nature portfolio

Peer Review File

Dysregulation of mTOR signaling is a converging mechanism in lissencephaly

Corresponding Author: Dr Angeliki Louvi

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data. This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this manuscript, Zhang et al. defined a clinically relevant molecular mechanism underlying lissencephaly, a major congenital brain malformation often linked to epilepsy and intellectual disabilities. Using an elegant and comprehensive collection of cerebral organoid models (derived from patient lines, mutation-corrected isogenic controls, knock-in lines from different genomic backgrounds), the authors demonstrate common structural and cellular abnormalities (smaller oRG population, increased neurogenesis, disrupted neuronal identity, expanded cortical plate) in organoids from different lissencephaly causing mutations (i.e., newly identified recessive mutations in PIDD1 and chromosome 17p13.3 microdeletion [MDLS]). Importantly, single-cell transcriptomic and LC-MS/MS-based proteomic analysis of these organoids indicate that the common cellular and structural phenotypes triggered by distinct genetic causes of lissencephaly converge onto a common set of molecular mechanisms previously not implicated in lissencephaly, i.e., dysregulation of protein translation, metabolism, and mTOR pathway. Leveraging this finding, the authors show that the hypoactive mTOR pathway in lissencephaly and the associated structural abnormalities can be rescued with a readily administrable, brain-selective mTROC1 activator, NV-5138.
Although the mechanisms of lissencephaly have been explored before, this study is unique in its approach and the diverse organoid, transcriptomic, and proteomic tools it brought to bear on this issue. Overall, this well-designed study with high-quality data defines a clinically addressable common mechanism underlying lissencephaly with diverse genetic underpinnings. It makes a significant contribution to our understanding of the developmental malformations of the cerebral cortex. Addressing the following points, with new data where necessary and possible, will help clarify and strengthen the main conclusions and impact of this work.
Major points:
1. The cerebral organoids used in this study have a much limited cellular repertoire and are lacking some critical cell types essential for brain development (e.g., interneurons, microglia). How this limitation of the organoid model may have affected/skewed the pathway analysis and the potential applicability of these findings to lissencephaly, in general, should be addressed.
2. The rescue effect of NV-5138 was demonstrated only with changes in pS6 immunolabeling intensity and CTIP2+ (and other neuronal marker) organoid cortical plate thickness. Since the rescue of lissencephaly with NV-5138 is a critical point of this manuscript, adding data on the rescue of altered protein translation, metabolism, and mTOR pathways with NV-5138, using transcriptomic and proteomic approaches, will strengthen the main conclusions of this work.
3. Additionally, related to Figure 6, it will be important to demonstrate the NV-5138 effects with organoids from knock-in and patient rescue lines as well.

Minor points:
1. Figure 4, comparing changes between patient vs. control, knock-in, or MDLS organoids: in B and D- ‘oxidative phosphorylation’ is both up and downregulated. In D, ‘myc targets V2’ is both up and downregulated. Same for ‘mTORC1 signaling’ in F. Please clarify this.
2. Indicate five bin demarcations in organoid images of Fig. 2a and c. It will help to clearly demonstrate the effect shown in Fig. 2d.
3. Abstract and discussion are a bit on the long side. The core findings are summarized multiple times in the discussion.

Referee #2

(Remarks to the Author)
ms 2023-09-16741A
Dysregulation of mTOR signaling is a converging mechanism in lissencephaly
Zhang et al.

The authors present an investigation of the molecular pathogenesis of what was reported by coauthors in 2009 as a lissencephaly (smooth brain) phenotype called pachygyria, due to mutation in PIDD1 (P53-induced death domain protein 1). They have made iPSCs from PIDD1 patients (R331X or c.2042-2A>G) and also created isogenic controls by CRISPR repair of the mutations. In addition they used patient iPSCs from a Miller Dieker Lissencephaly Syndrome (MDLS) patient. They produced cortical organoid cultures to interrogate the molecular underpinning of the lissencephaly phenotype. Through expression analyses, they found a connection with dysregulated mTOR signaling and tested it using pharmacologic manipulation.

The experiments seem to be well done, including important aspects as isogenic controls, single cell transcriptomics, in parallel with LC-MS/MS proteomics. Histochemical analyses of the organoids suggest premature neurogenesis, especially of SVZ cells, that depletes those progenitors. They also found the PIDD1 mutations reduced apoptosis in the organoids, with both neurogenesis and cell death effects leading to cortical (CTIP2- and SATB2-labeled neurons) expansion, which they equate to thickening of the cortical mantle in lissencephaly cases. Their extensive expression analyses suggested that mutant cells suffer dysfunction of the mTOR pathway and they show that pharmacologic activation of mTORC1 prevents the morphological and molecular abnormalities in PIDD1 organoids. They interpret their data to indicate mTOR dysregulation is a previously unrecognized contributor to lissencephaly syndromes and that the mTOR data provide an avenue toward disease specific treatment of rare lissencephalies.

There are a number of concerns regarding author interpretations that need to be addressed.

1. Is the phenotype really lissencephaly. The 3 MR images in Supplementary figure 1 of patients with PIDD1 R331X, W589X, and c.2042-2A>G variants do not appear to manifest a classical lissencephaly phenotype, but are more in line with a simplified gyral pattern. The frontal gyri in patient R331X may have a thickened cortical grey (elsewhere it looks pretty normal), but this is often misleading in frontal gyri. It would take 1mm slices and several cuts above and below that shown as well as additional imaging sequences to ensure it is not apparent thickening due to polymicrogyria (PMG) or simply the imaging sequence used. It is all the more suspicious, when the W589X patient appears to have a patch of PMG in the left fronto-parietal cortex. With the diffuse white matter abnormality in the splice defect patient, it is unclear whether there is grey matter thickening or not. Thus, it is debatable whether this is a lissencephaly condition. It would be prudent to call it a brain malformation phenotype or cortical dysplasia.

2. Interpretation of cortical thickening. The comparable time frame of observation in these models is short relative to cortical development. While the increase in neurogenesis along with reduction in apoptosis observed may well account for a relative thickening of the later generated SATB2+ neurons, the organoids only go to d120. Especially since the SVZ is depleted of progenitors more quickly, one would expect that the overall upper layer neuron numbers would eventually be lower than controls. And what does the morphology of the neurons look like?

3. Comparison to lissencephaly. The authors compare their findings in cortical organoids with a more widely recognized classical lissencephaly, MDLS. While technically a microdeletion syndrome, MDLS results from deletion of a substantial number of contiguous genes, several of which have been shown through comparative genetics to contribute to the severity of the lissencephaly phenotype [1, 2]. In addition to the PAFAH1B1 (LIS1) gene responsible for lissencephaly (tending to manifest as smooth cortex in the occipital pole with frontal pachygyria), genes deleted in the MDLS critical region such as MNT and 14-3-3-epsilon increase the extent of the agyria. If the intent were to compare with a classical lissencephaly, they might better have used cells with a point mutation in or targeted knockout of LIS1 [3]. Therefore the effects of mTOR pathway function may have more to do with other genes in the MDLS critical region than lissencephaly per se.

