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An *N*-ethyl-*N*-nitrosourea mutagenesis recessive screen identifies two candidate regions for murine cardiomyopathy that map to chromosomes 1 and 15

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Abstract

N-ethyl-*N*-nitrosourea (ENU) mutagenesis screens have been successful for identifying genes that affect important biological processes and diseases. However, for heart-related phenotypes, these screens have been employed exclusively for developmental phenotypes, and to date no adult cardiomyopathy-causing genes have been discovered through a mutagenesis screen. To identify novel disease-causing and disease-modifying genes for cardiomyopathy, we performed an ENU recessive mutagenesis screen in adult mice. Using noninvasive echocardiography to screen for abnormalities in cardiac function, we identified a heritable cardiomyopathic phenotype in two families. To identify the chromosomal regions where the mutations are localized, we used a single nucleotide polymorphism (SNP) panel for genetic mapping of mouse mutations. This panel provided whole-genome linkage information and identified the mutagenized candidate regions at the proximal end of chromosome 1 (family EN1), and at the distal end of chromosome 15 (family EN25). We have identified 94 affected mice in family EN1 and have narrowed the candidate interval to 1 Mb. We have identified 20 affected mice in family EN25 and have narrowed the candidate interval to 12 Mb. The identification of the genes responsible for the observed phenotype in these families will be strong candidates for disease-causing or disease-modifying genes in patients with heart failure.

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Introduction

Heart failure is a significant cause of morbidity and mortality in the United States. Variation in outcome for patients with heart failure is in part due to the influence of modifier genes whose multiple alleles differentially affect the heart failure phenotype. To identify genes that modify susceptibility to heart failure, mouse models that rely on variation in genetic background show promise (Le Corvoisier et al. 2003). Another approach is to induce genetic variation by random mutagenesis of the mouse genome using *N*-ethyl-*N*-nitrosourea (ENU) as the mutagen (Clark et al. 2004; Hagge-Greenberg et al. 2001). This approach has the added advantage that it can also identify novel candidate genes for Mendelian forms of heart disease.

Dilated cardiomyopathy is a common and largely irreversible form of heart muscle disease; it is the third most common cause of heart failure in the young and a major cause of heart transplantation (Hershberger et al. 2009). Approximately half of the patients with the disease are found to have idiopathic dilated cardiomyopathy (Hershberger et al. 2009). Inheritance patterns of familial dilated cardiomyopathy include autosomal dominant, autosomal recessive, and X-linked. Dilated cardiomyopathy exhibits high genetic heterogeneity as mutations in more than 20 genes have been associated with the disease, such as desmin, tafazzin, δ-sarcoglycan, dystrophin, and metavinculin, and nuclear envelope proteins such as emerin and lamin A/C (Colombo et al. 2008). Lamin A/C is the most frequent disease-associated gene for familial dilated cardiomyopathy with conduction system disease. Mutations in the sarcomere genes, which are responsible for causing hypertrophic cardiomyopathy, are also associated with dilated cardiomyopathy (Colombo et al. 2008). Gene mutations in sarcomere-related genes such as myosin-binding protein C (MYBPC3), β-myosin heavy chain (MYH7), troponins T and I, and others account for approximately 10–16% of familial dilated cardiomyopathy (Colombo et al. 2008). Thus, while a number of genes that cause dilated cardiomyopathy have been identified, it appears there are many unknown genes that likely play a role in the development of dilated cardiomyopathy.

Several large-scale mutagenesis projects using ENU have been established with the aim of generating large numbers of mutants that will allow gene functions to be systematically investigated in vivo (Cordes 2005; Hagge-Greenberg et al. 2001). Only two groups (The Jackson Laboratory and The Toronto Centre for Modeling Human Disease) have cardiovascular function in adult mice as part of their phenotypic screen (Svenson et al. 2003). Recently, an ENU recessive mutagenesis screen for identifying genes that contribute to congenital heart defects was performed (Yu et al. 2004). A number of cardiovascular anomalies were detected, including arrhythmias, outflow regurgitation, increased outflow velocity, heart failure, hypertrophy, and ectopia cordis. Mutations from five of these families were mapped, and the mutant genes in two of these families were identified as semaphorin and connexin43 (Yu et al. 2004). However, to date, no genes have been identified from an ENU mutagenesis screen for adult dilated cardiomyopathy.

In this study we tested whether a recessive ENU-induced mutagenesis screen can be performed to identify novel genes that cause adult cardiomyopathy. We describe the identification and genetic mapping of the first two monogenic mutants with a heritable cardiomyopathic phenotype by using noninvasive echocardiography in conscious mice.

Material and methods

Animals

Animals were handled according to protocols and animal welfare regulations approved by the Institutional Review Board at Duke University Medical Center. DBA/2 J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA).

Mutagenesis and breeding

We treated 25 wild-type (WT) DBA males between the ages of 8 and 12 weeks with a single dose of 250 mg/kg ENU (Sigma) via intraperitoneal (IP) injection, the dose recommended for this strain (Davis et al. 1999; Justice et al. 2000; Percec et al. 2003; Weber et al. 2000). ENU is a potential health hazard because it is a potent mutagen and carcinogen and procedures were approved by the Institutional IACUC.

The 25 WT DBA treated males (G0) were mated to WT DBA females to generate G1 progeny. The G1 mice were bred to C57BL/6 J (B6) WT mice to generate G2 progeny. By introducing a different strain (B6) in the second generation, we have significantly accelerated our ability to map the mutated genetic region since we can use microsatellite or single nucleotide polymorphism (SNP) markers. Back-crossing the G2 progeny to the parental G1 or intercrossing G2 siblings generated G3 progeny for phenotype screening.

