

Quality control of glycogen through direct ubiquitylation by RNF213

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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:

Referee #1

(Remarks to the Author)

In this manuscript the authors report that the ubiquitin E3 ligase RNF213 directly ubiquitylates polyglucosan, i.e. abnormally branched, poorly soluble glycogen-like polymers, which are then degraded via autophagy. The manuscript such adds another novel non-proteinaceous target to the list of substrates for ubiquitylation for mammalian cells. Actually, the same group previously showed that RNF213 directly ubiquitylates LPS (specifically the lipid A portion), thus breaking the dogma that ubiquitylation is limited to proteins. The concept that the carbohydrate binding domain CBM20 of RNF213 has a proofreading function to exclude regular glycogen as a substrate of RNF213, is very interesting. The finding that RNF213 initiates the removal of polyglucosans is also clinically relevant because accumulation of polyglucosans causes different disorders such as Andersen's disease and Adult Polyglucosan Body Disease, which usually affects multiple organs including brain and muscle. The statistical tests appear appropriate. Overall, the manuscript is clearly written, a pleasure to read, and also gives appropriate credit to previous work.

Some questions/ideas came into my mind to which the authors may comment: It is surprising that the same E3 ligase can ubiquitylate so different targets such as polyglucosans and LPS in the bacterial wall. Can this be explained by different "co-factors"? Any hint from tissue-specific interactomes of RNF213?

More specifically:

Figure 1: According to common knowledge, typical organs for glycogen storage are the liver and skeletal muscle and not the brain. Therefore, the reported findings suggest that also in the brain a limited storage of glycogen takes place and I wonder, which cell types are affected: neurons, glia or other cell types? Are these PAS-positive deposits intracellular? Does this lead to neuron loss in the long term? Are these deposits ubiquitin-negative or positive? This would suggest that also some other E3 ligases may be involved in the ubiquitination of polyglucosans. Are these deposits nevertheless subject to autophagy? This could be easily answered by co-staining with SQSTM1 or LC3B.

Figure S1: GBE1 mutations also manifest in skeletal muscle, liver, heart, ... RNF213 is also broadly expressed. If there is no accumulation of polyglucosans in other tissues than the brain, this suggests that other E-ligases than RNF213 are responsible for the ubiquitination of polyglucosans in other tissues, which should be discussed.

Related to the section "The CBM20 domain proofreads RNF213 substrates": Is there consensus that MoyaMoya is associated with RNF213 gain-of-function? An association has also been reported with loss-of-function mutations. There is one report that RNF213 loss-of-function is associated with altered angiogenesis because of activation of the hippo pathway. Is there any evidence for MoyaMoya-like alterations in heterozygous or homozygous RNF213 KO mice? Is there any report of accumulation of polyglucosans in such patients? If so, this would confirm the findings reported in this study for the human system.

Figure 2C: Here you see a large glycogen complex in WT, which is not apparent in b. Is c in the presence of shGBE1? If so, please label accordingly.

Figure 3e: Please, indicate the cell borders. Why are the glycogen deposits much larger for the W382A and Y434V variant?

Referee #2

(Remarks to the Author)

The manuscript reports that RNF213, a large AAA+ E3 ligase previously shown to ubiquitinate microbial glycans, also ubiquitinates aberrant glycogen, i.e. polyglucosan, to promote its autophagic clearance. Using an engineered cell system in which polyglucosan is induced, the authors show RNF213 recruitment to inclusions, loss of ubiquitin signal in RNF213-deficient cells, and amylase- and hydroxylamine-sensitive glycogen-ubiquitin conjugates, consistent with non-protein ubiquitylation of the glucan polymer. They combine biochemistry with cryo-EM to define a carbohydrate-binding module within RNF213 that state functions as a “proofreading” domain, restricting E3 activity toward normal glycogen while allowing targeting of polyglucosan. CBM mutants mis-target physiological glycogen and enhance ubiquitination. In Rnf213^{-/-} and catalytic-dead knock-in mice, they document age-dependent, amylase-resistant PAS-D-positive inclusions in hippocampus, cerebellum, and pons, which they interpret as polyglucosan bodies (PGBs) arising from impaired glycogen quality control in the brain. They conclude that non-protein ubiquitination of aberrant glycogen by RNF213 is a key safeguard against pathological glucan accumulation and propose that direct ubiquitination of aberrant glycogen is a general strategy used by several E3 ligases to preserve organism-wide homeostasis. While the results are very intriguing, there are a number of gaps to be addressed. The biochemical and cryo-EM work are largely rigorous, and the engineered cell system is a clear strength. In contrast, the mouse data stop at describing polyglucosan burden without defining cell type, mechanism, or functional consequences. If the *in vivo* mechanism and physiological impact were more fully established, this would represent a conceptually important advance in non-protein ubiquitination and glycogen quality control.

Major:

- Comprehensively define glycogen metabolism: This is the first report of Rnf213^{-/-} mice displaying polyglucosan inclusions in the brain and it is not clear what drives polyglucosan formation in these mice. Thus, the following is needed:
 - oProtein level and/or activity profile of glycogenin (Gyg), glycogen synthase 1 (Gys1), glycogen phosphorylase (PYGB), and glycogen branching enzyme (Gbe) in Rnf213^{-/-} brain versus WT
 - o What is the activity status of lysosome and specifically acid α -glucosidase (GAA) in Rnf213^{-/-} brain versus WT?
 - Do the inclusions colocalize with ubiquitin, LC3, and p62, similar to PGBs observed in APBD mouse brain.
 - o Fig 1- The data supporting polyglucosan inclusion the brain are not convincing in its current form.
 - The authors should include whole-brain PAS, PAS-D, and Cy5-labeled glycogen probe staining from WT, RNF213^{+/-}, and RNF213^{-/-} animals to demonstrate regional distribution of glycogen/polyglucosan
 - PAS/PAS-D quantification is missing in Fig. 1a/b. In Fig. 2b, WT mice should be included with PAS-D staining to determine what proportion of PAS-D–positive signal is age related (impact of ageing) rather than deficiency of RNF213
 - o The authors should clarify whether polyglucosan inclusions are ubiquitin-positive?
 - Extended data fig 1c- It appears that amylase digestion may not have been effective on frozen brain sections, because glycogen staining appears more punctate after amylase treatment in Rnf213^{+/+} hippocampus and cerebellum. This experiment should be repeated and shown with clean pre and post digestion data, along with quantification for Fig. 1c-d and Extended Fig. 1c. There are also other inconsistencies as cerebellar staining in Rnf213^{-/-} sections differs between Fig. 1c and Extended Fig. 1c. Moreover, visually, polyglucosan appears more abundant in Rnf213C4462S brains than in Rnf213^{-/-}, whereas the quantification suggests the opposite. Is this driven by a possible outlier in one Rnf213C4462S mouse?
- The central claim that brain polyglucosan is cleared by an RNF213-dependent ubiquitination and autophagy pathway is extrapolated from the engineered cell system rather than directly established in tissue. The *in vivo* mechanism is not demonstrated, only inferred from the cell system.
 - o The authors make a strong mechanistic case in cells that RNF213 ubiquitinates polyglucosan and directs it to autophagy.
 - o But *in vivo*, they only demonstrate that Rnf213^{-/-} mice accumulate PAS-D positive and amylase resistant polyglucosan in specific brain regions.
 - o Thus, the link b/w RNF213 loss to impaired brain glycogen autophagy to increased brain polyglucosan accumulation is speculative. Needed:
 - Co-stain brain sections with:
 - CBM20 glycogen probe or PAS-D
 - Ubiquitin and SQSTM1 (p62), LC3
 - RNF213
- No cell-type identity or whole-brain mapping of polyglucosan inclusions. The cellular identity and brain regions of polyglucosans *in vivo* are unresolved.
 - o are the inclusions neuronal, astrocytic, microglial, oligodendroglial, or in other cell types?
 - o co-staining with NeuN, GFAP, Iba1, Olig2, etc.
 - o The authors identify cerebellum, pons, and hippocampus as “specific brain regions” with polyglucosan accumulation, but they do not provide a systematic survey or quantification across other brain regions such as cortex or striatum.
 - o Quantify puncta in all figures.
- Ester linkages: The manuscript states that these are ester linkages w/o any definitive evidence and instead relies primarily on hydroxylamine sensitivity and amylase release and differences in mobility.
 - o There is no MS confirming Ub modified at its C-term, with carbohydrate adducts.
 - o Controls are lacking with Ub G76A or C-terminally blocked Ub to show loss of conjugation.

•Essential role: The authors state multiple times about the “essential role” of this pathway to maintain organism homeostasis. Yet there is no functional or behavioural phenotype despite strong claims about “safeguarding brain health” and “organismal homeostasis.”

o While they demonstrate that specific brain regions of Rnf213^{-/-} mice exhibit increased polyglucosans, there is no data suggesting a negative phenotype in these mice exhibit survival curves, neurological phenotypes (seizures, ataxia, cognition), or systemic physiology.

o Lack of phenotypic data

Even if full behavioural/EEG work is not possible, some indication that polyglucosan accumulation in Rnf213^{-/-} brains has consequences in order to claim it is a generalized safeguard: Neurological phenotype scoring (gait, rotarod, simple motor tasks), Seizure threshold (e.g., PTZ challenge), or Age-dependent progression of inclusion load vs any functional decline.

o Given that PGB accumulation in other models (e.g., Lafora disease, APBD, GBE1 hypomorphs) is typically associated with seizures and neurodegeneration, the absence of any functional phenotyping is a major weakness. This weakness is especially highlighted when the Discussion asserts an “essential role of non-protein ubiquitylation in maintaining organismal homeostasis.” Those conclusions are not supported by the current in vivo data.

•Figure 4, The authors show several indirect pieces of data that polyglucosan is targeted to lysosomes. Direct evidence, like TEM images of polyglucosan surrounded by double membranes would greatly strengthen the manuscript.

o These findings are puzzling and appear to contradict what has been reported in Pompe disease mouse model (GAA-KO). GAA is the only lysosomal enzyme known to degrade glycogen and polyglucosan inside lysosomes. So, one would expect lysosomes in GAA-KO mice to accumulate both soluble glycogen and polyglucosan. Yet only soluble glycogen has been detected in Pompe mice, and there are no reports describing lysosomal polyglucosan accumulation in Pompe patients. Do the authors have a hypothesis that could reconcile these observations? This point could strengthen the discussion section, which is currently quite brief.

o In addition, knocking out GAA in the PGB-producing engineered cell line could be envisioned. This cell line should contain even more PGBs, if the hypothesis described in the manuscript is correct.

•Lack of acknowledgement of existing RNF213 mouse phenotype data.

o Published Rnf213^{-/-} mice already exhibit CNS-related phenotypes, including blood–brain barrier fragility, altered cortical microvasculature, increased susceptibility to chronic hypoperfusion, and exaggerated responses to infection and angiogenic stimuli.

o How does the glycogen QC role integrate with published data?

•Overstating claims

o Phrases such as “essential for organismal homeostasis,” “safeguards brain health,” and “direct ubiquitylation of aberrant glycogen is a general strategy in mammals” are not fully justified. The data show an important role for RNF213 in limiting age-dependent brain polyglucosan in mice and compelling evidence for direct glycogen ubiquitination in cells, but they do not demonstrate systemic failure when RNF213 is lost.

o If RNF213 is truly a major “glycogen QC” gatekeeper, and its loss causes substantial PGB accumulation in hippocampus and cerebellum, you’d reasonably predict:

at least some EEG abnormalities or sensitivity to epilepsy inducing drugs
motor/coordination deficits (rotarod, gait, balance),
clear age-dependent neurological decline or neurodegeneration

•Human disease relevance: Test a small panel of bona fide Moyamoya RNF213 variants in the glycogen reporter system:

o Do they shift specificity toward/away from physiological glycogen?

o Do they alter autophagic clearance of polyglucosan?

o The CBM20 mutants (W382A, Y434A) are interpreted as “gain-of-function” toward physiological glycogen, then compared to Moyamoya: “This situation is reminiscent of Moyamoya disease... Since mutations causing Moyamoya disease cluster in the C-terminal E3 module..., even mild gain-of-function effects on E3 ligase activity... could get magnified...”

The disease-causing variants they reference cluster in the C-terminal E3 module, NOT the CBM20; they don’t test any bona fide Moyamoya mutations in their glycogen system.

There’s no demonstration that Moyamoya variants have gain-of-function activity toward glycogen or any defined substrate. Stronger structural/biochemical characterization of “polyglucosan”

•The data relied upon to define the aggregates as polyglucosan are rather elementary. While this reviewer does agree the aggregates are a polyglucosan, the chain-length distribution and branching frequency of the reporter-cell material vs brain inclusions should be defined.

Define engineered cell lines more completely.

•What are the protein levels of GYG1, GYS1 and GBE1 (with and without shGBE1) in the transduced MEFs compared to the transduced ones?

•What is the quantitative impact of shGBE1 on GBE1 expression as well as on glycogen and PGBs?

•How long does it take for the cells to accumulate PGBs? Is there any toxicity?

•Do the MEFs express basal levels of RNF213 expression?

•Does RNF213 overexpression significantly reduces glycogen/PGBs accumulations in those cell lines?

•What is the knockdown efficiency in all experiments?

Minor:

- Abstract: “organismic homeostasis” in the last sentence of the abstract is unusual; “organismal homeostasis” is more standard.
- Abstract: “heavily glucosidated glycogenin” is odd verbiage; “glycosylated” is more standard.
- “Since defects in other E3 ubiquitin ligases ... also cause polyglucosan accumulation, direct ubiquitylation of aberrant glycogen may be a general strategy in mammals to maintain glycogen quality.”
softening this to “may represent one strategy”

Referee #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #4

(Remarks to the Author)

In the present work, Yip et al report that the giant E3 ligase RNF213 ubiquitinates aberrant glycogen (polyglucosan), promoting its clearance via selective autophagy. Based on experiments in RNF213 knockout and ligase-deficient knock-in mice, a synthetic cellular system, in vitro ubiquitination assays, and cryo-EM analysis of RNF213 bound to maltoheptaose, the authors conclude that RNF213 functions as a glycogen quality-control factor required to prevent polyglucosan accumulation in the brain.

RNF213 itself is an unusual E3 ligase that has come to prominence through its roles in innate immunity, vascular disease, and non-protein ubiquitination, yet its physiological substrates remain poorly defined. In this context, the study by Yip et al is timely and intriguing, as it points to a potential link between RNF213 and glycogen biology. Nonetheless, despite the broad experimental range, the study does not converge on a coherent mechanistic model, and several key experiments are lacking. In particular, prior work showing HOIL-1/LUBAC-mediated glucan ubiquitination is not adequately considered, leaving central questions unresolved regarding how RNF213 relates to this quality-control (QC) pathway and how it would selectively target aberrant glycogen.

Major concerns

1. Conceptual advance relative to established glycogen QC pathway?

The manuscript presents RNF213-mediated glycogen ubiquitination as a novel glycogen QC mechanism. However, Kelsall et al. (EMBO J. 2022) already demonstrated that HOIL-1 directly ubiquitinates unbranched glucans, including glycogen and maltoheptaose, and that this activity is essential to prevent polyglucosan accumulation in vivo. Despite this, the Introduction describes the role of HOIL-1 as uncertain, even though the prior study provided detailed biochemical characterization, MS-based mapping, and a knock-in mouse model. Thus, the conceptual advance of the present work remains unclear.

2. Unresolved relationship between RNF213 and LUBAC

RNF213 knockout and ligase-deficient knock-in mice show phenotypes that closely resemble those reported for HOIL-1(C458S) mice and for human LUBAC-deficiency syndromes, including PAS-positive inclusions in hippocampus, cerebellum, and pons. This apparent overlap is particularly relevant in light of recent work (Bhardwaj et al, Nature 2025) showing that RNF213 ubiquitinates CYLD and SPATA2, thereby activating LUBAC and linear ubiquitin signalling. Thus, an alternative model is that RNF213 affects glycogen quality control indirectly by regulating LUBAC and HOIL-1, rather than acting as the primary glycogen-modifying ligase. Surprisingly, this possibility is not addressed in the manuscript. No experiments test linear ubiquitin chain formation, RNF213–LUBAC interplay, or the dependence of polyglucosan clearance on LUBAC activity in vivo or in the cellular system. Accordingly, the manuscript does not resolve whether RNF213 acts as an autonomous glycogen QC ligase or instead functions upstream or in concert with the LUBAC/HOIL-1 pathway. Because the phenotypes, proposed substrates, and disease associations of RNF213 and HOIL-1 are so closely overlapping, distinguishing between these models is not a secondary mechanistic detail but a central conceptual question.

3. Concerns regarding physiological relevance of the cellular system

The cellular experiments rely on a highly engineered MEF system involving overexpression of glycogenin (glycogen carrier) together with GBE1 (branching enzyme), combined with GBE1 knockdown to induce polyglucosan formation. However, it is not explained why such an elaborate setup is necessary. If MEF cells do not produce sufficient levels of glycogen, why did the authors pick this cell line for studying glycogen QC? Given that polyglucosan accumulation is restricted to brain tissues in mice, neuronal or glial cells may have been the better model system. Furthermore, their setup generates prominent polyglucosan–protein clusters (Fig.2a,c), raising the possibility that RNF213 recruitment is indirect and reflects a general aggregation or stress response rather than a specific glycogen QC mechanism. The authors should test the composition of these clusters and their relationship to disease-relevant neuronal polyglucosan inclusions. Finally, overexpression of RNF213 itself may affect its localization, activity and assembly and need to be controlled as well. For all these reasons, it is not clear to what extent the engineered cellular system reflects physiological glycogen QC, limiting the relevance of the mechanistic conclusions drawn from it.

4. Structural analysis does not explain substrate recognition or selectivity

The cryo-EM structures reveal maltoheptaose bound to a CBM20-like domain of RNF213, providing evidence that this domain can engage short linear glucans. However, CBM20 mutants that disrupt maltoheptaose binding still localize to and ubiquitinate polyglucosan in cells, indicating that carbohydrate binding by this domain is not required for RNF213 recruitment or activity. So, is there another sugar binding site in RNF213, or is the interaction with glycogen in cells indirect? With respect to the tested CBM20 mutants, the authors should demonstrate that the proteins are structurally intact to exclude local misfolding as reason for the observed phenotypes. It is concerning that both CBM20 mutants display strongly increased E3 activity compared to WT (Fig.3f), pointing to non-specific or pleiotropic effects rather than a clean separation-of-function phenotype. Moreover, maltoheptaose-like segments are expected to occur in both physiological glycogen and poorly branched polyglucosan, given the known branching frequency of glycogen. As such, the current data do not explain how RNF213 would selectively discriminate between normal and aberrant glycogen, a distinction that should be central to the QC mechanism. Consequently, the proposed model of CBM20-mediated proofreading extrapolates far beyond the presented evidence, and the suggested "auto-inhibitory proofreading module" remains a conceptual hypothesis rather than a mechanism supported by experimental data.

5. Insufficient evidence for direct ubiquitination of glycogen by RNF213

Direct glycogen ubiquitination is inferred from ubiquitin release experiments performed on STBD1 (CBM20)-captured aggregates containing cellular glycogen and from in vitro ubiquitination of maltoheptaose. However, STBD1 capture is expected to co-purify glycogen associated or co-aggregating proteins, meaning that hydroxylamine sensitivity could reflect ester-linked ubiquitination of proteins as well. Interactome data could resolve this concern, revealing bystander and QC partner proteins. Also, the small-size difference observed upon amylase treatment (Fig.2g) is not sufficient to propose sugar modification. Definitive evidence would require chemical or MS-based mapping of the ubiquitin adducts. In addition, the in vitro maltoheptaose ubiquitination assay relies on remarkably high substrate concentrations of 20 mM and yields only weak modification, raising concerns about the specificity and efficiency of the reaction. Testing CBM mutants would help to show specific targeting via the sugar binding site. It also needs to be shown to what extent maltoheptaose reflects the physiological substrate, for example by analysing other carbohydrates that more closely resemble the different glycogen forms.

6. Autophagy involvement without a defined initiating ubiquitin signal

The manuscript shows colocalization of polyglucosan with p62 and LC3 and increased polyglucosan accumulation upon autophagy inhibition, consistent with autophagy contributing to clearance. Which is good. However, these observations do not establish RNF213-dependent ubiquitination as a critical signal for selective autophagy and are equally consistent with ubiquitination of associated proteins or secondary recruitment of autophagy receptors. Analysis of ubiquitin linkage types and interactions with known partner E3 ligases would be required to support this interpretation. It also remains unclear whether the ubiquitin signal resides on glycogen itself or on associated proteins, a distinction that is essential for interpreting the mechanism. With regards to the assay, the group introduces a flow cytometry-based approach using TMR-labelled glycogenin to distinguish cytosolic from lysosomal polyglucosan. While the data appear technically sound, its interpretation remains ambiguous in the absence of proper controls. For example, the reduced TMR signal in the presence of RNF213 is interpreted in different ways. In Extended Data Fig.8a, reduced in-gel TMR-labelled glycogenin is taken to reflect RNF213-dependent clearance. In contrast, in the flow cytometry assay (Fig.4d), a similar reduction in TMR signal is linked to altered subcellular localization, attributed to reduced TMR labelling in the acidic lysosomal environment. To support the claim that the flow cytometry assay specifically reports on subcellular localization rather than protein abundance, glycogenin levels should be directly quantified under the same conditions.

7. Speculation on disease relevance

The Discussion speculates on the relevance to Moyamoya disease (MMD) and interprets CBM20 mutants as proxies, yet RNF213 variants linked to MMD have not been associated with glycogen storage defects or polyglucosan pathology. In contrast, HOIL-1 and HOIP mutations are directly linked to polyglucosan accumulation in patients, indicating that the established disease biology lies within the LUBAC pathway rather than with RNF213. Moreover, the manuscript argues that Moyamoya mutations are gain-of-function because patients are heterozygous, yet the here presented data show that RNF213 heterozygous mice accumulate polyglucosan, which is more consistent with partial loss-of-function. Given these inconsistencies, the disease-related interpretations are not supported by current evidence.

Minor points

- 1) Cellular levels of used RNF213 constructs should be quantified, potential effects of mutations on protein stability assessed, and expression tuned closer to endogenous levels where feasible. In addition, the Walker B3 (E2449) mutant should be included.
- 2) Beyond histological analysis, it would be informative to test whether RNF213-deficient or mutant mice show behavioral or neurological phenotypes consistent with brain polyglucosan accumulation, such as impairments in motor coordination, cognition, or seizure susceptibility.
- 3) Terminology relating to glycogen, polyglucosan, amylopectinosis, and Lafora bodies should be used consistently and aligned with the glycogen-storage literature. A schematic illustrating the different glycogen species and indicating which assays probe which species would greatly improve clarity.
- 4) Figure 3d switches to human HEK293ET cells and uses a truncated RNF213 "CBM-stalk" construct (human or mouse protein?), whereas most other experiments use MEFs and full-length RNF213. The rationale for this change in cell type and

model system should be explained.

5) In Fig. 4a, the baseline number of polyglucosan puncta differs between WT and CA cell lines, even in the –Dox condition, complicating interpretation of the rescue experiment. Considering errors between experiments, effects do not look significant.

6) In Extended Data Fig. 8a, Coomassie staining suggests unequal loading between –Dox and +Dox samples for the mutants, making conclusions about unchanged polyglucosan levels less convincing.

7) Extended Data Figure 6 does not specify which sequences or organisms were used to compute conservation scores. In addition, the text states that all contact residues except H447 are highly conserved, whereas the figure indicates that Y434 is also not conserved. Thus, two key residues implicated in glycogen binding are not conserved, raising questions about the identity of the recognized substrate. Furthermore, the interpretation that carbohydrate-binding residues of CBM20 are highly conserved seems to contradict an earlier report from the same group that CBM20 underwent positive selection in pathogen recognition (Crespillo-Casado et al., EMBO Rep. 2024). These opposing models imply distinct evolutionary pressures shaping the CBM20 domain and its substrate spectrum, suggesting that at least one of the arguments requires revision.

