SUPPLEMENTARY INFORMATIONS

The myotubularin-amphiphysin 2 complex in membrane tubulation and centronuclear myopathies.

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SUPPLEMENTARY MATERIAL AND METHODS

Constructs

The constructs pEGFP BIN1 full length (isoform 8, 454 aa) and pEGFP BAR+PI (aa 1-281) were obtained from P. De Camilli (Yale University, New York). cDNA corresponding to MTM1 sequence (603 aa) was cloned into pEGFP-C1 (Clontech, N-ter GFP fusion) by PCR amplification followed by BamH1 restriction and into pCS2+ vector ([1], untagged). cDNA corresponding to the full length, BAR+PI, BAR (aa 1-255) and SH3 (aa 380-454) sequences of BIN1, and to the full length sequence of MTM1 were cloned into pENTR1A Gateway entry vector (Invitrogen) and recombined into several destination vectors: pEGFPgateway (N-ter GFP fusion), pSG5EYFP (N-ter YFP fusion), pSG5B10 (N-ter B10 fusion = 14aa epitope of the estrogen receptor alpha) and pDEST15 (N-ter GST fusion). Mutations were introduced in pEGFP BIN1 full length, in pCS2+ MTM1 and in the different pENTR1A constructs by primer-directed PCR mutagenesis. The full length open reading frames for human MTM1 (GenBank U46024), MTM1 GRAM (aa 1-162), MTM1 ΔGRAM (aa 146-603), MTM1 Cter (aa 545-603) and MTMR1, MTMR2 MTMR10 and MTMR12 were subcloned into pENTR Gateway entry vector and recombined into destination vector pGex4T3 (N-ter GST fusion) and pSG5B10 [2]. All the constructs were verified by Sanger sequencing.

Cell culture and transfections

African green monkey kidney fibroblast (COS-1 cells) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 1g/L glucose and supplemented with 5% fetal calf serum (FCS) and 40mg/L gentamycin. Mouse myoblast cell line (C2C12) was grown in DMEM containing 1g/l glucose and supplemented with 20% fetal calf serum (FCS) and 40 mg/L gentamycin; differentiated myotubes were obtained after culture for 5 to 8 days in DMEM containing 1g/l glucose and supplemented with 2% horse serum (FCS) and 40mg/L gentamicin. Knockdown (KD) *Mtm1* and control C2C12 myoblast cell lines used were a gift from A. Beggs (Children's Hospital, Boston) and generated as described [2]. COS-1 and C2C12 cells were grown on 100 mm diameter Petri dishes or on 22 mm squared glasses in 5% CO2 at 37°C until they reached 60–80% confluence; cells were transiently transfected with the appropriate plasmids using the Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol.

Production of GST-fusion proteins in bacteria

GST fusion proteins were expressed in the *Escherichia coli* strain BL21-Rosetta 2 (Novagen). Bacteria were lysed in extraction buffer (50mM Tris-HCl (pH8.0), 100mM NaCl, 5mM EDTA, 1mM EDTA)

supplemented with 1mg/ml lysosyme, and a cocktail of protease inhibitors (cOmplete EDTA free, Roche). After 30 min incubation on ice, detergents were added (0.01 % N-laurylsarcosine and 0,5% Triton-X100) and lysates were incubated O/N at 4°C to obtain high solubilization. Then, lysates were centrifuged at 16.000xg for 30 min. GST fusion proteins were purified from bacterial lysates by incubation with glutathione sepharose 4B beads (GE Healthcare) overnight followed by extensive washing with extraction buffer plus 0,5% Triton-X100. For additional analysis, proteins were eluted from beads in extraction buffer supplemented with 10mM of reduced Gluthathion.

Antibodies

For immunoprecipitation: mouse anti-B10 and anti-GFP antibodies (IGBMC) or mouse anti-MTM1 (1G1, IGBMC [3]) and anti-BIN1 (C99D, Sigma-Aldrich). For Western Blot: mouse anti-B10 (1/1000, IGBMC), mouse anti-GFP (1/1000, IGBMC), rabbit anti-MTM1 (1/400, R2827, IGBMC [4]), rabbit anti-BIN1 (1/800, R2444, IGBMC [5]), anti-RyR1 (1/1000 Sigma-Aldrich), anti-SERCA1 ATPase (1/1000, ABR) and goat anti-caveolin 3 (1/2000, BD Bioscience).

For Immunofluorescence: rabbit anti-MTM1 (1/1000 for cells or 1/200 for fibers, R2827, IGBMC [4]) and mouse anti-B10 and anti-β–actin (1/1000, IGBMC), anti-BIN1 (1/200 for cells or 1/50 for fibers, C99D Sigma-Aldrich), anti-DHPR antibody (1/150, Abcam), anti-α-actinin (1/1000, BM-75.2 Sigma-Aldrich) and/or anti-RYR1 (1/150, Sigma-Aldrich) primary antibodies. Goat anti-mouse and anti-rabbit secondary antibodies (for cells, 1/1000, Invitrogen) or donkey anti-mouse and anti-rabbit secondary antibodies (for fibers 1/250, Life technologies) coupled to Alexa Fluor 488/594.

