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***Drosophila melanogaster* models of galactosemia**

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Abstract

The galactosemias are a family of autosomal recessive genetic disorders resulting from impaired function of the Leloir Pathway of galactose metabolism. Type I, or classic galactosemia, results from profound deficiency of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme in the Leloir Pathway. Type II galactosemia results from profound deficiency of galactokinase (GALK), the first enzyme in the Leloir Pathway. Type III galactosemia results from partial deficiency of UDP galactose 4'-epimerase (GALE), the third enzyme in the Leloir Pathway. Although at least classic galactosemia has been recognized clinically for more than 100 years, and detectable by newborn screening for more than 50 years, all three galactosemias remain poorly understood. Early detection and dietary restriction of galactose prevent neonatal lethality, but many affected infants grow to experience a broad range of developmental and other disabilities. To date, there is no intervention known that prevents or reverses these long-term complications. *Drosophila melanogaster* provides a genetically and biochemically facile model for these conditions, enabling studies that address mechanism and open the door for novel approaches to intervention.

Keywords

galactose; galactosemia; metabolic disorder; newborn screening; motor defect

1. Galactose metabolism via the Leloir Pathway

Galactose is a naturally occurring monosaccharide that is found at high levels in milk and dairy products, and at low levels in many other foods. Galactose is also synthesized endogenously. In species ranging from *E. coli* to *Drosophila* and humans, galactose is metabolized predominantly via the Leloir Pathway (Figure 1, [15]), which is comprised of three enzymes.

The first Leloir pathway enzyme, galactokinase (GALK, EC 2.7.1.6), catalyzes the phosphorylation of galactose to galactose-1-phosphate (Gal-1P) at the expense of ATP. The second enzyme, galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12), catalyzes a two-step reaction. In the first step, GALT cleaves UDPglucose (UDPGlc), releasing glucose-1-phosphate (Glc-1P) and retaining UMP covalently bound to a histidine residue in the active site. In the second step, Gal-1P enters the active site and receives the UMP group,

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restoring the active site histidine and creating UDPgalactose (UDPGal), which is released. The third Leloir enzyme, UDPgalactose 4'-epimerase (GALE, EC 5.1.3.2) interconverts UDPgal and UDPglc. Acting together, the three enzymes of the Leloir Pathway therefore convert galactose into Glc-1P at the expense of ATP (Figure 1).

In many metazoans, including both humans and *Drosophila*, GALE also interconverts a larger substrate pair: UDP-N-acetylgalactosamine (UDPGalNAc) and UDP-N-acetylglucosamine (UDPGlcNAc). GALE therefore not only functions in the Leloir Pathway but also maintains the ratios of UDPGal to UDPGlc, and UDPGalNAc to UDPGlcNAc, and allows for the endogenous synthesis of galactose when exogenous sources are lacking. All four of the UDP sugars recognized by GALE serve as key substrates for the biosynthesis of glycoproteins and glycolipids in humans, *Drosophila*, and other species.

Alternate pathways of galactose metabolism

While the Leloir pathway is the predominant route of galactose metabolism, minor “bypass” pathways also exist (Figure 1). These include oxidation of galactose to galactonate by galactose dehydrogenase, reduction of galactose to galactitol by aldose reductase, and conversion of galactose-1P to UDPgalactose by UDPglucose pyrophosphorylase (UGP) (reviewed in [10]). These bypass pathways are generally considered insignificant when the Leloir pathway is functioning; but when the Leloir pathway is blocked by deficiency of GALK, GALT, or GALE these alternate pathways may become extremely important.

Three types of galactosemias

Inherited mutations leading to deficiency of any of the three enzymes of the Leloir Pathway (GALK, GALT, or GALE) result in a form of galactosemia. The clinical presentation and severity of the disorder may vary depending on which enzyme is affected, the degree of impairment, and other genetic and environmental factors – most of which remain poorly understood. Of note, while sometimes mistakenly confused with lactose intolerance, a common and generally mild adult-onset condition resulting from reduced ability to digest lactose into its constituent monosaccharides (glucose and galactose), galactosemia results from the inability to fully metabolize galactose itself, presents clinically in infancy, and can be lethal.