8) The phrase “enzymatically amplified recruitment” is vague and not helpful, as the mechanistic steps and/or catalytic activities underlying such amplification are not defined.

9) C4462S and C4462A mutations in the catalytic RZ domain are used in different parts of the study. The authors should clarify whether these mutations have equivalent effects on RNF213 E3 ligase activity and explain why both are used interchangeably.

Referee #5

(Remarks to the Author)

Ubiquitylation is an essential posttranslational modification that has mostly been described for its ability to control the function or stability of proteins. However, recent work suggested that ubiquitylation can also target non-proteinaceous biomolecules, including sugars or small molecules. The Randow lab has been at the forefront of this development with their discovery of RNF213 as an E3 ligase that helps cells defend against bacterial invasion by ubiquitylating lipopolysaccharides exposed on the bacterial membranes. Here, they report evidence that suggests that RNF213 can also ubiquitylate polyglucosan bodies that originate from aberrantly branched glycogen molecules. Ubiquitylation of polyglucosan bodies by RNF213 directs these structures into autophagosomes, although it seems that polyglucosan is not degraded. Loss of RNF213 leads to accumulation of polyglucosan bodies in specific regions of the brain, indicating that RNF213 is involved in polyglucosan body removal in vivo. While previous manuscripts had documented glycogen ubiquitylation in vitro, it is nice to see similar reactions in vivo, thus expanding the reach of ubiquitylation from regulating proteins to the control of other biomolecules. However, several questions would need to be addressed before this paper represents progress expected from a publication in Nature.

1. Most importantly, it is not clear whether this regulatory circuit is critical for preserving cellular fitness or survival. As a consequence, it remains to be shown whether the reactions described here primarily occur to prevent polyglucosan accumulation, or whether they are a side reaction of more important, still to be discovered, pathways. It would be critical that the authors identify a RNF213 mutant that is unable to bind polyglucosan and hence not recruited to these structures (different from their CBM20 mutants that result in a potential gain-of-function phenotype). They would then have to show that such mutants cause some fitness defect to provide evidence that the regulation reported here is important. The authors could also document the importance of RNF213-dependent polyglucosan ubiquitylation by showing genetic interactions between RNF213 loss of function and enzymes of glycogen processing or degradation, with a focus on substrate-binding mutants rather than catalytic mutations that could affect other processes as well.

2. What happens with polyglucosan bodies in autophagosomes? The way the manuscript is written suggests that these structures may be degraded in autophagosomes, but the presented data only shows degradation of associated proteins. If the polyglucosan body is not degraded, how then do the authors explain the accumulation of glycogen in the brain of RNF213 KO mice? Maybe the authors could follow polyglucosan bodies for longer periods of time? Maybe they did not use the proper cell line for their studies? This discrepancy should be addressed.

3. While their data (especially in Fig. 2g) is consistent with ubiquitin being attached to a glucose unit, they do not show direct evidence for such a reaction. Given the importance for this manuscript, they would need to provide direct evidence for polyglucosan ubiquitylation, potentially by mass spectrometry after amylase treatment.

4. Fig. 3 – the mechanism of RNF213 regulation via the CBM20 domain is discussed in a slightly confusing way. It is possible that the CBM20 domain acts in modulating autoinhibition of RNF213, but it may also regulate features such as E3 ligase oligomerization that could affect catalytic activity independently of autoinhibition. To address these issues, the authors should at least look at RNF213 dimerization/oligomerization as well as binding partners in CBM20-mutant cells. Such analyses may address the discrepancy between CBM20-mutants disrupting glycogen binding in pulldown experiments, while being competent to bind polyglucosan bodies by microscopy.

5. Fig. 3e – it seems that polyglucosan bodies are larger when CMB20-mutant RNF213 is being expressed. This

complicates interpretations of a gain-of-function phenotype regarding polyglucosan ubiquitylation, as enhanced recruitment of multiple RNF213 molecules to a larger polyglucosan body might result in aberrant ubiquitylation that is independent of intrinsic catalytic activity.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

To my mind, the authors addressed all questions raised by me and also raised by the other reviewers in an appropriate manner. Thereby, the manuscript has been further improved. As far as I have been able to assess the statistical tests applied, I think these were appropriate. In Fig.4 A, I would suggest to also state the number of cells analyzed per experiment or even show the quantification for each cell with different colours per experiment. This also applies to Ext. Data Fig. 3 d, e, g, h, Ext. Data Fig. 8c-g, j and k, and Ext. Data Fig. 9 d-g and l. Ext Figure 1g, please, specify n=3 mice each?

Referee #2

(Remarks to the Author)

The authors did an impressive job of addressing reviewer concerns. The revised manuscript is substantially improved and now provides a strong mechanistic and conceptual advance. The authors added important *in vivo*, biochemical, structural, and cell-biological data that address most major concerns. The central conclusion that RNF213 limits brain polyglucosan accumulation and can directly ubiquitylate aberrant glycogen is now very well supported. The remaining limitations focus around the extent to which the cell-based autophagy mechanism is directly demonstrated in brain tissue, the incomplete mechanistic explanation for CBM20-dependent substrate discrimination, and the lack of robust organismal phenotypes. IMO, these limitations can be addressed largely by textual clarification, rather than by new experiments.

1. The *in vivo* autophagy mechanism is inferred from the engineered cell system. The text should be explicit that the tissue data establish the phenotype and genetic requirement, while the mechanistic autophagy pathway is established primarily in cells.

2. The CBM20 "proofreading" mechanism still seems incomplete. The CBM20 data are interesting and strong, but it is not that simple. B/c RNF213 binds maltoheptaose via a CBM20-like domain, mutations in sugar-contact residues disrupt glycogen binding, and W382A/Y434A mutants gain the ability to ubiquitylate physiological glycogen. However, some CBM20 mutants still target polyglucosan. Therefore, CBM20 is not the only recruitment determinant for aberrant glycogen. It appears to suppress inappropriate activity toward physiological glycogen. I would suggest that the authors avoid verbiage saying that the CBM20 fully explains specificity. Instead: The CBM20 domain restricts RNF213 activity toward physiological glycogen and contributes to substrate discrimination.

3. The new LUBAC data are a very strong addition. RNF213 deletion prevents HOIP recruitment, HOIL1/HOIP loss does not prevent RNF213 recruitment, and LUBAC overexpression can bypass RNF213 in cells. This supports the hierarchy: RNF213 upstream of LUBAC under their experimental conditions. This is very strong in cells, but not proven *in vivo*. If possible, I would recommend framing this as the model rather than fully established *in vivo*.

4. I applaud the behavioral testing and data in the R2R. Agreed that it should not be included in this manuscript. I also agree that their mechanism does "safeguard the brain" in terms of molecular/histopathological rather than functional.

5. The Moyamoya sections are substantially improved and they acknowledge that the relationship to Moyamoya remains open.

6. Should the CBM20 mutant structural-integrity controls be included as extended data? SEC-MALS for WT/W382A/Y434A and cryo-EM structure of Y434A, arguing against misfolding. The authors can decide.

Referee #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #4

(Remarks to the Author)

The authors have undertaken an extensive revision, generating substantial new data (MALDI-TOF mass spectrometry, LUBAC epistasis analysis, cryo-ET, astrocyte validation). However, rather than reinforcing the mechanistic model, the additional experiments do more to weaken than to strengthen the central claims. While the data establish that RNF213 contributes to polyglucosan homeostasis in astrocytes, they do not show that RNF213 directly recognizes or ubiquitinates polyglucosan, that it acts as the priming E3 ligase, or that the mechanism constitutes a general quality-control system across tissues. Most critically, LUBAC overexpression rescues polyglucosan ubiquitination in RNF213 KO cells. Combined with the

absence of direct biochemical reconstitution of RNF213-catalysed polyglucosan ubiquitination, the data remain fully compatible with an alternative model in which LUBAC performs the central glucan ubiquitination while RNF213 is an upstream regulator. My remaining concerns are:

1. The data do not establish RNF213 as the priming E3 ligase for polyglucosan ubiquitination

The revised manuscript continues to position RNF213 as the primary E3 ligase that directly recognises and ubiquitinates polyglucosan. Four lines of evidence indicate this conclusion remains indirect and that the evidence is equally compatible with a model in which LUBAC performs the central glucan ubiquitination reaction while RNF213 serves as upstream regulatory factor.

First, LUBAC overexpression restores polyglucosan ubiquitination in RNF213 KO cells (ExtData Fig 8i–k), directly challenging the proposed epistatic hierarchy: if RNF213 were the essential initiating E3 ligase, bypassing it through overexpression of a nominally downstream enzyme would not be expected. The data instead indicate that polyglucosan ubiquitination can be established independently of RNF213.

Second, loss of polyglucosan ubiquitination in ATPase and E3-ligase dead RNF213 mutants is interpreted as evidence that these enzymatic activities are specifically required for ubiquitin transfer to polyglucosan. However, these same activities are required for RNF213 coat formation on bacteria and lipid droplets, i.e. contexts where the downstream function is recruitment and organizing a signaling hub rather than direct substrate ubiquitination. Likewise, in *Toxoplasma gondii*, ubiquitin ligase activity is dispensable for RNF213 recruitment, illustrating context-dependent contributions. The observed loss of polyglucosan ubiquitination in these mutants is therefore equally compatible with impaired recruitment, defective coat formation, or failure to engage downstream ubiquitination factors.

Third, direct biochemical reconstitution of RNF213-mediated polyglucosan ubiquitination has not been demonstrated. The only in vitro reconstitution experiment uses maltoheptaose, a generic α 1,4-linked glucan shared by physiological glycogen and pathological polyglucosan and lacking the structural features (extended chain length, reduced branching) that distinguish polyglucosan from normal glycogen. The data show that purified RNF213 can ubiquitinate a short linear glucan, but do not demonstrate recognition or ubiquitination of polyglucosan, nor do the data address substrate specificity. Showing direct RNF213-catalysed polyglucosan ubiquitination with appropriate substrate specificity is essential, as asked for by referees, yet this point was not addressed during revision.

Fourth, polyglucosan structures in RNF213 KO brains are p62-positive but ubiquitin-negative (Reviewer Fig 1). This is hard to reconcile with the proposed ubiquitin-dependent autophagy model, under which structures accumulating without RNF213 should be devoid of both ubiquitin and the ubiquitin-dependent receptor p62. The presence of p62 on ubiquitin-negative polyglucosan instead points to either ubiquitin-independent p62 recruitment or a clearance mechanism less ubiquitin-dependent than the model assumes - undermining a central premise of the proposed pathway.

2. Tissue restriction and mild phenotype do not support an essential quality-control mechanism

The in vivo phenotype is restricted to astrocytes in specific brain regions, despite RNF213's broad tissue expression. Liver, skeletal muscle, and heart – these are tissues where glycogen metabolism is highly active and where Jochem et al (2026) detect abundant endogenous glycogen ubiquitination - show no polyglucosan accumulation in RNF213 KO mice. This pattern challenges the generality of the quality-control mechanism proposed in the abstract and instead points to a context-dependent, tissue-specific role. The authors suggest compensation by other E3 ligases may account for this discrepancy. While plausible, this remains speculative and is equally consistent with a model in which LUBAC constitutes the principal glucan ubiquitination system and RNF213 becomes limiting only in specific cellular contexts.

The physiological phenotype in RNF213 KO mice is also notably mild compared with established disorders of glycogen and polyglucosan metabolism. At 8–10 months, polyglucosan accumulates in cerebellum, pons, and hippocampus (regions critical for motor coordination and memory) yet no behavioural deficits are detected. Defects in proteins that directly regulate glycogen architecture or polyglucosan clearance (GBE1, laforin, malin) result in progressive polyglucosan accumulation, pronounced neuroinflammation, and severe neurological phenotypes at comparable ages. Even HOIL-1 knock-in mice exhibit learning and motor coordination deficits. The presented evidence is thus not sufficient to state that RNF213-mediated glycogen ubiquitination is “essential for biomolecular quality control”. The mild phenotype suggests RNF213 plays a contributory rather than central role in polyglucosan homeostasis.

3. The CBM20 data do not explain substrate selectivity

The SEC-MALS and cryo-EM data rule out misfolding artefacts for one CBM20 mutant, supporting the interpretation that its phenotype reflects altered carbohydrate recognition. However, the data show only negative regulation — CBM20 prevents inappropriate targeting of normal glycogen — rather than a positive selectivity mechanism for polyglucosan. The manuscript itself states the recognition mechanism is unknown, yet the abstract claims that “the CBM20 domain confers specificity”, implying an active substrate selection that is not directly demonstrated.

There is also a discrepancy between RNF213 localisation and ubiquitin deposition: CBM20 mutant proteins penetrate throughout soluble glycogen particles, whereas wild-type RNF213 forms rim-like structures on polyglucosan. If the model held, ubiquitination should mirror this altered occupancy, yet the ubiquitin signal in Fig 3e remains largely confined to peripheral rims rather than showing the predicted homogeneous distribution, an inconsistency that is not further explained. Taken together, the central question underlying any quality-control function — how RNF213 distinguishes pathological polyglucosan from normal glycogen — remains unresolved.

4. Data restricted to the rebuttal, and claims without experimental support

A notable feature of the revision is the amount of experimental data presented only in the reviewer response and not incorporated into the manuscript, despite being central to the authors' mechanistic arguments. These include the finding that polyglucosan in RNF213 KO brains is p62-positive but ubiquitin-negative (Reviewer Fig 1), which questions whether clearance is ubiquitin-dependent; behavioural phenotyping (Reviewer Figs 8–11), performed to support claims of physiological significance; expression-level controls comparing RNF213 variants with endogenous levels (Reviewer Fig

14); and proteomics of polyglucosan composition (Reviewer Fig 13). The same applies to the fate of polyglucosan within lysosomes, which is likewise only partly resolved. ExtData Fig 9j–l shows glycogenin degradation in LAMP1-positive compartments while polyglucosan persists, and cryo-EM data shown only in the rebuttal (Reviewer Fig 18) reveal a dense-core/loose-periphery architecture interpreted as partial outer-layer degradation. This model — lysosomes degrading surface layers and smaller particles while the core persists — remains speculative, and the key supporting data will again be unavailable in the published manuscript.

Finally, some mechanistic arguments lack experimental support altogether. For example, a hypothetical ubiquitin-binding site in RNF213 is used to explain enzymatically amplified recruitment and a ubiquitin dependent feed-forward model of coat formation, yet no data supporting this site or its role in polyglucosan biology are presented in either the manuscript or the rebuttal. Given that the CBM alone does not account for polyglucosan targeting, providing such data is well within the scope of this study.

Taken together, these data are important to the authors' conclusions but invisible to readers — an unfortunate disconnect between the evidence used to support the model and what the published paper allows others to assess.

Referee #5

(Remarks to the Author)

The authors have added several nice experiments to strengthen their model in which RNF213 ubiquitylates aberrant glycogen for clearance through autophagy. This is an important discovery and may deserve publication. However, they did not address my main initial concern, i.e. whether removal of aberrant glycogen through RNF213 is important for cell fitness or survival. While their data clearly shows that RNF213 can modify polyglucosan with ubiquitin, and that RNF213-knockout mice accumulate polyglucosan bodies in the brain, they do not show that ubiquitin-dependent removal is important - they merely infer this from diseases, in which polyglucosan accumulates. However, it is dangerous to draw such conclusions as diseases often have multiple contributors. At this point, I am not fully convinced that RNF213-dependent glycogen ubiquitylation is critically important for cells. Given that the discovery of glycogen ubiquitylation (together with a recent paper from David Komander's lab) is important, maybe the authors could add a substantial paragraph to explicitly mention this shortcoming of their study, protecting them from potential overinterpretations.

Version 2:

Reviewer comments:

Referee #2

(Remarks to the Author)

The revised manuscript includes an important new experiment demonstrating RNF213 directly ubiquitinates cell-derived polyglucosan while comparatively sparing glycogen. This directly addresses important reviewers' concern that the earlier maltoheptaose assay established glucan ubiquitination but did not demonstrate recognition or modification of a disease-relevant polymer. Together with the added LUBAC epistasis, cryo-ET, and biochemical data, the revision substantially strengthens the central conclusion that RNF213 participates directly in polyglucosan quality control, while appropriately leaving the precise initial recognition mechanism and in vivo RNF213–LUBAC hierarchy unresolved. The authors have responded carefully and substantively to my previous comments; the remaining issues are primarily related to quantitative rigor, reporting, factual precision, and calibration of the strongest claims.

Moderate issues

- Quantification and normalization of the purified-substrate experiment. Figure 2i is central to the revised manuscript, but the legend does not state the number of independent substrate preparations or ubiquitylation reactions, and the glycogen-versus-polyglucosan comparison is presented without quantitative analysis. The authors should quantify the RNF213-dependent ubiquitin signal across independent experiments and specify the experimental unit.
- Substrate normalization in Figure 2i. Glycogen and polyglucosan appear to be normalized using TMR-labelled glycogenin. However, polyglucosan could contain more glucan per glycogenin molecule, potentially contributing to the greater apparent ubiquitylation.
- Reaction conditions for the new biochemical experiment. The Methods should provide complete reaction composition, substrate input, purification strategy, replicate structure, and normalization procedures for the direct glycogen/polyglucosan ubiquitination assay.
- Important response-only controls. Several datasets used to defend central mechanistic interpretations would be useful to readers as Extended Data, particularly the brain p62/ubiquitin/LC3 staining, RNF213 construct-expression controls, and SEC-MALS/Y434A structural-integrity controls. These data directly clarify the in vivo interpretation and exclude mutant instability as the cause of the CBM20 phenotype.
- Calibration of the central claims. The data establish direct glucan ubiquitination as a mechanism that limits astrocytic polyglucosan accumulation. They do not yet establish non-protein ubiquitination as broadly “essential for biomolecular quality control,” nor do they demonstrate complete degradation of the polyglucosan carbohydrate core after lysosomal delivery. These statements should be narrowed to distinguish autophagic targeting and partial lysosomal turnover from complete glucan degradation.

Minor issues

- Modify the description of the Jochem/Komander study. The manuscript states that polyglucosan ubiquitylation decreased by approximately 60% following HOIL-1 depletion. The published experiment used HOIP/RNF31 knockdown in GBE1-

deficient cells. This should be corrected.

- Revise the description of malin–laforin. The current text presents the pathway too narrowly as targeting glycogen synthase and PTG for proteasomal degradation. Malin also ubiquitylates PYGB and promotes its nuclear localization rather than degradation. “The malin–laforin pathway regulates glycogen metabolism through ubiquitination of glycogen-associated proteins, including glycogen synthase, PTG, and PYGB; depending on the substrate and context, this can alter protein abundance, localization, or activity rather than uniformly promoting proteasomal degradation.”
- Figure 1b legend. The panel appears to include age-matched wild-type and Rnf213-null sections, but the legend refers only to Rnf213-null mice.
- Figure 2i legend. Specify the number of independent reactions and independent substrate preparations.
- Extended Data Figure 3g,h legend. The text refers to “c, RNF213” and “d, ubiquitin”; these panel references appear to be copied incorrectly and should likely refer to panels g and h.

Referee #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #4

(Remarks to the Author)

The authors have added one new data point: They reconstituted the ubiquitination of polyglucosan and glycogen by RNF213 in vitro and could show that RNF213 can selectively ubiquitinate polyglucosan over glycogen under defined conditions. This is an important advance, addressing one of my major concerns. Moreover, I agree that the epistasis data together with the independent finding that HOIL-1 depletion reduces polyglucosan ubiquitination by only ~60% (Jochem et al, Nature 2026) make the alternative LUBAC-centric model harder to sustain and I consider these points adequately addressed. Nevertheless, I still have concerns that the revision does not resolve.

1. The glycogen QC mechanism remain unknown

The CBM20 domain has been re-framed as an autoinhibitory module that limits RNF213 activity towards physiological glycogen, which is supported by the data. However, this negative regulatory function does not explain how RNF213 is recruited to polyglucosan in the first place. The manuscript explicitly acknowledges that “the mechanism of RNF213 recruitment to polyglucosan remains to be elucidated”. The CBM-stalk pulldown data do not fill this gap, as efficient recruitment of full-length RNF213 requires the intact AAA core and E3 module that the fragment lacks. What structural or biochemical feature of polyglucosan triggers RNF213 engagement, for example reduced branching density, chain length, altered surface chemistry, is entirely open. Likewise, it is unclear how sugar binding to the CBM20 domain allows to secure glycogen, and thus it is still open how the surveillance machinery distinguishes normal from aberrant substrate. Without this, the paper describes a pathway but not the mechanism of the sugar QC system.

2. The p62 discrepancy between cells and brain tissue is not resolved

In the engineered cell system, p62 recruitment to polyglucosan bodies is RNF213-dependent. In RNF213 KO mouse brains, however, polyglucosan is p62-positive but ubiquitin-negative. The authors refer to ubiquitin-independent p62 recruitment in vivo as an explanation, which is plausible but leads to an additional question: if p62 can be recruited to polyglucosan without ubiquitin in vivo, why does this not trigger LC3 recruitment and at least partial autophagic clearance? The rebuttal raises this as an interesting topic for future research but does not reconcile it with the model, keeping an internal inconsistency between the cell-based mechanism and the in vivo observations.

3. Key mechanistic data remain outside the manuscript

Several experiments central to the authors' arguments are presented only in the reviewer response and will not be available for independent assessment in the published paper: the p62/ubiquitin co-immunofluorescence in RNF213 KO brain sections (directly relevant to the autophagy model) and the cryo-EM data showing a dense-core/loose-periphery architecture in lysosomes (the primary evidence for the partial degradation model). I would ask the authors to include at least these data as Extended Data figures.

4. The abstract conclusion is too broad

Given that the in vivo phenotype is restricted to astrocytes with no behavioural consequence and that the QC mechanism is unresolved, the concluding statement “establishing non-protein ubiquitylation as essential for biomolecular quality control” overstates what has been demonstrated. I would ask the authors to restrict this claim to what the data directly support, i.e. that RNF213-mediated ubiquitination is required for polyglucosan homeostasis in astrocytes.

Version 3:

Reviewer comments:

Referee #2

(Remarks to the Author)

I have reviewed the revised manuscript and rebuttal. The authors have satisfactorily addressed all of my remaining concerns. The new quantitative and biochemical data, particularly the direct reconstitution of preferential polyglucosan ubiquitylation by purified RNF213, substantially strengthen the central conclusion. The remaining mechanistic questions are appropriately acknowledged and represent future directions. This is a very strong, exciting and field-shifting piece of work.

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We thank the reviewers for their thorough and insightful evaluation of our manuscript.

Based on their suggestions, we obtained the following new insights:

1. Brain, but not liver, glycogen in *Rnf213*^{-/-} mice is poorly branched (**new data, Fig. 1f,g**), thus explaining the accumulation of polyglucosan in *Rnf213*^{-/-} mice
2. The levels of glycogen-metabolizing enzymes remain unchanged in *Rnf213*^{-/-} mice (**new data, Extended Data Fig. 1h,i**), consistent with a polyglucosan-directed role for RNF213 in glycogen quality control
3. Polyglucosan in *Rnf213*^{-/-} mice accumulates in astrocytes (**new data, Extended Data Fig. 1e**),
4. Mass spectrometry provides direct evidence for RNF213-catalyzed ubiquitylation of glucose in polyglucosan (**new data, Fig. 2h**)
5. Ubiquitin chains on polyglucosan are comprised of M1- and K48-, but not K63-linked monomers (**new data, Extended Data Fig. 3k**)
6. Epistasis analysis places RNF213 upstream of LUBAC in glycogen quality control (**new data, Extended Data Fig. 8**), revealing that RNF213 prevents polyglucosan accumulation through both direct ubiquitylation of aberrant polyglucosan and epistatic control of LUBAC
7. Cryo-electron tomography visualizes polyglucosan in autophagosomes, providing direct evidence for autophagic clearance of polyglucosan (**new data, Fig. 4c-e**)

Below we address all reviewers' comments in detail.

