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

Rebecca D. Sanders, *Emory University*
Jennifer M. I. Sefton, *Emory University*
Kenneth H Moberg, *Emory University*
Judith Fridovich-Keil, *Emory University*

Journal Title: Disease Models and Mechanisms

Volume: Volume 3, Number 9-10

Publisher: Company of Biologists | 2010-06, Pages 628-638

Type of Work: Article | Final Publisher PDF

Publisher DOI: 10.1242/dmm.005058

Permanent URL: http://pid.emory.edu/ark:/25593/cr7gj

Final published version: http://dx.crossref.org/10.1242%2Fdmm.005058

RESEARCH ARTICLE

Disease Models & Mechanisms 3, 628-638 (2010) doi:10.1242/dmm.005058

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

Rebecca D. Sanders[1], Jennifer M. I. Sefton[2], Kenneth H. Moberg[3] and Judith L. Fridovich-Keil[4,*]

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-acetylglucosamine. 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-acetylglucosamine (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

[1]Graduate Program in Biochemistry, Cell and Developmental Biology, and
[2]Graduate Program in Genetics and Molecular Biology, Emory University, Atlanta, GA 30322, USA
[3]Department of Cell Biology, Emory University, School of Medicine, Atlanta, GA 30322, USA
[4]Department of Human Genetics, Emory University, School of Medicine, Room 325.3, Whitehead Building, 615 Michael Street, Atlanta, GA 30322, USA
*Author for correspondence (jfridov@emory.edu)

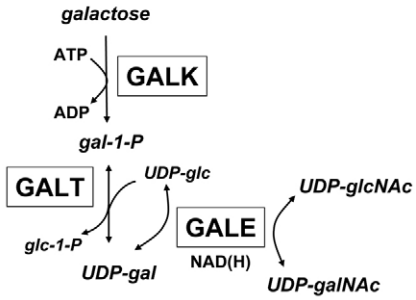

Fig. 1. The Leloir pathway of galactose metabolism. In both humans and *D. melanogaster*, GALE catalyzes the interconversion of UDP-gal and UDP-glc, as well as UDP-galNAc and UDP-glcNAc.

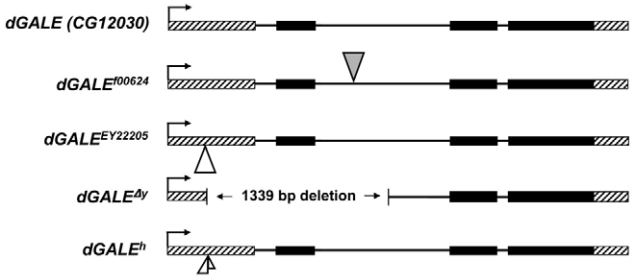

Fig. 2. *dGALE* alleles used in this study. Introns are shown as thin lines and exons as thick bars, with coding regions in filled black and non-coding regions cross-hatched. Transposon insertion sites are denoted by triangles, and deleted regions are indicated.

that *D. melanogaster* encode (http://superfly.ucsd.edu/homophila) 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 animals (Kalckar, 1965). Drosophila lacking *dGALE* die as embryos, and developing larvae from which *dGALE* has been conditionally knocked down die within days of *dGALE* loss. Of note, tissue-specific knockdown of *dGALE* in the gut primordium and Malpighian tubules is also lethal. Transgenic expression of human *GALE* (*hGALE*) restores viability, as does selective *hGALE* expression in the gut and Malpighian tubules of otherwise-*dGALE*-deficient animals. Finally, animals with partial GALE impairment survive development on food lacking galactose, but show reduced survival following exposure to 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, *PBac{WH}CG12030*^{f00624}, 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 for this allele demonstrated approximately 50% GALE enzymatic activity compared with age- and gender-matched wild-type animals (Table 1), indicating 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}CG12030*^{EY22205} insertion (Fig. 2) is homozygous viable. By mobilizing the *P{EPgy2}CG12030*^{EY22205} element through transient exposure to *Δ2-3* transposase in the germline of male flies (Ryder and Russell, 2003), as described in the Methods, we generated 278 excisions. In the work described here we have focused on two imprecise excision alleles identified from that cohort: *dGALE*^{Δy}, which is homozygous lethal, and *dGALE*^{h}, which is homozygous viable. As a control, we also studied a precise excision allele, *dGALE*^{d}, identified from the same cohort.