4. Novelty. Leaving that aside, it has been recognized for many years now that lissencephaly associated with LIS1 deficiency is not a pure migration problem, but overlaps with dysregulated spindle orientation, cytokinesis, cell polarity and cortical progenitor division (for recent example [4]). Authors provide evidence that PIDD1 mutations similarly alter expected cleavage plane orientation of dividing progenitors. It is not surprising then, that several pathways, among them mTOR, may recapitulate cellular level aspects of a lissencephaly/cortical malformation phenotype.

5. Therapeutic role for mTOR activation. The comment made in several locations in the text that pharmacologic activation of mTOR signaling may prove therapeutically useful for these patients is unduly optimistic. The critical window for significant intervention would be when cortical neurogenesis is going on in utero, and there are many events emerging in that period that are sensitive to alterations in mTOR pathway function. Indeed somatic mutational overactivity of mTOR signaling during corticogenesis is known to cause hemimegalencephaly or focal cortical dysplasia (FCD) [5, 6] and activating this pathway may do more harm than good. For couples carrying this recessively inherited PIDD1 mutation, preimplantation genetic diagnosis would be the most effective option. Authors should refrain from making statements regarding therapeutic potential for their mTOR link.

1. Leventer, R.J., et al., LIS1 missense mutations cause milder lissencephaly phenotypes including a child with normal IQ. Neurology, 2001. 57(3): p. 416-22.

2. Cardoso, C., et al., The location and type of mutation predict malformation severity in isolated lissencephaly caused by abnormalities within the LIS1 gene. Human Molecular Genetics, 2000. 9(20): p. 3019-3028.
3. Lo Nigro, C., et al., Point mutations and an intragenic deletion in LIS1, the lissencephaly causative gene in isolated lissencephaly sequence and Miller-Dieker syndrome. Hum Mol Genet, 1997. 6(2): p. 157-64.
4. Moon, H.M., et al., LIS1 determines cleavage plane positioning by regulating actomyosin-mediated cell membrane contractility. Elife, 2020. 9.
5. Lee, J.H., et al., De novo somatic mutations in components of the PI3K-AKT3-mTOR pathway cause hemimegalencephaly. Nat Genet, 2012. 44(8): p. 941-5.
6. Marin-Valencia, I., R. Guerrini, and J.G. Gleeson, Pathogenetic mechanisms of focal cortical dysplasia. Epilepsia, 2014. 55(7): p. 970-8.

Referee #3

(Remarks to the Author)
The authors have studied a new causative gene, PIDD1, in lissencephaly patients. Through the use of cerebral organoid models, they have demonstrated that PIDD1 deficiency leads to increased cortical thickness and layer disorganization in comparison to patient-rescue and control organoids. Notably, the combination of transcriptomic and proteomic data strongly supports the role of the mTOR pathway in influencing protein translation not only in PIDD1-deficient organoids but also in MDS organoids. By employing the mTOR activator NV-5138, the authors were able to prevent cortical thickening in PIDD1-deficient and MDS organoids. This discovery of the mTOR pathway's involvement in PIDD1-related and MDS-related lissencephaly holds promise for potential therapeutic options for these patients.
Major points:
In my opinion, conducting scRNAseq experiments following treatment with mTOR activators, along with performing functional assays like MEA or calcium imaging, would enhance the clinical relevance of the model.
Furthermore, I would recommend that several result panels be presented in a clearer way.
Specific Comments:
• With regard to the WES data and genetic investigation, it is advisable to provide a clearer distinction between what has previously been reported and what constitutes novel findings in this study. It appears that the genetic findings may have been previously published, which could warrant their relocation to the introduction section rather than the results section.
• In addition to the six patients with the described PIDD1 variants, have the authors explored other databases to identify additional cases with PIDD1 variants?
• Is it possible that the R331X mutation is predicted to trigger NMD-mediated transcript degradation? This aspect should be better discussed.
• Did the authors conclude on putative differences in genomic backgrounds and establish genotype-phenotype causality?
• The predicted impact of mutations on various domains and protein forms (PIDD1, PIDD1-C, PIDD1-CC) is not straightforward (the figure should be clarified). It would be helpful to clarify the expected effects of increased cell repair versus apoptosis, particularly since the death domain is part of PIDD1-C. Is there a potential link to the enrichment of "DNA repair" clusters in the proteomics analyses, as shown in Extended Data Fig. 1c-d (please clarify in Supplementary Figure 1d)?
• In Fig.1/2b-d-e, there is a lack of statistical comparison between patients and patient rescue (also in general in the ms). This information should be added.
• Fig.1a: The authors show a reduction in the number of Cleaved Caspase-3 (CC3)+ neural progenitor cells. At day 70, and even more so at day 50, this reduction seems to affect especially SVZ. Given that the SVZ houses oRG and IPCs, it would be valuable to determine which of these cell populations are impacted by the reduced apoptosis. To address this, conducting a co-labeling experiment that combines cleaved caspase with hopx, tbr2, and dapi could offer insights and help address this question.
• The authors demonstrate a reduction in the number of oRG (hopx+ cells) and mTOR hypoactivity in the PIDD1 and MDS organoids. A prior study (Cell Stem Cell 20, 435–449) has shown the crucial role of oRG in MDS and their sensitivity to mTOR alterations. It would be particularly interesting to quantitatively assess mTORC1 activity in oRG and the number of oRG when treated with and without the mTOR activator.
• In Extended Data Fig. 5c,d, the authors observe an increase in horizontal (asymmetric) divisions and a decrease in vertical divisions. Does this data suggest a premature increase in neurogenesis at the expense of progenitor cell numbers?
(1) Quantification should be done in the same way in all cell types (either DAPI+ counting or per mm2).
(2) If an increase in neurogenesis is anticipated, quantifying the total number of neurons, for instance, using Tuj1 staining, should be conducted. Furthermore, the authors note that the number of CTIP2 cells remains unaffected while the number of SATB2 cells increases. Since the latter cells are formed at a later stage, how does this align with the concept of premature neurogenesis?
• The specific persistence of PIDD1 expression in the patient's radial glia (RG) requires a more clearly articulated interpretation. The data presentation should be improved for better clarity.
• In Fig. 2c-e, the authors mention that the "layering" of the cortical plate is disrupted. Visually, it is evident that CTIP2 and SATB2 staining appears more dispersed, with SATB2 cells seemingly forming a second layer at D120. This suggests the possibility of migration defects. Is this phenomenon rescued by the mTOR activator NV-5138?
• In Supp Fig. 6e, the authors mention that a smaller proportion of CTIP2+ and SATB2+ cells is present in Patient and KI organoids, but the graph appears to show the opposite trend. Additionally, there is a missing legend for the X-axis.
• Cortical thickness and abnormal cortical folding are pivotal characteristics of lissencephaly and MDS. Recent advancements in the field of brain organoids have highlighted the ability to induce folding in cerebral organoids (doi: 10.4103/1673-5374.335789). To enhance their study, the authors might consider incorporating one of these engineering techniques to visually illustrate alterations in cerebral folding, thereby adding a valuable dimension to their research or at least referencing this development in the discussion.

