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

Rebecca D. Sanders¹, Jennifer M. I. Sefton², Kenneth H. Moberg³ and Judith L. Fridovich-Keil⁴,*

**SUMMARY**

UDP-galactose 4′ epimerase (GALE) catalyzes the interconversion of UDP-galactose and UDP-glucose in the final step of the Leloir pathway; human GALE (hGALE) also interconverts UDP-N-acetylgalactosamine and UDP-N-acetylgalactosamine. GALE therefore plays key roles in the metabolism of dietary galactose, in the production of endogenous galactose, and in maintaining the ratios of key substrates for glycoprotein and glycolipid biosynthesis. Partial impairment of hGALE results in the potentially lethal disorder epimerase-deficiency galactosemia. We report here the generation and initial characterization of a first whole-animal model of GALE deficiency using the fruit fly *Drosophila melanogaster*. Our results confirm that GALE function is essential in developing animals; *Drosophila* lacking GALE die as embryos but are rescued by the expression of a human GALE transgene. Larvae in which GALE has been conditionally knocked down die within days of GALE loss. Conditional knockdown and transgene expression studies further demonstrate that GALE expression in the gut primordium and Malpighian tubules is both necessary and sufficient for survival. Finally, like patients with generalized epimerase deficiency galactosemia, *Drosophila* with partial GALE loss survive in the absence of galactose but succumb in development if exposed to dietary galactose. These data establish the utility of the fly model of GALE deficiency and set the stage for future studies to define the mechanism(s) and modifiers of outcome in epimerase deficiency galactosemia.

**INTRODUCTION**

Galactose and its derivatives play a central role in higher eukaryotes in the biosynthesis of complex carbohydrates, glycoproteins and glycolipids (for a review, see Varki et al., 2009). In many organisms galactose also can serve as an important energy source, and galactose provides almost half of the sugar calories in mammalian milk. From bacteria to humans, galactose is metabolized through the three enzymes of the Leloir pathway (Fig. 1): galactokinase (GALK), galactose-1-phosphate uridylyltransferase (GALT), and uridine diphosphate (UDP)-galactose 4′-epimerase (GALE) (Holden et al., 2003). Collectively, these enzymes are responsible for metabolizing environmental galactose and for synthesizing endogenous UDP-galactose (UDP-gal) when environmental sources are limiting. Human GALE interconverts UDP-N-acetylgalactosamine (UDP-galNAc) and UDP-N-acetylgalactosamine (UDP-glcNAc), as well as UDP-gal and UDP-glucose (UDP-glc) (for a review, see Fridovich-Keil and Walter, 2008).

Impairment in any of the Leloir enzymes results in galactosemia (for a review, see Fridovich-Keil and Walter, 2008). The specific clinical and biochemical outcomes of the disease depend upon which enzyme is impaired, and the degree of catalytic impairment. Classic galactosemia results from a profound loss of GALT, whereas epimerase deficiency galactosemia results from a partial loss of GALE and ranges from the ostensibly benign ‘peripheral’ form to the potentially lethal ‘generalized’ form (Gitzelmann et al., 1976; Walter et al., 1999; Openo et al., 2006). Patients accumulate galactose metabolites, including galactose, UDP-gal and galactose-1-phosphate (gal-1-P), and may suffer acute and/or long-term complications, especially if exposed to dietary galactose (Holton et al., 1981; Sardharwalla et al., 1988; Alano et al., 1998; Walter et al., 1999). Of note, unlike loss of GALT or GALK, no patient completely lacking GALE activity has ever been reported; even the most severely affected patients demonstrate significant residual GALE activity, at least in some tissues. Noting the essential role of GALE in the biosynthesis of glycoconjugates, Kalckar postulated, more than 40 years ago (Kalckar, 1965), that a total absence of GALE activity would be incompatible with life in higher eukaryotes. The patient record has supported this hypothesis; however, the lack of a whole-animal model of GALE deficiency has prevented rigorous testing.

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 (Douglas and Hawthorne, 1964; Ross et al., 2004). GALE-deficient Chinese hamster ovary (CHO) cells [ldld cells (Krieger et al., 1989)] also demonstrate galactose-sensitive growth arrest, as well as defects in both N- and O-linked glycosylation (Kingsley et al., 1986; Schulz et al., 2005). Nonetheless, the relationship between yeast or tissue culture outcomes and the pathophysiology of epimerase deficiency galactosemia remains unclear.

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 (Bier, 2005), including metabolic disease (Bharucha, 2009; Zhang et al., 2009). 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 (for a review, see Ten Hagen et al., 2009). Further, we recently confirmed...
Disease Models & Mechanisms

that *D. melanogaster* encode (http://superfly.ucsd.edu/homopha) and express functional orthologs of all three Leloir enzymes, designated *dGALK* (CG5288), *dGALT* (CG9232) and *dGALE* (CG12030) (Kushner et al., 2010).

Here, we describe a *Drosophila melanogaster* model of GALE deficiency; this is the first whole-animal genetic model of epimerase deficiency galactosemia to be reported. Using this model, we have confirmed that, as predicted by Kalckar, GALE is essential in deficiency galactosemia to be reported. Using this model, we have confirmed that, as predicted by Kalckar, GALE is essential for fly development on food lacking galactose, thereby recapitulating the acute outcome reported for patients with generalized epimerase deficiency.

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 *dGALE* intron, *P{EPgy2}CG12030EY22205* (CG9232), referred to hereafter as *dGALE* f00624, was identified as part of the Exelixis collection (Thibault et al., 2004). Soluble protein lysates from adults that were heterozygous or homozygous (where viable) for each of these genotypes (Table 1), indicated that this insertion creates a strong loss-of-function allele and, considering the homozygous lethality of *dGALE* f00624, suggesting that profound loss of GALE is lethal in *D. melanogaster*.

