THE EMBO JOURNAL

The Glycine Arginine Rich Domain of the RNA Binding Protein Nucleolin Regulates its Subcellular Localization

Ella Doron-Mandel, Indrek Koppel, Ofri Abraham, Ida Rishal, Terika Smith, Courtney Buchanan, Pabitra Sahoo, Jan Kadlec, Juan Oses-Prieto, Riki Kawaguchi, Stefanie Alber, Eitan Zahavi, Pierluigi Di Matteo, Agostina Di Pizio, Didi-Andreas Song, Nataliya Okladnikov, Dalia Gordon, Shifra Ben-Dor, Rebecca Haffner-Krausz, Giovanni Coppola, Alma Burlingame, Pavel Jungwirth, Jeffery Twiss, and Mike Fainzilber
DOI: 10.15252/embj.2020107158

Corresponding author(s): Mike Fainzilber (mike.fainzilber@weizmann.ac.il)

Review Timeline:

Submission Date:	27th Oct 20
Editorial Decision:	14th Dec 20
Revision Received:	2nd Jun 21
Editorial Decision:	22nd Jul 21
Revision Received:	29th Jul 21
Accepted:	3rd Aug 21

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 14th Dec 2020

Dear Mike,

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting and support publication here. They raise a number of different issues that should be straightforward enough to address. I would therefore like to invite you to submit a revised version. I am happy to discuss the raised issues further either via email or video call if that is helpful.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. Looking forward to discussing/seeing the revised version

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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Referee #1:

This manuscript combines biochemistry, cell biology and mouse genetics to provide evidence that the GAR domain of nucleolin is important for interactions of the protein with the kinesin motor and the plasma membrane. The authors also build a case for the interaction of the GAR domain with kinesin promoting mRNA localization into axons, and thereby modulating axonal outgrowth. Overall, this is a high quality and comprehensive study that has broader implications for how GAR domains in other proteins, including several involved in post-transcriptional regulation, may contribute to protein targeting. Overall, I am enthusiastic about the study but some areas need to be strengthened before I can recommend publication.

Major points:

1. The authors conclude that the aptamer AS1411 targets the GAR domain of nucleolin based on in vitro experiments with peptides derived from the nucleolin GAR domain and control peptides that are not arginine-rich. This conclusion is important for interpreting some of the later cell biological experiments. Whilst the presented data are convincing, the in vitro binding studies would be strengthened by testing the selectivity of AS1411 for sequences from the GAR domain of nucleolin vs arginine-rich peptides from other GAR-domain containing proteins, or a 'scrambled' nucleolin GAR peptide with a similar charge but different sequence. In other words, does the interaction of AS1411 with short sequences derived from the GAR domain of nucleolin fully account for the

aptamer's specific targeting of nucleolin in vivo or might there by addition features of the broader GAR domain or other parts of nucleolin that are also important? These experiments, which should not be too onerous, should help with the interpretation of the cellular data involving the aptamer.

2. The work would be strengthened by showing that GAR-derived peptides of nucleolin bind recombinant kinesin (with control peptides such as those described in the previous point), as the interactions in pulldowns/immunoprecipitations could be indirect.

3. In Figure 6F, data are presented assessing the ratio of mRNA in the cell body vs axon. This analysis should also be performed for the data in Figure 5 E, G and I in order to back up the conclusion that the GAR domain contributes to axonal localization of these mRNAs (as opposed to mRNA levels in both the axon and cell body).

4. The authors need to be clearer with what n numbers refer to for each experiment (e.g. in the legends or a supplementary table). Does n refer to independent biological or technical replicates, number of cells, neurites etc.? If n refers to independent replicates, how many cells etc were analyzed by experiment?

Minor points:

1. In Figure 6, the authors use FISH with neurons {plus minus} AS1411 to demonstrate that Inpff5 localization is regulated by the GAR domain of nucleolin. This section would be strengthened by validating the localization defect (initially shown by RNA-seq) in wt vs GAR +/- neurons by FISH (including axon vs cell body data).

2. Perhaps the authors could explain why they focused on Kif5a. Is this because it has been implicated in axonal transport and ALS? Such clarification might help the reader follow this section more easily.

3. The title for Figure S3 is potentially misleading as no interaction between these proteins was detected.

4. I will leave it to the authors to decide but I would recommend including a caveat in the Discussion that they cannot rule out that the defects in nucleolar dynamics of nucleolin caused by GAR domain deletion contributes to the axonal localization and cell growth phenotypes (whilst recognizing that their alternative model is, however, supported by multiple lines of evidence).

5. Page 6. I would remove 'unfortunately' when describing the lethality of the homozygous GAR domain mutation. As the authors state in the discussion, this result was informative as it revealed the overall importance of this domain.

6. Page 8. It is premature to say that the removal of the GAR domain has equivalent effects in DT40 cells and mouse embryos unless the authors show that the mouse embryos die at the same stage as mutants for other genes with cell lethal phenotypes. Without these data, it is possible that the GAR domain deletion in embryos does not cause general cell lethality and this sentence should be toned down.

