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Peer Review File

Dietary cholesterol activates a Ral dependent pathway driving LDLR turnover

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

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
This manuscript describes the identification of a previously unrecognized role for RalA/RalB in regulating mouse plasma cholesterol levels through their impact on hepatic LDLR abundance under conditions of chronic high fat diet. The authors show that activation of the Ral pathway in hepatocytes promotes trafficking of LDLR from the plasma membrane to the endosome and ultimately, its lysosomal degradation by cathepsin A. Blocking of this pathway with a small molecule inhibitor, RBC8, reverses these phenotypes and leads to a post-transcriptional increase in LDLR protein levels (specifically at the plasma membrane).

Specific comments:

1. The authors refer to the diet used in the mouse studies as a 'high cholesterol diet' (HCD). But in the methods they cite the use of Research Diets D12492, which is a classic 60% fat containing high fat diet commonly referred to as HFD and used to induce obesity in mice. This diet is not only high in cholesterol but also in a variety of other fats. The construct put forth by the authors that the effects in the mice on this diet are due to 'chronic cholesterol exposure' is not necessarily accurate. The fundamental observations are sound, but the interpretation should be reassessed and the terminology of the diet in the manuscript should be reconsidered in light of the extensive use of this diet as a '60% high fat diet (HFD)' .

2. RalA and RalB were not changed after 4 weeks of 'HCD' but only at 20 weeks were upregulated. It is not clear what the time course of upregulation is between 4 and 20 weeks of HCD feeding. Yet in HepG2 cells cholesterol loading acutely increased RalA and RalB. How do the authors reconcile the acute effects of cholesterol in vitro with the substantially delayed effects of the high fat diet in vivo?

3. The authors show that blocking the CCC-WASH1 Retriever pathway (which favors recycling of LDLR to the plasma membrane rather than its degradation in the lysosome) leads to degradation of LDLR (later shown to be mediated by CTSA in the lysosome). Given the pulldown of the AP-2 complex with RalBP1 in the context of constitutively activated G23V RalA and the effects on LDLR, PCSK9, and vesicular trafficking, it would be interesting to know if sortilin (SORT1), which has been associated with regulation of LDL-C, is an effector of the activated Ral phenotypes.

4. The authors mention that GWAS has identified common non-coding SNPs that are associated with plasma LDL and show that they are hepatic eQTLs (for KRAS and REPS1) and colocalize with plasma lipid signals. Are either of these SNPs pQTLs for proteins that regulate LDL catabolism such as PCSK9 or ANGPTL3? Or any other circulating proteins? Are there rare coding variants in these pathways for which a gene burden indicates association with LDL-C levels?

Minor comments:

1. The qPCR data do not comport with the MIQE guidelines, which mandate the use of at least three normalizers (each of which should be shown empirically to be unaffected by the experimental treatment, genotype, or group).

2. The authors use the term ‘vehicle’ extensively in figures for a variety of treatments but do not adequately specify the composition of the various vehicles in the materials and methods. Similarly, abbreviations used but not defined (MβCD).

3. The figure legends are extremely terse. Given the large number of panels per figure, some additional narrative sentences beyond the title of the legend would make the paper more accessible.

4. Figure 6r: the convention is to show the right/major lobe on the left (ventral view).

Referee #2

(Remarks to the Author)
In this study, the authors describe a new pathway by which LDLR is degraded in the liver under normal and high-cholesterol diet (HCD). They show that chronic cholesterol treatment activates Ral GTPases in vivo, accompanied by a reduction in LDLR protein levels. They use a RalGAPB hepatocyte KO model to deduce a causality in the activation of RalA and RalB and the resulting reduction in LDLR abundance. Using hepatic cell lines, the authors infer a mechanism by which RalA induces the internalization of LDLR through a REPS1-clathrin-AP2 endocytosis pathway and enhances lysosomal degradation by cathepsin A. While the new regulation of LDLR is interesting, the manuscript is compromised by the need for more convincing mechanistic data and conceptual explanation on the regulation of LDLR under different physiological and pathophysiological conditions.

All experiments relating the hypothesis are done with the RalGAPB KO mice or cell lines, which is a GTP-activating protein for Ral A and B und thus induces an artificially overactivated Ral phenotype. The experiments that lead to the mechanism were performed in HepG2 or AML12 cells. Most of those data have not been confirmed in vivo or at least in primary hepatocytes.

In particular, the authors report on an activation of upstream Ras in vivo under HCD and claim specificity in Ral activation due to excluding MEK-ERK and PI3K-AKT activation in vitro. However, where does the specificity of Ras for Ral activation come from? Why are not other pathways of Ras induced? How are the activities of other Ras pathways in vivo?
Activated Ras is supposed to be recruited to caveolae by fluctuating membrane cholesterol levels. Is Ras more present on the plasma membrane upon HCD or in primary hepatocytes?

The authors claim that the degradation of LDLR is ligand independent, bypasses the recycling complexes known for LDLR trafficking such as CCC and WASH and is without the involvement of PCSK9. How are the activities of CCC and WASH in HCD diet relative to RalA? The authors report that knockdown of CCDC22, COMMD1, or WASH1 in RalGAPB-deficient cells did not rescue the reduction in LDLR protein observed upon RalGAPB loss, which suggests that Ral activation bypasses the CCC-WASH-Retriever recycling pathway to direct LDLR toward lysosomal degradation. This is interesting. The KD of the CCC complex seems to also reduce RalGAPB levels. Is there a cross-communication?
Why are these recycling pathways bypassed? Is there something changed in the NPxY motif in LDLR that is required for detection and binding? Is it blocked? These things should be investigated.
Most importantly, what is the physiological relevance of all these trafficking options for the LDLR, and which route is taken under normal condition and upon cholesterol overload?

The RalA pathway is ligand independent. How can this be explained, when no ligand is needed, but it’s activated by high cholesterol? Primary hepatocytes isolated from RalGAPB HKO mice show a significant reduction in LDLR protein levels compared to RalGAPB floxed (RalGAPBf/f) controls (Fig 1l), regardless of the presence or absence of extracellular lipoproteins. These western blots should be quantified, because it appears that with LDL in the medium there is even more reduction of LDLR protein levels. That would indicate an involvement of the ligand in LDLR degradation.

Cathepsin A was identified as a potential interacting protein with both wild-type RalA and the constitutively active mutant RalA-G23V. However, where is this interaction happening, in the lysosomes? Cathepsins are intra-lysosomal proteins. Does RalA stay bound to LDLR and gets degraded? No interaction of RalA and LDLR was seen by mass spectrometry, so its transient. It is puzzling to this reviewer, where the specificity for Cathepsin A to LDLR comes from? What about the other proteases in the lysosome? Are the lysosomes changed in abundance, acidity or number? What is the level and activation of CTSA in vivo under chow and HCD?

The interaction was shown in HEK293T cells and was supposed to be GTP-dependent (Fig 5c). But the level of the inputs of the different proteins in this figure are not comparable. There is less input of the S28N mutant relative to the others. So, no conclusion on the dependence of the GTP state can be drawn from these data.

Suggesting Cathepsin A, which also affects the abundance of hundreds of other proteins, as a potential target in the RalA pathway for treatment of cardiovascular disease should be carefully reconsidered. Also, the effects of pharmacologically blocking Ral by RBC8 on circulating LDL-C were minor in HCD fed mice. Such small changes will have no consequences on plaque formation. In addition, no safety and serum parameters are presented, where one could judge toxicity.

Minor:

The authors claim in Fig 2j, k that overexpressed constitutively active RalA-G23V or RalB-G23V livers have elevated

plasma TC level, primarily due to increased LDL levels. How can this be due to increased LDL levels, if the VLDL/LDL ratio is increasing?

In Fig. 3g, h the authors report an increase in co-localization between LAMP1 and LDLR in RalGAPB-deficient cells. However, the images don't show any co-localization with LAMP1. Not sure where the increase in PSC comes from? The findings were supposed to be recapitulated in RalGAPB knockout AML12 cells, however a surface marker (CD98) was used for colocalization analysis. What is the colocalization with LAMP1 in those cells?

The rescue of BafA1 treatment on LDLR levels RalGAPB knockout and knockdown hepatocytes seems rather incomplete in the Western Blot. There is only partial recovery of LDLR after BafA1, so they don't match the quantifications shown. These data imply that additional mechanisms to RalA dependent degradation are responsible for the reduction in LDLR protein levels.

Referee #3

(Remarks to the Author)
This study finds that Ral GTPases promote LDLR to lysosome for degradation. The mechanism is independent of PCSK9 or LDL binding. RalA and RalB interact with RalBP1-REPS1 complex to stimulate LDLR internalization and lysosomal targeting. The lysosomal protease Cathepsin A (CTSA) is required for LDLR degradation. Pharmacological inhibition of Ral by RBC8 increased LDLR level, but only slightly decreased plasma total cholesterol, much less potent than PCSK9i. While the findings are interesting and the study is well-organized, the reviewer has significant concerns about the physiological relevance, therapeutic potential, and the depth of mechanistic insight.

1. RalA/B are ubiquitously expressed and critical for numerous fundamental biological processes (exocytosis, cell migration, etc.). Their inhibition would likely have significant on-target toxicities. This is corroborated by the weak effect of the Ral inhibitor RBC8 on plasma cholesterol compared to the potent PCSK9 inhibitors. The relatively low expression of RalA/B in the liver further questions the pathway's importance in systemic cholesterol homeostasis. Any discussion of the potential advantage of inhibiting RalA/B for cholesterol lowering must be framed within the context of the highly potent and specific PCSK9 inhibitors (PCSK9i) already in clinical use. The minimal cholesterol-lowering effect observed with Ral inhibition is incomparable to the robust 50-60% reduction achieved by PCSK9i, significantly diminishing its perceived therapeutic value.
2. The LDLR protein level was not changed after 4 weeks of HCD feeding but decreased at 20 weeks. Similarly, RalA and RalB activities were not altered at 4 weeks but increased at 20 weeks. However, cholesterol loading in HepG2 cells increased RalA and RalB activities and decreased LDLR protein within hours. Why does it take 20 weeks for HCD to regulate RalA/RalB and LDLR in mice? The rapid effect of cholesterol loading on RalA/B and LDLR in HepG2 cells (hours) versus the very slow effect in HCD-fed mice (20 weeks) is a major point of contention. This suggests the in vivo mechanism may be indirect or fundamentally different from cellular mechanism.
3. The authors state that a "high-cholesterol diet (HCD, Research Diets, D12492)" was used throughout the study, including the analyses of LDLR expression, Ral activity, and efficacy evaluation of Ral inhibition (Figs. 1-5). However, D12492 is a standard high-fat diet (HFD) containing 60 kcal% Fat without added cholesterol, as clearly indicated on the Research Diets website (https://researchdiets.com/blog/posts/25-years-of-d12492-exploring-the-versatility-of-this-high-fat-diet) and in numerous publications. Commonly used HCDs typically contain 1-1.25% cholesterol, like D12109C (40 kcal% Fat, 1.25% cholesterol), TD92182 (1% cholesterol), modified TD88137 (21% fat, 1.25% cholesterol) (PMID: 32999215, 21995947, 35922515, 39111707). The current designation of D12492 as "HCD" is misleading and can not support the cholesterol-specific effects throughout the study. Given that D12492 lacks added cholesterol, the comparisons between 4-week and 20-week "HCD" and resulting statements, "prolonged cholesterol exposure may trigger ... (line 30 on page 4), activated by chronic cholesterol exposure (line 14 on page 5), cholesterol-induced Ras activation may preferentially promote chronic Ral activation (line 31 on page 5)" are not supported.
4. Ldlr and Srebp2 mRNA levels were significantly reduced after both 4 and 20 weeks of HCD, but LDLR protein levels declined only after 20 weeks (Fig 1 and Extended Fig 1). This observation raises several important questions: a) Is LDLR expression progressively decreased or declined abruptly after 20 weeks? Was it because hypercholesterolemia occurred at that time? Those time-course groups should be compared at same time at both RNA and protein levels. b) Why did the LDLR level remain unchanged at week 4 when Ldlr gene expression was decreased? Was the decrease in LDLR protein at week 20 due to the removal of some factors at week 4. c) What are the relative contributions of Ral-mediated degradation and transcriptional downregulation to cholesterol-induced LDLR reduction?
5. The authors reported that plasma insulin levels were unchanged between NCD- and HCD-fed mice (Extended Fig. 1q). Hyperinsulinemia is well-documented in long-term HFD-feeding. Fasting plasma insulin was elevated in HFD-fed mice by week 9 and exacerbated further in week 11, at which point it was nearly fourfold higher in the HFD-fed group compared with the CD-fed group (PMID: 25628421). In Extended Fig. 2a and 2d, body weight was almost unchanged after 20-week HFD compared with NCD. This result is unexpected, as a prolonged HFD typically induces significant weight gain.
6. Inconsistencies between blot images and statistical quantification. In Fig. 3i, the expression of LDLR was significantly decreased in BafA1-treated RalGAPB-KO AML12 cells compared with WT AML12 cells. In Extended Fig. 3h, LDLR levels appear not significantly changed in siCOMMD1-treated WT. In Extended Fig. 4g, h, REPS1 expression was comparable between RalA-G23V and GFP livers. In Fig. 5f, LDLR expression was decreased in S190A-infected WT cells compared to HA-infected WT cells.
7. The genetic associations presented are preliminary. To be compelling, they need validation in larger, independent datasets like the UK Biobank. The study does not investigate how the identified SNPs in KRAS and REPS1 affect gene expression or protein function to influence cholesterol levels.
8. It is known that many proteins are involved in LDLR internalization and degradation, such as PCSK9, IDOL, DAB2, and

