A Drosophila melanogaster model of classic galactosemia

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SUMMARY

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

INTRODUCTION

In higher eukaryotes, galactose and its derivatives serve as key constituents of glycoproteins, glycolipids and complex carbohydrates. As a component of lactose, galactose also provides close to half of the sugar calories in mammalian milk. Galactose is metabolized in mammals and other species by the enzymes of the Leloir pathway: galactokinase (GALK, EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12) and uridine diphosphate (UDP)-galactose 4′ epimerase (GALE, EC 5.1.3.2) (Fig. 1) (Holden et al., 2003). In humans, profound impairment of GALT results in the potentially lethal disorder classic galactosemia (OMIM #230400, reviewed in Fridovich-Keil and Walter (Fridovich-Keil and Walter, 2008)).

Classic galactosemia is an autosomal recessive disorder that affects around one in 60,000 live births. Following exposure to milk, affected infants experience a rapid clinical demise, with symptoms that escalate from vomiting, diarrhea and a failure to thrive, to jaundice, Escherichia coli sepsis and neonatal death. Untreated infants with classic galactosemia also accumulate dramatically elevated levels of galactose-1-phosphate (gal-1P) in their cells and tissues. Although careful dietary galactose restriction prevents or reverses the acute and potentially lethal symptoms of classic galactosemia, and also largely resolves the abnormal accumulation of gal-1P, significant long-term complications are common; these include cognitive impairment, speech difficulties, neuromuscular problems and premature ovarian insufficiency, among others (for a review, see Fridovich-Keil and Walter, 2008).

Despite decades of study, the underlying bases of the pathophysiology of both the acute and long-term complications of classic galactosemia remain unclear (for a review, see Fridovich-Keil and Walter, 2008) (Tyfield and Walter, 2002; Leslie, 2003). One of the principal factors limiting research progress has been the lack of an animal model that recapitulates either the acute or the long-term complications of the disorder. Studies conducted by ourselves and others using yeast and mammalian tissue culture models have provided biochemical insights into the consequences of GALT activity loss (e.g. Fridovich-Keil and Jinks-Robertson, 1993; Fridovich-Keil et al., 1995a; Fridovich-Keil et al., 1995b; Elsevier et al., 1996; Elsevier et al., 1996; Lai et al., 1996; Lai and Elsas, 2000; Riehman et al., 2001; Christacos and Fridovich-Keil, 2002; Lai et al., 2003; Ross et al., 2004), but the absence of an appropriate multicellular model has precluded studies into the timing and impact of GALT deficiency on organismal development, or on intact tissues and organ systems. Of note, more than a decade ago, Leslie and colleagues (Leslie et al., 1996) reported a Galt knockout mouse that demonstrated a complete loss of GALT activity and that accumulated gal-1P; however, these mice unexpectedly remained healthy and fertile despite dietary exposure to large quantities of galactose (Leslie et al., 1996; Ning et al., 2000; Leslie and Bai, 2001; Ning et al., 2001; Leslie et al., 2005). This outcome disparity between GALT-deficient humans and mice remains unexplained.

As an alternative to mice, we turned to the fruit fly Drosophila melanogaster. Fruit flies have facilitated genetic experiments for more than a century and, in recent years, have emerged as an extremely powerful system to model human genetic diseases (Bier, 2005), including complex metabolic disorders, such as diabetes and obesity (Lasko, 2002; Bharucha, 2009; Zhang et al., 2009). Sequence alignments show that greater than 75% of recognized human disease genes, including all three Leloir pathway genes (http://superfly.ucsd.edu/homophila/), have related sequences in the D. melanogaster genome (Bier, 2005).
Here, we report the establishment and first application of a *D. melanogaster* model of classic galactosemia. Like human patients, and unlike mice, GALT-deficient Drosophila survive if maintained on food that contains only glucose (glc), but die as larvae if exposed to food that contains both glucose and galactose. This galactose-dependent lethality is dose dependent, sugar specific and can be rescued by the expression of a human GALT transgene. GALT-deficient animals are also rescued from death by the initiation of a galactose-restricted diet early in development. Of note, larval animals transferred from a glucose diet to a glucose-plus-galactose diet die within days of the transfer, but adult flies do not, arguing for stage-specific or dose-dependent consequences to impaired galactose metabolism. As expected, metabolite studies of GALT-deficient larvae and adults exposed to galactose show an abnormal accumulation of gal-1P. Finally, GALT-deficient flies that are raised and maintained exclusively on a galactose-restricted diet demonstrate a clear deficiency in the normal negative geotaxic response seen in controls (Benzer, 1967; Akai, 1979). As with the acute galactose-dependent phenotype, this galactose-independent muscular or neuromuscular deficit is rescued by the expression of a human GALT transgene. These data confirm that GALT-deficient *D. melanogaster* mimic aspects of both the acute and long-term outcomes of classic galactosemia, paving the way for future studies to explore the genetic and environmental factors that underlie and modify these phenotypes.

