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A novel PtdIns3*P* and PtdIns(3,5)P_2 phosphatase with an inactivating variant in centronuclear myopathy

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In eukaryotic cells, phosphoinositides are lipid second messengers important for many cellular processes and have been found dysregulated in several human diseases. X-linked myotubular (centronuclear) myopathy is a severe congenital myopathy caused by mutations in a phosphatidylinositol 3-phosphate (PtdIns3*P*) phosphatase called myotubularin, and mutations in dominant centronuclear myopathy (CNM) cases were identified in the dynamin 2 gene. The genes mutated in autosomal recessive cases of CNMs have not been found. We have identified a novel phosphoinositide phosphatase (hJUMPY) conserved through evolution, which dephosphorylates the same substrates as myotubularin, PtdIns3*P* and PtdIns(3,5)P_2, *in vitro* and *ex vivo*. We found, in sporadic cases of CNMs, two missense variants that affect the enzymatic function. One of these appeared *de novo* in a patient also carrying a *de novo* mutation in the dynamin 2 gene. The other missense (R336Q) found in another patient changes the catalytic arginine residue of the core phosphatase signature present in protein tyrosine/dual-specificity phosphatases and in phosphoinositide phosphatases and drastically reduces the enzymatic activity both *in vitro* and in transfected cells. The inheritance of the phenotype with regard to this variant is still unclear and could be either recessive with an undetected second allele or digenic. We propose that impairment of hJUMPY function is implicated in some cases of autosomal CNM and that hJUMPY cooperates with myotubularin to regulate the level of phosphoinositides in skeletal muscle.

INTRODUCTION

In eukaryotic cells, spatio-temporal regulation of cellular organization requires tightly regulated messengers and microdomains. Phosphatidylinositol can be phosphorylated on the inositol ring into seven distinct phosphoinositides (PPIn) that act as second messengers. PPIn have been implicated in a variety of cellular processes, including signal transduction, actin cytoskeleton, actin cytoskeleton remodelling membrane trafficking and protein transport (1,2). They recruit signaling proteins to specific membrane domains, leading to their activation (3). Dysregulation of PPIn is implicated in several human diseases such as cancer, diabetes and bacterial/viral infections (4–6).

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Human myotubularins (MTM1 and MTMR1-13) are phosphoinositide 3-phosphatases or phosphatase-like proteins mutated in several neuromuscular disorders (5,7). *MTM1* is mutated in X-linked myotubular (centronuclear) myopathy (XLMTM) (8), whereas *MTMR2* and *MTMR13* are mutated in demyelinating Charcot–Marie–Tooth neuropathy types 4B1 and 4B2, respectively (9–11). Myotubularins share homology to the catalytic domain of protein tyrosine phosphatases and dual-specificity phosphatases (PTP/DSP). Catalytically active myotubularins dephosphorylate phosphatidylinositol 3-phosphate (PtdIns3*P*) and PtdIns(3,5)P_2 to produce PtdIns and PtdIns5*P*, respectively (12–17). However, some myotubularins, referred as dead phosphatases, lack key residues in the catalytic loop, especially the catalytic cysteine and arginine residues of the PTP/DSP signature CX_5R (where X can be any residue). These two residues are essential for the catalysis, and in other phosphatases, mutation of the cysteine nucleophile even lead to substrate-trapping properties (18,19).

The centronuclear myopathies (CNMs) are characterized by a high proportion of small myofibers with centrally placed nuclei. Although internalization of the nucleus can be observed in other muscle diseases, additional clinical and pathologic features define CNMs as a clear entity (20,21). Several forms have been distinguished on the basis of the clinical data and genetic inheritance. XLMTM is the most severe, and XLMTM patients have a generalized hypotonia at birth. Autosomal recessive patients have been classified into three subgroups: childhood onset with or without ophthalmoparesis and adolescent onset without ophthalmoparesis (22). Autosomal dominant families present a childhood or adult age of onset with a progressive myopathy, and mutations in dynamin-2 (DNM2) have been found very recently in some of these families (23).

As no genes are known to be implicated in the autosomal recessive forms of CNMs, we hypothesized that proteins with a similar function to myotubularin could be mutated in CNM patients. We identified a novel human protein, hJUMPY, which acts as a PtdIns3*P* and PtdIns(3,5)P_2 3-phosphatase *in vitro* and *ex vivo*. We found two heterozygous missense variants in CNM patients, including one variant which changes the catalytic arginine, leading to a drastic decrease in the phosphatase activity.

