Supporting Information

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SI Text

SI Materials and Methods ***Cloning and Site-Directed Mutagenesis.*** Most of constructs employed in this work have been described previously (1). Briefly, cDNA encoding for the last immunoglobulin domain of human titin (residues 34352-34350, henceforth M10 1-99), the first immunoglobulin domain of human obscurin (O1, residues 1-109), the first immunoglobulin domain of human obscurin-like 1 (OL1, residues 1-106) were amplified by PCR with custom oligonucleotides primers containing appropriate restriction enzyme recognition sites for cloning. Site-directed mutagenesis to generate M10 I55N (Belgian mutation, M10 Bel), OL1 F17R, O1 R15F, M10 A9Y variants was carried out using a single-primer modified version of the QuikChange protocol (Stratagene) with the following primers (mutated nucleotides are shown in bold capital letters): M10 I55N (Bel) 5′- agaacaggggaggttccaca**A**tgaaaacacagatgacctgac-3′; M10 A9Y 5′- gaattccgcctaaaattgaa-**TA**tcttccatctgatatcagca-3′; OL1 F17R 5′- ccccccgtgcttcctgcgc-**CG**cccgcggcctgtgcgggtggt -3′; O1 R15F 5′- gggcgccccgctttctcacc-**TTT**cccaaggccttcgtggtgtc -3′.

All sequences were verified by sequencing.

Protein Expression and Purification of Material Used for Crystallographic Studies and Isothermal Titration Calorimetry Measurements. All Ig domains were expressed as N-terminal His_6-tagged fusion proteins in Rosetta 2(DE3) cells (Novagen). Transformed cells were grown in Luria-Bertani (LB) medium supplemented with 100 $\mu g\,ml^{-1}$ ampicillin and 34 $\mu g\,ml^{-1}$ chloramphenicol at 37 °C until OD_{600} reached approximately 0.5. After decreasing the temperature to 18 °C, O/N protein expression was induced with 0.15 mM IPTG. Cells were harvested by centrifugation at 5000 g for 15 minutes at 4 °C and resuspended in PBS lysis buffer in which NaCl concentration was increased to 250 mM. The cell suspension was supplemented with 2.4 unit/ml of benzonase (Novagen), 0.25 mg/ml chicken egg white lysozyme (Novagen) and Complete EDTA-free protease inhibitor cocktail (Roche). Cell lysis was accomplished by two freeze-and-thaw cycles. Insoluble material was sedimented by centrifugation at 16500 g for 1 hour at 4 °C and the supernatant filtered using 0.22 μm prior to loading on a His-trap HP column (GE Healthcare) preequilibrated with the lysis buffer supplemented with 10 mM imidazole. His_6-tagged proteins were eluted with an imidazole step gradient. Fractions containing the target His_6-tagged domains were collected and dialysed overnight against PBS buffer. During dialysis the purification tag was cleaved using TEV protease. Uncleaved material was removed from the mixture sample loading the dialysed protein solution on a His-trap HP column (GE Healthcare). Untagged material was collected, concentrated and further purified by size-exclusion chromatography (SEC) on a 16/60 HiLoad Superdex 75 column (GE Healthcare) equilibrated with 20 mM Hepes pH 7.5, 50 mM NaCl, 1 mM DTT. M10-OL1 and M10-OL1 F17R complexes used for crystallization were obtained mixing the individual components with a 1:1 molar ratio and allowing incubation on ice incubating for 30 minutes. Complexes were further purified by SEC as described above. In isolation, M10, OL1 and OL1 F17R eluted from SEC as monomers. Stoichiometric mixing of the individual domains resulted in a sample that eluted from SEC earlier than the single components (Fig. S1), at a volume corresponding to a calculated molecular weight of 27 kDa indicative of the formation of heterodimers in solution.

