

## Supporting Information (SI Appendix)

### Myosin light chain phosphorylation enhances contractility of heart muscle via structural changes in both thick and thin filaments

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## SI Methods

### Preparation of cMLCK, and labelled cRLCs and cTnCs

The catalytic fragment of cMLCK, bifunctional rhodamine labelled cTnC (BR-cTnC-E) and bifunctional sulforhodamine labelled cRLCs (BSR-cRLCs) were prepared as previously described (1-3). Calmodulin (CaM) was kindly provided by Dr. Martin Reese (King's College London). Non-phosphorylatable and N-terminal truncated BSR-cRLCs were prepared by site-directed mutagenesis, expressed, purified and labelled with bifunctional sulforhodamine according to standard procedures.

### Preparation of Cardiomyofibrils

Cardiomyofibrils were prepared by homogenising freshly frozen ventricular tissue samples in myofibril buffer (composition in mmol/L: 20 Imidazole pH 7.4, 75 KCl, 2 MgCl<sub>2</sub>, 3 EDTA, 1 EGTA, 1 DTT, 1% (v/v) Triton X-100, protease inhibitor cocktail (ROCHE)) 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.

### *In vitro* Kinase Assays

RLCs were gel-filtered into cMLCK assay buffer (composition in mmol/L: 50 HEPES pH 7.0, 50 NaCl, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 1 DTT) and protein concentrations adjusted to 25 μmol/L. Reactions were started by adding 0.1 μmol/L cMLCK and 0.3 μmol/L CaM. The reactions were incubated for 1 h at 30°C, quenched with sample buffer containing 10 mol/L urea, and analysed by urea-glycerol-PAGE (4). Bands were visualized by rhodamine excitation and emission filter settings on a BIO-RAD Gel-Doc Imager, and total protein staining with Coomassie.

### Western-Blot

Trabeculae (see below) or cardiomyofibrils (CMF) were dissolved in SDS-PAGE loading buffer and run on either Phostag<sup>TM</sup>-SDS-PAGE gels (5) (containing 50 μmol/L Phostag<sup>TM</sup>-reagent and 100 μmol/L MnCl<sub>2</sub>) or standard SDS-PAGE gels for 1-2 h at 160 V. Phostag<sup>TM</sup> gels were washed first with transfer buffer (in mmol/L: 25 Tris-HCl pH 8.3, 192 glycine, 0.08% (v/v) SDS, 15% (v/v) methanol) containing 1 mmol/L EDTA for 10 min, followed by three washes in transfer buffer without EDTA. For standard gels the initial wash step was omitted. Proteins were blotted onto nitrocellulose (BIO-RAD) membranes for 2 h at 1 mA/cm<sup>2</sup> in transfer buffer using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). Post-blotting, membranes were blocked with Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBS-T)

and 5% (w/v) semi skimmed milk powder for standard antibodies, or TBS-T containing 5% (w/v) bovine serum albumin (BSA) for phospho-specific antibodies for 1h at room temperature. Membranes were incubated with the following primary antibodies (source; working dilution) overnight in TBS-T containing either 5% (w/v) semi skimmed milk powder or BSA: rabbit monoclonal anti-myosin light chain 2 (ABCAM; 1:10000), rabbit anti-cMyBP-C (kindly provided by Prof Mathias Gautel; 1:500), rabbit polyclonal anti-[pSer<sup>282</sup>] Myosin-binding protein C (cardiac type) (ENZO Lifescience; 1:1000), rabbit polyclonal anti-cardiac troponin I (ABCAM, 1:1000), rabbit polyclonal anti-cardiac troponin I (phospho S22 + S23) (ABCAM, 1:1000). Membranes were washed three times in TBS-T and incubated for 1 h at room temperature with secondary antibody (1:1000 dilution, HRP-conjugated donkey anti-rabbit IgG, GE Healthcare, NA934V) in TBS-T containing either 1% (w/v) semi-skimmed milk powder or BSA. Blots were washed in TBS-T, immersed in ECL Plus reagent (GE HEALTHCARE) and bands visualized on a BIO-RAD Gel-Doc Imager.

### **Reconstitution of Labelled cRLCs and cTnCs into Ventricular Trabeculae**

Wistar rats (male, 200-250 g) were sacrificed by cervical dislocation (Schedule 1 procedure in accordance with UK Animal Scientific Procedure Act, 1986) and demembrated right ventricular trabeculae were prepared as described previously (2).

BR-cTnC-E was reconstituted into demembrated trabeculae by overnight soak in relaxing buffer (composition in mmol/L: 25 Imidazole, 15 Na<sub>2</sub>Creatine phosphate (Na<sub>2</sub>CrP), 78.4 KPropionate (KPr), 5.65 Na<sub>2</sub>ATP, 6.8 MgCl<sub>2</sub>, 10 K<sub>2</sub>EGTA, 1 DTT, pH 7.1) containing 0.5 mg/ml of BR-cTnC-E at 4°C, replacing about 80% of the endogenous cTnC (6). The maximal calcium activated force after reconstitution with BR-cTnC-E was 34.7 mN/mm<sup>2</sup> ± 3.0 mN/mm<sup>2</sup> at 1.9 μm sarcomere length (mean ± SEM, n=13).

BSR-cRLCs were exchanged into demembrated trabeculae by extraction in CDTA-rigor solution (composition in mmol/L: 5 CDTA, 50 KCl, 40 Tris-HCl pH 8.4, 0.1% (v/v) Triton X-100) for 30 min followed by reconstitution with 40 μmol/L BSR-cRLC in relaxing solution for 1h, replacing 30%-50% of the endogenous cRLCs (3). The average maximal calcium activated force after cRLC exchange (36.4 ± 2.0 mN/mm<sup>2</sup>, mean ± SEM, n=25) was 88% ± 3% (mean ± SEM, n=25) of that before cRLC exchange. The average force recovery after exchange of N-terminal truncated cRLC (ND-BSR-cRLC-BC) was 69% ± 5% (mean ± SEM, n=5).