**RESULTS**

**D. melanogaster metabolize galactose via the Leloir pathway**

To explore the role of galactose metabolism in *D. melanogaster*, we first confirmed that flies encode and express orthologs of human GALK, GALT and GALE (Fig. 1). The *D. melanogaster* genes whose predicted protein products show the greatest amino acid sequence homology to the human Leloir enzymes are CG5288 (on chromosome III, designated here as *dgALK*), CG9232 (on chromosome II, designated here as *dgALT*), and CG12030 (on chromosome III, designated here as *dgALE*) (http://superfly.ucsd.edu/homophila/) (Chien et al., 2002). Both *dgALT* and *dgALE* show strong conservation with their corresponding human orthologs, having 57% sequence identity with 72% sequence similarity, and 60% sequence identity with 76% sequence similarity, respectively. *dgALK* demonstrates slightly lower conservation, with 27% amino acid sequence identity and 44% sequence similarity.

Publicly available in situ RNA hybridization data from the Berkeley Drosophila Genome Project (BDGP; http://www.fruitfly.org/) confirm that all three fly Leloir genes are expressed during embryogenesis. Assays of lysates prepared from wild-type (*w*) *D. melanogaster* adults also reveal the active presence of all three enzymes (Table 1). Finally, studies of animals carrying disruptions or deletions of each of the three fly genes (Table 1) (Sanders et al., 2010) (P.J.L, K. Garza and J.L.F.-K., unpublished) demonstrate loss of the corresponding enzymatic activity, thereby confirming a functional connection between each gene sequence and the enzymatic activity attributed to its predicted protein product.

The *dgALT* gene is approximately 2 kb in length and includes four exons, the first of which is noncoding (supplementary material Fig. S1); the predicted mRNA is ~1.5 kb. The fly *dgALE* and *dgALK* genes will be described in detail elsewhere (Sanders et al., 2010).

![Fig. 1. The Leloir pathway of galactose metabolism.](image)

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

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

We created an impaired allele of *dgALT* by imprecise excision of an existing P-element insertion, *Kg00049*, in the 5′-untranslated region (UTR) of the *CG9232* gene (supplementary material Fig. S1A). This *CG9232*<sup*Kg00049*</sup> allele fully complemented a strong *cup* allele (*cup<sup>01355*</sup>), suggesting that it has little if any effect on *cup* expression; female *cup<sup>01355*/<sup>*</sup>*GALT<sup>Kg00049*</sup> compound heterozygotes crossed to *w<sup>1118*</sup>* males produced viable embryos that developed into phenotypically normal flies. The *GALT<sup*Kg00049*</sup>* allele also failed to impair *dgALT* expression or function, as indicated by the wild-type level of GALT activity detected in lysates from homozygotes (Table 1).

Excision of the P-element was achieved by transient exposure to the Δ2-3 transposase in the germ line of male flies (Ryder and Russell, 2003), as described in the Methods. From the resulting excision alleles, we identified two, designated *dgALT<sup>Δ1AP2*</sup>* and *dgALT<sup>Δ1V2*</sup>* that each carried the same 1647-bp deletion removing almost the entire *dgALT* gene (supplementary material Fig. S1B). Flies carrying a precise excision of the *Kg00049* P-element insertion, designated *dgALT<sup>C2*</sup>, were also identified and characterized (supplementary material Fig. S1C). As expected, *dgALT<sup>Δ1AP2*</sup>* and *dgALT<sup>Δ1V2*</sup>* homozygotes demonstrated a complete lack of detectable GALT activity, whereas *dgALT<sup>C2*</sup> homozygotes demonstrated wild-type GALT activity (Table 1). Finally, all three excision alleles demonstrated normal GALE activity (Table 1) and complemented the *cup<sup>01355*</sup> allele, indicating that *cup* remained functional on these chromosomes. Of note, a small increase in GALK expression or activity, or alternatively an artifact of the assay,
Dietary galactose is lethal to \textit{dGALT-deficient} flies