RESULTS

hJUMPY, a novel protein with homology to myotubularin phosphoinositide phosphatases

In order to identify new genes implicated in the myotubularin pathway and in autosomal CNMs, we have searched for proteins which might perform similar functions to myotubularin, especially in muscle. We screened the human sequence databases with BLAST and TBLASTN using human myotubularin protein sequence as a bait. An uncharacterized gene was found, FLJ22405 (Gene ID: 64419, C3orf29), which encodes a hypothetical protein of 650 amino acids (accession no. AK074792) with strong homology to the catalytic loop of myotubularin (Fig. 1). Exons 17 and 18 appeared alternative, as they were independently found in a subset of ESTs and RNA sequences. The predicted protein was highly homologous to a *Drosophila melanogaster* protein called 'egg-derived tyrosine phosphatase' (EDTP) and encoded by the gene CG6542 on chromosome II. Interestingly, disruption of this gene in flies due to a P-element insertion produced the JUMPY phenotype: a muscle defect and a progressive loss of muscle control together with shaky and slower movements (24,25). We thus named the human protein hJUMPY.

hJUMPY harbored a predicted PTP/DSP domain and contains the canonical CX_5R consensus present in all active PTP/DSPs such as PTP1B and in PPIn phosphatases such as PTEN (26). hJUMPY catalytic loop sequence shows the closest resemblance to that of active myotubularins (Fig. 1B). hJUMPY defines a novel protein conserved through evolution together with the previously reported EDTP proteins that were annotated as tyrosine phosphatases, in *Drosophila* and in flesh flies (27,28). Members were found in slime mold and coelomates, which include arthropods, chordates and sea urchin, but were absent in nematodes, fungi and plants (Supplementary Material, Figs S1 and S2). In particular, the predicted phosphatase domain of hJUMPY shared 52% identity over 316 amino acids with the corresponding domain in *Dictyostelium discoideum*. Noteworthy, the predicted PTP/DSP domain was very conserved through evolution with identical CX_5R signatures, suggesting that the main function of JUMPY is a phosphatase activity.

hJUMPY is expressed in different tissues including skeletal muscle (Supplementary Material, Fig. S3), and this was confirmed by ESTs and results found in the SymAtlas (http://symatlas.gnf.org/SymAtlas/). Quantitative polymerase chain reaction (PCR) analysis showed that endogenous mouse *mJumpy* expression increased with C2C12 myotubes formation and differentiation in culture and reached more than a 3-fold increase after 5 days of differentiation (Fig. 1C). This was also the case for *Mtm1* (Fig. 1C) (29). *Mtm1* and *mJumpy* expression increased with the beginning of myotubes formation at day 2, but *Mtm1* expression continued to increase up to 13-folds at day 9, whereas *mJumpy* expression reached a plateau at day 5.

hJUMPY sequence variants in CNM

On the basis of the fact that hJUMPY shares some homology with myotubularin, which is mutated in the X-linked form of myotubular/CNM, we screened *hJUMPY* in 50 patients with CNM after PCR amplification of all exons and splice sites (primers in Supplementary Material, Table S1). This pool of patients included forms with neonatal, childhood and adult onsets; most of the cases were sporadic with a few recessive and dominant families and in whom no previous MTM1 mutation was found. Apart from intronic variants (Supplementary Material, Table S2), amino acid changes were detected in two Brazilian sporadic cases with early onset, DNM57 and DNM77 (Fig. 2). Patient DNM57 had a heterozygous missense R336Q. This 12-year-old boy had a neonatal hypotonia and hypoxia and has now a stable or slowly progressive disease with general hypotonia and diffuse weakness, affecting predominantly proximal portions of the limbs, together with ophthalmoparesis [case 5 in Zanoteli *et al*. (30)]. The arginine at position 336 is conserved in all the JUMPY protein orthologs and in all PTP/DSP phosphatases, as it is one of the compulsory amino acids for the enzymatic activity (Fig. 1B) (31,32). In PTP1B, for example, mutation of this catalytic

