Mis-regulation of the alternative splicing of BIN1 is associated with T-tubule alterations and muscle weakness in myotonic dystrophy.

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**Supplementary Figure 1**

### Table

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<th>Xray</th>
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- **b**
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- **f**
  - Scatter plot showing percentage of BIN1 and DMD exon 78 inclusion.

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Nature Medicine doi:10.1038/nm.2374
SUPPLEMENTAL FIGURE 1.
The alternative splicing of the exon 11 of BIN1 is altered in myotonic dystrophy.

(a) Skeletal muscle cell cultures derived from control and age-matched CDM1 (2000 CTG repeats) patients were differentiated into myotubes for 10 days, RNA was extracted from three independent cultures and hybridized on Human Exon 1.0 ST Array (Affymetrix), data were analyzed using XRAY software (Biotique systems) and visualized using FAST-DB database (http://www.fast-db.com/). Top 5 predicted splicing alterations (BIN1, MYOM1, NCAM1, DOCK11, KIF1B) were tested by RT-PCR, and we confirmed robust splicing alteration of BIN1, MYOM1 and NCAM1 pre-mRNAs. Among the various predicted mis-regulated targets, we also tested and confirmed splicing alteration of ATP2A2 (top 30) and PDLIM5 (top 50) pre-mRNAs.

- Myomesin (MYOM1) is a 185 kDa muscle protein which binds to myosin and titin, and is one of the main structural component of muscle fiber M-bands. Alternative exon 18 (also called exon EH) of MYOM1 is included in embryonic heart and in slow skeletal muscle fibers (Agarkova et al., 2004), where it increases the elasticity of the myomesin protein (Bertoncini et al., 2005).

- Neural cell adhesion molecule 1 (NCAM1) is found predominantly during development in nervous tissue and in developing muscle where it participates in muscle innervation and myogenesis through myoblast fusion (Dickson et al., 1990). Inclusion of the alternative exon 20 introduces a polyadenylation signal, which results in a shorter 125-kDa NCAM1 isoform, the expression of which correlates with muscle differentiation, and when overexpressed in transgenic mice, enhances myoblast fusion (Fazeli et al., 1996).

- PDZ and LIM domain 5 (PDLIM5) belongs to a family of adaptor proteins that interact with ACTN1 (α-Actinin) and have an important role in maintaining Z-disc stability in striated muscle (Zhou et al., 2001). Alternative splicing of the exon 9 introduces a polyadenylation signal, resulting in a shorter isoform of PDLIM5. Note that alterations of the alternative splicing of PDLIM3 (ALP), PDLIM6 (LDB3/ZASP/CYPHER) and PDLIM7 (ENIGMA/LMP1) in DM patients or CUG expressing mice have been reported previously (Lin et al., 2006; Du et al., 2010), suggesting a coordinated mis-regulation of the PDLIM family by alternative splicing in DM.

- ATP2A2 (SERCA2) encodes an ion pump that translocates calcium from the cytosol into the sarcoplasmic reticulum lumen of muscle cells, and is involved in regulation of the excitation-contraction coupling. Alternative splicing of the intron 20 introduces a
polyadenylation signal, resulting in a shorter isoform of ATP2A2. Alterations of the alternative splicing of both ATP2A1 and ATP2A2 pre-mRNAs in Myotonic dystrophic patients have been reported previously (Kimura et al., 2005; Lin et al., 2006). Mis-regulation of the splicing of ATP2A1, ATP2A2 and RYRI pre-mRNAs in DM results in subtle modifications of the channel-properties of these proteins (Kimura et al., 2005), which are probably not sufficient to fully explain the muscle weakness in DM, but point toward an alteration of the calcium homeostasis and/or of the excitation-contraction coupling in DM. A model consistent with alterations of BIN1 and T-tubules in DM.

• Lastly and topic of this study, BIN1 is involved in membrane remodeling, has a role in organization of muscle T-tubules and is mutated in autosomal recessive centronuclear myopathy (Nicot et al., 2007). Mammals possess two amphiphysin genes, amphiphysin 1 and amphiphysin 2 (BIN1). Amphiphysin 1 is a neuronal protein enriched in nerve terminals and is involved in endocytosis (David et al, 1996). In contrast, bridging integrator 1 or amphiphysin 2 (BIN1/AMPH2/SH3P9) is widely present in various tissues, but is most highly expressed in the brain and striated muscles (Butler et al, 1997). BIN1 is extensively regulated by alternative pre-mRNA splicing. All BIN1 splicing forms possess a common NH2-terminal BAR domain, by which BIN1 induces membrane curvature, and a common COOH-terminal SH3 domain (Ramjaun and McPherson, 1998), by which BIN1 binds to canonical PxxP sequences of other proteins, such as Synaptojanin and Dynamin 2 (Wigge and McMahon, 1998). In brain, the splicing form of BIN1 excludes the exon 11, includes the exon 7 (also called exon 6B) of unknown function and the brain-specific exons 13 to 16 (also named exon 12A to 12D) encoding a CLathrin-Associated Protein binding region (CLAP) bearing the clathrin- and AP-2-binding sites, which was why BIN1 has been first suggested to be involved in endocytosis (Butler et al, 1997; Wigge and McMahon, 1998). In contrast, in skeletal muscle, the splicing form of BIN1 (also called isoform 8) excludes its exon 7 and exons 13 to 16 (clathrin- and AP-2-binding sites), but includes the muscle-specific exon 11 (named exon 10 in some studies) encoding the basic amino-acid sequence RKKSKLFSRLRKRKN (Ramjaun and McPherson, 1998; Wechsler-Reya et al, 1998), which binds to phosphoinositides and is involved in skeletal muscle transverse tubule (T-tubule) biogenesis (Lee et al., 2002). T-tubules are specialized invaginations of the muscle plasma membrane that serve to propagate action potentials to the interior of muscle fibers (Flucher, 1992), ultimately resulting in muscle contraction through Ca2+ release from the sarcoplasmic reticulum. Therefore, structural alteration of the T-tubule network leads to excitation-
contraction coupling dysfunction, ultimately resulting in muscle weakness (Oddoux et al., 2009; Brotto et al., 2004; Ito et al., 2001).

