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Association of Variants in *BAG3* With Cardiomyopathy Outcomes in African American Individuals

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IMPORTANCE The prevalence of nonischemic dilated cardiomyopathy (DCM) is greater in individuals of African ancestry than in individuals of European ancestry. However, little is known about whether the difference in prevalence or outcomes is associated with functional genetic variants.

OBJECTIVE We hypothesized that Bcl2-associated anthanogene 3 (*BAG3*) genetic variants were associated with outcomes in individuals of African ancestry with DCM.

DESIGN This multicohort study of the *BAG3* genotype in patients of African ancestry with dilated cardiomyopathy uses DNA obtained from African American individuals enrolled in 3 clinical studies: the Genetic Risk Assessment of African Americans With Heart Failure (GRAHF) study; the Intervention in Myocarditis and Acute Cardiomyopathy Trial-2 (IMAC-2) study; and the Genetic Risk Assessment of Cardiac Events (GRACE) study. Samples of DNA were also acquired from the left ventricular myocardium of patients of African ancestry who underwent heart transplant at the University of Colorado and University of Pittsburgh.

MAIN OUTCOMES AND MEASURES The primary end points were the prevalence of *BAG3* mutations in African American individuals and event-free survival in participants harboring functional *BAG3* mutations.

RESULTS Four *BAG3* genetic variants were identified; these were expressed in 42 of 402 African American individuals (10.4%) with nonischemic heart failure and 9 of 107 African American individuals (8.4%) with ischemic heart failure but were not present in a reference population of European ancestry ($P < .001$). The variants included 2 nonsynonymous single-nucleotide variants; 1 three-nucleotide in-frame insertion; and 2 single-nucleotide variants that were linked in *cis*. The presence of *BAG3* variants was associated with a nearly 2-fold (hazard ratio, 1.97 [95% CI, 1.19-3.24]; $P = .01$) increase in cardiac events in carriers compared with noncarriers. Transfection of transformed adult human ventricular myocytes with plasmids expressing the 4 variants demonstrated that each variant caused an increase in apoptosis and a decrease in autophagy when samples were subjected to the stress of hypoxia-reoxygenation.

CONCLUSIONS AND RELEVANCE This study demonstrates that genetic variants in *BAG3* found almost exclusively in individuals of African ancestry were not causative of disease but were associated with a negative outcome in patients with a dilated cardiomyopathy through modulation of the function of *BAG3*. The results emphasize the importance of biological differences in causing phenotypic variance across diverse patient populations, the need to include diverse populations in genetic cohorts, and the importance of determining the pathogenicity of genetic variants.

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Supplemental content

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Heart failure (HF) secondary to dilated cardiomyopathy (DCM) affects an estimated 2.5 million Americans 20 years or older.[1] The epidemiology of DCM differs by race and ethnicity, with American individuals of African ancestry having the highest incidence and prevalence of HF and a preponderance of idiopathic dilated cardiomyopathy (IDC).[2,3] This contrasts with American individuals of European ancestry, who most commonly have DCM secondary to ischemic heart disease.[4-7] Consistent with the epidemiology of IDC in American individuals of African ancestry, IDC is far more common and the age at which the disease is first recognized is substantially lower in sub-Saharan Africa than in the United States or in Europe.[8,9]

The increased prevalence of DCM in US people of African ancestry has been attributed to a diverse set of medical and sociologic factors, including neighborhood,[10] and a higher burden of cardiovascular risk factors, including diabetes mellitus, hypertension, cholesterol, smoking status, and ventricular hypertrophy.[11] Several observations, however, suggest that the increased incidence of IDC may also be attributed to genetic risk factors. For example, HF hot spots are found in geographic regions across sub-Saharan Africa.[12] The presence of hypertension does not correlate strongly with DCM in sub-Saharan African individuals.[13] Truncating variants in *TTN*, the gene most commonly associated with DCM,[14] are more prevalent in women of African ancestry with postpartum cardiomyopathy than in women of European ancestry with postpartum cardiomyopathy.[15]

Genetic variants in more than 40 genes have been linked with DCM. One such gene encodes Bcl-2-associated athanogene 3 (*BAG3*), an evolutionarily conserved protein that is expressed predominantly in the heart and skeletal muscle and in many cancers.[16] The BAG3 protein has pleotropic effects in the heart[17,18] and has emerged as a major DCM locus.[19] Several observations suggest that *BAG3* mutations might be prevalent in African American individuals.[20] The Genome Aggregation Database (gnomAD) shows marked differences between the allele frequency of *BAG3* variants found commonly in individuals of African ancestry when compared with the allele frequency of *BAG3* variants in individuals of European ancestry.[21]

To investigate whether *BAG3* variants contribute to either the increased prevalence or the clinical outcomes of IDC in African American individuals, we sequenced *BAG3* in DNA samples obtained from participants who were enrolled in 1 of 3 clinical trials.

Key Points

Question Are functional variants in Bcl2-associated anthanogene (*BAG*) found in individuals of African ancestry with dilated cardiomyopathy, and are they associated with specific outcomes?

Findings This cohort study demonstrates the presence of unique genetic variants in *BAG3* found almost exclusively in African American individuals that are associated with a nearly 2-fold greater risk of having an adverse cardiovascular outcome. In addition, this study assesses the pathogenicity of genetic variants and their association with protein levels in the human heart.

Meaning Per this analysis, mutations in *BAG3* may play an important role in cardiovascular pathobiology in individuals of African ancestry.

Methods

The primary objective of this study was to determine via a retrospective analysis whether genetic variants in *BAG3* found in individuals of African ancestry were associated with either the prevalence of the disease or disease outcome in patients with heart failure because of reduced ejection fraction (HFrEF). The study began with an exploratory assessment of *BAG3* variants in patients enrolled in the Genetic Risk Assessment of African Americans With Heart Failure (GRAHF) trial. Subsequent analysis in African American individuals who participated in 2 additional HFrEF trials provided a larger population with which to evaluate the study end points. A secondary objective of the study was to determine the pathogenicity of the variants that were identified.

