nature portfolio

Peer Review File

Combining dynamin 2 myopathy and neuropathy mutations rescues both phenotypes

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
DNM2 is a ubiquitously expressed GTPase with mutations leading to two human congenital diseases: Charcot-Marie-Tooth neuropathy (CMT) and centronuclear myopathy (CNM). Currently, there are no therapeutics available for DNM2-associated CMT.
In this work, Goret et al. demonstrate the opposing effects of DNM2 mutations derived from CMT and CNM using in vitro and in vivo experiments. Importantly, they discover compensatory effects in mice bearing CNM and CMT mutant DNM2 simultaneously. The rescuing effects via the combination of two disease-associated DNM2 mutations in mice provide surprising and exciting evidence for a promising therapeutic strategy for DNM2-associated CNM and CMT.

However, the major concern of this work is the limited neurological phenotype of Dnm2K562E/+ mice, which hinders the full support of the restoration effects. Therefore, in addition to the G-ratio, further examination of neuron physiology, such as nerve conductance velocity measurement or compound muscle action potentials (Pereira et al., 2020), could significantly strengthen the conclusions regarding nerve physiology in Dnm2K562E/S619L mice relative to Dnm2K562E/+ or wild type mice.
Moreover, some results are not thoroughly examined and interpreted. For example, the increase of Chrna and Chrng in both Dnm2S619L/+ and Dnm2K562E/+ mice suggests muscle denervation. Further explanation and examination on this new phenotype are necessary.
Additionally, the mouse phenotypes are examined at 8 weeks of age. It is important to determine whether mice behavior and activities decline or worsen with age.

Another concern is the lack of mechanistic insight. The authors have established a robust system to examine the effects of Flag-Dnm2S619L and His-Dnm2K562E in vitro, and they have obtained excitingly proof-of-concept results. The biochemical defects of these two proteins have been examined and published by several groups: Dnm2K562E is defective in membrane binding, whereas Dnm2S619L is insensitive to membrane (Kenniston and Lemmon, 2010). Therefore, it is important and will be informative to test the effect of these two mutant proteins on each other's biochemical abnormalities. Revealing the mechanism by which these two mutant proteins restore each other would provide valuable insight into the molecular action of dynamin on membranes.

Finally, the embryonic lethality of Dnm2K562E and Dnm2S619L homozygous mice is intriguing. Further description of the developmental stage or defects in these embryos would be valuable and informative.

Reviewer #2

(Remarks to the Author)
Mutations within a single gene can lead to a variety of diseases. Dominant mutations in the DNM2 gene result in distinct neuromuscular disorders: centronuclear myopathy (CNM) and Charcot-Marie-Tooth neuropathy (CMT). The authors present in vitro assays demonstrating opposing effects of DNM2 mutations, with gain-of-function in CNM and loss-of-function in CMT. They explored the potential compensatory effects of these mutations by breeding Dnm2S619L/+ CNM and

Dnm2K562E/+ CMT mouse models. The offspring, Dnm2S619L/K562E, exhibit significantly improved motor coordination and muscle structure and function. This study reveals that two distinct disease-causing mutations within the DNM2 gene can compensate for each other in vivo. These findings are novel and significant and they were obtained through what is overall a well-conducted study. I have the following comments:

1. The studies were conducted on young mice (8 weeks old). It will be important to show that these compensatory effects of the Dnm2S619L/K562E mice are maintained in older mice, ideally at least 6 months old mice. Additionally, insights into whether there might be sex differences should be provided.
2. The functional assays that were performed on the mice are limited. Please provide insights in the running performance of the mice, either free wheel running or treadmill running
3. The muscle histology and ultrastructural results of Figure 3 need to be more extensively quantified
4. Molecular insights are overall lacking. Please provide a figure showing where the mutations are located and their possible effect on protein structure.
5. The authors should provide a more detailed discussion of how insights from the present study can be used therapeutically.
6. Line 48: Patients experience…
7. Line 143, provide a reference.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have soundly addressed my questions. I am excited about the revised manuscript and endorse the publication of it.

Reviewer #2

(Remarks to the Author)
The authors have been responsive to the earlier comments. I am satisfied with the revision.

Response to reviewers

Subject: Submission of revised manuscript NCOMMS-24-34539-T "Combining dynamin 2 myopathy and neuropathy mutations rescues both phenotypes".

We would like to thank the reviewers for their insightful comments. We have carefully reviewed all feedback and revised the manuscript accordingly. We hope that the updated paper is now suitable for publication.

