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Decreased Levels of BAG3 in a Family With a Rare Variant and in Idiopathic Dilated Cardiomyopathy

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The most common cause of dilated cardiomyopathy and heart failure (HF) is ischemic heart disease; however, in a third of all patients the cause remains undefined and patients are diagnosed as having idiopathic dilated cardiomyopathy (IDC). Recent studies suggest that many patients with IDC have a family history of HF and rare genetic variants in over 35 genes have been shown to be causative of disease. We employed whole-exome sequencing to identify the causative variant in a large family with autosomal dominant transmission of dilated cardiomyopathy. Sequencing and subsequent informatics revealed a novel 10-nucleotide deletion in the BCL2-associated athanogene 3 (*BAG3*) gene (Ch10:del 121436332_12143641: del. 1266_1275 [NM 004281]) that segregated with all affected individuals. The deletion predicted a shift in the reading frame with the resultant deletion of 135 amino acids from the C-terminal end of the protein. Consistent with genetic variants in genes encoding other sarcomeric proteins there was a considerable amount of genetic heterogeneity in the affected family members. Interestingly, we also found that the levels of BAG3 protein were significantly reduced in the hearts from unrelated patients with end-stage HF undergoing cardiac transplantation when compared with non-failing controls. Diminished levels of BAG3 protein may be associated with both familial and non-familial forms of dilated cardiomyopathy.

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Heart failure (HF) secondary to systolic dysfunction and cardiac dilatation affects over 5 million individuals in the United States and is an important cause of both morbidity and mortality. Approximately 30% of these patients have non-ischemic disease or idiopathic dilated cardiomyopathy (IDC). Although in the majority of patients with IDC the causative factors have remained undefined, emerging evidence suggests that up to 35% of individuals with IDC have an affected first-degree relative (Jefferies, 2010) and IDC can be associated with genetic abnormalities in 20–35% of individuals—leading to the use of the nomenclature familial dilated cardiomyopathy (FDC) (Judge and Johnson, 2008; Hershberger et al., 2010). Indeed, mutations in more than 30 genes have been identified as causative factors (Hershberger et al., 2009a). And the most common pattern of inheritance is autosomal dominant with reduced penetrance and variable expressivity (Morales and Hershberger, 2013).

Mutations causing FDC are found in genes encoding a wide spectrum of proteins (Chang and Potter, 2005); however, a large number of the mutations that cause FDC occur in genes that encode sarcomere proteins or the complex network of proteins in the Z-disc (Chang and Potter, 2005; Selcen, 2011). Recently, mutations in Bcl-2 associated anthanogene-3 (*BAG3*), a 575 amino acid anti-apoptotic protein that serves as a co-chaperone of the heat shock proteins (HSPs), have also been associated with FDC (Selcen et al., 2009; Odgerel et al., 2010; Lee et al., 2012). For example, Norton et al. (2011) recently identified a deletion of *BAG3* exon 4 as a rare variant causative of FDC in a family without neuropathy or peripheral muscle weakness. Subsequent sequencing of *BAG3* in subjects diagnosed with IDC identified four additional mutations that segregated with all relatives affected by the disease. A genome-wide association study conducted in patients with HF

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secondary to IDC implicated a non-synonymous single nucleotide polymorphism (SNP) (c.757T>C, [p. Cys151Arg]) located within the *BAG3* gene as contributing to sporadic dilated cardiomyopathy (Villard et al., 2011).

In the present study, we identified a novel *BAG3* mutation in a family with adult-onset FDC. Furthermore, we report for the first time that BAG3 protein levels are significantly decreased in unrelated patients with non-familial IDC suggesting that altered levels of BAG3 protein may participate in the progression of HF.