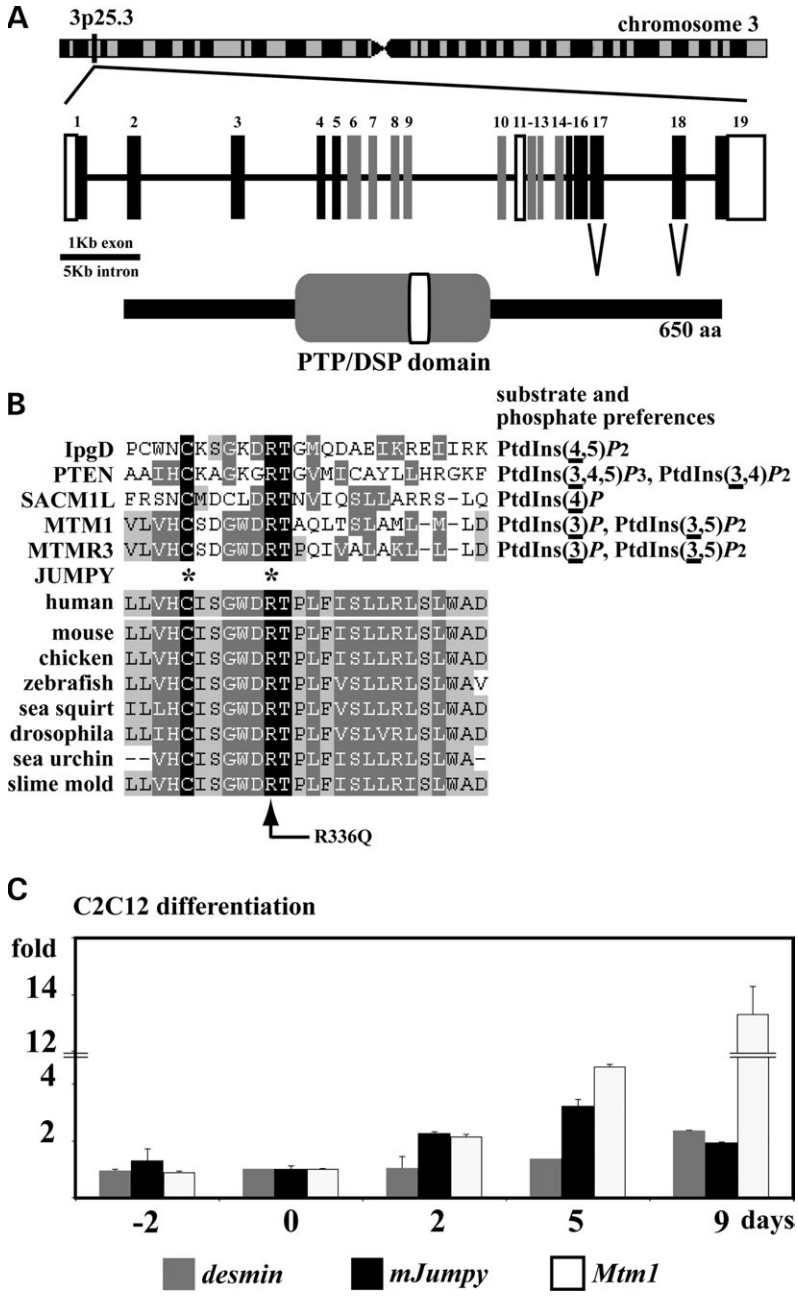


Figure 1. The hJUMPY gene and protein. (**A**) *hJUMPY* is composed of 19 exons; exons 17 and 18 are alternative on the basis of EST data. Exons and introns are represented to scale (scale is 1 kb for exons and 5 kb for introns). Exons in gray (6–14) encode a PTP/DSP from amino acids 180–409, whereas the catalytic consensus is encoded by exon 11 in white (the CX_5R signature is from amino acids 330–336). (**B**) Active-site homology with other PPIn phosphatases and in different species. IpgD is from *Shigella flexneri*, while the human sequences are shown for PTEN (phosphatase and tensin homolog), SACM1L (suppressor of actin mutations 1), MTM1 (myotubularin) and MTMR3 (myotubularin-related protein 3). The catalytic cysteine and arginine residues of the CX_5R motif are indicated with asterisks and the arrow shows the R336Q variant found in patient DNM57. On the right is indicated the preferred phosphoinositides substrate, and the phosphate removed by the reaction is underlined. Sequences of eight JUMPY orthologs are shown below. Shading reflects the conservation from black (100% conserved) to white (not conserved). (**C**) Quantitative PCR on total cDNAs extracted from differentiating C2C12 mouse muscle cells. Days of differentiation are indicated below: −2 (proliferating myoblasts), 0 (confluent myoblasts, when the differentiation was started by lowering the serum), 2, 5 and 9 days. Values were normalized to *hprt* and displayed as fold changes compared with day 0. Desmin is a control.

arginine of the CX_5R signature leads to a strong decrease in the phosphatase activity (19). The variant was found in the father who did not complain of myopathic signs but for whom no biopsy was performed. Apart from the father, this amino acid change was not found in unaffected individuals, including ethnically matched DNA, screened by AvaII restriction enzyme digestion and DHPLC (820 chromosomes). Possible deletions were not detected by Southern blot using the complete open-reading frame of *hJUMPY* as a probe (data not shown), and RNA material was not available,