We are providing the revised manuscript in two versions, with and without yellow highlighting, to indicate the added or modified text. Regarding the novel data, we addressed all the reviewers' points. We now present the mutants' binding sensitivity to PS and PI4,5P_2, both alone and in combination (Fig. 1E). We added Patricio Aguirre-Pineda and Sylvie Friant with whom these experiments were performed as co-authors, with consent of all authors. We have better commented on muscle denervation (Fig.4C, Extended Data Fig. 9E). Additionally, we confirmed that most phenotypes were still improved in older mice, with detailed phenotyping of 1-year-old mutants and performed EMG at this age (Fig. 5-7, Extended Data Fig. 10A-B). We also show the localization of both mutants on the open-close dynamin structure (Extended Data Fig. 1A). The embryonic lethality of homozygous mutants has been explored (Extended Data Fig. 3). To support fibrosis deposition observed by electron microscopy in single mutants, we performed complementary immunofluorescence of collagen VI in muscle and quantified the staining thickness (Extended Data Fig. 7F). Finally, quantifications of electron microscopy images have been performed (Extended Data Fig. 8A-C). Methods have been updated to include descriptions of the new experiments, and we have added several references to support our findings.

Responses to the reviewers are given below.

RESPONSE TO REVIEWER A

DNM2 is a ubiquitously expressed GTPase with mutations leading to two human congenital diseases: Charcot-Marie-Tooth neuropathy (CMT) and centronuclear myopathy (CNM). Currently, there are no therapeutics available for DNM2-associated CMT.

In this work, Goret et al. demonstrate the opposing effects of DNM2 mutations derived from CMT and CNM using in vitro and in vivo experiments. Importantly, they discover compensatory effects in mice bearing CNM and CMT mutant DNM2 simultaneously. The rescuing effects via the combination of two disease-associated DNM2 mutations in mice provide surprising and exciting evidence for a promising therapeutic strategy for DNM2-associated CNM and CMT.

Response: Thank you for your comments on our paper. Please find below our answers point-by-point.

Comment 1: "However, the major concern of this work is the limited neurological phenotype of Dnm2K562E/+ mice, which hinders the full support of the restoration effects. Therefore, in addition to the G-ratio, further examination of neuron physiology, such as nerve conductance velocity measurement or compound muscle action potentials (Pereira et al., 2020), could significantly strengthen the conclusions regarding nerve physiology in Dnm2K562E/S619L mice relative to Dnm2K562E/+ or wild type mice".

Response: We agree that this is an important point. In response, we have performed additional electromyography (EMG) analyses to examine nerve conduction velocity (NCV) and sensory/motor function in our mouse cohorts at 1 year of age (new Fig. 6A-D, Extended Data Fig. 10B). In summary, compound sensory NCV was slightly decreased in *Dnm2*$^{K562E/+}$ and increased in both *Dnm2*$^{S619L/+}$ and

Dnm2$^{S619L/K562E}$ double mutants compared to WT. Sensory amplitude, however, was reduced in both single mutants and was not improved in double mutants, whereas motor conduction remained unaffected. These findings suggest that while the double mutants exhibit partial improvements in conduction velocity, they fail to rescue sensory amplitude deficits.

The following text was added: "At 1 year, electromyography (EMG) analysis revealed decreased compound sensorial nerve conduction velocity (csNCV) in *Dnm2*$^{K562E/+}$ mice, consistent with the observed reduced thermal sensitivity (Fig. 5G-H, Fig. 6A). csNCV was increased in *Dnm2*$^{S619L/+}$ and *Dnm2*$^{S619L/K562E}$ compared to *Dnm2*$^{K562E/+}$ and to WT. In contrast, the sensory amplitude was decreased in all *Dnm2*$^{S619L/+}$, *Dnm2*$^{K562E/+}$ and *Dnm2*$^{S619L/K562E}$ groups compared to WT (Fig. 6B). The motor NCV and amplitudes were not affected in any of the groups (Fig. 6C-D, Extended Data Fig. 10B). These findings suggest that while the double mutant exhibit improvement in conduction velocity, they fail to rescue sensory amplitude deficits." (page 5 line 193-199). The "Electrophysiology" material and method section was added (page 9, line 350-362).

Comment 2: "Moreover, some results are not thoroughly examined and interpreted. For example, the increase of Chrna and Chrng in both Dnm2S619L/+ and Dnm2K562E/+ mice suggests muscle denervation. Further explanation and examination on this new phenotype are necessary."