To test the impact of galactose exposure on \textit{dGALT-deficient} \textit{Drosophila}, we assessed the survival of F1 progeny from crosses of homozygous \textit{dGALT}^{+/+} or \textit{dGALT}^{1A12} virgin females to heterozygous \textit{dGALT}^{1AP2}/\textit{CyO} or \textit{dGALT}^{1A12}/\textit{CyO} males. Crosses were performed on fly food containing 555 mM glucose, or 555 mM glucose plus 222 mM galactose (Fig. 2; Table 2). Surviving adult progeny were genotyped by the presence or absence of the balancer chromosome (\textit{CyO}), which conferred a dominant curly wing phenotype. Viability of the \textit{dGALT}-deficient progeny resulting from each cross was quantified in terms of the percentage of surviving F1 animals that were unbalanced (exhibiting straight wings).

The result was striking: on food that contained glucose as the sole sugar, unbalanced \textit{dGALT-deficient} flies accounted for close to the expected 50% of viable offspring, but on food supplemented with 222 mM galactose, \textit{dGALT-deficient} flies accounted, on perhaps resulting from increased stability of the GALK reaction product, gal-1P, when GALT is not present to metabolize it.

**Table 1. Leloir enzyme activities in \textit{D. melanogaster}**

<table>
<thead>
<tr>
<th>STOCK (comments)</th>
<th>GALK activity (pmol/µg/min)</th>
<th>GALT activity (pmol/µg/min)</th>
<th>GALE activity (pmol/µg/min)</th>
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<tr>
<td>\textit{w}^{111}/\textit{w}^{118} (Wild-type control)</td>
<td>19.91±1.61</td>
<td>24.31±2.03</td>
<td>81.70±2.80</td>
</tr>
<tr>
<td>\textit{CG9232}^{+} \textit{w}^{111}/\textit{CG9232}^{+} (Starting P-element insertion in \textit{dGALT})</td>
<td>11.56±2.06</td>
<td>22.50±3.60</td>
<td>65.77±10.21</td>
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<tr>
<td>\textit{dGALT}^{+/+}/\textit{dGALT}^{1A12} (Precise excision)</td>
<td>20.87±3.62</td>
<td>29.17±1.43</td>
<td>73.48±17.36</td>
</tr>
<tr>
<td>\textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} (Imprecise excision)</td>
<td>42.78±4.74</td>
<td>–0.07±0.02</td>
<td>87.38±10.47</td>
</tr>
<tr>
<td>\textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} α\textit{UAS-hGALT}^{1AP2}/α\textit{UAS-hGALT}^{1AP2} (Low expression transgenic rescue)</td>
<td>38.29±3.33</td>
<td>0.02±0.29</td>
<td>80.90±7.86</td>
</tr>
<tr>
<td>\textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} α\textit{UAS-hGALT}^{1AP2}/α\textit{UAS-hGALT}^{1AP2} (High expression transgenic rescue)</td>
<td>12.22±2.46</td>
<td>1.01±0.06</td>
<td>81.18±15.15</td>
</tr>
<tr>
<td>\textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} α\textit{UAS-hGALT}^{1AP2}/β\textit{tub-GAL4} (High expression transgenic rescue)</td>
<td>18.70±8.65</td>
<td>172.41±60.80</td>
<td>98.00±1.65</td>
</tr>
<tr>
<td>\textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} α\textit{UAS-hGALT}^{1AP2}/β\textit{tub-GAL4} (High expression transgenic rescue)</td>
<td>14.71±3.54</td>
<td>651.93±21.49</td>
<td>66.45±3.97</td>
</tr>
<tr>
<td>\textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} α\textit{UAS-hGALT}^{1AP2}/β\textit{tub-GAL4} (High expression transgenic rescue)</td>
<td>15.47±2.08</td>
<td>260.63±35.86</td>
<td>74.22±12.28</td>
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Enzyme activities were measured on lysates prepared from cohorts of ten male flies raised on molasses food and harvested 8–48 hours after eclosion. The values presented are the average ± S.E.M. (n=3 sets of duplicate samples).

