Imperial College

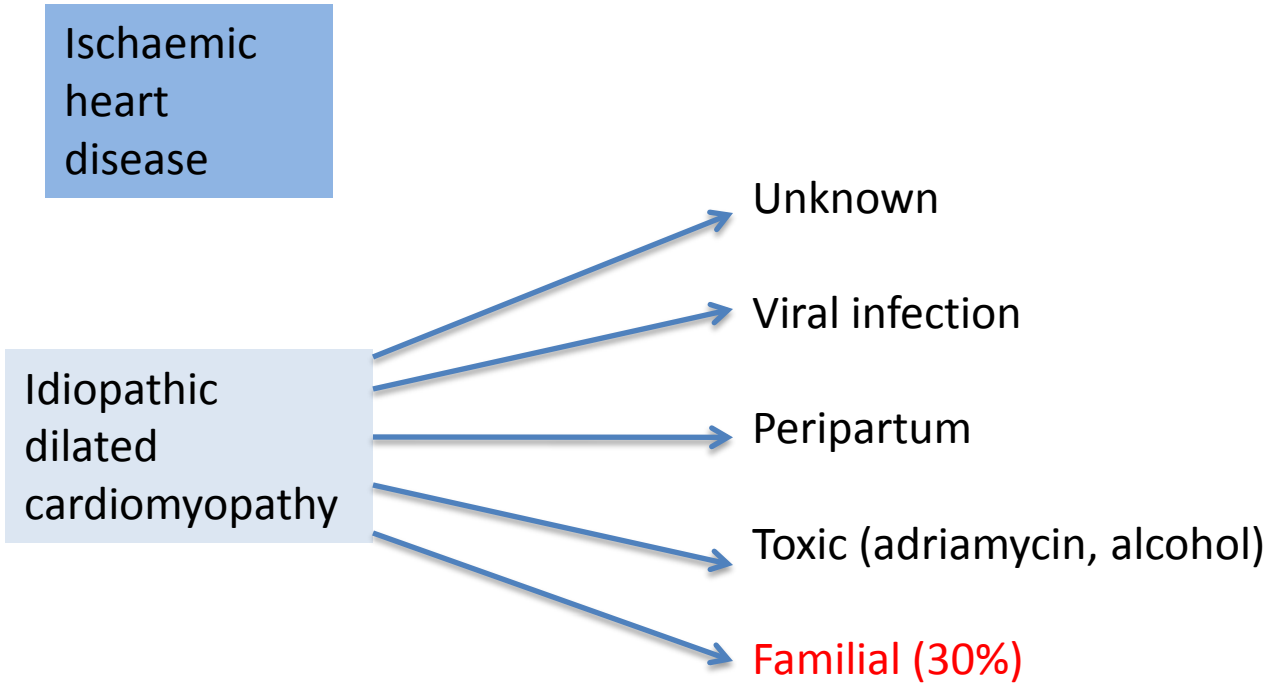
OF SCIENCE, TECHNOLOGY AND MEDICINE

Contractile proteins in Heart Failure

Steven Marston

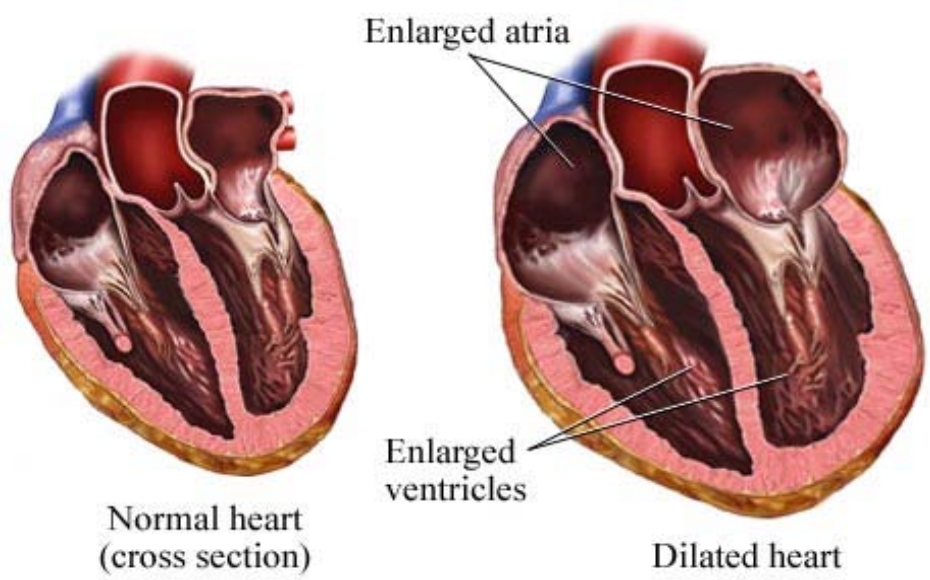
24 Oct 2011

Contractile proteins have been studied in explanted human hearts with IDC and end-stage heart failure

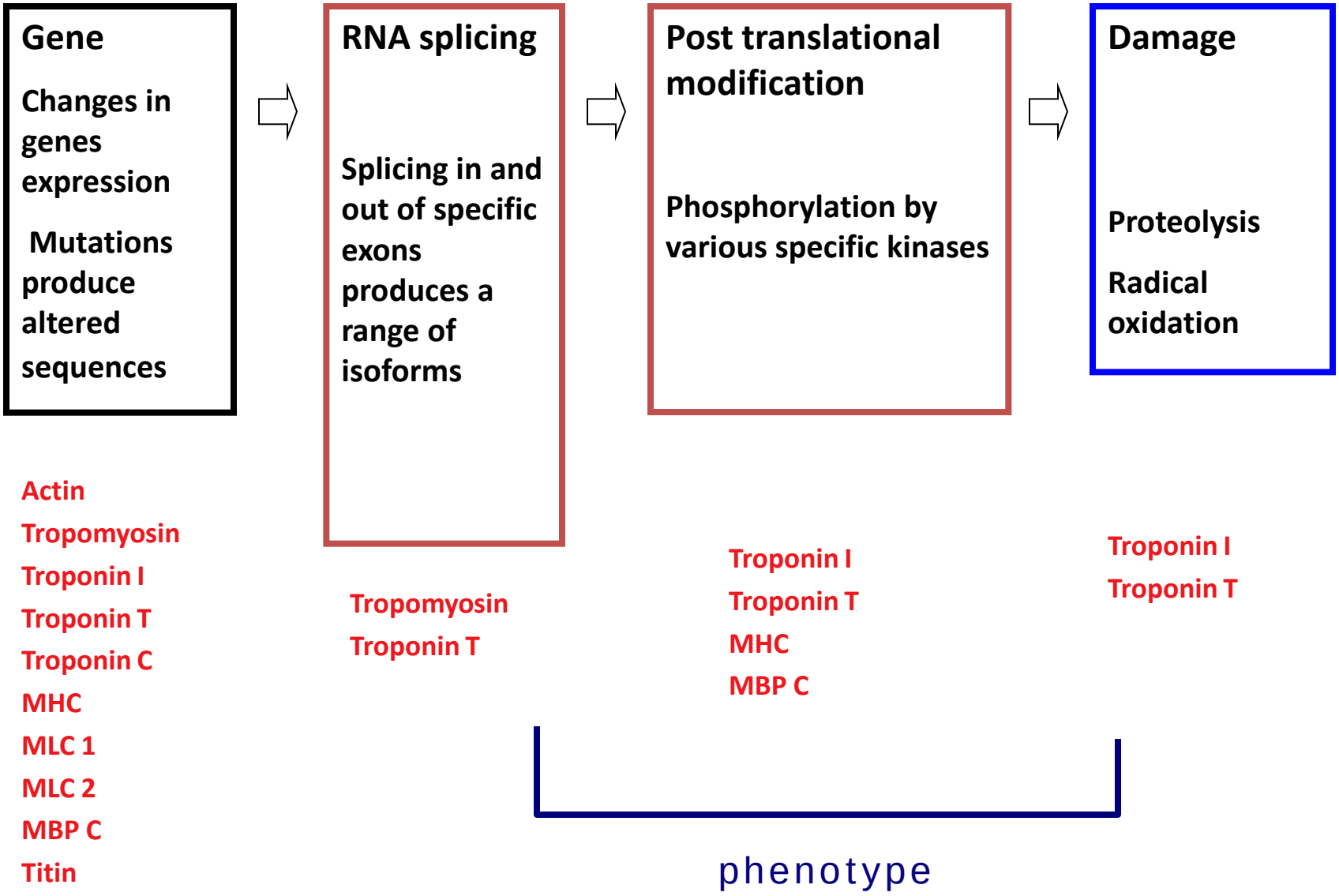


Dilated cardiomyopathy (DCM)

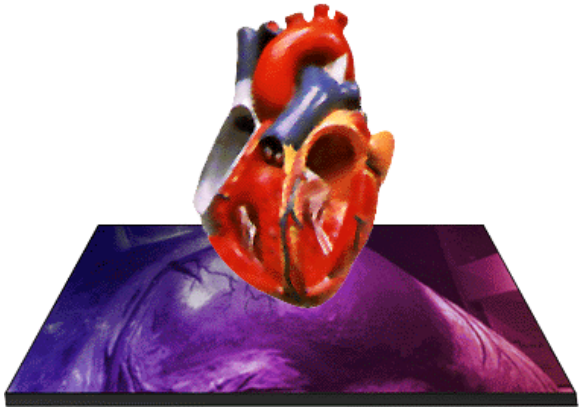
- Most common cardiomyopathy
- Dilatation and impaired contraction of ventricles
- Up to 30% of cases are genetically inherited

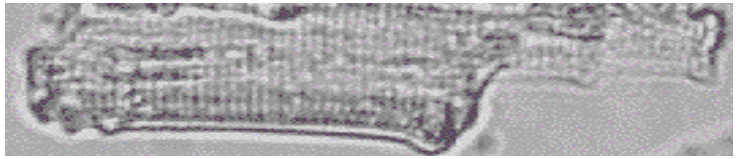


Molecular basis of protein polymorphisms



Methods to study muscle diseases



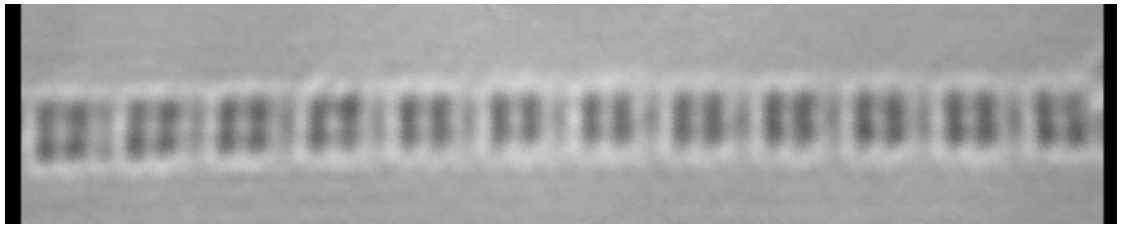


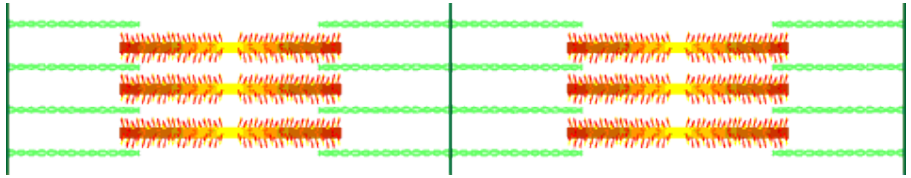
Muscle biopsy- most physiological but very limited material

Transgenic mouse- physiological but non-human background

Transfection of cardiomyocytes(rat)- easier than transgenic complete replacement possible

Skinned fibres, myofibrils- Easy for troponin I and TnC, possible for TnT. controlled replacement, can measure force and shortening





Synthetic actomyosin assayed by ATPase or *in vitro* motility assay.

Complete control of components allows fully human- cardiac synthetic system.

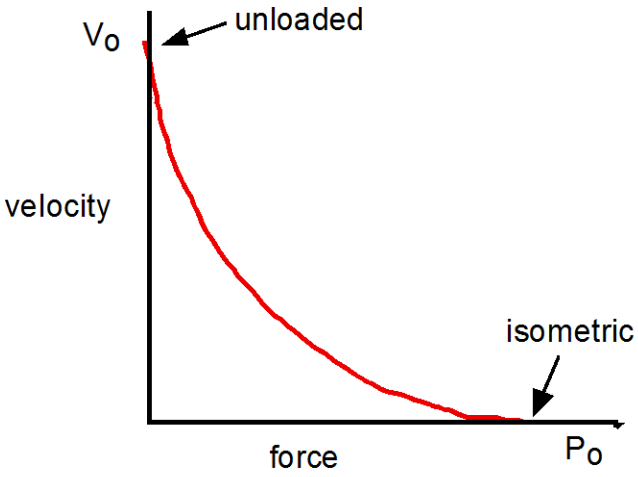
ATPase a non-specific assay but *in vitro* motility will measure unloaded velocity and crossbridge recruitment: modified assay can measure force.

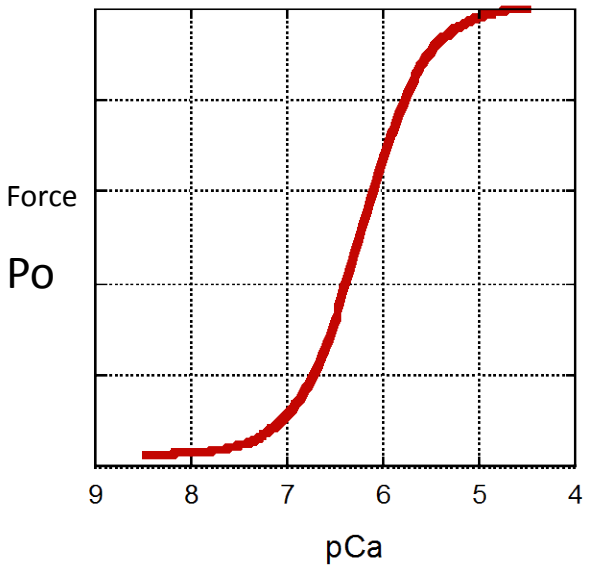
Diagrams by Michael Ferenczi

http://npbsn41.nimr.mrc.ac.uk/protocol_html/maf_home.html

Basic parameters of contractility and regulation

(P + a) (V + b) = (Po + a)b
P= isotonic load
a and b = constants
Po = maximal isometric tension
V = velocity





CHANGES IN CONTRACTILE PROTEIN PERFORMANCE IN FAILING HEART.

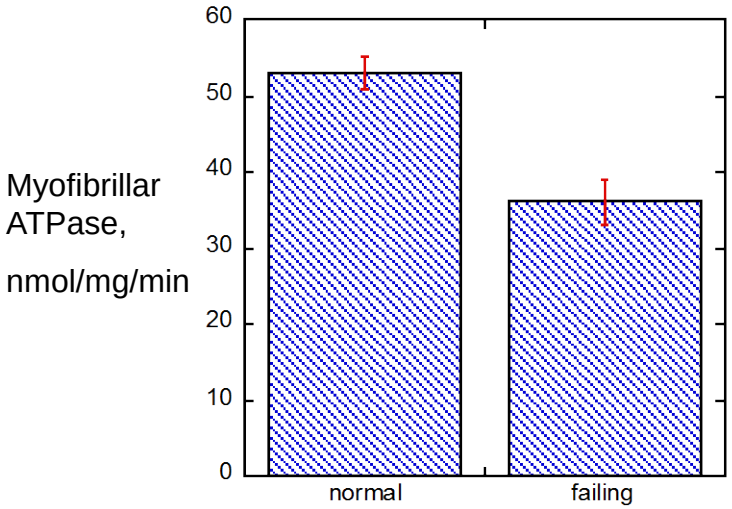
Vo, unloaded velocity is reduced ⬇

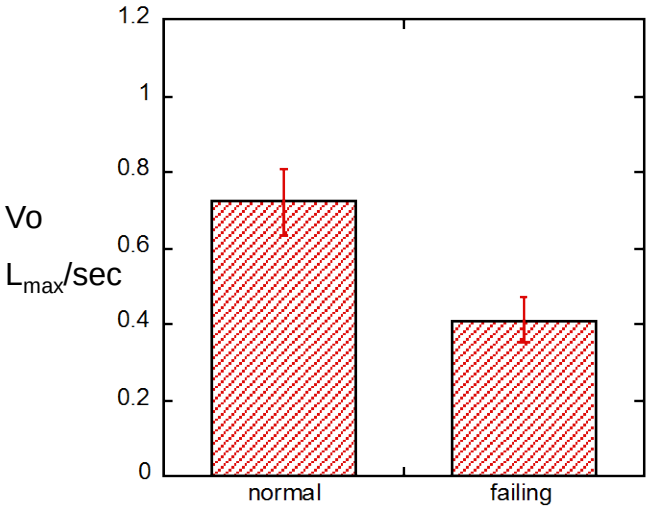
Po, isometric force is not significantly changed ➡

Ca2+-sensitivity is increased ⬆

This phenotype is distinct from the phenotype of foetal heart, Ischaemic heart and HOCM heart

Reduced maximum rate of crossbridge cycling in failing human heart





Data redrawn from Solaro et al. Circulation 87:VII38 (1993)

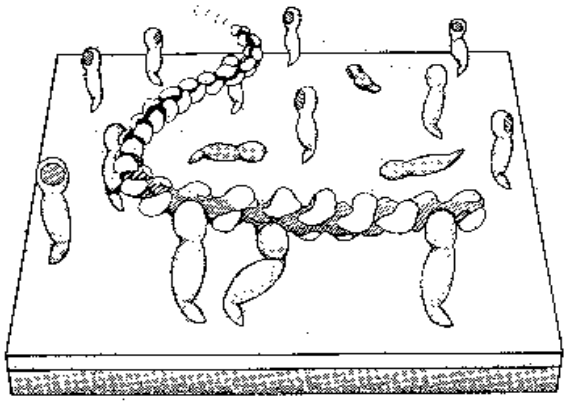
Data redrawn from Hajjar et al. Circulation 86:1819 (1992)

