Roles of developmentally regulated KIF2A alternative isoforms in cortical neuron migration and differentiation

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MS TITLE: Roles of developmentally regulated KIF2A alternative isoforms in cortical neuron migration and differentiation

AUTHORS: Cansu Akkaya, Dila Atak, Altug Kamacioglu, Busra Aytul Akarlar, Gokhan Guner, Efil Bayam, Ali Cihan Taskin, Nurhan Ozlu, and Gulayse Ince-Dunn

I have now received the reports of two referees on your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, both referees express great interest in your work, but they also have serious criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they both request that you provide further mechanistic insights into how KIF2a regulates dendritic development or neuronal migration, or into how KIF2a splice variants alter cellular functions. Referee 2 also requests that you examine the subcellular localisation of KIF2a variants at higher resolution. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript describes roles of alternatively spliced isoforms of Kif2a in cortical development and dendrite morphogenesis. It shows alternative splicing dependent and independent roles of Kif2a in neurons and reveals interactome of Kif2a in alternative splicing dependent manner, using bioID and mass spectrometry. Authors also describe how cortical malformation associated mutations of Kif2a alter its interacting partners. Overall, interacting partners of Kif2a change drastically in Kif2a.3 splice variant and in disease associated mutations. Revealing molecular and cellular roles of alternatively spliced protein variants is very important. I am very intrigued by the physiological roles of Kif2a in neurons described here and the use of bioID method to determine differential interactome of alternatively spliced forms of Kif2a is impressive. This study is potentially suitable for publication, if authors can expand on their interesting findings and clearly discuss these findings in relation to human conditions, where Kif2a is mutated.

Comments for the author

Comments:

In this study authors show that all splice isoforms of Kif2a can support dendrite arborisation, while only Kif2a.1 and Kif2a.2 can support the radial migration of excitatory neurons. In parallel authors describe interesting novel interactions of Kif2a, for example with reticulon-4 by bioID, confirmed by PLA.

1) Could authors expand on the mechanism by which Kif2a mediates its functions in dendrite development or neuronal migration by making use of their interactome data? Certainly, there may be multiple different mechanisms leading to the Kif2a loss of function phenotype, however expanding on one such mechanism would be of great interest. For example, could authors correlate expression, localization or transport of RTN4 with Kif2a phenotypes they describe using shRNA and mutant expression.

2) Authors also describe disease-associated mutations of Kif2a. Authors should better describe these patient phenotypes and the known effects of these mutations on Kif2a protein. Authors should discuss how phenotypes in humans with Kif2a mutations may be related to the inability of Kif2a to perform its functions described in this manuscript.

I have noticed a typo in the text:
“In the nervous system RTN4 acts as is an inhibitor of neurite outgrowth”.

Reviewer 2

Advance summary and potential significance to field

This paper by Akkaya et al. analyzes the function of alternative splice variants and disease mutants of KIF2A in cortical neurons. The authors identify three KIF2A isoforms expressed in the postnatal mouse cerebral cortex and analyze their expression profiles in developing cerebral cortices. The authors first demonstrate that all three isoforms have equal activity in regulating dendrite formation in cortical neurons in culture, while only two of them can rescue migration of KIF2A knockdown neurons in the cortex. Next, they perform a high throughput screening of interaction partners of individual isoforms and identify novel interactors in addition to some known binding

partners. They further characterize disease mutants of KIF2A, applying the interactome mapping and assays for dendritic development in culture.

Comments for the author

The authors do bring to bear novel interactome approach for mapping binding partners to address a difficult question of how partial sequence differences in splice variants affect protein function. They perform many different experiments and present some interesting results implying functional differences of KIF2A splice variants, but the value of the work for understanding physiological mechanism is questionable, as none of the analyses present sufficient evidence to reach a conclusion. This study would be more interesting if the authors clarify at least either the cellular mechanisms of the regulation of dendrite formation and neuronal migration by KIF2A variants and mutants, or the molecular bases of how differential sequences of the variants affect cellular functions and molecular interaction revealed in the interactome.

Specific comments
Dendritic arborization in cortical neurons occurs during postnatal development when KIF2A expression is already downregulated (Fig. 1B). Authors should examine the expression of endogenous KIF2A in primary cortical neurons and judge the cell types expressing KIF2A. In addition, subcellular localization of transfected KIF2A subtypes cannot be evaluated at the spatial resolution of presented images in Fig. 2A. It is of extremely important to examine the localization of each isoform, e.g. in dendritic tips and protrusions, axonal growth cones and shaft, microtubules, organelles, etc., to discern the mechanism of differential function of these isoforms.

shRNA experiments in Figure 2 successfully show the enhanced complexity of dendritic processes by KIF2A deficiency. (The image of NS control in Fig. 2D should be revised to show the entire dendritic processes). In order to prove the authors' hypothesis that KIF2A deficiency hampers dendritic pruning by altering MT dynamics in dendrites, MT distribution in dendrites with or without KIF2A should be compared. Also, time-lapse imaging of growing dendrites will provide direct evidence whether the excess branch formation in knockdown cells is due to overgrowth or the suppression of pruning.

