Supplemental Materials
Molecular Biology of the Cell

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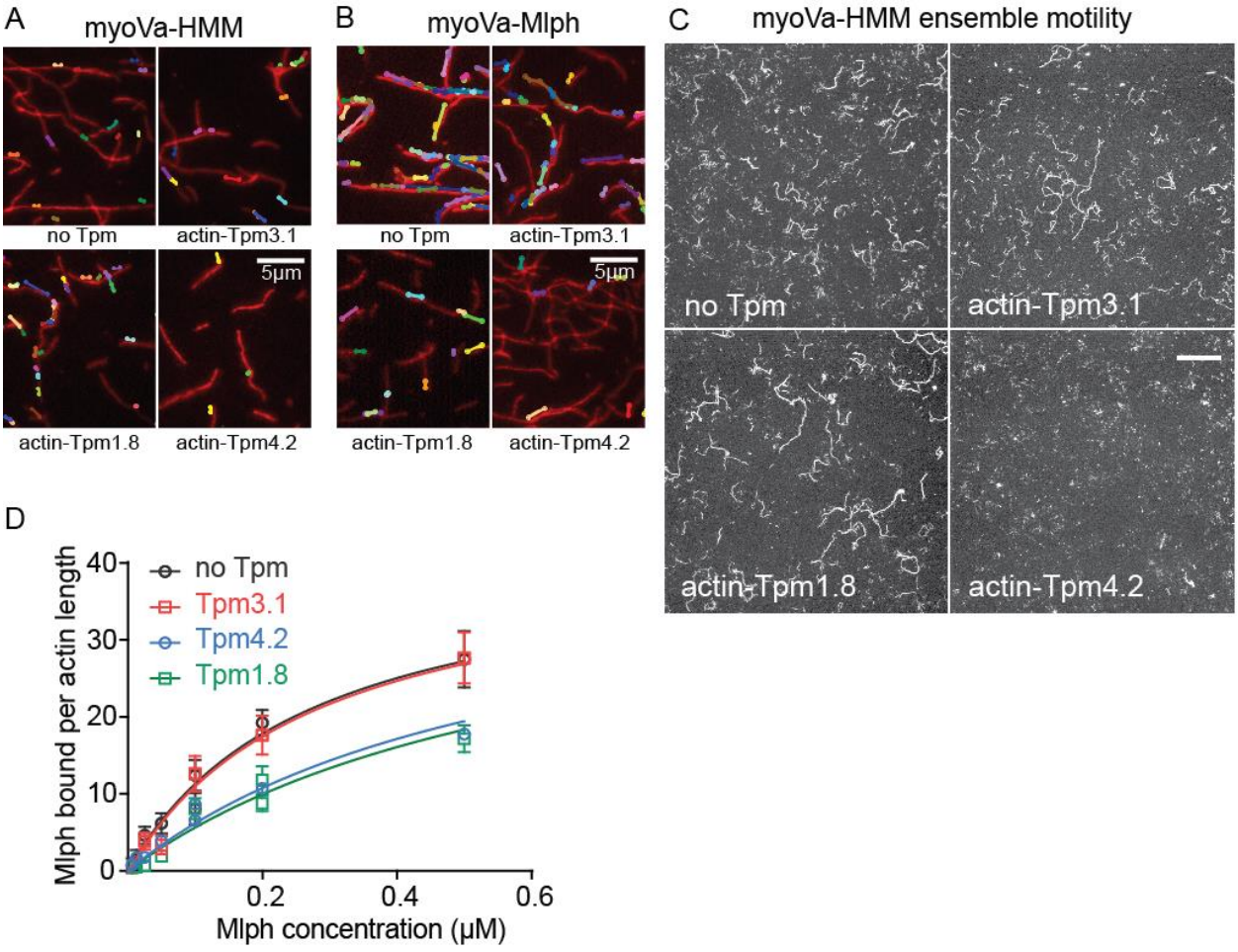

Supplemental Figure S1. Myosin and Mlph differentiate actin-Tpm tracks

(A) Projection of all movement tracks of myoVa-HMM or **(B)** myoVa-Mlph over the course of 100s, with actin filaments shown in red, and the indicated Tpm isoforms. This figure is the same as Figure 2B and 2H in the main text, but also shows the actin filaments in red. **(C)** Maximum projection of ensemble motility movies over the course of 50s show long trajectories for robust filament movement and noise like particles for filaments that only engage with the track briefly or drift in and out of focus. MyoVa-HMM is adhered to the surface, and rhodamine-phalloidin actin filaments are being moved in solution. **(D)** Apparent dissociation constants of Mlph for bare actin or actin decorated with various Tpm isoforms. Data points show average number of Atto-488-Mlph decorating surface bound rhodamine-phalloidin labeled actin by TIRF-microscopy. Values are normalized to actin length. Error bars are standard deviation, n=25. Line shows the best fit to the data with a one site specific binding model to estimate an apparent binding constant $K_{d,\,app}$: no Tpm, 0.27 μM (black); Tpm3.1, 0.28 μM (red); Tpm4.2, 0.58 μM (blue); Tpm1.8, 0.65 μM (green).

