Supplemental material

Clinical Characteristics and Natural History of Dilated Cardiomyopathy due to BLC2-associated Athanogene 3 (*BAG3)* Mutations

Short Title: DCM caused by *BAG3* mutations.

Fernando Domínguez, et al.

Supplemental material

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Appendix 1.

European genetic cardiomyopathies initiative investigators:

Aarhus University Hospital: Hans Eiskjær; **Complexo Hospitalario Universitario de A Coruña. Spain:** Roberto Barriales, Xusto Fernández Fernández; **Health in Code. A Coruña, Spain**: Marcos Cicerchia, Lorenzo Monserrat, Juan Pablo Ochoa, Joel Salazar-Mendiguchia; **Hospital San Pedro de Alcántara, Cáceres, Spain**: Maria Victoria Mogollón; **Hospital Universitario Son Llatzer. Majorca, Spain:** Tomás Ripoll; **Hôpital Universitaire Pitié-Salpêtrière. Paris, France:** Philippe Charron, Pascale Richard, Eric Villard; **Hospital Universitario 12 de Octubre, Madrid, Spain**: Julian Palomino Doza; **Hospital Universitario Marqués de Valdecilla Santander, Spain:** Ana Fontalba; **Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain**: Luis Alonso-Pulpón, Marta Cobo-Marcos, Fernando Domínguez, Pablo Garcia-Pavia, Manuel Gómez-Bueno, Esther González-López, Aitor Hernández-Hernández, Francisco José Hernández-Pérez, Ángela López-Sainz, Alejandra Restrepo-Córdoba, Javier Segovia-Cubero; **Hospital Universitario Puerta del Mar. Cádiz, Spain**: Rocio Toro, David de Gonzalo-Calvo, Félix Rosa Longobardo; **Hospital Universitario Vall d'Hebrón. Barcelona, Spain:** Javier Limeres, Jose F Rodriguez-Palomares; **Hospital Universitario Virgen de la Victoria. Málaga, Spain:** Jose Manuel Garcia-Pinilla, Miguel A López-Garrido; **Hospital Universitario Virgen de las Nieves. Granada, Spain:** Juan Jiménez-Jaimez; **Hospital de Valme. Seville, Spain:** Dolores Garcia-Medina; **Hospital Virgen del Rocío. Seville, Spain:** Diego Rangel Sousa, Maria Luisa Peña; **Odense University Hospital, Denmark:** Jens Mogensen, Thomas Morris-Hey; **Royal Brompton Hospital and Imperial College London, United Kingdom:** Paul J. Barton, Stuart A. Cook, William Midwinter, Angharad M. Roberts, James S Ware, Roddy Walsh; **St. Bartholomews' Hospital. London, United Kingdom:** Mohammed

Akhtar, Perry M. Elliott; Luis Rocha-Lopes, Konstantinos Savvatis; Petros Syrris.; **The Cardinal Stefan Wyszyński Institute of Cardiology. Warsaw, Poland**: Ewa Michalak, Rafal Ploski, Malgorzata Sobieszczanska-Malek, Zofia Bilińska; **University Hospital Giessen and Marburg GmbH, Germany**: Sabine Pankuweit; **University Medical Center Utrecht, Utrecht, The Netherlands**: Folkert Asselbergs, Annette Baas, Dennis Dooijes, Arjan Sammani.