Response: We appreciate the reviewer's comment and have further examined the potential issue of muscle denervation in the *Dnm2*$^{S619L/+}$ and *Dnm2*$^{K562E/+}$ mice. We evaluated the expression of *Chrne* using RT-qPCR. *Chrne* encodes the adult epsilon subunit of the nicotinic acetylcholine receptor, which replaces *Chrng* after birth. An increase in *Chrne* expression would rule out muscle denervation, while a stable or reduced level would support it. Our results showed an increase in *Chrne* expression in *Dnm2*$^{K562E/+}$ (Fig. 4C), suggesting that there is no significant denervation in this group. Therefore, the increased expression of *Chrna* and *Chrng* in the *Dnm2*$^{K562E/+}$ group likely reflects enhanced sub-synaptic nuclei activity rather than muscle denervation. Additionally, aspecific esterase staining showed no evidence of denervated fibers in muscle sections from this group (Extended Data Fig. 9F).

However, during *in situ* muscle force measurements, we stimulated either the sciatic nerve or the muscle. By comparing the ratio of maximal force produced by direct muscle stimulation versus nerve stimulation, we observed that WT mice generated similar muscle force following both stimulations. However, in *Dnm2*$^{S619L/+}$ mice, force production was lower following nerve stimulation than muscle stimulation, indicating a nerve conduction defect in this group (Fig. 6H at 1-year, Extended Data Fig. 9E at 8 weeks). This is consistent with the increased expression of *Chrna* and *Chnrg* and stable expression of *Chrne* in this group (Fig. 4A-C). However, to affirm a muscle denervation in *Dnm2*$^{S619L/+}$ mice, we would expect to see evidence of denervated fibers on aspecific esterase staining or a decrease in CMAP amplitude in this group, neither of which was observed.

We have updated the manuscript to reflect this new interpretation:

- We removed: "~~as well as enhanced muscle innervation"~~ (page 1, line 36).
- We added the text : " In most genotypes, TA muscle force measurements were similar whether stimulated directly (Fig. 2G) or via the sciatic nerve (Extended Data Fig. 9D). However, *Dnm2*$^{S619L/+}$ mice showed lower muscle force following nerve stimulation compared to muscle stimulation, suggesting potential conduction impairments (Extended Data Fig. 9E). This observation may indicate muscle denervation, supported by increased expression of acetylcholine receptor subunits α (*Chrna1*) and γ (*Chrng*), with stable levels of ε (*Chrne*)[21] (Fig. 4A-C). In contrast, the increased expression of *Chrna1* and *Chrng* in *Dnm2*$^{K562E/+}$ mice, along with elevated *Chrne* levels, likely reflects enhanced sub-synaptic nuclei activity rather than

denervation. Despite these indications of potential conduction impairments and muscle denervation in *Dnm2*$^{S619L/+}$ mice, aspecific esterase staining of TA muscle sections did not reveal significant morphological anomalies at the neuromuscular junctions nor the presence of denervated fibers (Extended Data Fig. 9F)." (page 4 line 161 to page 5 line 171).

Comment 3: "Additionally, the mouse phenotypes are examined at 8 weeks of age. It is important to determine whether mice behavior and activities decline or worsen with age."

Response: Thank you for the suggestion. To address this, we conducted a longitudinal study, phenotyping a full cohort of WT, single mutants, and double mutants at 1 year of age (Fig 5-7). We found that, aside from the TA muscle mass, all muscle phenotypes remained improved or fully rescued in double mutants at this age (Summary in Extended Data Fig. 11E). Concerning neurological phenotypes, single mutants exhibited sensory deficits that were not seen at 8 weeks (increased withdrawal latency in the hot plate and tail immersion tests). Double mutants showed a trend toward improvement in these tests. However, sensory amplitude measured by EMG was not restored (Summary in Extended Data Fig. 11F).

A result paragraph was added to discuss the novel results at 1 year showed in new Fig. 5, 6, 7: "**Combining CNM and CMT mutations shows long-term improvement of both phenotypes**