### **Fluorescence Polarization Experiments**

Composition of experimental solutions and activation protocols were identical to those described previously for fluorescence polarization experiments (3). Polarized fluorescence intensities were measured as described previously for skeletal muscle fibres (7, 8). Fluorescence emission from BSR-cRLCs and BR-cTnCs 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 sarcomere length of trabeculae was measured by laser diffraction in relaxing solution prior to each activation. Activating solution contained (in mmol/L): 25 Imidazole, 15 Na<sub>2</sub>CrP, 58.7 KPr, 5.65 Na<sub>2</sub>ATP, 6.3 MgCl<sub>2</sub>, 10 CaCl<sub>2</sub>, 10 K<sub>2</sub>EGTA, 1 DTT, pH 7.1. Each activation was preceded by a 2-min incubation in pre-activating solution (composition in mmol/L: 25 Imidazole, 15 Na<sub>2</sub>CrP, 108.2 KPr, 5.65 Na<sub>2</sub>ATP, 6.3 MgCl<sub>2</sub>, 0.2 K<sub>2</sub>EGTA, 1 DTT, pH 7.1). Solutions with varying concentrations of free [Ca<sup>2+</sup>] were prepared by mixing relaxing and

activating solutions using MAXCHELATOR software ([maxchelator.stanford.edu](http://maxchelator.stanford.edu)). Isometric force and steady-state fluorescence polarization values were measured once steady force had been established. The dependence on force and order parameters on free calcium concentration was fitted to data from individual trabeculae using non-linear least-squares regression to the modified Hill equation:

$$F = Y_0 + A \times ([Ca^{2+}]^{n_H} / (-\log_{10}[pCa_{50}]^{n_H} + [Ca^{2+}]^{n_H}))$$

where  $pCa_{50}$  is the negative logarithm of  $[Ca^{2+}]$  corresponding to half-maximal change in  $F$ ,  $n_H$  is the Hill coefficient,  $Y_0$  is the baseline, and  $A$  is the amplitude (please note that for normalized force data:  $Y_0=0$  and  $A=1$ ). Trabeculae which showed a decline in maximal calcium activated force after the pCa titrations of more than 15% were discarded.

### ***In situ* cRLC phosphorylation**

RLCs in demembrated trabeculae (and CMFs) were phosphorylated *in situ* with a protocol modified from that described previously (10, 11). Briefly, trabeculae were incubated in relaxing buffer containing 30 mmol/L 2,3-butanedione monoxime (BDM) for five minutes at room temperature, briefly washed in activating solution (pCa 4.5) containing 30 mmol/L BDM and finally incubated for 1 h at 22°C in activating solution containing 30 mmol/L BDM, 1  $\mu$ mol/L cMLCK and 1  $\mu$ mol/L CaM. After *in situ* phosphorylation of cRLCs, trabeculae were washed three times for 10 min in relaxing solution and the sarcomere length re-adjusted for experiments.

### **Measurement of cRLC Phosphorylation Levels after Experiments**

After experiments trabeculae were dismantled from the experimental setup and dissolved in SDS-PAGE loading buffer at 95°C for 2 min. Phospho-species were separated by Phostag™-SDS-PAGE at 160 V for 2 h. After electrophoresis, gels were washed in transfer buffer containing 1 mmol/L EDTA for 10 min and twice in transfer buffer. Proteins were blotted onto nitro-cellulose membranes (BIO-RAD) at 1 mA/cm<sup>2</sup> for 1.5 h. Post-blotting, BSR labelled cRLCs were visualized using rhodamine excitation and emission filter settings on BIO-RAD Gel-Doc Imager. Membranes were washed with TBS-T and blocked with 5% (w/v) semi-skimmed milk powder in TBS-T for 1h. The blocked membranes were briefly washed in TBS-T and then incubated overnight at 4°C with primary antibody against cRLC (rabbit monoclonal anti-myosin light chain 2, ACAM, ab92721) in a 1:10000 dilution in TBS-T containing 5% (w/v) semi-skimmed 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) semi-skimmed milk powder. Blots were washed in TBS-T, immersed in ECL Plus reagent (GE HEALTHCARE) and bands visualized on a BIO-RAD Gel-Doc Imager.

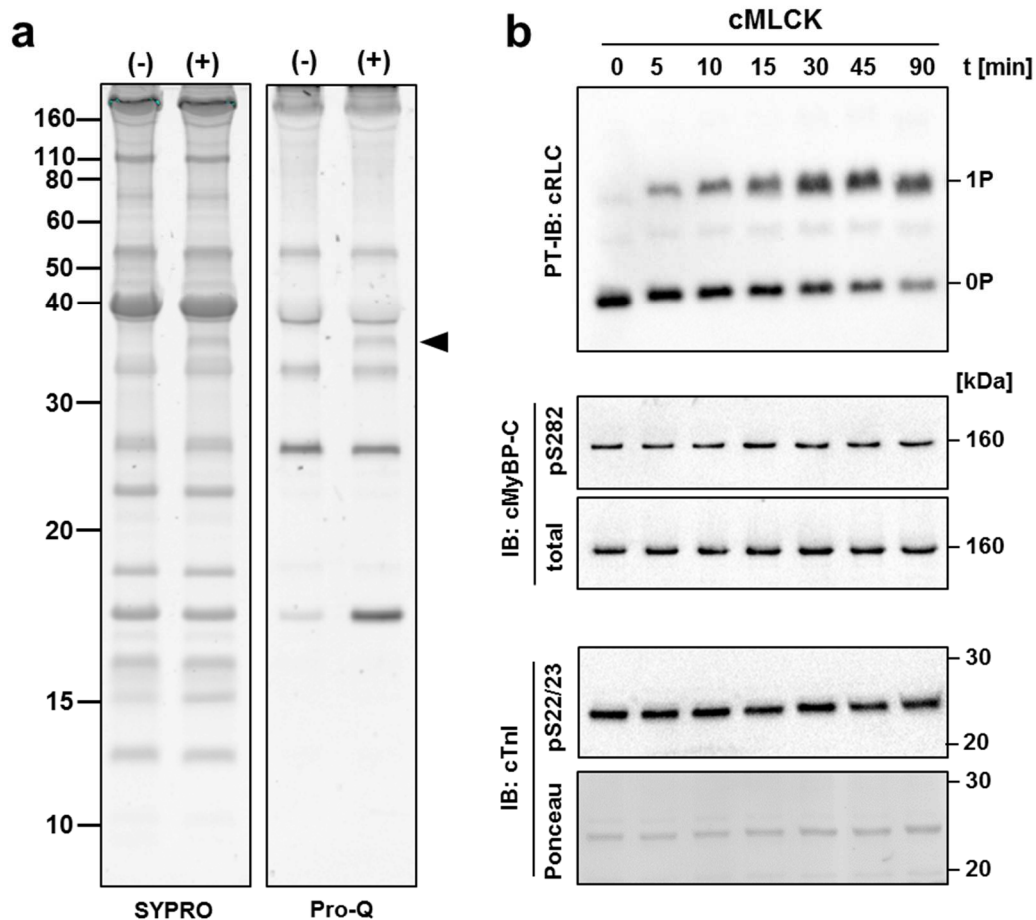
### **Phospho-protein Staining**

CMFs or trabeculae were dissolved in SDS-PAGE loading buffer, and samples denatured by boiling at 100°C for two minutes and run on either 15% (v/v) acrylamide SDS-PAGE gels or 4%-20% (v/v) gradient gels (BIO-RAD) for 1-2 h at 160 V. Post-running, gels were stained with ProQ-Diamond phospho-protein stain and SYPRO Ruby total protein stain according to manufacturer's instructions (Life Technologies).

