

# Supporting Information

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## SI Materials and Methods

**Mutagenesis, Expression, and Purification of Regulatory Light Chain (RLC).** Wild-type human smooth muscle RLC (CAG33124) has one Cys at residue 108 that was mutated to Ala for a cysteineless background. Single Cys mutants for spin-labeling were prepared using the Quick Change kit (Stratagene) using primers from Integrated DNA Technology, Inc. The smooth muscle RLC sequence was introduced in the pET7-7 vector (with ampicillin resistance gene) via heat shock (30 sec at 42 °C). For mutagenesis, DH5 $\alpha$  super competent cells were used. For large protein expression pET7-7 were transformed into BL21 (DE3) (1). All constructs were verified by sequencing. Bacteria were grown in LB media supplemented by 50  $\mu$ g/mL ampicillin (37 °C/160 rpm) for 9–11 h, auto-induced. RLC was purified on a Q-Sepharose column (Amersham Biosciences). The RLC was eluted using a 0–500 mM NaCl gradient at 2 mL/min. Thrombin was added to remove the N-terminal His-tag (5 U/mg of RLC) for 3 h at 25 °C. A second Q-Sepharose chromatography was performed to separate RLC from His-tag and thrombin. The purity of RLC was confirmed by SDS-PAGE gels. The proteins were expressed at 20–50 mg per liter of media. Spin-labeling was performed with MTSSL (Sigma–Aldrich) by incubating with a fourfold excess of spin label at 4 °C for 12 h. Excess free spin label was removed by dialysis. The labeling level for RLC was 0.75–1.0 labels per RLC. RLC concentration was determined using the bicinchoninic acid (BCA) protein assay, smooth muscle myosin (SMM) concentration was determined by both BCA and UV absorption at 280 nm using 0.56 (mg/mL)<sup>-1</sup> cm<sup>-1</sup> as the extinction coefficient (2).

**Reconstitution of SMM with Spin-Labeled RLC.** SMM was purified from frozen chicken gizzards (3) and heavy meromyosin (HMM) was prepared as described (4). A modification of the method of ref. 5 was used to exchange the native RLC with the mutant-labeled RLC. SMM (10 mg/ml) was placed to a stripping buffer containing 20 mM imidazole (pH 6.5), 0.4 M KCl, 5 mM EDTA, 2 mM EGTA, 3 mM ATP, 20 mM DTT, 0.05% Triton and 5 mM trifluoroperazine to weaken the binding of the RLC to the heavy chain. The mixture was incubated on ice for 2–3 h and applied to a 5 mL spin column of Sephacryl S400 HR (Sigma). Effluents were collected at 4 °C after two consecutive centrifugations at 475 g and 1000 g for 7 and 5 min respectively. A G-25 column eluting at 1.0 mL/min was used to change to a buffer containing 0.4 M KCl, 10 mM imidazole, 6 mM MgCl<sub>2</sub>, 2 mM EGTA, pH 6.5. The spin-labeled RLC, dialyzed overnight in the above buffer, was added to the RLC-deficient SMM at a four- to sixfold molar excess of RLC and incubated on ice for 4 h. The incubation mixture also contained essential light chain (ELC) prepared from purified SMM at a two- to threefold molar excess. of RLC. For underlabeled samples, the time of incubation was decreased to 30 min. The solution was dialyzed against low salt buffer [10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 0.2 mM EGTA, 2 mM MgCl<sub>2</sub>, 20 mM KCl, pH 7.0] to induce the precipitation of filaments, which were obtained by centrifuging at 10,000 g for 10 min. The pellet was rinsed three times with the same buffer.

The reconstituted myosin pellet was either dissolved in high salt buffer (0.5 M KCl, 0.1 mM EGTA, 10 mM MOPS, 50% glycerol, pH 7.5) and used for monomer experiments, or was resuspended in 35 mM KCl, 2 MgCl<sub>2</sub>, 10 mM MOPS, pH 7.5 for filament experiments. HMM samples were prepared similarly to SMM, using Sephacryl S300 HR and without the filament formation step, in 2 MgCl<sub>2</sub>, 10 mM MOPS, 50% glycerol, pH 7.5 with 35 mM KAc (sites 48, 108) or without any salt (sites 59,

96, and 108). All reconstituted samples were P by gizzard myosin light chain kinase as previously described (6). Complete phosphorylation was verified by 2D gels and by single-turnover ATPase. Prior to the acquisition of EPR data the proteins were frozen by plunging into a liquid N<sub>2</sub> bath. During acquisition the protein concentration was typically 50–150  $\mu$ M. Samples with greater than 0.7 spins per head contained sufficient spins to analyze.