Supplemental Methods

Tissue processing protocol

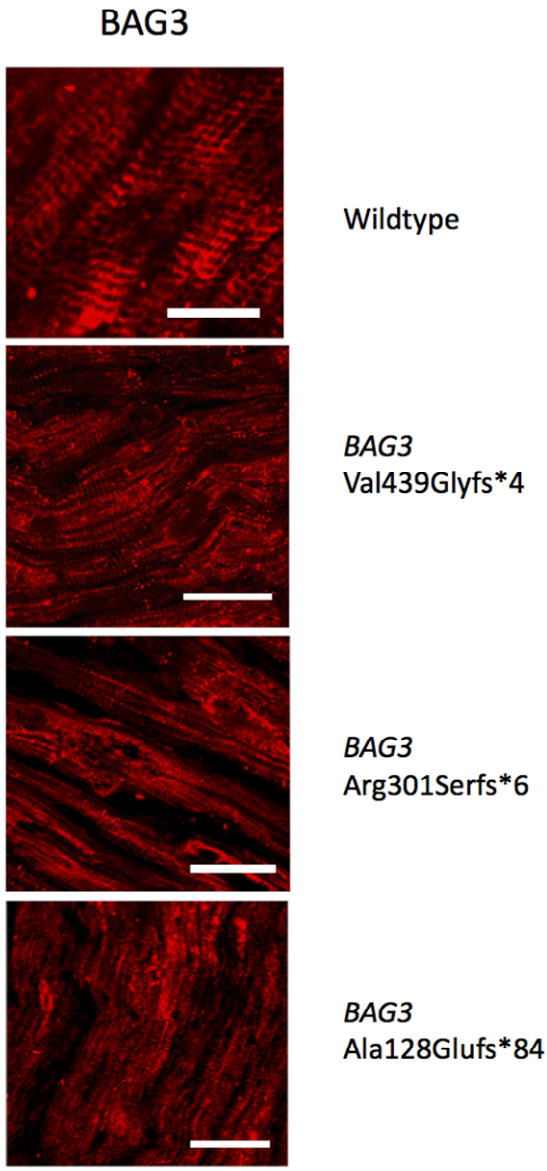
Explanted heart tissue samples were rinsed with phosphate buffered saline (PBS) immediately after extraction and fixed in 10% neutral buffered formalin. Samples were dehydrated and embedded in paraffin using an automatic tissue processor (Shandon Excelsior ES, ThermoFisher Scientific). Paraffin blocks were sectioned in 4-μm slices.

Antigen retrieval and rehydration were performed on the Dako PT Link instrument with Envision™ FLEX Target Retrieval Solution, high pH (Dako, Glostrup, Denmark). Tissue sections were first incubated with a BAG3 antibody (Abcam ab 47124, Cambridge, UK) at 1/50, overnight at 4ºC, washed with PBS and incubated with Alexa Fluor 546 anti-rabbit (Invitrogen Life Technologies, 1/500) for 45 minutes at room temperature. Sections were then blocked with 10% fetal calf serum and incubated with Actinin (Abcam ab 9465) 1/10, for 1 hour. After washing with PBS, sections were stained with Alexa fluor 488 anti-mouse (Invitrogen Life Technologies, 1/500). Nuclei were stained with Topro-3 (Invitrogen Life Technologies 1/1000) 15 minutes and the sections were mounted with PBS glycerol.

Images of the specimens were collected with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with 40× HCX PL APO (1.25 numerical aperture) oil-immersion optics. The three channels were acquired sequentially with the following excitation and emission parameters: (488 nm, 500–540 nm) for Alexa 488, (546 nm, 543–611nm) for Alexa 546 and (633 nm, 645–750 nm) for Topro-3. Gains were adjusted to avoid saturation in pixel intensity. Negative controls, in which primary antibodies were substituted with isotypic non-immune IgGs, did not result in any

detectable labeling. Z-series images were obtained through the collection of serial, confocal sections at 1-μm intervals.

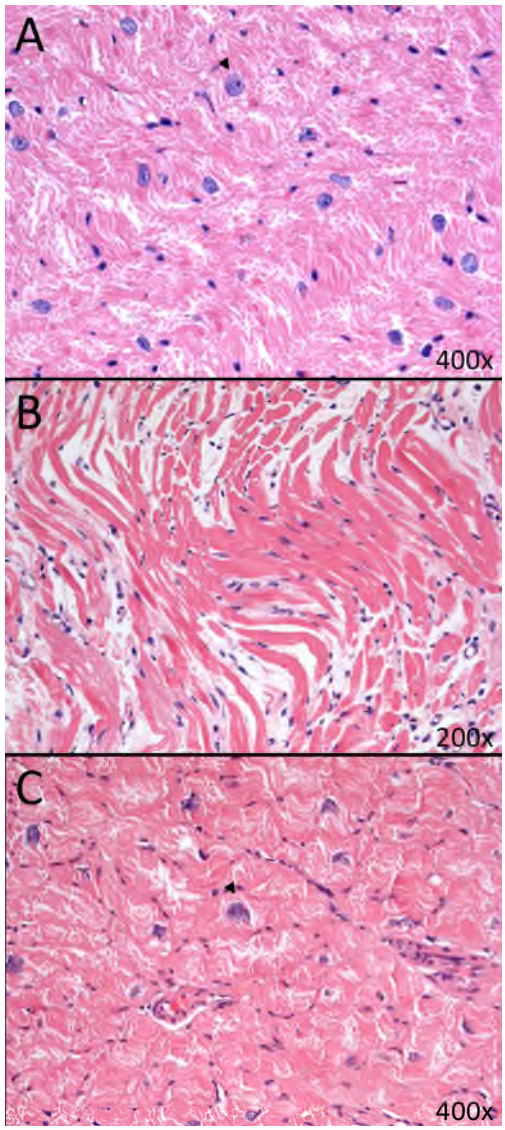
Online figure 1. BAG3 localization in explanted hearts (zoom).