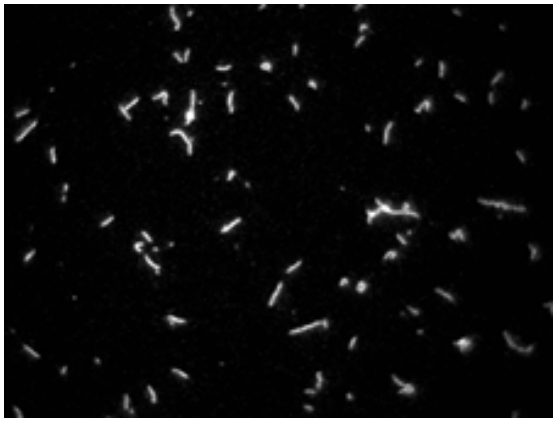
ATPase

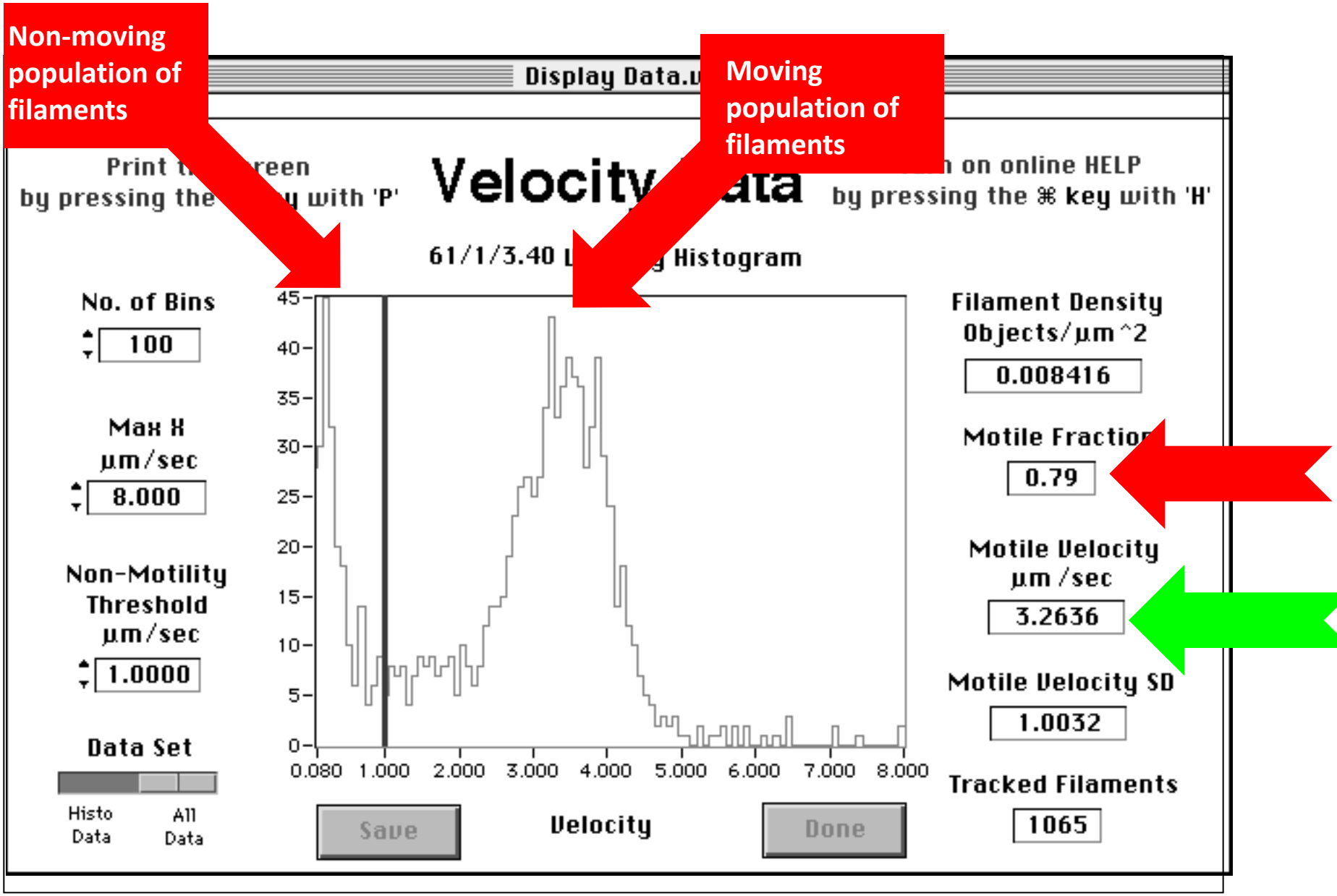
Shortening speed

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

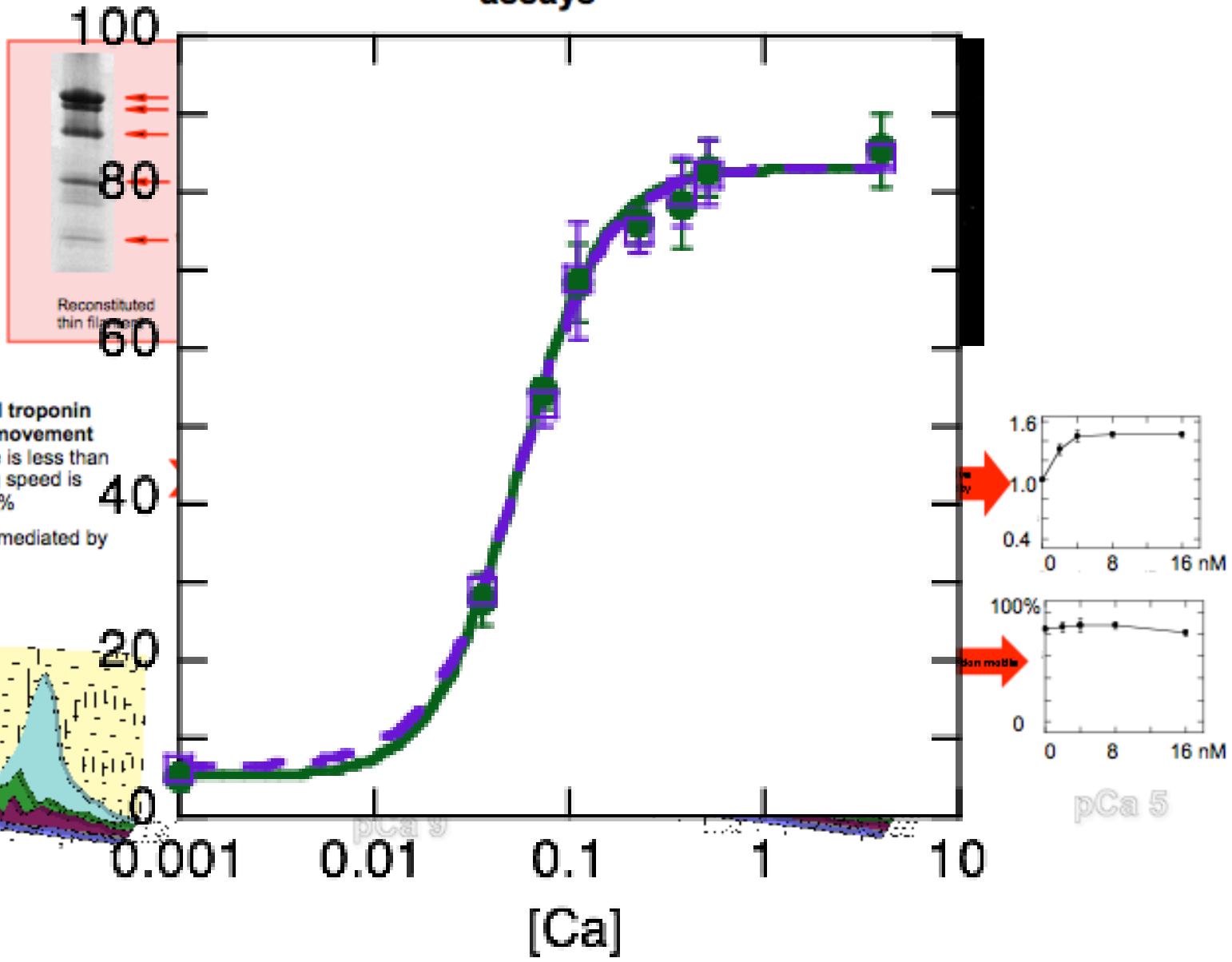
Thick and thin filament proteins are assembled on a microscope cover glass. The thin filaments move over the immobilised myosin in the presence of ATP and movement is controlled by Ca^{2+}. The assay therefore reproduces the functional properties of unloaded muscle in a synthetic system that can incorporate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





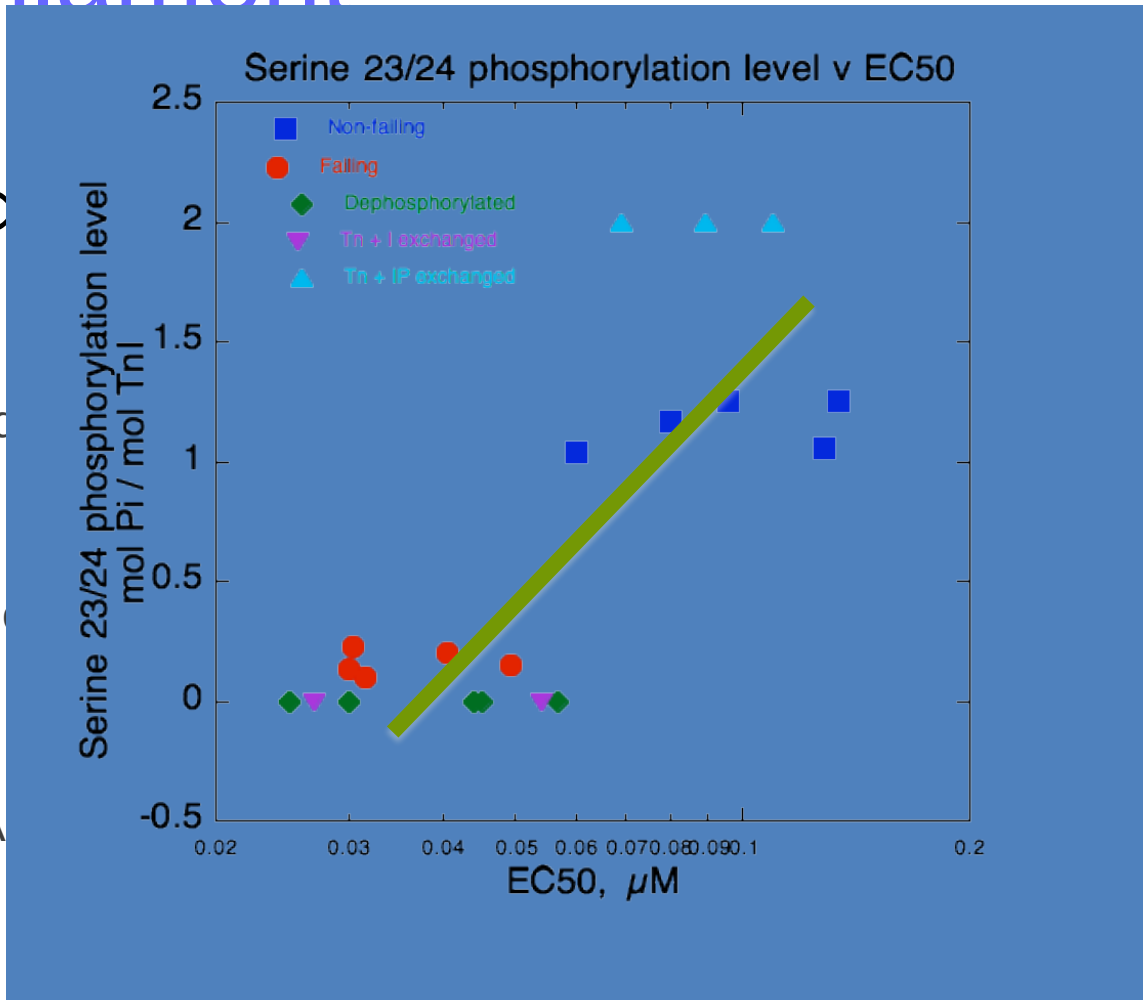

Non-moving population of filaments
Moving population of filaments
Display Data.u
Velocity
by pressing the
with 'P'
by pressing the ⌘ key with 'H'
61/1/3.40
Histogram
No. of Bins
100
Max X
µm/sec
8.000
Non-Motility
Threshold
µm/sec
1.0000
Data Set
Histo Data
All Data
45
40
35
30
25
20
15
10
5
0
0.080
1.000
2.000
3.000
4.000
5.000
6.000
7.000
8.000
Velocity
Save
Done
Filament Density
Objects/µm^2
0.008416
Motile Fraction
0.79
Motile Velocity
µm/sec
3.2636
Motile Velocity SD
1.0032
Tracked Filaments
1065

Human heart troponin subunits and tropomyosin are expressed and reconstituted into thin filaments which are studied by actomyosin ATPase and *in vitro* motility assays



phosphorylation modulates the Ca^{2+}-sensitivity of the thin filament

- Phosphorylation of TnI was manipulated in three ways
- Troponin from donor and failing heart were compared

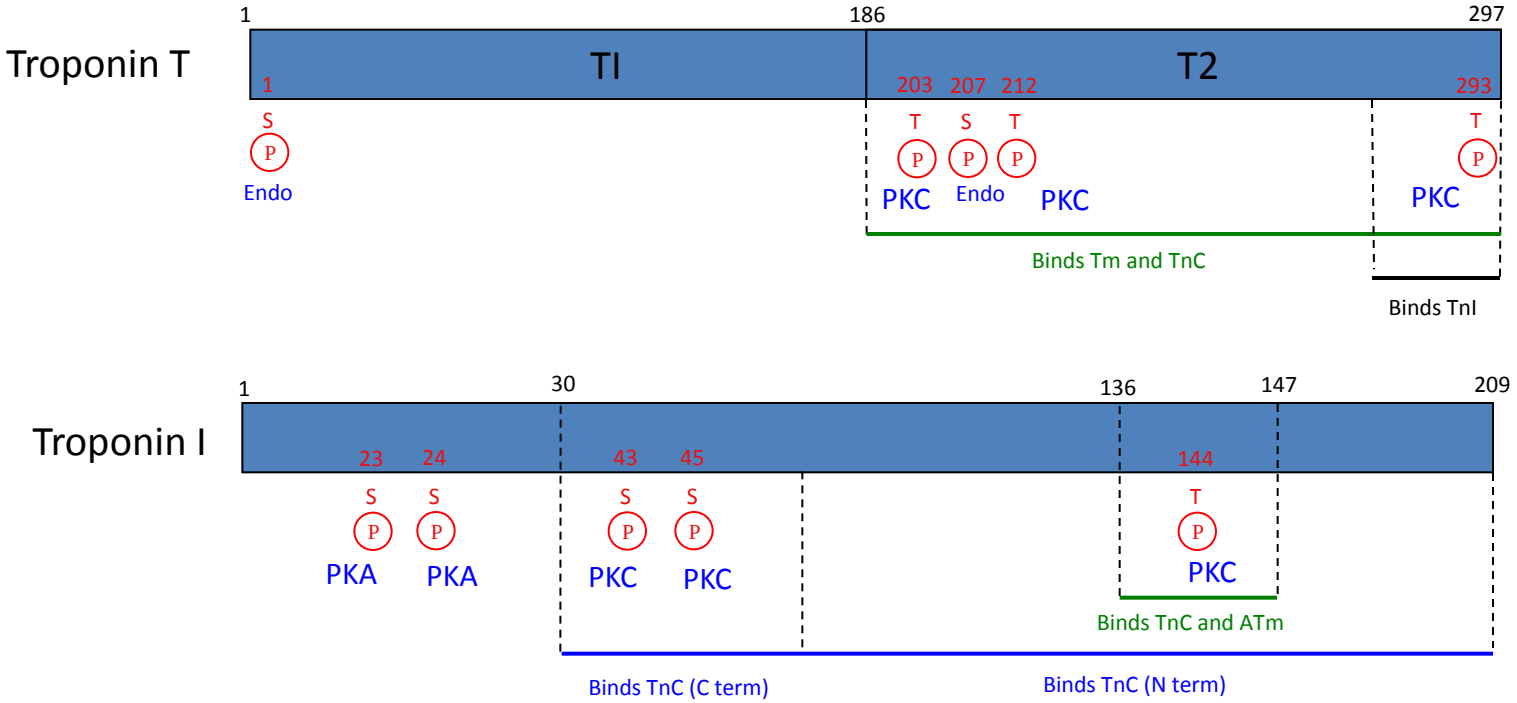


- Troponin from donor heart treated with phosphatase and compared untreated
- Troponin from donor heart had the TnI subunit exchanged for recombinant TnI.
- Recombinant TnI was either PKA treated or untreated

Changes in Troponin Phosphorylation Levels Account for the Differences in Troponin Function

Several phosphorylation sites have been proposed on Troponin T and I

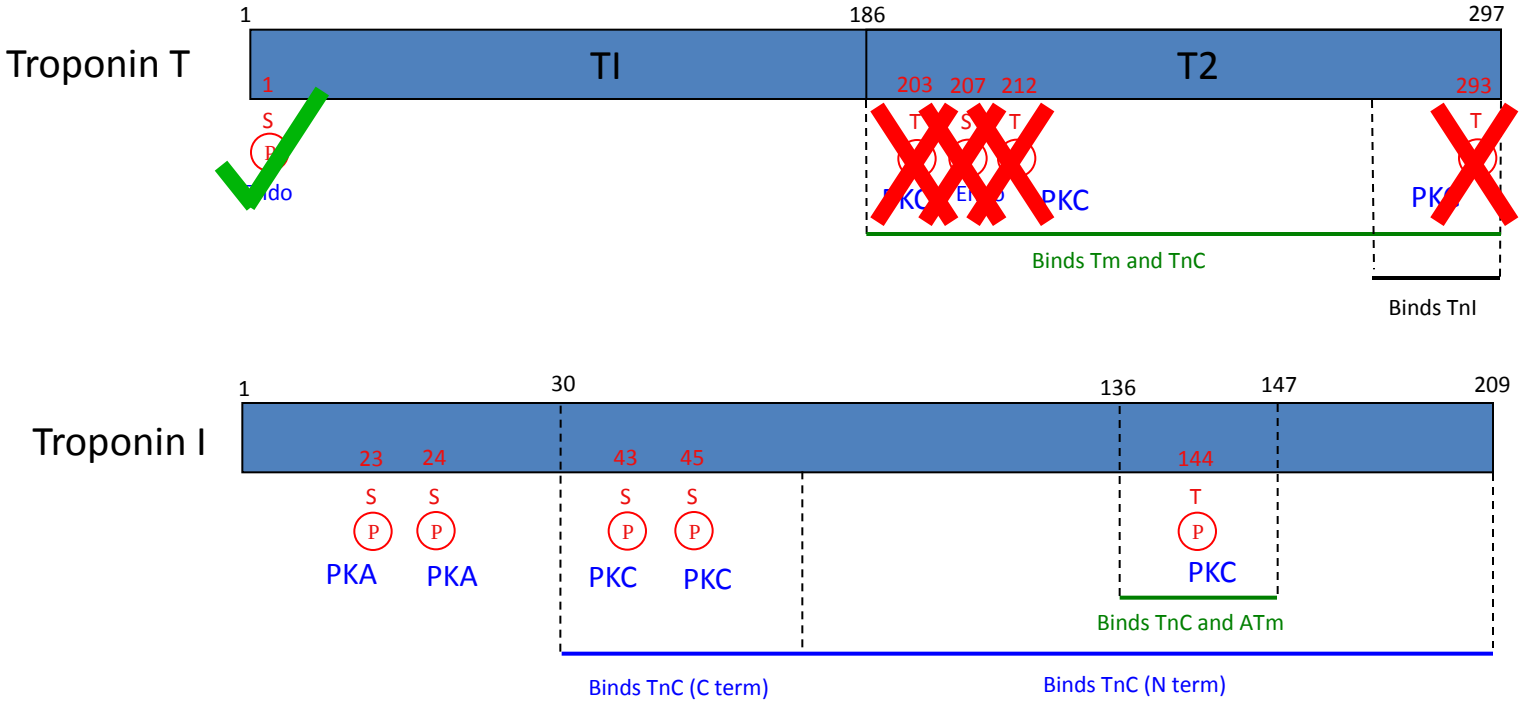
(but not C) by *in vitro* experimentation



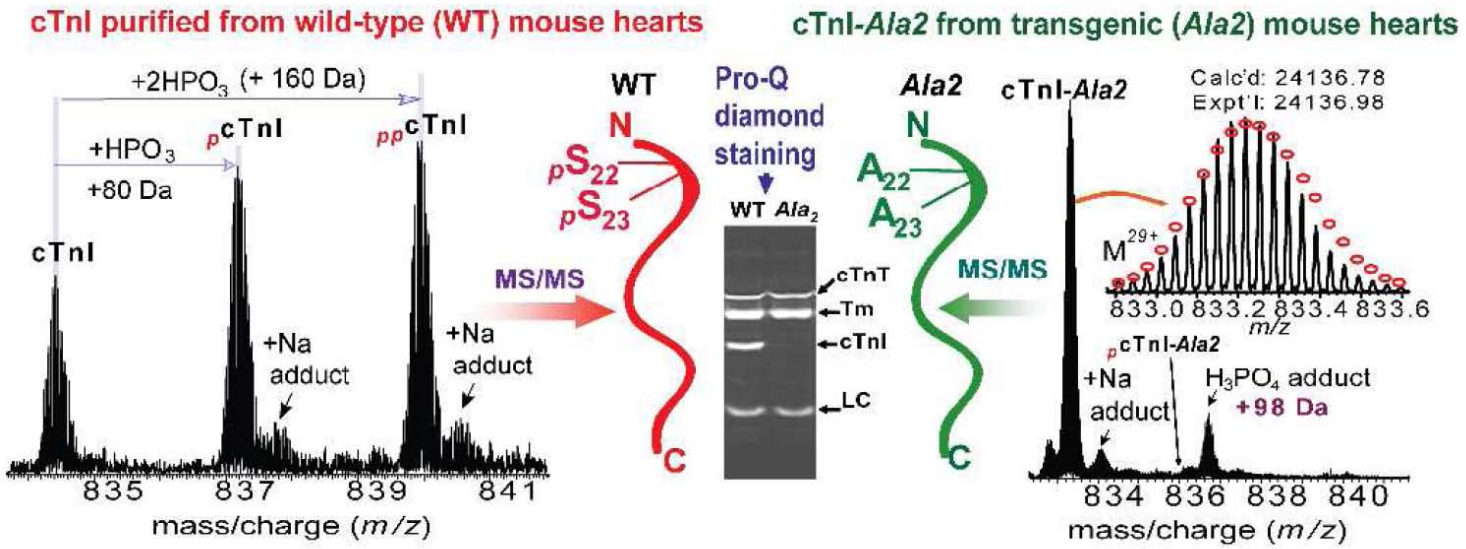
Troponin T- in human heart Ser 1 is constitutively phosphorylated and is not involved in regulation