7. Figure 1D. In these plots it would be helpful to know if the molecular weights are estimated based on standards or are precise measures based on the proteins being assayed. This information could be included in the methods.

8. The authors should be more specific in the methods or a supplementary table about the potential off-targets for the sgRNAs used for CRISPR. How many mismatches are present in the off-targets and are the off-target sites in genes (if so, which ones?) or intergenic regions? Are the off-target sites on different chromosomes to nucleolin, in which case they should segregate away from the nucleolin allele quite quickly.

Referee #2:

The manuscript by Doron-Mandel & Koppel et al. provides new mechanistic insight into how nucleolin, a RNA binding protein, is subcellularly localized within various cell types, with further suggestive links to RNA localization and axon growth. Overall the data are quite convincing and this is an impressive piece of work that will advance the field. Many proteins contain GAR domains, and the authors determine that this domain is required for subcellular localization to the nucleolus, the plasma membrane, and neuronal projections and axons. The authors utilize a variety of advanced biochemical and microscopy methods to dissect GAR role in cellular localization of nucleolin through direct Kif5A binding, plasma membrane association and in potentially in mRNA localization. They show that nucleolin is not associated with Lamp1, which indicates membrane-free transport, in contrast to recent findings of endosomal or lysosomal RBP transport (Cioni et al., 2019, Liao et al., 2019). While some of those functions of GAR domain were demonstrated or hinted at in other RBPs before (Darnell et al., 2001), there was no study up to date that examined them comprehensively. However, there are some concerns on interpretive issues that extrapolate to RNA localization and other minor issues that that should be addressed.

Major comments:

The authors show impairments of mRNA localization using GAR+/- heterozygous mice.
The authors state, " as shown in Fig. 5F-I, there are significant reductions in axonal levels of both Kpnb1 and mTOR mRNAs in GAR+/- axons in vivo....Thus, the GAR domain is required for axonal localization of nucleolin and its cargo mRNAs in vivo." I am missing the connection to the GAR domain. The authors should show that a GAR domain mutant fails to rescue defects in RNA localization but wild type construct can rescue. These suggested experiments can be done in their cell culture model.

The role of nucleolin, and in particular the GAR domain, on axon growth needs to be clarified. Neurons from GAR +/- heterozygous mice have accelerated growth (loss of function), as also do neurons with expression of a GAR domain mutant (dominant negative gain of function). The authors conclude that "Thus, perturbation of GAR-mediated axonal localization of nucleolin enhances axon growth". However, this extrapolation of logic is not clear. The authors need to further discuss this in the context of the proposed axon sensing model, whereby deficits in RNA localization lead to enhanced axon growth. The effects of the GAR domain mutant on RNA localization need to be examined.

In the discussion, the authors conclude that deletion of the GAR domain is lethal in vivo. Although this is likely the case, the authors provide little data to support this conclusion. The authors state that 30 embryos were genotyped with no homozygous embryos observed. The conclusion would be better supported if the authors provide data regarding the number of founders used in their crosses and a power analysis to indicate that the sample size is sufficient.

The GAR domain is required for localization to the plasma membrane and neuronal projections. The authors do not test if the GAR domain is sufficient to promote protein localization to any subcellular compartments.

The data within the manuscript show that the GAR domain is required for localization to plasma membrane, axons, and the nucleolus. It would be interesting for the authors to comment or speculate as to where nucleolin is localized in the absence of the GAR domain, if not the before mentioned compartments. Perhaps the authors could use the data already provided to comment on this point in the discussion.

Other specific comments to figures:

Figure 1C needs statistics associated with each of the comparisons.

Figure 1C, text states 15 kinesin-complex component but Figure 1C shows 16.

The description of the experiment associated with Figure 2C-2E is vague in the main text. "...the R4, but not its N4 control, significantly perturbed endogenous Kif5a-nucleolin interaction..." This is an important experiment, but it could be clearer if the authors explicitly state that the R4 peptide functions as a dominant negative and competes with endogenous nucleolin for Kif5a interaction. Similar strategies are nicely described on page 8 for Figure 7C and 7D.

Figure 3F-H. GAPDH and Importin beta 1 function as negative controls. If possible, this figure should have a positive control to confirm the fraction isolated contains axoplasma membrane proteins.

Abbreviation, AAV-PHP.S, in Figure 4C legend needs defining.

Figure 5 D, E, F: and 6 D: Authors need to enlarge panels, enhance brightness and contrast and increase font size. Alternatively, changing color pair to cyan/red or something more contrasted would make it more readable. Labels should be present in all panels.

In Figure 5F and 5H, the RNA channel is difficult to see any signal.

Figure 5D, 5F, and 5H - the labels associated with the panels need enlarging.

The legend for figure 5F is labeled as "5F-I" but there are no G, H, or I panels.