Samples with fluorescent label BAG3 (red). Scale bar: 16 μm.

The first image (top) is from a patient without mutations in *BAG3* gene. The remaining images are from samples belonging to patients with *BAG3* truncating mutations. In patients with *BAG3* mutations, disorganization of muscular fibers is evident and BAG3 protein is diminished in the Z-disks as compared with controls, and is arranged in a more disorganized and diffuse pattern. BAG3 aggregates are also shown in the Ala128Glufs*84 variant.

Online Figure 2. Cardiac tissue of DCM patients with *BAG3* mutations: Hematoxylin and eosin staining.



Cardiac tissue from the left ventricle of three patients with DCM caused by truncating BAG3 mutations: A: p.Val439Glyfs*; B: p.Arg301Serfs*; and C: p.Ala128Glufs*84. The three images show a loss of the normal alignment of cardiomyocytes, with curved-shape foci surrounding connective tissue. Nuclear irregularity is also evident mainly in images A and C, with some examples of hypertrophied nuclei (black arrowheads).

Table 1S. *BAG3* mutations and frequency

Genomic position	Coding DNA reference (NM_004281.3)	Protein reference (NP_004272.2)	Variant type	MAF ExAC	rs	ACMG criteria	ClinVar	HGMD	n
g.121411259_ 121411260insC	c.72_73insC	**p.Gly25Argfs*33**	Frame-shift (truncating)	0.00002	rs772351208	Pathogenic	NP	NP	3
g.121411276_121411278 delTCGinsACC	c.89_91delTCGinsACC	**p.Ile30_Asp31delinsAsnHis**	Indel (non-truncating)	NP	-	Likely pathogenic	NP	NP	1
g.121411295G>A	c.108G>A	**p.Trp36***	Nonsense (truncating)	NP	-	Likely pathogenic	NP	NP	1
g.121411356_121411358delGAGinsAA	g.121411356_121411358delGAGinsAA	**p.Glu57Lysfs*154**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	2
g.121411360delG	c.173delG	**p.Gly58Alafs*153**[1]	Frame-shift (truncating)	NP	-	Likely pathogenic	NP	NP	1
g.121429543C>T	c.361C>T	**p.Arg121***	Nonsense (truncating)	NP	-	Pathogenic	NP	NP	2
g.121429549C>T	c.367C>T	**p.Arg123***	Nonsense (truncating)	0.000016	rs387906875	Pathogenic	Pathogenic:4 Likely path:1	CM111934 Pathogenic	2
g.121429564_ 121429565insAG	c.382_383insAG	**p.Ala128Glufs*84**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	13
g.121429590_121429596delACCTCTG	c.408_414delACCTCTG	**p.Pro137Glyfs*72**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	2

g.121429639C>T	c.457C>T	**p.Gln153***	Nonsense (truncating)	NP	-	Pathogenic	NP	NP	6
g.121431790dupT	c.531dup	**p.Asp178***	Nonsense (truncating)	NP		Likely pathogenic	NP	NP	1
g.121431911C>T	c.652C>T	**p.Arg218Trp**	Missense (non-truncating)	0.000066	rs397514506	Pathogenic	Pathogenic:1 Likely path:1	CM1110061 Pathogenic	1
g.121431986delC	c.727delC	**p.His243Thrfr*64**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	29
g.121432080C>A	c.821C>A	**p.Ser274***	Nonsense (truncating)	NP	-	Pathogenic	NP	NP	3
g.121432162delG	c.903delG	**p.Arg301Serfs*6**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	4
g.121435991C>T	c.925C>T	**p.Arg309***	Nonsense (truncating)	NP	rs869248137	Pathogenic	Pathogenic	CM1111349 Pathogenic	3
g.121436093delC	c.1027delC	**p.Arg343Alafs**	Frame-shift (truncating)	NP	-	Likely pathogenic	NP	NP	1
g.121436121delG	c.1055delG	**p.Gln353Argfs*10**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	4
g.121436153G>T	c.1087G>T	**p.Glu363*[2]**	Nonsense (truncating)	NP	-	Likely pathogenic	NP	NP	1
g.121436200delG	c.1135delG	**p.Gly379Alafs*45**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	4