The gel filtration steps ensured that the samples did not contain appreciable free labeled RLC as determined by the room temperature conventional EPR spectra. Free RLC results in an EPR signal with splitting of 35 Gauss due to motional averaging, bound RLC that moves on ms timescale (6) has a splitting of 70 Gauss. The largest observed fraction of narrowed spectrum in the reconstituted samples was 15% of total signal. The free RLC contributes to the total EPR signal but it does not contribute to either broadening of the spectra or time evolution of the echo (signals used the determination of distances). At 10–50  $\mu$ M free RLC, the distance between the molecules is expected to be larger than the limits of EPR distance sensitivity. Only at the concentrations of 0.5–1 mM did we observe modulation of an echo from water soluble proteins, e.g control experiments with 100  $\mu$ M free RLC confirmed no dipolar coupling between free RLC.

**Single-Turnover of Formycin Triphosphate in the Presence of Actin.** Single-turnover assays to measure the rate of phosphate release from the acto-HMM formycin diphosphate Pi complex was performed as described at the indicated actin concentration (7). The temperature was 25 °C and the buffer was 10 mM MOPS (pH 7.0), 150 mM NaCl, 0.1 mM EGTA, 0.4 mM MgCl<sub>2</sub>, 1 mM DTT.

**EPR Spectroscopy.** Room temperature (298 K) cw-EPR spectra recorded on an EMX series spectrometer (Bruker Biospin, Billerica) were used to estimate the labeling efficiency. Low temperature (150 K) cw-EPR and double electron resonance (DEER) (at 65 K) were recorded on a Bruker Elexys 680 equipped with a Bruker dielectric ring resonator (Bruker, ER 4118X-MD5). Glycerol (25% final concentration) was added to the sample to ensure a homogeneous distribution of protein by avoiding water crystallization-induced phase separation. Samples were transferred into 4/3.2 mm o.d./i.d. quartz capillaries and flash frozen in liquid nitrogen. Instrumental parameters for cw experiments were: 9.7 GHz frequency, 0.02 mW microwave power, 1 G modulation amplitude, 100 kHz modulation frequency, 21 ms time constant, 41 ms of conversion time, 1024 points/scan and sweep width between 200 and 300 G. For four-pulse DEER experiments, the temperature was set to 65 K and the dielectric resonator was over coupled (low Q factor) for obtaining a broad resonator resonance line and minimizing the interference between the ringing coming from the pulses and our signal (8). The pumping frequency was set to the central manifold of the <sup>14</sup>N spectrum whereas the observing frequency was located on the low field shoulder of the nitroxide signal, with a 60–70 MHz downfield from pumped position. This arrangement maximizes the fraction of coupled spin as well as minimizing the orientational selectivity (9, 10). Shot repetition time was set within 2–3 ms and typical  $\pi/2$  pulse length was 16 ns with  $\pi$  electron double resonance pulse length of 32 ns.

**Analysis of EPR Data.** Cw-EPR and DEER data were analyzed using CWdipFit and DEFit dipolar broadening programs devel-

oped in our laboratory. Both programs assume models of multi-Gaussian shaped distance distributions between spins and utilizes Simplex/Monte Carlo curve fitting to actual data. The background subtracted DEER signals are fitted to the function (8):

$$\text{DEER}(t,r) = \int_{r=-\Delta/2}^{\Delta/2} \exp\left(-0.693 \frac{(r-r_o)^2}{\Delta r^2}\right) \times \int_0^{\pi/2} I_o \sin \theta \cos\left(\frac{\mu_{\text{Bohr}}^2 g^2}{\hbar r^3} (3 \cos^2 \theta - 1)t\right) d\theta dr.$$

The variables  $\mu_{\text{B}}$ ,  $g$ , and  $\hbar$  are Bohr magneton,  $g$ -factor and Planck constant,  $I_o$  is initial echo amplitude,  $\theta$  is the angle between the interspin vector and magnetic field. The two parameters of interest here are  $r$  the distance between the spin and  $\Delta r$  the width of distribution. The Student F test was applied to determine the number of Gaussians needed to fit the experimental curves. For detailed information see ref. 11. All data analysis were performed with the same evolution time (2  $\mu\text{s}$ ), as well as an analogous stretched exponential baseline subtraction. The evolution time between the  $\pi$  pulses was varied for many samples between  $T_2$  and  $2 * T_2$  relaxation time to ascertain no changes in the estimated distances due to limited evolution time (Fig. S7). The fraction of the signal used for the baseline estimate was routinely varied to avoid its influence on the estimated distances (Fig. S7). For all spectra the modulation depth was more than 2%, which is the limit of the amplitude of the DEER signal due to potential aggregation as measured in singly labeled samples and samples precipitated with ammonium sulfate. An underlabeled mutant (30%) was used as a single-labeled reference (one RLC labeled), in which the probability of double labeling (two RLC labeled) was approximately 9%.

