Supplemental Material

Supplemental Materials and Methods

Preparation of BSR-cRLC and cMLCK

Human cRLCs labelled with bifunctional sulforhodamine (BSR) were prepared as described previously [1]. Mutants of the human ventricular cRLC (UniProtKB entry: MLRV_HUMAN; P10916) with pairs of cysteines introduced at positions 6 and 10 in the N-terminal extension, 97 and 110 on helix E, 131 and 138 on helix G, 117 and 125 on helix F or 120 and 136 crosslinking helices F and G (**Fig. 1**) were obtained by site-directed mutagenesis. The mutants were expressed in BL21(DE3) cells (Stratagene) as N-terminal fusion proteins with a Histidine tag and TEV protease site from a pET6a vector. The N-terminal tag-sequence was removed by digestion with TEV protease leaving three additional amino acids (glycine-serine-serine) at the N-terminus. Each of the cRLC double-cysteine mutants was labelled with BSR-I_2 (Invitrogen, B-10621) and purified by ion-exchange chromatography on a MonoS column to 95% homogeneity. The catalytic fragment of human cMLCK [UniProtKB entry: Q32MK0] spanning amino acids 492-819 was expressed as an N-terminal fusion protein to Histdine-tag and TEV protease site in *Spodoptera frugiperda* 9 (Sf9) cells according to manufacturer's instructions (BaculoDirect^{TM} Baculovirus Expression System, Invitrogen). The fragment was purified by affinity chromatography on HIsTrapFF column (Invitrogen), followed by ion-exchange chromatography on CM-Sepharose (GE Healthcare) and gel filtration on a Superdex 75 HR 10/30 (GE Healthcare). The protein was concentrated to over 1 mg/ml and stored in 25 µl aliquots at -80ºC for further use. Calmodulin was kindly provided by Dr. Martin Reese (King's College London).

In-vitro Kinase Assays for cRLCs

In vitro kinase assays for cRLCs were performed in cMLCK assay buffer (composition in mM: 25 HEPES pH 7, 50 NaCl, 2 $MgCl_2$, 2 DTT, 1 ATP) with or without 1 mM Ca^{2+} in a total volume of 50 µl containing 0.5 mg/ml (25 µM) cRLC as substrate. EGTA and/or calmodulin were added to final concentrations of 1 mM and 6 µg/ml, respectively. The reactions were started by adding the C-terminal fragment of cMLCK at stoichiometries given in the relevant sections below. The reactions were incubated for 30 min at 30°C, quenched with 5× sample buffer containing 10 M urea, and analysed by urea-glycerol-PAGE [2].

Preparative Thiophosphorylation of BSR-cRLCs

BSR-cRLCs were gel-filtered into cMLCK assay buffer without ATP, protein concentration determined by absorbance spectroscopy at 528 nm (ε= 52,000 $M^{-1}cm^{-1}$) and adjusted to 1 mg/ml. Adenosine-5-thiotriphosphate (ATPγS, Jena Bioscience, NU-406-25) and calmodulin were added to final concentrations of 1 mM and 12 µg/ml, respectively. The reaction was started by adding the catalytic fragment of cMLCK to a final molar stoichiometry of 1:100 (cMLCK:cRLC). The samples were incubated at 30°C and the reaction followed by ESI mass spectrometry and urea-glycerol PAGE. The measured (calculated) masses in Da for thiophosphorylated BSR-cRLC-N, -E, -F, -G, and -FG were 19766.3 (19765.2), 19721.1 (19721.3), 19691.1 (19691.2), 19707.1 (19707.2) and 19637.1 (19636.7), respectively. The reactions were usually complete after 2h to 4h. After complete thiophosphorylation BSR-cRLCs were-gel-filtered into EDTA-rigor-solution and the concentration adjusted to 0.5 mg/ml.

Preparation of Tissue Samples and Cardiomyofibrils

Animals were sacrificed by cervical dislocation (Schedule 1 procedure in accordance with UK Animal Scientific Procedure Act, 1986), ventricular tissue removed within two minutes

and snap frozen in liquid nitrogen. Frozen tissue samples were homogenised without thawing the preparation, resuspended in SDS-PAGE loading buffer, boiled for 2 min at 95°C, centrifuged at 18,000 g to remove debris and the clear supernatant stored at -80°C until further use. Cardiomyofibrils were prepared by homogenising freshly frozen ventricular tissue samples in myofibril buffer (composition in mM: 20 Imidazole pH 7.4, 75 KCl, 2 $MgCl_2$, 3 EDTA, 1 EGTA, 1 DTT, 1% (v/v) Triton X-100, protease inhibitor cocktail (ROCHE), PhosStop cocktail (ROCHE), 0.02 H-89 kinase inhibitor, 0.001 Staurosporine) followed by centrifugation at 18,000 g for 5 min. Cardiomyofibrils were washed and homogenised three more times in the same buffer without Triton X-100, resuspended in SDS-PAGE loading buffer and stored at -80°C until further use. cRLC phosphorylation levels in samples were measured by Phostag™-SDS-PAGE as described below.