Type I, or classic galactosemia—The most clinically severe form of galactosemia in most populations is called Type I, or classic galactosemia (CG; reviewed in [4, 10]). CG results from profound deficiency of GALT, the middle enzyme of the Leloir pathway (Figure 1). Population-based newborn screening demonstrates that the prevalence of CG is about 1/50,000 in the United States [27]; however, prevalence can vary markedly among different ancestral groups.

Infants with CG may appear normal at birth but develop a rapid progression of potentially lethal sequelae within days of beginning to consume milk – either breast milk or a milk-based formula. Symptoms can escalate in days from vomiting and diarrhea to jaundice, liver disease, failure to thrive, and *E. coli* sepsis, which is a common cause of neonatal death.

If diagnosed early, an affected infant may be spared the trauma of acute disease by rapid dietary restriction of galactose, generally achieved by switching the baby from milk to a low galactose soy or elemental formula [3]. This simple intervention prevents or reverses the potentially lethal symptoms of CG, but fails to prevent most affected infants from experiencing a constellation of complications as they grow. Common complications of CG despite early and continued dietary restriction of galactose include growth delays in both boys and girls, cognitive and/or behavioral disabilities, speech difficulties, and motor disturbance. At least 80% or more of affected girls and young women also experience primary or premature ovarian insufficiency. At present, residual GALT activity is the only documented modifier of outcome severity in CG [31, 35]. Patients with even trace residual GALT activity detectable in a model system (yeast [28]) tend to show milder outcomes, and those with 10% or more GALT activity may be very mildly affected (reviewed in [4, 10]). Other modifiers of outcome, beyond galactose exposure in infancy and residual GALT activity, likely exist but remain undefined.

Type II, or galactokinase (GALK)-deficiency galactosemia—Type II galactosemia results from profound deficiency of GALK (reviewed in [10]). Because many newborn screening programs are not designed to detect GALK deficiency, the population prevalence and natural history of this condition remain poorly understood.

Based on the limited information available, profound GALK deficiency was long considered extremely rare with common clinical sequelae restricted to cataracts that could be prevented by early and continued dietary restriction of galactose [5]. This conclusion was challenged in 2011, however, when newborn screening and follow-up results from Germany reported a population prevalence of GALK deficiency close to that of GALT deficiency [14]. Of note, the prevalence of GALK deficiency appears to vary considerably by ancestral group, and the increased prevalence detected in Germany was attributed to changing demographics of the newborn population rather than altered screening procedures. Of concern, follow-up studies of the infants diagnosed with GALK-deficiency in Germany demonstrated that close to 30% exhibited cognitive disabilities by childhood, and this long-term complication was associated with continued galactose exposure, but not consanguinity [14]. Further studies will be required to confirm the generalizability of these results.

Type III, or epimerase (GALE) deficiency galactosemia—Type III galactosemia results from partial deficiency of GALE, the third Leloir pathway enzyme [9]. Like GALK deficiency, GALE deficiency goes undetected by many newborn screening programs so what is known about the prevalence and natural history of the disorder is limited.

Like CG, clinical severity in GALE-deficiency reflects many factors, including the level of residual GALE activity present. Unlike CG, however, no live births have been reported with complete loss of GALE, and the most severely affected patients described [39] nonetheless demonstrate detectable GALE, at least in some tissues [26, 40, 41]. GALE deficiency is now considered a continuum disorder, ranging from the apparently benign peripheral epimerase deficiency [11], characterized by biochemical impairment apparently restricted to the red blood cells (RBC) and circulating white blood cells [12, 25], through intermediate epimerase deficiency [26], in which biochemical impairment is profound in RBC, but partial in other

cell types, to generalized epimerase deficiency [16], which presents with symptoms reminiscent of CG following neonatal milk exposure, and GALE deficiency that is profound, but not complete, in all tissues examined.

Patients with peripheral or intermediate GALE deficiency generally remain clinically well despite milk exposure in infancy, and patients with generalized GALE deficiency respond quickly to dietary restriction of milk. The long-term outcomes associated with different levels of GALE deficiency remain unclear, complicated by consanguinity among many of the patients reported with generalized epimerase deficiency [39], and the small numbers and limited follow-up information available for patients with the intermediate disorder [9].

