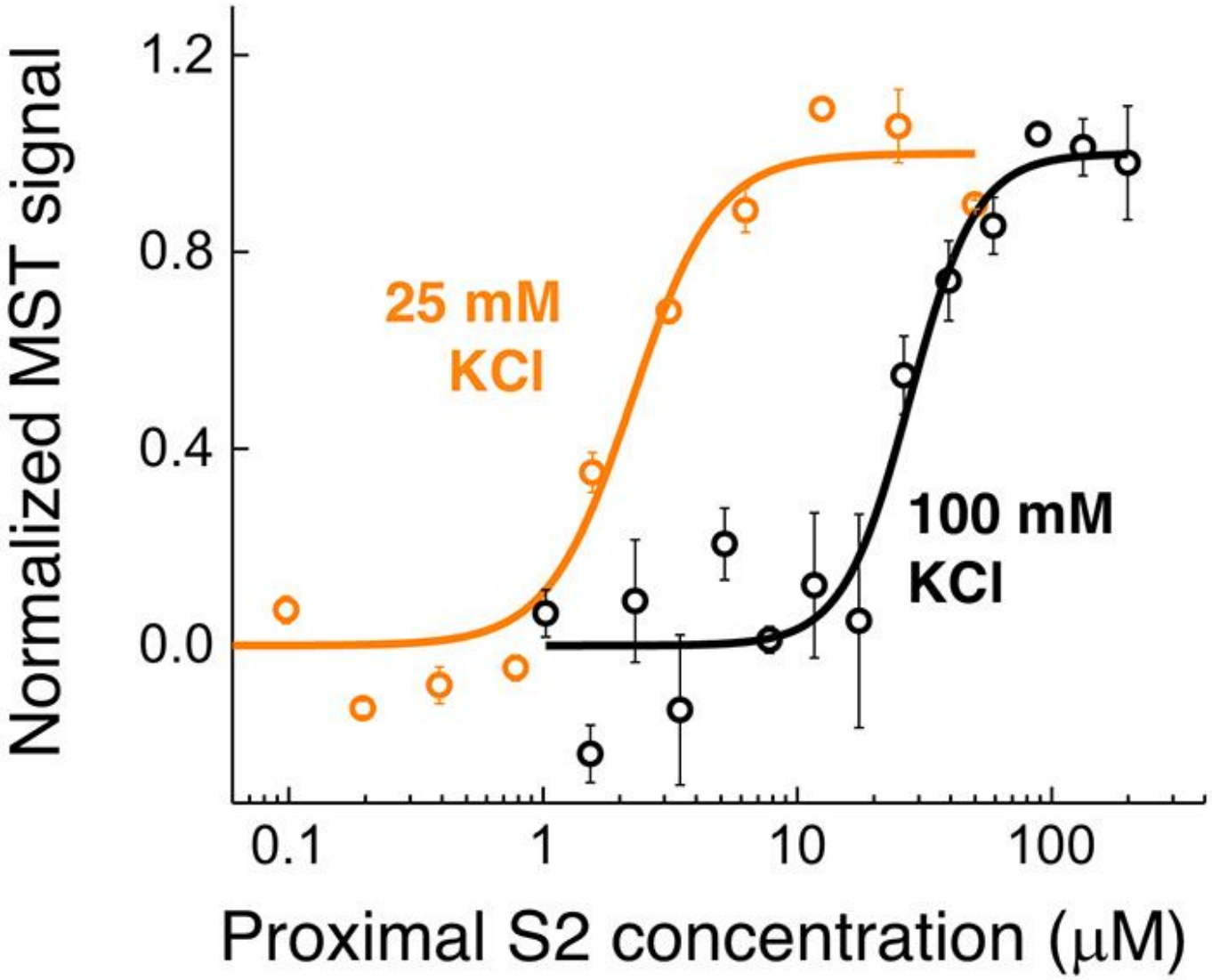