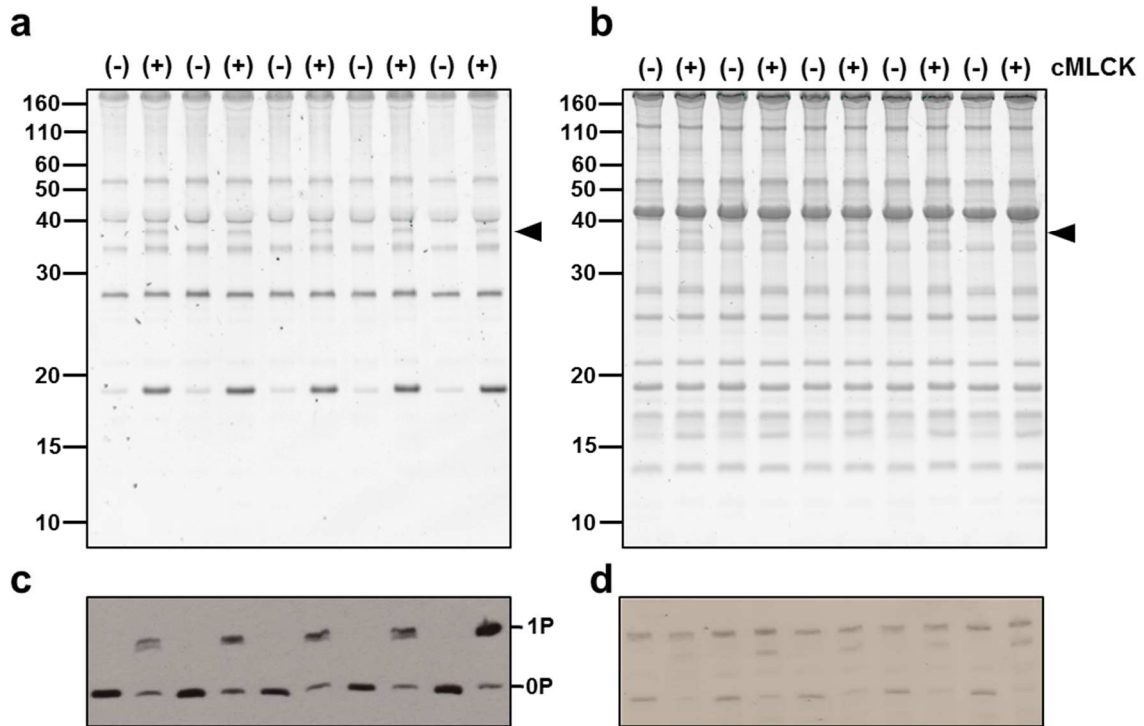
## Supplemental References

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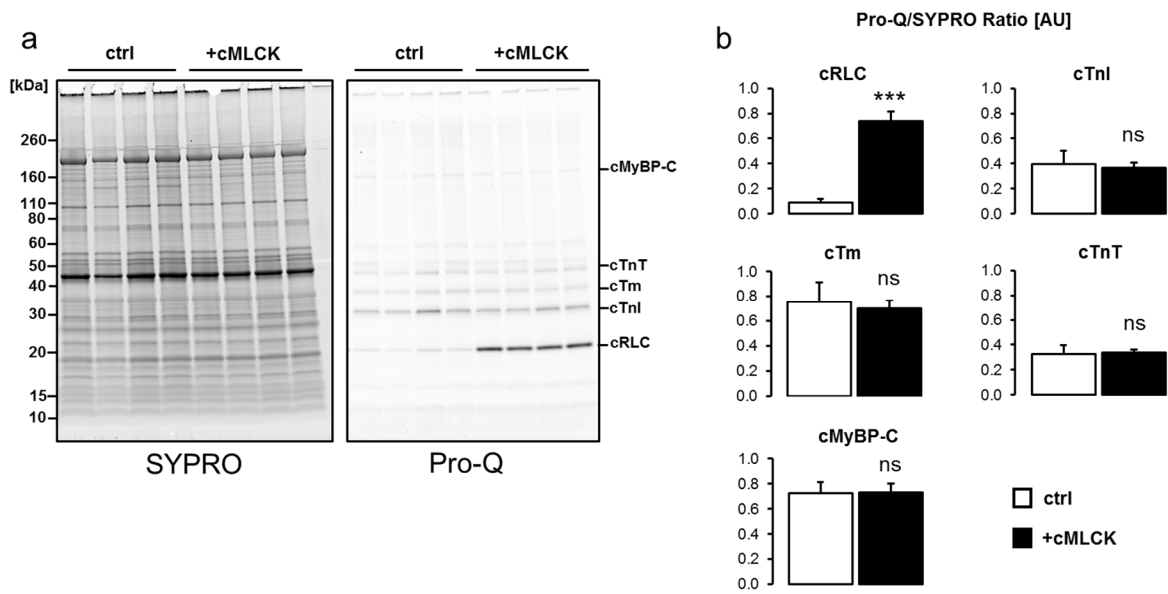
## SI Appendix Supplemental Figures and Tables