Methods

We identified a family with adult-onset familial dilated cardiomyopathy. After obtaining informed consent, participating family members underwent a physical examination by an HF cardiologist and blood was collected for subsequent DNA analysis. DNA was extracted using a DNA extraction kit (Qiagen, Valencia CA) and stored at −70°C. Whenever possible, electrocardiograms were obtained from affected family members who had not undergone heart transplantation. Family members who had not had a recent echocardiogram, underwent a transthoracic echocardiogram using a SonoHeart Elite (SonoSite, Inc., Bothell, WA) portable echocardiographic system. Medical records were obtained from one individual who had died. Affection status was determined on the basis of consensus guidelines (Mestroni et al., 1999). Participating family members provided written informed consent prior to evaluation and the protocols were approved by the Internal Review Boards of Thomas Jefferson University and of the University of Colorado.

Human heart tissue was obtained from nine subjects unrelated to our study family with end-stage HF undergoing heart transplant at Temple University Hospital (six male, three female, mean age $47.6 + 5.7$ years), from one affected family member at the time of heart transplantation at the University of Colorado and from seven organ donors (one male, six female, mean age 59.3 ± 3.7 years) whose hearts were unsuitable for donation owing to blood type, age or size incompatibility. All of the patients undergoing transplantation had severe left ventricular dysfunction and cardiac dilation with a mean left ventricular ejection fraction (LVEF) of $12.8 + 1.4\%$. Two of the transplant recipients had HF secondary to ischemic cardiomyopathy and the remainder had non-ischemic IDC. Four of the transplant recipients were receiving dobutamine alone, five were receiving milrinone alone and one was receiving both milrinone and dobutamine at the time of the transplantation. Echocardiography was performed on all of the organ donors prior to organ donation and all had normal left ventricular function by echocardiography with a mean LVEF of $57.5 + 1.6\%$. Tissue aliquots were removed from the left ventricular free wall, rapidly frozen in liquid nitrogen and stored at −70°C as described previously (Bristow et al., 1993). The Institutional Review Boards of the University of Colorado and Temple University approved the tissue study and consent was obtained for all subjects.

Exome sequencing and bioinformatics

DNA from five affected family members and one unaffected family member was selected for exome sequencing with a target depth of >100×. Exome enrichment was performed using the Agilent SureSelect Human Exon 51Mb kit (Agilent, Santa Clara, CA). Paired-end 100 nucleotide exome sequencing was performed using an Illumina HiSeq 2000 platform (San Diego, CA). Sequence reads passing *Illumina chastity filter*, were subjected to a quality filter step, trimmed and retained if the trimmed reads for each pair exceeded 50 nucleotides. Paired reads were then mapped to the reference human genome sequence (hg19) with gSNAP (Wu and Nacu, 2010). Sequence calls for variants (single-nucleotide polymorphisms [SNPs)] insertions and deletions [indels]) were performed using the Broad's Genome Analysis Toolkit (McKenna et al., 2010).

After variant detection, the program Annotate Variation (ANNOVAR) was used to classify variants (e.g., exonic, intronic, synonymous, non-synonymous, splice variant, stop gain, stop loss, insertion, or deletion) and to cross reference all the variants across various genetic variation databases (e.g., dbSNP, 1000 genomes database, AVSIFT) to isolate rare variants (variants with mean allele frequencies of <1% not found in dbSNP, 1000 genomes database, aVSIFT) (Wang et al., 2010). Only non-synonymous changes (SNPs and in-dels), those that cause an alternate splice site, and/or an aberrant stop codon, were considered for further analysis. For non-synonymous changes, all insertion and deletion variants were considered damaging, whereas SNP variants were cross-referenced to the dbNSFP database to determine whether the changes to the protein structure would be considered tolerable or damaging using four algorithms (Sorting Intolerant From Tolerant (SIFT), PolyPhen2, *likelihood ratio test* [LRT], or MutationTaster) (Liu et al., 2011). Putative mutations identified were confirmed with traditional Sanger sequencing in both affected and unaffected family members (primers and conditions available upon request).