Supplemental Fig 2C should have percentages of all categories on the diagram.

In Figure 6D, it is unclear what the top representative cell is showing "Src (grey)". This panel needs a description in the main text or figure legend as to why it is included.

Figure 6F states F-I. However, there is only Figure 6F.

Figure 7 A, C: To make figures consistent with the previous ones, authors should choose the same color pattern as in figure 5 and 6. Again, panels are too small. Authors could crop the example neuron to enlarge it.

Referee #3:

The GAR domain, like many other low-complexity domains, is implicated in LLPS, a topic of interest to cell and molecular biologists alike. Whether and how LLPS and active transport by motor proteins may be connected remains elusive/is unknown. This study provides an interesting example of a protein - nucleolin - involved in both processes. The experiments and data are of good quality and convincing, and support the authors' conclusions.

That said, the order and manner in which some of the data are presented is confusing. The sections of the manuscript presents (and read as) a list of interesting but seemingly disconnected facts concerning nucleolin. The authors should revise the manuscript to smooth the flow, in a way that explains their reasoning and the logic of the series of experiments. This would facilitate the reader's understanding of the data, and greatly improve the manuscript. A final model representing the authors' hypothesis would be helpful. The authors attribute/link an array of versatile functions to the GAR domain, but offer little insight or speculation as to how these functions may be connected or regulated.

In contrast to the Results section of the manuscript, which needs serious revision (both writing and reorganisation), the Discussion is particularly interesting and clear, and was a pleasure to read. Overall, this is an important study that will be of interest to the RNA field and beyond.

Concerns:

Title: The title is a bit obscure and should mention the name of the protein under study: nucleolin.

Page 4: "We chose to validate the interaction for Kif5a...". The authors should mention the method(s) they employed for the validation. The reader only learns which method was used when reading figure legend 1D, which names "Automated capillary electrophoresis", but no further explanation is provided. The relevant section in the Methods (Western blot and Wes capillary immunoanalysis) gives no insight, beyond "Automated capillary electrophoresis immunoquantification runs were conducted on a WES instrument (ProteinSimple) as described (Harris, 2015).", and Harris, 2015 is not listed among the References. Finally, the results obtained using the assay are first shown in Figure 1, whereas the assay scheme is shown in Figure 2.

Pages 13-15, Figures 1 and 2:
It is unclear why the authors chose to present their data in the order they did. Figure 2, in which data regarding the full-length nucleolin and deletion and mutation of the GAR-domain in the full-length context are presented, should in our opinion be shown first, before presenting the data shown in Figure 1, which deal with the isolated GAR domain as a whole and in detail.
Figure 1C shows cartoon models of nucleolin binding to kinesin via its GAR-domain. All these model complexes also contain vesicles, even though the data the authors present (Fig S3) do not provide evidence of co-transport with vesicles (endosomes/lysosomes). This is confusing, as it suggests that the mechanism of the GAR-kinesin interaction may be based on hitchhiking on membranes, especially because membrane-binding is one of the properties of the GAR-domain described in the following section. Only when reading the discussion did it become clear that the authors favour the idea of a direct interaction between nucleolin-GAR and kinesin.

Page 5: The subtitle "Nucleolin localizes to the plasma membrane via the GAR domain" is a

somewhat strong statement given the data presented in this section. What is actually shown is that the GAR domain anchors nucleolin at the membrane. Furthermore, the order in which the data are presented is odd: it would seem more logical to first present the experimental proof/biochemistry of the GAR membrane interaction and following that, the MD simulations which provide more detail on the interaction.

Page 15, Figure 3: The first sentence of the legend to Figure 3 A should include the word "dynamics", as in "Molecular dynamics simulation.." (rather than "Molecular simulation"). Also in the legend, the abbreviations PC:PS should be explained.

Page 6: the section heading "GAR contributions to nucleolar localization of nucleolin" suggests more than the weak contribution that the authors describe.

Figure 5: While overall the data are of quality, the images shown in figure 5 D-H are difficult to understand by persons not familiar with the tissue shown. The identity of Tuj1 (a neuron-specific class III beta-tubulin) which is used as a marker and loading control should be indicated in the legend. With regard to panels D-H, dashed lines to outline the axons would help; also, three colors in one panel make it difficult to appreciate the data. In some images, there is no visible DAPI signal. Is this just obscured by the other signals? What are the large patches of nucleolin signal in 5 D?

Page 9, Discussion: "GAR-mediated transport of nucleolin is likely dependent on direct interaction of the GAR domain with kinesin complexes." Ideally the authors would test this presumably direct interaction between nucleolin-GAR and kinesin using the GAR peptides used in this study and a recombinant kinesin. Indeed, pull-downs do not exclude that the interaction could be mediated by a membrane-enclosed vesicle. Demonstration of a direct interaction using purified components should be straightforward, would allow for a stronger conclusion and add significantly to this study.