I do not understand the significance of the results showing RTN4 binding to KIF2A isoforms. Although RTN4 is a known regulator of neuronal migration and axonal extension, it would not explain the differential activities of KIF2A isoforms in migration, as it binds to all three isoforms. Again, the subcellular co-localization of RTN4 and KIF2A in Neuro2a cells (Suppl. Fig. 3) does not provide any information about their sites of function at this resolution without co-staining with other organelle markers. These results could be deleted unless the authors provide evidence for functional interaction of RTN4 and KIF2A in the developing brain.

Although the disease mutations KIF2AS317N and KIF2AH321D cause neuronal migration defects in human brain, and the authors discuss about some common features in interactome of these mutants and KIF2A.3 defective in rescuing neuronal migration, the authors somehow focus on the effect of the mutations on dendritic development and found no apparent defects. If I understand correctly, the authors expect a dominant-negative effect of these mutants on endogenous KIF2A in cultured neurons. I wonder if they checked the expression level of mutants in pEGFP expressing cells, as co-transfected constructs do not necessarily express at equal levels. To confirm if the mutations do not affect KIF2A function in dendrite development, the authors should also test overexpression of these molecules in shRNA knockdown background as was done in Fig. 1. It will also strengthen the reliability of the present study if neuronal migration defects are reproduced by expression of these disease mutants.

Minor comments
Is the lower band (~80kDa) in Fig. 1B an artifact or a truncated KIF2A isoform by alternative splicing?
'We found the expression dynamics of nElavl and KIF2A protein abundance to correlate and generally exhibit elevated expression levels in embryonic cortex and to be downregulated postnatally (Fig.1B and C), suggesting that nElavl RNABP might regulate Kif2a mRNA stability in addition to its AS'

This speculation is not supported by the presented results, as KIF2A is downregulated much (2wks) earlier than nElavls.

Some images in Suppl. Fig. 1 and Fig.6B are identical. It is not desirable to use the same images for different comparisons, even if the experiments were done at the same time.

TuJ1 should be strongly localized in fibrous microtubules in neurons in cortical cultures, but the levels and patterns vary conspicuously among cultures in Suppl Fig.4A. For instance, signals become diffusive in the cell expressing pEGFPC1, and the levels in background cells in KIF2A.1 and H321D cultures are very different.

The method for cDNA cloning of KIF2A variants should be described.

First revision

Author response to reviewers' comments

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This manuscript describes roles of alternatively spliced isoforms of Kif2a in cortical development and dendrite morphogenesis. It shows alternative splicing dependent and independent roles of Kif2a in neurons and reveals interactome of Kif2a in alternative splicing dependent manner, using bioID and mass spectrometry. Authors also describe how cortical malformation associated mutations of Kif2a alter its interacting partners. Overall, interacting partners of Kif2a change drastically in Kif2a.3 splice variant and in disease associated mutations. Revealing molecular and cellular roles of alternatively spliced protein variants is very important. I am very intrigued by the physiological roles of Kif2a in neurons described here and the use of bioID method to determine differential interactome of alternatively spliced forms of Kif2a is impressive. This study is potentially suitable for publication, if authors can expand on their interesting findings and clearly discuss these findings in relation to human conditions, where Kif2a is mutated.

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We thank the reviewer for their insightful comments. In the revised version we have now expanded upon KIF2A-mitochondrial interactions, which is an interesting and unexpected finding that has arisen from our BioID results. Our BioID results had revealed mitochondrial proteins in the interactome datasets from the two isoforms (KIF2A.1 and KIF2A.2), but very few for the third isoform (KIF2A.3) or the mutant form ($KIF2A^{H321D}$). We asked whether different KIF2A isoforms indeed displayed different levels of co-localization with mitochondria. Towards this aim, in primary cortical neurons we co-transfected individual KIF2A isoforms or KIF2A mutants ($KIF2A^{S317N}$ and $KIF2A^{H321D}$) fused to EGFP together with a construct encoding an mCherry reporter fused to the mitochondrial outer membrane protein TOMM20. We then calculated co-localization of individual KIF2A isoforms with labeled mitochondria in different subcellular compartments of neurons by immunofluorescence confocal microscopy. We imaged n=65 neurons for pEGFPC1, KIF2A.1, and