As with the lethal transposon insertion *dGALE*^{f00624}, *dGALE*^{Δy}/+ heterozygotes retained around 50% of wild-type GALE activity (Table 1). Animals that were homozygous for the precise excision *dGALE*^{d} allele had fully wild-type GALE activity, and *dGALE*^{h} homozygotes demonstrated approximately 8% of wild-type GALE activity (Table 1). Finally, although *dGALE*^{h} was homozygous viable, both *dGALE*^{h} and *dGALE*^{Δy} alleles were 100% lethal in trans to chromosomal deficiencies [*Df(3L)emc-E12* and *Df(3L)4196*], which remove *CG12030*. Similarly, *dGALE*^{h} and *dGALE*^{Δy} were lethal in trans to each other, and both were also lethal in trans to *dGALE*^{f00624}.

Molecular characterization of *dGALE*^{Δy} revealed a 1339-bp deletion from *dGALE* that included the first coding exon (Fig. 2). Molecular characterization of *dGALE*^{h} revealed a partial P-element excision that left around 1500 bp of P-element sequence behind without further perturbation of *CG12030*. There is precedent for the creation of an impaired allele from the partial deletion of a P-element insertion located in a gene regulatory region (Secombe et al., 1998). Enzyme activities for animals that are heterozygous or homozygous (where viable) for each of these genotypes (Table 1), in concert with complementation and sequencing data, confirmed that *dGALE*^{f00624} and *dGALE*^{Δy} are both strong loss-of-function alleles and that *dGALE*^{h} is a hypomorphic allele of *dGALE*. We also measured GALK and GALT activities in each lysate; there was some minor variation in levels, but none of these differences was statistically significant [analysis of variance (ANOVA) P value >0.05].

Loss of *dGALE (CG12030)* is embryonic lethal in Drosophila

Trans-heterozygotes of *dGALE*^{f00624} and *dGALE*^{Δy} showed increased lethality during embryogenesis relative to wild-type animals; specifically, embryos were examined visually at least twice each day after dechorination, which was performed at 12-20 hours

Table 1. Leloir pathway enzyme activities

		Apparent enzyme activity (average ± S.E.M., n≥3) (pmol product/μg protein/min)		
Genotype (comments)		**GALE**	**GALK**	**GALT**
w^{1118} (wild-type, adult male)		81.42±2.42	19.30±1.68	24.31±2.03
dGALE^{d}/*dGALE*^{d} (precise excision, adult male)		91.65±6.58	26.79±7.47	29.64±2.50
dGALE^{f00624}/+ (P-element disruption, adult male)		39.24±4.18	16.50±1.39	25.99±6.06
dGALE$^{\Delta y}$/+ (imprecise excision, adult male)		42.95±4.01	15.44±4.68	25.02±2.57
dGALE^{h}/*dGALE*^{h} (hypomorphic, adult male)		6.62±0.48	14.28±0.51	24.06±0.63
UAS-hGALE/arm-GAL4; dGALE$^{\Delta y}$/*dGALE*^{f00624} (*hGALE* rescue, adult male)		*hGALE*^{32A}: 45.32±10.46 *hGALE*^{40B2}: 164.85±27.51	12.16 16.02±1.73	25.56±3.74 12.91±3.30
UAS-hGALE/Act5C-GAL4; dGALE$^{\Delta y}$/*dGALE*^{f00624} (*hGALE* rescue, adult male)		*hGALE*^{32A}: 666.34±40.11 *hGALE*^{40B2}: 2323.37 ±138.83	18.23±1.74 16.41±3.19	* *
GAL80^{ts}*/Act5C-GAL4; UAS-RNAi*^{dGALE}/+ (*dGALE* knockdown)		Larvae: 3.76±0.62	32.04; 30.01	10.30±2.92
	Male	Pupae: 5.69±0.21	NT	29.92±2.18
		Adults, 6d: 77.22±0.81	NT	NT
		Adults, 14d: 71.52; 73.77	NT	NT
	Female	Pupae: 2.96±0.04	NT	21.64±0.67
		Adults, 6d: 44.05±1.65	NT	NT
		Adults, 14d: 35.67; 39.08	NT	NT
GAL80^{ts}*; UAS-RNAi*^{dGALE}/*TSTL* (control for knockdown)		Larvae: 48.26±2.08	17.16; 22.07	17.25±1.59
	Male	Pupae: 78.09±1.14	NT	38.49±1.78
		Adults, 6d: 81.64±1.73	NT	NT
		Adults, 14d: 76.97	NT	NT
	Female	Pupae: not tested	NT	23.82±0.37
		Adults, 6d: not tested	NT	NT
		Adults, 14d: 71.64	NT	NT