Response to Reviewers

We thank the reviewers for their insightful and constructive comments, which we address point by point below. The main revisions are as follows:

1) We used surface plasmon resonance to show direct binding between a GAR-derived peptide and a kinesin light chain, with a measured Kd of 57.9 nM (Fig. 2F). These data further support direct interaction of nucleolin with kinesin motor complexes, without a requirement for membranous organelles.

2) We validated the role of the GAR domain in nucleolin-mediated mRNA localization to axons by siRNA knockdown of nucleolin in neuronal cultures, with rescue by transfection with siRNA-resistant full length nucleolin versus a GAR-deletion mutant of nucleolin. Nucleolin knockdown perturbed axonal localization of the three candidate mRNAs we tested, Kpnb1, mTor and Inpp5f; and the deficit was rescued for all three by siRNA-resistant full length nucleolin, but not the GAR deletion mutant (Figs 8, EV4 and EV5). These data support a requirement for the GAR domain in axonal localization of nucleolin cargo mRNAs.

3) Additional information and controls are now provided for different experiments as requested by the reviewers, and detailed below.

Referee #1:

This manuscript combines biochemistry, cell biology and mouse genetics to provide evidence that the GAR domain of nucleolin is important for interactions of the protein with the kinesin motor and the plasma membrane. The authors also build a case for the interaction of the GAR domain with kinesin promoting mRNA localization into axons, and thereby modulating axonal outgrowth. Overall, this is a high quality and comprehensive study that has broader implications for how GAR domains in other proteins, including several involved in post-transcriptional regulation, may contribute to protein targeting. Overall, I am enthusiastic about the study but some areas need to be strengthened before I can recommend publication.

Thank you for these kind comments.

Major points:

1. The authors conclude that the aptamer AS1411 targets the GAR domain of nucleolin based on in vitro experiments with peptides derived from the nucleolin GAR domain and control peptides that are not arginine-rich. This conclusion is important for interpreting some of the later cell biological experiments. Whilst the presented data are convincing, the in vitro binding studies would be strengthened by testing the selectivity of AS1411 for sequences from the GAR domain of nucleolin vs arginine-rich peptides from other GAR-domain containing proteins, or a 'scrambled' nucleolin GAR peptide with a similar charge but different sequence. In other words, does the interaction of AS1411 with short sequences derived from the GAR domain of nucleolin fully account for the aptamer's specific targeting

of nucleolin in vivo or might there by addition features of the broader GAR domain or other parts of nucleolin that are also important? These experiments, which should not be too onerous, should help with the interpretation of the cellular data involving the aptamer.

The selectivity of AS1411 for nucleolin has been addressed extensively in the literature, as summarized in a recent review by Bates et al (2017), cited in the introduction (page 3, bottom paragraph). Additional studies on other GAR domain interactions with G quadruplex nucleic acid structures are mentioned and cited in the same paragraph. Since the main focus of our study is GAR domain interactions with kinesin motors and cellular transport machinery, and we use the AS1411 aptamer only as a pharmacological tool in the initial phase of the work, we respectfully submit that further selectivity studies on the aptamer are not within the scope of the present work.

2. The work would be strengthened by showing that GAR-derived peptides of nucleolin bind recombinant kinesin (with control peptides such as those described in the previous point), as the interactions in pulldowns/immunoprecipitations could be indirect.

Point well-taken. We used surface plasmon resonance on a Biacore instrument to assess direct binding of a kinesin light chain (KLC2) and a kinesin heavy chain (Kif5c) to GAR-derived peptides. We observed specific high affinity (Kd = 57.9 nM) binding of KLC2 to the GAR-R4 peptide, but not to the corresponding N4 control (Figure 2F). Kif5c did not bind under these conditions. These data suggest that nucleolin interaction with kinesin complexes is via direct binding of the GAR domain to kinesin light chains.

3. In Figure 6F, data are presented assessing the ratio of mRNA in the cell body vs axon. This analysis should also be performed for the data in Figure 5 E, G and I in order to back up the conclusion that the GAR domain contributes to axonal localization of these mRNAs (as opposed to mRNA levels in both the axon and cell body).

The figure the reviewer refers to (now Figure 6 in the revised manuscript) shows *in vivo* FISH on DRG versus sciatic nerve from wild-type versus GAR deletion heterozygous mice. Since the DRG and nerve must be sectioned and imaged separately, it is not possible to link a given axon signal from a sciatic nerve section with the corresponding cell body in a DRG section, hence unfortunately the requested analysis is not possible. However, we have carried out targeted knockdown and rescue experiments to support the conclusion that the GAR domain is required for axonal localization of nucleolin cargo mRNAs – please see Figs 8, EV4 and EV5.

4. The authors need to be clearer with what n numbers refer to for each experiment (e.g. in the legends or a supplementary table). Does n refer to independent biological or technical replicates, number of cells, neurites etc.? If n refers to independent replicates, how many cells etc were analyzed by experiment?

n refers to independent biological replicates, unless otherwise mentioned. We have added this clarification at all relevant points in the figure legends, and in the statistical analyses section of the methods (page 38).

