Imperial College

OF SCIENCE, TECHNOLOGY AND MEDICINE

Contractile proteins in hypertrophic cardiomyopathy

Steven Marston

22 Oct 2010

Mutations in contractile proteins cause different phenotypes

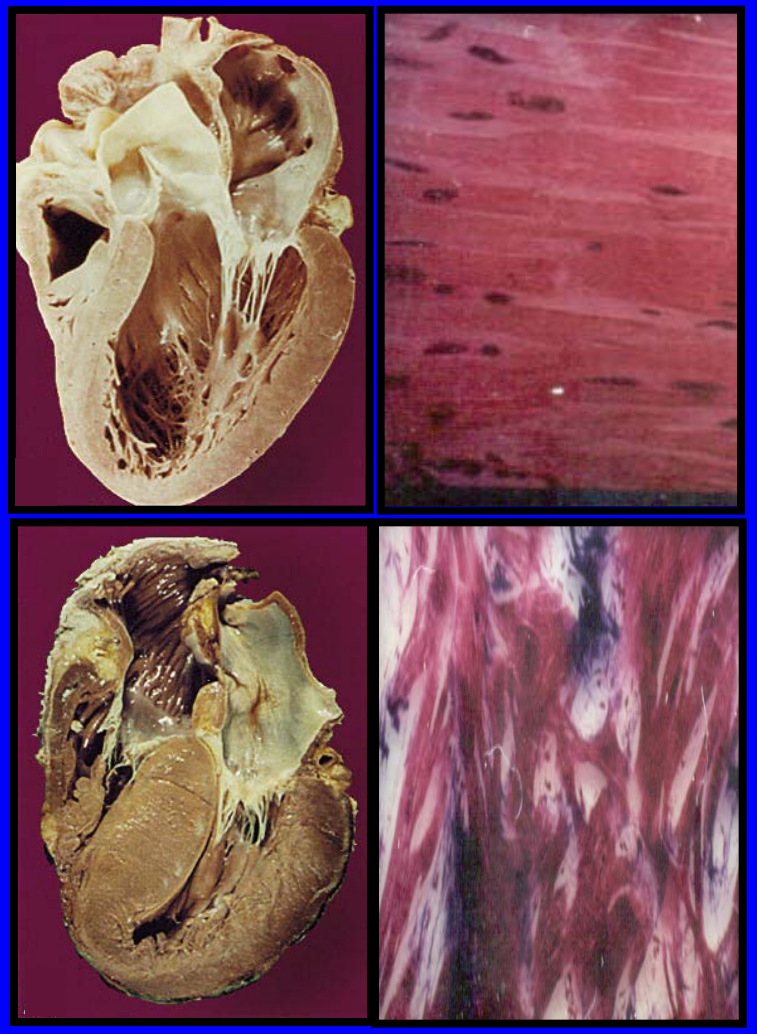
- ‘Classical’ HCM TnT, TnI, Tm, actin, MHC, MBPC, MLC, titin
- ‘Apical’ HCM TnI, Actin
- Restrictive cardiomyopathy TnI
- Dilated Cardiomyopathy TnT, TnC, Tm, actin, MHC, titin

Familial Hypertrophic Cardiomyopathy (HCM)

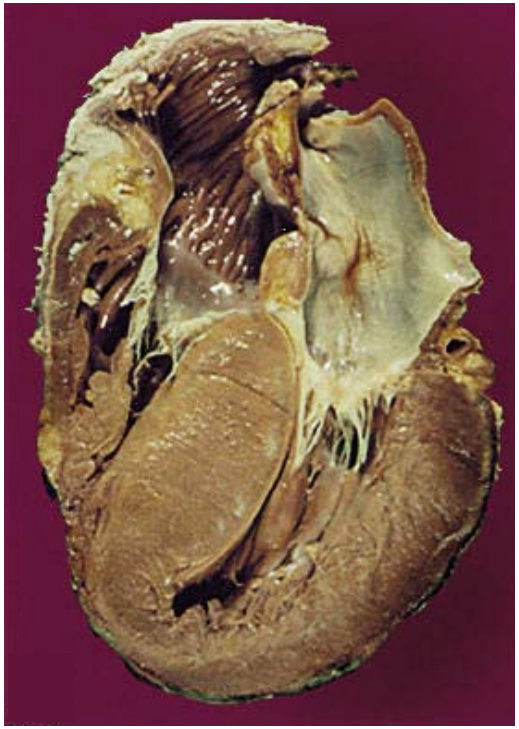
- Autosomal dominant disease
- Affects up to 1 in 500
- Onset occurs frequently during adolescence/early adulthood
- Symptoms may include chest pain, breathlessness and syncope
- Most common cause of sudden death in young adults
- Characterised by left ventricular hypertrophy and myocyte disarray

normal

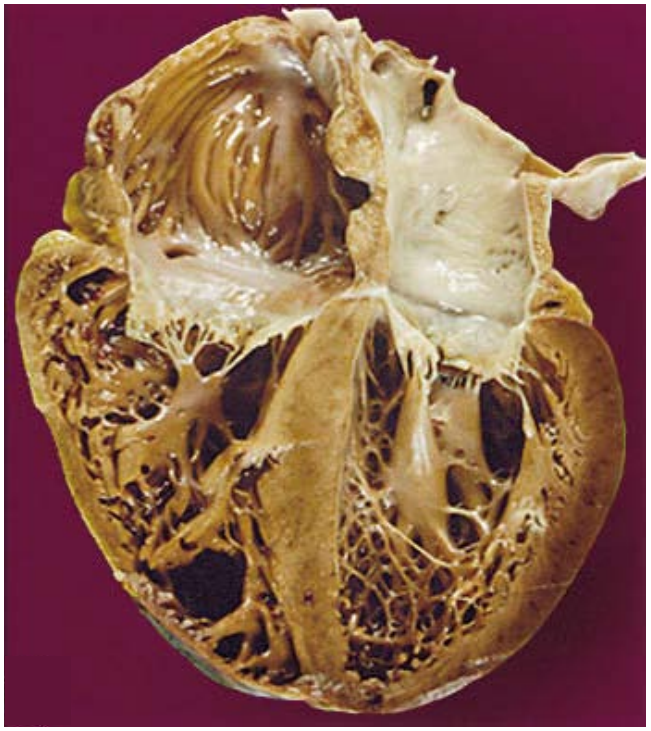
HCM



Contractile protein gene mutations can cause both Hypertrophic and Dilated Cardiomyopathies



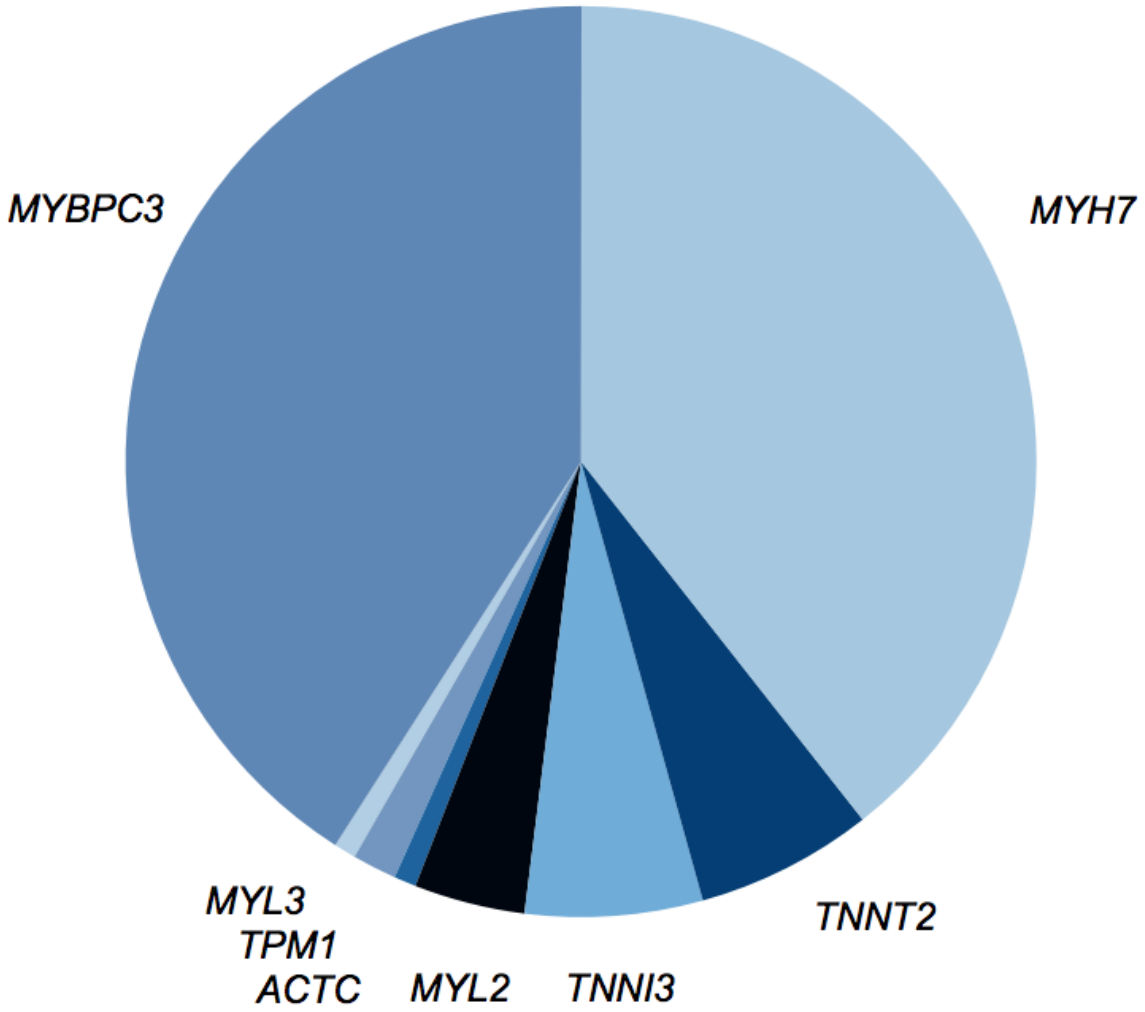
βMyHC
RLC
ELC
Titin
cMyBP-C
Actin
cTnT
cTnI
cTnC
αTM



HCM

DCM

Distribution of HCM –causing mutations in contractile protein genes
Over 600 different mutations have now been identified



Marston Figure 1

Hypertrophic Cardiomyopathy

Distribution of Disease Genes, Spectrum of Mutations, and Implications for a Molecular Diagnosis Strategy

Pascale Richard, PhD; Philippe Charron, MD, PhD; Lucie Carrier, PhD; Céline Ledeuil; Theary Cheav; Claire Pichereau; Abdelaziz Benaiche, MD; Richard Isnard, MD; Olivier Dubourg, MD; Marc Burban, MD; Jean-Pierre Gueffet, MD; Alain Millaire, MD; Michel Desnos, MD; Ketty Schwartz, PhD; Bernard Hainque, PhD; Michel Komajda, MD; for the EUROGENE Heart Failure Project

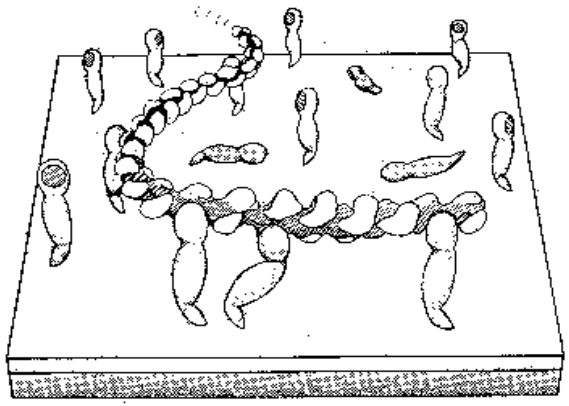
TABLE 1. Distribution of Genes in HCM-Genotyped Index Cases According to Familial or Sporadic Cases

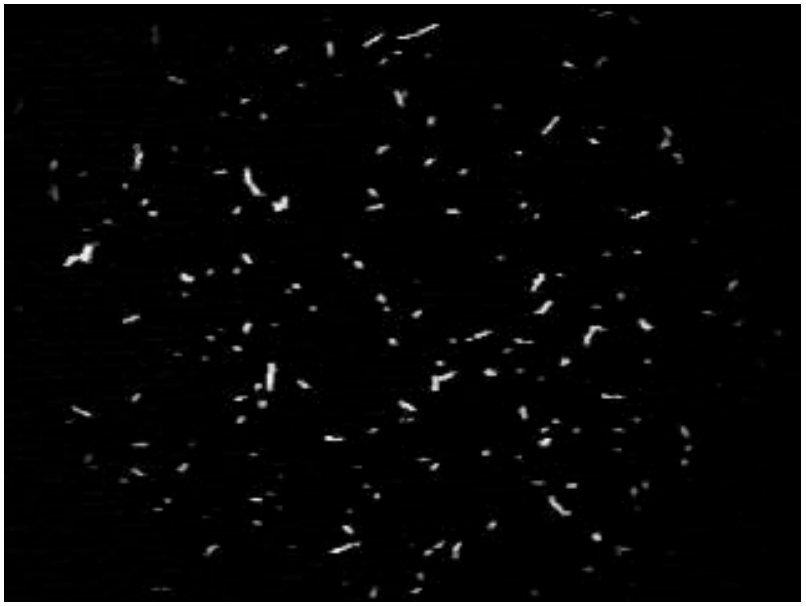
Gene	Total*	Familial HCM	Sporadic	Mutations (Novel)
Total	n=124	n=109	n=15	97 (60)
MYBPC3	52 (42%)	45 (41%)	7 (47%)	39 (25)
MYH7	50 (40%)	45 (41%)	5 (33%)	40 (24)
TNNT2	8 (6.5%)	5 (4.5%)	3 (20%)	7 (2)
TNNI3	8 (6.5%)	8 (7%)	0	7 (6)
MYL2	5 (4%)	5 (4.5%)	0	4 (2)
MYL3	1 (<1%)	1 (<1%)		1 (1)

*There were 120 initial index cases, but 2 different mutations within the same family were identified in 4 families. The distribution was therefore performed on 124 index cases.

