

## **Supplemental Materials and Methods**

### **Peptide Dot Blot Overlay**

Five peptides (1-5) were produced that covered overlapping portions of the last ~50 amino acids of the C10 domain (Biopolymer Facility, University of Maryland, Baltimore, MD). Specifically, they contained the following amino acid residues: peptide-1: 5' DDPRYRMFSNQGVCTLEI 3', peptide-2: 5' RKPSPYDGGTYCCKAV 3', peptide-3: NDLGTVEIECKLEVKIAQ, peptide-4: 5' SNQGVCTLEIRKPSPYDGG TYCCKAV 3' and peptide-5: 5' YDGGTYCCKAVNDLGTVEIECKLEVKIAQ 3'. The peptides were dissolved in ddH<sub>2</sub>O at 2 mg/ml; in the cases of peptides 3 and 5, DMSO was added dropwise to ensure solubility.

Dot blot overlays were performed by applying ~5 µg of each peptide onto a nitrocellulose membrane, which was allowed to dry completely. Blots were then incubated in buffer A, containing 50 mM Tris, pH 7.2, 120 mM NaCl, 3% BSA, 2 mM dithiothreitol, 0.5% NP-40, 0.1% Tween-20, for 3h at 25°C and subsequently with ~2.5 µg/ml of control GST, GST-Obscurin-Ig1/2, GST-Obscurin-Ig1, and GST-Obscurin-Ig2 diluted in buffer A for 16h at 4°C. Membranes were washed extensively with buffer A and blocked with buffer C (PBS, pH 7.2, 10 mM NaN<sub>3</sub>, 0.1 % Tween-20, 3% dry milk), before they were probed with rabbit antibodies to GST (100 ng/ml; prepared in our laboratory).

### **Generation of Antibodies Specific for MyBP-C slow variant-1**

Two additional sets of antibodies specific for the novel COOH-terminus of MyBP-C slow were produced. In the first set, two peptides present in the COOH-terminus of MyBP-C slow variant-1 were used as antigens to generate monoclonal antibodies in mice. One of the peptides contained sequences present in all MyBP-C slow variants, specifically within the C10 domain, and is referred to as common peptide: 5' CGGDDPRYRMFSNQG 3'; the other contained sequences present only in the unique COOH-terminus of variant-1, and is referred to as unique peptide: 5' CGGEGQQQLHNLDF 3' (Biosynthesis, Lewisville, TX). Sera (1:250 dilution) from the immunized mice and tissue culture supernatants (1:20 dilution) from clones of hybridoma cells that secreted specific antibodies to the unique peptide were subsequently tested

in immunofluorescent experiments. The unique peptide was also used for production of mouse polyclonal antibodies from a different commercial source (Genescript, Piscataway, NJ). Sera (1:200 dilution) from the immunized mice were subsequently tested in immunofluorescent experiments.

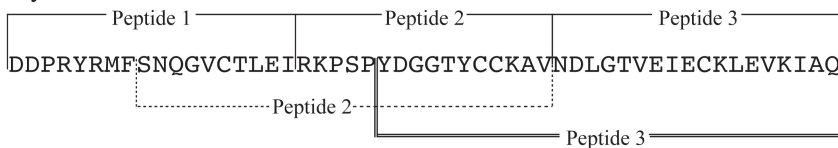
## Figure Legends

**Supplemental Figure 1:** Peptide binding assays indicate the minimal binding region on MyBP-C slow for obscurin is the second half of the C10 domain. **A:** To further refine the sequences within the C10 domain of MyBP-C slow variant-1 that bind to obscurin's Ig2, we generated a series of overlapping peptides containing successive or overlapping parts of the C10 domain. **B:** These were subsequently used in *in vitro* dot blot overlay assays and incubated with recombinant GST-Obscurin-Ig1/2, GST-Obscurin-Ig1, GST-Obscurin-Ig2, and GST. No single peptide was able to bind to the NH<sub>2</sub>-terminus of obscurin suggesting that the minimal binding site on MyBP-C slow variant 1 capable of binding the second Ig domain of obscurin is the last ~50 residues of the C10 domain.