Referee #1 (Remarks to the Author):

In this manuscript the authors report that the ubiquitin E3 ligase RNF213 directly ubiquitylates polyglucosan, i.e. abnormally branched, poorly soluble glycogen-like polymers, which are then degraded via autophagy. The manuscript thus adds another novel non-proteinaceous target to the list of substrates for ubiquitylation for mammalian cells. Actually, the same group previously showed that RNF213 directly ubiquitylates LPS (specifically the lipid A portion), thus breaking the dogma that ubiquitylation is limited to proteins. The concept that the carbohydrate binding domain CBM20 of RNF213 has a proofreading function to exclude regular glycogen as a substrate of RNF213, is very interesting. The finding that RNF213 initiates the removal of polyglucosans is also clinically relevant because accumulation of polyglucosans causes different disorders such as Andersen's disease and Adult Polyglucosan Body Disease, which usually affects multiple organs including brain and muscle. The statistical tests appear appropriate. Overall, the manuscript is clearly written, a pleasure to read, and also gives appropriate credit to previous work.

We thank the reviewer for their enthusiasm.

Some questions/ideas came into my mind to which the authors may comment: It is surprising that the same E3 ligase can ubiquitylate so different targets such as polyglucosans and LPS in the bacterial wall. Can this be explained by different "co-factors"? Any hint from tissue-specific interactomes of RNF213?

We agree with the reviewer that the broad substrate specificity of RNF213 is remarkable. In addition to polyglucosan and LPS, work from our group and others has shown that RNF213 also targets Gram-positive bacteria, apicomplexan parasites, and viruses, although the precise ubiquitylation substrates for the latter remain to be identified.

The deployment of cofactors to direct RNF213 towards its diverse substrates is therefore an attractive hypothesis. However, to the best of our knowledge, none has been identified yet, while some evidence suggests that RNF213 recognizes at least some of its substrates directly:

1. Purified RNF213 can ubiquitylate LPS (Otten et al., *Nature*, 2021) and glycogen-derived oligosaccharides in vitro, albeit with low efficiency (**Extended Data Fig. 4a**)
2. Human RNF213 expressed in cells from taxa that natively lack RNF213 (e.g. all insects) retains functionality towards *Salmonella* and *Listeria*, consistent with direct substrate recognition (unpublished observation)
3. RNF213 in simian monkeys has undergone episodes of strong positive selection, indicative of an evolutionary arms race between pathogens and RNF213, consistent with direct physical contact between RNF213 and at least some of its diverse substrates (Crespillo-Casado et al., *EMBO Rep.*, 2024)

Further experimentation will be needed to settle the issue. We agree with the reviewer that a tissue-specific interactome of RNF213 may reveal novel insights into RNF213 regulation. However, we believe that a comprehensive interactome analysis falls outside the scope of the current manuscript.

More specifically:

Figure 1: According to common knowledge, typical organs for glycogen storage are the liver and skeletal muscle and not the brain. Therefore, the reported findings suggest that also in the brain a limited storage of glycogen takes place and I wonder, which cell types are affected: neurons, glia or other cell types? Are these PAS-positive deposits intracellular? Does this lead to neuron loss in the long term? Are these deposits ubiquitin-negative or positive? This would suggest that also some other E3 ligases may be involved in the ubiquitination of polyglucosans. Are these deposits nevertheless subject to autophagy? This could be easily answered by co-staining with SQSTM1 or LC3B.

We thank the reviewer for these interesting questions.

Affected cell types: In brain, glycogen is found predominantly in astrocytes. In mouse models of Lafora disease, polyglucosan aggregates are present in two distinct forms – ‘corpora amylacea-like’ (CAL) bodies in astrocytes, and neuronal Lafora bodies (nLB). In histological sections, CAL bodies constitute the majority of the polyglucosan deposits. Due to their heterogeneity in size and shape, CAL bodies have a ‘dusty’ appearance. In contrast, nLBs are present as large, spherical polyglucosan bodies in the cell bodies of neurons.

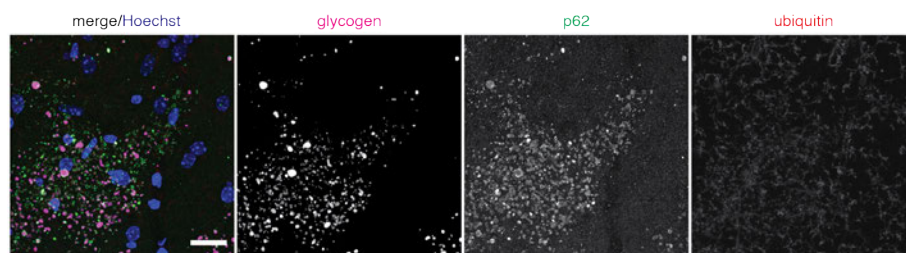
In *Rnf213*^{-/-} mice, we observe polyglucosan deposits exclusively in astrocytes (**new data, Extended Data Fig. 1e**). We infer that these deposits are intracellular, because they are frequently surrounded by the astrocyte-specific cytoskeletal protein GFAP.

Neuron loss: We have not observed obvious neuron loss in histological sections, consistent with the astrocyte-specific accumulation of polyglucosan. However, *Rnf213*^{-/-} mice develop reactive astrogliosis (**new data, Extended Data Fig. 1f-g**).

Ubiquitylation status of polyglucosan and the role of other E3 ligases: Polyglucosan bodies in *RNF213*^{-/-} mice appear ubiquitin-negative by immunofluorescence microscopy

(Reviewer Fig. 1). This result is consistent with RNF213 acting as the principal polyglucosan-directed E3 ligase in astrocytes (**Extended Data Fig. 1e**) and functioning upstream of LUBAC, another potential polyglucosan-directed E3 ligase (**new data, Extended Data Fig. 8**).

Autophagy of polyglucosan in *Rnf213*^{-/-} mice: Polyglucosan bodies in *Rnf213*^{-/-} mice are p62 positive, despite the absence of ubiquitin (**new data, Reviewer Fig. 1**), suggesting the existence of additional quality control pathways. We are excited to explore the role of p62 in greater mechanistic detail in the future. However, given the substantial experimental effort required to address this question, we consider this aspect of glycogen quality control outside the scope of this manuscript.



Reviewer Fig. 1 | immunofluorescence microscopy of p62 (green), ubiquitin (red), and polyglucosan (magenta) in brain. Scale bar: 20 μ m.

Figure S1: GBE1 mutations also manifest in skeletal muscle, liver, heart, ... RNF213 is also broadly expressed. If there is no accumulation of polyglucosans in other tissues than the brain, this suggests that other E-ligases than RNF213 are responsible for the ubiquitination of polyglucosans in other tissues, which should be discussed.

We agree with the reviewer that the tissue-specific accumulation of polyglucosan implies roles for other E3 ligases.

Inspired by the reviewer's comment, we experimentally addressed the relationship between RNF213 and LUBAC. Deletion of RNF213 prevented LUBAC recruitment to polyglucosan, whereas defects in the LUBAC subunits HOIL1 or HOIP did not affect RNF213 recruitment (**new data, Extended Data Fig. 8**), revealing that RNF213 acts upstream of LUBAC and controls its recruitment. Consistent with this hierarchy, polyglucosan ubiquitylation was abolished in RNF213 KO cells, but persisted in cells lacking HOIL1 or HOIP, albeit at reduced levels (**new data, Extended Data Fig. 8e,g,h**). However, overexpression of LUBAC in RNF213 KO cells restored polyglucosan ubiquitylation (**new data, Extended Data Fig. 8i-k**). Our new data are consistent with reports on HOIL-1 ubiquitylating glycogen in vitro (Kelsall et al., *EMBO J.*, 2022) and defects in LUBAC causing a partial reduction in polyglucosan ubiquitylation in cells (Jochem et al., *Nature*, 2026). Taken together, our new data confirm an essential function for RNF213 in glycogen quality control, manifested through both direct ubiquitylation of polyglucosan and epistatic control of LUBAC. At the same time, our data highlight the importance of LUBAC expression levels, which may explain the tissue-specific phenotype observed in HOIL1-deficient mice.

Related to the section "The CBM20 domain proofreads RNF213 substrates": Is there consensus that MoyaMoya is associated with RNF213 gain-of-function? An association has also been reported with loss-of-function mutations. There is one report that RNF213 loss-of-function is associated with altered angiogenesis because of activation of the hippo pathway.

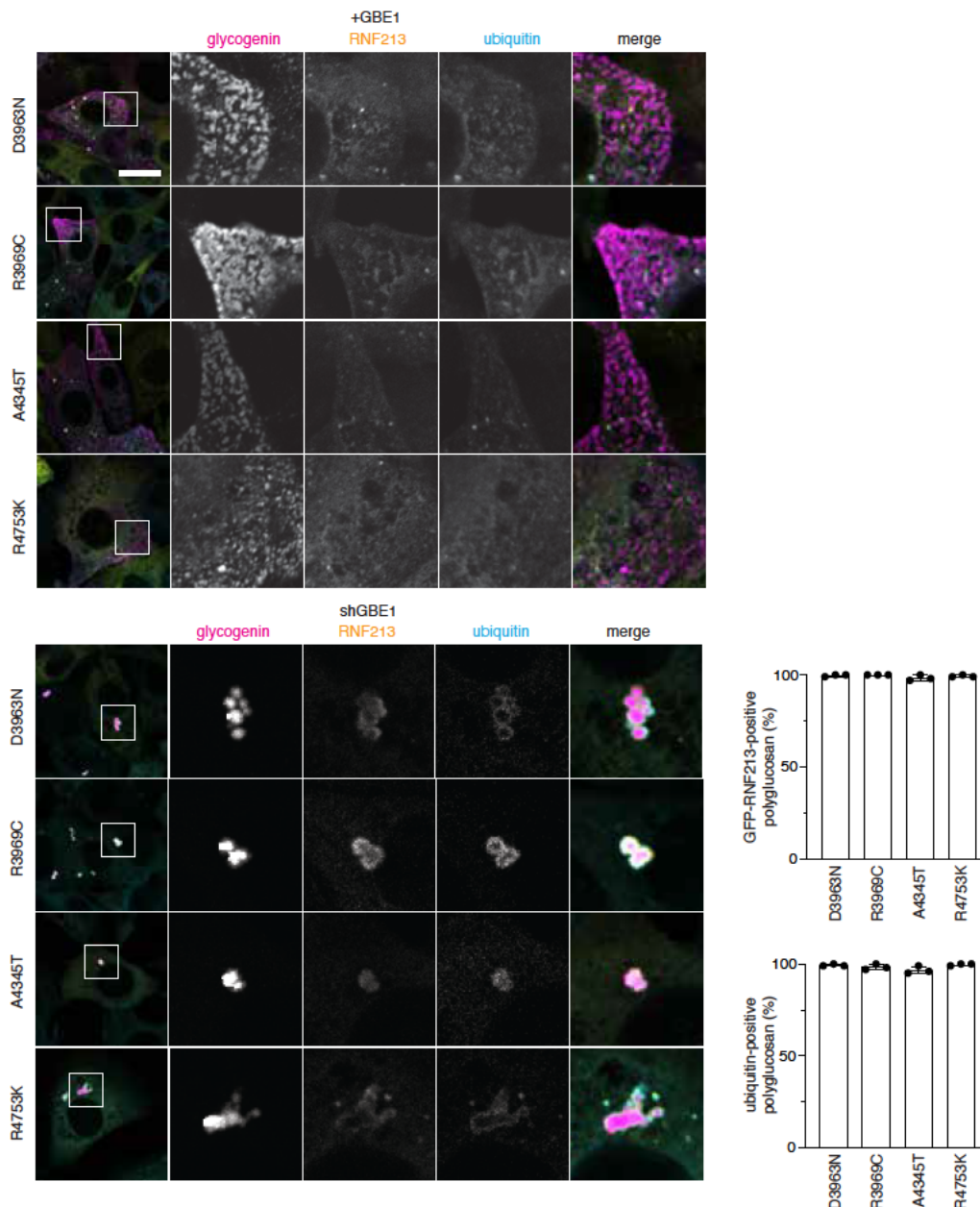
Is there any evidence for MoyaMoya-like alterations in heterozygous or homozygous RNF213 KO mice? Is there any report of accumulation of polyglucosans in such patients? If so, this would confirm the findings reported in this study for the human system.

We agree with the reviewer that the mechanism by which RNF213 causes Moyamoya disease remains unclear.

Regarding Moyamoya-like alterations in *Rnf213*^{-/-} mice, we have not observed any gross cerebrovascular defects. However, we have not directly assessed pulmonary artery hypertension or angiogenesis, as these fall outside the scope of our animal license.

Regarding human patients, we are not aware of reports on polyglucosan accumulation in human MMD patients, although the absence of such reports should not be interpreted as evidence that polyglucosan accumulation does not occur. We also note that Moyamoya disease is caused by missense mutations in RNF213, which could conceivably leave the glycogen quality control function of RNF213 intact. To experimentally address this question, we tested a panel of Moyamoya-predisposing RNF213 alleles for their effect on glycogen quality control (**new data, Reviewer Fig. 2**); indeed all alleles correctly distinguished polyglucosan from glycogen. Therefore, while our manuscript establishes an essential role for RNF213 in glycogen quality control through robust in vivo and in vitro experimentation, the relationship between RNF213-dependent glycogen quality control and Moyamoya disease remains an important open question that warrants further investigation.

Regarding the nature of mutations predisposing to Moyamoya, a recent review highlights that both gain- and loss-of-function mechanisms have been proposed (Kawakami et al., *J. Cardiol.*, 2026). Given this lack of consensus, we have toned down our speculation and moved it to the Discussion section.



Reviewer Fig. 2 | Immunofluorescence microscopy showing recruitment of a panel of Moyamoya disease alleles (yellow) and ubiquitin (cyan) to glycogen (top) or polyglucosan (bottom) (magenta).

Figure 2C: Here you see a large glycogen complex in WT, which is not apparent in b. Is c in the presence of shGBE1? If so, please label accordingly.

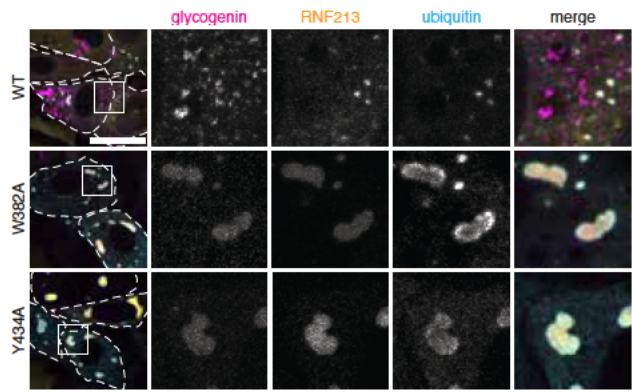
We thank the reviewer for pointing out the ambiguity. **Fig. 2c** illustrates the recruitment of RNF213 to polyglucosan, produced upon treatment with shGBE1. The figure has been re-labelled to improve clarity.

Figure 3e: Please, indicate the cell borders. Why are the glycogen deposits much larger for the W382A and Y434V variant?

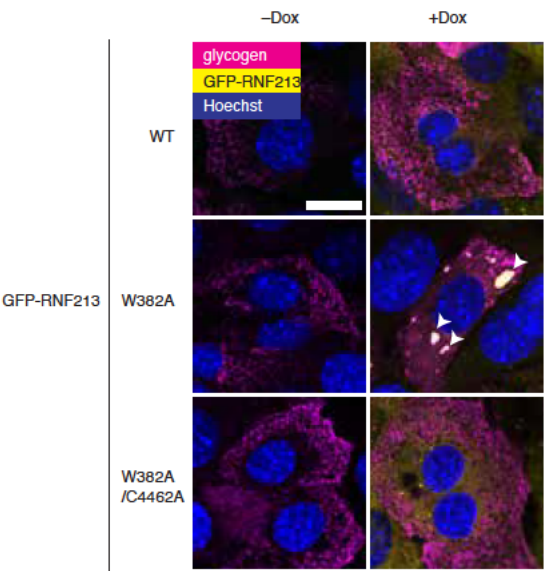
We have labelled cell borders in **Reviewer Fig. 3**.

RNF213 W382A and Y434A are gain-of-function alleles that ubiquitylate soluble glycogen (**Fig. 3f**). The formation of glycogen aggregates requires E3 ligase activity in RNF213

(Reviewer Fig. 4), suggesting it is the result of ubiquitin-mediated aggregation, a well-established phenomenon for other biomolecules.



Reviewer Fig. 3 | Fig. 3e in main text, highlighting cell borders.



Reviewer Fig. 4 | Expression of RNF213 W382A results in aggregation of ubiquitylated glycogen (white arrowheads). RNF213 W382A/C4462A, lacking RZ-mediated E3 ligase activity, fails to cause glycogen aggregation.

Referee #2 (Remarks to the Author):

The manuscript reports that RNF213, a large AAA+ E3 ligase previously shown to ubiquitinate microbial glycans, also ubiquitinates aberrant glycogen, i.e. polyglucosan, to promote its autophagic clearance. Using an engineered cell system in which polyglucosan is induced, the authors show RNF213 recruitment to inclusions, loss of ubiquitin signal in RNF213-deficient cells, and amylase- and hydroxylamine-sensitive glycogen–ubiquitin conjugates, consistent with non-protein ubiquitylation of the glucan polymer. They combine biochemistry with cryo-EM to define a carbohydrate-binding module within RNF213 that state functions as a “proofreading” domain, restricting E3 activity toward normal glycogen while allowing targeting of polyglucosan. CBM mutants mis-target physiological glycogen and enhance ubiquitination. In *Rnf213*^{-/-} and catalytic-dead knock-in mice, they document age-dependent, amylase-resistant PAS-D-positive inclusions in hippocampus, cerebellum, and pons, which they interpret as polyglucosan bodies (PGBs) arising from impaired glycogen quality control in the brain. They conclude that non-protein ubiquitination of aberrant glycogen by RNF213 is a key safeguard against pathological glucan accumulation and propose that direct ubiquitination of aberrant glycogen is a general strategy used by several E3 ligases to preserve organism-wide homeostasis. While the results are very intriguing, there are a number of gaps to be addressed. The biochemical and cryo-EM work are largely rigorous, and the engineered cell system is a clear strength. In contrast, the mouse data stop at describing polyglucosan burden without defining cell type, mechanism, or functional consequences. If the *in vivo* mechanism and physiological impact were more fully established, this would represent a conceptually important advance in non-protein ubiquitination and glycogen quality control.

We thank the reviewer for their concise summary of our work and for highlighting the conceptual advances reported in our manuscript.

(For ease of reference, we have numbered the reviewer's comments below.)

Major:

1. Comprehensively define glycogen metabolism: This is the first report of *Rnf213*^{-/-} mice displaying polyglucosan inclusions in the brain and it is not clear what drives polyglucosan formation in these mice. Thus, the following is needed:

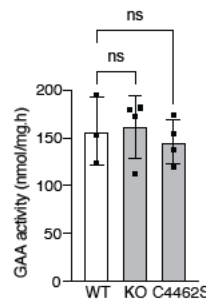
Protein level and/or activity profile of glycogenin (Gyg), glycogen synthase 1 (Gys1), glycogen phosphorylase (PYGB), and glycogen branching enzyme (Gbe) in *Rnf213*^{-/-} brain versus WT

We followed the reviewer's suggestion to further characterize polyglucosan formation in *Rnf213*^{-/-} mice. We find that all mice (WT, KO, and C4462S) express PYGB, GYS1, GBE1 and GYG1 at similar levels (**new data, Extended Data Fig. 1f,g**), revealing that polyglucosan accumulation in RNF213 KO and RNF213 C4462S mice is not due to changes in the levels of glycogen-metabolizing enzymes, consistent with glycogen quality control through polyglucosan ubiquitylation by RNF213.

2. What is the activity status of lysosome and specifically acid α -glucosidase (GAA) in *Rnf213*^{-/-} brain versus WT?

We addressed the reviewer's comment experimentally. We measured the activity of lysosomal alpha-glucosidase (GAA) in brain lysates from WT, RNF213 KO and RNF213 C4462S mice

using a commercial GAA activity assay (<https://www.abcam.com/en-us/products/assay-kits/lysosomal-alpha-glucosidase-activity-assay-kit-fluorometric-ab252887>). All lysates contained similar GAA activity (**new data, Reviewer Fig. 5**), revealing that polyglucosan accumulation in RNF213 KO and RNF213 C4462S mice are not due to changes in the activity of GAA, consistent with glycogen quality control through polyglucosan ubiquitylation by RNF213.



Reviewer Fig. 5 | GAA activity levels in brain lysates from the indicated mouse strains

3. Do the inclusions colocalize with ubiquitin, LC3, and p62, similar to PGBs observed in APBD mouse brain.

Polyglucosan bodies in Adult Polyglucosan Body Disease form due to defects in GBE1, resulting in the synthesis of poorly branched glycogen. Detection of aberrant glycogen results in colocalization with ubiquitin, p62, and LC3. In contrast, polyglucosan bodies in *Rnf213*^{-/-} mice appear ubiquitin-negative by immunofluorescence microscopy (**new data, Reviewer Fig. 1**). This result is consistent with an essential role for RNF213 in glycogen quality control through polyglucosan ubiquitylation (**Fig. 2 and 4**) and its epistatic control over LUBAC, another polyglucosan-directed E3 ligase (**new data, Extended Data Fig. 8**). Interestingly, despite the lack of ubiquitin deposition, p62 is present on polyglucosan bodies in *Rnf213*^{-/-} mice (**new data, Reviewer Fig. 1**), suggesting that additional glycogen quality control pathways may operate independently of RNF213-mediated ubiquitylation. We are excited to explore the role of p62 in mechanistic detail in the future. However, addressing this question comprehensively would require substantial additional experimentation and therefore falls outside the scope of this manuscript.

4. Fig 1- The data supporting polyglucosan inclusion the brain are not convincing in its current form.

The authors should include whole-brain PAS, PAS-D, and Cy5-labeled glycogen probe staining from WT, RNF213^{+/-}, and RNF213^{-/-} animals to demonstrate regional distribution of glycogen/polyglucosan

Following the reviewer's request, we include a whole-brain sagittal section of WT and RNF213 KO brains (**new data, Fig. 1c**), which confirms our conclusion of polyglucosan aggregates in specific brain regions, as reported in our original manuscript.

PAS/PAS-D quantification is missing in Fig. 1a/b. In Fig. 2b, WT mice should be included with PAS-D staining to determine what proportion of PAS-D-positive signal is age related (impact of ageing) rather than deficiency of RNF213

We addressed the reviewer's request experimentally (**new data, Fig. 1b**). We conclude that the vast majority of polyglucosan accumulation is caused by defects in RNF213.

5. The authors should clarify whether polyglucosan inclusions are ubiquitin-positive?

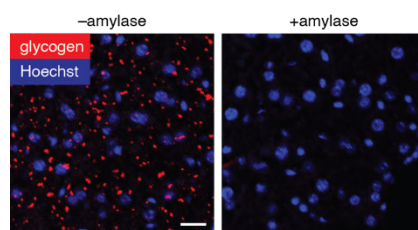
As mentioned in response to question #3 above, polyglucosan bodies in *Rnf213*^{-/-} brains appear ubiquitin-negative by immunofluorescence (**new data, Reviewer Fig. 1**).

