Glycogen-storage disease type IV (GSD-IV) is an autosomal recessive disease caused by a deficiency in glycogen-storage disease type IV (GSD-IV) is an autosomal recessive disease caused by a deficiency in glycogen-branching enzyme (GBE1) activity that results in the accumulation of amylopectin-like polysaccharide, which presumably leads to osmotic swelling and cell death. This disease is extremely heterogeneous in terms of tissue involvement, age of onset and clinical manifestation. The most severe fetal form presents as hydrops fetalis; however, its pathogenetic mechanisms are largely unknown. In this study, mice carrying a stop codon mutation (E609X) in the Gbe1 gene were generated using a gene-driven mutagenesis approach. Homozygous mutants (Gbe−/− mice) recapitulated the clinical features of hydrops fetalis and the embryonic lethality of the severe fetal form of GSD-IV. However, contrary to conventional expectations, little amylopectin accumulation and no cell degeneration were found in Gbe−/− embryonic tissues. Glycogen accumulation was reduced in developing hearts of Gbe−/− embryos, and abnormal cardiac development, including hypertrophi cation and noncompaction of the ventricular wall, was observed. Further, Gbe1 ablation led to poor ventricular function in late gestation and ultimately caused heart failure, fetal hydrops and embryonic lethality. We also found that the cell-cycle regulators cyclin D1 and c-Myc were highly expressed in cardiomyocytes and likely contributed to cardiomyocyte proliferation and trabeculation/compaction of the ventricular wall. Our results reveal that early molecular events associated with Gbe1 deficiency contribute to abnormal cardiac development and fetal hydrops in the fetal form of GSD-IV.

INTRODUCTION

Glycogen-storage disease type IV (GSD-IV; OMIM232500), also known as Andersen disease, is an autosomal recessive genetic disorder that results from a deficiency in the activity of the glycogen-branching enzyme GBE1, an amylo-(1,4-1,6)-transglycosylase (EC 2.4.1.18). The glycogen-branching enzyme adds short glucosyl chains in α-1,6 glycosidic links to the glycogen molecule to yield a branched polymer with increased water solubility and glycogen synthetic activity. A deficiency of this enzyme results in tissue accumulation of abnormal glycogen with fewer branch points and longer outer branches that resembles an amylopectin-like structure, also known as polyglucosan. This structurally abnormal glycogen has low solubility and forms precipitates in the liver, heart and muscle (1–3).

GSD-IV is a clinically heterogeneous disorder. Since the original description of the classical progressive hepatic form of GBE deficiency by Andersen in 1952, many variants of GSD-IV with different tissue involvement and variable clinical manifestations have been observed. The age of onset ranges from fetus to adulthood and is divided into four groups: (i) perinatal, presenting as fetal akinesia deformation sequence and perinatal death (4,5); (ii) congenital, with hydrops fetalis, neuronal involvement and death in early infancy (6–8); (iii) childhood, with myopathy or...
cardiomyopathy (9,10); and (iv) adult, with isolated myopathy or adult polyglucosan body disease (11,12).

Despite the fact that this disease has been known for many years, questions concerning its pathogenesis and molecular mechanisms have remained unanswered. It has been suggested that accumulation of the less soluble abnormal polysaccharide causes a foreign body reaction in the cell, leading to osmotic swelling and cell death (1). Mutations identified in patients cause a foreign body reaction in the cell, leading to osmotic swelling and cell death (1). Further studies are needed to understand the underlying molecular mechanisms of the various presentations of GSD-IV.

Although GBE1 deficiency has been reported in horses and cats (14,15), there has been no mouse model for GSD-IV. Here, we report the generation of a GSD-IV mouse model carrying a stop mutation (E609X) in the Gbe1 gene using a gene-driven ENU (N-ethyl-N-nitrosourea)-mutagenesis approach. The GSD-IV Gbe−/− mice exhibited hydrops fetalis and lethality between mid and late gestation, recapitulating the clinical features of severe fetal neuromuscular forms of human GSD-IV. Contrary to conventional expectations, Gbe−/− embryos exhibited less glycogen accumulation than their wild-type (WT) littermates. The morphological abnormalities of embryonic Gbe−/− hearts and underlying mechanisms were identified.