Western blot analysis of human heart tissue

Frozen tissue was homogenized in 40 mM Tris buffer, pH 7.5 containing 150 mM NaCl, 1% NP40, 1 mM DTT, and 1 mM EDTA. The sample was then centrifuged at 10,000*g* at 4°C for 30 min and the supernatant was collected and re-suspended in 350 μM Tris buffer, pH 6.8 containing 25% beta-mercaptoethanol, 30% glycerol, 10% SDS, and 2% bromophenol blue. The protein concentration was measured using the method of Bradford and the samples were stored at −80°C. Equal amounts of protein (10 μg) were fractionated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked in 10% nonfat dry milk/tris-buffered saline (pH 7.6) plus 0.1% Tween-20 (TBS-T) for 1 h and then incubated with polyclonal BAG3 antibody (Proteintech, Chicago, IL) in 5% nonfat dry milk with PBST for 2 h. Membranes were then incubated with goat-anti-rabbit 800 and goat-anti-mouse secondary antibody for 1 h and scanned on a LI-COR Odyssey imaging system (Lincoln NE). All Western blot procedures were carried out at room temperature. BAG3 signal intensity was normalized to GAPDH.

Results

Family history

The proband (Fig. 1, III-5) was a 65-year-old woman of Eastern European ancestry who was referred in June 2003 to the HF clinic at Thomas Jefferson University because of a family history of HF. She had first been noted to have a dilated cardiomyopathy at 45 years of age. She was largely asymptomatic while receiving a diuretic, a β-adrenergic receptor antagonist (β-blocker) and an angiotensin converting enzyme (ACE) inhibitor, Her vital signs were within normal limits and her physical examination was notable only for a soft S3 heart sound. She had no peripheral muscle weakness and her neurologic examination was unremarkable. Her electrocardiogram revealed normal sinus rhythm with mild LV hypertrophy and non-specific ST-T wave changes. Her LVEF was 20% by echocardiography. As seen in Figure 1 and Table I, the proband had two female siblings, one of whom (III-7) was asymptomatic with a normal physical examination; however, her ejection fraction by echocardiography was 44%. A second

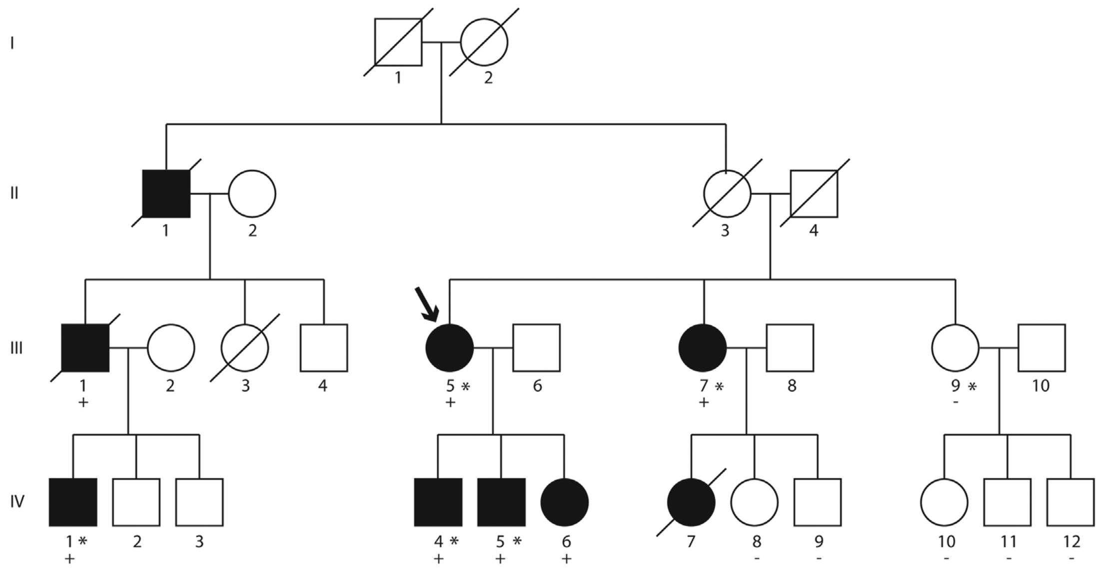

Fig. 1. BAG3-associated dilated cardiomyopathy pedigree. Males are represented by squares. Circles indicate females. Open symbols represent unaffected individuals and black symbols represent affected individuals. The presence or absence of the 10-nucleotide deletion in BAG3 is indicated by either a (+) or a (−), respectively. An arrow denotes the proband. An asterisk is used to denote individuals whose DNA was used for whole exome sequencing. A diagonal line is used to denote individuals who are deceased.

