New Dyscalc loci for myocardial cell necrosis and calcification (dystrophic cardiac calcinosis) in mice

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Ivandic, Boris T., H. Friedrich Utz, Piotr M. Kaczmarek, Zouhair Aherrahrou, Susanne B. Axtner, Carola Klepsch, Aldons J. Lusis, and Hugo A. Katus. New Dyscalc loci for myocardial cell necrosis and calcification (dystrophic cardiac calcinosis) in mice. Physiol Genomics 6: 137–144, 2001.—Dystrophic cardiac calcinosis (DCC) occurs among certain inbred strains of mice and involves necrosis and subsequent calcification as response of myocardial tissue to injury. Using a complete linkage map approach, we investigated the genetics of DCC in an F2 intercross of resistant C57BL/6J and susceptible C3H/HeJ inbred strains and identified previously a major predisposing quantitative trait locus (QTL), Dyscalc1, on proximal chromosome 7. Analysis of inheritance suggested, however, that DCC is influenced by additional modifier QTL, which have as yet not been mapped. Here, we report the identification by composite interval mapping of the DCC loci Dyscalc2, Dyscalc3, and Dyscalc4 on chromosomes 4, 12 and 14, respectively. Together, the four Dyscalc loci explained 47% of the phenotypic variance of DCC, which was induced by a high-fat diet. Additive epistasis between Dyscalc1 and Dyscalc2 enhanced DCC. Examining recombinant inbred strains, we propose a 10-cM interval containing Dyscalc1 and discuss potential candidate genes.

lead to heart failure in severe conditions (4, 5, 21). Although the pathogenesis of DCC is largely unknown, evidence from ultrastructural studies implied that calcium precipitation initiates in the mitochondria of irreversibly injured cells, irrespective of the etiology (14, 30). Hormones, high-fat and purified diets, cytomegaly and Coxsackie B virus infection, and even freeze-thaw injury may increase the incidence and severity of DCC (1, 5, 6, 10). The pivotal role of calcium for normal cell function and for necrosis and apoptosis are well established, and a large number of calcium-handling proteins must be considered as potential candidate genes for DCC (7, 27).

Employing quantitative trait locus (QTL) analysis based on a complete linkage map, we investigated the genetics of DCC in an F2 intercross of resistant C57BL/6J and susceptible C3H/HeJ strains and identified a major locus, Dyscalc1, predisposing to DCC (15). Independent linkage studies subsequently confirmed the location of Dyscalc1 on proximal chromosome 7 and demonstrated its pathogenetic relevance for the DBA/2 strain (2, 29). DCC is not a monogenic disorder, but a complex disorder influenced by multiple genes, which have as yet not been identified (2, 5).

We now report the mapping of major additional QTL for DCC using composite interval mapping (CIM), which is particularly suited for the analysis of composite QTL models and epistasis (9, 25). In addition, recombinant inbred (RI) strains were examined to analyze QTL interactions in vivo and to map Dyscalc1 more precisely.

METHODS

Animals. BXH RI strains 2, 8, 10, and 12 and C57BL/6J and C3H/HeJ inbred mice were purchased from Jackson Laboratories (Bar Harbor, ME). An F2 intercross between resistant C57BL/6J and susceptible C3H/HeJ strains was constructed. Only female F2 mice (n = 197) were examined, because spontaneous DCC was observed predominantly, but not exclusively, in female retired breeders (5). All animals had free access to water and food and were maintained in a pathogen-free environment with even light-dark cycle. At the age of 3 mo, regular chow (12% of calories as fat) was changed to a high-fat, high-cholesterol diet (TD 90221; Harlan-Tek-
laid, Madison, WI) containing 30% of calories as fat to induce DCC prematurely. After 8 wk on the high-fat diet, mice were anesthetized and killed by cervical dislocation before the heart and proximal aorta were removed for histological analysis. In addition, DCC was produced in 8-wk-old mice of strains BXH-8 and BXH-10 by freeze-thaw injury as described by Brunnert (1). Briefly, the abdomen of a deeply anesthetized animal was opened in midline for about an inch. A steel rod (1/5-inch in diameter), precooled in liquid nitrogen, was then pressed slightly, for about 10 s, against the base of the heart visible through the diaphragm. The mice recovered quickly after wound closure and were killed 2 wk after surgery.