Crystal Preparation. M10-OL1 and M10-OL1 F17R complexes were concentrated to 13 mg/ml and 15 mg/ml, respectively. Crystallization was achieved by the vapour diffusion set-up at 18 °C using a 1:1 protein:precipitant ratio in 400 nl sitting drops dispensed with the aid of Mosquito crystallization robot (TTP LabTech). Crystals of the M10-OL1 complex belonging to space group $P3_1$ were obtained using 0.10 M Bis-Tris pH 5.5, 2.0 M ammonium sulphate as precipitant. Crystals of the same complex belonging to space group $P1$ were obtained in 0.10 M MIB buffer pH 5.0, 25% (w/v) PEG 1500. The trigonal crystal form (Form I) contains one molecule of M10-OL1 in the crystallographic asymmetric unit (a.u.) whereas the triclinic form (Form II) contains two copies of the complex in the unit cell. The M10-OL1 F17R complex crystallized in the $C2$ monoclinic space group from a 0.1 M Bis-Tris pH5.5, 0.2 M Ammonium sulphate, 25% (w/v) PEG 3350 reservoir solution with one molecule of the complex in a.u.

Data Collection and Processing. For data collection crystals were cryoprotected by soaking them in their respective reservoir solutions supplemented with 20% glycerol. An initial dataset on a M10-OL1 complex trigonal form crystal was collected in-house on an Oxford Diffraction Nova system at the 2.1 Å resolution and processed using the CrysAlis package. This dataset was used to solve the structure of the M10-OL1 complex by the molecular replacement technique (*vide infra*). A higher-resolution dataset (1.48 Å resolution) on the same crystal form was later collected at the I04 beamline of Diamond Light Source. Data sets for the triclinic form of M10-OL1 and for M10-OL1 F17R were also collected at the Diamond Light Source at the I03 beamline. Synchrotron data were processed with *MOSFLM* (2) and *SCALA* (3) packages.

Structure Solution and Refinement. The structure of the M10-OL1 complex in the trigonal $P3_1$ space group was determined using the molecular replacement technique using the program *MOLREP* (4) and employing the structure of telokin (PDB code 1FHG) as search model. A clear solution was obtained with one M10-OL1 complex in the a.u. Density improvement and side-chain docking was carried out using *ARP/wARP* (5). The M10-OL1 model was subsequently completed manually using *COOT* (6) and refined using *REFMAC5* (7, 8). The structure of the M10-OL1 complex was then used as search probe in *MOLREP* (4) to solve the structures of the same complex in the alternative $P1$ space group and of the M10-OL1 F17R complex in the $C2$ space group. The quality of the final models was assessed with the program *MOLPROBITY* (9). A summary of data collection and refinement statistics are shown in Table S1.

Isothermal Titration Calorimetry (ITC). Samples for ITC measurements were extensively dialysed in ITC buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine-HCl (TCEP)). ITC experiments were conducted on a MicroCal VP ITC (MicroCal Inc.) instrument at a temperature of 20 °C. M10 (514 μM) and M10 Bel (465 μM) were loaded in the syringe and used as titrants while O1 and O1 R15F, OL1 and OL1 F17R were loaded in the cell and used as analytes at the final concentration of 50 μM. Data were corrected for heats of dilution of the protein solution. Binding constants and other thermodynamic parameters were calculated by fitting the integrated titration

data assuming a single set of binding sites using the Origin software package.

