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► **To cite this version:**

Felix W. Friedrich, Silke Reischmann, Aileen Schwalm, Andrea Unger, Deepak Ramanujam, et al.. FHL2 expression and variants in hypertrophic cardiomyopathy. *Basic Research in Cardiology*, 2014, 109, pp.451. 10.1007/s00395-014-0451-8 . hal-01110561

HAL Id: hal-01110561

<https://hal.sorbonne-universite.fr/hal-01110561v1>

Submitted on 28 Jan 2015

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FHL2 expression and variants in hypertrophic cardiomyopathy

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Received: 22 May 2014/Revised: 4 October 2014/Accepted: 22 October 2014/Published online: 31 October 2014
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Abstract Based on evidence that *FHL2* (four and a half LIM domains protein 2) negatively regulates cardiac hypertrophy we tested whether *FHL2* altered expression or variants could be associated with hypertrophic cardiomyopathy (HCM). HCM is a myocardial disease characterized by left ventricular hypertrophy, diastolic dysfunction and increased interstitial fibrosis and is mainly caused by mutations in genes coding for sarcomeric proteins. *FHL2* mRNA level, *FHL2* protein level and I-band-binding density were lower in HCM patients than control individuals. Screening of 121 HCM patients without mutations in

established disease genes identified 2 novel (T171M, V187L) and 4 known (R177Q, N226N, D268D, P273P) *FHL2* variants in unrelated HCM families. We assessed the structural and functional consequences of the nonsynonymous substitutions after adeno-associated viral-mediated gene transfer in cardiac myocytes and in 3D-engineered heart tissue (EHT). Overexpression of *FHL2* wild type or nonsynonymous substitutions in cardiac myocytes markedly down-regulated α -skeletal actin and partially blunted hypertrophy induced by phenylephrine or endothelin-1. After gene transfer in EHTs, force and velocity of both contraction and relaxation were higher with T171M and V187L *FHL2* variants than wild type under basal conditions. Finally, chronic phenylephrine stimulation depressed EHT function in all groups, but to a lower extent in

Electronic supplementary material The online version of this article (doi:10.1007/s00395-014-0451-8) contains supplementary material, which is available to authorized users.

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T171M-transduced EHTs. These data suggest that (1) *FHL2* is down-regulated in HCM, (2) both *FHL2* wild type and variants partially protected phenylephrine- or endothelin-1-induced hypertrophy in cardiac myocytes, and (3) *FHL2* T171M and V187L nonsynonymous variants induced altered EHT contractility. These findings provide evidence that the 2 novel *FHL2* variants could increase cardiac function in HCM.

Keywords Hypertrophic cardiomyopathy · Hypertrophy · *FHL2* · Engineered heart tissue · Hypercontractility

Introduction

The family of four and a half LIM (FHL) proteins is composed of FHL1–4, ACT (activator of CREM) and ARA55, and is characterized by an N-terminal half LIM domain followed by four complete LIM domains [12, 30]. FHL proteins are components of adhesion complexes, can act as transmitters of Rho signaling pathways and are involved in tissue-specific gene regulation [5, 39, 47, 60]. The second member, FHL2 plays a role in cell cycle regulation, differentiation and apoptosis, assembly of extracellular matrix, bone formation, and wound healing (for review, see [30]). *FHL2* is highly expressed in the heart throughout embryonic development and in adults [13, 32]. *FHL2* has been first shown to be located in the Z-disk and to a lesser extent in the M-band of the sarcomere in neonatal rat cardiac myocytes (NRCMs) [50] and further analysis indicated I-band localization in papillary muscle [33]. It interacts with several components, particularly with titin in the cardiac-specific N2B domain (I-band) and the IS2 region (M-band), where it couples cardiac metabolic enzymes to sites of high energy consumption [33]. A body of evidence indicates that *FHL2* inhibits cardiac hypertrophic pathways, such as calcineurin–NFAT (nuclear factor

of activated T cells)-dependent gene expression via binding of calcineurin [28], or the MEK1–ERK1/2 signaling cascade by binding to ERK2 [44]. Additionally, *FHL2* inhibited serum response factor (SRF)-dependent transcription in a Rho-dependent manner in embryonic stem cells and heart [42]. *FHL2* deficient mice exhibited normal response to short-term TAC [11], but developed exaggerated cardiac hypertrophy under chronic isoprenaline stimulation [32], suggesting that the implication of *FHL2* in heart failure depends on the trigger inducing heart failure. Whereas it was reported that *FHL2* is up-regulated upon adrenergic stimulation *in vivo* in rodents [28], *FHL2* protein abundance was markedly reduced in angiotensin II (AngII)-induced cardiac hypertrophy in mice [40] and in human heart failure [4].

Integrating the predominantly heart-specific expression of *FHL2*, its suggested antihypertrophic role and its lower expression in human heart failure, we hypothesized that *FHL2* altered expression or genetic variants could be associated with HCM. HCM is the most prevalent myocardial disease (1:500; [17]). Its hallmarks are hypertrophy, predominantly in the interventricular septum, diastolic dysfunction, myocardial fibrosis and disarray. The phenotype is very variable, and diastolic dysfunction can be the first feature of the disease. Symptoms include dyspnea, chest pain, palpitations, lightheadedness, fatigue, and syncope. HCM is a leading cause of sudden cardiac death (SCD) in young athletes and is connected with a significant risk of heart failure [24, 34]. HCM is a genetic disease mainly transmitted as an autosomal dominant trait. It is caused by mutations in at least 14 genes coding for sarcomeric components (for reviews, see [20, 45, 49, 51]). More recently, mutations in *FHL1* have been shown to be associated with HCM [21]. We evaluated *FHL2* expression in patients and mouse models of HCM and screened the *FHL2* gene for genetic variants in a cohort of HCM patients devoid of mutations in established disease genes. We identified six *FHL2* genetic variants and analyzed the molecular and/or functional impact of the nonsynonymous substitutions after gene transfer in rat cardiac myocytes and engineered heart tissues (EHTs).

Methods

A detailed description of materials and methods can be found in the Supplemental Material.

Patients and human samples

We enrolled 121 HCM index cases without mutations in *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, or *MYL2* (data not

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shown). They were selected out of 299 HCM index cases recruited from the Eurogene Heart Failure cohort supported by the Leducq Foundation [19, 21]. Diagnosis was grounded on medical history, physical examination, electrocardiogram, and echocardiogram (left ventricle (LV) wall thickness ≥ 15 mm in probands and >13 mm in relatives) [8–10]. Controls consisted of 262 individuals.

Human myocardial samples were obtained from HCM patients who underwent septal myectomy or heart transplantation, from a patient undergoing aortic valve implantation due to aortic stenosis, and from individuals who had no cardiac disease but died of another cause. All materials from patients and controls were taken with informed consent of the donors and with approval of the local ethical boards.

Mouse models

The study complies with the Guide for the Care and Use of Laboratory Animals published by the NIH (Publication No. 85–23, revised 1985). *Mybp3*-targeted knock-out (KO) and knock-in (KI) mice were developed previously and maintained on the Black swiss genetic background [6, 59].