KIF2A.3, n=63 for KIF2A.2, n=49 for KIF2A^{S317N} and KIF2A^{H321D} and calculated Manders' co-localization coefficients (now presented as Figure 8). As a negative control we used cytoplasmic EGFP. For KIF2A.1 and KIF2A.2 we observed a clear increase in co-localization with mitochondria in the axonal and dendritic compartments, but not the soma, when compared to EGFP only. In contrast KIF2A.3 co- localization with mitochondria was less pronounced in the axonal compartment and was not significantly different from the EGFP control in the dendrite compartment, although we observed a general trend for increased co-localization. Moreover, the two mutant forms KIF2A^{S317N} and KIF2A^{H321D}, both of which impair neuronal migration in the developing cortex, displayed no more additional levels of co-localization with mitochondria in either the soma, axon or dendrites in comparison to the EGFP control samples. These interesting results were in support of our results from BioID revealing unexpected enrichment for mitochondrial proteins in KIF2A.1 and KIF2A.2, but not KIF2A.3 and KIF2A^{H321D}. We have now included these additional data in Fig. 8 and added additional text explaining these results in pages 9-10. Paradoxically, KIF2A does not harbor a mitochondrial localization signal, is not a resident protein of the mitochondria (Calvo et al, *Nucleic Acids Res*, 2016) and *Kif2a* knockout neurons do not have a defect in mitochondrial distribution (Homma et al, *Cell*, 2003). Most mitochondrial proteins have long been thought to be translated by cytoplasmic ribosomes and imported to the mitochondria upon completion of translation.
Several important studies have challenged this notion and instead suggested that mRNAs for mitochondrial proteins can be targeted to the cytoplasmic face of the mitochondrial outer membrane and co-translationally inserted into the mitochondria (Williams et al, *Science*, 2014; Fazal et al, *Cell*, 2019; Lesnik et al, *RNA Biol*, 2014). It is possible that a KIF2A-dependent mechanism has a role for the targeting of the mitochondrial mRNA- protein-ribonucleoprotein complex to the outer surface of the mitochondria. Our identification of a large number of ribonucleoproteins would also support this mechanism. Even though our data is consistent with these predictions, currently we do not have experimental evidence directly testing this intriguing hypothesis and will be following up on it in the future.

As for the RTN4-KIF2A interaction, our original aim in verifying the proximal localization of these two proteins was to verify interaction for at least one of the hits we identified in our BioID screen. Our KIF2A antibody recognizes all isoforms (as well as the mutants), so we selected RTN4, as it was identified as an interactor in all of our datasets. We indeed were able to verify co-localization of KIF2A and RTN4, supporting the validity of our BioID experiments. As RTN4 is a well-characterized regulator of axonal growth and KIF2A is an axonal microtubule depolymerizing factor, it would be very interesting to study this interaction and its functional consequences for axonal growth and pruning. However, we prefer to expand on this particular question in a separate study in the future. We have now reworded the relevant section (page 8, lines 234-243) to better reflect our original intentions.

2) Authors also describe disease-associated mutations of Kif2a. Authors should better describe these patient phenotypes and the known effects of these mutations on Kif2a protein. Authors should discuss how phenotypes in humans with Kif2a mutations may be related to the inability of Kif2a to perform its functions described in this manuscript.

Both KIF2A mutations are localized at or nearby the ATP binding pocket within the motor domain. Both alternative exons investigated here (exon 5 and exon 18) are outside of the motor domain. We have not tested if the presence of these exons somehow regulate depolymerization activity of KIF2A, however given that all three isoforms can carry out dendritic pruning, we suggest that depolymerization activity is not affected significantly.
Recently three studies have demonstrated a role for KIF2A in regulation of primary cilia coupled with cell cycle control (Broix et al, 2018; Zhang et al, 2019; Miyamoto et al, 2015). Briefly, the depolymerization activity of KIF2A is necessary to maintain cilia length, such that upon compromise of this activity cilia length is abnormally long. Wild-type KIF2A is localized to the basal body of the primary cilium in neural progenitors and this subcellular localization is reduced in the two disease mutants. Mis-regulated cilia length is associated with delayed cell cycle progression, decreased NPC proliferation and premature neuronal differentiation (Broix et al, 2018; Zhang et al, 2019). How the primary cilium localization of the three individual KIF2A isoforms changes is an open question, but definitely one we would pursue in the future. Our BioID setup is not well-suited for investigating KIF2A interactors during cilia regulation as we have conducted these experiments in the neuronal cell line Neuro2A, which are dividing cells and do not have primary cilia. Hence our comparison of

our BioID dataset with cilia proteome compiled from several studies identified only found one overlapping gene (Ppp1cc) found in the KIF2A.1 interactome (http://www.sfu.ca/~leroux/ciliome_database.htm).