Western blot analysis

After separation on SDS-containing 8–10% polyacrylamide gels (SDS–PAGE), proteins were transferred on a nitrocellulose membrane (Protran, Whatman). After blocking the non-specific sites with 5% non-fat milk in Tris buffered saline, the membrane was incubated overnight with primary antibodies. Membranes were then incubated with anti-mouse or anti-rabbit HRP-conjugated antibodies (1/10000, Jackson Immunoresearch). The membranes were revealed with ECL Plus Western Blotting Detection System (Amersham, GE Healthcare) and exposed on autoradiographic film (BioMax MR films, Eastman-Kodak).

***In vitro* lipid phosphatase assay**

The recombinant proteins (1 pmol) were pre-incubated rotating overnight at 4°C in the phosphatase assay buffer (50mM ammonium acetate pH6.0). The fluorescent lipids C6-BODIFY FL-PtdIns(3,5)P_2 or C6-BODIFY FL-PtdIns3*P* (Echelon Inc.) in choroform/methanol 1/1 were evaporated under nitrogen, resuspended in 50mM ammonium acetate, 1mM DTT and added to the recombinant proteins to a final concentration of 2 ng/µl. After a 30 min incubation at 30°C, the reaction was stopped by addition of chloroform/methanol 1/1 and centrifugation at 13000xg for 3 min. The upper phase was collected, added to acetone and evaporated under nitrogen. The lipid were separated on Silica Gel 60

(Merck) by Thin Layer Chromatography in a chloroform/methanol/acetone/acetic acid/H2O (70/50/20/20/20) buffer. Fluorescence was detected with a Typhoon 9400 (GE Healthcare) and analyzed with the Image Quant TL software. Main experiment was repeated 4 times and supplementary experiment was repeated 2 times.

Malachite Green assay

MTM1 activity was assayed on recombinant proteins (GST-tagged) using the Malachite green Assay kit (echelon biosciences inc.) according to the manufacturer's instructions and the previous detailed protocol [6]. Briefly, the assay was first performed with increasing concentration of MTM1 recombinant protein (0 to 25 nM) in order to determine optimal activity. In the following assays, 10 nM MTM1 were used to measure the activity in the presence of increased concentration of BIN1 recombinant protein (0 to 100 nM). Recombinant proteins were diluted in phosphatase reaction buffer (50 mM Ammonium acetate pH 6) to reach the appropriate concentration, then incubated O/N at 4°C for complex formation. MTM1 substrate (DiC8 PI3*P* and DiC8 PI(3,5)P_2, Echelon biosciences inc.) and a control phosphoinositide (DiC8 PI(4,5)P_2) were incubated with the proteins for 10 min at 30 °C. The reaction was stopped by adding 10 µl of 10mM sodium orthovanadate. The malachite green solution was added for 30 min at RT and absorbance was measured at 620 nm. Measurement were performed in triplicate, in 2 independent experiments.

Muscle subcellular fractionation

This protocol is based on previously reported methods used in triad isolation from rabbit muscle membranes with slight modifications [7]. Twelve grams of rabbit skeletal muscle were minced into 2-4 mm2 pieces and fat and tendon were removed. Muscles were homogenized in 10 ml ice cold homogenization buffer (20mM tris-HCl pH 7.4, 250 mM sucrose, 1mM EDTA, 1mM pepstatin and 1mM leupeptin) using a polytron homogenizer at low speed (5000 rpm). Homogenates were centrifuged for 20 min at 12000 g in fixed angle rotor at 4°C. The supernatant (S1) was collected and pellet resuspended in 5 ml homogenization buffer and submitted to a second centrifugation 20 min at 12000 g. The resulting supernatant (S2) was pooled with S1 and the pellet was solubilized in homogenization buffer with 8M urea. An aliquot was mixed with 4X Laemmli buffer and freezed for analysis (pellet fraction, P). The S1 and S2 mixture was submitted to a step of myofibril solubilization by adding KCl to a final concentration of 0.6M and incubation during 1h at 4°C on an orbital shaker. A microsomal fraction (M) from S1+S2 was obtained by centrifugation during 2h at 110000 g at 4°C. Microsomes were resuspended in homogenization buffer using a Dounce homogenizer and layered onto a sucrose gradient. The gradient steps, 8 ml each, consisted of 45% (wt/wt) sucrose (1.6M), 38% (1.3M), 32% (1.1M) and 27% (0.8M) all buffered with 20mM tris-HCl pH 7.4. After a 16h centrifugation at 77000 g at 4°C (in Beckman SW27 rotor), fractions at the interfaces of the gradient steps were collected and diluted two fold with homogenization buffer and further centrifuged 2h at 120000 g at 4°C. Fraction 1 (top of the 27%) contained mostly longitudinal SR, with some T-tubules and sarcolemma, fraction 2 (27/32% interface), was enriched with longitudinal SR and sarcolemma, fraction 3 (32/38% interface) contained a mixture of longitudinal and junctional SR and some sarcolemma, and fraction 4 (38/45% interface) consisted of highly enriched junctional SR cisternae.