RESULTS

Generation of mice carrying Gbe1 mutations

Both mouse and human GBE1 are 702-amino-acid proteins encoded by 16 exons; their amino acid sequences share 95% similarity and 91% identity (Supplementary Material, Fig. S1). To isolate appropriately mutated Gbe1 clones, we screened an ENU-mutagenized mouse DNA archive, focusing on Gbe1 exons 5, 7, 12 and 14, which contain longer amino acid coding sequence and more identified mutations associated with GSD-IV in humans (Supplementary Material, Fig. S1). As shown in Supplementary Material, Figure S1 and Table S1, these screens revealed one nonsense mutation (E609X), six missense mutations (M302L, H319Q, Y329F, M522K, H608Q and G609R), three silent mutations (A214A, L506L and G532G) and three intronic mutations (intron 5, intron 7 and intron 11). Several nonsense and splice site mutations led to prematurely truncated Gbe1 proteins, which are known to cause the severe perinatal/congenital neuromuscular forms of GSD-IV in humans (1,16). Therefore, we established a mouse line in a C3HeB/FeJ background using the E609X mutant—a G-to-T nonsense mutation located within exon 14 that created a stop codon at amino acid position 609. No obvious phenotypes were observed in heterozygous Gbe+/− mice. Second-generation heterozygous mice were outcrossed to WT C3HeB/FeJ mice for two more generations to dilute potential unrelated mutations. Fourth-generation Gbe+/− pairs, which shared an estimated 1.25 common mutations (17), were then intercrossed to generate homozygous Gbe−/− mice.

Perinatal mortality of homozygous E609X mice

No live-born Gbe−/− mice were recovered (Table 1). To investigate the timing of in utero Gbe−/− lethality, we performed timed matings of Gbe−/− mice and examined embryos on embryonic days 10.5 (E10.5) to term by high-frequency ultrasound biomicroscopy. Embryonic survival was determined on the basis of the observation of beating hearts. The survival rate of Gbe−/− embryos decreased with increasing stage of embryonic development; at E10.5, survival was 67%, and decreased to 57% at E12.5–E15.5, 36% at E16.5–E19.5 and 0% at term (Table 1). The latest survivors were observed at E17.5. We conclude that Gbe−/− embryos died predominantly at mid-to-late gestational stages.

Decreased heart rate and diminished accumulation of glycogen in mid-gestation Gbe−/− embryos

Heart rates in developing Gbe−/− embryos (30–65 b.p.m.) were markedly decreased compared with those of their WT littermates (114–121 b.p.m.) at E10.5 (Fig. 1A). To explore the causes of slow heart rate and embryonic lethality of Gbe−/− mice in greater detail, we first examined glycogen accumulation in the developing embryos by periodic acid-Schiff (PAS) staining. At E10.5, there was a major accumulation of glycogen in the ventricular and atrial chamber wall of the developing hearts of WT embryos (Fig. 1Bc and e), but no glycogen staining was detected in the heart chamber of Gbe−/− embryos (Fig. 1Bd and f). Significantly lower levels of glycogen staining were also evident in the hearts of Gbe−/− embryos at subsequent developmental stages E14.5 (Fig. 2; compare b and d, and a and c in A) and E16.5 (Fig. 2; compare b and d, and a and c in B). The glycogen levels in the hearts of E16.5 WT and Gbe−/− embryos were further analyzed. The glycogen levels in the hearts of E16.5 Gbe−/− embryos were found to be significantly lower compared with those in WT embryos (Fig. 2C). At E11.5, glycogen also accumulated in the main bronchus of the lung bud in WT embryos (Supplementary Material, Fig. S2A) and could be detected in hepatic primordium in WT and Gbe−/− embryos (Supplementary Material, Fig. S2A–D). Glycogen accumulation in the liver and skeletal muscle was not significantly different between Gbe−/− and WT embryos at late gestational stages (Supplementary Material, Fig. S2; compare E and F, and I and J). We further tested for abnormal glycogen accumulation in the hearts, liver and skeletal muscle of Gbe−/− embryos by pretreating the tissue with diastase before PAS staining. No PAS-positive, diastase-resistant deposits were detected in the hearts of E11.5 or E12.5 Gbe−/− embryos (Supplementary Material, Fig. S3). Small amounts of PAS-positive, diastase-resistant material were observed in the hearts at E14.5 (Fig. 2; compare h and g in A) and E16.5 (Fig. 2; compare h and g in B), in the liver at E14.5 and in the skeletal muscle of stillborn Gbe−/− embryos (Supplementary Material, Fig. S2; compare G and H, and K and L), but no obvious cell disruption or structure defects were observed.