To further analyze the organismal requirement for GALE, we generated additional *dGALE* alleles by imprecise excision of a pre-existing P-element insertion in the 5’ untranslated region of *dGALE* [Berkeley Drosophila Genome Project (BDGP); www.fruitfly.org/]; unlike the *dGALE* f00624 insertion, this *P{EPgy2}CG12030EY22205* insertion (Fig. 2) is homozygous viable. By mobilizing the

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

In both humans and *D. melanogaster*, GALK catalyzes the interconversion of UDP-gal and UDP-glc, as well as UDP-galNAc and UDP-glCNAC.

that *D. melanogaster* encode (http://superfly.ucsd.edu/homopha) and express functional orthologs of all three Leloir enzymes, designated *dGALK* (CG5288), *dGALT* (CG9232) and *dGALE* (CG12030) (Kushner et al., 2010).

Here, we describe a *Drosophila melanogaster* model of GALE deficiency; this is the first whole-animal genetic model of epimerase deficiency galactosemia to be reported. Using this model, we have confirmed that, as predicted by Kalckar, GALE is essential for fly development on food lacking galactose, thereby recapitulating the acute outcome reported for patients with generalized epimerase deficiency.

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 *dGALE* intron, *P{EPgy2}CG12030EY22205* (CG9232), referred to hereafter as *dGALE* f00624, was identified as part of the Exelixis collection (Thibault et al., 2004). Soluble protein lysates from adults that were heterozygous or homozygous (where viable) for each of these genotypes (Table 1), indicated that this insertion creates a strong loss-of-function allele and, considering the homozygous lethality of *dGALE* f00624, suggesting that profound loss of GALE is lethal in *D. melanogaster*.

To further analyze the organismal requirement for GALE, we generated additional *dGALE* alleles by imprecise excision of a pre-existing P-element insertion in the 5’ untranslated region of *dGALE* [Berkeley Drosophila Genome Project (BDGP); www.fruitfly.org/]; unlike the *dGALE* f00624 insertion, this *P{EPgy2}CG12030EY22205* insertion (Fig. 2) is homozygous viable. By mobilizing the

![Fig. 2. *dGALE* alleles used in this study.](Image)

Introns are shown as thin lines and exons as thick black lines, with coding regions in filled black and non-coding regions cross-hatched. Transposon insertion sites are denoted by triangles, and deleted regions are indicated.
Table 1. Leloir pathway enzyme activities

<table>
<thead>
<tr>
<th>Genotype (comments)</th>
<th>GALE (pmol product/µg protein/min)</th>
<th>GALK</th>
<th>GALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>w1118 (wild-type, adult male)</td>
<td>81.42±2.42</td>
<td>19.30±1.68</td>
<td>24.31±2.03</td>
</tr>
<tr>
<td>dGALEf00624/dGALEf00624 (precise excision, adult male)</td>
<td>91.65±5.58</td>
<td>26.79±7.47</td>
<td>29.64±2.50</td>
</tr>
<tr>
<td>dGALEf00624e (P-element disruption, adult male)</td>
<td>39.24±4.18</td>
<td>16.50±1.39</td>
<td>25.99±6.06</td>
</tr>
<tr>
<td>dGALEf00624 (imprecise excision, adult male)</td>
<td>42.95±4.01</td>
<td>15.44±4.68</td>
<td>25.02±2.57</td>
</tr>
<tr>
<td>dGALEf00624/hypomorphic (adult male)</td>
<td>6.62±0.48</td>
<td>14.28±0.51</td>
<td>24.06±0.63</td>
</tr>
<tr>
<td>UAS-hGALE/actSC-GA4; dGALEf00624e (hGALE rescue, adult male)</td>
<td>hGALEe,h: 45.32±10.46</td>
<td>12.16</td>
<td>25.56±3.74</td>
</tr>
<tr>
<td>UAS-hGALE/actSC-GA4; dGALEf00624e (hGALE rescue, adult male)</td>
<td>hGALEe,h: 66.34±40.11</td>
<td>18.23±1.74</td>
<td>*</td>
</tr>
<tr>
<td>GAL80+/actSC-GA4; UAS-RNAiGALT/+/dGALE knockdown</td>
<td>Larvae: 3.76±0.62</td>
<td>32.04; 30.01</td>
<td>10.30±2.92</td>
</tr>
<tr>
<td>GAL80+/UAS-RNAiGALT/TSTL (control for knockdown)</td>
<td>Male: Pupae: 5.69±0.21</td>
<td>NT</td>
<td>29.92±2.18</td>
</tr>
<tr>
<td></td>
<td>Adults: 6d: 77.22±0.81</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Adults: 14d: 71.52; 73.77</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Female: Pupae: 2.96±0.04</td>
<td>NT</td>
<td>21.64±0.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adults: 6d: 44.05±1.65</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Adults: 14d: 35.67; 39.08</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Lethal dGALE alleles were assayed in heterozygotes (mutant/wild-type). Expression of hGALE transgenes was driven by arm-GAL4 for physiological or low-level expression and by Act5C-GAL4 for high-level expression, all in dGALEe,d/dGALEe,d trans-heterozygotes. Details concerning the ages and environmental exposures of each cohort of animals are presented in the Methods. *GALT activity measurements for animals overexpressing hGALE using an actSC-GA4 driver were quantitatively compromised by the extraordinarily high background levels of UDP-glc produced from UDP-gal (by GALE). NT, not tested.

after laying. Embryos that failed to hatch into L1s (larvae at the first instar stage of development), eventually becoming cloudy or discolored, were scored as ‘dead’. Those larvae that did hatch did not survive past the second instar (L2) stage of development; surviving L1s and L2s were small (Fig. 3) and lethargic. Larvae that failed to respond when touched, and that eventually turned cloudy or discolored were scored as dead.