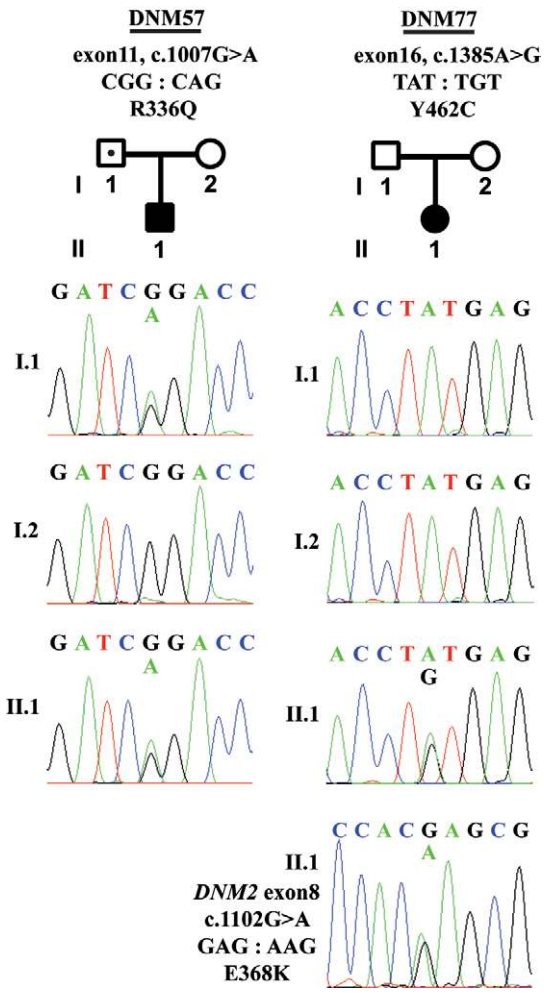


Figure 2. *hJUMPY* variants in CNM patients. Pedigree of families DNM57 and DNM77 is displayed: square for males, circles for females, white for unaffected and black for affected. The dot in individual DNM57/I.1 means that he carries the variant. Note that both parents of DNM77 proband do not have the variant. Numbering of the nucleotide change starts at the first nucleotide of the ATG in sequence AK074792. The chromatograms are shown below for each member of the two families. In addition, for patient DNM77, the chromatogram for exon 8 of *DNM2* is shown at the bottom.

which precludes the analysis of the splicing and level of the *hJUMPY* transcripts. Patient DNM77 had a non-conservative heterozygous Y462C missense. This 36-year-old female had a neonatal hypotonia followed by a stable muscle involvement and also presented ophthalmoparesis (33). The tyrosine 462 lies outside the PTP/DSP domain and is conserved at least in mammals and birds. However, this amino acid change was found at the heterozygous state in one control individual from Brazil out of 700 chromosomes tested by SSCP and DHPLC (data not shown). Moreover, sequencing of the dynamin-2 gene revealed a heterozygous c.1102G>A, leading to the amino acid change E368K. This variant was previously reported as a *de novo* mutation in a patient with sporadic CNM (23). Astonishingly, neither of the parents had the *hJUMPY* nor the *DNM2* variants (paternity and maternity confirmed by microsatellites analysis on several chromosomes); we concluded that both variants occurred *de novo*. Taken together, our results in these two patients suggested that impairment of hJUMPY functions could be implicated in CNM, as a direct cause or as a modifier.

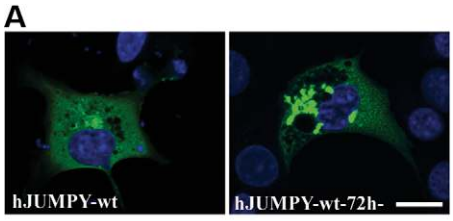
hJUMPY is a new phosphoinositide 3-phosphatase with an inactivating variant in CNM

We investigated the possible roles of hJUMPY and the impact of the two missense variants found in CNM patients (R336Q and Y462C). In addition, we generated a C330S variant, changing the catalytic cysteine of the predicted phosphatase signature, which was shown to impair drastically the enzymatic activity in other phosphatases and, in some examples, created substrate-trapping properties (19). Through transient transfection in COS-1 cells, EYFP-tagged hJUMPY localized to cytoplasmic reticular structures and displayed a concentration near the nucleus, and plasma membrane ruffles also contained hJUMPY (Fig. 3A and Supplementary Material, Fig. S4). Localization was similar in U373MG cells and using another tag (data not shown). We noticed that overexpressed hJUMPY is forming foci over time. Nearly 100% of transfected cells showed intense cytoplasmic foci close to the nucleus after 72 h of expression (Fig. 3). All the different variants described, C330S, R336Q and Y462C, displayed a similar localization pattern to the wild-type construct and also showed cytoplasmic protein foci with time (Fig. 3C; Supplementary Material, Fig. S4) (data not shown).

We noted that hJUMPY was more concentrated in structures positive for giantin, a golgi marker, by confocal microscopy (Supplementary Material, Fig. S4). We further observed that after 72 h of transfection, foci of hJUMPY formed and disrupted giantin-positive structures (Fig. 3C), but these foci were not labeled with ubiquitin (data not shown), suggesting that they were not part of a degradation process due to overexpression. This effect was not dependent on the phosphatase enzymatic activity, as formation of foci with the different mutants also disrupted giantin labeling. Interestingly, an inactive cysteine to serine mutant of hMTMR3 was shown as well to disrupt the golgi structure (34).