To determine whether the lower level of glycogen in the hearts of Gbe−/− embryos was directly correlated with Gbe1 expression, we measured Gbe1 transcripts in the hearts.
suggest that the stop codon mutation in the Gbe1 gene compromises Gbe1 transcription, affecting Gbe1 protein level and glycogen synthesis in the developing heart, and ultimately leading to the lethality of Gbe2/− embryos.

**Heart defects in Gbe1-deficient embryos**

Our examination of embryos for gross abnormalities revealed a congested venous system in the peripheral vasculature and generalized edema in E14.5 Gbe2/− embryos (Fig. 3; compare A and B). A further analysis of organ sections from E14.5 Gbe2/− embryos showed a congested venous system in the lung (Fig. 3; compare G and H) and liver (Fig. 3; compare I and J). At E15.5, prominent livers were also evident in Gbe2/− embryos in addition to edema (Fig. 3; compare C and D). The hearts of stillborn Gbe2/− mice showed significantly smaller ventricles compared with those of WT hearts, were nearly filled with cells (Fig. 4; compare G and H), the interventricular septa of Gbe2/− hearts were smaller than those of WT hearts and, unlike those of WT hearts, were nearly filled with cells (Fig. 4; compare K and L, and M and N). Frank ventricular septum defects (VSD) were observed in some stillborn Gbe2/− mice (Fig. 4; compare O and P). These results suggest that abnormalities in heart development contribute to embryonic lethality in Gbe2/− embryos.

**Cardiomyocyte hyperproliferation accounts for the hypertrabeculation and ventricular noncompaction in Gbe2/− hearts**

To identify the cells that occupied the nearly filled ventricular cavities of late-gestation (E16.5) Gbe2/− hearts, we performed immunofluorescence staining. Using the myocardial marker MF-20, we confirmed that these cells were cardiomyocytes (Fig. 5A). Confocal microscopy revealed hypertrabeculation and a thin compact zone with no significant Z-line/ body or organized sarcomeres in E16.5 Gbe2/− hearts (Fig. 5A and B), suggesting extensive trabecula and nonmaturation/noncompaction of the compact wall in Gbe2/− hearts. To address the possibility that this phenotype might be secondary to increased proliferation of cardiomyocytes at an earlier stage, we stained heart sections for phosphorylated histone H3, an M-phase-specific marker, and found a marked increase in the number of positive-stained cells in the ventricular wall area of E14.5 Gbe2/− hearts (Fig. 5C). These observations suggest that the cell-filled ventricles observed in late-gestation Gbe2/− hearts result from hypertrabeculation caused by altered regulation of cell proliferation and maturation during ventricular wall formation, which, in turn, led to a decrease in ventricular wall compaction. Therefore, the heart failure observed in Gbe2/− embryos is the result of hyperproliferation of cardiomyocytes and subsequent hypertrabeculation/noncompaction.

