

Table S1: SAXS data collection, analysis and derived structural parameters

Construct	Ig1Ig2 ^{250-444*}	Ig1Ig2 ²⁵⁰⁻⁴⁹⁸	Ig1Ig2 ²²⁰⁻⁴⁵²	Trx-MYOT	Ig1Ig2 ^{220-452[†]}	Ig1Ig2 ^{220-452 R405K}
Data collection parameters						
Radiation source	ESRF (Grenoble, France)		DESY (Hamburg, Germany)			
			Doris		Petra III	
Beamline	BM29 BioSAXS		EMBL X33		EMBL P12	
Detector	Pilatus 1M		Pilatus 1M-W	Pilatus 2M		Pilatus 6M
Beam geometry [mm, FWHM]	0.10 × 0.20		n.d.	0.12 × 0.20		0.12 × 0.20
Wavelength [nm]	0.099		0.126	0.124		0.124
Sample–detector distance [m]	2.867		2.7	3.1		3.0
Momentum of transfer <i>s</i> range [nm ^{−1}]	0.04–4.0		0.1–6.0	0.08–3.5		0.15–7.3
Exposure time [s]	15		15	1 (SEC-SAXS)		0.145
Temperature [°C]	20		11	20		20
Buffer	20 mM HEPES, 150 mM NaCl, 5% glycerol, 1 mM DTT, pH 7.4		20 mM MES, 200 mM NaCl, 3% glycerol, pH 6.0	20 mM Tris 400 mM NaCl, 250 mM arginine, 5% glycerol, pH 7.5	20 mM HEPES, 150 mM NaCl, 5% glycerol, 1 mM DTT, pH 7.4	
Overall parameters						
Conc. range measured [§]	1–14.12 mg/ml (0.05–0.64 mM)	1–42.8 mg/ml (0.04–1.53 mM)	1–52 mg/ml (0.04–1.96 mM)	n.a.	2.1–36.6 mg/ml (0.08–1.38 mM)	2.3–45.5 mg/ml (0.09–1.72 mM)
<i>R_g</i> (Guinier) [Å]	28 (0.64 mM)	32 (0.54 mM)	31 (0.04 mM)	51	30 (0.08 mM)	33 (0.09 mM)
		37 (1.53 mM)	37 (0.79 mM)		33 (0.19 mM)	34 (0.27 mM)
			39 (1.96 mM)		33 (0.37 mM)	34 (0.47 mM)
					34 (0.69 mM)	35 (0.82 mM)
					36 (1.38 mM)	36 (1.72 mM)
<i>R_g</i> from PDDF [Å]	29 (0.64 mM)	34 (0.54 mM)	32 (0.04 mM)	53	33 (0.08 mM)	34 (0.09 mM)
		38 (1.53 mM)	39 (0.79 mM)		34 (0.19 mM)	36 (0.27 mM)
			41 (1.96 mM)		35 (0.37 mM)	36 (0.47 mM)
					37 (0.69 mM)	37 (0.82 mM)
					37 (1.38 mM)	38 (1.72 mM)
<i>D_{max}</i> [Å]	101 (0.64 mM)	140 (0.54 mM)	107 (0.04 mM)	200	115 (0.08 mM)	120 (0.09 mM)
		152 (1.53 mM)	151 (0.79 mM)		125 (0.19 mM)	130 (0.27 mM)
			152 (1.96 mM)		130 (0.37 mM)	140 (0.47 mM)
					140 (0.69 mM)	140 (0.82 mM)
					140 (1.38 mM)	140 (1.72 mM)
<i>M_w</i> (RALLS) [kDa]	n.a.	n.a.	n.a.	70	n.a.	n.a.
<i>M_w</i> (DATMOV) [kDa]	24.3 (0.64 mM)	31.2 (0.54 mM)	27.3 (0.04 mM)	63.7	30.8 (0.08 mM)	29.1 (0.09 mM)
		44.7 (1.53 mM)	44.7 (0.79 mM)		30.7 (0.19 mM)	31.9 (0.27 mM)
			58.1 (1.96 mM)		33.7 (0.37 mM)	35.6 (0.47 mM)
					37.8 (0.69 mM)	40.6 (0.82 mM)
					43.2 (1.38 mM)	45.7 (1.72 mM)

(continued)

(continued)