Pre-embedding immunoelectron microscopy

Human septal myectomy and mouse ventricular tissues used for the study were fixed in 4 % paraformaldehyde, 15 % saturated picric acid in 0.1 M phosphate buffer (PB) pH 7.4 overnight at 4 °C. Sections were cut on a vibratome (Leica VT 1000S) at a thickness of 50 μ m, blocked in 20 % NGS in PBS and were incubated with *FHL2* (Abcam 12327) primary antibody in phosphate-buffered saline (PBS) containing 5 % normal goat serum (Vector Laboratories, Burlingame, CA, USA) overnight at 4 °C. After washing in PBS, sections were incubated with 1.4-nm gold-coupled secondary antibodies (diluted 1:100 in PBS; Nanoprobes, Stony Brook, NY, USA) overnight at 4 °C. After several washings sections were postfixed in 1 % glutaraldehyde in PBS for 10 min and then incubated with HQ Silver kit (Nanoprobes). After treatment with OsO_4 , sections were stained with uranyl acetate, dehydrated and embedded in Durcupan resin (Fluka, Switzerland). Ultrathin sections were prepared (Ultracut S; Leica, Germany) and examined with a ZEISS 910 electron microscope.

Screening of *FHL2* for genetic variants

FHL2 gene has eight exons, of which five (exons 4–8) are coding. There are four *FHL2* variants mainly differing in the 5'UTR, but encoding the same isoform. Therefore, only the five coding exons for *FHL2*, including neighboring intron boundaries were screened by PCR amplification

performed on 30 ng of genomic DNA from peripheral lymphocytes using specific primer pairs (Supplemental Table 2). Sequences were examined using Codon Code Aligner Software®. Reference *FHL2* sequence was taken from NCBI (NG_008844.2) with +1 designing the A of the ATG codon.

Rat cardiac myocytes culture, transduction, and hypertrophy stimulation

Isolation of neonatal rat cardiac myocytes (NRCMs) and stimulation with hypertrophy stimuli, followed by subsequent automated cell size determination was performed as defined previously [29]. In detail, cells were transduced with adeno-associated virus serotype 6 (AAV6)-*FHL2* at a MOI of 100,000, serum was reduced to 0.2 % during 24 h and then NRCMs were stimulated for 48 h with phenylephrine (PE; 50 μ M), endothelin-1 (ET1; 100 nM) or DMSO ($n = 3$ different experiments, each in triplicates). Afterwards, cells were stained for alpha-actinin and automated cell size and number quantification were executed.

For mRNA analysis, NRCMs were transduced with AAV6-*FHL2* at a MOI of 30,000 ($n = 5$ per condition), serum was reduced to 0.2 % during 24 h and then NRCMs were stimulated for 48 h with PE (50 μ M) or without. Subsequently, cells were harvested and RNA was extracted as described in the appropriate section thereafter.

Engineered heart tissue generation, transduction, contraction measurements, and immunofluorescence

Generation of EHTs from neonatal rat heart cells was performed as previously described [14, 21, 25]. EHTs were transduced with AAV6 encoding *FHL2* WT or variants at a MOI of 1,000. Briefly, transduction was performed directly in the reconstitution mix before pipetting it into the agarose slots. Constructs were then cultured at 37 °C in 7 % CO_2 humidified cell culture incubator for 14–21 days. EHT medium for the first 10 days of culture consisted of DMEM (Biochrom), 10 % horse serum inactivated (Gibco), 2 % chick embryo extract, 1 % penicillin/streptomycin (Gibco), insulin (10 lg/mL, Sigma-Aldrich), and aprotinin (33 μ g/mL, Sigma-Aldrich). To exclude any hypertrophic influence by the serum we applied a protocol with horse serum in the medium being successively replaced by triiodothyronine (T_3) and hydrocortisone after day ten of culture [26]. Therefore, horse serum medium content was reduced to 4 % between day 10 and 13. Up to day 13 medium was changed three times per week, afterwards twice daily. From day 13 onwards EHTs were kept in serum-free medium, i.e. the above medium without horse serum plus T_3 (0.5 ng/mL, European Commission-Joint Research Centre IRMM-469) and low concentrations of

hydrocortisone (50 ng/mL, Sigma-Aldrich). EHTs started to beat coherently one week after casting. For chronic PE stimulation, phenylephrine (PE, 20 μ M, powder dissolved in H₂O) was added to the medium every 12 h for seven consecutive days starting day 14. Contraction measurements were performed on day 8, 10, 13, 14, 17, 20 and 21 as previously described [14, 21, 25, 48]. Subsequently after chronic stimulation, EHTs were PBS washed three times and directly processed or frozen in liquid nitrogen.

For immunofluorescence analysis, the entire EHTs were analyzed using confocal imaging as specified [21]. Immunofluorescence was performed as described above (primary antibodies against FLAG 1:800 and cMyBP-C, custom 1:200, nuclear staining by Draq5, Axxor, 1:1000).

Statistical analysis

Data are presented as mean \pm SEM. Statistical analyses were performed by one-way or two-way ANOVA followed by Dunnett's or Bonferroni's post test, and by Student's *t* test as indicated in the figure legends, using the GraphPad software (GraphPad Software), version 5.02. A value of $P < 0.05$ was considered significant. Quantitative PCR data analyses were carried out using the $\Delta\Delta C_t$ method.

Results

Reduction of *FHL2* expression and I-band binding signal in human and mouse HCM tissue

It has been shown that FHL2 protein level is markedly reduced in human failing hearts [4]. To examine whether *FHL2* expression is altered in HCM we measured both mRNA and protein levels in ventricular tissue of HCM patients carrying *MYBPC3* mutations [52]. *FHL2* mRNA and FHL2 protein levels were $>50\%$ lower in HCM patients than in controls (Fig. 1a, d, e). Similarly, *FHL2* mRNA levels were markedly reduced in two HCM mouse models, the homozygous *Mybpc3*-targeted knock-in (Hom-KI) [59], and both heterozygous (Het-KO) and homozygous (Hom-KO) *Mybpc3*-targeted knock-out mice (Fig. 1b, c; [6]). Moreover, whereas no difference was observed in the right ventricle between WT, Het-KO and Hom-KO, *FHL2* mRNA level was lower merely in the hypertrophied parts of the Het-KO (=septum) and Hom-KO (=septum + LV; Online Fig. 1).

We then investigated the subcellular localization of *FHL2* by immunogold labeling in cardiac myofibres on ultrathin sections of ventricular tissue obtained from of a human donor, a patient with aortic stenosis, two HCM patients with a *MYBPC3* mutation, and from WT and Hom-KI mice (Fig. 1f–k). The highest density of immunogold