Galactose metabolism in *Drosophila melanogaster*

Like humans, *Drosophila melanogaster* metabolize galactose predominantly via the Leloir pathway. The *D. melanogaster* genes that encode GALK, GALT, and GALE are *CG5288* on chromosome 3, referred to here as *dGALK* and in FlyBase (<http://flybase.org/>) as *Galk*, *CG9232* on chromosome 2, referred to here as *dGALT* and in FlyBase (<http://flybase.org/>) as *Galt*, and *CG12030* on chromosome 3, referred to here as *dGALE* and in FlyBase (<http://flybase.org/>) as *Gale*, respectively [6, 23]. The predicted protein product of *dGALK* shows 27% amino acid sequence identity and 44% amino acid sequence similarity with human GALK. The predicted protein product of *dGALT* shows 57% amino acid sequence identity and 72% amino acid sequence similarity with human GALT. Finally, the predicted protein product of *dGALE* shows 60% amino acid sequence identity and 76% amino acid sequence similarity with human GALE. While the three Leloir genes in *D. melanogaster* were originally identified on the basis of sequence homology, they have all now been confirmed based on loss of the expected enzyme activity following disruption or deletion of the corresponding gene (explained below, and Daenzer et al, in preparation). *D. melanogaster* is therefore an excellent model system in which to study the galactosemias.

2. A *D. melanogaster* model of classic galactosemia

2.1 Creating GALT-null *Drosophila*

In 2010, Kushner and colleagues [23] reported the creation of a *dGALT* null allele (*dGALT*^{AP2}) by imprecise excision of an existing P-element insertion, KG00049, in the 5′-untranslated region (UTR) of the *CG9232* gene. The excision removed 1647 bp of sequence, including almost the entire *dGALT* gene [23]. The mutated allele was designated *dGALT*^{AP2}. Because *dGALT* is encoded within an intron of *cup*, a fly gene important for female fertility [22], Kushner and colleagues further tested whether a chromosome carrying the *dGALT*^{AP2} allele could complement a strong allele of *cup* (*cup*⁰¹³⁵⁵); it did [23]. Biochemical studies of lysates prepared from *dGALT*^{AP2} homozygotes confirmed complete loss of GALT activity and normal, or near-normal, levels of GALK and GALE activity [23]. Further studies, described below, also demonstrated that a human *GALT* transgene expressed in these *dGALT*-null flies complemented both the loss of GALT activity and the mutant phenotypes observed [23].

2.2 Characterizing the galactose-sensitivity of GALT-null larvae

Patients with classic galactosemia typically die as infants if galactose, generally in the form of milk, is not removed from their diet [4]. Once switched to a low-galactose diet such as soy formula, these infants survive and, at least initially, appear to thrive.

To determine whether a similar sensitivity to dietary galactose would be evident in GALT-null *Drosophila*, Kushner and colleagues tested the ability of larvae homozygous for the *dGALT*^{AP2} allele to survive to adulthood in the presence versus absence of galactose [23]. First, crosses of heterozygotes on molasses food were tested; these yielded viable homozygotes in almost the expected proportion, confirming that loss of GALT in flies, as in humans, is not incompatible with life.

Next, Kushner and colleagues tested the ability of GALT-null larvae to survive on food containing glucose spiked with different levels of galactose. Their results demonstrated a clear dose-response sensitivity of the GALT-null, but not control, larvae. Specifically, on food containing 555mM glucose the majority of GALT-null larvae, like controls, survived to adulthood. On food containing 555mM glucose plus 222mM galactose, however, while the control animals continued to thrive, none, or almost none, of the GALT-null larvae survived to adulthood. When an intermediate level of galactose was used, the surviving fraction was intermediate. When a comparable level of mannose was spiked into the food in place of galactose there was no detectable impact on survival of either GALT-null or control animals, demonstrating specificity of the result for galactose.

To confirm that the galactose sensitivity of *dGALT*^{AP2} homozygotes resulted from loss of GALT and not some other off-target effect of the deletion, Kushner and colleagues created a human *GALT* transgene and crossed this allele, with an appropriate driver, into flies lacking endogenous GALT. As expected, expression of human GALT rescued survival of *dGALT*^{AP2} homozygotes on food containing otherwise lethal levels of galactose.