Patients

Genomic DNA was obtained from 509 African American individuals with DCM enrolled in 3 independent US studies: 342 patients from the GRAHF trial[22-25]; 109 patients from the Intervention in Myocarditis and Acute Cardiomyopathy Trial-2 (IMAC-2) study[26]; and 58 patients from the Genetic Risk Assessment of Cardiac Events (GRACE) study.[27,28] Patients were excluded from analysis if they had acute myocarditis, peripartum cardiomyopathy, or any potentially reversible cause for DCM.[29] Patients in the GRAHF and IMAC-2 studies were followed up to an end point of death or HF hospitalization adjudicated by an end point committee. Participants in the GRACE study were followed up to the end point of heart transplant or death. Samples of DNA were also acquired from the left ventricular (LV) myocardium of participants of African ancestry who underwent heart transplantation at the University of Colorado (n = 15) or at the University of Pittsburgh (n = 31). Nonfailing human heart tissue that could not be used for transplant served as nonfailing control samples.[30]

Written informed consent was obtained from all patients (or appropriate family members) who contributed DNA or tissue to the aforementioned study repositories. The study protocols were approved by the institutional review board at each of the participating institutions.

We used 3 reference populations. First, we sequenced DNA from individuals of African ancestry with ischemic cardiomyopathy who were enrolled in the GRAHF, IMAC-2, and GRACE studies (eTable 1 in the Supplement). Second, we obtained *BAG3* sequence data from a cohort of 359 individuals of European ancestry with both familial and sporadic DCM, collected at the Brigham and Women's Hospital, Boston, Massachusetts, from clinics throughout the United States.[31] Third, population genetic data from gnomAD were accessed online, including data from 123 136 exome sequences and 15 496 whole-genome sequences from unrelated individuals. Of these, 7509 whole genomes and

55 860 exomes are from individuals of non-Finnish European ancestry, and 4368 whole genomes and 7652 exome sequences are from individuals of African ancestry.[21]

DNA Sequencing and Analyses

The DNA samples from the GRAHF trial were sequenced in the Genetics Resources Core Facility at the McKusick-Nathans Institute of Genetic Medicine at the Johns Hopkins University School of Medicine, as described in detail in the eMethods in the Supplement. Targeted genotyping was performed to confirm the results of the GRAHF cohort and to identify single-nucleotide variants (SNVs) in each of the subsequent cohorts using real-time polymerase chain reaction (PCR) and single-nucleotide polymorphism (SNP)-specific reagents (eMethods in the Supplement). The SNVs were chosen for confirmatory genotyping and functional analysis if they (1) had an allele frequency of more than 0.005 in the GRAHF study, (2) were nonsynonymous, and (3) were more common in participants of African ancestry than in those of European ancestry. To determine whether 2 *BAG3* SNVs in the same sample were arranged in *cis*, the *BAG3* locus was amplified, cloned into a plasmid, and subjected to Sanger sequencing, as described in the eMethods and eFigure 1 in the Supplement.

Western Blot Analysis and Quantitative PCR of Human Heart Tissue

Protein levels of BAG3 in human failing heart were quantified by Western blot and messenger RNA (mRNA) *BAG3* levels were determined by quantitative PCR. This approach has been described previously[32] and in the eMethods in the Supplement.

Measurement of Autophagy and Apoptosis in AC16 Cells

Autophagy was measured in transformed human ventricular cells (AC16) that were transfected with plasmids containing either wild-type *BAG3* or a *BAG3* variant (eTable 2 in the Supplement) and cotransfected with the adenovirus-red fluorescent protein-green fluorescent protein-microtubule–associated protein 1A/1B light chain 3 (Adv-RFP-GFP-LC3) reporter construct, as described in detail in the eMethods in the Supplement and in previous studies.[18,33] In a second group of experiments, cells were stained for apoptosis with Annexin-V (Dead Cell Apoptosis kit with AnnexinV Alexa fluoro 488 and propidium iodide; Thermofisher Scientific) and propidium iodide, as described previously[34] and in the eMethods in the Supplement.

Measurement of Autophagy in Adult Myocytes Isolated From cBAG3$^{+/-}$ Mice

Cardiac myocytes were isolated from the septum and left ventricular (LV) free wall of male mice aged 10 to 12 weeks who were heterozygotic for the murine version of the constitutive (c) deletion of *BAG3* (*cBAG3*$^{+/-}$). These cells were infected with an adenovirus containing *BAG3*$^{\text{wild-type}}$ or *BAG3*$^{p.P63A+P380S}$, a *BAG3* variant having 2 heterozygous SNVs that result in substitution of an alanine for a proline at amino acid 63 and substitution of a serine for a proline at amino acid 380 and an autophagy reporter construct, as described previously[18,33] and in the eMethods in the Supplement.

Physiological Associations of a *BAG3* Mutation on Left Ventricular Function in *cBAG3*$^{+-}$ Mice

To confirm the pathogenicity of the p.P63A+P380S variant in vivo, we generated an adeno-associated virus serotype 9-BAG3$^{p.P63A+P380S}$ and injected 1×10^{12} particles into the retroorbital plexus of *cBAG3*$^{+/-}$ and *cBAG3*$^{+/+}$ mice, as described previously.[33,35] Control mice were injected with preparations that included adeno-associated virus 9-green fluorescent protein and adeno-associated virus 9-*BAG3*$^{\text{wild-type}}$. Mice were examined on follow-up with echocardiography for 6 weeks, as described previously and in the eMethods in the Supplement.[36]

Statistical Analysis

Statistical analyses were conducted based on stratification or partitioning by *BAG3* SNV (SNV vs no SNV), ischemia (ischemic patients vs nonischemic patients), and the concomitant interaction. Continuous variables were assessed using the *t* test or analysis of variance as appropriate, and categorical variables were assessed using χ^2 or Fisher exact tests. For the time-to-event analyses, an event was defined as death, transplant, or HF-associated hospitalization. Survival was assessed using the Kaplan-Meier method; the resulting curves were assessed using the log-rank test. Hazard ratios were estimated using Cox proportional-hazards models. All statistical analyses were conducted using SAS version 9.4 (SAS Institute). Statistical significance was defined as $P < .05$. All reported *P* values are 2-sided.