Several phosphorylation sites have been proposed on Troponin T and I

(but not C) by *in vitro* experimentation



Troponin I phosphorylation investigated by mass spectroscopy

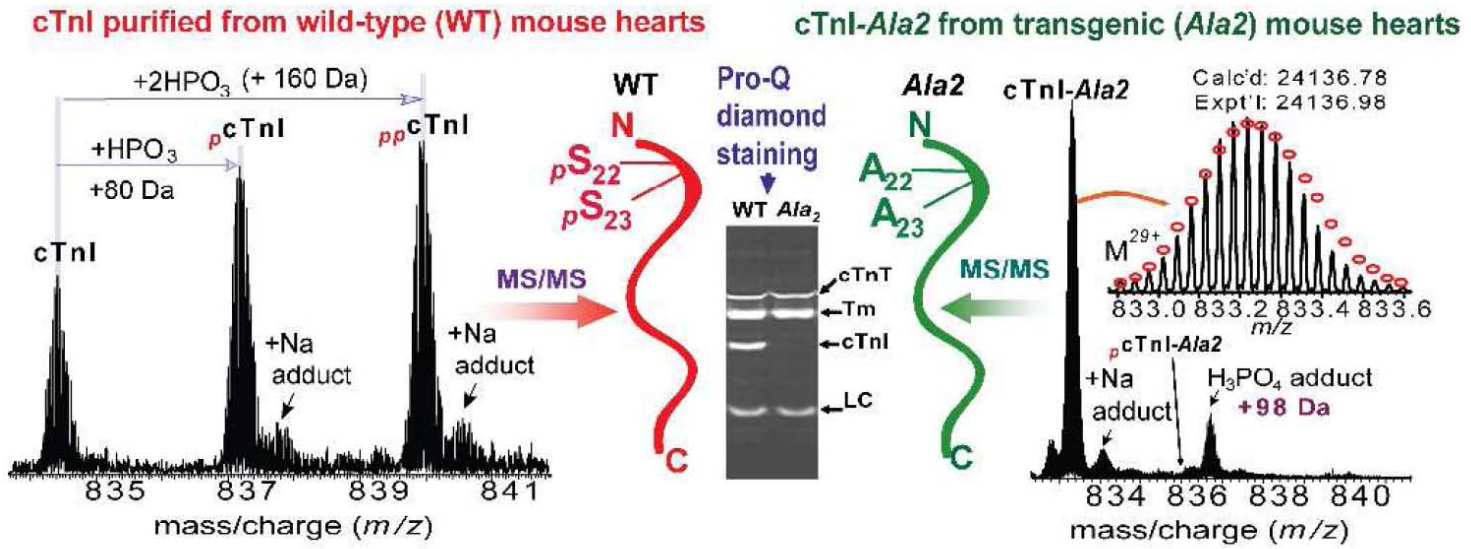


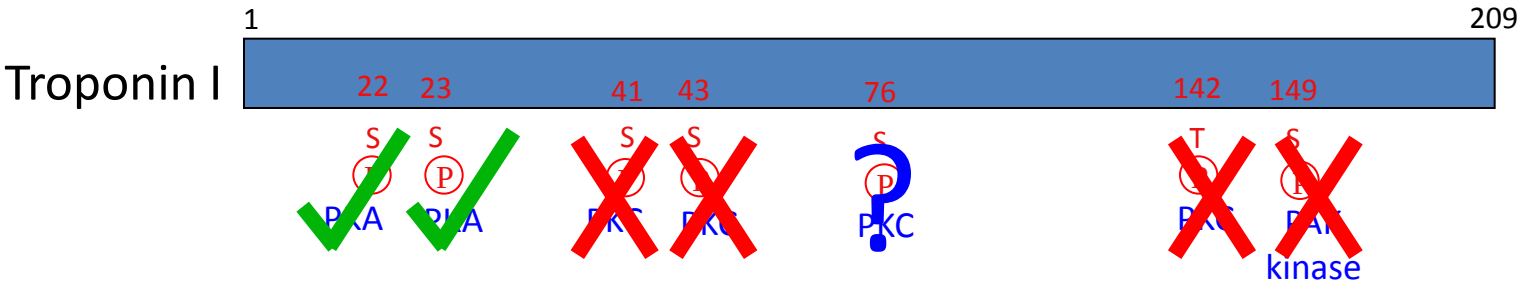
Unphosphorylated, mono-phosphorylated and bis-phosphorylated troponin I are present in normal heart.

Serines 22 and 23 (23,24) are the only sites (PKA specific)

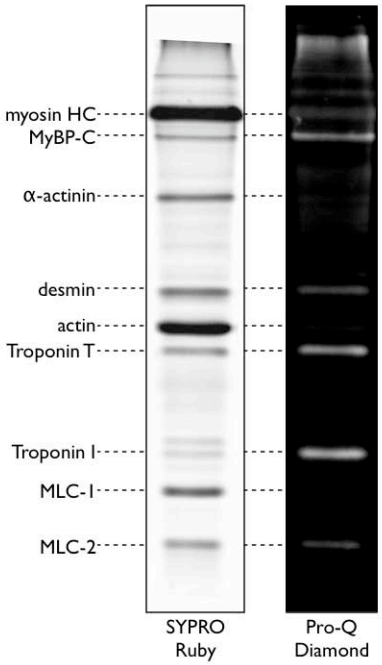
Ayaz-Guner S, Zhang J, Li L, Walker JW & Ge Y. (2009). In Vivo Phosphorylation Site Mapping in Mouse Cardiac Troponin I by High Resolution Top-Down Electron Capture Dissociation Mass Spectrometry: Ser22/23 Are the Only Sites Basally Phosphorylated. *Biochemistry*. **48**, 8161

Troponin I phosphorylation investigated by mass spectroscopy



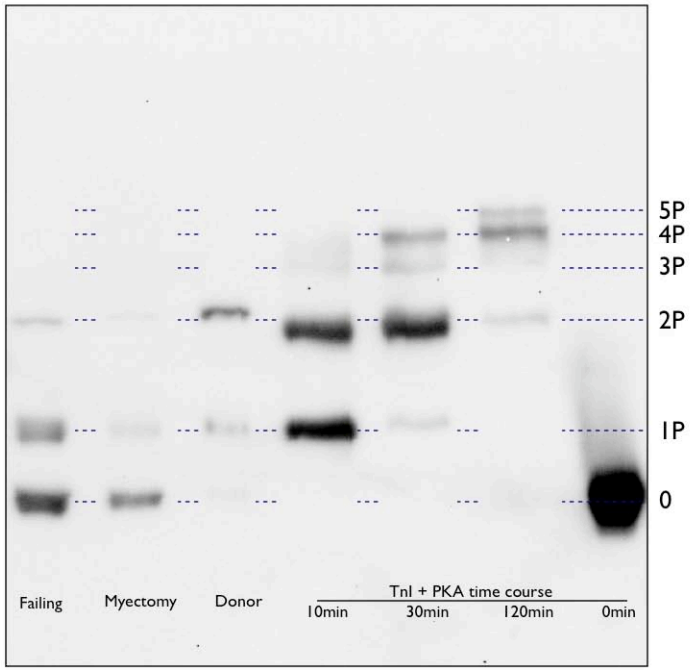


Two techniques are used to measure protein phosphorylation in human heart muscle myofibrils



Pro-Q Diamond

Specifically stains phosphoproteins in SDS-PAGE

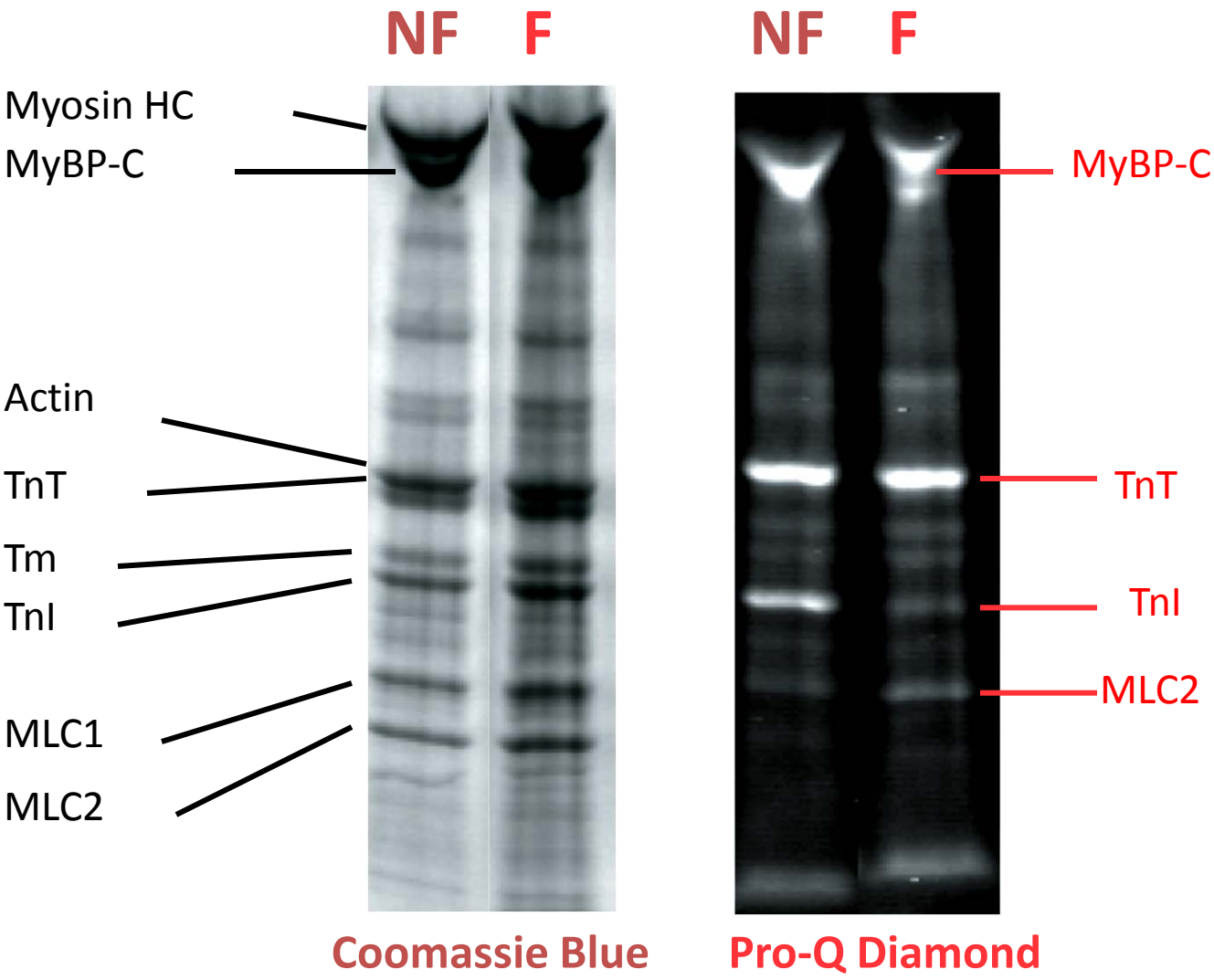


Phosphate affinity SDS-PAGE

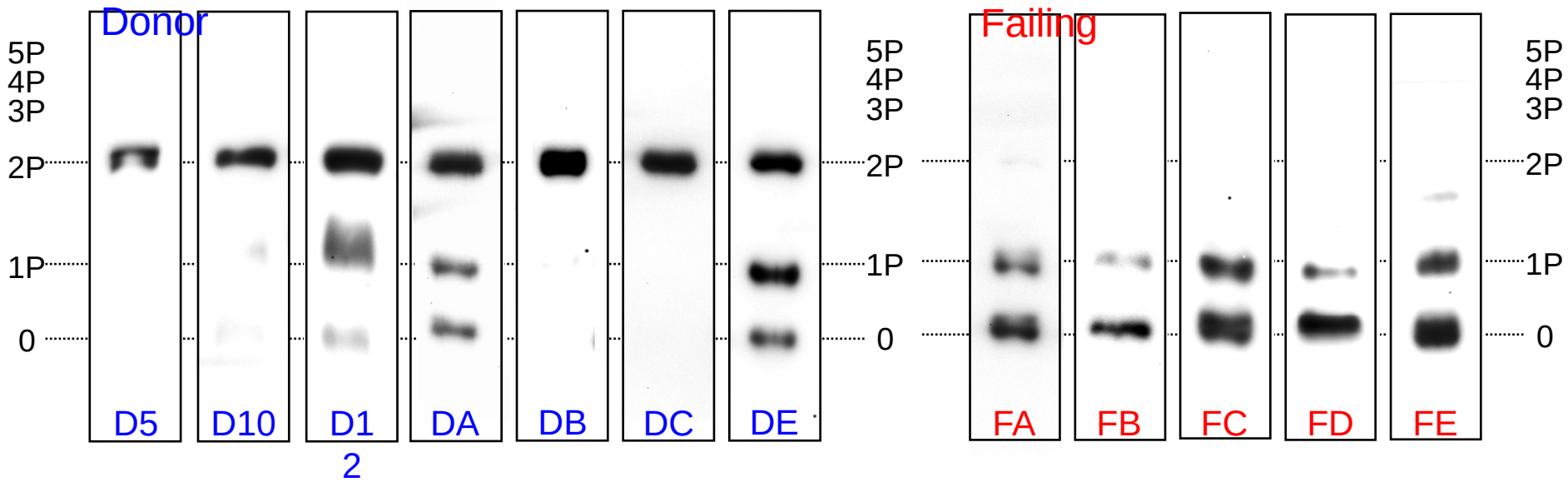
Separates phosphospecies according to amount Pi incorporated

Phosphorylated proteins in human heart muscle myofibrils

Reduced phosphorylation of MyBP-C and TnI in failing heart



Phosphate affinity SDS-PAGE separates phosphorylated species

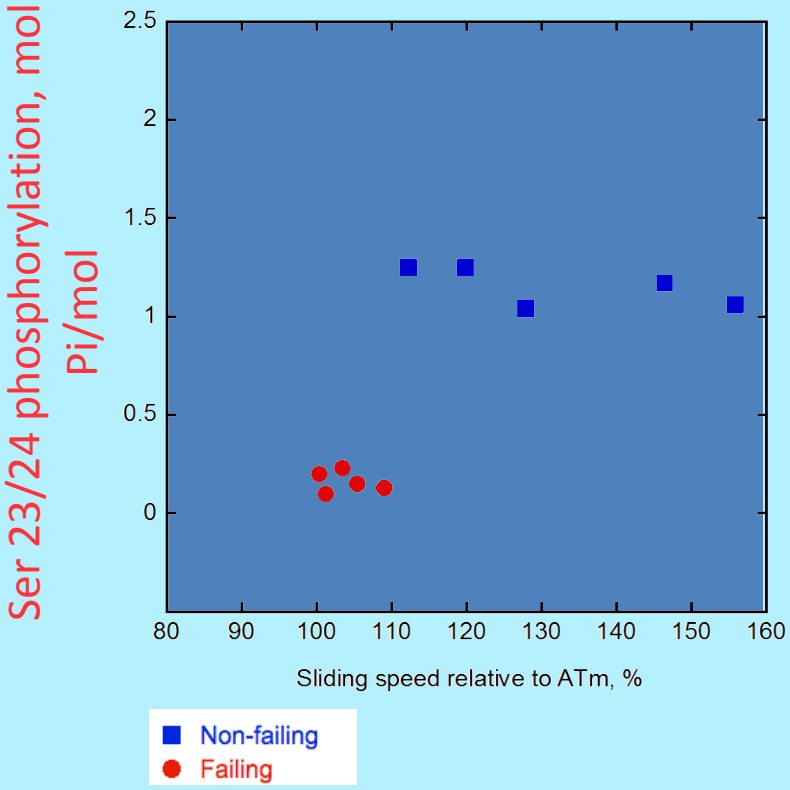


Donor is predominantly 2P.
No more than 2P found.

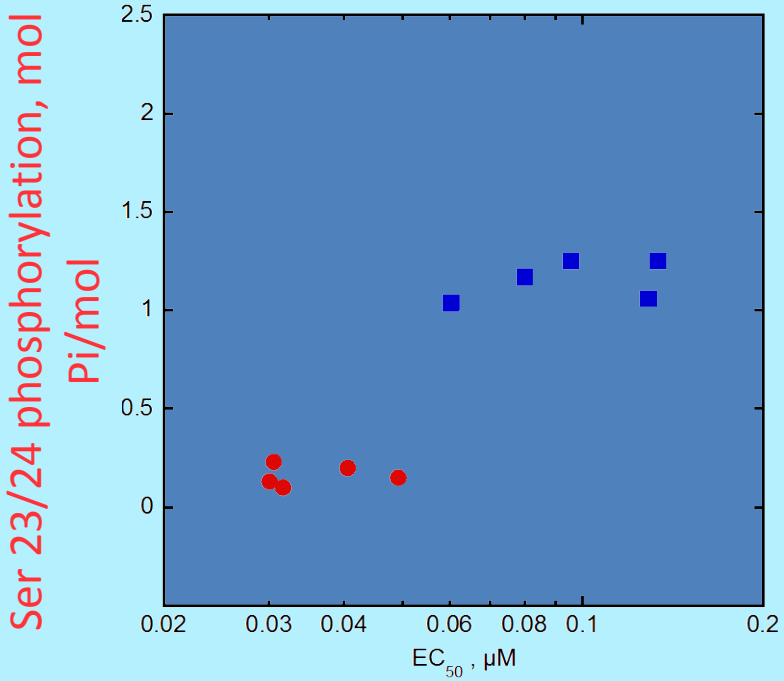
Failing is predominantly 0P and 1P

Crossbridge turnover rate and Ca^{2+} sensitivity are correlated with troponin I phosphorylation at Ser 23/24 in failing and non-failing heart muscle.

