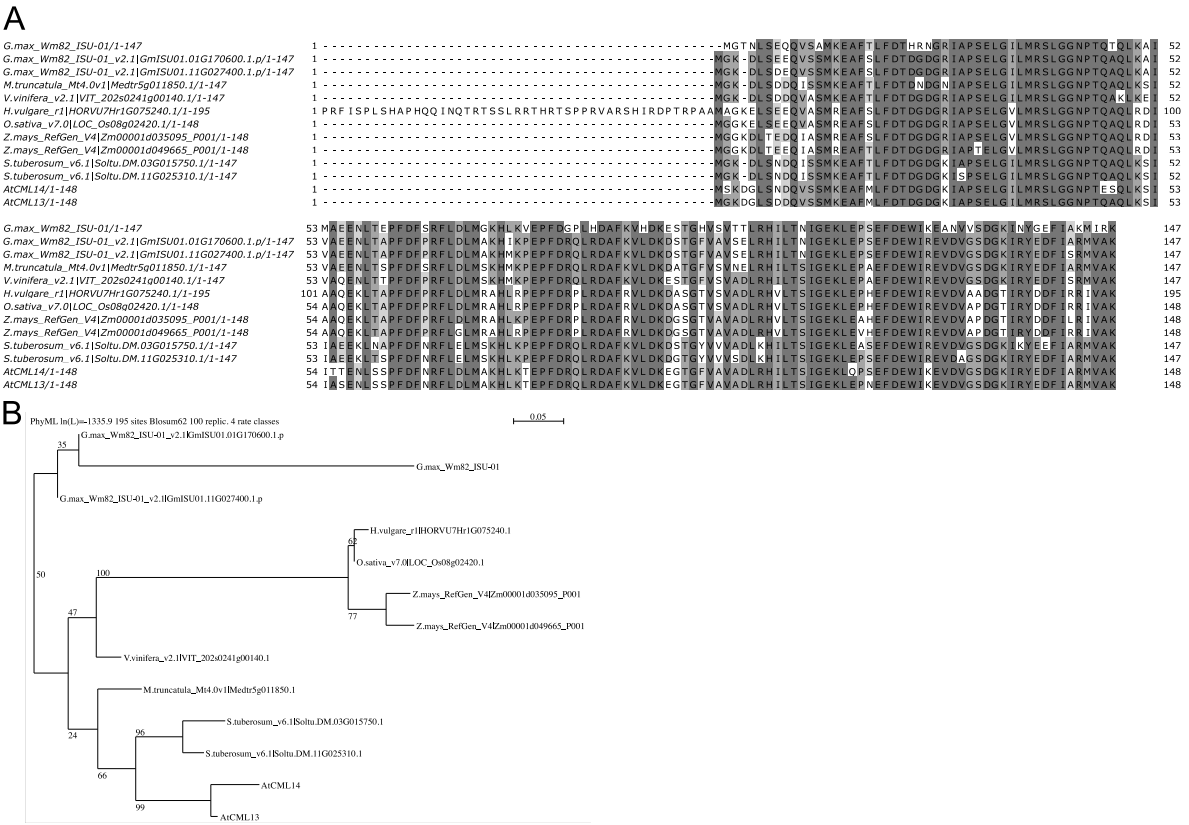
Supplementary Table S1. List of primers used in this study

Primer name	Sequence (5'-3')	Restriction Enzyme
ATM1 F	GGCCCATATGCGTACTCTCCACGGC	NdeI
ATM1 IQ1 R	GGCCGGTCGACAATAGAAATTCCCCTTTTAAGTTC	SalI
ATM1 IQ2 F	GGCCGGATCCATGTGTCTCCTGAAGGAACTTAAAAG	BamHI
ATM1 IQ2 R	GGCCGGTCGACTTAAGTAGCAGCAGCCTTATGCC	SalI
ATM1 IQ3 F	GGCCCATATGAAAGAGTTTGCTGAGTTACG	NdeI
ATM1 IQ3 R	GGCCGGTCGACTTACACAACTGATGCATCG	SalI
ATM1 IQ4 F	GGCCGGATCCATGATACAGTATAAGGGCATAG	BamHI
ATM1 R	GGCCGGATCCTTAATCCCCTGAACATCTTC	BamHI
ATM2 F	GGCCCATATGAGGAAAAAGGTTCTTCAAGG	NdeI
ATM2 IQ1 R	GGCCGGTCGACAACCAATGTTACTTTCCGC	SalI
ATM2 IQ2 F	GGCCGGATCCATGGCATACTTCCAAAATATGCGG	BamHI
ATM2 IQ2 F	GGCCCATATGGCATACTTCCAAAATATGCGG	NdeI
ATM2 IQ2 R	GGCCGGTCGACGTGTATGACTGCACTCAACTC	SalI
ATM2 IQ3 F	GGCCGGATCCATGAGATTGTTTGACACTGAAGC	BamHI
ATM2 IQ3 F	GGCCCATATGAGATTGTTTGACACTGAAGC	NdeI