In addition to KIF2A's role in the regulation of cell cycle and cilia length, our data now suggests that mitochondrial functions should also be investigated in *Kif2a* disease models and patient IPSC-derived fibroblasts or neurons. Very little is known how mitochondrial dynamics and mitochondrial protein import has a role in radial neuron migration and cortical lamination. Mice knockout for *Ant1* gene, which encode the mitochondrial adenine nucleotide transferase, display no defects in radial migration (Lin-Hendel et al, *Cell Reports*, 2016), however overexpression of *Mgarp* gene, encoding mitochondrial glutamic acid-rich protein, in neural progenitor cells have resulted in reduced radial migration, dendritic complexity and mitochondrial localization to axons and dendrites (Jia et al, *Mol Neurobiol*, 2014). In initial experiments we do not observe a defect in mitochondrial targeting to axons and dendrites in neurons expressing individual KIF2A isoforms. We hypothesize that KIF2A might have a role in trafficking proteins or mRNA/ribonucloprotein complexes destined for mitochondria. However, currently we do not have experimental evidence to support for this hypothesis.

We have now added these interesting points to the Discussion section of the manuscript (mostly discussed in the final paragraph in page 12-13).

I have noticed a typo in the text:
"In the nervous system RTN4 acts as is an inhibitor of neurite outgrowth".

Thank you for noticing, we have fixed this typo.

Reviewer 2 Advance Summary and Potential Significance to Field:
This paper by Akkaya et al. analyzes the function of alternative splice variants and disease mutants of KIF2A in cortical neurons. The authors identify three KIF2A isoforms expressed in the postnatal mouse cerebral cortex and analyze their expression profiles in developing cerebral cortices. The authors first demonstrate that all three isoforms have equal activity in regulating dendrite formation in cortical neurons in culture, while only two of them can rescue migration of KIF2A knockdown neurons in the cortex. Next, they perform a high throughput screening of interaction partners of individual isoforms and identify novel interactors in addition to some known binding partners. They further characterize disease mutants of KIF2A, applying the interactome mapping and assays for dendritic development in culture.

Reviewer 2 Comments for the Author:
The authors do bring to bear novel interactome approach for mapping binding partners to address a difficult question of how partial sequence differences in splice variants affect protein function. They perform many different experiments and present some interesting results implying functional differences of KIF2A splice variants, but the value of the work for understanding physiological mechanism is questionable, as none of the analyses present sufficient evidence to reach a conclusion. This study would be more interesting if the authors clarify at least either the cellular mechanisms of the regulation of dendrite formation and neuronal migration by KIF2A variants and mutants, or the molecular bases of how differential sequences of the variants affect cellular functions and molecular interaction revealed in the interactome.

Specific comments
Dendritic arborization in cortical neurons occurs during postnatal development when KIF2A expression is already downregulated (Fig. 1B). Authors should examine the expression of endogenous KIF2A in primary cortical neurons and judge the cell types expressing KIF2A. In addition, subcellular localization of transfected KIF2A subtypes cannot be evaluated at the spatial resolution of presented images in Fig. 2A. It is of extremely important to examine the localization of each isoform, e.g. in dendritic tips and protrusions, axonal growth cones and shaft, microtubules, organelles, etc., to discern the mechanism of differential function of these isoforms.

We examined the expression of KIF2A in neurons and glia cells in our primary cortical cultures

prepared from E14.5 cortices and analyzed at 4 DIV. Neurons were labeled with an antibody against the neuronal transcription factor NEUROD2 and glia were labeled with anti-GFAP antibody. We observed that neurons expressed high levels of KIF2A, whereas KIF2A expression levels were very low or absent in glia cells. We now present these images as Fig.S3 and have added additional text to the second paragraph on page 5 (lines 139-142). Therefore, we conclude that at the stage when we carry out our dendrite experiments by Sholl analysis, KIF2A is expressed primarily by neurons but not glia.

We also examined the subcellular localization of KIF2A in primary cortical neurons in better resolution. We transfected in EGFP-KIF2A fusion proteins for all three isoforms and examined subcellular localization in 34 neurons for pEGFPC1 and KIF2A.1, 35 neurons for KIF2A.2 and KIF2A.3 from two biological replicates. In agreement with our previous findings, we observed that all three isoforms were localized to both soma, axons, and dendrites in all neurons examined. Our results demonstrate that none of the three KIF2A isoforms exhibit a strong preference for somal, axonal, dendritic or growth cone localization. We have provided representative images as Suppl. Fig.2 and added additional text (page 5, lines 127-134).

shRNA experiments in Figure 2 successfully show the enhanced complexity of dendritic processes by KIF2A deficiency. (The image of NS control in Fig. 2D should be revised to show the entire dendritic processes). In order to prove the authors' hypothesis that KIF2A deficiency hampers dendritic pruning by altering MT dynamics in dendrites, MT distribution in dendrites with or without KIF2A should be compared. Also, time-lapse imaging of growing dendrites will provide direct evidence whether the excess branch formation in knockdown cells is due to overgrowth or the suppression of pruning.