Sliding speed at pCa5



EC_{50}, Ca^{2+}



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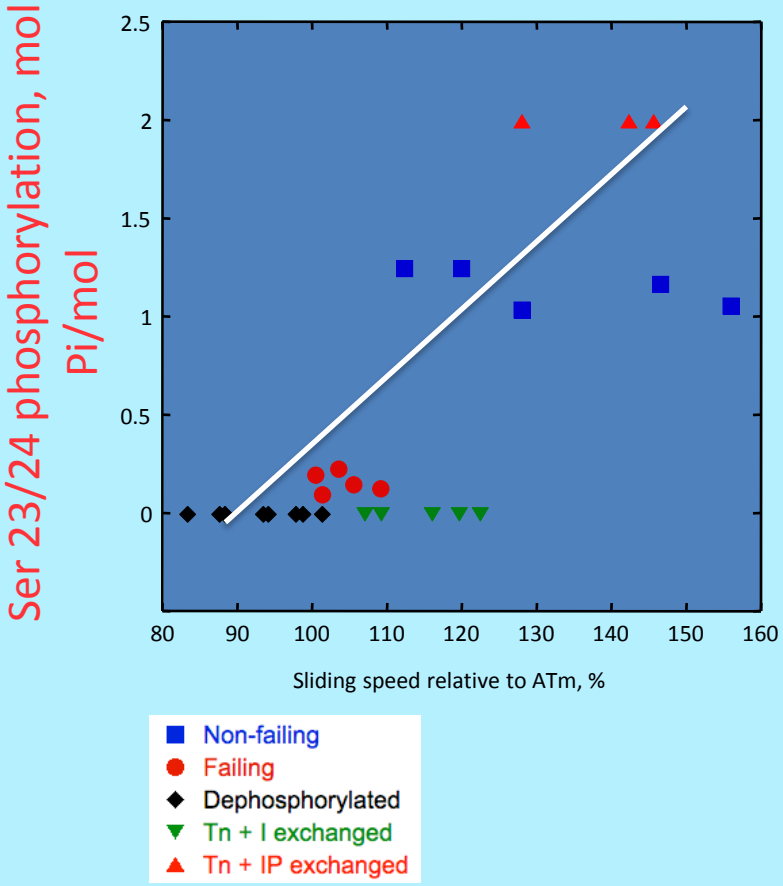
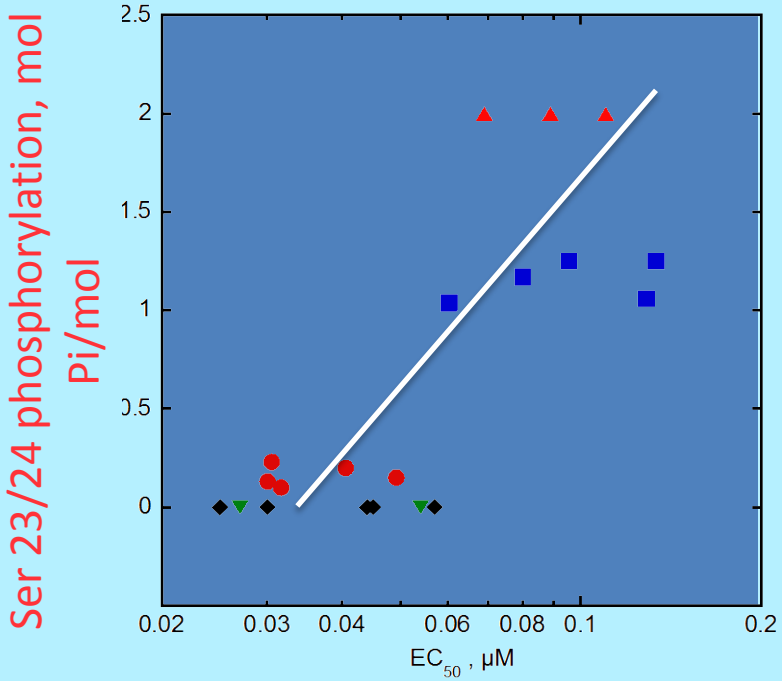
Sliding speed at pCa5



EC_{50}, Ca^{2+}



CONCLUSIONS

- **Troponin I phosphorylation in failing heart muscle is 1/6 the amount found in non-failing heart muscle**

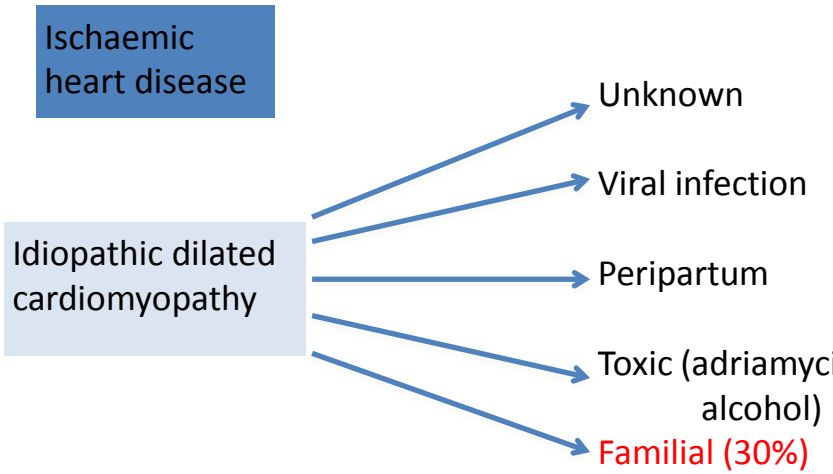
- **The low phosphorylation level of troponin I Ser23/24 is responsible for the decreased crossbridge cycling rate and increased Ca^{2+}-sensitivity observed in failing heart muscle**

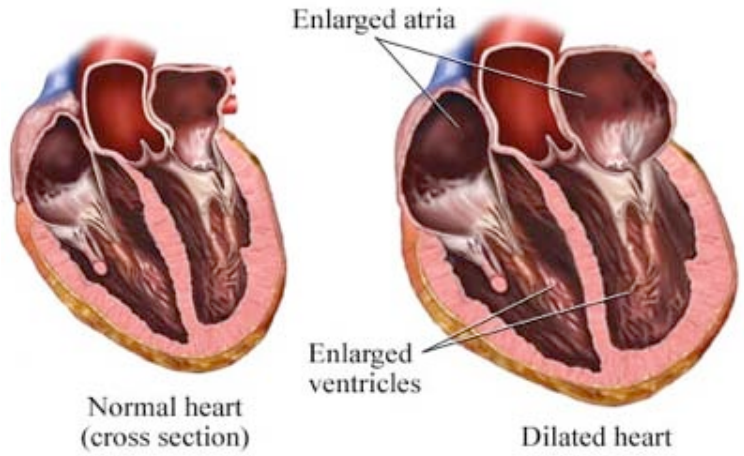
- **The low level of troponin I phosphorylation is due in part to activation of Troponin-I specific Phosphatase PP2a activity associated with myofibrils**

Imperial College London

Molecular Mechanism For Familial Dilated Cardiomyopathy

British Heart Foundation





- IDIOPATHIC DILATED CARDIOMYOPATHY

Dilatation and impaired contraction of ventricles
Common cause of heart failure
Up to 30% of cases are genetically inherited
Mutations in cytoskeletal or contractile proteins
Contractile protein mutations are associated with ‘pure’ DCM phenotype

Potential mechanisms for familial dilated cardiomyopathy

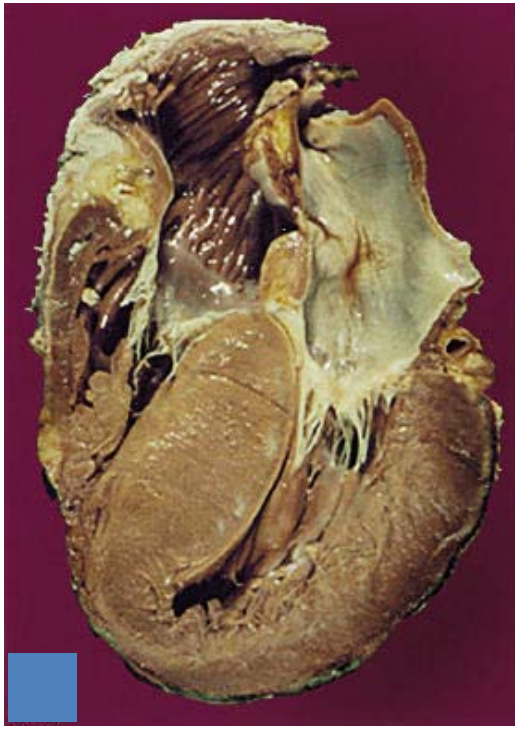
- Defective force generation
- Defective force transmission
- Defective force sensing/mechanotransduction

The diagnostic criteria for DCM are defined as:

- An ejection fraction <50% on echocardiographic analysis,
- regional fractional shortening <27% on M-mode analysis, or both
- presence of a left ventricular internal diastolic diameter >2.7 cm/m2 of body surface area.
- A diagnosis of HCM was excluded based on the demonstration of a left ventricular wall thickness <13 mm by echocardiography.
- In addition, other conditions that may simulate DCM or HCM were excluded, including coronary heart disease, myocarditis, hypertension, and valvular heart disease.

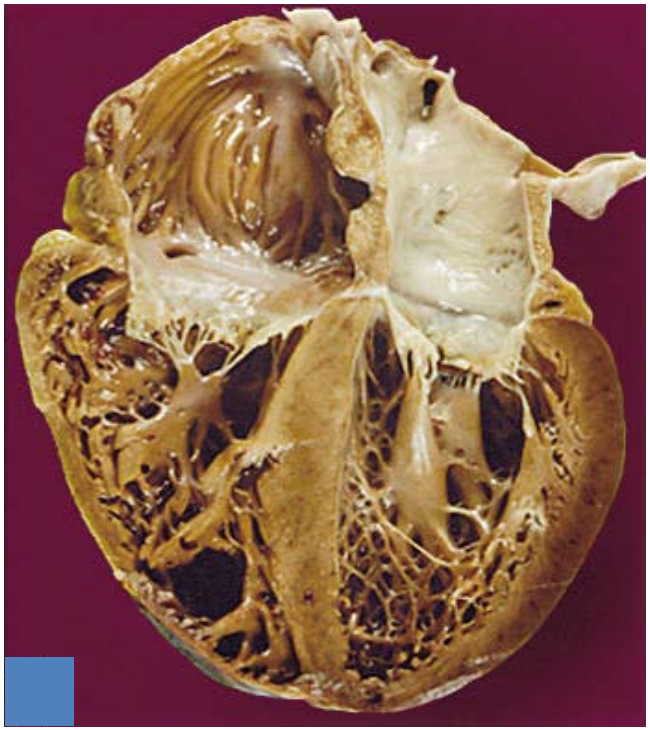
Figure 1.
The pedigree of the involved family. Squares: male family members; circles: female family members; symbols with slash: deceased individuals; open symbols: unaffected individuals; solid symbols: individuals affected by dilated cardiomyopathy; checkered symbols: individuals who died suddenly; plus signs: presence of mutation; minus signs: absence of mutation; arrow: individual described in this report.

Contractile protein mutations that cause Dilated Cardiomyopathy are in the same genes that cause Hypertrophic cardiomyopathy



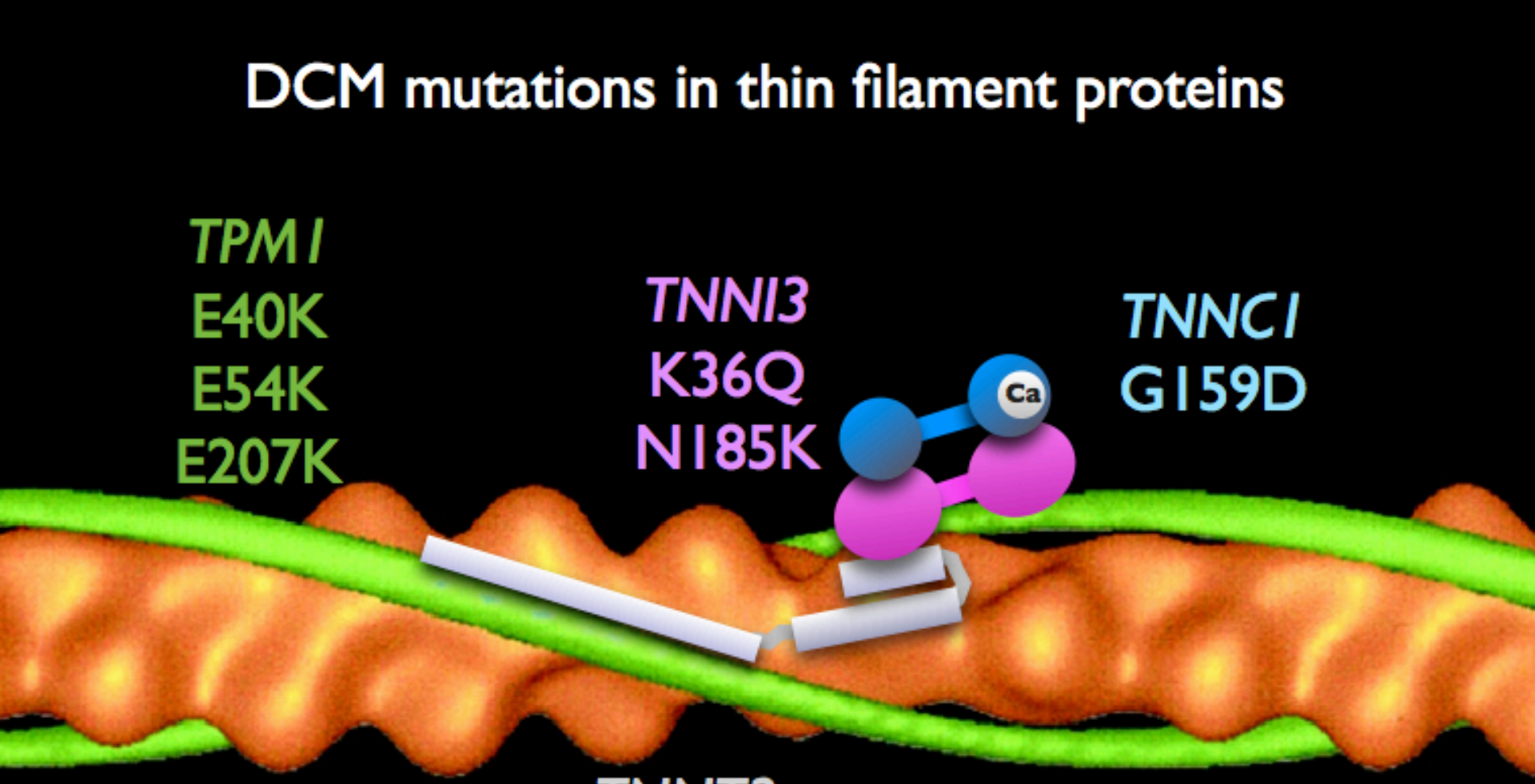
HCM

®MyHC
RLC
ELC
Titin
cMyBP-C
Actin
cTnT
cTnI
⟨TM
(AMPK□2)

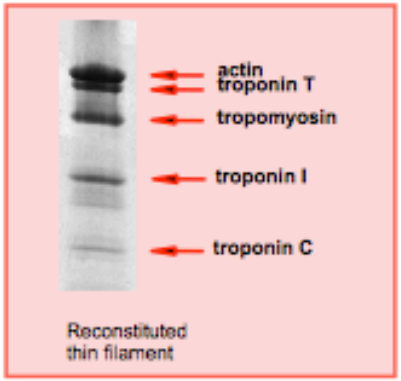


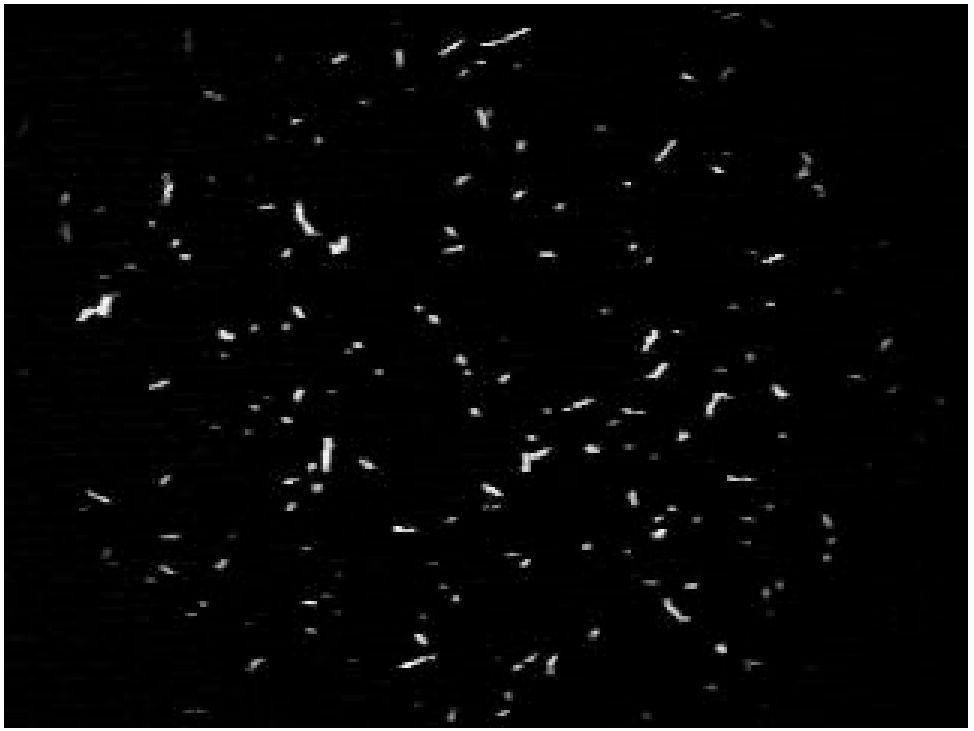
DCM

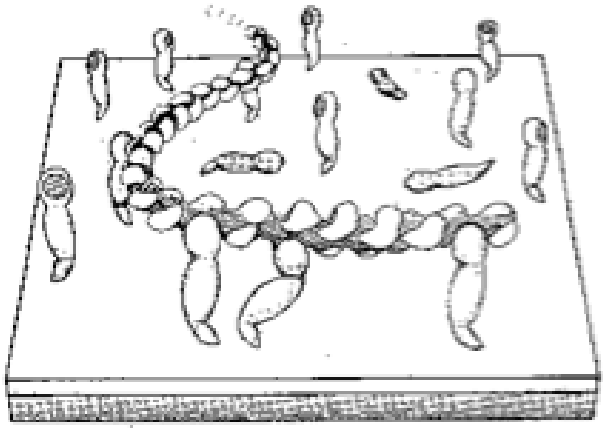
DCM mutations in thin filament proteins



Human heart troponin subunits and tropomyosin are expressed and reconstituted into thin filaments which are studied by actomyosin ATPase and *in vitro* motility assays