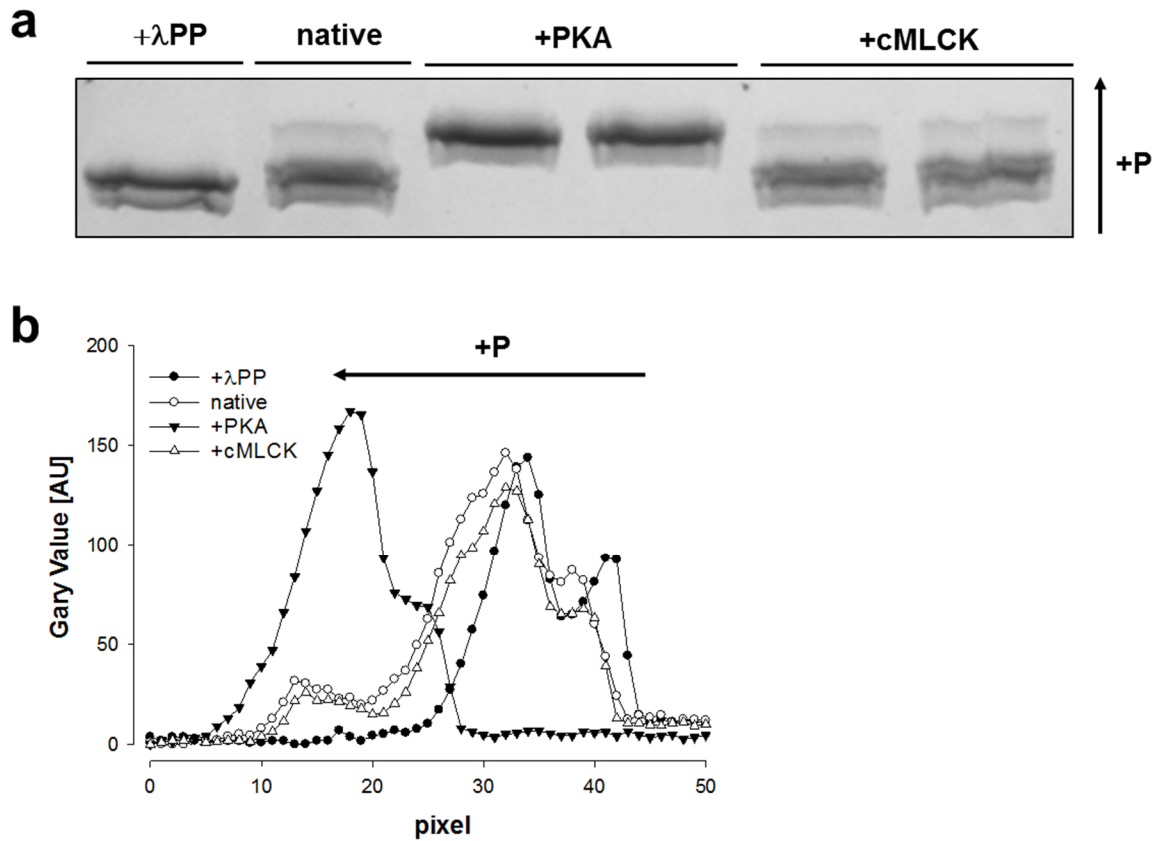
**Figure S1.** cMLCK phosphorylates cRLC specifically *in situ*. (a) Rat cardiomyofibrils were incubated in activating solution (pCa 4.5) containing 30 mmol/L BDM and 16  $\mu\text{g/ml}$  calmodulin without (-) and with 1  $\mu\text{mol/L}$  cMLCK (+). Proteins were separated by SDS-PAGE and stained for total protein with SYPRO (left) and phospho-proteins with Pro-Q Diamond staining (right). The arrowhead indicates the position of added cMLCK. (b) Time-dependent phosphorylation of cRLC by cMLCK *in situ* (top) was confirmed by Phostag<sup>TM</sup>-SDS-PAGE followed by Western-Blot (PT-IB) against cRLC. Note that in the same samples cardiac myosin binding protein-C (cMyBP-C; middle) and cardiac troponin I (cTnI; bottom) are not significantly phosphorylated by cMLCK.



**Figure S2.** Cardiac myofibrils (2 mg/ml) were incubated in activating solution (pCa 4.5) containing 30 mmol/L BDM and 16  $\mu$ g/ml calmodulin without (-) and with (+) 1  $\mu$ mol/L cMLCK for 1 h. Cardiomyofibrillar protein was separated by SDS-PAGE (12.5  $\mu$ g per lane) and stained for phospho-proteins with ProQ-Diamond (a) and total protein with SYPRO Ruby (b). Only a protein with an apparent molecular weight of about 18 kDa was significantly phosphorylated. The band representing cMLCK is indicated by an arrowhead. The same samples were run on Phos-tag-SDS-PAGE, blotted onto nitrocellulose membrane and probed with an anti-cRLC antibody (c) confirming the specific phosphorylation of cRLC by cMLCK. The total protein stain of the membrane with Ponceau is shown in (d).

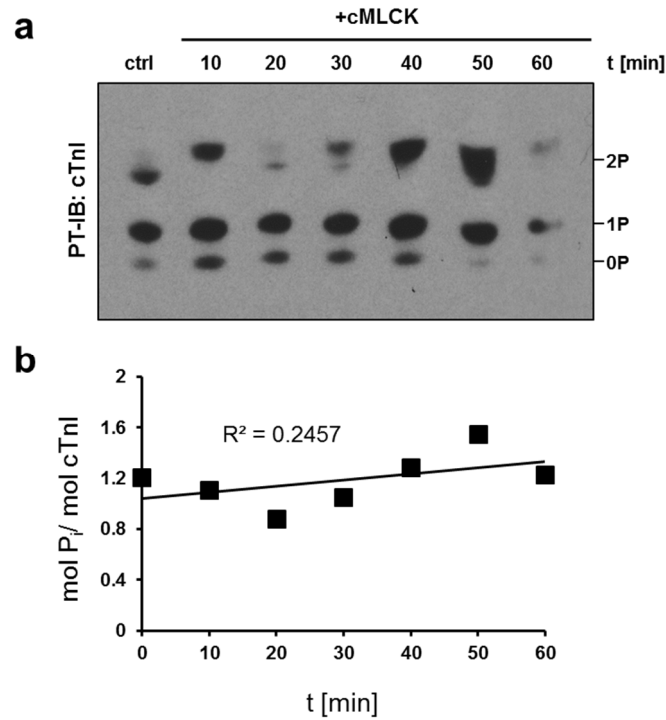


**Figure S3.** (a) Rat ventricular trabeculae were incubated in activating solution (pCa 4.5) containing 30 mmol/L BDM and 16  $\mu\text{g/ml}$  calmodulin without (ctrl) and with 1  $\mu\text{mol/L}$  cMLCK for 1 h (+cMLCK). Trabeculae proteins were separated by 4%-20% gradient SDS-PAGE and stained for total proteins with SYPRO Ruby (*left*) and phospho-proteins with ProQ-Diamond (*right*). (b) Densitometric analysis of protein phosphorylation levels of control trabeculae (white bars) and trabeculae incubated with cMLCK (black bars) expressed as ratios of ProQ and SYPRO band intensities (cRLC, cardiac myosin regulatory light chain; cTnI, cardiac troponin I; cTm, tropomyosin; cTnT, cardiac troponin T; cMyBP-C, cardiac myosin binding protein-C). Statistical significance of differences was assessed using two-tailed unpaired Student's t-test: ns - not significant; \*\*\* $p < 0.001$  (means  $\pm$  SEM;  $n=4$ ).