Assessment of phenotypes. Details of histological analysis have been described previously (15, 23). Briefly, serial 10-μm cryosections throughout the myocardium were prepared, and every fifth section, adding up to 40–60 sections per heart, was collected. Slides were usually stained with hematoxylin or with alizarin red S to detect calcium deposits. In F2 animals DCC was scored semiquantitatively by counting the number of sections per heart displaying calcified lesions upon light microscopy. Before further analysis, scores were squared to normalize the distribution of score data. In contrast, only the prevalence of DCC was assessed in BXH RI strains. In general, a higher DCC prevalence was noted in RI strains exhibiting severe calcification. RI animals were considered positive for DCC if they exhibited at least three single-cell calcifications or one larger confluent lesion.

Heparinized plasma was obtained by retroorbital bleeding from mice of strains BXH-2, -10, and -12 after overnight fasting before changing the diet and before death. Plasma cholesterol and triglyceride concentrations were measured in fasted animals DCC was scored semiquantitatively by counting the number of sections per heart displaying calcified lesions upon light microscopy. Before further analysis, scores were squared to normalize the distribution of score data. In contrast, only the prevalence of DCC was assessed in BXH RI strains exhibiting severe calcification. RI animals were considered positive for DCC if they exhibited at least three single-cell calcifications or one larger confluent lesion.

**RESULTS**

**QTL analysis.** We analyzed the F2 intercross data with the intention to map QTL modifying the penetrance and expression of DCC. For this purpose, we employed multiple regression analysis, selecting markers as cofactors to test composite QTL models. Based on permutation analysis, LOD scores exceeding 4.2
and 5.2 were considered significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively. CIM confirmed the existence of Dyscalc1 on proximal chromosome 7, mapping it 4 cM proximal of D7Mit229 with a highly significant LOD score peak of 14.8 (Fig. 1A, Table 1), very similar to the results reported previously (LOD score peak 14.6) (15). This locus accounted for 30.8% of the phenotypic variance (coefficient of determination calculated as partial correlation of the QTL with the observed DCC score adjusted for other QTL cofactors). We also identified three new QTL, designated Dyscalc2, Dyscalc3, and Dyscalc4, which contributed to DCC and mapped on chromosomes 4, 12, and 14, respectively (Table 1). Dyscalc2 was located on distal chromosome 4, 2 cM proximal of D4Mit16, with a significant LOD score peak of 4.7 (Fig. 1B, Table 1). This locus was responsible for 11.4% of the DCC variance. Dyscalc3 was identified very close to the centromere of chromosome 12, about 2 cM distal of D12Mit37, with a suggestive LOD score peak of 3.5 (Fig. 2A, Table 1). Another suggestive QTL, Dyscalc4, mapped at D14Mit133 on proximal chromosome 14 with a LOD score peak of 3.7 (Fig. 2B, Table 1). Dyscalc3 and Dyscalc4 accounted for 8.3% and 8.8% of the phenotypic DCC variance, respectively. Significant distortion of genotype segregation, which may have influenced the mapping results, was excluded by $\chi^2$ analysis, which is implemented in the PLABQTL program (data not shown).

### Analysis of allele effects.

To examine the allele effects on calcification, scores were grouped by genotype at the Dyscalc loci and analyzed by ANOVA (Figs. 3 and 4). Homozygous inheritance of susceptible C3H/HeJ alleles (“HH”) at Dyscalc1 and Dyscalc2 predisposed to DCC among F2 progeny (Fig. 3). Consistent with the autosomal recessive mode of inheritance of DCC, detailed analysis by Fisher’s PLSD revealed that F2 mice with the “HH” genotype exhibited significantly more calcification than those with a resistant C57BL/6J allele (“BH” or “BB”) (Fig. 3). In striking contrast, at Dyscalc3 and Dyscalc4, DCC appeared to be promoted by a homozygous C57BL/6J genotype (“BB”) rather than a C3H/HeJ allele (“H”) (Fig. 4). Therefore, alleles from the resistant parental strain contribute to DCC as well.