Western blot was performed using rabbit anti-MTM1 (1/400, R2827, IGBMC), rabbit anti-BIN1 (1/800, R2444, IGBMC), anti-RyR1 (1/1000 Sigma-Aldrich), anti-SERCA1 ATPase (1/1000, ABR) and goat anti-caveolin 3 (1/2000, BD Bioscience) antibodies.

Statistical analysis

Statistical analysis was performed using the unpaired student's *t* test or the one-way ANOVA test followed by the Bonferroni's Multiple Comparison Test for length tubule measurement. p-values of <0.05 were considered significant.

SUPPLEMENTAL FIGURES

Figure S1

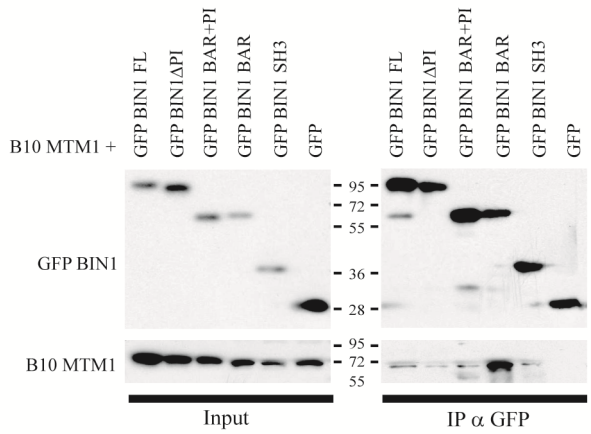

Figure S1. MTM1 and BIN1 interact *in vitro.* Co-immunoprecipitation assays using anti-GFP antibodies on lysates from COS-1 cells expressing B10-MTM1 and GFP-fused BIN1 domains. Top panel: Immunoblot hybridized with anti-GFP antibodies. Bottom panel: Immunoblot hybridized with anti-MTM1 antibodies.