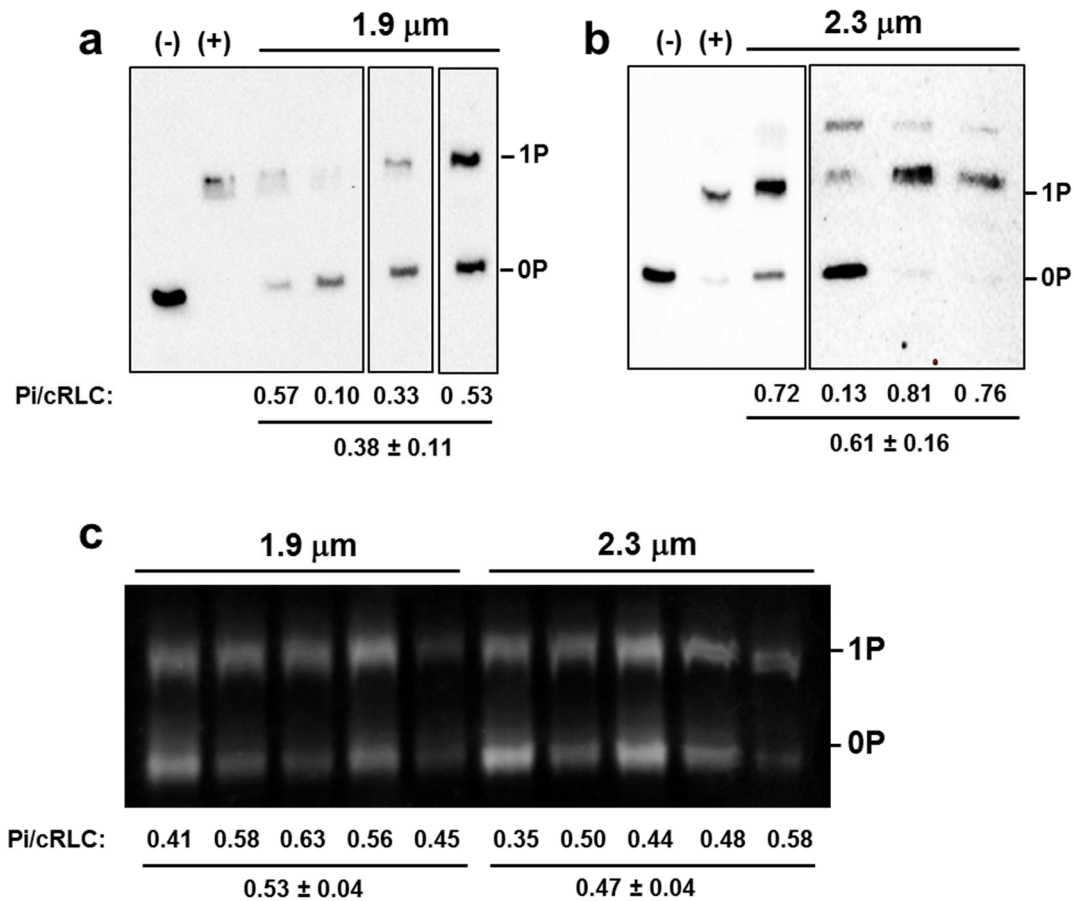


**Figure S4.** cMLCK does not phosphorylate rat cMyBP-C *in vitro*. (a) cMyBP-C was extracted from rat ventricular tissue and phospho-species separated by Phostag<sup>TM</sup>-SDS-PAGE (lane 2; native). Native cMyBP-C was dephosphorylated by lambda protein phosphatase (lane 1; +λPP) as negative control and phosphorylated with PKA as positive control (lanes 3 and 4; +PKA). Incubation with calcium and calmodulin activated cMLCK in cMLCK assay buffer (lanes 5 and 6; +cMLCK) did not change the phospho-species distribution of native cMyBP-C, indicating that cMLCK does not phosphorylate native cMyBP-C *in vitro*. (b) Overlay of line scans from lanes 1, 2, 3 and 5.