Results

BAG3 Genetic Variants

Sanger sequencing of the GRAHF study cohort revealed 18 variants (eTable 3 in the Supplement), 8 of which were synonymous. Four SNVs met the criteria for inclusion in this study: p.Pro63Ala (10:121429369 C/G; rs133031999); p.His83Gln (10:151331972; rs151331972); Pro380Ser (10:121436204 C/T; rs144692954); and Ala479Val (10:121436502 C/T, rs34656239). We also identified a 3-nucleotide in-frame insertion that added an alanine to the protein at position 160 (p.Ala160dup;10:121429647 A/AGCG).

A total of 51 participants carried a *BAG3* SNV (10%), and 458 did not (90.0%); this included 42 of 402 individuals (10.4%) with nonischemic heart failure and 9 of 107 individuals (8.4%) with ischemic heart failure. The characteristic demographics of the human cohorts with HF with and without the 4 identified *BAG3* variants were not different, as shown in eTable 4 in the Supplement. Briefly, 30 of 51 individuals with *BAG3* variants (59%) were male, as were 280 of those without *BAG3* variants (61.1%); mean (SD) ages of those with and without the variants were 55.6 (13.4) years and 54.5 (13.6) years, respectively.

Every individual who harbored the p.Pro63Ala variant also carried the p.Pro380Ser variant, and the corresponding allele frequencies for these 2 SNVs in gnomAD were almost identical, suggesting that the 2 SNVs were linked. This was confirmed by Sanger sequencing (eFigure 1 in the Supplement). The linked SNVs are designated as p.Pro63Ala+Pro380Ser.

Table. Frequency of *BAG3* Mutation by Pathology and Data Source

Patients per Study Cohort and Pathology Type	No./Total No. (%)				Prevalence, %	
	Patients With Variant 63/380[a]	Patients With Ala160dup	Patients With His83Gln	Patients With Ala479V	Unadjusted[c]	Adjusted for Multiple Alleles[d]
GRAHF						
Nonischemic	4/255 (1.6)	18/249 (7.2)	9/246 (3.7)	4/255 (1.6)	14.0	11.4
Ischemic	0/87	6/87 (6.90)	2/87 (2.3)	0/87	9.2	9.2
GRACE						
Nonischemic	1/88 (1.1)	5/89 (5.6)	0/89	1/88 (1.1)	7.9	7.9
Ischemic	0/20	0/20	1/20 (5.0)	0/19	5.0	5.0
IMAC-2						
Nonischemic	0/58	3/58 (5.2)	2/58 (3.5)	1/58 (1.7)	10.3	10.3
All studies						
Nonischemic	5/401 (1.3)	26/396 (6.6)	11/393 (2.8)	6/401 (1.5)	12.1	10.5
Ischemic	0/107	6/107 (5.6)	3/107 (2.8)	0/106	8.4	8.4
Total	5/508 (0.98)	32/503 (6.4)	14/500 (2.8)	6/507 (1.2)	11.3	10.0
European American reference data[e]						
Nonischemic	0/359	0/359	0/359	0/359	0	0
gnomAD population data						
Individuals of African ancestry	263/12 015 (2.19)	627/11 682 (5.4)	250/12 015 (2.1)	76/12 019 (0.63)	10.3	9.1
Individuals of European ancestry	9/63 271 (0.01)	0/62 112	5/63 345 (0.01)	0/63 354	0.02	0.02

Abbreviations: gnomAD, the Genome Aggregation Database; GRACE, Genetic Risk Assessment of Cardiac Events; GRAHF, the Genetic Risk Assessment of African Americans With Heart Failure; IMAC-2, Myocarditis and Acute Cardiomyopathy Trial-2.

[a] 63/380 is the double heterozygous p.Pro63Ala+p.Pro380Ser *BAG3* variant in which both variants are linked in *cis*.

[b] The percentage of individuals with a given SNV was calculated as the number of individuals with the SNV divided by the number sequenced; not all individuals could be sequenced for every SNV, so the denominator varied by SNV and cohort.

[c] Calculated as the sum of 4 percentages.

[d] Calculated as the total number of individuals with 1 or more SNV, divided by the total number of individuals in that cohort.

[e] These reference data are from the Brigham and Women's Hospital data set.

As seen in eFigure 2 in the Supplement, the amino acids (His83, Ala160, Pro380, and Ala479) affected in the *BAG3* variants are highly conserved across mammals.

The prevalence of the 4 *BAG3* variants in the 402 patients with IDC in the cohorts from the GRAHF, IMAC-2, and GRACE studies (n = 42; 10.4%) was not greater than the prevalence of the 4 variants in patients with ischemic HF (n = 9 of 107; 8.41%) when adjusted for multiple alleles (**Table**). Similarly, the proportion of patients in these 3 cohorts with 1 of the 4 *BAG3* variants was not significantly different than the proportion of patients of African ancestry with a *BAG3* variant in the sum of the corresponding gnomAD data set for each variant (9.06%). In contrast, the proportion of patients in the 3 cohorts who harbored a *BAG3* variant (10%) was significantly higher than the prevalence of the 4 *BAG3* variants among more than 60 000 individuals of European ancestry in the gnomAD European data set (0.02%; $P < .001$) and was significantly greater than that of a reference population (collected at the Brigham and Women's Hospital) of 359 individuals with European ancestry and IDC, of whom 0 individuals had *BAG3* variants ($P < .001$).