To assess the long-term effects of combining both CNM and CMT mutations, we analyzed key phenotypes in *Dnm2*$^{S619L/K562E}$ mice at 1 year of age. The improvements in body weight and length observed at 8 weeks were maintained at 1 year, while the hanging test was more challenging at this age due to increased fat (Fig. 5A-C). Stride was reduced in *Dnm2*$^{S619L/+}$ mice and rescued in *Dnm2*$^{S619L/K562E}$ (Fig. 5D). Contrary to what was observed at 8 weeks, no coordination defects were seen in *Dnm2*$^{K562E/+}$ mice at 1 year (Fig. 5D-E). Rear activity was significantly decrease at 1 year in both mutants, and there was a tendency towards improvement in *Dnm2*$^{S619L/K562E}$ (Fig. 5F). Sensory tests revealed thermal, but not mechanical, sensitivity deficits which were absent in younger mice (Fig. 5G-I). In the hot plate test, both mutants showed higher withdrawal latencies, with a tendency toward improvement in *Dnm2*$^{S619L/K562E}$ mice. In the tail immersion test, the increased tail flick latency in *Dnm2*$^{K562E/+}$ mice was fully normalized in *Dnm2*$^{S619L/K562E}$. X-ray images taken at 1.5 years revealed kyphosis and reduced body size in both single mutants, with a clear improvement in the *Dnm2*$^{S619L/K562E}$ (Fig. 5J).

At 1 year, electromyography (EMG) analysis revealed decreased compound sensorial nerve conduction velocity (csNCV) in *Dnm2*$^{K562E/+}$ mice, consistent with the observed reduced thermal sensitivity (Fig. 5G-H, Fig. 6A). csNCV was increased in *Dnm2*$^{S619L/+}$ and *Dnm2*$^{S619L/K562E}$ compared to *Dnm2*$^{K562E/+}$ and to WT. In contrast, the sensory amplitude was decreased in all *Dnm2*$^{S619L/+}$, *Dnm2*$^{K562E/+}$ and *Dnm2*$^{S619L/K562E}$ groups compared to WT (Fig. 6B). The motor NCV and amplitudes were not affected in any of the groups (Fig. 6C-D, Extended Data Fig. 10B). These findings suggest that while the double mutant exhibit improvement in conduction velocity, they fail to rescue sensory amplitude deficits.

The TA muscle weakness in *Dnm2*$^{S619L/+}$ mice persisted at 1 year, as well as the rescue provided when combining both CNM and CMT mutations (Fig. 6E-G). Of note, *Dnm2*$^{S619L/+}$ mice still develop lower TA force following nerve stimulation compared to direct muscle stimulation, supporting conduction impairments (Fig. 6H).

TA muscle mass reduction in both *Dnm2*$^{S619L/+}$ and *Dnm2*$^{K562E/+}$ mice persisted in *Dnm2*$^{S619L/K562E}$ mice at 1 year (Fig. 7A). However, reductions in Gastrocnemius mass in *Dnm2*$^{S619L/+}$ and Soleus mass in *Dnm2*$^{K562E/+}$ mice were fully rescued in *Dnm2*$^{S619L/K562E}$ at 1 year (Fig. 7B-C). Despite no improvement in

TA mass, myofiber hypotrophy was fully rescued in this muscle in *Dnm2*$^{S619L/K562E}$ mice, as shown by normalized fiber size (Fig. 7D-F).

In the peripheral nerves, the g-ratio remained decreased in *Dnm2*$^{K562E/+}$ mice compared to WT, due to thicker myelin (Fig. 7G-J). In contrast, *Dnm2*$^{S619L/+}$ mice showed an increased g-ratio compared to WT, driven by larger axons. In *Dnm2*$^{S619L/K562E}$, the g-ratio was also increased compared to *Dnm2*$^{K562E/+}$ mice, resulting from both an increase in axon diameter and thicker myelin.

Overall, the rescue of motor performance and muscle organization through the combination of CNM and CMT mutations was maintained at 1 year of age. In addition, several peripheral nerve defects were also improved." (page 5 line 178 to page 6 line 215).

We also completed some of the material and methods sections:

- "All the experiments were performed either at 8 weeks of age or between 52 and 66 weeks of age." (page 8, line 327).
- "The tail suspension test was performed on 52-week-old mice by briefly suspending them. X-ray images were taken in old mice, aged 1.5 years." (page 9, lines 345-346).

Comment 4: "Another concern is the lack of mechanistic insight. The authors have established a robust system to examine the effects of Flag-Dnm2S619L and His-Dnm2K562E in vitro, and they have obtained excitingly proof-of-concept results. The biochemical defects of these two proteins have been examined and published by several groups: Dnm2K562E is defective in membrane binding, whereas Dnm2S619L is insensitive to membrane (Kenniston and Lemmon, 2010). Therefore, it is important and will be informative to test the effect of these two mutant proteins on each other's biochemical abnormalities. Revealing the mechanism by which these two mutant proteins restore each other would provide valuable insight into the molecular action of dynamin on membranes."