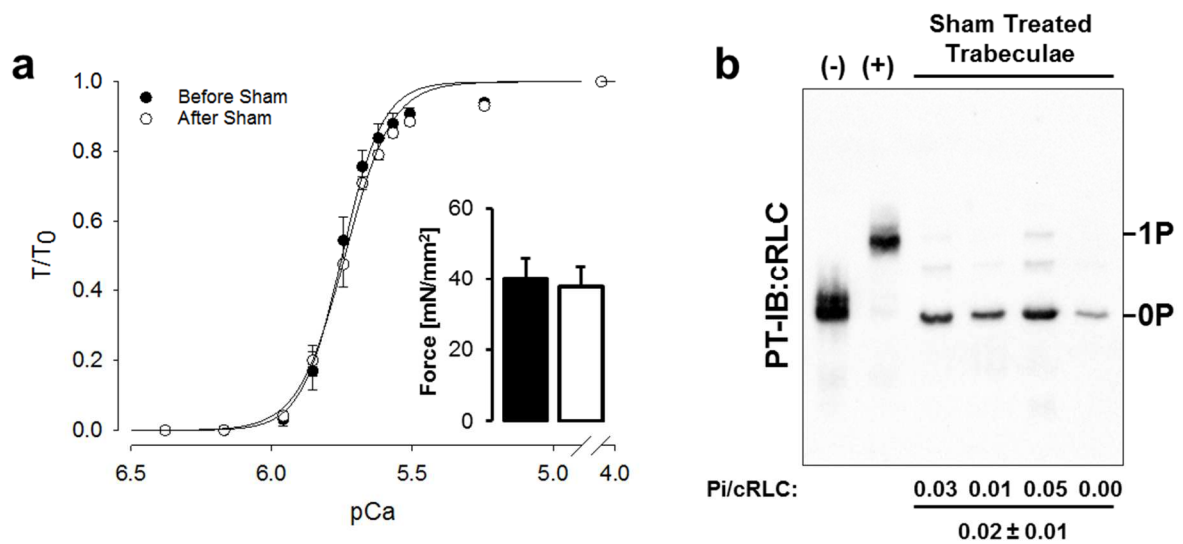




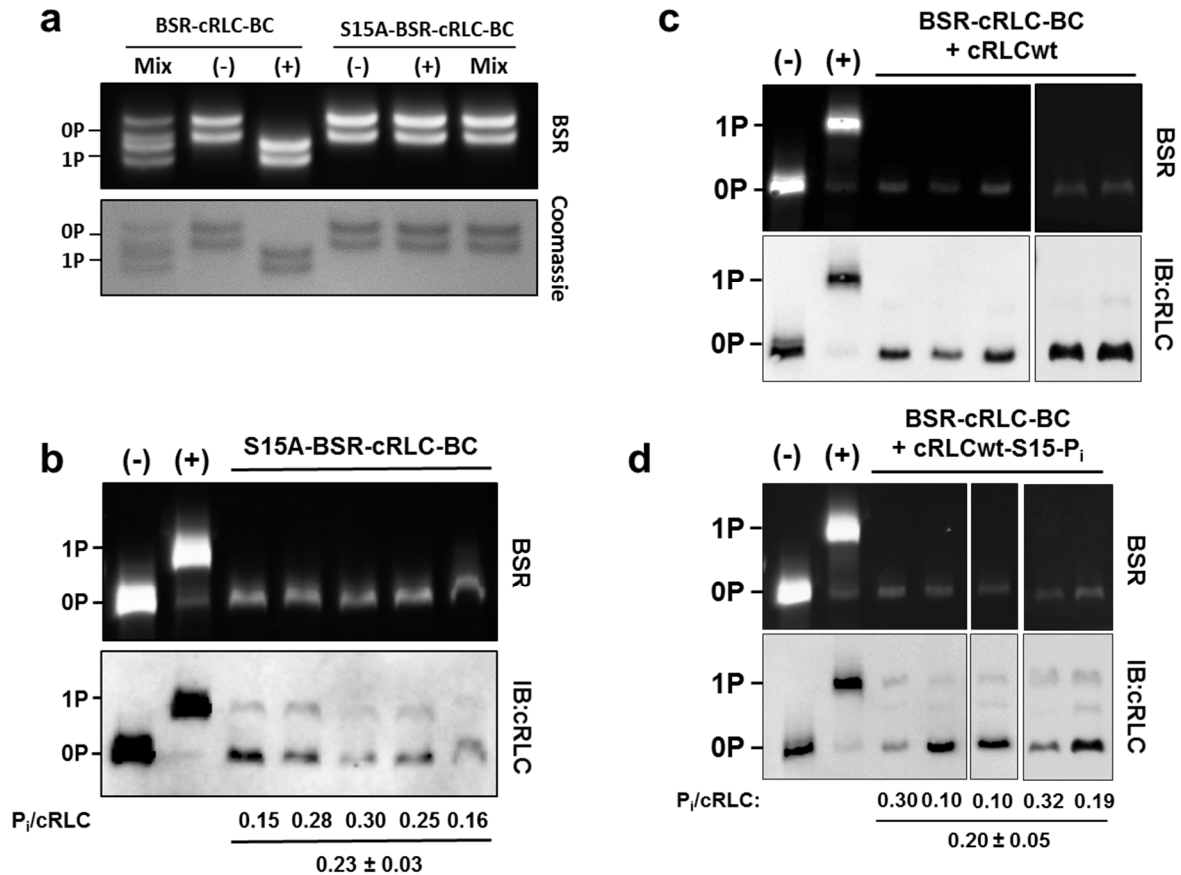
**Figure S5.** Phosphorylation levels of cardiac troponin I are not altered by cMLCK treatment. (a) Skinned ventricular trabeculae were incubated in activating buffer containing 30 mmol/L BDM, 16  $\mu$ g/ml calmodulin and 1  $\mu$ mol/L cMLCK and samples taken at the indicated time points. Trabeculae were dissolved in SDS-PAGE loading buffer and cTnI phosphorylation levels determined by Phostag<sup>TM</sup>-SDS-PAGE followed by Western-Blot against cTnI. (b) cTnI phosphorylation levels plotted against time (continuous line: linear regression; Pearson Product Mean Correlation  $P=0.258$ ).



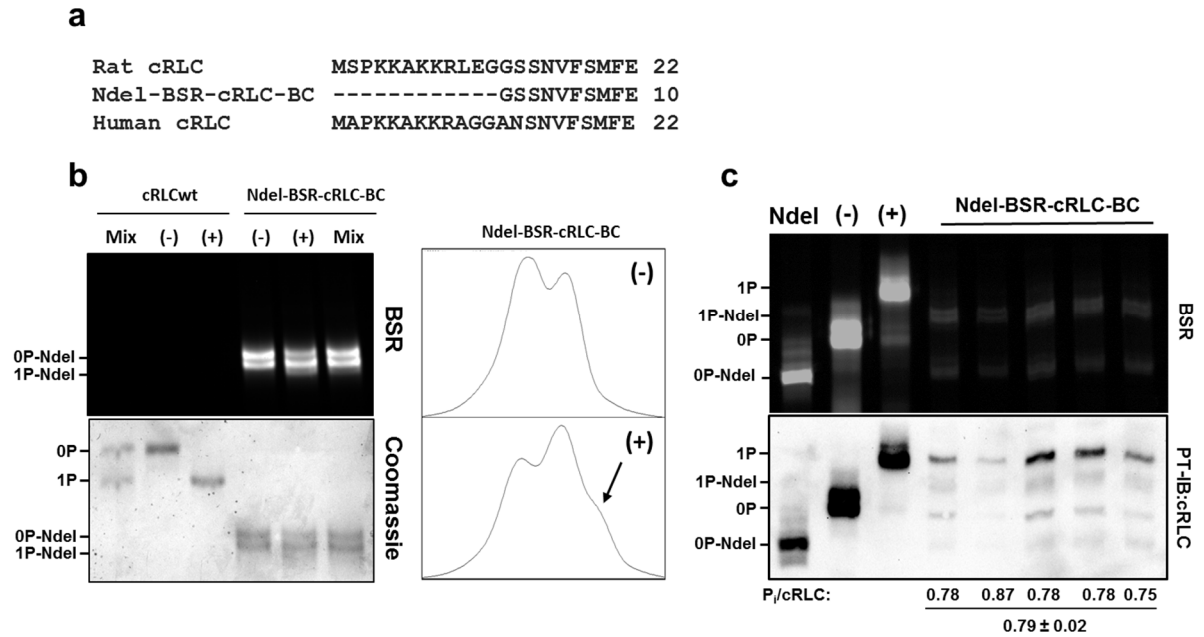
**Figure S6.** cRLC phosphorylation level of experimental trabeculae. (a) and (b): cRLC phosphorylation level of native, skinned trabeculae after incubation with cMLCK (see Materials and Methods) used for experiments summarized in Table S1. Recombinant unphosphorylated and cMLCK mono-phosphorylated cRLC were used as negative (-) and positive control (+), respectively. (c) Phosphorylation level of BSR-cRLC-BC exchanged experimental trabeculae used for data shown in Figures 2 and 6 were assessed by Phostag<sup>TM</sup>-SDS-PAGE and bands visualized by rhodamine fluorescence under UV illumination. Values are expressed as mole  $\text{P}_i$  per mole cRLC. Means  $\pm$  SEM (n=4-5).