**Fig. 2. Loss of \textit{dGALT} results in galactose sensitivity of \textit{D. melanogaster}.** (A) Percentage of viable \textit{dGALT}-deficient progeny resulting from crosses of \textit{dGALT}^{+/–} virgin females with \textit{dGALT}^{+/–} males on the food sources indicated (glc, 555 mM glucose food; + gal, 555 mM glucose plus 222 mM galactose food; + man, 555 mM glucose plus 222 mM mannose). The fractions over the bars indicate the combined numbers of homozygous adult flies over the total numbers of progeny counted in each category; n=the number of crosses from which those progeny were derived. Each cross produced at least 75 viable F1 adults. Flies carrying a precise excision (\textit{dGALT}^{+/–}) rather than an imprecise excision (\textit{dGALT}^{1AP2}) allele of \textit{dGALT} served as the positive control. (B) Expression of a human \textit{GALT} transgene rescued the viability of \textit{dGALT}^{1AP2}/\textit{dGALT}^{1AP2} \textit{D. melanogaster} exposed to galactose. Note that these data were generated using the \textit{hGALT} transgene alleles \textit{9A12}, \textit{10A12} and \textit{10B22}, which were minimally, if at all, leaky. Asterisks (*) indicate statistical significance, P<0.0001 for a two-tailed \textit{t}-test at the 95% confidence interval.
Average, for <10% of viable offspring (Fig. 2A; Table 2). When the galactose concentration was raised to 278 mM, the number of viable dGALT-deficient animals plummeted nearly to zero (data not shown). This same result was also obtained using the dGALT1V2 allele, as well as using dGALTΔAP2/dGALTΔV1V2 transheterozygotes.

As a control for galactose specificity of the apparent food sensitivity, we repeated each cross on food containing 555 mM glucose plus 222 mM galactose, and food containing 555 mM glucose plus 111 mM galactose. The transgenic rescue data further demonstrated that overexpression of hGALT appears benign, although we have not ruled out subtle effects.

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

To confirm that the galactose sensitivity of dGALT-deficient Drosophila resulted from the loss of GALT activity, we attempted transgenic rescue using a UAS-driven human GALT transgene (UAS-hGALT). The rationale for selecting a transgene rather than a fly GALT transgene was to test whether dGALT and hGALT are true functional orthologs. Male flies that are homoygous for dGALTΔAP2 and heterozygous for a β-tubulin-GAL4 driver were crossed to virgin females that were heterozygous for dGALTΔAP2 and homozygous for one of the three UAS-hGALT transgenes, UAS-hGALTΔA12, UAS-hGALTΔA11, or UAS-hGALTΔB2. These crosses were conducted in parallel on foods containing 555 mM glucose or 555 mM glucose plus 222 mM galactose. As illustrated in Fig. 2B and Table 1, the presence of an hGALT transgene together with the β-tubulin-GAL4 driver resulted in overexpression of hGALT activity and fully rescued the viability of dGALTΔAP2 homozygous Drosophila that were exposed to galactose. The transgenic rescue data further demonstrated that overexpression of hGALT appears benign, although we have not ruled out subtle effects.

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

To identify the stage of development at which GALT-deficient D. melanogaster succumb to galactose toxicity, we followed the fates of cohorts of >100 individual progeny derived from crosses of dGALTΔAP2 homozygous virgin females and males. In brief, embryos less than 12-hours old were harvested from egg-laying plates and transferred to the wells of 96-well plates that were preloaded with each of three types of fly food: food containing 555 mM glucose, food containing 555 mM glucose plus 111 mM galactose, and food containing 555 mM glucose plus 222 mM galactose. Each day the numbers of live and dead animals were recorded; the results (Fig. 3) revealed four important points.

First, wild-type dGALT animals that were homozygous for the precise excision allele (dGALTΔA12) remained viable and developed normally throughout the 11-day course of the experiment, regardless of the presence or absence of galactose in their food. By day 11, close to 90% of these animals had eclosed as viable adults, compared with 90% of controls. Of note, this difference did not appear to be as pronounced in standard culture vials, suggesting that the 96-well plate format was not itself a major cause of delayed development or death.

Second, even in the absence of dietary galactose, animals that were homozygous for the imprecise excision allele (dGALTΔA12) were slightly less robust than their precise excision counterparts. By day 11, only 74% of these homozygotes had eclosed as viable adults, compared with 90% of controls. Of note, this difference did not appear to be as pronounced in standard culture vials, suggesting that the 96-well plate format may have disproportionately stressed the dGALT-deficient animals, although this possibility remains unexplained.