**Effects of Mutations on Phosphorylation-Dependent Regulation of the ATPase.** To date approximately 50 different point mutations of the RLC of SMM and HMM have been studied with regard to regulation of the actin-activated ATPase (6, 12–17). These mutations represent sites distributed over the entire surface of the subunit. The general conclusion from these and other studies is that point mutations have little effect, even if the mutant is a cysteine mutant labeled with an extrinsic probe such as a fluorophore, photo-cross-linker, or spin probe (6, 12–17). The mutations have similar negligible effects in both HMM and SMM, because both are regulated proteins containing the same head domains with the same RLC and ELC subunits.

Here we check and confirm the above and show that three of the constructs studied here, S59C, T96C, and C108, also had normal regulation of the actin-activated ATPase activity (see Table S1) as determined by a single-turnover transient kinetic assay (16). The turnover rates for the unphosphorylated reconstituted samples reflected full inhibition and were similar to native SMM or HMM. Transients from the phosphorylated samples were fit to two exponentials. The effect of phosphorylation was essentially the same as found in our previous study for native SMM (13) using a similar reconstitution procedure. Thus, the reconstitution procedure itself, or the presence of the labeled mutated RLC did not alter the phosphorylation-dependent regulation of the ATPase activity. Other sites used in this study have been previously studied and shown to retain full regulation when labeled with various probes (see Table S1). Sites 31, 38, and 48 were not measured because the activity was not expected to be perturbed by mutagenesis/labeling. They are nonconserved amino acids with side chains exposed to the surface but not interacting with the other RLC in the Wendt model.

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**Table S1. ATPase data for myosin with point mutations on the RLC**

Sample <sup>‡</sup>	Actin (μM)	uP		P*	
		Rate <sup>‡</sup> (s <sup>-1</sup> )	Fractionalamplitude (%)	Rate <sup>‡</sup> (s <sup>-1</sup> )	Fractionalamplitude (%)
SMM(native unlabeled)	5	0.001	100	0.001	71
				0.114	29
	12.5	0.001	100	0.010	31
SMM (WT, C108)	5	0.001	100	0.178	69
				0.0016	68
				0.109	32
SMM S59C	5	0.001	100	0.004	70
SMM T96C	5	0.001	100	0.095	30
				0.004	29
	12.5	0.001	100	0.155	71
HMM (native unlabeled)	5	0.004	100	0.12	60
HMM (WT, C108)	5	0.005	100	0.52	40
				0.08	63
				0.45	37
HMM Q15C	labeled with benzophenone-iodoacetamide, normal regulation (1)				
HMM A23C					
HMM S59C					
HMM C108					
HMM C108	labeled with 3-(5-f luoro-2,4-dinitroanilino)-PROXYL spin probe; normal regulation (2)				
HMM G165C					
SMM C108	labeled with InVSL spin label; normal regulation (3)				
	labeled with biotinylated benzophenone-iodoacetamide; normal regulation (4)				
	labeled with benzophenone-iodoacetamide; fully inhibited in the uP state (5)				

The first 4 entries in this table show data from this study using the single-turnover kinetic assay. Also listed are data from similar studies relating to the other mutants used in this study

\*For phosphorylated (P) samples, the data was best fit to two exponentials, giving two rates and their fractional amplitudes. See ref. 6 for a plot of the actin-dependence of the turnover rates for native HMM.

<sup>‡</sup>All samples were labeled with MTSSL unless otherwise indicated. WT, wild-type.

<sup>‡</sup>Each value is the average of at least three measurements. Variations in both the rates and the fractional amplitudes were approximately ±5%. Total amplitudes of all measurements were similar.