Response: To provide mechanistic insight on the phenotypic in vivo compensation between the S619L CNM mutation and the K562E CMT mutation, we previously confirmed that the DNM2 K562E GTPase activity is not induced by lipid, while the DNM2 S619L is insensitive to lipid together with an increased activity under basal (no lipid) condition compared to DNM2 WT (Fig. 1A). To test the effect of these two mutant proteins on each other's biochemical abnormalities, we performed GTPase activity tests without and with lipids, and found that their combination partially rescued the overactivation of the S619L mutant (Fig. 1C-D). We indeed verified the 2 mutants can interact (Fig. 1B), supporting that the phenotypic rescue is due to the formation of DNM2 hetero-oligomers and normalization of the GTPase activity.

To investigate this mechanism in more details, and as Kenniston and Lemmon showed the DNM2 K562E is defective in membrane binding, we performed a liposome-binding assay using PS (phosphatidylserine) or PI(4,5)P2 liposomes.

The following text was added: "Previous studies reported that *DNM2* mutants have an impact on lipid binding[12]. Using liposome co-sedimentation experiments with different composition, PC 100% or bearing 3% of PI(4,5)P2 (PIP2) or 3% of PS in a PC background and recombinant DNM2 proteins, we confirmed rDNM2-WT binds liposomes and this binding is enhanced by the addition of PIP2 (Fig. 1E, Extended Data Fig. 2). Noteworthy, it binds equally to PS and PIP2. The data show that the rDNM2-KE is defective in PIP2 binding, consistent with the localization of this mutation in the lipid-binding loop. To determine the effect of combining the rDNM2-SL CNM mutant with the rDNM2-KE CMT mutant on

the membrane binding, we either co-expressed both mutants in insect cells and purified the proteins as a complex (Coexpr), or we analyzed an equimolar mixture of purified rDNM2-SL and rDNM2-KE (Mix). We found that the SL&KE hetero-oligomer normalized the binding to PIP2 liposomes to WT level compared to rDNM2-KE alone, similarly in the Coexpr and Mix conditions." (page 3, lines 85-95).

We modified the conclusion of this first result paragraph: "Overall, we confirmed gain and loss-of-function impact of *DNM2*-CNM and CMT mutations, respectively, and revealed the combination of both mutations rescued the abnormally high GTPase activity of the S619L mutation and the defective lipid binding of the K562E mutation; restoring their respective major biochemical abnormalities." (page 3, lines 97-99).

We also updated the material and methods, raw data and unedited gels supplementary files and we added reference N°41.

Comment 5: "Finally, the embryonic lethality of Dnm2K562E and Dnm2S619L homozygous mice is intriguing. Further description of the developmental stage or defects in these embryos would be valuable and informative."

Response: We agree that the embryonic lethality observed in *Dnm2*^{K562E} and *Dnm2*^{S619L} homozygous mice is an interesting aspect of the phenotype. We investigated the developmental stage at which lethality occurs. Concerning *Dnm2*$^{S619L/S619L}$ homozygous mice, in the reference n°14 (Muñoz, X. M. et al., 2020), they observed homozygous *Dnm2*$^{S619L/S619L}$ mice at E18.5 but not anymore at P2 or P10 after birth (see Fig. 1E of this reference below). We found a *Dnm2*$^{S619L/S619L}$ pup dead the day of the birth, confirming these previous results.

[FIGURE REDACTED]

Regarding the K562E CMT mutation, no *Dnm*$^{K562E/K562E}$ pups were found at P10 among the 36 genotyped (Extended Data Fig. 3A). Three litters were examined before birth (two at E18.5 and one around E14.5), and again, among the 19 embryos, none were *Dnm*$^{K562E/K562E}$ homozygous (Extended Data Fig.3B-C).

In summary, *Dnm2*$^{S619L/S619L}$ homozygous pups exhibit perinatal lethality. *Dnm*$^{K562E/K562E}$ homozygotes show early embryonic lethality, as no homozygotes were found at E18.5. Noteworthy, Ferguson and De Camilli (Dev Cell 2009) and us (Cowling JCI 2014) found the constitutive *Dnm2* knockout is embryonic lethal. Our novel data support that the K562E mutation is a loss-of-function similar to the complete lack of DNM2, while the S619L is compatible with complete embryogenesis and thus behaves differently than a loss-of-function.