Third, in the presence of increasing concentrations of galactose, we saw a decreasing percentage of dGALT-deficient animals that

<table>
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<tr>
<th>Experimental cross</th>
<th>Genotype</th>
<th>Anticipated mendelian proportion of F1 with this genotype</th>
<th>Observed viable F1 with this genotype in gluc food</th>
<th>Observed viable F1 with this genotype in gluc+gal food</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGALTΔ/Δ × dGALTΔ/Δ</td>
<td>dGALTΔ/Δ (dGALT control)</td>
<td>0.50</td>
<td>0.466±0.003</td>
<td>0.454±0.003</td>
</tr>
<tr>
<td>dGALTΔAP2/ΔAP2 × dGALTΔV1V2/ΔV1V2</td>
<td>dGALTΔAP2/ΔV1V2 (dGALT null)</td>
<td>0.50</td>
<td>0.403±0.005</td>
<td>0.062±0.008</td>
</tr>
<tr>
<td>dGALTΔAP2/ΔAP2 × UAS-ΔGALTΔA11/ΔA11</td>
<td>dGALTΔAP2/ΔA11 (dGALT null with a leaky hGALT transgene)</td>
<td>0.50</td>
<td>0.443±0.006</td>
<td>0.160±0.015</td>
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</table>

Expression of about 4% of the wild-type levels of human GAL T partially rescues the viability phenotype in dGALT-null Drosophila raised on glucose-plus-galactose food (*t* test, *P*<0.05; Glc, glucose; gal, galactose. Values represent the average of ≥3 independent replicates ± S.E.M.)
survived to adulthood. Specifically, on food containing 111 mM galactose, only 50% of the animals eclosed as viable adults, whereas on food containing 222 mM galactose, none of the dGALT-deficient animals eclosed as viable adults.

Finally, the timing of death of the dGALT-deficient Drosophila that were exposed to galactose occurred during mid- to late-larval stages. Essentially, all the mutant embryos visibly hatched into viable first instar (L1) larvae and began to eat food, as shown by the presence of coloring from the food in their gut, but none of these larvae survived to pupation. It is difficult to distinguish the precise stage of lethality, because the dying animals were stunted relative to controls (supplementary material Fig. S2). Whether this small organismal size reflected a true developmental delay or simply a growth defect remains unclear.

Window of galactose sensitivity of GALT-deficient D. melanogaster

To define the stage of development at which GALT-deficient D. melanogaster are most sensitive to galactose, we performed dietary ‘cross-over’ experiments (Fig. 4) in which cohorts of dGALT\textsuperscript{D1AP2} homozygotes (GALT deficient) were transferred, as embryos or larvae, from food containing only 555 mM glucose to food containing 555 mM glucose plus 222 mM galactose, or from food containing 555 mM glucose plus 222 mM galactose to food containing only 555 mM glucose. As a control for the transfer process, animals were also transferred each day from glucose food to fresh galactose-supplemented food. By contrast, animals homozygous for the dGALT\textsuperscript{D1AP2} precise excision allele remained predominantly viable in the absence of galactose, but died in increasing numbers during larval development when exposed to increasing levels of dietary galactose.

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

To test whether GALT-deficient Drosophila that are exposed to dietary galactose accumulate abnormal levels of gal-1P, we exposed developing larvae and newly eclosed adults to food containing either 555 mM glucose alone or 555 mM glucose plus 222 mM galactose for four days. Extracts of these animals were analyzed as described in the Methods. In the absence of dietary galactose, dGALT\textsuperscript{D1AP2} homozygous larvae and adults had 0.75±0.13 (n=3) and 0.22±0.09 (n=3) pmol gal-1P/µg protein, respectively, and in the presence of dietary galactose, these animals accumulated 11.82 or 12.09 (n=2) and 7.95±4.25 (n=3) pmol gal-1P/µg protein, respectively. Parallel samples from dGALT\textsuperscript{C2} homozygous larvae and adults had only 0.37±0.09 and 0.10±0.04 (n=3/group, glucose-only food) and 0.85±0.12 and 0.30±0.10 (n=3/group, glucose-plus-galactose food) pmol gal-1P/µg protein, respectively.

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

To address whether GALT-deficient D. melanogaster might, like their human counterparts, exhibit long-term complications despite lifelong dietary restriction of galactose, we assessed the negative geotaxic response of dGALT\textsuperscript{D1AP2} homozygous, dGALT\textsuperscript{C1V2} homozygous and dGALT\textsuperscript{C2} homozygous flies. All of these animals were raised on standard molasses food, which contains negligible
Fig. 4. Window of galactose sensitivity of dGALTΔ1AP2 imprecise excision homozygotes. The day of development is plotted on the x-axis; the percentage survival in each cohort is plotted on the y-axis. dGALT-deficient embryos and larvae were transferred daily in cohorts of >20 animals each from 555 mM glucose-only food to 222 mM galactose-supplemented food (dashed lines in A), or from 222 mM galactose-supplemented food to 555 mM glucose-only food (dashed lines in B), for six days, after which the animals in each cohort were allowed to complete development without further interference. To the right of each plotted curve is a key illustrating the food exposure of the corresponding cohort of animals; each open box represents one day of development on glucose food and each shaded box represents one day of development on food supplemented with 222 mM galactose. The shading of the arrow to the right of each key indicates whether subsequent development occurred on glucose (open arrow) or glucose-plus-galactose (shaded arrow) food. Of note, only live animals were transferred, which led to an apparent but reversible response to galactose exposure during early larval development.

