired galactose metabolism, infertility, or neurological disorders were also enrolled in the study. Controls were solicited via announcements posted electronically and in hard copy on the Emory University (Atlanta, GA) campus. Anonymous plasma sample discards

representing controls between the ages of <1 to 21 years were obtained from the Emory Genetics Laboratory (Atlanta, GA). This study was approved by the Institutional Review Board of Emory University. With the exception of anonymous controls, informed consent was obtained from all subjects or their legal guardians. None of the authors has a conflict of interest regarding this work.

Assays

Sample Collection/Storage: All blood samples were drawn without regard to menstrual cycle day. Blood draws on patients were performed either at the 2006 Parents of Galactosemic Children, Inc. (PGC) conference or in conjunction with routine clinical venipuncture. All blood samples were collected and stored overnight (or shipped overnight), then aliquotted and frozen. Using a small number of control bloods we compared AMH and FSH levels in samples frozen immediately or after overnight storage; storage for ≤24 hours had no effect on AMH or FSH values.

For measurements from serum, ~5mL of blood drawn into a serum gel separator tube was allowed to clot at room temperature (RT) and then centrifuged for 5 minutes at 1315 rcf in a fixed-angle rotor centrifuge. Each serum sample was transferred to a fresh tube, stored for no more than 24 hours at 0-4°C, and then aliquotted to smaller volumes and stored at -85°C until use.

For measurements from plasma, ~5mL of blood drawn into a sodium heparin tube was allowed to sit at RT or 0-4°C for no more than 24 hours and then centrifuged at 1800 rpm for 15 minutes in a swinging bucket rotor; plasma was collected and aliquots were stored at -85°C until use. Anonymous pediatric control plasma samples were obtained pre-frozen and stored at -85°C until use.

AMH measurements: AMH levels in plasma or serum were determined using the AMH/MIS ELISA kit from Diagnostic Systems Laboratories (cat.# DSL-10-14400). Samples were assayed in duplicate following the manufacturer's instructions. For many samples both serum and plasma were available; independent assays of both sample types from the same study subject demonstrated indistinguishable results. Assays were performed by the Biomarkers Core Facility of Yerkes Primate Research Center at Emory University (Atlanta GA), or by the Reproductive Endocrine Unit Laboratory, Massachusetts General Hospital (Boston MA). Duplicate samples sent to both laboratories yielded indistinguishable results. Lower limit of detection for this assay was 0.01 ng/mL, and inter-assay CV value was 11.42%.

FSH measurements: Levels of FSH in plasma or sera were quantified by immunoradiometric assay using a kit from Diagnostic Systems Laboratories (cat.# DSL-4700). Samples were assayed in duplicate according to the manufacturer's protocol. Assays were performed by the Biomarkers Core

Facility of Yerkes Primate Research Center at Emory University (Atlanta GA). As for AMH studies, comparisons of FSH results from plasma and sera from the same study subjects yielded indistinguishable results. Lower limit of detection for this assay was 1.50 mIU/mL; inter-assay and intra-assay CV values were 3.56% and 7.71%, respectively.

For both AMH and FSH measurements, samples were batched to minimize assay-to-assay variation. Each batch contained both control and galactosemic samples. In all cases, persons performing the assays were blinded as to whether specific samples represented patients or controls.

FSH bioactivity: Bioactivity measurements were performed on samples collected from a subset of subjects ≥8 years of age who were receiving no exogenous hormones. Bioactivity of FSH in plasma samples was determined using Chinese Hamster Ovary (CHO) cells transfected to express the human FSH receptor (FSHR) (manuscript in preparation). Briefly, the assay is a modification of other transfected cell assays in which cells produce cAMP in response to receptor activation by FSH (31-34). CHO-FSHR cells were incubated with patient samples at a final concentration of 12.5% plasma with added phosphodiesterase inhibitor (IBMX). cAMP production was quantified by a commercial RIA kit (Perkin-Elmer Life Sciences, Boston MA) and compared to a cAMP standard curve generated by recombinant hFSH at doses of 0.18 to 56mIU/ml. Total

bioactivity of a given sample was then divided by total quantity of FSH in that sample to obtain the B/I ratio, allowing for bioactivity comparisons between samples containing different amounts of FSH.

Statistical Analyses:

Analyses of AMH and FSH Levels: We applied regression procedures to assess the relationship between galactosemia and hormone levels of interest. To circumvent the non-normality of the distributions of both AMH and FSH, we created dichotomized forms of these two outcomes. Specifically, we developed a binary outcome of abnormal AMH (1 if AMH < 1.5, 0 otherwise) and also created a binary outcome of abnormal FSH (1 if FSH > 10, 0 otherwise). We then applied logistic-regression procedures that regressed each of these binary outcomes on galactosemia status (1 if the subject is galactosemic, 0 if the subject is a control), adjusting for the effects of age. Given the apparent non-linear relationship between age and hormone levels, we modeled the former as a four-level categorical covariate (with each level containing an equal number of subjects) and treated the youngest level as the baseline category. To ensure that our results were robust to small-cell counts in the data, we avoided the use of asymptotic theory for significance and instead based significance on 10,000 random permutations of the data. We also used exact procedures to calculate

odds ratios (OR) and 95% confidence intervals (CI). We conducted these analyses using appropriate components of the *SASv.9.1* and *R* software packages.

Analysis of FSH Bioactivity: For FSH bioactivity, we calculated mean B/I ratios for both patient and control groups. We then compared the means of the two groups using a two-tailed Student's T-test assuming equal variances of the groups; this assumption was supported by an F test of variance equality. A p-value of <0.05 was considered statistically significant. We conducted these analyses using appropriate components of the Microsoft Excel software package.

2.4 RESULTS

Study Volunteers

We enrolled a total of 35 girls and women with classic galactosemia ranging in age from 10 months to 46 years, and 43 control girls and women ranging in age from 6 weeks to 51 years (Table 2.1). Mean age of the galactosemic group was 14.3 years; mean age of the control group was 24 years. The majority of enrolled subjects in both groups were ≥8 years old; in this subset we identified 10 patients and 8 controls who were not taking oral contraceptives or receiving hormone replacement therapy. For all parameters measured, the values detected in our control group corresponded to previously published normal ranges (27, 28, 35).

Subjects Enrolled	Controls		Galactosemics	
	n	**Mean age (range)**	**n**	**Mean age (range)**
Total subjects	43	24.0 (0.1-51)	35	14.3 (0.8-46)
<8 yrs old	7	3.6	11	3
≥8yrs old	36	28	24	19.5
not on OCPs or HRT	8	35.8 (20-51)	10	14.3 (8-29)

Table 2.1 **Subjects enrolled in the study**. The difference in mean ages between patients and controls was accommodated by regression analysis.

AMH levels in most galactosemic girls and women are abnormally low

We measured serum or plasma AMH as an indicator of functional follicular reserve in girls and women with classic galactosemia. Levels obtained for 33 patients and 13 controls were below the assigned cutoff value of ≥1.50ng/mL for normal AMH (Figure 2.2, Table 2.2); this is a conservative cut-off based on previously reported ranges seen in girls and reproductive-age women (27, 28, 35). Across all age groups, AMH values in our patients were significantly lower than in our controls (Table 2.2). Age-adjusted logistical regression analysis for AMH value below 1.5 ng/mL yielded an exact odds ratio of 104.22 (95% CI 12.48 to >1000, p-value <0.0001) for patients as compared to controls.

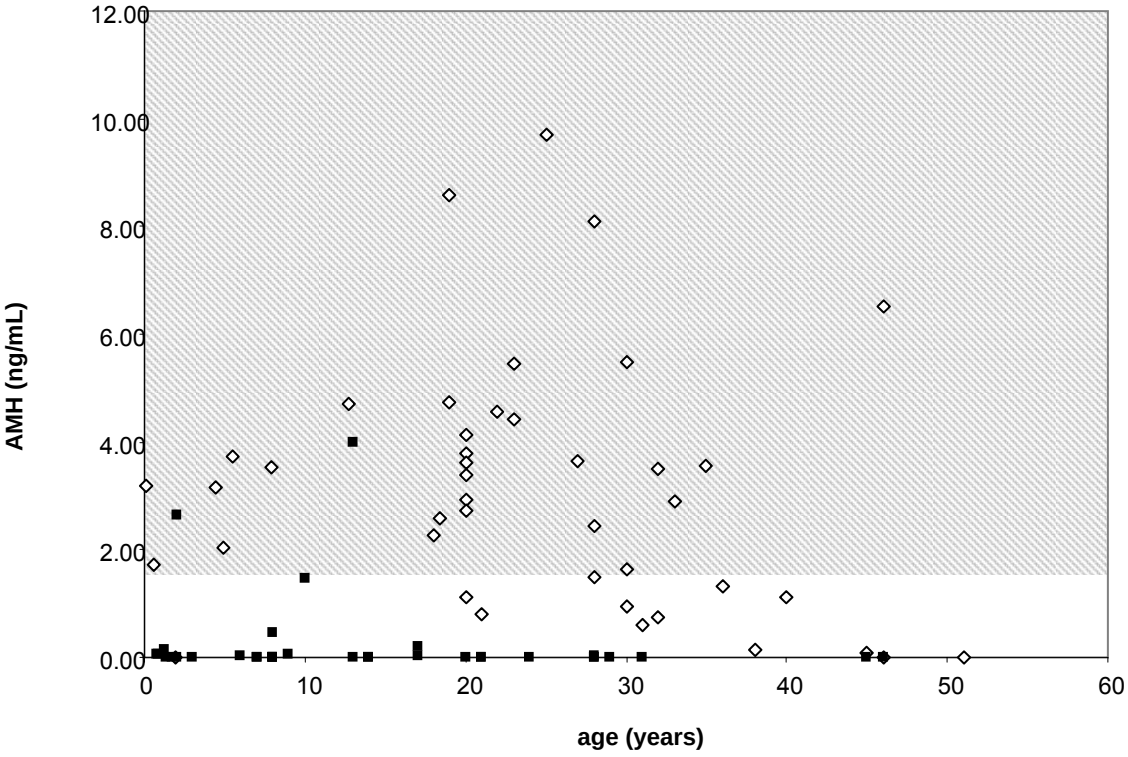

Figure 2.2 **AMH levels** of girls and women with classic galactosemia (filled squares) and controls (open diamonds) between the ages of <1 and 52 yrs. Normal AMH range is shaded.

FSH levels in many galactosemic girls and women are high

We also evaluated gonadal function indirectly by measuring circulating FSH levels in our subjects. 16 patients and 4 controls had FSH values above the assigned cutoff of 10mIU/mL for normal FSH (Figure 2.3, Table 2.2); all controls with FSH >10mIU/mL were either <2 yrs or >40 yrs old, ages at which FSH is either unreliable or expected to rise. Using logistical regression analysis and adjusting for age, we confirmed the expected outcome — that having galactosemia was significantly associated with abnormally high FSH levels.