(b-c) RT-PCR analysis using primers located within \textit{BIN}1 exon 6 and 8 confirms Exon 1.0 ST Array prediction and demonstrates an increase of exon 7 inclusion in skeletal muscle biopsies of CDM1, DM1 and DM2 patients. Further RT-PCR experiments using primers located within \textit{BIN}1 exon 6 and 12 demonstrate that the aberrant inclusion of the exon 7 is independent of the abnormal skipping of the muscle-specific exon 11, thus, resulting in expression of three abnormal splicing forms of \textit{BIN}1 in skeletal muscles of DM patients: \textit{BIN}1 plus exons 7 and 11 (+ex7-ex11), \textit{BIN}1 plus exon 7, but minus exon 11 (+ex7-ex11), and \textit{BIN}1 minus exons 7 and 11 (-ex7-ex11). All three isoforms, plus the splicing form of \textit{BIN}1 found in normal muscles (-ex7-ex11), were cloned and tested for tubulation \textit{in cellulo} and for phosphoinositides binding \textit{in vitro} (data not shown). All tests were consistent with no or little function of the exon 7 for tubulation and phosphoinositides binding, while presence of the exon 11 is required for \textit{BIN}1 to tubulate membrane and to bind to PtdIns5P and PtdIns3P. Therefore we focused our study on \textit{BIN}1 exon 11.

(d) Skipping of \textit{BIN}1 exon 11 in DM recapitulates an embryonic situation. RT-PCR analyses demonstrated that alternative splicing of \textit{BIN}1 exon 11 is developmentally regulated with progressive inclusion of the exon 11 during human skeletal muscle development. Similar results were obtained by western-blotting analysis using an antibody directed specifically against exon 11 (data not shown). (e) Finally, progressive inclusion of exon 11 during normal muscle development but not in age-matched CDM1 patients, was confirmed by real-time RT-PCR.

(f) Skipping of \textit{BIN}1 exon 11 in DM1 patients correlates with skipping of \textit{DMD} (Dystrophin) exon 78, which was previously shown to be altered in DM1 patients (Nakamori et al., 2007), suggesting that a common mechanism contributes to their splicing alterations.
Supplementary Figure 2

a

β-globin

FL

58bp

11

45bp

12

GUUCUGCACCUGGCCUUUCUCCAGAAUGAAGGCCUCCACCUCCCGUCCGUCCCCACAG

AAAGAAAAGUAAACUGUUUUCGCGGCUGCGCAGAAAGAAGAACAG

GUACCGGCAGUGAGUGCUGCGGAGGGGCGCAGAGGCCCGCGCC

CUGGCUGGCCCUGUGCAUGCGCCUUGCGCCCUGCUCCCAGGUGCCACUAAC

CCGUAAUCUGGCUCUGUGUGCAGUGCUGCCCGGCAGGGCUGUCGUGUGCGUGUUGGG

b

BIN1 #D: AGGGGCGCAGAGGCCCGCGCCCUGGCUGGCCCUGUGCAUGCGCCUUGCGCCCUGCUCCCAGGUGCCACUAAC

CUGUGCAUGC

CUGCG

CCUUGCGC

CCUGCUC

CCUGUC

UCGCUUUUCCC

CUCCGC

UGCGG

BIN1 RNA

#D

BIN1 hs

UCUGCJ

UCGJG

UUAAGUUAUUUCGCUU

MBNL1

UCUGC

UGCG

UCGCG

ATP2A1

GUCUGC

CCUGC

UGC

TNNT2

CCUUGC

UCGCCUUUCCC

CUCUGC

UGC

MBNL1

UCUGC

UGCGJ

UCGUGA

UGCCAU

UGCUU

MBNL1

UCUGC

UGCGJ

UCCUGA

UGCG

INSR

UCUGC

UGCGC

AGG

GRIN1

UCUGC

UCCUGA

UGC

INSR

UCUGC

UGCGC

CUGCG

CUGCG

UGCG

e

CUG 10x

BIN1 hs

BIN1 mm

BINS RNA

#D

BIN1 hs

UCUGC

UCG

UCG

BIN1 mm

Nature Medicine doi:10.1038/nm.2374
SUPPLEMENTAL FIGURE 2.