**Gbe1-deficiency alters the expression of cell-cycle regulators**

To explore the mechanism by which Gbe1 deficiency might affect cardiac trabeculation and/or compaction, we first analyzed bone morphogenetic protein 10 (Bmp10). Bmp10, a peptide growth factor that belongs to the TGF-β superfamily, has been suggested to be a key morphogenetic growth factor in the regulation of cardiac trabeculation and/or compaction (18,19). Bmp10 transcript levels decreased in the developing heart during late gestational stages (Supplementary Material, Fig. S4), as described previously (19). However, no significant differences in Bmp10 transcript levels were observed in the hearts of Gbe2/− and WT embryos at E14.5 or at term (Supplementary Material, Fig. S4). We further analyzed cyclin D1 and c-Myc, the cell-cycle regulators, which have been shown to drive cardiomyocyte proliferation (20). Cyclin D1 expression levels were increased in Gbe2/− hearts compared with WT hearts (Fig. 6A and B). An analysis of c-Myc levels in heart sections of Gbe2/− and WT showed that c-Myc was highly expressed in the cardiomyocytes of Gbe2/− embryos, but was expressed in only a small restricted area of

**Table 1. Survival of mutant embryos**

<table>
<thead>
<tr>
<th>Age</th>
<th>WT</th>
<th>Gbe2+/−</th>
<th>Gbe2−/−</th>
<th>Survival rate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5</td>
<td>22 (1)</td>
<td>34 (1)</td>
<td>15 (5)</td>
<td>67%</td>
<td>71</td>
</tr>
<tr>
<td>E12.5–15.5</td>
<td>7 (0)</td>
<td>16 (0)</td>
<td>7 (3)</td>
<td>57%</td>
<td>30</td>
</tr>
<tr>
<td>E16.5–19.5</td>
<td>13 (0)</td>
<td>17 (1)</td>
<td>11 (7)</td>
<td>36%</td>
<td>41</td>
</tr>
<tr>
<td>Newborn</td>
<td>11 (1)</td>
<td>19 (0)</td>
<td>15 (15)</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>86</td>
<td>48</td>
<td>187</td>
<td></td>
</tr>
</tbody>
</table>

Shown are embryos obtained from timed heterozygous matings. The numbers of embryos of each genotype obtained at the indicated time are listed. The numbers in the last column are the total number of embryos collected at each gestational age. The numbers in the last row indicate the total number of embryos of each genotype collected.

1The survival rate of Gbe2−/− mice at each gestational age.

2Parenthetic values indicate embryos without a detectable heart beat, determined by ultrasound microscopy.
the ventricular trabecular myocardium in WT hearts (Fig. 6C). These results suggest that the dysregulation of cell-cycle regulators, cyclin D1 and c-Myc, might contribute to hyperproliferation of cardiomyocytes and subsequent hypertrabeculation/noncompaction of the ventricular wall in Gbe<sup>−/−</sup> embryos.

**Figure 1.** Heart rate, morphology, glycogen accumulation and Gbe<sub>1</sub> expression in E10.5 WT and Gbe<sup>−/−</sup> embryos. (A) E10.5 WT and Gbe<sup>−/−</sup> embryo echocardiograms (left panel) and heart rates (right panel). Heart rates in E10.5 Gbe<sup>−/−</sup> embryos were slower than those in E10.5 WT embryos (P < 0.001; n = 5 embryos each for Gbe<sup>−/−</sup> and WT, both from the same three pregnant mothers). (B) Morphological and histological analyses of E10.5 embryos. Both WT (a) and Gbe<sup>−/−</sup> (b) embryos exhibited normal morphological development. PAS staining of sections from E10.5 embryos showed substantial glycogen accumulation in the developing heart chamber wall in E10.5 WT embryos (c and e), but no glycogen accumulation in Gbe<sup>−/−</sup> embryos (d and f). v.c., ventricular chamber; a.c., atrial chamber. (C) Relative Gbe<sub>1</sub> mRNA levels in the hearts of WT and Gbe<sup>−/−</sup> mice at E14.5 and newborns (WT)/stillborns (Gbe<sup>−/−</sup>) analyzed by real-time RT-PCR (n = 3).
Herein we describe the first mouse model of GSD-IV disease. This Gbe\textsuperscript{-/-} mouse model recapitulates the clinical features of hydrops fetalis and the embryonic lethality of severe fetal neuromuscular forms of human GSD-IV. Using it, we identify the underlying mechanisms that lead to hydrops fetalis. The deficiency of Gbe1 led to reduced glycogen accumulation in developing hearts. Furthermore, our studies found that expression of cyclin D1 and c-Myc, two factors which have a role in cardiomyocyte proliferation during heart development, was increased in Gbe\textsuperscript{-/-} embryos. The hyperproliferation of cardiomyocytes impaired trabeculation/compaction of ventricular wall, leading to poor ventricular function in late gestation and, ultimately, to heart failure, fetal hydrops and embryonic lethality.