Minor points:

1. In Figure 6, the authors use FISH with neurons {plus minus} AS1411 to demonstrate that Inpff5 localization is regulated by the GAR domain of nucleolin. This section would be strengthened by validating the localization defect (initially shown by RNA-seq) in wt vs GAR +/- neurons by FISH (including axon vs cell body data).

The localization deficit has been validated in nucleolin knockdown neurons with rescue of the deficit by full length nucleolin, but not the GAR deletion mutant (Figs 8 and EV5).

2. Perhaps the authors could explain why they focused on Kif5a. Is this because it has been implicated in axonal transport and ALS? Such clarification might help the reader follow this section more easily.

Kif5a is indeed a major motor in axonal transport, and was a focus in our previous work on nucleolin (Perry et al., 2016, *Cell Reports*). Sentence to this effect have been added or expanded (Page 3, paragraph 3 and page 4, bottom paragraph).

3. The title for Figure S3 is potentially misleading as no interaction between these proteins was detected.

Point well-taken, the title of this figure (now Appendix Figure S2) has been modified.

4. I will leave it to the authors to decide but I would recommend including a caveat in the Discussion that they cannot rule out that the defects in nucleolar dynamics of nucleolin caused by GAR domain deletion contributes to the axonal localization and cell growth phenotypes (whilst recognizing that their alternative model is, however, supported by multiple lines of evidence).

This caveat has been added to the opening paragraph of the Discussion section (page 9, paragraph 2).

5. Page 6. I would remove 'unfortunately' when describing the lethality of the homozygous GAR domain mutation. As the authors state in the discussion, this result was informative as it revealed the overall importance of this domain.

Done.

6. Page 8. It is premature to say that the removal of the GAR domain has equivalent effects in DT40 cells and mouse embryos unless the authors show that the mouse embryos die at the same stage as mutants for other genes with cell lethal phenotypes. Without these data, it is possible that the GAR domain deletion in embryos does not cause general cell lethality and this sentence should be toned down.

We have toned down the sentence as requested (page 9, paragraph 2). In addition, we now provide quantitative data and statistical analyses for genotyping of embryos from multiple

breeding of different founders to further support our conclusion of homozygous lethality (Appendix Figure S5 and associated text on page 7, paragraph 2).

7. Figure 1D. In these plots it would be helpful to know if the molecular weights are estimated based on standards or are precise measures based on the proteins being assayed. This information could be included in the methods.

The indicated molecular weights are determined based an internal standard spiked into every capillary run. This detail has been added to the methods (page 28, paragraph 2).

8. The authors should be more specific in the methods or a supplementary table about the potential off-targets for the sgRNAs used for CRISPR. How many mismatches are present in the off-targets and are the off-target sites in genes (if so, which ones?) or intergenic regions? Are the off-target sites on different chromosomes to nucleolin, in which case they should segregate away from the nucleolin allele quite quickly.

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Thank you for these kind comments.

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The authors show impairments of mRNA localization using GAR+/- heterozygous mice.
The authors state, " as shown in Fig. 5F-I, there are significant reductions in axonal levels of both Kpnb1 and mTOR mRNAs in GAR+/- axons in vivo....Thus, the GAR domain is required for axonal localization of nucleolin and its cargo mRNAs in vivo." I am missing the connection to the GAR domain. The authors should show that a GAR domain mutant fails to rescue defects in RNA localization but wild type construct can rescue. These suggested experiments can be done in their cell culture model.

The requested experiment was carried out by siRNA-mediated knockdown of nucleolin in cultured neurons (validated as shown in Appendix Figure S7), with attempted rescue of RNA localization by transduction of full length nucleolin versus GAR deleted nucleolin. As shown in Figures 8, EV4 and EV5, nucleolin knockdown perturbed axonal localization of KPNB1, mTor and Inpp5f mRNAs; and the deficit was rescued by expression of exogenous full length nucleolin, but not the GAR deletion mutant.

The role of nucleolin, and in particular the GAR domain, on axon growth needs to be clarified.
Neurons from GAR +/- heterozygous mice have accelerated growth (loss of function), as also do neurons with expression of a GAR domain mutant (dominant negative gain of function). The authors conclude that "Thus, perturbation of GAR-mediated axonal localization of nucleolin enhances axon growth". However, this extrapolation of logic is not clear. The authors need to further discuss this in the context of the proposed axon sensing model, whereby deficits in RNA localization lead to enhanced axon growth. The effects of the GAR domain mutant on RNA localization need to be examined.

We have added text to clarify the logic of this claim in the Results on pages 8-9 (paragraph starting at bottom of page 8 and continuing to page 9) and in the discussion (page 11, paragraph 2). The effect of GAR deletion on axonal mRNA localization has now also been tested, as detailed in our response to the preceding comment immediately above.