MBNL1 regulates the alternative splicing of the exon 11 of BIN1.

(a) Illustration of the human BIN1 minigene and sequence of the fragments tested for MBNL1 binding by UV-crosslinking assays. BIN1 exon 11 (fragment B) is bordered by 58 nts of its upstream intron 10 (fragment A) and 157 nts of its downstream intron 11 (fragment C, D and E). Putative MBNL1 binding sites (UGC motifs) are indicated in blue.

(b) Identification of the MBNL1 binding site on BIN1 intron 11 fragment D by RNase T1 structure probing. Identification of MBNL1 binding site within BIN1 intron 11 fragment D. Binding of recombinant purified GST-MBNL1Δ protects four guanosines (G58, G62, G69 and G76; indicated in blue) from RNase T1 cleavage. Protected guanosines are inserted within the single-stranded sequence YGC(U/G)Y, which is close to the MBNL1 binding consensus sequences determined previously (Ho et al., 2004, Goers et al., 2010). Note that the GST-MBNL1ΔCter, which contains its four zinc fingers but is deleted of the last 101 amino acids of the C-terminal part of MBNL1, has identical affinity and specificity but better solubility and stability for production and purification in E. Coli than full-length GST-MBNL1-HIS (data not shown).

(c) The protection of four single-stranded regularly spaced YGC motifs within BIN1 RNA fragment D is consistent with the crystal structure analysis of MBNL1 (Teplova and platel., 2008), which demonstrates that the guanosine of each YGC motif is buried inside each zinc finger of MBNL1. According to the opposite orientation of ZnF3 and ZnF4 domains, binding of MBNL1 to its RNA dictates an antiparallel orientation to the bound YGC sequence (Teplova and platel., 2008), suggesting that binding of MBNL1 to BIN1 intron 11 provokes a looped RNA conformation, whose importance for splicing regulation remains to be determined. Furthermore, such peculiar zinc finger architecture suggests that MBNL1 may bind either to one looped RNA molecule, or to two separated RNA molecules, resulting in MBNL1-mediated intra- or inter-strand RNA bridges. A model, in which MBNL1 loops or links different RNA molecules composed of expanded CUG repeats, is consistent with the formation of unsoluble CUG aggregates and the consequent sequestration of MBNL1 in DM.
(d) Alignment of MBNL1 binding sites within human BIN1, mouse Tnnt3 (sTNI) (Yuan et al., 2007), human TNNT2 (cTNT) (Ho et al., 2004; Warf et al., 2007), human MBNL1, MBNL2, INSR, GRIN1 and ATP2A1 RNAs (Goers et al., 2010; Grammatikakis et al., 2010), reveals presence of conserved YGC motifs regularly positioned. Presence of 3 to 4 YGC motifs correlates with a high affinity of MBNL1 for its target RNA (KD < 20 nM). In contrast, presence of two (INSR, KD of 120 nM) or only one (GRIN1, KD of 280 nM) YGC motif correlates with lower affinities to MBNL1. The KD of MBNL1 for MBNL1, MBNL2, ATP2A1, INSR(1), and GRIN1 RNAs are from the study of Goers et al., 2010. The KD of MBNL1 for INSR(2) RNA is from Grammatikakis et al., 2010. Affinity of recombinant purified GST-MBNL1Δ to expanded CUG (10 repeats), expanded CCUG (10 repeats) and BINI intron 11 fragment D RNAs were measured by gel-shift assays (KD of 2.0 +/- 0.2 nM, 3.1 +/- 0.1 nM and 7 +/- 0.4 nM, respectively).

(e) The YGC motifs located downstream of BIN1 exon 11 are not fully conserved between human and mouse. UV-cross-linking assays demonstrated that recombinant purified GST-MBNL1Δ binds with higher efficiency to human BIN1 compared to mouse Bin1 sequence. These data suggest that MBNL1 may weakly promote inclusion of exon 11 in mouse compared to human. An hypothesis consistent with modest exon 11 splicing alterations (10 to 30% of exon 11 skipping) observed in RNA samples of skeletal muscle of mice deficient for Mbnl1 (Mbnl1^{AE3/AE3} mice, gift from Maury Swanson), or in mice expressing expanded CUG repeats (HSA^{LR} mice, gift from Charles Thornton and DM300 mice, gift from Genevieve Gourdon).
Supplementary Figure 3

(a) COS1 cells and C2C12 cells showing GFP-BIN1 + ex11 and GFP-BIN1 - ex11.

(b) Graph showing pmol of PtdIns for BIN1 + exon 11 and BIN1 - exon 11.

(c) Images showing GFP-ING2PHD, GFP-P40PX, and GFP-PLC5PHD.

(d) Images showing BIN1 H100 and BIN1 99D.

(e) Images showing BIN1 and ACTN1 with DM1 and control conditions.

(f) Bar graph showing % cells with tubules for different conditions.
SUPPLEMENTAL FIGURE 3.
The exon 11 of BIN1 binds to PtdIns5P and is required for membrane tubulation.