GST Pull-Down Assays and Densitometric Analysis. M10, M10 Bel, and M10 A9Y were subcloned into an in-house modified pGEX-2TK bacterial expression vector and plasmids were transformed into *E. coli* BL21(DE3) cells. Bacteria were grown at 37 °C in LB medium supplemented with ampicillin until OD_{600} reached 0.5–0.6 prior to induction with 0.15 mM IPTG for protein production. After 16 hrs at 18 °C, the cells were harvested by centrifugation (10 mins/8,000 g/4 °C). Cell pellets were suspended in PBS pH 7.4, 1 mM DTT supplemented with 0.25 mg/ml lysozyme and 10 μg/ml DNAseI. After 30 minutes incubation on ice to facilitate the cells lysis, cell suspensions were sonicated 5 × for 5 seconds (Sonics & Materials VC130, output 20). Soluble fractions were collected by centrifugation (30 mins/15,000 g/4 °C) and incubated with Glutathione-Sepharose 4B beads (GE Healthcare) for 30 minutes at RT. Beads were washed 4 times with PBS pH 7.4 supplemented with 1 mM DTT and stored at 4 °C. Frozen stock of COS-1 cells were plated on 90 mm Petri dishes and cultured in COS-1 maintenance medium (10% fetal calf serum, 1% penicillin/streptomycin, 4 mM glutamine in DMEM) in 37 °C/6%CO_2 incubator up to 50–70% confluence according to standard procedures. Subsequently, COS1 cells were transfected with pEGFPC2 constructs expressing prey proteins using Escort™ IV (Sigma-Aldrich) following the manufacturer's manual. Cells were cultured for 48 hrs to promote protein expression. Cells were then lysed on ice with 1 ml IP buffer (10 mM Tris-HCl pH 7.9, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, supplemented with Complete protease inhibitor cocktail (Roche)) per dish for a few minutes. After extraction, cell lysates were collected by centrifugation (20 mins/10,000 g/4 °C). Pull-down assays were performed with 380 pmol GST-tagged protein immobilized on 20 μl of Glutathione-Sepharose 4B beads (GE Healthcare). After equilibration with IP buffer, the beads were incubated with 50 μl of COS-1 cells lysates expressing GFP-tagged prey proteins for 1.5 hrs at 4 °C. The concentration of all GFP-tagged proteins in COS-1 cell lysates were equalized by dilution with IP buffer and equalization was confirmed by densitometry analysis of western blot films by the ImageJ software. Following 4× washing of the beads with 200 μl of IP buffer, all proteins retained on the beads particles were separated on SDS-PAGE and blotted on nitrocellulose membranes. Following membrane blocking with 5% skimmed milk/TBST buffer for 30 minutes at RT, blots were incubated O/N with primary antibodies (1:8000 diluted, Mouse monoclonal anti-GFP (Roche)) in 1% skimmed milk/TBST buffer at 4 °C. Subsequently, blots were washed with 1% skimmed milk/TBST buffer 3× and treated with secondary antibodies (1:4000 diluted, Horseradish peroxidase-conjugated antimouse IgG (DAKO Cytomation, Glostrup, Denmark) for 1 hr at RT. After 3 × washing with TBST buffer, GFP-tagged protein signals were detected by ECL™ western blotting analysis system following the manufacture's manual (GE Healthcare). The x-ray films were scanned and analyzed by ImageJ software.

Confocal Microscopy Competition Assay in Neonatal Rat Cardiomyocytes (NRCs) and Image Ratiometry. NRCs were isolated from rats and cultured essentially as described in (10). NRCs were transfected with pEGFPC2 constructs using Escort™ III (Sigma-Aldrich) in transfection medium (20% M199, 73% DBSS-K, 4% horse serum, 4 mM glutamine) for 8 hrs in 37 °C/10%CO_2 incubator following the manufacture's manual. Subsequently, NRCs were cultured in maintenance medium (78% DMEM, 19.4% M199 medium, 4% horse serum, 1% penicillin/streptomycin, 4 mM glutamine, 0.1 mM phenylephrine) for 48 hrs to promote protein expression. NRCs on dishes were double-immunostained with myomesin B4, a mouse monoclonal antibody raised against myomesin domain My12 (11), and Ob58-59 (former Ob48-49, renamed after comprehensive genomic analysis (12)), a rabbit polyclonal antibody raised against two Ig domains; Ob58 (exon Ob48) and Ob59 (exon Ob49) and then observed with Zeiss LSM510 confocal microscope as described (1). All fluorescent-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (USA). To investigate dominant negative effects of exogenous proteins to endogenous obscurin, images of 9 to 11 randomly chosen GFP-positive cells were ratiometrically analyzed as described (1) and differences analyzed for significance using Students one-tailed paired t-test. All image-processing steps were performed using Mathematica 7. To determine the GFP mask, GFP channels were background-subtracted as follows. An image histogram for the GFP channel was determined and the mean number of pixels from all intensity bins calculated. The first intensity level that had more pixels than 5 percent of the mean pixel value was adjudged to be background. This value was subtracted from the GFP channel. A new intensity histogram was then generated and low-pass filtered (averaged) to smooth it out. The histogram was then differentiated and the first zero crossing point determined. The intensity value at this point was used to threshold the background-subtracted GFP image to produce a mask for further image-processing steps. The outline of the mask was determined using the Mathematica command "MorphologicalPerimeter."

Atomic Force Microscopy. The human titin M10 domain and the first Ig-domain of human obscurin or obscurin-like 1 (*domain boundaries* as above) were connected by a flexible, 22 aa linker composed of GGSG repeats and sandwiched between 6 concatenated ubiquitin domains, carrying a C-terminal 6 × His-tag by standard molecular biology techniques (13). All constructs were verified by DNA sequencing. The effective linker between the two proteins including unstructured residues of the protein domains was 33 aa for titin-obscurin and 35 as for titin/obscurin-like 1. Soluble protein was expressed in E. coli and purified by Nickel-affinity chromatography followed by gel filtration. To prevent intermolecular disulfide bridges from surface-exposed cysteines, all protein solutions were stored in PBS containing 1 mM TCEP.