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 *dGALE*^{f00624}/*dGALE*$^{\Delta y}$ 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 *Act5C-GAL4* driver were quantitatively compromised by the extraordinarily high background levels of UDP-gal produced from UDP-glc (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 ceased all movement (observed over multiple days), 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 *dGALE*^{f00624}, as described in the Methods. With the maternal *dGALE* component removed, *dGALE*^{f00624}, *P{FRT}*^{2A}*/dGALE*$^{\Delta y}$ 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 (*dGALE*^{f00624}, *P{FRT}*^{2A}/+) 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}*^{2A}/+) 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-RNAi*^{dGALE} knockdown construct (*12030-R2*, National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan) to drive ubiquitous knockdown of *dGALE*. These animals phenocopied Drosophila carrying the strong loss-of-function alleles *dGALE*^{f00624} and *dGALE*$^{\Delta y}$; no viable *Act5C-GAL4>UAS-RNAi*^{dGALE} offspring were observed out of 894 animals scored.

Human *GALE* rescues viability of *dGALE*-deficient Drosophila

To confirm that the homozygous lethality of *dGALE*^{f00624} and *dGALE*$^{\Delta y}$ alleles resulted from loss of GALE activity, and not from some 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 *dGALE*^{f00624}*/dGALE*$^{\Delta y}$ animals. Two separate *UAS-hGALE* transgene insertions were tested, each driven by either *arm-GAL4*

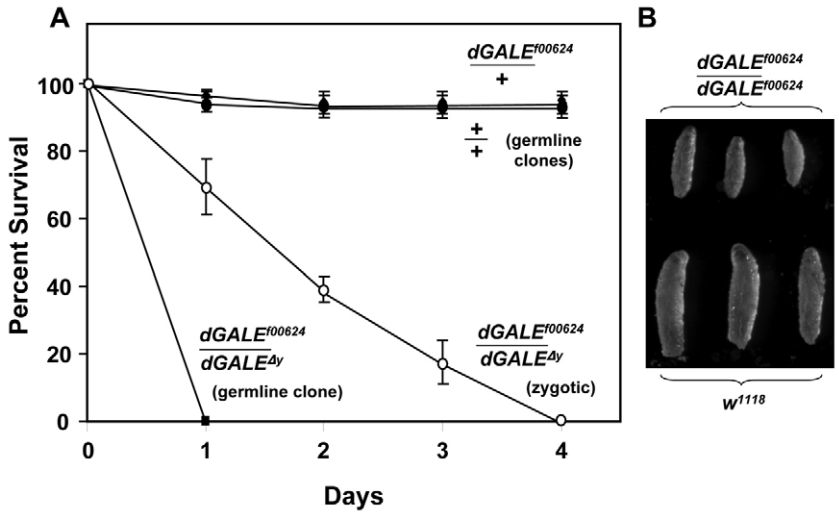

Fig. 3. Compromised viability of *dGALE* mutants. (A) Some zygotic $dGALE^{f00624}/dGALE^{\Delta y}$ mutants (open circles) died as embryos, whereas others survived to mid-larval stages; however, all $dGALE^{f00624}/dGALE^{\Delta y}$ germline clone mutants (filled squares) died as embryos. By contrast, the survival rates of germline clone $dGALE^{f00624}$/+ heterozygotes (filled triangles) and their germline clone homozygous wild-type counterparts (filled circles) were indistinguishable. (B) GALE-deficient zygotic larvae (top three animals), which were hatched and maintained on grape juice agar plates with yeast paste for 48 hours, were smaller than their wild-type counterparts (bottom three animals).

(Ahmad and Henikoff, 2001) to achieve low-level expression, or by *Act5C-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: $UAS\text{-}hGALE^{32A}$, *n*=15/258 (observed) and *n*=52/258 (expected); $UAS\text{-}hGALE^{40B2}$, *n*=102/556 (observed) and *n*=111/556 (expected). When these transgenes were driven by *Act5C-GAL4*, the following numbers of observed and expected rescued animals were obtained: $UAS\text{-}hGALE^{32A}$, *n*=174/916 (observed) and *n*=131/916 (expected); $UAS\text{-}hGALE^{40B2}$, *n*=418/1540 (observed) and *n*=308/1540 (expected). That the observed number of rescued animals in the *Act5C* 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 *Act5C-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 $UAS\text{-}hGALE^{40B2}$ and $UAS\text{-}RNAi^{dGALE}$ driven by *Act5C-GAL4* resulted in the expected ratio of 124 viable progeny from a total of 274; no viable progeny expressing only $RNAi^{dGALE}$ 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 $UAS\text{-}RNAi^{dGALE}$ expression, and therefore *dGALE* knockdown, in a total of five different tissues or tissue combinations, including: salivary gland (using *Sgs3-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 $dGALE^{f00624}/dGALE^{\Delta y}$ 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 $dGALE^{f00624}/dGALE^{\Delta y}$ 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* ($GAL80^{ts}$) 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