Exchange of BSR-cRLC into skinned Ventricular Trabeculae

Wistar rats were sacrificed by cervical dislocation (Schedule 1 procedure in accordance with UK Animal Scientific Procedure Act, 1986) and demembranated right ventricular trabeculae were prepared as described previously [3]. BSR-cRLCs were exchanged into skinned trabeculae preparations with a procedure modified from that described previously [1], resulting in replacement of about 10%-30% of the endogenous rat cRLCs with thiophosphorylated, BSR-labelled human cRLCs. Briefly, suitable trabeculae (free running, unbranched, diameter ≤ 250 mm) were dissected from the right ventricle in Krebs-Henseleit solution containing 25 mM 2,3-butanedione-monoxime, demembranated in relaxing solution (composition in mM: 25 imidazole, 5 MgATP, 1 free Mg^{2+}, 10 EGTA, 1 dithiothreitol (DTT) and 0.1% (v/v) protease inhibitor cocktail (P8340, Sigma); ionic strength adjusted to 200 mM with KPropionate; pH 7.1 at 20°C) containing 1% (v/v) Triton X-100 for 60 min on ice and stored in relaxing solution containing 50% (v/v) glycerol at -20°C for experiments. Trabeculae were used for experiments within two days of dissection. BSR-cRLCs were introduced by incubating demembrabated trabeculae for 30 min at 22°C in an EDTA-rigor-extract solution (10 mM K_2PO_3H, pH 7.1, 20 mM EDTA, 115 mM KPropionate) containing 0.5 mg/ml BSR-cRLC and 10 mM DTT. The trabeculae were then washed for 5 min in relaxing solution (composition in mM: 25 imidazole, 5 MgATP, 1 free Mg^{2+}, 10 EGTA, 1 dithiothreitol and 0.1% (v/v) protease inhibitor cocktail (P8340, Sigma); ionic strength adjusted to 200 mM with KPropionate; pH 7.1 at 20°C) and subsequently bathed in relaxing solution containing 0.5 mg/ml recombinant human cardiac troponin C and troponin complex (kindly provided by Dr. Mitla Garcia) for 15 min and 1h, respectively. Trabeculae were then washed in relaxing solution for 30-45 min, the sarcomere length adjusted to 2.1 μm and activated again. The maximal isometric force was recorded, and trabeculae showing less than 75% force recovery compared to that before cRLC exchange were discarded. The maximal calcium activated force was 88% ± 9% (mean ± S.D., n= 23) of that before cRLC exchange (26.8 ± 11.9 mN/mm^2, mean ± S.D., n = 23).

Exchange of thiophosphorylated BSR-cRLCs was quantified by SDS-PAGE followed by either Coomassie staining or Western-Blot against cRLC. Exchanged trabeculae were dissolved in SDS-PAGE sample buffer and run on 15% (v/v) SDS-acrylamide (30:1 acrylamide:bis-acrylamide) gels [4]. After electrophoresis, gels were equilibrated for 15 min in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol). Gels were blotted for 1 h at 50 mA in transfer buffer onto nitrocellulose membranes (BIO-RAD) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). Post-blotting, membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T, Calbiochem) containing 5% (w/v) non-fat dried milk powder. The blocked membranes were briefly washed with TBS-T and then incubated overnight at 4°C with primary antibody against cRLC (rabbit monoclonal anti-myosin light chain 2, ABCAM, ab92721) in a 1:10000 dilution in TBS-T containing 2.5% (w/v) non-fat dried milk powder. Membranes were washed with TBS-T and incubated for 1h at room temperature with secondary antibody (1:1000 dilution, HRP-conjugated donkey anti-rabbit IgG, GE Healthcare, NA934V) in TBS-T containing 1% (w/v) non-fat dried milk powder. Blots were

washed in TBS-T, immersed in ECL Plus reagent (GE Healthcare) and bands visualized by developing the blot with BioMax MR film (Kodak) or BIO-RAD Gel Imager. Phospho-species were separated by SDS-PAGE gels containing 50 μM Phos-tag™-Acrylamide (NARD Insitute, Hyogo, Japan) [5] and 100 μM $MnCl_2$. Subsequently, gels were washed for 10 min in transfer buffer containing 1 mM EDTA and two times with transfer buffer without EDTA, and blotted under identical conditions as standard SDS-PAGE gels.

4%-20% gradient gels (BIO-RAD) of native and BSR-cRLC exchanged trabeculae were stained with Pro-Q Diamond phospho-protein stain and SYBPRO Ruby total protein stain (Invitrogen) according to manufacturer's instructions and bands visualized on a BIO-RAD Gel-Doc Imager.

Fluorescence Polarization Experiments

Composition of experimental solutions and trabecular activation protocols were identical to those described previously for cTnC-experiments [3]. Polarized fluorescence intensities were measured as described previously [6-8]. Fluorescence emission from thiophosphorylated BSR-cRLCs in trabeculae was collected by a 0.25 N.A. objective using excitation light beams either in line with or at 90° to the emission path. The polarization of the excitation and emitted beams was set either parallel or perpendicular to the trabecular axis, allowing determination of the three order parameters, $\langle P_{2d} \rangle$, $\langle P_2 \rangle$ and $\langle P_4 \rangle$ that describe the dipole orientations in the trabeculae [9]. The mean orientations of the BSR fluorescence dipoles with respect to the filament axis (θ_{ME}) were calculated using one-dimensional maximum entropy (ME) analysis [9] using the measured order parameters. The orientation of the cRLC C-lobe was calculated using two-dimensional maximum entropy (ME) analysis, combining the data from four BSR-cRLCs in each case [9]. The orientation of the C-lobe in the laboratory frame was then described by the angles β_{EG} and γ_{EG}, where β_{EG} is the angle between the E-helix and the trabecular/thin filament axis, and γ_{EG} describes the rotation of the lobe around the E-helix, with γ_{EG} =0° when the plane containing the E- and G-helices coincides with that containing the E-helix and filament axis. An increase in γ_{EG} indicates a counter-clockwise rotation of the C-lobe viewed from the + end of the E-helix.