To test whether GALT-null *Drosophila* larvae, like neonatal patients, could be rescued by switching from high (222mM) to low (0mM) galactose food, Kushner and colleagues conducted a time course experiment in which larvae were physically transferred from one food source to another – and then followed to see if they survived. Specifically, cohorts of L1 larvae were collected from egg-laying plates manually using a small spatula and transferred to the desired food (e.g. glucose food) for the desired length of time, then transferred again into individual wells of a 96 well plate containing the second food type (e.g. glucose plus 222mM galactose food) where they could be monitored daily for viability and development. The results clearly demonstrated that GALT-null larvae could be rescued by transferring to 0mM galactose food after 2 days on high galactose food (L1 to L2 stage). After four days on high galactose food only a fraction could be rescued (L2 to L3 stage). After 6 days on high galactose food (L3 stage) almost none could be rescued. Conversely, a fraction of GALT-null larvae transferred to high galactose food after up to 4 days on 0mM galactose food (L2 to L3) nonetheless survived, and larvae transferred to high galactose food after 6 days on 0mM galactose food (L3s) almost uniformly survived. These data suggest that the window of greatest galactose sensitivity is in the first days of life, and that the “damage” done by early galactose exposure is largely reversible if caught sufficiently early.

This result is fully consistent with studies documenting that outcomes of patients diagnosed with classic galactosemia as infants after beginning to drink milk are comparable to outcomes of affected infants who never drank milk [17].

2.3 A motor defect in adult GALT-null *Drosophila*

A significant fraction of patients with classic galactosemia demonstrate motor complications, especially as adults [10, 38], despite early detection and continued dietary restriction of galactose. To test whether GALT-null flies also exhibit some form of motor disturbance, Kushner and colleagues subjected cohorts of mutant and control animals raised on low galactose food to a repetitive climbing task using a “counter current” device first introduced by S. Benzer in the 1960s [2]. Consistent with the results of Benzer, Kushner and colleagues found that >60% of control flies climbed well, ending up in the final tube of the device. In contrast, cohorts of GALT-null flies ended up distributed across the series of tubes, with only about 20% making it to the final tube. As above, expression of a human *GALT* transgene rescued the climbing defect otherwise seen in *dGALT*^{AP2} homozygotes.

2.4 Mediators of long-term outcome severity in GALT-null *Drosophila*

One of the striking features of classic galactosemia is the variability of long-term outcomes among patients. Ryan and colleagues [30] applied the GALT-null *Drosophila* model in a set of experiments testing the impact of two candidate modifiers of long-term outcome severity: trace residual GALT activity and low-level dietary exposure to galactose. The outcome followed was ability of adult flies to traverse a counter-current device to reach the final tube.

Trace GALT activity was achieved by using a leaky human *GALT* transgene, *UAS-hGALT*^{10A11}, in the absence of a *GAL4* driver. When present in single copy this transgene conferred 2.3% wild-type GALT activity; when present in double copy this transgene conferred 6.5% wild-type GALT activity [30]. In both cases, adult flies expressing these trace levels of GALT activity demonstrated significantly improved climbing ability as measured using the counter-current device. Consistent with this result, subsequent studies in patients with classic galactosemia demonstrated that *GALT* genotypes linked with at least 0.4% predicted residual GALT activity were associated with significantly milder scholastic [31] and ovarian [35] outcomes.

Impact of low-level galactose exposure on long-term outcome was tested by comparing the climbing phenotypes of flies raised on food containing either glucose as the sole sugar, or glucose plus a sub-lethal dose of galactose (50mM). Of note, while insufficient to cause mortality in GALT-null larvae, 50mM galactose added to the fly food did cause a >20 fold increase in the level of Gal-1P detected in lysates [30]. Nonetheless, being reared in the presence of 50mM galactose did not cause any significant impact on the climbing phenotype of control or GALT-null flies [30]. This result was consistent with anecdotal data from patients, suggesting that more lenient diets that include slightly increased galactose are not deleterious to long-term outcomes [19, 37]. These data are also consistent with reports demonstrating a lack of association between patient Gal-1P levels and long-term outcome severity (reviewed in [10]).