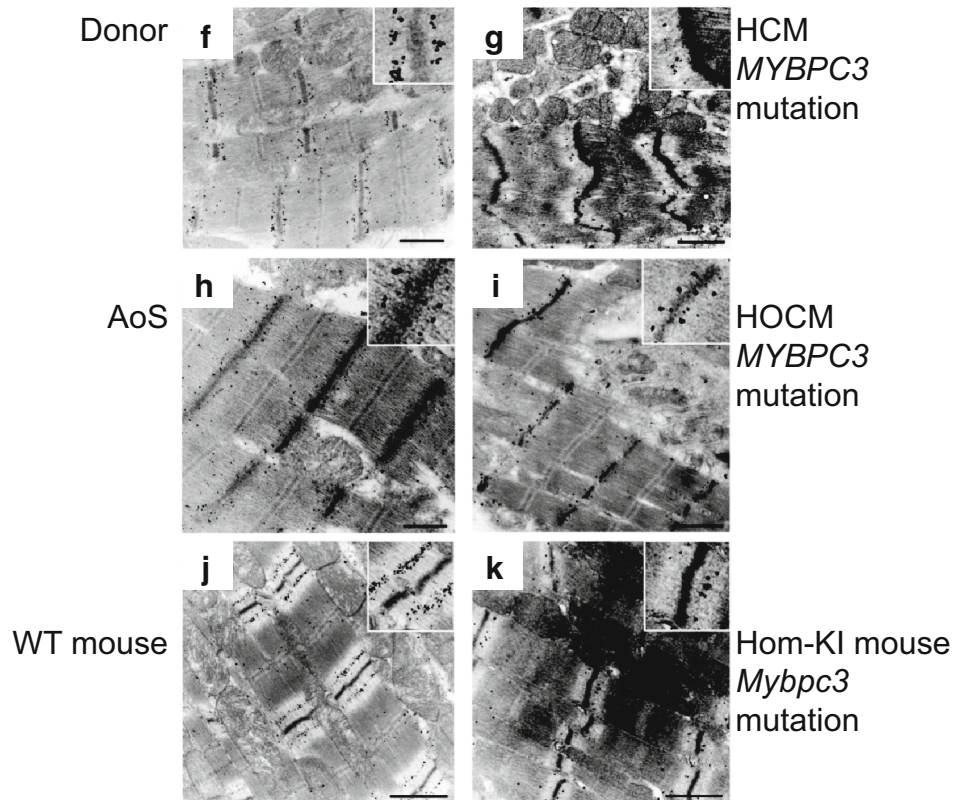
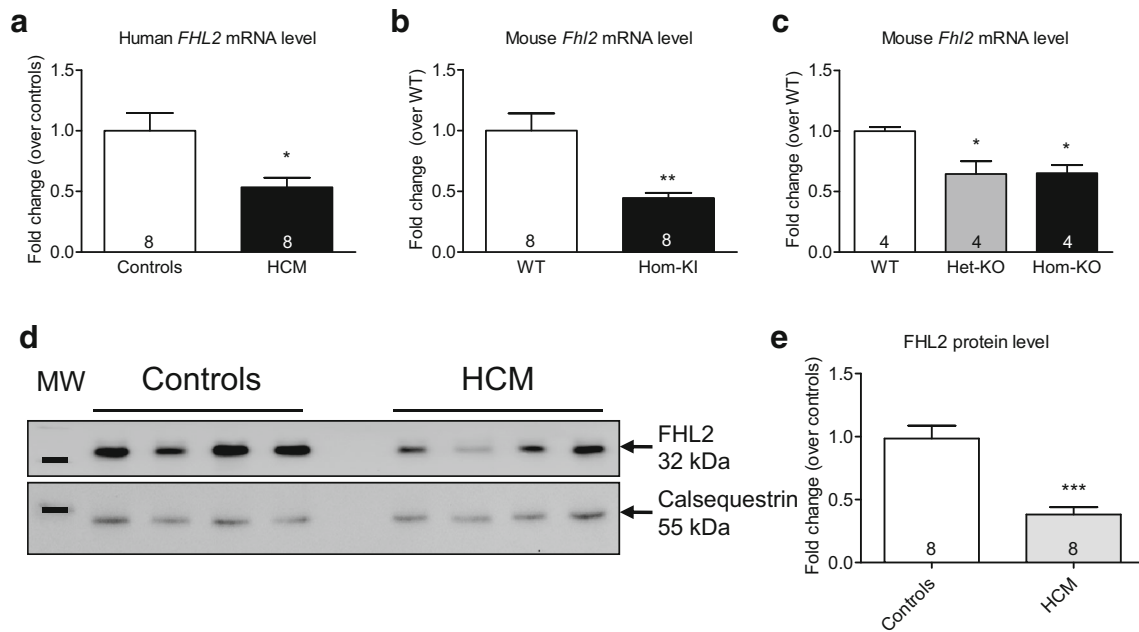
Fig. 1 *FHL2* expression and sarcomeric localization in human and mouse hearts. *FHL2* mRNA level in human (a) and mouse (b, c) HCM and normal hearts ($n = 8$ for human, $n = 4–8$ for mouse). Data are expressed as mean \pm SEM. * $p < 0.05$ vs. healthy controls/WT, unpaired Student's *t* test (in a and b) or one-way ANOVA with Dunnett's multiple comparison vs WT (in c). **d–e** FHL2 protein level in human HCM and control hearts. **d** Representative Western blot stained with an antibody recognizing FHL2 and with an antibody directed against calsequestrin, used as an endogenous control. Molecular weight markers (MW) indicate 25 and 55 kDa. **e** Protein levels of FHL2, normalized to calsequestrin levels and indexed to protein levels of healthy controls. Data are expressed as mean \pm SEM. *** $p < 0.001$ vs. healthy controls, unpaired Student's *t* test (in e). **f–l** Immunoelectron microscopy using an anti-FHL2 antibody in control and disease cardiac sections showing a specific labeling pattern in the I-band (zoom-in right upper corner of each image) of the sarcomeres; **f** human donor heart; **g** patient with HCM; **h** patient with aortic stenosis; **i** patient with obstructive HCM; **j** WT mouse; **k** *Mybpc3*-targeted knock-in mouse. Scale bars 1 μ m. *AoS* aortic stenosis, *Hom-KI* homozygous *Mybpc3*-targeted knock-in mice, *Het-KO* heterozygous *Mybpc3*-targeted knock-out, *Hom-KO* homozygous *Mybpc3*-targeted knock-out mice, *HOCM* obstructive HCM

particles for FHL2 was observed in the middle of the I-band (inserts, higher magnification, Fig. 1f–k), suggesting that most of the cytosolic FHL2 is bound to the spring region of titin. Some immunolabelling was also observed in the cytosol, but the density of the enhanced gold particles was consistently lower than in the I-band. Overall, the abundance of gold particles was $\sim 50\%$ lower in HCM and Hom-KI cardiac sections than in donor tissue.

Identification of *FHL2* genetic variants in patients with HCM

Next we evaluated whether *FHL2* genetic variants could be associated with HCM. We screened a cohort of 121 HCM patients devoid of mutations in five of the major HCM disease genes (*MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, or *MYL2*). We identified two novel and one known *FHL2* genetic variants and three other known *FHL2* synonymous polymorphisms (Fig. 2; Table 1, Online Table 1).

Variant I is a c.530G>A transition in exon 7 (p.Arg177Gln (R177Q)), which affects the LIM 3 domain. It was detected in three individuals of a French family (F-19949). The index patient (III.3) was diagnosed with HCM at the age of 13. Heart transplantation was required at the age of 19 because of congestive heart failure with pulmonary edema. Variant I was found in 2/262 control individuals and in 201 of 12,805 alleles (1.5%; rs1131188481) in the NHLBI Exome variant server (<http://evs.gs.washington.edu/EVS/>), a freely available database comprising about 13,006 alleles of European American and African American populations. In silico analysis using the prediction programs Mutation Taster (<http://www.mutationtaster.org>) and PolyPhen-2 (<http://genetics.bwh>



harvard.edu/pph2/) classified variant I as a benign polymorphism (rs1131188481).