• It is greatly appreciated that the authors generated appropriate control organoids, including a mutation-corrected version of the patient-derived hiPSC and a KI introduced in the control iPSC. However, in the manuscript, there is a tendency to compare the patient organoids with the control organoids rather than with the patient-rescue organoids. Even though significant differences in cellular analyses between the control and patient rescue organoids are not observed, it would be sensible to include these comparisons. Interestingly, in the sc-RNA sequencing analysis (Fig. 3d, GOBP analysis of upregulated DEGs in RG and oRG), the enrichment of terms related to oxidative phosphorylation appears to be specific to comparisons with the control organoids and absent in the comparison of patient vs. patient rescue organoids, so it may not warrant significant emphasis.
• In Fig. 3, the authors have primarily focused on the analysis of oRG and RG scRNA-seq data. However, Fig. 3d suggests potentially interesting differences in IPs as well. It may be worthwhile to include this analysis in the manuscript or provide it as supplementary data.
• Fig. 3d: Given that the mutation is a stop gain, it is expected that the PIDD1 transcript would be subject to degradation by NMD. However, some cell types express it. Is there a predicted cell-type-specific action of NMD? Please discuss this point.
• In Fig. 3f, the comparison presented lacks clarity. It is unclear which PIDD1-expressing versus non-expressing cell types were compared and how the data are presented (e.g., pseudo-bulk). What is the rationale for this approach, and what are the potential interpretations or biases?
• In Fig. 3f, the authors state that "downregulated GO biological processes for mutant PIDD1-expressing cells were related to neurogenesis, neuron development, and neuron differentiation," but Fig. 3f seems to show terms related to metabolic processes. Please discuss this subtle inconsistency.
• Regarding the dysregulated mTOR signaling, the comparisons are not clearly explained.
(1) In the cortical plate (CP) of the brain organoids, the pS6 staining appears to be less intense in the patients and MDS organoids. Could the authors also quantify this observation?
(2) Have the authors found any evidence supporting the same phenomenon in human patients with lissencephaly/MDS, such as previously published RNA-seq data or postmortem brain samples of MDS patients?
• Regarding the MTOR activator as a potential therapeutic target for lissencephaly patients, the authors have demonstrated the drug's ability to prevent cortical thickening. Do they have data that supports a curative effect of the drug, for example, by administering treatment at D50–70 and assessing cortical thickness at D120? Moreover, since epilepsy is a major clinical manifestation and mTOR overactivity is known to play a role in epilepsy, please discuss what is the predicted effect on seizures? In vitro, testing with functional readouts such as MEA or calcium imaging would be needed to assess potential effects on neuronal hyperexcitability.
• Important experiments appear to be missing: scRNA-seq data after NV-5138 rescue.
Minor comments:
• The term "genetic causation" is used multiple times in the text. I believe this term may not be commonly utilized.
• Text on page 12: Add "PIDD1-expressing" to the sentence, "Strikingly, lissencephaly was the second most significant disease process enriched for differentially expressed genes (DEGs) that were downregulated in patients vs. control organoids."
• Text p13: "Furthermore, we identified 848 DEGs in patient vs. control organoids in scRNA-seq (oRG cell-type) (…) mass spectrometry datasets.". Please include a brief sentence explaining why selecting the oRGs sc-RNA seq dataset.
• Fig. 2b: Could the authors provide a more detailed explanation of what is meant by "relative thickness (CTIP2)"? It is clear for the CP, but it is not evident how CTIP2 staining helps quantify the thickness of the SVZ and VZ. The description in the methods section lacks clarity..
• Fig. 3: For the scRNA-seq data, a color scale with a stronger contrast between positive and negative average expression would improve the figure's interpretability.
• Fig. 3: Consider placing figure 3e after figure 3f.
• Fig. 6c: In the MDLS organoids in the SVZ, the color is brown instead of grey.
• Some of the images and graphs could be transitioned from the supplementary figures to the main figures. For example, none of the information about the patients or PIDD1 expression is found in the main figures.
• Supp Fig. 3j: The method description for this heterologous expression experiment appears to be missing. It would be helpful to explain the significance of V5/6His.
• Supp figure 3k: Please provide better annotations for the figure (PIDD1-FL, PIDD1-C, PIDD1-CC, and other bands). Alternatively, consider semi-quantitative analysis and include a bar plot for clearer interpretation.
• Supplementary Figure 6e-f: The "+" and "-" symbols are small, and the y-axis legend is missing in Supplementary Figure 6e. In Figure 6f, simplifying the representation of double-stained cells might facilitate interpretation.
• Supplementary Figure 7a: Write out the abbreviations in full in the figure legends.
• Supplementary Figure 7c: Consider capturing a subpart of the fetal cortical tissue with higher magnification and adding it to this figure for improved visualization of the in situ hybridization result.
• Supplementary Figure 9b: Include the quantification of pS6+/Hopx+ and pS6+/Tbr2+ cells.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
Appropriately revised to address all the major concerns with additional new data.

One minor point: regarding the same pathways that are both up and down regulated in the bioinformatic analyses in Figure

4- the authors may want to annotate the figure legend to indicate that different members of the same pathway are up or down regulated, thus a given pathway can appear both up- and down-regulated in the bioinformatic analysis.

Referee #2

(Remarks to the Author)
Referee #2 (Remarks to the Author):

In their revised manuscript, the authors present additional data and further clarification of their rationale for interpretations is provided. On the whole, it continues to be an interesting manuscript, though there are several points of interpretation that should be further modified as, in this reviewer's opinion, they are overstated.

Reviewer point 1. Is the phenotype really lissencephaly. The 3 MR images in Supplementary figure 1 of patients with PIDD1 R331X, W589X, and c.2042-2A>G variants do not appear to manifest a classical lissencephaly phenotype, but are more in line with a simplified gyral pattern. The frontal gyri in patient R331X may have a thickened cortical grey (elsewhere it looks pretty normal), but this is often misleading in frontal gyri. It would take 1mm slices and several cuts above and below that shown as well as additional imaging sequences to ensure it is not apparent thickening due to polymicrogyria (PMG) or simply the imaging sequence used. It is all the more suspicious, when the W589X patient appears to have a patch of PMG in the left fronto-parietal cortex. With the diffuse white matter abnormality in the splice defect patient, it is unclear whether there is grey matter thickening or not. Thus, it is debatable whether this is a lissencephaly condition. It would be prudent to call it a brain malformation phenotype or cortical dysplasia.