ARH. Their protein levels in mice fed NCD or HCD for 4 or 20 weeks should be analyzed on the same gel. The involvement of these proteins should be analyzed.
9. RalGAPB HKO may affect ABCA1 and ABCG1 plasma membrane localization and consequently influence cholesterol levels. This possibility should be tested.
10. The authors should directly measure the LDL clearance rate in different mice (HCD-4w vs HCD-20w, WT vs RalGAPB HKO).
11. The interaction between RalA and LDLR should be investigated in more detail. For example, the binding sites should be mapped, and whether their interaction is regulated by LDL should be determined.
12. The authors showed that Cathepsin A (CTSA) interacted with RalA and that the interaction was enhanced by GTP (ED Fig 5a). This result was confusing because CTSA is a luminal protein and RalA is cytosolic. What is the biological meaning of this interaction?
13. CTSA is a lysosomal protease. If RalA promotes LDLR endocytosis and lysosomal targeting, one would naturally observe an interaction between LDLR and CTSA (as well as other lysosomal proteases). Overexpression of a lysosomal protease causing LDLR degradation is potentially a non-specific, artifactual effect of overwhelming the lysosomal system, rather than proof of a specific biological role in this pathway.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have responded to reviewer comments and the revised manuscript has extensive additional data and is improved.

The new data on pharmacologic CTSA inhibition are particularly interesting.

However, I remain unclear as to how inhibiting CTSA-mediated degradation of the LDLR in the lysosome promotes increased plasma membrane LDLR protein. Does the preserved LDLR somehow recycle back to plasma membrane from the lysosome? Or does the CTSA-mediated degradation of the LDLR occur in the endosome, thus preserving the ability of the LDLR to be recycled? In the schematic model in ext fig 8, the LDLR recycling process should be depicted as it is a critical component of the dynamics of intracellular LDLR homeostasis and the above question should be addressed in the model.

Given the increased emphasis on CTSA in the revised manuscript, it would be of interest to extend the analysis of rare coding variant burden in humans to CTSA and the association with LDL-C (and perhaps apoB).

Referee #2

(Remarks to the Author)
The authors have substantial improved their manuscript, provided compelling evidences for the mechanism in primary hepatocytes and conducted additional strong in vivo experiments to prove the requirement of RalA in LDLR degradation under chronic high cholesterol exposure. Thus, all my points have been addressed. The discussion is nicely streamlined now and I enjoyed reading it.

My only small question remaining would be, how they envisage cathepsin A, as a luminal lysosomal protein, to interact with RalA, which is only associated with the outside of lysosomes? Or is this interaction mediated via additional proteins? Maybe one could address this point in the discussion or in the result section.

Referee #3

(Remarks to the Author)

While the authors have made some effort to revise the manuscript, the revisions fail to address the fundamental, fatal flaws of this study. The core claims remain unsupported by adequate and rigorous evidence, rendering the work unsuitable for publication in a journal of Nature's standing. My major concerns, which are critical to the validity of the entire study, remain largely unresolved.
RalA/B GTPases are master regulators of fundamental cellular processes. Pharmacological inhibition is therefore predicted to cause substantial on-target toxicities. The limited toxicity data presented (body weight, ALT) are grossly insufficient to rule out this critical concern. Without comprehensive in vivo toxicity profiling across multiple organ systems, the translational premise of targeting Ral is severely undermined.
Even setting aside toxicity concerns, the observed cholesterol-lowering effects of both the Ral inhibitor and the CSTA inhibitor are marginal at best. This calls into question the biological significance and therapeutic relevance of the entire proposed pathway.
The proposed CTSA-RalA interaction and its mechanistic link to cholesterol regulation remain obscure. The data presented

are correlative and fail to establish a direct, causative relationship. A clear, stepwise molecular mechanism is absent, leaving the central hypothesis unsubstantiated.
The association of SNPs in KRAS and REPS1 with disease phenotype constitutes a key point of the study, yet the authors provide no functional validation. It is imperative to experimentally assess the impact of these SNPs on gene expression, protein localization, and function. These are standard and feasible experiments. In their absence, the human genetic data are merely suggestive and too shaky to support their function in human lipid metabolism.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)
No further comments

Referee #2

(Remarks to the Author)
The authors have addressed all my points.

Referee #3

(Remarks to the Author)

I thank the authors for the effort invested in revising the manuscript. The additional experiments and analyses have strengthened several aspects of the study. In particular, the revised manuscript provides more support for the involvement of CTSA in LDLR regulation and expands the human genetic analyses. However, after reviewing the revision and rebuttal carefully, I remain unconvinced that the manuscript meets the standard required for publication in Nature.

1. The manuscript is framed around a RalA-CTSA-LDLR regulatory axis. However, the key mechanistic step—how cytosolic RalA controls the function or maturation of the lysosomal luminal protease CTSA—remains unresolved. The revised manuscript acknowledges the topological problem and proposes an indirect model involving CI-M6PR/IGF2R or related trafficking machinery. This is plausible, but it remains hypothetical. No direct evidence is provided to establish the proposed intermediate step, either physically or functionally. For a study whose main claim is the identification of a new mechanistic axis, this unresolved link is not a peripheral issue. At present, the manuscript supports an association between Ral signaling, CTSA-dependent processing, and LDLR abundance, but it does not yet establish the full mechanistic chain with the level of rigor expected here.

2. The revised human genetic analyses are a useful addition, but they remain largely associative. The rare-variant and gene-level data are directionally consistent with the proposed pathway, yet they do not establish causality, nor do they functionally connect the implicated human variants to the specific mechanism proposed in the manuscript. This is particularly important given the emphasis placed on the genetics as support for translational relevance.

Referee #1 (Remarks to the Author):

This manuscript describes the identification of a previously unrecognized role for RalA/RalB in regulating mouse plasma cholesterol levels through their impact on hepatic LDLR abundance under conditions of chronic high fat diet. The authors show that activation of the Ral pathway in hepatocytes promotes trafficking of LDLR from the plasma membrane to the endosome and ultimately, its lysosomal degradation by cathepsin A. Blocking of this pathway with a small molecule inhibitor, RBC8, reverses these phenotypes and leads to a post-transcriptional increase in LDLR protein levels (specifically at the plasma membrane).

Specific comments:

1. The authors refer to the diet used in the mouse studies as a 'high cholesterol diet' (HCD). But in the methods they cite the use of Research Diets D12492, which is a classic 60% fat containing high fat diet commonly referred to as HFD and used to induce obesity in mice. This diet is not only high in cholesterol but also in a variety of other fats. The construct put forth by the authors that the effects in the mice on this diet are due to 'chronic cholesterol exposure' is not necessarily accurate. The fundamental observations are sound, but the interpretation should be reassessed and the terminology of the diet in the manuscript should be reconsidered in light of the extensive use of this diet as a '60% high fat diet (HFD)'.

We thank the reviewer for pointing out this important issue and for the careful examination of our Methods section. Upon review, we realized that we mistakenly listed an incorrect catalog number for the diet used in our study. The correct diet is D12109Ci (Research Diets), which contains 20% fat and 1.25% cholesterol and is classified as a high cholesterol diet. We apologize for this error and for the confusion it may have caused. We have corrected the catalog number in the Methods section. In addition, we have included measurements of total cholesterol (TC) and VLDL/LDL levels (Extended Fig 1f, g), showing that both TC and VLDL/LDL were increased in the HCD group compared with the NCD group, further validating the metabolic impact of the cholesterol-enriched diet.

2. RalA and RalB were not changed after 4 weeks of 'HCD' but only at 20 weeks were upregulated. It is not clear what the time course of upregulation is between 4 and 20 weeks of HCD feeding. Yet in HepG2 cells cholesterol loading acutely increased RalA and RalB. How do the authors reconcile the acute effects of cholesterol in vitro with the substantially delayed effects of the high fat diet in vivo?

We thank the reviewer for this insightful question. To define better the chronic regulation of Ral signaling *in vivo*, we performed an additional intermediate time-point analysis between 4 and 20 weeks of HCD feeding. We found that RalA activity was increased at 12 weeks of HCD, and both RalA and RalB were upregulated after prolonged (20-week) HCD exposure (Fig 1c-e), supporting a progressive increase of Ral signaling during chronic HCD feeding *in vivo*. In addition, we examined RAS activation at 4, 12, and 20 weeks of HCD compared with NCD. RAS activation also showed a sustained increase over time (Fig 1i), suggesting prolonged activation of signaling upstream of Ral during HCD feeding.

As the reviewer points out, it is difficult to extrapolate the temporal changes that occur *in vitro* to those *in vivo*. In cultured cells, cholesterol is delivered directly and acutely, producing immediate, "all-or-none" activation of Ral signaling and LDLR downregulation. In contrast, *in vivo* HCD feeding involves gradual uptake of dietary cholesterol through intestinal absorption, chylomicron assembly, and stepwise lipoprotein transport before delivery to the liver, while hepatic export and remodeling of VLDL to LDL further shape hepatic cholesterol exposure. This multistep process results in a time-dependent cumulative increase in hepatic RAS and Ral signaling (Fig 1c-e, i). Increases in plasma cholesterol or LDL-C are modest during the early stages of HCD feeding, reaching approximately twofold after 4 weeks (Extended Fig 1f, g). Only after prolonged HCD exposure, when hepatic cholesterol pools and chronic metabolic signals reached higher levels (Extended Fig 1f, g), RAS and RalA activities were increased (Fig 1d, e, i), leading to LDLR

reduction (Fig 1a, b). Thus, the delayed *in vivo* response likely reflects the stepwise delivery and gradual accumulation of dietary cholesterol in the liver, in contrast to the effects of direct and acute cholesterol exposure used in cultured cells.

We appreciate this perspective and thank the reviewer for prompting us to add the intermediate time point. We have added these new data (Fig 1a, b, d, Extended Fig 1b, f, g) and revised the relevant section in the Results.

3. The authors show that blocking the CCC-WASH1 Retriever pathway (which favors recycling of LDLR to the plasma membrane rather than its degradation in the lysosome) leads to degradation of LDLR (later shown to be mediated by CTSA in the lysosome). Given the pulldown of the AP-2 complex with RalBP1 in the context of constitutively activated G23V RalA and the effects on LDLR, PCSK9, and vesicular trafficking, it would be interesting to know if sortilin (SORT1), which has been associated with regulation of LDL-C, is an effector of the activated Ral phenotypes.

We thank the reviewer for this insightful suggestion. Although SORT1 has been reported to regulate PCSK9 secretion and could be involved in LDL-C metabolism[1-5], our data demonstrate that PCSK9 is not involved in Ral-driven LDLR trafficking and degradation (Fig 3n-p, Extended Fig 4k, l). Moreover, to our knowledge, there is no direct evidence in the literature showing that SORT1 directly regulates LDLR itself. We now also show here that SORT1 protein (Fig a) and mRNA levels (Fig b) are comparable between NCD and 20-week HCD livers, and knockdown of SORT1 in WT or RalGAPB knockout hepatocytes does not affect LDLR protein levels or rescue the decrease in LDLR produced by Ral activation (Fig c-e). Together, these data indicate that SORT1 does not mediate the effects of activated Ral signaling on LDLR.

While we appreciate the reviewer's suggestion, we prefer not to include the following data to maintain the focus of the manuscript. However, we will be glad to include these if requested.