Screening

We phenotyped 2400 G3 mice for dilated cardiomyopathy using echocardiography performed in conscious mice at two time points: 8 and 16 weeks of age. Parameters measured were left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and % fractional shortening. All G3 mice at 8 and 16 weeks with a value greater than 2 SD from the mean were considered cardiomyopathy mutants and were followed with echocardiograms every two months.

The noninvasive technique of transthoracic echocardiography is an efficient method by which cardiac morphology and performance in animals can be determined without invasive damage or the requirement of terminal histopathologic analysis. Based on our work and others, echocardiography is now the standard for defining cardiac phenotype in mice and rats (Chin et al. 2007; Tanaka et al. 1996). Moreover, we have previously validated this method in anesthetized and conscious mice and currently routinely use echocardiography in conscious mice for phenotyping (Esposito et al. 2000).

Echocardiograms were performed in conscious adult mice obviating the need for anesthesia or sedation. Moreover, it allowed a nearly high-throughput phenotypic screen because each echocardiogram took about 8–10 min to perform. Importantly, the echo operators and the echo readers were blinded to the genotype (heterozygous or homozygous) of the mice, and also which mice belonged to the control or affected group. In addition, the echo operators and readers underwent an intense training period prior to the study where inter- and intraobserver variability was assessed and it was confirmed that the reproducibility of our screening technique was sufficiently robust to allow genetic mapping of this trait.

Fine-mapping strategy

The whole-genome SNP panel was genotyped using the Illumina GoldenGate Platform at The Broad Institute Center for Genotyping and Analysis. The 768-SNP genotyping panel indicated the chromosomal region of the gene, but in order to identify the gene, we performed fine mapping. To narrow the interval and reduce the number of candidate genes, two resources have been necessary: a higher-density map using additional polymorphic markers in the region and additional informative meioses (crossovers). The creation of a higher-density map of the region has required additional polymorphic markers. We have used microsatellite markers and additional SNPs that are polymorphic between the DBA/2 J and C57BL/6 strains. This information is available on the following databases: Jackson Labs Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/), NCBI (http://www.ncbi.nlm.nih.gov/), UCSC genome bioinformatics (http://genome.ucsc.edu/), SNPview at GNF (http://snp.gnf.org/GNF10K/), the Mouse Phenome Database

(http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home), and the Mouse SNP Database at Roche (http://mousesnp.roche.com/). Using these genomic resources, we were able to achieve a high density of markers in the chromosomal region of the gene. These microsatellite markers were used to define the recombinant interval as precisely as possible in all the available affected animals. As additional G3 affected mice are identified, they are genotyped with these markers to determine the minimal recombinant interval.

Results

Overview of the mutagenesis screen

To identify heritable cardiomyopathy mutants induced through random ENU mutagenesis in the mouse we mutagenized 25 G0 DBA/2 J (DBA) males. The G0 males were bred to wild-type DBA females to generate the first-generation progeny (G1 mice), whereby each G1 defined a unique family. We then bred the G1 mice to C57BL/6 WT mice to generate G2 progeny in order to use polymorphic microsatellite (or SNP) markers to facilitate mapping of the mutagenized gene. Backcrossing the G2 progeny to the parental G1 mice or intercrossing G2 siblings generated G3 progeny for phenotype screening (Fig. 1). In the initial screen we generated G1 mice from 23 of the original 25 G0 mutagenized males. However, as we went forward with our screen we concentrated on 6 of the original 25 G0 mice. These six mutagenized G0 mice, gave rise to G1's that later gave rise to affected G3 mice. In our study we focused on two G1 mice that gave rise to progeny with abnormal cardiac function, establishing two distinct mutant lines. One G1, EN1, gave rise to 13 affected siblings, and another G1, EN25, gave rise to 8 affected siblings.

To screen for a dilated cardiomyopathy phenotype we used noninvasive echocardiography in conscious mice. Mice underwent echocardiography in the awake state at 8 and 16 weeks of age. The parameters measured were left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and % fractional shortening. In the total population, we screened over 2400 G3 mice derived from G1 males (Fig. 2). Based on our population data, we defined a G3 mouse to be affected if it showed at 8 or 16 weeks an echocardiographic parameter ±2 SD (standard deviations) from the mean.

Identification of a heritable cardiomyopathic phenotype in Family EN1

Based on our phenotypic screen we identified 13 affected siblings derived from the G1 mouse EN1. We followed the 13 affected progeny with serial echocardiography at 8 weeks of age and extended to 72 weeks of age (Fig. 3). For all echocardiographic parameters, affected progeny showed significant enlargement of cardiac chambers and progressive decline in cardiac function compared to age and genetic background-matched wild-type controls (Fig. 3A–C).

Examples of M-mode echocardiograms from two G3 siblings from family EN1 at 16 weeks of age is shown in Fig. 3D. ENX 2036 is an example of a G3 mouse with a value greater than 2 SD from the mean and would be considered affected, while ENX 2040 had normal values. Mapping of Family EN1 reveals a potential locus on chromosome 1

Since we performed a recessive screen, all affected progeny must have two copies of the mutated gene (homozygous) to produce the cardiomyopathic phenotype. Our strategy to mutagenize DBA males and then cross these animals to a different strain (B6) allowed us to use homozygosity mapping to identify chromosomal regions most likely to contain the mutated gene (Lander and Botstein 1987). Importantly, this strategy also excluded chromosomal regions that were unlikely to carry the mutation and allowed the mapping of recessive mutations with a small number of affected progeny (Beier and Herron 2004).