Single-molecule force spectroscopy was performed on a custom-built atomic force microscope. Gold-coated cantilevers (Biolever Type A, Olympus) with a typical spring constant of 30 pN/nm were used in all experiments. Cantilevers were calibrated individually employing the equipartition theorem. In a typical experiment, 1–2 μl of the purified protein were applied to a freshly activated Nickel-NTA surface and force-extension traces were recorded at a pulling velocity of 1 μm/s and a sampling frequency of 20 kHz. Contour lengths increases (ΔL) were analyzed by fitting worm-like chain (WLC) curves at a fixed persistence length p of 0.5 nm. Contour length increases were used to calculate the number of amino acids involved in an unfolding event by $\Delta L = n \cdot d_{aa} - d_{intermediate} + d_{folded}$. Here, $d_{intermediate}$ and d_{folded} denote the distance between the points of force application in the intermediate and the native state, respectively.

The correlation of free energy and affinity constants follows the equation $\Delta G/k_BT = \ln(K_D)$, where ΔG is the change in free energy (Gibbs), k_B the Boltzmann constant, T the absolute temperature, and K_D the dissociation constant. For a difference in K_D of 3.4, the difference of k_BT is therefore $\ln(3.4) = 1.22$.

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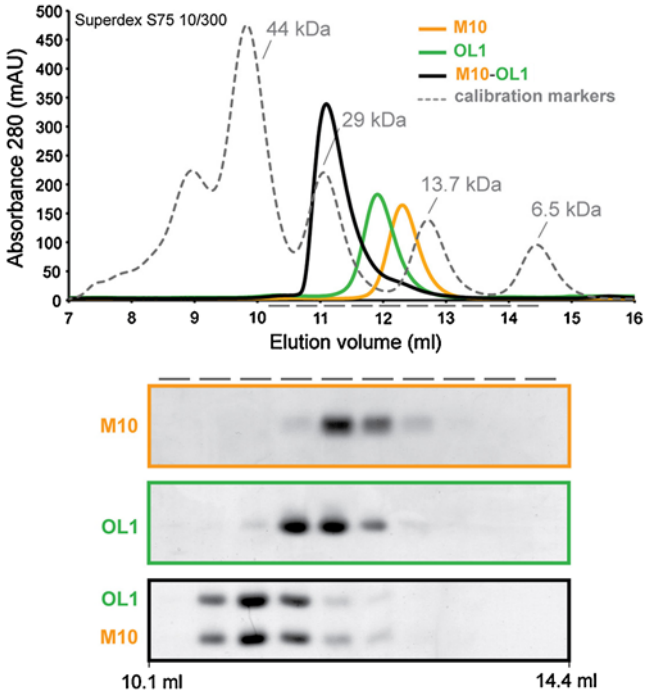

Fig. S1. *(A)* The M10 (*Orange Trace*) and OL1 (*Green Trace*) domains elute between the 13.7 kDa and the 29 kDa markers from a Superdex 75 size-exclusion column. When M10 and OL1 are mixed stoichiometrically they form a complex (*Black Trace*) that elutes slightly after the 29 kDa calibration marker consistent with a dimeric M10-OL1 complex. Ten fractions were analyzed by SDS–PAGE and Coomassie staining.

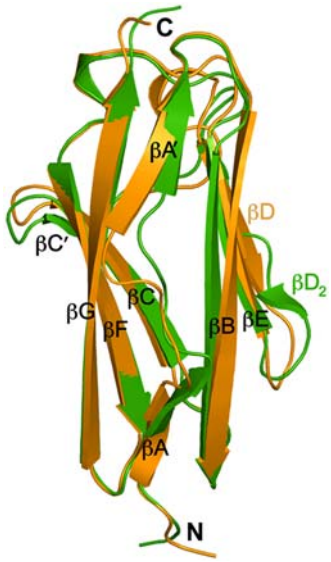

Fig. S2. Ribbon representation of the M10 (*Orange*) and OL1 (*Green*) domains superimposed onto each other highlighting the common I-set Ig-fold. The most divergent regions are those involved in establishing the M10-OL1 interface.