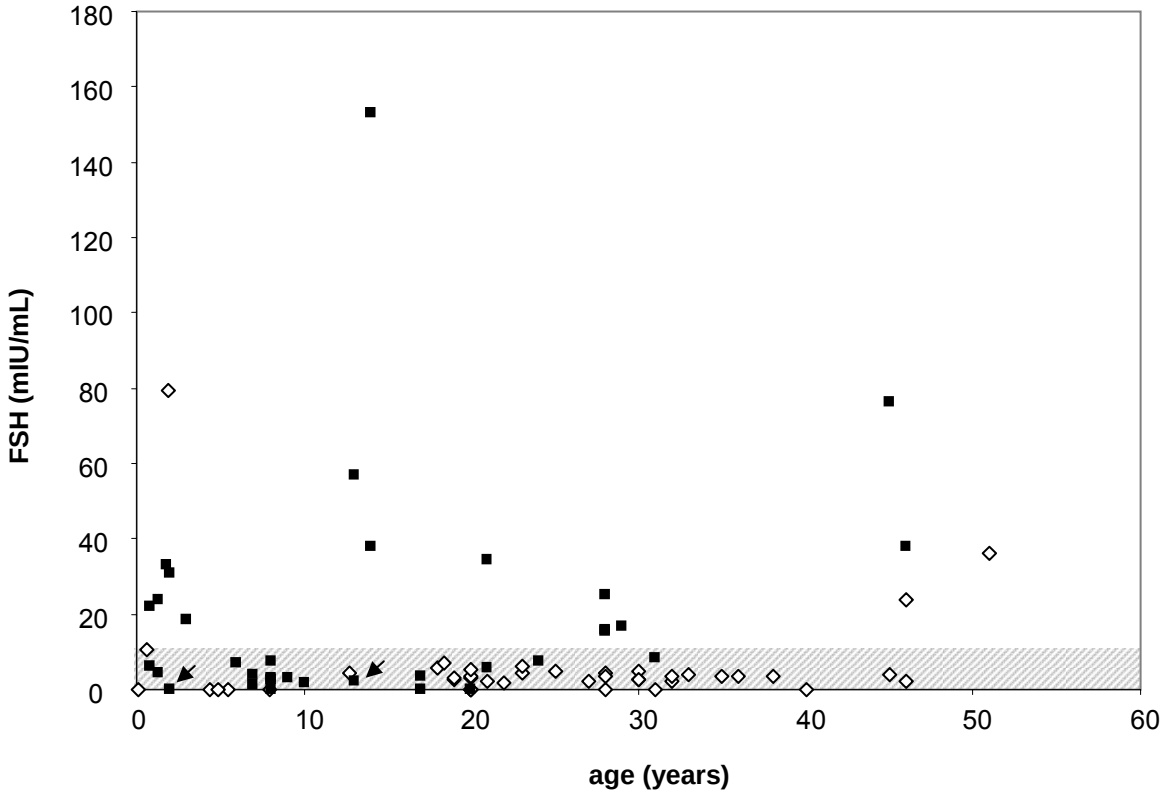

Figure 2.3 **FSH levels** of girls and women with classic galactosemia (filled squares) and controls (open diamonds) between the ages of <1 and 51 yrs. Small arrows indicate the two patients in the study with normal AMH values (Figure 2.2). Normal FSH range in reproductive-age women is shaded. Note that FSH levels in very young girls (<2 yrs) may vary, and women over 40 yrs are expected to demonstrate elevated FSH levels as they approach and enter menopause.

The exact odds ratio for high FSH (>10mIU/mL) in patients relative to controls was 10.58 with a 95% confidence estimate of 2.36-68.51 (p-value = 0.0006). As expected, the two galactosemic girls with normal AMH values (Figure 2.2) also demonstrated normal FSH levels (small arrows, Figure 2.3). Of note, despite the

elevated FSH level of galactosemic patients as a group, more than half of all patients individually demonstrated normal FSH levels (Figure 2.3, Table 2.2).

FSH bioactivity in galactosemic girls and women is normal

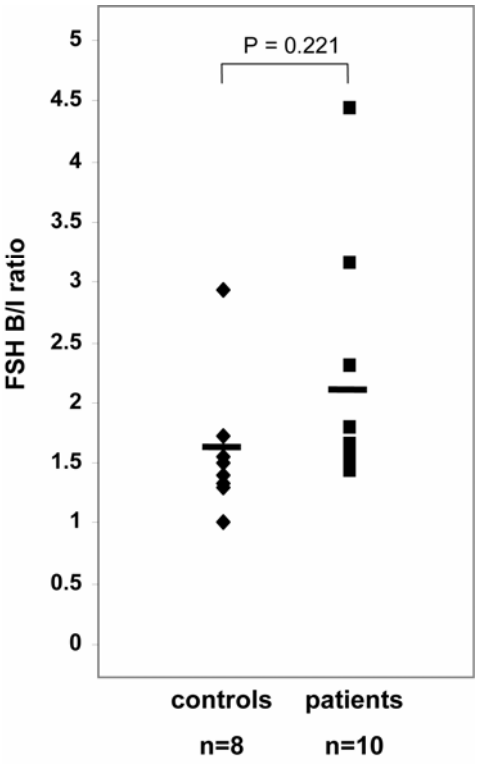

Figure 2.4 **FSH bioactivity** represented by B/I ratio (activity divided by the circulating FSH level) in patients and controls ≥8 yrs of age who were not on oral contraceptives or hormone therapy.

To determine whether the FSH produced by galactosemic girls and women is functional, we assessed FSH bioactivity in a subset of subjects ≥8 years of age who were not on oral contraceptives or hormone replacement (Figure 2.4, Table 2.2). The mean B/I ratio for the galactosemic group was 2.09 (range, 1.44-4.43) compared to a mean B/I ratio of 1.59 (range, 1.00-2.94) for controls. The difference in mean B/I ratio between the two groups was not statistically significant (p=0.221).

Table 2.2 **Biomarker and clinical outcome data** for 35 girls and women with classic galactosemia. Cases are presented in terms of age range to protect the privacy of individual patients. *This patient had spontaneous menarche but also had elevated FSH and LH clinical values. ** Unk (unknown)--mutation was not identified by a clinical screen for S135L, T138M, Q188R, L195P, Y209C, N314D, L218L, K285N, IVS2-2A>G, and the 5kb GALT deletion. ***Classic galactosemia self-reported; supporting data unavailable.

age (years)	GALT genotype**	AMH (ng/mL)	FSH (mIU/ml)	clinical evidence of ovarian function	hormone therapy
0-2	K285N/unk	0.05	22.04	NA	no
0-2	Q188R/Q188R	0.05	6.39	NA	no
0-2	Q188R/Q188R	0.15	4.56	NA	no
0-2	Q188R/unk	0.01	23.64	NA	no
0-2	Q188R/R148P	0.00	33.09	NA	no
0-2	K285N/unk	0.00	31.03	NA	no
0-2	D197G/ Q188R-N314D-L218L	2.64	<1.50	NA	no
3-7	K285N/5kb del	0.00	18.43	NA	no
3-7	Q188R/ K285N	0.04	7.23	NA	no
3-7	Q188R/Q188R	0.00	1.52	NA	no
3-7	Q188R/Q188R	0.00	3.98	NA	no
8-14	Q188R/Q188R	0.01	7.34	pre-menarche	no
8-14	Q188R/Q188R	0.00	2.51	pre-menarche	no
8-14	Q188R/Q188R	0.00	3.00	pre-menarche	no
8-14	Q188R/ K285N	0.45	2.78	pre-menarche	no
8-14	Q188R/ A320T	0.00	0.00	pre-menarche	no
8-14	Q188R/ Q188R	0.07	3.12	pre-menarche	no
8-14	Q188R/ Q188R	1.45	1.77	pre-menarche	no
8-14	unk/ unk	3.98	2.38	pre-menarche	no
8-14	Q188R/ Q188R	0.00	56.77	spontaneous menarche*	no
8-14	Q188R/ Q188R	0.00	38.10	oligomenorrhea	no
8-14	Q188R/ Q188R	0.00	153.20	secondary amenorrhea	no
15-39	Q188R/ unk	0.00	5.95	oligomenorrhea	no
15-39	Q188R/ Q188R	0.00	16.76	secondary amenorrhea	no
15-39	Q188R/ Q188R	0.00	8.43	secondary amenorrhea	yes
15-39	Q188R/ unk	0.19	3.62	primary amenorrhea	yes
15-39	Q188R/ Q188R	0.03	0.00	primary amenorrhea	yes
15-39	Q188R/ unk***	0.00	0.00	primary amenorrhea	yes
15-39	Q188R/ Q188R	0.00	34.20	primary amenorrhea	yes
15-39	Q188R/ unk	0.00	7.58	primary amenorrhea	yes
15-39	Q188R/ unk	0.00	15.48	primary amenorrhea	yes
15-39	Q188R/ Q188R	0.00	25.36	no data	yes
15-39	Q188R/ Q188R	0.04	15.93	no data	no
≥40	Q188R/ Q188R	0.00	76.47	history of oligomenorrhea	no
≥40	Q188R/ Q188R	0.00	37.81	history of infertility	yes

2.5 DISCUSSION

The purpose of this study was to utilize serum or plasma biomarkers to explore the basis of hypergonadotrophic hypogonadism in girls and women with classic galactosemia. Specifically, we compared the predictions of two hypotheses that suggested either (1) that most galactosemic girls and women have diminished follicular function, or (2) that most galactosemic girls and women have functionally abnormal FSH, leading to secondary or apparent gonadal failure (4, 5, 9, 10, 22, 36, 37). Our data overwhelmingly support the first hypothesis, and contradict the second.

Across age groups, the vast majority of our female galactosemic subjects demonstrated extraordinarily low AMH levels relative to age-matched controls, suggesting that follicular dysfunction occurs unusually early in life, ostensibly prior to menarche, and perhaps even prior to birth. Of note, there was no clear relationship between AMH value and GALT genotype in our cohort, although we did not have any patients in this study who carried mutations that have been recognized previously as mild (e.g. S135L, reviewed in (1)). Of further note, Knauff and colleagues (38) recently reported that serum AMH levels in galactosemia *carriers* are normal, consistent with the clinical reality that heterozygotes show no evidence of ovarian insufficiency.

The percentage of galactosemic girls and women demonstrating abnormally low AMH levels in our study was >90% -- a number in striking agreement with the percentage of girls and women with classic galactosemia who demonstrate *clinical* evidence of POI (>80%) (4-6). Of note, both of the patients in our study who demonstrated normal AMH levels are pre-menarche (at ages 2 yrs and 13 yrs), as is a third patient (10 yrs) whose AMH level (1.45 ng/mL) just missed the normal cut-off (1.5 ng/mL). It will be particular interesting to track the AMH levels and clinical progression of these 3 girls over time to see if their AMH levels remain within or near the normal range, and also to see whether these girls grow and mature to experience normal puberty and regular menses.

Our data also demonstrated that while the FSH level in our cohort of galactosemic girls and women, as a group, was significantly elevated, this marker was not elevated in all patients, especially in the pre-pubertal state. For example, of 11 galactosemic girls ages <8 yrs, 6 demonstrated normal FSH, yet of these 6 only 1 demonstrated normal AMH. Were a normal FSH level a reliable indicator of ovarian *sufficiency* in galactosemic girls, these data would predict a prevalence of POI in galactosemia of <50%, a value clearly at odds with the clinical data. Further, bioactivity measurements of FSH in our subjects with galactosemia were statistically indistinguishable from those of age-matched

controls, thereby contradicting the hypothesis that galactosemic girls and women produce "functionally abnormal" FSH. Of course, our limited data set cannot rule out the possibility that studies of larger numbers of samples might identify a subtle difference in FSH bioactivity not detected here. However, given that 8 of the 10 patients studied here demonstrated FSH bioactivities well within the control range, our data suggest that any potential small difference in FSH bioactivity in some patients would be unlikely to account for the ovarian insufficiency seen in >80% of girls and women with galactosemia. Of course, it is also possible that there may be additional FSH function(s) or receptors in humans other than the one represented by the assay we used here; we may therefore have missed some difference between cases and controls with regard to that function.

Our findings regarding AMH and FSH do not support previous hypotheses of a resistant ovary syndrome in galactosemia (10, 37); in contrast, it appears that the decreased follicular function or reserve is present from a very young age, at least in some patients. The cause for the variability in ovarian function among galactosemic women is not clear. Previous authors have proposed that higher amounts of galactose in the diet may positively correlate with FSH levels in metabolically normal women of late reproductive age (39). However, to what extent rigorous adherence to a galactose-restricted diet might

delay follicular atresia in girls with galactosemia is uncertain. Studies in rats have shown that a high galactose diet, but not a high lactose diet, reduces the follicular count in subsequently harvested ovaries, possibly via down regulation of GDF-9 on the developing follicle (40, 41). Given the abnormal AMH levels in even our youngest galactosemic subjects (< 1 year) it seems likely that ovarian insufficiency in patients may be evident at birth, suggesting that the ootoxic effects of gal-1-P or other galactose metabolites likely begin *in utero*. This hypothesis is supported by evidence from pregnant rats fed a high concentration of galactose, who subsequently gave birth to female pups with significantly decreased numbers of oocytes (14).