It is correct that we do not directly demonstrate a change in MT polymerization/depolymerization dynamics in KIF2A isoform expressing neurons in our experiments. KIF2A is a very well-described MT depolymerizing and axonal pruning factor (Homma et al., 2003; Maor-Nof et al., 2013; Noda et al., 2012). Therefore, the most straight forward explanation of increased dendritic growth upon Kif2a knockdown was to attribute it to its MT depolymerization properties. However we do understand the reviewers concern and in order to faithfully report our findings we have now deleted the use of the terminology "dendritic pruning" throughout the manuscript and replaced it with "dendrite arbor development" or "dendrite arborization". Only in one instance we claim that our results are consistent with a defect in dendritic pruning (page 5, line 148).

To answer the question of how KIF2A isoforms affect MT distribution and dynamics we immunostained EGFP-KIF2A (all three isoforms individually) expressing primary cortical neurons with Tuj1 antibody, detyrosinated alpha-tubulin antibody (for stable MTs) (Abcam, cat.no. ab48389, 1:100) or tyrosinated alpha-tubulin antibody (for dynamic MTs) (Abcam, cat.no. ab6160, 1:500). These results suggest to us that individual KIF2A isoforms display similar levels of co-localization with Tuj1, stable and dynamic MTs. As the staining patterns of all three of these antibodies (Tuj1, detyrosinated MT and tyrosinated MT) look similar and we are not completely confident that there is no cross reactivity of antibody stainings, at this stage we prefer to submit the figure displaying these results only to the reviewer. It would be of interest to answer this question also by carrying out in vitro MT depolymerzation assays in the future, as described in (Noda et al., 2012).

We agree with the reviewer that time-lapse imaging would have provided additional valuable insight into how individual isoforms regulate dendrite branch dynamics, however severe limitation of access to our lab and microscopy setups due to Covid-19 restrictions have prohibited us from carrying out these experiments. It would have been difficult to complete time-lapse imaging and quantification in a reasonable time-frame for this review process. We hope to be able to focus on this important point in the future.

Finally, we have revised Fig.2D to include the entire dendritic process.

I do not understand the significance of the results showing RTN4 binding to KIF2A isoforms. Although RTN4 is a known regulator of neuronal migration and axonal extension, it would not explain the differential activities of KIF2A isoforms in migration, as it binds to all three isoforms. Again, the subcellular co-localization of RTN4 and KIF2A in Neuro2a cells (Suppl. Fig. 3) does not

provide any information about their sites of function at this resolution without co-staining with other organelle markers. These results could be deleted unless the authors provide evidence for functional interaction of RTN4 and KIF2A in the developing brain.

We agree that the initial way we have presented the KIF2A-RTN4 interaction data may mislead the reader to expect that we further investigate the functional consequences of this interaction for either neuronal migration or dendrite development. However, our original rationale for including this data was to demonstrate the validity of our BioID approach for identifying novel proteins that are proximal to KIF2A isoforms. We selected RTN4 as it displayed robust interaction with all three isoforms. We wished to verify endogenous interactions of KIF2A and as our KIF2A antibody detects all three isoforms, KIF2A-RTN4 interaction was a good candidate to confirm. We have now reworded this section of the manuscript to reflect these intentions (page 8, lines 234-243).

Although the disease mutations KIF2AS317N and KIF2AH321D cause neuronal migration defects in human brain, and the authors discuss about some common features in interactome of these mutants and KIF2A.3 defective in rescuing neuronal migration, the authors somehow focus on the effect of the mutations on dendritic development and found no apparent defects. If I understand correctly, the authors expect a dominant-negative effect of these mutants on endogenous KIF2A in cultured neurons. I wonder if they checked the expression level of mutants in pEGFP expressing cells, as co-transfected constructs do not necessarily express at equal levels. To confirm if the mutations do not affect KIF2A function in dendrite development, the authors should also test overexpression of these molecules in shRNA knockdown background as was done in Fig. 1. It will also strengthen the reliability of the present study if neuronal migration defects are reproduced by expression of these disease mutants.