The fetal and infantile forms of GSD-IV are relatively rare. A complete absence of GBE1 enzyme activity results in the development of fetal hydrops and akinesia during the second trimester. Brain examinations of affected fetuses have shown intraventricular hemorrhage. Diastase-resistant amylopectin accumulates exclusively in fetal skeletal muscle cells and the heart, but not in the liver or neurons (5,21). However, the mechanisms responsible for human GSD-IV fetal hydrops and akinesia have remained unclear; notably, the structure and function of fetal hearts have not been examined in detail. Our findings based on the Gbe\textsuperscript{-/-} mouse model suggest that morphological and functional abnormalities of fetal hearts might be the underlying cause of fetal hydrops. Several cases of perinatal neuromuscular forms of GSD-IV have been reported by different groups (7,16,21,22). Severe hypotonia and dilated cardiomyopathy (which were attributed

**Figure 2.** Glycogen accumulation in the hearts of E14.5 and E16.5 embryos from Gbe\textsuperscript{-/-} and WT mice. Heart sections of Gbe\textsuperscript{-/-} embryos showed less PAS-positive staining (glycogen accumulation) than those from WT embryos at E14.5 (A) and E16.5 (B). Only a small amount of diastase-resistant PAS-positive staining (abnormal glycogen) was detected in the hearts of E14.5 and E16.5 Gbe\textsuperscript{-/-} embryos. Scale bars: 200 μm (a, b, e and f) and 20 μm (c, d, g and h). Diastase: PAS staining after diastase incubation. (C) Glycogen content was determined in the hearts of E16.5 WT and Gbe\textsuperscript{-/-} embryos (n = 3 biological samples, P < 0.001).
to amylopectin deposits in cardiomyocytes) with subsequent extensive injury of myocardial fibers leading to heart failure were observed in these cases (7). In our Gbe<sup>−/−</sup> mouse model, fetal hydrops was observed, but little diastase-resistant amylopectin deposition was detected in the heart and no myocardial cell injury was observed. Therefore, this model allowed us to investigate the early molecular events involved in the pathogenesis of fetal-type GSD-IV before cell death occurred.

Glycogen occupies ≈2% of the cell volume in adult cardiac myocytes and >30% of the cell volume in fetal cardiac myocytes (23). Cardiac glycogen is present at high levels during early-to-mid gestation before falling to low levels at the time of birth (24–26), suggesting that glycogen might have a specific role in heart function and development. However, the physiologic role of glycogen in the fetal heart is still not completely understood. It has been shown that glycogen plays an important role as an energy source for developing cardiac myocytes, allowing them to proliferate and function to support adequate circulation for the developing embryo (27). In the present work, glycogen, which was highly accumulated in cardiomyocytes of E10.5 WT mice, was undetectable in cardiomyocytes of E10.5 Gbe<sup>−/−</sup> mice, and remained significantly lower during mid-to-late gestation stages (to newborn/stillborn) compared with WT mice. Heart rate was significantly decreased in E10.5 Gbe<sup>−/−</sup> mice compared with that in WT mice, and hyperproliferation of cardiomyocytes and hypertrabeculation/noncompaction of the ventricular wall were observed in late-gestation Gbe<sup>−/−</sup> embryos. These results suggest that, in addition to serving as an energy reserve, cardiac glycogen might also play a critical role in regulating normal heart development and maturation. A mouse model in which the ability to store glycogen in skeletal muscle and cardiac muscle was eliminated by disruption of the glycogen synthase 1 gene (Gys1) as reported previously (28). In these mice, glycogen was undetectable in cardiac muscle, and thin ventricular walls and reduced trabecular structures, attributable to a decrease in cell proliferation, were observed. Most Gys1-null animals died soon after birth due to impaired cardiac function, but ≈10% survived (27). Although glycogen was low in both Gys1-null and our Gbe<sup>−/−</sup> mouse models, the phenotypes of impaired heart development were quite different. These results imply that the abnormal heart development observed in Gbe<sup>−/−</sup> embryos is not simply due to insufficient glycogen-based energy to maintain normal cardiomyocyte growth, and suggest that glycogen homeostasis might play a critical role in regulating heart development and maturation. Gbe<sup>−/−</sup> mice exhibited hypertrabeculation and a lack of ventricular wall compaction, which led to nearly filled ventricular cavities, poor heart function and, ultimately, heart failure—the major cause of embryonic lethality at late gestational stages. Ventricular trabeculation is one of the most critical steps of ventricular chamber development. The trabecular myocardium of embryonic mouse hearts rapidly expands during E9.5–E13.5, followed by maturation, a slowing of the cell-cycling rate, and compaction to form a thicker, compact ventricular wall after E14.5. The balance between proliferation and maturation is critical to the formation of