ATM2 R	GGCCGGTCGACTTATTTTTGTCTTTGCATACTATTG	SalI
VIII-A IQs F	GGCCGGATCCATGAGGAATCGCACTCTGCATGGC	BamHI
VIII-A IQs R	GGCCGGTCGACTTAACTAAGCCATCCAATATCCCC	SalI
VIII-B IQs F	GGCCGGATCCATGCGTGGCATACTTGGATTACAG	BamHI
VIII-B IQs R	GGCCGGTCGACTTAACTATTTAAGAGTTTACGAG	SalI
LBb1.3	ATTTTGCCGATTTCGGAAC	N/A
ATM1-4IQ F bacvirus	AAACCATGGCTCAGAAGGTTACTCC	NcoI
ATM1-4IQ R bacvirus	TTTACCGGTTCCAATATCCCCTGAACATC	AgeI
ATM2-3IQ F bacvirus	AAACCATGGCATTATCGGCATCGCCG	NcoI
ATM2-3IQ R bacvirus	TTTACCGGTTTGTCTTTGCATACTATTGAAATG	AgeI
ATM1 gF	GGGAAACGTTTCCTTCACCG	Genotyping
ATM1 gR	AATTTATACAGAACTGCTCGAAG	Genotyping
ATM2 gF	CCCGGAGTTCCAGTAGACATCG	Genotyping
ATM2 gR	TCCGTGGTCACTTGTCTCGTG	Genotyping
VIII-A gF	ctctgactatgagctagatcg	Genotyping
VIII-A gR	GTTACCGTTTGGAAGTTGGAC	Genotyping
VIII-B gF	TGCACAGCTGAGCCACTCTTG	Genotyping
VIII-B gR	TGCAGTGGCAGATGCAGCTTA	Genotyping