Suspecting that the prolonged and somewhat variable period of death might reflect the variable persistence of maternally loaded dGALE mRNA and protein in individual animals, we created germline clone mutants of dGALEf00624, as described in the Methods. With the maternal dGALE component removed, dGALEf00624, P(FRT)235/dGALEb trans-heterozygotes died uniformly in late embryogenesis, confirming an absolute requirement for GALE at this stage of development (Fig. 3). Of note, unfertilized eggs were identified by visual inspection and removed from all cohorts prior to further analysis; embryonic death was scored only among apparently fertilized eggs. Further, although they all died prior to hatching, the germline clone GALE-deficient embryos displayed no obvious morphological defects; cuticle deposition and patterning, and tracheal development, appeared indistinguishable from controls (data not shown).

Interestingly, dGALE germline clone mutant embryos fertilized by a wild-type paternal dGALE allele (dGALEf00624, P(FRT)235/) were viable and eclosed as healthy fertile adults. Furthermore, these paternal allele dGALE heterozygotes did not differ from their wild-type dGALE homozygous (P(FRT)235/) counterparts in their survival rates (Fig. 3). Paternal rescue of germline clone embryos demonstrates that, although GALE may be essential in late embryogenesis, it is not essential in early embryogenesis prior to the onset of zygotic transcription.

As a final test of the requirement for dGALE function in developing Drosophila, we used an Act5C-GAL4 driver in combination with an inducible UAS-RNAiGAL80 transgene to rescue the trans-heterozygous lethality of dGALE. These animals phenocopied Drosophila carrying the strong loss-of-function alleles dGALEf00624 and dGALEb; no viable Act5C-GAL4>UAS-RNAiGAL80 offspring were observed out of 894 animals scored.

Human GALE rescues viability of dGALE-deficient Drosophila

To confirm that the homozygous lethality of dGALEf00624 and dGALEb alleles resulted from loss of GALE activity, and not from any cryptic off-target effect of the disruption or intragenic deletion, we demonstrated the ability of human GALE (UAS-hGALE) transgenes to rescue the trans-heterozygous lethality of dGALEf00624/dGALEb animals. Two separate UAS-hGALE transgene insertions were tested, each driven by either arm-GAL4
GALE is essential for fly development

(Ahmad and Henikoff, 2001) to achieve low-level expression, or by ActSC-GAL4 to achieve high-level expression. In all four contexts, hGALE rescued the viability of the dGALE-deficient animals, albeit to different extents. When hGALE expression was driven by arm-GAL4 the following numbers of observed and expected rescued animals were obtained: UAS-hGALE<sup>52A</sup>, n=15/258 (observed) and n=52/258 (expected); UAS-hGALE<sup>1082</sup>, n=102/556 (observed) and n=111/556 (expected). When these transgenes were driven by ActSC-GAL4, the following numbers of observed and expected rescued animals were obtained: UAS-hGALE<sup>52A</sup>, n=174/916 (observed) and n=131/916 (expected); UAS-hGALE<sup>1082</sup>, n=418/1540 (observed) and n=308/1540 (expected). That the observed number of rescued animals in the ActSC crosses was often higher than predicted from the mendelian ratio may reflect the relatively enhanced fitness of animals overexpressing hGALE, or the relatively reduced fitness of animals carrying balancer chromosomes, or both. Rescued animals from all crosses were viable, fertile and appeared morphologically normal.

As expected, biochemical analyses of lysates from rescued animals carrying the arm-GAL4 driver demonstrated low to near-physiological levels of hGALE expression, whereas lysates from rescued animals carrying the ActSC-GAL4 driver demonstrated up to 25-fold overexpression of hGALE (Table 1). Combined, these data confirm that hGALE is a functional ortholog of dGALE, that sub-physiological expression of hGALE provides partial rescue of viability in dGALE-deficient Drosophila, and that overexpression of hGALE fully rescues viability with no apparent negative consequences.

Transgenic expression of hGALE similarly rescued the lethality of dGALE RNA interference (RNAi) knockdown animals, confirming that the lethality observed in these animals is a specific effect of GALE loss and not an off-target RNAi effect. Strong, ubiquitous expression of both UAS-hGALE<sup>1082</sup> and UAS-RNAi<sup>hGALE</sup> driven by ActSC-GAL4 resulted in the expected ratio of 124 viable progeny from a total of 274; no viable progeny expressing only RNAi<sup>hGALE</sup> without hGALE were observed. Again, rescued animals were fertile and appeared morphologically normal.

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

To determine which developing organ systems require GALE function, we used tissue-specific knockdown of dGALE. We tested the impact of UAS-RNAi<sup>hGALE</sup> expression, and therefore dGALE knockdown, in a total of five different tissues or tissue combinations, including: salivary gland (using Sga3-GAL4, FlyBase); neurons [elav-GAL4 (Lin and Goodman, 1994) and pros-GAL4 (Pearson and Doe, 2003)]; larval brain and fat body [P{GawB}c754 (Harrison et al., 1995)]; eye [gmr-GAL4 (Perrin et al., 2003) and eyeless-GAL4, FlyBase]; and embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubules and small intestine (drm-GAL4 driver, FlyBase). Of these five, only dGALE knockdown in the embryonic proventriculus, anterior midgut, posterior midgut, Malpighian tubule and small intestine, driven by drm-GAL4, resulted in a clear phenotype; 100% of these animals died at 28-29°C (no viable knockdowns from a total of 128 animals scored).