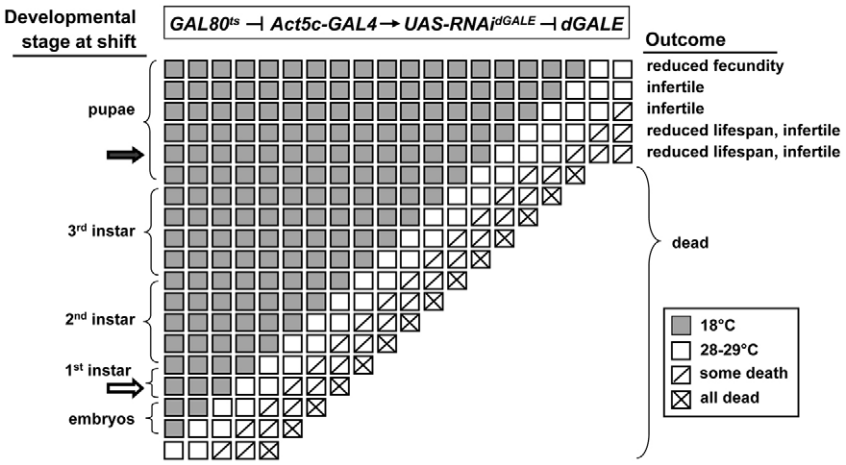

Fig. 4. *dGALE* is required throughout development. Each row represents a cohort of animals in which *dGALE* knockdown was initiated on a particular day in development by shifting the animals from the *GAL80*^{ts} permissive temperature of 18°C, to the restrictive temperature of 28-29°C. Shaded boxes represent days at the permissive temperature (*dGALE* expressed) and open boxes represent days at the restrictive temperature (*dGALE* knockdown). Of note, knockdown was not complete and varied by age, as presented in Table 1 and described in the Results. The arrows at the left of the figure indicate cohorts of larvae and pupae that were harvested for enzymatic assays (Table 1). Slashed and crossed boxes denote the outcomes observed for a given cohort on a given day, as indicated in the key. Final outcomes are summarized to the right of each row.

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 *GAL80*^{ts} 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 *GAL80*^{ts} 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 *Act5C-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. Cohorts 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

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

	Gal-1P (pmol/mg protein)	
Genotype	**555mM glc food**	**555mM glc + 111 mM gal food**
Act5C-GAL4/GAL80^{ts}; UAS-RNAi^{dGALE}/+ (*dGALE* knockdown)	Larvae: 104.30±18.99 Adults: 45.40±13.22	Larvae: 2386.20±365.65 Adults: 129.66±16.55
(GAL80^{ts}; UAS-RNAi^{dGALE})/T(2;3)TSTL, CyO: TM6B (controls, *dGALE* expressed)	Larvae: 93.11±6.90 Adults: 54.59±18.05	Larvae: 213.05±58.64 Adults: 72.19±3.94

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 *dGALE^{h}*, 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 *dGALE^{d}*. 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 *dGALE^{h}* and *dGALE^{d}* 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 *dGALE^{h}* crosses, but remained near 50% for *dGALE^{d}* crosses; this difference was highly significant (P<0.000001). The reduction in viability of *dGALE^{h}* 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 dysmorphology.

The *GAL80^{ts}* system allowed us to modulate the expression of an RNAi cassette at will, but the extent and timing of GALE loss was a

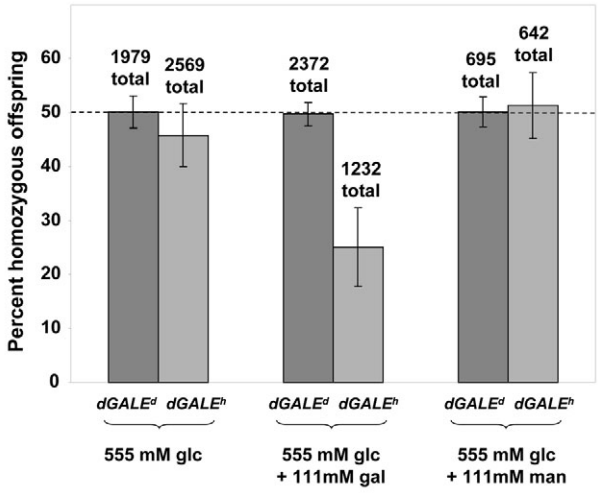

Fig. 5. Hypomorphic *dGALE* mutants are sensitive to dietary galactose. Crosses between homozygotes and heterozygotes for both the precise excision *dGALE^{d}* allele (dark columns) and the hypomorphic *dGALE^{h}* allele (light columns) yielded the indicated percentages of homozygous offspring on food containing glucose, glucose plus galactose, and glucose plus mannose. The number above each column represents the total number of progeny scored for that condition. The expected proportion of 50% homozygotes is represented by a dashed line. Crosses involving the *dGALE^{h}* hypomorphic allele produced significantly fewer homozygotes on food containing galactose than crosses involving the control *dGALE^{d}* allele.