Author response. Thank you for this comment. We revised Extended Data Fig. 1 to include additional images of the index patient (NG375-1) of the NG375 family, who donated somatic cells from which the iPSCs and organoids analyzed in our study were derived. The patient presented with bilateral diffuse pachygyria and enlarged ventricles; the upper and middle gyri in the temporal lobes are diffusely and symmetrically thickened and smooth; no microgyria was observed. The sister of the index patient also displayed a similar, albeit more subtle, pattern of bilateral fronto-temporal pachygyria. Extended Data Fig. 2 contains additional imaging sequences from the patients in our families.

Respectfully, we note that the lissencephaly spectrum comprises a continuum of structural brain abnormalities. Indeed, the patterns of malformations seen with the multiple (>19) genes causally associated with lissencephaly spectrum are increasingly diverse, prompting an update in the imaging and clinical classification of lissencephaly, which also includes patients with malformations intermediate between lissencephaly and polymicrogyria (dysgyria) (Di Donato 2017, PMID: 28440899).

Consistent with the appreciated phenotypic diversity of lissencephaly spectrum, previously reported neuroradiological characteristics of patients with validated PIDD1 mutations (Zaki et al., 2021 PMID: 34163010; Sheikh et al., 2021 PMID: 33414379) include predominant anterior pachygyria, dysgyria/simplified gyral pattern (Zaki et. al, 2021), and subtle smooth gyration and shallow sulcation affecting the frontal and temporal lobes, compatible with lissencephaly (Sheikh et al., 2021). Reports of patients harboring mutations in CRADD, an established direct interactor of PIDD1 in the tripartite CASP2-PIDDosome protein complex, describe a "thin lissencephaly" variant (TLIS) of relatively mild lissencephaly characterized by megalencephaly, anterior-predominant pachygyria with shallow and unusually wide sulci and mildly thick cortex especially in the frontal regions (Di Donato et al., 2016; PMID: 27773430), fronto-temporal predominant pachygyria (Polla et al., 2019; PMID: 30914828), hypogyria with frontal predominance, and delayed cortical sulcation suggestive of lissencephaly (Harel et al., 2017; PMID: 28686357). Finally, a recent study (published when our manuscript was under review) reports recessive truncating variants in CASP2 (the third member of the CASP2-PIDDosome) in individuals with lissencephaly, with neuroimaging revealing bilateral pachygyria and mild cortical thickening in the frontotemporal lobes (Uctepe et al., 2023; PMID: 37880421). For these reasons, the patients identified in our study are within the diverse lissencephaly spectrum.

Considered together, the neuroradiological findings in patients with recessive mutations in the three genes encoding the core proteins of the CASP2-PIDDosome (PIDD1, CRADD, and CASP2) support their classification in the lissencephaly spectrum.

Reviewer 2 response: The authors are thanked for their detailed review, in which they reinforce the point of the original comment. To describe a phenotype simply as "lissencephaly" tends to undercut the highly heterogeneous nature of the term and implies the more classical appearance of highly thickened cortex—whether agyric or pachygyric. The images provided in this manuscript are quite variable and include multiple forms of dysgyria, with either thickened or thinned cortical gray matter, asymmetric ventricles, areas of polymicrogyria, etc. It would be better for the phenotype to be described as dysgyria, but since earlier manuscripts have used the term lissencephaly for mutations encompassing the PDD1 region, the authors for this paper should refer to the phenotype as a "lissencephaly spectrum disorder" or "cortical dysplasia in the lissencephaly spectrum".
In addition, the abstract acknowledges that lissencephaly spectrum comprises a group of genetically heterogeneous congenital brain malformations. In the next sentence (starting line 96) they state incorrectly that the cellular pathogenesis is thought to be defective neuronal migration. Substantial literature includes mechanisms such as progenitor proliferation defects, extracellular signaling and matrix abnormalities. In fact, this manuscript does not touch on migration abnormalities at all. It is recommended that authors either omit the sentence or replace with something like: "The cellular pathogenesis is multi-factorial and may encompass abnormalities of progenitor proliferation and survival, displacement of proliferation zones, neuronal migration and guidance mechanisms."

Reviewer point 2. Interpretation of cortical thickening. The comparable time frame of observation in these models is short relative to cortical development. While the increase in neurogenesis along with reduction in apoptosis observed may well account for a relative thickening of the later generated SATB2+ neurons, the organoids only go to d120. Especially since the SVZ is depleted of progenitors more quickly, one would expect that the overall upper layer neuron numbers would eventually be lower than controls. And what does the morphology of the neurons look like?

Author response. Thank you for this comment. We respectfully note that 120 days in culture, even though short relative to fetal cortical development, represents a significant period for organoid modeling of structural brain abnormalities.

Relevant previous studies employing organoid models to investigate structural brain disorders performed analyses at significantly earlier stages of organoid differentiation:

a) day 22 (Lancaster et al., 2013; PMID: 23995685; CDK5RAP2-associated microcephaly)
b) day 22 (Krenn et al., 2021; PMID: 33838105; virus-induced microcephaly)
c) day 25 (Iefremova et al., 2017; PMID: 28380362; Miller-Dieker lissencephaly syndrome)
d) day 30 (Dell'Amico et al., 2023; PMID: 37272619; WDR62-associated microcephaly)
e) day 35 (Bershteyn et al., 2017; PMID: 28111201; Miller-Dieker lissencephaly syndrome).

The studies yielded insight into the cellular mechanisms underlying these structural brain malformations, even though the analyses were confined to early stages of organoid differentiation. Considering these previous studies, day 70 and day 120 represent significantly more advanced timepoints of analysis. In this context, it is also worth noting that a recent study demonstrated no further utility in extending time in culture beyond 120 days (i.e., to 150 days) when whole organoids are used as structural brain disease models, as cortical organization does not improve after the 120-day timepoint (Qian et al., 2020; PMID: 32142682). Considering current practice in the field and these findings, we performed most of our analyses at days 70 and 120. Cortical thickening was obvious already at day 70 and remained a striking feature of day 120 organoids.

The question of whether there would be more upper layer neurons in control is indeed interesting but would require another advancement in cerebral organoid technology (e.g., organoids with expanded oSVZ) to test experimentally. To answer the question of neuronal morphology, we performed Golgi-Cox staining of organoids at D120. These qualitative results are presented in Extended Data Fig. 6g.