To specify Drosophila tissues in which GALE function might be sufficient for viability, we selectively expressed a UAS-hGALE transgene in an otherwise dGALE-deficient background using the same collection of tissue-specific GAL4 drivers listed above. We observed that drm-GAL4-driven hGALE was minimally sufficient to rescue dGALE<sup>10024*/dGALE<sup>n</sup></sup> trans-heterozygotes, demonstrating a ratio of 15 rescued out of 484 offspring scored; the expected mendelian ratio was 1:3, or 161:484. By contrast, hGALE expressed in the nervous system by elav-GAL4, or in the larval brain and fat body by P{GawB}c754, was completely insufficient to rescue or delay lethality in dGALE<sup>10024*/dGALE<sup>n</sup></sup> trans-heterozygotes (zero rescued out of >400 offspring scored).

**dGALE function is required at many stages of Drosophila development**

By employing a temperature-sensitive allele of GAL80 (GAL80<sup>ts</sup>) that negatively regulates GAL4-dependent transgene expression at the permissive temperature of 18°C, but not at the restrictive temperature of 28-29°C, we established conditional knockdown of dGALE and applied this system to identify the developmental window of GALE requirement in Drosophila. As described in the Methods and illustrated in Fig. 4, we generated a staggered series
of cohorts, each of which experienced loss of dGALE beginning one day later in development. Of note, flies develop at different rates at 18°C and 28-29°C; the relevant stages of development of each cohort of animals at the time they were shifted are indicated in Fig. 4. As controls, some crosses were maintained at 18°C for the duration of the experiment; in these animals, functional GAL80ts suppressed dGALE knockdown and the animals survived to adulthood. Other crosses were maintained at the restrictive temperature of 28-29°C for the duration of the experiment; in these animals GAL80ts did not suppress dGALE knockdown and, as expected, the animals died in development.

By staging the dGALE knockdown schedule and recording the outcomes of resulting cohorts of animals, we determined that GALE is required at multiple stages of Drosophila development from embryogenesis through to pupation (Fig. 4). Animals that were shifted during pupation produced viable adults that demonstrated some negative effects on life span and fertility (Fig. 4). Females shifted early in pupation laid few, if any, embryos when crossed to control males; however, their ovaries (germaria) and eggs appeared morphologically normal (data not shown). Similarly, crosses of control females to dGALE knockdown males that were shifted early in pupation resulted in few, if any, viable embryos being laid. Whether these apparent male and female fertility deficits reflect true reproductive dysfunction or behavioral disturbance (mating), or neuromuscular deficits that inhibit effective mating or egg deposition, remains unclear.

Enzyme assays of animals shifted as larvae (Fig. 4, unshaded arrow) or pupae (Fig. 4, shaded arrow) to the non-permissive temperature demonstrated strong knockdown of GALE with, relative to controls, less than 8% of GALE activity remaining after four days (larvae) or six days (pupae) at the restrictive temperature (Table 1). Considering that most larvae in the knockdown cohort died before the fourth day, but only live animals were harvested for biochemical analysis, these residual GALE activity values probably represent overestimates of the mean residual dGALE activity in the population.

However, in contrast to larvae and pupae, adult flies shifted to the non-permissive temperature after eclosion demonstrated only marginal knockdown when assayed either six or 14 days after the temperature shift (Table 1). The reason for this unexpected resistance to dGALE knockdown in adult flies remains unclear, but may reflect extraordinary stability of the dGALE protein or RNA in adults relative to developing animals. Similarly, the basis for the apparent disparity of knockdown efficiency in male versus female flies remains unclear. The minimal knockdown observed in adult flies suggests that animals shifted to the non-permissive temperature late in pupation may also have experienced only limited knockdown. The progressively milder phenotypes observed in animals shifted later in pupation may therefore reflect successively weaker degrees of knockdown rather than successively muted responses to GALE loss.

**Loss of GALE results in abnormal accumulation of gal-1P**

As a first step toward exploring the mechanism(s) of morbidity and mortality in GALE-deficient Drosophila, we asked whether GALE-deficient larvae and adult flies accumulate abnormal levels of gal-1P. Prior studies have demonstrated that GALE-impaired yeast (Douglas and Hawthorne, 1964; Ross et al., 2004), mammalian tissue culture cells (Schulz et al., 2005) and patients (Openo et al., 2006) all accumulate elevated levels of gal-1P upon exposure to high levels of environmental galactose, ostensibly because, when GALE is deficient, GALT activity is compromised by an accumulation of product (UDP-gal) and a depletion of substrate (UDP-glc).

To test whether gal-1P also accumulates in epimerase-deficient Drosophila we harvested animals that were shifted to knockdown conditions (28–29°C) as larvae and as newly eclosed adults (Table 2). Matched cohorts of animals were maintained on food containing 555 mM glucose alone or 555 mM glucose plus 111 mM galactose. As a control, we also prepared cohorts of animals that lacked the ActSC-GAL4 driver, so that dGALE was continuously expressed regardless of the temperature; these controls were maintained on the same foods and shifted to the same temperatures as their conditional knockdown counterparts.