In the discussion, the authors conclude that deletion of the GAR domain is lethal in vivo. Although this is likely the case, the authors provide little data to support this conclusion. The authors state that 30 embryos were genotyped with no homozygous embryos observed. The conclusion would be better supported if the authors provide data regarding the number of founders used in their crosses and a power analysis to indicate that the sample size is sufficient.

This information has now been added as Appendix Figure S5.

The GAR domain is required for localization to the plasma membrane and neuronal projections. The authors do not test if the GAR domain is sufficient to promote protein localization to any subcellular compartments.

We have now tested this point by comparing axonal localization of wild type GAR domain or GAR(N) mutant fused to photoconvertible Dendra fluorescent protein in transfected sensory neurons in culture. As shown in Figure 2G, photoconversion in the cell body leads to accumulation of photoconverted Dendra-GAR(WT), but not Dendra-GAR(N), in an axonal region of interest monitored at 60-90 µm distance from the cell body.

The data within the manuscript show that the GAR domain is required for localization to plasma membrane, axons, and the nucleolus. It would be interesting for the authors to comment or speculate as to where nucleolin is localized in the absence of the GAR domain, if not the before mentioned compartments. Perhaps the authors could use the data already provided to comment on this point in the discussion.

Figure 4 shows that GAR deleted nucleolin is still found within the nucleus and the nucleolus, with less pronounced nucleolar segregation. We have added text to clarify this point (page 6, bottom paragraph).

Other specific comments to figures:

Figure 1C needs statistics associated with each of the comparisons.

This is stated in the figure legend. All proteins identified in the figure pass cutoffs of fold change >2 and probability score > 0.95 in SAINTexpress analysis.

Figure 1C, text states 15 kinesin-complex component but Figure 1C shows 16.

Corrected, thank you.

The description of the experiment associated with Figure 2C-2E is vague in the main text. "...the R4, but not its N4 control, significantly perturbed endogenous Kif5a-nucleolin interaction..." This is an important experiment, but it could be clearer if the authors explicitly state that the R4 peptide functions as a dominant negative and competes with endogenous nucleolin for Kif5a interaction. Similar strategies are nicely described on page 8 for Figure 7C and 7D.

The text has been edited as suggested (Page 5, upper paragraph).

Figure 3F-H. GAPDH and Importin beta 1 function as negative controls. If possible, this figure should have a positive control to confirm the fraction isolated contains axoplasma membrane proteins.

A GIRK channel has now been added as a positive control (Appendix Figure S3C).

Abbreviation, AAV-PHP.S, in Figure 4C legend needs defining.

AAV-PHP.S is a recently published AAV serotype, and the lettering is a designation, not an abbreviation. We have added a few words regarding the peripheral neuron specificity of the serotype in the legend to Figure 4, in addition to the previously provided citation of the paper describing this useful AAV in the Methods section.

Figure 5 D, E, F: and 6 D: Authors need to enlarge panels, enhance brightness and contrast and increase font size. Alternatively, changing color pair to cyan/red or something more contrasted would make it more readable. Labels should be present in all panels.
In Figure 5F and 5H, the RNA channel is difficult to see any signal.
Figure 5D, 5F, and 5H - the labels associated with the panels need enlarging.
The legend for figure 5F is labeled as "5F-I" but there are no G, H, or I panels.

We have revised all figure panels for size and clarity, and taken care to maintain uniform presentation throughout.

Supplemental Fig 2C should have percentages of all categories on the diagram.

Done. This is now Figure EV1C.

In Figure 6D, it is unclear what the top representative cell is showing "Src (grey)". This panel needs a description in the main text or figure legend as to why it is included.

Scr refers to scrambled FISH probe, which is a control for specificity of the FISH signal. This is now clarified in the legend (now Figure 7D).

Figure 6F states F-I. However, there is only Figure 6F.

Apologies. Now corrected (updated Figure 7F)

Figure 7 A, C: To make figures consistent with the previous ones, authors should choose the same color pattern as in figure 5 and 6. Again, panels are too small. Authors could crop the example neuron to enlarge it.

The requested corrections have all been carried out.

Referee #3:

The GAR domain, like many other low-complexity domains, is implicated in LLPS, a topic of interest to cell and molecular biologists alike. Whether and how LLPS and active transport by motor proteins may be connected remains elusive/is unknown. This study provides an interesting example of a protein - nucleolin - involved in both processes. The experiments and data are of good quality and convincing, and support the authors' conclusions.

That said, the order and manner in which some of the data are presented is confusing. The sections of the manuscript presents (and read as) a list of interesting but seemingly disconnected facts concerning nucleolin. The authors should revise the manuscript to smooth the flow, in a way that explains their reasoning and the logic of the series of experiments. This would facilitate the reader's understanding of the data, and greatly improve the manuscript. A final model representing the authors' hypothesis would be helpful. The authors attribute/link an array of versatile functions to the GAR domain, but offer little insight or speculation as to how these functions may be connected or regulated.