To map the mutation in EN1 we used a whole-genome panel of 768 SNPs to genotype the affected mice. By whole-genome linkage we identified a region with the highest retention of DBA alleles that localized to an approximately 28-Mb interval at the proximal end of chromosome 1. Higher-density mapping using microsatellite markers and additional SNPs was used to narrow the candidate region, and 2 of the 13 mutant G3 progeny (ENX 261 and ENX 2533) allowed us to narrow the interval to approximately 9 Mb (13.4–21.2 Mb), defined by markers *D1Mit67* and SNP 21.274. Mapping the locus to this provisional region of chromosome 1 enabled us to follow the inheritance of the mutation using genetic markers. Animals exhibiting the DNA haplotype over this region of chromosome 1 must also retain the mutation within this region of the chromosome. Inheritance of the mutant allele could then be followed even in the heterozygous state.

Increasing the number of informative recombinants and renewable source of crossovers

To narrow the EN1 candidate interval on chromosome 1 we generated a renewable source of crossovers. Breeding G2 mice heterozygous for the mutant allele, or affected G3 mice to wild-type B6, and intercrossing the progeny from these crosses has provided a continual supply of mutant mice homozygous for the DBA mutagenized allele. We have identified an additional 81 affected progeny from these two mutant lines by means of noninvasive echocardiography at 16 weeks. The new mutants' echocardiographic parameters were compared to the initial 13 identified G3 mutants and age-matched wild-type controls of the same genetic background (Supplementary Fig. 1). The progeny from the new lines showed an increase in chamber dimensions and decrease in % fractional shortening, similar to the echocardiography data of the initial 13 identified G3 mutants, thus demonstrating heritability of the cardiomyopathic phenotype.

Candidate interval in proximal end of chromosome 1

Using a higher-density map with additional polymorphic markers in the region, we have used the additional affected mice to fine map the mutation. Twenty-three of the total 94 mutant mice have informative crossovers, allowing us to narrow the candidate region on the proximal end of chromosome 1 to less than 1 Mb. The interval is defined by the SNP rs6404446 and the SNP rs1347575 (Fig. 4A). Examination of unaffected progeny for crossovers has allowed us to employ unaffected progeny that show crossovers that have crossed out the mutation to aid in fine-mapping the mutation (Fig. 4B). Crossovers in the unaffected mice were completely consistent with the location determined using the affected mice; this validated our localization of the mutant allele. The conserved syntenic region for the murine region of chromosome 1 (<1 Mb) in humans is localized to chromosome 6 (49.9–52.7 Mb). Interestingly, a locus near this conserved syntenic region in humans (6q12–16) has been identified in a French family with nine individuals affected by a pure form of autosomal dominant dilated cardiomyopathy (Sylvius et al. 2001).

Our minimal interval contains five annotated genes: three well defined and two poorly defined. The three well-defined genes in our interval are *Tram2* (translocating chain-associating membrane protein 2), *Tmem14a* (transmembrane protein 14A), and *Gsta3* (glutathione S-transferase alpha 3). TRAM2 appears to be involved in controlling the posttranslational processing of secretory and membrane proteins at the endoplasmic reticulum through its interaction with SERCA2b (Stefanovic et al. 2004). Tmem14a is expressed in the brain and is a protein integral to the cell membrane (http://www.informatics.jax.org/). The gene *GSTA3* encodes a glutathione S-transferase belonging to the alpha class genes (Board 1998). Genes of the alpha class are highly related and encode enzymes with glutathione peroxidase activity. The enzymes encoded by these genes are involved in cellular defense against toxic, carcinogenic, and pharmacologically active electrophilic compounds (Tetlow et al. 2004). While none of these genes are known to be cardiomyopathy-causing genes or sarcomeric

protein genes, all are good candidates and will be explored as genes previously not described to cause dilated cardiomyopathy.

Identification and mapping of heritable cardiomyopathy in family EN25

In family EN25 we identified eight affected siblings derived from the G1 mouse EN25. These eight affected mice had a value greater than 2 SD from the mean in at least one of the three parameters measured and have been followed with echocardiograms every 2 months (Fig. 5).

To identify the chromosomal region where the mutation is localized for the EN25 family we used the same SNP genotyping panel described for family EN1 for genetic mapping of mouse mutations (Moran et al. 2006). Whole-genome linkage information for the eight affected G3 mice identified the region with the highest retention of DBA alleles in the distal end of chromosome 15. Higher-density mapping using microsatellite markers and additional SNPs has been used to narrow initially the candidate region to approximately 12 Mb (Fig. 6). The interval is defined by the SNP rs4230884 and the SNP rs6285067 on chromosome 15 (Fig. 6). This region of chromosome 15 is supported by 11 different affected animals, with a single animal discordant with the crossovers supporting the 12-Mb interval. The conserved syntenic region is localized to chromosome 12 (33.4–53.3 Mb) and chromosome 22 (34.3–49.6 Mb) in humans (Supplementary Fig. 2).

Similar to the strategy for family EN1, we generated a renewable source of crossovers for EN25 by breeding affected G3 mice to wild-type B6 and intercrossing the progeny to supply a continual source of mutant mice homozygous for the DBA mutagenized allele. The progeny from the new lines showed an increase in chamber dimensions and decrease in % fractional shortening, similar to the echocardiography data of the initial eight identified G3 mutants, thus demonstrating heritability of the cardiomyopathic phenotype (Supplementary Fig. 3).

Discussion

In this study we performed a recessive screen in adult mice for dilated cardiomyopathy using ENU mutagenesis. Based on our phenotypic screen we identified two families, EN1 and EN25, with heritable cardiomyopathy. In the first family we identified 94 affected siblings derived from the G1 mouse EN1. The affected progeny showed significant enlargement of cardiac chambers and progressive decline in cardiac function compared to age- and genetic background-matched wild-type controls. In the second family we identified 20 affected siblings derived from the G1 mouse EN25. These 20 affected mice had a value greater than 2 SD from the mean in at least one of the three parameters measured.