As presented in Table 2, dGALE knockdown resulted in a greater than tenfold accumulation of gal-1P in larvae raised on food containing galactose, but not in larvae raised on food lacking galactose. Coefficients of dGALE-knockdown adult flies maintained on food containing galactose, but not on food lacking galactose, also accumulated statistically significant elevations of gal-1P, although the levels accumulated in adults were modest in comparison to larvae. The difference in gal-1P accumulation in galactose-exposed dGALE-knockdown larvae versus adults may
GALE is essential for fly development

Table 2. Accumulation of gal-1P in Drosophila exposed to galactose

<table>
<thead>
<tr>
<th>Genotype</th>
<th>555mM glc food</th>
<th>555mM glc + 111 mM gal food</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActSC-GAEL/GAL80Δ; UAS-RNAiGAEL/+</td>
<td>Larvae: 104.30±18.99</td>
<td>Larvae: 238.60±36.65</td>
</tr>
<tr>
<td>(dGALE knockdown)</td>
<td>Adults: 45.40±13.22</td>
<td>Adults: 129.66±16.55</td>
</tr>
<tr>
<td>(GAL80Δ; UAS-RNAiGAL80Δ(T2;3))</td>
<td>Larvae: 93.11±6.90</td>
<td>Larvae: 213.05±58.64</td>
</tr>
<tr>
<td>(controls, dGALE expressed)</td>
<td>Adults: 54.59±18.05</td>
<td>Adults: 72.19±3.94</td>
</tr>
</tbody>
</table>

Metabolites were extracted from cohorts of animals of the indicated genotypes and developmental stages that were maintained on food, with or without galactose supplementation as indicated, and shifted from the permissive temperature (18°C) to the restrictive temperature (28-29°C) for four days (larvae) or six days (adults), as described in the Methods. Values presented are average ± S.D. (n=3). *t*-test comparisons demonstrated that, on galactose-containing food, the gal-1P levels that accumulated in knockdown versus control adult flies, and in knockdown versus control larvae, were significant (P<0.01); the same comparisons on glucose food were not significant.

reflect the markedly different degrees of knockdown achieved in these populations (Table 1), or the different amounts of food consumed per body weight by these populations, or both. Of note, gal-1P was measured in mixed-gender populations (Table 2), whereas enzyme assays were performed on gender-segregated populations (Table 1).

Partial impairment of dGALE results in galactose sensitivity of Drosophila

To determine the impact of dietary galactose on Drosophila expressing minimal dGALE activity we quantified the survival rates of progeny from crosses between flies that were homozygous for the hypomorphic excision allele dGALEH, and balanced flies that were heterozygous for the same allele (Fig. 5). As a control, we performed parallel crosses involving homozygotes and heterozygotes for the precise excision allele dGALED. All crosses were set up on each of three different foods: food containing 555 mM glucose as the sole sugar, food containing 555 mM glucose plus 111 mM galactose, and food containing 555 mM glucose plus 111 mM mannose. On glucose-only food, both test and control crosses produced the expected 50:50 ratios of viable balanced and unbalanced flies, demonstrating that both dGALEH and dGALED homozygotes remain viable in the absence of galactose exposure. On food supplemented with galactose, however, the proportion of unbalanced offspring was reduced to around 25% for dGALEH crosses, but remained near 50% for dGALED crosses; this difference was highly significant (P<0.000001). The reduction in viability of dGALEH homozygous progeny was not seen in crosses conducted on food containing 555 mM glucose plus 111 mM mannose (Fig. 5), indicating that the impact was galactose specific.

DISCUSSION

We report here the development and application of a D. melanogaster genetic model of GALE deficiency. This is the first whole-animal genetic model of GALE deficiency and as such represents a major step forward for the field.

Using this fly model system we have confirmed what Kalckar postulated over 40 years ago – that a complete loss of GALE is incompatible with metazoan survival (Kalckar, 1965). 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 for survival? Finally, in an effort to model epimerase deficiency galactosemia, we have begun to explore the relationship between partial GALE impairment and viability in the presence of galactose.

When is dGALE required?

The results of our conditional dGALE knockdown experiments demonstrate that epimerase is required for organismal viability, continuously from late embryonic development through to mid-pupation. GALE is also required in late pupation for the normal fertility of both males and females, and for normal life span. The apparent compromised fertility of both male and female flies that are partially depleted of GALE in mid- to late-pupation might reflect reproductive, behavioral or neuromuscular defects. Of note, the dissection of female flies demonstrating infertility or reduced fecundity revealed apparently normal germaria and eggs, suggesting that the problem does not result from gross morphomorphology.

The GAL80Δ system allowed us to modulate the expression of an RNAi cassette at will, but the extent and timing of GALE loss was a...
function of many factors, including the abundance and half-life of any dGALE mRNA and/or protein pre-existing at the time of knockdown. The span of time from temperature shift to death, which was two to five days in most instances (Fig. 3), therefore probably represents an overestimate of the span of time that 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 germline clone mutant lethality (Fig. 3). This window of early GALE independence suggests either that GALE truly is not required for the key processes of early development, or alternatively that damage resulting from GALE deficiency in the earliest stages of development is not immediately lethal and can be reversed upon the expression of paternally derived GALE. At this time, the possibility cannot be ruled out that early embryonic development proceeds in GALE-deficient embryos owing to some cryptic maternal loading of galactose metabolites, perhaps derived by scavenging from hemolymph or other non-ovarian tissue. Finally, whether dGALE is required for adult viability remains untested.

Where is dGALE required?

In situ RNA hybridization studies demonstrate that dGALE transcripts are enriched in the salivary glands, proventriculus and developing gut of Drosophila embryos (www.fruitfly.org/cgi-bin/ex/insitu.pl). We addressed the question of the tissue-specific requirement for epimerase function from the perspectives of both necessity and sufficiency using conditional knockdown and conditional add-back of GALE, respectively. In short, we found that dGALE expression in the embryonic proventriculus, midgut, Malpighian tubules and small intestine (using a drm-GAL4 driver) is both necessary and sufficient for survival, and that none of the other tissue-specific drivers tested demonstrated either necessity or sufficiency.