Our study represents a first step toward understanding the basis of POI in girls and women with classic galactosemia. In the near future we plan to increase the size of our subject and control populations. In addition, we will implement longitudinal studies to follow the progression of AMH, FSH, and clinical outcomes for galactosemic girls ascertained at a young age, which will be especially important for the few girls who demonstrate *normal* AMH levels in childhood. We also plan to conduct genetic and biochemical studies to investigate *why* some girls with classic galactosemia demonstrate normal AMH levels—and presumably preserved ovarian function—while most do not.

Our studies to elucidate the pathophysiology of impaired ovarian function in galactosemia may ultimately provide insight into other mechanisms of POI that do not involve galactosemia. Association with POF or POI has been described in autoimmunity, disruption of germ-cell migration, other genetic disorders, Turner's syndrome, and possibly environmental or infectious etiologies. Some of these causes may share an as yet uncharacterized common pathway with POI in classic galactosemia. One recently proposed mechanism is *via* gal-1-P activation of the short form of the prolactin receptor in the ovary, which leads to premature follicular depletion in mice (42). Combined, these patient and animal studies may provide greater insight into normal processes of ovarian development and function, as well as causes of ovarian dysfunction in galactosemia and other conditions.

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Chapter 3

ROLE OF UDP-GALACTOSE 4' EPIMERASE (GALE) IN *DROSOPHILA MELANOGASTER* DEVELOPMENT AND HOMEOSTASIS

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3.1 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.

3.2 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 3.1): 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

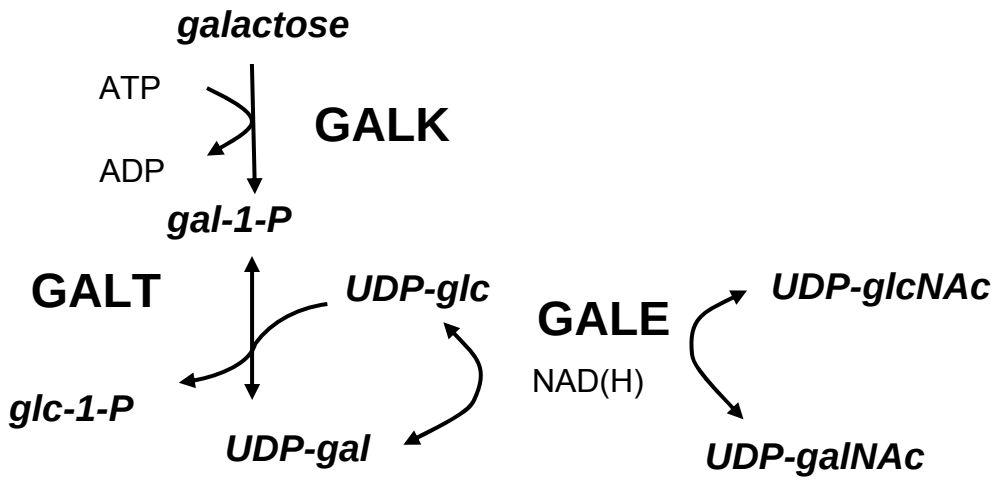

Figure 3.1 **The Leloir Pathway of Galactose Metabolism.** In both humans and Drosophila, GALE catalyzes UDP-galNAc/UDP-glcNAc interconversion in addition to UDP-gal/UDP-glc interconversion.

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 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.

3.3 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.

3.4 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 3.1), 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 *dGALE*; this insertion (*P{EPgy2}CG12030*^{EY22205}, Figure 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 3.1). 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}.

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 3.1 **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.

Molecular characterization of *dGALE*^{y} revealed a 1339bp deletion from *dGALE* that included the first coding exon (Figure 3.2). 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 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.*

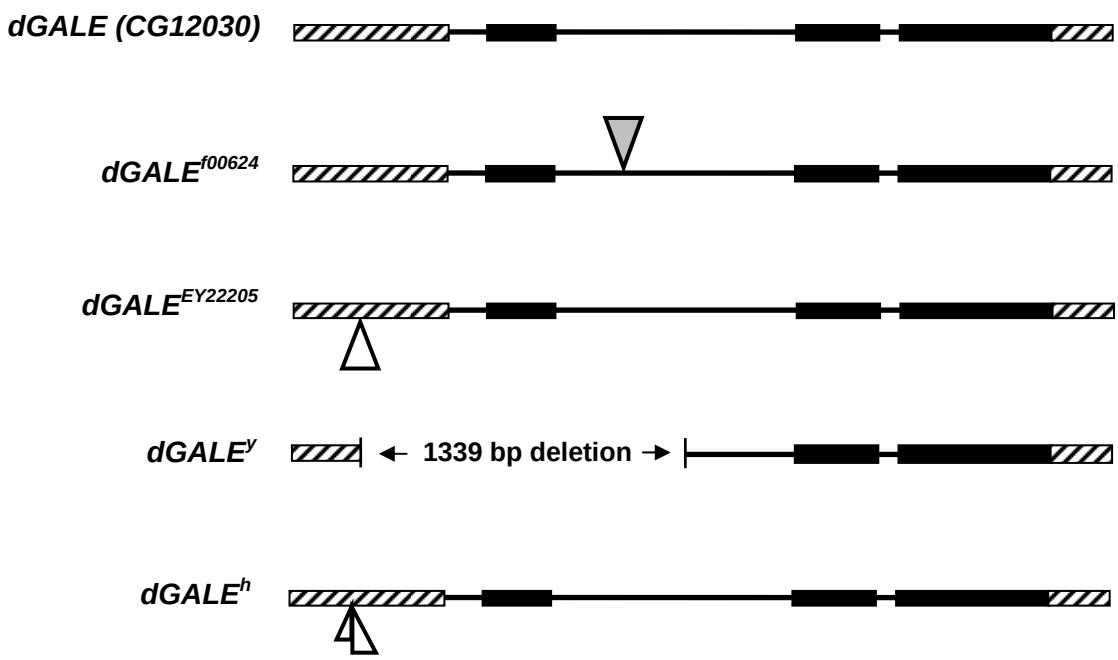

Figure 3.2 **Schematic of *dGALE* and Alleles.** Introns are shown as thin lines and exons as thick lines, with coding regions in solid black and non-coding regions cross-hatched. Transposon insertion sites are denoted by triangles.

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 3.3). 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 3.3). 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. Furthermore, these *dGALE* heterozygotes did not differ from their wild-type *dGALE* homozygous (*P{FRT}^{2A} /+)* counterparts in survival rates (Figure 3.3).

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.

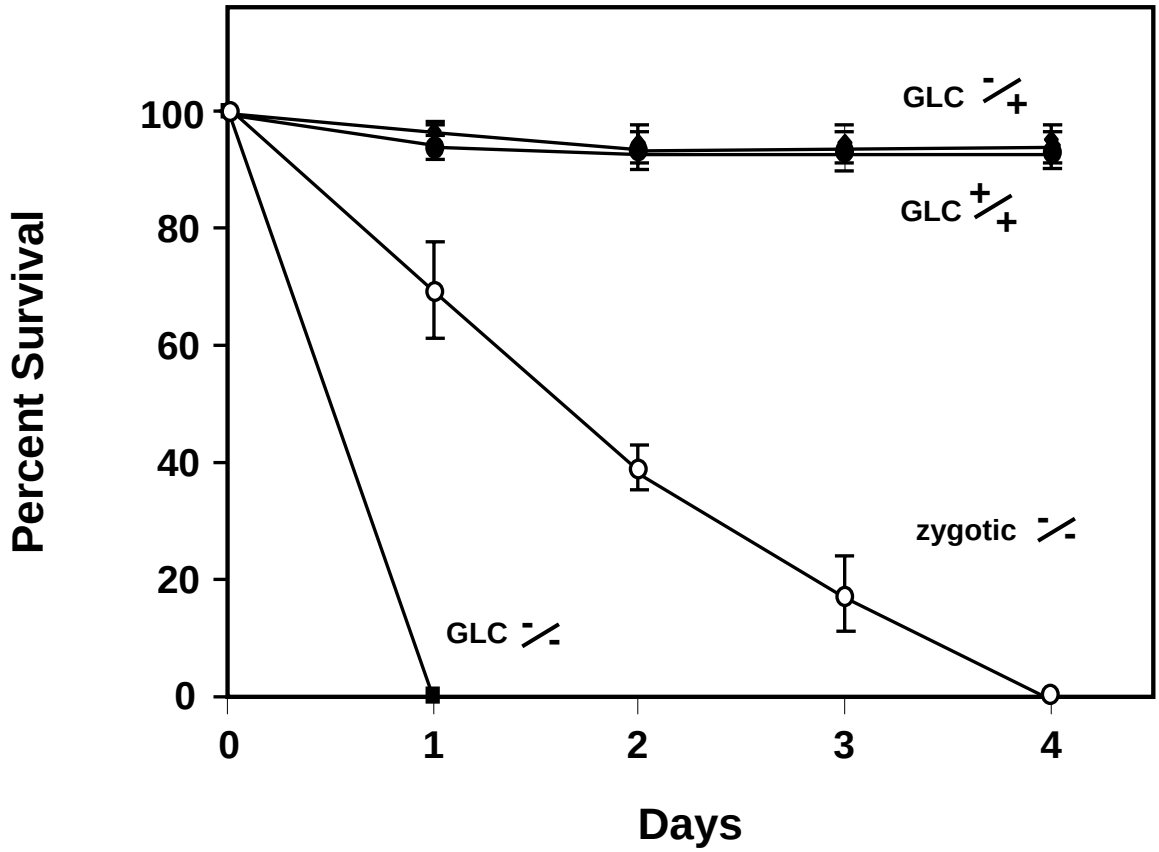

Figure 3.3 **Viability of *dGALE* Mutants.** Some zygotic $dGALE^{f00624}/dGALE^{y}$ (open circles, denoted -/-) mutants die as embryos while others survive through L1 or L2, while all $dGALE^{f00624}/dGALE^{y}$ germ line clone mutants (filled squares, denoted GLC -/-) die as embryos. Survival of germ line clone $dGALE^{f00624}$/+ heterozygotes (filled triangles, denoted GLC -/+) was not distinguishable from germ line clone wild-type homozygotes (filled circles, denoted GLC +/+).

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 3.1). 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 3.4). 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 3.4). Although females shifted in early pupal stages laid few or no embryos, their ovaries and egg chambers appeared morphologically normal (data not shown).