In contrast to the Results section of the manuscript, which needs serious revision (both writing and reorganisation), the Discussion is particularly interesting and clear, and was a pleasure to read. Overall, this is an important study that will be of interest to the RNA field and beyond.

Thanks for these kind comments.

Concerns:

Title: The title is a bit obscure and should mention the name of the protein under study: nucleolin.

Modified as requested.

Page 4: "We chose to validate the interaction for Kif5a...". The authors should mention the method(s) they employed for the validation. The reader only learns which method was used when reading figure legend 1D, which names "Automated capillary electrophoresis", but no further explanation is provided. The relevant section in the Methods (Western blot and Wes capillary immunoanalysis) gives no insight, beyond "Automated capillary electrophoresis immunoquantification runs were conducted on a WES instrument (ProteinSimple) as described (Harris, 2015).", and Harris, 2015 is not listed among the References. Finally, the results obtained using the assay are first shown in Figure 1, whereas the assay scheme is shown in Figure 2.

We apologize for some potential confusion. The scheme in Fig. 2 refers to Fig. 2, not Fig. 1. We have now added an appropriate scheme to Fig. 1 (panel 1D) for clarification.

Pages 13-15, Figures 1 and 2:
It is unclear why the authors chose to present their data in the order they did. Figure 2, in which data regarding the full-length nucleolin and deletion and mutation of the GAR-domain in the full-length context are presented, should in our opinion be shown first, before presenting the data shown in Figure 1, which deal with the isolated GAR domain as a whole and in detail.

We respectfully disagree with this suggestion, and have done our best to improve the Results section for clarification.

Figure 1C shows cartoon models of nucleolin binding to kinesin via its GAR-domain. All these model complexes also contain vesicles, even though the data the authors present (Fig S3) do not provide evidence of co-transport with vesicles (endosomes/lysosomes). This is confusing, as it suggests that the mechanism of the GAR-kinesin interaction may be based on hitchhiking on membranes, especially because membrane-binding is one of the properties of the GAR-domain described in the following section. Only when reading the discussion did it become clear that the authors favour the idea of a direct interaction between nucleolin-GAR and kinesin.

Point well-taken, thank you. The figure has been modified accordingly. We also now provide additional data (as detailed in the response to Reviewer 1, point 2, above) showing direct binding of the GAR domain to a kinesin light chain.

Page 5: The subtitle "Nucleolin localizes to the plasma membrane via the GAR domain" is a somewhat strong statement given the data presented in this section. What is actually shown is that the GAR domain anchors nucleolin at the membrane. Furthermore, the order in which the data are presented is odd: it would seem more logical to first present the experimental proof/biochemistry of the GAR membrane interaction and following that, the MD simulations which provide more detail on the interaction.

The subtitle has been toned down as suggested.

Page 15, Figure 3: The first sentence of the legend to Figure 3 A should include the word "dynamics", as in "Molecular dynamics simulation.." (rather than "Molecular simulation"). Also in the legend, the abbreviations PC:PS should be explained.

Done.

Page 6: the section heading "GAR contributions to nucleolar localization of nucleolin" suggests more than the weak contribution that the authors describe.

Point well-taken, the section heading has been modified accordingly.

Figure 5: While overall the data are of quality, the images shown in figure 5 D-H are difficult to understand by persons not familiar with the tissue shown. The identity of Tuj1 (a neuron-specific class III beta-tubulin) which is used as a marker and loading control should be indicated in the legend. With regard to panels D-H, dashed lines to outline the axons would help; also, three colors in one panel make it difficult to appreciate the data. In some images, there is no visible DAPI signal. Is this just obscured by the other signals? What are the large patches of nucleolin signal in 5 D?

We now specify that Tuj1 is a neuronal marker in the legend (this figure is now Fig. 6) and have enlarged and contrasted the images to improve clarity.

Page 9, Discussion: "GAR-mediated transport of nucleolin is likely dependent on direct interaction of the GAR domain with kinesin complexes." Ideally the authors would test this presumably direct interaction between nucleolin-GAR and kinesin using the GAR peptides used in this study and a recombinant kinesin. Indeed, pull-downs do not exclude that the interaction could be mediated by a membrane-enclosed vesicle. Demonstration of a direct interaction using purified components should be straightforward, would allow for a stronger conclusion and add significantly to this study.

Point well taken. We used surface plasmon resonance to show direct binding between a GAR-derived peptide and a kinesin light chain, with a measured Kd of 57.9 nM (Fig. 2F). Please see more details in our response to Reviewer 1, major point 2, above.

Dear Mike,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referees #1 and 2. As you can see below both referees are happy with the introduced changes and support publication here. Referee #1 has a remaining minor issue, but that is it.

When you submit the final version will you also take care of the following points:

You can only have 5 keywords

Please relabel "Declaration of Interests" as "Conflict of Interest"

Tables EV1 and 2 need to have their legends inserted into xlsx sheet in a separate tab.