Figure 3. Blood congestion in Gbe<sup>−/−</sup> embryos. Morphological (A–F) and histological (G–J) examination of blood distribution in WT and Gbe<sup>−/−</sup> embryos. A morphological examination of E14.5 embryos showed congested vessels (indicated by arrows) and edema in Gbe<sup>−/−</sup> mice (B) compared with WT mice (A). At E15.5, Gbe<sup>−/−</sup> embryos exhibited prominent liver (D; indicated by arrow) in addition to edema (compare with WT mice in C). The hearts from stillborn Gbe<sup>−/−</sup> mice (F) showed smaller ventricles and congested blood in atria compared with those from WT newborns (E). H&E-stained sections of E14.5 Gbe<sup>−/−</sup> embryos (H) showed evidence of blood congestion in the pulmonary parenchyma (compare with WT embryos in G). H&E staining showed a similar degree of blood congestion in liver sections of E14.5 Gbe<sup>−/−</sup> (J) and WT (I) embryos. Scale bars: 100 μm (G–J).
a functionally competent ventricular wall. To identify mechanisms by which the lack of Gbe1 might lead to hypertrabeculation/noncompaction of the ventricular wall, we examined genes implicated in regulating cardiac trabeculation and ventricular compaction. We found that the basic cell-cycle regulators cyclin D1 and c-Myc were highly expressed in the ventricular myocardium of Gbe1−/− embryos compared with that of WT embryos. Although the details of the molecular mechanisms responsible for cardiomyocyte hyperproliferation have not been fully examined, our findings provide a starting point for further investigation. Furthermore, the possibility that physiologic and molecular changes in other organs (e.g. liver secretion of growth factors) could lead to secondary changes in the hearts of Gbe1−/− mice also needs to be further investigated.

In summary, using a gene-driven ENU-mutagenesis approach, we have generated a Gbe1-deficient mouse model that recapitulates the clinical features of hydrops fetalis and the embryonic lethality of severe fetal forms of GSD-IV. Contrary to expectations, Gbe1−/− embryos showed less glycogen accumulation than their WT littermates, little amyllopectin accumulation and no evidence of cell degeneration. Our data indicate that, in addition to the deposition of amyllopectin-like polysaccharides, which presumably leads to cell death in the tissues, a novel mechanism leading to uncontrolled cardiomyocyte proliferation and trabeculation/compaction of the ventricular wall contributes to abnormal cardiac development and fetal hydrops in GSD-IV. Further studies on the mechanisms that control cardiomyocytes proliferation/maturation can be expected to enhance our ability to repair heart injury in adults and induce cardiac regeneration.