As the predicted phosphatase domain of hJUMPY contains a sequence similar to myotubularin catalytic signature, we tested whether hJUMPY had the phosphoinositide phosphatase activity *in vitro*. Although hJUMPY did not dephosphorylate PtdIns4*P*, PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Fig. 4A), it efficiently dephosphorylated PtdIns3*P* and PtdIns(3,5)P_2 (Fig. 4A). hJUMPY has thus a PPIn 3-phosphatase activity and also shares the same substrate specificity than myotubularin. Interestingly, we noted a strong decrease in the enzymatic activity of the R336Q mutant, to a level similar to the C375S mutant, indicating that the missense variant found in patient DNM57 has lost most of its phosphatase activity (Figs 4B–D). Moreover, the enzymatic activity of the Y462C mutant was also decreased, although to a lesser extent (to ~80% compared with the wild-type for PtdIns3*P*, Fig. 4D). The residual activity of the C330S and R336Q mutants (at ~22% of the wild-type) is most probably due to co-immunoprecipitation of a cellular phosphatase activity.

To confirm this phosphatase activity *ex vivo*, we used the 2XFYVE PtdIns3*P*-specific biosensor developed by the group of Stenmark (35). PtdIns3*P* localized to early



B

time (h)	% cells with aggregates
18	6.5
24	26.9
36	50.2
48	76.3
72	92.5

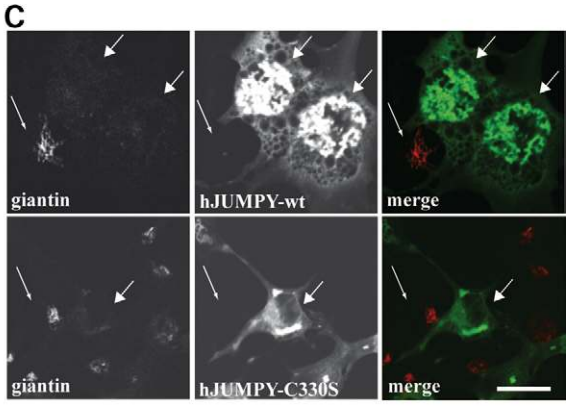


Figure 3. Subcellular localization of hJUMPY and impact on golgi structure. (**A**) Cos-1 cells were grown on glass slides and transiently transfected with YFP-tagged hJUMPY wild-type. Cells were fixed after 24 h (left) or after 72 h (right) and analyzed by confocal microscopy. Nuclear DNA labeled with DAPI is shown in blue. (**B**) Proportional increase of cells showing aggregates with time. At least 200 cells were counted per timepoint. (**C**) Cos-1 cells were grown on glass slides and transiently transfected with YFP-hJUMPY wild-type or C330S mutant. Cells were fixed after 72 h and analyzed by confocal microscopy after labeling of the Golgi with Giantin antibodies. Note that a golgi staining is present in the untransfected cells (thin arrows) and not in the transfected cells (thick arrows). The merge pictures depict giantin in red and hJUMPY in green. There is no yellow signal because of the lack of giantin labeling in cells transfected with hJUMPY. Results are representative of three independent experiments with about 150 cells analyzed, all showing giantin disruption to some extent. Scale bar is 20 μm.

endosomes as seen in the untransfected cell in Figure 5A, and as previously described (17,35). Overexpression of wild-type hJUMPY strongly decreased the PtdIns3*P* biosensor labeling. As expected, the catalytic C330S mutant did not affect the cellular level of PtdIns3*P*. Strikingly, the R336Q variant, found in one of the CNM patients, did not affect the PtdIns3*P* level, similar to the C330S construct (Fig. 5B). In agreement with the *in vitro* data, the Y462C variant was able to decrease PtdIns3*P* level when overexpressed in cells, but not to the extent of the wild-type construct, as some PtdIns3*P*-positive endosomes remained.

In conclusion, hJUMPY is a PPIn 3-phosphatase as sustained by both *in vitro* and *ex vivo* experiments and has a similar substrate specificity to myotubularin. Importantly, the R336Q variant and, to a lesser extent, the Y462C variant, found in CNM patients, impaired the enzymatic function of hJUMPY.

DISCUSSION

We have identified a novel protein with a phosphatase catalytic loop highly similar to the one found in myotubularins. Using *in vitro* and *ex vivo* approaches, we have shown that hJUMPY is a PPIn 3-phosphatase which dephosphorylates the same substrates as myotubularin, PtdIns3*P* and PtdIns(3,5)P_2. Sequencing of patients with CNM, a disorder closely related to XLMTM due to mutations in myotubularin, revealed two heterozygous missense variants, one of them affecting the catalytic arginine and drastically inactivating the phosphatase function.