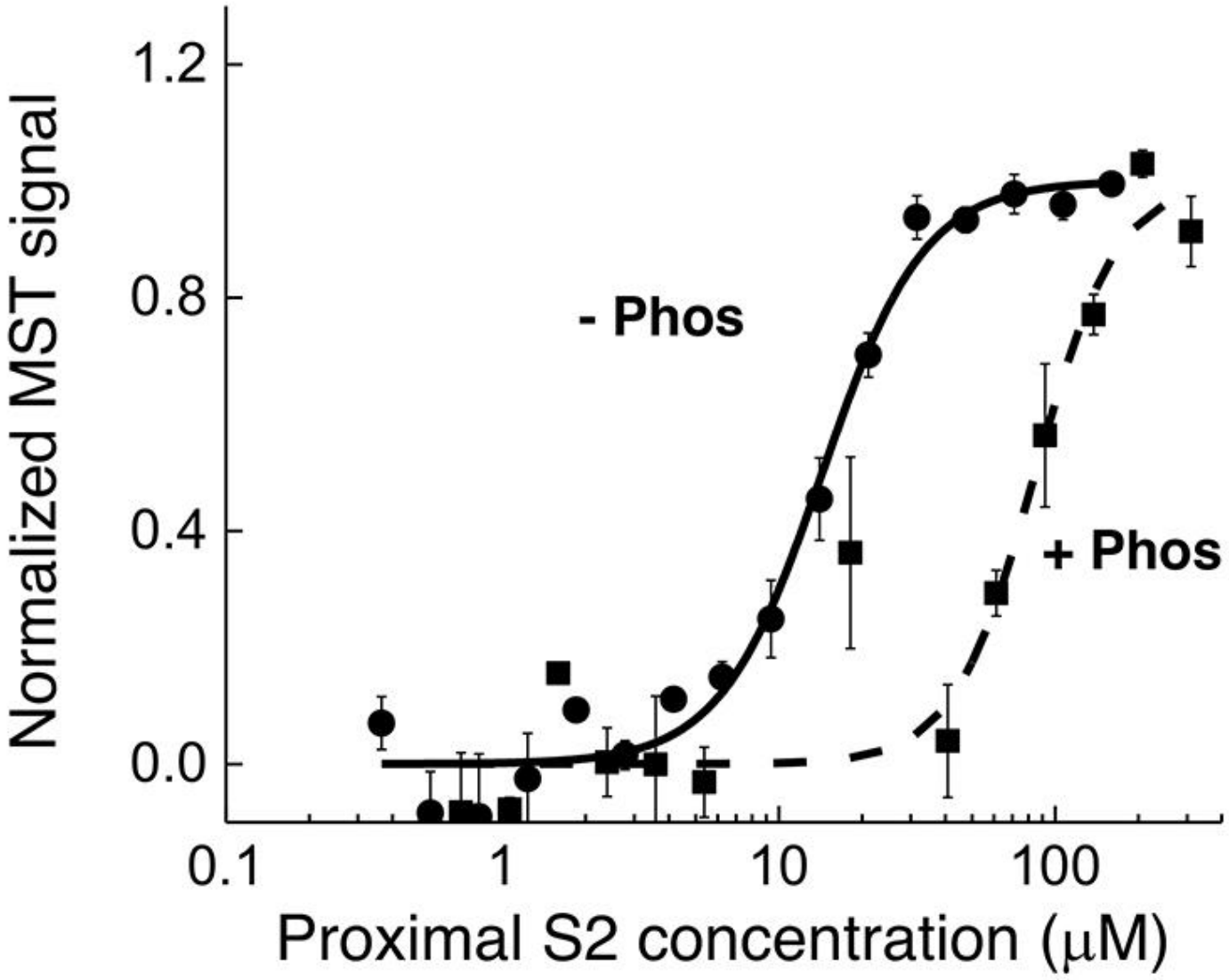