Fig R1

4. The authors mention that GWAS has identified common non-coding SNPs that are associated with plasma LDL and show that they are hepatic eQTLs (for KRAS and REPS1) and colocalize with plasma lipid signals. Are either of these SNPs pQTLs for proteins that regulate LDL catabolism such as PCSK9 or ANGPTL3? Or any other circulating proteins? Are there rare coding variants in these pathways for which a gene burden indicates association with LDL-C levels?

As the reviewer requested, we evaluated the colocalizing eQTLs associated with hepatic KRAS (rs75667995) and REPS1 (rs72974722 and rs62441842) gene expression for association with circulating PCSK9 or ANGPTL3 protein levels. When examined in a serum proteomics study of 35,559 individuals from the deCODE cohort[6], none of the variants had significant association with circulating PCSK9 or ANGPTL3 protein levels.

Additionally, we examined the effect of rare coding variation burden in KRAS and REPS1 association with LDL-C in 394,841 exome sequenced individuals in the UK Biobank[7]. We identified 50 rare coding variants in KRAS and 336 rare coding variants in REPS1. Across a series of burden masks including predicted loss-of-function (pLoF) variants, missense variants predicted to be damaging, and synonymous variants, we detected no significant association between rare coding variation in KRAS and LDL-C. However, we

observed a nominally significant association between a burden of deleterious missense variants in REPS1 and both LDL-C and total cholesterol. These results are summarized in the table below:

We thank the reviewer for this suggestion that bolsters our findings and have incorporated it into our updated manuscript.

Minor comments:

1. The qPCR data do not comport with the MIQE guidelines, which mandate the use of at least three normalizers (each of which should be shown empirically to be unaffected by the experimental treatment, genotype, or group).

We thank the reviewer for this helpful comment. While our qPCR experiments used a single normalizer, we carefully confirmed that its expression was stable across treatments, genotypes, and experimental groups prior to analyzing target genes, and thus we believe the results reliably reflect changes in gene expression. We greatly appreciate the suggestion, and in future experiments, we will follow MIQE guidelines by normalizing all samples to the average of at least three housekeeping genes. This clarification has been added to the revised Methods section.

2. The authors use the term ‘vehicle’ extensively in figures for a variety of treatments but do not adequately specify the composition of the various vehicles in the materials and methods. Similarly, abbreviations used but not defined (MβCD).

We thank the reviewer for this helpful comment. We have clarified the composition of all vehicles used in our experiments in both the Figure Legends and the Methods sections, specifying the appropriate solvent or buffer for each treatment. In addition, all abbreviations, including methyl-β-cyclodextrin (MβCD), have been defined at first use to ensure clarity for the reader.

3. The figure legends are extremely terse. Given the large number of panels per figure, some additional narrative sentences beyond the title of the legend would make the paper more accessible.

We thank the reviewer for this helpful suggestion. We have revised all Figure Legends to include additional descriptive sentences for each panel to improve clarity and accessibility.

4. Figure 6r: the convention is to show the right/major lobe on the left (ventral view).

We thank the reviewer for noting the conventional orientation of the liver lobes. In Fig 6r (now Extended Fig 8), we replaced the whole liver illustration with a hepatocyte. The Ral-dependent pathway identified in this study specifically regulates LDLR in hepatocytes. Therefore, we believe that depicting a hepatocyte provides a clearer and more precise representation than showing the entire liver, avoiding unnecessary complexity while highlighting the relevant cell type.

Referee #2 (Remarks to the Author):

In this study, the authors describe a new pathway by which LDLR is degraded in the liver under normal and high-cholesterol diet (HCD). They show that chronic cholesterol treatment activates Ral GTPases in vivo, accompanied by a reduction in LDLR protein levels. They use a RalGAPB hepatocyte KO model to deduce a causality in the activation of RalA and RalB and the resulting reduction in LDLR abundance. Using hepatic cell lines, the authors infer a mechanism by which RalA induces the internalization of LDLR through a REPS1-clathrin-AP2 endocytosis pathway and enhances lysosomal degradation by cathepsin A. While the new regulation of LDLR is interesting, the manuscript is compromised by the need for more convincing mechanistic data and conceptual explanation on the regulation of LDLR under different physiological and pathophysiological conditions.

1. All experiments relating the hypothesis are done with the RalGAPB KO mice or cell lines, which is a GTP-activating protein for Ral A and B und thus induces an artificially overactivated Ral phenotype. The experiments that lead to the mechanism were performed in HepG2 or AML12 cells. Most of those data have not been confirmed in vivo or at least in primary hepatocytes.

We thank the reviewer for this important comment. While some experiments were performed using RalGAPB knockout models or cell lines to probe the mechanistic effects of Ral activation, not all experiments rely on this system. Key findings have been confirmed in primary hepatocytes or *in vivo* in C57BL/6J mice, and we also employed expression of constitutively active or dominant negative mutants of RalA and B *in vitro* and *in vivo* (Fig 1f, Extended Fig 2h-m, Extended Fig 3p-s). It is also important to note that we documented the finding that Ral activation increases naturally under physiological or metabolic stress, as demonstrated in HCD-fed mice, where RalA/B activity rose progressively over time (Fig 1c-e). To test directly the role of Ral in regulating LDLR under these conditions, we treated HCD-fed C57BL/6J mice with the highly specific Ral inhibitor RBC8 (Fig 6h-j, Extended Fig 7e-n). Inhibition of Ral activity *in vivo* attenuated LDLR degradation and improved downstream metabolic defects, further supporting the physiological relevance of the Ral pathway in regulating LDLR.

In light of the reviewer's comment, and to further strengthen our findings, we have now included extensive new data from *in vivo* experiments and from primary hepatocytes (Fig 5b, c, 5i-t; Extended Fig 6e, f; Extended Fig 6m-s; Fig 6k-s; Extended Fig 7o, p), which confirm key aspects of the Ral-CTSA-LDLR regulatory pathway under more physiological conditions. These new data show that the lysosomal expression of CTSA (CTSA-lyso) was elevated in the livers of C57BL/6J mice fed HCD for 20 weeks (Fig 5b, c), whereas the precursor form of CTSA (CTSA-pre) was more abundant in the livers of mice fed HCD for 4 weeks compared with NCD controls (Extended Fig 6e, f), indicating increased CTSA activity upon Ral activation. We also used AAV-mediated *in vivo* gene editing to inactivate CTSA specifically in hepatocytes. SpCas9 knock-in mice carrying a loxP-STOP-loxP cassette were injected with AAV8 expressing a TBG-Cre-driven sgRNA targeting either a control sequence (sgLacZ) or CTSA (sgCTSA). Knockdown of lysosomal CTSA in the liver was verified and was shown to increase LDLR protein levels without altering *Ldlr* mRNA expression (Fig 5i, j, Extended Fig 6m). Body weight, liver-to-body weight ratio, plasma glucose, and plasma TG levels were unchanged (Extended Fig 6n-q), whereas plasma total cholesterol (TC) (Fig 5k) and VLDL/LDL levels (Fig 5l) were significantly reduced in CTSA knockdown mice compared with controls. Moreover, primary hepatocytes isolated from CTSA knockdown mice exhibited a prolonged LDLR half-life (Fig 5m, n). Immunofluorescence analysis further revealed increased LDLR localization at the plasma membrane (Fig 5o, p) and enhanced LDL uptake (Fig 5q, r) in CTSA knockdown hepatocytes. Importantly, CTSA knockdown restored the cholesterol-induced reduction in plasma membrane LDLR abundance in primary hepatocytes (Fig 5s, t). We also evaluated the effect of CTSA inhibition using the small-molecule inhibitor SAR164653 (CTSAi) [8-11]. In hepatocytes, CTSAi increased LDLR protein levels (Fig 6k, l), restored LDLR abundance (Fig 6m, n) and plasma membrane localization after cholesterol

loading (Fig 6o, p). To assess *in vivo* effects, RalGAPB$^{f/f}$ and RalGAPB^{HKO} mice were administered with either vehicle or CTSAi via oral gavage for 4 weeks following 4 weeks of HCD feeding. In livers from RalGAPB^{HKO} mice, CTSAi rescued hepatic LDLR protein levels (Fig 6q, r), reversed the elevation of plasma VLDL/LDL (Fig 6s) and TC (Extended Fig 7o) to levels observed in RalGAPB$^{f/f}$ mice, while HDL levels remained unchanged (Extended Fig 7p). Collectively, these results support a model in which CTSA functions downstream of cholesterol-triggered Ral signaling to promote lysosomal LDLR degradation and associated metabolic defects.

2. In particular, the authors report on an activation of upstream Ras in vivo under HCD and claim specificity in Ral activation due to excluding MEK-ERK and PI3K-AKT activation in vitro. However, where does the specificity of Ras for Ral activation come from? Why are not other pathways of Ras induced? How are the activities of other Ras pathways in vivo?

We thank the reviewer for this comment; this is a very good question that we are unable to answer directly and can only speculate. As mentioned, we did not observe notable changes in MEK-ERK or PI3K-AKT signaling at the time points analyzed in our study (after 20 weeks of HCD). We bolstered this finding with new data showing that the interaction between RAS and RGL2, a Ras effector and RalGEF[12], was increased upon cholesterol treatment (Fig 1k), which likely contributes to Ral activation. In addition, overexpression of RGL2 decreased LDLR protein expression (Extended Fig 2i, j), consistent with the Ral activation phenotype. While this does not directly answer the issue of effector specificity; we suspect that the differences lie in the fact that Raf/MEK and PI3K are transiently activated at earlier times during cholesterol feeding and not sustained as is the RGL2/Ral pathway. Alternatively, it is possible that cholesterol activation of RAS proteins selectively enhances RGL2 binding.

We have added these new data (Fig 1k, Extended Fig 2i, j) and revised the relevant section in the Results. To avoid overstating our conclusions, we have revised the manuscript to present these observations without implying exclusive or highly specific activation of Ral and have moderated the tone in the Results section accordingly. Nevertheless, this is an important and intriguing question that will require significant additional experimentation for us in future studies.

3. Activated Ras is supposed to be recruited to caveolae by fluctuating membrane cholesterol levels. Is Ras more present on the plasma membrane upon HCD or in primary hepatocytes?

We thank the reviewer for this important question. While activated Ras has been proposed to associate with cholesterol-rich membrane microdomains, the GTPase is lipid-anchored and already predominantly localized to the plasma membrane at steady state[13]. Accumulating evidence indicates that changes in membrane cholesterol regulate RAS signaling primarily by modulating its nanoscale organization and possibly effector coupling within zones of the plasma membrane, rather than by increasing bulk plasma membrane recruitment[14-17]. To address directly whether high cholesterol feeding increases RAS plasma membrane localization *in vivo*, we performed subcellular fractionation of livers from C57BL/6J mice fed either NCD or HCD for 20 weeks. RAS was predominantly detected in the plasma membrane fraction under both conditions, and HCD did not increase RAS abundance in this fraction (Extended Fig 2f). Consistent with these data, immunofluorescence staining showed that HA-KRAS was already mainly localized to the plasma membrane, with no detectable increase in membrane enrichment upon cholesterol treatment (Extended Fig 2d, e). These data indicate that RAS plasma membrane localization in hepatocytes is not increased by cholesterol. Instead, the cholesterol-dependent increase in RAS-RGL2 interaction (Fig 1k) produces enhanced effector engagement. The precise molecular interactions that are altered by cholesterol loading have been studied by others and have been extensively reviewed. Further work will be required to determine whether cholesterol loading produces increased RGL2 binding of RAS in lipid raft microdomains or caveolae of hepatocytes.

We have included the new data in Extended Fig 2d-f and further discuss this point in Discussion.

4. The authors claim that the degradation of LDLR is ligand independent, bypasses the recycling complexes known for LDLR trafficking such as CCC and WASH and is without the involvement of PCSK9. How are the activities of CCC and WASH in HCD diet relative to RalA? The authors report that knockdown of CCDC22, COMMD1, or WASH1 in RalGAPB-deficient cells did not rescue the reduction in LDLR protein observed upon RalGAPB loss, which suggests that Ral activation bypasses the CCC-WASH-Retriever recycling pathway to direct LDLR toward lysosomal degradation. This is interesting. The KD of the CCC complex seems to also reduce RalGAPB levels. Is there a cross-communication?