A novel PtdIns3*P* phosphatase

Inclusion of proteins in the myotubularin family was primary based on the extensive sequence homology and not on shared enzymatic properties, as some myotubularins are dead phosphatase lacking the enzymatic activity. The human genome encompasses 107 phosphatases or phosphatase-like genes and hJUMPY corresponds to the predicted protein named *MTMR14* in the review by Alonso *et al*. (32). Although hJUMPY shares a conserved phosphatase signature with myotubularin, it is not part of the myotubularin family, as it lacks the GRAM-PH domain and the Rac-induced recruitment domain (or myotubularin-related domain, PF06602), characteristic of the MTM family (36). Despite the previously reported homology between myotubularin and hJUMPY (32), it was not possible to predict the substrate specificity. Here, we show that the conservation of the catalytic site between myotubularin and hJUMPY is reflected by the similar enzymatic activity, and in both cases, the CX3WDR signature is associated with PtdIns3*P* and PtdIns(3,5)P_2 3-phosphatase activity. We hypothesize that the conserved aspartate of this motif could be the catalytic aspartate commonly found on the so-called WPD loop in the other phosphatases such as PTP1B and PTEN, as this was first suggested by the group of Dixon (36) for MTMR2.

hJUMPY and myotubularin both dephosphorylate PtdIns3*P* and PtdIns(3,5)P_2, membrane-anchored lipids which act as second messengers. These PPIn are mainly localized to vesicles of the endocytic machinery and recognized by specific effector proteins which fulfill important roles in vesicle fusion and sorting and in protein transport (37). PtdIns3*P* is concentrated on early endosomes, is produced by the class III VPS34 PPIn 3-kinase and binds, for example, EEA1 and Hrs, two proteins implicated in endosome fusion and receptor sorting, respectively. The role of PtdIns(3,5)P_2 is less characterized but is implicated at least in receptor degradation and membrane retrieval. With its newly described enzymatic activity, hJUMPY could antagonize the PPIn 3-kinase activity and regulate the level of these second messengers, thus acting as a new regulator of membrane

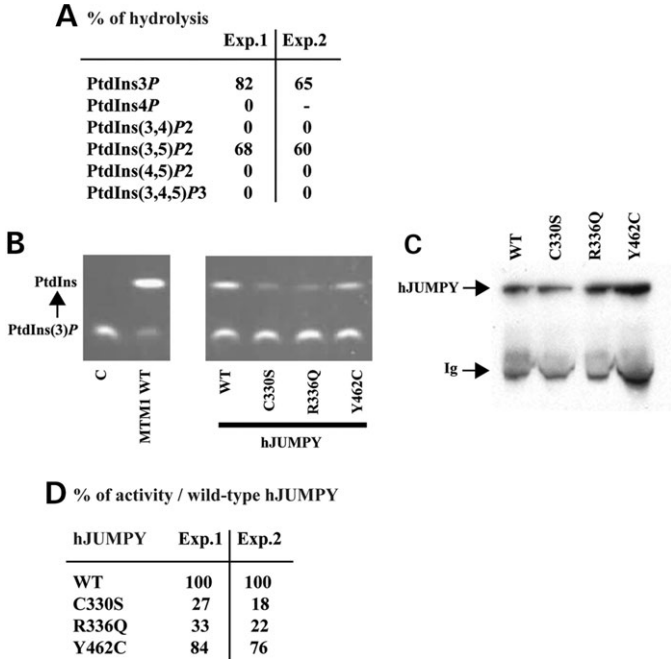


Figure 4. hJUMPY is a phosphoinositide 3-phosphatase *in vitro*. (**A**) Constructs were transiently expressed in HEK 293 cells and immunoprecipitated using the B10-tag antibody. The immunoprecipitates were tested for *in vitro* phosphatase activity on the indicated substrates, and the percentage of hydrolysis is shown for two independent experiments. (**B**) Different tagged hJUMPY constructs, indicated at the bottom, were transfected and the immunoprecipitates were tested for *in vitro* phosphatase activity on PtdIns3*P*. MTM1 wild-type was also tested as a positive control. C stands for a negative control (no immunoprecipitant antibody). (**C**) Western blot of immunoprecipitates from experiment in (B) with an anti-B10-tag antibody revealing the expressed protein constructs. (**D**) Percentage of activity compared with wild-type hJUMPY is reported for each construct, from two independent experiments.

trafficking processes. Interestingly, both myotubularin and hJUMPY are not directly localized to endosomes in transfected cells. Either the colocalization with their substrates is strictly regulated or they act on a minor pool of phosphoinositides. Myotubularin homologs (MTMRs) were proposed to act on different PPIn subpools (16,29). Myotubularin (MTM1) was also recruited to different compartments upon several conditions: to the late endosomes by EGF treatment (38), to the plasma membrane upon overexpression of a dominant activated Rac1 GTPase (39) and to MTMR12/3-PAP localization through heterodimerization (40). We hypothesize that hJUMPY acts on a specific PPIn subpool to regulate trafficking between intracellular membranes, for example, between the golgi and the endoplasmic reticulum or the endosomes. However, although overexpression of hJUMPY affects the normal golgi structure, as noted with the disruption of giantin labeling, this effect was not linked to the phosphatase activity and might depend on another yet uncharacterized protein domain in hJUMPY.