We added Extended Data Fig. 3 and the text: "Of note, *Dnm2*$^{S619L/S619L}$ mice exhibit perinatal lethality[14] while *Dnm2*$^{K562E/K562E}$ died during early embryogenesis before E18.5 (Extended Data Fig. 3). Noteworthy, Ferguson et al.[17] and Cowling et al.[18] found the constitutive *Dnm2* knockout is embryonic lethal, suggesting the critical role of a DNM2 activity for mammalian development. However, our study

demonstrates the viability of *Dnm2*$^{S619L/K562E}$ mice for at least 52 weeks in the absence of any WT protein." (page 3, lines 103-108).

RESPONSE TO REVIEWER B

Mutations within a single gene can lead to a variety of diseases. Dominant mutations in the DNM2 gene result in distinct neuromuscular disorders: centronuclear myopathy (CNM) and Charcot-Marie-Tooth neuropathy (CMT). The authors present in vitro assays demonstrating opposing effects of DNM2 mutations, with gain-of-function in CNM and loss-of-function in CMT. They explored the potential compensatory effects of these mutations by breeding Dnm2S619L/+ CNM and Dnm2K562E/+ CMT mouse models. The offspring, Dnm2S619L/K562E, exhibit significantly improved motor coordination and muscle structure and function. This study reveals that two distinct disease-causing mutations within the DNM2 gene can compensate for each other in vivo. These findings are novel and significant and they were obtained through what is overall a well-conducted study. I have the following comments.

Response: Thank you for reviewing our paper. Please find below our answers to your comments point-by-point.

Comment 1 part 1 : "The studies were conducted on young mice (8 weeks old). It will be important to show that these compensatory effects of the Dnm2S619L/K562E mice are maintained in older mice, ideally at least 6 months old mice.

Response: Thank you for the suggestion. To address this, we conducted a longitudinal study, phenotyping a full cohort of WT, single mutants, and double mutants at 1 year of age (Fig 5-7). We found that, aside from the TA muscle mass, all muscle phenotypes remained improved or fully rescued in double mutants at this time point (Summary in Extended Data Fig. 11E). Concerning neurological phenotypes, single mutants exhibited sensory deficits that were not seen at 8 weeks (increased withdrawal latency in the hot plate and tail immersion tests). Double mutants showed a trend toward improvement in these tests. However, sensory amplitude measured by EMG was not restored (Summary in Extended Data Fig. 11F).

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To assess the long-term effects of combining both CNM and CMT mutations, we analyzed key phenotypes in *Dnm2*$^{S619L/K562E}$ mice at 1 year of age. The improvements in body weight and length observed at 8 weeks were maintained at 1 year, while the hanging test was more challenging at this age due to increased fat (Fig. 5A-C). Stride was reduced in *Dnm2*$^{S619L/+}$ mice and rescued in *Dnm2*$^{S619L/K562E}$ (Fig. 5D). Contrary to what was observed at 8 weeks, no coordination defects were seen in *Dnm2*$^{K562E/+}$ mice at 1 year (Fig. 5D-E). Rear activity was significantly decrease at 1 year in both mutants, and there was a tendency towards improvement in *Dnm2*$^{S619L/K562E}$ (Fig. 5F). Sensory tests revealed thermal, but not mechanical, sensitivity deficits which were absent in younger mice (Fig. 5G-I). In the hot plate test, both mutants showed higher withdrawal latencies, with a tendency toward improvement in *Dnm2*$^{S619L/K562E}$ mice. In the tail immersion test, the increased tail flick latency in *Dnm2*$^{K562E/+}$ mice was fully normalized in *Dnm2*$^{S619L/K562E}$. X-ray images taken at 1.5 years revealed kyphosis and reduced body size in both single mutants, with a clear improvement in the *Dnm2*$^{S619L/K562E}$ (Fig. 5J).

At 1 year, electromyography (EMG) analysis revealed decreased compound sensorial nerve conduction velocity (csNCV) in *Dnm2*$^{K562E/+}$ mice, consistent with the observed reduced thermal sensitivity (Fig. 5G-H, Fig. 6A). csNCV was increased in *Dnm2*$^{S619L/+}$ and *Dnm2*$^{S619L/K562E}$ compared to *Dnm2*$^{K562E/+}$ and to WT.

In contrast, the sensory amplitude was decreased in all *Dnm2*$^{S619L/+}$, *Dnm2*$^{K562E/+}$ and *Dnm2*$^{S619L/K562E}$ groups compared to WT (Fig. 6B). The motor NCV and amplitudes were not affected in any of the groups (Fig. 6C-D, Extended Data Fig. 10B). These findings suggest that while the double mutant exhibit improvement in conduction velocity, they fail to rescue sensory amplitude deficits.