Reviewer 2 response: The authors are thanked for their defense of 3-D organoids as an investigative system. There is no doubt that this in vitro method is relevant to pursue mechanisms that impact progenitor proliferation/survival and early neural specification. It is these aspects of cell biology that their observations relating to mTOR signaling appears to influence. The appropriate use of cortical organoids in mechanistic investigations is certainly supported by the 5 studies that the authors list in response to the comment, each of which interrogates aspects of radial glial cell, oRG, and intermediate progenitor proliferation and differentiation. Nevertheless, now relatively standard organoid protocols do not adequately model other aspects of cortical neogenesis, including neuronal migration, neurite elaboration, generation of multiple cell types including but not limited to interneurons and microglia. Therefore, the contribution of mTOR signaling in the clinical syndromes associated with lissencephaly spectrum disorders is far from settled and will likely vary among the disorders associated with now nearly 2 dozen different genes. Furthermore, the study has investigated only 2 genetic associations with a lissencephaly spectrum phenotype, when there are at least 17 more. The manuscript should avoid over-generalization of the mTOR pathway importance to dysgyria in the clinical picture and should avoid language that implies that mTOR is now established as a final common pathway to lissencephaly.
The inclusion of Golgi-cox stains in the extended data figure 6g is appreciated, but it is far too limited to draw an inference much less a conclusion about the contribution of neuritogenesis in the variably observed cortical thickening associated with PIDD1-lissencephaly spectrum

Reviewer point 3. Comparison to lissencephaly. The authors compare their findings in cortical organoids with a more widely recognized classical lissencephaly, MDLS. While technically a microdeletion syndrome, MDLS results from deletion of a substantial number of contiguous genes, several of which have been shown through comparative genetics to contribute to the severity of the lissencephaly phenotype [1, 2]. In addition to the PAFAH1B1 (LIS1) gene responsible for lissencephaly (tending to manifest as smooth cortex in the occipital pole with frontal pachygyria), genes deleted in the MDLS critical region such as MNT and 14-3-3-epsilon increase the extent of the agyria. If the intent were to compare with a classical lissencephaly, they might better have used cells with a point mutation in or targeted knockout of LIS1 [3]. Therefore, the effects of mTOR pathway function may have more to do with other genes in the MDLS critical region than lissencephaly per se.

Author response. Thank you for this comment. It is correct that the microdeletion region of chromosome 17p13.3 contains several genes that have been associated with lissencephaly. By extending our analyses beyond PIDD1-associated lissencephaly, we intended to investigate whether mTOR pathway dysregulation represents a converging molecular mechanism in lissencephaly spectrum, despite differences in the underlying genetic lesions.

We studied MDLS for the following reasons:

a) MDLS was the first identified genetic cause of severe lissencephaly (Dobyns et al., 1983; PMID: 6834189)
b) Abnormally thick cortex is a common characteristic of MDLS (Dobyns 1991 et al.; PMID 1671808; Dobyns et al., 2003;

PMID 7607669) and PIDD1-lissencephaly (herein and previously reported patients with “PIDD1-lissencephaly” [please see our response to point 1 above for details])
c) We elected to utilize patient-derived iPSCs, rather than generating a point mutation in LIS1 by gene editing, because, in our view, patient material is preferable over genetic manipulation in a non-patient line, and, to the best of our knowledge, iPSCs (or somatic cells/fibroblasts) from patients with LIS1 (or other lissencephaly-associated genes) are not available in public repositories.
d) MDLS organoids (Iefremova et al., 2017; PMID: 28380362; Bershteyn et al., 2017; PMID: 28111201) and PIDD1 organoids (our study) share cellular abnormalities (e.g., increased horizontal divisions and enhanced neurogenesis). The observations reported in these earlier studies were critical, as they validated MDLS organoids as the optimal experimental system modeling lissencephaly in which to test the generality of our findings.

Our analyses of MDLS organoids demonstrated downregulation of mTOR signaling within the progenitor zones. In other words, MDLS and PIDD1-mutant organoids share not only cellular, but also molecular phenotypes. This finding suggested to us that despite their different genetic causes, MDLS and “PIDD1-lissencephaly” both converge to a common molecular mechanism.

Reviewer 2 response. Somewhat related to point 2 above, it is well established that LIS1 deficiency alters cortical progenitor proliferation and radial glial cleavage plane orientation and that mTOR signaling impacts progenitor proliferation in the setting of LIS1 deficiency. Previous studies, including a recent investigation in iPSCs (Moon et al., 2020, doi: 10.7554/eLife.51512) indicate the LIS1 effect is mediated through perturbation of its role in microtubule stability, dynein protein motor function or localization, mitotic spindle orientation, chromosomal segregation, and nuclear migration. Use of proliferation outcomes is a relatively crude way of examining molecular mechanisms and any manipulation (such as of the mTOR pathway) that can enhance or suppress progenitor proliferation may mimic or compensate for a mutation-associated phenotype such as cell division. However, there are published observations that mTOR dysregulation has consequences for oRG proliferation in an organoid model of MDLS (the Bershteyn et al., 2017article authors mention above). The present work indicates an impact of PDD1 mutation on mitotic cleavage plane and oRG proliferation. Thus, demonstration of similar sensitivity of their PIDD1 and MDLS organoids to NV-5138 mTOR activator is not particularly indicative of the “generalizability” of the mTOR pathway to lissencephaly. The claim of generalizability and of a final common pathway to lissencephaly is not proven in this study.

Reviewer point 4. Novelty. Leaving that aside, it has been recognized for many years now that lissencephaly associated with LIS1 deficiency is not a pure migration problem, but overlaps with dysregulated spindle orientation, cytokinesis, cell polarity and cortical progenitor division (for recent example [4]). Authors provide evidence that PIDD1 mutations similarly alter expected cleavage plane orientation of dividing progenitors. It is not surprising then, that several pathways, among them mTOR, may recapitulate cellular level aspects of a lissencephaly/cortical malformation phenotype.

Author response. We respectfully disagree with this assessment. Hypoactivity of mTOR signaling has not been associated with lissencephaly previously and is reported here for the first time. More importantly, though, the novelty of our study lies in the finding that mTOR pathway dysregulation is a converging molecular mechanism in lissencephaly spectrum of diverse genetic causation. We also provide a thorough and unbiased characterization of patient-derived lissencephaly organoids by single-cell transcriptomics and mass spectrometry, followed by orthogonal validation of the findings, as well as pharmacological manipulation of mTORC1 signaling to rescue the observed phenotypes, a comprehensive approach never undertaken for lissencephaly.