6. Extended data fig 1c- It appears that amylase digestion may not have been effective on frozen brain sections, because glycogen staining appears more punctate after amylase treatment in *Rnf213*^{+/+} hippocampus and cerebellum. This experiment should be repeated and shown with clean pre and post digestion data, along with quantification for Fig. 1c-d and Extended Fig. 1c. There are also other inconsistencies as cerebellar staining in *Rnf213*^{-/-} sections differs between Fig. 1c and Extended Fig. 1c. Moreover, visually, polyglucosan appears more abundant in *Rnf213*^{C4462S} brains than in *Rnf213*^{-/-}, whereas the quantification suggests the opposite. Is this driven by a possible outlier in one *Rnf213*^{C4462S} mouse?

The reviewer voices concerns that amylase may be unable to digest glycogen in fixed frozen tissue sections. We therefore provide fixed frozen liver sections processed in parallel as a positive control, where amylase treatment abolished staining by the Cy5-glycogen probe (**new data, Reviewer Fig. 6**). We conclude that amylase efficiently digests glycogen under our experimental conditions.

Regarding the reviewer's comment about '... differences in cerebellar staining ...between Fig1.c and Extended Fig.1c', we repeated the staining of Extended Data Fig.1c, including samples with and without amylase treatment as requested. These data are now displayed as **Extended Data Fig. 1d**.

Regarding differences in polyglucosan abundance between *Rnf213*^{-/-} and C4462S brains, we do not observe any significant difference between the two strains (**Fig. 1d-e, Extended Data Fig. 1d**). We would like to emphasize that the key finding is the marked polyglucosan accumulation in both mutant strains compared with wild-type mice (**Fig. 1c-e, Extended Data Fig. 1d**). To further illustrate the similarity of polyglucosan levels in *Rnf213*^{-/-} and C4462S brains, we replaced the images in **Extended Data Fig. 1d** (previously Extended Data Fig. 1c) with alternative images from the same experiment.



Reviewer Fig. 6 | Frozen liver sections from PFA-perfused mice were subjected to amylase treatment, showing loss of glycogen signal (red). Scale bar: 20 μ m.

7. The central claim that brain polyglucosan is cleared by an RNF213-dependent ubiquitination and autophagy pathway is extrapolated from the engineered cell system rather than directly established in tissue. The in vivo mechanism is not demonstrated, only inferred from the cell system.

The manuscript is based on a combination of in vivo observations and in vitro mechanistic experiments. The assessment of autophagy in polyglucosan clearance in vivo is difficult due to the premature death of autophagy-deficient mice.

Polyglucosan accumulates not only in the brains of *Rnf213*^{-/-} but also in mice expressing catalytically dead RNF213 C4462S (**Fig. 1d,e, Extended Data Fig. 1d**), providing in vivo evidence that the accumulation of brain polyglucosan is antagonized by an RNF213-dependent ubiquitylation pathway. The in vivo data also indicate that glycogen-metabolizing enzymes are unlikely targets of RNF213, as they are present at normal levels in brain lysates of RNF213-deficient mice (**new data, Extended Data Fig. 1h,i**), and that lack of RNF213 skews the chain-length distribution of brain glycogen towards longer chains (**new data, Fig. 1f,g**), observations consistent with a role for autophagy in polyglucosan clearance.

Direct functional importance for the importance of autophagy in clearing polyglucosan was obtained in vitro because autophagy-deficient mice die within hours after birth, i.e. months before polyglucosan accumulates (**Fig. 1b**). Even inducible ATG7^{loxP} mice that live for some time after birth do not survive long enough to assess polyglucosan accumulation. Our discovery of polyglucosan accumulating specifically in astrocytes of *Rnf213*^{-/-} mice (**new data, Extended Data Fig. 1e**) will allow us to test the role of autophagy in vivo in the future. However, the scale of this experiment is incompatible with the time available for this revision.

8. The authors make a strong mechanistic case in cells that RNF213 ubiquitinates polyglucosan and directs it to autophagy.

But in vivo, they only demonstrate that *Rnf213*^{-/-} mice accumulate PAS-D positive and amylase resistant polyglucosan in specific brain regions.

As stated by the reviewer, we provide evidence for the accumulation of PAS-D positive and amylase-resistant polyglucosan in specific brain regions of RNF213^{-/-} mice.

Importantly, our analysis of RNF213 C4462S mice establishes an in vivo requirement for RNF213 E3 ligase activity, as these mice likewise accumulate polyglucosan (**Fig. 1d,e, Extended Data Fig. 1d**).

And during revision we have obtained further supportive in vivo data:

- the levels of glycogen-metabolizing enzymes are unchanged in the brains of *Rnf213*^{-/-} mice (**new data, Extended Data Fig. 1f,g**), indicating that polyglucosan accumulation is not caused by altered expression of glycogen-metabolizing enzymes and is instead consistent with glycogen quality control through RNF213-dependent polyglucosan ubiquitylation.
- the chain-length distribution of brain, but not liver, glycogen is shifted towards longer chains in *Rnf213*^{-/-} mice (**new data, Fig. 1f,g**), consistent with RNF213 targeting poorly branched glycogen.

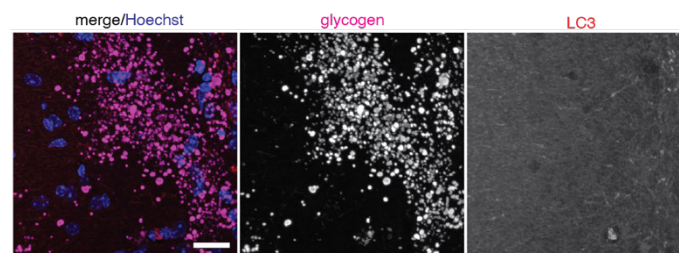
Thus, the link b/w RNF213 loss to impaired brain glycogen autophagy to increased brain polyglucosan accumulation is speculative. Needed:

Co-stain brain sections with:

- CBM20 glycogen probe or PAS-D
- Ubiquitin and SQSTM1 (p62), LC3
- RNF213

We followed the reviewer's suggestion to stain brain slices from *Rnf213*^{-/-} mice. We also refer the reader to question #3 (asking for the colocalization of polyglucosan with ubiquitin, p62, and LC3) and question #5 (asking for the colocalization polyglucosan with ubiquitin).

By immunofluorescence microscopy, polyglucosan bodies in *Rnf213*^{-/-} mice appear negative for RNF213, ubiquitin, and LC3, consistent with a defect in polyglucosan ubiquitylation and autophagy targeting in *RNF213*^{-/-} mice (**new data, Reviewer Fig. 1 and 7**). Interestingly, despite the lack of ubiquitin deposition, p62 is present on polyglucosan bodies in *Rnf213*^{-/-} mice (**new data, Reviewer Fig. 1**), suggesting that additional glycogen quality control pathways may operate independently of RNF213-mediated ubiquitylation. We are excited to explore the role of p62 in mechanistic detail in the future. However, addressing this question comprehensively would require substantial additional experimentation and therefore falls outside the scope of this manuscript.



Reviewer Fig. 7 | immunofluorescence microscopy of LC3 (red), and polyglucosan (magenta) in brain.
Scale bar: 20 μ m.

8. No cell-type identity or whole-brain mapping of polyglucosan inclusions. The cellular identity and brain regions of polyglucosans in vivo are unresolved.

are the inclusions neuronal, astrocytic, microglial, oligodendroglial, or in other cell types?

co-staining with NeuN, GFAP, Iba1, Olig2, etc.

Our original manuscript provides evidence for the accumulation of polyglucosan in specific brain regions (**Fig. 1c-e, Extended Data Fig.1d**). As requested, we now provide whole-brain sagittal sections from WT and *RNF213* KO mice (**new data, Fig. 1c**), confirming that polyglucosan aggregates accumulate to high levels specifically in the cerebellum, hippocampus, and pons. Polyglucosan aggregates are also present in the molecular layer of the piriform cortex (**new data, Extended Data Fig. 1d**).

We followed the reviewer's suggestion to investigate the identity of the polyglucosan-accumulating cell type. Staining of brain sections from *Rnf213*^{-/-} mice revealed polyglucosan bodies within GFAP-positive astrocytes but not within other cell types, including Iba1+ microglia or NeuN+ neurons (**new data, Extended Data Fig. 1e**).

9. The authors identify cerebellum, pons, and hippocampus as “specific brain regions” with polyglucosan accumulation, but they do not provide a systematic survey or quantification across other brain regions such as cortex or striatum.

As suggested by the reviewer, we now provide whole-brain sagittal sections from WT and RNF213 KO mice (**new data, Fig. 1c**), confirming that polyglucosan aggregates accumulate to high levels specifically in the cerebellum, hippocampus, and pons. Polyglucosan aggregates are also present in the molecular layer of the piriform cortex (**new data, Extended Data Fig. 1d**).

10. Quantify puncta in all figures.

Puncta are now quantified

(**Extended Data Fig. 1b** quantifies **Fig. 1a,b**;

Fig. 1d,e quantifies **Extended Data Fig. 1d**;

Extended Data Fig. 1g quantifies **Extended Data Fig. 1f**;

Fig. 2d,e quantifies **Fig. 2c**;

Extended Data Fig. 3d,e quantifies **Extended Data Fig. 3c**;

Extended Data Fig. 3g,h quantifies **Extended Data Fig. 3f**;

Extended Data Fig. 8c,d,f,g quantifies **Extended Data Fig. 8b,e**;

Extended Data Fig. 8j,k quantifies **Extended Data Fig. 8i**;

Extended Data Fig. 9d-g quantifies **Fig. 4b** and **Extended Data Fig. 9a-c**).

11. Ester linkages: The manuscript states that these are ester linkages w/o any definitive evidence and instead relies primarily on hydroxylamine sensitivity and amylase release and differences in mobility.

There is no MS confirming Ub modified at its C-term, with carbohydrate adducts.

Controls are lacking with Ub G76A or C-terminally blocked Ub to show loss of conjugation.

We addressed the reviewer’s comment experimentally, and we now provide MS data that confirm RNF213-mediated ubiquitylation of glucose in polyglucosan (**new data, Fig. 2h**).

We did not follow the reviewer’s suggestion to deploy C-terminally blocked ubiquitin or ubiquitin G76A, as these variants cannot be activated the E1 enzyme, an essential step in for ubiquitylation by any known mammalian E3 ligase.

12. Essential role: The authors state multiple times about the “essential role” of this pathway to maintain organism homeostasis. Yet there is no functional or behavioural phenotype despite strong claims about “safeguarding brain health” and “organismal homeostasis.”

While they demonstrate that specific brain regions of Rnf213^{-/-} mice exhibit increased polyglucosans, there is no data suggesting a negative phenotype in these mice exhibit survival curves, neurological phenotypes (seizures, ataxia, cognition), or systemic physiology.

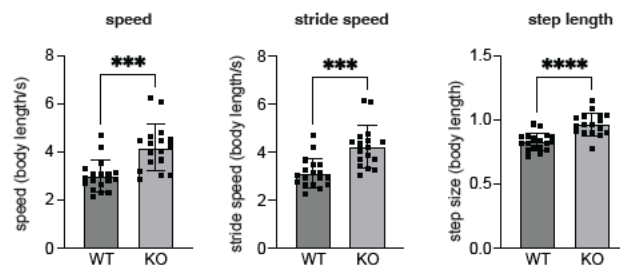
Lack of phenotypic data

Even if full behavioural/EEG work is not possible, some indication that polyglucosan accumulation in Rnf213^{-/-} brains has consequences in order to claim it is a generalized safeguard: Neurological phenotype scoring (gait, rotarod, simple motor tasks), Seizure threshold (e.g., PTZ challenge), or Age-dependent progression of inclusion load vs any functional decline.

We thank the reviewer for highlighting this important point. We followed their advice to investigate behavioural phenotypes linked to the accumulation of polyglucosan bodies. As these experiments fall outside our core expertise, they were conducted in collaboration with Anne Bertolotti and Lucia de los Reyes-Ramirez.

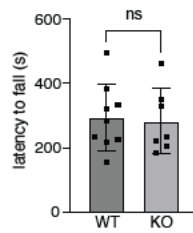
As recommended by the reviewer, we investigated motor function (MouseWalker and rotarod) as well as anxiety-like behaviour (open field) and memory (object recognition memory and spatial memory experiments) in 8-10 month old WT or RNF213 KO mice. Seizure threshold experiments (PTZ challenge) also suggested by the reviewer could not be performed as they fall strictly outside the scope of our animal licence.

Mouse Walker. We performed Mouse Walker experiments to quantitatively assess coordination and motor deficits. RNF213 KO mice maintained a normal, symmetrical trotting gait compared to age- and sex-matched WT controls. However, RNF213 KO mice exhibited altered gait dynamics, and moved faster than the WT mice due to increases in both stride speed and stride length, suggesting increased locomotor activity (**new data, Reviewer Fig. 8**). While we are excited by these findings, we feel that including them in the manuscript would be premature, as further experiments are required to determine the mechanism underlying this behavioural phenotype.



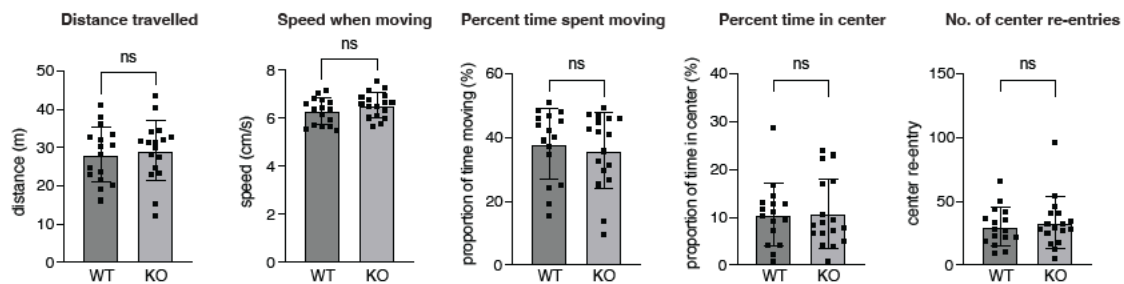
Reviewer Fig. 8 | MouseWalker experiments. A MouseWalker, constructed by the LMB mechanical workshop from schematics by Mendes et al. (PMID: 26197889), was used to assess locomotor phenotypes in WT or Rnf213^{-/-} mice. Mice were habituated to the mouse walker and trained to walk across it by attaching their home cage to the end of the apparatus. Five walks were performed during training, with a 1 min rest between attempts. Tests were performed 24 h after training. Ten attempts were performed, with a 1 min rest between attempts, until 3 successful walks were recorded. Videos were analysed by training a neural network from the open-source package provided by DeepLabCut, from which parameters such as mouse speed, step length, and stride speed were measured.

Rotarod. We performed rotarod experiments to assess balance, motor coordination / learning, and fatigue resistance. No difference in the latency to fall between WT and RNF213 KO mice was observed, indicating that the cerebellar and hindbrain polyglucosan burden does not impair motor function at this age (**new data, Reviewer Fig. 9**)



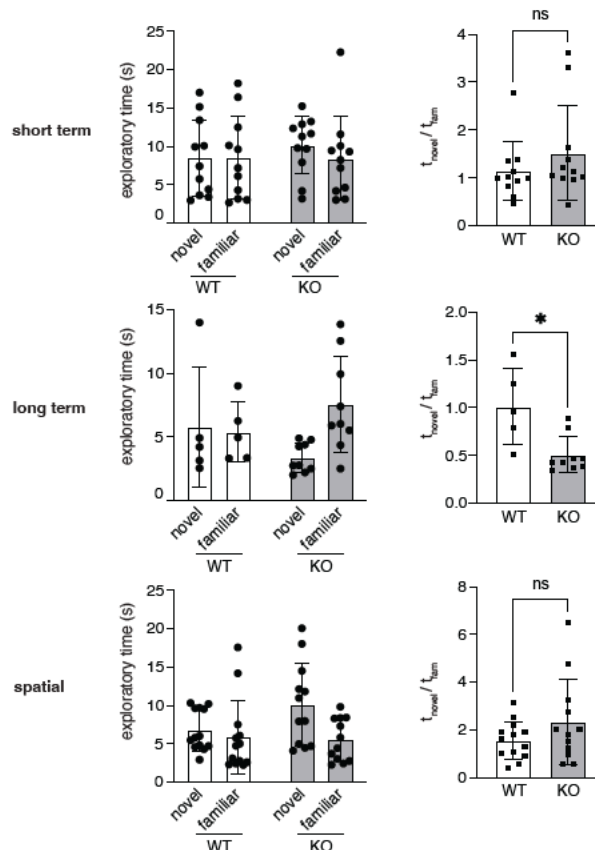
Reviewer Fig. 9 | Rotarod experiments. Mice were first habituated for 1 min on a static rotor and 1 min at constant speed (4 rpm) on two occasions and then tested for motor function and learning over three trials performed on three consecutive days (one trial per day). Each trial consisted of three test sessions with a 15 min rest between sessions. For each session, three or four mice were placed on an accelerating rotor (4 to 40 rpm) and the latency to fall was recorded, with a maximum limit for individual animals set at 900 seconds.

Open field experiments. We performed Open Field experiments to assess anxiety-like behaviour, exploratory behaviour, and baseline locomotor activity. We did not observe any statistically significant differences in anxiety-related behaviour between WT and RNF213 KO mice, as assayed by distance travelled, speed, percentage of time spent moving, percentage of time in the center of the arena, or number of re-entries into the center (**new data, Reviewer Fig. 10**).



Reviewer Fig. 10 | Open field experiments. An open field arena consisting of a 40 cm x 40 cm Perspex box was used, with a camera situated above the arena at the center of the box. A single mouse was placed in the middle of the arena, using cupping to minimise stress. A 15 min recording was captured. Videos were analysed by training a neural network from the open-source package provided by DeepLabCut to assess parameters including distance travelled, mouse speed, percentage of time spent moving, percentage of time spent in the center of the box, and number of re-entries into the center.

Novel Object Recognition (NOR) and spatial memory experiments. We performed NOR experiments to assess short- and long-term recognition memory in WT and RNF213 KO mice. We did not observe robust cognitive defects in NOR experiments (**new data, Reviewer Fig. 11**). Regarding long-term memory, RNF213 KO mice slightly preferred the familiar object over the novel object. However, we note that the WT controls did not explore the novel object more than the familiar object, as would be expected, and therefore these findings should be interpreted with caution.



Reviewer Fig. 11 | Novel object recognition and spatial memory experiments. A 40 cm x 40 cm arena was used for NOR experiments. Mice were habituated to the arena for 15 min on the first day. On the following day, mice were habituated to the empty arena for 15 min, before two identical objects were introduced. The mouse was allowed to explore them for 10 min. Testing for short-term memory was performed one hour later. The mouse was returned to the arena with one familiar object and one novel object and allowed to explore them for 5 min. Testing for long-term memory was performed the next day with one familiar and a new novel object. Spatial memory was tested an hour after that, with the same objects but with the position of one object changed. Videos were analysed by training a neural network using the open-source package provided by DeepLabCut, and the time spent exploring the novel and familiar objects was measured. Mice that explored either object for less than 2 seconds were removed from analysis.

We conclude that 8-10 months old RNF213 KO mice do not display cognitive or motor defects in the Mouse Walker, the Rotarod, Open Field, Novel Object Recognition, or spatial memory experiments. Such phenotypes may manifest at later ages, when the polyglucosan burden is greater. To reflect these new data, we have edited the manuscript to avoid overstating claims about general brain health and to focus on the molecular mechanism of glycogen quality control through non-protein ubiquitylation and autophagy.

13. Given that PGB accumulation in other models (e.g., Lafora disease, APBD, GBE1 hypomorphs) is typically associated with seizures and neurodegeneration, the absence of any functional phenotyping is a major weakness. This weakness is especially highlighted when the Discussion asserts an “essential role of non-protein ubiquitylation in maintaining organismal homeostasis.” Those conclusions are not supported by the current in vivo data.

We thank the reviewer for their comment.

As discussed in our answer to Comment #12, we have edited the manuscript to avoid overstating claims about general brain health and to instead focus on the molecular mechanism of glycogen quality control through non-protein ubiquitylation and autophagy.

14. Figure 4, The authors show several indirect pieces of data that polyglucosan is targeted to lysosomes. Direct evidence, like TEM images of polyglucosan surrounded by double membranes would greatly strengthen the manuscript.

We thank the reviewer for the suggestion. We now provide cryo-electron tomograms of FIB-milled lamella showing polyglucosan enclosed within double-membrane autophagosomes, thus providing direct evidence that macroautophagy targets polyglucosan (**new data, Fig. 4c-e**).

15. These findings are puzzling and appear to contradict what has been reported in Pompe disease mouse model (GAA-KO). GAA is the only lysosomal enzyme known to degrade glycogen and polyglucosan inside lysosomes. So, one would expect lysosomes in GAA-KO mice to accumulate both soluble glycogen and polyglucosan. Yet only soluble glycogen has been detected in Pompe mice, and there are no reports describing lysosomal polyglucosan accumulation in Pompe patients. Do the authors have a hypothesis that could reconcile these observations? This point could strengthen the discussion section, which is currently quite brief.

We agree with the reviewer that lysosomes in GAA-KO mice should accumulate soluble glycogen and polyglucosan. However, polyglucosan comprises only a minor proportion of cellular glycogen, unless glycogen synthesis is compromised or glycogen quality control is impaired by genetic defects. GAA-KO lysosomes are therefore expected to contain predominantly soluble glycogen and only trace amounts of polyglucosan. In addition, glycogen quality control mechanisms are likely to monitor glycogen structure continuously. In the presence of functional glycogen quality control, structural aberrancies are likely detected early, preventing the formation of large aggregates. Therefore, these aberrant species may not appear as large polyglucosan aggregates but rather as smaller, pre-pathologic species in the lysosomes of GAA-deficient mice, and would therefore be extremely difficult to detect by electron microscopy, thus explaining the apparent lack of aberrant glycogen in GAA-KO mice.

16. In addition, knocking out GAA in the PGB-producing engineered cell line could be envisioned. This cell line should contain even more PGBs, if the hypothesis described in the manuscript is correct.

We agree with the reviewer's hypothesis that lysosomal defects should aid in the accumulation of polyglucosan in RNF213 KO cells. In addition to evidence for the targeting of polyglucosan to autophagy already present in the manuscript (**Fig. 4**), we now provide additional evidence for autophagy-dependent lysosomal degradation of polyglucosan (**new data, Extended Data Fig. 9j-l**). Genetic ablation of ATG5, pharmacological PI3K inhibition, and pharmacological inhibition of lysosome acidification all impair polyglucosan degradation, indicating that polyglucosan bodies are targeted to and degraded within lysosomes.

17. Lack of acknowledgement of existing RNF213 mouse phenotype data.

Published *Rnf213*^{-/-} mice already exhibit CNS-related phenotypes, including blood–brain barrier fragility, altered cortical microvasculature, increased susceptibility to chronic hypoperfusion, and exaggerated responses to infection and angiogenic stimuli.

How does the glycogen QC role integrate with published data?

The precise molecular function of RNF213 in CNS-related phenotypes (other than polyglucosan accumulation) remains entirely unknown. These include Moyamoya disease, blood-brain barrier fragility, altered cortical microvasculature, increased susceptibility to chronic hypoperfusion, and exaggerated responses to angiogenic stimuli. RNF213 alleles that predispose to Moyamoya do not cause defects in LPS ubiquitylation (Otten, *Nature* 2021) or in glycogen quality control (**new data, Reviewer Figure 2**).

In contrast, glycogen quality control as reported in this manuscript and resistance to infection (Otten et al., *Nature*, 2021, Crespillo Casado et al., *EMBO Rep.*, 2024) show striking similarities. Both responses are dependent on RZ-dependent E3 ligase activity, require ATPase activity in RNF213 (**new data, Extended Data Fig. 3c-e, 4b,c**), result in activation of the LUBAC complex (**new data, Extended Data Fig. 8**), and are mediated by RNF213-controlled autophagy (**Fig. 4**).