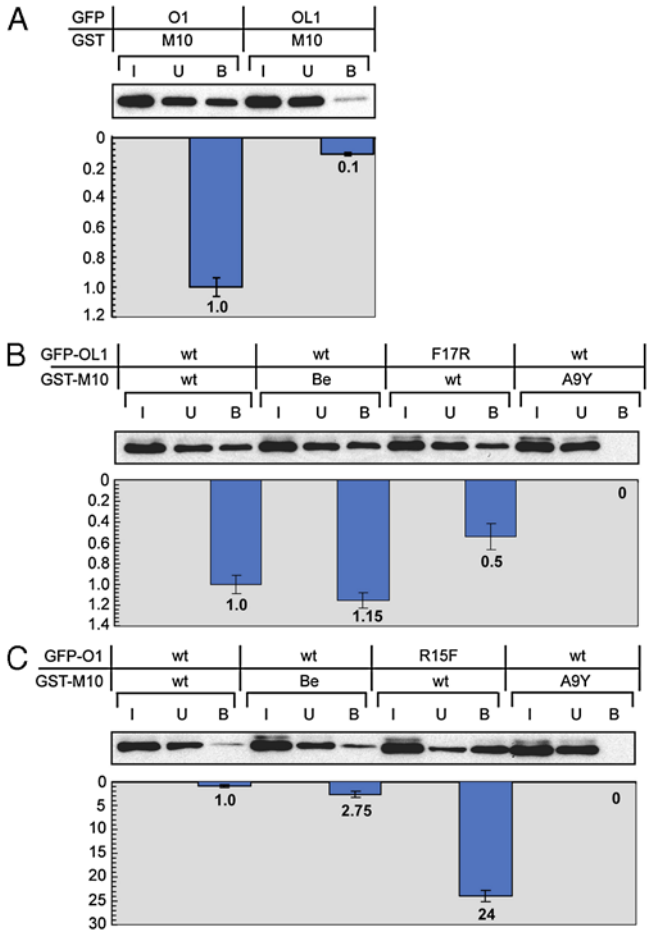

Fig. S3. (*A*) In vitro pull-down assay of GFP-OL1 and GFP-O1 using GST-tagged M10. IB was performed using an anti-GFP Ab. Labels I, U, B indicate the input, unbound, and bound fractions, respectively. The lower panel shows a densitometric quantification of the bound fractions (lanes 1,2) normalized to the GST-M10-GFP-OL1 level (lane 1). (*B*) In vitro pull-down assay of GFP-OL1 and GFP-OL1 F17R using the GST-tagged M10 constructs indicated. Detection was as in (*A*). Normalization is relative to the GST-M10-GFP-OL1 level (lane 1). Labels are as in (*A*). (*C*) In vitro pull-down assay of GFP-O1 and GFP-O1 R15F using the GST-tagged M10 constructs indicated. Detection was as in (*A*). Normalization is relative to the GST-M10-GFP-O1 level (lane 1). Labels are as in (*A*).