We believe that the identification of a common downstream pathway in lissencephaly significantly enhances our understanding of this heterogenous spectrum of severe cortical malformations and serves as a starting point for further mechanistic study. Moreover, our findings extend mTORopathies, classically considered to be malformations of cortical development underlain by hyperactivation of mTOR signaling (Crino 2015; PMID: 25833943), to include the lissencephaly spectrum converging to hypoactivation of mTOR signaling, thereby providing strong evidence that mTOR pathway dysregulation (hyper- or hypo-activity) is detrimental to human brain development.

Reviewer 2 response. The authors themselves make the point that critical molecular signaling pathways often have a bimodal relationship to important developmental events. Thus, just as hyperactivity of a crucial pathway may result in brain malformation, reduced activity of the same pathway may be expected to have deleterious consequences. This appears to be the case in their examples of PIDD mutations. It is not a compelling insight to provide additional evidence that mTOR dysregulation is detrimental to brain development. The value of the present manuscript is in the multi-omic investigation of transcript and protein expression changes in the wake of these mutations.
I take exception to authors' comment here that “… the novelty of our study lies in the finding that mTOR pathway dysregulation is a converging molecular mechanism in lissencephaly spectrum of diverse genetic causation.” The finding that pharmacologic manipulation of mTOR activity can mitigate progenitor proliferation abnormalities in two genetic conditions—the second comparative example having been selected precisely because of previously demonstrated mTOR dysfunction sensitivity of proliferation in mutant progenitors—does not make this a unifying mechanism in lissencephaly spectrum disorders any more than downstream consequences for GTPase dysregulation or HIPPO pathway dysfunction constitutes a final common pathway for this malformation spectrum.

I recommend that the language in the abstract be modified to replace the last sentence with something like: “These findings illuminate an unexpected molecular mechanism contributing to several genetically caused cortical malformations in the

lissencephaly spectrum."

This also would require a modification of the title of the article replacing "Dysregulation of mTOR signaling is a converging mechanism in lissencephaly" with something like "Dysreguation of mTOR signaling contributes to several genetically caused malformations in the lissencephaly spectrum"

5. Therapeutic role for mTOR activation. The comment made in several locations in the text that pharmacologic activation of mTOR signaling may prove therapeutically useful for these patients is unduly optimistic. The critical window for significant intervention would be when cortical neurogenesis is going on in utero, and there are many events emerging in that period that are sensitive to alterations in mTOR pathway function. Indeed somatic mutational overactivity of mTOR signaling during corticogenesis is known to cause hemimegalencephaly or focal cortical dysplasia (FCD) [5, 6] and activating this pathway may do more harm than good. For couples carrying this recessively inherited PIDD1 mutation, preimplantation genetic diagnosis would be the most effective option. Authors should refrain from making statements regarding therapeutic potential for their mTOR link.

Thank you for this remark. We agree that the critical period for intervention would be in-utero; we are not suggesting that our findings can be directly and immediately translated to clinical trials. Rather, we posit that the identification of a molecular mechanism represents the first step for exploring a potential (and targeted) therapeutic intervention. Even though mTOR activation is known to disrupt cortical development (e.g., as the referee notes, mosaic somatic activation in hemimegalencephaly and focal cortical dysplasia), one could envision a scenario in which activation of mTOR pathway is carefully calibrated in the setting of an inherited recessive disorder, in which pathway hypoactivity is global. This aside, the words of caution by the referee are much appreciated, prompting us to moderate statements of immediate therapeutic application throughout the manuscript and in the discussion and to remove references to potential pre- or postnatal clinical intervention to treat lissencephaly of various genetic etiologies.

Reviewer 2 response. The authors are thanked for their moderation of statements implying immediate therapeutic application of mTOR activation as a treatment strategy in these lissencephaly spectrum disorders. This should include modification of the abstract last sentence as recommended in point 4 above.

Version 3:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors are thanked for the further elaboration of their position on concerns regarding the messaging of their studies. There is probably little to be gained by additional debate, as the authors appear to be unwilling to further modify their implied significance of the outcomes.

In the interest of accuracy, they should refer to PIDD1 as associated with cortical malformation in the lissencephaly spectrum or as a lissencephaly spectrum disorder, much as gene mutations leading to autistic behaviors are termed associated with autism spectrum disorder.

The authors contend that they do not in fact assert that mTOR signaling is a final common pathway to lissencephaly. But the use of the term "converging mechanism" will be construed as exactly that…a common final pathway. The "unrelated" lissencephaly they use for comparison to PIDD1 was, by the authors' admission, chosen because of cellular proliferation commonalities of the two conditions. If they insist on using the term converging in the title they should indicate that mTOR dysregulation is a convergent mechanism for two genes associated [or two distinct gene associations] with lissencephaly spectrum. The final sentence in the abstract should be similarly modified.

The authors see the novelty of their findings in the insight that mTOR hypoactivity can produce cortical thickening. Why don't they take that as their lead message in the title and abstract that hypoactive mTOR signaling is associated with proliferation phenotypes in two lissencephaly spectrum disorders.

They can still speculate in the discussion that it is yet to be determined whether mTOR signaling dysregulation is a common contributor to a broad swath of malformations in the lissencephaly spectrum. Given the heterogeneity of lissencephaly malformations at the cellular level, that seems unlikely. But these kinds of assertions based on limited observations (only 2 genotypes) often lead to generalized assumptions in the field that are challenging to overcome once ingrained in the discipline.

Dear Editor,

Thank you for inviting us to resubmit our manuscript. We wish to thank our referees for their time and insightful comments and suggestions. We have performed additional experiments to address the referees' concerns, which we have incorporated in the manuscript. We believe that the revised manuscript is now much stronger.

Please find below our point-by-point response to the points raised by the referees.

Referee #1 (Remarks to the Author):

In this manuscript, Zhang et al. defined a clinically relevant molecular mechanism underlying lissencephaly, a major congenital brain malformation often linked to epilepsy and intellectual disabilities. Using an elegant and comprehensive collection of cerebral organoid models (derived from patient lines, mutation-corrected isogenic controls, knock-in lines from different genomic backgrounds), the authors demonstrate common structural and cellular abnormalities (smaller oRG population, increased neurogenesis, disrupted neuronal identity, expanded cortical plate) in organoids from different lissencephaly causing mutations (i.e., newly identified recessive mutations in PIDD1 and chromosome 17p13.3 microdeletion [MDLS]). Importantly, single-cell transcriptomic and LC-MS/MS-based proteomic analysis of these organoids indicate that the common cellular and structural phenotypes triggered by distinct genetic causes of lissencephaly converge onto a common set of molecular mechanisms previously not implicated in lissencephaly, i.e., dysregulation of protein translation, metabolism, and mTOR pathway. Leveraging this finding, the authors show that the hypoactive mTOR pathway in lissencephaly and the associated structural abnormalities can be rescued with a readily administrable, brain-selective mTROC1 activator, NV-5138.