(a) Representative confocal images of COS1 and non-differentiated C2C12 cells transfected with cDNA constructs encoding control (+ex11) or DM (-ex11) isoforms of BIN1 fused to eGFP. As noted previously, the over-expression of the normal muscle isoform of BIN1 (+ex11) in cells structures the cell membrane in numerous narrow tubules, which are different from microtubules, actin and intermediate filaments (Lee et al., 2002; Peter et al., 2004; Kojima et al., 2004; Casal et al., 2006; Löw et al. 2008; Meunier et al., 2009; Spiegelhater et al., 2010). In contrast, the expression of the DM splicing isoform of BIN1 (-ex11) does not induce formation of membrane tubules and BIN1 is diffuse within the cytoplasm. Scale Bars: 10 μm.

(b) Binding of purified bacterial recombinant full-length GST-tagged BIN1 control (+ex11) or DM (-ex11) isoform to PIP arrays (Echelon Biosciences) containing the serial dilutions of the indicated PtdInsPs. BIN1 was detected by immunoblot using a pan-isoform antibody (99D, Upstate). In contrast to a previous study (Lee et al., 2002), little binding of full-length GST-BIN1 to PtdIns4P or to PtdIns(4,5)P2 was observed, which is probably due to differences in techniques (no PtdIns3P or PtdIns 5P in Lee et al.) or constructs (full-length versus truncated BIN1 in Lee et al., 2002).

(c) Representative confocal images from human skeletal muscle cells derived from a control individual, differentiated 6 days and transduced with recombinant adenovirus (MOI of 1500) expressing GFP fusion constructs fused to three copies of the PHD domain of ING2, the PX domain of P40 or the PH domain of PLCγ, which recognized PtdIns5P, PtdIns3P and PtdIns(4,5)P2, respectively. BIN1 was immunostained using a pan-isoform antibody (99D, Upstate). Localization of the GFP-P40^{PX} grip within cytoplasmic vesicles is consistent with the known localization of PtdIns3P in early and late endosomes. Localization of the GFP-PLCγ^{PH} grip is consistent with the known localization of PtdIns(4,5)P2 to the plasmic membrane. Localization of the GFP-ING2^{PHDx3} grip suggest co-localization of PtdIns5P within membrane tubules containing BIN1. Scale bars: 10 μm.
(d) Immunofluorescence labeling of BIN1 with the polyclonal H100 (SantaCruz) and monoclonal 99D (upstate) antibody in paraffin-embedded longitudinal sections of skeletal muscles from WT mice revealed perfect co-localization. Note that the normal localization and organization of BIN1 with CACNA1C or RYR1 in T-tubules can be inconsistent and of poor quality in frozen muscle sections, and that paraffin-embedded sections are better to preserve BIN1 structures (data not shown).

(e) Representative confocal images of immunofluorescence labeling of BIN1 (polyclonal H100, SantaCruz) and ACTN1 (α-Actinin, clone SA20, Abnova) in paraffin-embedded longitudinal sections of skeletal muscles from adult DM1 and age-matched control individual.

(f) Quantification of the number of muscle cells presenting significant BIN1 tubules. CDM1 (2000 CTG repeats) or DM1 (1300 CTG repeats) myotubes differentiated 6 days were transduced (MOI of 1000) with an adenovirus expressing either the normal muscle form of BIN1 (+ex11) or the DM (-ex11) isoform of BIN1 fused to the eGFP. Control GFP-BIN1 (+ex11) induced numerous BIN1 tubules in CDM1 and DM1 myotubes whereas expression of the DM splicing variant of GFP-BIN1 (-ex11) did not. Data are presented as means ± standard deviation.
SUPPLEMENTAL FIGURE 4.

The skipping of the exon 11 of Bin1 leads to muscle weakness in adult mice.

(a) Efficiency of the U7-ex11AS construct. Increasing amount of a plasmid expressing either a control or an antisense sequence of BIN1 exon 11 (U7-ex11AS) was co-transfected in C2C12 cells with human BIN1 minigene. Inclusion of BIN1 exon 11 was tested by RT-PCR 48 hours after transfection.

(b) Skipping of Bin1 exon 11 did not induced major muscle degeneration 4 months after injection. Hematoxylin and eosin staining and muscle fiber size quantification of transversal sections of tibialis anterior muscles injected with AAV2/1 expressing control or U7-ex11AS construct demonstrated no sign of regeneration, significant atrophy or central nuclei. Data are presented as means ± standard deviation. Note that a similar absence of central nuclei was observed in a knock-in mouse model of CNM expressing a R465W muted Dynamin 2 (Durieux et al., 2010), suggesting that mice may only partially reproduce CNM.

(c) Osmium tetroxide-potassium ferricyanide staining of T-tubules in longitudinal sections of TA muscle injected with U7-ex11AS-AAV2/1 revealed alterations of T-tubules structures in absence of major degeneration of the muscle. 300 to 500 T-tubules from muscles injected with CTL (n=3) or U7-ex11AS (n=3) AAV2/1 were analyzed.