### Epistasis.

The PLABQTL software was used to test several models of epistasis between all possible pairs of Dyscalc loci. Significant two-loci additive interaction was observed between Dyscalc1 and Dyscalc2 (Table 2). This relationship was also evident comparing mean calcification scores grouped by genotype (Fig. 5). A C3H/HeJ genotype (H) at Dyscalc2 significantly enhanced the predisposing effect of the C3H/HeJ genotype (HH) at Dyscalc1 (ANOVA, $P < 0.0001$) and only

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**Table 1. Dyscalc QTL with LOD score peaks exceeding 3.0 (composite interval mapping)**

<table>
<thead>
<tr>
<th>Dyscalc Locus</th>
<th>Chromosome</th>
<th>Closest Marker</th>
<th>Position Relative to Marker, cM</th>
<th>LOD Score Peak</th>
<th>Coefficient of Determination, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>D7Mit229</td>
<td>-4</td>
<td>14.8</td>
<td>30.8</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>D4Mit16</td>
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<td>4.7</td>
<td>11.4</td>
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<td>3</td>
<td>12</td>
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<td>8.3</td>
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<tr>
<td>4</td>
<td>14</td>
<td>D14Mit133</td>
<td>0</td>
<td>3.7</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Positive and negative genetic distances (“Position Relative to Marker”) are given in centimorgans (cM) and refer to mapping positions distal and proximal of the closest marker, respectively. LOD (log of the likelihood odds ratio) scores exceeding 4.2 and 5.2 were considered significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively. “Coefficient of Determination” describes the part of the trait variance explained by the quantitative trait loci (QTL).
one of 13 F2 mice displaying HH genotypes at both QTL was free of calcification. Dominance × dominance and additive × dominance models were calculated as well but did not produce significant results, probably because the number of F2 progeny was too small (data not shown). In the final regression, the combined effects of all four Dyscalc loci explained 46.8 ± 5.4% of the trait variance (coefficient of determination), corresponding to an overall LOD score of 25.3.

Analysis of BXH RI strains. We examined members of the set of BXH RI strains, hypothesizing that a high prevalence of DCC would be observed predominantly among the strains displaying predisposing Dyscalc genotypes (Table 3). On a high-fat diet, strains BXH-4 and -11 exhibited a high DCC prevalence of 75% and 100%, respectively, and susceptible H genotype at the two most influential DCC loci, Dyscalc1 and Dyscalc2. This observation was consistent with the additive in-

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**Fig. 3.** Squared calcification scores (means ± SD) grouped by genotypes BB, BH, and HH at Dyscalc1 (A) and Dyscalc2 (B). B and H denote alleles from parental strains C57BL/6J and C3H/HeJ, respectively. Overall, genotype groups differed significantly (ANOVA: \( P < 0.0001 \) for D7Mit229, \( P = 0.0046 \) for D4Mit16). Comparison between groups by Fisher’s PLSD is indicated by horizontal brackets.