Although the mechanisms of lissencephaly have been explored before, this study is unique in its approach and the diverse organoid, transcriptomic, and proteomic tools it brought to bear on this issue. Overall, this well-designed study with high-quality data defines a clinically addressable common mechanism underlying lissencephaly with diverse genetic underpinnings. It makes a significant contribution to our understanding of the developmental malformations of the cerebral cortex. Addressing the following points, with new data where necessary and possible, will help clarify and strengthen the main conclusions and impact of this work.

Major points:

1. The cerebral organoids used in this study have a much-limited cellular repertoire and are lacking some critical cell types essential for brain development (e.g., interneurons, microglia). How this limitation of the organoid model may have affected/skewed the pathway analysis and the potential applicability of these findings to lissencephaly, in general, should be addressed.

Thank you for this point. It is, indeed, true that the organoids have a much-limited cell repertoire at the stages analyzed, and prompted by your remark, we discuss this limitation in the revised manuscript (please see Discussion). However, because lissencephaly is a neurodevelopmental

disorder first detected in utero, at early stages of fetal brain development (cellular abnormalities have been reported to occur as early as 10-14 gestational weeks [Dobyns 1991; PMID: 1671808] and prenatal MRI can detect cortical thickening by 27-28 gestational weeks [Lerman-Sagie et al., 2021; PMID: 34390998]), it is likely that neural progenitors represent the earliest cell type affected. For this reason, organoids are still a valuable model system in which to study the early stages of (ab)normal fetal brain development.

2. The rescue effect of NV-5138 was demonstrated only with changes in pS6 immunolabeling intensity and CTIP2+ (and other neuronal marker) organoid cortical plate thickness. Since the rescue of lissencephaly with NV-5138 is a critical point of this manuscript, adding data on the rescue of altered protein translation, metabolism, and mTOR pathways with NV-5138, using transcriptomic and proteomic approaches, will strengthen the main conclusions of this work.

Thank you for this comment. We now include single-cell RNA-Seq data from control, patient, and MDLS organoids treated with NV-5138 in Extended Data Fig 10. These results demonstrate changes in RG and oRG of C, P, and MDLS organoids following treatment with NV-5138.

In more detail, treatment with NV-5138 decreases neuron-related gene expression in RG and oRG of C, P, and MDLS organoids compared to untreated counterparts. This is consistent with the results of immunostaining of drug-treated organoids. In addition, treatment with NV-5138 leads to upregulation of mTOR signaling and translation/metabolism-related GOs, consistent with the known role of mTORC1 in translation and metabolism. Compared to RG and oRG of untreated control organoids, P and MDLS counterparts treated with NV-5138 display upregulation of mTOR, metabolism, and translation (please see Extended Data Fig. 10g), consistent with our findings detailed in Fig. 3.

We note that NV-5138 treatment does not alter the gene expression profile of P or MDLS organoids relative to control counterparts (please see Extended Data Fig. 10); mTOR, translation, and metabolic pathway gene expression remains upregulated. This is not surprising to us since the scRNA-seq data show that the drug directly upregulates translation/metabolism GOs and given the mode of action of NV-5138 (Sengupta et al., 2019; PMID: 30858438). Briefly, NV-5138 acts by directly binding to Sestrin 2, a stress-inducible protein that impacts multiple biological processes and signaling pathways, including mTORC1, AMP-activated protein kinase (AMPK), insulin/AKT, and redox signaling (Kim et al., 2021; PMID: 33113341). In the context of mTOR signaling, by directly binding to Sestrin 2, NV-5138 facilitates the dissociation of Sestrin 2 from GATOR2, and subsequently activates mTORC1. In the absence of NV-5138, Sestrin 2 direct binding to GATOR2 releases the GTPase-activating protein GATOR1 from GATOR2-mediated inhibition; in turn, this results in GATOR1-mediated inhibition of Rag GTPases, and subsequently, inhibition of mTORC1. We note that Sestrins are also thought to inhibit mTORC1 signaling via activation of AMPK and Tuberous Sclerosis Complex (TSC).

In response to this comment, we now provide details on NV-5138, in addition to presenting and discussing the new single-cell transcriptomic data.

3. Additionally, related to Figure 6, it will be important to demonstrate the NV-5138 effects with organoids from knock-in and patient rescue lines as well.

Thank you for this suggestion, these data are now presented in the revised Fig. 6.

Minor points:
1. Figure 4, comparing changes between patient vs. control, knock-in, or MDLS organoids: in B and D- 'oxidative phosphorylation' is both up and downregulated. In D, 'myc targets V2' is both up and downregulated. Same for 'mTORC1 signaling' in F. Please clarify this.

This is correct. As these complex pathways comprise multiple members that individually may be either up- or down-regulated, as well as positive and negative feedback loops, pathway enrichment analysis can result in seemingly contradictory findings (i.e., a given pathway can appear to be both up- and down-regulated). In our view, bioinformatic analyses of transcriptomic and proteomic datasets are powerful and unbiased approaches to reveal dysregulated pathways, providing an entry point for experimental validation. We confirmed at the protein level via immunostaining and western blot analyses that mTOR signaling is downregulated.

2. Indicate five bin demarcations in organoid images of Fig. 2a and c. It will help to clearly demonstrate the effect shown in Fig. 2d.

Thank you for this suggestion – the 5-bin demarcation has been added to Figure 2c; the boundaries of CP, SVZ, and VZ, used to calculate the relative thickness of CTIP2-expressing CP, SZV (CTIP2-negative) and SOX2-expressing VZ, were already indicated in Figure 2a.

3. Abstract and discussion are a bit on the long side. The core findings are summarized multiple times in the discussion.

Thank you for this comment. We have shortened the abstract and revised the discussion.

Referee #2 (Remarks to the Author):

The authors present an investigation of the molecular pathogenesis of what was reported by coauthors in 2009 as a lissencephaly (smooth brain) phenotype called pachygyria, due to mutation in PIDD1 (P53-induced death domain protein 1). They have made iPSCs from PIDD1 patients (R331X or c.2042-2A>G) and also created isogenic controls by CRISPR repair of the mutations. In addition they used patient iPSCs from a Miller Dieker Lissencephaly Syndrome (MDLS) patient. They produced cortical organoid cultures to interrogate the molecular underpinning of the lissencephaly phenotype. Through expression analyses, they found a connection with dysregulated mTOR signaling and tested it using pharmacologic manipulation.