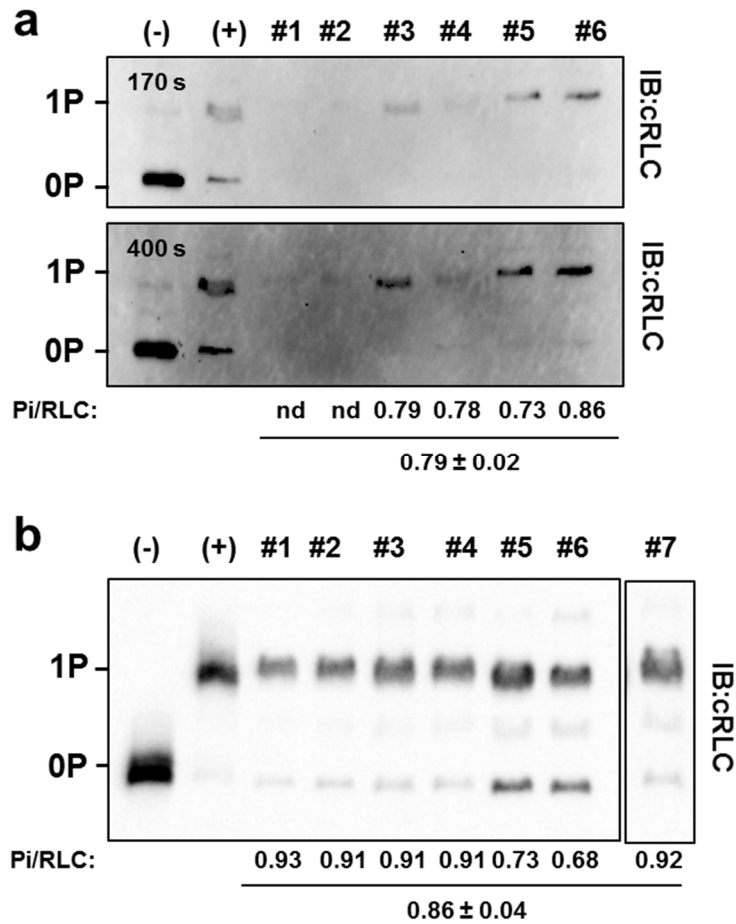
**Figure S7.** Sham-treatment of skinned trabeculae. (a) Force-pCa relation of skinned trabeculae before (closed circles) and after sham-treatment (open circles) in activating solution (pCa 4.5) containing 30 mmol/L BDM without cMLCK for 1 h at room temperature. Note that sham-treatment did not significantly change the calcium sensitivity, cooperativity and maximal calcium activated force of skinned trabeculae ( $P > 0.05$  for two-tailed, unpaired Student's T-test). Values indicate mean  $\pm$  SEM ( $n=4$ ). (b) cRLC phosphorylation level (expressed as mole  $P_i$  per mole cRLC) of sham treated trabeculae measured by Phostag<sup>TM</sup>-SDS-PAGE and Western-Blot against cRLC. Recombinant unphosphorylated and cMLCK mono-phosphorylated cRLC were used as negative (-) and positive control (+), respectively.



**Figure S8.** (a) *In vitro* kinase assays with BSR-cRLC-BC and S15A-BSR-cRLC-BC ((-) control without cMLCK; (+) with cMLCK; Mix-mixture of both samples). Note that S15A-BSR-cRLC-BC is not phosphorylated by cMLCK *in vitro*. (b) Phostag<sup>TM</sup>-SDS-PAGE of experimental trabeculae from Figure 3A and 3B ((-) negative control: unphosphorylated BSR-cRLC-BC; (+) positive control: mono-phosphorylated BSR-cRLC-BC. Bands were visualized by either UV illumination (upper panel) to estimate the extent of phosphorylation of BSR labelled cRLCs or by Western-Blot against total cRLC (lower panel). (c) and (d): Phostag<sup>TM</sup>-SDS-PAGE and Western-Blot of experimental trabeculae co-exchanged with BSR-cRLC-BC and either unphosphorylated wildtype cRLC (cRLCwt) or mono-phosphorylated wildtype cRLC (cRLCwt-S15-P<sub>i</sub>), respectively. Note that mono-phosphorylated wildtype cRLCs are only detected in (d).



**Figure S9.** (a) Sequence alignment of the N-terminal extension of wildtype rat and human cRLC, and Ndel-BSR-cRLC-BC. (b) *In vitro* kinase assays with wildtype cRLC (cRLCwt) and Ndel-BSR-cRLC-BC ((-) control without cMLCK; (+) with cMLCK; Mix-mixture of both samples) analysed by urea-glycerol-PAGE. Bands were visualized by excitation of BSR under UV and total protein staining with Coomassie. Line scans of lanes 4 and 5 are shown on the right. Note that Ndel-BSR-cRLC-BC is a poor substrate for cMLCK *in vitro* (phosphorylated Ndel-BSR-cRLC-BC is indicated by an arrow). (C) Phostag<sup>TM</sup>-SDS-PAGE of experimental trabeculae exchanged with Ndel-BSR-cRLC-BC after *in situ* phosphorylation from Figure 4 (Ndel: Ndel-BSR-cRLC-BC; (-) negative control: unphosphorylated BSR-cRLC-BC; (+) positive control: mono-phosphorylated BSR-cRLC-BC). Bands were visualized under UV (upper panel) to estimate the extent of phosphorylation of Ndel-BSR-cRLC-BC or subsequently by Phostag<sup>TM</sup>-Western-Blot (PT-IB) against total cRLC (lower panel).



**Figure S10.** cRLC phosphorylation level of experimental trabeculae from Figure 4 after cMLCK treatment measured by Phostag<sup>TM</sup>-SDS-PAGE and Western-blot against cRLC. ((-) negative control: unphosphorylated cRLCwt; (+) positive control: phosphorylated cRLC). (a) Samples from trabeculae used in Figures 4a and 4b (nd = not determined). Exposure times are shown in the upper left corner. (b) Samples from trabeculae used in Figures 5C and 5D. Means  $\pm$  SEM (n=4-7).

**Table S1.** Summary of force-pCa and  $\langle P_2 \rangle$ -pCa parameters for native and BSR-cRLC-BC exchanged trabeculae before (-) and after cMLCK treatment (+) at 1.9  $\mu\text{m}$  and 2.3  $\mu\text{m}$  sarcomere length (SL).

		1.9 $\mu\text{m}$ SL		2.3 $\mu\text{m}$ SL			
		(-)	(+)	(-)	(+)		
<b>Native</b>	<b>(n=4)</b>	<b>Force</b>	pCa <sub>50</sub>	5.63 $\pm$ 0.01	5.70 $\pm$ 0.01*	5.69 $\pm$ 0.01	5.76 $\pm$ 0.01**
			$\eta_{\text{H}}$	5.99 $\pm$ 0.53	5.30 $\pm$ 0.41	5.96 $\pm$ 0.70	3.54 $\pm$ 0.15**
			F <sub>MAX</sub> [kPa]	32.40 $\pm$ 3.87	33.60 $\pm$ 0.99	42.41 $\pm$ 2.67	43.17 $\pm$ 1.58
			P <sub>i</sub> /cRLC	<0.05	0.38 $\pm$ 0.11	<0.05	0.61 $\pm$ 0.16
<b>BSR-cRLC-BC</b>	<b>(n=5)</b>	<b>Force</b>	pCa <sub>50</sub>	5.56 $\pm$ 0.01	5.63 $\pm$ 0.01***	5.65 $\pm$ 0.01	5.74 $\pm$ 0.02*
			$\eta_{\text{H}}$	5.63 $\pm$ 0.52	5.31 $\pm$ 0.39	6.45 $\pm$ 0.33	4.35 $\pm$ 0.16***
			F <sub>MAX</sub> [kPa]	28.86 $\pm$ 1.35	34.95 $\pm$ 2.40	47.41 $\pm$ 5.09	53.37 $\pm$ 4.38*
			P <sub>i</sub> /cRLC	<0.05	0.53 $\pm$ 0.04	<0.05	0.47 $\pm$ 0.04
	<b>&lt;P<sub>2</sub>&gt;</b>	pCa <sub>50</sub>	5.61 $\pm$ 0.01	5.71 $\pm$ 0.01***	5.70 $\pm$ 0.01	5.87 $\pm$ 0.02**	
		$\eta_{\text{H}}$	8.13 $\pm$ 0.80	7.61 $\pm$ 0.48	8.01 $\pm$ 0.49	6.15 $\pm$ 0.48***	
		Baseline (Y <sub>0</sub> )	-0.0177 $\pm$ 0.0096	0.0381 $\pm$ 0.0038***	-0.0015 $\pm$ 0.0092	0.0623 $\pm$ 0.0031**	
		Amplitude (A)	0.0763 $\pm$ 0.0150	0.0538 $\pm$ 0.0080	0.1009 $\pm$ 0.0112	0.0538 $\pm$ 0.0062**	