Using our strategy of introducing a different genetic background early in the breeding scheme, we were able to map the chromosomal regions responsible for the dilated cardiomyopathy phenotype in these two families to the proximal end of chromosome 1 (EN1) and to the distal end of chromosome 15 (EN25). In family EN1 we narrowed the candidate region on the proximal end of chromosome 1 to less than 1 Mb. Plausible candidate genes mapping within this region include translocating chain-associating membrane protein 2, transmembrane protein 14A, and glutathione S-transferase alpha 3. In family EN25 we narrowed the candidate region to an interval of approximately 12 Mb in the distal end of chromosome 15. Plausible candidate genes mapping within this region include endothelial cell growth factor 1, prickle 1, and alpha 1,4-galactosyltransferase.

There are several points worth noting in our screen. First, we decided to focus our screen on a cardiomyopathy phenotype. We believe a narrowly focused, disease-related phenotypic screen offered the best chance of finding heritable mutants of cardiovascular interest. Second, echocardiograms were performed in conscious mice, obviating the need for anesthesia or

sedation which can be a confounding variable. Moreover, because each echocardiogram takes about 8–10 min to perform, our method allows for a relatively high-throughput screen. Third, by introducing a different strain (B6) in the second generation, we significantly accelerated our ability to map the mutated genetic region.

In conclusion, we report the identification of two families with heritable dilated cardiomyopathy. While the identification of the genes responsible for this cardiomyopathic phenotype is ongoing, once identified they should increase our understanding of the way genetic factors contribute to heart failure and facilitate the development of novel diagnostic and therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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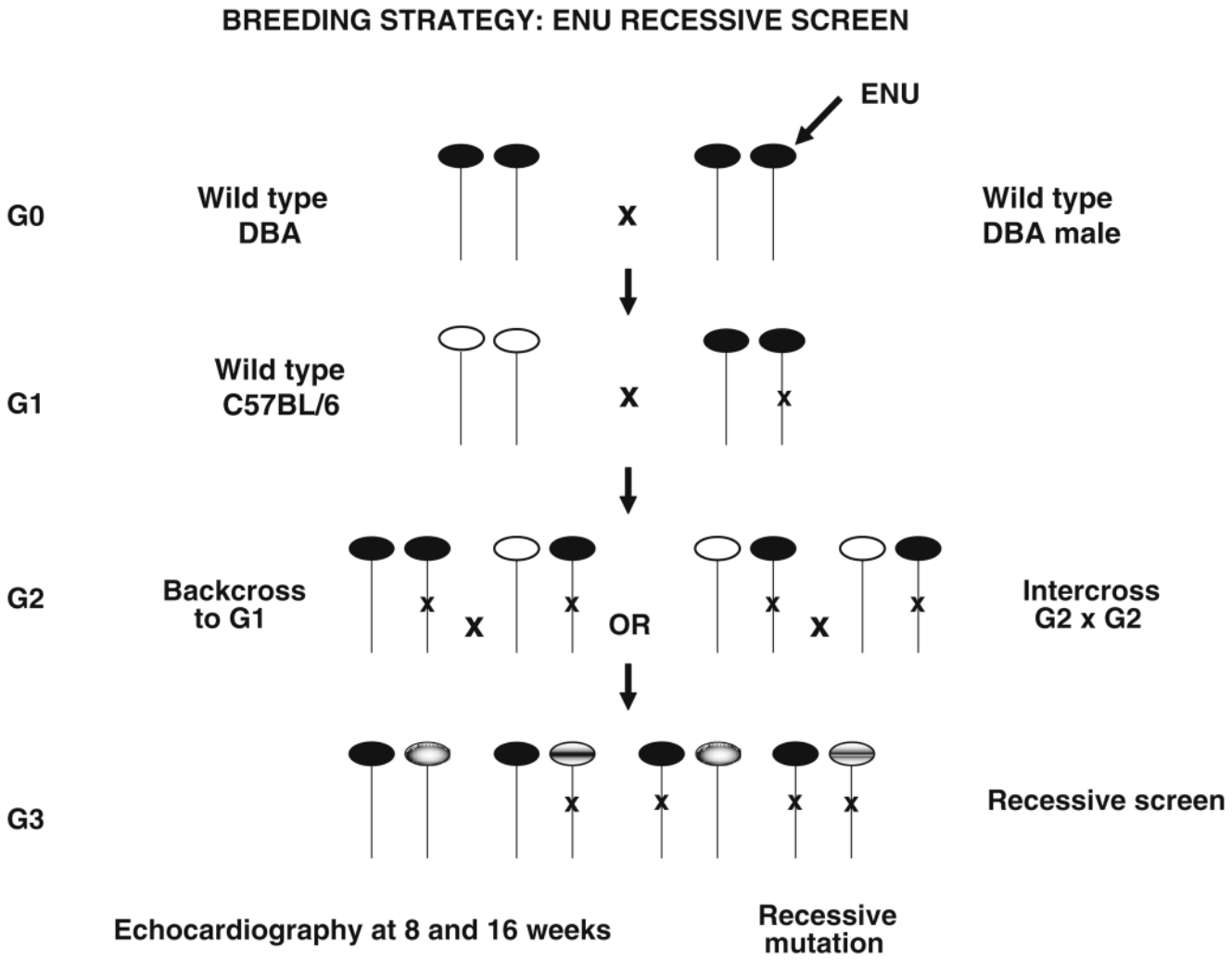

Fig. 1.