Given that the midgut and tubules are enriched for dGALE mRNA in control animals (Tomancak et al., 2002; Chintapalli et al., 2007), and are thus apparent sites of UDP-gal metabolism, it is not completely unexpected that the expression of dGALE (or hGALE) should rescue dGALE\textsuperscript{y} /dGALE\textsuperscript{y} trans-heterozygotes in these tissues. However, we cannot rule out the possibility that dGALE expression might be necessary and/or sufficient in additional tissues not tested here, or in combinations of specific tissues, as our experimental design required that we test the drivers 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 Drosophila midgut and tubule express specific UDP-galNAc transferases that are involved in mucin-type O-linked glycosylation reactions (Tian and Ten Hagen, 2006), and that these tissues demonstrate elevated glycans production during embryogenesis (Tian and Ten Hagen, 2007a). It has been suggested that, during tubulogenesis in these tissues and in the trachea, glycan expression is required for proper establishment of apical-luminal cell polarity (Tian and Ten Hagen, 2007a; Tian and Ten Hagen, 2007b). Since dGALE supplies the cell with UDP-galNAc, the obligate first sugar donor in all mucin-type O-linked glycosylation, it stands to reason that the requirement for GALE in the gut and tubules may be related to glycan expression in these tissues. By extension, human GALE may play a similar role in the development and maintenance of cell polarity; the highest level of expression of human GALE is in the bronchial epithelium (http://biogps.gnf.org/gene/2582), a site with strong apicobasal polarity (Su et al., 2004).

How much GALE is required?

Our results help to define a lower limit of epimerase function required for viability in Drosophila. The galactose sensitivity of dGALE\textsuperscript{y} homozygotes, which express about 8% of wild-type GALE activity, and the lethality of dGALE\textsuperscript{y}/dGALE\textsuperscript{y} or dGALE\textsuperscript{y}/dGALE\textsuperscript{y} compound heterozygotes, each of which should express about 4% residual GALE activity, together imply that the minimum level of GALE activity that is sufficient for survival is between 4 and 8%.

The arm-GAL4-driven hGALE transgenic experiments imply that about half of the normal levels of hGALE expression affords only partial rescue; however, this result may be misleading. If transgene expression levels vary between animals, then the animals expressing lower levels are more likely to die in development, and the animals expressing higher levels are more likely to survive to adulthood. Since only surviving adults were assayed for GALE activity, the values obtained may therefore present an overestimate of the mean of the original population.

The fact that dGALE\textsuperscript{y} homozygotes are sensitive to galactose exposure during development also suggests that metabolite accumulation might be detrimental to larvae with low levels of GALE activity. These results are fully consistent with reports of generalized epimerase deficiency patients who survive but are quite galactose sensitive (Walter et al., 1999). All of the cases reported to date are homozygotes for the V94M mutation, which reduces GALE activity to around 5% of wild-type levels with regard to UDP-gal and around 24% with regard to UDP-galNAc (Wohlers et al., 1999; Wohlers and Fridovich-Keil, 2000).

We addressed the possibility of a GALE overexpression phenotype using a human GALE transgene plus driver combination, which produced expression levels that were up to 25 times higher than 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 the levels of enzyme are sufficient to manage the substrate and product pools, excess enzyme should not perturb the equilibrium.

Why is dGALE required in development?

GALE plays a multifaceted role in the cell: as the third enzyme of the Leloir pathway it assists in the conversion of dietary galactose to gluc-1P, enabling the endogenous biosynthesis of UDP-gal and UDP-galNAc when dietary sources are insufficient, and maintaining appropriate ratios of UDP-gal:UDP-glcl and UDP-galNAc:UDP-glcl:UDP-glCNac to enable appropriate glycan biosynthesis. When GALE is deficient, each of these pathways may be perturbed, raising the question of which missing function leads to the detrimental phenotypes observed. Our data address this question from several angles.

First, our germline clone dGALE-deficient animals died uniformly as embryos despite the fact that they were not yet eating. This result rules out the possibility that dietary galactose could have accounted for the problem, and instead implicates an essential role
for GALE in the endogenous biosynthesis of UDP-gal and UDP-galNAc, and in the maintenance of proper UDP-gal:UDP-glc and UDP-galNAc:UDP-glcNAc ratios.

The galactose sensitivity resulting from partial GALE deficiency in developing Drosophila further implies that, although sensitivity to environmental galactose may not explain the full pathophysiology of GALE deficiency, the abnormal accumulation of metabolites in response to galactose exposure is nonetheless important. Indeed, our results demonstrate that GALE-deficient Drosophila, like epimerase-impaired galactosemia patients following exposure to galactose (Fridovich-Keil and Walter, 2008), accumulate elevated levels of gal-IP. This result further confirms that studies of the fly model system may provide an explanation of the spectrum of outcomes and the underlying mechanism of pathophysiology in epimerase deficiency galactosemia.

METHODS
Drosophila stocks and maintenance
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. Stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University unless noted otherwise. For experiments involving a wild-type control, the isogenic strain w1118 (FBst0005905) was used.

Stocks used for complementation testing in the excision screen were PBac[WH]CG12030f00624 (FBst1016354), Harvard Exelixis Collection; Df(3L)emc-E12/TM6B, Tb1 ca1 (FBst0002577); and w1118; Df(3L)ED4196, P[3’RS;3.3]ED4196/TM2 (FBst008050).