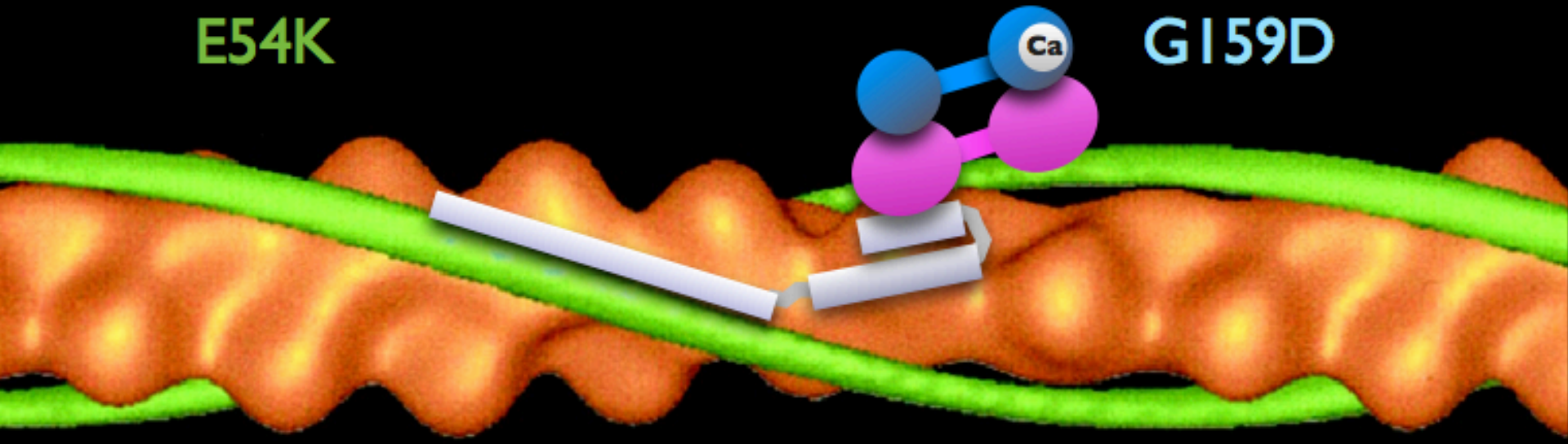




DCM mutations studied in troponin studied with recombinant protein

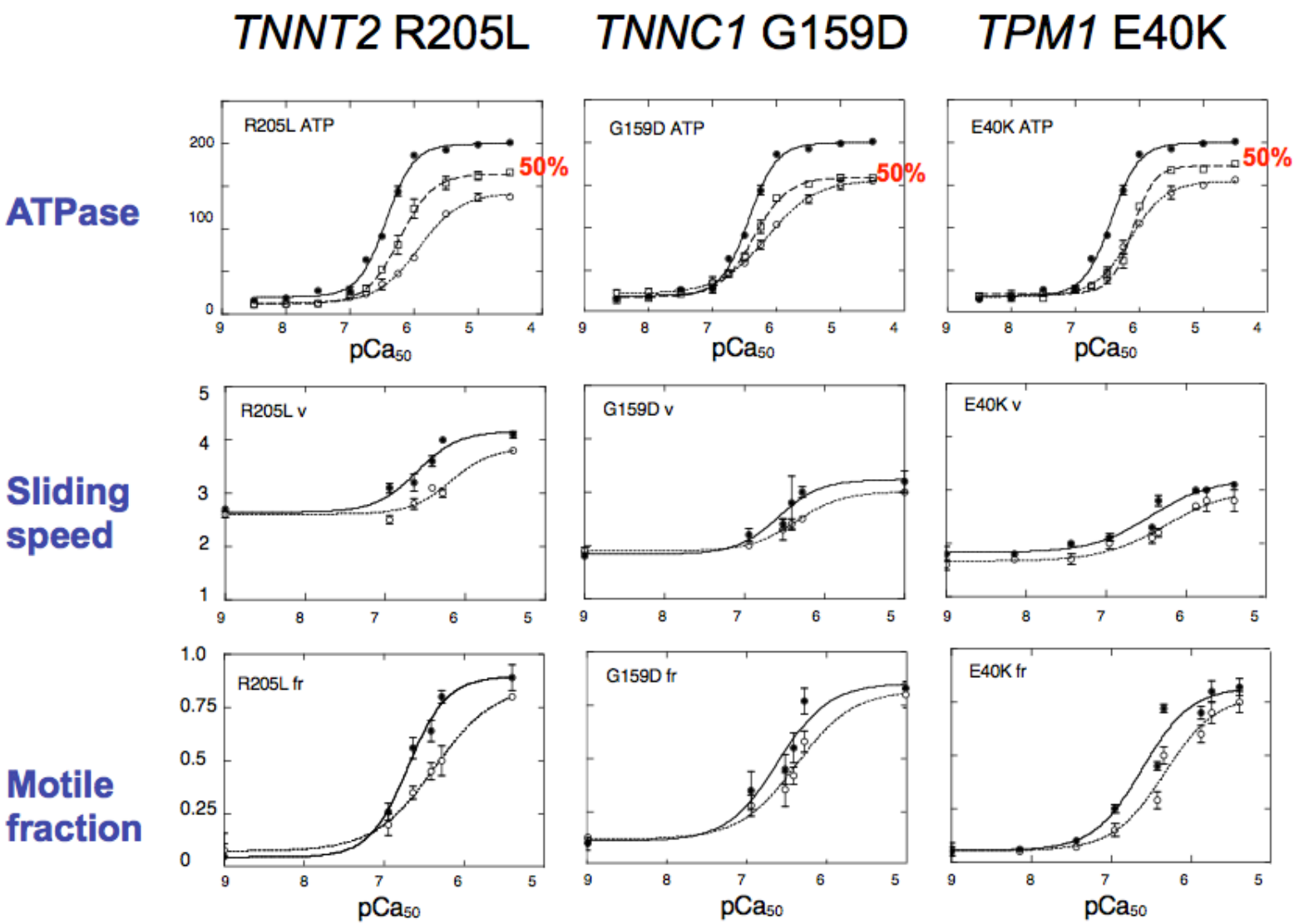
TPM1
E40K
E54K

TNNC1
G159D



TNNT2
R131W R141W
R205L ΔK210
D270N

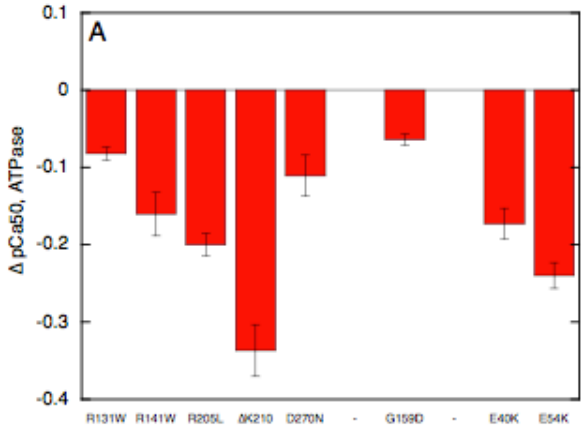
Representative Ca^{2+}-activation curves, thin filaments assembled with recombinant DCM mutant protein



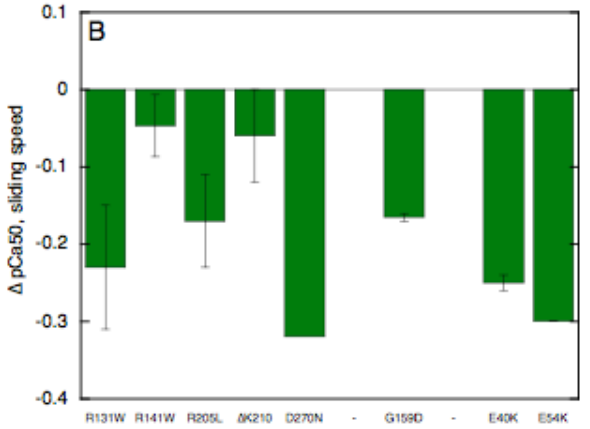
Mirza Fig 3

All mutations cause a decrease in pCa_{50} in 50:50 mixtures with wild-type protein.

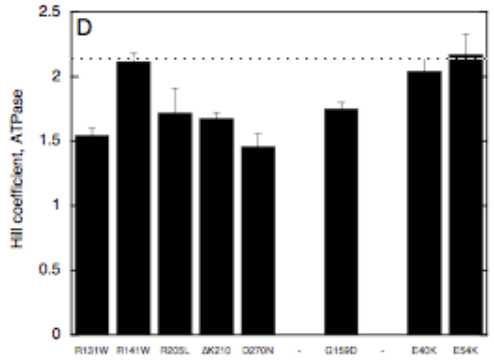
ΔpCa_{50}, ATPase



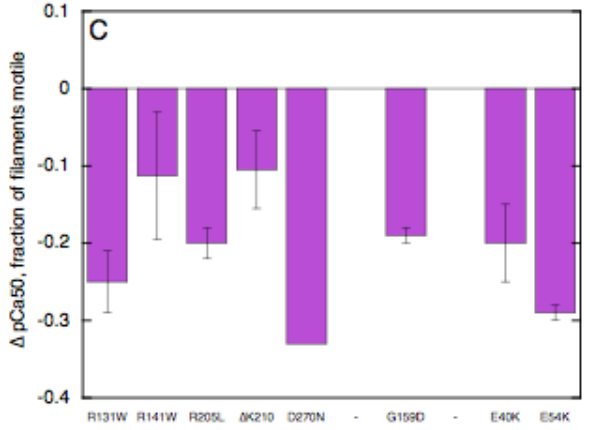
ΔpCa_{50}, sliding speed



Hill coefficient, ATPase

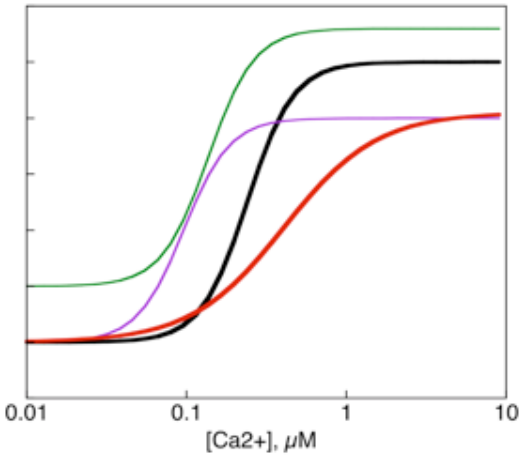


ΔpCa_{50}, motile fraction



DCM investigation through in vitro motility assay

- First approach: recombinant proteins expressed in E.coli
- Mutants showed decrease in Ca^{2+}-sensitivity and maximum sliding speed



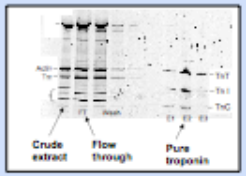
- The recombinant protein studies have indicated a clear molecular phenotype common to all familial DCM mutations (red line): lower Ca^{2+}-sensitivity, crossbridge turnover rate and cooperativity compared with wild-type (black line).

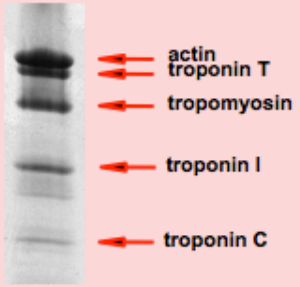
- The DCM molecular phenotype is the opposite of the molecular phenotype of HCM (green line) and also differs from acquired heart failure (purple line) where reduced crossbridge turnover rate is associated with higher Ca^{2+}-sensitivity

- The consistent pattern of results obtained with synthetic thin filaments suggests that the lower Ca^{2+}-sensitivity and slower crossbridge turnover could be the cause of the familial DCM phenotype
- To confirm this we need to study mutations in intact muscle.
- HOW?
- - Study human tissue samples with known mutations
- - Express disease-causing mutations in transgenic mouse

Native troponin and tropomyosin are isolated from human and mouse hearts and reconstituted into thin filaments which are studied by *in vitro* motility assay

Troponin was isolated from high salt extract of myofibrils by affinity chromatography using 0.5 ml anti TnI monoclonal 14G5 (epitope in the centre of TnI) conjugated to Sepharose CL4B.



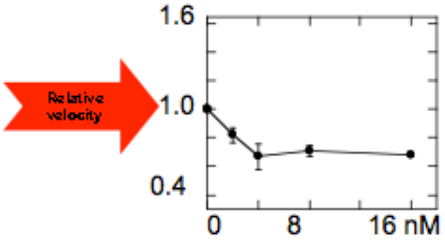


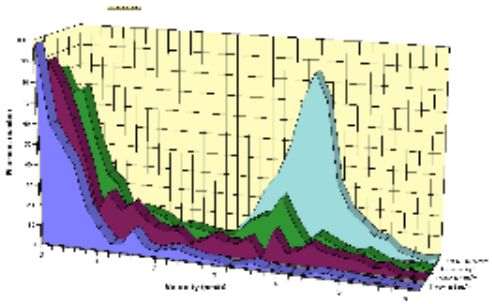
Reconstituted thin filament

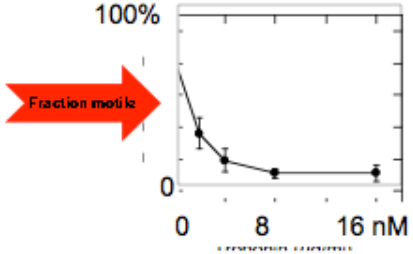
At pCa9 10nM troponin switches off movement

Fraction motile is less than 5% and sliding speed is reduced by 30%

"Switch-off" is mediated by troponin I





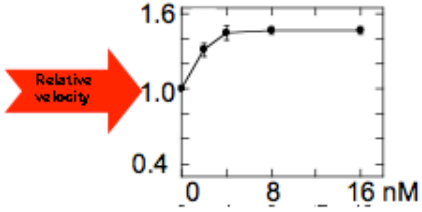


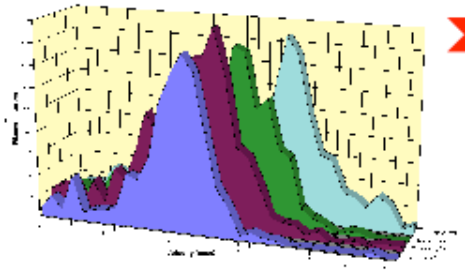
pCa 9

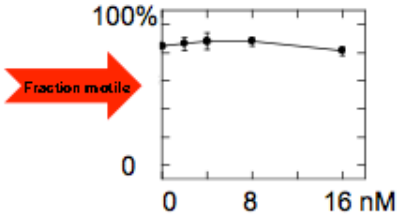
At pCa5 10nM troponin increases sliding velocity up to 50%

fraction of filaments motile remains high

"Switch-on" is mediated by troponin T



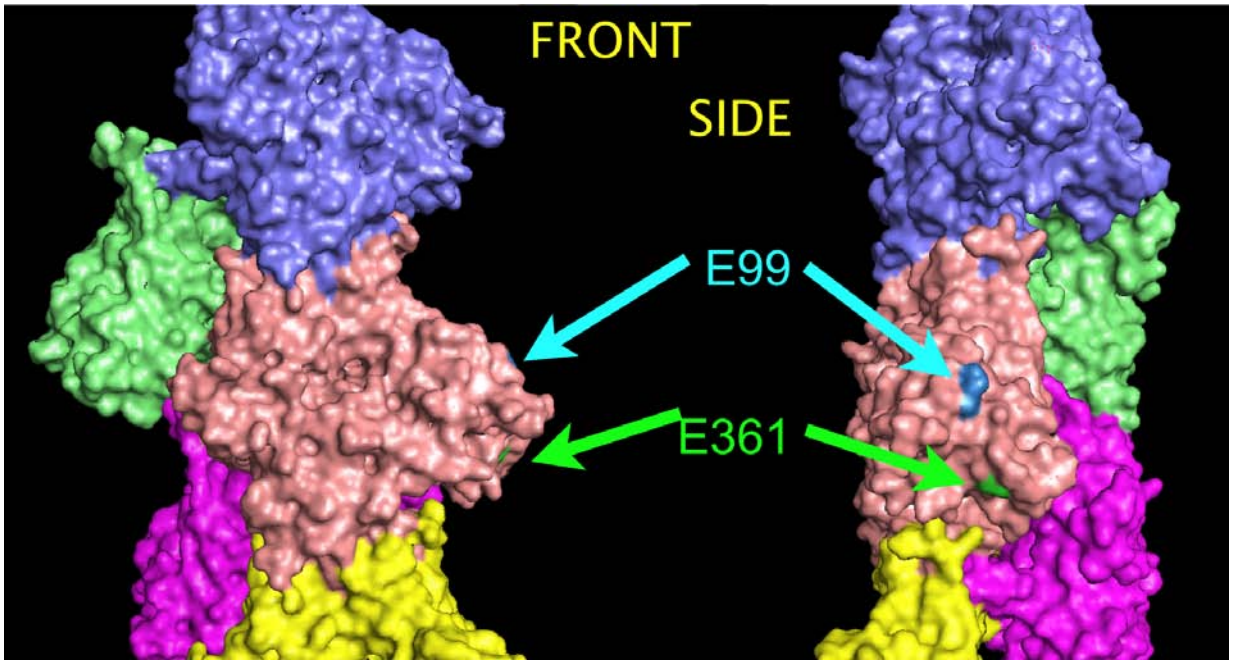




pCa 5

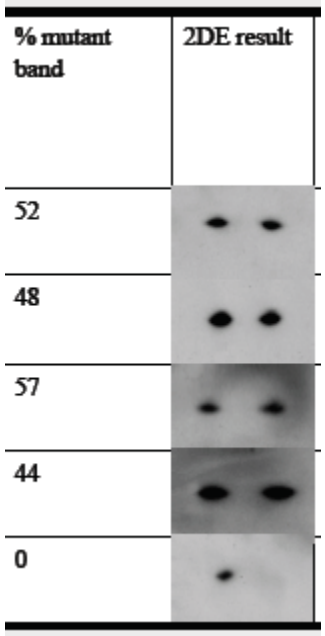
Actin E361G linkage to DCM

- Actin E361G was described in two generations of a small family
- We produced a TG mouse model
- E361 is located on the surface of subdomain 1



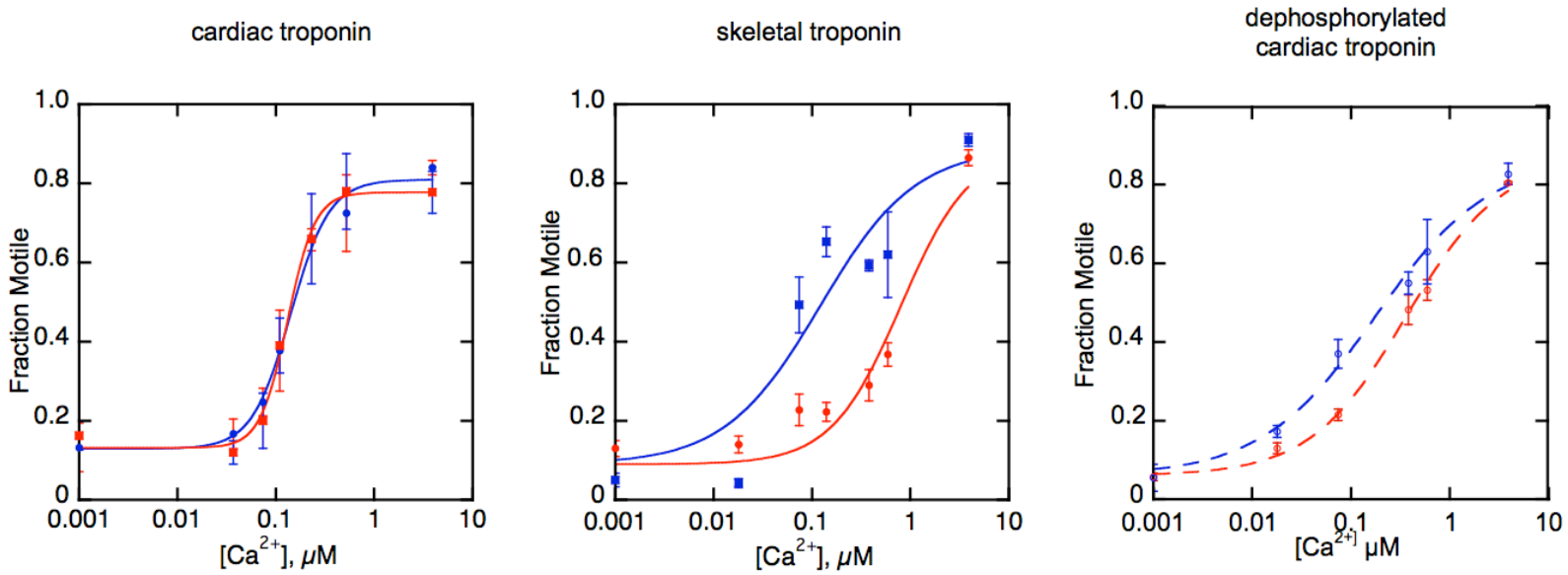
The E361G Transgenic Mice

- Transgenic (TG) mice expressing 50% E361G mutant cardiac actin in their hearts were generated