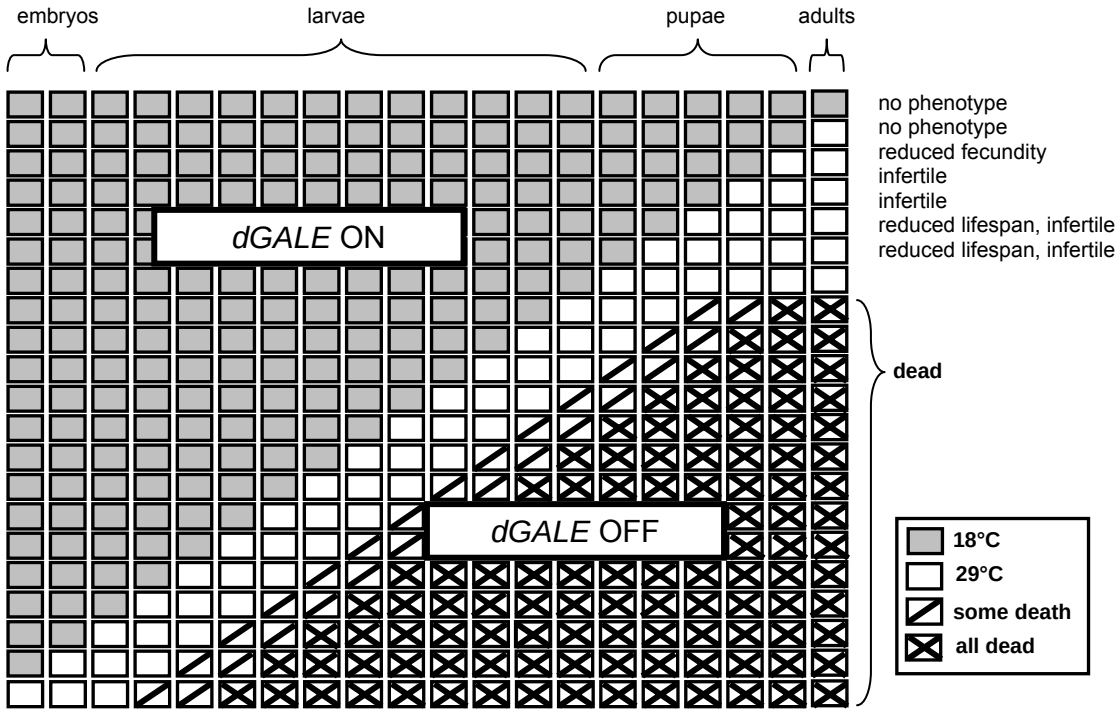

Figure 3.4 ***dGALE* Is Required Throughout Development.** Each row represents a cohort of animals in which GALE knockdown was initiated on a particular day in development. Shaded boxes represent days in which dGALE knockdown was prevented by GAL80(ts) at 18°; open boxes represent days after animals 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.

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 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 3.5), indicating that the impact was galactose-specific.

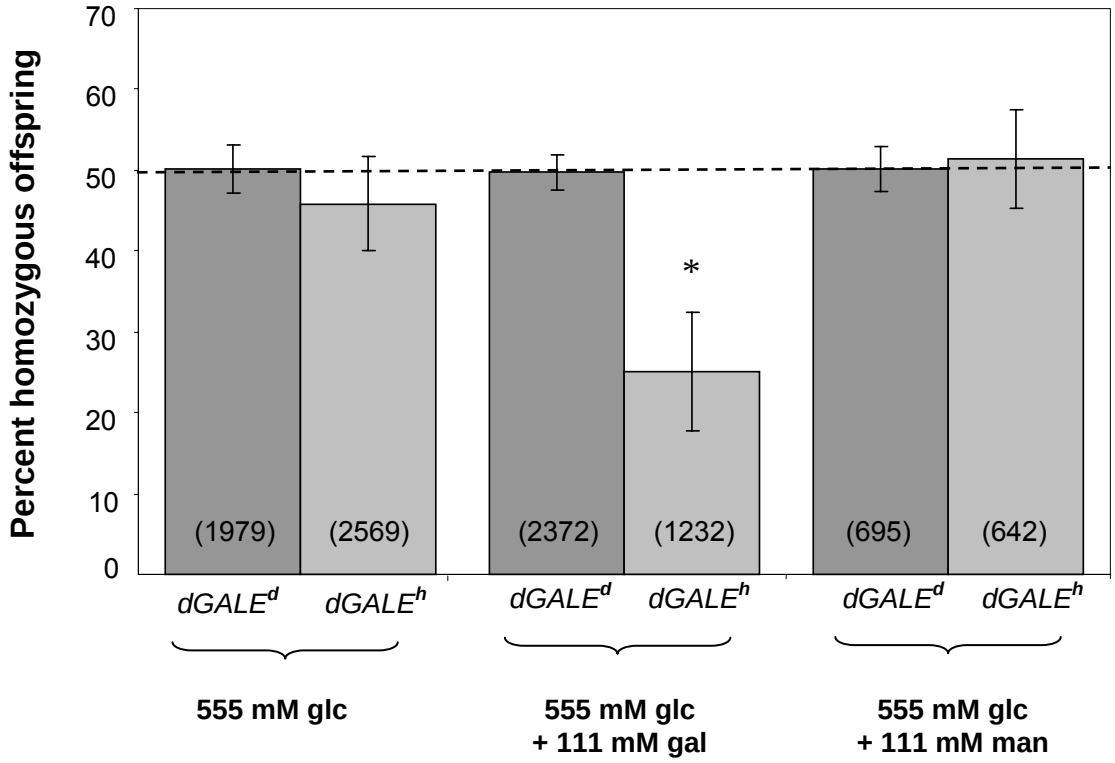

Figure 3.5 **Hypomorphic *dGALE* Mutants Are Sensitive To Dietary Galactose.** The percentage of homozygous offspring which eclosed as adults is shown for both the precise excision *dGALE*^{d} (dark columns) and the hypomorph *dGALE*^{h} (light columns) on food containing dextrose, dextrose and galactose, 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}.

3.5 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.

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 3.3), 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 3.3). 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 4

CONCLUSION

4.1 SUMMARY

This dissertation addresses several issues pertinent to galactosemia caused by impairment in either GALT or GALE. A key step towards understanding the pathophysiology of these disorders is identifying which tissues of the body are most affected by impaired galactose metabolism, and at what points in life Leloir pathway function is most critical.

In chapter 2, these questions were addressed in relation to POI, a specific long-term complication of classical galactosemia. We determined that POI in classical galactosemia arises from dysfunction within the ovary, and not from the production of ineffective FSH signal by the pituitary. Diminished follicular function was observed in classical galactosemics less than two years of age, indicating that ovarian insufficiency has its root in early, perhaps even prenatal, development.

Chapter 3 is a broader exploration of the timing and tissue-specific requirements for GALE, using the model organism *D. melanogaster*. In these animals, we identified the gut and Malpighian tubule as necessary and sufficient sites of GALE expression. Furthermore, we determined that GALE was required throughout development but not in adulthood, at least in the absence of dietary galactose consumption.

4.2 POI IN CLASSICAL GALACTOSEMIA

Summary

Chapter 2 examines the underlying cause of POI, the most common long-term complication developed by women with classical galactosemia. Although the predominance of POI in galactosemic women is well-documented (1,2,3) the underlying cause is not known. Some previous studies have suggested that galactose metabolite accumulation or reduced incorporation of glycolipids and glycoproteins in the ovary might be the source of the dysfunction (1,4,5). Other reports have suggested that FSH has reduced incorporation of galactosyl residues in N-linked glycan chains and is therefore unable to activate its receptor in the ovary (6,7).

Using the serum biomarkers FSH and AMH, we were able to evaluate both of these potential explanations. We compared overall abundance of AMH and FSH in a group of 35 girls and women with classical galactosemia and 43 age-matched controls, and tested FSH bioactivity in a subset of 8 controls and 10 galactosemics who were ≥ 8 years old and not on hormonal therapy.

FSH levels, an indirect indicator of ovarian dysfunction in individuals with a functioning hypothalamic-pituitary-ovarian axis, were high in our galactosemic group relative to controls. This finding was expected, given the high rate of POI in this population, and is consistent with previous reports (2,8).

FSH bioactivity, a measure of the hormone's ability to bind and activate its receptor, was not significantly different between the two groups. By contrast, AMH levels, an indicator of the number and maturity of ovarian follicles, were strikingly low in the galactosemic group. In many affected individuals, AMH could not be detected. Furthermore, AMH was very low across all age groups, including girls younger than two years old. From the group of 35, only 3 girls with galactosemia had normal or near-normal AMH. This proportion mirrors the prevalence of POI reported in previous studies of classical galactosemia (1,2,3).

Implications and Future Directions

These results indicate that POI in classical galactosemia arises within the ovary, and is not caused by improper function of circulating FSH. Although AMH levels normally decline in the post-pubertal period, usually reaching undetectable levels post-menopause (9), in our galactosemic population they were universally low, regardless of age. Very low AMH measurements were obtained from girls younger than two, suggesting that ovarian damage caused by impaired galactose metabolism occurs very early in life, and raising the possibility that ovarian dysfunction is the result of prenatal insult.

AMH is produced by preantral and antral follicles in the ovary (10,11), and is therefore clinically useful as a proxy of ovarian reserve. Though our study pinpoints the site of ovarian dysfunction, it does not explain the mechanism. The diminished follicular function we observed could have several explanations. One possibility is that the ovaries of women with classical galactosemia have low initial oocyte pools or undergo accelerated depletion of follicles early in development. This explanation is supported by studies in rats, in which prenatal exposure to high amounts of galactose led to failure of germ cell migration to the developing ovary (12), and to reduced oocyte pools (13). Another possibility is that the ovaries of galactosemics do not have fewer follicles, but that they are stuck in earlier stages of development, prior to the production of AMH.

The recruitment of new study subjects is an ongoing effort in our lab. By increasing the size and age diversity of our study population, we hope to more sharply define the ovarian status of girls with galactosemia, particularly the very young. Our results suggest that ovarian damage is already present at birth or shortly thereafter; obtaining more infant samples will allow us to conclusively demonstrate whether ovarian dysfunction is acquired or congenital. Additionally, our first cohort included 3 girls with normal or near-normal AMH levels. POI is not a universal finding in classical galactosemia, and the

identification of more girls in whom ovarian function is preserved might enable us to identify factors that distinguish this group from the majority.

This first study was limited to girls and women with classical galactosemia. No large-scale studies of reproductive function in women with intermediate GALT impairment have been undertaken. In order to determine whether POI extends to milder forms of galactosemia, we have begun collecting samples from young girls with Duarte galactosemia. We are specifically interested in whether AMH levels in these girls will be normal or somewhat depressed. If AMH levels are normal, we would conclude that there is a minimal threshold for GALT activity above which ovarian dysfunction does not occur; if AMH levels are low, we would conclude that the same processes which limit follicular function in classical galactosemia are at play in Duarte galactosemia, though perhaps not to the same extent.

We also plan to collect repeat samples from as many study subjects as possible. Our initial measurements constitute a "snapshot" of ovarian function in each individual; sampling additional time points would allow us to draw conclusions about the consistency of AMH levels over time. Since our study was published, a single case report of pregnancy in a woman with galactosemia and low AMH has been reported (14). Extrapolation of a single case to the general condition of galactosemia-asssociated POI is not possible, but this report does

raise the possibility that follicles are present but not normally able to mature. If follow-up of our study population revealed marked fluctuation in AMH levels from individual subjects over time or a high instance of spontaneous conception, this might suggest that ovarian insufficiency is related to defects in follicular maturation, and not depletion of ovarian reserve.

For girls with normal levels of AMH, repeat measurements will allow us to determine whether follicular function is sustained over time. In combination with clinical data, longitudinal AMH data will reveal whether normal values correlate with positive outcomes such as spontaneous menarche and preserved fertility. If we are able to identify a sufficient number of girls with normal AMH, we plan to investigate biochemical and genetic differences between galactosemics with low and normal AMH, as a step toward understanding why some, but not all, galactosemic women develop POI.

4.3 GALE IN DROSOPHILA DEVELOPMENT AND HOMEOSTASIS

Summary

Chapter 3 describes the generation and characterization of a model of GALE-deficiency galactosemia, using the fruit fly *D. melanogaster*. This novel system enabled us to answer several questions relating to GALE's role in organismal development and homeostasis. Using strong-loss of function alleles as well as

hypomorphic alleles, we were able to generate models of both profound and intermediate GALE deficiency. As we suspected, complete loss of GALE was incompatible with life. By controlling the timing and location of GALE loss, we were then able to identify developmental stages and tissues in which GALE was most critical.