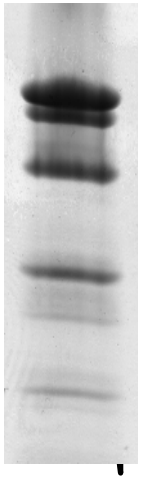
To study the contractile apparatus at the single filament level we have developed a quantitative in vitro motility assay

Thick and thin filament proteins are assembled on a microscope cover glass. The thin filaments move over the immobilised myosin in the presence of ATP and movement is controlled by Ca^{2+}. The assay therefore reproduces the functional properties of unloaded muscle in a synthetic system that can incorporate mutant proteins

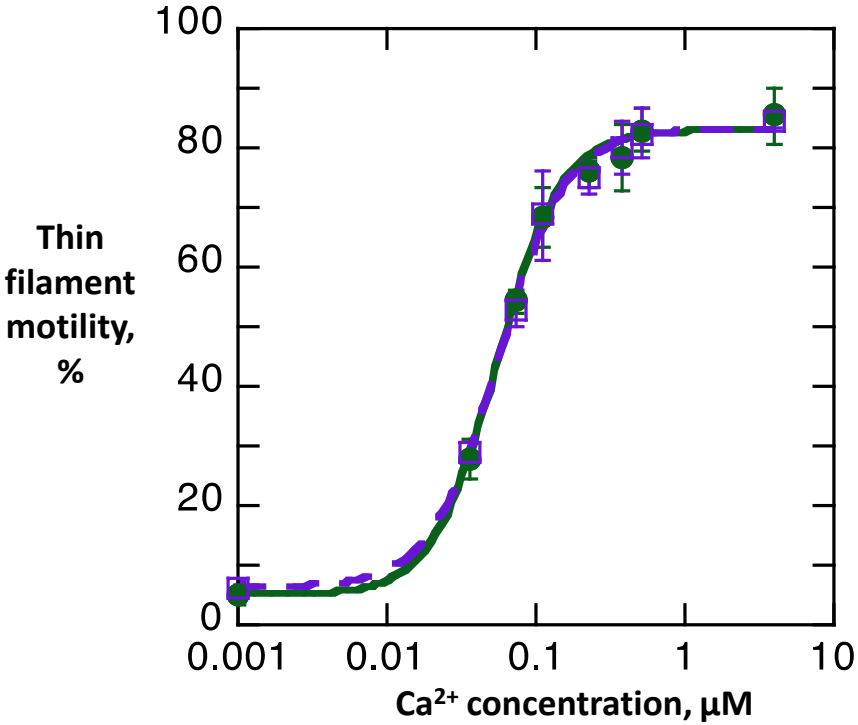


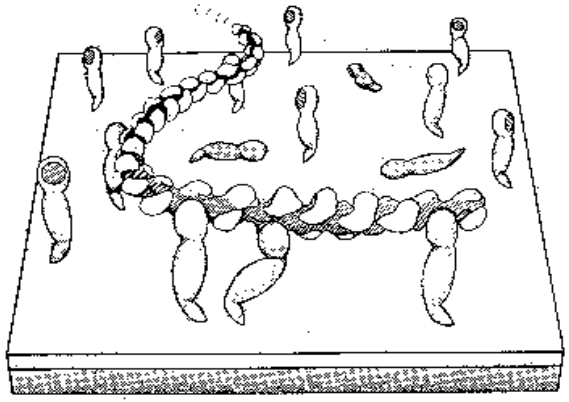


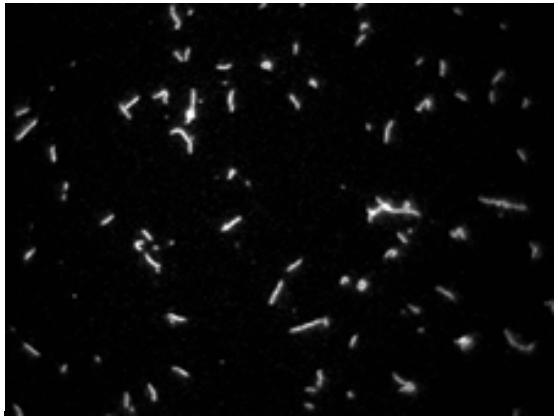
Ca^{2+}-regulation of synthetic thin filament activity studied by the *in vitro* motility assay



Reconstituted thin filament







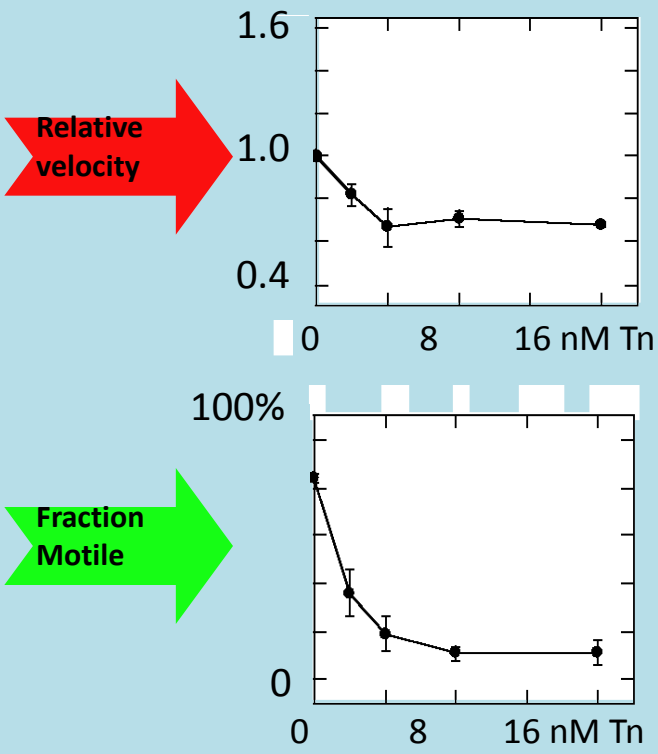
Motility of Thin Filaments is Regulated by Calcium

At pCa9 10nM troponin switches off movement

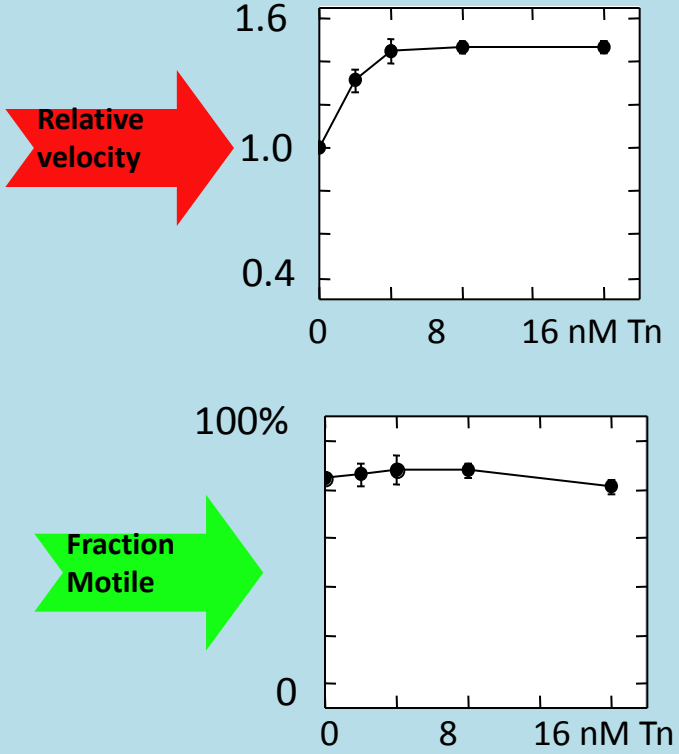
Sliding velocity is reduced by 30% and the Fraction motile is reduced to less than 5%

At pCa5 10nM troponin switches on movement

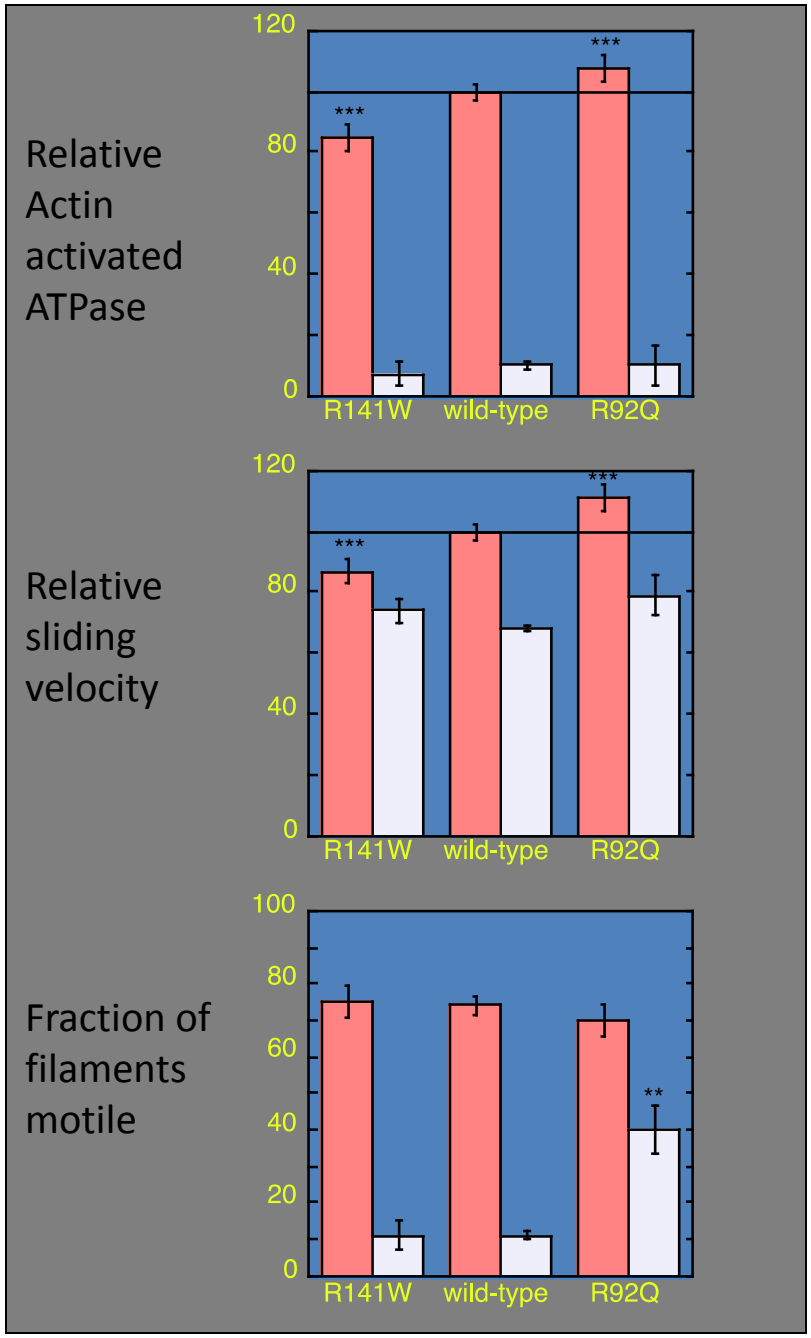
Sliding velocity increased by up to 50%**.** Fraction motile remains high



pCa9



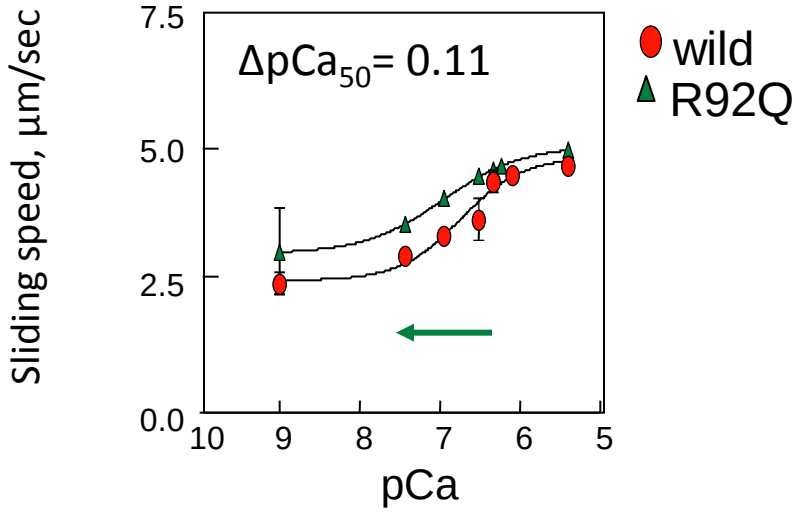
pCa5



HCM mutation TnT R92Q increases ATPase and *sliding speed* at pCa5

- The HCM mutation R92Q reproducibly gave enhanced activation of ATPase activity and thin filament sliding velocity.
- At pCa9 R92Q thin filaments gave less inhibition of ATPase and filament motility than wild type

TnT R92Q (HCM) mutation increases Ca^{2+}-sensitivity



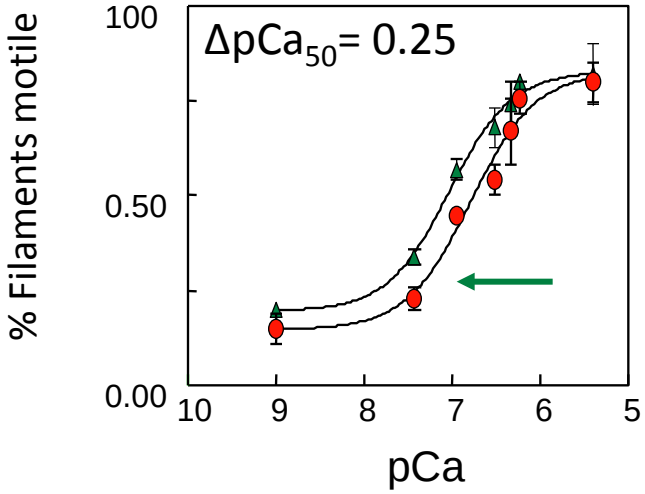


Table 2: Functional effects of sarc

All HCM mutations INCREASE myofibrillar Ca^{2+}-sensitivity

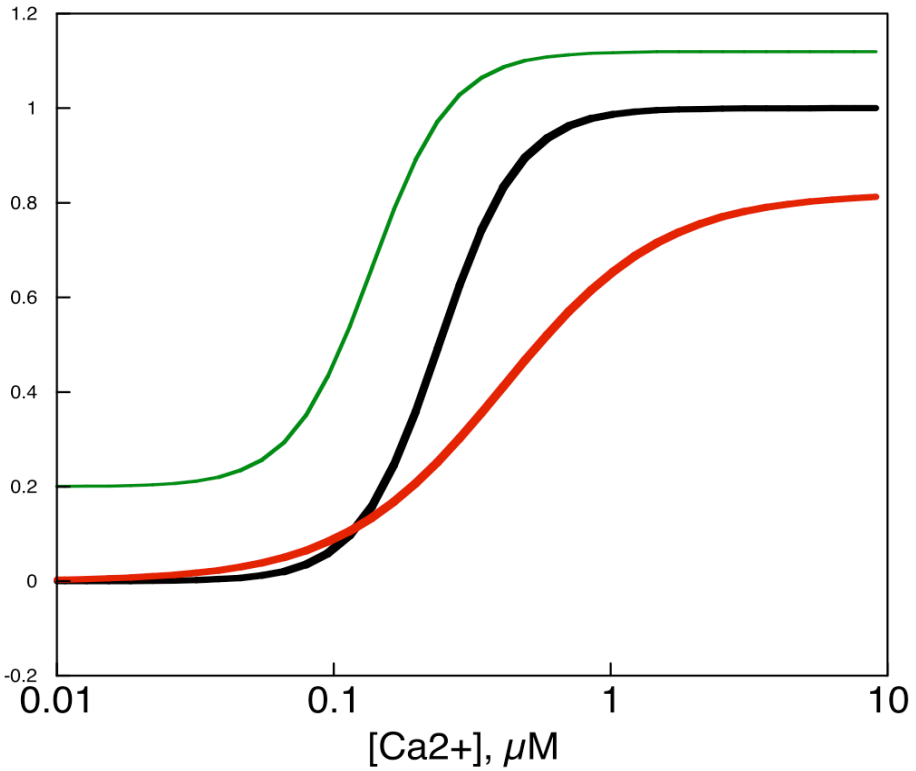
GENE	MUTATION	System	Ca^{2+}-sensitivity, ΔpCa_{50}	Switch-off at pCa9	Max turnover rate	Reference
ACTC	E99K	TG mouse tissue, motility	+0.39	normal	normal	[56]
		Human tissue, motility	+0.12	normal	normal	[56]
		TG mouse tissue, force	+0.11	normal		[56]
		Baculovirus, motility	+0.05	normal		[63]
MYBPC3	IVS17+4A>T Truncated at 868	Human tissue, skinned cells	+0.1			[19]
	c.2864_2865delCT Truncated at 860	Human tissue, skinned cells	+0.06			[20]
MYL2	R58Q	Recombinant Exchange, force	+0.11			[64]
	D166V	TG mouse tissue, force	+0.25		reduced	[35]
MYH7	R403Q	Human tissue, motility			increased	[25]
		Recombinant, motility			increased	[65]
		TG mouse tissue, force	+0.15	normal		[29]
	R453C	TG mouse tissue, force	+0.30	normal		[30]
	R719Q	Recombinant, motility			normal	[65]
	R719W	Recombinant, motility			normal	[65]
	D778G	Recombinant, motility			increased	[65]
	L908V	Human tissue, motility			increased	[25]
TNNI3	R145G	Recombinant, ATPase	+0.56	NONE		[66]
		Recombinant, ATPase	+0.32	incomplete	increased	[67]
		Recombinant Exchange, force	+0.16			[67]
	R145Q	Recombinant, ATPase	+0.23	incomplete	increased	[67]
		Recombinant Exchange, force	+0.10			[67]
	R162W	Recombinant, ATPase	+0.11	incomplete	normal	[67]
		Recombinant Exchange, force	+0.06			[67]
		Recombinant, ATPase	+0.13	incomplete		[66]
	ΔK182	Recombinant, ATPase	+0.18	normal	normal	[67]
		Recombinant Exchange, force	+0.1			[67]
	K206Q	Recombinant, ATPase	+0.18	normal		[67]
		Recombinant Exchange, force	+0.04			[67]
	G203S	Recombinant, ATPase	+0.10	normal		[67]
		Recombinant Exchange, force	+0.02			[67]
TNNT2	Exon 16/17del (truncated at 267)	Recombinant, ATPase	+0.43	NONE	normal	[68]
	R92Q	Recombinant, ATPase	+0.24	incomplete		[52]
		Recombinant Exchange, force	+0.18			[69]
	I79N	Recombinant Exchange, force	+0.15	incomplete		[69]
	R94L	Recombinant Exchange, force	+0.11			[70]
	F110I	Recombinant Exchange, force	+0.37	incomplete	decreased	[69]
	ΔE160	Recombinant Exchange, force	+0.15			[70]
	E163K	Recombinant Exchange, force	+0.07	incomplete	increased	[69]
	R278C	Recombinant Exchange, force	+0.34	incomplete	increased	[69]
TPM1	A63V	Recombinant, ATPase, motility	+0.30			[71]
	K70T	Recombinant, ATPase, motility	+0.33			[71]
	D175N	Recombinant, motility	+0.082	normal	normal	[72]
		Human tissue (skeletal), Force	+0.09	normal	normal	[62]
		TG mouse tissue, force	+0.10			[73]
	E180G	Recombinant, motility	+0.115	normal	normal	[72]

in the 25 cases published to date, the Ca^{2+}-sensitivity is increased in every one.