18. Overstating claims

Phrases such as “essential for organismal homeostasis,” “safeguards brain health,” and “direct ubiquitylation of aberrant glycogen is a general strategy in mammals” are not fully justified. The data show an important role for RNF213 in limiting age-dependent brain polyglucosan in mice and compelling evidence for direct glycogen ubiquitination in cells, but they do not demonstrate systemic failure when RNF213 is lost.

We thank the reviewer for pointing out phrases considered not fully justified. We followed their advice and edited the text accordingly.

Regarding the lack of ‘... systemic failure when RNF213 is lost’, defects in three E3 ligases (RNF213, Malin/Laforin, and LUBAC) can cause polyglucosan accumulation. In each case, different tissues are affected, revealing tissue specific roles for individual E3 ligases and suggesting cross-regulation and partial redundancy.

Inspired by the reviewer’s comment, we experimentally addressed the relationship between RNF213 and LUBAC. Deletion of RNF213 prevented LUBAC recruitment to polyglucosan, whereas defects in the LUBAC subunits HOIL1 or HOIP did not affect RNF213 recruitment (**new data, Extended Data Fig. 8a-g**), revealing that RNF213 acts upstream of LUBAC and controls its recruitment. Consistent with this hierarchy, polyglucosan ubiquitylation was abolished in RNF213 KO cells, but persisted in cells lacking HOIL1 or HOIP, albeit at reduced levels (**new data, Extended Data Fig. 8h**). However, overexpression of LUBAC in RNF213 KO cells restored polyglucosan ubiquitylation (**new data, Extended Data Fig. 8i-k**). Our new data are consistent with reports on HOIL-1 ubiquitylating glycogen in vitro (Kellsall et al., *EMBO J.*, 2022) and defects in LUBAC causing a partial reduction in polyglucosan ubiquitylation in cells (Jochem et al., *Nature*, 2026). Taken together, our new data confirm an essential function for RNF213 in glycogen quality control, manifested through both direct ubiquitylation of polyglucosan and epistatic control of LUBAC. At the same time, our data highlight the

importance of LUBAC expression levels, which may explain the tissue-specific phenotype observed in HOIL1-deficient mice.

19. If RNF213 is truly a major “glycogen QC” gatekeeper, and its loss causes substantial PGB accumulation in hippocampus and cerebellum, you’d reasonably predict:

at least some EEG abnormalities or sensitivity to epilepsy inducing drugs

motor/coordination deficits (rotarod, gait, balance),

clear age-dependent neurological decline or neurodegeneration

As described in detail in our response to Comment #12, we investigated cognitive and motor/coordination deficits using mouse walker, rotarod, open field, and novel object recognition assays (**new data, Reviewer Fig. 8-11**). We conclude that 8-10 months old RNF213 KO mice do not display detectable cognitive or motor defects. However, we are limited to relatively young animals, and we cannot apply epilepsy-inducing drugs, due to legal requirements. To reflect the new data, we edited the manuscript to avoid overstating claims about brain health.

20. Human disease relevance: Test a small panel of bona fide Moyamoya RNF213 variants in the glycogen reporter system:

Do they shift specificity toward/away from physiological glycogen?

Do they alter autophagic clearance of polyglucosan?

We addressed the reviewer’s suggestion experimentally.

We tested four Moyamoya RNF213 alleles (R4753K, A4345T, D3963N, R3969C) in the glycogen reporter system (**new data, Reviewer Fig. 2**). All four alleles exhibited wild-type behavior, i.e. they were recruited to and ubiquitylated polyglucosan but not glycogen. We conclude that glycogen quality control is not affected by mutations predisposing to Moyamoya, consistent with previous findings for LPS ubiquitylation (Otten et al., *Nature*, 2021).

21. The CBM20 mutants (W382A, Y434A) are interpreted as “gain-of-function” toward physiological glycogen, then compared to Moyamoya: “This situation is reminiscent of Moyamoya disease... Since mutations causing Moyamoya disease cluster in the C-terminal E3 module..., even mild gain-of-function effects on E3 ligase activity... could get magnified...”

The disease-causing variants they reference cluster in the C-terminal E3 module, NOT the CBM20; they don’t test any bona fide Moyamoya mutations in their glycogen system.

There’s no demonstration that Moyamoya variants have gain-of-function activity toward glycogen or any defined substrate.

We apologise for the lack of clarity.

As stated by the reviewer, we tested mutants in the CBM20 domain, which had lost the ability to bind glycogen (**Fig. 3d**) and, surprisingly, had gained the ability to ubiquitylate physiological glycogen (**Fig. 3e-f**).

We did not intend to suggest that mutations in the CBM20 domain may cause Moyamoya disease, nor that Moyamoya alleles are defective in glycogen quality control. In fact, we have now tested four Moyamoya alleles, none of which showed any aberrant behaviour towards glycogen or polyglucosan in cells (**new data, Reviewer Fig. 2**).

Instead, we intended to draw a conceptual link between how CBM20 mutants misdirect RNF213 towards soluble glycogen and how Moyamoya mutations within the E3 module may similarly produce a gain-of-function phenotype towards an unknown substrate. To avoid confusion, we have moved the comments on Moyamoya disease to the discussion.

22. Stronger structural/biochemical characterization of “polyglucosan”

The data relied upon to define the aggregates as polyglucosan are rather elementary. While this reviewer does agree the aggregates are a polyglucosan, the chain-length distribution and branching frequency of the reporter-cell material vs brain inclusions should be defined.

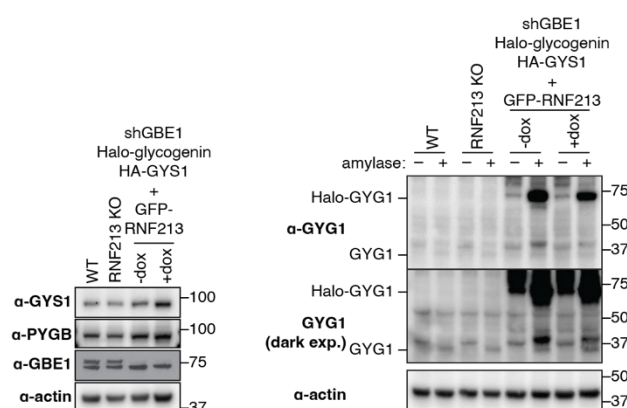
We performed the experiments suggested by the reviewer.

We analysed the chain length distributions of glycogen extracted from WT and *Rnf213*^{-/-} mice (**new data, Fig. 1f,g**). Glycogen in *Rnf213*^{-/-} brains is significantly less branched than glycogen in wild-type brains, consistent with the proposed role for RNF213 in glycogen quality control. In contrast, no difference in the chain length distribution of liver glycogen extracted from RNF213 KO or WT mice was apparent, consistent with the histological appearance of polyglucosan in the brains but not the livers of RNF213 KO mice. We conclude that in the absence of RNF213 impaired glycogen quality control results in the accumulation of poorly branched glycogen and the formation of polyglucosan bodies.

23. Define engineered cell lines more completely.

•What are the protein levels of GYG1, GYS1 and GBE1 (with and without shGBE1) in the transduced MEFs compared to the transduced ones?

We analyzed the levels of GYG1, GYS1 and GBE1 (**Reviewer Fig. 12**).



Reviewer Fig. 12 | expression of GYS1, PYGB, GBE1 and GYG1 in WT, RNF213 KO, or RNF213 KO cells complemented with doxycycline-inducible GFP-RNF213 that produce polyglucosan. Exogenous GYS1 is not detectable by the antibody since the antibody recognises an epitope within the C-terminus of GYS1 that was truncated to remove inhibitory phosphorylation residues.

24. What is the quantitative impact of shGBE1 on GBE1 expression as well as on glycogen and PGBs?

shGBE1 significantly reduces GBE1 expression (**new data, Reviewer Fig. 12**), resulting in the formation of polyglucosan from otherwise soluble glycogen in cells (**Fig. 2a-b**).

25. How long does it take for the cells to accumulate PGBs? Is there any toxicity?

Cells were transduced with lentiviruses encoding shGBE1, Halo-GYG1 and GYS1, followed by selection with Blasticidin and G418 for one week. At this time point, cells had already accumulated amylase-resistant polyglucosan bodies detectable by immunofluorescence. We did not observe any overt toxicity, although it remains formally possible that cells with a particularly large PGB burden may have died. Cells can be cultured for 20+ passages without losing polyglucosan expression.

26. Do the MEFs express basal levels of RNF213 expression?

MEFs express RNF213 basally. In **Fig. 2c** and **Extended Data Fig. 8e**, we assay recruitment of endogenous RNF213 to polyglucosan. In **Fig. 2g** and **Extended Data Fig. 8h**, we assay ubiquitylation of polyglucosan dependent on endogenous RNF213.

27. Does RNF213 overexpression significantly reduces glycogen/PGBs accumulations in those cell lines?

The number of cytosolic polyglucosan puncta is reduced upon RNF213 overexpression in a manner dependent on its E3 activity (**Fig. 4a**). Similarly, the level of polyglucosan (assayed by the levels of Halo-GYG1) is reduced upon RNF213 overexpression in an E3 and autophagy-dependent manner (**new data, Extended Data Fig. 9j-l**).

28. What is the knockdown efficiency in all experiments?

shGBE1 knockdown efficiency is almost complete, judged by Western blotting (**new data, Reviewer Fig. 12**).

29. Minor:

- Abstract: “organismic homeostasis” in the last sentence of the abstract is unusual; “organismal homeostasis” is more standard.

- Abstract: “heavily glucosidated glycogenin” is odd verbiage; “glycosylated” is more standard.

- “Since defects in other E3 ubiquitin ligases ... also cause polyglucosan accumulation, direct ubiquitylation of aberrant glycogen may be a general strategy in mammals to maintain glycogen quality.”

softening this to “may represent one strategy”

We thank the reviewer for the suggestions. We have modified the text accordingly. However, we prefer ‘glucosylated’ over ‘glycosylated’ as it specifically refers to the modification of glycogenin with glucose. We note that both terms have been used in the literature.

Referee #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We thank Reviewer 3 for their time.

Referee #4 (Remarks to the Author):

In the present work, Yip et al report that the giant E3 ligase RNF213 ubiquitinates aberrant glycogen (polyglucosan), promoting its clearance via selective autophagy. Based on experiments in RNF213 knockout and ligase-deficient knock-in mice, a synthetic cellular system, in vitro ubiquitination assays, and cryo-EM analysis of RNF213 bound to maltoheptaose, the authors conclude that RNF213 functions as a glycogen quality-control factor required to prevent polyglucosan accumulation in the brain.

RNF213 itself is an unusual E3 ligase that has come to prominence through its roles in innate immunity, vascular disease, and non-protein ubiquitination, yet its physiological substrates remain poorly defined. In this context, the study by Yip et al is timely and intriguing, as it points to a potential link between RNF213 and glycogen biology. Nonetheless, despite the broad experimental range, the study does not converge on a coherent mechanistic model, and several key experiments are lacking. In particular, prior work showing HOIL-1/LUBAC-mediated glucan ubiquitination is not adequately considered, leaving central questions unresolved regarding how RNF213 relates to this quality-control (QC) pathway and how it would selectively target aberrant glycogen.

We thank the reviewer for their concise summary of our manuscript, judging it ‘... timely and intriguing’.

The respective roles of RNF213 and LUBAC in glycogen quality control have been addressed experimentally. As explained in more detail below, epistasis analysis places RNF213 upstream of LUBAC (**new data, Extended Data Fig. 8a-h**).

Major concerns

1. Conceptual advance relative to established glycogen QC pathway?

The manuscript presents RNF213-mediated glycogen ubiquitination as a novel glycogen QC mechanism. However, Kelsall et al. (EMBO J. 2022) already demonstrated that HOIL-1 directly ubiquitinates unbranched glucans, including glycogen and maltoheptaose, and that this activity is essential to prevent polyglucosan accumulation in vivo. Despite this, the Introduction describes the role of HOIL-1 as uncertain, even though the prior study provided detailed biochemical characterization, MS-based mapping, and a knock-in mouse model. Thus, the conceptual advance of the present work remains unclear.

We appreciate the reviewer’s perspective regarding the study by Kelsall et al. (EMBO J 2022). However, we respectfully disagree that our manuscript lacks significant conceptual advance.

We note that while Kelsall et al. elegantly demonstrate that purified HOIL-1 can ubiquitylate glycogen and maltoheptaose in vitro, and that *Hoil1*^{-/-} mice accumulate polyglucosan, their study did not demonstrate that polyglucosan ubiquitylation actually occurs in cells. Nor did they establish the mechanism by which HOIL1 deficiency leads to polyglucosan accumulation. In other words, HOIL-1-dependent polyglucosan ubiquitylation and selective autophagy were proposed as a glycogen quality control pathway, but their occurrence and physiological relevance were not demonstrated. Our work thus advances the field conceptually by demonstrating that polyglucosan is directly and selectively ubiquitylated in cells and subsequently removed by selective autophagy. We furthermore demonstrate that RNF213 is

essential for the recruitment of LUBAC (HOIP) to polyglucosan and that the overall contribution of LUBAC to polyglucosan ubiquitylation is relatively limited, as confirmed by the 60% reduction in HOIL-1 mediated ubiquitylation reported very recently by the Komander lab (Jochem et al., *Nature*, 2026). These findings place RNF213 upstream of LUBAC in glycogen quality control and identify RNF213-mediated ubiquitylation as the initiating event in the pathway.

2. Unresolved relationship between RNF213 and LUBAC

RNF213 knockout and ligase-deficient knock-in mice show phenotypes that closely resemble those reported for HOIL-1(C458S) mice and for human LUBAC-deficiency syndromes, including PAS-positive inclusions in hippocampus, cerebellum, and pons. This apparent overlap is particularly relevant in light of recent work (Bhardwaj et al, *Nature* 2025) showing that RNF213 ubiquitinates CYLD and SPATA2, thereby activating LUBAC and linear ubiquitin signalling. Thus, an alternative model is that RNF213 affects glycogen quality control indirectly by regulating LUBAC and HOIL-1, rather than acting as the primary glycogen-modifying ligase. Surprisingly, this possibility is not addressed in the manuscript. No experiments test linear ubiquitin chain formation, RNF213–LUBAC interplay, or the dependence of polyglucosan clearance on LUBAC activity in vivo or in the cellular system. Accordingly, the manuscript does not resolve whether RNF213 acts as an autonomous glycogen QC ligase or instead functions upstream or in concert with the LUBAC/HOIL-1 pathway. Because the phenotypes, proposed substrates, and disease associations of RNF213 and HOIL-1 are so closely overlapping, distinguishing between these models is not a secondary mechanistic detail but a central conceptual question.

We thank the reviewer for this conceptually important comment.

We agree with the reviewer on the importance of clarifying the epistatic relationship between RNF213 and LUBAC. To experimentally address this question, we compared cells deficient in either RNF213, HOIP, or HOIL1 (**new data, Extended Data Fig. 8a-h**). Lack of RNF213 prevented the recruitment of endogenous LUBAC to polyglucosan bodies and abolished polyglucosan ubiquitylation. In contrast, deficiency in HOIL-1 or HOIP did not affect RNF213 recruitment to polyglucosan bodies, and polyglucosan ubiquitylation persisted, albeit at reduced levels. These findings place RNF213 upstream of LUBAC in glycogen quality control.

However, we also note that overexpression of LUBAC in RNF213 KO cells can bypass the requirement for RNF213 (**new data, Extended Data Fig. 8i-k**), suggesting that while LUBAC normally requires RNF213, it can function independently of RNF213 if expressed at sufficiently high levels. This observation may provide a clue to the tissue specificity of RNF213 deficiency, potentially explaining why polyglucosan accumulates in the brains of RNF213 KO mice but not in other organs. One possibility is that higher expression of alternative glycogen quality control E3 ligases, including LUBAC, can compensate for the absence of RNF213 in certain tissues.

3. Concerns regarding physiological relevance of the cellular system

The cellular experiments rely on a highly engineered MEF system involving overexpression of glycogenin (glycogen carrier) together with GBE1 (branching enzyme), combined with GBE1 knockdown to induce polyglucosan formation. However, it is not explained why such an

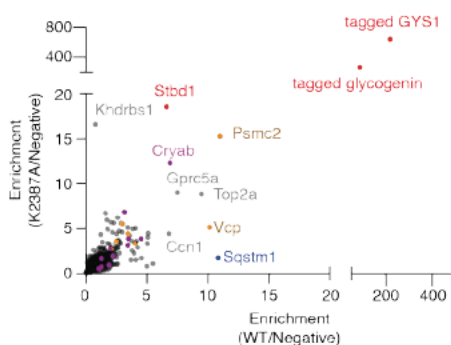
elaborate setup is necessary. If MEF cells do not produce sufficient levels of glycogen, why did the authors pick this cell line for studying glycogen QC? Given that polyglucosan accumulation is restricted to brain tissues in mice, neuronal or glial cells may have been the better model system. Furthermore, their setup generates prominent polyglucosan–protein clusters (Fig.2a,c), raising the possibility that RNF213 recruitment is indirect and reflects a general aggregation or stress response rather than a specific glycogen QC mechanism. The authors should test the composition of these clusters and their relationship to disease-relevant neuronal polyglucosan inclusions. Finally, overexpression of RNF213 itself may affect its localization, activity and assembly and need to be controlled as well. For all these reasons, it is not clear to what extent the engineered cellular system reflects physiological glycogen QC, limiting the relevance of the mechanistic conclusions drawn from it.

We appreciate the reviewer's questions regarding the engineered nature of our MEF system.

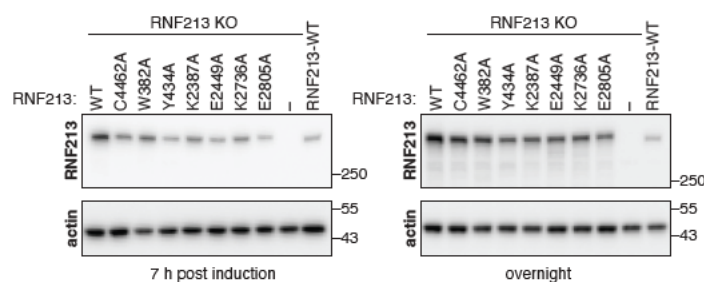
We have performed several validation experiments, which establish that our findings reflect physiologic glycogen quality control, rather than artifacts of an engineered system or non-specific protein aggregation:

- Overexpression of glycogenin and glycogen synthase is not essential to trigger polyglucosan formation in MEFs, and knocking down GBE1 alone is sufficient to induce polyglucosan accumulation (**new data, Extended Data Fig. 3a**). Such polyglucosan bodies became ubiquitin-positive in an RNF213-dependent manner, similar to the engineered cellular system. However, while MEFs produce endogenous glycogen, its abundance is insufficient for robust quantitative analysis. In contrast, the engineered system provides a robust and tractable platform for quantitative studies, enabling sensitive monitoring of polyglucosan dynamics through the Halo-GYG1 reporter.
- Since polyglucosan accumulates in astrocytes of *Rnf213*^{-/-} mice (**new data, Extended Data Fig. 1e**), we tested our genetic system in the astrocyte cell line C8-D1A. Endogenous RNF213 is recruited to and ubiquitylates polyglucosan bodies in C8-D1A cells, thus confirming our previous observations in astrocytes (**new data, Extended Data Fig. 3f-i**).
- To further characterize polyglucosan bodies, we attempted purification from cells expressing either WT RNF213 or the ATPase mutant K2387A, using the STBD1 glycogen probe as a bait (**new data, Reviewer Fig. 13**). We observe a large enrichment of HA-GYS1 and mCherry-GYG1 (introduced to produce polyglucosan), as well as endogenous Stbd1 (enriched relative to cells not expressing polyglucosan) and Sqstm1 (p62) (enriched when WT but not K2387A RNF213 are expressed). With the exception of the small heat shock protein Cryab, no other chaperones (e.g. heat shock proteins) were strongly enriched, suggesting that the formation of polyglucosan bodies does not result in non-specific protein aggregation.
- Regarding RNF213 levels, we demonstrate in key experiments that endogenous RNF213 is recruited to polyglucosan (**Fig. 2c** and **Extended Data Fig. 8e**), and that endogenous RNF213 mediates polyglucosan ubiquitylation (**Fig. 2g** and **Extended Data Fig. 8h**). Where exogenous RNF213 was used, for example in rescue experiments, levels of exogenously expressed RNF213 alleles were comparable to each other and broadly similar to endogenous RNF213 levels, particularly when doxycycline induction was kept brief (**new data, Reviewer Fig. 14**). For instance, in

the live-cell microscopy experiment (Fig. 4b), imaging commenced 6h post RNF213 induction, when RNF213 levels are similar to endogenous levels.



Reviewer Fig. 13 | Enrichment of proteins on polyglucosan precipitated from cells expressing WT RNF213 or the ATPase mutant K2387A. Negative refers to cells not producing polyglucosan. Glycogen metabolism related (red); proteasome-related (orange); chaperones (purple); Sqstm1 (blue)



Reviewer Fig. 14 | Expression levels of RNF213 after 7 h or overnight doxycycline induction

4. Structural analysis does not explain substrate recognition or selectivity

The cryo-EM structures reveal maltoheptaose bound to a CBM20-like domain of RNF213, providing evidence that this domain can engage short linear glucans. However, CBM20 mutants that disrupt maltoheptaose binding still localize to and ubiquitinate polyglucosan in cells, indicating that carbohydrate binding by this domain is not required for RNF213 recruitment or activity. So, is there another sugar binding site in RNF213, or is the interaction with glycogen in cells indirect? With respect to the tested CBM20 mutants, the authors should demonstrate that the proteins are structurally intact to exclude local misfolding as reason for the observed phenotypes. It is concerning that both CBM20 mutants display strongly increased E3 activity compared to WT (Fig.3f), pointing to non-specific or pleiotropic effects rather than a clean separation-of-function phenotype. Moreover, maltoheptaose-like segments are expected to occur in both physiological glycogen and poorly branched polyglucosan, given the known branching frequency of glycogen. As such, the current data do not explain how RNF213 would selectively discriminate between normal and aberrant glycogen, a distinction that should be central to the QC mechanism. Consequently, the proposed model of CBM20-mediated proofreading extrapolates far beyond the presented evidence, and the suggested “auto-inhibitory proofreading module” remains a conceptual hypothesis rather than a mechanism supported by experimental data.

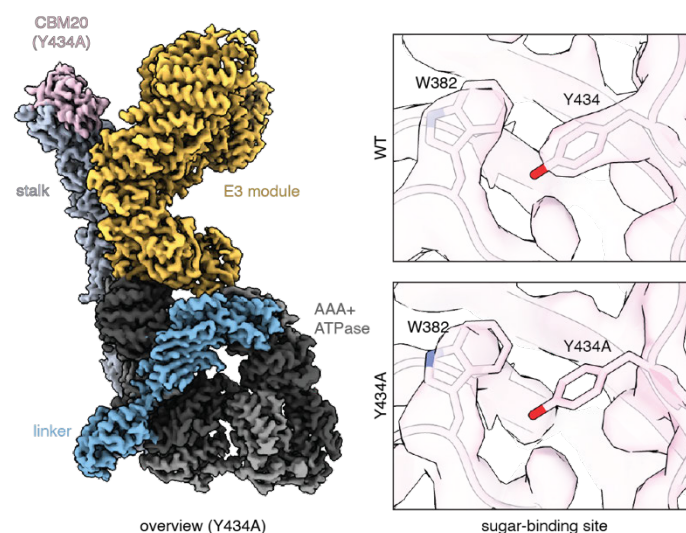
We experimentally addressed the reviewer's concern of local unfolding in the mutant CBM20 domain through SEC-MALS and direct structural analysis:

[Text redacted]

- We solved the cryo-EM structure of the Y434A mutant, which revealed that its CBM20 domain is structurally intact and virtually indistinguishable from the wild-type WT protein, except that the side-chain density for Y434 is absent (**Reviewer Fig. 16**).