Breeding scheme for an ENU recessive screen. Male mice are injected with ENU and a large number of G1 offspring are produced by mating the treated males to unaffected females. The resulting G1 progeny, which are heterozygous for many different newly induced mutations distributed throughout the genome, are mated to wild-type B6 mice to establish families of G2 siblings. These G2 animals share the same set of mutations derived from their father, and each G2 animal has a 50% chance of inheriting any single mutation carried by the G1 male. To generate G3 animals, G2 s are backcrossed to the G1, or, alternatively, random intercrossing between G2 siblings can be performed. In this recessive screen the phenotype is followed only in the animals that are homozygous for the mutation

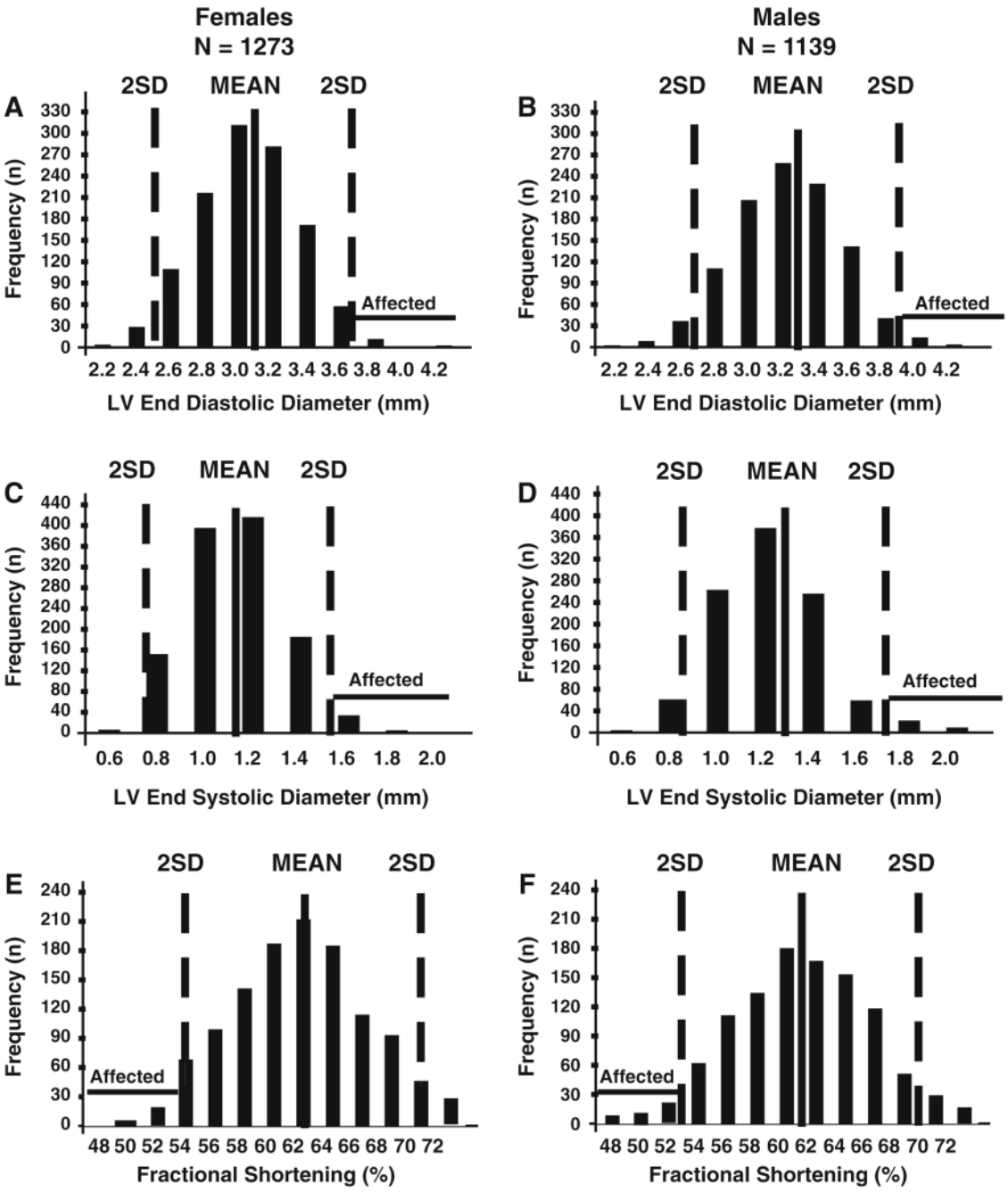

Fig. 2.

Phenotypic screen of G3 mice at 16 weeks of age. We phenotyped G3 mice for dilated cardiomyopathy using echocardiography performed in conscious mice at two time points: 8 and 16 weeks of age. Parameters measured were left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and % fractional shortening. G3 mice with a value greater than 2 SD from the mean in any of the parameters measured were considered cardiomyopathy mutants. Data for over 2400 G3 mice at 16 weeks is shown for (**a**) left ventricular end-diastolic diameter for females; (**b**) left ventricular end-diastolic diameter for males; (**c**) left ventricular end-systolic diameter for females; (**d**) left ventricular end-systolic diameter for males; (**e**) fractional shortening for females; (**f**) fractional shortening for males