Association of *BAG3* Variants With Heart Failure Outcomes

We next sought to determine whether the presence of any 1 of the 4 *BAG3* variants was associated with a worse outcome, as reflected by the combined outcome variable of an HF hospitalization, heart transplant, or death. As seen in **Figure 1**A, when compared with patients with HF with either ischemic or nonischemic disease who did not carry 1 of the 4 *BAG3* variants (n = 458), patients who had a *BAG3* variant (n = 51) had a significantly (83 events in 458 patients [18.1%] vs 15 events in 51 patients [29.4%]; $P = .01$) higher incidence of an adverse event. Similarly, individuals with nonischemic HF who carried a *BAG3* variant had a worse outcome compared with patients with nonischemic disease who did not have a *BAG3* variant (Figure 1B; 11 events in 42 patients [26.1%]; $P = .02$). In patients with ischemic HF and a *BAG3* SNV, outcomes were not significantly worse (Figure 1C); 4 of the 98 patients without a mutation experienced an event (4.1%), while 4 of the 9 patients with a mutation had an event (44.4%; $P = .18$).

Using a 2-variable Cox proportional hazard analysis (*BAG3* variant and ischemic HF), we determined that the risk of a carrier of 1 of the 4 *BAG3* variants having an adverse event was 1.97 times higher than for an individual with HF who did not have a *BAG3* variant (hazard ratio, 1.97 [95% CI, 1.19-3.24]; $P = .01$), and that the risk of an individual with ischemic HF having an adverse event was 1.76 times higher than the risk of an individual with nonischemic HF (hazard ratio, 1.76 [95% CI, 1.18-2.60]; $P = .01$). Based on additional

modeling using a 3-term Cox proportional hazard analysis (*BAG3* variant, ischemic HF, and the interaction between these 2 variables), the interaction between the *BAG3* variant and ischemic HF variables was not statistically significant.

BAG3 Levels in Failing Human Heart

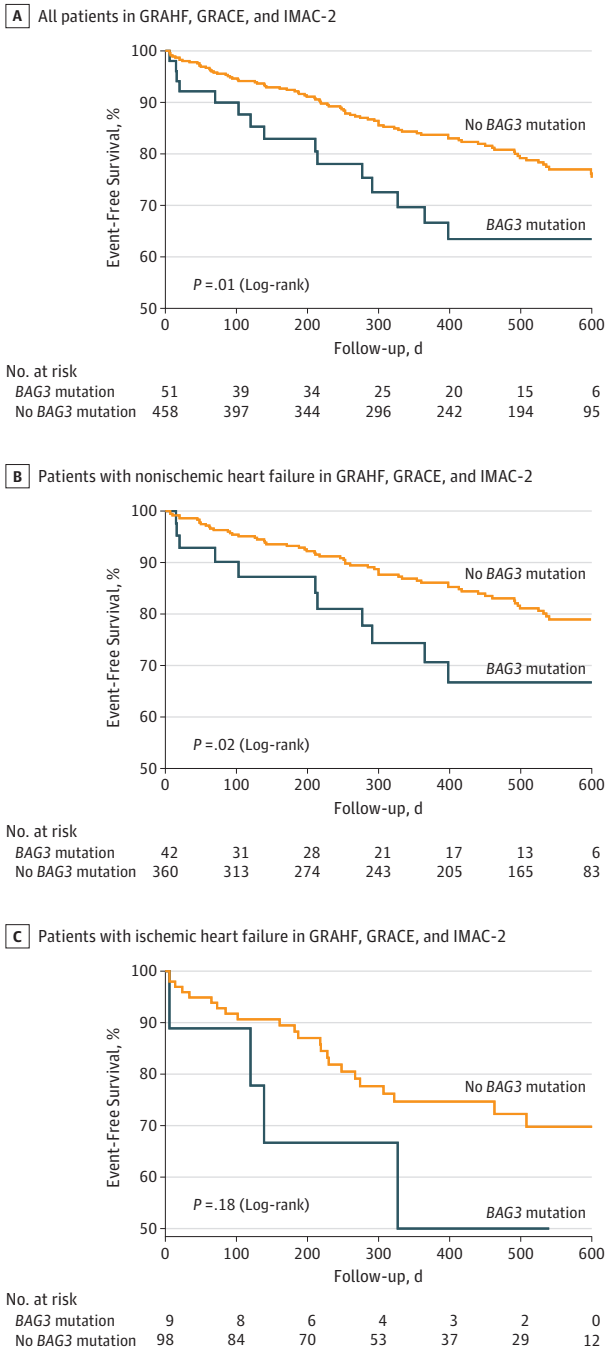
Western blot analysis of BAG3 protein levels and quantitative PCR to quantify *BAG3* mRNA were performed in failing human hearts from participants of African ancestry and nonfailing hearts from control participants of African ancestry (cohorts from the University of Colorado and University of Pittsburgh). As we reported previously in a cohort of individuals of largely European ancestry,[32] *BAG3* levels were significantly reduced in hearts extracted from transplant recipients who had IDC (n = 23; $P = .001$) or ischemic HF (n = 16; $P < .001$) compared with control participants with nonfailing hearts (n = 4; **Figure 2**A and B). By contrast, *BAG3* mRNA levels from the hearts of patients with IDC (n = 18) and from the hearts of patients with ischemic DCM (n = 13) were not different from those of control participants (n = 4; Figure 2C). The levels of BAG3 protein were higher than normal in the 2 hearts of patients with p.Pro63Ala+Pro380Ser variants and were unchanged in the heart of the patient with a p.His83Gln variant. By contrast, the level of BAG3 protein in the heart of a patient harboring the p.Ala160dup variant was comparable with levels seen in the patients without *BAG3* variants.