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 $dGALE^{\Delta y}/dGALE^{f00624}$ 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 glycan 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 $dGALE^{h}$ homozygotes, which express about 8% of wild-type GALE activity, and the lethality of $dGALE^{h}/dGALE^{\Delta y}$ or $dGALE^{h}/dGALE^{f00624}$ 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 $dGALE^{h}$ 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 glc-1P, enabling the endogenous biosynthesis of UDP-gal and UDP-galNAc when dietary sources are insufficient, and maintaining appropriate ratios of UDP-gal:UDP-glc and UDP-galNAc: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-1P. This result further confirms that future 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 mls propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol). For experiments in which the levels and types of sugar were to be varied, we used a glucose-based food [5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l glucose, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol)] (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 w^{1118} (FBst0005905) was used.

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

For viability assays, the alleles *dGALE*^{y} and *dGALE*^{f00624} were balanced using w^{1118}*; Dr*^{Mio}*/TM3, P{GAL4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb*^{1} *Ser*^{1} (FBst0006663). The following stocks were used to generate germline clone mutants: *w*; P{FRT(w*^{hs}*)}2A* (FBst0001997); *P{hsFLP}1, y*^{1} w^{1118}*; Dr*^{Mio}*/TM3, ry* Sb*^{1} (FBst0000007); and *w*; P{ovoD1-18}3L P{FRT(w*^{hs}*)}2A/st*^{1} β*Tub85D*^{D} *ss*^{1} *e*^{s}*/TM3, Sb*^{1} (FBst0002139).

The stocks used to determine timing, tissue-specific requirements and sufficiency of GALE were the UAS-RNAi stock *12030R-2,* (NM_138200.2), National Institute of Genetics Fly Stock Center, Mishima, Shizuoka, Japan; *w*; P{tubP-GAL80*^{ts}*}20; TM2/TM6B, Tb1* (FBst0007019); *y*^{1} *w*; P{Act5C-GAL4}25FO1/CyO, y*$^{+}$ (FBst0004414); w^{1118}*; P{drm-GAL4.7.1}1.1/TM3, Sb*^{1} (FBst0007098); *P{GawB}elav*^{C155} (FBst0000458); *P{GawB}c754,* w^{1118} (FBst0006984); w^{1118}; *P{Sgs3-GAL4.PD}TP1* (FBst0006870); w^{1118}*; P{GMR-GAL4.w*$^{-}$*}2/CyO* (FBst0009146); and *y*^{1} w^{1118}*; P{ey1x-GAL4.Exel}3* (FBst0008227).

Generation and characterization of excision alleles

Excision alleles were generated by mobilizing a P-element insertion in *CG12030, P{EPgy2}CG12030*^{EY22205} (FBst0022544) through exposure to a transposase source, *H{PDelta2-3}HoP8, y*^{1}*w*;;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)4196*, and to the lethal *PBac* insertion *PBac{WH}CG12030*^{f00624}. Homozygous lethal excisions that failed to complement these three stocks were selected as potential strong loss-of-function alleles. Homozygous viable excisions that demonstrated a failure to complement were selected as potential hypomorphs, and designated for further characterization for environmental galactose sensitivity and GALE enzyme activity levels. Homozygous viable excisions that did not fail to complement were retained as probable precise excisions. The strong loss-of-function excision allele *dGALE*^{Δy}, the hypomorphic allele *dGALE*^{h}, and the precise excision allele *dGALE*^{d} were further characterized at the molecular level. The breakpoints for the excisions *dGALE*^{h} and *dGALE*^{Δy} were determined by PCR amplification and sequencing using the following primers: RSexcF119, 5′-GTCAGCCTGCTGCTAGCACTTG-3′; RSexc105, 5′-AGTGCTATCGTGTGCTAACC-3′; RSexcR720, 5′-GAATGGTATCCAGTTGGCAC-3′; and RSexc1767, 5′-CTACCTTGTCAGACTTGCAC-3′. These primers were also used to confirm that *CG12030* was not disrupted in the precise excision *dGALE*^{d}. For *dGALE*^{d} and *dGALE*^{h}, genomic DNA was extracted from homozygous adults. Because *dGALE*^{Δy} homozygotes were not viable, genomic DNA was obtained from *dGALE*^{Δy}/+ heterozygotes. Amplicons were separated by size by performing electrophoresis through a 1.5% agarose gel, and the *dGALE*^{Δy} amplicon 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 CO_2 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: 2 mM $MgCl_2$, 40 mM Tris pH 8.0, 40 µM dithiothreitol, 4 mM galactose and 4 mM ATP. Lysates were diluted 1:10.