In contrast, relaxation may be impaired but is often normal, and crossbridge turnover rate is not always increased by HCM mutations.

Marston. *How do mutations in contractile proteins cause the primary familial cardiomyopathies?*. Journal of cardiovascular translational research (2011) vol. 4 (3) pp. 245-55

Molecular phenotype for HCM mutations in thin filament proteins: gain of function



- Increased crossbridge turnover rate, increased Ca^{2+}-sensitivity and incomplete relaxation.

Conclusion from *in vitro* motility studies

We find a distinctive molecular phenotype associated with each pathology

Contractility parameter	Max crossbridge cycling rate	relaxation	Ca^{2+}-sensitivity
Genetic HCM	↑	↓	↑
Genetic DCM	↓	→	↓
Acquired DCM	↓	→	↑

How is this molecular phenotype related to the phenotype in whole hearts?

Genetic cardiomyopathy

The consistent pattern of results we have obtained with synthetic thin filaments suggests the distinct molecular phenotypes observed with HCM and DCM mutations are responsible for initiating the disease.

- To test this hypothesis this we need to study mutations in intact muscle.

HOW ?

- Study human tissue samples with known mutations (e.g myectomy samples from HOCM patients)

- Express disease-causing mutations in transgenic mouse that replicate the HCM phenotype

Investigations of hypertrophic cardiomyopathy using intact tissue

- HCM mutation in *MYBPC3* from a human myectomy sample
- HCM mutation in *ACTC* from a transgenic mouse and a human biopsy

Mode of action: dominant-negative or haploinsufficiency?

Most studies have suggested that the mutant proteins produced are incorporated into the sarcomere where they presumably act as “poison polypeptides”

However it is possible that a mutant protein might be unstable and thus be absent from the heart thus causing disease due to hapoinsufficiency

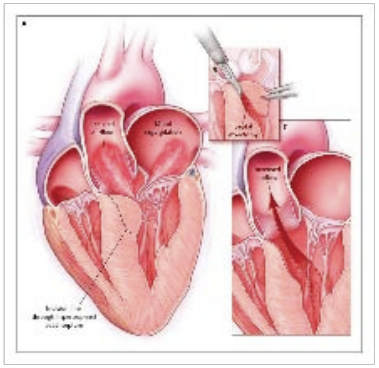
Both mechanisms have been found in HCM

→ How do these mutant proteins affect the contractility of the heart?

→ How do the changes in contractility give rise to disease?

Heart samples with known mutations obtained from septal myectomy

- The surgical septal myectomy operation removes 3-7g of myocardium from the interventricular septum in the region of the obstruction and the excised muscle is a unique source of human heart muscle with HCM. We have compared the functional and structural properties of myectomy muscle with non-failing donor heart muscle as a control to determine the molecular phenotype of HCM muscle and ultimately to link this to the disease causing mutation.



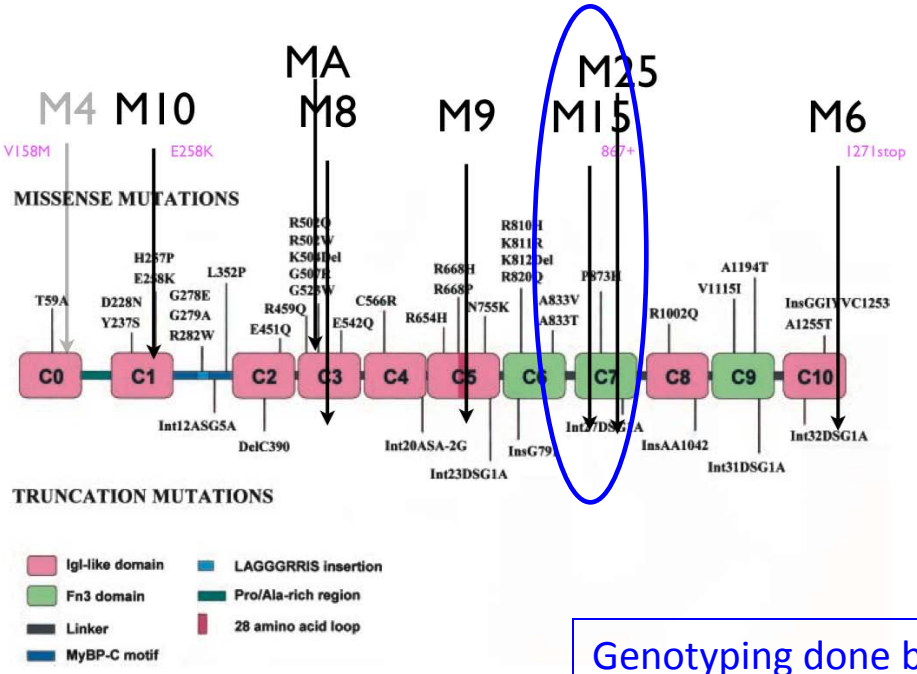
- We studied myectomy samples from a patient M15 with a mutation in MyBP-C

Clinical details, myectomy patient M15

Biopsy sample	M15
Diagnosis	HCM PPM ASA 2004
Age	42
Gender	M
Diagnosed	1993
Family History	Father died 49yrs ? SCD Paternal grandfather died young ? SCD
Current Treatment	Verapamil Disopyramide Warfarin Salbutamol
ECHO appearance	ASH
Max LVWT (mm)	23
Max. ST(mm)	23
LVEDD (mm)	460
LVESD (mm)	300
LA (mm)	49
FS (%)	35
SAM	Incomplete
MR	Mild
Resting LVOT Gradient	44mmHg (Valsalva 116mmHg)
VT on Holter Monitor	No
ETT MVO2 (ml/min/kg)	28.1 78% predicted No arrhythmias BP rise
ECG	Paced
NYHA Class	III

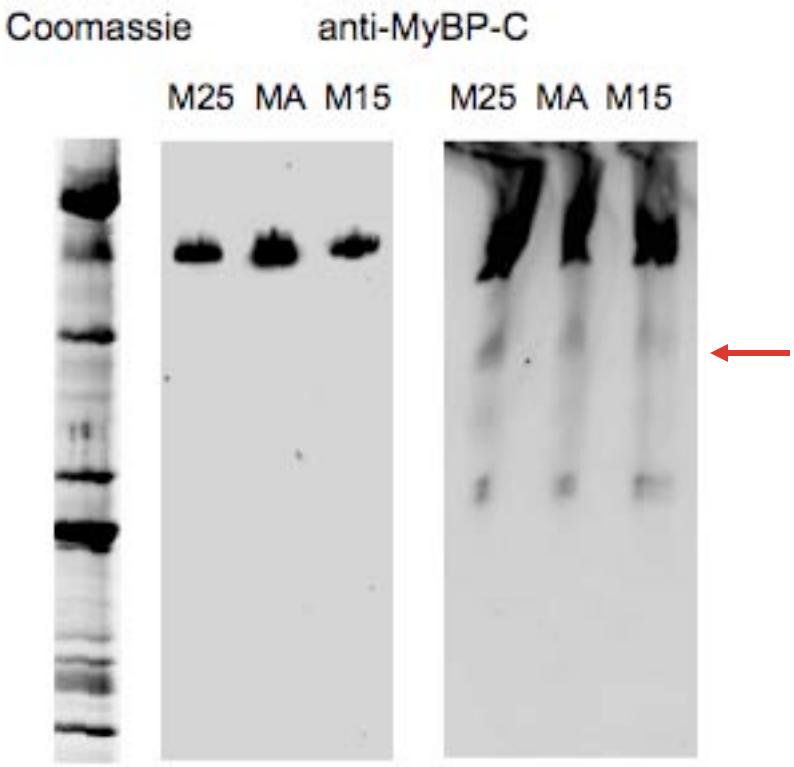
We have studied septal muscle from myectomy patient M15

This patient has mutation in MYBPC3 gene which is predicted to cause premature truncation in domain C7



Genotyping done by Hugh Watkins' lab, Oxford

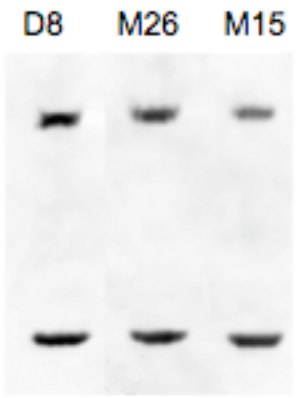
Tissue samples were separated on SDS-PAGE and probed with antibody to the N-terminus of MyBP-C

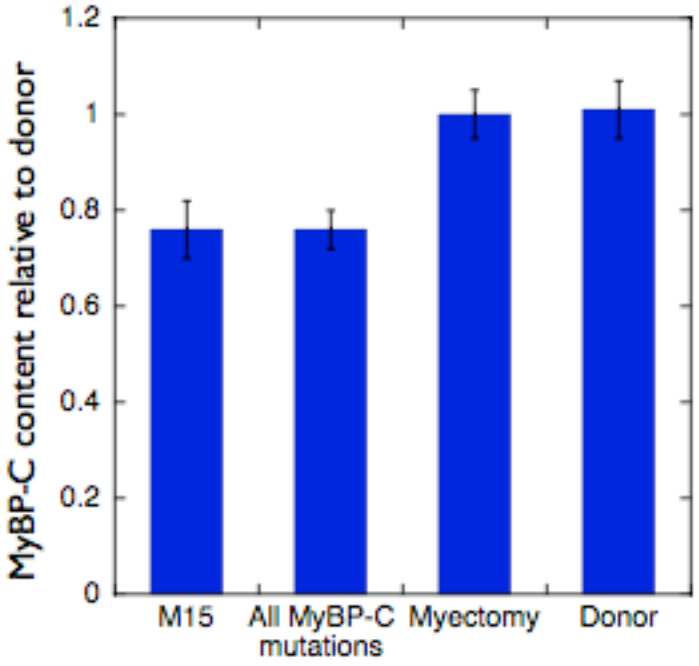


Normal loading overloaded

The mutant protein is not expressed in the patient's muscle

The quantity of MyBP-C relative to actin was measured

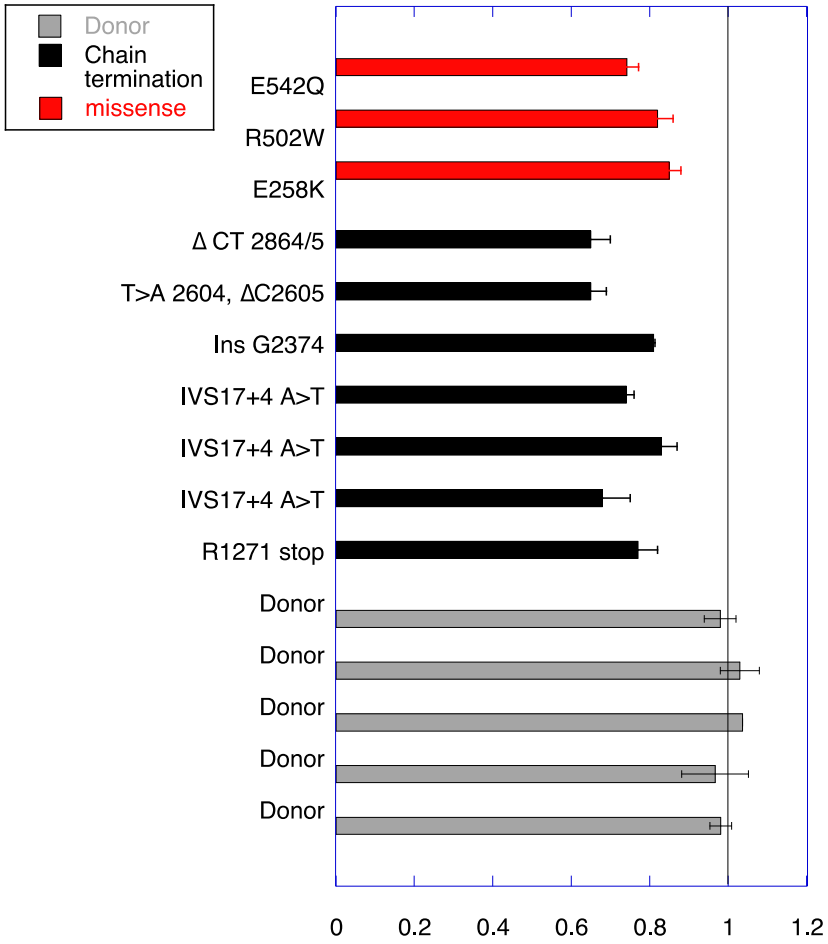




Total MyBP-C expression is 22% less than in donor heart

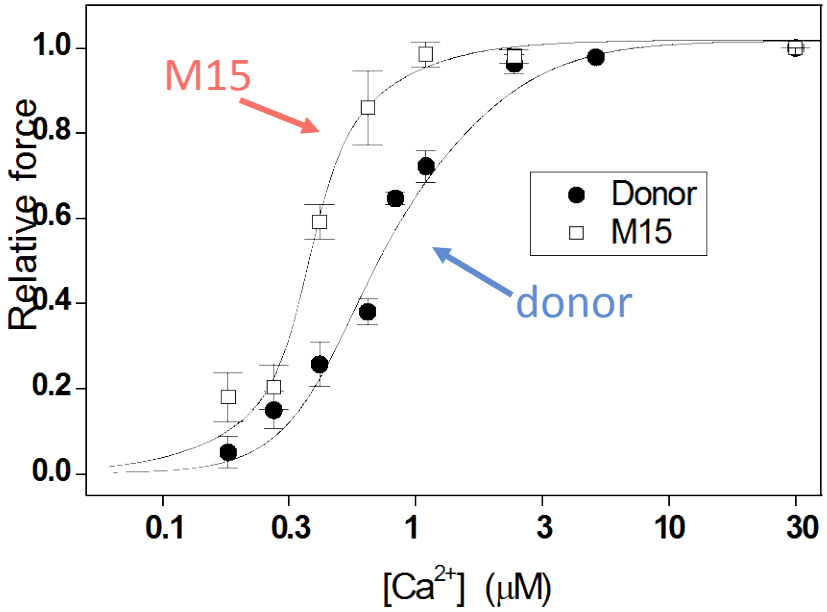
Evidence for haplo-insufficiency that may be disease-causing