We thank the reviewer for these insightful questions. We examined both mRNA and protein levels of CCC and WASH components in livers from mice fed NCD or HCD for 20 weeks and found no dramatic differences (Extended 4m-o), indicating that high cholesterol feeding does not substantially alter the abundance of these complexes. In our experiments, knockdown of CCC components modestly increased, rather than decreased, RalGAPB levels in a single experiment. However, this effect was not reproducible in an independent batch of samples and is therefore not considered biologically meaningful.

We have added these new data (Extended 4m-o) and revised the relevant section in the Results.

5.Why are these recycling pathways bypassed? Is there something changed in the NPxY motif in LDLR that is required for detection and binding? Is it blocked? These things should be investigated. Most importantly, what is the physiological relevance of all these trafficking options for the LDLR, and which route is taken under normal condition and upon cholesterol overload?

We thank the reviewer for these important questions. In response, we now show that the cytosolic C-terminal tail of LDLR, particularly the NPxY motif, is critical for interaction with RalA, as deletion of the C-tail (C-del) or mutation of the NPxY motif to 4A (NPxY-4A) largely decreased LDLR-RalA binding (Fig 4f). We also observed that cholesterol or LDL loading further enhanced the interaction between LDLR and RalA (Fig 4e). The NPxY motif is a versatile binding hub that can interact with multiple trafficking proteins, including components of the CCC-WASH-Retriever machinery such as COMMD1[18]. These findings suggest that under conditions of cholesterol overload or Ral activation, the NPxY motif of LDLR may preferentially engage within the RalA complex, providing a mechanistic explanation for why LDLR is diverted toward lysosomal degradation rather than being recycled through the canonical CCC-WASH pathway. In the absence of Ral activation, LDLR is predominantly recycled via CCC-WASH-Retriever to maintain cholesterol homeostasis.

We have added these data (Fig 4e, f) in the revised figures and did our best to provide a clearer explanation of the trafficking routes with and without cholesterol overload. We agree that the bypassing of the CCC-WASH-Retriever complex by Ral activation is of great interest and may provide a clue as to how Ral directs LDLR to lysosomes. Our plan is to dive into this mechanism in more detail in future studies.

7. The RalA pathway is ligand independent. How can this be explained, when no ligand is needed, but it's activated by high cholesterol? Primary hepatocytes isolated from RalGAPB HKO mice show a significant reduction in LDLR protein levels compared to RalGAPB floxed (RalGAPBf/f) controls (Fig 1l), regardless of the presence or absence of extracellular lipoproteins. These western blots should be quantified, because it appears that with LDL in the medium there is even more reduction of LDLR protein levels. That would indicate an involvement of the ligand in LDLR degradation.

We thank the reviewer for raising this important point, and this comment has led us to temper our statements about ligand dependence. Our data demonstrate that LDL binding to LDLR is not required once RalA is activated, as occurs in RalGAPB-KO cells. Mechanistically, cholesterol acts as a physiological stimulus that

triggers intracellular signaling to activate RalA, although RalA is constitutively active in RalGAPB-KO cells without additional stimulation. Once the Ral pathway is activated, LDLR degradation proceeds without any ligand requirement. Consistent with this, primary hepatocytes isolated from RalGAPB^{HKO} mice exhibited a significant reduction in LDLR protein levels compared to RalGAPB$^{f/f}$ controls when cultured in lipoprotein-deficient (LPDS) medium (Fig 1g). Quantification of the Western blots confirmed that this reduction also occurs in the absence of extracellular LDL (Fig 1h), supporting the ligand-independent nature of pathway activation. While the presence of LDL can further enhance the interaction between LDLR and RalA (Fig 4e) and modestly increase the extent of LDLR degradation (Fig1g, h), this enhancement is not essential. Even in the absence of LDL, LDLR and RalA binding occurred (Fig 4e) and LDLR protein levels were decreased, although the presence of LDL increased LDLR and RalA binding (Fig 4e), probably due to enhanced LDLR internalization when LDL is present. Taken together, these observations indicate that ligand binding to LDLR is not necessary for RalA-mediated LDLR degradation.

We have added the quantification (Fig1h) in the manuscript. To avoid potential confusion, we have removed the sentences stating that RalA-dependent LDLR degradation is ligand independent. We appreciate these comments and opportunity to clarify this confusion.

8. Cathepsin A was identified as a potential interacting protein with both wild-type RalA and the constitutively active mutant RalA-G23V. However, where is this interaction happening, in the lysosomes? Cathepsins are intra-lysosomal proteins. Does RalA stay bound to LDLR and gets degraded? No interaction of RalA and LDLR was seen by mass spectrometry, so its transient. It is puzzling to this reviewer, where the specificity for Cathepsin A to LDLR comes from? What about the other proteases in the lysosome? Are the lysosomes changed in abundance, acidity or number? What is the level and activation of CTSA in vivo under chow and HCD?

We thank the reviewer for these insightful comments.

As a small GTPase involved in membrane trafficking, RalA is dynamically associated with intracellular membranes and can be transported along endosomal pathways to late endosomes and lysosomes. Previous studies have shown that both endogenous and GFP-tagged RalA localize to late endosomal compartments positive for Lysotracker[19]. Consistent with this, our immunofluorescence analysis demonstrates that RalA colocalizes with CTSA in TMEM192-enriched lysosomes (Extended Fig 6c), a widely used lysosomal marker[20,21], supporting the notion that a fraction of RalA interacts with lysosomal CTSA during LDLR trafficking. As a trafficking GTPase, RalA likely facilitates LDLR delivery to lysosomes and may subsequently recycle back to other cellular compartments.

We agree with the reviewer that the RalA-LDLR interaction is transient and discussed this in the Discussion section of the original manuscript: "Although co-immunoprecipitation and pulldown assays support a functional interaction between Ral proteins and LDLR, the absence of LDLR peptides in our mass spectrometry dataset suggests that this interaction may be indirect, transient, or highly dynamic. This is especially relevant given that LDLR trafficking is a tightly regulated and temporally dynamic process, involving rapid internalization, endosomal sorting, and either recycling or degradation." However, lack of detection by mass spectrometry does not rule out a functional relationship, as only a small fraction of molecules may interact at any given time, and membrane or luminal regions, such as the LDLR luminal domain, are often underrepresented in standard cytosolic MS analyses[22,23].

Moreover, the lysosomal active form of CTSA (CTSA-lyso) was elevated in RalGAPB knockout cells (Fig 5a, Extended Fig 6d) and in the livers of mice fed HCD for 20 weeks (Fig 5b, c), whereas the precursor form of CTSA (CTSA-pre) was more abundant in the livers of mice fed HCD for 4 weeks compared with NCD controls (Extended Fig 6e, f), indicating increased CTSA activity upon Ral activation. This is important as CTSA activation, which involves cleavage to an active form, is highly pH sensitive, and occurs upon

lysosomal entry. Thus, the specificity of CTSA toward LDLR likely arises from the combination of RalA-mediated LDLR trafficking to lysosomes and the increased activity of CTSA in lysosomes upon cholesterol loading (Fig 5b, c) or Ral activation (Fig 5a). In this context, LDLR is positioned in proximity to activated CTSA, facilitating its degradation. While we do not exclude contributions from other lysosomal proteases, our data with the highly specific CTSA inhibitor support a key role for this protease (Fig 6q-s).

To assess lysosomal function, we performed live-cell imaging using LysoTracker to measure lysosome number and intensity in primary hepatocytes from RalGAPB$^{f/f}$ or RalGAPB^{HKO} mice and observed no differences (Fig 3k-m). Because LysoTracker is a pH-sensitive dye, this also indicates that lysosomal acidity is unchanged. Similar results were obtained in WT and RalGAPB-KO AML12 cells (Extended Fig 4h-j). To rule out effects of CTSA itself on lysosomes, we performed the same analysis in primary hepatocytes from sgLacZ or sgCTSA mice and observed no changes (Extended Fig 6r, s). We also assessed expression levels of lysosomal marker genes (LAMP1, P62) and other proteins known to undergo lysosomal degradation, including transferrin receptor (TFR), LXRα, and EGFR, and found no changes upon CTSA overexpression (Extended Fig 6g). These results indicate that CTSA affects LDLR without broadly altering lysosomal function. While the molecular basis underlying the relative specificity of CTSA toward LDLR remains unclear, elucidating the mechanisms that confer this selectivity will be an important direction for future studies.

We have added these new data (Fig 5b, c; Extended Fig 6d-g; Extended Fig 6r, s) and revised the relevant section in the Results.

9. The interaction was shown in HEK293T cells and was supposed to be GTP-dependent (Fig 5c). But the level of the inputs of the different proteins in this figure are not comparable. There is less input of the S28N mutant relative to the others. So, no conclusion on the dependence of the GTP state can be drawn from these data.

We appreciate the reviewer's careful examination of this point. We agree that, in the original blot, the input level of the S28N mutant was lower than that of the other constructs. To address this concern, we repeated the experiment and ensured equal expression of RalA-G23V, and RalA-S28N in the input samples. The revised data was included in Fig 5e and showed a markedly reduced interaction for the GDP-bound S28N mutant compared with WT and the GTP-bound G23V mutant. These data support a GTP-dependent interaction.

10. Suggesting Cathepsin A, which also affects the abundance of hundreds of other proteins, as a potential target in the RalA pathway for treatment of cardiovascular disease should be carefully reconsidered.

We appreciate the reviewer's concern and agree that targeting CTSA requires careful consideration. It is likely that CTSA has lysosomal substrates in addition to LDLR, and we have not addressed this in great detail. CTSA is an intriguing molecule; in addition to acting as a protease, it serves a scaffolding function in which it binds to and stabilizes β-galactosidase and neuraminidase within the lysosomal multienzyme complex (LMC)[24]. This scaffolding function is independent of its protease activity[25] and confounds previous attempts to understand CTSA activity. Complete loss of CTSA leads to severe systemic phenotypes due to disruption of its scaffolding functions[26]. Importantly, our approach focuses on selective inhibition of CTSA's protease activity rather than complete protein loss.

We note that the selective small-molecule CTSA inhibitor, SAR164653[8-11], was previously advanced to clinical investigation and underwent rigorous preclinical safety and toxicological studies for up to 13 weeks in two species. Moreover, the drug progressed through Phase I clinical studies in healthy volunteers, showing a manageable safety profile with no drug related adverse events. Single and repeat oral doses up to 800 mg for several weeks were well tolerated, and a maximum tolerated dose was not reached, indicating

a favorable safety and pharmacokinetic profile[11]. Although Sanofi initially explored this compound for heart failure in diabetic patients, the program was discontinued when the company strategically shifted focus to immunology and vaccines.

In the revised manuscript, we synthesized SAR164653 (CTSAi) and evaluated its effect on Ral pathway-regulated LDLR degradation and cholesterol metabolism. In hepatocytes, CTSAi increased LDLR protein levels (Fig 6k, l), restored LDLR abundance (Fig 6m, n) and plasma membrane localization after cholesterol loading (Fig 6o, p). To assess *in vivo* effects, RalGAPB$^{f/f}$ and RalGAPB^{HKO} mice were administered either vehicle or CTSAi via oral gavage for 4 weeks following 4 weeks of HCD feeding. In livers from RalGAPB^{HKO} mice, CTSAi rescued hepatic LDLR protein levels (Fig 6q, r), reversed the elevation of plasma VLDL/LDL (Fig 6s) and TC (Extended Fig 7o) to levels observed in RalGAPB$^{f/f}$ mice, while HDL levels remained unchanged (Extended Fig 7p). These results indicate that CTSA acts as a key downstream effector of Ral-mediated LDLR degradation and that pharmacological inhibition of CTSA can selectively reverse Ral-driven dyslipidemia.

Using the small-molecule CTSA inhibitor SAR164653, we demonstrate that pharmacological inhibition of CTSA lowers plasma cholesterol levels *in vivo* by reversing Ral-driven LDLR degradation in hepatocytes, without broadly affecting lysosomal homeostasis. These findings suggest that it is possible to target CTSA in a selective and controlled manner to modulate LDLR and cholesterol metabolism, while minimizing the systemic consequences associated with complete loss of the protein. We wish to emphasize these findings that highlight the therapeutic potential of targeting CTSA for hypercholesterolemia and cardiovascular disease. Indeed, these new data on the CTSA inhibitor may represent the most exciting aspect of our paper, at least with respect to a potential translational impact, and we thank the reviewer for prompting us to study this in more detail. We have added these new data (Fig 6k-s, Extended Fig 7o, p) and revised the relevant section in the Results.

11. Also, the effects of pharmacologically blocking Ral by RBC8 on circulating LDL-C were minor in HCD fed mice. Such small changes will have no consequences on plaque formation. In addition, no safety and serum parameters are presented, where one could judge toxicity.