Supplementary Figure 1

Binding of sS1 to proximal S2 using MST.

Binding of Cy5-tagged sS1 to proximal S2 at 25 mM (orange) and 100 mM (black) KCl. All data shown in the figure are representative curves; data points are mean and s.e.m. from n=3 readings from a single protein preparation; source data are in Supplementary Table 2. Data from multiple preparations are summarized in Supplementary Table 1.



Supplementary Figure 2

Binding of 2-hep HMM to proximal S2 at 100 mM KCl using MST.

Non-phosphorylated (-Phos) and phosphorylated (+Phos) 2-hep HMM are compared. All data shown in the figure are representative data points are mean and s.e.m. from n=3 readings from a single protein preparation; source data are in Supplementary Table 2.

Supplementary Table 1

Prep # (#readings)	S1-Cy5 with S2 25 mM KCl	S1-Cy5 with S2 100 mM KCl	S1-GFP with S2 25 mM KCl	S1-GFP with S2 100 mM KCl	R403Q S1 with S2	R453C S1 with S2	R249Q S1 with S2	R870H S2 with S1	D906G S2 with S1	S1 with MyBPC (-P)	S1 with MyBPC (+P)	S1 with C0C2 (-P) 100 mM KCl	S1 with C0C2 (+P) 100 mM KCl	S2 with HMM (-P) 25 mM KCl	S2 with HMM (+P) 25 mM KCl	S2 with HMM (-P) 100 mM KCl	S2 with HMM (+P) 100 mM KCl
1 (4-6)	1	30	5	47	46	>90	>300	60	nb	17	34	35	60	5	70	25	60
2 (4-6)	2	35	3	30	60	>200	>300	57	nb	19	44	32	65	11	60	20	>80
3 (4-6)		45		35	56	>200				14	32					15	>60
4 (4-6)		26		50													
5 (4-6)				70													
Mean ± sem	**1.5±0.5***	**34±4***	**4±1$^{\#}$**	**46±7$^{\#}$**	**54±4**	**cd**	**cd**	**59±2**	**cd**	**17±2$^{\%,+}$**	**37±4$^{\%,-}$**	**33±2$^{@,+}$**	**63±3$^{@,-}$**	**8±3$^{!,\$}$**	**65±5$^{!}$**	**20±3$^{\$}$**	**cd**

* p<0.01; $^{\#}$ p<0.05; $^{\%}$ p<0.05; $^{@}$ p<0.05; $^{!}$ p<0.05; $^{\$}$ p<0.1; $^{+}$ p<0.05; $^{-}$ p<0.05

Table. S1. Equilibrium dissociation constants for different protein-protein interactions as measured by MST. The equilibrium dissociation constant value, K_d (in µM) for different experiments are tabulated. The stoichiometry of binding across all the binding experiments varied between 1-3. Each row denotes one set of binding conditions, and various K_d values under that are different biological repeats from different fresh protein preparations. For each fresh pair of proteins (prep #), 4-6 readings were carried out on each capillary, which were averaged and then the average was fit to get the K_d shown in each cell of the table. The mean values are shown as the last row. nb denotes 'no binding' and cd denotes 'cannot determine'. p values were calculated using a student's unpaired t-test. Errors shown are s.e.m.

Supplementary Note

Protein Constructs, Expression, Purification and Labeling procedures

Human β-cardiac sS1: WT and mutant (R403Q and R453C) human β-cardiac sS1 were constructed and produced using a modified AdEasy™ Vector System (Qbiogene, Inc). A complete cloning, expression and purification methodology is described in detail elsewhere [1]. Briefly, cDNA for MYH7 (human β-cardiac myosin) and MYL3 (human ventricular essential light chain; ELC) were purchased from Open Biosystems (Thermo). Two different truncated versions of MYH7 (residues 1-808), corresponding to a short S1 (sS1), followed by a flexible GSG (Gly-Ser-Gly) linker were made: a) a carboxy-terminal eGFP linker (for binding studies with proximal S2) or b) a carboxy-terminal 8-residue (RGSIDTWV) PDZ binding peptide (for binding studies with both proximal S2 and MyBP-C). Human ventricular essential light chain (ELC) with an N-terminal FLAG tag (DYKDDDDK) and TEV protease site was co-expressed with the heavy chain using an adenoviral vector/mouse myoblast C2C12 system (purchased from ATCC), as described previously [1]. Purified fractions of the protein were either dialyzed or buffer–exchanged using Amicon centrifugal filter units (Millipore) in binding buffer containing 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and either 100 mM KCl or 25 mM KCl at pH 7.5. Before any experiment, the myosin constructs were sedimented by centrifugation at 350,000 × g for 15 minutes to remove any aggregated protein. Protein concentrations were quantified by measuring absorbance at 488 nm on a Nanodrop spectrophotometer for GFP-fused sS1 or by the Bradford assay (Bio-Rad) on a Multiskan GO Microplate Spectrophotometer (Thermo Scientific) for PDZ-binding-peptide tagged myosin. All binding experiments were done with fresh preparations of sS1 at 23°C.