Haploinsufficiency was found in every MYBPC-3 mutation studied, including mis-sense mutations



The force-pCa relationship was measured in skinnned myocytes

(Anita Hoskins and Jon Kentish, KCL)



Increase in Ca^{2+}-sensitivity and in rate of crossbridge turnover

Typical HCM molecular phenotype

M15- HCM due to MyBP-C mutation

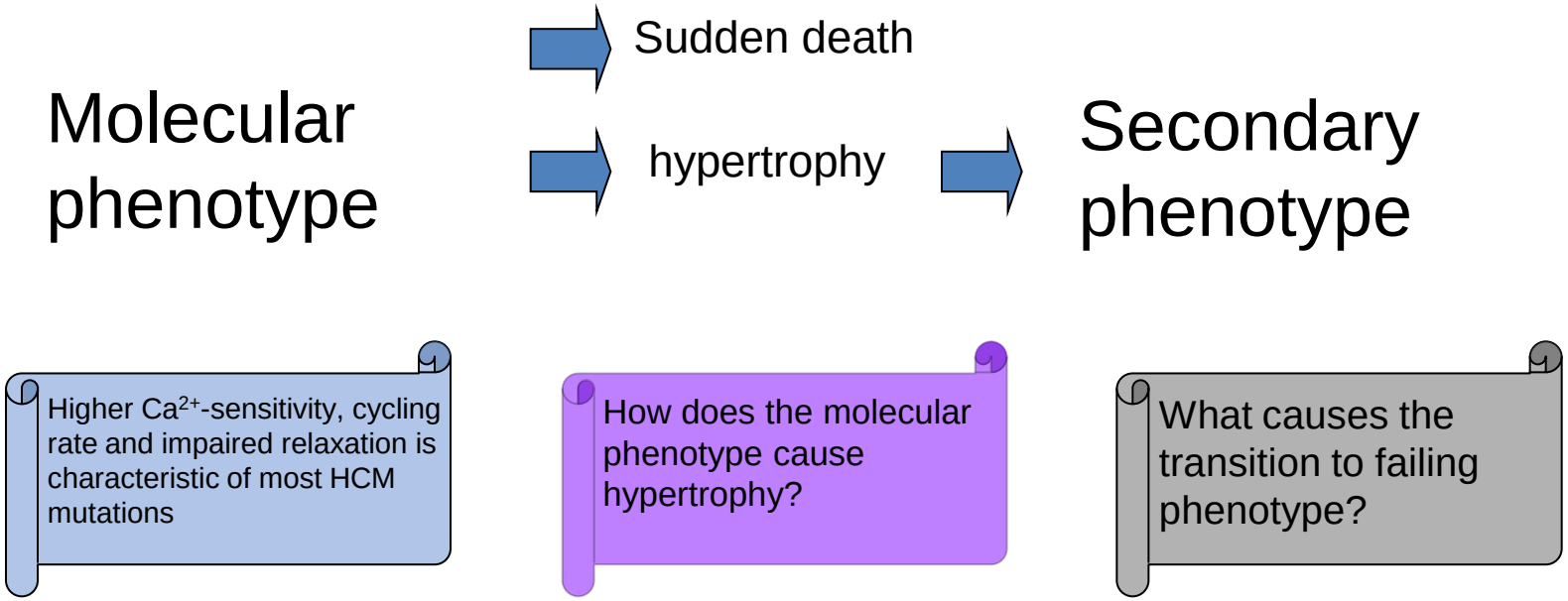
In measurements at the molecular level the M15 MyBP-C Mutation is associated with haplo-insufficiency and an increase in Ca^{2+}-sensitivity and in rate of crossbridge turnover.

Typical HCM molecular phenotype

BUT

Studies of intact muscle indicate severe contractile dysfunction that resembles failing heart muscle

The HCM phenotype is complex

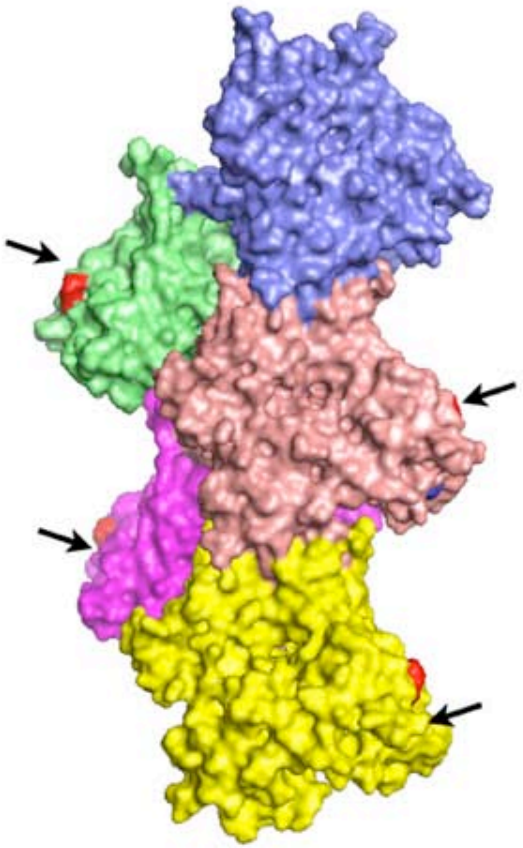


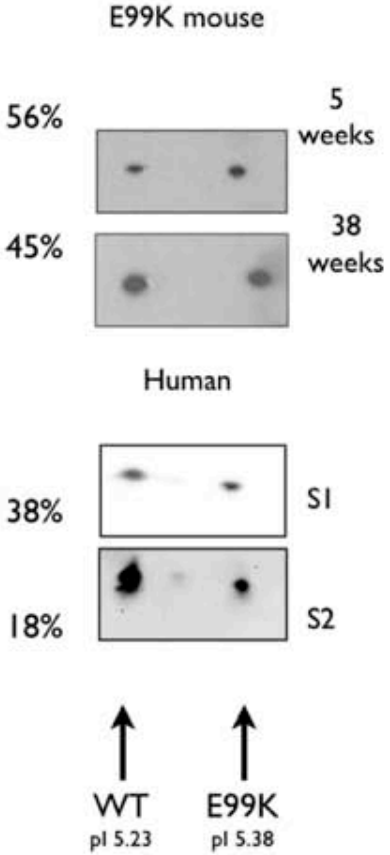
Cardiac actin E99K mutation

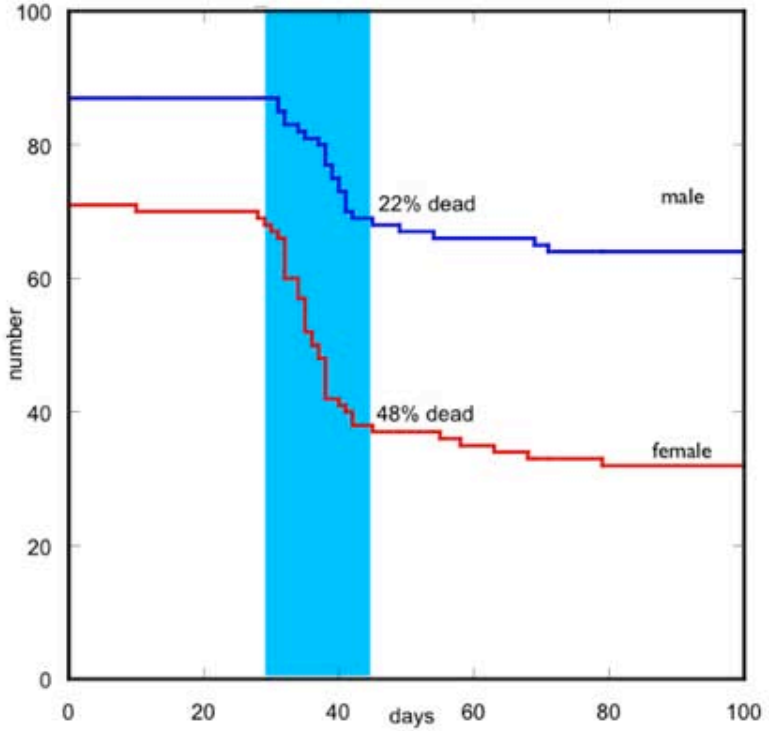
- An *ACTC* E99K actin transgenic mouse was developed as a model of hypertrophic cardiomyopathy.
- A small piece of biopsy tissue was obtained from an HCM patient with the *ACTC* E99K mutation who underwent a operation to repair an atrial-septal defect.
- A direct comparison between human diseased tissue and mouse model was made.

Transgenic mouse model of HCM

The mutation Gly99lys (E99K) in the cardiac actin (ACTC) gene is reported to cause Hypertrophic Cardiomyopathy