Pathogenicity of *BAG3* Variants

We next undertook studies to assess the association of *BAG3* variants with the 2 primary actions of BAG3 protein in the cell: autophagy and apoptosis. As seen in **Figure 3**A and eFigure 3 in the Supplement, when AC16 cells were transfected with either the empty plasmid or with wild-type *BAG3*, there was a significant increase in autophagy in response to hypoxia-reoxygenation; however, the autophagy response to hypoxia-reoxygenation was significantly diminished in AC16 cells that overexpress each of the 4 *BAG3* variants, because there was no significant increase in the LC3 reporter construct (Figure 3A and eFigure 3 in the Supplement). In addition, after hypoxia-reoxygenation, cells transfected with *BAG3* variants showed significantly more Annexin-V–positive cells than did cells transfected with a plasmid expressing wild-type *BAG3* (Figure 3B and eFigure 3 in the Supplement). Thus, by contrast with wild-type *BAG3*, the *BAG3* variants were unable to impede hypoxia-induced apoptosis. To demonstrate that the measurement of autophagy in the presence of the different *BAG3* variants was not compromised by variable levels of expression of each variant, the plasmids containing wild-type *BAG3* and each *BAG3* variant were transduced into AC16 cells. Each *BAG3* variant was tagged with myc, which allowed endogenous *BAG3* to be separated from transduced *BAG3* by assessing the levels of myc. The levels of myc appeared comparable across all plasmids (eFigure 4 in the Supplement).

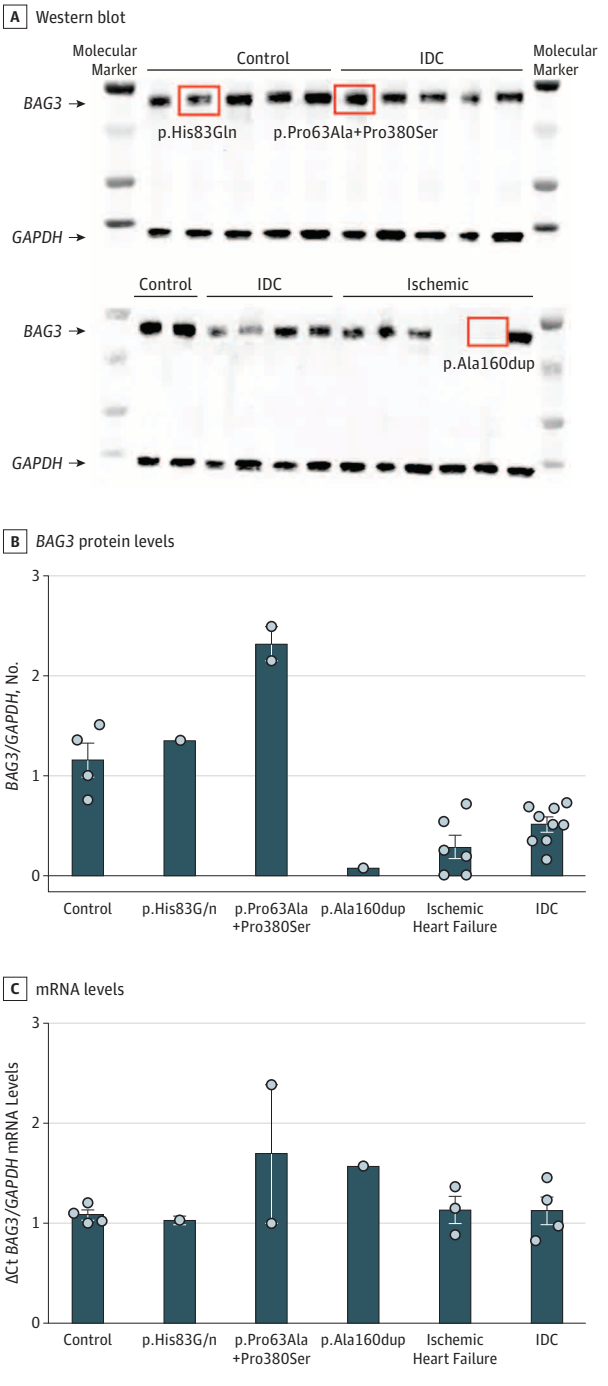
Because AC16 cells are transformed and thus might not adequately represent cardiac myocytes, we confirmed these findings using adult myocytes isolated from *cBAG3*$^{+/-}$ mice. When lysosome-autophagasome fusion was inhibited by the addition of bafilomycin A1, there was a marked increase in total LC3 reporter construct in *cBAG3*$^{+/-}$ myocytes transfected with Adenovirus-*BAG3*$^{wild\text{-}type}$ (Adv-*BAG3*$^{wild\text{-}type}$; eFigure 5 in the Supplement), suggesting that autophagy was occurring. By contrast, transfection

Figure 1. Kaplan-Meier Curves Showing Event-Free Survival in Patients With or Without a *BAG3* Genetic Variant



Kaplan-Meier curves show event-free survival in all patients (A), patients with nonischemic heart failure (B), and patients with idiopathic dilated cardiomyopathy (C), with or without a BAG3 variant. GRACE indicates the Genetic Risk Assessment of Cardiac Events trial; GRAHF, the Genetic Risk Assessment of African Americans With Heart Failure trial; IMAC-2, Myocarditis and Acute Cardiomyopathy Trial-2.

Figure 2. Levels of *BAG3* Protein and Messenger RNA (mRNA) in Failing Human Hearts



A, A representative Western blot of protein isolated from human hearts with severe left ventricular dysfunction secondary to idiopathic dilated cardiomyopathy (IDC; n = 23) or ischemic heart disease (n = 16), contrasted with control participants with nonfailing hearts (n = 4). Samples obtained from hearts that were found to carry a *BAG3* variant are indicated by the red squares. The unlabeled lanes represent molecular weight markers. B, Quantification of multiple Western blots. C, Quantification of quantitative polymerase chain reaction assessment of mRNA levels in a subset of the same hearts (non-IDC, n = 13; IDC, n = 18; and nonfailing human hearts from control participants, n = 4). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control for both the Western blot and the quantitative polymerase chain reaction.

with Adv-*BAG3*$^{P63A+P380S}$ had no significant effect on LC3 reporter construct levels, suggesting that the variant could not support autophagy. In the heterozygous *BAG3* knockout mouse, autophagasome production is so low that blockade of autophagasome-lysosome fusion is associated with little change in the abundance of LC3, whereas restitution of normal *BAG3* levels increases the amount of autophagy.