Supplemental Figures and Tables

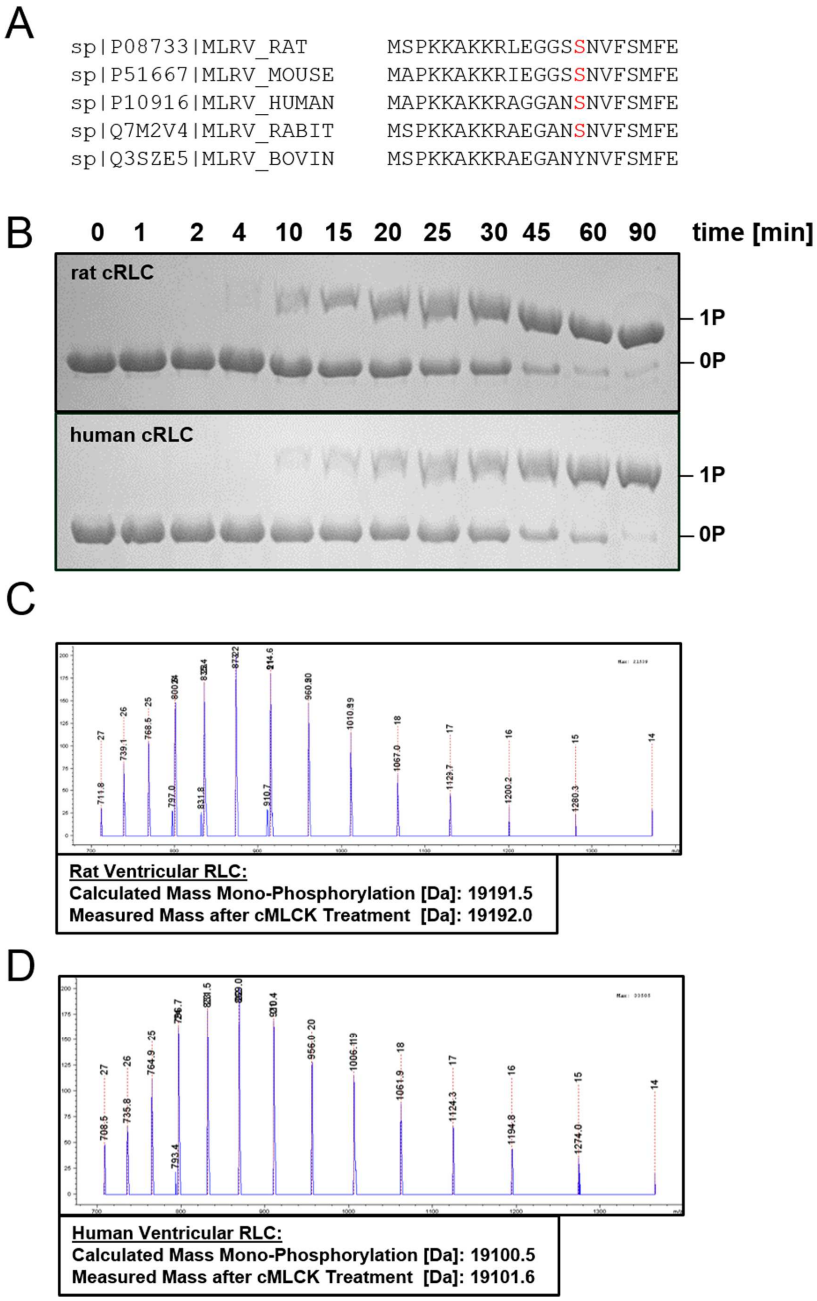

Figure S1. (A) Sequence alignment of the N-terminus of ventricular RLCs from different species. The serine residue phosphorylated by cMLCK is highlighted in red. (B) In-vitro phosphorylation of rat (top) and human (bottom) ventricular RLC with 0.1 µM cMLCK (1:400 stoichiometry, cMLCK:cRLC) analysed by Phostag™-SDS-PAGE and stained with Coomassie. Both cRLCs are mono-phosphorylated by cMLCK within 90 min. ESI mass spectrometry of rat (C) and human (D) ventricular RLC after phosphorylation by cMLCK. The measured molecular masses (predicted m/z values for mono-phosphorylated cRLCs are shown as red dashed lines) correspond to mono-phosphorylated RLCs.