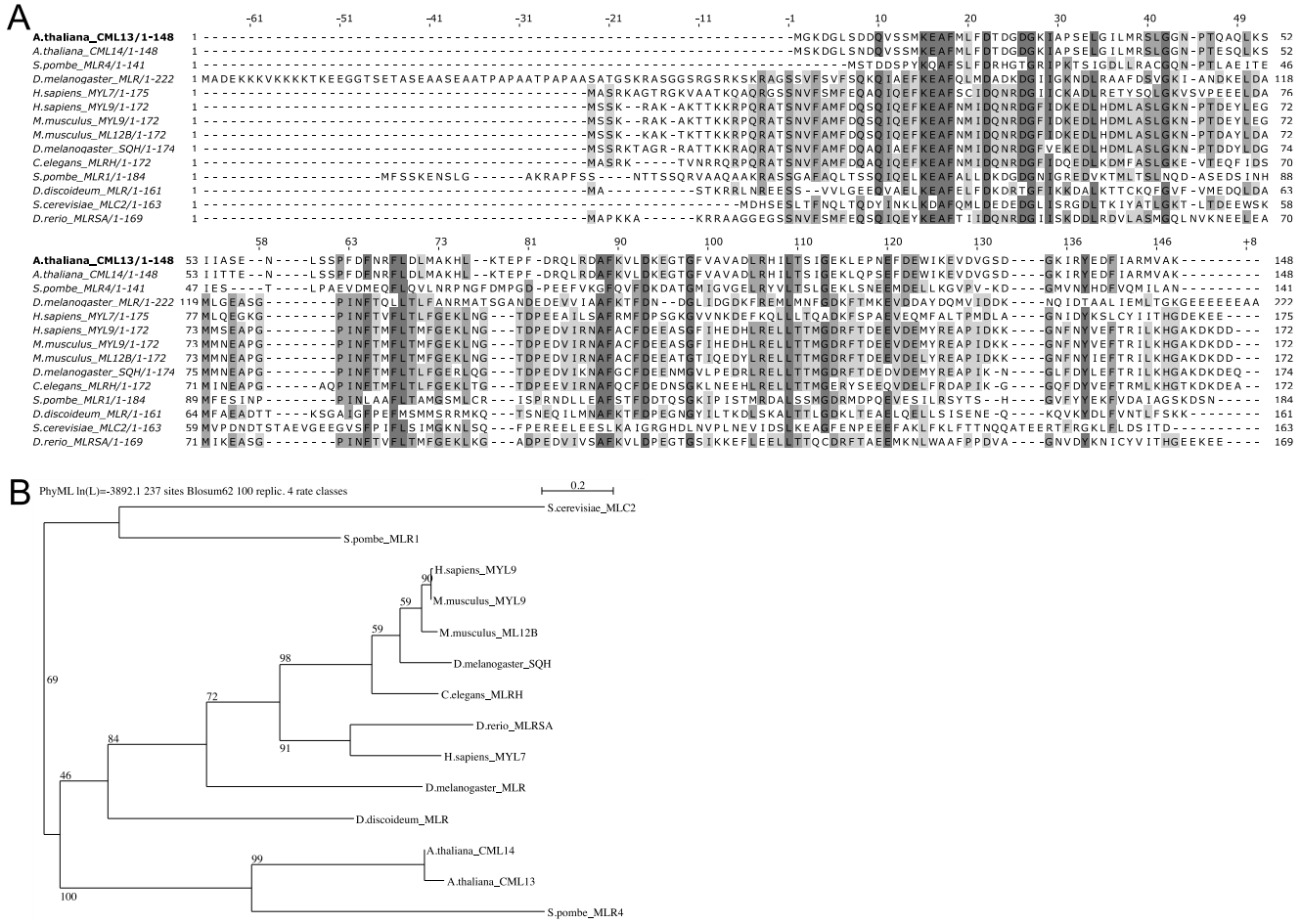
Supplementary Table S2. Description of plasmid constructs used in this study

Construct Name	AGI	Amino Acids	Vectors
ATM1 IQ1	At3G19960	848-878	Nluc
ATM1 IQ1+2	At3G19960	848-904	Nluc, pGEX-4-T3
ATM1 IQ2	At3G19960	867-904	Nluc
ATM1 IQ3	At3G19960	890-927	Nluc
ATM1 IQ3+4	At3G19960	890-943	Nluc, pGEX-4-T3
ATM1 IQ4	At3G19960	916-943	Nluc
ATM1 Neck	At3G19960	848-943	Nluc, pET28b-GB1
ATM2 IQ1	At5G54280	878-910	Nluc
ATM2 IQ1+2	At5G54280	878-948	Nluc, pGEX-4-T3
ATM2 IQ2	At5G54280	899-948	Nluc
ATM2 IQ3	At5G54280	922-967	Nluc, pGEX-4-T3
ATM2 Neck	At5G54280	878-967	Nluc, pET28b
CaM81	M80836 (Genbank Accession)	Full	Cluc, pET5a
CML13	At1G12310	Full	Cluc, pET30a
CML14	At1G62820	Full	Cluc, pET30a
CML15	At1G18530	Full	Cluc
CML19	At4G37010	Full	Cluc
CML24	At5G37770	Full	Cluc
CML35	At2G41410	Full	Cluc