if any galactose, and tested using a previously described ‘countercurrent’ apparatus (Benzer, 1967; Akai, 1979) that quantifies negative geotaxic response. Normal flies exhibit a strong negative geotaxic response and a quick response to stimulus, meaning they begin to move shortly after being tapped to the bottom of a tube, and they prefer to run ‘up’ the sides of a tube rather than stand still or run ‘down’.

The apparatus and procedure that we applied offered cohorts of approximately 60 male flies, aged 2–3 days, six sequential opportunities each to walk or run up the sides of a tube in a well-lit room within a specified period of time (15 seconds). Flies that moved to the top of inverted tube one within the first 15-second period were tapped to the bottom of tube two, and the next round was initiated. At the end of the six sequential rounds, the ‘fastest’ flies would be found in tube six and the ‘slowest’ flies would be found in tube one.

Control flies subjected to this countercurrent test presented a final distribution in which around 60% of the flies were in tube six, with the remainder distributed almost evenly between the other five tubes (Fig. 5A); this result is fully consistent with the original report of this behavior (Benzer, 1967). By striking contrast, both GALT-deficient stocks demonstrated an almost-random distribution of flies among the six tubes, with no enrichment in tube six (Fig. 5B,C). This differential distribution profile was highly statistically significant (P<0.01) and was rescued by the expression of the human GALT transgene (Fig. 5D), thereby demonstrating that this impairment was rescued by the expression of a human GALT transgene (D).

**DISCUSSION**

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

**Galactose sensitivity of GALT-deficient flies**

Our data establish two main points about galactose sensitivity. First, loss of dGALT in developing *D. melanogaster* results in a galactose-dependent lethality, and this outcome is rescued by transgenic expression of human GALT. Although obvious in its simplicity, this result is also profound because it largely puts to rest a nagging concern that has plagued the field since Leslie and colleagues first reported that loss of GALT in mice is apparently benign (Leslie et al., 1996). In short, the lack of a phenotype in Galt-deficient mice raised the unlikely, but disturbing, possibility that the clinical outcomes of patients with classic galactosemia might result from some cryptic impact of their mutations, rather than from the detected loss of GALT enzymatic activity. With the work presented here, the causal relationship between loss of GALT activity and galactosemia-related phenotypes in a multicellular organism is confirmed.

Our data presented here further establish that even trace expression (~4%) of human GALT can at least partially rescue the galactose-sensitivity of dGALT-deficient Drosophila. This result is especially notable in light of patient data; humans who are homozygous for the *S135L-hGALT* allele, which is associated with about 5% residual GALT activity, tend to show a relatively mild clinical course (Lai et al., 1996) (for a review, see Fridovich-Keil and Walter, 2008). This result is also notable in contrast to GALE. The patients who are most severely affected with GALE-deficiency galactosemia express at least 5% residual GALT activity (for a review, see Fridovich-Keil and Walter, 2008). This result is also remarkable in light of patient data; humans who are homozygous for the *S135L-hGALT* allele, which is associated with about 5% residual GALT activity, tend to show a relatively mild clinical course (Lai et al., 1996) (for a review, see Fridovich-Keil and Walter, 2008). The fly data are nonetheless compelling, because they suggest that future experiments using this model system may reveal, with greater precision, both the nature of the damage and the mechanism by which it is reversed or overcome when galactose is removed from the diet.

Despite the significance of these findings, it is important not to over-interpret the fly data. For example, although exposure of Drosophila adults or late-stage larvae to galactose was not lethal, we do not know that it was completely benign. It would be premature, therefore, to conclude that dGALT-deficient flies, or classic galactosemia patients, do not require lifelong dietary restriction of galactose. Nevertheless, there are intriguing anecdotal reports from the literature (Bosch et al., 2004; Panis et al., 2006) that describe a similar finding in patients; namely, that older children and adults with classic galactosemia appear to be able to consume dietary galactose with no acute negative clinical consequences.