**Supplemental Figure 2:** MyBP-C slow variant-1 preferentially accumulates at the M-band and possibly flanking regions in developing and adult myofibers, as indicated by two additional antibodies specific for the novel COOH-terminus of variant-1. **A:** Immunolabeling of rat adult soleus muscle with serum from mice immunized with a mixture of MyBP-C slow peptides (top panel, green) that contained sequences present either in all known forms or only in the unique COOH-terminus of MyBP-C slow variant-1 and antibodies to the COOH-terminus of obscurin that localizes at the M-band and Z-disks (middle panel, red). As shown in the overlay (bottom panel), the serum stains the entire A-band, including the M-band, which appears yellow in the middle of the A-band. **B1:** Double immunostaining of rat adult soleus muscle with tissue culture supernatant that contains antibodies specific to the unique COOH-terminus of MyBP-C slow variant-1 (top panel, green) and antibodies to sAnk1 (middle panel, red) that localizes at Z-disks and M-bands. Notably, the supernatant specifically stains M-bands and possibly flanking regions, as shown in the overlay (bottom panel), where it co-distributes with sAnk1. **B2:** Co-staining of P1 rat skeletal myotubes with supernatant that contains antibodies specific to MyBP-C slow variant-1 (top panel, green) and antibodies to the COOH-terminus of obscurin (middle panel, red) also demonstrated that the two proteins co-localize at M-bands. **C:** Immunolabeling of rat adult soleus muscle with serum from mice immunized with a peptide present in the unique COOH-terminus of MyBP-C slow variant-1 (top panel, green) and antibodies to the NH<sub>2</sub>-terminus of titin located at the Z-disk (middle panel, red) demonstrated that MyBP-C slow

variant-1 localizes at M-bands, as shown by the alternating staining pattern with titin's NH<sub>2</sub>-terminus (overlay, bottom panel).

MyBP-C slow C10



MyBP-C Peptide 1

MyBP-C Peptide 2

MyBP-C Peptide 3

MyBP-C Peptide 4

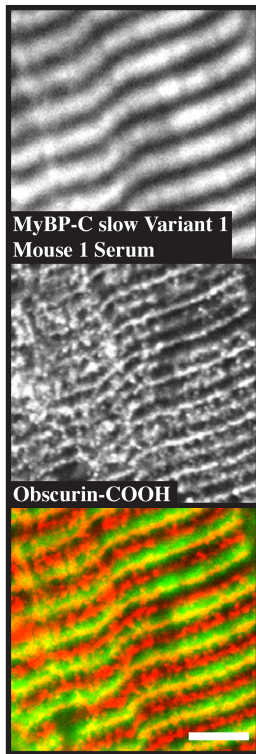
MyBP-C Peptide 5

GST-ObscIg 1/2	+	-	-	-
GST-ObscIg 1	-	+	-	-
GST-ObscIg 2	-	-	+	-
GST	-	-	-	+

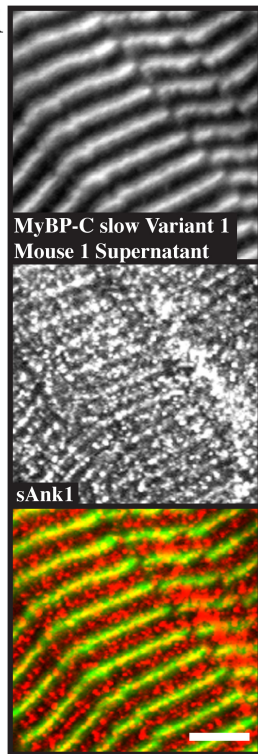
Western:  $\alpha$ -MyBP

Supplemental Figure 1

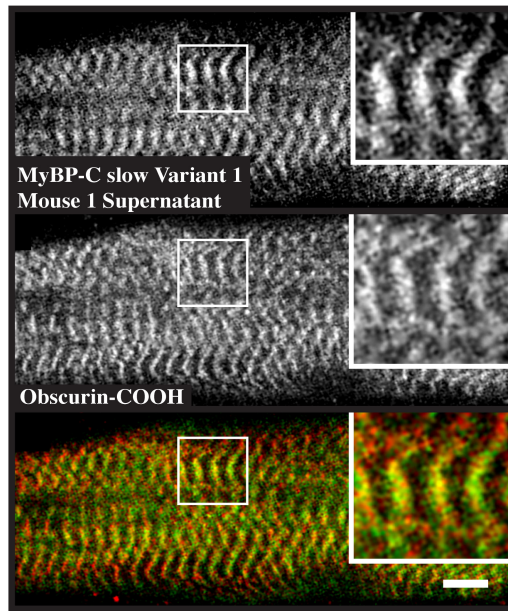
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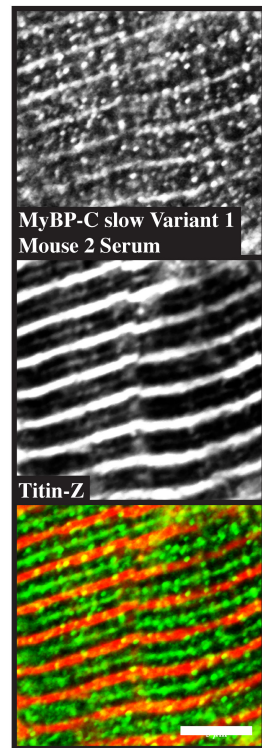
B1



B2



C



Supplemental Figure 2