- 1 Wu XD, Clack BA, Zhi G, Stull JT, Cremo CR (1999) Phosphorylation-dependent structural changes in the regulatory light chain domain of smooth muscle heavy meromyosin. *J Biol Chem* 274:20328–20335.
- 2 Nelson WD, Blakely SE, Nesmelov YE, Thomas DD (2005) Site-directed spin labeling reveals a conformational switch in the phosphorylation domain of smooth muscle myosin. *Proc Natl Acad Sci USA* 102:4000–4005.
- 3 Li HC, Song L, Salzameda B, Cremo CR, Fajer PG (2006) Regulatory and catalytic domain dynamics of smooth muscle myosin filaments. *Biochemistry* 45:6212–6221.
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Table S2 Summary of EPR data and modeling results

RLC site	Modeled parameters from MMCM*		Sample <sup>†</sup>	Phosphorylation <sup>‡</sup>	ATP <sup>‡</sup>	EPR parameters <sup>§</sup>			Modeled state		
	$r_{ave}$ (Å)	$\Delta$ (Å)				$r_{ave}$ (Å)	$\pm$	$\Delta$ (Å)		$\pm$	
15	NA	NA	monomers	—	—	30	5	28	12	—	
				p	—	41	5	38	10	—	
23	43	4	monomers	—	—	44	5	33	7	off/on	
31	11	4	monomers	—	—	10(50%) <sup>¶</sup>	1	5	2	off	
				—	—	27(50%)	5	35	7	on	
38	11	2	monomers	—	—	15(50%)	1	8	4	off	
				—	—	34(50%)	3	45	5	on	
				P	—	no broadening <sup>¶</sup>	—	—	—	—	—
				P	—	38	3	42	6	—	
				—	—	44	4	43	7	—	
				—	+ATP	40	3	36	7	—	
48	36	4	monomers	—	—	46	2	34	4	off/on	
				—	—	44	3	42	4	—	
				—	—	45	5	43	8	—	
				—	+ATP	44	6	41	11	—	
				—	—	36	4	27	7	—	
				—	P	39	4	28	5	—	
59	30	16	monomers	—	—	30(67%)	2	14	4	off	
				—	—	50(33%)	6	35	9	on	
				P	—	28	5	24	11	—	
				—	—	37	6	38	8	—	
				—	—	32	4	41	7	—	
				—	P	30	4	45	7	—	
96	36	4	monomers	—	—	34(31%)	2	4	2	off	
				—	—	43(69%)	2	19	3	on	
				P	—	42	4	26	8	—	
				—	—	41	3	29	5	—	
				—	—	36(45%)	3	6	3	—	
				—	P	40(55%)	3	33	9	—	
108	49	6	monomers	—	—	51	2	31	4	off/on	
				—	—	49	6	35	10	—	
				—	+ATP	46	5	40	8	—	
				—	—	44	4	43	8	—	
				—	P	38	3	34	6	—	
				—	+ATP	38	4	40	8	—	
165	NA	NA	monomers	—	—	43	2	23	3	—	
				—	—	38	4	23	8	—	
				—	+ATP	37	4	26	6	—	

\* $r_{ave}$  and  $\Delta$  designate the spin-spin distance and distance distribution width, respectively. The uP HMM structure (PDB ID code 1I84) from Wendt et al. (1) was used here except for A23C where the calcium-free scallop myosin RD (2) (PDB ID code 3JTD) was used. The most probable rotamer conformations for MTSSL on the RLC were found using MMCM for each of labeled sites. Note that the distance distribution width is much smaller than the widths measured by EPR.

<sup>†</sup>Monomers and filaments refer to SMM under high and low salt conditions, respectively. Filaments were formed in the presence of 35 mM KCl. For sites 48, 108, and 165, filaments were also formed in the presence of 35 mM KAc (KCl data are quoted here). The differences in the results with the two salts were found to be smaller than the measurements errors. HMM samples were prepared in 35 mM KAc (sites 48 and 108) or without any salt (sites 59, 96, and 108), 2 MgCl<sub>2</sub>, 10 mM MOPS, 50% glycerol, pH 7.5.

<sup>‡</sup>P (P) or not (-), in the presence (+ATP) or absence (—) of 2 mM MgATP.

<sup>§</sup>The  $\pm$  symbol indicates 67% confidence limit. The values in parentheses refer the relative fractions of the double Gaussian populations.

<sup>¶</sup>Only for site 31 and 38 in monomers have we observed an appreciable broadening of conventional spectra. For all other sites the distances quoted are from DEER experiments. Coupled spins (spin labels that see each other) broaden the conventional spectra of uncoupled spins if they are at a distance smaller than 20 Å. Above this distance the effect is too small to observe but can be extended to a range of 20–70 Å using pulsed EPR DEER.

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