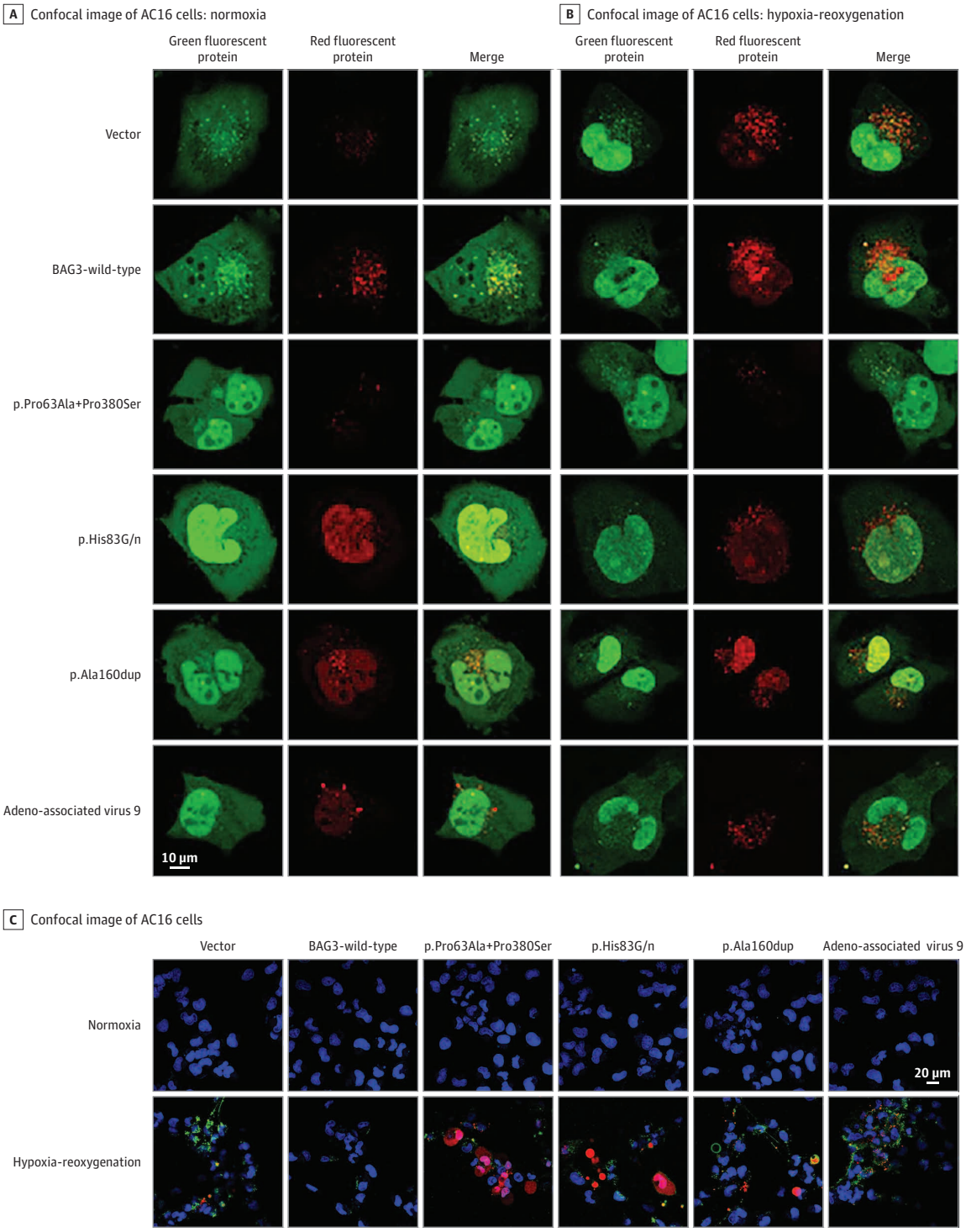
When the adult *cBAG3*$^{+/-}$ myocytes were stained for apoptosis, there were significantly fewer apoptotic cells when the cells were infected with Adv-*BAG3*$^{wild\text{-}type}$ than when the cells were infected with either Adv-null (an empty vector) or Adv-BAG3$^{p.P380A+P380S}$ (eFigure 6 in the Supplement). By contrast with AC16 cells, we did not use hypoxia and reoxygenation to induce apoptosis in the *cBAG3*$^{+/-}$ cells, because under the culture conditions used, the loss of a single *BAG3* allele led to a marked intolerance to even modest amounts of hypoxia, thereby making it difficult to find live cells after this process was initiated.

To further confirm the findings in AC16 cells, we administered adeno-associated virus 9 (rAAV9)-*BAG3*$^{wild\text{-}type}$ or rAAV9-green fluorescent protein (GFP) by retroorbital injection to *cBAG3*$^{+/-}$ mice aged 8 to 10 weeks. As shown in **Figure 4**A, infection with rAAV9-*BAG3*$^{wild\text{-}type}$ did not alter the ejection fraction (EF) of mice homozygous for *cBAG3* (n = 3) with a normal complement of *BAG3* (mean [SD] EF, 76.3% [9.5%]) compared with mice transfected with rAAV9-GFP (71.0% [6.1%]). However, consistent with results in AC16 cells, administration of rAAV9-BAG3$^{p.P63A+p.P380S}$ significantly (P = .05) reduced the EF (mean [SD] EF, 49.7% [4.4%]) compared with the outcomes of AAV9-GFP or AAV9-BAG3$^{wild\text{-}type}$, suggesting that the p.P63A+p.P380S variant has a dominant-negative effect. By contrast, in mice with haploinsufficiency of *BAG3*, mice with wild-type *BAG3* (n = 4) improved left ventricular function (mean [SD] function, 59.4% [2.2%]) when compared with mice in the rAAV9-GFP group (n = 4; 50.5% [4.6%]). Consistent with results in mice with a full complement of *BAG3*, infection with rAAV9-BAG3$^{p.P63A+p.P380S}$ reduced the EF in mice who were heterozygous for *cBAG3* (n = 4; mean [SD] EF, 38.6% [5.1%]; P = .05) compared with mice who were heterozygous for *cBAG3* who were infected with rAAV9-*BAG3*$^{wild\text{-}type}$. In aggregate, these results were consistent with the observations in AC16 cells. Furthermore, these results could not be explained by differences in the level of *BAG3* expression that were comparable across all groups (eFigure 7 in the Supplement).