GALT assay conditions

Activity was calculated from the conversion of gal-1-P to UDP-gal. The initial reaction mixture concentrations were: 125 mM glycylglycine pH 8.7, 0.8 mM UDP-glc and (when relevant) 1.6 mM gal-1-P. To account for the conversion of UDP-glc 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.

GALE assay conditions

Activity was calculated from the conversion of UDP-gal to UDP-glc. 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 $dGALE^{h}/dGALE^{h}$ animals, which were diluted 1:30, and those prepared from animals overexpressing *hGALE* transgenes, which were diluted to a greater extent. Lysates from $Act5C{>}hGALE^{32A}$ animals were diluted 1:200 and lysates from $Act5C{>}hGALE^{40B2}$ animals were diluted 1:600.

Viability assays

Zygotic mutants were generated by crossing $dGALE^{\Delta y}$ and $dGALE^{f00624}$ heterozygotes. Females carrying $dGALE^{f00624}$ germline clone embryos were generated by the FLPase-dominant female sterile (FLP-DFS) technique (Chou and Perrimon, 1996), and crossed either to heterozygous $dGALE^{\Delta y}$ males or to wild-type males. As a control, females carrying wild-type germline 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*$^{+mC}$*GAL4-twi.G}2.3, P{UAS-2xEGFP}AH2.3, Sb*^{1} *Ser*^{1} was used. Embryos were collected on grape juice agar plates (10% w/vol glucose, 3% w/vol agar in organic, unfiltered Concord grape juice) spread with yeast paste. Embryos were dechorinated in 50% bleach for 3 minutes and rinsed with 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 *Eco*RI and *Sal*I to release the *hGALE* insert, which was then cloned into the expression vector *pP{UAST}* (Brand and Perrimon, 1993) using *Eco*RI and *Xho*I sites located in the multiple cloning region. Transgenic lines were created by standard techniques using the helper plasmid *pP{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 $P\{tubP\text{-}GAL80^{ts}\}10$ and *12030R-2.* The $GAL80^{ts}$ 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 $GAL80^{ts}$, 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 $GAL80^{ts}$. 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 eclosing 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. $dGALE^{h}/dGALE^{h}$ or $dGALE^{d}/dGALE^{d}$ virgin females were crossed to $dGALE^{h}$ or $dGALE^{d}$ 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 $dGALE^{d}$ and $dGALE^{h}$ crosses raised on a given type of food. Based on the results of an F-test of variance equality, a two-tailed, 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 $dGALE^{h}$ crosses compared with $dGALE^{d}$ crosses.

TRANSLATIONAL IMPACT

Clinical issue

UDP-galactose 4′ epimerase (GALE) plays key roles in the metabolism of dietary galactose, in the production of endogenous galactose when exogenous sources are lacking, and in maintaining the ratios of key substrates for glycoprotein and glycolipid biosynthesis. GALE catalyzes the interconversion of UDP-galactose and UDP-glucose in the final step of the Leloir pathway of galactose metabolism and, in humans, also interconverts UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. No patients completely lacking GALE activity have been found, suggesting that loss of GALE must be lethal. However, partial loss of GALE can be frequent, at least in some populations, and results in epimerase deficiency galactosemia. This spectrum disorder ranges in severity from benign to potentially lethal, depending, among other factors, on the degree and tissue-specificity of GALE impairment. The prognosis and treatment for patients with epimerase deficiency galactosemia are currently ill-defined, partly because affected individuals are often either missed or misdiagnosed, but also owing to the lack of an appropriate animal model.

Results

This paper reports the generation and initial characterization of the first whole-animal model of *GALE* deficiency, using the fruit fly *Drosophila melanogaster*. Drosophila lacking *GALE* died as embryos, confirming that GALE function is essential in developing animals. Larvae in which Drosophila *GALE* (*dGALE*) expression was conditionally inhibited by *dGALE*-specific RNA interference (RNAi) also died within days of GALE loss. Both types of affected embryos were rescued by expression of a human *GALE* (*hGALE*) transgene, showing the specificity of the mutation. To examine possible tissue specificity of the lethal GALE phenotype, conditional knockdown and *hGALE* transgene expression were used to show that *GALE* expression in the gut primordium and Malpighian tubules was both necessary and sufficient for Drosophila survival. Finally, like patients with generalized epimerase deficiency galactosemia, Drosophila with a partial loss of GALE activity survived in the absence of galactose, but succumbed during development if exposed to dietary galactose.