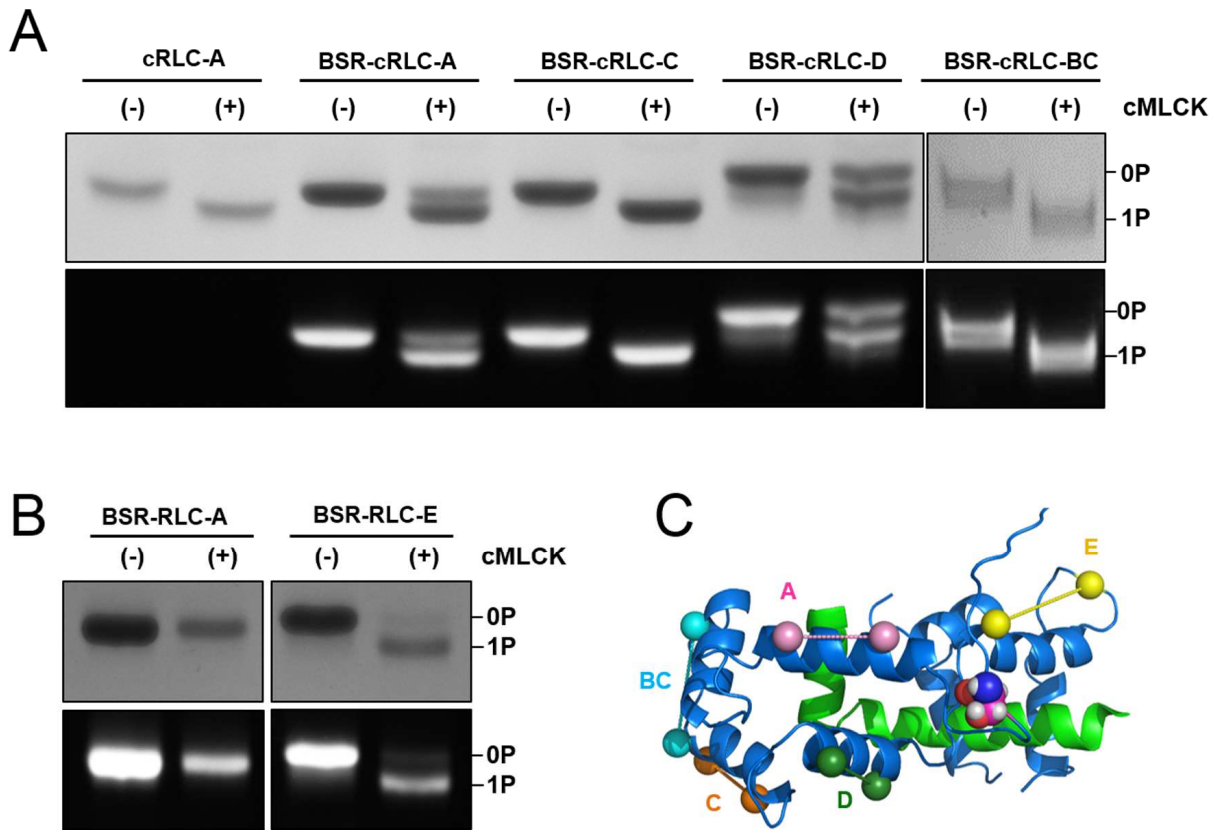

Figure S2. Phosphorylation of BSR-labelled cRLCs by cMLCK. (A) Kinase assay in the presence of ATP using unlabelled cRLC-A mutant and BSR-labelled cRLCs A, C, D and BC as substrate. Unlabelled RLC-A mutant, and BSR-cRLCs C and BC could be entirely phosphorylated by cMLCK. BSR-cRLC-A and -D could not be phosphorylated to homogeneity. (B) Kinase assay in the presence of ATPγS using BSR-cRLC-A and BSR-cRLC-E (control) as substrates showing that the labelled A-helix mutant could not be thiophosphorylated. (C) The regulatory light chain (blue) shown bound to the myosin heavy chain (green). The Cβ-atoms (or Cα-atoms in case of glycine residues) of mutated residues in the RLC are shown as coloured spheres (A-pink; BC-cyan; C-orange; D-green, E-yellow) and the expected probe dipole orientations are indicated by sticks. The phosphorylatable serine residue 15 is shown in Van-der-Waals representation.

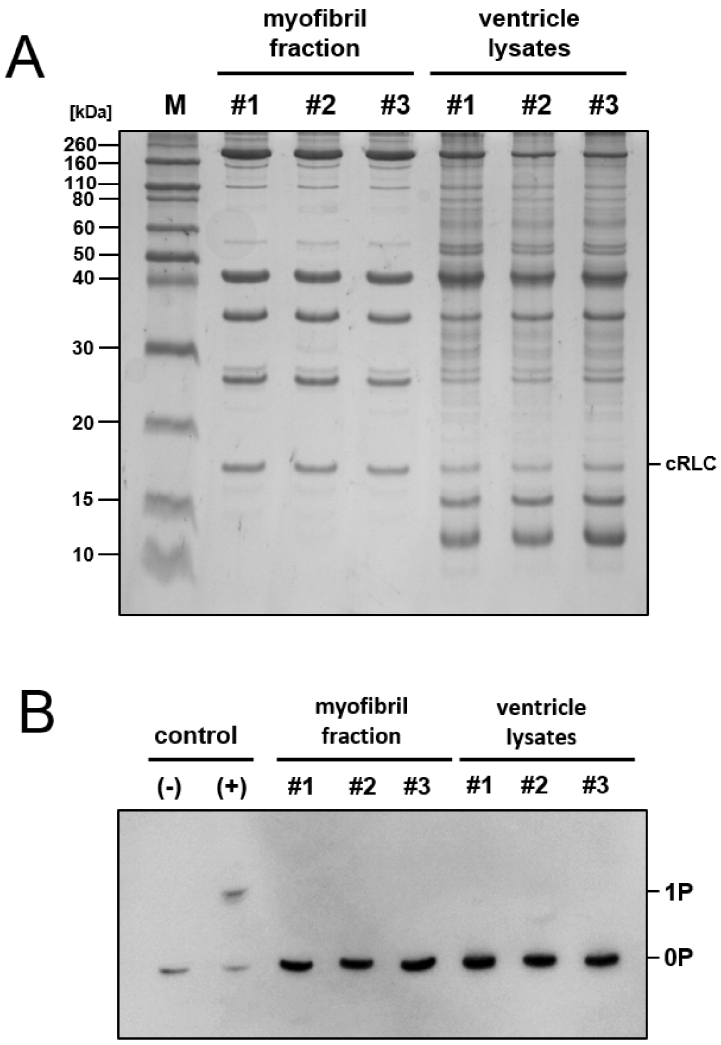

Figure S3. cRLC Phosphorylation level in mouse hearts. (A) SDS-PAGE of enriched myofibrillar fraction and ventricular lysates prepared as described in *Materials and Methods*. (B) Phostag™-SDS-PAGE followed by Western-Blot against ventricular RLC from the same samples as in (A). The negative (-) and positive (+) control are untreated and cMLCK treated cardiomyofibrils.