We thank the reviewer for this comment. The modest changes in circulating LDL-C following RBC8 treatment in HCD-fed mice likely reflect the short-term nature of the study (only 9 days RBC8 treatment) and may also depend on the dose used. RBC8 treatment did not affect body weight, liver-to-body weight ratio, or plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Extended Fig 7f-i), indicating minimal hepatic toxicity. More importantly, our primary goal was to block the Ral pathway using RBC8 to determine clear mechanistic insights into Ral-mediated LDLR regulation. Whether RBC8 could serve as a therapeutic agent for atherosclerosis would require additional preclinical and clinical evaluation, including pharmacokinetics, pharmacodynamics, and safety studies.

We have added these new data (Extended Fig 7h, i) and revised the relevant section in the Results. In the Discussion, we also comment on the potential of RBC8 as a therapeutic agent, while noting that further studies on pharmacokinetics, pharmacodynamics, efficacy, and safety would be required before considering its use in treating atherosclerosis. Again, we emphasize that while Ral inhibitors have the potential for therapeutic use in cholesterol management, in comparison CTSA is far advanced as a therapeutic target for small molecule or antisense/siRNA approaches.

Minor:

1. The authors claim in Fig 2j, k that overexpressed constitutively active RalA-G23V or RalB-G23V livers have elevated plasma TC level, primarily due to increased LDL levels. How can this be due to increased LDL levels, if the VLDL/LDL ratio is increasing?

We thank the reviewer for pointing this out. We have corrected the description in the Results section to accurately reflect the data: the increase in total cholesterol is due to an increased VLDL/LDL ratio.

2. In Fig. 3g, h the authors report an increase in co-localization between LAMP1 and LDLR in RalGAPB-deficient cells. However, the images don't show any co-localization with LAMP1. Not sure where the increase in PSC comes from? The findings were supposed to be recapitulated in RalGAPB knockout AML12 cells, however a surface marker (CD98) was used for colocalization analysis. What is the colocalization with LAMP1 in those cells?

We thank the reviewer for pointing this out. We would like to clarify that in Fig 3g, we used LysoTracker staining rather than LAMP1, and the labeling and description have been corrected accordingly. In the images, the arrows in the first column indicate lysosomes labeled by LysoTracker. When LDLR colocalizes with lysosomes, more arrows in the second column can be observed pointing to LDLR signals like in the RalGAPB-deficient group. We quantified this using ImageJ and Pearson's correlation coefficient (Fig 3h). Additionally, we have now included colocalization staining of LDLR and LAMP1 in both WT and RalGAPB knockout AML12 cells (Extended Fig 4e), further supporting these observations.

3. The rescue of BafA1 treatment on LDLR levels RalGAPB knockout and knockdown hepatocytes seems rather incomplete in the Western Blot. There is only partial recovery of LDLR after BafA1, so they don't match the quantifications shown. These data imply that additional mechanisms to RalA dependent degradation are responsible for the reduction in LDLR protein levels.

We thank the reviewer for pointing this out. We would like to clarify that LDLR quantification in this figure was performed by normalizing its band intensity to that of the internal control HSP90 (Extended Fig 4f, g). This approach is more meaningful than assessing the LDLR band alone, particularly because the internal control bands are not perfectly even across samples. Thus, while the LDLR band may appear to show only partial recovery after BafA1 treatment (Extended Fig 4f), the normalized quantification accurately reflects full recovery (Extended Fig 4g). To further support this, we included new data from primary hepatocytes isolated from RalGAPB$^{f/f}$ and RalGAPB^{HKO} mice, showing that BafA1 treatment fully restored LDLR levels (Fig 3i, j). Additionally, we have updated the Figure Legend to indicate that the quantification was performed using the ratio of LDLR protein levels to HSP90.

Referee #3 (Remarks to the Author):

This study finds that Ral GTPases promote LDLR to lysosome for degradation. The mechanism is independent of PCSK9 or LDL binding. RalA and RalB interact with RalBP1-REPS1 complex to stimulate LDLR internalization and lysosomal targeting. The lysosomal protease Cathepsin A (CTSA) is required for LDLR degradation. Pharmacological inhibition of Ral by RBC8 increased LDLR level, but only slightly decreased plasma total cholesterol, much less potent than PCSK9i. While the findings are interesting and the study is well-organized, the reviewer has significant concerns about the physiological relevance, therapeutic potential, and the depth of mechanistic insight.

1. RalA/B are ubiquitously expressed and critical for numerous fundamental biological processes (exocytosis, cell migration, etc.). Their inhibition would likely have significant on-target toxicities. This is corroborated by the weak effect of the Ral inhibitor RBC8 on plasma cholesterol compared to the potent PCSK9 inhibitors. The relatively low expression of RalA/B in the liver further questions the pathway's importance in systemic cholesterol homeostasis. Any discussion of the potential advantage of inhibiting RalA/B for cholesterol lowering must be framed within the context of the highly potent and specific PCSK9 inhibitors (PCSK9i) already in clinical use. The minimal cholesterol-lowering effect observed with Ral inhibition is incomparable to the robust 50-60% reduction achieved by PCSK9i, significantly diminishing its perceived therapeutic value.

We thank the reviewer for these thoughtful comments. We agree that RalA and RalB are ubiquitously expressed and play essential roles in fundamental cellular processes, which raises the possibility of on-target toxicities. Our study focuses on the observation that chronic high cholesterol exposure led to increased Ral activity (Fig 1c-e). Importantly, RalA/B activation specifically regulates LDLR degradation under conditions of elevated Ral activity rather than through basal expression. Although RalA/B expression in the liver is relatively low, their activity as GTPases is more relevant than absolute expression levels.

Supporting this, treatment with the highly specific Ral inhibitor RBC8 did not affect body weight, liver-to-body weight ratio, or plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Extended Fig 7f-i), indicating minimal hepatic toxicity. While we agree that RBC8 treatment produced modest reductions in plasma cholesterol compared to PCSK9 inhibitors, the primary goal of using RBC8 was to elucidate the mechanistic role of the Ral pathway in regulating LDLR trafficking and degradation, rather than to propose it as an effective cholesterol-lowering drug. These findings expand our understanding of cholesterol regulation beyond the well-characterized PCSK9 pathway and describe these changes in Ral signaling in specific pathological contexts.

We have added these new data (Extended Fig 7h, i) and revised the relevant section in the Results. In the Discussion, we also comment on the potential of RBC8 as a therapeutic agent, while noting that further studies on pharmacokinetics, pharmacodynamics, efficacy, and safety would be required before considering its use in treating atherosclerosis. As we discuss below and in response to Reviewer 2, we are far more enthusiastic about CTSA as a therapeutic target based on our new data.

2. The LDLR protein level was not changed after 4 weeks of HCD feeding but decreased at 20 weeks. Similarly, RalA and RalB activities were not altered at 4 weeks but increased at 20 weeks. However, cholesterol loading in HepG2 cells increased RalA and RalB activities and decreased LDLR protein within hours. Why does it take 20 weeks for HCD to regulate RalA/RalB and LDLR in mice? The rapid effect of cholesterol loading on RalA/B and LDLR in HepG2 cells (hours) versus the very slow effect in HCD-fed mice (20 weeks) is a major point of contention. This suggests the in vivo mechanism may be indirect or fundamentally different from cellular mechanism.

We appreciate the reviewer's point regarding the different kinetics of cholesterol-induced Ral activation in HepG2 cells versus HCD-fed mice. As mentioned above in response to Reviewer 1, it is difficult to extrapolate the temporal changes that occur *in vitro* to those *in vivo*. To define better the chronic regulation of Ral signaling *in vivo*, we performed an additional intermediate time-point analysis between 4 and 20 weeks of HCD feeding. We found that RalA activity was increased at 12 weeks of HCD, and both RalA and RalB were upregulated after prolonged (20-week) HCD exposure (Fig 1c-e), supporting a progressive increase of Ral signaling during chronic HCD feeding *in vivo.* In addition, we examined RAS activation at 4, 12, and 20 weeks of HCD compared with NCD. RAS activation also showed a sustained increase over time (Fig 1i), suggesting prolonged activation of signaling upstream of Ral during HCD feeding.

In cultured cells, cholesterol is delivered directly and acutely, producing immediate, "all-or-none" activation of Ral signaling and LDLR downregulation. In contrast, *in vivo* HCD feeding involves gradual uptake of dietary cholesterol through intestinal absorption, chylomicron assembly, and stepwise lipoprotein transport before delivery to the liver, while hepatic export and remodeling of VLDL to LDL further shape hepatic cholesterol exposure. This multistep process results in a time-dependent cumulative increase in hepatic RAS and Ral signaling (Fig 1c-e, i). Increases in plasma cholesterol or LDL-C are modest during the early stages of HCD feeding, reaching approximately twofold after 4 weeks (Extended Fig 1f, g). Only after prolonged HCD exposure, when hepatic cholesterol pools and chronic metabolic signals reached higher levels, RAS and RalA activities were increased (Extended Fig 1f, g), leading to LDLR reduction (Fig 1a, b). Thus, the delayed *in vivo* response likely reflects the stepwise delivery and gradual accumulation of dietary

cholesterol in the liver, in contrast to the effects of direct and acute cholesterol exposure used in cultured cells. We appreciate this perspective and thank the reviewer for prompting us to add the intermediate time point.

We have added these new data (Fig 1a, b, d, Extended Fig 1b, f, g) and revised the relevant section in the Results.

3. The authors state that a “high-cholesterol diet (HCD, Research Diets, D12492)” was used throughout the study, including the analyses of LDLR expression, Ral activity, and efficacy evaluation of Ral inhibition (Figs. 1-5). However, D12492 is a standard high-fat diet (HFD) containing 60 kcal% Fat without added cholesterol, as clearly indicated on the Research Diets website (https://researchdiets.com/blog/posts/25-years-of-d12492-exploring-the-versatility-of-this-high-fat-diet) and in numerous publications. Commonly used HCDs typically contain 1-1.25% cholesterol, like D12109C (40 kcal% Fat, 1.25% cholesterol), TD92182 (1% cholesterol), modified TD88137 (21% fat, 1.25% cholesterol) (PMID: 32999215, 21995947, 35922515, 39111707). The current designation of D12492 as “HCD” is misleading and can not support the cholesterol-specific effects throughout the study. Given that D12492 lacks added cholesterol, the comparisons between 4-week and 20-week “HCD” and resulting statements, “prolonged cholesterol exposure may trigger ... (line 30 on page 4), activated by chronic cholesterol exposure (line 14 on page 5), cholesterol-induced Ras activation may preferentially promote chronic Ral activation (line 31 on page 5)” are not supported.

We thank the reviewer for pointing out this important issue and for the careful examination of our Methods section. We realized that we mistakenly listed an incorrect catalog number for the diet used in our study. The correct diet is Research Diets D12109Ci, which contains 20% fat and 1.25% cholesterol and is classified as a high cholesterol diet. We apologize for this error and for the confusion it may have caused. We have corrected the catalog number in the Methods section. In addition, we have included measurements of total cholesterol (TC) and VLDL/LDL levels (Extended Fig 1f, g), showing that both TC and VLDL/LDL are increased in the HCD group compared with the NCD group, further validating the metabolic impact of the cholesterol-enriched diet.

4. Ldlr and Srebp2 mRNA levels were significantly reduced after both 4 and 20 weeks of HCD, but LDLR protein levels declined only after 20 weeks (Fig 1 and Extended Fig 1). This observation raises several important questions:

a) Is LDLR expression progressively decreased or declined abruptly after 20 weeks? Was it because hypercholesterolemia occurred at that time? Those time-course groups should be compared at same time at both RNA and protein levels.

We thank the reviewer for this important point. To clarify the dynamics of LDLR regulation, we added new data from an intermediate 12-week HCD time point (Fig 1a, b, d, i, Extended Fig 1b), showing the progression of LDLR protein decline alongside RAS-Ral activation.

b) Why did the LDLR level remain unchanged at week 4 when Ldlr gene expression was decreased? Was the decrease in LDLR protein at week 20 due to the removal of some factors at week 4.

We think that LDLR protein remained unchanged at week 4 despite decreased *Ldlr* mRNA because LDLR protein is relatively stable, and short-term reductions in mRNA do not immediately translate into protein loss. In addition, plasma PCSK9 levels were lower at week 4 in HCD-fed mice compared with NCD controls (Extended Fig 1d), which may have transiently limited LDLR degradation. By week 20, prolonged Ral activation enhances LDLR degradation, overcoming these compensatory mechanisms and resulting in the observed decline in protein levels.