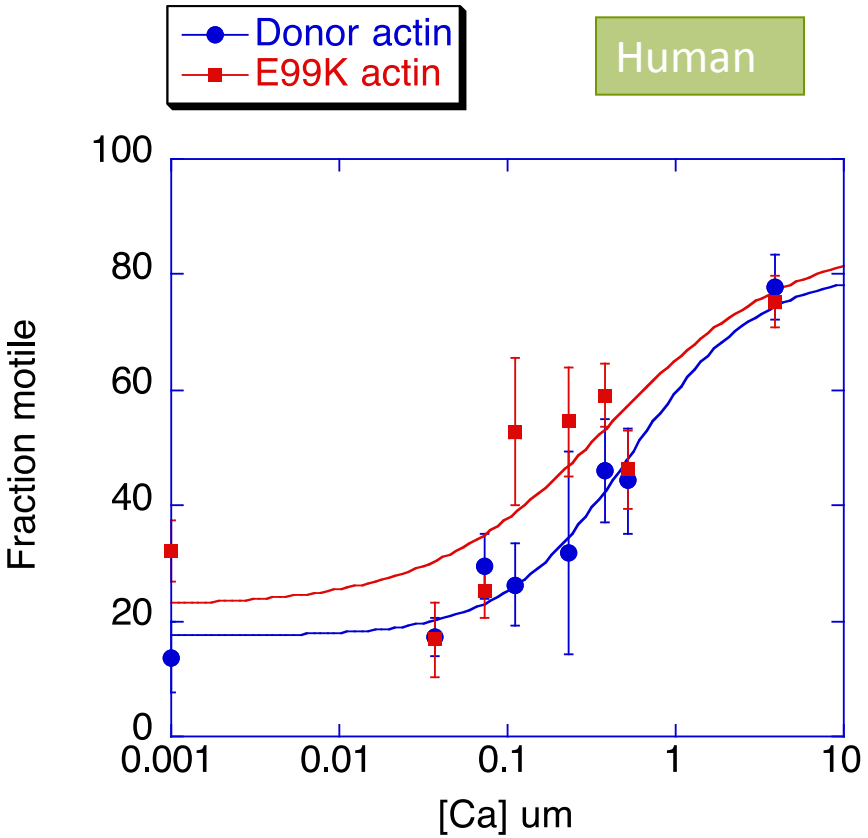


Results: E99K vs Donor actin with native donor troponin

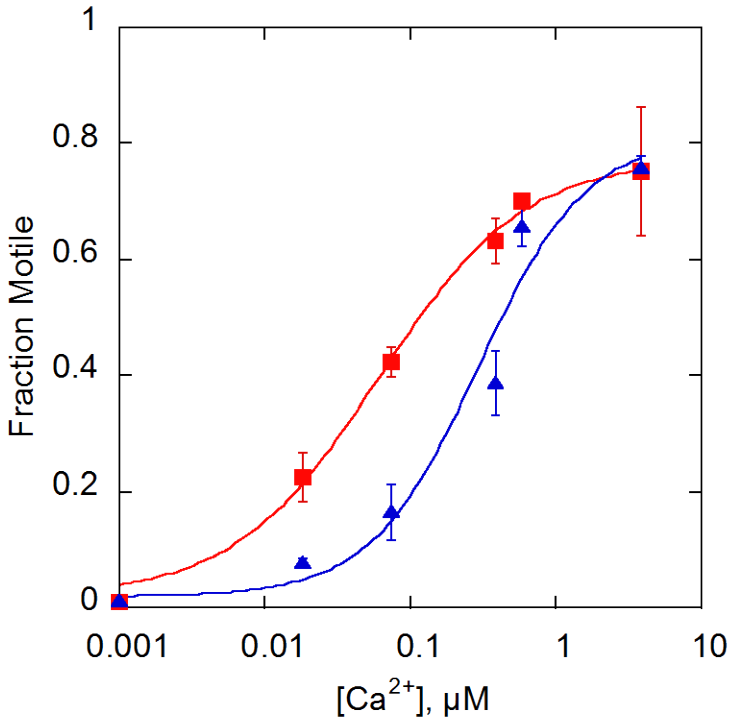
Donor troponin: 1.62 molsPi/molsTnI

Increased Ca2+-sensitivity

Human



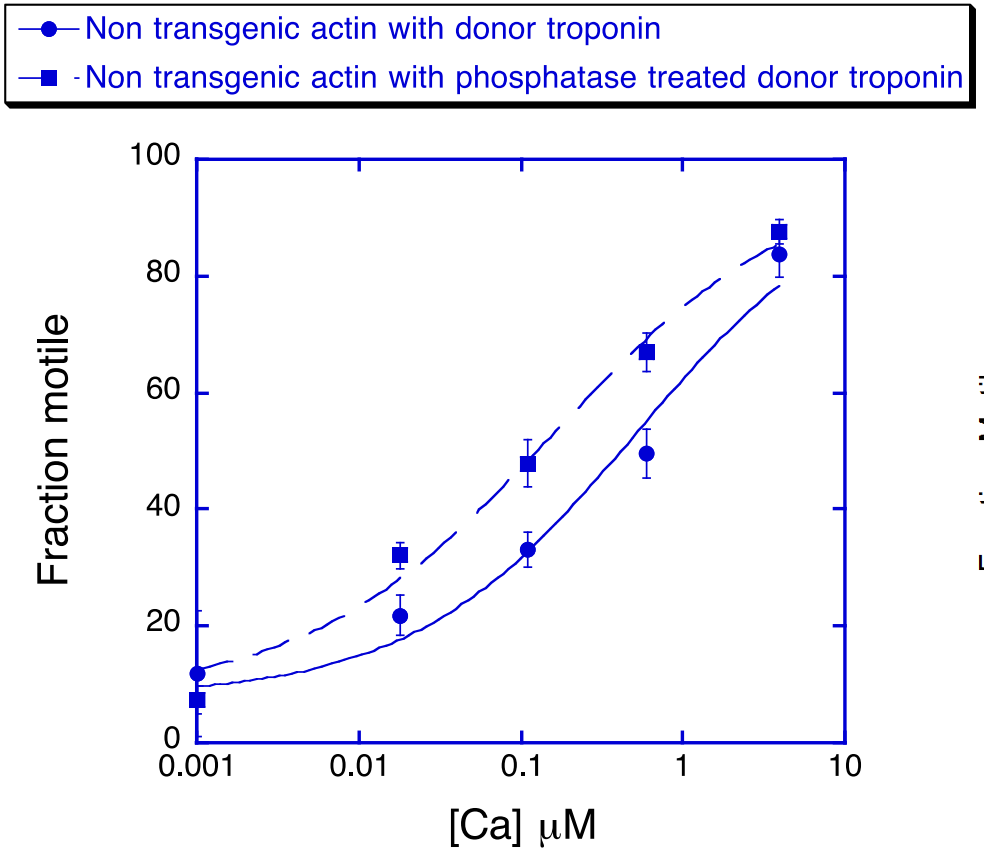
Mouse

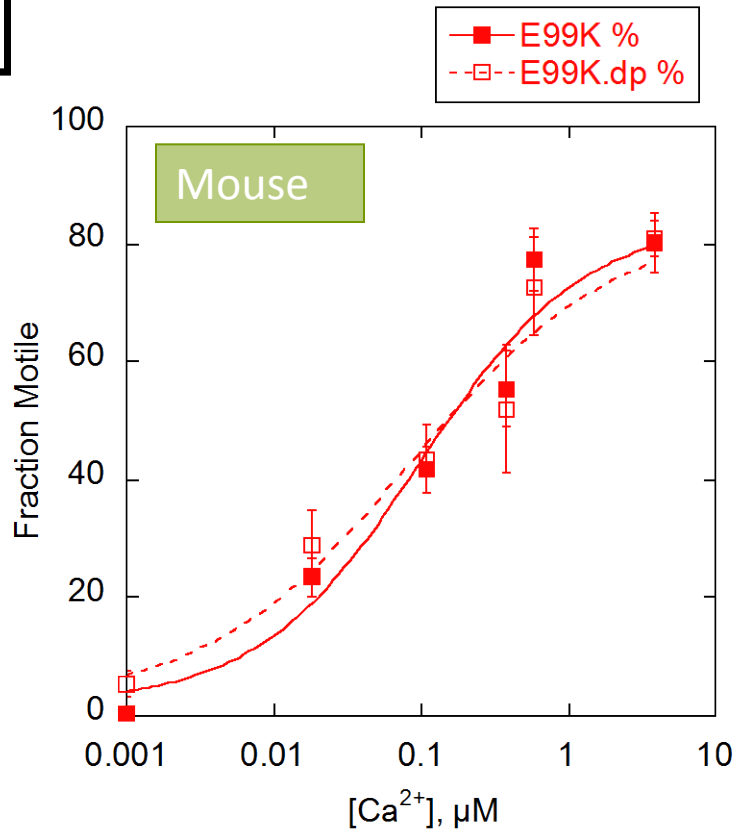


Donor EC_{50} (µM)	SD	E99K EC_{50} (µM)	SD	Donor/E99K	SD	n	t-test vs one
0.42	± 0.10	0.34	± 0.12	1.33	± 0.26	5	P = 0.05

Results: E99K actin with phosphorylated and unphosphorylated troponin

Uncoupling of Ca2+-sensitivity from phosphorylation

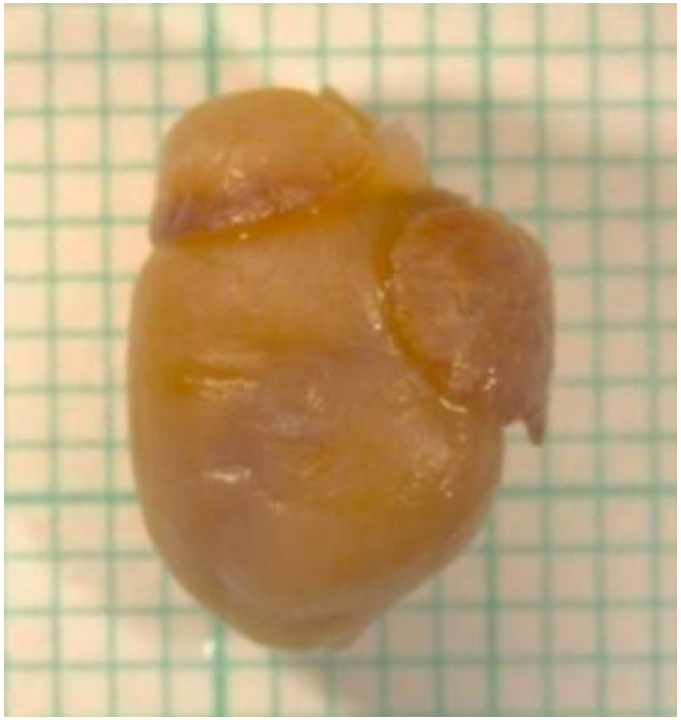


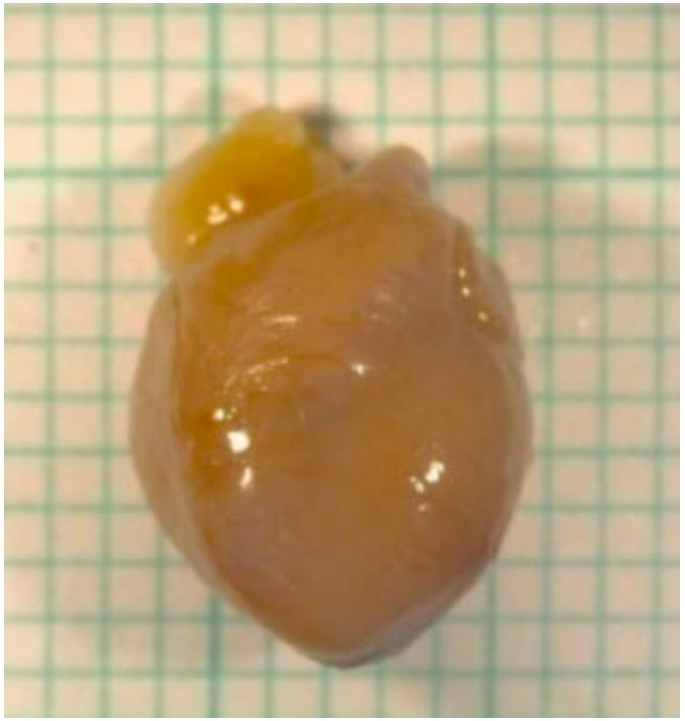


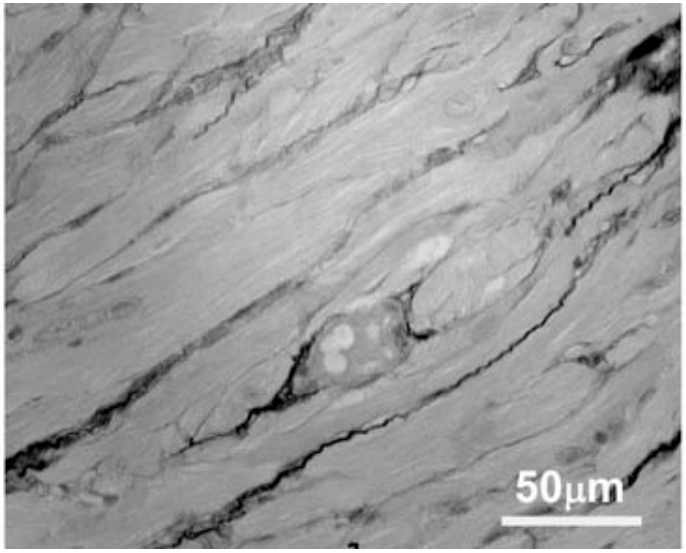
- There was no difference when E99K actin was reconstituted with donor and failing troponin.