Implications and future directions

These data establish the utility of the fly model of generalized *GALE* deficiency and set the stage for future studies to define the mechanism(s) and modifiers of outcome in epimerase deficiency galactosemia.

doi:10.1242/dmm.005835

Measuring gal-1P accumulation in GALE-deficient larvae and adult flies

Cohorts of *Act5C-GAL4/GAL80^{ts};UAS-RNAi^{dGALE}*/+ 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 CO_2, 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; 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) 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* construct; and Doug Rennie 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 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.

Received 2 December 2009; Accepted 17 April 2010.

REFERENCES

Ahmad, K. and Henikoff, S. (2001). Modulation of a transcription factor counteracts heterochromatic gene silencing in Drosophila. *Cell* **104**, 839-847.

Alano, A., Almashanu, S., Chinsky, J. M., Costeas, P., Blitzer, M. G., Wulfsberg, E. A. and Cowan, T. M. (1998). Molecular characterization of a unique patient with epimerase-deficiency galactosaemia. *J. Inher. Metab. Dis.* **21**, 341-350.

Bharucha, K. (2009). The epicurean fly: using Drosophila melanogaster to study metabolism. *Pediatr. Res.* **65**, 132-137.

Bier, E. (2005). Drosophila, the golden bug, emerges as a tool for human genetics. *Nat. Rev. Genet.* **6**, 9-23.

Brand, A. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007). Using FlyAtlas to identify better Drosophila melanogaster models of human disease. *Nat. Genet.* **39**, 715-720.

Chou, T. B. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster. *Genetics* **144**, 1673-1679.

Douglas, H. C. and Hawthorne, D. C. (1964). Enzymatic expression and genetic linkage of genes controlling galactose utilization in Saccharomyces. *Genetics* **49**, 837-844.

Fridovich-Keil, J. and Walter, J. (2008). Galactosemia. In *The Online Metabolic and Molecular Bases of Inherited Disease – OMMBID* (ed D. Valle, A. Beaudet, B. Vogelstein, K. Kinzler, S. Antonarakis and A. Ballabio), chapter 72. New York: McGraw-Hill. www.ommbid.com.

Gitzelmann, R., Steinmann, B., Mitchell, B. and Haigis, E. (1976). Uridine diphosphate galactose 4′-epimerase deficiency. *Helv. Paediatr. Acta* **31**, 441-452.

Harrison, D. A., Binari, R., Nahreini, T. S., Gilman, M. and Perrimon, N. (1995). Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *EMBO J.* **14**, 2857-2865.

Holden, H. M., Rayment, I. and Thoden, J. B. (2003). Structure and function of enzymes of the Leloir pathway for galactose metabolism. *J. Biol. Chem.* **278**, 43885-43888.

Holton, J. B., Gillett, M. G., MacFaul, R. and Young, R. (1981). Galactosemia: a new severe variant due to uridine diphosphate galactose-4-epimerase deficiency. *Arch. Dis. Child* **56**, 885-887.