sister (III-9) was phenotypically normal and had a normal echocardiogram.

The proband had three children. A son underwent cardiac transplantation at the age of 20 secondary to IDC (IV-5), a second son was diagnosed with IDC at the age of 20 but remained asymptomatic at age 32 despite an ejection fraction of 33% (IV-4). A daughter had no cardiac symptoms; however, her LVEF by echocardiography was 48% and she had mild dilatation of the left ventricle and the aortic root without obvious aortic valve disease (IV-6). Her echocardiogram met the criteria for diagnosis of a dilated cardiomyopathy. Her electrocardiogram was normal. Neurologic function was normal in all three children. The proband's affected sister (III-7) had one daughter who died of progressive HF secondary to IDC at the age of 22 (IV-7): two other children had normal echocardiograms. A cousin underwent cardiac transplantation because of IDC at 42 years of age after diagnosis at the age of 40 (III-1) and one of his sons also underwent cardiac transplantation for IDC at the University of Colorado at the age of 30 (IV-1). Healthy subjects were defined as "non-affected" if they had reached the age of 40 without symptoms and had a normal echocardiogram that did not meet the criteria for diagnosis of a cardiomyopathy. Ten-year follow-up of all participants demonstrated that functional capacity had remained stable in all family members.

Genetic analysis

As seen in Figure 1, the pedigree and clinical data were compatible with autosomal dominant adult-onset familial IDC. Exome sequencing of the DNA from five affected (III-5, 7: IV-1,4,5) and one unaffected (III-9) family members had an average of 11.8 + 0.96 Gb of past-filter sequence reads per sample. After bioinformatics filtering a 10-nucleotide deletion in the coding portion of exon 4 of *BAG3* (Ch10:del 121436332_12143641: del. 1266_1275 [NM 004281]) was noted to be present in all tested affected subjects and absent in the one healthy sister of the proband (III-9) (Fig. 2) Additional

TABLE I. Phenotype of study subjects with and without a 10-nucleotide deletion in the *BAG3* gene

Subject	Age eval/onset/death or transpl	Gender	EF (%)	ECG	Mutation	Comment
II-1	na/na/70+	M				Died late 1970s, hx of HF
II-3	na/na/80	F				Hx of HBP and CVA
II-4	na/na/29	M				Motor vehicle accident
III-1	62/40/42	M			Yes	Transplant at 42
III-5	65/45/na	F	20	NS-ST-T changes	Yes	
III-7	67/47/na	F	44	nl	Yes	Asymptomatic
III-9	68/na/na	F	58	nl	No	
IV-1	30/30/30	M			Yes	Transplant at 30
IV-4	39/20/na	M	33	Sinus brady, IVCD	Yes	Asymptomatic
IV-5	35/20/20	M			Yes	Transplant at 20
IV-6	34/34/na	F	48	nl	Yes	Mild aortic root dilat, LVDD 5.8
IV-7	na/18/22	F				Died—worsening HF
IV-8	38	F	nl		No	
IV-9	42	M	nl		No	
IV-10	41	F	nl		No	
IV-11	44	M	nl		No	
IV-12	45	M	nl		No	