MATERIALS AND METHODS
Screening for Gbe1 mutations and generation of Gbe1−/− mice
ENU mutagenesis, screening of mutation-bearing mice and in vitro fertilization for mutant mice generation were performed by Ingenium Pharmaceuticals, Germany (17). Briefly, C3HeB/FeJ male mice were treated with ENU. Genomic DNA from G1 male mice was archived for mutation
Figure 5. Hypertrabeculation and noncompaction in heart ventricles of late-gestation Gbe\(^{-/-}\) embryos caused by hyperproliferation of cardiomyocytes. (A) Cardiomyocytes in the hearts of WT and Gbe\(^{-/-}\) E16.5 embryos were identified using the myocardial marker MF-20. Images were acquired using a confocal microscope. Hypertrabeculation, noncompaction and thin ventricular walls are evident in Gbe\(^{-/-}\) hearts. The red lines indicate the trabeculation regions, and the white lines show the compaction regions of the ventricular wall. A significant Z-line/body (arrows and inset), indicating the presence of well-organized sarcomeres, was observed in the hearts of E16.5 WT but not in the hearts of E16.5 Gbe\(^{-/-}\). Scale bars: 50 \(\mu\)m. (B) Left panel: quantification of the thickness of the trabecular zone in Gbe\(^{-/-}\) and WT hearts at E16.5 (\(n = 3\) biological samples); right panel: quantification of the thickness of the compact zone in Gbe\(^{-/-}\) and WT hearts at E16.5 (\(n = 3\) biological samples). (C) Proliferating cells in the hearts of WT and Gbe\(^{-/-}\) E14.5 embryos identified by staining for phosphorylated histone H3. Sections were stained with an anti-phospho-histone H3 antibody. Scale bars: 200 \(\mu\)m (upper panel) and 100 \(\mu\)m (lower panel). (D) Percentage of phospho-histone H3-positive cells at E14.5 in the interventricular septum region (IVS) and ventricular wall in Gbe\(^{-/-}\) and WT hearts (\(n = 3\) biological samples). The percentage of proliferating cardiomyocytes in Gbe\(^{-/-}\) hearts was increased significantly.
screening, and the corresponding sperm samples were frozen for recovery of mutant mice. The mutations of interest in the collected DNA archives were screened by temperature-gradient capillary electrophoresis, and mutant lines (G2 generation) were established by in vitro fertilization using the banked sperm. After receiving the mice in our animal facility, G2 mice were outcrossed with WT C3HeB/FeJ for two more generations, and G4 heterozygous mice were intercrossed to generate homozygous mice. Mice were housed in a temperature- and humidity-controlled room with a 12 h light/12 h dark cycle under specific pathogen-free conditions. All animal protocols were approved by the institutional animal safety committee.

**PCR and DNA sequencing**

Genomic DNA from mice and developing embryos was prepared from tail and yolk sac, respectively. GeneBank accession number NT_039625 was used as the reference sequence for the mouse Gbe1 gene. Mutations were genotyped by PCR amplification and sequencing. The sequences of the primers used for PCR amplification and DNA sequencing were 5′-AAA CTG TAG AGC TCA GAG ATT CCA-3′ (forward) and 5′-CAT CTT AAT AGG CAC ATG CAC TG-3′ (reverse).

**Assessment of embryonic heart rate by ultrasound biomicroscopy Doppler**

Embryonic Doppler echocardiography was performed noninvasively from day 10.5 to 19.5 post-coitum (E10.5–E19.5). After removing abdominal hair, the pregnant female was allowed to inhale 2% isoflurane and was then intubated via a nose cone with 1–1.5% isoflurane to maintain anesthesia. The mice were kept warm by placing on a heated platform, and body temperature and electrocardiograms were continuously monitored. For heart rhythm assessment, fetal heart images and blood velocities were obtained using a Vevo 660 scanner equipped with a 30 MHz ultrasound probe (VisualSonics, Inc., Ontario, Canada).

**RNA isolation and quantitative real-time RT-PCR analysis**

Total RNA from the hearts of various gestational age embryos was isolated using the TRizol reagent (Invitrogen, Taipei, Taiwan) and further purified using an RNaseasy Mini Kit (QIAGEN, Hilden, Germany). RNA was then treated with DNase I (30 U/µg total RNA; QIAGEN) and reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Expression of Gbe1 mRNA was quantified using SYBR Green PCR Master Mix and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA).