Construct	Ig1Ig2 ^{250-444*}	Ig1Ig2 ²⁵⁰⁻⁴⁹⁸	Ig1Ig2 ²²⁰⁻⁴⁵²	Trx-MYOT	Ig1Ig2 ^{220-452†}	Ig1Ig2 ^{220-452 R405K}
M _w from Porod volume [kDa]	21.4 (0.64 mM)	26.4 (0.54 mM)	27.5 (0.04 mM)	77.3	26.9 (0.08 mM)	24.5 (0.09 mM)
		37.9 (1.53 mM)	36.7 (0.79 mM)		25.9 (0.19 mM)	26.3 (0.27 mM)
			48.2 (1.96 mM)		28.3 (0.37 mM)	29.6 (0.47 mM)
					30.9 (0.69 mM)	33.6 (0.82 mM)
					36.2 (1.38 mM)	38.2 (1.72 mM)
M _w (monomer sequence) [kDa] ^{&}	21.9	28.0	26.5	69.7	26.5	26.5
Software employed						
Primary data red.	SaxsAnalysis pipeline system			SASFLOW	SaxsAnalysis pipeline system	
Data processing	PRIMUS			PRIMUS / Cromixs	PRIMUS	
Calculation and comparison of scattering data	Crysol / Oligomer				n. a.	
<i>Ab initio</i> modelling	DAMMIF				n. a.	
Addition of missing residues	CORAL				n. a.	
Ensemble modelling	EOM				n. a.	
SASDB accession code	SASDF38	SASDF48	SASDF28	SASDFZ7		




^{*}, the data for Ig1Ig2²⁵⁰⁻⁴⁴⁴ were already measured and described previously (Puz et al., 2017)

[§], for Ig1Ig2²⁵⁰⁻⁴⁹⁸ and Ig1Ig2²²⁰⁻⁴⁵² structural parameters at various concentrations, relevant for the comparison, were calculated separately

[&], M_w (monomer sequence) denotes molecular weight calculated from the amino acid sequence of the monomeric species

[†], measured at the same experimental settings/conditions as Ig1Ig2^{220-452 R405K}

Table S2: List of major cross-links found between myotilin and F-actin (using DMTMM)

Myotilin constructs used		(A) 		(B) 		(C) 	
From Protein	From Residue	To Protein	To Residue	Myotilin region	Myotilin fragment identified	Score	
myotilin	K 474	actin	E 102	C-ter.	C	212.6	
myotilin	K 462	actin	D 27	C-ter.	C	199.5	
myotilin	K 411	actin	D 27*	Ig2	A, B	A) 157.5, B) 175.5	
myotilin	K 469	actin	E 102	C-ter.	C	169.4	
myotilin	D 236	actin	K 330	N-ter.	B, C	B) 138.4, C) 76.2	
myotilin	D 236	actin	K 328*	N-ter.	B	134.9	
myotilin	K 246	actin	E 336	N-ter.	B, C	B) 105.9, C) 111.2	
myotilin	E 245	actin	K 330	N-ter.	B	107.1	
myotilin	K 246	actin	D 26	N-ter.	C	106.3	
myotilin	K 452	actin	D 27*	C-ter.	C	103.9	
myotilin	D 241	actin	K 330*	N-ter.	B	102.4	
myotilin	K 469	actin	D 26*	C-ter.	C	102.0	
myotilin	K 452	actin	D 26	C-ter.	C	100.9	
myotilin	D 239	actin	K 330*	N-ter.	B	97.0	
myotilin	D 239	actin	K 328	N-ter.	B	88.1	
myotilin	K 474	actin	D 27	C-ter.	C	84.6	
myotilin	K 474	actin	E 336*	C-ter.	C	82.7	
myotilin	D 241	actin	K 328*	N-ter.	B	78.2	
myotilin	K 462	actin	D 26*	C-ter.	C	77.7	
myotilin	K 354	actin	D 27*	Ig2	C	76.7	
myotilin	K 246	actin	D 27*	N-ter.	B, C	B) 54.5, C) 73.9	
myotilin	K 415	actin	D 27	Ig2	A, B	A) 66.1, B) 57.6	
myotilin	K 303	actin	D 27*	Ig1	A	65.9	
myotilin	K 367	actin	D 365	Ig2	B	63.6	
myotilin	K 367	actin	E 366	Ig2	B	60.2	
myotilin	K 452	actin	E 363	C-ter.	C	48.2	
myotilin	K 411	actin	D 26	Ig2	A	47.8	
myotilin	K 474	actin	D 26	C-ter.	C	35.4	

*, cross-links shown on [Figure 3A](#); N-ter., region flanking Ig1Ig2 N-terminally; C-ter., region flanking Ig1Ig2 C-terminally; Score, computed as $-10 \log_{10}$ (E-value) representing best identification for a crosslink pair. The higher values of the score indicate more reliable identifications.