Human β-cardiac 2-hep and 25-hep HMM: Similar to human β-cardiac sS1, both human β-cardiac 2-hep and 25-hep HMM constructs were produced using a modified AdEasy™ Vector System (Qbiogene, Inc). The human β-cardiac 2-hep HMM cDNA consists of a truncated version of MYH7 (residues 1-855), corresponding to S1 and first two heptad repeats of S2, followed by a GCN4 leucine zipper[2] to ensure dimerization. This is further linked to a flexible GSG (Gly-Ser-Gly) linker then a GFP moiety followed by another GSG linker and finally ending with a 8-residue (RGSIDTWV) PDZ binding peptide. The human β-cardiac 25-hep HMM construct was similar to the 2-hep HMM, excepting that S1 is now linked by a 25 heptad repeats (175 amino acids) of S2 region (upto residues 1016). Expression and purification of human β-cardiac 2-hep and 25-hep HMM were very similar to that of the sS1, except an additional step in the purification protocol was required for the exchange of human ventricular regulatory light chain (RLC, MYL2), which was expressed separately in E. coli using a pET-28b vector. The RLC construct had an N-terminal His-tag followed by TEV protease site. The purification protocol of human cardiac RLC was similar to the one described for the purification of proximal S2 (see below). The recombinant 2-hep or 25-hep HMM construct was co-expressed with a FLAG-tagged human ventricular ELC construct in C2C12 mouse myoblast cells using adenoviral vectors, as described previously for sS1 [1]. The expressed 2-hep or 25-hep HMM had human ELC and mouse RLC, which was subsequently exchanged with human cardiac RLC during the purification process as follows. The exchange process is described for the 2-hep HMM below, a similar protocol was followed for the 25-hep HMM construct[3]. Mouse RLC-bound 2-hep HMM was treated with 0.5% Triton X-100, 5 mM CDTA, 200 mM KCl and 20

mM Tris pH 7.5 at 4°C for 1 hr to deplete mouse RLC from the human 2-hep HMM. Complete depletion of mouse RLC was confirmed by disappearance of the band for mouse RLC after the treatment in a 15% SDS PAGE gel. Human RLC binding to the heavy chain was performed by incubation of an excess amount of human cardiac RLC for 2 hrs at 4°C. The depletion of mouse RLC and binding of human RLC was performed while 2-hep HMM was bound to an anti-FLAG resin through the FLAG tag epitope of ELC. Subsequently, the protein was eluted with FLAG peptide, followed by ion exchange purification, as described previously for sS1 [1]. Analysis of a 15% SDS PAGE gel confirmed a 1:1:1 stoichiometric complex of 2-hep HMM with both the human light chains. Purified protein had dephosphorylated RLC which was phosphorylated by incubation in phosphorylation assay buffer (20 mM imidazole pH 7.5, 4 mM $MgCl_2$, 150 mM NaCl, 0.1 mM $CaCl_2$, 2 mM ATP) with 0.01mg/mL sea urchin calmodulin and 0.01 mg/mL myosin light chain kinase (MLCK2) (SignalChem) at 23°C for 40 min. RLC phosphorylation was quantitated by urea-SDS-glycerol gel electrophoresis [4], and confirmed that more than 90% of the RLC was phosphorylated [5]. Phosphorylated and dephosphorylated protein was always centrifuged at 350,000 ×g for 15 min to get rid of any aggregates before using for binding studies. All binding experiments were done within 2-3 days of fresh preparations of 2-hep HMM.

Human MyBP-C: Full length human cardiac MyBP-C: A plasmid containing the full-length human cardiac MyBP-C coding sequence was obtained from Open Biosystems. The sequence verified plasmid was then cloned into pFastBac1 transfer plasmid (Invitrogen). The expressed protein contained the N-terminal FLAG tag epitope (DYKDDDDK) followed by a TEV protease site followed by the native human MyBP-C sequence. The resulting vector encoding full-length MyBP-C was used for the site-specific transposition of an expression cassette into a bacmid. Isolation of recombinant bacmid DNA, transfection of Sf9 cells (ThermoFisher) and isolation of high titer viral stocks were carried out according to manufacturer's instructions. For protein expression, ~100-150 mL of Sf9 cells growing in suspension (~$2x10^6$ cells/ml) were infected with ~10 ml of P2 virus. Cells were collected ~48 hr post-infection. Cells were harvested in lysis buffer that contained 10 mM Tris-HCl pH 7.4, 200 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.01 mg/ml Aprotinin, Leupeptin and Pepstatin along with the protease inhibitor tablets (Roche protease inhibitor cocktail tablet) supplemented with 5% sucrose and 0.5% igepal. The cells were then lysed by dounce homogenization. The cell lysate was clarified by centrifugation at 45,000×g for 1 hour, and bound to anti-FLAG resin (Sigma-Aldrich) for 1 hr. The resin was then washed with 12-15 column volumes of wash buffer (similar to lysis buffer but without sucrose and igepal). Protein was then eluted by addition of FLAG peptide (Sigma-Aldrich) to 0.1 mg/mL. Protein quality was checked by SDS-PAGE and no further purification was required. Purified fractions of the protein were dialyzed in binding buffer containing 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100 mM KCl at pH 7.5, before further concentration using Amicon centrifugal filter units (Millipore). After purification, the protein was fully de-phosphorylated. The concentrated MyBP-C was then treated with protein kinase-A (PKA) and ATP which phosphorylates the MyBP-C on the M domain. As a control, lambda phosphatase treated MyBPC was used as described [6]. Dephosphorylation of MyBP-C was carried out in an EDTA- and Mg^{2+}-free binding buffer, containing 2 mM $MnCl_2$. Phosphorylation was qualitatively assessed by ProQ Diamond stained SDS-PAGE. Phosphorylated or dephosphorylated MyBP-C was then centrifuged at 350,000 ×g for 15 min to get rid of any aggregates before using for Microscale Thermophoresis. Protein concentrations were quantified by the Bradford assay (Bio-Rad) on a Multiskan GO Microplate Spectrophotometer (Thermo