CML38	At1G76650	Full	Cluc
CML42	At4G20780	Full	Cluc, pET5a
CML6	At4G03290	Full	Cluc
CML8	At4G14640	Full	Cluc
VIII-A Neck	At1G50360	828-930	Nluc
VIII-B Neck	At4G27370	834-913	Nluc

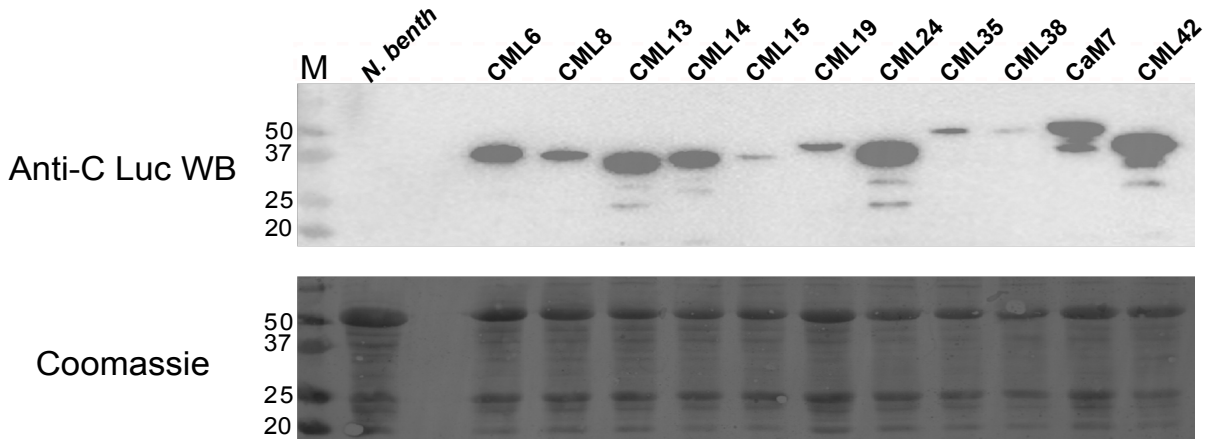
See Methods for the vectors used for microscopy



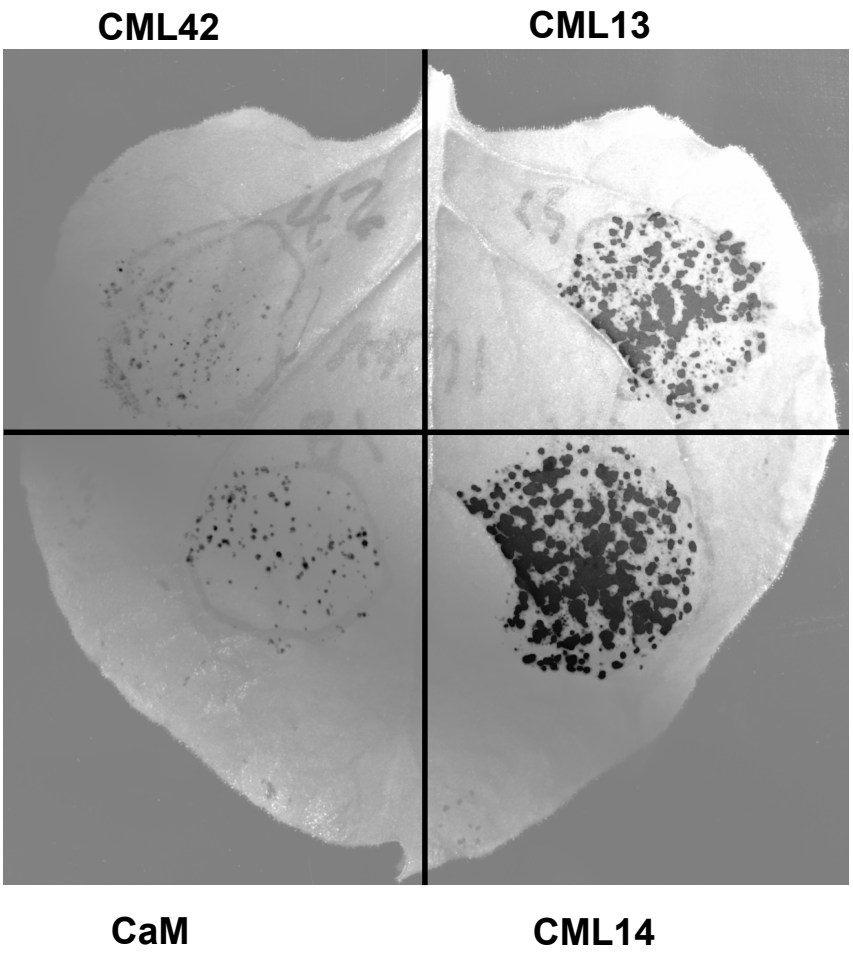
Supplemental Figure S1. Comparison of Arabidopsis CML13 and CML14 with predicted orthologs. (A) Protein alignment of CML13 and CML14 with orthologs from various plant species. Amino acid residues were shaded based on their percent identity, dark grey if identical, and progressively lighter grey until white as unconserved. ClustalΩ was used for alignment (Sievers and Higgens, 2014) and images were generated using Jalview Version 2.11.2.6. (B) Phylogenetic tree showing relatedness among the proteinss compared in panel A. SeaView (v 5.0, Gouy et al., 2021) was used to generate the tree using neighbour-joining with bootstrapping analysis (1000 reiterations).