Figure S2

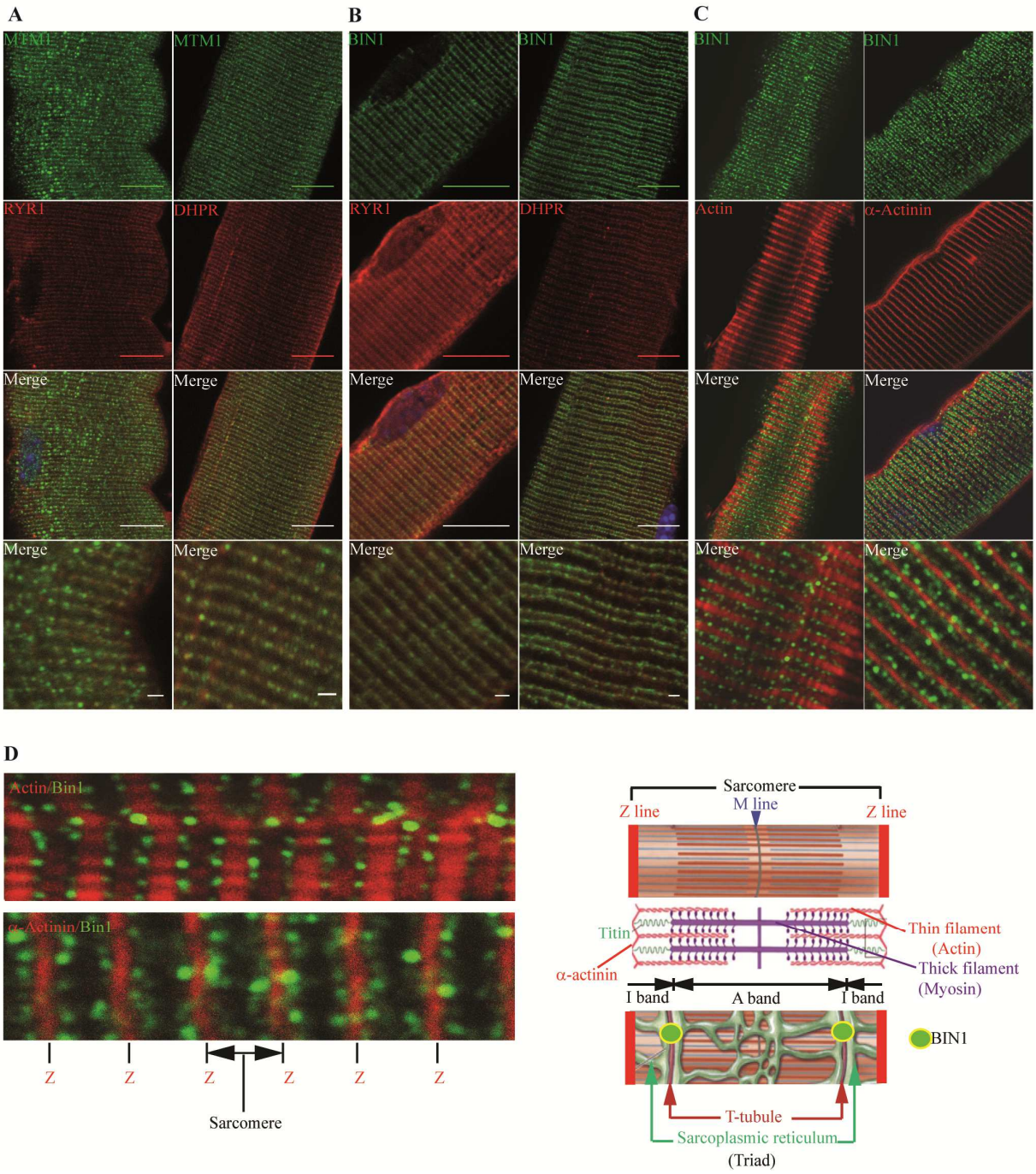

Figure S2. MTM1 and BIN1 both co-localize with the triad markers RYR1 and DHPRα, and BIN1 non extensively co-localise with sarcomeric actin and z-line marker α–actinin. Muscle fibers isolated from wild type mice were stained with anti-MTM1 and anti-RYR1 antibodies (**A**, left panel), anti-MTM1 and anti-DHPRα (**A**, right panel), anti-BIN1 and anti-RYR1 antibodies (**B**, left panel), anti-BIN1 and anti-DHPR antibodies (**B**, right panel), anti-BIN1 and anti-actin antibodies (**C**, left panel), anti-BIN1 and anti-α-actinin antibodies (**C**, left panel).Confocal planes in the middle of fibers. Scale Bar: 10µm or 1µm for magnifications. (**D**) Schematic representation of BIN1 localization in the sarcomere.