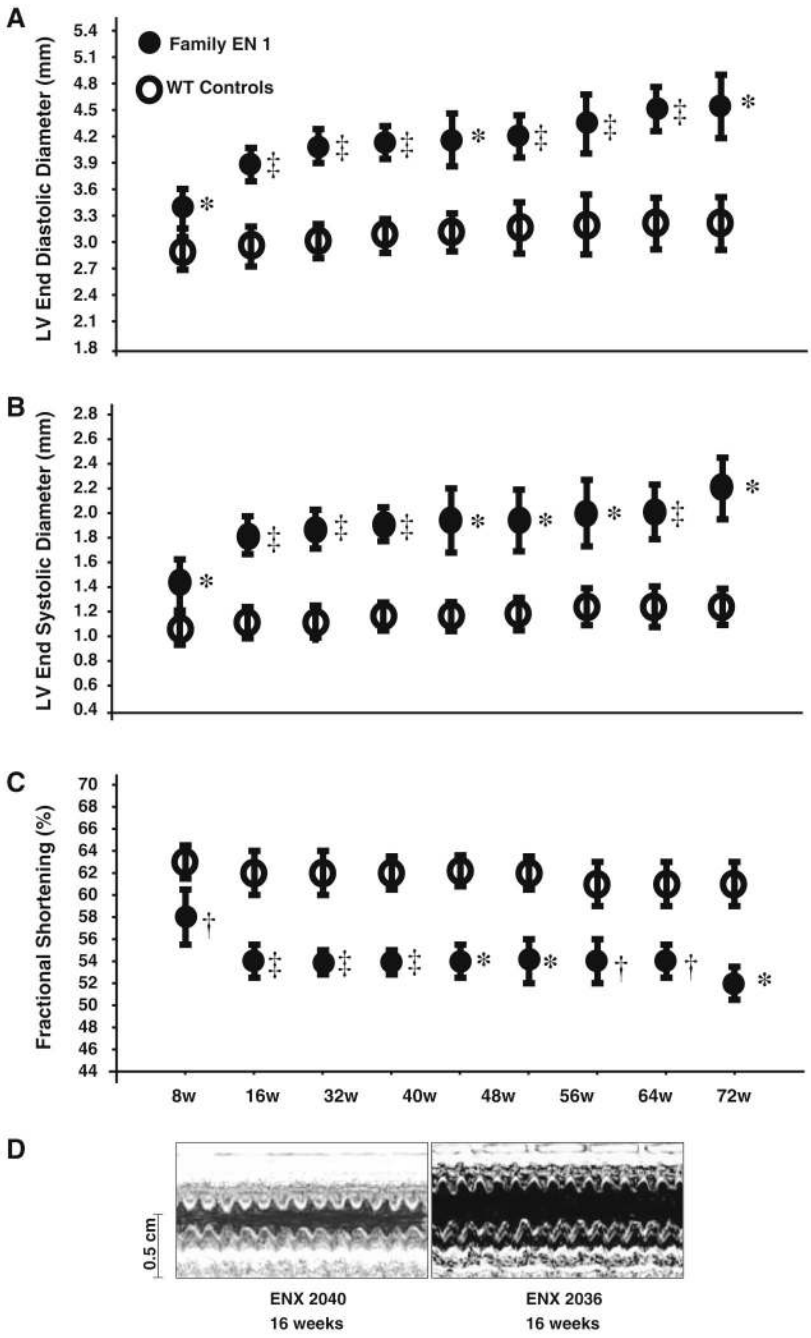

Fig. 3.

Progressive cardiac dysfunction in G3 ENU mutants from family EN1. Thirteen siblings derived from EN1 (G1) and with abnormal cardiac function were identified in our phenotypic screen. Progressive cardiac dysfunction of these affected G3 mice with values 2 SD outside the mean were compared to age-matched wild-type (WT) control mice of the same genetic background (DBA/B6). Echocardiograms were performed at 8, 16, 24, 32, 40, 48, 56, 64, and 72 weeks in EN1 mutants (N = 10–13) and WT controls (N = 19–24). Note the progressive increase in chamber dimensions and fall in % fractional shortening in the mutagenized G3 mice indicating progressive cardiomyopathy. Shown are data for (**a**) left ventricular end-diastolic diameter; (**b**) left ventricular end-systolic diameter; (**c**) fractional shortening (FS), $^{*}p <$

0.0001; $^{\dagger}p < 0.01$; $^{\ddagger}p < 0.0000001$. **d** Examples of M-mode echocardiograms from two G3 siblings at 16 weeks of age are shown. ENX 2036 is an example of a G3 mouse with a value greater than 2 SD from the mean. Its heart is dilated and shows depressed cardiac function compared with its sibling ENX 2040, which is normal