**Fig. 4.** Calcification scores (means ± SD) grouped by genotypes BB, BH, and HH at Dyscalc3 (A) and Dyscalc4 (B). B and H denote alleles from parental strains C57BL/6J and C3H/HeJ, respectively. Overall, genotype groups differed significantly (ANOVA: \( P = 0.0029 \) at D12Mit37, \( P = 0.0248 \) at D14Mit133). Comparison between groups by Fisher’s PLSD is indicated by horizontal brackets.
interaction between these loci detected by QTL analysis of the F2 mice. No calcification was found in strains BXH-7, -8, and -9, concordant with a resistant B genotype at Dyscalc1. As previously emphasized, BXH-10 appeared resistant to diet-induced DCC despite predisposing H genotypes at Dyscalc1 (D7Mit229) (15). Therefore, we examined 17 additional BXH-10 mice to confirm this striking finding. Only two mice exhibited spurious, insignificant calcifications, whereas the remaining animals appeared free of calcified lesions after high-fat diet. Next, plasma cholesterol concentrations were compared in strains BXH-2 (high DCC prevalence), BXH-10 (DCC resistance), and BXH-12 (low DCC prevalence), to determine whether DCC prevalence in general and the resistance of strain BXH-10 to diet-induced DCC in particular might depend on alterations of lipid metabolism. Upon high-fat diet, plasma cholesterol levels increased from 2.2 ± 0.5 to 5.3 ± 1.0 mmol/l in BXH-2 mice (n = 4; P = 0.0679, Wilcoxon signed-ranks test), from 1.6 ± 0.2 to 2.4 ± 0.6 mmol/l in BXH-10 mice (n = 8; P = 0.0173, Wilcoxon signed-ranks test), and from 2.2 ± 0.1 to 6.5 ± 1.7 mmol/l in BXH-12 mice (n = 9; P = 0.0077, Wilcoxon signed-ranks test). In contrast, plasma triglyceride levels did not change (0.37 ± 0.16 mmol/l on chow vs. 0.38 ± 0.06 mmol/l on high-fat diet) in BXH-2 mice (n = 4; P = not significant (NS), Wilcoxon signed-ranks test) and even decreased from 0.51 ± 0.09 to 0.25 ± 0.04 mmol/l in BXH-10 mice (n = 8; P = 0.0117, Wilcoxon signed-ranks test) and from 0.86 ± 0.11 to 0.29 ± 0.03 mmol/l in BXH-12 mice (n = 9; P = 0.0076, Wilcoxon signed-ranks test). Although we analyzed only a few mice, a smaller increase of plasma cholesterol levels in strain BXH-10 was evident, compared with strains BXH-2 (P = 0.0174, Mann-Whitney U) and BXH-12 (P = 0.0005, Mann-Whitney U), and may have contributed to the resistance of strain BXH-10 to diet-induced DCC. For comparison, no significant relationship (rho corrected for ties: −0.18, tied P value: 0.7987; Spearman’s rank correlation) was found between plasma cholesterol levels and calcification scores in the F2 intercross mice after high-fat diet.

Since the issue of DCC resistance of strain BXH-10 was not resolved after all, we examined five additional BXH-10 mice by employing freeze-thaw injury to produce DCC independent of plasma cholesterol levels. Two weeks after freeze-thaw injury these mice all exhibited severely calcified myocardial lesions close to the apex. Histological analysis showed a pattern of calcification comparable to the histopathological results reported previously (1, 15). Calcification was confined to the freezing spot characterized by fibrosis and little cellular infiltration, whereas diet-induced calcification was rather multifocal and smaller, without evidence of cellular infiltration or fibrosis.

The susceptibility to DCC of strain BXH-10 suggested that Dyscalc1 mapped distal of D7Mit266 (10.0 cM), where a transition of resistant to susceptible ge-
Percentage and raw number of affected animals per group (n) after high-fat diet unless indicated otherwise (asterisks). *In this strain, the n7/9 4/8 6/8 1/4 0/8 0 0 0 100 100 19 29 14

“B” and “H” denote homozygous alleles inherited from either resistant C57BL/6 or susceptible C3H/He progenitor strains, respectively. Markers representing Dyscalc1 QTL are shown in italics. Map positions of the markers are given in cM. “DCC Prevalence” is expressed as percentage and raw number of affected animals per group (n) after high-fat diet unless indicated otherwise (asterisks). *In this strain, the susceptibility to DCC was tested by freeze-thaw injury (see RESULTS). In contrast, none of 22 BXH-10 and none of 3 BXH-8 mice exhibited calcifications upon high-fat diet. DCC, dystrophic cardiac calcification; RI, recombinant inbred.

To define an interval containing Dyscalc1, we examined six additional BXH-8 mice, which also appeared resistant to diet-induced DCC and exhibited a transition of resistant to susceptible genotypes between D7Mit229 (23.0 cM) and D7Mit230 (24.5 cM) (Table 3). Careful histological analysis of these animals revealed no evidence of calcium deposition in freeze-thaw lesions, which were otherwise comparable with regard to fibrosis and cellular infiltration. Under the assumption that DCC requires a predisposing H genotype at Dyscalc1, a recombination in strain BXH-6 between D7Mit224 (15.0 cM) and D7Mit230 (16.0 cM) even further limited the chromosomal interval potentially containing Dyscalc1 to less than 10 cM between markers D7Mit224 (15.0 cM) and D7Mit230 (24.5 cM) (Table 3).