Variant II is a c.512C>T transition in exon 7, which results in the exchange of a threonine with a methionine at position 171 in the third LIM domain (p.Thr171Met (T171M)). This nonsynonymous substitution was found in a

female index patient from Spain (F-19170, II.7) with a wall thickness of 22 mm. The father of the proband (I.1) died at the age of 79 years of stroke and the mother (I.2) passed away in the course of an acute coronary syndrome at the age of 78 years. One of the sisters (II.4) and her son (III.2) presented with HCM and received an implantable

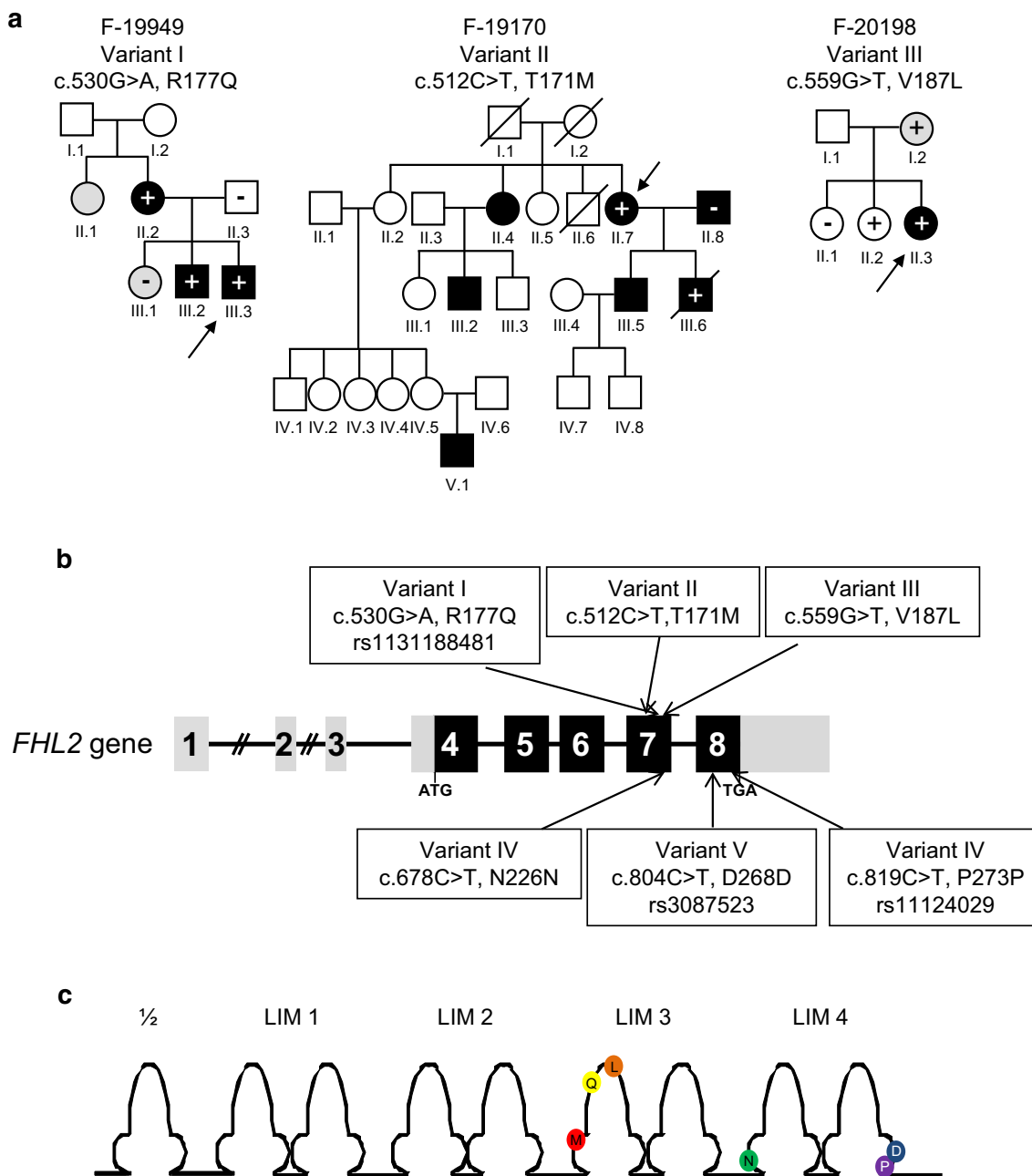


Fig. 2 *FHL2* genetic variants detected in patients with hypertrophic cardiomyopathy. **a** Pedigrees of families with HCM carrying *FHL2* missense variants. Roman numerals symbolize generations. Arabic numerals mark individuals within each generation. Males are symbolized as squares, females as circles. Individuals with HCM are indicated by black symbols, individuals with an intermediate phenotype by gray symbols, unaffected individuals by empty symbols, and deceased individuals by a diagonal line. Index cases

are indicated by arrows. Family members with a plus sign (+) carry the respective genetic variant at the heterozygous state; non-carriers are marked with a minus sign (-); individuals without signs were not genotyped. **b** Variants in the *FHL2* gene. Exon numbers, and translational start (ATG) and stop (TGA) codons are indicated. Light gray squares represent noncoding regions. The *FHL2* variants identified in HCM patients are depicted. **c** Localization of *FHL2* genetic variants in the LIM domains

cardioverter defibrillator (ICD). One grandchild (V.1) also presented with HCM. One son (III.6) was positive for the variant and had earlier been diagnosed with HCM, requiring ICD implantation. He died of SCD in February 2011. The second son (III.5) also presented with HCM and received an

ICD. Several affected individuals of family 19170 were not available for molecular diagnosis, which reduced the power of the co-segregation analysis. In addition, the husband of individual II.7 (II.8) also presented with HCM but does not carry the variant; he also does not have any variant in

Table 1 Clinical and genetic features of HCM index cases and relatives (with missense mutations)

Family	Ind.	Sex	Birth Year	Age at diagnosis procedure	Proband	Clinical status	Age at onset	PWth (mm)	SWth (mm)	ECG	Other symptoms	Var.	FHL2 variant	FHL2 protein consequence
19949	III.3	M	1984	18	P	A	HCM age 13 Heart transplant age 19	13	29	Abnormal	No	I	c.530G>A	p.Arg177Gln
	II.2	F	1959	43	R	A	n.a	8	17	Abnormal	No		c.530G>A	p.Arg177Gln
	II.3	M	1955	47	R	NA	n.a	9	10	Normal	No		No	
	III.2	M	1988	14	R	A	10	8	16	Abnormal	Myalgia hypophonia		c.530G>A	p.Arg177Gln
	III.1	F	1997	5	R	I	n.a	3	5	Abnormal	No		No	
19170	II.7	F	1942	66	P	A	n.a		22		NYHA II, dyspnea, angina, no subaortic obstruction	II	c.512C>T	p.Thr171Met
	II.8	M	1940	70	R	A	n.a		19		NYHA II, pacemaker, dynamic gradient of 36 mmHg		No	
	III.6	M	1975		R	A	n.a		19		Asymptomatic, subaortic obstruction		c.512C>T	p.Thr171Met
20198	II.3	F	1957	39	P	A	28	16	20	Abnormal Q wave, negative T wave, LVH/Romhilt Este score 6	NYHA III, dyspnea, syncope, angina	III	c.559G>T	p.Val187Leu
	II.1	F	1954	49	R	NA	n.a			Normal except LVH/Romhilt Este score 5	No		No	
	II.2	F	1956	47	R	NA	n.a	6	10	n.a	No		c.559G>T	p.Val187Leu
	I.2	F	1932	71	R	I	n.a			Normal except LVH/Romhilt Este score 5	No		c.559G>T	p.Val187Leu