Discussion

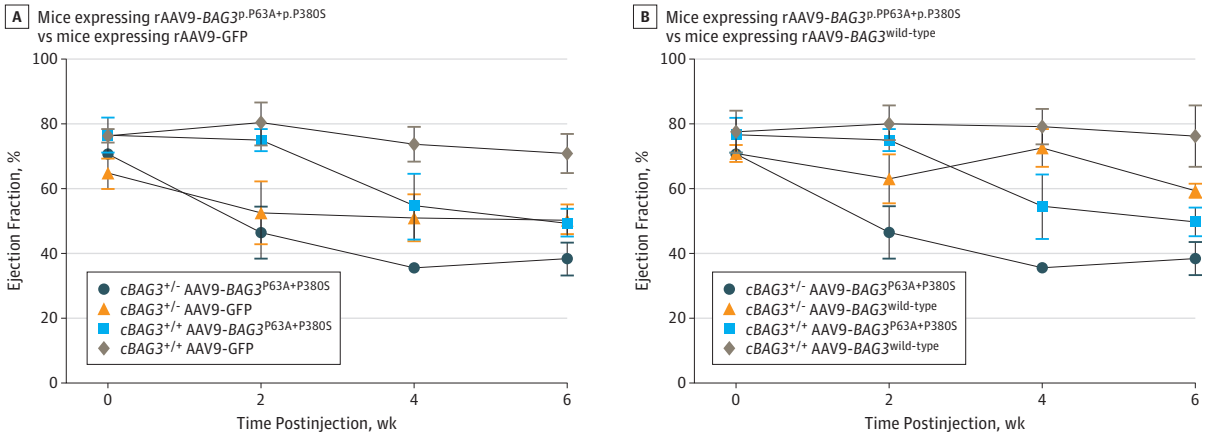
BAG3 is an evolutionarily conserved protein that is expressed largely in the heart and the skeletal muscle.[37] Its importance lies in its regulation of important cellular functions, including protein quality control, apoptosis, and excitation/contraction coupling.[17] Genetic variants in *BAG3*, including deletions, truncations, and SNVs, have met the criteria for causation of familial DCM.[38] The levels of *BAG3* are reduced in families with DCM and *BAG3* truncations or deletions[32,39] and individuals with nonfamilial end-stage HF.[32,39] The levels of *BAG3*

Figure 3. Confocal Images of Autophagy Flux and Apoptosis in AC16 Cells



Representative confocal images from AC16 cells that were transfected with an empty plasmid or one containing wild-type *BAG3* or c.187C→G+c.1138C→T (p.Pro63Ala + p.Pro380Ser), c.249C→A (p.His83Gln), c.474_476dupGGC (p.Ala160dup), or c.1436C→T (p.Ala479Val) and then cotransfected with adenovirus-red fluorescent protein-green fluorescent protein-microtubule--associated protein 1A/1B light chain 3. A, Red puncta represent increased LC3 in autophagolysosomes in which green fluorescent protein has been quenched by the increased acidity after lysosomal-autophagasome fusion. B, Hypoxia-reoxygenation increased autophagy in cells with empty vectors or wild-type *BAG3*, as seen by an increase in yellow-red LC3 puncta; expression of any *BAG3* variants resulted in impaired autophagy response and a diminished increase in total LC3 puncta compared with wild-type *BAG3*. C, A representative confocal image of AC16 cells stained with Annexin-V (green) and propidium iodide (red) to distinguish apoptotic cells (green), late apoptotic and necrotic cells (red), and nonviable cells (green and red). Few apoptotic cells are seen in AC16 cells transfected with wild-type *BAG3*; there was a marked increase in apoptotic cells in the presence of an empty vector or any *BAG3* variant.

Figure 4. Hemodynamic Effects of Infection With rAAV9-BAG3 vs rAAV9-BAG3$^{p.P63A+P380S}$ in Mice With BAG3 Haploinsufficiency (cBAG3$^{+/-}$) or in Nontransgenic Wild-Type Mice (cBAG3$^{+/+}$)



cBAG3$^{+/-}$ mice aged 4 to 6 weeks received either a retro-orbital injection of 1x10^{12} particles of adeno-associated virus 9 (rAAV9)–*BAG3*$^{p.P63A+P380S}$, rAAV9-green fluorescence protein (GFP) or rAAV9-*BAG3*$^{wild\text{-}type}$ and were then given echocardiograms every other week. In the same experiment, 6-week-old *cBAG3*$^{+/+}$ mice received an injection of either rAAV9-*BAG3*$^{p.P63A+P380S}$, rAAV9-GFP, or rAAV9-*BAG3*$^{wild\text{-}type}$. After 6 weeks, left ventricular myocardium was harvested for subsequent analysis. A, A comparison of serial measures of left ventricular ejection fraction by echocardiogram in mice infected with the rAAV9-*BAG3*$^{p.P63A+P380S}$ (*cBAG3*$^{+/+}$: n = 4; *cBAG3*$^{+/-}$: n = 3) with mice infected with rAAV9-GFP (*cBAG3*$^{+/+}$: n = 8; *cBAG3*$^{+/-}$: n = 4) shows statistical significant differences at 2, 4, and 6 weeks (P = .01). B, A comparison of mice infected with the rAAV9-*BAG3*$^{p.P63A+P380S}$ (*cBAG3*$^{+/+}$: n = 4; *cBAG3*$^{+/-}$: n = 3) with mice expressing rAAV9-*BAG3*$^{wild\text{-}type}$ (*cBAG3*$^{+/+}$: n = 4; *cBAG3*$^{+/-}$: n = 5) shows statistically significant differences at 4 and 6 weeks (P = .05).