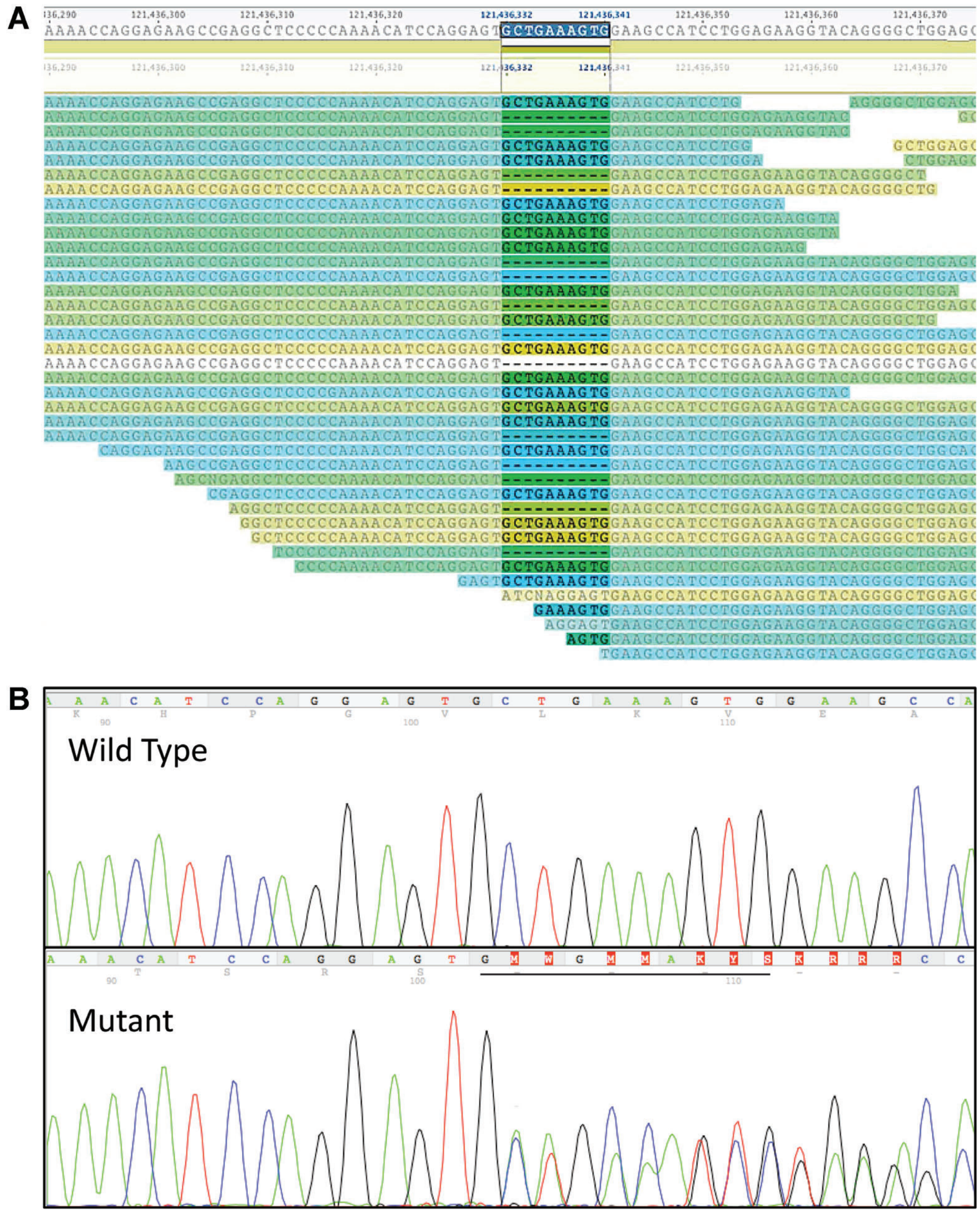

Fig. 2. **A: Sequencing alignment for *BAG3* 10-nucleotide deletion. B: Representative Sanger sequencing shows the deletion in the *BAG3* gene in an affected individual.**