We thank the reviewer for bringing up this point. Transfection efficiency is very low in primary neurons and we cannot collect sufficient lysate for western blotting. However, we have looked at the levels of expression of EGFP fusion proteins of KIF2A.1, KIF2A^{S317N} and KIF2A^{H321D} in Neuro2A cells. We observe similar levels of expression in all three of these constructs, perhaps a slightly increased amount of KIF2A^{H321D}. We have now generated shRNA resistant forms of KIF2A^{S317N} and KIF2A^{H321D} and carried out the experiment suggested by the reviewer here. Specifically, we have knocked down endogenous *Kif2a* expression with shRNA transfection in neurons and introduced mutant *Kif2a* constructs by co-transfecting shRNA-resistant forms. We have then carried out Sholl analysis as has been described in the manuscript. Interestingly, we have found that expression of only the mutant KIF2A constructs in the background of silenced endogenous KIF2A expression impairs dendrite arbor development. The phenotype is similar to the increased dendritic branching and growth we have observed in neurons where endogenous *Kif2a* has been knocked down. Therefore, we conclude that KIF2A^{S317N} and KIF2A^{H321D} mutations may influence dendrite morphology and depending on the expression levels of wild-type KIF2A. These results are now presented in Fig. 6.

Minor comments
Is the lower band (~80kDa) in Fig. 1B an artifact or a truncated KIF2A isoform by alternative splicing?

Upon further evaluation we have decided to remove the western blotting results where we have compared the developmental protein expression dynamics of KIF2A and nELAVL, as it is difficult to make a sound hypothesis of how nELAVL might be regulating KIF2A at the mRNA stability level from these results. But for the reviewer's information, the datasheet for this antibody (Abcam cat.no.ab71160) makes a remark of the lower band being unspecific.

'We found the expression dynamics of nElavl and KIF2A protein abundance to correlate and generally exhibit elevated expression levels in embryonic cortex and to be downregulated postnatally (Fig.1B and C), suggesting that nElavl RNABP might regulate Kif2a mRNA stability in addition to its AS'

This speculation is not supported by the presented results, as KIF2A is downregulated much (2wks) earlier than nElavls.

We agree with the reviewer, we have now deleted this section, as explained in the previous comment.

Some images in Suppl. Fig. 1 and Fig.6B are identical. It is not desirable to use the same images for different comparisons, even if the experiments were done at the same time.

We apologize for this mistake on our end, we have now fixed it.

TuJ1 should be strongly localized in fibrous microtubules in neurons in cortical cultures, but the levels and patterns vary conspicuously among cultures in Suppl Fig.4A. For instance, signals become diffusive in the cell expressing pEGFPC1, and the levels in background cells in KIF2A.1 and H321D cultures are very different.

We thank the reviewer for noticing this. This kind of difference in Tuj1 expression is certainly not something we observe upon transfection with either different isoforms or mutants. It has just been a bad coincidence. We have now repeated these kinds of Tuj1 staining and always get consistent staining patterns. Representative images are provided in Fig.6.

The method for cDNA cloning of KIF2A variants should be described.

We have now added additional details to the “Cloning” section under Material and Methods.

Second decision letter

MS ID#: DEVELOP/2020/192674

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AUTHORS: Cansu Akkaya, Dila Atak, Altug Kamacioglu, Busra Aytul Akarlar, Gokhan Guner, Efil Bayam, Ali Cihan Taskin, Nurhan Ozlu, and Gulayse Ince-Dunn

I have now received the reports of the two referees who reviewed the earlier version of your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The reviewers' evaluation is overall positive but the two referees require further revisions to fully support the conclusion of the manuscript. We would therefore like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of the referees, including the addition of a positive control by using wildtype Kif2a to rescue dendritic branching (referee 1), and the demonstration that loss of Kif2A affects the expression or localisation of one of the mitochondrial Kif2A interactors (referee 2). Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

Akkaya et al describe roles of the kinesin motor Kif2A isoforms in neuronal migration and dendrite arborization in rodents. They then take on a challenging quest to determine the functional significance of different splice isoforms of Kif2A. The manuscript has improved by addition of new data analysis and text. Discussion points are added as well. Overall, the quality improved and reasoning and flow is very clear. I believe the manuscript is suitable for publication by addressing minor points.

Comments for the author

My main criticism was the lack mechanistic explanation of the neuronal migration or dendrite development phenotypes.
In their revised version authors added new data in Figure 8, showing that KIF2A isoforms associate differentially with mitochondria. These experiments nicely confirm their findings from BioID experiments. In Figure 8, using fluorescent imaging they demonstrate that KIF2A.1 and KIF2A.2 show colocalization with mitochondria, but KIF2A.3 or mutant KIF2A isoforms do not show colocalization with mitochondria. This new data allows authors to speculate in discussion how mitochondrial protein mRNA localization and translation could be affected by Kif2a isoforms. Examples of magnified colocalizations of Figure 8 should be included in supplements.
The new data does not however mechanistically explain migration or dendrite development phenotypes. Authors argue that previous literature on Kif2a's effect on microtubules is strong enough to confidently propose that this role of Kif2a contribute to migration and dendrite deficits. This is acceptable.