By preventing the deposition of maternally loaded GALE mRNA or protein in embryos, we were able to determine that GALE is first required in late embryogenesis, after zygotic transcription would normally begin. Furthermore, we were able to sequentially knock-down GALE in advancing stages of development to determine if GALE was required post-embryonically. We found that GALE was essential for survival at all stages of development through pupation. Loss of GALE during pupation was associated with adverse outcomes in adulthood; these adult flies had shorter lifespan and females deposited few or no embryos. Surprisingly, flies in which GALE was not knocked down until adulthood appeared unaffected by the loss; these animals were able to produce viable offspring and did not have diminished lifespan.

Flies with partial impairment in GALE, corresponding to approximately 8% of wild-type activity, were viable and apparently healthy and fertile. However, when these animals were raised on a diet supplemented with galactose, significantly fewer were able to survive to adulthood. Compound

heterozygotes for this intermediate allele and a strong loss of function allele, which would be predicted to have approximately 4% of wild-type activity, were not viable even in the absence of environmental galactose.

In order to identify those tissues most affected by GALE loss, we selectively expressed an RNAi targeting GALE in specific tissues. Strong, ubiquitous expression of the knock-down construct phenocopied the strong loss-of function genomic alleles and was able to be rescued by transgenic expression of hGALE. Isolated knock-down of GALE in the nervous system, eye, salivary gland, fat body or ovary did not affect viability, nor did it have an appreciable effect on morphology or fertility. GALE knockdown in the proventriculus, midgut and Malpighian tubule, however, was lethal. Furthermore, selective expression of hGALE in these tissues was enough to rescue the strong loss of function alleles to viability. Expression of hGALE in the nervous system or fat body was not sufficient to permit survival.

Implications and future directions

GALE has a dual role in the cell, participating in the conversion of dietary galactose to a readily usable energy source as a part of the Leloir pathway, and regulating the supply of UDP-sugars necessary for the synthesis of complex carbohydrates, glycoproteins and glycolipids. In this second role, GALE

catalyzes two separate interconversion reactions. The establishment of a fly model provides the necessary tools to begin dissecting the relative importance of these roles.

Our germ line clone mutant embryos, which had no GALE, including no maternally loaded GALE protein or message, died in embryogenesis. Since these animals died before they began to eat, death from accumulation of toxic galactose metabolites seems unlikely. Rather, we hypothesize that these animals died because they could not synthesize galactose, and were unable to manufacture proper glycoproteins and glycolipids essential for organogenesis. To determine whether they do indeed have glycosylation defects, we have prepared a large sample of germline clone mutant embryos for mass spectrometric analysis of both N- and O-linked glycans.

In hypomorphic mutants and animals in which GALE is knocked down in later stages of development, we suspect that accumulation of galactose metabolites may exacerbate phenotypes we observe. GALE deficiency has only recently been recognized as a disease spectrum (15), but humans with either intermediate or severe GALT deficiency accumulate galactose metabolites (15-20) Glycoprotein studies of a cell line derived from a single patient with severe GALE impairment suggest that even low levels of enzyme activity allow sufficient endogenous galactose synthesis (31). Studies of glycan synthesis and

metabolite accumulation in our hypomorphic mutants would help determine the mechanisms of galactose toxicity across the spectrum of GALE deficiency.

We are especially interested in the apparent insensitivity of animals to knock-down of GALE in adulthood. We hypothesize that the role of GALE in regulating UDP-sugar pools for glycan synthesis is essential for development, but may be less important for adult homeostasis. Rather, we anticipate that loss of GALE in adulthood will only be detrimental in the context of galactose exposure. To determine whether GALE knock-down adults are galactose sensitive, we have placed them on galactose supplemented media and are planning to assay them for accumulation of Leloir pathway intermediates.

Our studies of selective GALE expression or knock-down in various tissues revealed that GALE is essential in the gut and Malpighian tubule of flies. GALE expression is enriched in these tissues throughout development (21,22,23), and both play a role in metabolism. The fly gut is similar to the human intestine with regards to functional organization, development, and maintenance (24). The Malpighian tubule is analogous to the human kidney and is essential for osmoregulation, but also performs functions relating to metabolism and detoxification of toxic compounds that are similar to those of the human liver (25). Our experimental design did not permit us to evaluate the requirement for GALE in these tissues separately, but these findings warrant future

investigations of the impact of GALE loss on functions of the gut and tubule, in tandem and independently.

GALE may also be essential for development and maintenance of these tissues. Both the gut and tubule have an epithelial layer with strong apical-luminal polarity, the organization of which depends upon glycan synthesis during development (26,27). The ratio of UDP-gal:UDP-glc and UDP-galNAc:UDP-glcNAc is tightly regulated by GALE (28), and it stands to reason that this role of GALE would be essential in tissues that depend upon glycan synthesis for proper organization and function. This possibility could be investigated through a detailed examination of morphology and cellular organization of these tissues in the context of impaired or absent GALE activity.

The relative import of GALE activity toward UDP-gal or UDP-galNAc is not known, and has potential bearing on glycan synthesis as it relates to development and organization of tissues. Mutations in human GALE have been demonstrated to differentially affect activity towards the two substrates (29,30), but it is not known whether one activity has greater influence on patient outcome than the other. Our lab has selectively reconstituted GALE activity towards either UDP-gal or UDP-galNAc in flies that lack endogenous GALE and found that activity towards both substrates is required for viability (Jennifer Sefton,

unpublished data). Work is currently ongoing to determine if requirement for both substrates is necessary at all stages of life and in all tissues.

The studies presented in chapter 3 are a first step in elucidating the pathophysiology of GALE deficiency galactosemia, and provide valuable information about the contributions of GALE to normal development and homeostasis. The creation of a new model of GALE deficiency lays the groundwork for further investigations into the specific cellular and biochemical consequences of GALE impairment, and how those consequences affect the entire body.

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Appendix

ORIGINS, DISTRIBUTION, AND EXPRESSION OF THE DUARTE-2 (D2) ALLELE OF GALACTOSE-1-P URIDYLYLTRANSFERASE (GALT)

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A.1 ABSTRACT

Duarte galactosemia is a mild to asymptomatic condition that results from partial impairment of galactose-1-phosphate uridylyltransferase (GALT). Patients with Duarte galactosemia demonstrate reduced GALT activity and carry one profoundly impaired GALT allele (G) along with a second, partially impaired GALT allele (Duarte-2, D2). Molecular studies reveal at least five sequence changes on D2 alleles: a p.N314D missense substitution, three intronic base changes, and a 4-bp deletion in the 5′ proximal sequence. The four non-coding sequence changes are unique to D2. The p.N314D substitution, however, is not; it is found together with a silent polymorphism, p.L218(TTA), on functionally normal Duarte-1 alleles (D1, Los Angeles, LA). The HapMap database reveals that p.N314D is a common human variant, and cross-species comparisons implicate D314 as the ancestral allele. The p.N314D substitution is also functionally neutral in mammalian cell and yeast expression studies. In contrast, the 4-bp 5′ deletion characteristic of D2 alleles appears to be functionally impaired in reporter gene transfection studies. Here we present allele-specific qRT-PCR evidence that D2 alleles express less mRNA *in vivo* than their wild-type counterparts; the difference is small but statistically significant. Furthermore, we characterize the prevalence of the 4-bp deletion in GG, NN, and DG populations; the deletion appears exclusive to Duarte-2 alleles. Combined,

these data strongly implicate the 4-bp 5′ deletion as a causal mutation in Duarte galactosemia and suggest that direct tests for this deletion, as proposed here, could enhance or supplant current tests, which define D2 alleles on the basis of the presence and absence of linked coding sequence polymorphisms.

A.2 INTRODUCTION

Duarte galactosemia is a mild to asymptomatic condition that results from partial impairment of galactose-1-phosphate uridylyltransferase (GALT) (reviewed in (1)). Individuals with Duarte galactosemia are identified by newborn screening at an incidence as high as one in 4,000 live births (2,3), which is almost 10 times the detection rate of classic galactosemia (4). Patients with Duarte galactosemia carry one GALT allele (G) that is profoundly impaired, and a second GALT allele (Duarte-2, D2; sometimes called D) that is partially impaired. Hemolysates from patients with Duarte galactosemia demonstrate, on average, about 25% normal GALT activity, although there is a broad range in individual values (3). Duarte hemolysates also have a characteristic pattern of altered GALT isozyme mobility on native or isoelectric focusing gels (5,6,7).

Prior studies have shown that D2 alleles carry the amino acid substitution p.N314D (c.940A>G) (8,9,10,11), which fully accounts for the altered isozyme mobility (12), but this amino acid substitution does not cause the partial impairment of activity (11,12). Of note, p.N314D is also found on Duarte-1 alleles (D1, also called Los Angeles or LA alleles), which exhibit normal or even above normal activity (13,14,15). Based on HapMap comparative sequence studies (http://www.hapmap.org/index.html.en), p.N314D is now considered a common variant or polymorphism (16) with an allele frequency of ~11% in European

populations (CEPH, or Centre d'Etude du Polymorphisme Humain) and lower frequencies in other populations, for a "pan-ethnic" frequency near 8% (4).

A number of different approaches have been taken to the question of why D2 alleles are functionally compromised, whereas D1 alleles are not. Andersen and colleagues (17) used immunochemistry to reveal that the different activities attributed to D1 and D2 alleles reflects differential GALT protein abundance, rather than differential specific activity. Later studies of the coding and non-coding nucleotide sequences of D1 and D2 alleles revealed that p.N314D exists on both alleles in linkage disequilibrium with other sequence variants, and that these variants differ between D1 and D2 alleles. Specifically, D1 alleles carry a c.652C>T nucleotide change which results in a silent substitution at codon 218 (p.L218) (CTA to TTA, p.L218, sometimes called L218L) (14,15,18,19), while D2 alleles carry a 4-bp 5' deletion (c.-119_-116delGTCA) (20) along with three intronic base changes (c.378-27G>C or IVS4-27G>C, c.508-24G>A or IVS5-24G>A, and c.507+62G>A or IVS5-62G>A (20,21,22)). Both D1 and D2 alleles also carry an extended sequence of adenine nucleotides in intron 10 (22). Large-scale studies of *GALT* alleles tested for the presence or absence of the p.D314 and p.L218(TTA) sequence variants estimated the pan-ethnic frequencies of D1 and D2 alleles at 2.7% and 5.1%, respectively (4).

The question of which reported base changes account for the up to 50% reduction in hemolysate D2 GALT protein levels has remained a point of controversy for many years. One group proposed that the intronic base changes in D2 alleles might compromise processing or expression of the encoded message (15). Another group (14,23) compared the *GALT* mRNA and protein levels in NN versus DD human cell lines using RNase protection and western blots and concluded that the D2 GALT message levels were normal, but that the p.N314D GALT protein was destabilized. These authors proposed that D1 alleles, which also carry p.D314, might fail to manifest destabilization due to improved translation of the p.L218(TTA) codon (14,23). However, mammalian and yeast expression studies found no evidence of compromised p.D314 GALT protein expression or function (11,12).

The plot thickened when Kozak and colleagues (20) speculated that the 5′ 4-bp deletion specific to D2 alleles might be functionally significant, because it disrupted the predicted binding sites of two transcriptional activators (AP1Q2 and AP1Q4). Two years later, GALT promoter-luciferase reporter gene studies confirmed that the D2 promoter was indeed less active than the wild-type promoter in transient transfection experiments (24,25). One study (25) also reported diminished total GALT message in lymphoblasts cultured from a Duarte carrier (DN) and a Duarte patient (DG) compared with lymphoblasts

cultured from five (NN) controls. These results implicated the 4-bp 5′ deletion and perhaps the other D2 non-coding sequence changes as functionally significant. Nonetheless, variations in GALT message levels between the individual samples in the study combined with the inability to distinguish p.N314 from p.D314 *GALT* messages in individual samples meant that altered D2 message abundance could not be confirmed as the basis for diminished D2 GALT expression.