**Long-term complications**

Although we have just begun to test our dGALT-deficient flies for long-term phenotypes, abnormalities clearly exist (Fig. 5) and these can be rescued by expression of a human GALT transgene. The galactose-independent phenotype that we report here, impaired negative geotaxis response, is a complex trait that could reflect any of a number of underlying muscular or neuromuscular abnormalities. Although many patients with classic galactosemia do experience apparent neuromuscular complications, the relationship between those patient outcomes and the fly phenotype reported here remains to be explored. Future studies will address the extent and nature of other potential abnormalities in dGALT-deficient flies, as well as the genetic and environmental factors that underlie them.

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

The finding that GALT-deficient Drosophila exposed to galactose accumulate elevated levels of gal-1P is fully consistent with metabolic abnormalities reported in patients (for a review, see Fridovich-Keil and Walter, 2008). What is striking, however, is that the levels of gal-1P accumulated from four days of galactose exposure in larvae and adults is fairly comparable [11.82 or 12.09 (n=2) and 7.95±4.25 (n=3) pmol gal-1P/µg protein, respectively], yet the larvae are progressing rapidly toward death, whereas the adults are not. There are many possible explanations for this apparent disparity, but we must also consider that gal-1P itself may not be the primary cause of symptoms. Rather, the symptoms may result from stage-specific ways in which the body responds to an elevated gal-1P level. This observation, in essence, parallels the lesson of the Galt knockout mouse, which also accumulated high...
levels of gal-1P in response to dietary galactose exposure, and which also did not die as a result (Leslie et al., 1996). Hence, regardless of age, mice do not succumb in response to galactose exposure, whereas flies only succumb to galactose-exposure within an apparent developmental window of sensitivity. Clearly, if we are to understand the underlying pathophysiology of galactosemia we must find out what occurs downstream of gal-1P accumulation in developing animals. With a fly genetic model that recapitulates aspects of the patient phenotype, at long last we have tools to begin the work.

**METHODS**

**Fly stocks and maintenance**
Details about the stocks of *D. melanogaster* used in this study are provided in supplementary material Table S1. Stocks were maintained at 25°C on a molasses-based food that contained 43.5 g/l cornmeal, 17.5 g/l yeast extract, 8.75 g/l agar, 54.7 ml/l molasses, 10 ml propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol). For experiments in which the levels and types of sugar were to be varied, we used a glucose-based food [5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l glucose, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol)] (Honjo and Furukubo-Tokunaga, 2005) supplemented with galactose or mannose, as indicated. For some experiments, liquid food coloring was also added to facilitate confirmation that larvae were eating.

**Creation and molecular characterization of the dGALT1AP2, dGALT4I1V2 and dGALT2C alleles**

Excision alleles of dGALT were generated by mobilizing an existing SUPor-P insertion in the 5`-UTR of the CG9232 locus (KG00049) via transient expression of the Δ2-3 transposase enzyme in the male germ line, according to standard methods (Ryder and Russell, 2003). Flies carrying excision alleles were identified by loss of the associated mini-+ marker (white eyes), and homozygous stocks derived from those flies were sorted according to the level of GALT enzymatic activity; those stocks were designated as imprecise excision stocks tested, five demonstrated a profound loss of GALT enzymatic activity; those stocks were designated as precise excision candidates, whereas stocks demonstrating wild-type GALT activity were designated as precise excision candidates.

Given the genomic location of dGALT within the second intron of cup, we further tested all excision alleles of interest for their ability to complement a strong mutant allele of cup (cup01355). Of note, the KG00049 P-element insertion allele was itself homozygous viable and female fertile, and also complemented the cup01355 allele. Similarly, female flies carrying the cup01355 allele in trans to either dGALT1AP2, dGALT4I1V2 or dGALT2C remained viable and fertile, confirming that cup remained functional in each genotype.

To characterize the dGALT1AP2, dGALT4I1V2 and dGALT2C alleles at the molecular level, we amplified the relevant genomic sequences using primers that annealed within the flanking exons of cup. The primer sequences were 5’-GCTGACTGCTGATCTCAGCGGTGT3’ and 5’-CCAAGGGGACCTTGTATGCCCT-3’. Control PCR amplification of the corresponding region from w118 flies revealed the anticipated 4-kb amplicon. The dGALT2C stock also produced a 4-kb amplicon, whereas both the dGALT1AP2 and dGALT4I1V2 stocks produced ~2.4-kb amplicons.

Direct sequencing of the amplicons from all three excision templates revealed that the presumed precise excision allele was indeed precise (supplementary material Fig. S1C), and that each of the presumed imprecise excision alleles carried the same 1647-bp deletion removing virtually the entire coding region of the dGALT gene, with a small remnant of the P-element sequence left behind (supplementary material Fig. S1A,B).