Abnormal ECG corresponds to abnormal negative T-waves

A affected, ECG electrocardiogram, F female, HCM hypertrophic cardiomyopathy, I intermediate, Ind. individual number, LVH left ventricular hypertrophy, M male, P proband, NA not affected, n.a. not available, PWth posterior wall thickness, R relative, SCD sudden cardiac death, SWth septal wall thickness, Var. variant number

MYBPC3, *MYH7*, *TNNT2*, *TNNI3*, *MYL2* and *FHL1*, suggesting that he has potentially a mutation (not yet identified) in another gene. The presence of HCM in both parents II.7 and II.7 suggests the existence of double heterozygous mutations that could explain the severity of the clinical phenotype (and SCD) of individual III.6 in one of the two children. Variant II was not detected in 262 control individuals and was not found in the NHLBI Exome variant server, indicating that it is a very rare variant (<1:1000). Mutation Taster indicates this variant to be disease causing with a probability of 99 % and PolyPhen-2 envisages damaging consequences (prediction sensitivity 0.83/specificity 0.93).

Variant III is a c.559G>T transversion in exon 7, leading to a full-length mutant protein with an amino acid exchange in the third LIM domain in a highly conserved region (p.Val187Leu (V187L)). It was detected in a German patient (F-20198, II.3), presenting with a 20 mm septal wall thickness. Whereas one sister carrying the variant (II.1) was not clinically affected at the time of diagnosis, the mother (I.2) presented some LVH signs in the ECG, suggesting an intermediate phenotype. Variant III was found in 1/262 control individuals (1/524 alleles), but not in the 13,000 alleles of NHLBI Exome variant server. Like variant II, this is a very rare variant (<1:1000).

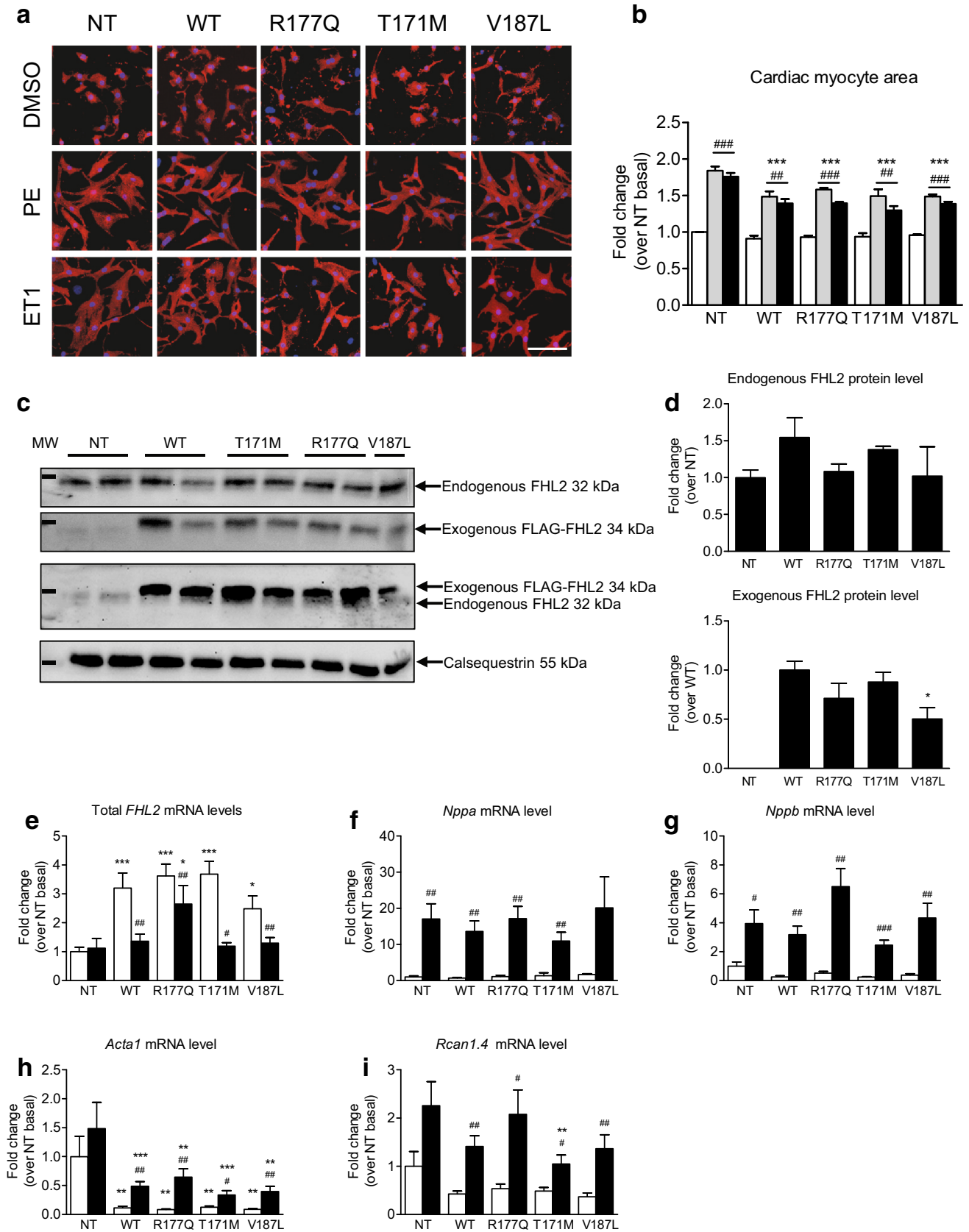
Finally, variants IV–VI were detected in the cohort but are known to be *FHL2* synonymous polymorphisms (Fig. 2, Online Table 1). Variant IV is a c.678C>T transition in exon 7 (p.Asn226Asn (N226N)), which was also found in 1/262 control individuals. Frequency in the NHLBI Exome variant server is 126 in 13,328 alleles (1 %; rs137869171). Variant V is a c.804C>T transition in exon 8 (p.Asp268Asp (D268D)) with a frequency of 9 % in the Exome variant server (rs3087523). Variant VI is a c.819C>T transition in exon 8 (p.Pro273Pro (P273P)) with a frequency of 16 % in the Exome variant server (rs11124029). Variants V and VI had already formerly been identified in healthy controls and suggested to be polymorphisms [2]. Since the three synonymous polymorphisms do not affect the amino acid sequence in contrast to the nonsynonymous variants they were not analyzed further.