DISCUSSION

We previously reported genetic studies that led to the identification of the major genetic determinant of DCC in C3H/HeJ and DBA/2J mice. This QTL was designated Dyscalc1 and mapped on proximal chromosome 7 in a region containing a large number of candidate genes potentially carrying the mutation predisposing to DCC (15). Analysis of inheritance revealed, however, that DCC is in fact influenced by a major QTL and at least one modifier QTL (15). This hypothesis was supported by suggestive, but nonsignificant LOD score peaks near markers D4Mit16 (LOD score 2.4), D8Mit190 (LOD score 2.2), and D12Mit37 (LOD score 2.7) obtained previously using simple interval mapping (15). Therefore, we sought to map these potential QTL by employing a different approach, CIM, to account for the complex genetics of DCC. Haley and Knott (11) developed CIM by multiple regression of trait data on genotype data. Jansen and Stam (16, 17) improved this method, incorporating markers as cofactors in the regression to identify QTL and to test their interaction. CIM has been applied successfully for the analysis of complex disorders (8, 25, 26). Using the PLABQTL software package for CIM, we confirmed the location of the major Dyscalc1 QTL, mapped Dyscalc2 and Dyscalc3 on chromosomes 4 and 12, and identified a novel QTL, Dyscalc4, on chromosome 14. A suggestive QTL mapping previously near D8Mit190 (see above) was lost during CIM analysis. Compared with previous QTL analysis (see above), Dyscalc2 now attained a significant LOD score peak of 4.7. LOD score results for Dyscalc3 and Dyscalc4 were considered suggestive with respect to the significance thresholds defined after permutation analysis (3).

Apart from presenting evidence for additional Dyscalc loci, we examined epistasis influencing DCC expression and penetrance. Using the PLABQTL software, we obtained a composite QTL model explaining nearly 50% of the phenotypic variance of DCC and detected additive epistatic interactions between Dyscalc1 and Dyscalc2. Homozygous C3H/HeJ alleles at Dyscalc1 doubled the extent of myocardial calcification, if associated with C3H/HeJ alleles at Dyscalc1. Dyscalc2 appeared to explain, in large part, the presence of DCC in 5 of 30 F2 mice, which displayed calcification despite a resistant C57BL/6J allele at Dyscalc1. This indicated a small, but independent effect of Dyscalc2 on DCC. The observation of DCC in 12 of 13 F2 progeny with susceptible alleles at Dyscalc1 and Dyscalc2 suggested that the combined influence of both QTL may be almost sufficient to cause DCC, although this issue requires further investigation. Therefore, Dyscalc2 was not another major predisposing locus, like Dyscalc1, but may be a modifier QTL as postulated in our previous report (15). Very interestingly, we identified also two QTL in the resistant C57BL/6J strain background, which appeared to promote DCC indepen-
dently, yet were not sufficient to cause DCC in parental C57BL/6J mice.

RI strains have been developed for gene mapping and QTL analysis and basically constitute an inbred F$_2$ generation. They allow studying a trait in multiple, genetically identical animals of a single RI strain with a unique genotype. The BXH RI strains have been derived from C57BL/6 and C3H/He progenitor strains. We genotyped all available BXH RI strains to determine whether DCC prevalence was concordant with predisposing genotypes at the Dyscalc loci. Although significant diet-induced DCC was found in 4 of 12 BXH RI strains, none of these RI strains exhibited susceptible alleles at all Dyscalc loci. As discussed earlier, BXH-10 appeared resistant to diet-induced DCC despite exhibiting predisposing alleles in a large part of proximal chromosome 7 including the 99% confidence interval for Dyscalc1 (15). This striking contradiction could be explained by rare double recombination events creating a tiny, resistant chromosomal region, which contains Dyscalc1 and has eluded detection by genotyping. To exclude phenotyping errors explaining this contradiction, we examined 17 additional BXH-10 mice, which had received a high-fat diet. Careful histological analysis revealed that almost all BXH-10 mice were resistant. However, two mice exhibited nonsignificant single-cell calcifications, suggesting that the penetrance of DCC might be largely inhibited by other loci in this strain. Such loci may have influenced plasma lipid concentrations indirectly determining the susceptibility to DCC. Indeed, in BXH-10 mice cholesterol levels increased only 1.5-fold ($P < 0.05$) upon high-fat challenge. However, even a 3.3-fold increase in plasma cholesterol produced DCC in only 19% of BXH-12 mice, whereas a 2.4-fold increase was sufficient to cause 78% prevalence in BXH-2 animals. These findings neither prove nor exclude an influence of plasma cholesterol levels on DCC expression. Examination of lipid metabolism and DCC in congenic strains, in which the Dyscalc loci have been isolated separately on a simpler, uniform genetic background, may help to explain the relationship between DCC and lipids. Clearly, incomplete penetrance, the small number of BXH RI strains, and the polygenic basis account for the lack of genotype-phenotype correlation in diet-induced DCC.