(d-f) Skipping of Bin1 exon 11 induced muscle weakness also in adult mice. AAV2/1 expressing control or U7-ex11AS constructs were injected in the tibialis anterior muscles of adult (2 months old) mice and isometric tetanic force, muscle mass, BIN1 localization and muscle structure were analyzed 4 months later. U7-ex11AS-AAV adult injected mice demonstrate a significant (p<0.05) decrease of ~18% of their muscle force (d), but no significant changes in their muscle mass (e). Immunofluorescence labeling demonstrates alteration of the BIN1 organization in U7-ex11AS-AAV2/1 injected TA muscles of adult mice but not in control contralateral injected muscles (f). In contrast, immunofluorescence labeling of Desmin or α-Actinin revealed no alterations of the Z band structures (data not shown). Electron microscopy analysis revealed that similarly to injected new-born mice, adult injected mice presented alterations of T-tubules structures in absence of major muscle
degeneration (data not shown). These data suggest that BIN1 is required for the biogenesis and the preservation of T-tubules both post-natally and in adult life.

(g) Skipping of Bin1 exon 11 induced mis-expression of key regulators of the excitation-contraction coupling process. Quantitative RT-PCR analysis demonstrated significant down-regulation of Cacna1s, Ryr1 and Atp2a1 mRNAs, while the levels of the chloride channel Clcn1, the sodium-calcium exchanger Ncx1, the calcium channel subunit b1 (Cacnb1 or Dhprβ1), Triadin (Trdn), Calsquestrin (Casq1), Troponin 3 (Tnnt3) and amphiphysin 2 (Bin1) mRNAs were normal. Data are presented as means ± standard deviation. *** p<0.001, * p<0.05.

(h) Western blotting analysis of TA muscle from control or U7-ex11AS-AAV2/1 injected mice using antibodies directed against Bin1 exon 17 (99D, Upstate), Bin1 exon 11 (R2406, Nicot et al., 2007), Cacna1s (mAB-1a, Chemicon), Ryr1 (34C, Sigma) and Gapdh (9484, Abcam). Data are presented as means ± standard deviation. * p<0.05. These data suggest that, similarly to the Mtm1 knock-out model (Al-Qusairi et al., 2009), the mis-regulation of Bin1 leads to specific alteration of key regulators of the excitation-contraction (E-C) coupling process. Altered E-C coupling gives rise to muscle weakness (Ito et al., 2001; Brotto et al., 2004; Oddoux et al., 2009; Piétri-Rouzel et al., 2010).
SUPPLEMENTAL FIGURE 5.

Model of T-tubule alterations and muscle weakness in myotonic dystrophic patients. In DM, expanded CUG or CCUG repeats sequester MBNL1 within intranuclear aggregates, resulting in decrease levels of free MBNL1 available to regulate alternative splicing. Consequently, the exon 11 of BIN1 pre-mRNA, which is normally included in adult skeletal muscle, is skipped in DM patients and results in expression of a fetal isoform of BIN1 unable to bind to PtdIns5P and to tubulate membranes, leading to misorganization of the T-tubule network and alteration of the excitation-contraction (E-C) coupling.
SUPPLEMENTAL METHODS

Human Exon 1.0 ST Array profiling and analysis. Total RNA of control and CDM1 primary culture of muscle cells differentiated into myotubes was extracted using RNeasy columns (Qiagen). RNA quality was verified by analysis on the 2100 Bioanalyzer (Agilent). All samples displayed a 28S/18S ratio >1.5 and RNA Integrity Number > 9.0. Biotinylated cDNA targets were prepared and hybridized according to the GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (P/N 701880 Rev.2, Affymetrix, Santa Clara, USA), using 2 µg of total RNA as starting material. Hybridization was performed on GeneChip® Human Exon 1.0 ST array using 5 µg of biotinylated target at 45°C for 16 hours. The chips were washed and stained in the GeneChip® Fluidics Station 450 (Affymetrix) using the FS450-0001 script. The arrays were scanned with the GeneChip® Scanner 3000 7G (Affymetrix) and raw data (.CEL Intensity files) were extracted from the scanned images using the Affymetrix GeneChip® Operating Software (GCOS) version 1.4. Data were analyzed using Xray (biotique Systems) and the EASANA visualization module (GenoSplice technology). Data are available in the GEO database http://www.ncbi.nlm.nih.gov/geo under the accession number GSE21795.

RT-PCR analysis of patient’s samples.
Total RNA was isolated from control or CDM1 differentiated muscle cells or skeletal muscle biopsies from control, ALS or DM patients with Trizol Reagent (Invitrogen). Reverse transcription, using 1 µg of total RNA, was performed with SuperScript II (Invitrogen), according to the manufacturer’s instructions. 2 µl of reverse transcription were used for PCR using Taq polymerase according to the manufacturer’s instructions, and primers located within BIN1 exon 10 (FWD: 5’-AGAACCTCAATGATGTGCTGG-3’) and BIN1 exon 12 (REV: 5’-TCGTGGTTGACTCTGATCTCGG-3’). The reaction mixture was heated to 94°C for 5 min and followed by 25 PCR cycles: 1 min at 94°C, 45 sec at 58°C and 1 min at 72°C. PCR products from BIN1 plus and minus exon 11 mRNAs, which are 208 and 163 bp, respectively, were resolved on 6% nondenaturing polyacrylamide gels, BET stained and quantified by PhosphorImager.
**Real-time RT-PCR analysis of patient’s samples.**