Scientific). For all experiments, fresh preparations of MyBP-C were used (less than 3 days old), and all binding experiments were performed at 23°C.

Human proximal S2 and C0-C2: Both constructs contain an N-terminal His-tag followed by a TEV protease recognition site. Both were expressed using the pET 21a expression system in E. coli. The proximal S2 construct (839-968) begins 4 residues before the end of S1 and includes the first 126 amino acids of S2. Proximal S2 spans the 18 N-terminal heptad repeats of the S2 coiled-coil region of myosin. For all of our binding studies, we use this construct of S2, which we interchangeably term as S2 or proximal S2. The C0-C2 construct was codon optimized and gifted by MyoKardia, Inc. It contains residues 1-451 of human cardiac MyBP-C. Bacterial cells containing the recombinant DNA were grown, induced and harvested as described by the manufacturer (Qiagen, Germany). The cells were then lysed using an Emulsiflex (EmulsiFlex-C5, Avestin, Canada) and the lysate was clarified by centrifugation at 35,000×g for 30 min. The supernatant was then loaded on a Ni-NTA column (GE) on a FPLC. A step and gradient protocol was followed to wash and elute the protein of interest. The column was extensively washed in steps with buffer containing 20 mM, 40 mM, 80 mM and 100 mM imidazole. Elution with a gradient of 100-400 mM imidazole was then performed followed by a final wash with 500 mM imidazole. All fractions were analyzed by SDS-PAGE and fractions containing the purest protein were pooled together. The proteins were then concentrated and buffer exchanged using the Amicon centrifugal filter units. Similar to MyBP-C, C0-C2 was then treated either with protein kinase-A or lambda phosphatase to produce phosphorylated and desphosphorylated samples of C0-C2, respectively. All proteins were centrifuged at 350,000 ×g for 15 min to get rid of any aggregates before using for Microscale Thermophoresis. Protein concentrations were quantified by the Bradford assay (Bio-Rad) on a Multiskan GO Microplate Spectrophotometer (Thermo Scientific). Fresh preparations (less than 4 days old) of proximal S2 and C0-C2 were used for all binding experiments, which were performed at 23°C.

Actin: Purified bovine α-cardiac G-actin was gifted to us by Myokardia, Inc. Actin was cycled from G- to F-actin freshly for binding assays and used only for up to one week before being re-cycled again. After preparation, actin was stored in its F-form in binding buffer containing 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and either 100 mM KCl or 25 mM KCl. F-actin was stored at 4°C, and G-actin was frozen at - 80°C for future use.

PDZ18: The SNAP-PDZ18 fusion construct was expressed in bacterial cells, as described elsewhere [7]. Eluted protein was concentrated and the buffer was exchanged to 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100 mM KCl, at pH 7.5 before performing any binding experiments.