My second comment was on how disease-causing mutations may disrupt Kif2A function. Authors responded to my comments and added further points in Discussion.
In addition, they also now tested how disease-causing mutants of Kif2a affect dendrite arborization. They present new data in Figure 6, showing that increased branching caused by Kif2a shRNA cannot be rescued by mutant Kif2A expression. These experiments are very informative. Here it would be useful to also report rescue with wild type Kif2a as a positive control. I am not sure if authors will be able to add this dataset during COVID period, but it would be ideal to see a rescue with wt Kif2a. If possible adding a statistical comparison at a single distance from the soma (e.g. 50 um) would also be ideal.

Reviewer 2

Advance summary and potential significance to field

The authors have added new experiments to further support their hypothesis, and refined their conclusions to fit with their data. Especially they have added new experiments showing the differential interactions between KIF2A variants and mitochondria in line with the interactome data.

Comments for the author

Although the changes that they made significantly improved the manuscript, the causal link between neuronal phenotypes and differential interactions of KIF2A variants/mutants with mitochondria still remain ambiguous. I think it is critical to show how mitochondria change in the absence of normal KIF2A function. The authors have added discussion that KIF2A is not required for mitochondria transport but it may have a role in the regulation of targeting of proteins and mRNAs in mitochondria. This paper will be suitable for publication in Development if the authors would show that KIF2A loss alters expression and/or localization of at least one of the mitochondrial KIF2A interactors identified by the interactome, or of their possible downstream target(s) in mitochondria which they mentioned in the revised manuscript.

Second revision

Author response to reviewers' comments

Reviewer 1 comment:

Reviewer 1 Advance Summary and Potential Significance to Field:

Akkaya et al describe roles of the kinesin motor Kif2A isoforms in neuronal migration and dendrite arborization in rodents. They then take on a challenging quest to determine the functional significance of different splice isoforms of Kif2A. The manuscript has improved by addition of new data analysis and text. Discussion points are added as well. Overall, the quality improved and reasoning and flow is very clear. I believe the manuscript is suitable for publication by addressing minor points.

Reviewer 1 Comments for the Author:

My main criticism was the lack mechanistic explanation of the neuronal migration or dendrite development phenotypes. In their revised version authors added new data in Figure 8, showing that KIF2A isoforms associate differentially with mitochondria. These experiments nicely confirm their findings from BioID experiments. In Figure 8, using fluorescent imaging they demonstrate that KIF2A.1 and KIF2A.2 show colocalization with mitochondria, but KIF2A.3 or mutant KIF2A isoforms do not show colocalization with mitochondria. This new data allows authors to speculate in discussion how mitochondrial protein mRNA localization and translation could be affected by Kif2a isoforms. Examples of magnified colocalizations of Figure 8 should be included in supplements.

Response:
We have now added this data as Suppl. Fig. 8.

Reviewer 1 comment:

The new data does not however mechanistically explain migration or dendrite development phenotypes. Authors argue that previous literature on Kif2a's effect on microtubules is strong enough to confidently propose that this role of Kif2a contribute to migration and dendrite deficits. This is acceptable.

My second comment was on how disease-causing mutations may disrupt Kif2A function. Authors responded to my comments and added further points in Discussion. In addition, they also now tested how disease-causing mutants of Kif2a affect dendrite arborization. They present new data in Figure 6, showing that increased branching caused by Kif2a shRNA cannot be rescued by mutant Kif2A expression. These experiments are very informative. Here it would be useful to also report rescue with wild type Kif2a as a positive control. I am not sure if authors will be able to add this dataset during COVID period, but it would be ideal to see a rescue with wt Kif2a. If possible adding a statistical comparison at a single distance from the soma (e.g. 50 um) would also be ideal.

Response:
We have now repeated the same dendrite quantification experiment, which we had shown in Fig. 6. Briefly, we silence endogenous *Kif2a* and then reintroduce shRNA resistant *Kif2a*^{S317N} and *Kif2a*^{H321D} mutant constructs in cultured cortical neurons. As the reviewer has suggested we have now added a control sample which is transfected with a shRNA resistant wild-type *Kif2a.1*. In this experiment we have replicated our previous findings demonstrating that *Kif2a*^{S317N} and *Kif2a*^{H321D} mutants cannot rescue a dendrite overgrowth phenotype, however a wild-type *Kif2a.1* is able to do so. Finally, we have now plotted a statistical comparison at a single distance from the soma at 50 um. An updated version of Figure 6 is now provided.