Figure S3

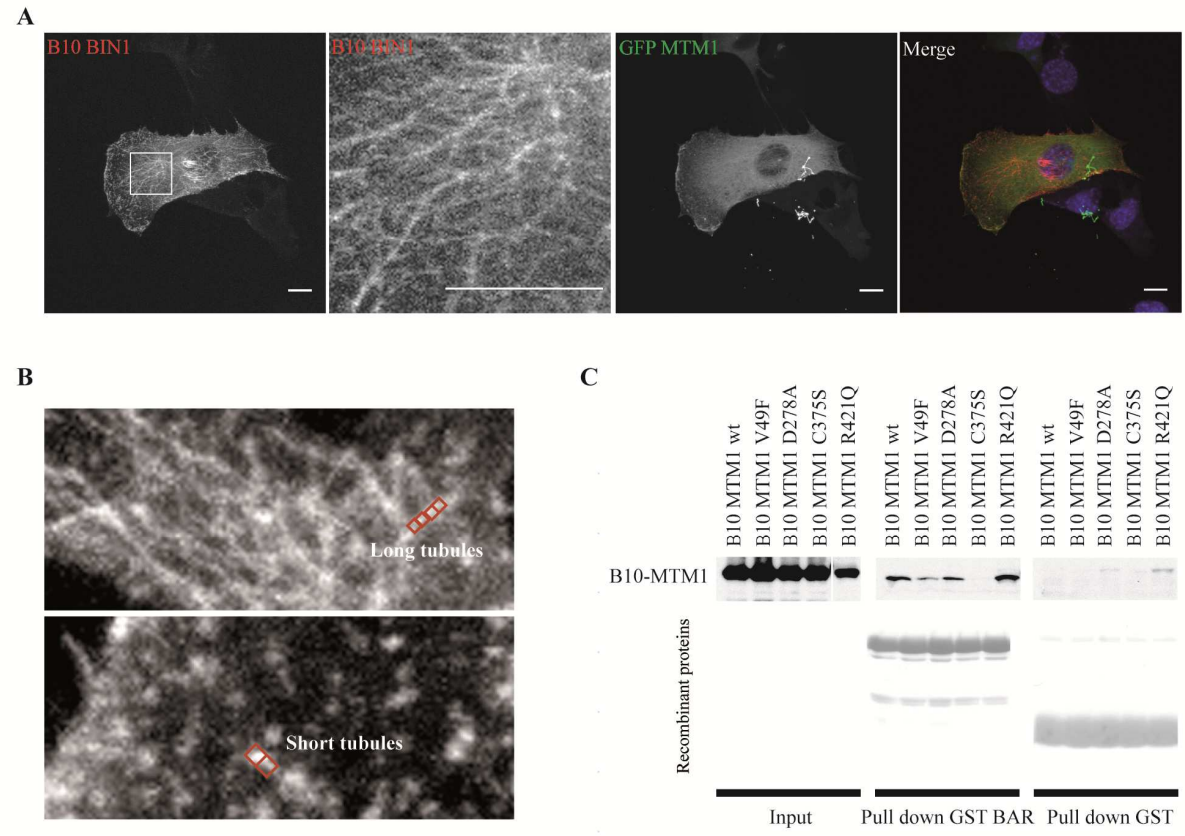

Figure S3. (**A**) GFP-fusion (tag) did not alter MTM1 regulation of BIN1 membrane tubulation compared to untagged MTM1 in Fig. 3. C2C12 cells were transfected with cDNAs encoding B10-BIN1 and GFP-MTM1. Images represent confocal z-stack projections. Scale Bars: 10µm. (**B**) Difference between short and long tubules. Confocal images of membrane tubules in cell expressing GFP BIN1 with (top) or without (bottom) MTM1. A square represents the diameter of the tubule. Tubules were considered short when they were shorter or equal to twice their diameter, 2 squares on the example (bottom). Tubules were considered long when they were more than twice longer than large, more than 2 squares in the example (top). (**C**) Impact of inactivating MTM1 mutations on its interaction with BIN1. Most of the inactivating MTM1 mutations, apart from C375S, did not alter MTM1 pull down with BIN1. GST (negative control) and GST-BAR recombinant proteins were used to pull down B10-MTM1 overexpressed in COS-1 cells. Top panel: Immunoblot hybridized with anti-B10 antibody. Bottom panel: Ponceau Red detecting recombinant proteins.