family members were tested for the *BAG3* deletion by Sanger sequencing confirming appropriate co-segregation of the deletion with the phenotype among affected (III-1,5,7 and IV-1,4,5,6) and unaffected (III-9 and IV-8,9,10,11,12) individuals. This deletion was not found in existing databases and introduces a frame shift and premature stop codon after 13 amino acids that predicts truncation of BAG3 at the carboxy terminal end by 140 amino acids. Thus, the abnormal BAG3 protein is predicted to have 435 amino acids instead of 575 amino acids. In addition, the amino acid sequence distal to the deletion (K P S W R R Y R G W S R L) is predicted to be different from that found in the normal protein. Only one additional variant was found by exome sequencing and after bioinformatics filtering. The variant (rs8192669), found in the *IKZF5* gene did not segregate according to the IDC phenotype in other family members. An analysis of 52 genes previously associated with monogenic IDC for rare variants (<1%) identified only non-synonymous mutations in *TTN*, *GATAD1*, *MYPN*, *ANKRD1*, and *RBM20*: none of these variants segregated with the disease phenotype.

BAG3 expression in failing human heart

In order to determine whether the *BAG3* deletion (*BAG3* del. NM_004281) found in this patient cohort resulted in a decrease in the levels of BAG3 protein, Western blot analysis was performed on cardiac muscle obtained from one affected family member (IV-1) who underwent cardiac transplantation.

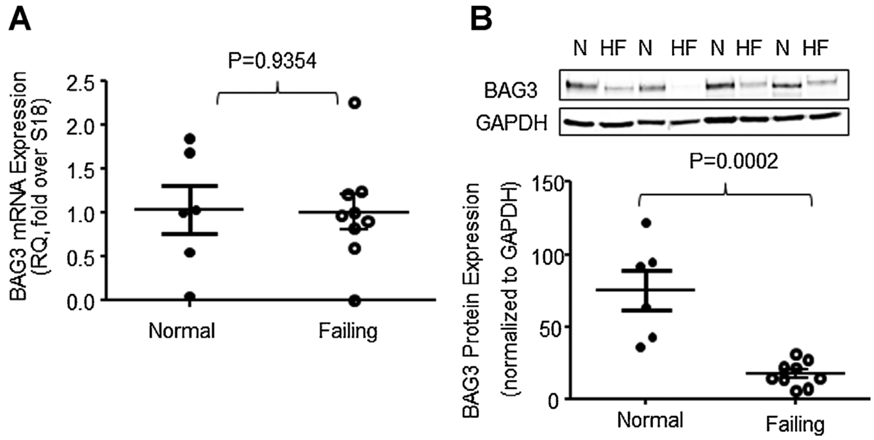

Fig. 3. A. mRNA levels in non-failing and failing human heart. B. Representative Western blot of BAG3 and GAPDH levels in non-failing (N/F) and failing (HF) human heart (Upper Panel). Quantification of BAG3 protein levels in non-failing and failing heart (Lower Panel). Values are normalized to the level of GAPDH in order to account for variations in protein loading. Horizontal lines represent mean and standard error of the mean. Statistical analysis was performed using unpaired *t*-test with Welch's correction for unequal variance.

The level of BAG3 protein in subject IV-1 was less than half that seen in heart tissue obtained from organ donors whose heart could not be utilized for transplantation. As seen in Figure 3, BAG3 levels in failing human heart from patients with end stage HF without known *BAG3* mutations were significantly ($P = 0.0002$) less than that found in non-failing control hearts. Thus, it appears that decreased levels of BAG3 protein can be found both in individuals with a *BAG3* mutation as well as in end-stage failing human heart.