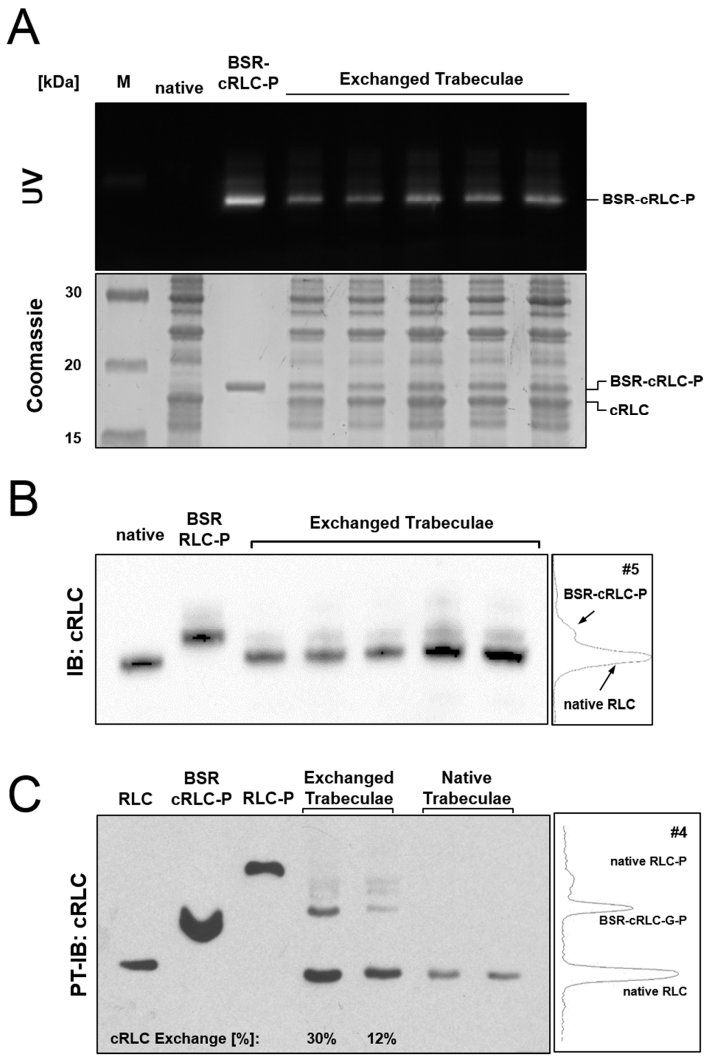

Figure S4. Exchange of thiophosphorylated BSR-cRLCs into demembranated right ventricular trabeculae. (A) Separation of endogenous (cRLC) and thiophosphorylated BSR-cRLC-G (BSR-cRLC-P) in exchanged trabeculae by SDS-PAGE. Bands were visualized by UV illumination (top row) or by Coomassie staining (bottom row). The fraction of exchanged phosphorylated BSR-cRLC was estimated by densitometric analysis as 33% ± 2%. (B) Separation of endogenous and thiophosphorylated BSR-cRLC-G in exchanged trabeculae by SDS-PAGE followed by Western-Blot against cRLC (lane #1: native trabeculae; lane #2: thiophosphorylated BSR-cRLC-G (BSR-cRLC-P), lanes #3-7: trabeculae exchanged with thiophosphorylated BSR-cRLC-G). The density profile of lane 5 is shown on the right. The fraction of exchanged phosphorylated BSR-cRLC was estimated by densitometric analysis as 18% ± 2%. (C) Wild-type human cardiac RLC (lane 1; RLC), thiophosphorylated BSR-cRLC-G (lane 2; BSR-RLC-G-P), mono-phosphorylated human cardiac RLC (lane 3; RLC-P), right ventricular trabeculae exchanged with thiophosphorylated BSR-cRLC-G (lanes 4 and 5) and native trabeculae (lanes 6 and 7) were separated by Phostag^{TM}-SDS-PAGE followed by Western Blot against cardiac RLC. The native unphosphorylated, native phosphorylated and thiophosphorylated BSR-cRLC were clearly separated and allowed determination of the distribution of phosphorylated RLC species. The density profile of lane 4 is shown on the right.