Table S3: List of constructs

Name	⁽¹⁾ Protein	Residues	Mutations	Vector	⁽²⁾ Tag	Expression in	Origin
Trx-MYOT	myotilin	1-498	-	pETM-20	N-Trx-His ₆ -TEV	<i>E. coli</i>	this study
Trx-MYOT-NEECK	myotilin	1-498	F96E, L97E, L101E	pETM-20	N-Trx-His ₆ -TEV	<i>E. coli</i>	this study
Ig1Ig2¹⁸⁵⁻⁴⁹⁸	myotilin	185-498	-	pETM-20	N-Trx-His ₆ -3C C-Strep	<i>E. coli</i>	this study
⁽³⁾Ig1Ig2^{185-498*}	myotilin	185-498	-	pDB-HisGST	N-His ₆ -GST-TEV	<i>E. coli</i>	this study
Ig1Ig2²²⁰⁻⁴⁵²	myotilin	220-452	-	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{220-452 R405K}	myotilin	220-452	R405K	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
⁽⁴⁾Ig1Ig2²⁵⁰⁻⁴⁹⁸	myotilin	250-498	-	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2²⁵⁰⁻⁴⁴⁴	myotilin	250-444	-	pETM-14	N-His ₆ -3C	<i>E. coli</i>	Puz et al., 2017
Ig1Ig2^{K354A}	myotilin	250-444	K354A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{Q356A}	myotilin	250-444	Q356A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{K358A}	myotilin	250-444	K358A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{K359A}	myotilin	250-444	K359A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{K367A}	myotilin	250-444	K367A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{R405K}	myotilin	250-444	R405K	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{K411A}	myotilin	250-444	K411A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{K354/359A}	myotilin	250-444	K354A, K359A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1Ig2^{K354/358/359A}	myotilin	250-444	K354A, K358A, K359A	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
Ig1²⁵⁰⁻³⁴⁴	myotilin	250-344	-	pET-3d(+)	N-His ₆ -TEV	<i>E. coli</i>	this study
Ig2³⁴⁹⁻⁴⁵⁹	myotilin	349-459	-	pET-3d(+)	N-His ₆ -TEV	<i>E. coli</i>	this study
MYOT^{WT}	myotilin	1-498	-	pEGFP-N1	N-EGFP	C2C12	this study
MYOT^{K354A}	myotilin	1-498	K354A	pEGFP-N1	N-EGFP	C2C12	this study
MYOT^{K359A}	myotilin	1-498	K359A	pEGFP-N1	N-EGFP	C2C12	this study
MYOT^{K354/359A}	myotilin	1-498	K354A, K359A	pEGFP-N1	N-EGFP	C2C12	this study
MYOT^{K354/358/359A}	myotilin	1-498	K354A, K358A, K359A	pEGFP-N1	N-EGFP	C2C12	this study
ACTN2-WT	α -actinin-2	1-894	-	pET-3d(+)	N-His ₆ -TEV	<i>E. coli</i>	Ribeiro et al., 2014
ACTN2-NEECK	α -actinin-2	1-894	R268E, I269E, L273E	pET-3d(+)	N-His ₆ -TEV	<i>E. coli</i>	Ribeiro et al., 2014
ACTN2-EF14	α -actinin-2	746-894	-	pETM-14	N-His ₆ -3C	<i>E. coli</i>	this study
palladin Ig3	palladin	1022-1126	-	pTBSG	N-His ₆ -TEV	<i>E. coli</i>	Yadav et al., 2016
Doc2b	Doc2b	125-412	-	pGEX4T1	N-GST-Thr	<i>E. coli</i>	Groffen et al., 2010
DVD-actin	actin-5C	1-376	D287A, V288A, D289A	pFastBacHT	N-His ₆	<i>Sf9</i>	Zahm et al., 2013
tropomyosin	tropomyosin					<i>E. coli</i>	von der Ecken et al., 2015

(1), myotilin (human, Uniprot-ID Q9UBF9); α -actinin-2 (human, P35609), palladin (Mus musculus, Q9ET54), Doc2b (Rattus norvegicus, P70610), actin-5C (Drosophila melanogaster, P10987), tropomyosin (Tpm1.1st, Homo sapiens, NP_001018005.1)

(2), in many cases removed during purification; N, N-terminal fusion; Trx, Thioredoxin-tag; His₆, 6xHis-tag, TEV; TEV protease cleavage site; 3C, HRV-3C protease cleavage site; C, C-terminal fusion; Strep, Strep-tag II; Thr, thrombin cleavage site, GST, Glutathione s-transferase-tag; EGFP, Enhanced green fluorescent protein-tag

(3), Ig1Ig2^{185-498*} was used to produce Ig1Ig2¹⁸⁵⁻⁴⁵⁴, and GST

(4), Ig1Ig2²⁵⁰⁻⁴⁹⁸ was used to produce Ig1Ig2²⁵⁰⁻⁴⁶⁶