Reviewer 2 comment:

Reviewer 2 Advance Summary and Potential Significance to Field: The authors have added new experiments to further support their hypothesis, and refined their conclusions to fit with their data. Especially they have added new experiments showing the differential interactions between KIF2A variants and mitochondria in line with the interactome data.

Reviewer 2 Comments for the Author:
Although the changes that they made significantly improved the manuscript, the causal link between neuronal phenotypes and differential interactions of KIF2A variants/mutants with mitochondria still remain ambiguous. I think it is critical to show how mitochondria change in the absence of normal KIF2A function. The authors have added discussion that KIF2A is not required for mitochondria transport but it may have a role in the regulation of targeting of proteins and mRNAs in mitochondria. This paper will be suitable for publication in Development if the authors would show that KIF2A loss alters expression and/or localization of at least one of the mitochondrial KIF2A interactors identified by the interactome, or of their possible downstream target(s) in mitochondria which they mentioned in the revised manuscript.

Response:
We thank the reviewer for finding our revised version of the manuscript significantly improved, and we understand their viewpoint that additional functional experiments would add useful mechanistic insight into our study. Due to the COVID-19-related restrictions our access to the laboratories, to the core microscopy facility and our ability to receive timed-pregnant mice for primary neuronal cultures has been severely reduced. During the second revision period we requested access to research facilities from facility managers and were granted only limited access (several days) and timed-pregnant mice were provided only once. Despite these severe limitations we carried out an initial experiment where we transfected primary cortical neurons with non-silencing or shKif2a together with mitochondrially targeted mCherry (mCherry- TOMM20). We quantified mCherry-Mito signal in neuronal soma, axons and dendrites. We carried out the experiment twice in parallel, derived from two independent pregnancies. We quantified approximately 35-40 neurons in total. Our results suggest that mitochondrial localization in *Kif2a* silenced cortical neurons in axons and dendrites are similar to control levels. These results are in line with published data from *Kif2a* knockout mice (Homma et al., *Cell*, 2003) and suggest that KIF2A is not required for mitochondrial targeting to axons and dendrites. These results are now provided as. Fig. S9 and explanatory text has been added to the manuscript (pg 10, lines 312-322).

Whether KIF2A has roles in mitochondrial biogenesis, protein targeting, fusion, fission or other functions are open questions. Additional experiments suggested by the reviewer which focus on the levels or localization of mitochondrially localized KIF2A interactors identified in our screen would be useful in answering these relevant questions. However, we believe that these experiments are now beyond the scope of this initial characterization of our isoform-specific and disease-mutant-specific KIF2A interactome analysis. Furthermore, the practical difficulties in our access to the research facilities make it even more questionable whether these experiments could be finished in a reasonable time-frame. Nevertheless, we thank the reviewer for pointing at these interesting directions for the future development of this project.

Third decision letter

MS ID#: DEVELOP/2020/192674

MS TITLE: Roles of developmentally regulated KIF2A alternative isoforms in cortical neuron migration and differentiation

AUTHORS: Cansu Akkaya, Dila Atak, Altug Kamacioglu, Busra Aytul Akarlar, Gokhan Guner, Efil Bayam, Ali Cihan Taskin, Nurhan Ozlu, and Gulayse Ince-Dunn
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This manuscript describes the roles of Kif2a splice variants as well as disease-associated Kif2a mutants in neuronal migration, dendrite arborisation and mitochondrial localisation.

Comments for the author

All my concerns have been addressed. The is manuscript suitable for publication.

Reviewer 2

Advance summary and potential significance to field

My request in the last peer review was to see if any of the mitochondrial molecules identified in the interactome would change in KIF2A deficient cells. The authors claim that they were unable to do the suggested experiments due to the severe activity restrictions, and this is understandable. Instead, they have performed new experiments to confirm that mitochondrial distribution is not changed in KIF2A deficient cells in line with the previous findings by other groups.

Comments for the author

As the new data provide neither new findings nor causal links of the phenotypes and genotypes, the results in Figure 8 showing the differential binding of KIF2A isoforms have become more important. Judging from the magnified views in Figure S8 however, I am afraid that they are unable to quantitatively determine the co-localization of KIF2A variants and mitochondrial outer membrane labeled with mCherry-TOMM2. It must be said that the results in Figure 8 are unreliable and may lead to wrong conclusions. Differential binding would be more accurately evaluated by proximity ligation assays and biochemical analyses.