Figure S4

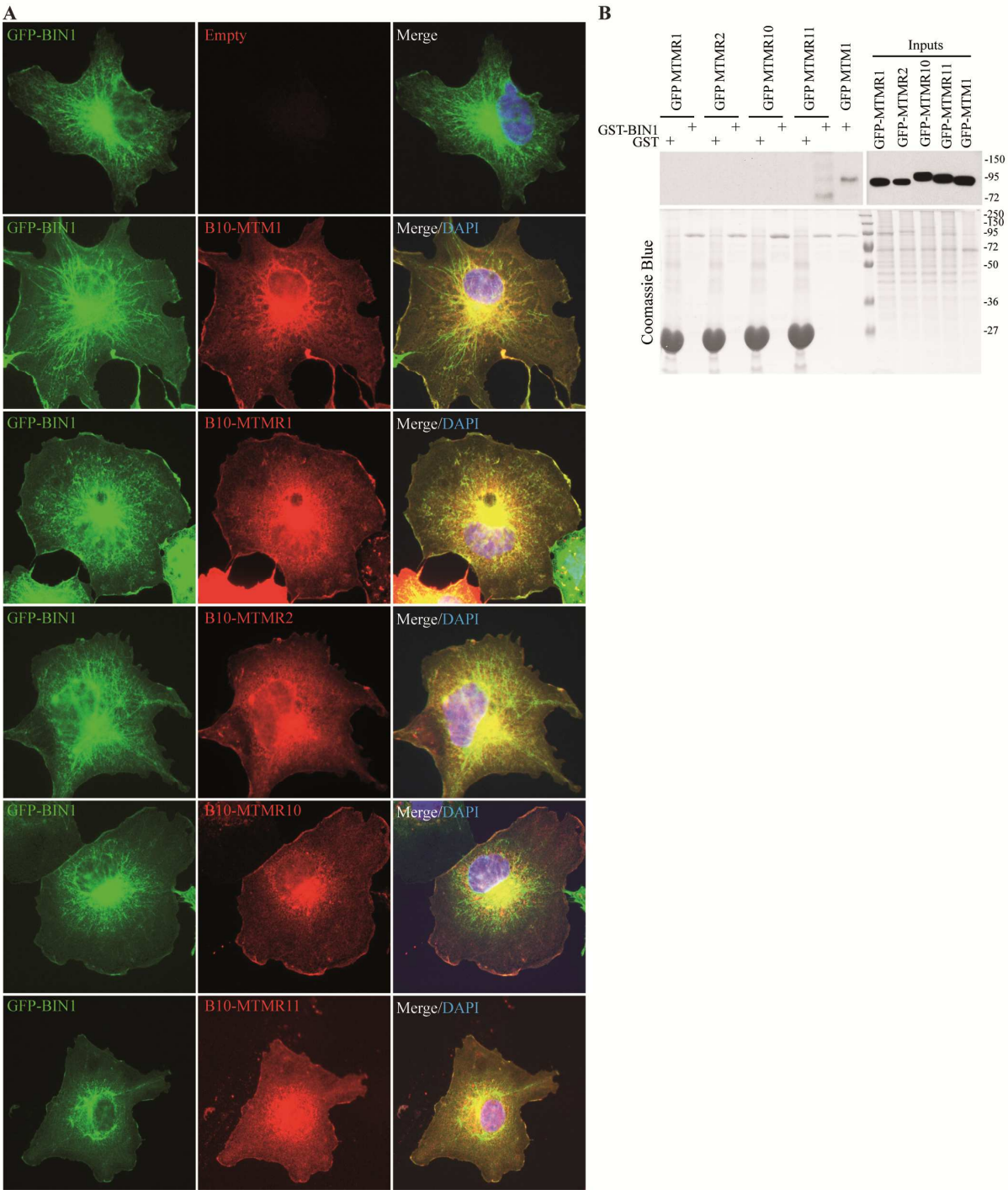

Figure S4. Impact of myotubularins on BIN1-mediated membrane tubulation. (**A**) Catalytically active (MTMR1 and MTMR2) or inactive (MTMR10 and MTMR11) myotubularins do not enhance BIN1 membrane tubulation. Co-expression in COS-1 cells of GFP-BIN1 with B10-MTM1, B10-MTMR1, B10-MTMR2, B10-MTMR10 or B10-MTMR11. Epifluorescence images. (**B**) Myotubularin related proteins do not bind to BIN1. GST (negative control) and GST-BIN1 recombinant proteins were used to pull down GFP tagged MTM1, MTMR1 , MTMR2, MTMR10, MTMR11. Top panels: Immunoblot hybridized with anti-GFP antibody. Bottom panel: Coomassie Blue staining showing recombinant proteins used for the pull-down.