FHL2 mutants do not induce cardiac myocyte hypertrophy

It has been shown that *FHL2* overexpression reduced PE-induced hypertrophy in cardiac myocytes [44], suggesting an antihypertrophic role of *FHL2*. Therefore, we sought to evaluate whether *FHL2* mutants would act differently. NRCMs were transduced with AAV6 encoding FLAG-tagged *FHL2* WT or mutants, and treated with PE or ET1 for 48 h. Cell area was determined using an automated

Fig. 3 Effect of phenylephrine or endothelin-1 on hypertrophic parameters after transduction of cardiac myocytes with *FHL2* wild-type or variants. **a, b** Cardiac myocytes were isolated from neonatal rats, transduced with AAV6 (MOI 100,000) encoding FLAG-tagged *FHL2* WT or mutants (R177Q, T171M, V187L) and then subjected to phenylephrine (PE; gray bars), endothelin-1 (ET1, black bars) or DMSO (white bars) for 48 h ($n = 3$, experiments performed in triplicates). Cell area was determined using an automated microscopic edge detection algorithm. **c–i** Cardiac myocytes were isolated from neonatal rats cardiac myocytes, transduced with AAV6 (MOI 30,000) encoding FLAG-tagged *FHL2* WT or mutants (R177Q, T171M, V187L) and then subjected to either 50 μ M phenylephrine (black bars) or H₂O (white bars) for 48 h ($n = 5$). **c** Representative Western blots stained with antibodies directed against endogenous rat *FHL2* (only stained with antibody against endogenous *FHL2*), exogenous FLAG-tagged human *FHL2*, a membrane showing endogenous *FHL2* (lower band) and exogenous *FHL2* (stronger FLAG signal above endogenous *FHL2*), or calsequestrin (protein loading control). Molecular weight marker (MW) indicates 37 and 55 kDa. **d** Quantification of protein levels of endo- and exogenous *FHL2*, normalized to calsequestrin, and related to *FHL2* NT and WT, respectively ($n = 5–6$ wells per group). Levels of mRNA of **(e)** total (with primers detecting rat and human) *FHL2*, **f** *Nppa*, **g** *Nppb*, **h** *Acta1*, and **i** *Rcan1.4*. Values are related to non-transduced (NT) cardiac myocytes in basal conditions. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. non-transduced (NT) cells in the same condition, two-way ANOVA followed by Bonferroni's comparison post test. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. basal conditions, unpaired Student's *t* test, for protein quantification one-way ANOVA followed by Dunnett's post test * $p < 0.05$ vs. WT. Scale bars 100 μ m

microscopic edge detection algorithm and mRNA levels of *FHL2* and markers of hypertrophy were quantified by RT-qPCR (Fig. 3). Under basal conditions, cell area did not differ between the groups (Fig. 3a, b), although total *FHL2* (exogenous human and endogenous rat) mRNA levels were ~two- to fourfold higher in *FHL2*-transduced NRCMs (Fig. 3e). To evaluate an overexpression on protein level, we used an antibody detecting the endogenous, native *FHL2* directed against the N-terminal regions and an antibody only recognizing the exogenous FLAG-tagged *FHL2*. Staining with the *FHL2* antibody revealed only one band in all samples (Fig. 3c). This suggests that the *FHL2* antibody only recognized the endogenous *FHL2* protein, likely because of the presence of the FLAG tag at the N-terminal end, which could hinder the binding of the *FHL2* antibody. The endogenous *FHL2* level did not differ significantly between the groups (Fig. 3d). We then stained the membranes with the antibody detecting the FLAG tag. Exogenous FLAG-tagged *FHL2* was overexpressed in all groups (except for NT) with some slight difference (Fig. 3c, d). Due to differences in binding affinities of the antibodies no quantification of overexpression (endogenous + exogenous *FHL2*) was possible. Furthermore, mRNA levels of atrial natriuretic peptide (*Nppa*), brain natriuretic peptide (*Nppb*) and the NFAT-target gene regulator of calcineurin (*Rcan1.4*) did not differ between all groups, whereas α -skeletal actin (*Acta1*) mRNA levels



were ~90 % lower in *FHL2*-transduced than non-transduced NRCMs (Fig. 3f–i). Both PE and ET1 increased cardiac myocyte area in all groups, but to a lower extent in *FHL2*-transduced than non-transduced NRCMs (Fig. 3b). This was associated with a lower activation of *Acta1* gene expression in *FHL2*-transduced NRCMs. Conversely, the PE-induced increased mRNA levels of *Nppa*, *Nppb* and *Rcan1.4* did not differ between the groups, except for a partial inhibition on *Rcan1.4* in T171M-transduced NRCMs (Fig. 3f–i). The amount of overexpressed *FHL2* was also lower in PE-treated NRCMs. These data suggest that FHL2 WT has an antihypertrophic effect and FHL2 mutants do not lose this feature.

FHL2 mutants affect contraction parameters in rat-engineered heart tissue

Since expression of *FHL2* variants did not induce an exaggerated hypertrophy after gene transfer in cardiac myocytes, we sought to evaluate whether they would affect contraction parameters of EHTs. EHTs were derived from neonatal rat heart cells, transduced or not with AAV6 encoding FHL2 WT or mutants. After 14 days of culture and transduction, EHTs were subjected to PE or control medium for 7 days. Force of contraction did not differ significantly between groups under basal conditions (no PE), although a tendency to higher force was observed in EHTs expressing T171M and V187L mutants (and $p < 0.05$ vs WT using Student's *t* test; Fig. 4a). Furthermore, T171M EHTs exhibited higher velocity of both contraction and relaxation than WT EHTs (and $p < 0.01$ vs WT using Student's *t* test; Fig. 4b–c). V187L EHTs showed a trend to higher contraction velocity (and $p < 0.05$ vs WT using Student's *t* test; Fig. 4b). EHTs transduced with R177Q did not differ to WT EHTs. Chronic PE stimulation reduced all parameters in WT-, R177Q- and V187L-transduced EHTs, but had a blunted effect in T171M-transduced EHTs (Fig. 4a–c). As observed in NRCMs, mRNA levels for *Nppa*, *Nppb*, *Acta1* and *Rcan1.4* did not differ between groups under basal conditions (Online Fig. 2). Chronic PE stimulation induced accumulation of *Nppa* and *Nppb* mRNAs in all groups, but had no major effect on *Rcan1.4* mRNA level (Online Fig. 2).

Under basal conditions, total *FHL2* mRNA levels were ~twofold to threefold higher in *FHL2*-transduced EHTs than in non-transduced EHTs, suggesting a slight overexpression (Online Fig. 3). As in NRCMs, staining with the FHL2 antibody revealed only one band in all samples (Online Fig. 3b). The endogenous FHL2 level did not differ significantly between the groups (Online Fig. 3b, c). FLAG-tagged FHL2 proteins were detected by Western blot only in *FHL2*-transduced EHTs, but no major