To overcome the influence of loci potentially inhibiting DCC penetrance, we employed a freeze-thaw injury to produce calcifications. Then, even BXH-10 mice exhibited large calcified lesions comparable to those observed in C3H/He mice after freeze-thaw injury. In contrast, strain BXH-8 was completely resistant to DCC, even after freeze-thaw injury. Under the assumption that DCC requires a predisposing genotype at Dyscalc1, recombination events in strains BXH-6 and -8 allowed us to define a 10-cM interval on proximal chromosome 7 potentially containing the Dyscalc1 gene. This interval was limited by markers D7Mit224 (15.0 cM) and D7Mit230 (24.5 cM) and overlapped with the previously reported 99% confidence interval for Dyscalc1 ranging from D7Mit247 (16.0 cM) to D7Mit82 (25.0 cM) (15). For comparison, Brunnert et al. (2) mapped Dyscalc1 in a 19-cM interval between markers D7Mit57 (4.0 cM) and D7Mit229 (23.0 cM), analyzing freeze-thaw lesions in a backcross of (C57BL/6 $\times$ DBA/2) $\times$ DBA/2 mice.

Proximal chromosome 7 contains many potential candidate genes for Dyscalc1. We discussed some of these in the previous report, focusing mainly on myocyte development and calcium homeostasis (15). Based on the current genetic maps, our data exclude candidate genes proximal of the interval proposed for Dyscalc1, such as the dystrophia myotonica-related genes Dm9 and Dm15 (4.0 cM), the sodium/calcium exchanger Slc8a2 (4.0 cM), muscle creatine kinase Ckmm (4.5 cM), the calmodulin locus Calm (4.5 cM), the osteosarcoma oncogene Fosb (5.0 cM), and the locus for cardiac troponin 1 Tnmi (9.0 cM). Using radiation hybrid mapping, additional candidate genes with unknown or uncertain mapping position will be excluded in the future. Several candidate genes map within the 99% confidence interval. The histidine-rich calcium binding protein, Hrc (20.4 cM), a sarcomplasmic reticulum protein, remains particularly attractive, and the recent cloning of murine Hrc may help to clarify its role for DCC (2, 12, 13, 24, 29). The bcl-2 associated X-protein, Bax (23.0 cM), determines survival or death following an apoptotic stimulus (22). At present, it is not clear whether calcification is a specific form of apoptosis induced by sublethal injury. Bone morphogenetic proteins have been implicated in the regulation of bone formation (31). Therefore, potential candidate genes for Dyscalc2 also include the bone morphogenetic protein-8a, Bmp8a (57.4 cM), on chromosome 4 and, for Dyscalc4, the bone morphogenetic protein 4, Bmp4 (15.0 cM), on chromosome 14. However, no obvious candidate gene for Dyscalc3 was found on chromosome 12.

Based on results reported previously and other evidence from literature, we suggested a pathogenetic model for DCC in which Dyscalc1 enhanced the susceptibility for irreversible injury and led subsequently to calcium deposition in necrotic tissue (15). Now we presented evidence for modifier genes in susceptible C3H/HeJ strain and even in resistant C57BL/6J strain backgrounds. At least one of them, Dyscalc2, enhanced the influence of Dyscalc1 considerably. Examining BXH RI strains, we proposed a chromosomal region containing potential candidate genes for Dyscalc1, which have to be tested by additional biochemical analyses and by the isolation and mapping of Dyscalc loci in congenic mouse models. The dissection of the genetic basis of DCC may improve our understanding of various calcifying cardiac diseases including infarction-associated myocardial calcification in humans, which shows striking ultrastructural similarity to DCC (19).

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