Protein labeling: Both sS1 and proximal S2 were labeled using standard cysteine labeling procedures using Cy5 maleimide. In each case, a concentrated stock of Cy5 maleimide (2-10 mM) was made in DMSO, followed by addition of either 10 fold excess (for labeling proximal S2) or equimolar amounts (for labeling sS1) into protein stocks in a DTT-free binding buffer (10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM TCEP and 100 mM KCl at pH 7.5). This mixture was mixed thoroughly, wrapped in aluminum foil and kept on ice overnight. The labeling reaction was terminated by addition of a 10-fold molar excess of DTT. The labeled protein was dialyzed into 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100

mM KCl at pH 7.5. The labeling efficiency was measured using an extinction coefficient of 250000 $M^{-1}cm^{-1}$ at 650 nm for Cy5 and by calculating the protein concentration by the Bradford assay. Labeling efficiency for proximal S2 was estimated to be 95%, and for sS1 was ~ 50-75%. The low labeling efficiency for the sS1 was on purpose, so as to avoid interference in the binding assay due to multiple Cy5 dye molecules on a single sS1 molecule. Fluorescently labeled actin filaments were obtained by incubating F-actin with Alexa Fluor 647 phalloidin at an equimolar ratio in binding buffer (10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 100 mM KCl at pH 7.5).

Estimating the physiological concentration of S1and S2: Since S1 and S2 are connected in an intact myosin molecule, we estimated the effective concentrations of S1 and S2 in the intact molecule to be ~60 μM (proximal S2 is 19 nm long and to sweep through 180° from an extended to the folded form of the molecule, the molecule need be in a sphere of radius 19 nm [(1 molecule/2.9 x 10^{-20} L) (1 mole/6.02 x 10^{23} molecules)]).

Estimating a relationship between K_d and N_a: Let's assume a very simplistic scenario of the folded myosin heads (S1) binding to its own S2

Consider the reaction:

$$S1 + S2 \overset{K_d}{\rightleftharpoons} S1.S2$$

At equilibrium

$$K_d = \frac{[S1_{free}][S2_{free}]}{[S1.S2]}$$

Also from the mass conservation theorem, we get the following equations

$$[S1_{free}] = [S1_{total}] - [S1.S2]$$

$$[S2_{free}] = [S2_{total}] - [S1.S2]$$

Since the S1 and S2 are the part of the same molecule; $[S1_{total}] = [S2_{total}]$

Therefore the binding constant deduces to

$$K_d = \frac{([S1_{total}] - [S1.S2])^2}{[S1.S2]}$$

The above quadratic equation can be solved for $[S1.S2]$ assuming $[S1_{total}]$ to be 60 μM (see Estimating the physiological concentration of S1and S2) and a K_d of ~ 40 μM for WT S1 binding to WT S2. Such a calculation yields a value of 33 μM for $[S1_{free}]$ which is roughly a ball park estimate of the number of free accessible myosin heads available to interact with actin (N_a). In this example it is ~ 55% of the heads. Now for example if a mutation, say R453C, diminishes the S1-S2 affinity by say 5 fold (K_d of ~ 200 μM), then the same calculation yields a value of

48 μM for $[S1_{free}]$. *This means that now there are 25% more accessible heads, which can interact with actin and could be the cause for the hyper-contractility observed in HCM cases.*
This calculation can be made more rigorous by introducing three more known interactions in addition to the one above:

$$S1 + MyBPC \overset{K_d}{\rightleftharpoons} S1.MyBPC \text{ with its } K_d = \frac{[S1_{free}][MyBPC_{free}]}{[S1.MyBPC]} = 20 \text{ μM}$$

$$S2 + MyBPC \overset{K_d}{\rightleftharpoons} S2.MyBPC \text{ with its } K_d = \frac{[S2_{free}][MyBPC_{free}]}{[S2.MyBPC]} = 5 \text{ μM}$$

and

$$S1 + S1 \overset{K_d}{\rightleftharpoons} S1.S1 \text{ with its } K_d = \frac{([S1_{free}])^2}{[S1.S1]} = \text{ double digit μM (say 100 μM; just a rough estimate)}$$

Applying appropriate mass conservation theorem for these four coupled reactions, and assuming $[MyBPC_{total}] = \frac{1}{6}[S1_{total}]$ [8] one can again calculate the $[S1_{free}]$ for WT to be 27 μM (~ 45% of the heads are free and accessible to interact with actin). This increases to be ~ 33 μM if there is a 5-fold diminishing of either the $[S1.S2]$ interaction by a mutation (say R453C) or $[S1.S1]$ interaction (as is predicted by R403Q) respectively. *In either cases there are now ~ 10% of more heads available and could be the basis of hyper-contractility exhibited by these HCM-causing mutations.*

Supplemental references

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