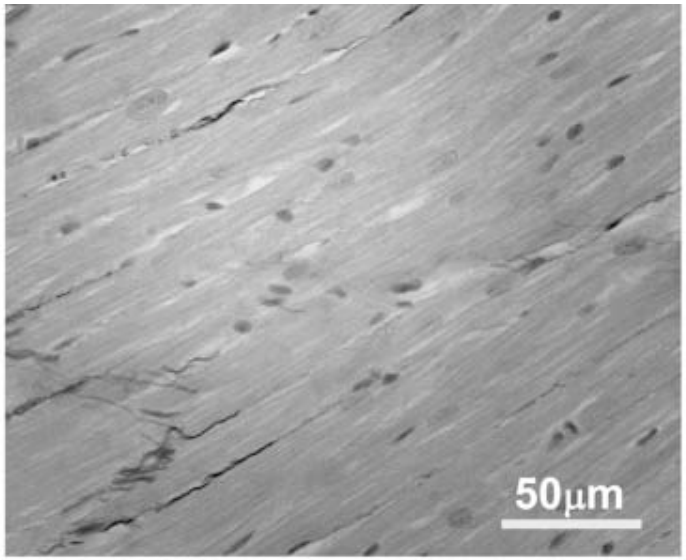
ACTC E99K

NTG



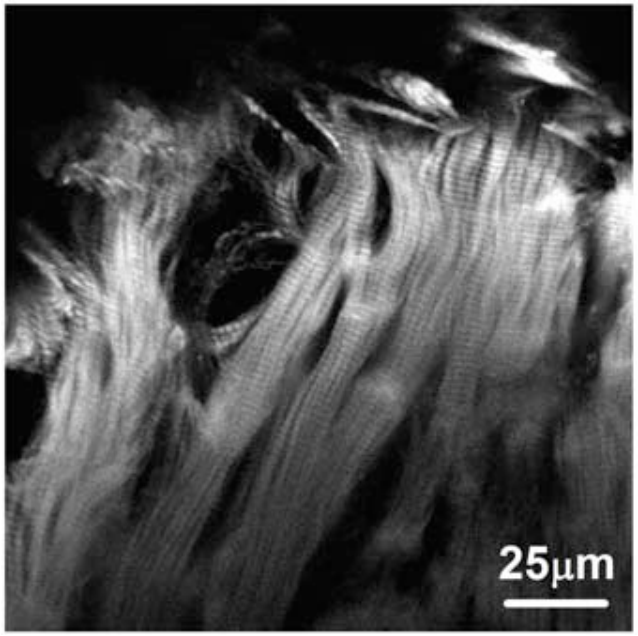


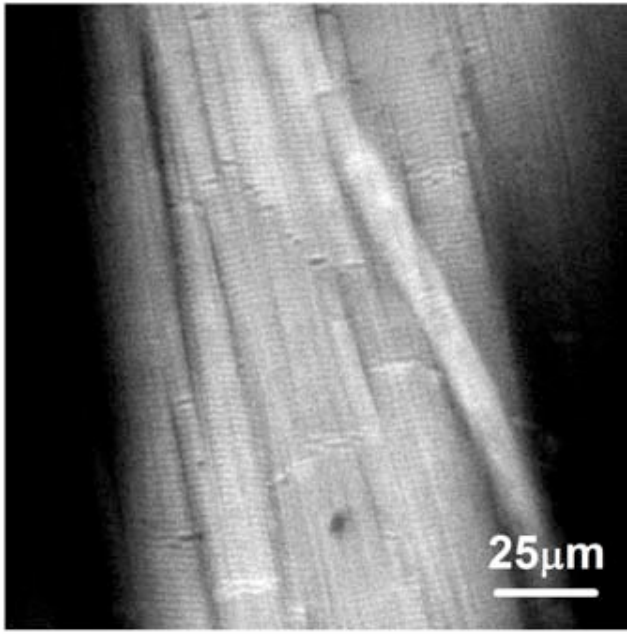


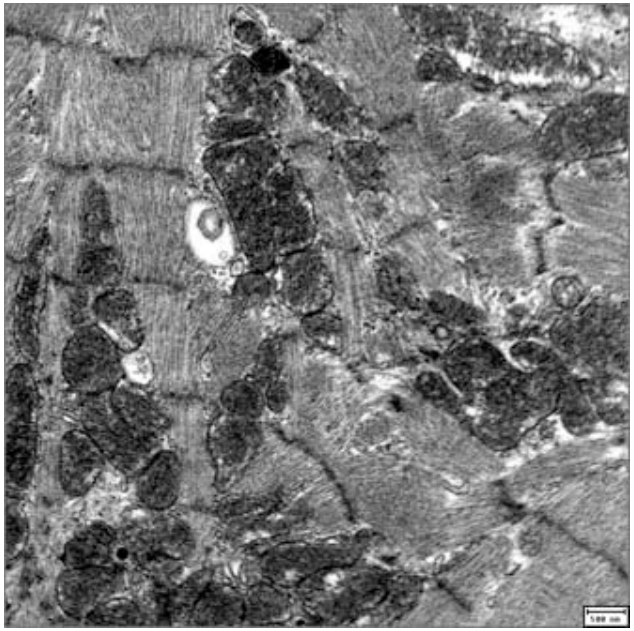


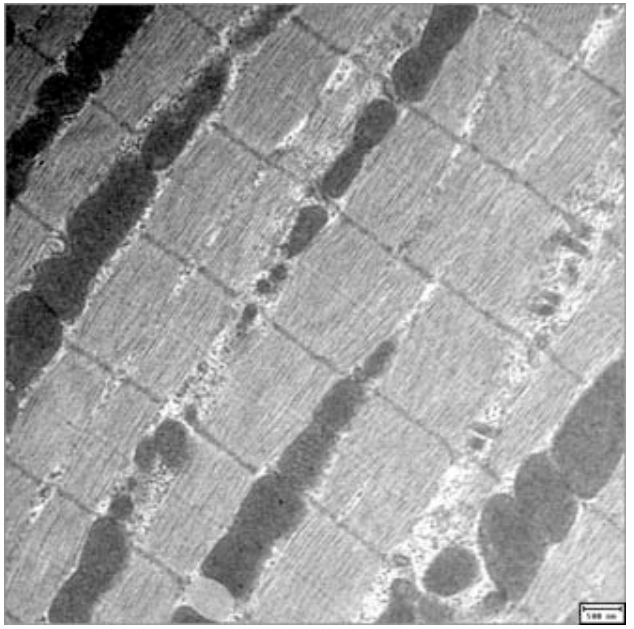
ACTC E99K

NTG

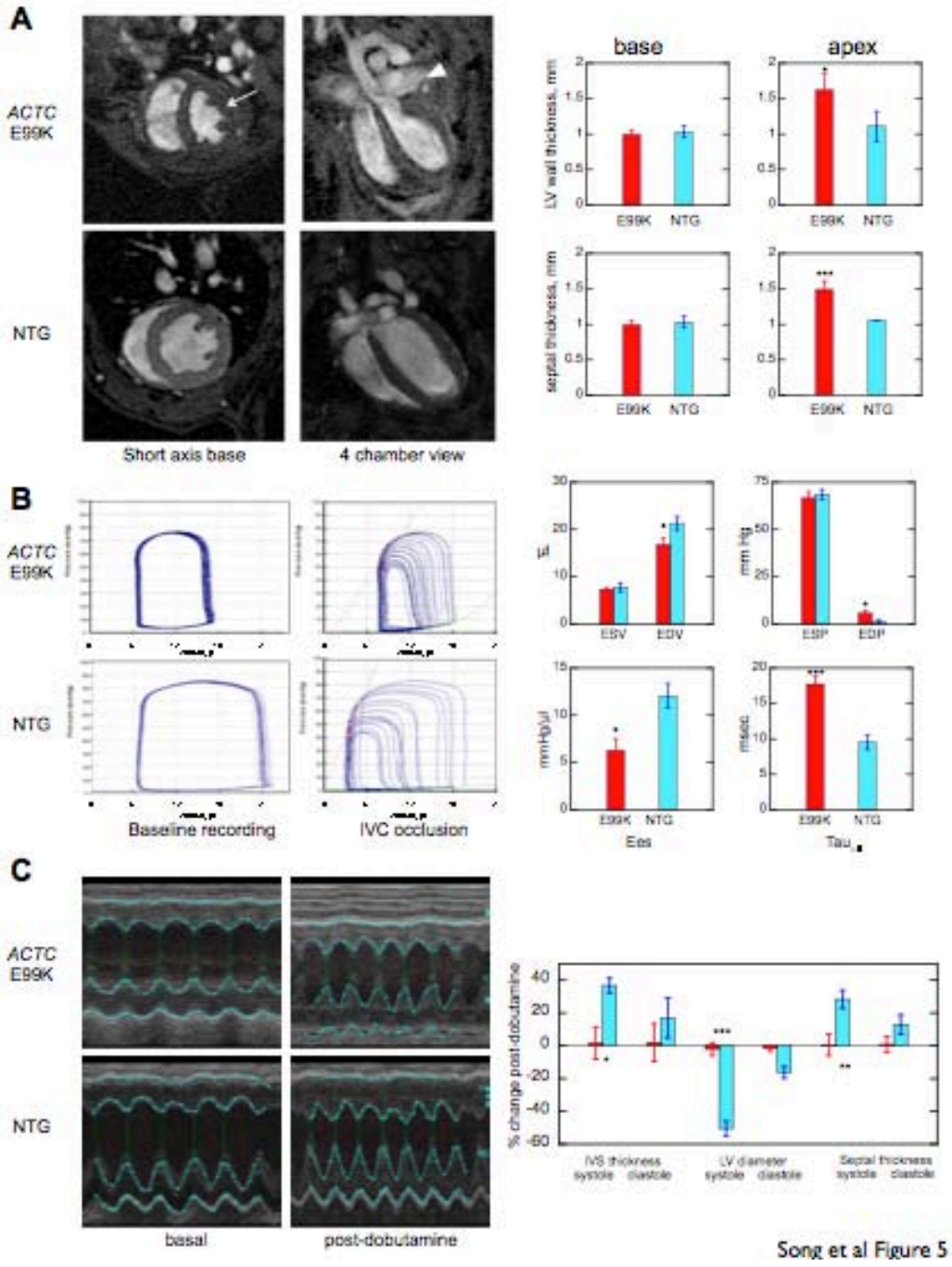






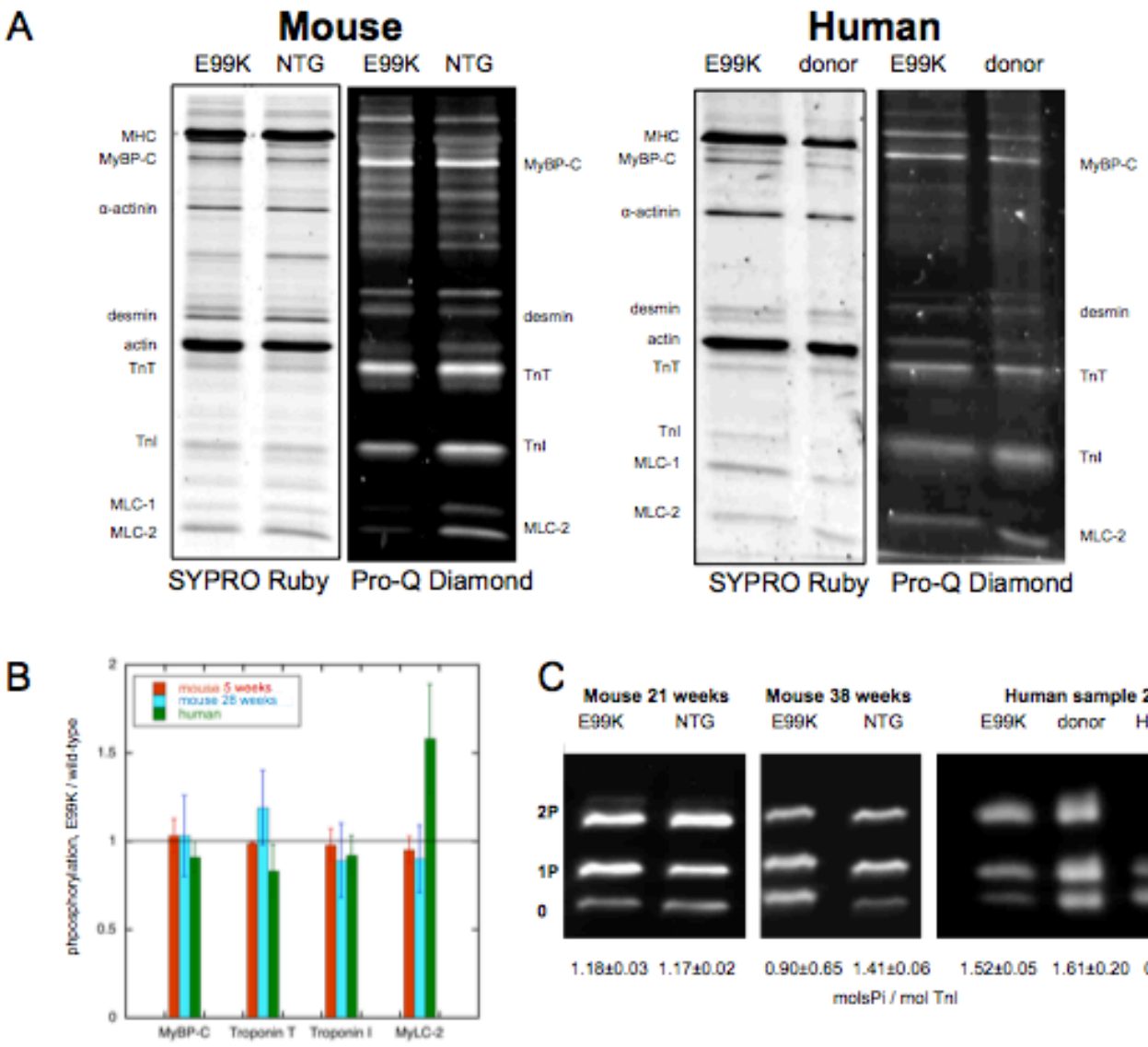


Song et al. Figure 2



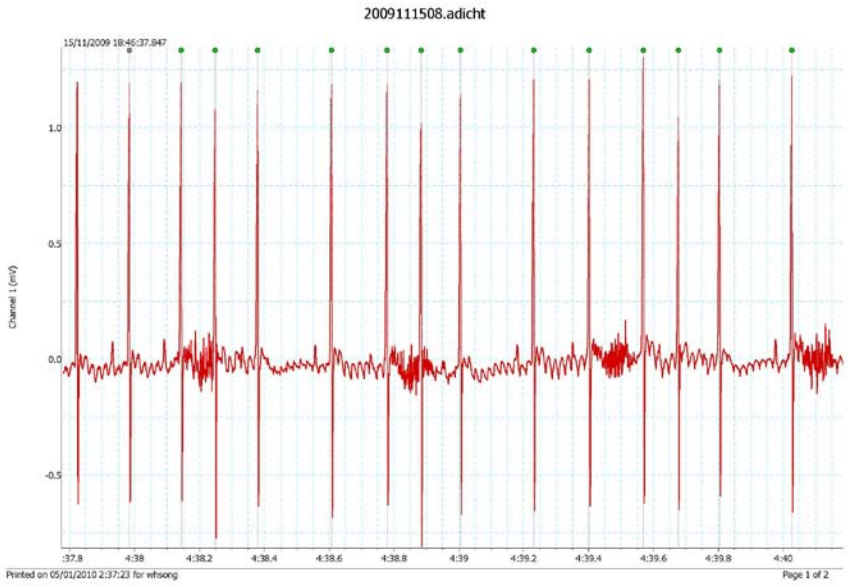
Song et al Figure 5

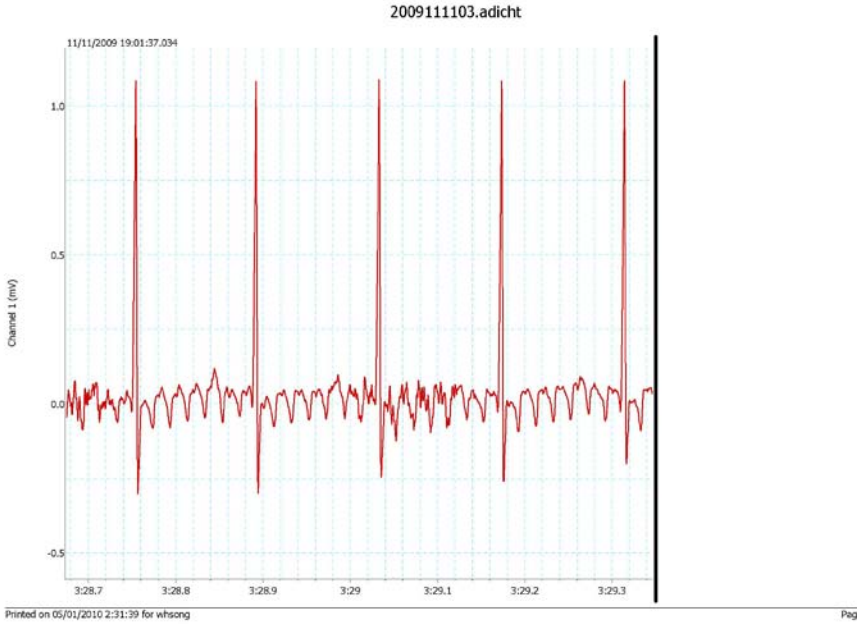
Phosphorylation of myofibrillar proteins



ECG of 7 month *ACTC* E99K mice

Frequent ectopic beats and atrial flutter





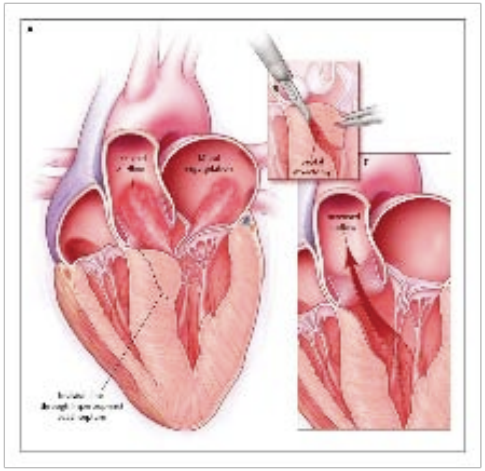
Conclusions: molecular mechanisms of HCM mutations in intact tissue

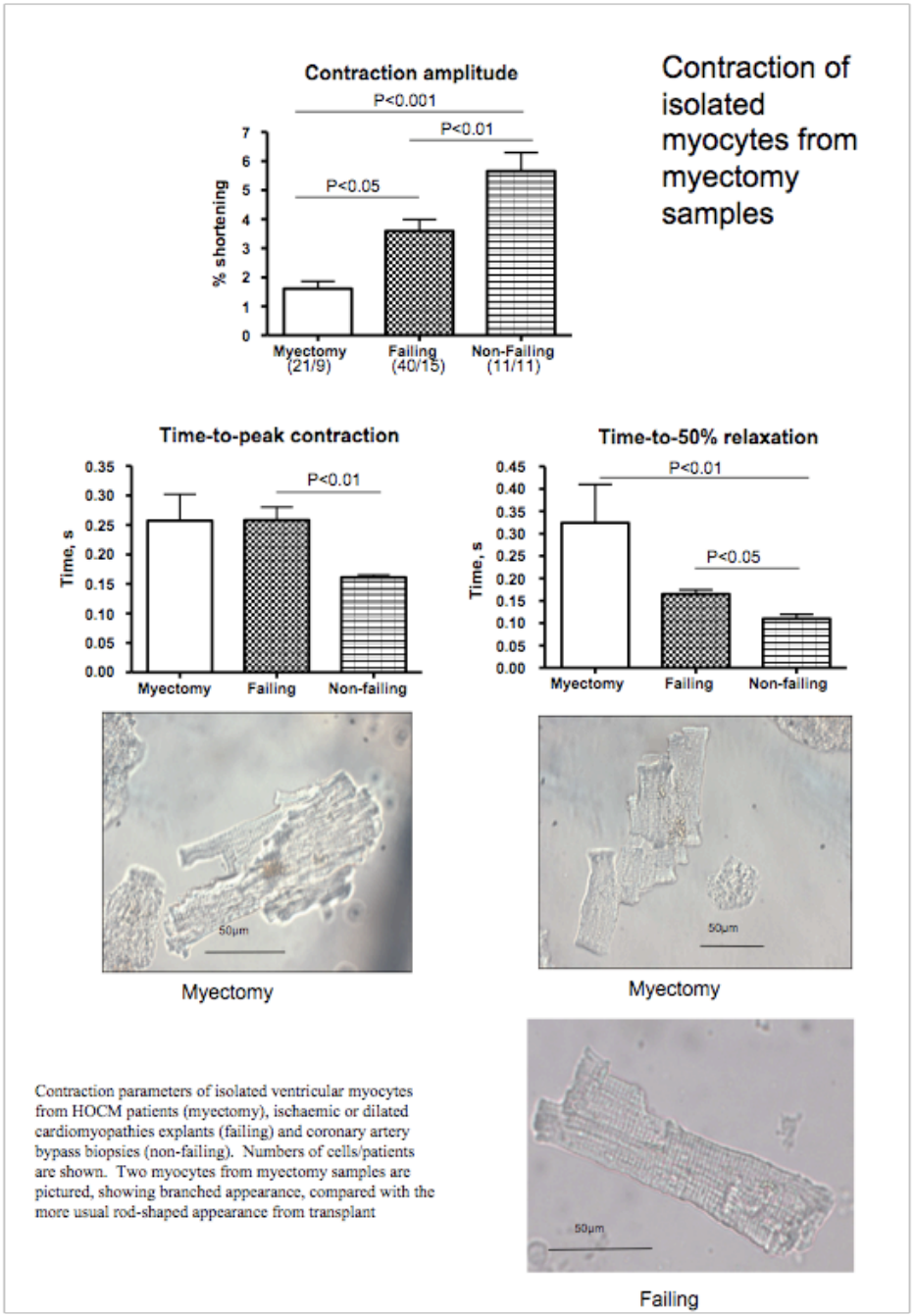
- The E99K mutation causes sudden cardiac death at 5 weeks, hypertrophy with diastolic dysfunction but normal contractility at 5 months and dilated cardiomyopathy with systolic dysfunction at 9months
- At the molecular level the E99K mutation causes an increase in thin filament Ca^{2+}-sensitivity, replicating results of previous investigations of HCM mutations in thin filament proteins.
- The E99K mutant also causes uncoupling of the relationship between Ca^{2+}-sensitivity of the thin filament and constituent TnI phosphorylation. This uncoupling must be a direct effect of the mutation.
- Transgenic mouse and human sample give the same result at the single filament level

.

Heart muscle samples obtained from septal myectomy show abnormal contractility independent of the HCM-causing mutation

This complicates interpretation of functional experiments





Contraction parameters of isolated ventricular myocytes from HOCM patients (myectomy), ischaemic or dilated cardiomyopathies explants (failing) and coronary artery bypass biopsies (non-failing). Numbers of cells/patients are shown. Two myocytes from myectomy samples are pictured, showing branched appearance, compared with the more usual rod-shaped appearance from transplant

Contractile dysfunction myocytes

Isolated myocytes had abnormal shapes,
contractile amplitude was low and both contraction and relaxation was slow.