Here we present studies that address the origins, distribution, and expression of the D2 *GALT* allele. Specifically, we have compared *GALT* sequences among humans, non-human hominids, non-hominid primates, and non-primate placental mammals, and these clearly implicate p.D314 as the ancestral allele and p.N314 as a recent sequence variant that may be unique to humans. We also report that the 4-bp 5' deletion in D2 alleles represents a one-unit contraction of a GTCA tetranucleotide repeat whose repeat number has fluctuated through evolution. To track the distribution of this 4-bp deletion within cohorts of patient and control samples, we developed and applied a robust PCR-based assay that confirmed complete linkage of the 4-bp deletion with *GALT* alleles that also carry p.D314+L218(CTA), and complete repulsion of the 4-bp deletion with *GALT* alleles that carry both p.D314+p.L218(TTA). Finally, we applied an allele-specific qRT-PCR approach to quantify the relative

abundance of "normal" versus p.D314 *GALT* messages in four separate DN individuals. Our results confirm a small but statistically significant under-representation of p.D314 *GALT* message in each of these carriers. These data revealed that under-expression, at the mRNA level, contributes to the compromised function of the D2 *GALT* allele.

A.3 MATERIALS AND METHODS

Study subjects

All DNA samples were obtained either from consenting volunteers from an ongoing IRB-approved study of galactosemia (Emory IRB Protocol # 618-99, PI: Fridovich-Keil) or as anonymized discards from the Emory Genetics Laboratory. All of the samples studied were tested by the Emory Genetics Laboratory to detect the presence of p.N314D, p.L218(TTA), and a set of common *GALT* gene mutations associated with classic galactosemia.

Quantitative allele-specific RT-PCR

RNA samples were prepared from whole blood collected in Tempus™ Blood RNA Tubes using the PerfectPure™ RNA Purification Kit according to the manufacturer's instructions (5 Prime Inc., Gaithersburg, MD); RNA was quantified by UV absorbance. Whole blood was collected and stored at -20°C

until RNA isolation. RNA was stored at -80°C, until it was used to prepare cDNA for quantitative PCR. SuperScript III First-Strand Synthesis System for RT-PCR using Oligo(dT)20 primers was used to prepare cDNA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Each cDNA synthesis reaction contained 250 ng of RNA.

Relative abundance of the D2 transcript in four DN individuals, of whom three were unrelated, was determined by comparison of the results of qRT-PCR reactions from each cDNA sample using primers that specifically amplified a 225-bp fragment of the D314 sequence versus primers that specifically amplified the corresponding 225-bp fragment of the N314 sequence. The primers used in these reactions were h*GALT*.D314.f3, h*GALT*.N314.f3, and h*GALT*.end.r2 (Table A.1). The specificity/selectivity of each allele-specific primer set was confirmed by qRT-PCR with RNA derived from NN or DD homozygous individuals, and subsequent analyses of DN mixed message populations were corrected for the low level of background cross-reactivity (1.5% for D314 primers and 3.5% for N314 primers). All qRT-PCR reactions were performed using SYBR Green for the LightCycler® 480 in a 96-well plate format (Roche, Indianapolis, IN). The absolute abundance of the D314 and N314 messages in each DN sample was calculated using titration curves of DN cDNA amplified with both primer sets. The relative abundance of the D314 and N314 messages in each sample was calculated as

D/(D+N) and N/(D+N), respectively. Assays were performed in triplicate or more, with results reported as mean ± SD.

Primer name	Sequence (5′ to 3′)	Comments
h*GALT*.extwt (primer 1)	TACAAAGTGAAAGTACTTCTA AAATTGTTTTGGTTACAGGTG GTGCTGGATACATTGGTTCAC ACACTGTGGTAGAGCTAATTG AGAATGGggcagcccagtcagtcagt	anneals only to alleles that do not carry the 4-bp 5′ deletion
h*GALT*.shrtdel (primer 2)	ggcagcccagtcagtcacg	anneals only to alleles that carry the 4-bp 5′ deletion
h*GALT*.481rev (primer 3)	gagcgttccaaccttcggaggg	Reverse primer anneals to both alleles
h*GALT*.N314.f3	gaggctggggccaactgga	anneals to p.N314 encoding message
h*GALT*.D314.f3	gaggctggggccaactggg	anneals to p.D314 encoding message
h*GALT*.end.r2	aggtggtaatgaacctcaggaagtgc	reverse primer anneals to both p.N314 and p.D314 encoding messages

Table A.1 **Primers used in this study**. Uppercase letters represent non-human sequence (*S. cerevisiae*) used to extend the length of the wild-type amplicon. Lowercase letters represent human *GALT* sequence.

Detection of the 4-bp GTCA proximal promoter deletion

The presence or absence of the 4-bp *GALT* promoter deletion was determined by allele-specific genomic PCR with primers h*GALT*.extwt (specific for the WT allele), h*GALT*.shrtdel (specific for the deletion allele), and h*GALT*481rev (reverse primer that recognizes both alleles). Each 20-μL PCR reaction contained 3.75 pmol of h*GALT*.extwt (Table A.1), 10 pmol of

h*GALT*.shrtdel (Table A.1), 20 pmol of h*GALT*481rev (Table A.1), between 1 and 50 ng of genomic DNA, 4 μL of 5X GoTaq™ buffer (Promega), 3.2 μL of 2.5 mM dNTPs, 0.4 μL DMSO, and 0.5 units of GoTaq (Promega). All reactions were assembled in 0.5-mL tubes on ice and transferred directly to a thermal cycler pre-heated to 95°C. Reactions were melted at 95°C for 5 minutes, followed by 38 cycles of 94°C for 45 seconds, 65°C for 45 seconds, and 74°C for 1 minute. In the final cycle reactions were extended at 72°C for 5 minutes, and then held at 16°C for at least 10 minutes. Following amplification of each sample, 2 μL of PCR product were diluted 1:5 in ddH_2O and run at 90 volts for 55 minutes on a 2% agarose/TAE gel in conjunction with an appropriate marker (100-bp ladder, New England Biolabs); bands were visualized by staining with ethidium bromide.

A.4 RESULTS

Origins of the p.N314D and p.L218(TTA) polymorphisms and the 4-bp 5′ deletion in hGALT

As a first step toward understanding the origins and relationship of the p.N314D (rs2070074 at position 34,639,442 of chromosome 9 in NCBI Build 36.1) and 5' variations in human *GALT*, we performed cross-species comparisons of the appropriate coding and non-coding sequences from representative human, non-human hominid (e.g. chimpanzee), non-hominid primate (e.g. macaque),

and non-primate placental mammalian (e.g. mouse) species. All species examined, other than human, encoded D rather than N at position 314 (Table A.2), strongly implicating D314 as the ancestral *GALT* allele; the variant that is predominant among modern humans is therefore most appropriately termed p.D314N (Figure A.1).

Sequence	Humans (European)*	Other hominids	Other primates	Other placental mammals
Amino acid at residue 314	asparagine (N) ~89% aspartate (D) ~11%	aspartate (D)	aspartate (D)	aspartate (D)
Sequence at residue 218	CTA (Leu) ~96% TTA (Leu) ~4%	CTA (Leu)	CTA (Leu)	some CTA (Leu) some TTA (Leu)
GTCA repeats	$(GTCA)^3$ in control & D1 alleles $(GTCA)^2$ in D2 alleles	$(GTCA)^3$	$(GTCA)^2$	$(GTCA)^1$

*(http://hapmap.org/cgiperl/gbrowse/hapmap_B35/?name=Sequence:NM_147131

Table A.2 **Cross-species sequence comparisons** suggest origins of the p.N314D and p.L218(TTA) polymorphisms and 5' promoter deletion characteristic of the D1 and D2 alleles of *GALT*. Species examined that encode D at residue 314 included: chimp, gibbon, gorilla, orangutan, rhesus, galago, tree shrew, mouse, rat, guinea pig, shrew, dog, cat, horse, cow, and armadillo. Species examined encoding N at residue 314 included human only. Species examined with p.L218(CTA) included: chimp, gibbon, gorilla, orangutan, rhesus, galago, tree shrew, dog, cat, horse, and armadillo. Species examined with p.L218(TTA) included: mouse and rat. Of note, cow *GALT* encodes p.L218(CTT), whereas guinea pig and rabbit *GALT* encode p.L218(CTG). Species examined that carry a 5' $(GTCA)^3$ included: chimp, gibbon, gorilla, and orangutan. Species examined that carry a 5' $(GTCA)^2$ included: rhesus and macaque. Species examined that carry a 5' $(GTCA)^1$ included: tree shrew, mouse, rat, guinea pig, shrew, hedgehog, dog, cat, cow, elephant, and armadillo. Notably, horses carry one copy of ATCA in place of the GTCA sequence found in other species.

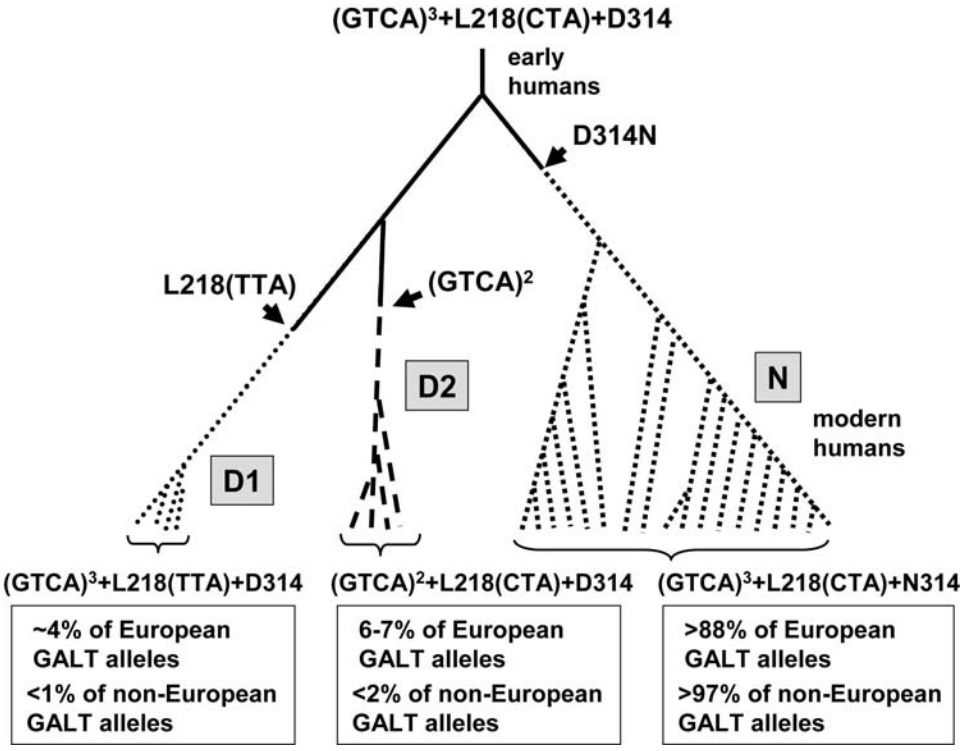

Figure A.1 Proposed origins and relationship of the p.N314, Duarte-1, and Duarte-2 alleles of human *GALT*. The predicted ancestral human *GALT* allele carries the $(GTCA)^3$+p.L218(CTA)+p.D314 sequences found in other hominid species. The p.D314N substitution occurs early in human evolution, and the 4-bp 5' deletion and p.L218(TTA) silent substitution occur later, on distinct branches of the tree. The three intronic base changes reported to exist in *cis* with D2 alleles (c.378-27G>C or IVS4-27G>C, c.508-24G>A or IVS5-24G>A, and c.507+62G>A or IVS5-62G>A (20,21,22), not illustrated here) presumably occurred subsequent to, or concurrently with, the 4-bp 5' deletion on the D2 branch of the tree.