**Figure 6.** Cyclin D1 and c-Myc expression in the hearts of Gbe<sup>−/−</sup> and WT embryos. (A) Increased cyclin D1 in the hearts of Gbe<sup>−/−</sup> embryos. Sections were stained with anti-cyclin D1 antibody (brown) and then counterstained with hematoxylin to identify nuclei (blue). Note the overall increase in cyclin D1 staining in E16.5 Gbe<sup>−/−</sup> hearts. Scale bars: 200 µm (upper panel) and 50 µm (lower panel). (B) Percentage of cyclin D1-positive cells at E16.5 in the interventricular septum region and ventricular wall in Gbe<sup>−/−</sup> and WT embryonic hearts (n = 3 biological samples). (C) Increased c-Myc in the hearts of Gbe<sup>−/−</sup> E16.5 embryos. Sections were stained with anti-c-Myc antibody (brown) and then counterstained with hematoxylin to identify nuclei (blue). Note the overall increase in c-Myc staining in Gbe<sup>−/−</sup> hearts compared with WT hearts, where staining was restricted to a small area of trabecular myocardium. Scale bars: 200 µm (upper panel) and 50 µm (lower panel). The higher magnification images in (B) and (D) are demarcated in lower magnification panels.
Taipei, Taiwan). Gbe1 expression data were normalized to β-actin mRNA levels and presented as fold changes. The regions used to design Gbe1 primers are indicated in Figure 2C. The primers used for amplifying β-actin mRNA were 5′-GGA CTC CTA TGT GGG TGA CG-3′ (forward) and 5′-CTT CTC CAT GTC GTC CCA GT-3′ (reverse). Primers for amplifying Bmp10 were 5′-ACA TCA TCC GGA GCT TCA AGA ACG-3′ (forward) and 5′-AAC CGC AGT TCA GCC ATG ACG-3′ (reverse).

Histological analysis of embryos and mice
Embryos collected from timed heterozygous matings were immediately submerged in 3.6% neutral buffered paraformaldehyde solution and stored at 4°C until embedding in paraffin wax by standard methods. Placental tissue was collected for genotyping. The sections were stained with hematoxylin–eosin (H&E) or PAS (with or without pretreatment with diastase) by standard methods.

Determination of glycogen content
Glycogen content was estimated by measuring glucose released by Glycogen Assay Kit (BioVision, CA, USA), as described by the manufacturer. Briefly, glycogen was hydrolyzed with glycogen hydrolysis enzyme released by the kit, and the glycogen content was determined as glucose units analyzed colorimetrically with appropriate standard curve. The fresh samples were used for the determination, and the glucose background in the samples was also determined and subtracted from the glycogen content.

Immunohistochemical and immunofluorescence staining
For immunohistochemical staining of phosphorylated histone H3, cyclin D1 and c-Myc, paraffin-embedded sections were treated with 0.3% H2O2 and incubated with anti-phospho-histone H3 (1:200; Cell Signaling), anti-cyclin D1 (1:100; Epitomics, Inc., CA, USA) and anti-c-Myc (1:50; Epitomics, Inc.) antibodies, respectively. After incubation, the slides were washed and further incubated with a biotinylated secondary antibody (1:200; Vector Laboratories) for 1 h. Signals (brown) were detected using an avidin–biotin complex assay following the manufacturer’s protocol (Vector Laboratories). Sections were counterstained with hematoxylin to identify nuclei (blue) and visualized by standard light microscopy. For immunofluorescence staining, paraffin-embedded heart sections were stained with a monoclonal anti-MF-20 antibody (1:500; Developmental Studies Hybridoma Bank, IA, USA). The signals were then detected with a fluorescein isothiocyanate-conjugated secondary antibody. Images were acquired using a Zeiss LSM 510 laser confocal microscope (Carl Zeiss MicroImaging, Inc., Hamburg, Germany).

Statistical analyses
A two-tailed Student’s t-test was used to test for differences between treatments. A P-value <0.05 was considered statistically significant (*P-value <0.05, **P-value <0.01, ***P-value <0.001).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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