We have added these new data (Extended Fig 1d) and revised the relevant section in the Results.

c) What are the relative contributions of Ral-mediated degradation and transcriptional downregulation to cholesterol-induced LDLR reduction?

Our data suggest that both transcriptional downregulation and Ral-mediated degradation contribute to cholesterol-induced LDLR reduction, but their relative contributions differ over time. Early in HCD feeding at week 4, *Ldlr* mRNA was already decreased, but LDLR protein was largely maintained, likely due to protein stability and compensatory mechanisms such as lower PCSK9 levels (Extended Fig 1d). With prolonged cholesterol exposure at week 12 and week 20, Ral activation drives enhanced LDLR degradation, which becomes the dominant mechanism contributing to the observed decline in protein levels. Thus, in our view, transcriptional downregulation initiates the process, while Ral-mediated degradation amplifies and sustains LDLR reduction over time.

We have discussed these transcriptional versus post-translational contributions in the Discussion. We thank you for this important perspective.

5. The authors reported that plasma insulin levels were unchanged between NCD- and HCD-fed mice (Extended Fig. 1q). Hyperinsulinemia is well-documented in long-term HFD-feeding. Fasting plasma insulin was elevated in HFD-fed mice by week 9 and exacerbated further in week 11, at which point it was nearly fourfold higher in the HFD-fed group compared with the CD-fed group (PMID: 25628421). In Extended Fig. 2a and 2d, body weight was almost unchanged after 20-week HFD compared with NCD. This result is unexpected, as a prolonged HFD typically induces significant weight gain.

We thank the reviewer for this comment and apologize for the confusion. We realize that the expectation of hyperinsulinemia and weight gain arose because we previously listed the diet as D12492 (60% fat). In fact, the correct diet used in our study was D12109Ci (20% fat, 1.25% cholesterol). The moderate fat content in this diet explain why fasting plasma insulin and body weight were unchanged after 20 weeks, despite elevated plasma cholesterol. Thus, our model primarily reflects prolonged cholesterol exposure rather than obesity-driven metabolic changes.

6. Inconsistencies between blot images and statistical quantification. In Fig. 3i, the expression of LDLR was significantly decreased in BafA1-treated RalGAPB-KO AML12 cells compared with WT AML12 cells. In Extended Fig. 3h, LDLR levels appear not significantly changed in siCOMMD1-treated WT. In Extended Fig. 4g, h, REPS1 expression was comparable between RalA-G23V and GFP livers. In Fig. 5f, LDLR expression was decreased in S190A-infected WT cells compared to HA-infected WT cells.

We thank the reviewer for pointing out these observations. All statistical quantifications for LDLR and other proteins shown were performed using band intensity measurements quantified with ImageJ and normalized to appropriate internal controls (e.g., HSP90 or tubulin) across independent biological replicates. This approach is more meaningful than assessing the LDLR or REPS1 band alone, particularly because the internal control bands are not perfectly even across samples. Minor differences in loading may cause individual bands (e.g., LDLR or REPS1) to appear less consistent with the quantified data. We have carefully re-examined all blots and corresponding quantifications and confirmed that they are consistent with the reported statistical results. Additionally, we have updated the Figure Legends to indicate that quantification was performed by normalizing LDLR or REPS1 protein levels to HSP90.

7. The genetic associations presented are preliminary. To be compelling, they need validation in larger, independent datasets like the UK Biobank. The study does not investigate how the identified SNPs in KRAS and REPS1 affect gene expression or protein function to influence cholesterol levels.

We appreciate the reviewer's comments and the opportunity to clarify this point. The SNPs highlighted in our manuscript, rs75667995 for KRAS and rs72974722 and rs62441842 for REPS1, have been identified

as genome-wide significant lead variants associated with plasma lipid traits in the Global Lipids Genetics Consortium (GLGC) meta-analysis[27], with rs75667995 associated with total cholesterol ($p = 3.37 \times 10^{-20}$), rs72974722 associated with LDL-C ($p = 4.84 \times 10^{-23}$), and rs62441842 associated with total cholesterol ($p = 2.5 \times 10^{-21}$). This meta-analysis included over 1.6 million participants and incorporated the UK Biobank demonstration robust and replicable findings across multiple independent cohorts.

With respect to the effects of these SNPs on KRAS and REPS1 gene expression and protein function, we share the reviewer's enthusiasm for evaluating the genomic mechanism which would entail re-engineering these variants in vitro. However, these experiments are beyond the scope of the current manuscript, and we would defer them to future work.

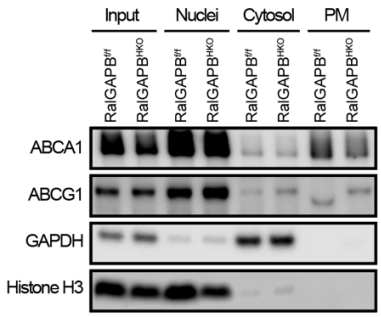
8. It is known that many proteins are involved in LDLR internalization and degradation, such as PCSK9, IDOL, DAB2, and ARH. Their protein levels in mice fed NCD or HCD for 4 or 20 weeks should be analyzed on the same gel. The involvement of these proteins should be analyzed.

We thank the reviewer for this suggestion. In response, we analyzed the protein levels of key LDLR regulatory factors, including PCSK9, IDOL, DAB2, and ARH, in liver lysates from mice fed NCD or HCD for 4 and 20 weeks. All samples were run on the same gels for direct comparison (Extended Fig 1d). We found that IDOL and DAB2 protein levels were not significantly altered by HCD at either time point. PCSK9 protein was transiently reduced at 4 weeks of HCD, consistent with the preservation of LDLR protein at this stage (Fig 1a, b), despite a decrease in *Ldlr* mRNA levels (Extended Fig 1a). ARH protein levels decreased at both 4 and 20 weeks. This reduction would be expected to impair LDLR internalization[28] rather than promote LDLR degradation. Therefore, the decline in LDLR protein observed after prolonged HCD feeding cannot be explained by changes in these canonical LDLR regulatory proteins.

We have added these new data (Extended Fig 1d) and revised the relevant section in the Results.

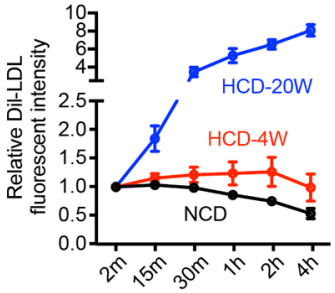
9. RalGAPB HKO may affect ABCA1 and ABCG1 plasma membrane localization and consequently influence cholesterol levels. This possibility should be tested.

We thank the reviewer for this suggestion. We performed cellular fractionation and examined ABCA1 and ABCG1 plasma membrane localization in livers of RalGAPB$^{f/f}$ and RalGAPB^{HKO} mice under NCD. We did not observe significant changes in RalGAPB^{HKO} compared with controls, as shown below. ABCA1 and ABCG1 primarily regulate cholesterol efflux to HDL[29]. In our study, RalGAPB^{HKO} affected VLDL/LDL levels but not HDL, consistent with our observation that ABCA1 and ABCG1 plasma membrane localization was unchanged. These results indicate that the effect of RalGAPB^{HKO} on plasma cholesterol is unlikely to involve ABCA1/ABCG1-mediated cholesterol efflux. We did not include these data in the manuscript because they do not directly contribute to the main findings regarding Ral-mediated regulation of LDLR. However, we will be happy to include them upon request.



10. The authors should directly measure the LDL clearance rate in different mice (HCD-4w vs HCD-20w, WT vs RalGAPB HKO).

We thank the reviewer for the suggestion. We directly measured plasma clearance of injected DiI-LDL at multiple time points. In 4-week HCD-fed mice, DiI-LDL began to be cleared by 4 hours, as shown below. In 20-week HCD-fed mice, plasma DiI-LDL levels unexpectedly increased over time after injection, and this was observed even when we lowered the injected dose. We propose several possible contributing factors for this increase in 20-week HCD mice: (a) severely impaired hepatic LDLR-mediated clearance due to Ral-mediated LDLR degradation; (b) slow redistribution of injected LDL from peripheral compartments. In 20-week HCD mice, some DiI-LDL initially distributed to peripheral tissues but was not internalized due to low LDLR and gradually returns to the plasma over time; and (c) saturation effects. In prolonged HCD, excess endogenous lipoproteins may compete for the few remaining LDLR on the hepatocyte surfaces, slowing LDL removal. Despite this, our data clearly showed that LDL clearance is more efficient in 4-week HCD-fed mice compared with 20-week HCD-fed mice. Because the unexpected increase of DiI-LDL in 20-week HCD mice appears unusual, we have decided not to include this data in the manuscript.



We also performed the same experiment in RalGAPB^{HKO} mice under NCD conditions (Fig 2e). Compared with RalGAPB$^{f/f}$ controls, the RalGAPB^{HKO} mice showed a slower LDL clearance rate, consistent with impaired LDLR-mediated uptake (Fig 3c, d) due to reduced LDLR on the liver surface in RalGAPB-deficient mice (Fig 3e, f).

We have added these new data (Fig 2e) and revised the relevant sections in the Results and Methods.

11. The interaction between RalA and LDLR should be investigated in more detail. For example, the binding sites should be mapped, and whether their interaction is regulated by LDL should be determined.

We thank the reviewer for this valuable suggestion. In addition to the pulldown assays already shown using RalA mutants (RalA-G23V and RalA-S28N, Fig 4d), we further mapped the interaction and found that the cytosolic C-terminal tail of LDLR, particularly the NPxY motif, is critical for interaction with RalA, as deletion of the C-tail (C-del) or mutation of the NPxY motif to 4A (NPxY-4A) largely decreased LDLR-RalA binding (Fig 4f). We also observed that cholesterol or LDL loading further enhanced the interaction between LDLR and RalA (Fig 4e).

We have added these new data (Fig 4e, f) and revised the relevant section in the Results.

12. The authors showed that Cathepsin A (CTSA) interacted with RalA and that the interaction was enhanced by GTP (ED Fig 5a). This result was confusing because CTSA is a luminal protein and RalA is cytosolic. What is the biological meaning of this interaction?

We thank the reviewer for these insightful comments. As a small GTPase involved in membrane trafficking, RalA is dynamically associated with intracellular membranes and can be transported along endosomal pathways to late endosomes and lysosomes. Previous studies have shown that both endogenous and GFP-

tagged RalA localize to late endosomal compartments positive for Lysotracker[19]. Consistent with this, our immunofluorescence analysis demonstrates that RalA colocalizes with CTSA in TMEM192-enriched lysosomes (Extended Fig 6c), a widely used lysosomal marker[20,21], supporting the notion that a fraction of RalA interacts with lysosomal CTSA during LDLR trafficking.

We propose that RalA interacts with CTSA to facilitate LDLR degradation by making LDLR more structurally accessible for lysosomal processing. While RalA-mediated delivery of LDLR to lysosomes does not require CTSA, this interaction may enhance the efficiency of LDLR degradation once it reaches the lysosome. Moreover, the lysosomal active form of CTSA (CTSA-lyso) was elevated in RalGAPB knockout cells (Fig 5a, Extended Fig 6d) and in the livers of mice fed HCD for 20 weeks (Fig 5b, c), whereas the precursor form of CTSA (CTSA-pre) was more abundant in the livers of mice fed HCD for 4 weeks compared with NCD controls (Extended Fig 6e, f), indicating increased CTSA activity upon Ral activation. This is important as CTSA activation, which involves cleavage to an active form, is highly pH sensitive, and occurs upon lysosomal entry. Fully elucidating the molecular details of this interaction would require structural studies, which are beyond the scope of the current work and will be addressed in future studies.

We have added these new data (Fig 5b, c, Extended Fig 6c-f) and revised the relevant section in the Results.

13. CTSA is a lysosomal protease. If RalA promotes LDLR endocytosis and lysosomal targeting, one would naturally observe an interaction between LDLR and CTSA (as well as other lysosomal proteases). Overexpression of a lysosomal protease causing LDLR degradation is potentially a non-specific, artifactual effect of overwhelming the lysosomal system, rather than proof of a specific biological role in this pathway.