For viability assays, the alleles dGALEA and dGALEB were balanced using w1118; DfAtio/TM3, P[GA4-twi.G]2.3, P[UA5-2xEFGP]AH2.3, Sb1 Ser1 (FBst0006663). The following stocks were used to generate germline clone mutants: w*; P[FRT(w1118)]2A (FBst0001997); P[hsFLP]1, y1 w1118; DfAtio/TM3, ry* Sb1 (FBst0000007); and w*; P[ovoD1-18]3L P[FRT(w1118)]2A/st1[βTub65D]s3 e/TM3, Sb1 (FBst0002139).

The stocks used to determine timing, tissue-specific requirements and sufficiency of GALE were the UAS-RNAi stock I2030R-2 (NM_138200.2), National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan; w*; P[tub-GAL80]20; TM2/TM6B, Tbl (FBst0007019); y1 w*; P[Act5C-GAL4]2SF01/Cyo, y1 (FBst0004414); y1 w*; P[drm-GAL4.7.1]/1.1/TM3, Sb1 (FBst0007098); P[GawB]elavC155 (FBst000458); P[GawB]elV54, w1118 (FBst0006984); y1 w1118; P[Sgs3-GAL4.PD]/TP1 (FBst0006870); w1118; P[GMR-GAL4.w1]2/Cyo (FBst0009146); and y1 w1118; 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[EpFyg2]/CG12030EY22205 (FBst0022544) through exposure to a transposase source, H[Pdelta2-3]HoP8, y1 w*; Dr/TM3,Sb (a gift from Dr Subhabrata Sanyal, Emory University). Two hundred and seventy-eight excisions were identified by loss of the white marker gene and tested for complementation to the chromosomal deficiencies Df(3L)emc-E12 and Df(3L)I4196, and to the lethal PBac insertion PBac[WH]CG12030f00624. Homozygous lethal excisions that failed to complement these three stocks were selected as potential strong loss-of-function alleles. Homozygous viable excisions that did not fail to complement were retained as probable precise excisions. The strong loss-of-function excision allele dGALEA, the hypomorphic allele dGALEB, and the precise excision allele dGALEA were further characterized at the molecular level. The breakpoints for the excisions dGALEA and dGALEB were determined by PCR amplification and sequencing using the following primers: RSexcF119, 5’-GTCAGGCTCTGCTTAGCATTTG-3’; RSexc105, 5’-AGTGCTATCGTGTGTAACCC-3’; RSexcR720, 5’-GAATTGTTACGAAGTGGCAAC-3’; and RSexc1767, 5’-CTACCTTTCATGACTTGAC-3’. These primers were also used to confirm that CG12030 was not disrupted in the precise excision dGALEB. For dGALEA and dGALEB, genomic DNA was extracted from homozygous adults. Because dGALEB homozygotes were not viable, genomic DNA was obtained from dGALEB/+ heterozygotes. Amplicons were separated by size by performing electrophoresis through a 1.5% agarose gel, and the dGALEB ampiclon was excised and purified (QiAquick gel extraction kit, Qiagen) prior to sequencing.

GALK, GALT and GALE enzymatic assays
Animals harvested for analysis
All animals were maintained on molasses food prior to harvest. Unless specified otherwise, crude lysates were prepared from pools of six to ten adult males harvested eight to 48 hours after eclosion. We assayed only males to avoid possible complications from females harboring developing embryos. RNAi knockdown and control larvae developed for three to four days at 18°C, followed by four days at 28-29°C. RNAi knockdown and control pupae developed at 18°C from embryos until pupation (~13 days), followed by six days at 28-29°C, during which time they eclosed as adults. RNAi knockdown and control adult flies developed at 18°C from embryos until eclosion, followed by either six days or 14 days, as indicated, at 28-29°C.

Preparation of lysates
Cohorts of six to ten flies each were anesthetized with CO2 and resuspended in lysis buffer [one complete mini protease inhibitor cocktail pellet, EDTA-free (Roche) dissolved in 10 ml of 100 mM glycine, pH 8.7] at 10 µl/fly. Samples were ground on ice for 15 seconds using a Teflon micropestle and handheld micropestle motor (Kimble Chase Life Science and Research Products LLC, Vineland, NJ) and centrifuged at 16,110 × g for 5 minutes 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 an appropriate concentration (to maintain the linear range of the assay). 30 µl of diluted protein and 20 µl of a cocktail of substrates and cofactors were combined to start each reaction. The 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 minutes and then quenched by the addition of 450 μl of ice-cold high-performance liquid chromatography (HPLC)-grade water (Fisher). Particulates were removed from the samples by centrifugation at 4000 × g for 4 minutes at 4°C through 0.22-μm Costar Spin-X centrifuge tube filters (Corning); reactants and products were quantified by HPLC, as described previously (Ross et al., 2004), and the specific activity reported as pmol of product formed per μg protein per minute of reaction time. 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. The initial reaction mixture concentrations were: 125 mM glycglycine pH 8.7, 0.8 mM UDP-glc and (when relevant) 1.6 mM gal-1-P. To account for the conversion of UDP-glucose to UDP-gal by GALE in the lysates, assays were performed both with and without added gal-1-P. UDP-gal formed in the absence of added gal-1-P was subtracted from UDP-gal formed in the presence of gal-1-P, and the net UDP-gal production was used to calculate GALT-specific activity. Lysates were diluted 1:10.