Missense changes in CNM

We obtained several evidences that impairment of hJUMPY function could be a cause or a modifier of CNM. hJUMPY CX_5R catalytic loop shares extensive homology with the one of myotubularin and acts on the same PPIn substrates. Moreover, the two missense variants found in CNM patients decrease the enzymatic activity *in vitro* and *ex vivo*. In particular, the R336Q variant is changing the compulsory arginine found in the catalytic site of all active PTP/DSP and PPIn phosphatases and drastically decreases the enzymatic activity of hJUMPY to the level of the artificial C330S mutant, *in vitro* and *ex vivo*. Similarly, mutation of this arginine residue to lysine, in the CX_5R signature of PTP1B, dramatically decreased the enzymatic activity, but this mutant still bound the substrate normally (19). Moreover, disruption of the *Drosophila* ortholog produced a progressive muscle defect (24).

For patient DNM57, the R336Q variant is present at the heterozygous state in the unaffected father. This is in favor of a recessive inheritance where the father would be a healthy carrier (frequency of 1/820 normal chromosomes). We have not detected a second sequence change on the other allele in this patient, by sequencing all the coding exons and splice sites. Southern blot analysis using the full open-reading frame of *hJUMPY*, and sequencing of putative additional exons found in unique ESTs and located in introns 1 and 2, did not reveal additional sequence changes (data not shown). RNA material was not available. We hypothesize that the other allele is mutated in intronic sequences of *hJUMPY* or in upstream sequences important for its transcriptional regulation. Alternatively, one may propose a digenic mode of inheritance where haploinsufficiency of *hJUMPY* would interact with a mutation in another gene. We excluded the myotubularin (*MTM1*) and dynamin-2 genes by direct sequencing of all exons and splice sites.

For patient DNM77, we found two *de novo* heterozygous variants, Y462C in *hJUMPY* and E368K in *DNM2*. The *DNM2* variant previously reported in a CNM patient is most likely disease causing (23). Patients with other characterized mutations in *DNM2* appear usually to have an age of onset in childhood or adulthood, whereas in our case, the patient presented with neonatal hypotonia. It is thus possible that partial impairment of hJUMPY function accelerated in this case the onset of the disease.

Linking phosphoinositides and skeletal muscle functions

It is noteworthy that both hJUMPY (this study) and myotubularin are PtdIns3*P* and PtdIns(3,5)P_2 3-phosphatases (14,17,29). This would suggest a common physiological mechanism, involving the regulation of these two PPIn. Although their implication in membrane trafficking, especially for PtdIns3*P*, is well established in yeast and mammalian cells (37), little is known about their roles in skeletal muscle.

In muscle cells in culture, insulin is believed to stimulate the production of PtdIns3*P* and PtdIns(3,5)P_2 (41,42) and, subsequently, GLUT4 translocation to the plasma membrane (43). Myotubularin and PtdIns3*P* are implicated in GLUT4 glucose receptor translocation (44). Moreover, PtdIns (3,5)P_2 might also play a role in GLUT4 trafficking as insulin activation of Akt/PKB stimulates PIKfyve, the kinase producing PtdIns(3,5)P_2, and plays an important role in the regulation

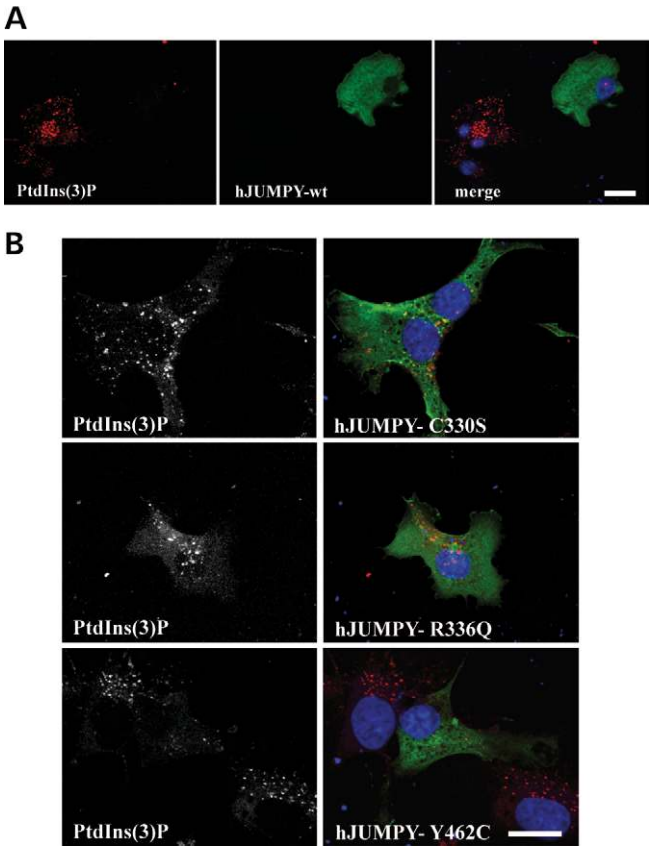


Figure 5. hJUMPY dephosphorylates endosomal PtdIns3*P* in cells. Cos-1 cells were grown on glass slides and transiently transfected with YFP-hJUMPY variants. After fixation, PtdIns3*P* was detected using a biotinylated GST-2xFYVE biosensor. (**A**) Confocal pictures of a cell expressing transfected, enzymatically active YFP-hJUMPY wild-type protein, nearby an untransfected cell on the left. (**B**) Cells transfected with hJUMPY mutants as indicated on the pictures. The merge pictures depict PtdIns3*P* in red and transfected constructs in green. Nuclear DNA labeled with DAPI is shown in blue. Representative cells from two independent experiments are shown. Scale bar is 20 μm.