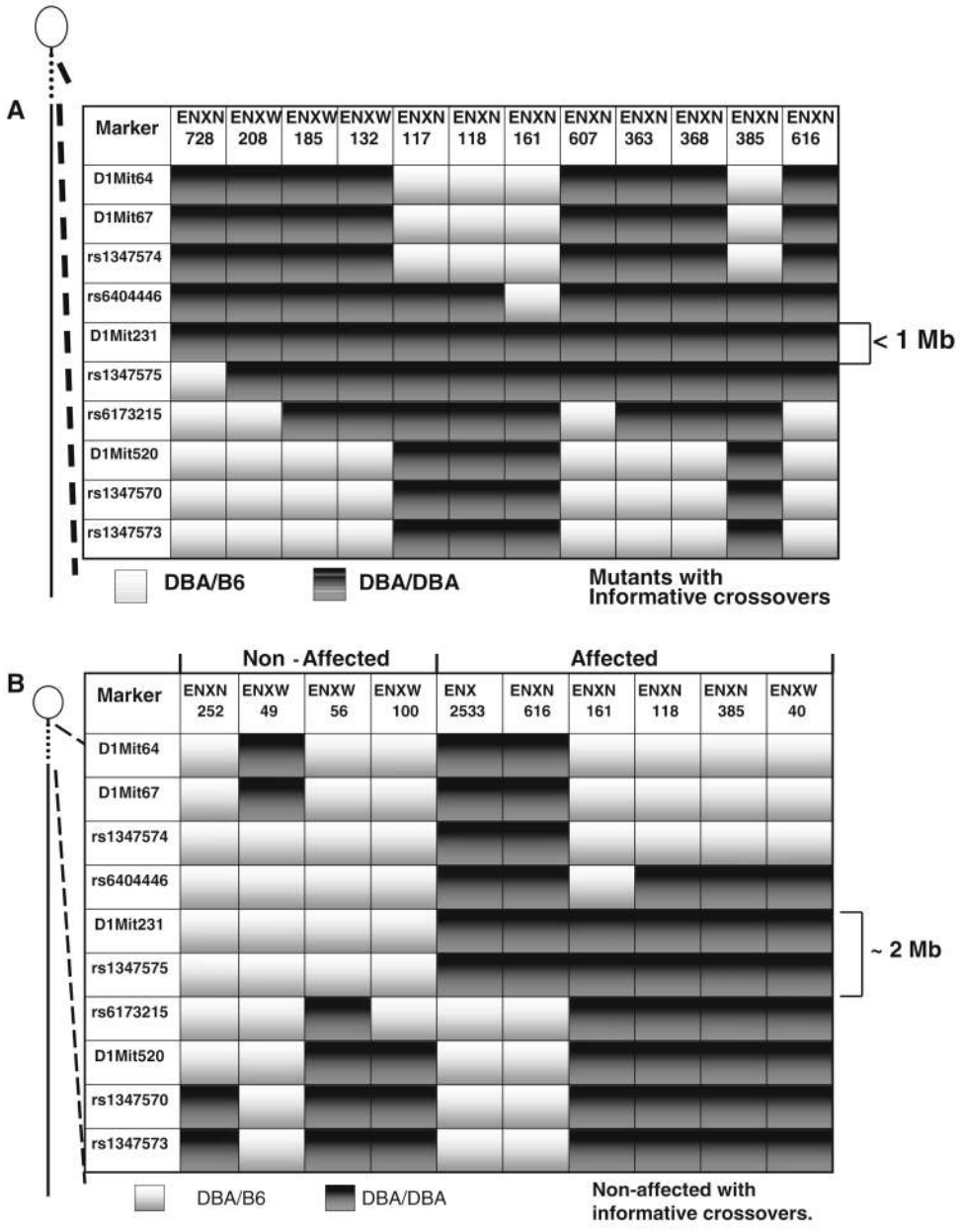

Fig. 4.

Candidate interval in the proximal end of chromosome 1. The SNP genotyping panel indicated a provisional location for the mutation. We performed fine mapping toward the eventual identification of the causative mutation and gene. **a** Fine mapping of the candidate region on chromosome 1. Twenty-three of the 94 mutant mice have informative crossovers that have allowed us to narrow the interval to approximately 1 Mb. This interval is defined by the SNPs rs6404446 and rs1347575. **b** Examination of unaffected progeny for crossovers. Unaffected mice from this intercross that are homozygous DBA over our interval, but lack the phenotype, have essentially crossed out the mutagenized allele

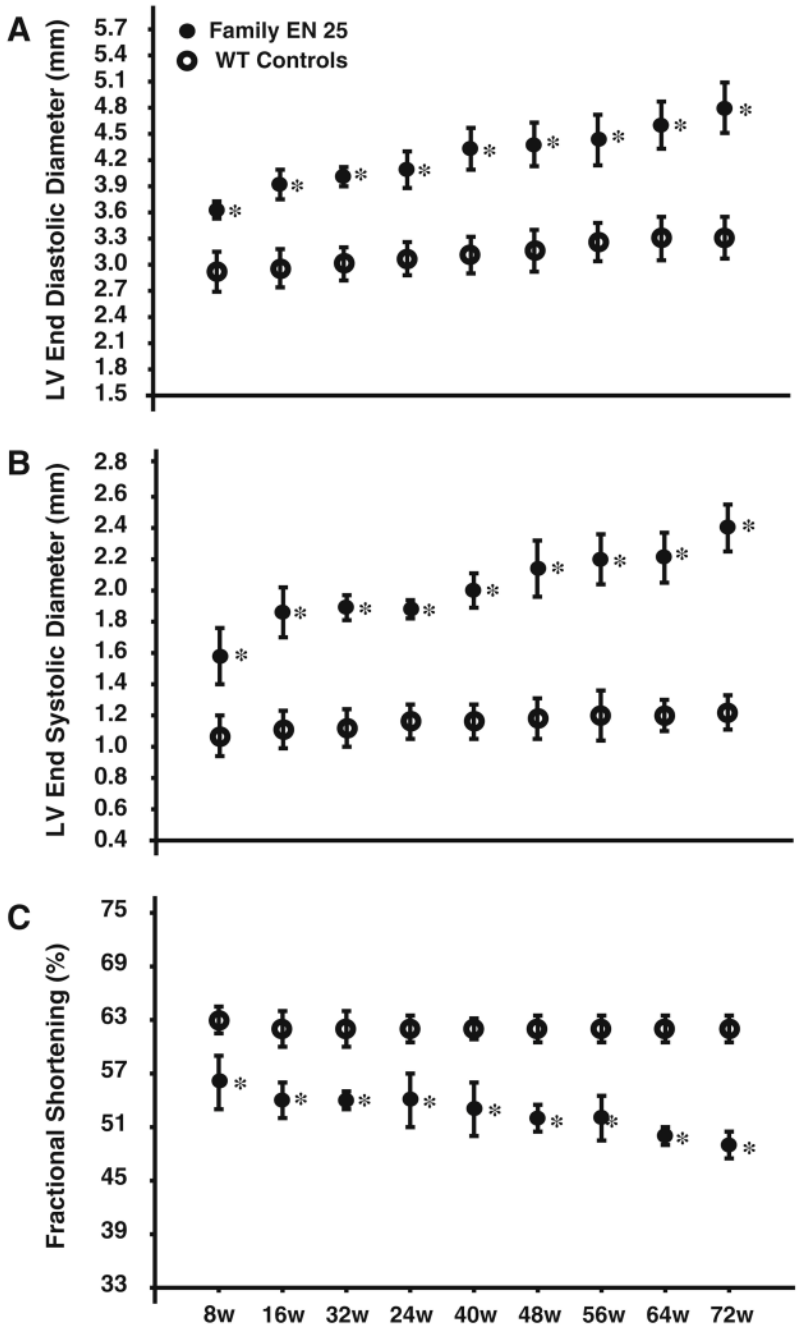

Fig. 5.