Within the CEPH population, the frequency of the ancestral p.D314 allele is approximately 11.3% (http://hapmap.org), which is unusually high compared with other human populations; Yoruba, Chinese, and Japanese populations each exhibit a frequency of p.D314 well under 3%. Thus, it seems that the common

allele p.D314 is not only the derived state, but also that the ancestral allele is nearly absent among non-European populations.

We also explored the origins of the codon 218 sequence; a silent substitution in this codon constitutes the p.L218 variation (CTA to TTA, rs2070075 at position 34,638,418 of chromosome 9 in NCBI Build 36.1) found on D1 but not D2 alleles. In non-human hominids and non-hominid primates, the CTA codon also predominates. Among non-primate placental mammals, some published sequences carry CTA (e.g. dog, cat, horse), whereas others carry TTA (e.g. mouse, rat). These data implicate CTA as the ancestral allele in humans, but also suggest that the TTA silent substitution may have arisen more than once through the course of evolution.

As summarized in Table A.2, the ancestral CTA (Leu) codon accounts for ~95.5% of alleles in the CEPH population, while the derived TTA (Leu) codon accounts for about 4.5% of alleles. The derived TTA allele is even rarer in non-European populations, with an observed frequency of approximately 1% in the HapMap Chinese sample and a complete absence in both the Yoruba and Japanese samples (http://hapmap.org).

Cross-species comparisons of the *GALT* proximal 5' sequence also reveal an interesting pattern (Table A.2). *GALT* alleles are predominant in humans, and non-human hominids carry three tandem GTCA repeats $(GTCA)^3$,

but non-hominid primates carry only two repeats $(GTCA)^2$, and non-primate placental mammals carry only one repeat unit $(GTCA)^1$. Hence this repeat sequence appears to have expanded through the course of evolution, and the 4-bp deletion seen in human D2 alleles represents a contraction by one repeat unit.

A PCR-based assay to detect the 4-bp 5′ deletion associated with D2 GALT

To facilitate rapid and direct detection of the 4-bp 5′ deletion in human genomic DNA, we designed a multiplex allele-specific PCR-based assay (Figure A.2); allele-specific amplicons are generated using forward primers specific for either the wild-type $(GTCA)^3$ or mutant $(GTCA)^2$ *GALT* sequences, together with a shared reverse primer. As illustrated in Figure A.2, the $(GTCA)^3$-specific primer includes a 91-bp "tail" of non-human (*Saccharomyces cerevisiae*) sequence that extends the length of the amplicon without compromising annealing specificity. The wild-type amplicon is 403 bp, whereas the corresponding $(GTCA)^2$-specific amplicon, which lacks the 91-bp tail, is 308 bp. This size difference makes for easy visual distinction between the two amplified fragments following agarose gel electrophoresis. Figure A.3A illustrates the application of this method to a set of controls, including one sample homozygous for the $(GTCA)^3$ wild-type allele, one homozygous for the $(GTCA)^2$ D2 allele, and one heterozygous for the $(GTCA)^2$ and $(GTCA)^3$ alleles.

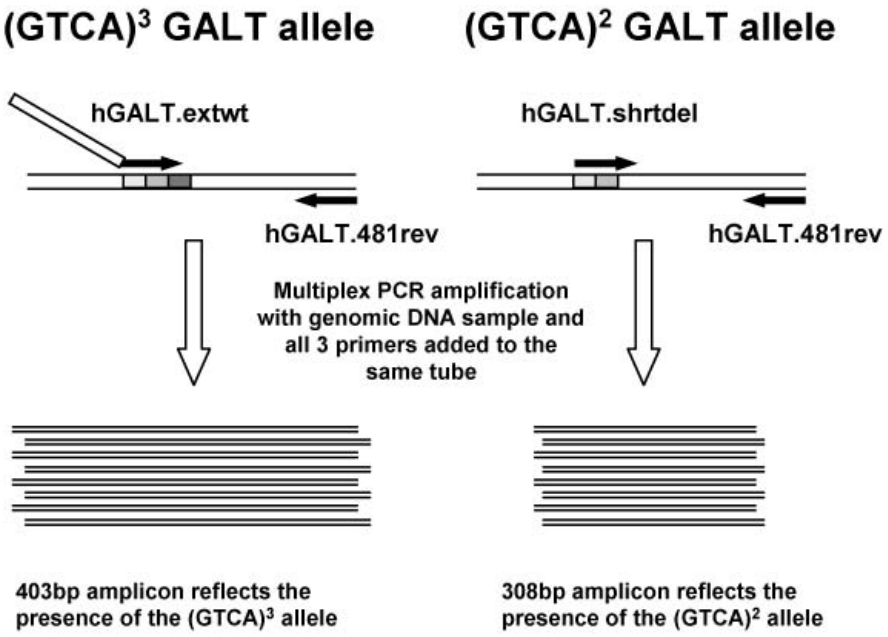

Figure A.2: Strategy for allele-specific amplification of $(GTCA)^2$ versus $(GTCA)^3$ 5' *GALT* sequences. As illustrated, the forward primer specific for the $(GTCA)^3$ allele includes a 91-bp "tail" of *S. cerevisiae* sequence that increases the size of the corresponding amplicon.

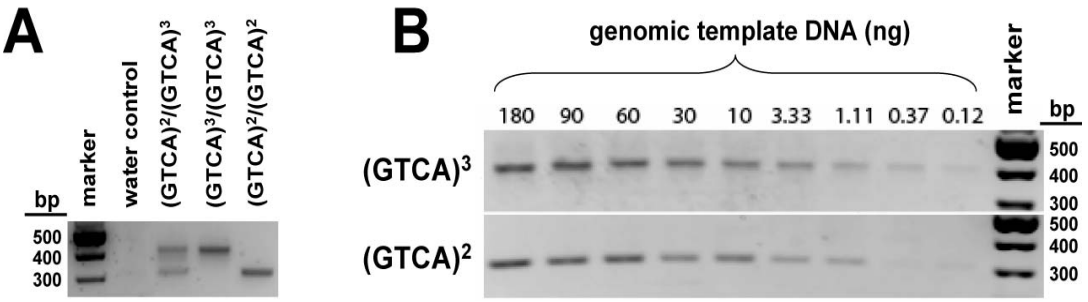

Figure A.3: Multiplex allele-specific amplification of $(GTCA)^2$ and $(GTCA)^3$ 5' *GALT* sequences. (A) Amplification of $(GTCA)^2/(GTCA)^2$ and $(GTCA)^3/(GTCA)^3$ templates demonstrates the specificity of the primer sets. (B) A dilution series of template levels establishes the tolerance of both amplification reactions to a wide range of template concentrations.

To test the sensitivity of this method to template concentration, we performed assays on wild-type and D2 homozygous genomic DNAs ranging in concentration from 1 ng/reaction to 180 ng/reaction. As illustrated (Figure A.3B), bands of the expected sizes, and the expected sizes only, were easily detected in all reaction lanes, demonstrating tolerance of the assay for a wide range of template concentrations.

Distribution of the 4-bp 5′ deletion among D1 and D2 GALT alleles

To explore the distribution of the $(GTCA)^2$ variant among D1 and D2 *GALT* alleles, we applied our assay to 215 genomic samples representing 430 *GALT* alleles, of which 238 were known to encode p.D314, and 192 were known to encode p.N314 (Table A.3). Of these 238 p.D314 alleles, 11 had previously tested positive for the p.L218(TTA) silent base substitution; the remainder were L218(CTA). Our results demonstrated the anticipated one-to-one correspondence between the *presence* of the 4-bp 5' deletion and the *absence* of the p.L218(TTA) variation among the D2 alleles (Table A.3). Our results also revealed a perfect one-to-one correspondence between the *absence* of the 4-bp 5' deletion and the *presence* of the p.L218(TTA) variation among D1 alleles (Table A.3). As expected, these results were also fully consistent with biochemical data associated with

each sample, with D2 alleles being partially impaired, and D1 alleles not impaired at all (data not shown).

GALT genotype of patient (biochemically confirmed)	# samples	# D2 alleles p.D314+ p.L218(CTA)	# D1 alleles p.D314+ p.L218L(TTA)	other alleles (N or G)	# alleles carrying $(GTCA)^2$	# alleles carrying $(GTCA)^3$
D2/D2	18	36	0	0	36	0
D2/N	52	52	0	52 N	52	52
D2/G	109	109	0	109 G	109	108*
D2/D1	1	1	1	0	1	1
D1/G	3	0	3	3 G	0	6
TOTALS	**183**	**198**	**4**	**164**	**198**	**167**

Table A.3: Distribution of the 4-bp 5' deletion among D1 and D2 alleles of *GALT*. D1 alleles were identified on the basis of p.D314+p.L218L(TTA); D2 alleles were identified on the basis of p.D314+p.L218(CTA). N alleles were identified on the basis of N314, no detected mutations, and normal biochemical activity. *One G allele in this cohort was the 5-kb deletion, which removes the 5' *GALT* sequence.

Absence of the 4-bp 5′ deletion among N (control) and G (classic galactosemia) GALT alleles that carry N314

To test the distribution of the 4-bp 5' deletion among control (N) and classic galactosemia (G) *GALT* alleles that encode p.N314, we applied our assay to 98 control genomic DNAs (196 control alleles) and 48 genomic DNAs derived from GG patients (96 G alleles). Of note, each of these alleles had already been shown to *not* carry p.D314 or p.L218(TTA). As expected, none of these alleles demonstrated the presence of the 4-bp 5' deletion (Table A.4).

GALT genotype of volunteer	# samples	# alleles	# alleles carrying $(GTCA)^2$	# alleles carrying $(GTCA)^3$
N/N (normal) (p.N314+p.L218(CTA))	98	196	0	196
G/G (classic) (p.N314+p.L218(CTA))	48	96	0	96

Table A.4: Distribution of the 4-bp promoter deletion among normal (N) and classic (G) galactosemia alleles of *GALT*.

Distribution of the 4-bp 5′ deletion among classic galactosemia (G) GALT alleles that also carry D314

From the many classic galactosemia patient DNA samples tested, we identified eight unrelated samples that encoded p.D314 on one or both alleles in *cis* with a classic (G) mutation. Indeed, of the 16 alleles represented, 11 carried p.D314 (Table A.5). The *GALT* genotypes identified in these samples illustrate two important points. First, classic galactosemia (G) mutations are found on both D1 and D2 genetic backgrounds. Second, we found no apparent association of the D1 or D2 background with a specific G mutation.

D2 GALT mRNA is under-represented in the blood of DN carriers

To test the hypothesis that D2 alleles express a lower level of message *in vivo* than wild-type alleles, we performed allele-specific quantitative RT-PCR on blood-derived RNA samples from DN (D2 carrier) volunteers, as well as from

Patient	G mutation genotype	D1 or D2-related variations
A	p.R204X / p.P265A	p.D314+ $(GTCA)^2$ / p.D314+ $(GTCA)^2$
B	p.Q207X / p.Q207X	p.D314+ $(GTCA)^2$ / p.D314+ $(GTCA)^2$
C	p.H132Q, p.R204X, p.P265A *cis*/*trans* relationships unknown	p.D314 / p.N314 $(GTCA)^2$ / $(GTCA)^3$
D	p.Q188R, p.R259Q, p.D273H *cis*/*trans* relationships unknown	p.D314+p.L218(TTA)+$(GTCA)^3$ / p.N314+$(GTCA)^3$
E	p.Q188R / p.R333W	p.D314+p.L218(TTA)+$(GTCA)^3$ / p.N314+$(GTCA)^3$
F	p.R204X / unknown	p.D314+$(GTCA)^2$ / p.D314+ $(GTCA)^2$
G	p.Q188R / unknown	p.D314+p.L218(TTA)+$(GTCA)^3$ / p.N314+$(GTCA)^3$
H	unknown / unknown	p.D314+ $(GTCA)^2$ / p.N314+$(GTCA)^3$

Table A.5: Distribution of the D1- and D2-related variations among *GALT* alleles that also carry a classic galactosemia (G) mutation. If the codon at 218 is not specified, it is CTA.