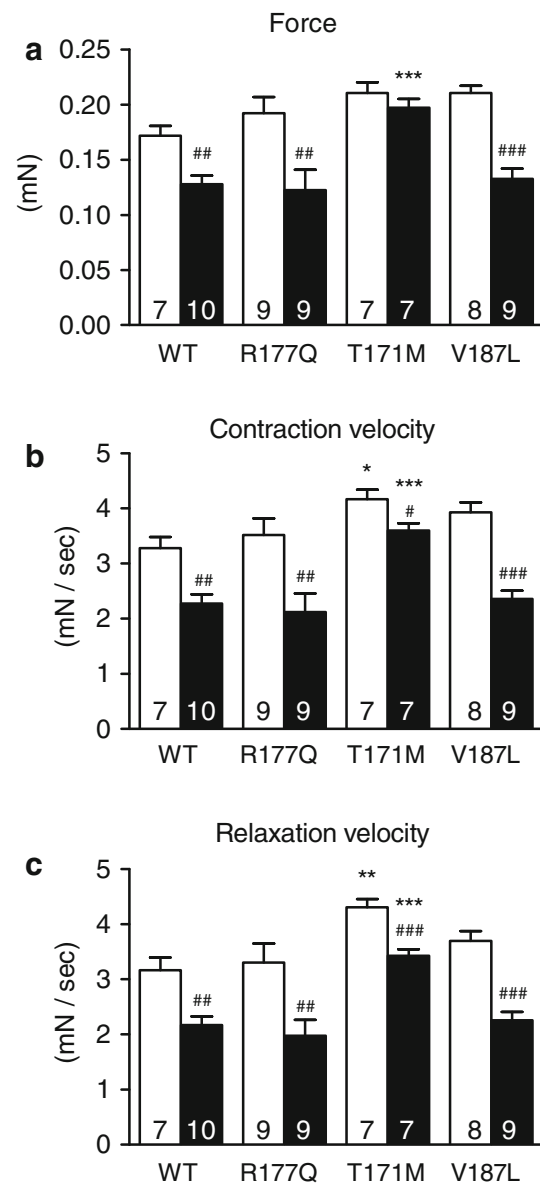


Fig. 4 Evaluation of chronic phenylephrine stimulation on contractile parameters of rat-engineered heart tissue transduced with *FHL2* wild-type or variants. Rat EHTs were transduced at day 0 with AAV6 (MOI 1,000) encoding FLAG-tagged FHL2 wild type (WT) or mutants (R177Q, T171M, V187L). On day 13, serum content was reduced to 0 %, on day 14 EHTs were treated with 20 μ M phenylephrine (black bars) or without (white bars) for 7 days. Measurements of **a** force of contraction, **b** contraction velocity, and **c** relaxation velocity at day 21 are shown. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs WT in the same condition, two-way ANOVA followed by Bonferroni's multiple comparison post test. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. H₂O, unpaired Student's *t* test. Number of EHTs is indicated in the bars

difference was observed between groups (Online Fig. 3b, c). Due to differences in binding affinities of the antibodies no quantification of total (endogenous + exogenous FHL2) was possible. Immunofluorescence analysis

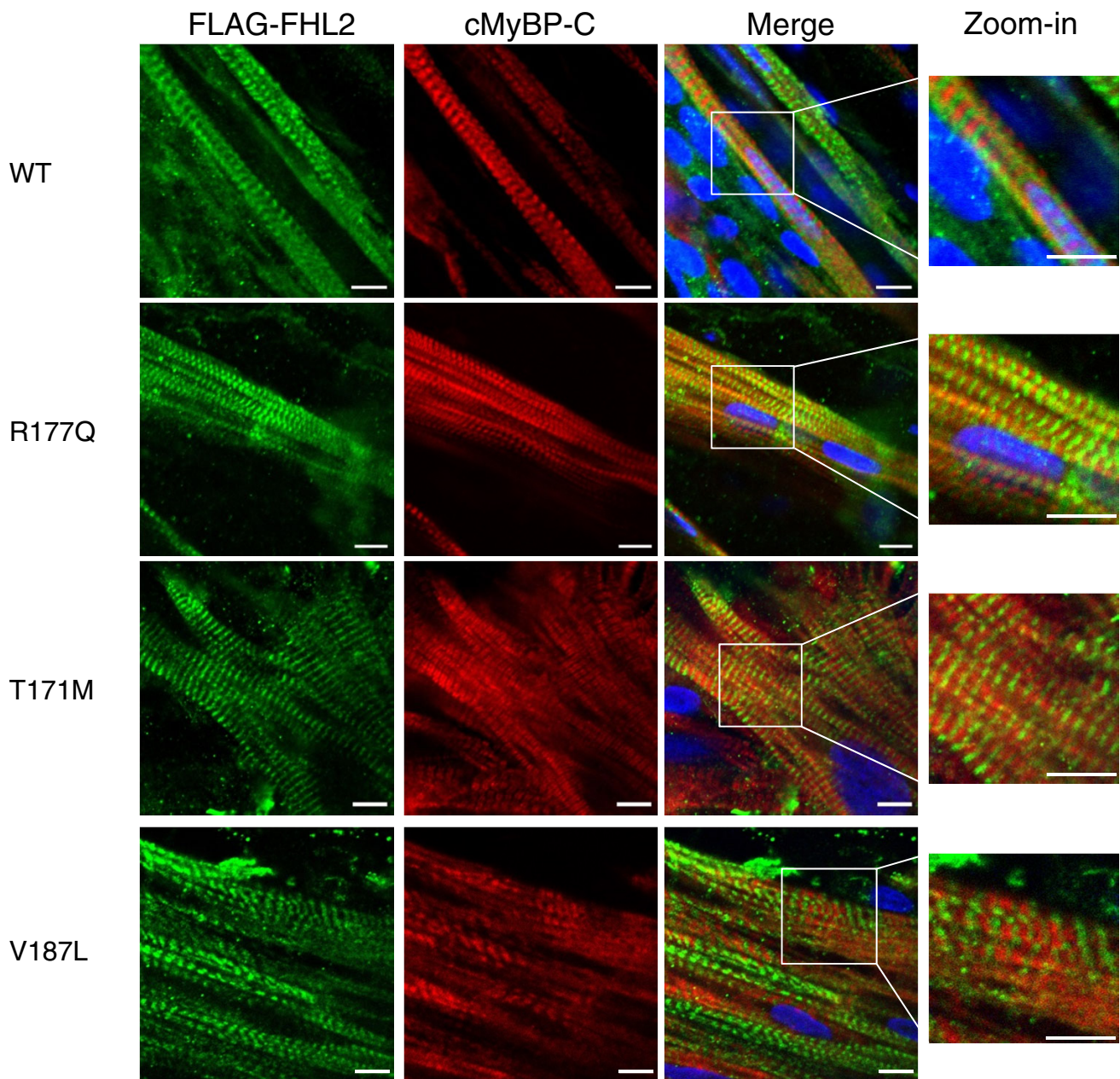


Fig. 5 Immunofluorescence analysis of rat-engineered heart tissue transduced with *FHL2* wild type or variants. EHTs were transduced with AAV6 encoding FLAG-tagged *FHL2* wild type (WT) or mutants (R177Q, T171M, V187L) at a MOI of 1,000. After fixation at day 14,

revealed a striated pattern for FLAG-FHL2 WT, R177Q, T171M and V187L proteins, mainly in alternation with cMyBP-C doublets, but also within the cMyBP-C doublets, suggesting an additional M-band incorporation (Fig. 5).