Progressive cardiac dysfunction in G3 ENU mutants from family EN25. Eight siblings, derived from EN25 (G1), with abnormal cardiac function were identified in our phenotypic screen. Progressive cardiac dysfunction of these affected G3 mice with values 2 SD outside the mean were compared to age-matched wild-type (WT) control mice of the same genetic background (DBA/B6). Echocardiograms were performed at 8, 16, 24, 32, 40, 48, 56, 64, and 72 weeks in EN25 mutants ($N = 8$) and WT controls ($N = 19–24$). Note the progressive increase in chamber dimensions and fall in % fractional shortening (FS) in the mutagenized G3 mice indicating progressive cardiomyopathy. Shown are data for (**a**) left ventricular end-diastolic diameter; (**b**) left ventricular end-systolic diameter; (**c**) fractional shortening (FS); $^{*}p < 0.0001$

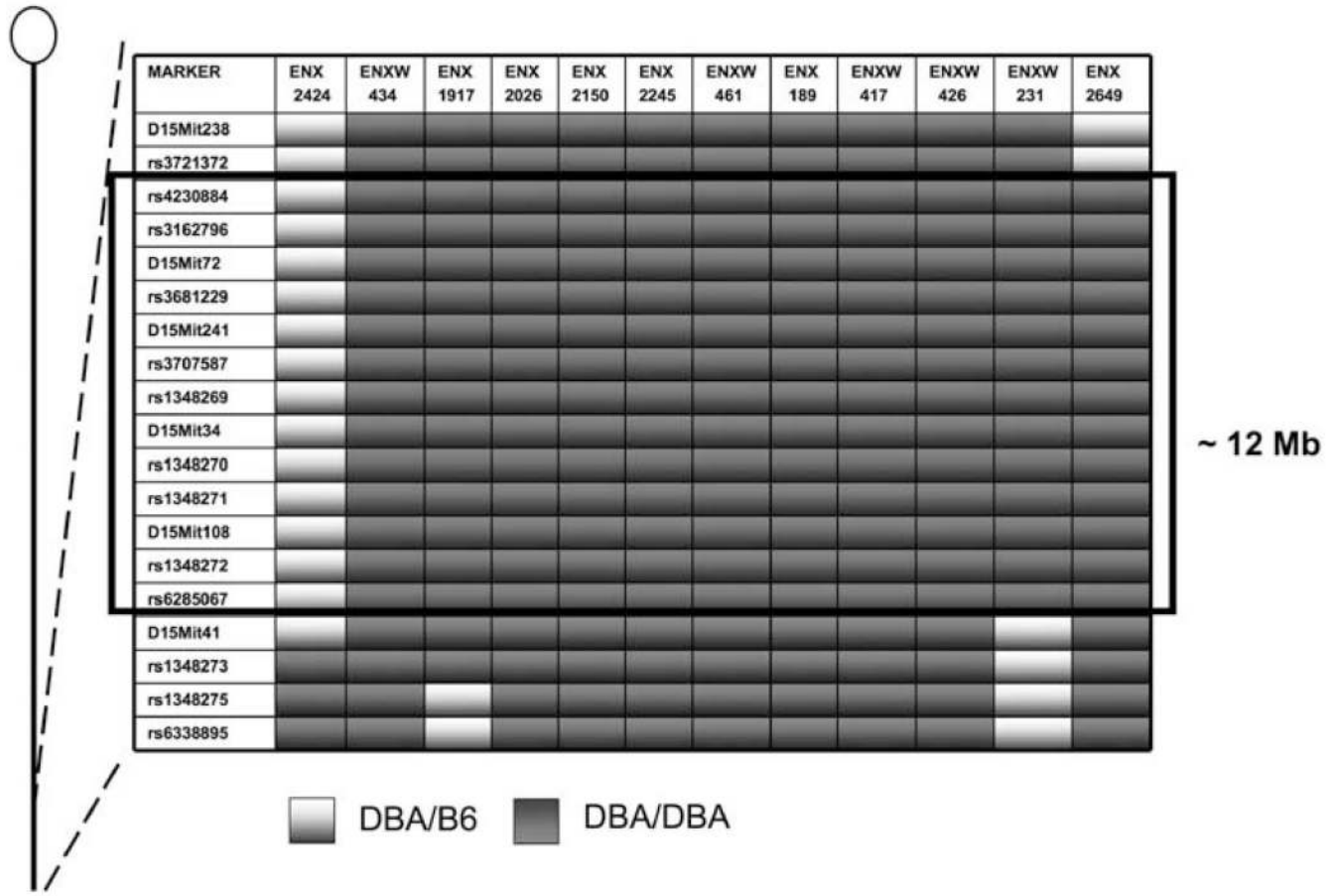

Fig. 6.

Fine mapping of the candidate interval in the distal end of chromosome 15. The SNP genotyping panel indicates a provisional location for this mutation. We performed fine mapping toward the eventual identification of the causative mutation and gene. Three of the 20 mutant mice have informative crossovers that have allowed us to narrow the interval to approximately 12 Mb