Specific primers were selected by Lightcycler Probes Design2 software (Roche), and were located in the ubiquitous exon 1 (Fwd: 5’-GAGATGGGCAGTAAAGGG-3’) and exon 2 (Rev: 5’-CTTGTTGAAATCTCTTGGACGCA-3’) to quantify the total amount of BIN1. While to quantify BIN1 exon 11, the primers were located in exon 10 (Fwd: 5’-AATGATGTGCTGGTCCGCT-3’) and exon 11 (Rev: 5’-GTCTTTGTCTTCTGCGACG-3’). Real-time PCR was performed using a Lightcycler 480 (Roche). Reactions were performed in a 20 µl final volume with 0.3 µm primers and MgCl2 concentration optimized between 2-5 mM. Nucleotides, Taq DNA Polymerase and buffer were included in the QuantiTect SYBR Green PCR kit (QIAGEN) according to the manufacturer’s instructions. PCR cycles were a 15 min denaturation step followed by 50 cycles with a 94°C denaturation for 15 sec, 55°C annealing for 20 sec, and 72°C extension for 20 sec. RPLP0 mRNA was used as standart (Fwd: 5’-GAAGTCACTGTGCCAGCCCA-3’ and Rev: 5’-GAAGGTGTAATCCGTCTCCA-3’). Data were analysed with the Lightcycler 480 analysis software and the relative quantification of BIN isoforms were obtained with 2ΔCt method.

**Constructions.**

BIN1 (+ex7+ex11+ex17; a rare DM muscle isoform), (+ex7-ex11+ex17, a rare DM muscle isoform), (-ex7-ex11+ex17, a common isoform in DM skeletal muscle) (-ex7+ex11+ex17, the normal skeletal muscle isoform) were cloned by depletion or insertion of exon 7, exon 11 or exon 17 from BIN1 NM_139343.1 (Bridging Integrator 1 isoform 1, +ex7-ex11+ex13-16+ex17) and BIN1 NM_004305.2 (Bridging Integrator 1 isoform 8, -ex7+ex11-ex13-16-ex17) by primer-directed PCR mutagenesis from eGFP-BIN1 (gift from Pietro De Camilli). Presence of the alternative exon 17 was determinant for further recognition by the anti-BIN1 99D (Upstate) antibody. All BIN1 isoforms were cloned in peGFP or in pGEX. eGFP-BIN1 fusion constructs were PCR amplified and cloned into pENTR-D Topo vector (Invitrogen), then in pAd-DEST using the ViralPower Adenoviral Expression System (Invitrogen). eGFP-P40(PX) (gift from Karen Anderson) and eGFP-ING2(PHD) (gift from Or Gozani) constructs were PCR amplified, cloned into pENTR-D Topo vector (Invitrogen), then in pAd-DEST (Invitrogen).
**Cell culture and BIN1 transfection.**

COS1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FCS and 40 mg/l gentamicin in a 5% CO2 incubator at 37°C. Cells were grown on 22-mm2 cover slips and were transfected at 80% confluence with 1 µg of DNA constructs using the Fugene-6 reagent, following the manufacturer’s instructions (Roche). After 24 h of transfection, cells were fixed with 4% paraformaldehyde for 24 h at 4°C. C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20 % FCS and 40 mg/l gentamicin in a 5% CO2 incubator at 37°C. Cells were grown on 22-mm2 cover slips. For C2C12 myoblasts, cells were transfected at 60% confluence with 1 µg of DNA constructs using lipofectamine reagent, following the manufacturer’s instructions (Invitrogen). After 3 h of transfection, cells were fixed with 4% paraformaldehyde for 24 h at 4°C. For C2C12 myotubes, cells were transfected at 60% confluence with 1 µg of DNA constructs using lipofectamine supplemented with +Reagent reagents, following the manufacturer’s instructions (Invitrogen). After 3 h of transfection, cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% Horse Serum and 40 mg/l gentamicin in a 5 % CO2 incubator at 37°C during 5 days of differentiation. Cells were then fixed with 4% paraformaldehyde for 24 h at 4°C. Fluorescence was examined with Leica SP2-AOBS confocal microscope.

**Muscle tissue lysate preparation**

Lysate of whole tissue was prepared from mouse (freshly dissected) skeletal muscle. Tissue was homogenized with a polytron (3 times 5 sec., speed 5) in lysis buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP40 and 1 protease inhibitor cocktail tablet (1/10ml buffer, Roche, Indianapolis, IN)). Homogenate was centrifuged (10 min, 14 000 x g) and the pellet discarded. Protein concentration was determined by Bradford assay (Biorad).

**Western blot.**

Proteins were extracted from muscle biopsies of two control foetuses (20 and 31 weeks) and two age-matched CDM1 foetuses. The total proteins extracts were separated on 10% SDS-PAGE gel, and transferred onto nitrocellulose membranes that were blocked with 5% non-fat dry milk in TBS-T buffer (Tris buffer saline plus 0.1% Tween-20). Incubation with primary antibodies was performed for 1 hour in TBS-T buffer containing 5% non-fat dry milk. BIN1 was detected with antibody directed etheir against the phosphoinositides binding domain encoded by exon 11 (1:10000, polyclonal antibody anti-BIN1 R2406, Nicot et al., 2007) or
against the myc-binding domain encoded by exon 17 (1:250, monoclonal antibody 99D, Upsate). Secondary antibodies (anti-rabbit-Horseradish Peroxidase or anti-mouse-HRP, Jackson ImmunoResearch) were incubated for 1 hour, followed by autoradiography. Equal loading was monitored by Ponceau red and Coomassie staining.