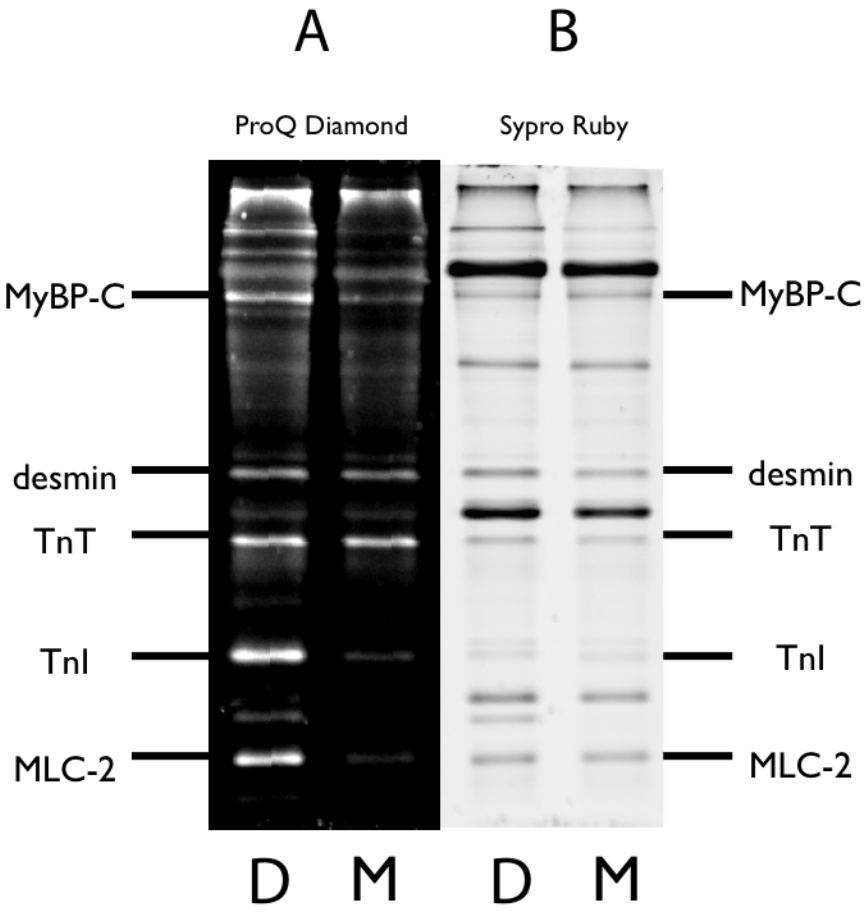
This contractile dysfunction resembles failing heart although the defect in myectomy muscle is more pronounced.

The dysfunction was found in all myectomy samples and thus is not related to the genotype

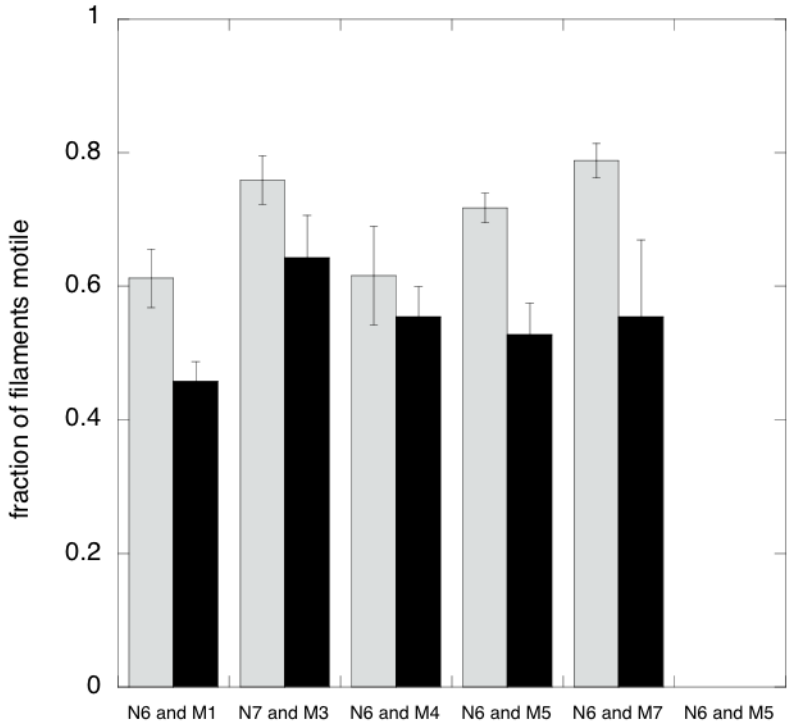
Data of Sian Harding, Edwin Garcia and Gaelle Kikonda Kanda

Reduced contractile protein phosphorylation in HOCM muscle

- 50% reduction in Myosin-binding protein C phosphorylation
- 10% reduction in troponin T phosphorylation
- 85% reduction in troponin I phosphorylation
- 44% reduction in MLC-2 phosphorylation



Functional abnormality in myosin from HOCM muscle:16% lower motility than donor myosin in the *in vitro* motility assay



Functional changes cannot be explained by changes in phosphorylation; may be due to oxidative stress

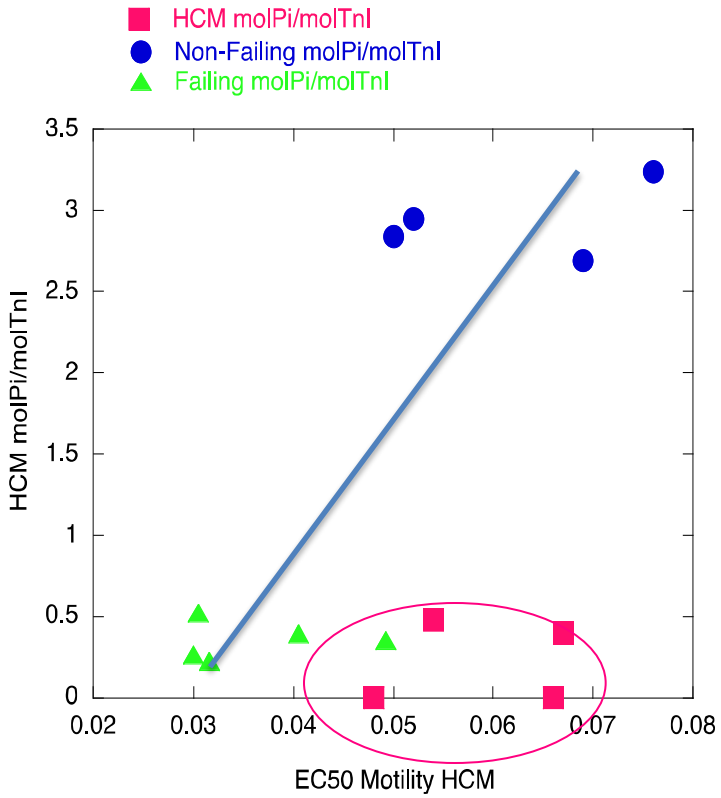
Functional abnormality in troponin from HOCM muscle

PARADOX:

In previous work we showed that Ca^{2+}-sensitivity and crossbridge turnover rate are dependent upon the level of troponin I phosphorylation. This is sufficient to account for the difference between failing and non-failing heart troponin.

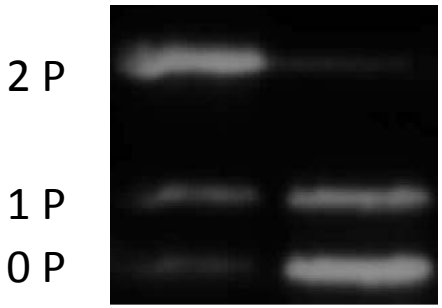
BUT

In HCM muscle we observe very low phosphorylation levels (as in failing heart troponin) yet EC_{50} and crossbridge turnover rate are the same as non-failing heart troponin.

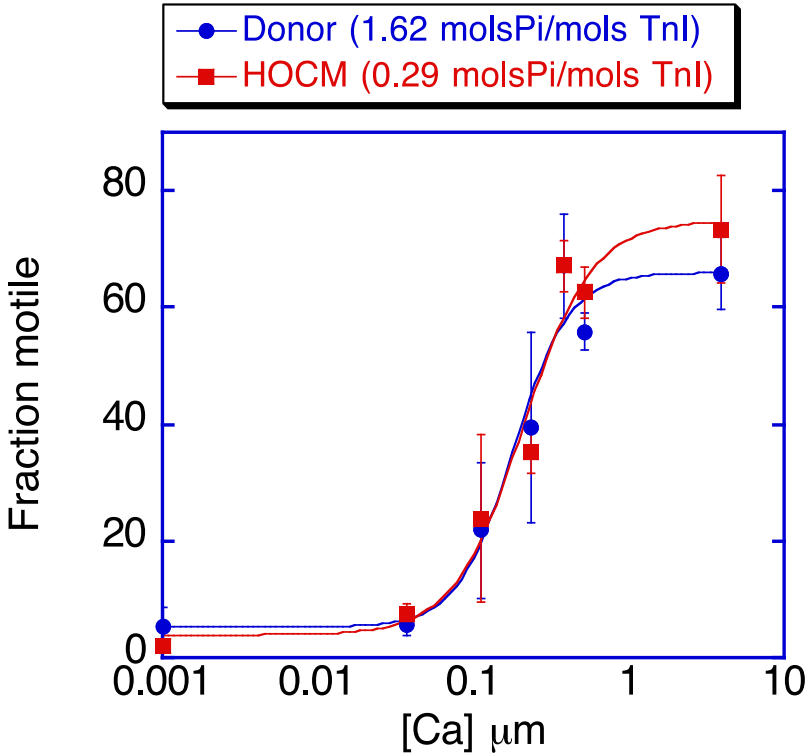


Donor vs HOCM troponin

- TnI Phos-tag Western blots



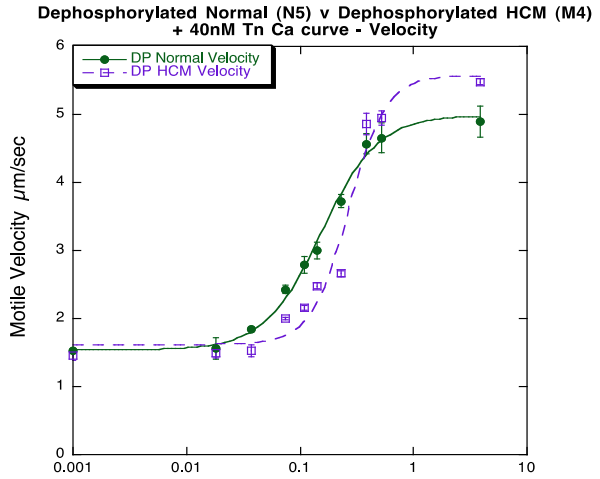
Donor HOCM

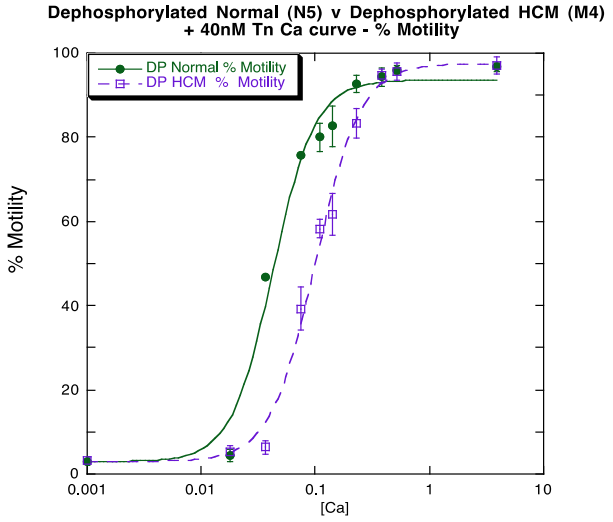


Donor EC_{50} (μM)	SD	HOCM EC_{50} (μM)	SD	HOCM/ Donor	SD	n	t-test
0.19	± 0.08	0.16	± 0.05	0.88	± 0.22	8	P = 0.15

Donor and HOCM troponin behave differently when not phosphorylated.

Dephosphorylation produced a marked increase in Ca^{2+} sensitivity and a decrease of 15% in filament sliding speed in donor, but not in HOCM troponin.

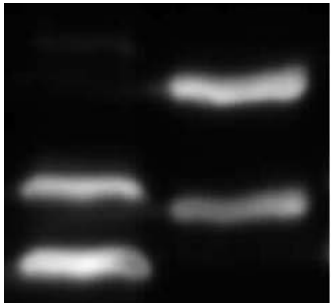


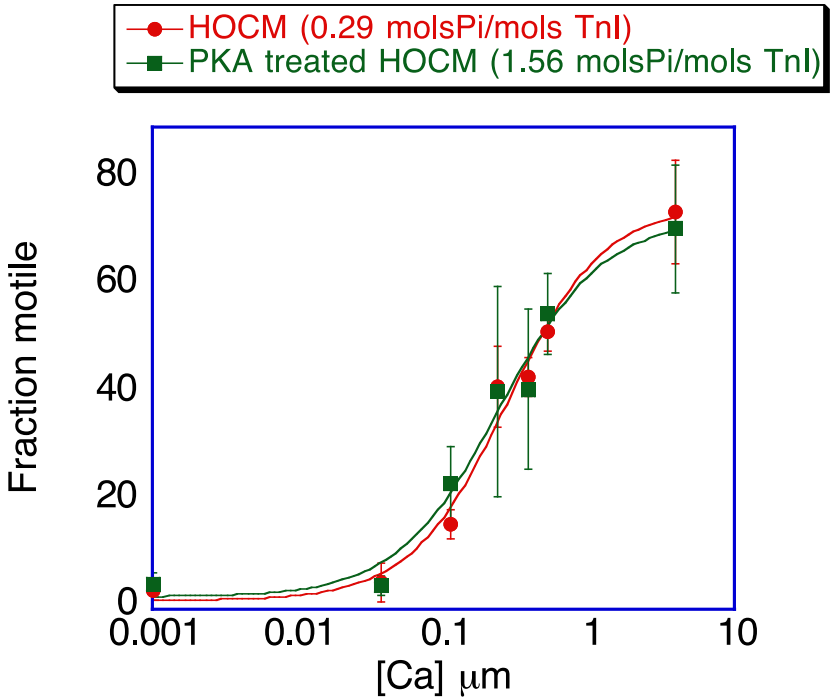


HOCM vs PKA-treated HOCM troponin

myectomy tissue troponin is not responsive to changes in troponin I phosphorylation level

- TnI Phos-tag Western blots





HOCM EC_{50} (μM)	SD	PKA-HOCM EC_{50} (μM)	SD	HOCM/ PKA-HOCM	SD	n	t-test
0.16	± 0.07	0.15	± 0.05	1.08	± 0.25	8	p= 0.37

Uncoupling may be a default result of many forms of structural perturbations

Molecular basis of protein polymorphisms

Gene

Changes in genes expression

Mutations produce altered sequences

RNA splicing

Splicing in and out of specific exons produces a range of isoforms

Post translational modification

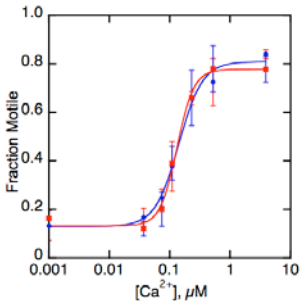
Phosphorylation by various specific kinases

Damage

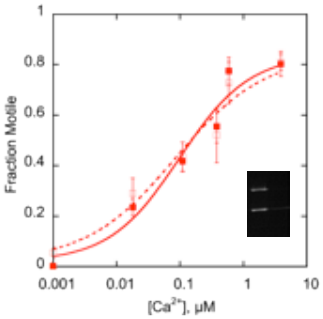
Proteolysis

Radical oxidation

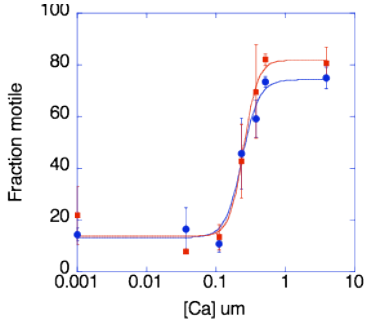
DCM ACTC E361G



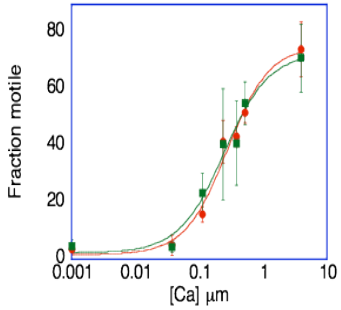
HCM ACTC E99K



HCM TNNT2 K280N



HCM myectomy (Mybp-c mut)