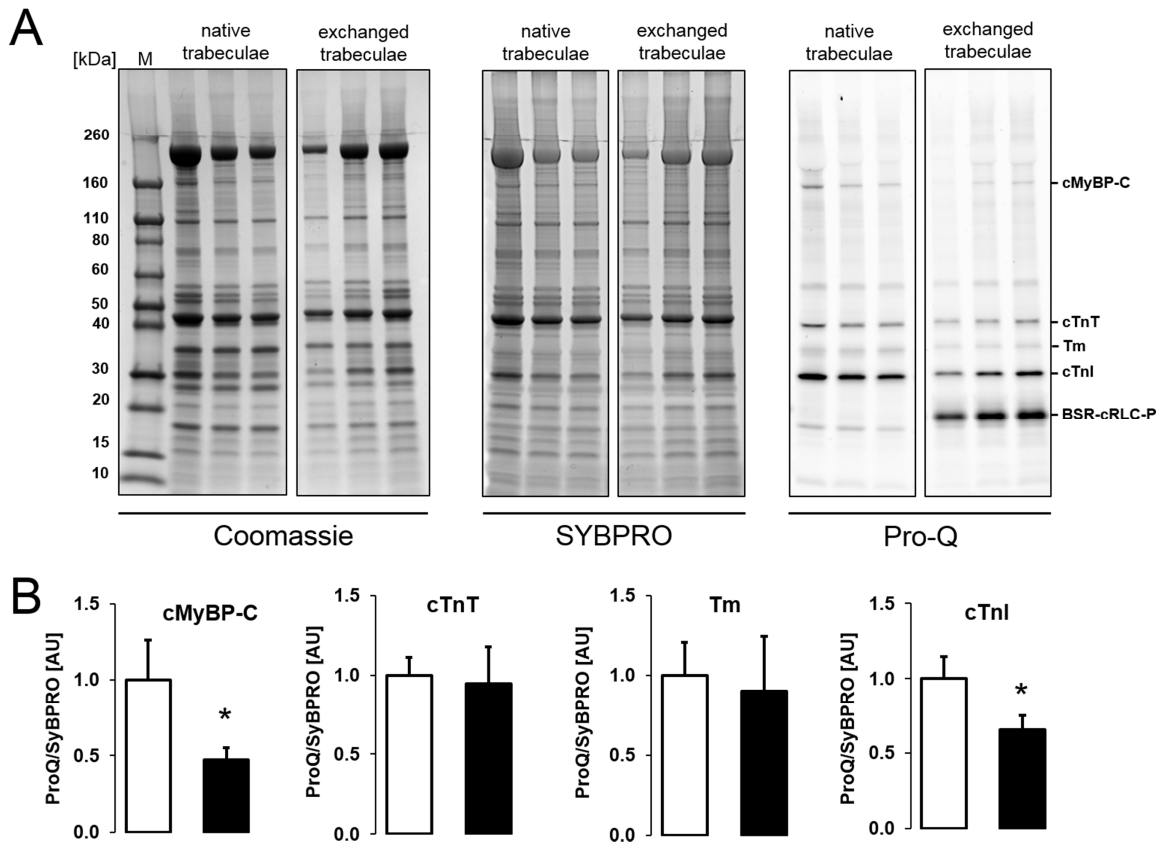

Figure S5. Effects of BSR-cRLC incorporation into demembranated cardiac trabeculae on protein content and phosphorylation levels. (A) Three native and three thiophosphorylated BSR-cRLC exchanged trabeculae were dissolved in SDS-PAGE loading buffer and proteins separated by SDS-PAGE on a 4%-20% gradient gel. Total protein was visualized by Coomassie (left) and SYBPRO Ruby stain (middle). Total protein contents were not affected by cRLC exchange. Phosphorylated proteins were visualized with Pro-Q Diamond phospho-protein stain (right). The intense bands in the lower part of the Pro-Q Diamond stain of exchanged trabeculae correspond to incorporated thiophosphorylated BSR-cRLCs (BSR-cRLC-P). Note that BSR and Pro-Q Diamond have almost identical excitation and emission spectra and therefore BSR-cRLC bands appear very intense. (B) Densitometric analysis of protein phosphorylation levels before (white bars) and after cRLC exchange (black bars) expressed as ratios of ProQ and SYBPRO intensity normalized to native trabeculae. Statistical significance of differences was assessed using two-tailed unpaired Student's t-test: *$p<0.05$ (mean ± SD; n=3).

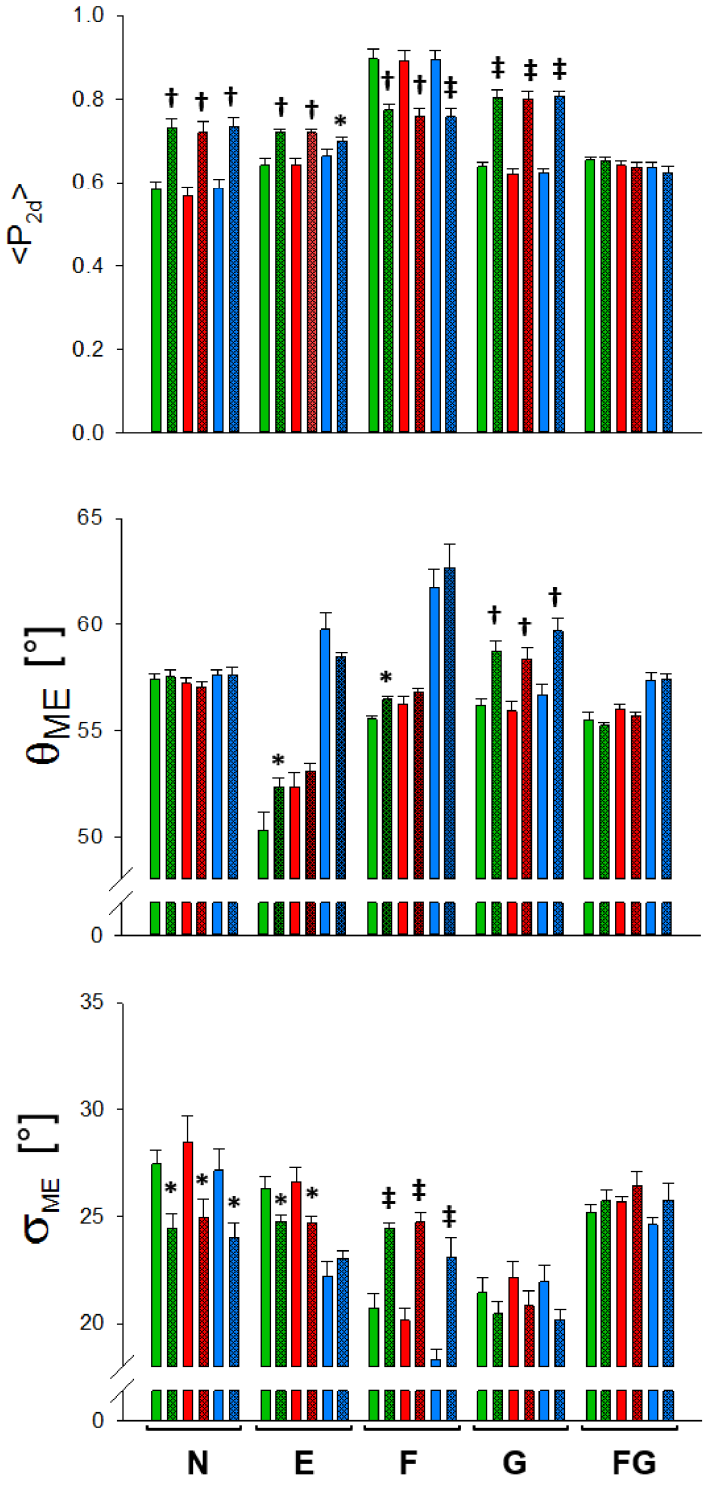

Figure S6. Orientation parameter $\langle P_{2d} \rangle$, and average probe dipole orientation with respect to the filament axis θ_{ME} and standard deviation σ_{ME} calculated from measured order parameters for unphosphorylated (light colours) and phosphorylated (dark colours) BSR-cRLC-N, -E, -F, -G and -FG during relaxation (green), active isometric contraction (red) and rigor (blue) (mean ± SEM, n=4-5). Statistical significance of differences were assessed using unpaired two-tailed Student's t-test: *P < 0.05; †P < 0.01; ‡P < 0.001.