2.5 Oxidative stress and GALT-deficiency

Jumbo-Lucioni and colleagues [20] applied the GALT-null *Drosophila* model to test the hypothesis that galactose exposure leads to increased oxidative stress, and that GALT deficiency makes animals more vulnerable to that stress. A number of different experimental approaches were applied. First, these authors tested the impact of oxidants (paraquat and DMSO) and antioxidants (vitamin C and α -mangostin) on survival of control and GALT-null *Drosophila* larvae exposed to food containing 555mM glucose with or without 200mM galactose. Both oxidants compromised the survival of GALT-null but not control larvae, and both antioxidants improved survival of GALT-null but not control larvae. Of note, these changes in survival were not accompanied by significant changes in the levels of Gal-1P accumulated, suggesting that the mechanism of oxidant or antioxidant impact was either independent, or downstream, of Gal-1P.

Finally, Jumbo-Lucioni and colleagues [20] tested the impact of galactose, paraquat, and vitamin C on the levels and ratios of oxidized and reduced glutathione and cysteine, and on expression of two glutathione-S-transferase genes known to be responsive to oxidative stress. The results demonstrated that galactose exposure alone caused modest changes in markers of oxidative stress, and that the combination of galactose plus paraquat appeared almost synergistic. While changes were seen in both controls and GALT-null *Drosophila*, the levels of these changes differed between cases and controls, suggesting that loss of GALT may impact the pathways of either sensing or response to oxidative stress. These results also raised the possibility that patients with classic galactosemia might be unusually vulnerable to environmental oxidants or other exposures.

In a follow-up study [21], Jumbo-Lucioni and colleagues extended from their earlier findings by testing the impact of each of two manganese porphyrin mimics of superoxide dismutase (SOD), MnTnBuOE-2-PyP5⁺ and MnTE-2-PyP5⁺ [1] on both acute and long-term outcomes in GALT-null *Drosophila*. Both SOD mimics significantly improved the survival rates of GALT-null but not control larvae exposed to galactose. As seen earlier, this impact occurred despite the continued accumulation of high Gal-1P in the galactose-exposed GALT-null animals. One mimic, MnTE-2-PyP5⁺, also notably improved the climbing phenotype of GALT-null adult flies suggesting that oxidative stress may contribute to long-term as well as acute outcomes in classic galactosemia.

2.6 Potential impact of GALT deficiency on synaptic architecture and synaptomatrix glycosylation at the neuromuscular junction in *Drosophila*

Recently, Jumbo-Lucioni and colleagues [18] reported further studies of GALT-null *Drosophila* raising the possibility of altered synaptic architecture in the mutant animals. Specifically, these authors found altered bouton number and also altered lectin binding at the neuromuscular junction in GALT-null larvae. These authors also reported an abnormal coordination/movement phenotype in GALT-null larvae. Surprisingly, many of the phenotypes reported were both relieved by deletion of GALK and also phenocopied in wild-type animals exposed to galactose. Given that infants who do not have galactosemia routinely drink large quantities of milk, which contains high levels of galactose, without experiencing neuromuscular problems, it is difficult to know how to relate these results in

Drosophila to human outcomes. Further studies will be required to connect these findings with potential mechanisms of outcome among galactosemia patients.

3. A *D. melanogaster* model of epimerase deficiency galactosemia

3.1 Creation of GALE-deficient *Drosophila*

Sanders and colleagues [32] developed the first whole-animal model of GALE deficiency using *Drosophila melanogaster*. These authors used two severe loss of function alleles and one hypomorphic allele of *dGALE* to characterize the effects of GALE loss. One loss-of-function allele, *dGALE*^{f00624}, was the result of a P-element insertion in the second intron of *dGALE*. This allele (*PBac{WH}CG12030*^{f00624}) was found in the Exelixis collection [36]. Flies homozygous for *dGALE*^{f00624} displayed 100% lethality, and lysates from flies heterozygous for this allele exhibited a 50% reduction in GALE activity compared to lysates from wild-type animals.