The HCM phenotype is complex

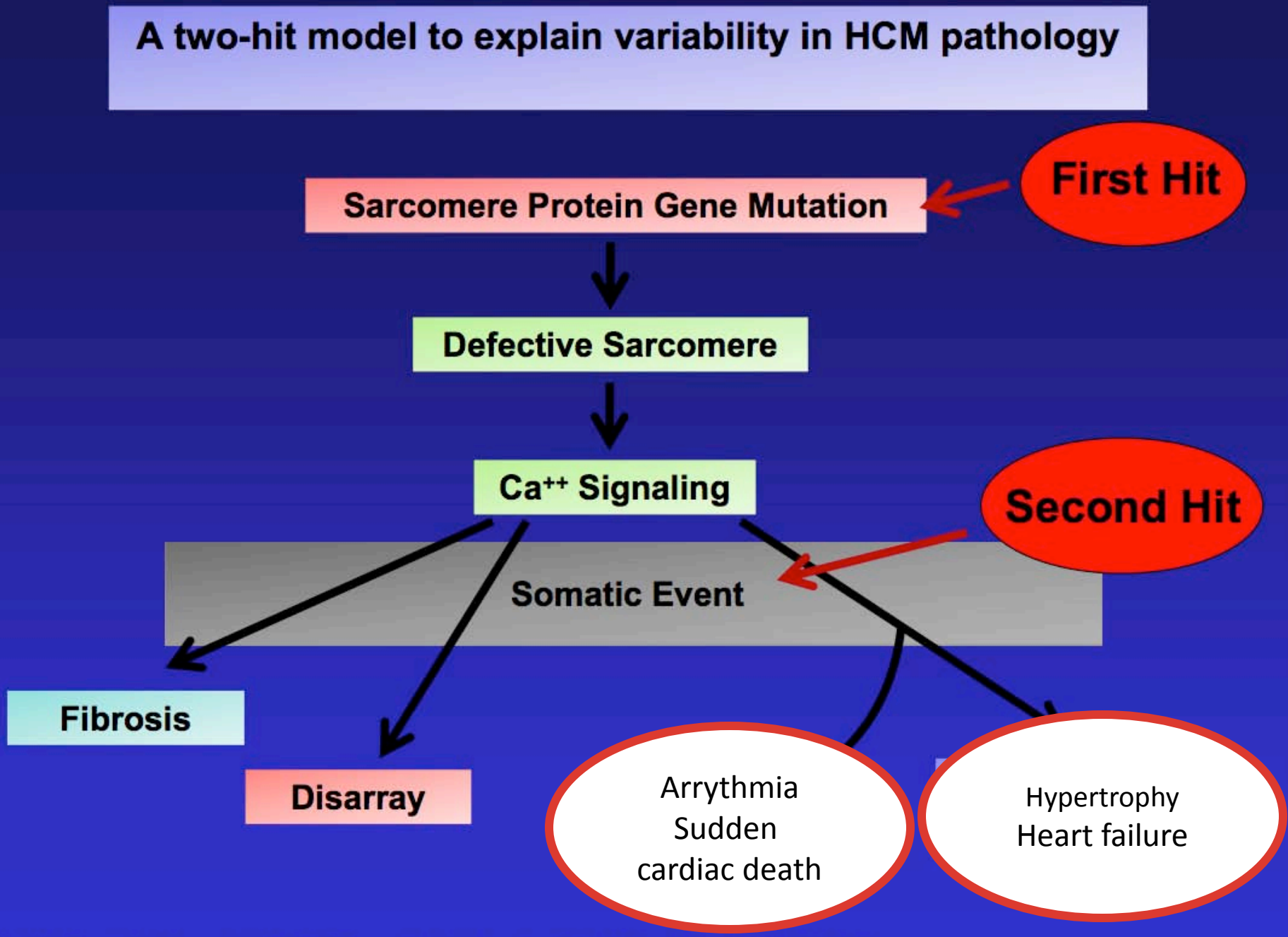
Molecular phenotype

→ Sudden death

→ hypertrophy →

Higher Ca^{2+}-sensitivity, cycling rate and impaired relaxation is characteristic of most HCM mutations

How does the molecular phenotype cause hypertrophy?



Wolf, Cordula M. et al. (2005) Proc. Natl. Acad. Sci. USA 102, 18123-18128

Hypotheses 1. sudden cardiac death

Key points:

(1) Baudenbacher et al. observed increased susceptibility to arrhythmia *in the absence of any detectable cardiac hypertrophy or fibrosis* when myofilaments sensitized with thiadiazinone derivative, EMD.

(2) Sensitization associated with shortening of action potential.

Baudenbacher et al. work leads to several proposals:

(1) Disturbance of intracellular Ca homeostasis alters the function of key Ca regulatory proteins in the SR and sarcolemmal membranes leading to alterations of the shape and duration of the action potential and Ca transient

(2) APD shortening decreases Ca influx (via L-type Ca channels) leading to decreased Ca release from the SR and *smaller intracellular Ca transient*.

(3) APD shortening results in shorter effective refractory period, greater beat-to-beat variability of APD and increased dispersion of ventricular conduction velocities. *These changes create an arrhythmogenic substrate.*

(4) Changes to APD *in parallel* with dysfunctional Ca regulation enhances arrhythmogenic probability

Baudenbacher et al. *Myofilament Ca2+ sensitization causes susceptibility to cardiac arrhythmia in mice*. Journal of Clinical Investigation (2008) vol. 118 (12) pp. 3893-903

Hypotheses 2. compromised energetics causing hypertrophy

- The hypercontractile phenotype in HCM would lead to inefficient contraction at rest, requiring the consumption of more ATP than usual and compromising the capacity of the cardiomyocyte to maintain energy levels.
- The hypothesis has the advantage of explaining not only familial HCM but also certain phenocopies such as mutations in AMPK and mitochondrial mutations.
- The PCr/ATP ratio in human heart tissue measured by MR is lower in HCM patient's heart than in controls, and interestingly, the low ratio is also present in HCM mutation carriers that do not have any hypertrophy [46], suggesting there is a link between the mutation and energy compromise.
- Direct measurements of energy efficiency in the ACTC E99K HCM mouse have confirmed there is reduced efficiency

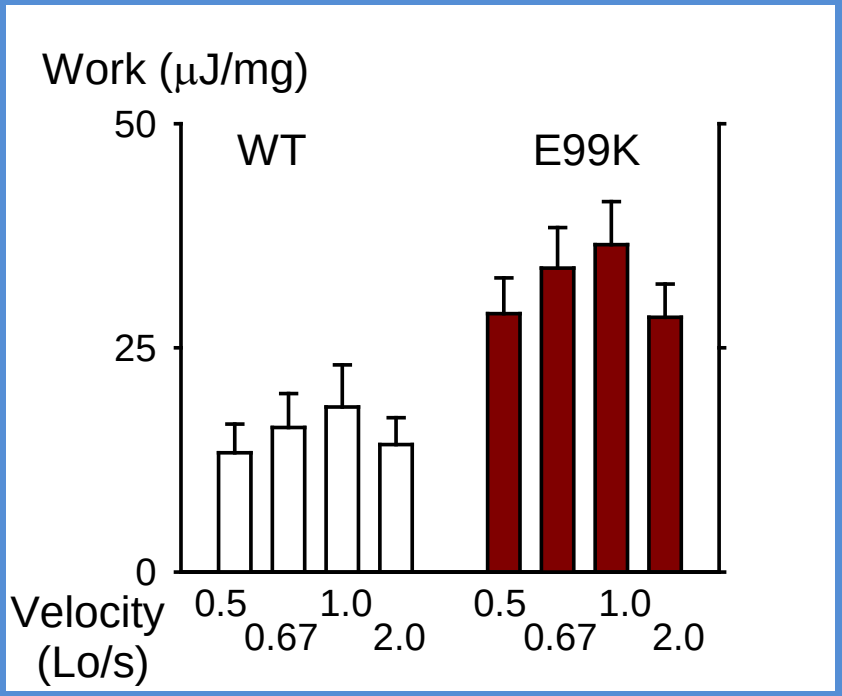
BUT- Correlation does not prove cause and effect

HYPERCONTRACTILE PHENOTYPE

Baseline un-stimulated force was greater than NTG,

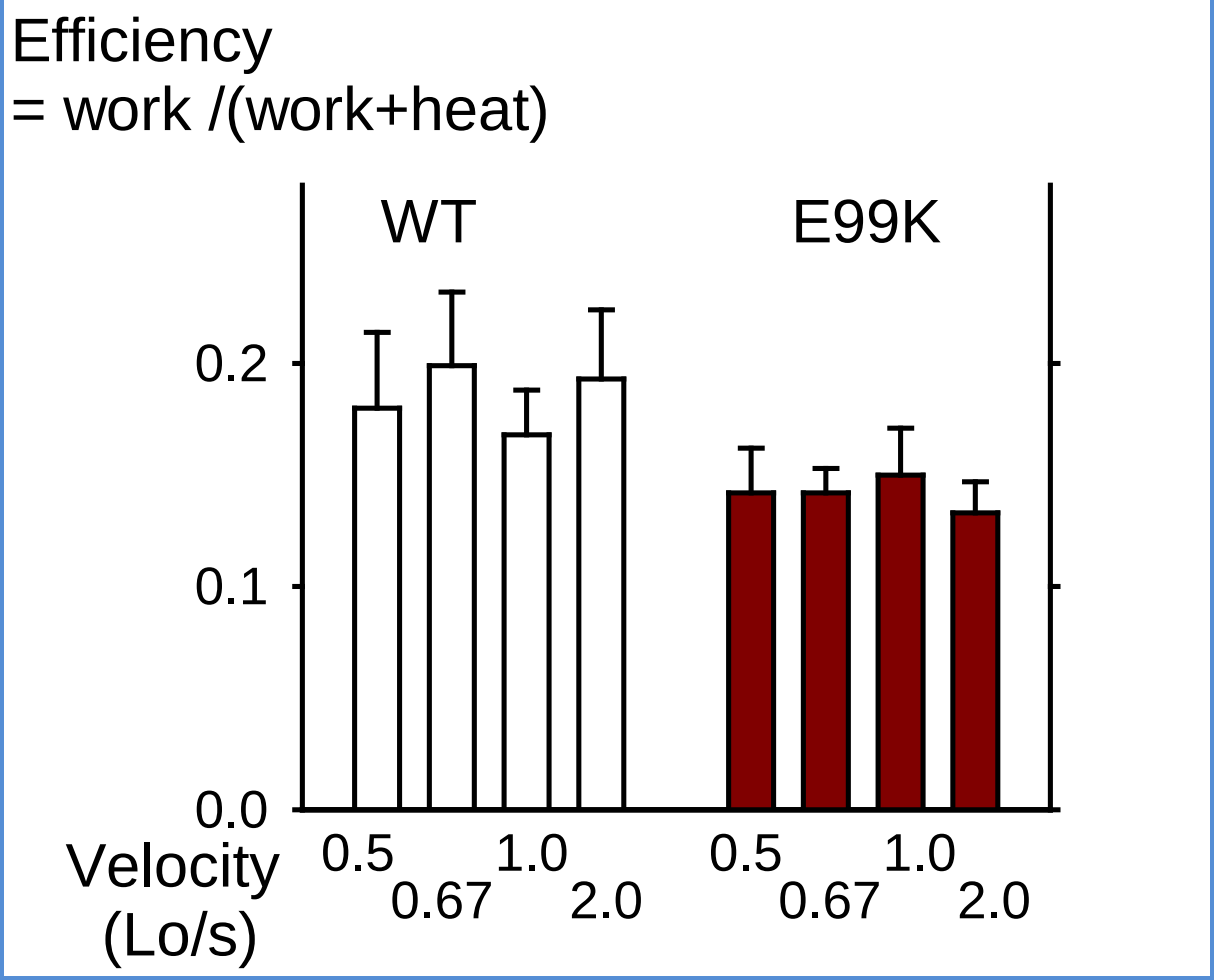
Isometric relaxation was incomplete

Isotonic force was greater than NTG

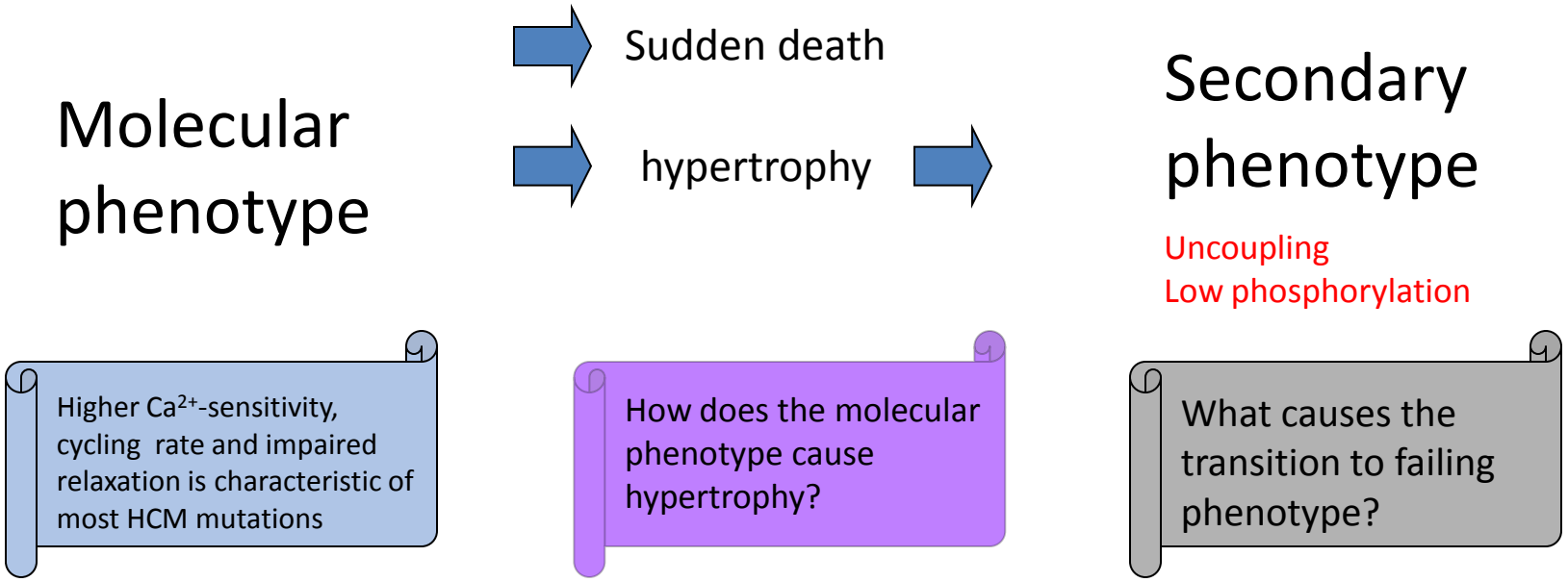


At each velocity the transgenic muscle did more work than the wt muscle

Transgenic muscle is less efficient: smaller fraction of the total energy turnover is converted to work



The HCM phenotype is complex



CONCLUSIONS

Studies of genetic DCM and HCM mutations in native proteins and in intact tissue show that in both cases the phenotype is complex and does not necessarily correspond to the simple molecular phenotypes determined *in vitro* with recombinant proteins

Increased Ca2+-sensitivity is the common feature of all HCM-causing mutations although the mechanism for this may vary.

In the interventricular septum of HOCM patients major secondary changes can mask the effects of the mutations that triggered the disease.

Acknowledgements

- MARSTON'S LAB

Steve Marston
Massimiliano Memo
Adam Jacques
Andrew Messer
Christopher Bayliss
Emma Dyer
O'neal Copeland
Weihua Song
Ross Wilkinson

COLLABORATORS

Kristen Nowak
Dominic Wells
Douglas Ward
Doug Lopes
Juan Pablo Kaski
Mike Burch
Chris Dos Remedios
Charles Redwood

Imperial College London

British Heart Foundation