**Transgenic lines**

A UAS-hGALT transgene was generated by subcloning the wild-type human GALT coding sequence, as an EcoRI-Sall fragment, into pUAST (Brand and Perrimon, 1993) using the EcoRI and XhoI sites in the pUAST polylinker region. The resulting plasmid was confirmed by sequence analysis. UAS-hGALT stocks were generated using standard transgenic techniques by the Fly Core of the Massachusetts General Hospital, Charlestown, MA. Candidate insertion lines were mapped and balanced. Four homozygous viable insertions (dGALT9B12, dGALT10A11, dGALT10A12 and dGALT10B22) on chromosome III were used in this study.

**GALK, GALT and GALE enzyme assays**

Lysates were prepared and assays (n≥3) were performed (for Table 1), as described in Sanders et al. (Sanders et al., 2010).

**Measuring gal-1P in larvae and adults**

Cohorts of newly hatched dGALT1AP2 homozygous and dGALT2C homozygous larvae and newly eclosed adults were transferred to cages or vials containing either 555 mM glucose only or 555 mM glucose plus 222 mM galactose food. After 4 days, pools of 10 adult flies or ≥20 larvae were anaesthetized with CO2, suspended in 125 μl of ice-cold high-performance liquid chromatography (HPLC)-grade water, and ground on ice for 15 seconds using a Teflon micropestle and handheld micropestle motor (Kimble Chase Life Science and Research Products LLC, Vineland, NJ). Ten μl of each lysate was saved for protein quantification (using the BioRad DC assay with BSA as a standard). Intracellular metabolites were extracted from the remainder, as described previously (Ross et al., 2004; Openo et al., 2006). The extracted samples were then dried under vacuum with no heat (Eppendorf Vacufuge) until no liquid remained visible. Dried metabolite pellets were rehydrated with HPLC-grade water in volumes normalized for protein concentration, and centrifuged through 0.22-μm Costar Spin-X centrifuge tube filters (Corning Inc., Lowell, MA) at 4000 × g for four minutes to remove any insoluble matter. The soluble phase of each sample was transferred to a glass HPLC vial and metabolites were separated and quantified using a Dionex HPLC, as described previously (Ross et al., 2004). For each sample, 20 μl were injected into a 25-μl injection loop.

**Time of death experiments**

Embryos that were less than 24 hours old and homozygous for the dGALT1AP2 (imprecise excision) or dGALT2C (precise excision) alleles (n≥100 for each genotype) were harvested from egg-laying plates and deposited individually into the wells of 96-well plates, which had been preloaded to about three-quarters full with either 555 mM of glucose only, or 555 mM glucose plus 111 mM or 222 mM of galactose fly food. Once loaded, plates were tightly covered with a clear plastic wrap that was pricked with a needle to introduce
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a small hole over each well, to permit an exchange of air. All animals were monitored and scored for viability daily under a dissecting microscope until they either died or eclosed as adults.

Window of galactose sensitivity
Embryos homozygous for the dGALTΔAP2 (imprecise excision) and dGALTΔC2 (precise excision) alleles were deposited on plates containing either 555 mM glucose food or 555 mM glucose plus 222 mM galactose food. Every 24 hours, starting with first instar larvae (L1s), cohorts of more than 20 developing animals were transferred from glucose food individually into the wells of 96-well plates that had been preloaded with 555 mM glucose plus 222 mM galactose food, and similarly larvae were transferred from 555 mM glucose plus 222 mM galactose food into the wells of 96-well plates preloaded with 555 mM glucose-only food. To control for a potential impact of the transfer process itself, cohorts of larvae also were transferred from glucose-only food to wells containing glucose-only food, and from glucose plus galactose food to wells containing glucose plus galactose food. This transfer process was continued for the first six days of development, after which all animals were allowed to continue developing without further transfer.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
R.F.K. generated the dGALT excision alleles, and designed and performed the majority of the experiments presented in Figs 2-4 and Figs S1 and S2; E.L.R. generated some of the data for Fig. 2 and all of the data for Fig. 5; R.D.S. and J.M.I.S. generated most of the data presented in Table 1; P.J.L. generated some of the data for Tables 1 and 2; K.H.M. provided general oversight for experiments involving fly genetic manipulation; J.L.F.-K. conceived the project and provided oversight for much of its completion. All authors contributed to writing the final manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at

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REFERENCES


A fly model of classic galactosemia