The TA muscle weakness in *Dnm2*$^{S619L/+}$ mice persisted at 1 year, as well as the rescue provided when combining both CNM and CMT mutations (Fig. 6E-G). Of note, *Dnm2*$^{S619L/+}$ mice still develop lower TA force following nerve stimulation compared to direct muscle stimulation, supporting conduction impairments (Fig. 6H).

TA muscle mass reduction in both *Dnm2*$^{S619L/+}$ and *Dnm2*$^{K562E/+}$ mice persisted in *Dnm2*$^{S619L/K562E}$ mice at 1 year (Fig. 7A). However, reductions in Gastrocnemius mass in *Dnm2*$^{S619L/+}$ and Soleus mass in *Dnm2*$^{K562E/+}$ mice were fully rescued in *Dnm2*$^{S619L/K562E}$ at 1 year (Fig. 7B-C). Despite no improvement in TA mass, myofiber hypotrophy was fully rescued in this muscle in *Dnm2*$^{S619L/K562E}$ mice, as shown by normalized fiber size (Fig. 7D-F).

In the peripheral nerves, the g-ratio remained decreased in *Dnm2*$^{K562E/+}$ mice compared to WT, due to thicker myelin (Fig. 7G-J). In contrast, *Dnm2*$^{S619L/+}$ mice showed an increased g-ratio compared to WT, driven by larger axons. In *Dnm2*$^{S619L/K562E}$, the g-ratio was also increased compared to *Dnm2*$^{K562E/+}$ mice, resulting from both an increase in axon diameter and thicker myelin.

Overall, the rescue of motor performance and muscle organization through the combination of CNM and CMT mutations was maintained at 1 year of age. In addition, several peripheral nerve defects were also improved." (page 5 line 178 to page 6 line 215).

We also completed some of the material and methods sections:

- "All the experiments were performed either at 8 weeks of age or between 52 and 66 weeks of age." (page 8, line 327).
- "The tail suspension test was performed on 52-week-old mice by briefly suspending them. X-ray images were taken in old mice, aged 1.5 years." (page 9, lines 345-346).

Comment 1 part 2 : **"**Additionally, insights into whether there might be sex differences should be provided."

Response: Thank you for your comment, pointing out the lack of clarity in our text. As mentioned in the Statistical Analyses section of Materials and Methods, we initially performed a two-way ANOVA to assess any interaction between sex and genotype. No significant interaction was observed in any of the tests, indicating that the differences between genotypes are consistent across both sexes. To enhance the robustness of our findings, we therefore pooled the data from males and females for all analyses.

We clarified the sentence: "This analysis revealed significant effects of the genotype on most phenotypes. In some phenotypes (e.g. body weight and length), both genotype and sex impacted the measures; however, there was no significant interaction between these two factors, indicating that the genotype differences were consistent across both sexes (Extended Data Fig. 10C-D)." (page 12, line 480-484).

Comment 2 : **"**The functional assays that were performed on the mice are limited. Please provide insights in the running performance of the mice, either free wheel running or treadmill running".

Response: In our study, we believe we have performed a quite extensive analysis of the motor performance of the mice. During the hanging time test, we observed general motor defects in both single mutant groups, that were improved in the double mutants (Fig. 2C). We then conducted gait analysis using a treadmill, which revealed coordination impairments in *Dnm2*$^{K562E/+}$ mice that were improved with the addition of the S619L mutation (Fig. 2D-E). Additionally, the voluntary activity was not investigated by free wheel running as suggested, but through actimetry analysis over a 12-hour period and showed no major defects in free locomotion across any of the groups (Extended Data Fig. 5A-C).

Moreover, Pereira et al. (2020) previously performed rotarod and forepaw grip strength tests on *Dnm2*$^{K562E/+}$ mice and found no significant performance impairments compared to controls at 2 months of age. As the rotarod assay tests the exercise capacity and endurance, similarly to the treadmill running, we did not implement this test in our study.

Comment 3 : "The muscle histology and ultrastructural results of Figure 3 need to be more extensively quantified".

Response: Thank you for your thoughtful comment. The muscle histology in Fig. 3 has already been extensively quantified, including measurements of fiber size (Fig. 3B-C), fiber circularity (Extended Data Fig. 7A-B), internalized nuclei (Extended Data. Fig. 7C), and the percentage of fibers with abnormal staining from SDH images (Extended Data Fig. 7D).