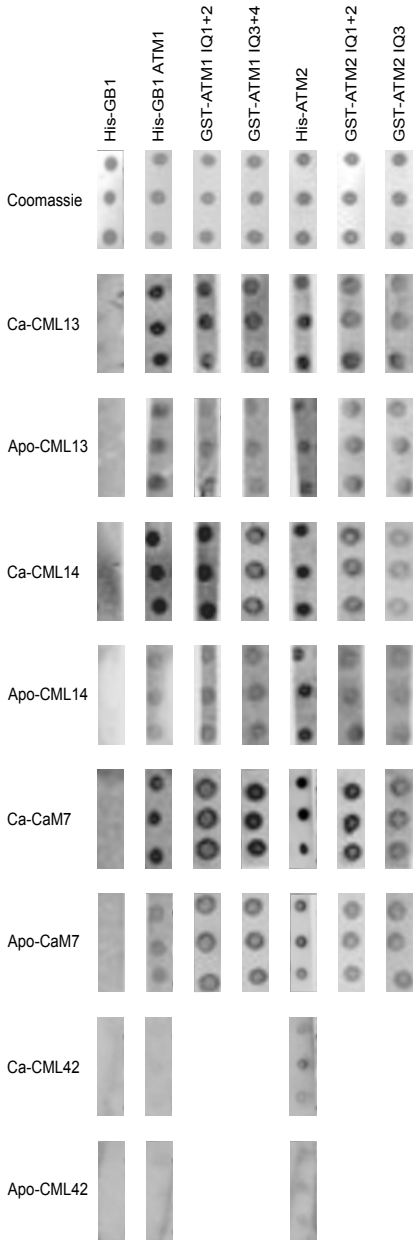
Supplemental Fig S2. Comparison of Arabidopsis CML13/14 with regulatory myosin light-chains (RLCs) from various species of eukaryotes. (A) Protein alignment of CML13 and CML14 with myosin RLCs from multiple species. Amino acid residues were shaded based on their percent identity, dark grey if identical, and progressively lighter grey until white as unconserved. ClustalΩ was used for alignment (Sievers and Higgens, 2014) and images were generated using Jalview Version 2.11.2.6. (B) Phylogenetic tree showing relatedness among myosin RLCs compared in panel A. SeaView (v 5.0, Gouy et al., 2021) was used to generate the tree using neighbour-joining with bootstrapping analysis (1000 reiterations).