of GLUT4 trafficking, at least in 3T3-L1 cells (41). Thus, hJUMPY could regulate membrane and protein trafficking in muscle and downstream pathways implicating PtdIns3*P* and PtdIns(3,5)P_2 protein effectors. For example, CKIP-1, the casein kinase 2-interacting protein-1, binds to PtdIns3*P* through its PH domain and participates to the regulation of muscle cell differentiation (45). This suggests that regulation of PtdIns3*P* could be important for several mechanisms, other than GLUT4 translocation in muscle fibers, which remain to be characterized.

In conclusion, we have identified a novel phosphoinositide phosphatase which might be important for skeletal muscle, as its enzymatic activity is strongly impaired in a patient with CNM. Analysis of additional patients with CNMs and characterization of animal models will confirm and precise the role of JUMPY phosphoinositide phosphatases in membrane trafficking and muscle function. Moreover, hJUMPY represents an interesting target for compensatory therapeutic approaches for XLMTM.

MATERIALS AND METHODS

Patients

Clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki. DNA was extracted by standard procedures from blood samples obtained after informed consent of the family. Patients were selected on the basis of their clinical and histopathological phenotypes (22). The histological criteria used for diagnosis were: nuclei centralized in >25% of the fibers, an increase of oxidative enzyme activity in the center of the fibers, type 1 fiber predominance, variable amount of connective tissue infiltration and the absence of other structural abnormalities in the muscle fibers such as nemaline bodies, ragged fibers, central core, etc. They also had no signs of active muscle degeneration and inflammation.

Mutation screening

All the exonic coding sequence and exon–intron boundaries were amplified by PCR using intronic primers (Supplementary Material, Table S1) and tested by SSCP and direct sequencing. Absence of the variants was checked in control DNA from non-myopathic individuals by AvaII digestion for exon 11 (only wild-type sequences are cleaved), by SSCP for exon 16 and by DHPLC for both exons. Sequencing of the MTM1 and DNM2 genes was performed as described (23,46).

Cell culture and fluorescence microscopy

Mammalian cell lines were maintained and transfected as described (17,39). The human IMAGE consortium (clone ID 5755887) cDNA clone [accession no. BC035690 (47)] was cloned and expressed from pSG5 hER-B10 tag and pSG5-EYFP vectors. Mutations were inserted by PCR- directed mutagenesis. Transfected cells were fixed in 4% paraformaldehyde. For colocalization, cells were permeabilized with 1% Triton and probed with a mouse monoclonal anti-giantin (Coger Reactifs) and a Cy3-conjugated goat anti-mouse IgG (Beckman Coulter France SA) or with a biotinylated GST-2xFYVE biosensor probe (5 ng/μl) followed by Alexa594 streptavidin. Pictures were recorded using a Leica SP2 AOBS confocal microscope system and processed with Adobe®Photoshop 7.0.

In vitro enzymatic assay

HEK293 cells were transfected with the different plasmids for 24 h and immunoprecipitated using the B10-tag antibody for the JUMPY constructs or the 1G6 antibody for MTM1. Phosphatase activity was analyzed in the immunoprecipitate using 1 μg of fluorescent NBD-C6-PPIn as described (17).

Expression analysis

Total RNAs were extracted from C2C12 cells at several differentiation stages using the TRIzoI reagent (Invitrogen). About 1 μg of total RNA was subjected to reverse transcription using both random hexamers and oligo-dT with Superscript Reverse Transcriptase (Invitrogen). Primers for the PCR

amplification were as follows: *mJumpy* (5′-CCGAAGAGTT CTGCCTGAAG-3′ with 5′-ATAGCTGAAGCTCCCCACAG -3′), *desmin* (5′-GCTCTCAACTTCCGAGAAACC-3′ with 5′-TGTGTAGCCTCGCTGACAAC-3′) and *hprt* (5′-GTAAT GATCAGTCAACGGGGGAC-3′ with 5′-CCAGCAAGCTTG CAACCTTAACCA-3′). Real-time PCR was performed with SYBR Green using the Light Cycler apparatus (Roche). Specificity of reactions was confirmed by melt curve analysis and fold changes were calculated after normalization to *hprt*.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors state that they have no conflict of interest.

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