are also reduced in mice with haploinsufficiency of *BAG3*[18] and in animal models of LV dysfunction.[17,35] In the present study, we identified a group of relatively common genetic variants (presented in more than 1% of individuals); 2 nonsynonymous SNVs, a 3-nucleotide in-frame insertion, and 2 linked SNVs. The 4 variants were found equally in African American individuals with nonischemic DCM and ischemic DCM, as well as in African American individuals without known cardiovascular disease. However, these variants were largely absent in individuals of European ancestry with or without HF. Most importantly, the presence of any 1 of the 4 variants was associated with a nearly 2-fold increase in the risk of death or HF hospitalization. Thus, while they were not a disease-initiating factor, these variants were significantly associated with modified response to disease.

Although it is axiomatic that large deletions or truncations of a protein will significantly alter the protein's function, the physiologic effect of SNVs is often far less obvious. That the 4 variants have functional significance in the heart was demonstrated by the fact that each altered both autophagy and apoptosis when transfected into AC16 cells. The relevance of the changes in the AC16 cells was supported by the finding that the variant that combines p.Pro63Ala with p.Pro380Ser was unable to support LV function in mice with haploinsufficiency of BAG3 as evidenced by a significant decrease in the EF in adult myocytes isolated from *cBAG3*$^{+/+}$ and *cBAG3*$^{+/-}$ mice that were infected withrAAV9-*BAG3*$^{p.P63A+p.P380S}$. Fang et al[40] have recently shown that when a human SNV (*E455K*) is knocked into mice, the resulting progeny have a loss of *BAG3* function and develop dilated cardiomyopathy; however, they only found this phenotype in mice with homozygosity.

Interestingly, the 4 variants identified in this study were annotated by ClinVar[41] as benign (p.Pro63Ala), benign or likely benign (p.Ala160dup; p.Pro380Ser), or with indeterminate pathogenicity (p.His83Gln and p.Ala479Val). The finding that each of these is pathogenic when evaluated in a biologic system emphasizes the lack of specificity of in silico pathogenicity prediction algorithms and the use of enhanced software and computational predictions in determining pathogenicity and providing annotation for SNVs.[42]

The variants identified in the present study have not been recognized previously in cohorts of probands with familial DCM. This is likely because of the paucity of African American individuals in genetic studies of HF. For example, in 4 studies[20,38,43,44] that identified *BAG3* variants in independent index cases with familial DCM, fewer than 16 participants in aggregate were identified as individuals of African ancestry. In a large exome-wide array-based association study, investigators identified a *BAG3* locus (c.451T→C, p.Cys151Arg) that conferred a reduced risk of DCM. Although we found the same variant in this study population, the variant is very common in populations of European ancestry (0.2163 allele frequency) and African ancestry (0.03 allele frequency) and therefore did not meet the criteria for inclusion in the present analysis.[31] The absence of racial/ethnic minority participants in genetic studies is an important disparity in cardiovascular research that needs to be addressed.[45]

Conclusions

In conclusion, unique variants in *BAG3* found almost exclusively in individuals of African descent were not associated with

the onset of DCM but were associated with a negative influence on the phenotypic response to the development of DCM and worse outcomes of patients with both ischemic and nonischemic disease. This study raises several points that are relevant to our understanding of the genetics of HF. First, biological differences should be considered along with environmental and social factors as important in understanding phenotypic differences across diverse patient populations.[46] Second, we cannot fully understand population-based differences without enhancing the diversity of the populations that are included in genomic studies.[45,47,48] Third, evidence supporting pathogenicity of these SNVs would be strengthened by a prospective study and an understanding of possible familial recurrence and/or segregation. Finally, in the era of big data, it is important to sometimes take a reductionist approach until such time as computer algorithms have the same efficacy as biological measurements for ascertaining the functional effects of SNVs.

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Correction: This article was corrected on September 25, 2019, to fix an error in Figure 4. The key for the graph in Figure 4B was left out. The key has been added back in, including the labels *cBAG3*$^{+/-}$AAV9-*BAG3*$^{P63A+P380S}$, *cBAG3*$^{+/-}$AAV9-*BAG3*$^{wild\text{-}type}$, *cBAG3*$^{+/+}$AAV9-*BAG3*$^{P63A+P380S}$, and *cBAG3*$^{+/+}$AAV9-*BAG3*$^{wild\text{-}type}$ for the gray circles, orange triangles, blue squares, and brown diamonds, respectively.

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Supervision: Madesh, Fisher, Kontos, Yancy, Khalili, J. G. Seidman, Feldman.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Dr Feldman reports being a cofounder of Renovacor, Inc, a startup company developing therapeutics that target deficiencies in *BAG3* found in patients with both hereditary and nonhereditary forms of dilated cardiomyopathy. Drs Feldman, Myers, Cheung, Tilley, McClung, and Kontos owns shares in Renovacor Inc. Dr Cheung also has patent 621205,990 pending, and exclusive rights to the patent have been optioned by Temple University to Renovacor Inc. Dr Feldman reports that, through Renovacor Inc, he has a patent pending for a *BAG3* therapy for patients with heart failure, a patent pending for a *BAG3* composition and method, and a patent pending for the role of *BAG3* in ischemia/reperfusion injury. Dr Kontos reports a patent pending (PCT/US2018/012962); serves as chief medical officer (unpaid) for Renovacor Inc; and reports grants from Duke University during the conduct of this study. Dr McClung reports grants from the National Heart Lung and Blood Institute during the conduct of the study and patent PCT/US2018/012962 pending. Dr C. Seidman reports grants from Howard Hughes Medical Institute and the National Institutes of Health. No other disclosures are reported.

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