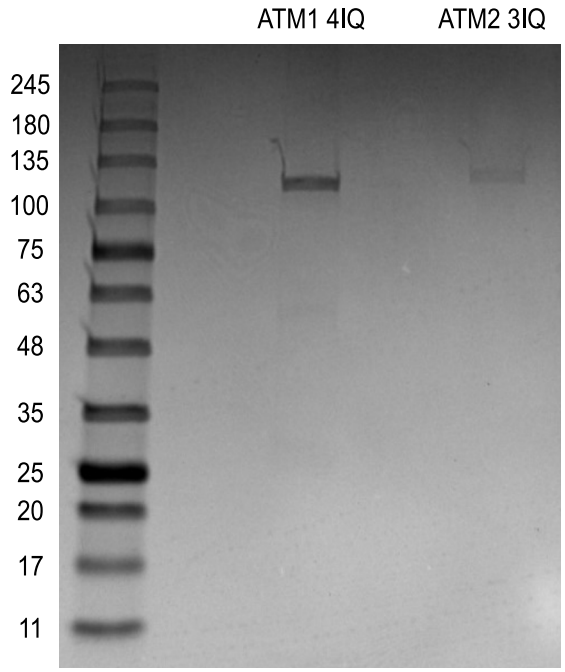
Supplemental Fig S3. Immunoblot showing expression of C-Luc-CaM/CML fusion proteins in N. benthamiana. Leaves of N. benthamiana were infiltrated with Agrobacteria expressing C-Luc (bait) vectors and N=Luc (prey) vectors as described in Materials and Methods. Immunoblots (upper panel) using anti-C-terminal luciferase antisera, or Coomassie-stained gels showing equal lane loading, following SDS-PAGE of samples of total, clarified protein extracts (~25ug) from leaves used in the split-luciferase assays.



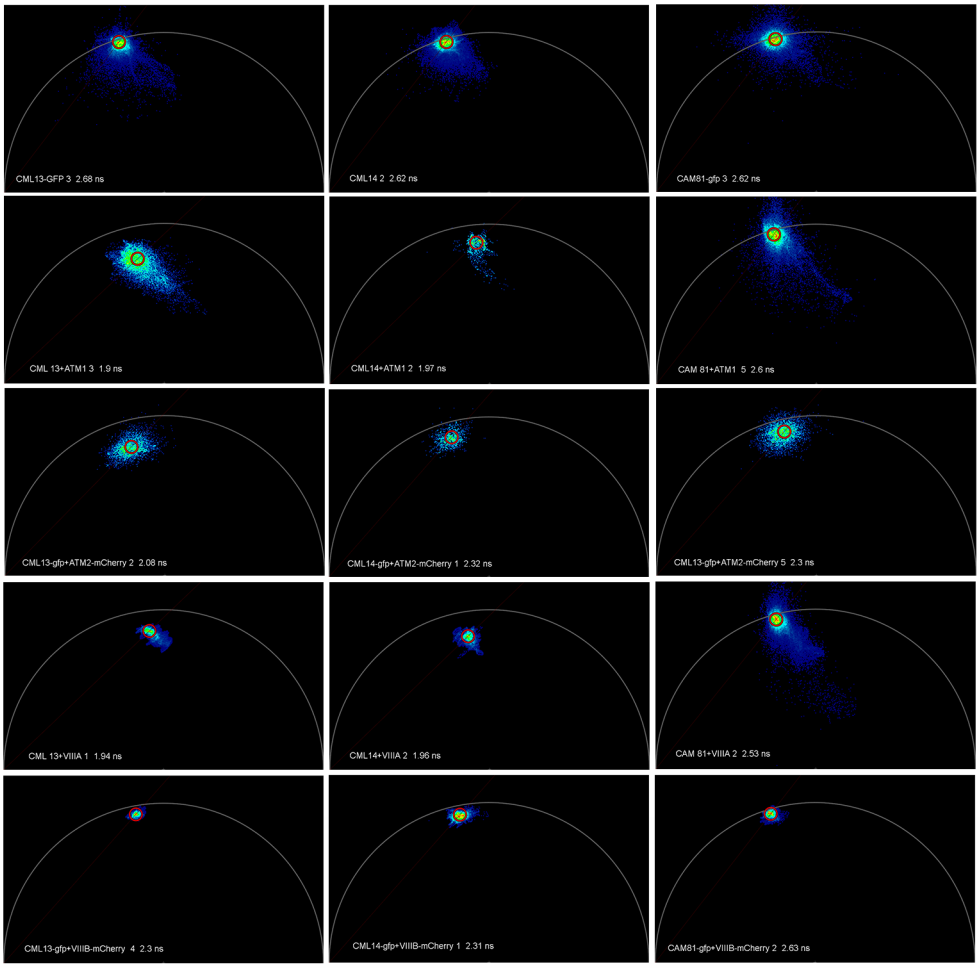
Supplemental Figure S4. Whole-leaf image of split-luciferase protein-protein interaction bioluminescent assays using transiently transformed N. benthamiana. A representative image is shown for interaction analysis of CML13, CML14, CaM, and CML42 (negative control) as baits (as C-Luc fusion proteins) with the neck region of ATM1 (as N-luc fusion) prey protein.



Supplementary Fig. S5. *In vitro* protein-interaction overlay assays of CaM, CML13, CML14, and CML42 with IQ domains of ATM1 and ATM2. Triplicate samples (200 ng) of pure, recombinant proteins ATM1 or ATM2 full-neck region (ATM1, ATM2), paired IQ domains (ATM1-IQ1+2, -IQ3+4, ATM2-IQ1+2), or the isolated IQ domain of ATM2 (ATM2-IQ3), were spotted onto nitrocellulose, blocked with 5% casein in TBST, and incubated with 200 nM of CaM, CML13, CML14, or CML42, as indicated, each of which was covalently labeled with