We thank the reviewer for raising this important point. While we cannot rule out interactions of LDLR with other proteases, the functional modulation of LDLR levels by CTSA without evidence of general lysosomal dysfunction supports a relatively specific, physiologically relevant role rather than a non-specific consequence of lysosomal overload. We assessed expression of lysosomal marker genes (LAMP1, P62) and other proteins known to undergo lysosomal degradation, including transferrin receptor (TFR), LXRα, and EGFR, and found no changes upon CTSA overexpression (Extended Fig 6g), indicating that CTSA-medicated LDLR degradation is not a consequence of overwhelming the lysosomal system.

Furthermore, Ral activation is associated with increased CTSA activity after 20 weeks of HCD (Fig 5b, c), as evidenced by enhanced levels of the cleaved, active form of the enzyme, suggesting that Ral promotes CTSA trafficking into lysosomes where it is processed and activated by the acidic environment. Together, these findings indicate that Ral enhances CTSA-mediated LDLR degradation in a physiologically relevant manner by promoting CTSA activation in lysosomes.

We also used AAV-mediated *in vivo* gene editing to inactivate CTSA specifically in hepatocytes. SpCas9 knock-in mice carrying a loxP-STOP-loxP cassette were injected with AAV8 expressing a TBG-Cre-driven sgRNA targeting either a control sequence (sgLacZ) or CTSA (sgCTSA). To rule out effects of CTSA on lysosomal function, we performed live-cell imaging using LysoTracker to measure lysosome number and intensity in primary hepatocytes from sgLacZ or sgCTSA mice and observed no changes (Extended Fig 6r, s).

More importantly, we synthesized the CTSA inhibitor SAR164653[8-11] (CTSAi) and evaluated its effect on Ral pathway-regulated LDLR degradation and cholesterol metabolism. In hepatocytes, CTSAi increased LDLR protein levels (Fig 6k, l), restored LDLR abundance (Fig 6m, n) and plasma membrane localization after cholesterol loading (Fig 6o, p). To assess *in vivo* effects, RalGAPB$^{f/f}$ and RalGAPB^{HKO} mice were administered with either vehicle or CTSAi via oral gavage for 4 weeks following 4 weeks of HCD feeding. In livers from RalGAPB^{HKO} mice, CTSAi rescued hepatic LDLR protein levels (Fig 6q, r), reversed the elevation of plasma VLDL/LDL (Fig 6s) and TC (Extended Fig 7o) to levels observed in RalGAPB$^{f/f}$ mice,

while HDL levels remained unchanged (Extended Fig 7p). These results indicate that CTSA acts as a key downstream effector of Ral-mediated LDLR degradation and that pharmacological inhibition of CTSA can selectively reverse Ral-driven dyslipidemia.

Although lysosomal proteases can theoretically degrade multiple substrates, our combined genetic, biochemical, and pharmacological evidence indicates that CTSA contributes to LDLR degradation under cholesterol-induced Ral activation, supporting a physiologically relevant and specific role rather than a non-specific lysosomal overload effect. As mentioned above in response to Reviewer 2, these findings leave us excited about the potential therapeutic repurposing of the CTSA inhibitor for cholesterol management, alone or in combination with existing therapies.

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Referee #1 (Remarks to the Author)

The authors have responded to reviewer comments and the revised manuscript has extensive additional data and is improved.

The new data on pharmacologic CTSA inhibition are particularly interesting.

However, I remain unclear as to how inhibiting CTSA-mediated degradation of the LDLR in the lysosome promotes increased plasma membrane LDLR protein. Does the preserved LDLR somehow recycle back to plasma membrane from the lysosome? Or does the CTSA-mediated degradation of the LDLR occur in the endosome, thus preserving the ability of the LDLR to be recycled?

We appreciate the reviewer's recognition of the improvements made in the revised manuscript. We also thank the reviewer for raising this important point and for prompting us to clarify how inhibition of CTSA-mediated degradation leads to increased plasma membrane LDLR levels. Our data support a model in which CTSA mediates LDLR degradation specifically in lysosomes rather than in earlier endosomal compartments.

First, we showed that knockdown of CTSA slowed LDLR degradation rate and extended its half-life (Fig 5m, n), confirming that CTSA contributes directly to LDLR turnover. Second, the reduction in LDLR protein levels observed under conditions of Ral activation can be largely rescued by lysosomal inhibitors (Fig 3i, j, Extended Fig S4f, g), indicating that LDLR degradation occurs through the lysosomal pathway. Consistent with this, CTSA is a lysosomal protease that becomes active following maturation in the acidic lysosomal lumen, and immunofluorescence experiments showed clear colocalization of CTSA and RalA in lysosomes (Extended Fig 6c), with lysosomal CTSA levels increasing upon Ral activation (Extended Fig 6d, Fig 5a-c). Together, these observations argue against the possibility that CTSA-mediated degradation of LDLR occurs in earlier endosomal compartments.

With respect to whether LDLR might recycle back to the plasma membrane from the lysosome, we agree that the lysosome is generally considered a terminal degradative compartment and that direct recycling from lysosomes is unlikely to represent the major mechanism. Indeed, our new data indicate that chronic activation of Ral diverts LDLR away from recycling endosomes to the lysosome for degradation. Instead, we propose that CTSA acts at the final degradative step. Inhibition of CTSA reduces lysosomal degradation of LDLR, extending the half-life and slowing the overall turnover of the receptor. Because LDLR normally resides predominantly at the plasma membrane while continuously cycling through endocytic and recycling pathways, slowing degradation increases the steady-state intracellular pool of LDLR, which allows a larger fraction of receptors to remain available for normal recycling and redistribution to the plasma membrane.

In the schematic model in ext fig 8, the LDLR recycling process should be depicted as it is a critical component of the dynamics of intracellular LDLR homeostasis and the above question should be addressed in the model.

We thank the reviewer for highlighting the importance of LDLR recycling in intracellular receptor homeostasis. To clarify further this pathway, we investigated the role of SNX17[1-5], a well-known adaptor that mediates LDLR recycling from endosomes back to the plasma membrane. Our study showed that the RalA complex interacts with the NPxY motif in the cytoplasmic tail of LDLR (Fig 4f), which is also the binding site for SNX17. Consistent with this, our new data demonstrate that overexpression of constitutively active RalA (RalA-G23V) reduced the interaction between SNX17 and LDLR (Extended Fig 4t), suggesting that Ral activation can impair SNX17-mediated recycling by competing for LDLR binding.

In line with these observations, knockdown of SNX17 alone promoted LDLR degradation, supporting its critical role in receptor recycling (Extended Fig 4r, s). Furthermore, in RalGAPB-KO cells, which had reduced LDLR due to increased Ral activity, additional knockdown of SNX17 did not further reduce LDLR levels (Extended Fig 4s), indicating that both manipulations act through the same recycling-degradation pathway, with Ral activation exerting the dominant effect. Immunofluorescence analysis revealed reduced LDLR levels in recycling endosomes, as indicated by decreased co-localization with RAB11 (Extended Fig 4u,v). These findings support a model in which SNX17 is a key mediator of LDLR recycling, and that Ral activation limits LDLR availability at the plasma membrane by interfering with this pathway. Taken together, these new data complement our findings on lysosomal CTSA-mediated degradation, highlighting that LDLR trafficking is determined by a balance between recycling and lysosomal turnover. While CTSA inhibition

primarily slows lysosomal degradation, SNX17 mediates recycling to the plasma membrane. Integrating both processes helps explain the overall dynamics of LDLR homeostasis.

To integrate these insights with our findings on lysosomal degradation, we have updated Extended Fig S8 to explicitly depict the LDLR recycling pathway alongside endocytosis and lysosomal degradation. LDLR is shown cycling between the plasma membrane and endosomal compartments, with lysosomal targeting mediated by CTSA highlighted as a terminal degradative step. The revised schematic shows how Ral competes with SNX17 for binding to the LDLR NPxY motif, reducing receptor recycling to the plasma membrane while simultaneously promoting lysosomal trafficking, thereby illustrating the balance between degradation and recycling.

Given the increased emphasis on CTSA in the revised manuscript, it would be of interest to extend the analysis of rare coding variant burden in humans to CTSA and the association with LDL-C (and perhaps apoB).

We thank the reviewer for this insightful suggestion. We agree and have extended the human genetic analysis to CTSA. In UK Biobank exomes, predicted loss-of-function (pLoF) variants in CTSA were associated with lower APOB (p=0.0276, beta=-0.00255) and lower total cholesterol (p=0.0176, beta=-0.00270), with a directionally concordant trend for lower LDL-C/LDL direct (p=0.0573, beta=-0.00220) (Table 2). These results are consistent with our experimental findings that reducing CTSA activity preserves LDLR function and improves cholesterol uptake.

Notably, the human genetic support for CTSA is not limited to rare pLoF variation: at the gene level, analyses aggregating broader common-variant signal across the locus showed even stronger associations, including MAGMA signals for APOB ($p=5.09\times10^{-9}$), LDL-C ($p=4.60\times10^{-7}$), and total cholesterol ($p=8.71\times10^{-4}$) (Table 3). In addition, CTSA also showed a significant gene-level association with coronary artery disease in the European Cardiogram-based coronary artery disease (CAD) meta-analysis ($p=5.97\times10^{-4}$) (Table 3). We have added these analyses to the revised manuscript (Table 2, 3).

REDACTED

Table3: Gene-level association of *CTSA* with lipid traits and coronary artery disease (CAD)

Gene symbol	P-value APOB	P-value Cholesterol	P-value LDL-C	P-value CAD
CTSA	5.09E-09	8.71E-04	4.60E-07	5.97E-04

Referee #2 (Remarks to the Author)

The authors have substantial improved their manuscript, provided compelling evidences for the mechanism in primary hepatocytes and conducted additional strong in vivo experiments to prove the requirement of RalA in LDLR degradation under chronic high cholesterol exposure. Thus, all my points have been addressed. The discussion is nicely streamlined now and I enjoyed reading it.

My only small question remaining would be, how they envisage cathepsin A, as a luminal lysosomal protein, to interact with RalA, which is only associated with the outside of lysosomes? Or is this interaction mediated via additional proteins? Maybe one could address this point in the discussion or in the result section.

We sincerely thank the reviewer for their positive and encouraging comments. We also thank the reviewer for this insightful comment regarding the topological separation of luminal CTSA and cytosolic RalA. We agree that a direct physical interaction across the lysosomal membrane is unlikely. Instead, we propose that this interaction is mediated by a transmembrane scaffolding or docking protein. A prime candidate for this role is the Cation-Independent Mannose-6-Phosphate Receptor (CI-M6PR), also called IGF2R. We have discussed this in the Discussion as described below:

"CTSA resides within the lysosomal lumen while RalA is associated with the cytosolic surface of endosomal and lysosomal membranes, suggesting that a direct interaction between the two is unlikely. Their coordination is more plausibly achieved through transmembrane elements of the trafficking pathway. One candidate is the cation-independent mannose-6-phosphate receptor (CI-M6PR, also known as IGF2R), which binds M6P-labeled cargo and plays a central role in trafficking cathepsins to lysosomes[6]. CTSA undergoes glycan modification to M6P in the Golgi, enabling its recognition by M6P receptors and subsequent lysosomal targeting[7]. IGF2R may provide a physical framework linking CTSA-containing vesicles to the cytosolic trafficking machinery, with its luminal domain potentially binding CTSA and its cytosolic tail possibly interacting with Ral proteins. Within this model, Ral-dependent regulation of endosomal-lysosomal trafficking could influence CTSA delivery or activity, thereby impacting LDLR degradation."

Referee #3 (Remarks to the Author):

While the authors have made some effort to revise the manuscript, the revisions fail to address the fundamental, fatal flaws of this study. The core claims remain unsupported by adequate and rigorous evidence, rendering the work unsuitable for publication in a journal of Nature's standing. My major concerns, which are critical to the validity of the entire study, remain largely unresolved.

RalA/B GTPases are master regulators of fundamental cellular processes. Pharmacological inhibition is therefore predicted to cause substantial on-target toxicities. The limited toxicity data presented (body weight, ALT) are grossly insufficient to rule out this critical concern. Without comprehensive in vivo toxicity profiling across multiple organ systems, the translational premise of targeting Ral is severely undermined. Even setting aside toxicity concerns, the observed cholesterol-lowering effects of both the Ral inhibitor and the CSTA inhibitor are marginal at best. This calls into question the biological significance and therapeutic relevance of the entire proposed pathway. The proposed CTSA-RalA interaction and its mechanistic link to cholesterol regulation remain obscure. The data presented are correlative and fail to establish a direct, causative relationship. A clear, stepwise molecular mechanism is absent, leaving the central hypothesis unsubstantiated.