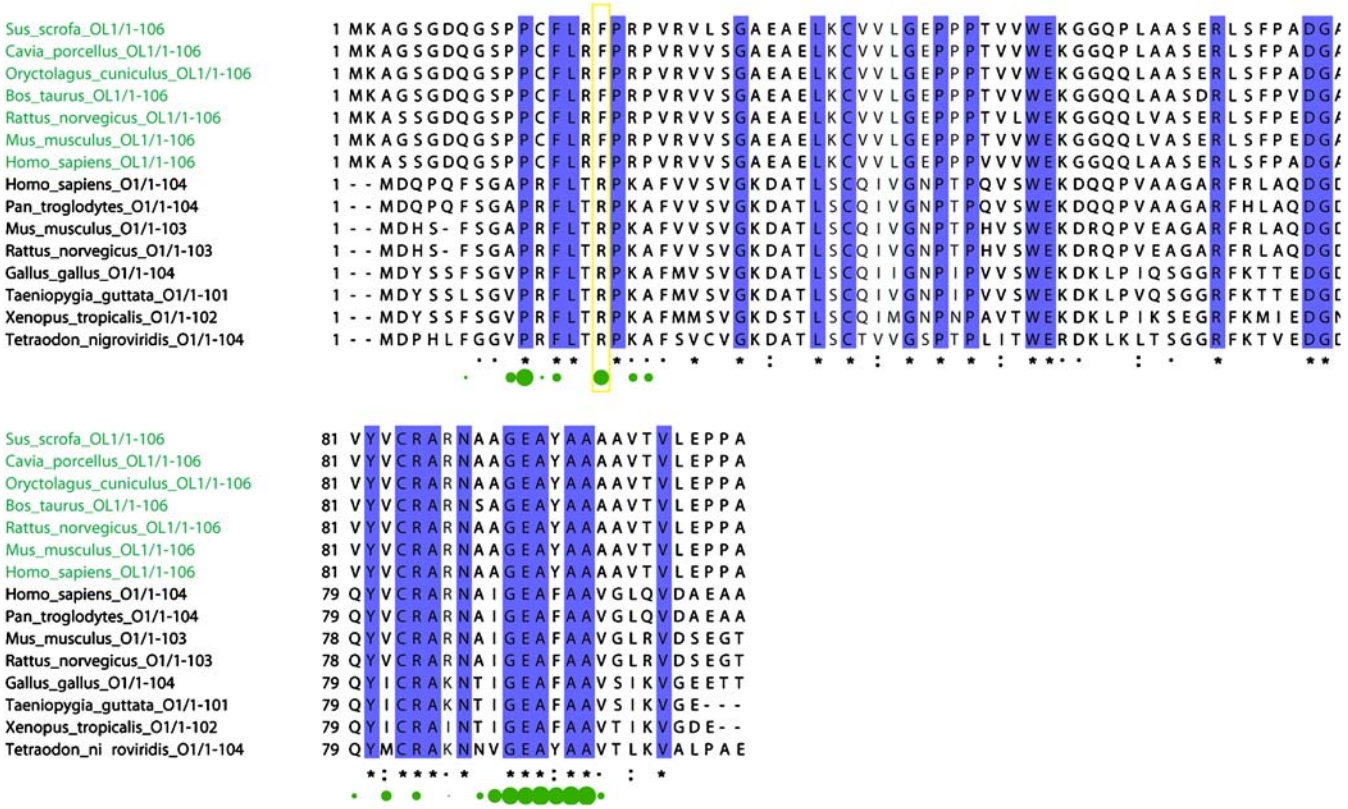

Fig. S4. Multiple sequence alignment of OL1 and O1 domains from various species. Identical, strongly conserved, and weakly conserved residues are indicated by an asterisk, a colon, and a dot, respectively. Identical residues are additionally highlighted by the blue background. Green balls have the same meaning as in Fig. 1*D* of the main text. The signature interface residues OL1 F17 and O1 R15 (*Homo sapiens* numbering) are boxed in yellow.