Discussion

The recent evidence that *FHL2* plays an antihypertrophic role and that its expression is reduced in human heart

EHTs were stained with antibodies directed against the FLAG epitope (green) and cardiac myosin-binding protein C (cMyBP-C, red). Scale bars 10 μ m

failure suggested that altered *FHL2* expression or variants could be associated with HCM. Therefore, we evaluated *FHL2* expression in human and mouse HCM cardiac tissue samples and screened *FHL2* for genetic variants in a cohort of 121 HCM unrelated index cases, who do not carry any other known mutations in major sarcomeric genes. The key findings are as follows: (1) *FHL2* expression and number of *FHL2* immunogold particles were lower in ventricular tissue of HCM patients; (2) out of six identified *FHL2* variants in unrelated HCM families, two were novel

(T171M, V187L); (3) gene transfer of *FHL2* WT or nonsynonymous variants in cardiac myocytes down-regulated *Acta1* and partially blunted the hypertrophic response induced by PE or ET1; (4) force and velocity of contraction or relaxation of EHTs were higher in the presence of T171M and V187L mutants than WT under basal conditions; (5) chronic PE stimulation reduced contractile force and velocities in all groups, but had no major effect in T171M-transduced EHTs. These findings support the view that *FHL2* expression is negatively associated with HCM and that FHL2 WT partially protects against PE- or ET1-induced hypertrophy, whereas T171M and V187L FHL2 mutants mainly induced hypercontractility.

The molecular mechanisms by which changes in *FHL2* expression and/or *FHL2* variants could contribute to HCM and associated cardiac dysfunction remain elusive at this point. One obvious hypothesis is a loss of antihypertrophic function [15, 28, 40, 42, 44]. The present study shows not only a downregulation of *FHL2* mRNA levels upon PE stimulation in NRCMs, but also lower *FHL2* mRNA and protein levels as well as a lower number of FHL2 immunogold particles in the I-band of the sarcomere in human and mouse models of HCM. This supports previous findings of reduced FHL2 protein levels in AngII-induced cardiac hypertrophy in mice [40] and in failing human hearts [4]. Reduced *FHL2* mRNA levels were also found in models of pathological hypertrophy induced by ET1, PE or afterload enhancement in rat EHT ([26] and Hirt, unpublished data). Besides a lower grade of antihypertrophic action, low *FHL2* expression could also have consequences on cardiac energy metabolism [33]. Whether *FHL2* downregulation is a dispensable by-product or whether the same mechanisms leading to hypertrophy and heart failure cause this concomitant downregulation is unclear. Altogether, these data suggest that *FHL2* expression is negatively associated with HCM and more generally with cardiac disease.

However, several arguments suggest that the hypothesis of the lack of antihypertrophic function of FHL2 mutants is unlikely. First, FHL2 mutants showed a stable expression on gene and protein level. Second, in the absence of hypertrophic stimuli, cardiac myocyte area and mRNA levels of *Nppa*, *Nppb* and *Rcan1.4* did not differ between non-transduced and *FHL2*-transduced NRCMs, indicating that neither FHL2 WT nor mutants exert growth effects per se. Conversely, *Acta1* gene expression was markedly down-regulated after gene transfer of FHL2 WT or mutants in NRCMs, whereas downregulation of *Fhl2* and up-regulation of *Acta1* coincided with the appearance of hypertrophy in *Mybpc3*-targeted KI HCM mice (Friedrich, unpublished data and [22, 35]). This supports the previously suggested role of FHL2 as a negative regulator of cardiac hypertrophy [28, 42, 44]. Finally, FHL2 mutants

had similar effects as WT on cardiac myocyte area and *Nppa*, *Nppb*, *Rcan1.4* and *Acta1* mRNA levels, which indicates that at least in the 48 h NRCM assay the genetic variants do not interfere with the antihypertrophic activity of FHL2.

Findings in EHTs rather indicate that the genetic variants may induce hypercontractility, which suggests a gain-of-function effect. Out of the six *FHL2* variants identified in HCM families, variants IV–VI are silent variants that were previously recognized as SNPs (rs137869171, rs3087523 and rs11124029), whereas variants I–III are *FHL2* nonsynonymous substitutions (R177Q, T171M, V187L). These nonsynonymous substitutions were found in HCM-affected individuals, they were expressed into stable proteins, correctly incorporated into the sarcomere and thereby may act in a dominant fashion on endogenous FHL2/sarcomere function. However, there are arguments against variant I (R177Q) as disease causing. First, variant I (recently named as rs1131188481) was found in 201 of 12,805 alleles (frequency 1.5 %) in the NHLBI Exome variant server and was classified as a benign polymorphism by Mutation Taster and Polyphen-2. Second, variant I was detected in a family, which also carries a mutation in *FHL1* [21]. Finally, the EHT contractile parameters did not differ to WT under basal conditions and after chronic PE. In contrast, the novel *FHL2* variants (T171M, V187L) could be associated with or contribute to HCM for the following reasons. First, variants II and III were not detected in the large cohort of the NHLBI Exome variant server and in silico analysis predicts harmful consequences for variant I. Second, the expression of both variants in EHT induced hypercontractility, characterized by either higher amplitude and/or shorter kinetics of force under basal conditions. Additionally, whereas chronic PE stimulation depressed EHT force of contraction in all groups as expected, it did not have any effect in T171M-transduced EHTs. One explanation could be that the T171M substitution alters post-translational modifications such as phosphorylation or oxidation. In fact, in silico analysis proposes a phosphorylation of FHL2 at position T171 (<http://www.phosida.com>). Which kinase performs this modification is unknown, but ERK2 and protein kinase D were previously excluded [44, 54]. Hypercontractility with consecutive higher LV systolic pressure is a common finding in patients with HCM [23, 41] and is in agreement with previous findings obtained for HCM-associated *FHL1* and *ANKRD1* variants in EHTs [14, 21]. It is also consistent with the increased myofilament Ca²⁺ sensitivity observed in human or mouse models of HCM with different sarcomere gene mutations [18, 31, 37, 38, 46, 57, 58], and with enhanced contractile function in muscle fibers, cardiac myofibrils, or cardiac myosins containing different *MYH7*-HCM mutations and HCM mouse native or engineered tissues [1, 3, 7,

37, 43, 53, 55]. Hypercontractility and diastolic dysfunction, but not LVH have also been observed in a transgenic *Tnnt2* HCM mouse model [56]. Similarly, functional changes (such as increased myofilament Ca^{2+} sensitivity, diastolic dysfunction) do exist without hypertrophy in heterozygous *Mybpc3*-targeted KI mice [18, 22, 55]. These data support the findings in human HCM patients who exhibited “supranormal” contractile function or diastolic dysfunction without accompanying LVH [16, 27, 36]. Hence, changes in contractility as induced by *FHL2* variants could precede or may even be independent of LVH development and consistent with the conception that compensatory mechanisms may play a role in the development of hypertrophy.

In conclusion, this study provides evidence for altered *FHL2* expression and novel *FHL2* genetic variants in HCM. However, whereas we confirmed that *FHL2* has an antihypertrophic role, our data suggest that *FHL2* genetic variants did not release this antihypertrophic effect of *FHL2*. Instead, our data support the view that *FHL2* genetic variants could increase cardiac function in HCM.

Acknowledgments We are thankful to family members for collaboration. We would like to thank M. Komajda (Paris) for continuous support in the Eurogene Heart Failure network, M. Hirt (Hamburg) for fruitful discussion. We would also like to thank A. Eder, I. Vollert, C. Neuber and A. Stöhr (Hamburg) for help in contractility experiments, E. Kraemer and S. Schlossarek (Hamburg) for isolation of human ventricular RNA, and Charles Redwood (Oxford) for DNA sequencing of human HCM tissue samples. Funding sources: This work was supported by the Leducq Foundation (Eurogene Heart Failure), the seventh Framework Program of the European Union (Health-F2-2009-241577-Big-Heart project), and the Association Institut de Myologie (Paris).

Conflict of interest None.

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