Two additional alleles were created by imprecise excision of a P-element, *P{EPgy2}CG12030*^{EY22205}, located in the 5' untranslated region of the *dGALE* gene. One of these excisions, *dGALE*^Y, resulted in a 1339-bp deletion in *dGALE* that included the first coding exon. This allele displayed a homozygous lethal phenotype that was rescued by expression of a human *GALE* transgene (described below [32]), and lysates from flies heterozygous for *dGALE*^Y demonstrated approximately 50% GALE enzymatic activity compared with lysates from wild-type animals. Another allele, *dGALE*^h, resulted from partial excision of the P-element such that 1500 bp of P-element sequence was left behind in the 5' untranslated region; the rest of the *dGALE* gene remained unchanged. This disruption of the 5' UTR yielded a hypomorphic allele; flies homozygous for *dGALE*^h demonstrated approximately 8% of wild-type GALE activity.

3.2. Characterizing the phenotype of GALE deficiency in *Drosophila*

The work described in this section was published by Sanders and colleagues [32] unless otherwise noted.

3.2.1. *dGALE* is required throughout development—Both profound loss-of-function *dGALE* alleles, *dGALE*^{f00624} and *dGALE*^Y, demonstrated a homozygous lethal phenotype, and trans-heterozygotes were also not viable. These mutants displayed a variable period of death such that some animals died during embryonic development prior to hatching, while others survived to the second instar larval stage (L2). Because maternally loaded *dGALE* mRNA or protein could be present during early stages of development, Sanders et. al. created *dGALE*^{f00624} germline clone mutants which were unable to contribute maternal loading of GALE to their offspring. The GALE-null offspring from these mothers died uniformly in late embryogenesis. Notably, a wild-type paternal *dGALE* allele rescued the germline clone mutants. Together, these data demonstrated that *dGALE* is essential during late embryogenesis, but is not required before the onset of zygotic transcription.

To look at the requirement for *GALE* in developmental stages beyond embryogenesis, Sanders et. al. used RNAi knockdown of *dGALE* in combination with a temperature sensitive allele of *GAL80* to generate a series of *Drosophila* that experienced knockdown of

dGALE on successive days of development. Knockdown during larval or pupal stages of development resulted in less than 8% residual GALE activity compared to control animals. *GALE* loss was lethal at all stages of embryonic and larval development. During pupal stages of development, *GALE* knockdown was not lethal, however the adult flies displayed problems including a reduced lifespan and infertility. These adult phenotypes were dependent on the stage of pupal development at which *GALE* was lost, with the phenotype being more severe the earlier *GALE* knockdown was initiated. The effects of *GALE* knockdown initiated after eclosion of adult flies could not be evaluated, as the knockdown was inefficient when initiated at this stage.

3.2.2. GALE activity is required in specific tissues in *Drosophila*: To assess tissue-specific requirements for GALE, Sanders and colleagues [32] used two approaches: first, knocking down endogenous *dGALE* expression in specific tissues via RNAi; and second, expressing a human *GALE* transgene in various tissues of otherwise GALE-null animals. Knockdown of *dGALE* was tested using *GAL4* drivers specific to five tissues or tissue combinations. Knockdown of *dGALE* in the salivary gland, neurons, larval brain and fat body, or eye did not result in any clear phenotypes. In contrast, knockdown in the embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules and small intestine by the *drm-GAL4* driver resulted in lethality, indicating that GALE is required in some or all of these tissues. The same drivers were also used to express *hGALE* in a tissue-specific manner in *dGALE*-null animals to test whether human GALE (*hGALE*) expression in any of these tissues was sufficient for rescue. *hGALE* driven by *drm-GAL4* in the embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules and small intestine resulted in a partial rescue; however, the proportion of animals rescued was approximately one tenth the expected number. None of the other drivers tested resulted in any degree of rescue. The authors speculated that the requirement for *dGALE* in the gut and tubules might be related to the expression of glycans, since GALE produces UDP-galNAc, the obligate first sugar donor in mucin-type O-linked glycosylation.