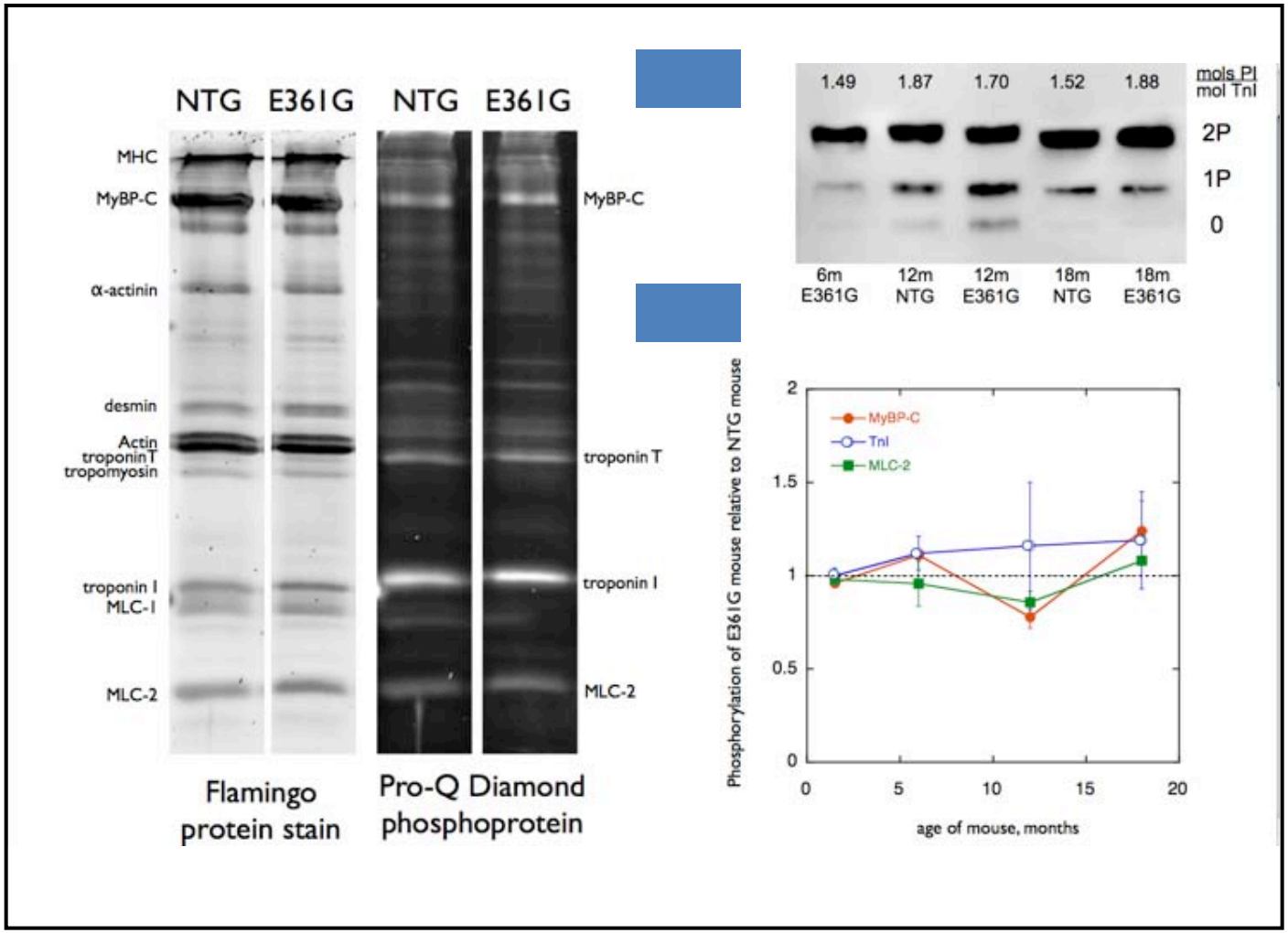
- Kristen Nowak (University of Western Australia)
- Charles Redwood (University of Oxford)
- Kim Wells (Imperial College London)
- Dominic Wells & Ke Liu (Imperial College London)

Ca^{2+} regulation using human troponin and tropomyosin is the same in thin filaments with NTG and E361G actin

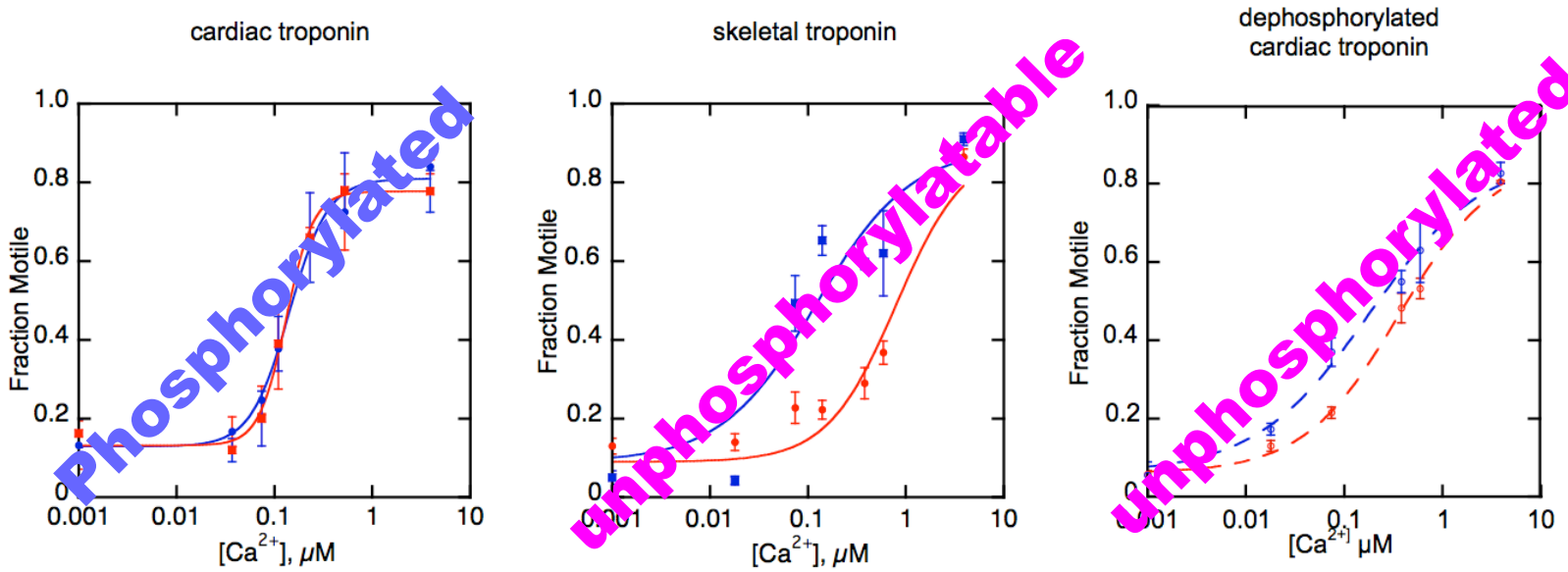


But Ca^{2+} sensitivity is lower in thin filaments with unphosphorylated troponin

Contractile protein phosphorylation is the same in NTG and E361G

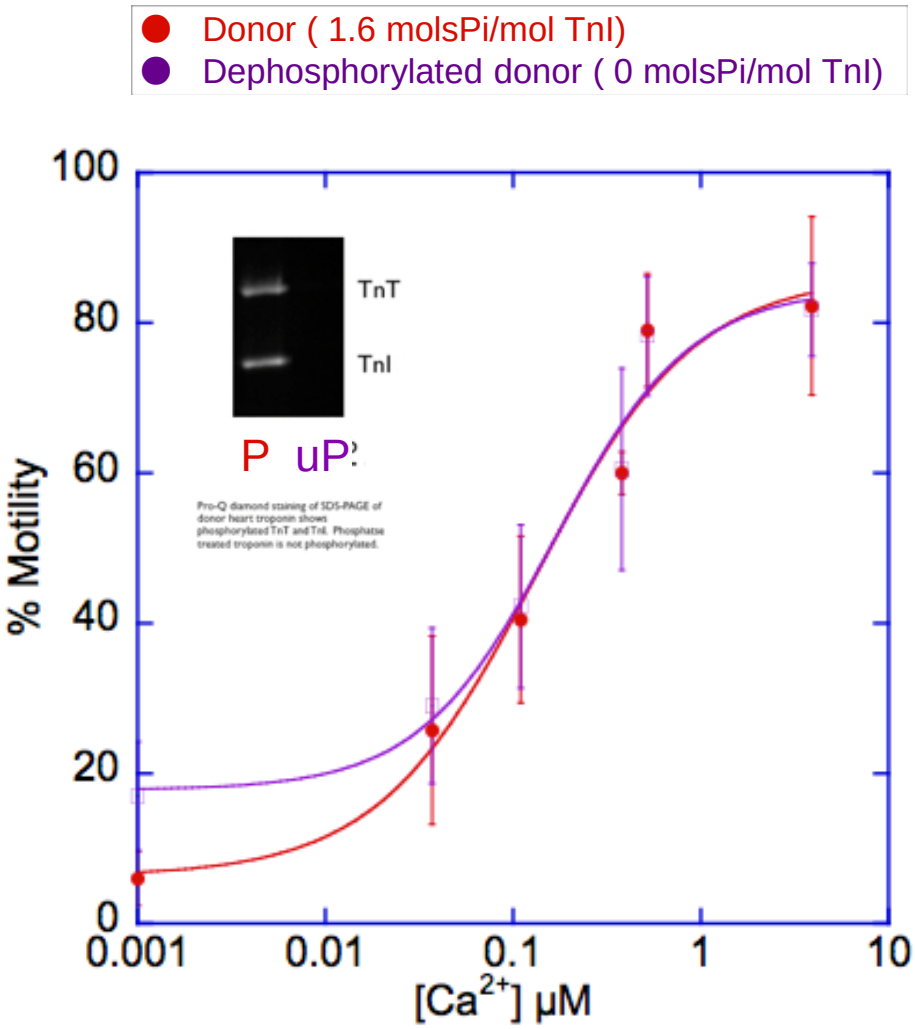


Ca^{2+} regulation using human troponin and tropomyosin is the same in thin filaments with NTG and E361G actin



Phosphorylation is not altered but the response to phosphorylation is absent in ACTC E361G thin filaments

UNCOUPLES TnI phosphorylation from change in Ca^{2+}-sensitivity



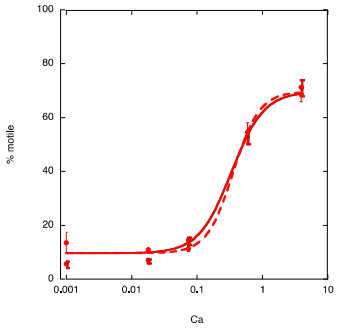
Mouse Line	EC_{50} Percentage Motility, µM		$\frac{EC_{50}\ D}{EC_{50}\ D.uP}$
	Donor Tn	UnP Donor Tm	
20.55.19	0.309 ± 0.091	0.331 ± 0.087	0.93
20.1.20.1	0.604 ± 0.160	0.623 ± 0.073	0.97
20.55.26	0.153 ± 0.046	0.120 ± 0.045	1.28
20.55.26	0.335 ± 0.058	0.358 ± 0.058	0.94
20.55.33	0.152±.052	0.149±.054	1.08
Mean: ± SE	0.311 ± 0.082	0.314 ± 0.091	1.04 ± 0.07
Student's t-test (paired)	p = 0.75		Single group, p = 0.58 (compare with 1)
(Unpaired)	p = 0.96		

Comparison of EC_{50}

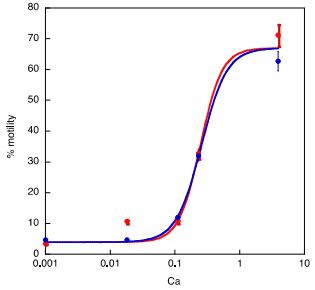
no significant difference in sliding speed at activating $[Ca^{2+}]$

Ca^{2+}-sensitivity of E361G actin-containing thin filaments is not responsive to TnI phosphorylation levels

E361G actin



Low



x 1.1

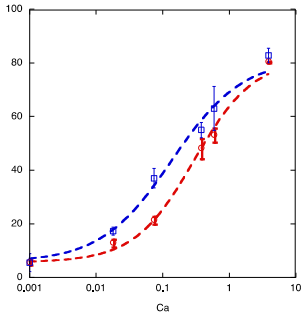
Low

P

x 0.9

x 2.6

uP

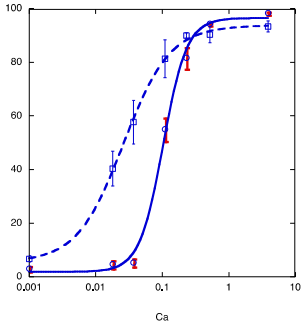


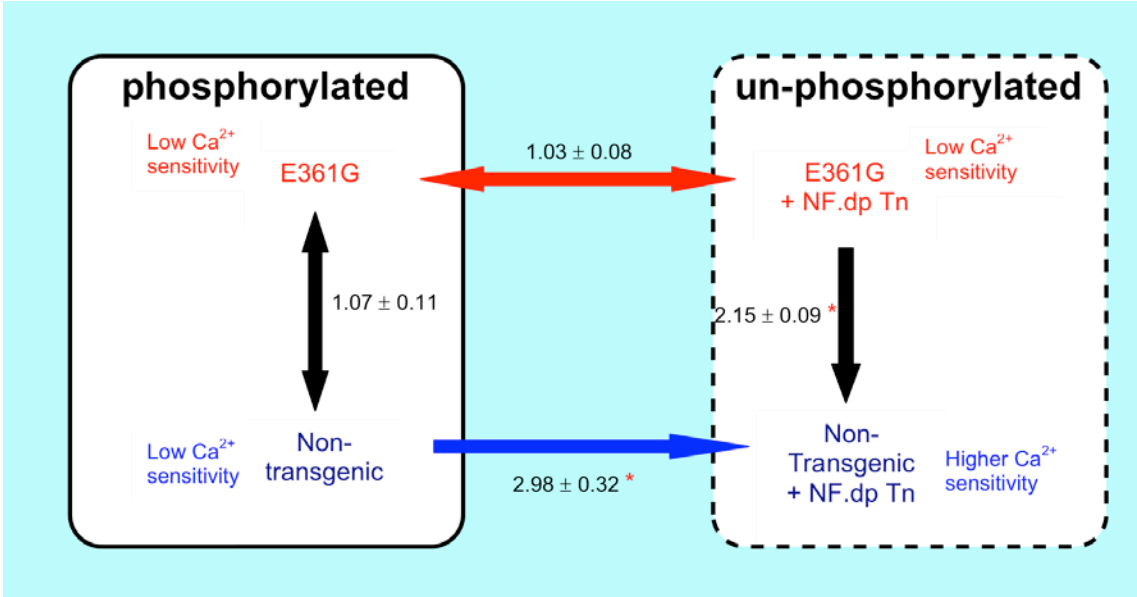
x 3.1

Low

High

NTG actin



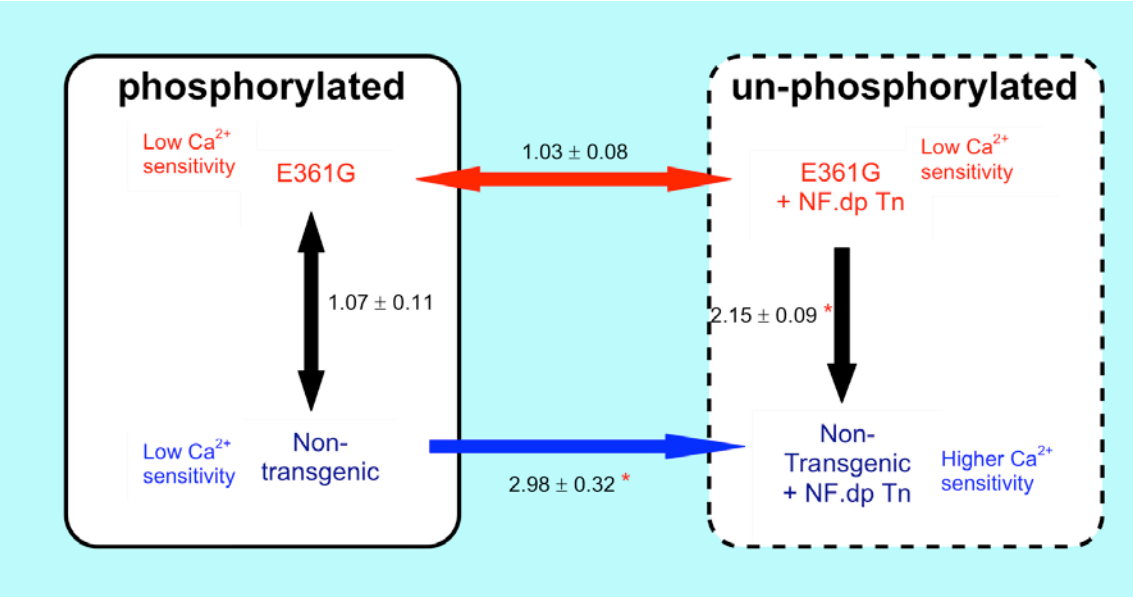


CONCLUSIONS

We conclude that the primary functional change induced by the E361G mutation in cardiac actin is a blunted response to troponin I phosphorylation. Ca^{2+}-sensitivity is low, characteristic of phosphorylated troponin I at all levels of TnI phosphorylartion.

The blunted response to troponin phosphorylation in E361G actin-containing filaments would be predicted to compromise the lusitropic response to catecholamines.

We propose that the chronic reduced cardiac reserve is the main factor that predisposes DCM mutant hearts to dilation and failure

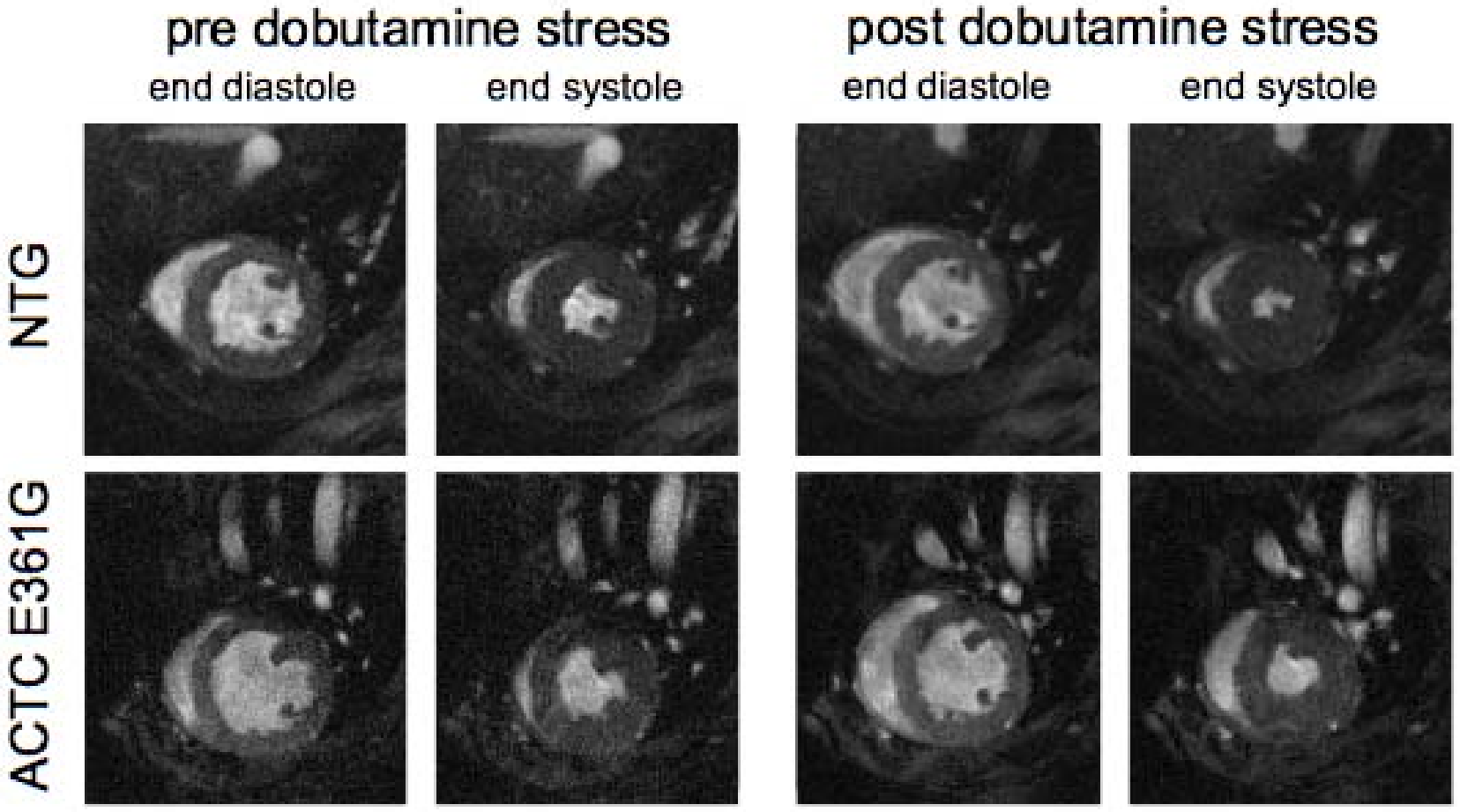


TEST OF HYPOTHESIS

✦ Is there a blunted response to β-adrenergic stimulus in hearts with DCM mutations?