g.121436218_ 12143625del	c.1153_1160 del	**p.Ser385Glnfs*56**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	6
g.12143271delC	c.1205delC	**p.Pro402Leufs*22**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	2
g.121436367_121436378 dupGGGCTGGAGC	c.1300_1309dup GGG CTGGAGC	**p.Gln437Argfs*10**	Frame-shift (truncating)	NP	-	Likely pathogenic	NP	NP	1
g.121436382_ 121436383delTA	c.1316_1317delTA	**p.Val439Glyfs*4**	Frame-shift (truncating)	NP	-	Pathogenic	NP	NP	2
g.121436419C>A	c.1353C>A	**p.Tyr451***	Nonsense (truncating)	NP	-	Pathogenic	NP	NP	4
g.121436429G>A	c.1363G>A	**p.Glu455Lys**	Missense (non-truncating)	NP	rs397516881	Likely pathogenic	Pathogenic:1 Likely path:1 VUS:1	CM1111351 Pathogenic	13
g.121436477G>A	c.1411G>A	**p.Glu471Lys**	Missense (non-truncating)	0.000016		Likely pathogenic	NP	NP	3
g.121431768_121436795del	c.508_1728del	**Deletion of exons 3 and 4** (17990 bp)	CNVs (deletion of two exons)	NP		Pathogenic	NP	NP	11
g.121431765A>G	c.508-2 A>G	**N/A**	Splicing mutation (truncating)	NP	-	Pathogenic	NP	NP	3

ACMG: American college of medical genetics.
HGMD: Human Gene Mutation Database
MAF: Mutation annotation format

1: This patient also presented the p.Glu57Lys missense variant in *BAG3*

2: The patient also presented the p.Pro368Ser missense variant in *BAG3*

Table 2S. Clinical predictors of heart transplant, LVAD and heart failure death in DCM patients with *BAG3* mutations

	DCM patients without HF-related events (n=58)	DCM patients with HF-related events (n=20)	p
Male sex (%)	53.4	95.0	**0.001**
Truncating mutation (%)	79.3	80.0	0.95
Non-truncating mutation (%)	20.7	20.0	0.95
Age at DCM onset	38.4 ± 12.6	31.6 ± 13.7	0.055
QRS width (ms) on 1st ECG	98.3 ± 21.0	98.9 ± 21.6	0.84
Negative T waves on 1st ECG (%)	18.4	30.8	0.33
LVEDD (mm) on 1st echo	61.5 ± 8.7	68.2 ± 8.7	**0.01**
LVEF (%) on 1st echo	40.9 ± 14.6	24.7± 10.7	**<0.001**
NSVT on Holter monitor (%)	33.3	57.1	0.26
CK (UI/L)	113.2 ± 67.6	99.6 ± 62.0	0.44

LVEF: Left ventricular ejection fraction, LVEDD: Left ventricular end-diastolic diameter, NYHA: New York Heart Association, SD: Standard deviation, TAPSE: tricuspid annular plane systolic excursion

Table 3S. Clinical predictors of serious arrhythmic events in DCM patients with *BAG3* mutations

	DCM patients without arrhythmic events (n=71)	DCM patients with arrhythmic events (n=7)	p
Male sex (%)	64.8	57.1	0.69
Truncating mutation (%)	78.9	85.7	0.67
Non-truncating mutation (%)	21.1	14.3	0.67
Age at DCM onset	35.4 ± 13.1	44.0 ± 12.1	0.10
QRS width (ms) on 1st ECG	98.6 ± 21.4	92.0 ± 7.5	0.51
Negative T waves on 1st ECG (%)	19.3	40.0	0.28
LVEDD (mm) on 1st echo	62.4 ± 9.1	68.0 ± 6.9	0.18
LVEF (%) on 1st echo	38.5 ± 15.3	23.4 ± 7.0	**0.01**
TAPSE (mm) on 1st echo	20.5 ± 5.3	15.5 ± 2.1	0.20
NSVT on Holter monitor (%)+	34.5	100	0.07
CK (UI/L)	112.7 ± 66.7	55.3 ± 19.9	0.15

Arrhythmic events: sustained ventricular tachycardia, ventricular fibrillation, appropriate ICD shock or sudden cardiac death.

CK: Creatine kinase, LVEDD: Left ventricular end diastolic diameter, LVEF: Left ventricular ejection fraction.

+: 29 patients without arrhythmic events and 2 with arrhythmic events have 24-Holter ECG data.