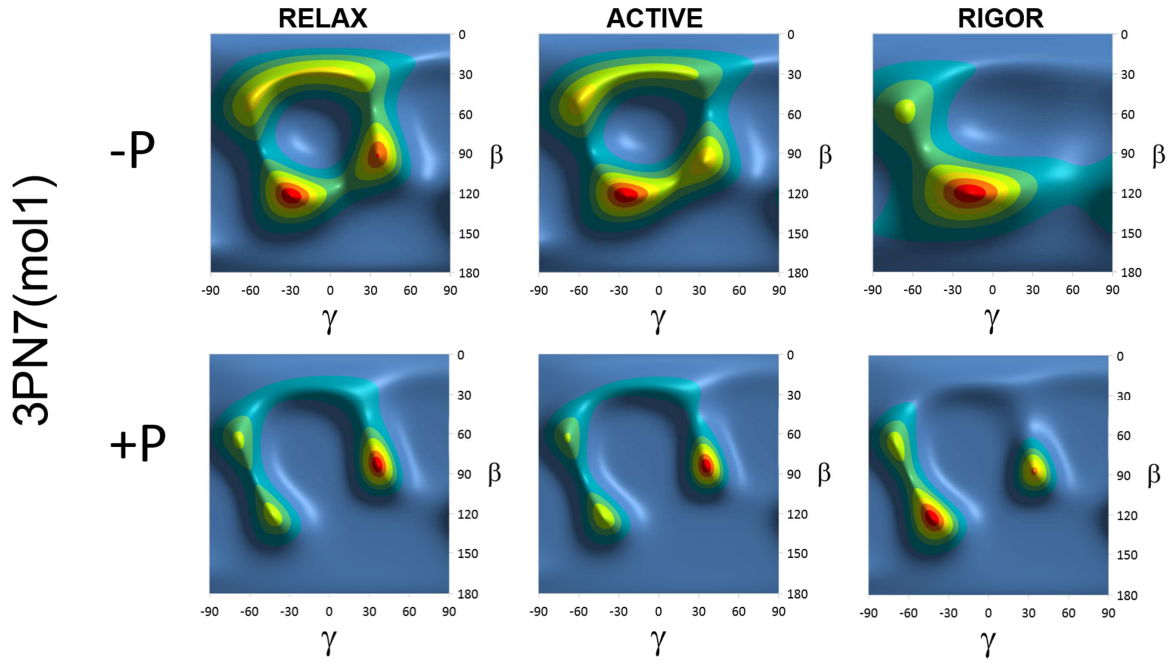

Figure S7. Maximum entropy distributions of the $((\beta,\gamma)_{EG})$ orientation of unphosphorylated and thiophosphorylated cRLC C-lobe calculated using reference structure 3PN7(mol1). Maximum entropy distributions were calculated from order parameters for the unphosphorylated and thiophosphorylated C-lobe RLC probes during relaxation, active isometric contraction and rigor (**Table S1**) using reference structure 3PN7(mol1).

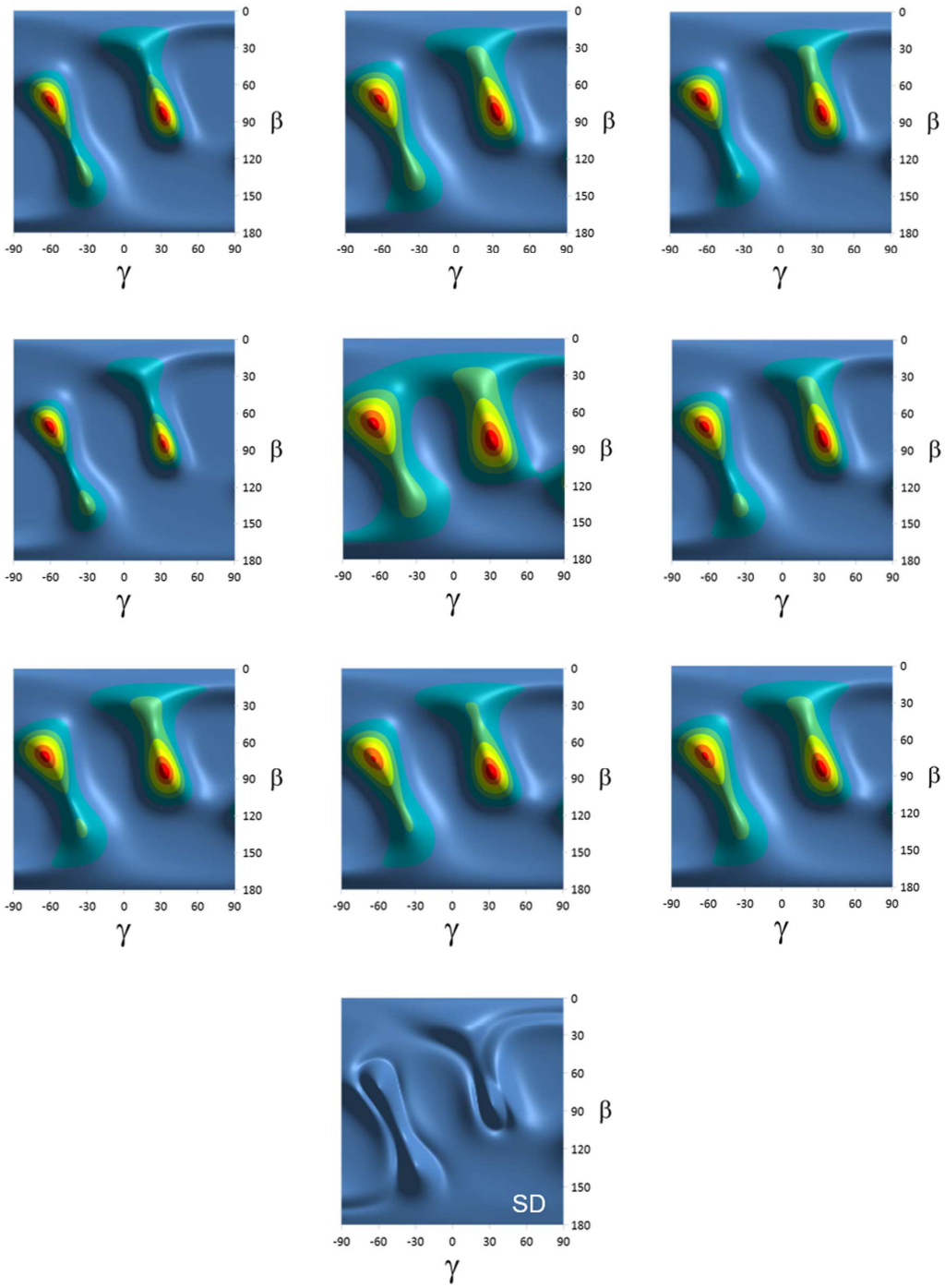

Figure S8. Reproducibility of maximum entropy orientation distributions of the thiophosphorylated RLC C-lobe ($(\beta,\gamma)_{EG}$) in relaxation. In order to assess the effects of measurement errors in the order parameters in **Table S1**, the mean values used for **Fig. 4** in the main text were replaced by values chosen at random from a normal distribution with the same mean and standard deviation as each order parameter in **Table S1** for each probe. This procedure was repeated nine times to give the nine contour maps shown in the top part of the Figure. The position and intensity of the peaks is very similar in the nine maps, but their dispersion is more variable. The standard deviation (SD) between those maps is shown at the bottom.