These data argue strongly against local unfolding of the CBM20 domain and indicate that the observed gain-of-function phenotype results from a specific disruption of the carbohydrate binding site, rather than from structural destabilization.

[Figure redacted]



Reviewer Fig. 16 | Cryo-EM structure of Y434A RNF213. The model of WT RNF213 is shown in the sugar binding site overlayed on cryo-EM maps from WT or Y434A.

Regarding the reviewer's concern that the mutations cause a non-specific increase in E3 ligase activity, we note that both W382A and Y434A catalyze polyglucosan ubiquitylation at

levels comparable to wt RNF213 (**Extended Data Fig. 7b**), and display higher activity than wt RNF213 only towards glycogen (**Fig. 3f**), clearly indicating a selective gain of activity towards glycogen rather than a general increase in E3 ligase activity.

Selectivity is also apparent from the microscopy-based analysis of RNF213 recruitment: WT and the CBM20 mutants are all recruited to polyglucosan, whereas only the CBM20 mutants, and not WT RNF213, cause ubiquitylation and clustering of soluble glycogen (**Fig. 3e,f**).

We conclude that wild-type RNF213 distinguishes polyglucosan from soluble glycogen, while the W382A and Y434A mutants have lost this discriminatory ability. This defect cannot be explained by unfolding of the CBM20 domain or by a non-specific increase in E3 ligase activity, but instead reflects a specific loss of substrate selectivity. We therefore conclude that a functional CBM20 domain is essential for the ability of RNF213 to distinguish polyglucosan from soluble glycogen, but that the CBM20 domain is not a recruitment module, because CBM20 mutants that no longer bind sugars are nevertheless recruited to polyglucosan.

The W382A and Y434A alleles thus reveal a mechanism by which RNF213 avoids targeting soluble glycogen, but they do not explain how RNF213 is recruited to polyglucosan. Identification of the polyglucosan recognition mechanism will require further investigation.

We speculate that polyglucosan does contain specific structures absent from soluble glycogen, such as extended helical segments formed by unbranched chains. However, despite extensive efforts, we have been unable to detect glycogen / polyglucosan binding by RNF213 through any domain other than CBM20. Nevertheless, we recently made progress towards understanding the enzymatically amplified recruitment mechanism of RNF213 by discovering a ubiquitin-binding site in RNF213, whose integrity is essential for the formation of RNF213 coats on all tested substrates, including polyglucosan. Our data reveal that progressive RNF213 recruitment depends on initial RNF213-mediated ubiquitin deposition, followed by recruitment of additional RNF213 monomers into the coat via ubiquitin binding, resulting in processive assembly of RNF213 coats. This mechanism is consistent with a low-affinity interaction between RNF213 and glycogen, as is typical for many enzyme-substrate interactions, and may therefore explain the apparent lack of binding in immunoprecipitation experiments, which typically require at least micromolar affinities. Much further work is required to verify this model, and we consider such studies outside the scope of this manuscript, which primarily reports on RNF213-mediated glycogen quality control through direct and selective ubiquitylation of polyglucosan.

5. Insufficient evidence for direct ubiquitination of glycogen by RNF213

Direct glycogen ubiquitination is inferred from ubiquitin release experiments performed on STBD1(CBM20)-captured aggregates containing cellular glycogen and from in vitro ubiquitination of maltoheptaose. However, STBD1 capture is expected to co-purify glycogen associated or co-aggregating proteins, meaning that hydroxylamine sensitivity could reflect ester-linked ubiquitination of proteins as well. Interactome data could resolve this concern, revealing bystander and QC partner proteins. Also, the small-size difference observed upon amylase treatment (Fig.2g) is not sufficient to propose sugar modification. Definitive evidence would require chemical or MS-based mapping of the ubiquitin adducts. In addition, the in vitro maltoheptaose ubiquitination assay relies on remarkably high substrate concentrations of 20

mM and yields only weak modification, raising concerns about the specificity and efficiency of the reaction. Testing CBM mutants would help to show specific targeting via the sugar binding site. It also needs to be shown to what extent maltoheptaose reflects the physiological substrate, for example by analysing other carbohydrates that more closely resemble the different glycogen forms.

As suggested by the reviewer, we analyzed ubiquitin adducts by mass spectrometry, which revealed direct ubiquitylation of glucose in polyglucosan by RNF213 (**new data, Fig. 2h**).

Regarding the reviewer's comments on the conditions of the in vitro maltoheptaose ubiquitylation assay, these are identical to those used by Kellsell et al. (EMBO J 2022) in their landmark paper demonstrating maltoheptaose and glycogen ubiquitylation by recombinant HOIL1 in vitro. The primary intent of this experiment was to test whether RNF213 has the principal chemical capacity to ubiquitylate non-amine groups in maltoheptaose.

6. Autophagy involvement without a defined initiating ubiquitin signal

The manuscript shows colocalization of polyglucosan with p62 and LC3 and increased polyglucosan accumulation upon autophagy inhibition, consistent with autophagy contributing to clearance. Which is good. However, these observations do not establish RNF213-dependent ubiquitination as a critical signal for selective autophagy and are equally consistent with ubiquitination of associated proteins or secondary recruitment of autophagy receptors. Analysis of ubiquitin linkage types and interactions with known partner E3 ligases would be required to support this interpretation. It also remains unclear whether the ubiquitin signal resides on glycogen itself or on associated proteins, a distinction that is essential for interpreting the mechanism.

Following the reviewer's request, we experimentally characterized ubiquitin-chain types on polyglucosan and investigated '... known partner E3 ligases'.

- We identify M1- and K48-, but not K63-linked ubiquitin chains on polyglucosan (**new data, Extended Data Fig. 3k**).
- RNF213-dependent ubiquitylation is critical for autophagy since autophagy cargo adapters (p62, TAX1BP1 and Optineurin) and the autophagy marker LC3 associate with polyglucosan only in WT and not in RNF213 KO cells (**Fig. 4b and Extended Data Fig. 9a-g**).
- To the best of our knowledge, RNF213 does not physically interact with other E3 ubiquitin ligases. However, RNF213 controls LUBAC recruitment to and ligase activity against bacteria (Otten et al., *Nature*, 2021) and polyglucosan (see Comment #2 above; **new data, Extended Data Fig. 8a-h**). Regarding the role of '... partner E3 ligases' specifically in autophagy, in HOIL1 and HOIP KO cells polyglucosan bodies remain positive for ubiquitin, autophagy cargo receptors, and LC3 (**new data, Extended Data Fig. 8e,g and 9a-g**). In contrast, in RNF213 KO cells, polyglucosan bodies are no longer ubiquitylated and do not attract LUBAC, autophagy cargo receptors, and LC3 (**Fig. 2c,e,g and 4b, and Extended Data Fig. 8b-d and 9b-g**), confirming an essential role for RNF213 in inducing autophagy of polyglucosan upstream of LUBAC.

Regarding the location of the autophagy-inducing ubiquitin signal, we agree with the reviewer that ubiquitylation is unlikely to target polyglucosan exclusively. Instead, we expect that ubiquitylation of both polyglucosan and associated proteins will contribute to cargo recognition and clearance.

7. With regards to the assay, the group introduces a flow cytometry-based approach using TMR-labelled glycogenin to distinguish cytosolic from lysosomal polyglucosan. While the data appear technically sound, its interpretation remains ambiguous in the absence of proper controls. For example, the reduced TMR signal in the presence of RNF213 is interpreted in different ways. In Extended Data Fig.8a, reduced in-gel TMR-labelled glycogenin is taken to reflect RNF213-dependent clearance. In contrast, in the flow cytometry assay (Fig.4d), a similar reduction in TMR signal is linked to altered subcellular localization, attributed to reduced TMR labelling in the acidic lysosomal environment. To support the claim that the flow cytometry assay specifically reports on subcellular localization rather than protein abundance, glycogenin levels should be directly quantified under the same conditions.

We apologise for the confusion.

The assay can indeed be used to measure glycogen degradation or glycogen localization, depending on the timing of Halo-TMR addition relative to RNF213 induction. To measure degradation, cells are pre-labelled with TMR, resulting in uniform glycogenin labelling. Loss of the TMR signal serves as a readout for glycogen degradation. Alternatively, because TMR-labelling is less efficient under acidic conditions, if TMR is added after induction of RNF213 expression / RNF213-dependent autophagy, TMR preferentially labels Halo-glycogenin in the cytosol, thereby distinguishing Halo-glycogenin localization. We edited the figure legends and revised the text to explain the two assay configurations clearly.

We also followed the reviewer's request to directly quantify glycogenin levels. Our new data confirm that glycogenin levels decrease upon expression of RNF213 in an autophagy-dependent manner (**new data, Extended Data Fig.9j-l**), revealing that RNF213-dependent autophagy promotes degradation of polyglucosan.

8. Speculation on disease relevance

The Discussion speculates on the relevance to Moyamoya disease (MMD) and interprets CBM20 mutants as proxies, yet RNF213 variants linked to MMD have not been associated with glycogen storage defects or polyglucosan pathology. In contrast, HOIL-1 and HOIP mutations are directly linked to polyglucosan accumulation in patients, indicating that the established disease biology lies within the LUBAC pathway rather than with RNF213. Moreover, the manuscript argues that Moyamoya mutations are gain-of-function because patients are heterozygous, yet the here presented data show that RNF213 heterozygous mice accumulate polyglucosan, which is more consistent with partial loss-of-function. Given these inconsistencies, the disease-related interpretations are not supported by current evidence.

We apologise for the lack of clarity.

We did not intend to suggest that mutations in the CBM20 domain may cause Moyamoya disease nor that Moyamoya alleles were known to be defective in glycogen quality control. In

fact, we have now tested four Moyamoya alleles, all of which showed wild-type behaviour towards glycogen / polyglucosan in cells (**new data, Reviewer Fig. 2**).

Instead, we intended to draw a conceptual link between how CBM20 mutants misdirect RNF213 activity towards soluble glycogen and how Moyamoya mutations within the E3 module may similarly produce a gain-of-function phenotype towards an unknown substrate. To avoid confusion, we have moved these comments on Moyamoya disease to the discussion.

Regarding LUBAC (as discussed in our answer to Comment #2) we have established that RNF213 controls LUBAC recruitment to and activity against polyglucosan (**new data, Extended Data Fig.8a-h**). However, overexpression of LUBAC can compensate for the absence of RNF213 (**Extended Data Fig. 8i-k**), raising the possibility that tissue-specific expression levels of individual E3 ligases may determine whether a genetic defect manifests as polyglucosan disease in a given organ.

Minor points

1) Cellular levels of used RNF213 constructs should be quantified, potential effects of mutations on protein stability assessed, and expression tuned closer to endogenous levels where feasible. In addition, the Walker B3 (E2449) mutant should be included.

We followed the reviewer's suggestions.

Levels of exogenously expressed RNF213 alleles were similar to each other and broadly similar to endogenous RNF213 levels, particularly when doxycycline induction was kept brief (**new data, Reviewer Fig. 14**). For instance, in the live-cell microscopy experiment (**Fig. 4b**), imaging commenced 6h post RNF213 induction, when RNF213 levels are very similar to endogenous levels.

As requested, we included the Walker B3 mutant in our analysis (**new data, Extended Data Fig. 3c-e and Extended Data Fig. 4c**).

2) Beyond histological analysis, it would be informative to test whether RNF213-deficient or mutant mice show behavioral or neurological phenotypes consistent with brain polyglucosan accumulation, such as impairments in motor coordination, cognition, or seizure susceptibility.

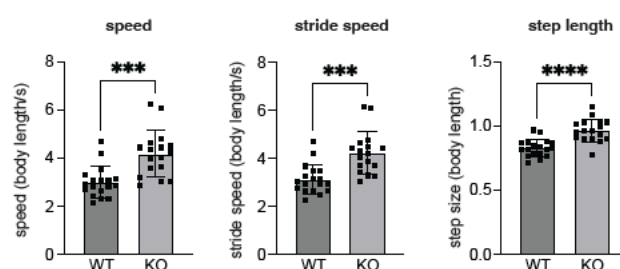
We thank the reviewer for the suggestion, which we addressed experimentally.

(This suggestions is similar to Comment #12 from Reviewer #2. For ease of reference we copy our reply:

"... We followed the reviewer's advice to investigate behavioural phenotypes linked to the accumulation of polyglucosan bodies. As these experiments fall outside our core expertise, they were conducted in collaboration with Anne Bertolotti and Lucia de los Reyes-Ramirez.

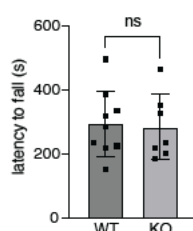
As recommended by the reviewer, we investigated motor function (MouseWalker and rotarod) as well as anxiety-like behaviour (open field) and memory (object recognition memory and spatial memory experiments) in 8-10 month old WT or RNF213 KO mice. Seizure threshold experiments (PTZ challenge) also suggested by the reviewer could not be performed as they fall strictly outside the scope of our animal licence.

Mouse Walker. We performed Mouse Walker experiments to quantitatively assess coordination and motor deficits. RNF213 KO mice maintained a normal, symmetrical trotting gait compared to age- and sex-matched WT controls. However, RNF213 KO mice exhibited altered gait dynamics, and moved faster than the WT mice due to increases in both stride speed and stride length, suggesting increased locomotor activity (**new data, Reviewer Fig. 8**). While we are excited by these findings, we feel that including them in the manuscript would be premature, as further experiments are required to determine the mechanism underlying this behavioural phenotype.



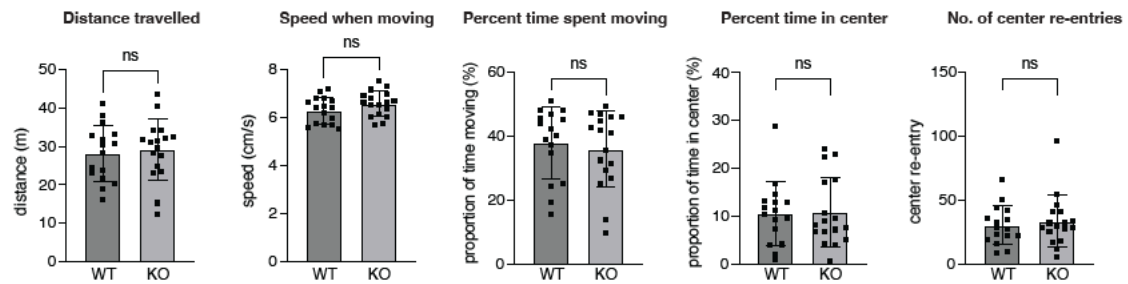
Reviewer Fig. 8 | MouseWalker experiments. A MouseWalker, constructed by the LMB mechanical workshop from schematics by Mendes et al. (PMID: 26197889), was used to assess locomotor phenotypes in WT or *Rnf213*^{-/-} mice. Mice were habituated to the mouse walker and trained to walk across it by attaching their home cage to the end of the apparatus. Five walks were performed during training, with a 1 min rest between attempts. Tests were performed 24 h after training. Ten attempts were performed, with a 1 min rest between attempts, until 3 successful walks were recorded. Videos were analysed by training a neural network from the open-source package provided by DeepLabCut, from which parameters such as mouse speed, step length, and stride speed were measured.

Rotarod. We performed rotarod experiments to assess balance, motor coordination / learning, and fatigue resistance. No difference in the latency to fall between WT and RNF213 KO mice was observed, indicating that the cerebellar and hindbrain polyglucosan burden does not impair motor function at this age (**new data, Reviewer Fig. 9**)



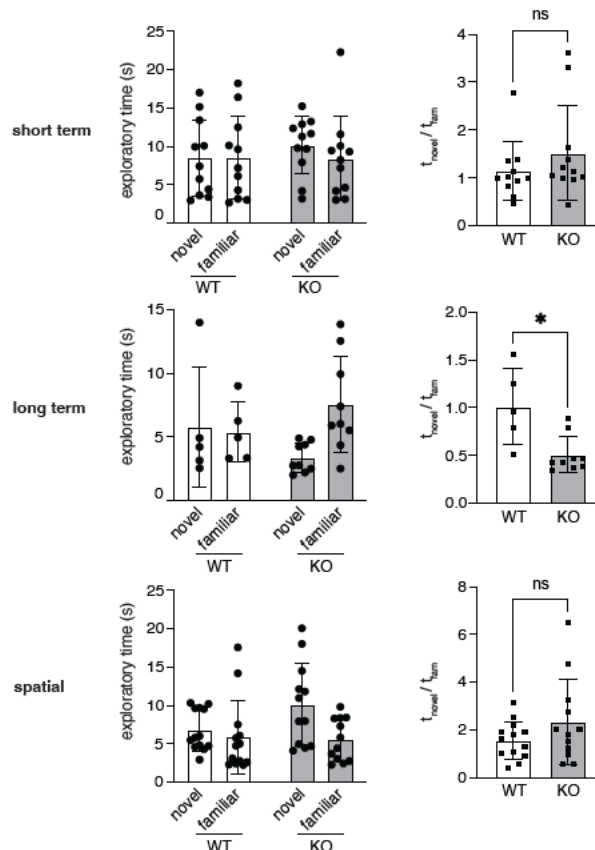
Reviewer Fig. 9 | Rotarod experiments. Mice were first habituated for 1 min on a static rotor and 1 min at constant speed (4 rpm) on two occasions and then tested for motor function and learning over three trials performed on three consecutive days (one trial per day). Each trial consisted of three test sessions with a 15 min rest between sessions. For each session, three or four mice were placed on an accelerating rotor (4 to 40 rpm) and the latency to fall was recorded, with a maximum limit for individual animals set at 900 seconds.

Open field experiments. We performed Open Field experiments to assess anxiety-like behaviour, exploratory behaviour, and baseline locomotor activity. We did not observe any statistically significant differences in anxiety-related behaviour between WT and RNF213 KO mice, as assayed by distance travelled, speed, percentage of time spent moving, percentage of time in the center of the arena, or number of re-entries into the center (**new data, Reviewer Fig. 10**).



Reviewer Fig. 10 | Open field experiments. An open field arena consisting of a 40 cm x 40 cm Perspex box was used, with a camera situated above the arena at the center of the box. A single mouse was placed in the middle of the arena, using cupping to minimise stress. A 15 min recording was captured. Videos were analysed by training a neural network from the open-source package provided by DeepLabCut to assess parameters including distance travelled, mouse speed, percentage of time spent moving, percentage of time spent in the center of the box, and number of re-entries into the center.

Novel Object Recognition (NOR) and spatial memory experiments. We performed NOR experiments to assess short- and long-term recognition memory in WT and RNF213 KO mice. We did not observe robust cognitive defects in NOR experiments (**new data, Reviewer Fig. 11**). Regarding long-term memory, RNF213 KO mice slightly preferred the familiar object over the novel object. However, we note that the WT controls did not explore the novel object more than the familiar object, as would be expected, and therefore these findings should be interpreted with caution.



Reviewer Fig. 11 | Novel object recognition and spatial memory experiments. A 40 cm x 40 cm arena was used for NOR experiments. Mice were habituated to the arena for 15 min on the first day. On the following day, mice were habituated to the empty arena for 15 min, before two identical objects were introduced. The mouse was allowed to explore them for 10 min. Testing for short-term memory was performed one hour later. The mouse was returned to the arena with one familiar object and one novel object and allowed to explore them for 5 min. Testing for long-term memory was performed the next day with one familiar and a new novel object. Spatial memory was tested an hour after that, with the same objects but with the position of one object changed. Videos were analysed by training a neural network using the open-source package provided by DeepLabCut, and the time spent exploring the novel and familiar objects was measured. Mice that explored either object for less than 2 seconds were removed from analysis.

We conclude that 8-10 months old RNF213 KO mice do not display cognitive or motor defects in the Mouse Walker, the Rotarod, Open Field, Novel Object Recognition, or spatial memory experiments. Such phenotypes may manifest at later ages, when the polyglucosan burden is greater. To reflect these new data, we have edited the manuscript to avoid overstating claims about general brain health and to focus on the molecular mechanism of glycogen quality control through non-protein ubiquitylation and autophagy.

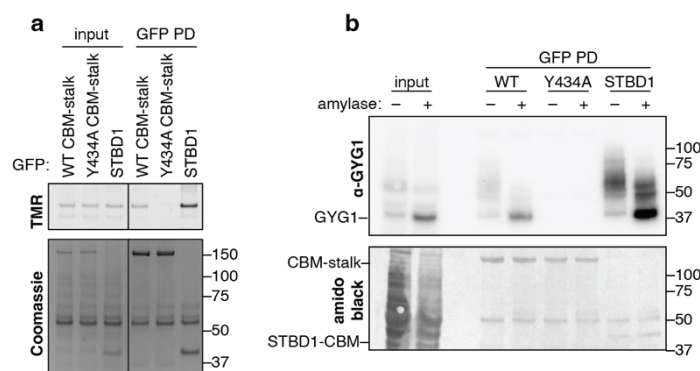
3) Terminology relating to glycogen, polyglucosan, amylopectinosis, and Lafora bodies should be used consistently and aligned with the glycogen-storage literature. A schematic illustrating the different glycogen species and indicating which assays probe which species would greatly improve clarity.

As suggested, we edited the text for clarity. A cartoon defining our experimental setup is shown in Fig. 2a.

4) Figure 3d switches to human HEK293ET cells and uses a truncated RNF213 “CBM-stalk” construct (human or mouse protein?), whereas most other experiments use MEFs and full-length RNF213. The rationale for this change in cell type and model system should be explained.

In **Fig. 3d**, murine RNF213 constructs were used. The legend has been edited for clarity.

HEK293ET cells were used for convenience. Similar results can be obtained from MEFs producing glycogen and from endogenous brain glycogen, using GYG1 post amylase digestion as a readout for glycogen (**Reviewer Fig. 17**).



Reviewer Fig.17 | **a**, purified GFP-tagged CBM-stalk or STBD1 probe constructs were used as bait proteins to pulldown glycogen (assayed by TMR-labelled Halo-GYG1 or GYG1) from **a**, cells or **b**, brain lysates

5) In Fig. 4a, the baseline number of polyglucosan puncta differs between WT and CA cell lines, even in the –Dox condition, complicating interpretation of the rescue experiment. Considering errors between experiments, effects do not look significant.

We thank the reviewer for highlighting this weakness. The baseline differed because cells had been complemented independently with wt and mutant RNF213. To address the reviewer’s comment directly, a new batch of cells has been generated, and the baseline is now consistent throughout (new **Fig. 4a**).

6) In Extended Data Fig. 8a, Coomassie staining suggests unequal loading between –Dox and +Dox samples for the mutants, making conclusions about unchanged polyglucosan levels less convincing.

We replaced the Coomassie panel with blotting for a loading control (Sec61β). We now provide densitometric data from three experiments (new **Extended Data Fig. 9j-l**), confirming our original observation.

7) Extended Data Figure 6 does not specify which sequences or organisms were used to compute conservation scores. In addition, the text states that all contact residues except H447 are highly conserved, whereas the figure indicates that Y434 is also not conserved. Thus, two key residues implicated in glycogen binding are not conserved, raising questions about the identity of the recognized substrate. Furthermore, the interpretation that carbohydrate-binding residues of CBM20 are highly conserved seems to contradict an earlier report from the same group that CBM20 underwent positive selection in pathogen recognition (Crespillo-Casado et

al., EMBO Rep. 2024). These opposing models imply distinct evolutionary pressures shaping the CBM20 domain and its substrate spectrum, suggesting that at least one of the arguments requires revision.

The alignments were performed using the Consurf server using RNF213 sequences from fish, reptiles, birds and mammals.

Regarding Y434, we apologize for the poor display of our data in the cartoon. We changed the colouring scheme and added a sequence logo to correctly display conservation. Y434 is scored as consurf grade 7 [F (4%), H (0.667%), W (1.333%) or Y (94%)], supporting its role in sugar binding.