3.2.3. Galactose sensitivity of *dGALE* hypomorphs—Infants with generalized epimerase deficiency galactosemia demonstrate acute sensitivity to dietary galactose [13, 16, 33]. Sanders and colleagues [32] tested whether *Drosophila* with low-level GALE activity would also display sensitivity to galactose. Specifically, flies homozygous for the hypomorphic allele *dGALE^h*, which retains approximately 8% GALE activity, were challenged in development with a diet containing 555 mM glucose plus 111 mM galactose. In progeny of crosses where the expected ratio of *dGALE^h* homozygous offspring was 50%, only about 25% of the surviving animals were homozygotes. However, when the food contained only glucose, or glucose plus mannose, rather than galactose, the expected proportion of homozygous offspring was observed. These results confirmed that galactose exposure caused lethality in development of *Drosophila* lacking normal GALE activity.

3.2.4. GALE-deficient *Drosophila* accumulate a high level of Gal-1P—*GALE* knockdown larvae developing on food containing 555 mM glucose plus 111 mM galactose accumulated approximately twentyfold more Gal-1P than corresponding animals developing on food containing only 555 mM glucose, with no galactose. This increase is much larger

than was seen in control larvae expressing wild-type *dGALE*, which accumulated only about twice as much Gal-1P on galactose-containing food compared with “glucose only” food. The elevated levels of Gal-1P in *GALE*-deficient flies was consistent with data from *GALE*-impaired yeast [8, 29], mammalian tissue culture cells [34] and patients [26], all of which accumulate Gal-1P upon exposure to high levels of environmental galactose

3.3 Using *Drosophila* to dissect the differential roles of *GALE* in development

The work described in this section is from Daenzer and colleagues [7] unless otherwise noted.

3.3.1. Uncoupling the two activities of *GALE* in *Drosophila*: *GALE* from both humans and *Drosophila* interconverts two sets of substrates: UDPgal/UDPglc and UDPgalNAc/UDPglcNAc (Figure 1). To uncouple and test the developmental roles of these activities individually, Daenzer and colleagues created flies with activity toward only one or the other substrate set. This substrate specificity was achieved by effectively replacing *dGALE* expression with expression of either of two prokaryotic epimerase genes, each encoding a product capable of interconverting only one substrate pair: *eGALE*, which interconverts only UDPgal/UDPglc, and *wbgU*, which interconverts only UDPgalNAc/UDPglcNAc. Expressing these microbial transgenes in *dGALE*-deficient *Drosophila* resulted in animals with only one or the other *GALE* activity.

3.3.2. Both *GALE* activities play essential roles during development—As explained above, Sanders and colleagues [32] showed that *GALE* is essential for *Drosophila* development, and that *dGALE*-deficient animals die in embryogenesis. By expressing *eGALE* only, *wbgU* only, or both *eGALE* and *wbgU* together in *dGALE*-deficient embryos, Daenzer and colleagues confirmed that *GALE* activity toward both substrate pairs is essential for survival of *Drosophila*. Specifically, neither microbial transgene alone enabled survival, but together they did, and further, *hGALE*, which like *dGALE* recognizes both substrate pairs, was also sufficient.

Using a modified version of the conditional knockdown technique described in Sanders et al. [32], Daenzer and colleagues [7] created a time course series of *Drosophila* that experienced *dGALE* knockdown with concurrent induction of either *hGALE*, *eGALE*, *wbgU*, or both *eGALE* and *wbgU* at successive stages of development. As expected, RNAi knockdown of *dGALE* during both embryonic and larval stages of development was lethal, but was rescued by expression of either *hGALE* or *eGALE* + *wbgU*. Surprisingly, expression of either *eGALE* or *wbgU* alone was also sufficient for survival when *dGALE* knockdown was initiated during the early stages of development, though to a lesser extent. This result suggested that residual *dGALE* activity remaining after knockdown, or existing substrate and product pools already accumulated in the animals, may have reduced the requirement for transgene activity. However, animals experiencing *dGALE* knockdown with replacement by either *eGALE* only, or *wbgU* only, at any stage prior to late pupation, exhibited marked fecundity defects. Interestingly, the fecundity defects differed between males and females, and were dependent on which *GALE* activity was missing. Specifically, *dGALE* knockdown during early to mid-pupation resulted in fecundity defects in both male

and female flies. GALE activity toward UDPgal/UDPglc alone, but not UDPgalNAc/UDPglcNAc alone, was sufficient to rescue the male defect. However, both GALE activities were required to rescue female fecundity.