Table S1. Order parameters $\langle P_{2d} \rangle$, $\langle P_2 \rangle$ and $\langle P_4 \rangle$ describing the orientation and mobility of the unphosphorylated and thiophosphorylated BSR-cRLC probes during relaxation, active isometric contraction and rigor in permeabilized right ventricular trabeculae. Values indicate mean ± S.E.M. (n = 4-5).

		Relaxed			Active			Rigor		
		$\langle P_{2d} \rangle$	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$\langle P_{2d} \rangle$	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$\langle P_{2d} \rangle$	$\langle P_2 \rangle$	$\langle P_4 \rangle$
BSR-cRLC-N	**-P**	0.5838 ± 0.0173	-0.0026 ± 0.0044	0.2076 ± 0.0206	0.5675 ± 0.0205	0.0030 ± 0.0033	0.2506 ± 0.0401	0.5863 ± 0.0229	-0.0065 ± 0.0038	0.2046 ± 0.3011
	+P	0.7312 ± 0.0213†	0.0061 ± 0.0070	0.0829 ± 0.0211*	0.7203± 0.0257†	0.0039 ± 0.0059	0.1010 ± 0.00282*	0.7341 ± 0.0216†	-0.0088 ± 0.0068	0.0751 ± 0.0218*
BSR-cRLC-E	**-P**	0.6424 ± 0.0153	0.1359 ± 0.0127	0.1259 ± 0.0175	0.6415 ± 0.0163	0.1003 ± 0.0138	0.1225 ± 0.0156	0.6637 ± 0.0166	-0.0508 ± 0.0163	0.0407 ± 0.0169
	+P	0.7281 ± 0.0084*	0.0988 ± 0.0099*	0.0733 ± 0.0116*	0.7275 ± 0.0093†	0.0850 ± 0.0093	0.0722 ± 0.0113*	0.7068 ± 0.0091*	-0.0277 ± 0.0026	0.0520 ± 0.0117
BSR-cRLC-F	**-P**	0.8972 ± 0.0235	0.0275 ± 0.0028	-0.0426 ± 0.0196	0.8922 ± 0.0240	0.0117 ± 0.0068	-0.0546 ± 0.0167	0.8948 ± 0.0217	-0.1025 ± 0.0153	-0.0416 ± 0.0199
	+P	0.7734 ± 0.0149†	0.0149 ± 0.0023‡	0.0794 ± 0.0077‡	0.7605 ± 0.0180†	0.0084 ± 0.0039	0.0921 ± 0.0146‡	0.7586 ± 0.0179‡	-0.1080 ± 0.0228	-0.1095 ± 0.0126‡
BSR-cRLC-G	**-P**	0.6380 ± 0.0096	0.0155 ± 0.0057	-0.0171 ± 0.0222	0.6215 ± 0.0124	0.0220 ± 0.0074	0.0038 ± 0.0257†	0.6227 ± 0.0104	0.0072 ± 0.0093	0.0019 ± 0.0281
	+P	0.8029 ± 0.0180‡	-0.0380 ± 0.0110†	-0.0232 ± 0.0115	0.7996 ± 0.0193‡	-0.0299 ± 0.0114†	-0.0156 ± 0.0152	0.8069 ± 0.0137‡	-0.0573 ± 0.0132†	-0.0204 ± 0.0132
BSR-cRLC-FG	**-P**	0.6541 ± 0.0084	0.0347 ± 0.0072	0.0982 ± 0.0110	0.6412 ± 0.0097	0.0262 ± 0.0051	0.1172 ± 0.0085	0.6371 ± 0.0117	-0.0026 ± 0.0075	0.0919 ± 0.0119
	+P	0.6528 ± 0.0086	0.0416 ± 0.0027	0.1152 ± 0.0163	0.6373 ± 0.0130	0.0331 ± 0.0032	0.1405 ± 0.0237	0.6238 ± 0.0147	-0.0018 ± 0.0060	0.1308 ± 0.0262

Statistical significance of differences between values obtained from unphosphorylated and phosphorylated BSR-cRLCs was assessed using unpaired Student's t-test: *P < 0.05; †P < 0.01; ‡P < 0.001

Supplemetal References

[1] Kampourakis T, Sun YB, Irving M. Orientation of the N- and C-terminal lobes of the Myosin regulatory light chain in cardiac muscle. Biophys J. 2015;108:304-14.

[2] Perrie WT, Perry SV. An electrophoretic study of the low-molecular-weight components of myosin. Biochem J. 1970;119:31-8.

[3] Sun YB, Lou F, Irving M. Calcium- and myosin-dependent changes in troponin structure during activation of heart muscle. J Physiol. 2009;587:155-63.

[4] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-5.

[5] Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics. 2006;5:749-57.

[6] Brack AS, Brandmeier BD, Ferguson RE, Criddle S, Dale RE, Irving M. Bifunctional rhodamine probes of Myosin regulatory light chain orientation in relaxed skeletal muscle fibers. Biophys J. 2004;86:2329-41.

[7] Romano D, Brandmeier BD, Sun YB, Trentham DR, Irving M. Orientation of the N-terminal lobe of the myosin regulatory light chain in skeletal muscle fibers. Biophys J. 2012;102:1418-26.

[8] Knowles AC, Ferguson RE, Brandmeier BD, Sun YB, Trentham DR, Irving M. Orientation of the essential light chain region of myosin in relaxed, active, and rigor muscle. Biophys J. 2008;95:3882-91.

[9] van der Heide UA, Hopkins SC, Goldman YE. A maximum entropy analysis of protein orientations using fluorescence polarization data from multiple probes. Biophys J. 2000;78:2138-50.