We thank the reviewer for their careful evaluation and for raising these important concerns.

1. Toxicity concerns related to Ral inhibition

We agree that Ral GTPases regulate fundamental cellular processes, and that systemic inhibition could raise concerns regarding on-target toxicity. However, we would like to clarify that the primary focus of this study using Ral inhibitor is mechanistic, identifying a previously unrecognized pathway linking Ral loss-of-function to LDLR trafficking and cholesterol regulation, rather than establishing a fully developed therapeutic strategy targeting Ral itself. In this context, the pharmacological inhibitor is used as a tool compound to complement genetic approaches and establish pathway causality.

While we previously discussed the possibility of targeting Ral as a therapeutic strategy, we now agree with the reviewer that systemic inhibition of Ral GTPases may cause substantial on-target toxicities. Therefore, we have removed all statements in the manuscript suggesting Ral as a druggable target. These changes are indicated in the revised manuscript using red text and strikethrough formatting for ~~deleted lines~~.

2. Translational relevance of CTSA

Importantly, our findings identify CTSA as a downstream effector mediating LDLR degradation. Targeting CTSA may provide a more selective approach to modulate this pathway while avoiding broader functions governed by Ral. We have added some new data to address this.

First, we use wild-type mice and fed them with high cholesterol diet (HCD) for 12 weeks following by another 4 weeks of vehicle or CTSA inhibitor (CTSAi) via oral gavage. CTSAi increased hepatic LDLR protein levels (Fig 6o, p) and reduced plasma VLDL/LDL by ~40% (from 201.8 mg/dL to 125.2 mg/dL; Fig 6q) without affecting HDL (Fig 6r).

Second, to extend the translational relevance, we performed experiments in human primary hepatocytes. Immunofluorescence staining showed increased plasma membrane LDLR upon CTSA inhibitor treatment in human hepatocytes (Extended Fig 7o, p). Cholesterol loading decreased LDLR protein levels, while treatment with a CTSA inhibitor partially rescued the reduction in LDLR (Extended Fig 7q). These results demonstrate that pharmacological inhibition of CTSA effectively stabilizes LDLR and improves cholesterol handling in both mice and human hepatocytes, supporting its potential as a therapeutic target, particularly given its favorable safety profile in humans in Phase 1 trials.

Third, we have extended the human genetic analysis to CTSA as suggested by Reviewer 1. In UK Biobank exomes, predicted loss-of-function (pLoF) variants in CTSA were associated with lower APOB (p=0.0276, beta=-0.00255) and lower total cholesterol (p=0.0176, beta=-0.00270), with a directionally concordant trend for lower LDL-C/LDL direct (p=0.0573, beta=-0.00220) (Table 2). These results are consistent with our experimental findings that reducing CTSA activity preserves LDLR function and improves cholesterol uptake.

Notably, the human genetic support for CTSA is not limited to rare pLoF variation: at the gene level, analyses aggregating broader common-variant signal across the locus showed even stronger associations, including MAGMA signals for APOB ($p=5.09\times10^{-9}$), LDL-C ($p=4.60\times10^{-7}$), and total cholesterol ($p=8.71\times10^{-4}$) (Table 3). In addition, CTSA also showed a significant gene-level association with coronary artery disease in the European Cardiogram-based coronary artery disease (CAD) meta-analysis ($p=5.97\times10^{-4}$) (Table 3). We have added these analyses to the revised manuscript (Table 2, 3).

Together, these findings support CTSA as a functionally and genetically validated regulator of LDLR and highlight its potential as a therapeutic target for lipid disorders and cardiovascular disease.

3. CTSA-RalA interaction and its mechanistic link to cholesterol regulation

To define the pathway connecting RalA activation to LDLR turnover, we performed the following experiments to establish direct, stepwise mechanisms:

a. RalA competes with SNX17 for LDLR binding.

Our study showed that RalA interacts with the NPxY motif in the cytoplasmic tail of LDLR (Fig 4f), which is also the binding motif for SNX17, a well-known adaptor that mediates LDLR recycling from endosomes back to the plasma membrane[1-5]. We observed that overexpression of constitutively active RalA (RalA-G23V) reduced the interaction between SNX17 and LDLR (Extended Fig 4t), suggesting that Ral activation can impair SNX17-mediated recycling by competing for LDLR binding. In line with these observations, knockdown of SNX17 alone promoted LDLR degradation, supporting its critical role in receptor recycling (Extended Fig 4r, s). Furthermore, in RalGAPB-KO cells, which had reduced LDLR due to increased Ral activity, additional knockdown of SNX17 did not further reduce LDLR levels (Extended Fig 4s), indicating that both manipulations act through the same recycling-degradation pathway, with Ral activation exerting the dominant effect. Immunofluorescence analysis revealed reduced LDLR levels in recycling endosomes, as indicated by decreased co-localization with RAB11 (Extended Fig 4u,v). These findings support a model in which SNX17 is a key mediator of LDLR recycling, and that Ral activation limits LDLR availability at the plasma membrane by interfering with this pathway and directing LDLR into the lysosomes for degradation. These new data highlights that LDLR trafficking is determined by a balance between recycling and lysosomal turnover. We have also updated our model figure to include the recycling process (Extended Fig 8).

b. Ral activation promotes lysosomal CTSA maturation and decreases CTSA secretion.

CTSA is initially synthesized as an inactive zymogen (precursor CTSA) and undergoes proteolytic processing into mature, enzymatically active forms under acidic lysosomal conditions. Notably, lysosomal CTSA expression (CTSA-lyso) was elevated in RalGAPB knockout cells (Fig 5a, Extended Fig 6d) and in the livers of mice fed HCD for 20 weeks (Fig 5b, c), whereas the precursor form of CTSA (CTSA-pre) was more abundant in the livers of mice fed HCD for 4 weeks compared with NCD controls (Extended Fig 6e, f), indicating increased CTSA activity upon Ral activation.

While CTSA is primarily localized to lysosomes, previous studies have reported that a fraction of CTSA can be secreted into the extracellular space, where it may play additional roles in extracellular protein processing and signaling[8]. Plasma from RalGAPB knockout mice contained lower levels of secreted CTSA compared with the control mice (Extended Fig 6g, h). Similarly, CTSA in the supernatant was reduced in RalGAPB knockout AML12 cells compared to WT cells, as measured by Western blot and ELISA (Extended Fig 6i, j).

These findings indicate that Ral activation directs CTSA toward lysosomes for maturation while reducing its secretion, similar to the fate of LDLR. Enhanced lysosomal CTSA activity maintains high catalytic capacity for LDLR degradation, providing a direct mechanistic link between Ral signaling, CTSA processing, LDLR turnover, and circulating LDL-C levels.

c. CTSA directly mediates LDLR turnover.

We have shown that genetic knockdown (Fig 5i-r) or pharmacological inhibition of CTSA (Fig 6k-w) stabilized LDLR and increased LDL uptake in mice or in primary hepatocytes. These manipulations also prevented cholesterol-induced reduction of LDLR abundance (Fig 5s, t, 6m,n), demonstrating that CTSA activity is required for lysosomal degradation of LDLR under physiological stimuli.

d. CTSA mediates Ral-dependent LDLR degradation.

We provided direct evidence that CTSA functions downstream of the Ral pathway in both cells and genetic mouse models. In hepatocytes, double knockout of CTSA and RalGAPB (DKO) restored LDLR protein levels (Fig 5h), plasma membrane LDLR abundance (Extended Fig 6m, n), and LDL uptake (Extended Fig 6o, p) compared with RalGAPB knockout alone.

In vivo, treatment of RalGAPB^{HKO} mice, which exhibit elevated Ral activity and reduced LDLR, with a CTSA inhibitor fully restored hepatic LDLR protein levels to those of control mice (Fig 6u, v). These findings establish that CTSA is both necessary and sufficient for Ral-mediated LDLR degradation, providing direct mechanistic evidence linking Ral activation, CTSA, and LDLR turnover.

e. the interaction between CTSA and Ral

Inspired by comments from Reviewer 2, we have expanded the Discussion to address the interaction between CTSA and Ral, as follows:

“CTSA resides within the lysosomal lumen while RalA is associated with the cytosolic surface of endosomal and lysosomal membranes, suggesting that a direct interaction between the two is unlikely. Their coordination is more plausibly achieved through transmembrane elements of the trafficking pathway. One candidate is the cation-independent mannose-6-phosphate receptor (CI-M6PR, also known as IGF2R), which binds M6P-labeled cargo and plays a central role in trafficking cathepsins to lysosomes[6]. CTSA undergoes glycan modification to M6P in the Golgi, enabling its recognition by M6P receptors and subsequent lysosomal targeting[7]. IGF2R may provide a physical framework linking CTSA-containing vesicles to the cytosolic trafficking machinery, with its luminal domain potentially binding CTSA and its cytosolic tail possibly interacting with Ral proteins. Within this model, Ral-dependent regulation of endosomal-lysosomal trafficking could influence CTSA delivery or activity, thereby impacting LDLR degradation.”

Our combined biochemical, genetic, and human association data support a central role for CTSA in this process. While further work will be required to optimize therapeutic strategies, these findings provide a strong conceptual and mechanistic foundation for future translational development, highlighting CTSA as a tractable and potentially safer target within the Ral-LDLR regulatory axis.

The association of SNPs in KRAS and REPS1 with disease phenotype constitutes a key point of the study, yet the authors provide no functional validation. It is imperative to experimentally assess the impact of these

SNPs on gene expression, protein localization, and function. These are standard and feasible experiments. In their absence, the human genetic data are merely suggestive and too shaky to support their function in human lipid metabolism.

We agree with the reviewer that direct functional testing of the specific common variants at the KRAS and REPS1 loci would further strengthen causal inference. We have therefore revised the manuscript to avoid implying functional validation of the individual common SNPs. At the same time, we note that these genes are also supported by distinct, protein-coding genetic instruments from exome sequencing, which are more directly interpretable functionally than the noncoding common-variant associations.

Specifically, rare predicted loss-of-function (pLoF) variants in KRAS were nominally associated with lower APOB (beta=-0.00531, p=0.0242). In addition, a burden of missense variation in REPS1 was associated with lower APOB (beta=-0.00163, p=0.00344), lower total cholesterol (beta=-0.00164, p=0.00277), and lower LDL-C/LDL direct (beta=-0.00175, p=0.00173) (Table 2). These coding-variant results are directionally concordant with our experimental model, in which KRAS and REPS1 promote LDLR loss and impaired cholesterol handling, and they provide orthogonal human genetic support that is independent of the common-variant association analyses.

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Referee #3 (Remarks to the Author):

I thank the authors for the effort invested in revising the manuscript. The additional experiments and analyses have strengthened several aspects of the study. In particular, the revised manuscript provides more support for the involvement of CTSA in LDLR regulation and expands the human genetic analyses. However, after reviewing the revision and rebuttal carefully, I remain unconvinced that the manuscript meets the standard required for publication in Nature.

The manuscript is framed around a RalA-CTSA-LDLR regulatory axis. However, the key mechanistic step—how cytosolic RalA controls the function or maturation of the lysosomal luminal protease CTSA—remains unresolved. The revised manuscript acknowledges the topological problem and proposes an indirect model involving CI-M6PR/IGF2R or related trafficking machinery. This is plausible, but it remains hypothetical. No direct evidence is provided to establish the proposed intermediate step, either physically or functionally. For a study whose main claim is the identification of a new mechanistic axis, this unresolved link is not a peripheral issue. At present, the manuscript supports an association between Ral signaling, CTSA-dependent processing, and LDLR abundance, but it does not yet establish the full mechanistic chain with the level of rigor expected here.

The revised human genetic analyses are a useful addition, but they remain largely associative. The rare-variant and gene-level data are directionally consistent with the proposed pathway, yet they do not establish causality, nor do they functionally connect the implicated human variants to the specific mechanism proposed in the manuscript. This is particularly important given the emphasis placed on the genetics as support for translational relevance.

Response: We thank Reviewer 3 for the careful evaluation of our revised manuscript. We also thank the editor for the guidance regarding Reviewer 3's remaining concerns. As requested, we have addressed these points textually in the revised manuscript. Specifically, we have substantially toned down the clinical translatability claims. We have also clarified that the proposed involvement of CI-M6PR/IGF2R or related trafficking machinery represents a model that will require future experimental investigation. In addition, we have revised the relevant text describing the human genetic analyses to avoid overstatement. These textual revisions were made in accordance with the editor's instructions and do not alter the scientific content, data, or conclusions of the manuscript.