**GALT assay conditions**
Activity was calculated from the conversion of gal-1-P to UDP-gal. The initial reaction mixture concentrations were: 100 mM glycine pH 8.7, 0.8 mM UDP-gal and 0.5 mM NAD. Lysates were diluted 1:60, except for those prepared from animals, which were diluted to a greater extent. Lysates from Act5C>y-GAL421 transgenic animals were diluted 1:200 and lysates from Act5C>y-GAL42082 animals were diluted 1:600.

**Viability assays**
Zygotic mutants were generated by crossing dGALEb4 and dGALEp6231 heterozygotes. Females carrying dGALEp6234 germ line clone embryos were generated by the FLPase-dominant female sterile (FLP-DFS) technique (Chou and Perrimon, 1996), and crossed either to heterozygous dGALEb4 males or to 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 needed to be genotyped for the presence or absence of a third chromosome balancer, TM3, P[w+1M]GAL4-tw1.G(2.3), P[UA.s-2xEGFP]AH2.3, Sb1 Ser1 was used. Embryos were collected on grape juice agar plates (10% w/v glucose, 3% w/v agar in organic, unfiltered Concord grape juice) spread with yeast paste. Embryos were dechorinated in 50% bleach for 3 minutes and rinsed with double deionized water before sorting. Trans-heterozygote embryos, identified as green fluorescent protein (GFP) negative, were placed under halocarbon oil overnight on grape juice plates spread with yeast paste. Any embryos that did not hatch as L1s by the following morning (~36 hours after egg-laying) were scored as dead embryos. Surviving L1s 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 EcoRI and SalI to release the hGALE insert, which was then cloned into the expression vector p[FUAST] (Brand and Perrimon, 1993) using EcoRI and Xhol sites located in the multiple cloning region. Transgenic lines were created by standard techniques using the helper plasmid p[wc Δ2,3] (Karess and Rubin, 1984). Transformants were selected by the presence of the white gene within pUAST and their insertions were mapped by standard methods. We confirmed that the transgenic lines were expressing hGALE by enzymatic assay, as described above.

**Timing of the 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 that were homozygous for both P[tubP-GAL80ts]10 and 12030R-2. The GAL80ts allele that we used allowed for maximal repression of GAL4 at 18°C, with progressive de-repression at increasing temperatures (McGuire et al., 2004). Crosses were maintained at 18°C to allow negative regulation of the UAS-GAL4 system by GAL80ts, and tapped at 24-hour intervals to fresh vials. When flies in the first vial began to eclose, all vials were shifted to 28-29°C to relieve repression of the UAS-GAL4 system by GAL80ts. In this manner, we created cohorts of flies in which GALE knockdown was initiated at 24-hour intervals in development. Vials were examined daily and scored for the presence of non-tubby larvae and pupae. Adult flies ecloseing from the vials were scored for the presence or absence of humeral.

**Galactose sensitivity**
For assays of galactose sensitivity and metabolite accumulation, crosses were maintained on cornmeal-agar-yeast extract food containing 555 mM of glucose. In some vials, galactose or mannose was added to a final concentration of 111 mM. To minimize the metabolism of sugars in the food by microbes, vials were not supplemented with live yeast. dGALEb4/dGALEb4 or dGALEb4/dGALEb4 virgin females were crossed to dGALEb4 or dGALEb4 heterozygous males balanced over TM6B, Tb, Hu. Offspring were scored for the presence or absence of humeral. The proportions of unbalanced offspring were compared in dGALEb4 and dGALEb4 crosses raised on a given type of food. Based on the results of an F-test of variance equality, a two-tailed, non-homoscedastic Student’s t-test was used to determine the statistical significance, for each type of food, of differences in the observed proportion of viable unbalanced offspring from dGALEb4 crosses compared with dGALEb4 crosses.
Measuring gal-1P accumulation in GALE-deficient larvae and adult flies

Co-horts of Act5C-GAL4/GAL80UAS-hGALE/+ flies were raised at 18°C on 555 mM glucose only or 555 mM glucose plus 111 mM galactose food. Animals to be assayed as larvae were transferred as L1s (on day three) to the restrictive temperature, 28-29°C, and were allowed to develop for another four days prior to harvest. Cohorts of male and female flies to be assayed as adults were transferred to fresh vials (of the same food type) within 48 hours of eclosion, and placed at the restrictive temperature for six days prior to harvest. As a control, corresponding cohorts lacking Act5C-GAL4 were also shifted to 28-29°C.

For analysis pools, ten adults or 20 larvae were anesthetized with CO₂, suspended in 125 μl of ice-cold 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). 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; Oopeno 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) 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 all samples, 20 μl were injected into a 25 μl injection loop. Based on the results of an F-test of variance equality, a two-tailed, homoscedastic Student’s t-test was used to determine, for both types of food, whether differences in the accumulation of gal-1P between the genotypes were statistically significant.

ACKNOWLEDGEMENTS

We thank Kerry Garza, Elisa Margolis and the members of the Moberg and Sanyal labs for helpful discussions concerning this project; Emily Ryan for help with statistical analyses; Michael Santoro for assistance with mapping of UAS-hGALE insertions; Jewels Chhay for contributions to the creation of the UAS-hGALE constructs; and Doug Hennig for embryo injection of the hGALE transgene. This work was supported by the National Institutes of Health grant DK046403 (to J.L.F.-K. and K.H.M.). Deposited in PMC for release after 12 months.

COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

R.D.S. created all of the dGALE excision alleles used in this work and also generated all of the data presented in Figs 2–5; J.M.I.S. and R.D.S. collaborated to generate 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 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. R.D.S. and J.L.F.-K. wrote the manuscript with editorial assistance from J.M.I.S. and K.H.M.

REFERENCES