**Protein purification.**

E. coli BL21(RIL) pRARE competent cells (Invitrogen) were transformed with pGEX-MBNL1-D101 (Nterminal GST tagged MBNL1ΔCter) or pGEX-BIN1 (Nterminal GST tagged BIN1 + or – exon 11) and grown at 37°C in 400 ml of LB medium supplemented with 1 mM ZnSO4 and ampicillin until OD600=0.5, 0.5 mM IPTG was added and the culture was further incubated 4 hours at 30°C. Harvested cells were sonicated in 50 mM Tris-Cl pH 7.5, 300 mM NaCl, 5% glycerol, 1 mM DTT, 5 mM EDTA, centrifuged 20 min at 20000 g and recombinant GST-BIN1 or GST-MBNL1ΔCter was purified using GST-Binds Kits (Novagen). The eluate was dialyzed against storage buffer (50 mM Na-HEPES pH 7.5 - 150 mM NaCl - 55% glycerol - 2 mM DTT) for 48 hours at 4°C. Protein concentration was determined by Coomassie protein assay (Pierce).

**RNA-protein UV-crosslinking.**

Wild type or mutated BIN1 RNAs were obtained by PCR including a T7 promoter in the forward oligonucleotide followed by T7 transcription kit (Ambion). 0.5 µg of recombinant GST-MBNL1ΔCter protein was incubated 10 min at 30°C with 20 000 cpm of internally αP32-CTP-labeled RNA in 10 µl containing 25 mM Na-HEPES pH 7.5, 0.3 mM MgCl2, 200 mM KCl, 0.2 mM Na-EDTA, 0.1 mg/ml BSA, 0.1 mg/ml total yeast tRNA, 0.1 mg/ml heparin and 0.5 mM DTT. Reactions were transferred on parafilm and irradiated 5 min, on ice, at 2.5 cm of a UV lamp (Vilberloumat VL-100C). 1 µg of RNase A1 was added and incubated 20 min at 37°C. RNA-protein complexes were resolved on 10% SDS-PAGE and analyzed by Coomassie protein assay (Pierce), dried and analyzed on Typhoon.

**Lipid dot-blot assays.**

Nitrocellulose membranes spotted with increasing amounts of various phosphoinositides (PIP arrays) were purchased from Echelon Biosciences and used according manufacturer’s instructions. Briefly, the membrane was incubated with 5 mL of purified full length GST-BIN1 at a final concentration of 10 µg/mL in TBS-TB (TBS supplemented with 0.1 % Triton X-100 and 1 mg/mL fat-free BSA) for 30 min at room temperature. After five washes with
TBS-TB, the membrane was incubated with BIN1 C99D antibody (Upstate) for 30 min, washed five times with TBS-TB, incubated with secondary antibody (peroxydase-labeled anti-mouse), and washed five times with TBS-TB. Detection was performed by chemiluminescent detection system (ECL, thermo scientific).

**Surface Plasmon Resonance (SPR) experiments.**

SPR experiments were performed using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; Fluka) liposomes spiked with 5% PtdIns3P, PtdIns5P or PtdIns(4,5)P2 (Echelon). Liposomes were prepared and coated on the L1 chip as described previously (Mortier, E., et al., 2005, EMBO J), and the experiments were carried out in a BIACORE 2000. The reference channel was coated with 5% PtdIns containing liposomes. Proteins were dialyzed to the running buffer (25 mM HEPES, 150 mM NaCl, pH 7.4) and perfused over the immobilized liposomes at a flow rate of 30 μl/min in a range of concentrations (0.01 -2 μM). The surface was regenerated by short pulses of 50 mM NaOH. The obtained sensograms were double reference subtracted and apparent KD values for PtdIns3P, PtdIns5P and PtdIns(4,5)P2 of GST-Bin1 (+ ex11) were determined by plotting the signals observed at equilibrium (Req) against the protein concentrations and fitting the data to a 1:1 Langmuir binding isotherm (GraphPad Prism).

**Human skeletal muscle cell culture and adenoviral infection.**

Control or DM1 myoblasts were grown and differentiated as previously described (Furling D. et al., 2001). For infection, myoblasts were differentiated on gelatin-coated coverglasses in 6-wells plates for 5-6 days. The EGFP-BIN1, eGFP-P40(PX) and eGFP-ING2(PHD) constructs were PCR amplified and cloned into pENTR-D Topo vector (Invitrogen), then in pAd-DEST using the ViralPower Adenoviral Expression System (Invitrogen). The recombinant adenoviruses were produced in 293A cells (invitrogen) using classical procedures (Lagrange M. et al., 2007). The myotubes were transduced with recombinant adenoviruses (MOI of 1000 to 2000) and the medium was replaced by fresh differentiation medium 17 hours post-infection. The cells were fixed 48 hours after in 4% paraformaldehyde,

**Electron Microscopy.**

Muscle samples were fixed in 2.5% glutaraldehyde and 0.1M sodium cacodylate buffer (PH=7.2) for 24h at 4°C, washed in 0.1M cacodylate buffer for 30 minutes and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hour at 4°C. For selective staining of T-tubules, the samples were postfixed with 2% OsO4, 0.8% K3Fe(CN)6 in 0.1 M cacodylate buffer.
buffer (pH 7.4) for 2 h at 4 °C and incubated with 5% uranyl acetate for 2 h at 4 °C. Following stepwise dehydration with increasing concentrations of ethanol and embedding in Epon 812. Semi-thin sections (2µm thick) were stained with toluidine blue for light microscopy, ultrathin sections (70nm) were contrasted with uranyl acetate and lead citrate and observed with a Philips Morgagni 268D electron microscope.