For the Appendix please label Appendix Table S.3 as Appendix Table S1.

Please make the mass spec and RNA seq data available (Data and Code Availability)

Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

That should be all!

Let me know if you have any further questions

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 20th Oct 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The manuscript has been improved by the revisions and I am now supportive of publication.

Minor point:
It would be helpful to add a label to the new Figure 2F to indicate that KLC2 is being assayed (so the reader does not have to refer to the legend).

Referee #2:

The authors have submitted a thoroughly revised manuscript with new data and changes to the text to clarify points. I particularly like the new data showing phenotype rescue by wild type exogenous full length nucleolin, but not the GAR deletion mutant. I am satisfied with this revision.

List of revisions and corrections requested by reviewers, editor, and publishing team:

Reviewer 1 Minor point:

- "It would be helpful to add a label to the new Figure 2F to indicate that KLC2 is being assayed (so the reader does not have to refer to the legend)".

- Point well taken. Figure revised accordingly.

Editor Comments

You can only have 5 keywords - Done.

Please relabel "Declaration of Interests" as "Conflict of Interest" – Done.

Tables EV1 and 2 need to have their legends inserted into xlsx sheet in a separate tab – Done.

For the Appendix please label Appendix Table S.3 as Appendix Table S1 – Done.

Please make the mass spec and RNA seq data available (Data and Code Availability) – Done.

Publisher issues listed in the "Data Edited Manuscript file":

1. *Fig 4B- make error bars visible* – Done.
2. *Fig 6F- correct the x axis labeling to mTOR* – Done.
3. *Fig 7C – add explanation why Inpp5f is in red* – Done.
4. *Fig 8 C-D – define error bars* – Done.
5. *9C – checked and approved* – Done.
6. *EV1 and EV3 –definition of the statistical test used to generate the p values* – Done.
7. *EV4- check publisher's correction* – Done.

2nd Revision - Editorial Decision 3rd Aug 2021

Dear Mike,

Thanks for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at it and everything looks good!

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Initial n was always =3 in order to reduce animal use (see 1.b.) or experimental cost. Whenever n=3 was not sufficient for the required power, n was increased to the appropriate computed minimum.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	When animal tissue was used, initial n was 3 in order to minimize the use of animals. In cases where n=3 was not sufficient for the required power, n was increased to the minimum required.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the analysis. In one case a sample was excluded from analysis based on outlier analysis (performed with the embedded ROUT method analysis in graphPad Prism, Q values set to 1%).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All samples (experimental versus control) were treated equally in each case. Whenever possible, sample order (such is in the case of loading electrophoresis gels) was shuffled to avoid bias.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was practiced.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes, figure legends describe the statistical tests used wherever relevant. Justification for the choice of statistical tests is detailed in the methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Shapiro-Wilk test was used via the graphPad Prism software to test for data normality
Is there an estimate of variation within each group of data?	Mean plus/minus standard errors of the mean were used unless otherwise mentioned. Figure legends state what error bars stand for.

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used in the study are commercially distributed and validated by the distributor. Antibody catalog numbers and references can be found in the material and methods section under 'antibodies'.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK-293 (human, female, RRID: CVCL_0045), U937 (human, male, RRID: CVCL_0007) and Neuro-2A (mouse, male, RRID: CVCL_0470) cells were purchased from ATCC (Cat# CRL-1573, CRL-1593, CCL-131, respectively). The cells lines were not authenticated. Mycoplasma contamination was tested upon thawing the cells and introducing them to the lab stocks. This infomation can be found under 'cell lines' in the materials and methods section of the manuscript.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Weizmann Institute of Science or the University of South Carolina. The following information is stated under the 'animals' section in the materials and methods section. C57BL6/OlaHSD and BALB/c mice were purchased from Harlan Laboratories (Envigo, Israel) and male Sprague Dawley rats (175-250 g) were purchased from Charles Rivers Laboratories. The C57BL/6YFP16 mice (Feng et al, 2000) were maintained at the Veterinary Resources of the Weizmann Institute. Nucleolin GAR+/- were generated and bred by the Weizmann Institute Veterinary Resources Department's core facility for transgenics and knockouts as described in the detailed methods section. All animals were housed in the Veterinary Resources Department of the Weizmann Institute or Animal Resource Facility of the University of South Carolina in a temperature-controlled room under a 12h light/dark cycle. Water and food were available ad libitum. Both female and male mice were used alternately in all experiments. Per biological replicate, mice for all conditions were sex and age matched, and littermates were used whenever possible. Tissue was extracted from animals 8-20 weeks of age.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Animal details as elaborated in section 8 and in the materials and methods section of the manuscript comply to the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Data and code availability are reported at the end of the materials and methods section, as follows: Gene expression analysis (RNA-seq) data generated from this paper have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number: GSE142576 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142576). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al, 2019) partner repository with the dataset identifier PXD020804 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD020804).
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G- Dual use research of concern

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