Figure S5

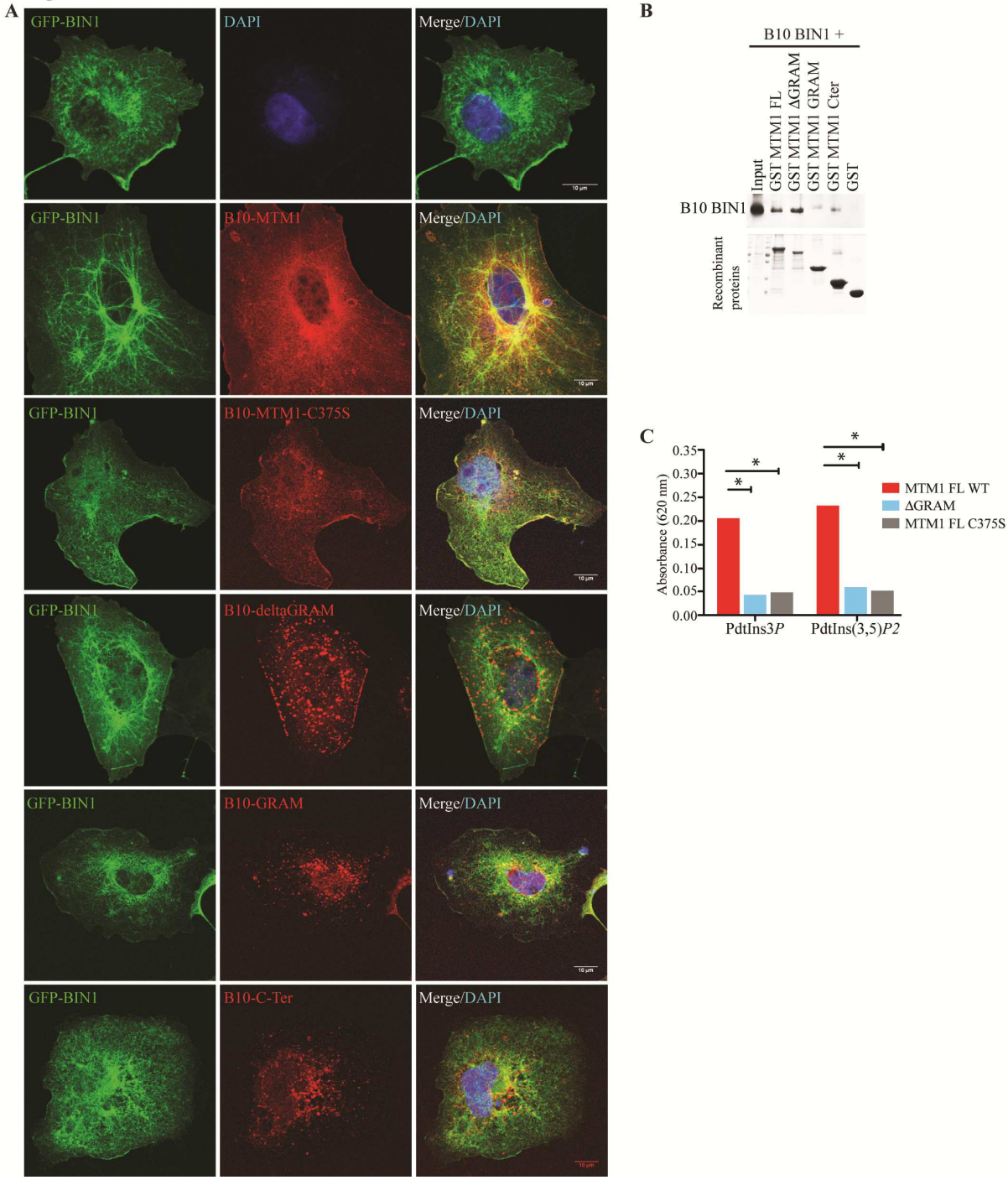

Figure S5. Impact of MTM1 domains on BIN1-mediated membrane tubulation. (**A**) Co-expression in COS-1 cells of GFP-BIN1 with B10-MTM1 (positive control), B10-MTM1 p.C375S (negative control), B10-ΔGRAM, B10-GRAM or B10-Cter. Epifluorescence images. (**B**) ΔGRAM is the binding domain of MTM1 to BIN1. GST (negative control), GST-MTM1 FL, GST-ΔGRAM, GST-GRAM and GSTCter recombinant proteins were used to pull down B10-BIN1. Top panels: Immunoblot hybridized with anti-B10 antibody. Bottom panel: Coomassie Blue staining showing recombinant proteins used for the pull-down. (**C**) MTM1 ΔGRAM has no catalytic activity. Malachite Green assay: dephosphorylation of PtdIns3*P* or PtdIns(3,5)P_2 substrates was quantified in presence of 10 nmoles of GST-MTM1 (positive control), GST-ΔGRAM and GST-MTM1 C375S (negative control).

Figure S6

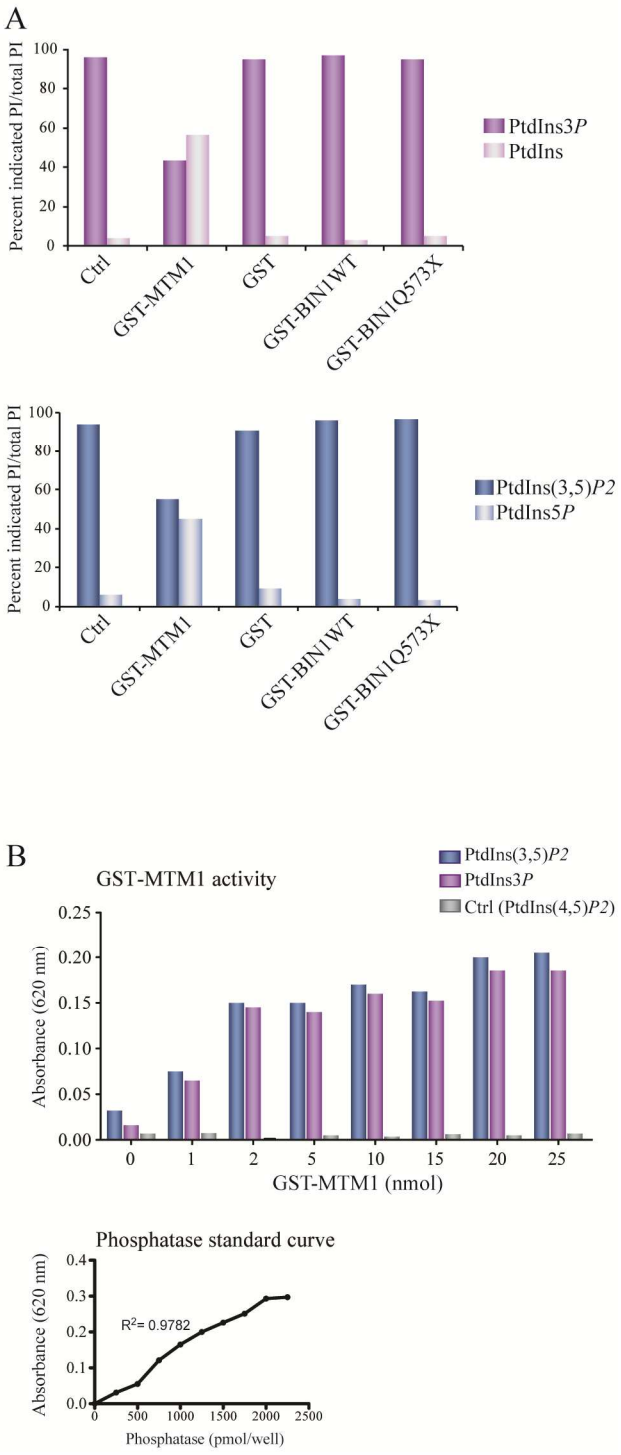

Figure S6. (**A**) BIN1 and GST have no phosphoinositide phosphatase activity. PtdIns and PtdIns5*P* were quantified relative to PtdIns3*P* (top panel) and PtdIns(3,5)P_2 (bottom panel), respectively, in the absence of protein (control) and in the presence of recombinant GST-MTM1, GST, GST-BIN1 wild type or GST BIN1 p.Q573X alone. PtdIns3*P* and PtdIns(3,5)P_2 were in excess, explaining the 50% of dephosphorylation observed with GST-MTM1. (**B**) Determination of MTM1 phosphatase activity by malachite green assay. Dephosphorylation of PtdIns3*P* and PtdIns(3,5)P_2 substrates, and PtdIns(4,5)P_2 (non-substrate control) in the presence of increasing concentration of GST-MTM1.