**Oligonucleotides for splicing analysis by RT-PCR.**

Splicing analysis by RT-PCR of the endogenous human *BIN1* exon 11:
FWD: 5’-AGAACCTCAATGATGTGCTGG-3’
REV: 5’-TCGTGTTGACTCTGATCTCGG-3’

Splicing analysis by RT-PCR of the ectopic human *BIN1* exon 11 minigene:
FWD: 5’-CATTCCACCACATTTGCTGTGC-3’
REV: 5’-AAGTGATCCTAGACTAGCCGCC-3’

Splicing analysis by RT-PCR of the endogenous mouse *Bin1* exon 11:
FWD: 5’-TCAATGATGTCCTGCAGC-3’
REV: 5’-GCTCATGGTTGACTCTGATC-3’

Splicing analysis by RT-PCR of the endogenous mouse *Mbnl1*:
FWD: 5’-GCTGCCCAATACCAGGTCAAC-3’
REV: 5’-TGGTGGGAGAAATGCTGTATGC-3’

Splicing analysis by RT-PCR of the endogenous mouse *Pdlim6 (Cypher, Zasp)*:
FWD: 5’-GGAAGATGAGGCTGATGAGTGG-3’
REV: 5’-TGCTGACAGTGGTAGTGCTCTTTC-3’

Splicing analysis by RT-PCR of the endogenous mouse *Atp2a1 (Serca1)*:
FWD: 5’-GCTCATGGTGCTCTCAAGATCTCAGG-3’
REV: 5’-GGGTCAGTGCCTCAGCTCTTTC-3’

Splicing analysis by RT-PCR of the endogenous mouse *Clcn1*:
FWD: 5’-GGAATACCTCACCACACTCAAGGCC-3’
REV: 5’-CACGGAACACAAAGGCACCTGATGT-3’
Oligonucleotides for expression analysis by quantitative RT-PCR.

**Human:**

*BIN1* exon 1 FWD: 5’-GAGATGGGCAGTAAAGGG-3’  
*BIN1* exon 2 REV: 5’-CTTGTTGAATTCTGGACGC-3’

*BIN1* exon 10 FWD: 5’-AATGATGTGCTGGTGGCCCT-3’  
*BIN1* exon 11 REV: 5’-GTTCTTTCTTCTGCACAGCC-3’

*RPLP0* FWD: 5’-GAAGGTCACTGTGCCAGCCCA-3’  
*RPLP0* REV: 5’-GAAGGTGAATTCCTGCTTCCA-3’

**Mouse:**

*Bin1* exon 1 FWD: 5’-CAGCAACGTACAGAAGAAGC-3’  
*Bin1* exon 4 REV: 5’-GCTCATACCTGCTCAAGGC-3’

*Cacnb1 (Dhprβ1)* FWD: 5’-CTTTGCCCTTTGAGCTAGACC-3’  
*Cacnb1 (Dhprβ1)* REV: 5’-GCACGTGCTCTGTCTTCTTA-3’

*Cacna1s (Dhprα1)* FWD: 5’-TCCAGCTACTGCCATGCTGAT-3’  
*Cacna1s (Dhprα1)* REV: 5’-CCTCAGCTTTGGCTGAGATG-3’

*Atp2a1 (Serca1)* FWD: 5’-TGGCTCATGCTTCTAGATGAT-3’  
*Atp2a1 (Serca1)* REV: 5’-CTTCTCTTCTTCTGGCCCT-3’

*Ryr1* FWD: 5’-GTTATCGTCATTCTGCTGCT-3’  
*Ryr1* REV: 5’-GCCTATTCCACAGATGAAGC-3’

*Tnnt3 (sTni)* FWD: 5’-CCCTCATGACAGCCAC-3’  
*Tnnt3 (sTni)* REV: 5’-CTTCTCTGCTCAGGAGAT-3’

*Ncx1* FWD: 5’-GGGAAGACCTTTGAGGACAC-3’  
*Ncx1* REV: 5’-TCTCTCTCTCTCCAGTCAAC-3’

*Clcn1* FWD: 5’-TCCCTATCTGCAGACC-3’  
*Clcn1* REV: 5’-TGGAGTCAACCCATTCTGTTAAAG-3’

*Trdn* FWD: 5’-CTTCACAGAGAAAGGAGG-3’  
*Trdn* REV: 5’-TGGGTTCACCTTTCTCCTGTT-3’

*Casq1* FWD: 5’-ATGGATGGAGATGGATAAC-3’  
*Casq1* REV: 5’-GGCAGAAAGGTCAACAAAGGTAA-3’
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