Supplemental Table S1. Run frequencies, speeds and run lengths from single molecule TIRF assays

	myosin	no Tpm	Tpm3.1	Tpm1.8	Tpm4.2	ANOVA
Normalized run frequency	myoVa-HMM	1 ± 0.08 *n* = 23	1.5 ± 0.11 *n* = 15	1.9 ± 0.27 *n* = 15	0.4 ± 0.06 *n* = 19	<0.0001
	myoVa-Mlph	1 ± 0.04 (3 ± 0.13) *n* = 13	0.9 ± 0.06 (2.7 ± 0.19) *n* = 14	0.4 ± 0.09 (1.2 ± 0.26) *n* = 8	0.2 ± 0.06 (0.6 ± 0.19) *n* = 16	<0.0001
Speed ± SD (nm/s)	myoVa-HMM	812 ± 289 *n* = 133	670 ± 214 *n* = 100	688 ± 221 *n* = 179	648 ± 259 *n* = 86	<0.0001
	myoVa-Mlph					P = 0.016
	slow	266 ± 163	213 ± 105	426 ± 184	216 ± 155	
	fast	719 ± 260	627 ± 260	835 ± 134	657 ± 166	
		n = 334	*n* = 263	*n* = 153	*n* = 119	
Run length (nm) (95% CI)	myoVa-HMM	531 (509-554) *n* = 181	567 (519-625) *n* = 112	601 (556-653) *n* = 178	483 (420-567) *n* = 117	<0.0001
	myoVa-Mlph					
	slow	1378 (1336-1422) *n* = 128	906 (864-951) *n* = 78	1168 (1082-1270) *n* = 44	1283 (1169-1421) *n* = 41	N.S.
	fast	658 (621-699) *n* = 144	635 (606-666) *n* = 140	601 (558-650) *n* = 66	556 (512-608) *n* = 63	N.S.
	combined	908 (889-927) *n* = 272	683 (666-701) *n* = 228	728 (700-758) *n* = 128	729 (706-754) *n* = 104	P = 0.020

Normalized run frequency of single molecular motors moving along actin. Values are –fold difference in frequency compared with bare actin ± standard error of the mean. (see Figure 2C, I in main text). Values in parenthesis are –fold difference in frequency relative to myoVa-HMM on bare actin (right y-axis in Figure 2I).

Speeds ± standard deviation (SD) of single molecular motors moving on actin or actin-Tpm were extracted from fits of speed histograms to a Gaussian distribution for myoVa-HMM, and to the sum of two Gaussian distributions for full length myoVa-Mlph (see Figure 3A, C in main text).

Run lengths of single molecular motors moving on actin or actin-Tpm were extracted from single exponential fits to histograms and correspond to the values graphed in Figure 3B, D in the main text. Values in parentheses represent the 95% confidence interval (CI). MyoVa-HMM moves with a homogeneous speed distribution so only one run length was extracted. Full length myoVa-Mlph has a bimodal speed distribution (Figure 3C of main text). Run length distributions were analyzed separately for motors belonging to the fast (>500 nm/s) or slow (<500 nm/s) population, and for the combined population.

Data are from 3 independent experiments (movies were 100s, field size 30 μm^2, total actin length per movie >100 μm) with 2 independent protein preparations. Frequency results are from

all moving motors counted in *n* movies, at least 2 fields were analyzed for each experiment. Speed and run length results are pooled from *n* motors tracked per condition. ANOVA was performed on pooled data. Results from post tests comparing results from individual conditions to results with bare actin are shown in the corresponding figures in the main text.
Conditions: pH 7.4, 150 mM KCl, 23°C

Supplemental Table S2. Speeds and probability of dissociation of actin filaments from myoVa-HMM in ensemble motility assays

	no Tpm	Tpm3.1	Tpm1.8	Tpm4.2	ANOVA
Speed ± SD (µm/s)	1.14 ± 0.18 *n* = 412	1.06 ± 0.16 *n* = 389	1.07 ± 0.32 *n* = 177	no interaction	N.S.
Median duration of filament movement $t_{1/2}$	2.6 *n* = 293	2.9 *n* = 306	3.6 *n* = 265	no interaction	P = 0.041

Speeds ± standard deviation (SD) of actin or actin-Tpm filaments moving on surface bound myoVa-HMM in an ensemble motility assay. Values are extracted from fits of histograms to a Gaussian distribution. Median time to filament detachment on ensembles of myoVa-HMM was determined from the exponential fit to a histogram. Values are averages from at least 3 independent experiments with 2 independent protein preparations. For each experiment, filaments moving in at least 4 different 64 µm^2 areas were analyzed. *n* is total number of filaments analyzed per condition. Conditions: pH 7.4, 150 mM KCl, 23°C