Figure S7

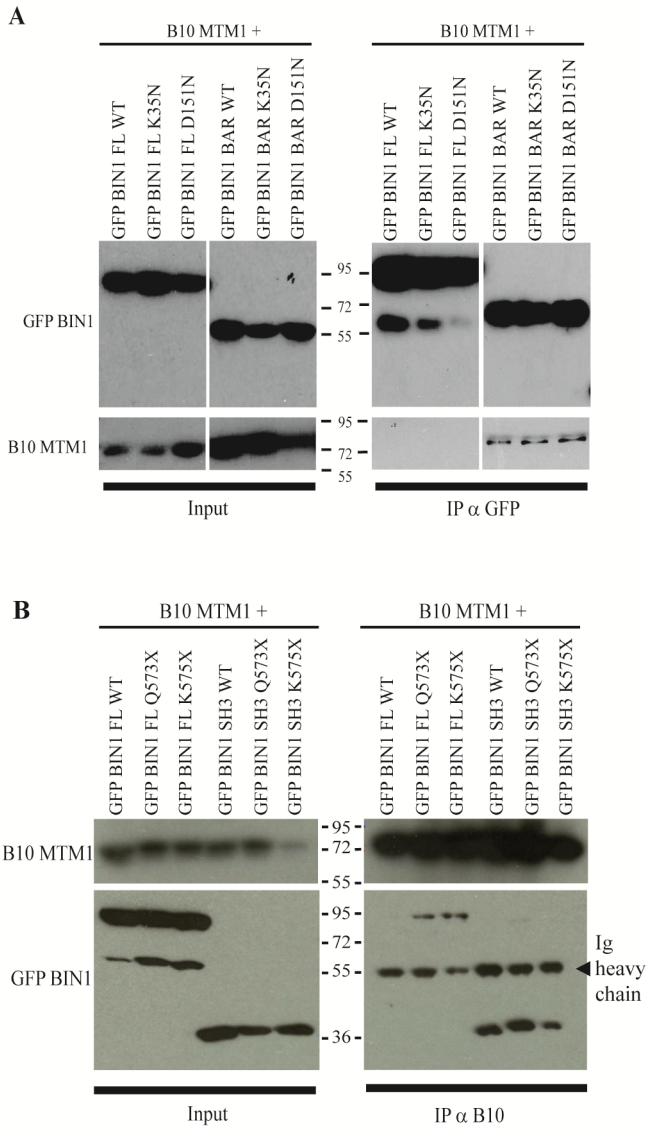

Figure S7. BIN1 mutations in the SH3 domain affect the interaction with MTM1, contrary to BIN1 mutation in BAR domain. (**A**) Autosomal recessive CNM mutations within the BAR domain of BIN1 do not affect co-immunoprecipitation with MTM1. Co-immunoprecipitation assays using anti-GFP antibodies on lysates from COS-1 cells co-transfected with B10-MTM1 and GFP-BIN1 full length and BAR domains presenting p.K35N and p.D151N patient mutations. Top panel: Immunoblot hybridized with anti-GFP antibody. Bottom panel: Immunoblot hybridized with anti-MTM1 antibody. (**B**) Autosomal recessive CNM mutations within the SH3 domain of BIN1 affect MTM1 co-immunoprecipitation. Co-immunoprecipitation assays using anti-B10 antibodies on lysates from COS-1 cells co-transfected with B10-MTM1 and GFP-BIN1 full length and SH3 domain presenting p.Q573X and p.K575X patient mutations. Top panel: Immunoblot hybridized with anti-B10 antibodies. Bottom panel: Immunoblot hybridized with anti-GFP antibodies. Note that all SH3 domains bind MTM1 with equal affinity, while the mutated BIN1 full length proteins have an increased affinity for MTM1 compared to BIN1 full length wild-type.

Table SI: Effect of catalytically inactive MTM1 mutants, MTM1 domains and myotubularin homologues on BIN1 binding and membrane tubulation when co-expressed in C2C12 muscle cells or in COS-1 cells. (*) designates artificial mutations, the R421Q is a patient mutation.

	Phosphatase activity	BIN1 binding	Enhance tubulation
WT FL	+ [1]	+	++
D278A*	- [1]	+	-
C375S*	- [1]	-	-
R421Q	- [8]	+	-
ΔGRAM	- (Fig.S5C)	+	-
GRAM		-	-
Cter		-	-
MTMR1	+ [9]	-	-
MTMR2	+ [9]	-	-
MTMR10	- [10]	-	-
MTMR11	- [10]	-	-

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