NN (control) and DD volunteers. Equivalent amounts of RNA derived from each sample were subjected to quantitative RT-PCR with each of two sets of primers: one specific for the p.N314 (normal) transcript and one specific for the p.D314 (D2) transcript. As anticipated, all NN samples amplified well with the N314-specific primers and showed only minimal (~3.5%) background signal when amplified with the p.D314-specific primers. Similarly, a DD sample amplified well with the p.D314-specific primers and showed only minimal (~1.5%) background signal when amplified with the N314-specific primers. As indicated (Table A.6), the ratio of p.N314:p.D314 signals, corrected for background, detected in each of the four DN samples was close to 54%:46%. Though small, the difference between this distribution and the null hypothesis (50%:50%) was quite statistically significant ($p < 0.002$). These data demonstrate that D2 alleles are

mildly under-expressed at the level of RNA *in vivo* relative to normal *GALT* alleles.

Sample	*GALT* genotype	Relative expression of p.N314 *GALT* message	Relative expression of p.D314 *GALT* message
1	p.D314+ $(GTCA)^2$ / N	0.525 ± 0.048	0.475 ± 0.048
2	p.D314+ $(GTCA)^2$ / N	0.573 ± 0.072	0.427 ± 0.072
3	p.D314+ $(GTCA)^2$ / N	0.541 ± 0.052	0.459 ± 0.052
4	p.D314+ $(GTCA)^2$ / N	0.530 ± 0.072	0.470 ± 0.072
	Total	0.540 ± 0.011 (n = 4)	0.460 ± 0.011 (n = 4, p<0.002))

Table A.6: Relative expression levels of the p.N314 and p.D314 *GALT* messages in DN carriers. These values were corrected for background cross-reactivity of the primers, which was measured at 1.5% for p.D314 signal and 3.5% for p.N314 signal.

GALT activity in hemolysates from DG patients

As a final test of the GALT activities attributable to D alleles, we examined GALT activity levels determined in the Emory Biochemical Genetics Lab for 133 NN samples, 90 DG samples, and 27 GG samples. As expected (Table A.7), average DG GALT values were about 21% of those in NN patients. Notably, however, the ranges of both sets were very large. There was a >2-fold range in the GALT levels of NN samples, and a >6-fold range in the GALT levels of DG samples. Indeed, "high-activity" DG samples had >60% of the GALT activity seen in "low-activity" NN samples. These ranges suggest there are likely many

factors, including GALT coding and non-coding sequences, and also factors beyond *GALT* sequence, that influence *GALT* expression or activity, or both, in human cells.

	GALT activity (μmol/hour/gram Hb)	
***GALT* genotype (n)**	**Average ± SD**	**range**
NN (133)	33.5 ± 6.8	20.1 → 47.8
DG (90)	7.2 ± 2.0	2.0 → 12.5
GG (27)	0.2 ± 0.6	0.0 → 2.4

Table A.7: GALT activities detected in hemolysates from GG, DG, and NN individuals.

A.5 DISCUSSION

The data presented here both extend and clarify our understanding of the origins, distribution, and expression of the D2 *GALT* allele associated with Duarte galactosemia.

Origins

To explore the origins of the p.D314, p.L218(TTA), and $(GTCA)^2$ variations found on D1 and/or D2 *GALT* alleles, we used interspecies sequence comparisons to reconstruct the variants most likely to represent the ancestral state. These analyses clearly implicated p.D314 as the ancestral allele and p.N314

as a polymorphism that may be predominant in modern humans, but which also appears unique to humans. Of note, p.D314 is not found with any significant frequency in populations of African descent, although it is found in African-Americans, presumably due to admixture (26). This distribution pattern suggests that the p.D314N variant arose in Africa very early in human evolution, and that the persistence of the p.D314 allele in European and, to a lesser extent, Asian populations may reflect a founder effect or other factors acting upon descendants of the early waves of human migration out of Africa.

We similarly noted that the 4-bp 5' deletion found in D2 *GALT* alleles is a one-unit contraction of a tetranucleotide repeat sequence that has otherwise expanded through evolution from placental mammals (one repeat unit), to primates (two repeat units), to hominids, including humans (three repeat units). That the repeat number of this tetranucleotide sequence has varied through evolution is consistent with the instability observed in other repeat tracts in the mammalian genome.

Distribution of the 4-bp 5′ deletion on human GALT alleles

To explore the distribution of the $(GTCA)^2$ sequence variant in human *GALT* alleles, we developed and applied a simple and robust PCR-based assay to identify and distinguish wild-type $(GTCA)^3$ from D2-associated $(GTCA)^2$ 5' sequences. Using this assay we confirmed the previously described strict linkage

of the $(GTCA)^2$ sequence with p.D314+L218(CTA) coding sequence alleles, and the strict repulsion of the $(GTCA)^2$ 5′ sequence with the p.N314 and p.D314+p.L218(TTA) coding sequence alleles. Although each of these relationships had been reported previously (e.g. (20,24,25)), to our knowledge ours is the largest pan-ethnic collection of GG, DG, and control samples genotyped with regard to the 4-bp 5′ deletion. It is also noteworthy that, though the HapMap database cites allele frequencies for the p.D314 and p.L218(TTA) polymorphisms, that study, which restricted itself to SNP polymorphisms, did not assay the $(GTCA)^2$ promoter allele in human *GALT*. Applying the apparently tight linkage of the $(GTCA)^2$ 5′ variation with *GALT* alleles that carry p.D314 but lack p.L218(TTA), we can estimate the likely allele frequency of this non-coding variation in the HapMap-tested European population at near 6-7% (the overall frequency of p.D314 (11%) minus the frequency of the p.L218(TTA) allele (4-5%)), with lower frequencies in other human populations.

It is also interesting to note that the p.D314 sequence, with or without p.L218(TTA), is also found in *cis* with many different G (classic galactosemia) mutations, suggesting that through the course of human history, although most G mutations have arisen on the predominant p.N314 *GALT* genetic background, others have arisen on p.D314+p.L218(TTA) backgrounds or on p.D314+$(GTCA)^2$ backgrounds. In short, with the exception of a small p.D314-G data set, we see no

clear evidence of preferential association between specific classic galactosemia mutations and any given *GALT* genetic background. Assuming that all classic galactosemia *GALT* mutations are younger than any of the *GALT* polymorphisms discussed, and assuming G mutations arise independently of genetic background, we therefore predict that in European populations approximately 89% of *GALT* mutations will be found on an p.N314 background, 6-7% on a D2 (p.D314+(GTCA)^2) background, and 4-5% on a D1 (p.D314+p.L218(TTA)) background. In non-European populations nearly all the *GALT* mutations will be found on an p.N314 background.

Expression

Another goal of this work was to clarify the basis of the ~50% decrease in GALT expression and activity typically associated with D2 alleles. As explained in the Introduction, earlier reports addressing this point were contradictory. Some studies claimed normal D2 *GALT* mRNA levels and attributed the decrease to altered p.N314D protein stability (14,23); others claimed decreased D2 GALT mRNA levels ostensibly result from impaired D2 promoter function (24,25). All of these studies were limited by extremely small sample sizes and by analyses of cultured transformed cell lines as opposed to primary tissues. Given the >6-fold difference in GALT activity attributed to Duarte alleles in different DG patients

(Table A.7), it is difficult to generalize from the <2-fold differences in results obtained using samples from individual study volunteers, much less individual cell lines derived from those individual volunteers. The decreased p.N314D protein stability hypothesis needed to account for the apparently normal or above normal levels of GALT protein and activity associated with D1 alleles, which also encode p.N314D. The explanation posed (but not tested) – namely, that improved translation efficiency of D1 messages due presence of the silent p.L218(TTA) variant (14,23) – is also problematic. Both the D1-associated TTA (Leu) and D2-associated CTA (Leu) codons occur at other places in N, D1, and D2 *GALT* alleles. Indeed, N and D2 alleles each carry a total of six CTA codons and one TTA codon, while D1 alleles carry a total of five CTA codons and two TTA codons.

To test whether D2 *GALT* alleles are under-expressed relative to N alleles in human blood, we applied allele-specific qRT-PCR to RNA samples derived from each of four individuals heterozygous for the D2 *GALT* allele. This study design had the advantages of primary human tissue samples and the inclusion of an internal control within each sample. Our results demonstrated a small but consistent and statistically significant under-representation of p.D314 encoding message relative to "normal" p.N314 encoding message in each sample tested. Specifically, the mRNA distribution detected was 54% p.N314 to 46% p.D314

encoding messages. While statistically distinct from the 50%:50% distribution predicted if there were no decrease in D2 *GALT* mRNA, this 54%:46% distribution also falls short of the 67% p.N314 to 33% p.D314 distribution predicted if mRNA deficits could account fully for the average 50% loss of expression/activity typically attributed to D2 alleles.

The reason for this "middle ground" result remains unclear, though there may be a small protein expression or stability effect. It is possible that the full effect is RNA-mediated, but that the primers and qRT-PCR technology applied here were unable to confirm it. Another possibility is that individual-to-individual naturally occurring variation between samples prevented unambiguous demonstration of the predicted 67%:33% p.N314:p.D314 message distribution.

When considering our results, it is also important to note that, unlike prior reports, our studies used mRNA samples isolated directly from whole blood and not from cultured transformed cell lines. These RNAs therefore derived predominantly from circulating white cells, whereas the GALT activity values listed here (Table A.7) or reported previously (e.g. (3,14)) were derived from red blood cells or from cultured transformed cells. To our knowledge, there have been no systematic studies of D2-associated GALT activity or abundance in circulating white cells. It is therefore possible that the 54%:46% distribution

reported here is an accurate reflection of the relative levels of p.N314 to p.D314 encoding *GALT* messages in the circulating white blood cells of DN carriers. Regardless, it is now clear that altered mRNA levels can indeed account for at least some of the "missing" GALT activity attributable to D2 *GALT* alleles.

Testing for the Duarte-2 allele

Most clinical diagnostic labs currently test for the presence or absence of a Duarte *GALT* allele using a combination of molecular and biochemical studies. The molecular studies generally involve a direct assay for p.N314D and/or p.L218(TTA) alleles, or full exon sequencing. The biochemical studies typically involve a GALT activity assay that will show high activity in the case of a D1 allele and low but detectable activity in the case of a D2 allele. Isozyme studies are used to detect the shifted banding pattern characteristic of both D1 and D2 GALT. These combined approaches can correctly identify and distinguish Duarte-1 from Duarte-2 *GALT* alleles; however, given the broad range of GALT activity values obtained for the suspected DG patient population, together with the realization that p.N314D and p.L218(TTA) are themselves most likely linked polymorphisms rather than causal mutations, an alternative diagnostic plan might prove beneficial.

We propose that DG patients could be accurately diagnosed via a combination of GALT enzyme assay and molecular studies to query the coding sequence and/or candidate G mutations, with an added test for the presence or absence of the 5' 4-bp deletion. If low but detectable activity were present, one G mutation found, and the 4-bp deletion seen, the patient would most likely have DG galactosemia. Defining the presence or absence of the p.N314D substitution or a shifted isozyme banding pattern would be superfluous. Given the prevalence of p.N314D in the general population and its linkage to D1, D2, and G *GALT* alleles, its utility as a clinically relevant marker is compromised at best. In fact, if the 5' 4-bp deletion is causative, tests that rely on the p.N314D polymorphism, with or without p.L218(TTA), could lead to faulty conclusions in any individual with a recent recombination between the 5' deletion and the tested coding sequence variants.

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CONFLICT OF INTEREST STATEMENT

All authors have submitted signed statements that they have no conflicts of interest for this project.

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