Honjo, K. and Furukubo-Tokunaga, K. (2005). Induction of cAMP response element-binding protein-dependent medium-term memory by appetitive gustatory reinforcement in Drosophila larvae. *J. Neurosci.* **25**, 7905-7913.
Kalckar, H. M. (1965). Galactose metabolism and cell "sociology". *Science* **150**, 305-313.
Karess, R. E. and Rubin, G. M. (1984). Analysis of P transposable element functions in Drosophila. *Cell* **38**, 135-146.
Kingsley, D., Kozarsky, K. F., Hobbie, L. and Krieger, M. (1986). Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell* **44**, 749-759.
Krieger, M., Reddy, P., Kozarsky, K., Kingsley, D., Hobbie, L. and Penman, M. (1989). Analysis of the synthesis, intracellular sorting, and function of glycoproteins using a mammalian cell mutant with reversible glycosylation defects. *Methods Cell Biol.* **32**, 57-84.
Kushner, R. F., Ryan, E. L., Sefton, J. M. I., Sanders, R. D., Lucioni, P. J., Moberg, K. H. and Fridovich-Keil, J. L. (2010). A *Drosophila melanogaster* model of classic galactosemia. *Dis. Model. Mech.* **3** (in press) [doi:10.1242/dmm.005041].
Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.
McGuire, S., Roman, G. and Davis, R. (2004). Gene expression systems in Drosophila: a synthesis of time and space. *Trends Genet.* **20**, 384-391.
Openo, K., Schulz, J., Vargas, C., Orton, C., Epstein, M., Schnur, R., Scaglia, F., Berry, G., Gottesman, G., Ficicioglu, C. et al. (2006). Epimerase-deficiency galactosemia is not a binary condition. *Am. J. Hum. Genet.* **78**, 89-102.
Pearson, B. and Doe, C. (2003). Regulation of neuroblast competence in *Drosophila*. *Nature* **425**, 624-628.
Perrin, L., Bloyer, S., Ferraz, C., Agrawal, N., Sinha, P. and Dura, J. M. (2003). The leucine zipper motif of the Drosophila AF10 homologue can inhibit PRE-mediated repression: implications for leukemogenic activity of human MLL-AF10 fusions. *Mol. Cell. Biol.* **23**, 119-130.
Ross, K. L., Davis, C. N. and Fridovich-Keil, J. L. (2004). Differential roles of the Leloir pathway enzymes and metabolites in defining galactose sensitivity in yeast. *Mol. Gen. Metab.* **83**, 103-116.
Ryder, E. and Russell, S. (2003). Transposable elements as tools for genomics and genetics in Drosophila. *Brief. Funct. Genomic. Proteomic.* **2**, 57-71.
Sardharwalla, I. B., Wraith, J. E., Bridge, C., Fowler, B. and Roberts, S. A. (1988). A patient with severe type of epimerase deficiency galactosemia. *J. Inher. Metab. Dis.* **11**, 249-251.
Schulz, J., Ross, K., Malmstrom, K., Krieger, M. and Fridovich-Keil, J. (2005). Mediators of galactose sensitivity in UDP-galactose 4′-epimerase-impaired mammalian cells. *J. Biol. Chem.* **280**, 13493-13502.
Secombe, J., Pispa, J., Saint, R. and Richardson, H. (1998). Analysis of a Drosophila cyclin E hypomorphic mutation suggests a novel role for cyclin E in cell proliferation control during eye imaginal disc development. *Genetics* **149**, 1867-1882.
Su, A. I., Wiltshire, T., Batalov, S., Lapp, H., Ching, K. A., Block, D., Zhang, J., Soden, R., Hayakawa, M., Kreiman, G. et al. (2004). A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc. Natl. Acad. Sci. USA* **101**, 6062-6067.
Ten Hagen, K. G., Zhang, L., Tian, E. and Zhang, Y. (2009). Glycobiology on the fly: developmental and mechanistic insights from Drosophila. *Glycobiology* **19**, 102-111.
Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L. et al. (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nat. Genet.* **36**, 283-287.
Tian, E. and Ten Hagen, K. G. (2006). Expression of the UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase family is spatially and temporally regulated during Drosophila development. *Glycobiology* **16**, 83-95.
Tian, E. and Ten Hagen, K. G. (2007a). O-linked glycan expression during Drosophila development. *Glycobiology* **17**, 820-827.
Tian, E. and Ten Hagen, K. G. (2007b). A UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase is required for epithelial tube formation. *J. Biol. Chem.* **282**, 606-614.
Tomancak, P., Beaton, A., Weiszmann, R., Kwan, E., Shu, S., Lewis, S. E., Richards, S., Ashburner, M., Hartenstein, V., Celniker, S. E. et al. (2002). Systematic determination of patterns of gene expression during Drosophila embryogenesis. *Genome Biol.* **3** [Epub] [doi:10.1186/gb-2002-3-12-research0088].
Varki, A., Freeze, H. and Manzi, A. (2009). Overview of glycoconjugate analysis. *Curr. Protoc. Protein Sci.* Chapter **12**, 12.1.1-8.
Walter, J. H., Roberts, R. E. P., Besley, G. T. N., Wraith, J. E., Cleary, M. A., Holton, J. B. and MacFaul, R. (1999). Generalised uridine diphosphate galactose-4-epimerase deficiency. *Arch. Dis. Child* **80**, 374-376.
Wohlers, T. and Fridovich-Keil, J. L. (2000). Studies of the V94M-substituted human UDP-galactose-4-epimerase enzyme associated with generalized epimerase-deficiency galactosemia. *J. Inher. Metab. Dis.* **23**, 713-729.
Wohlers, T. M., Christacos, N. C., Harreman, M. T. and Fridovich-Keil, J. L. (1999). Identification and characterization of a mutation, in the human UDP galactose-4-epimerase gene, associated with generalized epimerase-deficiency galactosemia. *Am J. Hum. Genet.* **64**, 462-470.
Zhang, H., Liu, J., Li, C., Momen, B., Kohanski, R. and Pick, L. (2009). Deletion of *Drosophila* insulin-like peptides causes growth defects and metabolic abnormalities. *Proc. Natl. Acad. Sci. USA* **106**, 19617-19622.