the infra-red dye, 680RD-NHS as described in Materials and Methods. Recombinant GB1 was tested as a negative control. Protein-protein interaction was assayed in the presence of 2 mM $CaCl_2$ (Ca) or 5 mM EGTA (Apo) and detected using the LI-COR Odyssey-XF infra-red imager. Representative Coomassie-stained blots are presented along the top row. Data are representative of a minimum of three independent experiments. See Supplementary Table 2 for a description of the primary sequence from the neck regions of ATM1 and ATM2 that were tested for binding.



Supplementary Fig. S6. SDS-PAGE of purified recombinant myosins. ATM1-4IQ and ATM2-3IQ were purified in High Five cells without co-expression of CaM and without addition of calmodulin during the purification process. The purified proteins were analyzed by SDS-PAGE using a 4-20% polyacrylamide gradient and stained with Coomassie brilliant blue. The positions of the molecular mass markers are indicated on the left (kDa).



Supplementary Figure S7. Representative phasor plots are shown corresponding to the FRET-FLIM analysis presented in Figure 3. This figure highlights the distinctions in the fluorescence decay of GFP (donor) fused to CMLs or CaM alone and when coexisting with RFP (acceptor) fused to the different class VIII myosin members. Each image represents one cell. Quantitative analysis of the whole experiment is shown in figure 3. CaM81 refers to the conserved CaM isoform (see Materials and Methods).