3.3.3. GALE activity toward UDPgal/UDPglc is required for normal lifespan of adult *Drosophila* exposed to dietary galactose—Sanders and colleagues [32] demonstrated that *dGALE* hypomorphs developing in the presence of environmental galactose displayed a reduction in viability. Daenzer and colleagues [7] extended from that finding by measuring the lifespans of *dGALE*-knockdown animals expressing neither, only one, or both GALE activities. Specifically, flies experiencing *dGALE* knockdown during early to mid-pupal stages were allowed to develop on a standard molasses food diet and, upon eclosion, were moved to food containing either 555 mM glucose or 555 mM glucose plus 175 mM galactose. The lifespan of *dGALE*-knockdown animals eating glucose-only food did not differ significantly from that of control animals. However, when exposed to galactose as adults, the *dGALE*-knockdown animals displayed a dramatic reduction in life span, such that in the absence of knockdown close to 50% of animals remained viable at 35 days but in the presence of knockdown close to half the animals had died by 10 days and almost no animals remained alive at 35 days. Expression of GALE activity toward UDPgalNAc/UDPglcNAc alone (*wbgU* transgene) was not sufficient to overcome the galactose-dependent reduction in lifespan. In contrast, *dGALE*-knockdown animals in which GALE activity toward UDPgal/UDPglc was restored (*eGALE* transgene) survived equally well in the presence and absence of dietary galactose. These data confirmed that loss of activity toward UDPgal/UDPglc was responsible for the galactose-induced reduction of lifespan in *dGALE*-knockdown animals.

3.3.4. The two GALE activities impact galactose metabolite levels differently—While it is clear that *dGALE*-impaired *Drosophila* experience a variety of acute and long-term outcomes, the pathophysiology of these outcomes remains unclear. Daenzer and colleagues gained some insights into pathophysiology from studying the galactose metabolite levels in their differentially impaired animals. For example, *Drosophila* in which *dGALE* knockdown occurred early in development accumulated very high levels of Gal-1P when exposed to galactose as larvae. These larvae also accumulated very high levels of UDPgal. Similarly, larvae deficient only in activity toward UDPgal/UDPglc accumulated very high levels of both Gal-1P and UDPgal when they developed in the presence, but not the absence, of high dietary galactose. In contrast, animals deficient only in GALE activity toward UDPgalNAc/UDPglcNAc did not accumulate abnormal levels of either Gal-1P or UDPgal. Nonetheless, these animals were not viable. Clearly, elevated Gal-1P and UDPgal cannot be the only cause of pathophysiology in Type III galactosemia.

4. A *D. melanogaster* model of kinase deficiency galactosemia

A deletion allele of *dGALK* was created by K. Garza and colleagues in the Fridovich-Keil lab by imprecise excision of a nearby P-element insertion; this allele was used by Jumbo-Lucioni and colleagues [18] and is described in a forthcoming publication from the Fridovich-Keil lab (Daenzer et al, in preparation). A number of different *dGALK* alleles or *GALK* knock-down experiments were also described recently by Lee and colleagues [24],

who reported that loss of GALK modified the cardiac outcomes otherwise seen in response to expression of constitutively active calcineurin in *Drosophila*. The mechanism of this interaction remains unclear, as does its relevance to galactosemia.

5. Conclusions

In the past 6 years, *Drosophila melanogaster* models of GALT and GALE deficiency have offered powerful insights into Type I and Type III galactosemia, and studies of GALK loss in *Drosophila* have raised the possibility that this gene and encoded enzyme may play important roles in biological processes beyond those typically associated with metabolic disease. Future studies leveraging the genetic and biochemical facility of these and additional models will be aimed at identifying genetic and environmental modifiers of outcomes beyond the Leloir pathway and galactose exposure, understanding mechanism, and seeking to identify pharmacological agents to prevent or reverse the complications of galactosemia in patients.

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Figure 1.

The Leloir Pathway of galactose metabolism (red) and recognized bypass routes (blue). The highly conserved Leloir Pathway is comprised of three enzymes: galactokinase (GALK), galactose-1-phosphate uridylyltransferase (GALT), and UDP galactose 4'-epimerase (GALE). Mutations resulting in deficiency of any of these three enzymes lead to a form of galactosemia.