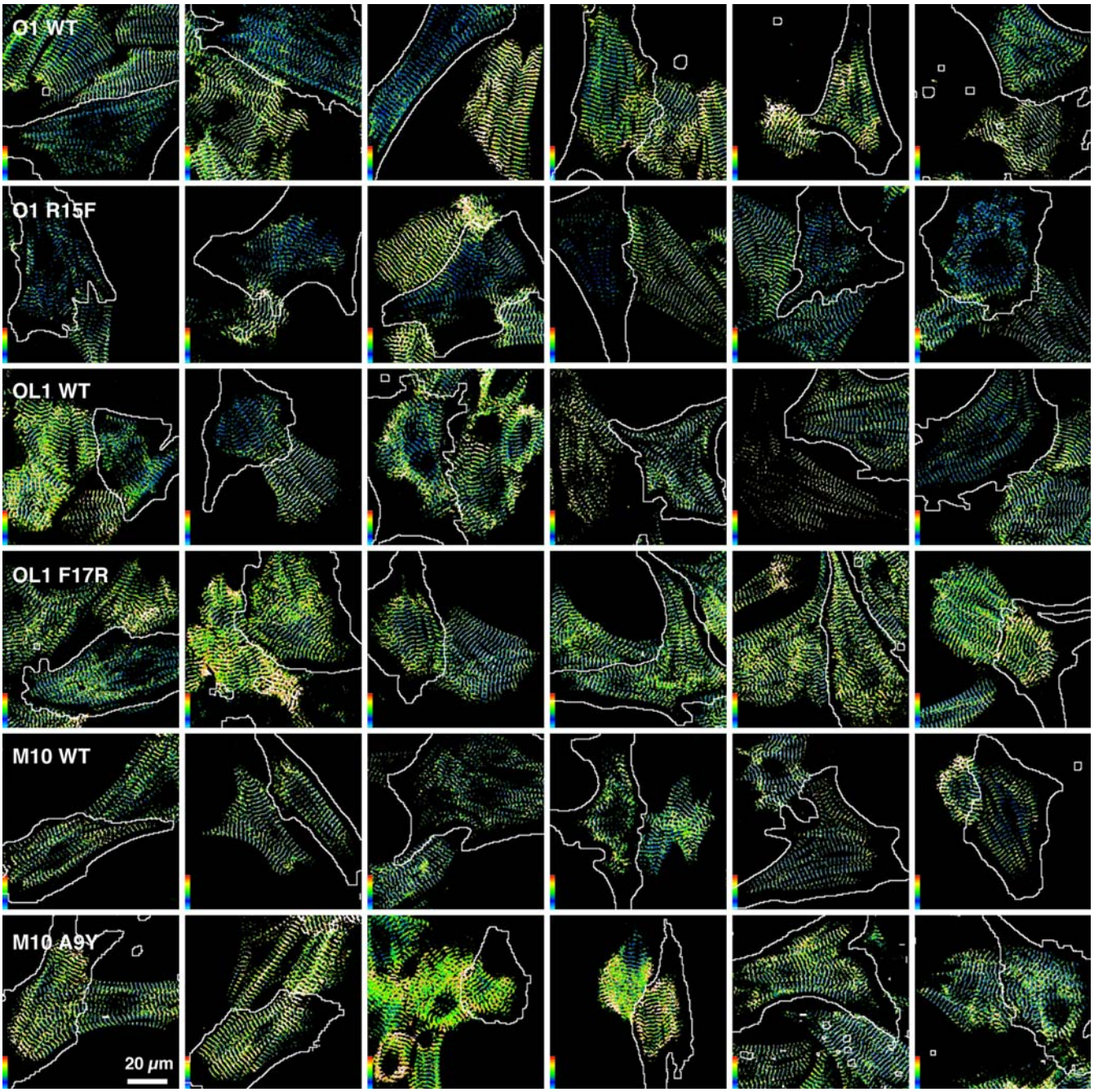

Fig. S5. Panel of six randomly chosen images for each Ig construct, showing the ratiometric imaging of endogenous obscurin to myomesin. The ratiometric falso-color image with the overlaid GFP mask for the outline of the transfected cells (*White Line*) are shown. The false-color scale range indicator shows a range of 0 (*Black*) to 3 (*White*). Scale bar: 20 μm.