In response to the request of the reviewer for more extensive quantification of ultrastructural defects, we have now added detailed analyses of Z-line misalignment (Extended Data Fig. 8A), and mitochondrial area and circularity (Extended Data Fig. 8B-C) from EM images. Additionally, to provide further insight into the fibrosis observed in EM images, we performed immunofluorescence for Collagen VI in transversal muscle sections and quantified the staining thickness (Extended Data Fig. 7F). We hope these additional analyses address these concerns and further strengthen our findings.

We added the text :"Ultrastructural analyses confirmed organelle misposition in *Dnm2*$^{S619L/+}$ fibers and identified fibrosis, which was further examined by immunofluorescence. Collagen thickness was found to be increased in both single mutants but normalized in double mutants (Fig. 3E, Extended Data Fig. 7F). Additionally, Z-line misalignment was observed in *Dnm2*$^{S619L/+}$ fibers (Fig. 3E, Extended Data Fig. 8A), while mitochondria were smaller in *Dnm2*$^{K562E/+}$ and rounder in both *Dnm2*$^{S619L/+}$ and *Dnm2*$^{K562E/+}$ fibers (Extended Data Fig. 8B-C). Notably, all these defects were either improved or fully rescued in *Dnm2*$^{S619L/K562E}$ muscles." (page 4, lines 142-148).

We added the following sentences in the "Electron microscopy" material and methods section : "Z-line misalignment in EM images was quantified using the angle tool in Fiji, as described in Extended Data Fig. 8A, from 2–4 mice per genotype and at least 220 sarcomeres. Mitochondrial area and circularity were assessed in QuPath via manual segmentation and annotation measurements, with 2–4 mice per genotype and at least 100 mitochondria analyzed." (page 11, lines 420-423).

Comment 4: "Molecular insights are overall lacking. Please provide a figure showing where the mutations are located and their possible effect on protein structure."

Response: In addition to the molecular data showing the impact of both mutations and their combination on the GTPase activity and the lipid sensitivity, including novel liposome binding experiments (Fig. 1A-E, Extended Data Fig. 1B-D and 2), we now provide Extended Data Fig. 1A with location of both mutations on dynamin 3D structure and commented in the text their possible effect

on protein structure: "Both DNM2 S619L-CNM and K562E-CMT mutations localize to the PH domain, albeit on distant sites. The S619L mutation likely disrupts PH-stalk interaction, leading to an open active conformation, while K562E likely affects lipid binding (Extended Data Fig. 1A)." (page 2 lines 64-66).

We also added "The 3D structure figure was performed on PyMOL, using the Crystal structure of nucleotide-free human dynamin 1 (PDB identifier: 3SNH)[42]" in the new "3D structure modelisation" material and method section (page 9 lines 347-349), and the reference n°42.

Comment 5: "The authors should provide a more detailed discussion of how insights from the present study can be used therapeutically."

Response: We agree with this and have incorporated this suggestion in the discussion part: "The inverse modulation of DNM2 activity emerges as promising therapeutic strategies to address *DNM2*-CNM and *DNM2*-CMT diseases. In *DNM2*-CNM, the pathomechanism is characterized by overactive GTPase activity. While earlier studies have established that reducing DNM2 levels can be therapeutic for *DNM2*-CNM in mice[14,33,34], our study highlights that targeting the GTPase activity might be sufficient to achieve therapeutic benefit, suggesting that the development of DNM2 GTPase inhibitors represent a future avenue**.** In contrast, our data support *DNM2*-CMT is rather a loss-of-function. A therapeutic strategy may aim to increase DNM2 activity through the development of pharmacological compounds. Gene therapy also emerges as a potential avenue for both diseases. AAV-shRNA-based therapies have already demonstrated efficacy in reducing DNM2 levels in CNM mouse models[35]. For CMT, whether increasing DNM2 level would improve the disease remains to be determined. Overall, our study introduces the inhibition of DNM2 activity to rescue *DNM2*-CNM and the upregulation of DNM2 activity as a novel therapeutic prospect for *DNM2*-CMT." (page 5 line 240 to page 6 line 251).

Comment 6 : "Line 48: Patients experience…."

Response: Thank you for bringing this mistake to our attention. We have corrected it (page 2 line 52).

Comment 7 : "Line 143, provide a reference."

Response: We added the reference n°21 : Liao, Z. *et al.* CHRNA1 induces sarcopenia through neuromuscular synaptic elimination. *Experimental Gerontology* 166, 111891 (2022). (page 15, line 562-563).