Values indicate mean  $\pm$  SEM. Statistical significance of differences between values was assessed using two-tailed paired Student's t-test: \*p<0.05; \*\*p<0.01; \*\*\*p<0.001



**Table S2.** Summary of force-pCa and  $\langle P_2 \rangle$ -pCa parameters for S15A-BSR-cRLC-BC exchanged trabeculae before (-) and after cMLCK treatment (+) at 2.1  $\mu\text{m}$  sarcomere length, and for trabeculae co-exchanged with BSR-cRLC-BC and either unphosphorylated (+cRLCwt) or mono-phosphorylated cRLC (+cRLCwt-S15-P<sub>i</sub>) at 2.1  $\mu\text{m}$  sarcomere length.

		<b>S15A-BSR-cRLC-BC</b>		<b>BSR-cRLC-BC</b>	
		(-)	(+)	+cRLCwt	+cRLCwt-S15-P <sub>i</sub>
<b>Force</b>	pCa <sub>50</sub>	5.69 ± 0.01	5.79 ± 0.01**	5.65 ± 0.01	5.69 ± 0.01*
	n <sub>H</sub>	5.17 ± 0.65	5.04 ± 0.43	6.38 ± 0.27	5.99 ± 0.21
	F <sub>MAX</sub> [kPa]	35.48 ± 3.41	38.89 ± 4.12	30.78 ± 2.46	31.56 ± 1.64
	P <sub>i</sub> /cRLC	<0.05	0.23 ± 0.03	<0.05	0.20 ± 0.05
<b>&lt;P<sub>2</sub>&gt;</b>	pCa <sub>50</sub>	5.76 ± 0.02	5.88 ± 0.02**	5.70 ± 0.01	5.74 ± 0.01*
	n <sub>H</sub>	7.94 ± 0.28	7.51 ± 0.34	8.83 ± 0.47	8.92 ± 0.31
	Baseline (Y <sub>0</sub> )	0.0074 ± 0.0007	0.0481 ± 0.0060**	-0.0139 ± 0.0060	0.0193 ± 0.0050**
	Amplitude (A)	0.0808 ± 0.0055	0.0493 ± 0.0040**	0.0826 ± 0.0041	0.0738 ± 0.0109

Values indicate mean ± SEM (n=5). Statistical significance of differences between values was assessed paired or unpaired Student's t-test: \*p<0.05; \*\*p<0.01.

**Table S3.** Summary of force-pCa and  $\langle P_2 \rangle$ -pCa parameters for Ndel-BSR-cRLC-BC exchanged trabeculae before (-) and after cMLCK treatment (+) at 1.9  $\mu\text{m}$  sarcomere length.

		<b>Ndel-BSR-cRLC-BC</b>	
		(-)	(+)
<b>Force</b>	pCa <sub>50</sub>	5.66 $\pm$ 0.02	5.68 $\pm$ 0.02
	n <sub>H</sub>	4.85 $\pm$ 0.58	3.22 $\pm$ 0.29*
	F <sub>MAX</sub> [kPa]	33.15 $\pm$ 2.45	33.11 $\pm$ 2.83
	P <sub>i</sub> /cRLC	<0.05	0.79 $\pm$ 0.02
$\langle P_2 \rangle$	pCa <sub>50</sub>	5.76 $\pm$ 0.03	-
	n <sub>H</sub>	9.06 $\pm$ 0.92	-
	Baseline (Y <sub>0</sub> )	0.0793 $\pm$ 0.0073	-
	Amplitude (A)	0.0490 $\pm$ 0.0080	-

Values indicate mean  $\pm$  SEM (n=5). Statistical significance of differences between values was assessed using two-tailed paired Student's t-test: \*p<0.05.

**Table S4.** Summary of force-pCa and  $\langle P_2 \rangle$ -pCa parameters for BR-cTnC-E exchanged trabeculae before (-) and after cMLCK treatment (+), and in the absence and in the presence of 25  $\mu\text{mol/L}$  Blebbistatin (+Blebb) at 1.9  $\mu\text{m}$  sarcomere length.

		BR-cTnC-E			BR-cTnC-E		
		(-)	(+)	(+), +Blebb	(-)	(-), +Blebb	(+), +Blebb
<b>Force</b>	pCa <sub>50</sub>	5.34 ± 0.02	5.44 ± 0.03 <sup>***</sup>	-	5.39 ± 0.03	-	-
	n <sub>H</sub>	3.78 ± 0.37	3.21 ± 0.17	-	4.54 ± 0.37	-	-
	F <sub>MAX</sub> [kPa]	28.34 ± 1.44	35.08 ± 1.97*	-	40.13 ± 3.49	-	-
	Pi/cRLC	<0.05	0.79 ± 0.02	0.79 ± 0.02	<0.05	<0.05	0.86 ± 0.04
<b>&lt;P<sub>2</sub>&gt;</b>	pCa <sub>50</sub>	5.34 ± 0.02	5.42 ± 0.02 <sup>**</sup>	5.32 ± 0.01	5.40 ± 0.02	5.31 ± 0.01 <sup>***</sup>	5.36 ± 0.01 <sup>***</sup>
	n <sub>H</sub>	3.78 ± 0.25	3.37 ± 0.16	2.93 ± 0.18*	4.66 ± 0.15	3.37 ± 0.06 <sup>***</sup>	3.34 ± 0.07
	Baseline (Y <sub>0</sub> )	0.1879 ± 0.0052	0.2008 ± 0.0050 <sup>***</sup>	0.2284 ± 0.0041 <sup>**</sup>	0.1698 ± 0.0087	0.2132 ± 0.0035 <sup>***</sup>	0.2198 ± 0.0034 <sup>***</sup>
	Amplitude (A)	0.0954 ± 0.0070	0.0976 ± 0.0062	0.0691 ± 0.0015 <sup>***</sup>	0.1266 ± 0.0098	0.0839 ± 0.0051 <sup>***</sup>	0.0908 ± 0.0046*

Values indicate mean ± SEM (n=6-7). Statistical significance of differences between values before and after cMLCK treatment, and before and after Blebbistatin was assessed by two-tailed paired Student's t-test: \*p<0.05; \*\*p<0.01; \*\*\*p<0.001