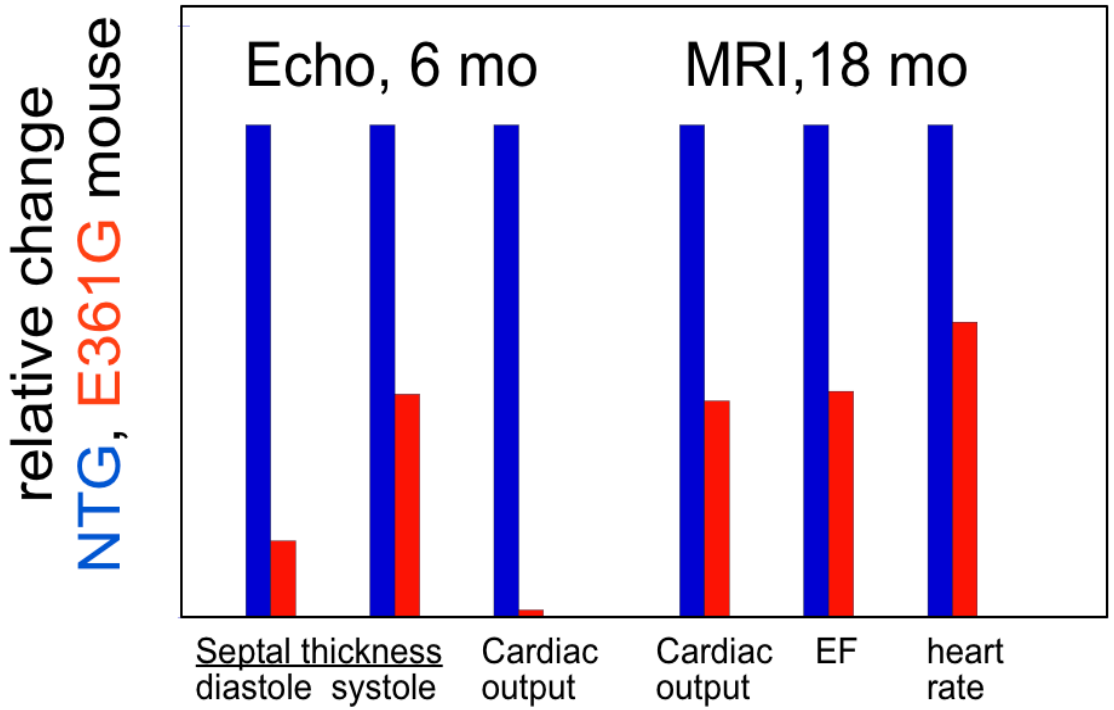
✦ Is this uncoupling of Ca^{2+}-sensitivity from phosphorylation observed in all DCM mutations?

In support of this hypothesis the E361G mouse heart is less responsive to dobutamine than NTG

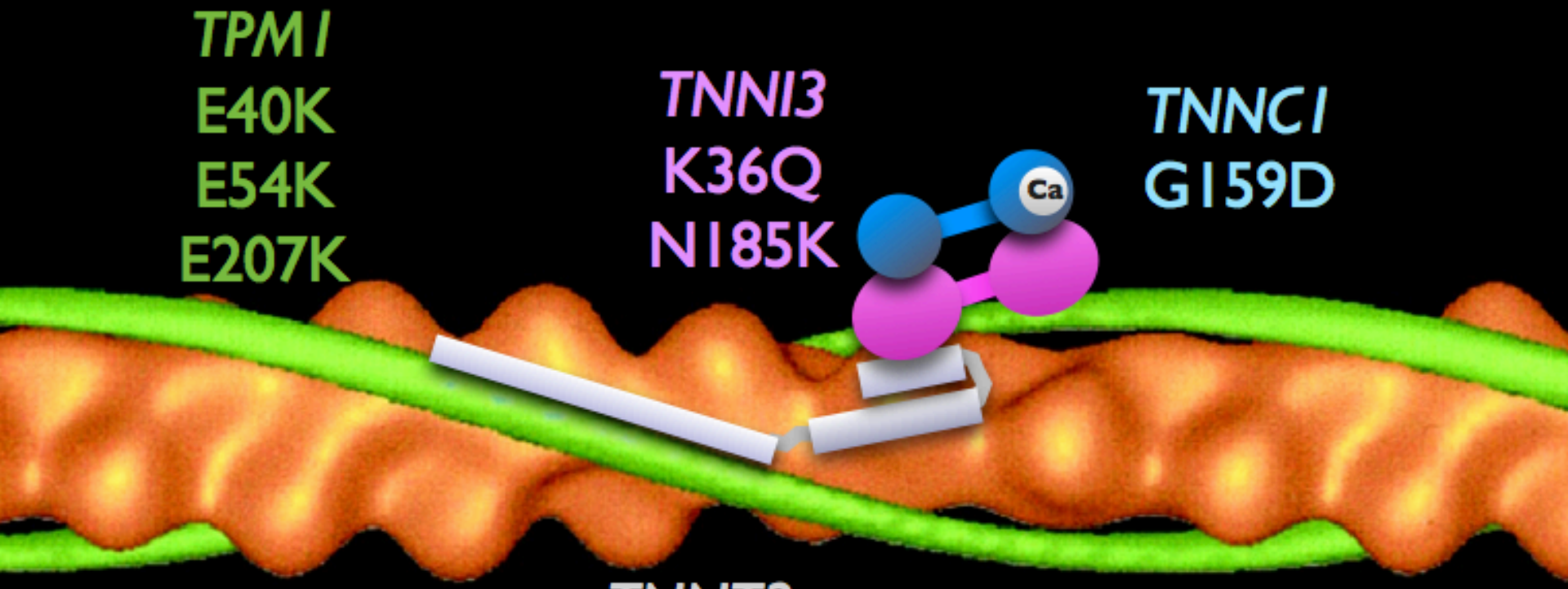


D. Stuckey

In support of this hypothesis the E361G mouse heart is less responsive to dobutamine than NTG



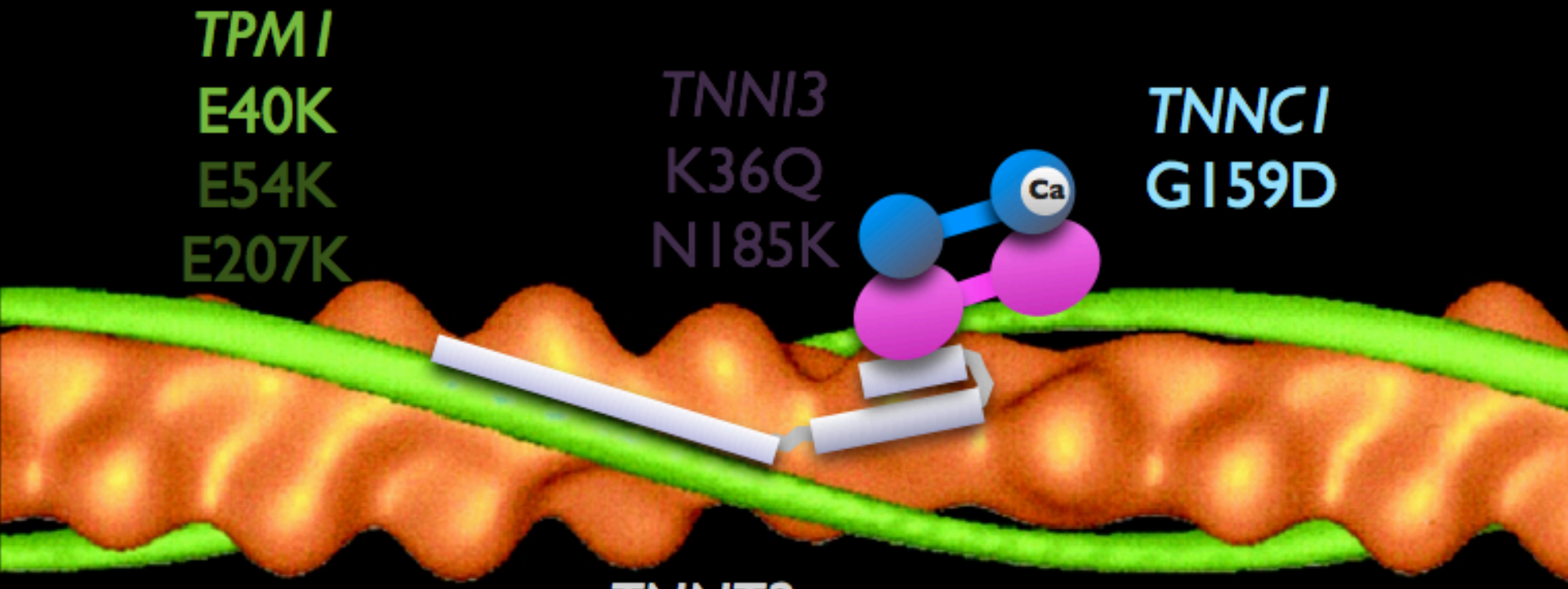
Is uncoupling or a decreased Ca^{2+}-sensitivity (or both) the molecular mechanism for familial DCM?



TNNT2
R131W R141W
ΔS171 R205L
ΔK210 K247R
D270N

ACTC
E361G
R312H

Is uncoupling or a decreased Ca^{2+}-sensitivity (or both) the molecular mechanism for familial DCM?



TNNT2

R131W R141W

ΔS171 R205L

ΔK210 K247R

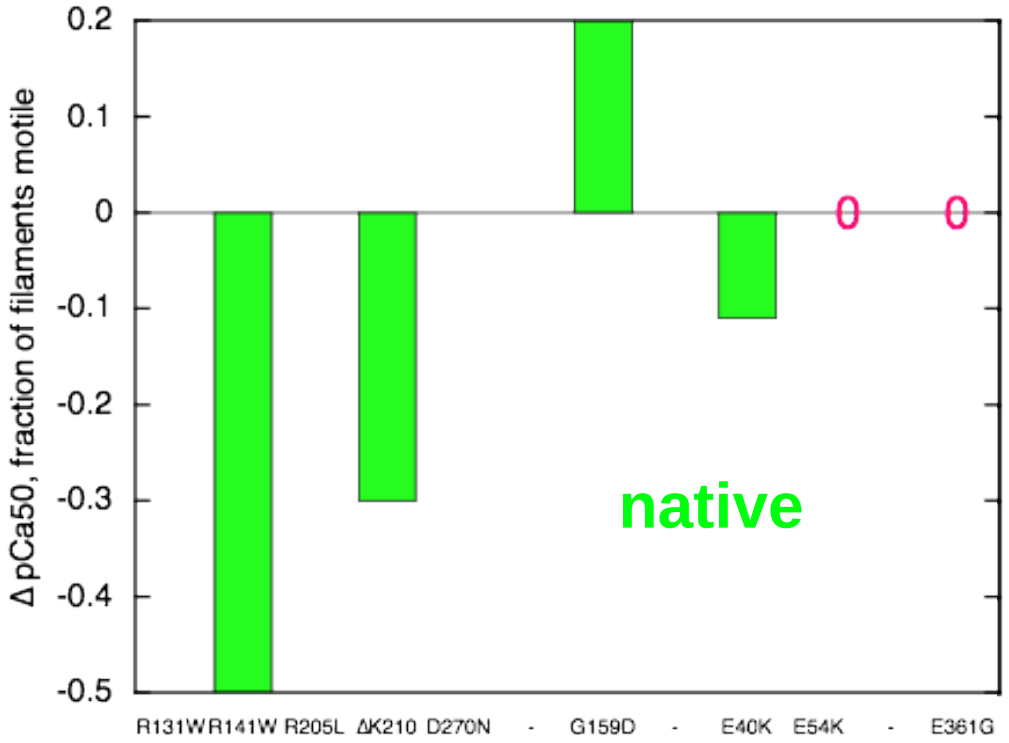
D270N

ACTC

E361G

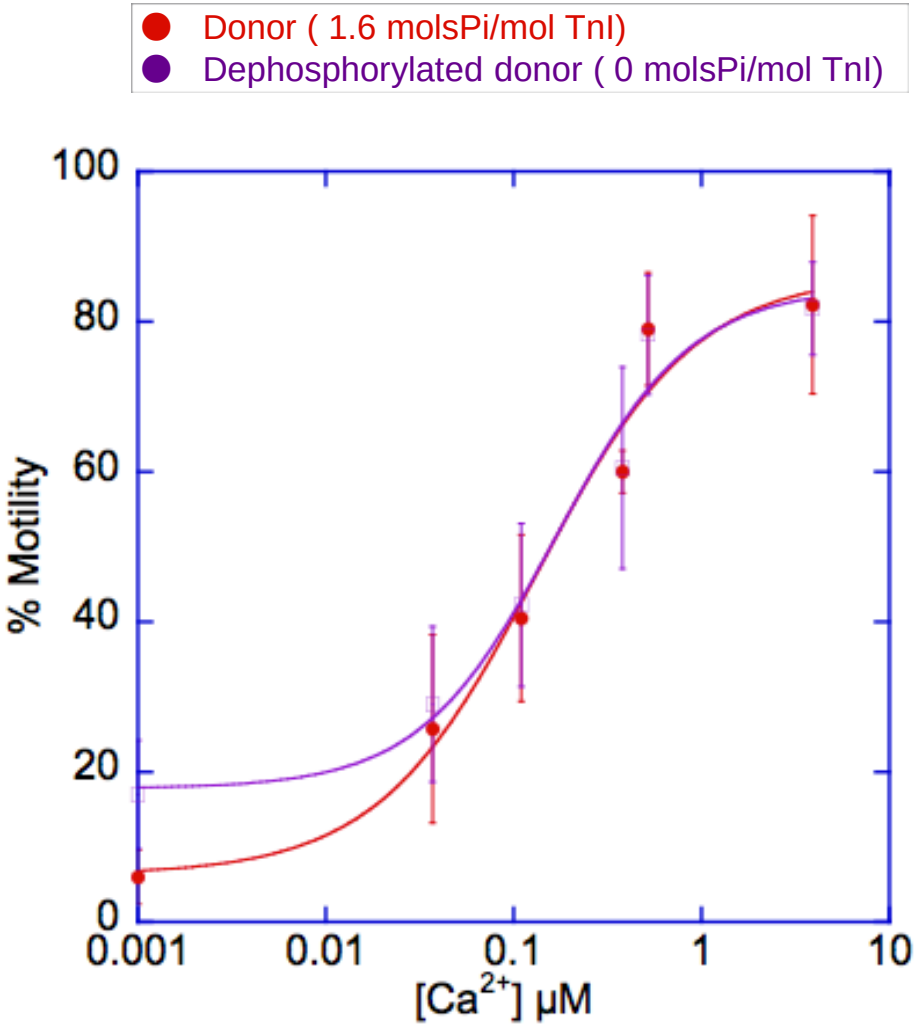
R312H

Ca^{2+}-sensitivity does not correlate with the DCM phenotype in native thin filament proteins



ACTC E361G actin plus human heart tropomyosin and troponin

Actin from transgenic mouse heart tested with human troponin, expression is 50%



Mouse Line	EC_{50} Percentage Motility, µM		$\frac{EC_{50}D}{EC_{50}D.uP}$
	Donor Tn	UnP Donor Tm	
20.55.19	0.309 ± 0.091	0.331 ± 0.087	0.93
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20.55.26	0.335 ± 0.058	0.358 ± 0.058	0.94
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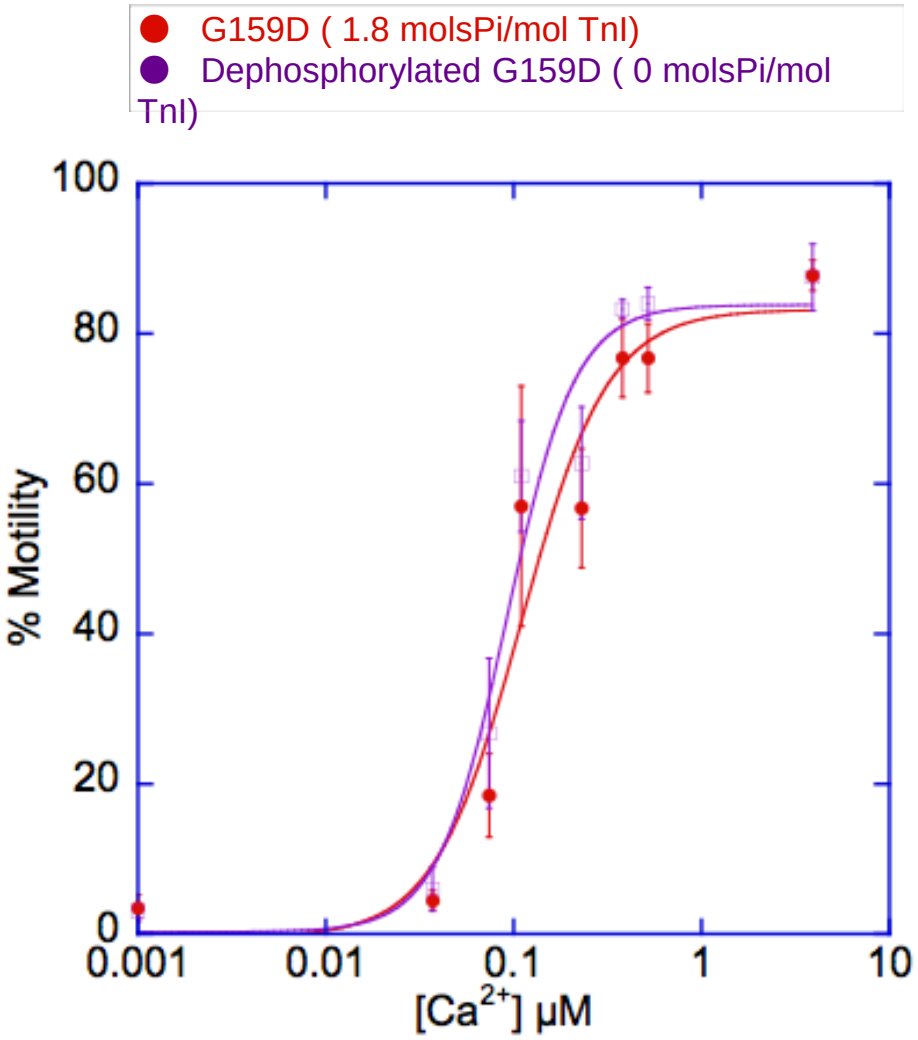
Mean: ± SE	0.311 ± 0.082	0.314 ± 0.091	1.04 ± 0.07
Student's t-test (paired)	p = 0.75		Single group, p = 0.58 (compare with 1)
(Unpaired)	p = 0.96		

Comparison of EC_{50}

no significant difference in sliding speed at activating $[Ca^{2+}]$

TNNC1 G159D troponin plus human heart tropomyosin

Troponin from explanted heart of patient with this mutation, mutant expression is 45%