H447 is scored as consurf grade 4, and is much more variable [D (0.667%), H (69.333%), I (4%), P (8.667%), Q (0.667%), V (0.667%), Y (10%)], as stated in the manuscript text.

8) The phrase “enzymatically amplified recruitment” is vague and not helpful, as the mechanistic steps and/or catalytic activities underlying such amplification are not defined.

“Enzymatically amplified recruitment” refers to a requirement for ATPase activity in AAA3 and AAA4 and for RZ-dependent E3 ligase activity in RNF213 coat formation on pathogens (*Salmonella*, *Listeria*, *Toxoplasma*) (Otten et al., *Nature*, 2021; Crespillo-Casado et al., *EMBO Rep.*, 2024). We edited the text accordingly.

9) C4462S and C4462A mutations in the catalytic RZ domain are used in different parts of the study. The authors should clarify whether these mutations have equivalent effects on RNF213 E3 ligase activity and explain why both are used interchangeably.

C4462S and C4462A have similar effects in abolishing RNF213 RZ-dependent E3 ligase activity. The enzyme requires C4462 to accept ubiquitin from its cognate E2. Replacing C4462 with either alanine or serine is incompatible with catalytic activity of the RZ domain, and similar mutations have been created for other transthiolating E3 ligases (e.g. HECTs and RBRs). C4462S and C4462A were used to generate transgenic mice and for cell experiments, respectively.

Referee #5 (Remarks to the Author):

Ubiquitylation is an essential posttranslational modification that has mostly been described for its ability to control the function or stability of proteins. However, recent work suggested that ubiquitylation can also target non-proteinaceous biomolecules, including sugars or small molecules. The Randow lab has been at the forefront of this development with their discovery of RNF213 as an E3 ligase that helps cells defend against bacterial invasion by ubiquitylating lipopolysaccharides exposed on the bacterial membranes. Here, they report evidence that suggests that RNF213 can also ubiquitylate polyglucosan bodies that originate from aberrantly branched glycogen molecules. Ubiquitylation of polyglucosan bodies by RNF213 directs these structures into autophagosomes, although it seems that polyglucosan is not degraded. Loss of RNF213 leads to accumulation of polyglucosan bodies in specific regions of the brain, indicating that RNF213 is involved in polyglucosan body removal in vivo. While previous manuscripts had documented glycogen ubiquitylation in vitro, it is nice to see similar reactions in vivo, thus expanding the reach of ubiquitylation from regulating proteins to the control of other biomolecules. However, several questions would need to be addressed before this paper represents progress expected from a publication in Nature.

We thank the reviewer for their concise summary of our findings and for acknowledging the significance of ‘... expanding the reach of ubiquitylation from regulating proteins to the control of other biomolecules’.

1. Most importantly, it is not clear whether this regulatory circuit is critical for preserving cellular fitness or survival. As a consequence, it remains to be shown whether the reactions described here primarily occur to prevent polyglucosan accumulation, or whether they are a side reaction of more important, still to be discovered, pathways. It would be critical that the authors identify a RNF213 mutant that is unable to bind polyglucosan and hence not recruited to these structures (different from their CBM20 mutants that result in a potential gain-of-function phenotype). They would then have to show that such mutants cause some fitness defect to provide evidence that the regulation reported here is important. The authors could also document the importance of RNF213-dependent polyglucosan ubiquitylation by showing genetic interactions between RNF213 loss of function and enzymes of glycogen processing or degradation, with a focus on substrate-binding mutants rather than catalytic mutations that could affect other processes as well.

Defects in glycogen metabolism have dire consequences, often resulting in embryonic lethality. Relatively ‘mild’ cases caused by defects in glycogen quality control still cause premature death, for example, in Lafora patients at ~25 years of age. The accumulation of insoluble, poorly branched polyglucosan is causative for the disease, highlighting the importance of glycogen quality control for organismic health and hence the urgent need to investigate the underlying mechanism.

We report the accumulation of polyglucosan in *Rnf213*^{-/-} and also in E3 ligase-deficient RNF213 C4462S mice, revealing the need for E3 ligase activity in RNF213 to prevent polyglucosan accumulation. We show that brain glycogen/polyglucosan from *Rnf213*^{-/-} mice is poorly branched and that polyglucosan accumulates specifically in astrocytes, resulting in reactive astrogliosis. Importantly, we show that RNF213 directly and selectively ubiquitylates

polyglucosan, thus demonstrating a role for non-protein ubiquitylation in the maintenance of organismic homeostasis.

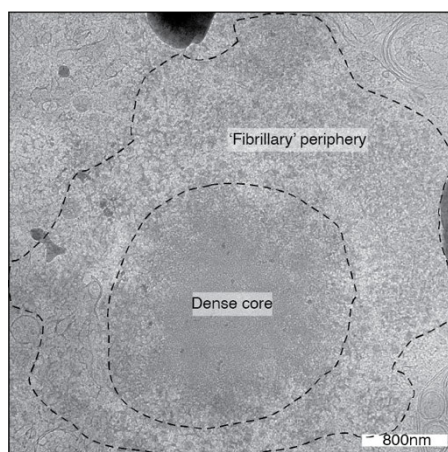
We addressed the reviewer's comment experimentally:

- we tested RNF213 alleles lacking ATPase activity in AAA3 and AAA4 (**new data, Extended Data Fig. 3b-e**). ATP coordination and ATP hydrolysis in both AAA3 and AAA4 are required for RNF213 recruitment to and ubiquitylation of polyglucosan
- we investigated the relationship between RNF213 and LUBAC in glycogen quality control. Polyglucosan ubiquitylation was abolished in RNF213 KO cells (**Fig. 2c,e,g**), while defects in HOIL-1 or HOIP only partially reduced polyglucosan ubiquitylation (**new data, Extended Data Fig. 8h**). Epistatic analysis revealed that RNF213 acts upstream of LUBAC, whereas defects in LUBAC did not affect RNF213 (**new data, Extended Data Fig. 8a-h**). However, overexpression of LUBAC in RNF213 KO cells restored polyglucosan ubiquitylation (**new data, Extended Data Fig. 8i-k**). Our new data are consistent with reports on HOIL-1 ubiquitylating glycogen in vitro (Kelsall et al., *EMBO J.*, 2022) and defects in LUBAC causing a partial reduction in polyglucosan ubiquitylation in cells (Jochem et al., *Nature*, 2026). Taken together, these findings reveal an essential function for RNF213 in glycogen quality control through both direct ubiquitylation of polyglucosan and epistatic control of LUBAC. At the same time, they highlight the importance of LUBAC expression levels, which may help explain the tissue-specific phenotype observed in HOIL1-deficient mice.

2. What happens with polyglucosan bodies in autophagosomes? The way the manuscript is written suggests that these structures may be degraded in autophagosomes, but the presented data only shows degradation of associated proteins. If the polyglucosan body is not degraded, how then do the authors explain the accumulation of glycogen in the brain of RNF213 KO mice? Maybe the authors could follow polyglucosan bodies for longer periods of time? Maybe they did not use the proper cell line for their studies? This discrepancy should be addressed.

We addressed the reviewer's comment experimentally.

Polyglucosan bodies are delivered to autophagosomes, where polyglucosan is degraded, as revealed by the loss of Halo-GYG1 signal (**new data, Extended Data Fig. 9j-l**). This degradation may preferentially affect smaller polyglucosan particles that are not easily detectable by light microscopy, resulting in an underestimation of the effect of RNF213 on the number of polyglucosan bodies. In addition, degradation may occur primarily at the periphery of larger polyglucosan bodies, where the glycogen network is less densely packed and hence more accessible to enzymatic hydrolysis (**new data, Reviewer Fig. 18**), again resulting in an underestimation of the effect of RNF213 on the number of polyglucosan bodies.



Reviewer Fig. 18 | cryo-EM image at 13,500× magnification of FIB-milled lamella in the cytosol of polyglucosan-producing cells. Note the dense core and looser peripheral material.

3. While their data (especially in Fig. 2g) is consistent with ubiquitin being attached to a glucose unit, they do not show direct evidence for such a reaction. Given the importance for this manuscript, they would need to provide direct evidence for polyglucosan ubiquitylation, potentially by mass spectrometry after amylase treatment.

We followed the reviewer's suggestions. We now provide mass spectrometry data revealing direct ubiquitylation of glucose in polyglucosan (**new data, Fig. 2h**).

4. Fig. 3 – the mechanism of RNF213 regulation via the CBM20 domain is discussed in a slightly confusing way. It is possible that the CBM20 domain acts in modulating autoinhibition of RNF213, but it may also regulate features such as E3 ligase oligomerization that could affect catalytic activity independently of autoinhibition. To address these issues, the authors should at least look at RNF213 dimerization/oligomerization as well as binding partners in CBM20-mutant cells. Such analyses may address the discrepancy between CBM20-mutants disrupting glycogen binding in pulldown experiments, while being competent to bind polyglucosan bodies by microscopy.

We followed the reviewer's advice to analyze the oligomeric state of RNF213.

SEC-MALS analysis revealed that WT RNF213 and the CBM20 mutants are primarily dimeric, with no evidence for aggregation, thus suggesting that the oligomeric status of RNF213 is unaffected by the CBM20 mutations (**new data, Reviewer Fig. 15**).

5. Fig. 3e – it seems that polyglucosan bodies are larger when CMB20-mutant RNF213 is being expressed. This complicates interpretations of a gain-of-function phenotype regarding polyglucosan ubiquitylation, as enhanced recruitment of multiple RNF213 molecules to a larger polyglucosan body might result in aberrant ubiquitylation that is independent of intrinsic catalytic activity.

We apologise for the lack of clarity.

The larger size of the glycogen species in cells expressing RNF213 W382A or Y434A is due to the aggregation of ubiquitylated glycogen, possibly mediated through autophagy cargo adapters such as p62, known to form so-called p62 bodies. This phenotype arises because the CBM20 mutants have lost specificity for polyglucosan and instead also ubiquitylate soluble

glycogen, resulting in clustering. In the absence of RNF213 expression, glycogen appears similar in all cells by immunofluorescence microscopy, and the larger glycogen structures appear only upon induction of the CBM20 mutant alleles and in an E3 ligase dependent manner (**new data, Reviewer Fig. 4**).

Referees' comments:

We are delighted that four reviewers concluded that no or only minor questions remain. We agree with these reviewers that their remaining points can be addressed through textual changes alone.

Below we respond to all comments in detail. To address concerns raised by Referee #4, we provide new data demonstrating the in vitro reconstitution of polyglucosan ubiquitylation by RNF213.

Referee #1 (Remarks to the Author):

To my mind, the authors addressed all questions raised by me and also raised by the other reviewers in an appropriate manner. Thereby, the manuscript has been further improved. As far as I have been able to assess the statistical tests applied, I think these were appropriate.

We thank the reviewer for their enthusiasm. According to Referee #1, all questions, including those raised by other reviewers, have been adequately addressed.

In Fig.4 A, I would suggest to also state the number of cells analyzed per experiment or even show the quantification for each cell with different colours per experiment. This also applies to Ext. Data Fig. 3 d, e, g, h, Ext. Data Fig. 8c-g, j and k, and Ext. Data Fig. 9 d-g and I. Ext Figure 1g, please, specify n=3 mice each?

The requested information has been added to the figure legends.

Referee #2 (Remarks to the Author):

The authors did an impressive job of addressing reviewer concerns. The revised manuscript is substantially improved and now provides a strong mechanistic and conceptual advance. The authors added important in vivo, biochemical, structural, and cell-biological data that address most major concerns. The central conclusion that RNF213 limits brain polyglucosan accumulation and can directly ubiquitylate aberrant glycogen is now very well supported.

We thank the reviewer for their support. According to Referee #2, the revised manuscript provides a '... strong mechanistic and conceptual advance' and central conclusions, especially that RNF213 limits brain polyglucosan accumulation and can directly ubiquitylate aberrant glycogen, are now '...very well supported'.

The remaining limitations focus around the extent to which the cell-based autophagy mechanism is directly demonstrated in brain tissue, the incomplete mechanistic explanation for CBM20-dependent substrate discrimination, and the lack of robust organismal phenotypes. IMO, these limitations can be addressed largely by textual clarification, rather than by new experiments.

The reviewer concludes that no further experiments are required. We will provide the requested textual clarifications, as detailed below.

1. The in vivo autophagy mechanism is inferred from the engineered cell system. The text should be explicit that the tissue data establish the phenotype and genetic requirement, while the mechanistic autophagy pathway is established primarily in cells.

As requested, we added a statement in the Discussion section:

"... Our engineered cell-based system of experimentally controlled polyglucosan synthesis suggests that polyglucosan accumulation in *Rnf213*^{-/-} mice is caused by lack of RNF213-mediated polyglucosan ubiquitylation and a downstream defect in ubiquitin-dependent autophagy. Further in vivo studies are required to test this model."

2. The CBM20 "proofreading" mechanism still seems incomplete. The CBM20 data are interesting and strong, but it is not that simple. B/c RNF213 binds maltoheptaose via a CBM20-like domain, mutations in sugar-contact residues disrupt glycogen binding, and W382A/Y434A mutants gain the ability to ubiquitylate physiological glycogen. However, same CBM20 mutants still target polyglucosan. Therefore, CBM20 is not the only recruitment determinant for aberrant glycogen. It appears to suppress inappropriate activity toward physiological glycogen. I would suggest that the authors avoid verbiage saying that the CBM20 fully explains specificity. Instead: The CBM20 domain restricts RNF213 activity toward physiological glycogen and contributes to substrate discrimination.

We did not intend to imply that our data on the CBM20 domain fully explain specificity and we agree with the reviewer's conclusion that '... the CBM20 domain restricts RNF213 activity toward physiological glycogen and contributes to substrate discrimination.' We edited the abstract to accommodate the reviewer's comment:

"... Cryo-EM analysis of RNF213 bound to glycogen-derived maltoheptaose revealed its CBM20 domain binds linear oligosaccharides. Disrupting carbohydrate binding results in gain

of E3 ligase activity towards physiological glycogen, indicating the CBM20 domain limits RNF213 activity."

3. The new LUBAC data are a very strong additions. RNF213 deletion prevent HOIP recruitment, HOIL1/HOIP loss does not prevent RNF213 recruitment, and LUBAC overexpression can bypass RNF213 in cells. This supports the hierarchy: RNF213 upstream of LUBAC under their experimental conditions. This is very strong in cells, but not proven in vivo. If possible, I would recommend framing this as the model rather than fully established in vivo.

We are delighted that the reviewer considers our new data on LUBAC 'very strong' and agrees with our conclusion regarding the hierarchy of RNF213 acting upstream of LUBAC. Our data were obtained in cells, thus no direct claims about the in vivo situation will be made. Following the reviewer's suggestion, we edited the discussion section:

"... While the epistatic relationship between RNF213 and LUBAC still requires in vivo validation, reconstituted glycogen / polyglucosan synthesis in cells provides an attractive tool to further dissect the mechanisms of glycogen quality control, including the role of other E3 ubiquitin ligases."

4. I applaud the behavioral testing and data in the R2R. Agreed that it should not be included in this manuscript. I also agree that their mechanism does "safeguard the brain" in terms of molecular/histopathological rather than functional.

We thank the reviewer for their support.

5. The Moyamoya sections are substantially improved and they acknowledge that the relationship to Moyamoya remains open.

We thank the reviewer for their support.

6. Should the CBM20 mutant structural-integrity controls be included as extended data? SEC-MALS for WT/W382A/Y434A and cryo-EM structure of Y434A, arguing against misfolding. The authors can decide.

We believe that including these data in the manuscript would distract from, rather than strengthen, the main message.

116 Referee #3 (Remarks to the Author):

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118 I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

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120 [We thank the reviewer for their time.](#)

Referee #4 (Remarks to the Author):

The authors have undertaken an extensive revision, generating substantial new data (MALDI-TOF mass spectrometry, LUBAC epistasis analysis, cryo-ET, astrocyte validation).

We thank the reviewer for appreciating the extent of the newly provided data.

However, rather than reinforcing the mechanistic model, the additional experiments do more to weaken than to strengthen the central claims. While the data establish that RNF213 contributes to polyglucosan homeostasis in astrocytes, they do not show that RNF213 directly recognises or ubiquitinates polyglucosan, that it acts as the priming E3 ligase, or that the mechanism constitutes a general quality-control system across tissues.

Regarding the reviewer's comment "...they do not show that RNF213 directly recognises or ubiquitinates polyglucosan":

The manuscript provides evidence that RNF213 is essential for the selective ubiquitylation of polyglucosan, while sparing glycogen (Fig. 2g), and that RNF213 can directly ubiquitylate maltoheptaose in vitro (Extended Data Fig. 4c). To further address the reviewer's concern, we have now reconstituted RNF213-mediated polyglucosan ubiquitylation in vitro, revealing direct ubiquitylation of polyglucosan while sparing glycogen (**new data, Fig. 2i**). Please see below (around line 277) for details.

Regarding the reviewer's concern about the role of RNF213 as the priming E3 ligase:

We would like to emphasize that the reviewer's remark does not represent a pre-existing question in the field but rather arose from discoveries added during revision. Because RNF213 is essential for polyglucosan ubiquitylation (Fig. 2g, Extended Data Fig. 8h) and for LUBAC recruitment (Extended Data Fig. 8b,c), while LUBAC makes a quantitatively limited contribution to polyglucosan ubiquitylation (Extended Data Fig. 8h) and does not control RNF213 recruitment (Extended Data Fig. 8e,f), we concluded that RNF213 acts upstream of LUBAC, which is consistent with its proposed role as the 'priming' E3 ligase. New data on the ability of RNF213 to selectively ubiquitylate polyglucosan are also consistent with its proposed role as the 'priming' E3 ligase (**new data, Fig. 2i**). We are not sure whether the reviewer disputes any of these conclusions or whether the concern relates solely to the label 'priming' E3 ligase. We would be happy to use different terminology if the reviewer feels that doing so would improve clarity.

Regarding the '... mechanism constituting a general quality-control system across tissues':

We report a specific role for RNF213 in glycogen quality control in the brain / in astrocytes. It is tempting to speculate that related mechanisms may contribute to quality control across tissues, but we have no data to support this possibility and we certainly do not claim such a role for RNF213.

Most critically, LUBAC overexpression rescues polyglucosan ubiquitination in RNF213 KO cells. Combined with the absence of direct biochemical reconstitution of RNF213-catalysed polyglucosan ubiquitination, the data remain fully compatible with an alternative model in which LUBAC performs the central glucan ubiquitination while RNF213 is an upstream regulator.

To address the reviewer's concern experimentally, we reconstituted polyglucosan ubiquitylation by RNF213 in vitro, revealing direct and selective ubiquitylation of polyglucosan by RNF213 (**new data, Fig. 2i-k and Extended Data Fig. 4d,e**).

We respectfully disagree with the alternative model proposed by the reviewer, in which LUBAC would perform the central glucan ubiquitylation. RNF213 deficiency abrogates polyglucosan ubiquitylation (Fig. 2g and Extended Data Fig. 8h) and prevents LUBAC recruitment (Extended Data Fig. 8b,c), whereas LUBAC deficiency (HOIL-1 or HOIP KO) causes only a mild reduction in polyglucosan ubiquitylation (Extended Data Fig. 8h) without affecting RNF213 recruitment (Extended Data Fig. 8e,f). These results are not compatible with the reviewer's model in which LUBAC performs the central polyglucosan ubiquitylation step. Rather, the genetic evidence demonstrates that RNF213 is essential for polyglucosan ubiquitylation, whereas LUBAC makes a more limited contribution to the overall extent of polyglucosan ubiquitylation downstream of RNF213. While LUBAC does not perform the central glucan ubiquitylation, LUBAC-derived ubiquitin may nevertheless be functionally important, for example by providing M1-linked chains.

1. The data do not establish RNF213 as the priming E3 ligase for polyglucosan ubiquitination. The revised manuscript continues to position RNF213 as the primary E3 ligase that directly recognises and ubiquitinates polyglucosan. Four lines of evidence indicate this conclusion remains indirect and that the evidence is equally compatible with a model in which LUBAC performs the central glucan ubiquitination reaction while RNF213 serves as upstream regulatory factor.

The genetics of polyglucosan ubiquitylation in RNF213-deficient and LUBAC-deficient cells is incompatible with the reviewer's model of LUBAC performing the '... central glucan ubiquitylation reaction while RNF213 serves as upstream regulatory factor'. Because RNF213 deficiency abolishes polyglucosan ubiquitylation (Fig. 2g and Extended Data Fig. 8h), whereas LUBAC deficiency causes only a partial reduction (Extended Data Fig. 8h), the hypothesis that LUBAC performs the central glucan ubiquitylation reaction is not supported by data.

First, LUBAC overexpression restores polyglucosan ubiquitination in RNF213 KO cells (Extended Data Fig. 8i-k), directly challenging the proposed epistatic hierarchy: if RNF213 were the essential initiating E3 ligase, bypassing it through overexpression of a nominally downstream enzyme would not be expected. The data instead indicate that polyglucosan ubiquitination can be established independently of RNF213.

Overexpression of LUBAC evaluates the potential of LUBAC to catalyze polyglucosan ubiquitylation, whereas knockout of LUBAC tests whether this potential is realized under physiological conditions.

Gene deletion reveals that LUBAC contributes to polyglucosan ubiquitylation (Extended Data Fig. 8h), whereas RNF213 is essential for the reaction (Fig. 2g and Extended Data Fig. 8h). Consistent with our findings, independent evidence for a quantitatively modest contribution of LUBAC has been published recently: HOIL1 deficiency reduces polyglucosan ubiquitylation by ~60% only (Jochem, *Nature* 2026). However, as revealed by LUBAC overexpression in RNF213 KO cells, LUBAC does have the capacity to mediate polyglucosan ubiquitylation on its own, if only present at sufficiently high levels. At endogenous LUBAC levels, however, LUBAC depends on RNF213 (Extended Data Fig. 8h).

The reviewer's argument therefore centers on the distinction between catalytic capacity and physiological function. Yes, LUBAC can catalyze the reaction in principle, i.e. when overexpressed, but this potential is not realized at physiological LUBAC concentrations. At endogenous expression levels, RNF213 is essential for polyglucosan ubiquitylation and controls LUBAC, which makes a quantitatively limited contribution in an RNF213-dependent manner. It remains possible that in a different biological context, for example in cell types

expressing much higher levels of LUBAC or in the presence of alternative upstream E3 ligases, LUBAC could mediate polyglucosan ubiquitylation independently of RNF213. Whether such situations exist is currently unknown, but defining the conditions under which LUBAC may fulfil this potential represents an interesting direction for future investigation.

Regarding the reviewer's statement that if "...RNF213 were the essential initiating E3 ligase, bypassing it through overexpression of a nominally downstream enzyme would not be expected", we respectfully disagree. In contrast to the reviewer's assertion, overexpression of signalling molecules can activate downstream pathways in the absence of their physiological upstream activators. For example, LUBAC overexpression activates NF- κ B in cells lacking upstream components (Tokunaga, *Nature Cell Biology* 2009), and overexpression of the downstream E3 ligase RNF168 bypasses the requirement for the upstream E3 ligase RNF8, restoring histone H2A ubiquitylation by RNF168 and recruitment of 53BP1 to sites of DNA double-strand breaks (Stewart, *Cell* 2009; Kato, *Molecular Cell* 2014). These examples demonstrate that bypass of an upstream genetic defect by overexpression of a downstream effector is a well-established phenomenon, thereby invalidating the assumption that overexpression of a downstream enzyme (i.e. LUBAC) cannot bypass an essential upstream E3 ligase (i.e. RNF213).