Discussion

It is being increasingly recognized that genetic mutations can account for as many as a third of cases of IDC. Indeed, investigators have begun to refer to these cases as familial dilated cardiomyopathy (FDC) (Judge and Johnson, 2008; Hershberger et al., 2010; Jefferies, 2010). Inheritance can occur in a variety of manners with the most common pattern of inheritance being autosomal dominant (Zeviani et al., 1991; Towbin et al., 1993; Murphy et al., 2004; Morales and Hershberger, 2013). Mutations are most commonly found in genes encoding the sarcomere leading to cardiac dysfunction, disintegration of the myofiber structure and accumulation of degraded material in autophagic granules (Chien and Olson, 2002; Selcen et al., 2004; Chang and Potter, 2005; Lange et al., 2006; Ferrer and Olive, 2008; Maloyan and Robbins, 2010). Here, we report a 10-bp deletion in the gene encoding the sarcomeric protein BAG3 that segregates completely with affected individuals in a family with an autosomal dominant pattern of FDC. We also report for the first time that BAG3 protein is substantially reduced in the hearts of unrelated patients who are undergoing heart transplantation when compared with normal hearts from transplant recipients.

BAG3 is a 575 amino acid anti-apoptotic protein that is constitutively expressed in the heart and serves as a co-chaperone of the HSPs (Rosati et al., 2011; McCollum et al., 2010). BAG3 binds to HSPs and regulates their ability to chaperone cytoskeletal proteins including desmin and also participate in degradation of cellular proteins through either the proteasome or autophagy pathways (McCollum et al., 2010). BAG3 also protects cells from apoptotic death (Maloyan et al., 2010) and inhibits myofibrillar degeneration in response to mechanical stress (Hishiya et al., 2010; Arimura et al., 2011). Knockdown of *BAG3* in zebrafish (Norton et al., 2011) or in neonatal cardiomyoctes (Hishiya et al., 2010) or homozygous disruption of *BAG3* in mice leads to cardiac dysfunction (Homma et al., 2006) and BAG3 levels are decreased in the skeletal muscle of spontaneously hypertensive rats (Bloemberg et al., 2013).

The results of the present study in a large family with FDC are consistent with earlier reports that demonstrated an association between mutations in *BAG3* and the development of muscle pathology. Mutations in *BAG3* were first shown to cause abnormal muscle function in two families with childhood-onset muscular dystrophy (Selcen et al., 2009; Odgerel et al., 2010) and the phenotype of IDC, diffuse myocardial fibrosis and sudden death was linked with markers in the chromosome 10q25–26 region which includes the *BAG3* locus (Ellinor et al., 2006). More recent studies have demonstrated a causative relationship between BAG3 mutations and the development of FDC without peripheral muscle weakness or neurologic findings (Arimura et al., 2011; Norton et al., 2011; Villard et al., 2011).

As seen with genetic variants in other sarcomeric genes, there was substantial genetic heterogeneity within this large family. For example, one of the proband's sons had an early onset of severe disease requiring transplantation whereas a sibling with moderate disease and a middle-aged daughter with very mild disease remain asymptomatic for over a decade. Indeed, the cardiac dysfunction in the proband's daughter would have gone unrecognized had it not been for careful phenotyping as part of this study. Identification of the causative mutation in this family provides an opportunity for guideline-driven genetic testing and counseling of family members and early identification of affected individuals (Hershberger et al., 2009b). The finding that use of an angiotensin converting enzyme inhibitor improved survival in a small group of patients with Duchenne muscular dystrophy suggests that early therapy in families with mutations in sarcomere genes might be beneficial; however, additional studies will be required to define the best treatment strategies (Duboc et al., 2005, 2007).

We report for the first time that the level of BAG3 protein is significantly reduced in the hearts of unrelated patients with end-stage HF who are undergoing heart transplant and who have no family history of heart muscle disease. This finding is interesting as it suggests that while mutations in *BAG3* can be causative of disease in FDC, changes in levels of BAG3 protein might participate in the progression of disease in patients with non-familial forms of IDC. However, it remains to be seen whether the decrease in BAG3 levels in patients with IDC participated in the progression of the disease or is secondary to the disease process itself. Mechanistic studies will also be needed to understand the cellular and molecular effects responsible for the myopathy in individuals with the rare *BAG3* deletion seen in this large family. Nonetheless, these results suggest that BAG3 protein might be a new target for therapeutic intervention in HF.

Acknowledgments

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