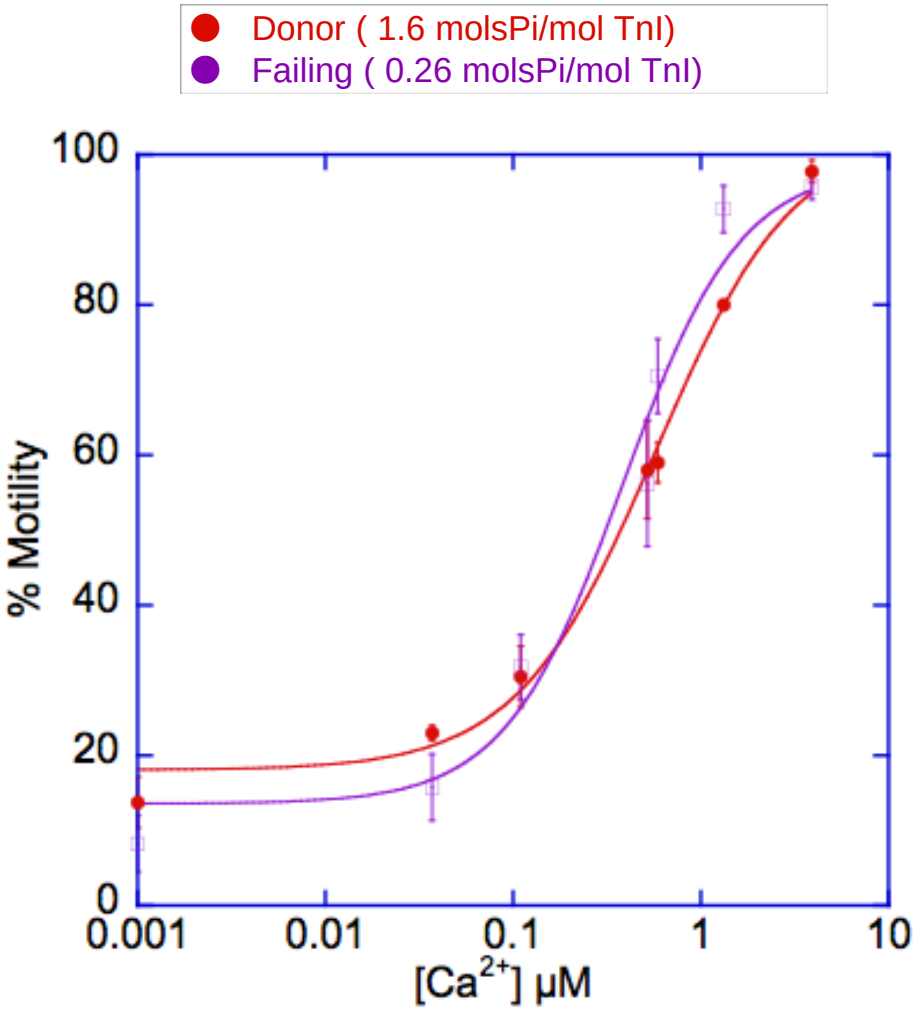
Expt. No.	EC_{50} Fraction motile , µmol/L		EC_{50} native / EC_{50} dephos-phorylated
	Native G159D	Dephosphorylated G159D	
1	0.036±0.014	0.033±0.009	1.09
2	0.019±0.005	0.018±0.003	1.05
3	0.033±0.018	0.021±0.010	1.57
4	0.065±0.021	0.063±0.007	1.03
5	0.109±0.030	0.092±0.016	1.18
Mean ± SD	0.052±0.036	0.045±0.032	**1.18±0.09** p=0.0003

Comparison of EC_{50}

no significant difference in sliding speed at activating [Ca^{2+}]

TPM1 E40K tropomyosin plus human heart troponin

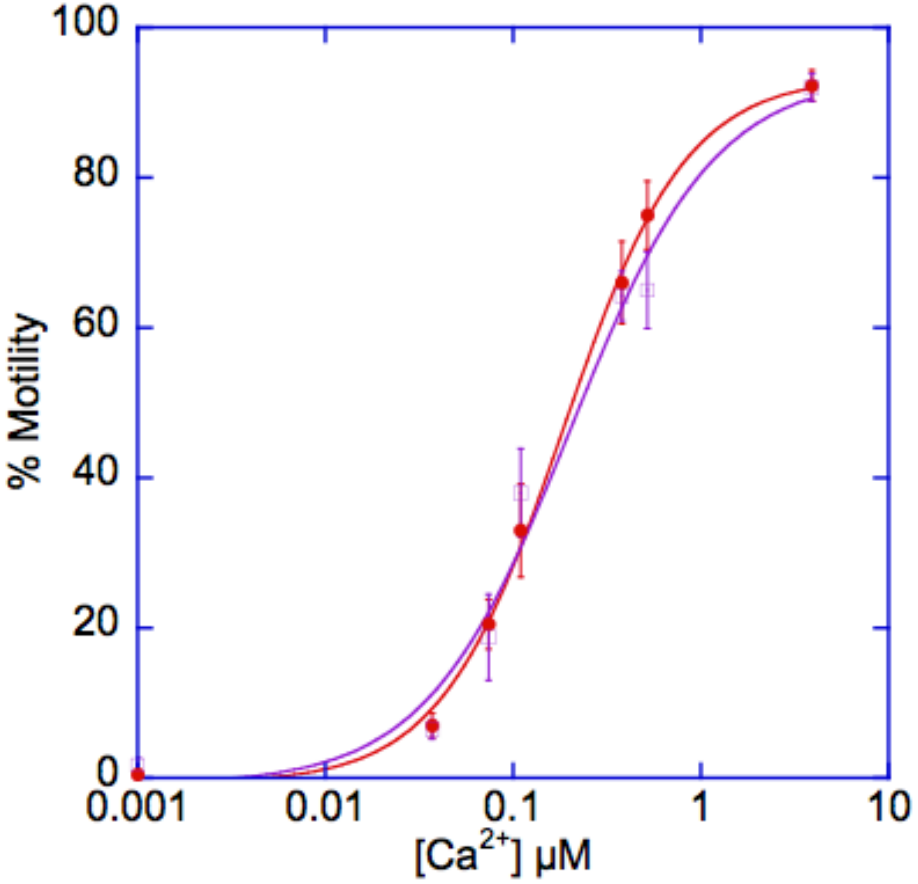
Tropomyosin is expressed in baculovirus/sf9 cells, tested with human troponin



TNNT2 R141W from transgenic mouse (Lianfeng Zhang)

Troponin is extracted from transgenic mouse heart, expression level not known)

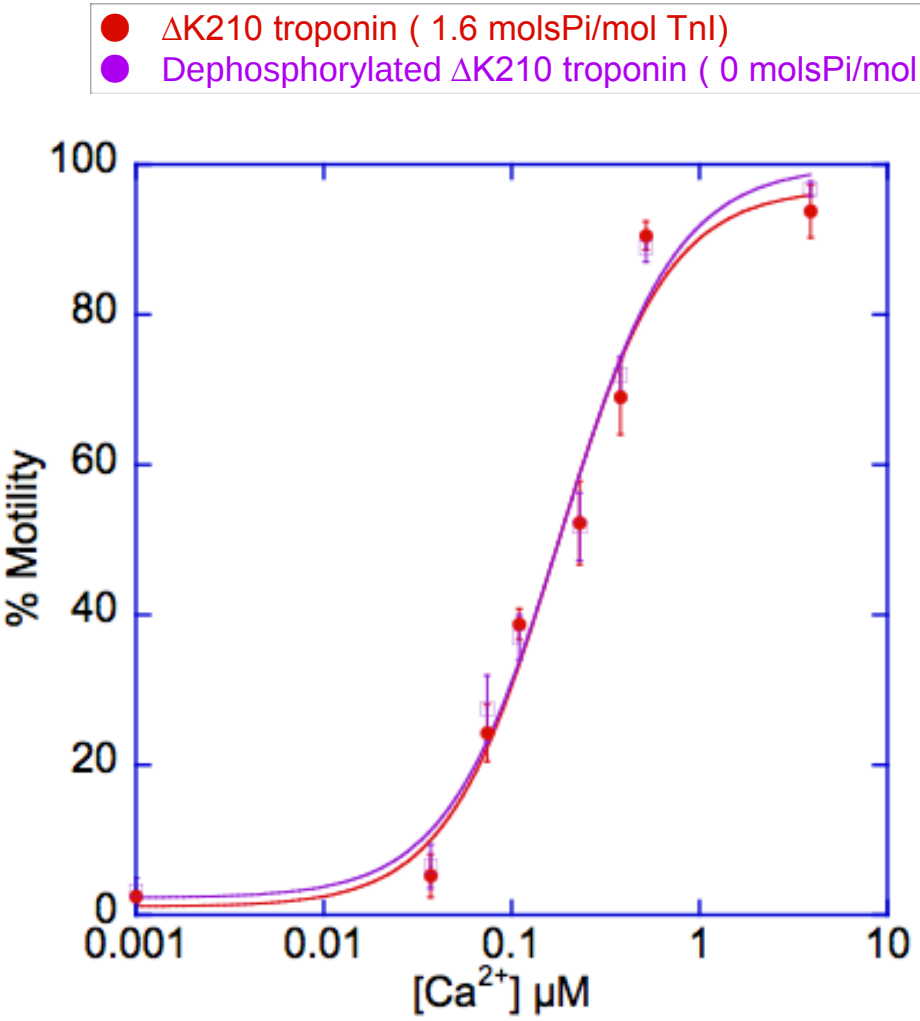
- R141W troponin (1.6 molsPi/mol TnI)
- Dephosphorylated R141W troponin (0 molsPi/mol TnI)



Tn R141W ANALYSIS	EC_{50} Percentage motility, µM		EC_{50} WT / EC_{50} mutant	Hill coefficient n	
DATE	R141W P	R141W dP		P	dP
09/02/11	0.17±0.07	0.16±0.06	1.06	1.15	1.11
09/02/11	0.16±0.08	0.18±0.1	0.89	1.02	1.19
10/02/11	0.18±0.01	0.2±0.06	0.9	**1.33**	**1.13**
MEAN±SE	0.17±0.01	0.18±0.01	0.95±0.06	1.17± 0.09	1.14± 0.02
Student's t-test (paired)	P=0.42		single group, p=0.46 (compared with one)		
(unpaired)	P=0.48				

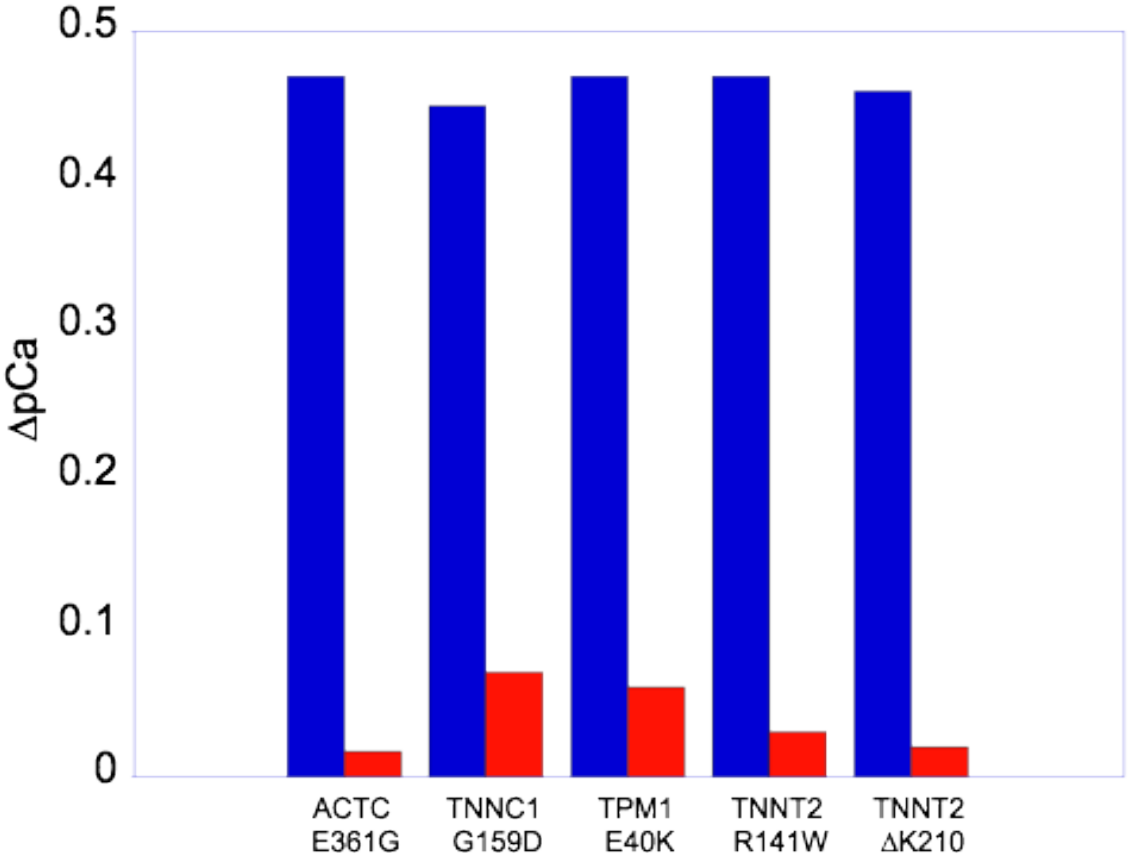
TNNT2 ΔK210 from transgenic mouse (Sachio Morimoto)

Troponin is extracted from homozygous KI transgenic mouse heart, expression level 100%



Tn ΔK210 ANALYSIS	EC_{50} Percentage motility, µM		EC_{50} WT / EC_{50} mutant	Hill coefficient n	
DATE	ΔK210 P	ΔK210 dP		P	dP
23/02/11	0.14±0.05	0.18±0.06	0.78	1.44	1.11
24/02/11	0.12±0.03	0.13±0.04	0.92	**1**	**1**
06/04/11	0.17±0.04	0.18±0.03	0.94	1.48	1.43
06/04/11	0.18±0.04	0.15±0.05	1.2	1	1
MEAN±SE	0.15±0.01	0.16±0.01	0.96±0.09	1.23± 0.13	1.14± 0.1
Student's t-test (paired)	P=0.638		single group, p=0.679 (compared with one)		
(unpaired)	P=0.698				

Summary: all DCM mutations tested uncouple Ca^{2+}-sensitivity and troponin I phosphorylation

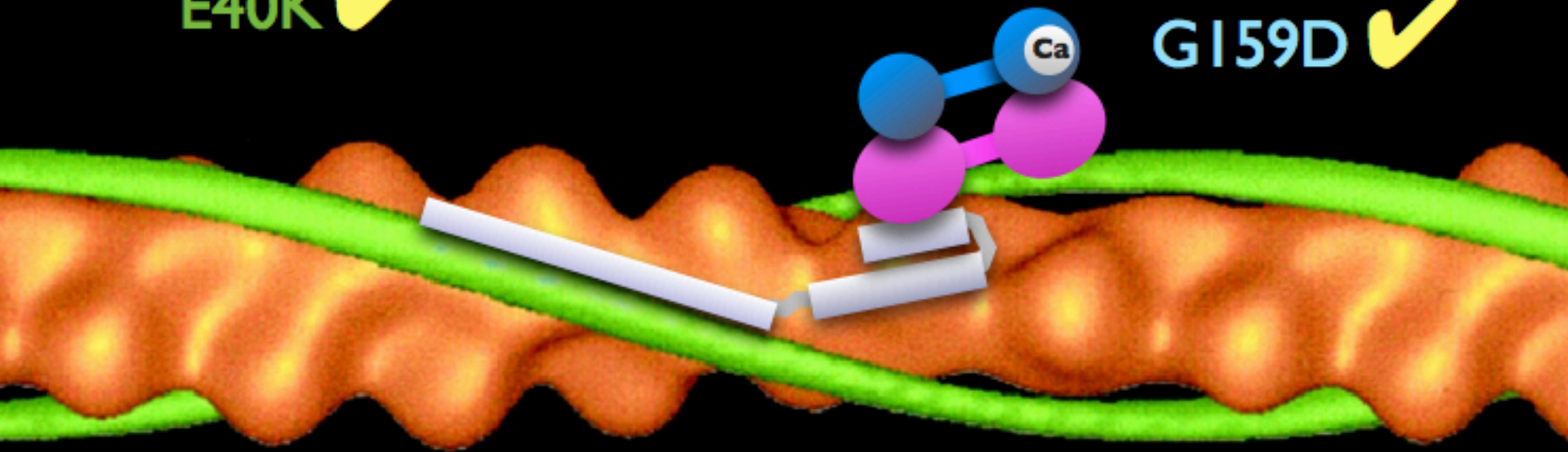


DCM mutations studied in troponin from intact heart

All the mutations uncouple

TPM1
E40K ✔

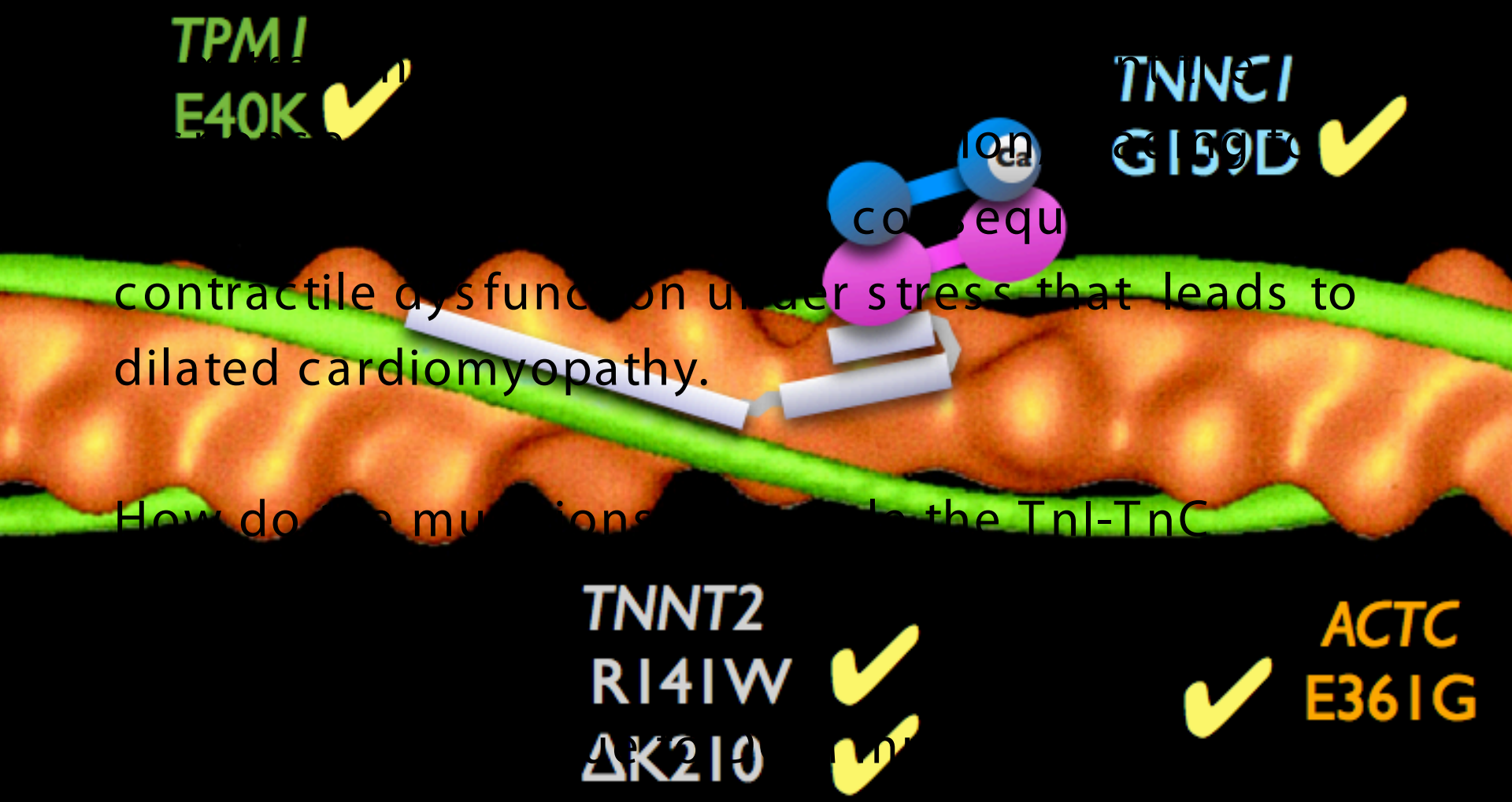
TNNC1
G159D ✔



TNNT2
R141W ✔
ΔK210 ✔

ACTC
✔ E361G

We conclude that DCM mutations in thin filament



ion

co equ

contractile dysfunction under stress that leads to dilated cardiomyopathy.

How do the mutations do the Tnl-TnC

CONCLUSION: DCM

Uncoupling Ca^{2+}-sensitivity from TnI phosphorylation is a causative mechanism of familial DCM

- This mechanism is compatible with previous measurements *in vitro*.
- Other changes measured *in vitro* may not be relevant to the DCM phenotype

Acknowledgements

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Imperial College London

British Heart Foundation