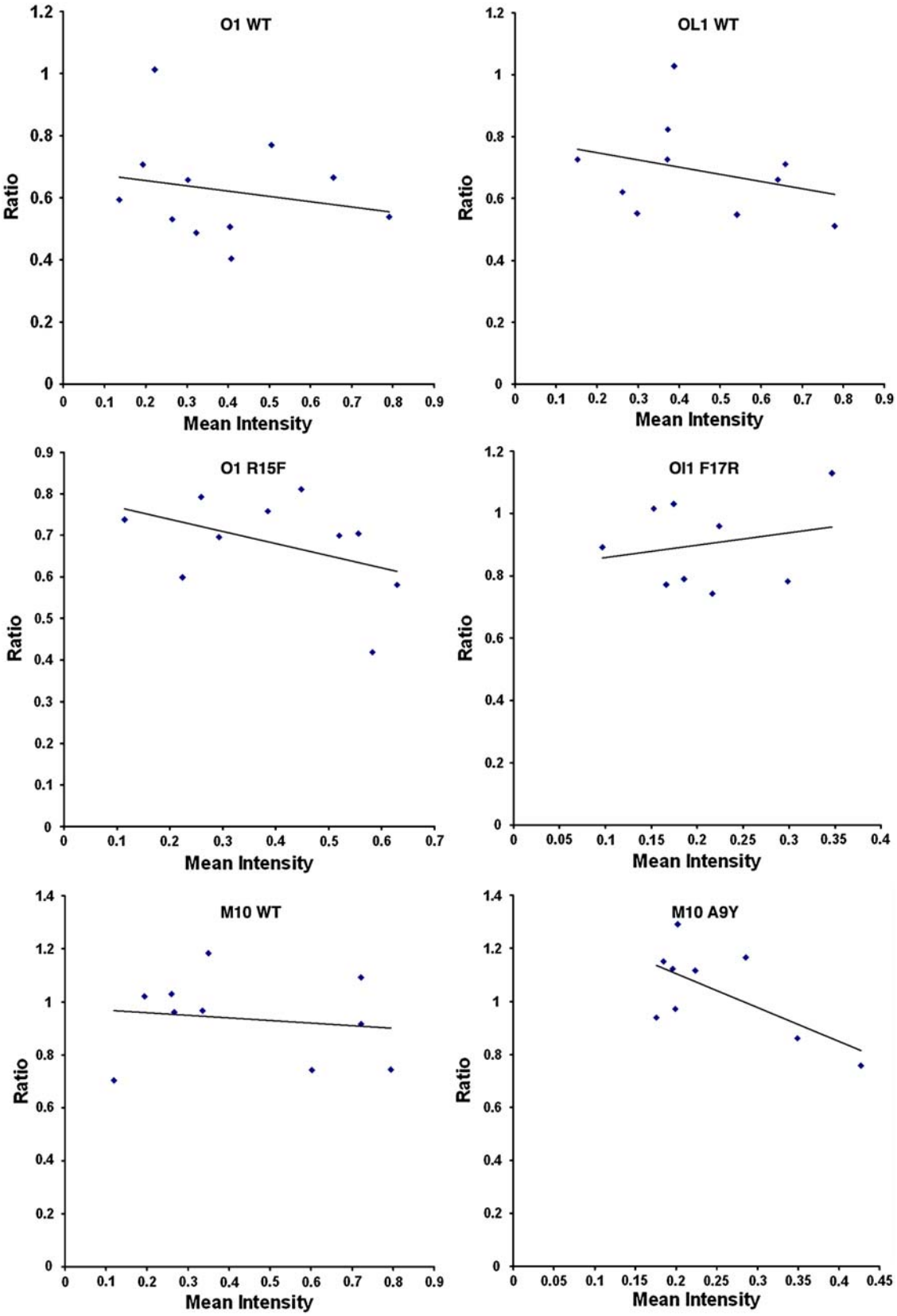

Fig. S6. Correlation of GFP intensity (*x*-axis) to obscurin/myomesin ratio for all Ig-domain constructs used. There is no indication of a systematic concentration-dependence towards lower GFP-levels, demonstrating that the differences in ratios measured are not caused by different expression levels. Between 9 to 11 cells were analyzed in these plots.

Table S1. Data collections and refinement statistics

Data collection			
Data set	M10-OL1 (Form I)	M10-OL1 (Form II)	M10-OL1 F17R
Beam Line	I04/Diamond	I03/Diamond	I03/Diamond
Wavelength (Å)	0.9745	0.9763	0.9763
Resolution range (Å)	53.38–1.48	36.13–2.30	34.79–1.70
Highest res. bin (Å)	(1.56–1.48)	(2.42–2.30)	(1.79–1.70)
Space group	$P3_1$	$P1$	$C2$
Cell dimensions (Å)			
a, b, c (Å)	61.67,61.67,42.35	37.65,44.94,68.01	89.01,37.80,68.49
α, β, γ (°)	90,90,120	87.29,86.91,68.79	90,92.99,90
Unique reflections	30142 (4398)	17094 (2514)	194802 (29257)
Overall redundancy	5.0 (3.9)	3.8 (3.8)	7.8 (8.0)
Completeness, (%)	99.9 (99.6)	92.6 (93.1)	98.7 (99.7)
R_{symm}*, (%)	5.9 (43.7)	5.7 (25.3)	7.9 (31.4)
$\langle I/\sigma(I)\rangle$	15.9 (4.6)	13.2 (4.3)	20.6 (6.0)
Refinement			
PDB code	2wp3	2wwm	2wwk
$R_{factor}(\%)/R_{free}(\%)$	17.7/21.7	20.0/25.2	21.9/25.9
# non-H atoms	1558	3069	1798
rms bond lengths[5] (Å)	0.019	0.010	0.015
rms bond lengths[5] (°)	1.78	1.23	1.56

*For the definitions of standard crystallographic quantities the reader is referred to Drenth, J (1999) Principles of X-ray Crystallography (Springer).