Second, loss of polyglucosan ubiquitination in ATPase and E3-ligase dead RNF213 mutants is interpreted as evidence that these enzymatic activities are specifically required for ubiquitin transfer to polyglucosan. However, these same activities are required for RNF213 coat formation on bacteria and lipid droplets, i.e. contexts where the downstream function is recruitment and organizing a signaling hub rather than direct substrate ubiquitination. Likewise, in *Toxoplasma gondii*, ubiquitin ligase activity is dispensable for RNF213 recruitment, illustrating context-dependent contributions. The observed loss of polyglucosan ubiquitination in these mutants is therefore equally compatible with impaired recruitment, defective coat formation, or failure to engage downstream ubiquitination factors.

We apologize for the confusion.

ATPase and E3 ligase activity of RNF213 are part of the general mechanism by which RNF213 attacks its substrates, including bacteria, lipid droplets, and now polyglucosan, as shown by us and others.

We therefore do not interpret the requirement for ATPase or E3 ligase activity in RNF213 as evidence that these enzymatic activities are specifically required for ubiquitin transfer to polyglucosan, as implied by the reviewer's comment. Rather, our interpretation is simply that these activities are required for RNF213 function irrespective of substrate. We are unsure how this misunderstanding arose and would appreciate guidance on how to make this point clearer, if the reviewer considers this necessary.

Third, direct biochemical reconstitution of RNF213-mediated polyglucosan ubiquitination has not been demonstrated. The only in vitro reconstitution experiment uses maltoheptaose, a generic α 1,4-linked glucan shared by physiological glycogen and pathological polyglucosan and lacking the structural features (extended chain length, reduced branching) that distinguish polyglucosan from normal glycogen. The data show that purified RNF213 can ubiquitinate a short linear glucan, but do not demonstrate recognition or ubiquitination of polyglucosan, nor do the data address substrate specificity. Showing direct RNF213-catalysed polyglucosan ubiquitination with appropriate substrate specificity is essential, as asked for by referees, yet this point was not addressed during revision.

To address the reviewer's question, we provide new experimental data, which demonstrate the reconstitution of polyglucosan ubiquitylation *in vitro* from purified components (**new data, Fig. 2i-k and Extended Data Fig. 4d,e**). We observe preferential ubiquitylation of polyglucosan versus glycogen and the formation of SDS- and heat-resistant ubiquitin adducts on polyglucosan through NH_2OH -sensitive bonds in an E1- and RNF213-dependent manner, thus demonstrating the ability of purified RNF213 to selectively and directly ubiquitylate polyglucosan.

Fourth, polyglucosan structures in RNF213 KO brains are p62-positive but ubiquitin-negative (Reviewer Fig 1). This is hard to reconcile with the proposed ubiquitin-dependent autophagy model, under which structures accumulating without RNF213 should be devoid of both ubiquitin and the ubiquitin-dependent receptor p62. The presence of p62 on ubiquitin-negative polyglucosan instead points to either ubiquitin-independent p62 recruitment or a clearance mechanism less ubiquitin-dependent than the model assumes - undermining a central premise of the proposed pathway.

We respectfully disagree with the reviewer. Polyglucosan accumulating in RNF213 KO brains appearing ubiquitin-negative is entirely consistent with the proposed function of RNF213 in glycogen quality control through ubiquitylation of aberrant polyglucosan. These structures are also LC3-negative, as expected, since the absence of RNF213-dependent ubiquitylation prevents autophagic clearance.

The presence of p62 on ubiquitin-negative polyglucosan indeed points to ubiquitin-independent p62 recruitment, as suggested by the reviewer above. Other autophagy cargo receptors are also known to respond to multiple 'eat-me' signals, for example NDP52, which independently senses both ubiquitin and glycans on damaged endomembranes (Thurston, *Nature* 2012). The identity of the ubiquitin-independent signal that recruits p62 to polyglucosan remains unknown and will be an interesting topic for future research.

However, we do not agree with the reviewer that the presence of p62 on ubiquitin-negative polyglucosan implies "... a clearance mechanism less ubiquitin-dependent than the model assumes." On the contrary, the accumulation of ubiquitin-negative polyglucosan in RNF213-deficient cells is precisely what our model predicts if RNF213-dependent ubiquitylation is required for productive autophagic clearance. The unexpected finding is therefore not the absence of ubiquitin, but the persistence of p62 in the absence of ubiquitin. This observation suggests that p62 can be recruited by an additional, ubiquitin-independent signal, but it does not challenge our conclusion that RNF213-dependent ubiquitylation is required for efficient clearance of aberrant polyglucosan.

2. Tissue restriction and mild phenotype do not support an essential quality-control mechanism

The *in vivo* phenotype is restricted to astrocytes in specific brain regions, despite RNF213's broad tissue expression. Liver, skeletal muscle, and heart – these are tissues where glycogen metabolism is highly active and where Jochem et al (2026) detect abundant endogenous glycogen ubiquitination - show no polyglucosan accumulation in RNF213 KO mice. This pattern challenges the generality of the quality-control mechanism proposed in the abstract and instead points to a context-dependent, tissue-specific role.

RNF213 is essential for glycogen quality control in the mouse brain (Fig. 1), specifically in astrocytes (Extended Data Fig. 1e). Such cell-type specific manifestation of RNF213 deficiency is not unexpected as other genetic polyglucosan diseases (caused by mutations in Laforin, Malin, or LUBAC) also manifest in a cell- and/or tissue-specific manner. For example,

in mice neither Hoil-1 nor Malin deficiency result in polyglucosan accumulation in the liver (Nitschke, *Brain* 2022; DePaoli-Roach, *JBC* 2010).

To further address the reviewer's comment we have edited the abstract to emphasize the astrocyte-specific manifestation of RNF213 deficiency in glycogen metabolism:

"...These findings identify RNF213 as a quality control factor preventing polyglucosan accumulation in astrocytes through direct ubiquitylation of aberrant glycogen, thus establishing non-protein ubiquitylation as essential for biomolecular quality control."

The authors suggest compensation by other E3 ligases may account for this discrepancy. While plausible, this remains speculative and is equally consistent with a model in which LUBAC constitutes the principal glucan ubiquitination system and RNF213 becomes limiting only in specific cellular contexts.

We respectfully disagree with the reviewer's conclusion that our data are "...equally consistent with a model in which LUBAC constitutes the principal glucan ubiquitination system and RNF213 becomes limiting only in specific cellular contexts." As discussed in more detail from line 210 onwards, lack of RNF213 prevents polyglucosan ubiquitylation and LUBAC recruitment to polyglucosan, while lack of LUBAC reduces polyglucosan ubiquitylation only mildly (Extended Data Fig. 8h) (see also Jochem, *Nature* 2026) and does not affect RNF213 recruitment. The reviewer's suggestion that LUBAC is the "...principal glucan ubiquitination system" is therefore not supported by the available experimental evidence.

The physiological phenotype in RNF213 KO mice is also notably mild compared with established disorders of glycogen and polyglucosan metabolism. At 8–10 months, polyglucosan accumulates in cerebellum, pons, and hippocampus (regions critical for motor coordination and memory) yet no behavioural deficits are detected. Defects in proteins that directly regulate glycogen architecture or polyglucosan clearance (GBE1, laforin, malin) result in progressive polyglucosan accumulation, pronounced neuroinflammation, and severe neurological phenotypes at comparable ages. Even HOIL-1 knock-in mice exhibit learning and motor coordination deficits. The presented evidence is thus not sufficient to state that RNF213-mediated glycogen ubiquitination is "essential for biomolecular quality control". The mild phenotype suggests RNF213 plays a contributory rather than central role in polyglucosan homeostasis.

RNF213 is essential for glycogen quality control in astrocytes (Fig. 1, Extended Data Fig. 8e). The accumulation of polyglucosan in astrocytes of RNF213 KO mice is profound (Fig. 1a-c) and certainly not "... notably mild compared with established disorders of glycogen and polyglucosan metabolism", as implied by the reviewer.

RNF213 KO mice at 8 months of age do not display severe neurological phenotypes. However, neither do LUBAC (*Rbck1*) deficient mice (Nitschke, *Brain* 2022), which develop behavioural abnormalities at a much older age (14-16 months) and only in females, despite the accumulation of polyglucosan in both sexes. Importantly, the behavioural phenotype in the female LUBAC deficient mice appears to depend on unknown genetic cofactors, as the behavioural phenotypes of the *Rbck1*^{-/-} females disappeared upon backcrossing (Nitschke, *Brain* 2022) despite the persistence of polyglucosan. Behavioural phenotypes are therefore not an obligatory consequence of polyglucosan accumulation in the brain and are context-sensitive.

Consistent with this conclusion, Malin KO mice, which are susceptible to kainate-induced epilepsy, accumulate polyglucosan bodies in both astrocytes and neurons. Experimental interference with polyglucosan accumulation specifically in astrocytes of Malin KO mice does not alter the kainate phenotype, suggesting that neuronal, rather than astrocytic, polyglucosan may have a greater impact on certain observable behavioural phenotypes (Duran, *Brain* 2021). We conclude that the accumulation of polyglucosan causes specific phenotypes depending on the affected cell type and that RNF213 is essential to prevent polyglucosan accumulation in astrocytes.

3. The CBM20 data do not explain substrate selectivity

The SEC-MALS and cryo-EM data rule out misfolding artefacts for one CBM20 mutant, supporting the interpretation that its phenotype reflects altered carbohydrate recognition. However, the data show only negative regulation — CBM20 prevents inappropriate targeting of normal glycogen — rather than a positive selectivity mechanism for polyglucosan. The manuscript itself states the recognition mechanism is unknown, yet the abstract claims that "the CBM20 domain confers specificity", implying an active substrate selection that is not directly demonstrated.

We apologize for the imprecise wording. We edited the abstract to accommodate the reviewer's comment:

"... Cryo-EM analysis of RNF213 bound to glycogen-derived maltoheptaose revealed its CBM20 domain binds linear oligosaccharides. Disrupting carbohydrate binding results in gain of E3 ligase activity towards physiological glycogen, indicating the CBM20 domain limits RNF213 activity towards physiological glycogen."

There is also a discrepancy between RNF213 localisation and ubiquitin deposition: CBM20 mutant proteins penetrate throughout soluble glycogen particles, whereas wild-type RNF213 forms rim-like structures on polyglucosan. If the model held, ubiquitination should mirror this altered occupancy, yet the ubiquitin signal in Fig 3e remains largely confined to peripheral rims rather than showing the predicted homogeneous distribution, an inconsistency that is not further explained. Taken together, the central question underlying any quality-control function — how RNF213 distinguishes pathological polyglucosan from normal glycogen — remains unresolved.

Regarding the ubiquitin labelling in Fig. 3e, the reviewer highlights the preferential labelling of ubiquitin on the surface of the glycogen structures, while RNF213 appears distributed throughout the particles. The difference is readily explained by limited antibody penetration into these dense structures, resulting in preferential detection of ubiquitin at the surface. In contrast, GFP-tagged RNF213, which became incorporated during particle formation and does not require antibody labelling, appears homogeneously distributed throughout the structures.

Regarding the specificity of RNF213 towards polyglucosan, our manuscript demonstrates glycogen quality control through RNF213-dependent selective ubiquitylation of polyglucosan and the induction of autophagy, thus establishing non-protein ubiquitylation as a mechanism for the quality control of biomolecules. In particular, we discovered a regulatory mechanism in which the carbohydrate-binding site of the CBM20 domain suppresses RNF213 activity towards physiological glycogen. Our data thus demonstrate that the functional substrate specificity of RNF213 depends on its CBM20 domain. Further work will be required to elucidate the underlying molecular mechanism, just as additional mechanistic work remains necessary for other proposed glycogen quality-control pathways (e.g. Malin and LUBAC).

4. Data restricted to the rebuttal, and claims without experimental support

A notable feature of the revision is the amount of experimental data presented only in the reviewer response and not incorporated into the manuscript, despite being central to the authors' mechanistic arguments. These include the finding that polyglucosan in RNF213 KO brains is p62-positive but ubiquitin-negative (Reviewer Fig 1), which questions whether clearance is ubiquitin-dependent; behavioural phenotyping (Reviewer Figs 8–11), performed to support claims of physiological significance; expression-level controls comparing RNF213 variants with endogenous levels (Reviewer Fig 14); and proteomics of polyglucosan composition (Reviewer Fig 13). The same applies to the fate of polyglucosan within lysosomes, which is likewise only partly resolved. ExtData Fig 9j–l shows glycogenin degradation in LAMP1-positive compartments while polyglucosan persists, and cryo-EM data shown only in the rebuttal (Reviewer Fig 18) reveal a dense-core/loose-periphery architecture interpreted as partial outer-layer degradation. This model — lysosomes degrading surface layers and smaller particles while the core persists — remains speculative, and the key supporting data will again be unavailable in the published manuscript.

Finally, some mechanistic arguments lack experimental support altogether. For example, a hypothetical ubiquitin-binding site in RNF213 is used to explain enzymatically amplified recruitment and a ubiquitin dependent feed-forward model of coat formation, yet no data supporting this site or its role in polyglucosan biology are presented in either the manuscript or the rebuttal. Given that the CBM alone does not account for polyglucosan targeting, providing such data is well within the scope of this study. Taken together, these data are important to the authors' conclusions but invisible to readers — an unfortunate disconnect between the evidence used to support the model and what the published paper allows others to assess.

During revision we added ~35 panels of new data to the manuscript that we considered particularly relevant and of general interest. Additional data in the point-by-point reply were provided to illustrate specific points in direct response to reviewers' questions. We believe most of those data would be of only marginal interest to most readers and would possibly even distract from the main message of our manuscript. However, we would be more than happy to include further data in the manuscript, space permitting, if the reviewer or editor advises us to do so.

Referee #5 (Remarks to the Author):

The authors have added several nice experiments to strengthen their model in which RNF213 ubiquitylates aberrant glycogen for clearance through autophagy. This is an important discovery and may deserve publication. However, they did not address my main initial concern, i.e. whether removal of aberrant glycogen through RNF213 is important for cell fitness or survival. While their data clearly shows that RNF213 can modify polyglucosan with ubiquitin, and that RNF213-knockout mice accumulate polyglucosan bodies in the brain, they do not show that ubiquitin-dependent removal is important - they merely infer this from diseases, in which polyglucosan accumulates. However, it is dangerous to draw such conclusions as diseases often have multiple contributors. At this point, I am not fully convinced that RNF213-dependent glycogen ubiquitylation is critically important for cells. Given that the discovery of glycogen ubiquitylation (together with a recent paper from David Komander's lab) is important, maybe the authors could add a substantial paragraph to explicitly mention this shortcoming of their study, protecting them from potential overinterpretations.

We thank the reviewer for highlighting the importance of our discovery and for their kind comment on the quality of the newly added data and the resulting improvement of our manuscript.

We followed the reviewer's advice by adding a statement to the Discussion section:

"...However, we infer the pathophysiological relevance of a failure in RNF213-mediated glycogen quality control from the neurological phenotypes observed in Lafora and other glycogen storage diseases. The contribution of non-protein ubiquitylation to overall organismal health therefore requires further investigation."

Referees' comments:

Referee #2 (Remarks to the Author):

The revised manuscript includes an important new experiment demonstrating RNF213 directly ubiquitinates cell-derived polyglucosan while comparatively sparing glycogen. This directly addresses important reviewers' concern that the earlier maltoheptaose assay established glucan ubiquitination but did not demonstrate recognition or modification of a disease-relevant polymer. Together with the added LUBAC epistasis, cryo-ET, and biochemical data, the revision substantially strengthens the central conclusion that RNF213 participates directly in polyglucosan quality control, while appropriately leaving the precise initial recognition mechanism and in vivo RNF213–LUBAC hierarchy unresolved. The authors have responded carefully and substantively to my previous comments; the remaining issues are primarily related to quantitative rigor, reporting, factual precision, and calibration of the strongest claims.

[We thank the reviewer for supporting our manuscript.](#)

Moderate issues

- Quantification and normalization of the purified-substrate experiment. Figure 2i is central to the revised manuscript, but the legend does not state the number of independent substrate preparations or ubiquitylation reactions, and the glycogen-versus- polyglucosan comparison is presented without quantitative analysis. The authors should quantify the RNF213-dependent ubiquitin signal across independent experiments and specify the experimental unit.

[We followed the reviewer's suggestion. The in vitro reactions were quantified and the number of independent substrate preparations and ubiquitylation reactions are provided in the figure legend.](#)

- Substrate normalization in Figure 2i. Glycogen and polyglucosan appear to be normalized using TMR-labelled glycogenin. However, polyglucosan could contain more glucan per glycogenin molecule, potentially contributing to the greater apparent ubiquitylation.

[Following the reviewer's suggestion, we compared the glucose content of our glycogen and polyglucosan preparations by quantifying the amount of glucose released upon glucoamylase digestion. We found that glycogen and polyglucosan preparations that had been normalized by TMR-glycogenin fluorescence released similar amounts of glucose. This information is now added to the methods section.](#)

- Reaction conditions for the new biochemical experiment. The Methods should provide complete reaction composition, substrate input, purification strategy, replicate structure, and normalization procedures for the direct glycogen/polyglucosan ubiquitination assay.

The requested information has been added.

- Important response-only controls. Several datasets used to defend central mechanistic interpretations would be useful to readers as Extended Dataa, particularly the brain p62/ubiquitin/LC3 staining, RNF213 construct-expression controls, and SEC-MALS/Y434A structural-integrity controls. These data directly clarify the in vivo interpretation and exclude mutant instability as the cause of the CBM20 phenotype.

We followed the reviewer's suggestion. Data previously in Reviewer Figures has been added (**Extended Data Fig. 3f, Extended Data Fig. 7a, Extended Data Fig. 9d,j**).

- Calibration of the central claims. The data establish direct glucan ubiquitination as a mechanism that limits astrocytic polyglucosan accumulation. They do not yet establish non-protein ubiquitination as broadly “essential for biomolecular quality control,” nor do they demonstrate complete degradation of the polyglucosan carbohydrate core after lysosomal delivery. These statements should be narrowed to distinguish autophagic targeting and partial lysosomal turnover from complete glucan degradation.

We followed the reviewer's suggestion. The sentence now reads "Ubiquitylated polyglucosan recruits the autophagy receptors SQSTM1, TAX1BP1, and optineurin, thereby triggering uptake into autophagosomes. These findings identify RNF213 as a quality control factor preventing polyglucosan accumulation in astrocytes through direct ubiquitylation of polyglucosan, revealing an essential role for non-protein ubiquitylation in glycogen quality control.

Minor issues

- Modify the description of the Jochem/Kommander study. The manuscript states that polyglucosan ubiquitylation decreased by approximately 60% following HOIL-1 depletion. The published experiment used HOIP/RNF31 knockdown in GBE1-deficient cells. This should be corrected.

We thank the reviewer for spotting our error. The sentence has been corrected.

- Revise the descriptionn of malin–laforin. The current text presents the pathway too narrowly as targeting glycogen synthase and PTG for proteasomal degradation. Malin also ubiquitylates PYGB and promotes its nuclear localization rather than degradation. “The malin-laforin pathway regulates glycogen metabolism through ubiquitination of glycogen-associated proteins, including glycogen synthase, PTG, and PYGB; depending on the substrate and context, this can alter protein abundance, localization, or activity rather than uniformly promoting proteasomal degradation.”

We followed the reviewer's suggestion. The text now reads "The malin-laforin complex, mutations in which cause Lafora disease^{10–12}, restricts polyglucosan synthesis by targeting glycogen-associated proteins including glycogen synthase,

the phosphatase subunit PTG (protein targeting to glycogen) and glycogen phosphorylase PYGB for degradation or nuclear localization depending on the context¹³⁻¹⁷."

- Figure 1b legend. The panel appears to include age-matched wild-type and Rnf213-null sections, but the legend refers only to Rnf213-null mice.

We thank the reviewer for spotting the omission. The information has been added.

- Figure 2i legend. Specify the number of independent reactions and independent substrate preparations.

The requested information has been added.

- Extended Data Figure 3g,h legend. The text refers to "c, RNF213" and "d, ubiquitin"; these panel references appear to be copied incorrectly and should likely refer to panels g and h.

We thank the reviewer for spotting this. The labels have been replaced.

Referee #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

[We thank the reviewer for their time.](#)

Referee #4 (Remarks to the Author):

The authors have added one new data point: They reconstituted the ubiquitination of polyglucosan and glycogen by RNF213 in vitro and could show that RNF213 can selectively ubiquitinate polyglucosan over glycogen under defined conditions. This is an important advance, addressing one of my major concerns. Moreover, I agree that the epistasis data together with the independent finding that HOIL-1 depletion reduces polyglucosan ubiquitination by only ~60% (Jochem et al, Nature 2026) make the alternative LUBAC-centric model harder to sustain and I consider these points adequately addressed.

We thank the reviewer for their positive assessment of our in vitro reconstitution experiment, and we are pleased that our interpretation of the epistatic relationship between RNF213 and LUBAC in polyglucosan ubiquitylation has been accepted by the reviewer.

Nevertheless, I still have concerns that the revision does not resolve.

1. The glycogen QC mechanism remain unknown The CBM20 domain has been re-framed as an autoinhibitory module that limits RNF213 activity towards physiological glycogen, which is supported by the data. However, this negative regulatory function does not explain how RNF213 is recruited to polyglucosan in the first place. The manuscript explicitly acknowledges that "the mechanism of RNF213 recruitment to polyglucosan remains to be elucidated". The CBM-stalk pulldown data do not fill this gap, as efficient recruitment of full-length RNF213 requires the intact AAA core and E3 module that the fragment lacks. What structural or biochemical feature of polyglucosan triggers RNF213 engagement, for example reduced branching density, chain length, altered surface chemistry, is entirely open. Likewise, it is unclear how sugar binding to the CBM20 domain allows to secure glycogen, and thus it is still open how the surveillance machinery distinguishes normal from aberrant substrate. Without this, the paper describes a pathway but not the mechanism of the sugar QC system.

We thank the reviewer for highlighting an important new question that directly arises from work reported here and that will be addressed in the future.

2. The p62 discrepancy between cells and brain tissue is not resolved In the engineered cell system, p62 recruitment to polyglucosan bodies is RNF213-dependent. In RNF213 KO mouse brains, however, polyglucosan is p62-positive but ubiquitin-negative. The authors refer to ubiquitin-independent p62 recruitment in vivo as an explanation, which is plausible but leads to an additional question: if p62 can be recruited to polyglucosan without ubiquitin in vivo, why does this not trigger LC3 recruitment and at least partial autophagic clearance? The rebuttal raises this as an interesting topic for future research but does not reconcile it with the model, keeping an internal inconsistency between the cell-based mechanism and the in vivo observations.

We thank the reviewer for raising another interesting new question that directly emerges from the findings reported here and which we hope to address in future work.

3. Key mechanistic data remain outside the manuscript

Several experiments central to the authors' arguments are presented only in the reviewer response and will not be available for independent assessment in the published paper: the p62/ubiquitin co-immunofluorescence in RNF213 KO brain sections (directly relevant to the autophagy model) and the cryo-EM data showing a dense-core/loose-periphery architecture in lysosomes (the primary evidence for the partial degradation model). I would ask the authors to include at least these data as Extended Data figures.

We followed the reviewer's request. A new panel has been added (**Extended Data Fig. 9d,j**).

4. The abstract conclusion is too broad

Given that the in vivo phenotype is restricted to astrocytes with no behavioural consequence and that the QC mechanism is unresolved, the concluding statement "establishing non-protein ubiquitylation as essential for biomolecular quality control" overstates what has been demonstrated. I would ask the authors to restrict this claim to what the data directly support, i.e. that RNF213-mediated ubiquitination is required for polyglucosan homeostasis in astrocytes.

We followed the reviewer's suggestion. The text now reads "These findings identify RNF213 as a quality control factor preventing polyglucosan accumulation in astrocytes through direct ubiquitylation of polyglucosan, revealing an essential role for non-protein ubiquitylation in glycogen quality control."