The experiments seem to be well done, including important aspects as isogenic controls, single cell transcriptomics, in parallel with LC-MS/MS proteomics. Histochemical analyses of the organoids suggest premature neurogenesis, especially of SVZ cells, that depletes those progenitors. They also found the PIDD1 mutations reduced apoptosis in the organoids, with both neurogenesis and cell death effects leading to cortical (CTIP2- and SATB2-labeled neurons) expansion, which they equate to thickening of the cortical mantle in lissencephaly cases. Their extensive expression analyses suggested that mutant cells suffer dysfunction of the mTOR pathway and they show that pharmacologic activation of mTORC1 prevents the morphological and molecular abnormalities in PIDD1 organoids. They interpret their data to indicate mTOR dysregulation is a previously unrecognized contributor to lissencephaly syndromes and that the mTOR data provide an avenue toward disease specific treatment of rare lissencephalies.

There are a number of concerns regarding author interpretations that need to be addressed.

1. Is the phenotype really lissencephaly. The 3 MR images in Supplementary figure 1 of patients with PIDD1 R331X, W589X, and c.2042-2A>G variants do not appear to manifest a classical lissencephaly phenotype, but are more in line with a simplified gyral pattern. The frontal gyri in patient R331X may have a thickened cortical grey (elsewhere it looks pretty normal), but this is often misleading in frontal gyri. It would take 1mm slices and several cuts above and below that shown as well as additional imaging sequences to ensure it is not apparent thickening due to polymicrogyria (PMG) or simply the imaging sequence used. It is all the more suspicious, when the W589X patient appears to have a patch of PMG in the left fronto-parietal cortex. With the diffuse white matter abnormality in the splice defect patient, it is unclear whether there is grey matter thickening or not. Thus, it is debatable whether this is a lissencephaly condition. It would be prudent to call it a brain malformation phenotype or cortical dysplasia.

Thank you for this comment. We revised Extended Data Fig. 1 to include additional images of the index patient (NG375-1) of the NG375 family, who donated somatic cells from which the iPSCs and organoids analyzed in our study were derived. The patient presented with bilateral diffuse pachygyria and enlarged ventricles; the upper and middle gyri in the temporal lobes are diffusely and symmetrically thickened and smooth; no microgyria was observed. The sister of the index patient also displayed a similar, albeit more subtle, pattern of bilateral fronto-temporal pachygyria. Extended Data Fig. 2 contains additional imaging sequences from the patients in our families.

Respectfully, we note that the lissencephaly spectrum comprises a continuum of structural brain abnormalities. Indeed, the patterns of malformations seen with the multiple (>19) genes causally associated with lissencephaly spectrum are increasingly diverse, prompting an update in the imaging and clinical classification of lissencephaly, which also includes patients with malformations intermediate between lissencephaly and polymicrogyria (dysgyria) (Di Donato 2017, PMID: 28440899).

Consistent with the appreciated phenotypic diversity of lissencephaly spectrum, previously reported neuroradiological characteristics of patients with validated PIDD1 mutations (Zaki et al., 2021 PMID: 34163010; Sheikh et al., 2021 PMID: 33414379) include predominant anterior pachygyria, dysgyria/simplified gyral pattern (Zaki et. al, 2021), and subtle smooth gyration and shallow sulcation affecting the frontal and temporal lobes, compatible with lissencephaly (Sheikh et al., 2021). Reports of patients harboring mutations in CRADD, an established direct interactor of PIDD1 in the tripartite CASP2-PIDDosome protein complex, describe a "thin lissencephaly" variant (TLIS) of relatively mild lissencephaly characterized by megalencephaly, anterior-predominant pachygyria with shallow and unusually wide sulci and mildly thick cortex especially in the frontal regions (Di Donato et al., 2016; PMID: 27773430), fronto-temporal predominant pachygyria (Polla et al., 2019; PMID: 30914828), hypogyria with frontal predominance, and delayed cortical sulcation suggestive of lissencephaly (Harel et al., 2017; PMID: 28686357). Finally, a recent study (published when our manuscript was under review) reports recessive truncating variants in CASP2 (the third member of the CASP2-PIDDosome) in individuals with lissencephaly, with neuroimaging revealing bilateral pachygyria and mild cortical thickening in the frontotemporal lobes (Uctepe et al., 2023; PMID: 37880421). For these reasons, the patients identified in our study are within the diverse lissencephaly spectrum.

Considered together, the neuroradiological findings in patients with recessive mutations in the three genes encoding the core proteins of the CASP2-PIDDosome (PIDD1, CRADD, and CASP2) support their classification in the lissencephaly spectrum.

2. Interpretation of cortical thickening. The comparable time frame of observation in these models is short relative to cortical development. While the increase in neurogenesis along with reduction in apoptosis observed may well account for a relative thickening of the later generated SATB2+ neurons, the organoids only go to d120. Especially since the SVZ is depleted of progenitors more quickly, one would expect that the overall upper layer neuron numbers would eventually be lower than controls. And what does the morphology of the neurons look like?

Thank you for this comment. We respectfully note that 120 days in culture, even though short relative to fetal cortical development, represents a significant period for organoid modeling of structural brain abnormalities.

Relevant previous studies employing organoid models to investigate structural brain disorders performed analyses at significantly earlier stages of organoid differentiation:

a) day 22 (Lancaster et al., 2013; PMID: 23995685; CDK5RAP2-associated microcephaly)
b) day 22 (Krenn et al., 2021; PMID: 33838105; virus-induced microcephaly)
c) day 25 (Iefremova et al., 2017; PMID: 28380362; Miller-Dieker lissencephaly syndrome)
d) day 30 (Dell'Amico et al., 2023; PMID: 37272619; WDR62-associated microcephaly)
e) day 35 (Bershteyn et al., 2017; PMID: 28111201; Miller-Dieker lissencephaly syndrome).

The studies yielded insight into the cellular mechanisms underlying these structural brain malformations, even though the analyses were confined to early stages of organoid differentiation. Considering these previous studies, day 70 and day 120 represent significantly more advanced timepoints of analysis. In this context, it is also worth noting that a recent study demonstrated no further utility in extending time in culture beyond 120 days (i.e., to 150 days) when whole organoids are used as structural brain disease models, as cortical organization does not improve after the 120-day timepoint (Qian et al., 2020; PMID: 32142682). Considering current practice in the field and these findings, we performed most of our analyses at days 70 and 120. Cortical thickening was obvious already at day 70 and remained a striking feature of day 120 organoids.

The question of whether there would be more upper layer neurons in control is indeed interesting but would require another advancement in cerebral organoid technology (e.g., organoids with expanded oSVZ) to test experimentally. To answer the question of neuronal morphology, we performed Golgi-Cox staining of organoids at D120. These qualitative results are presented in Extended Data Fig. 6g.

3. Comparison to lissencephaly. The authors compare their findings in cortical organoids with a more widely recognized classical lissencephaly, MDLS. While technically a microdeletion syndrome, MDLS results from deletion of a substantial number of contiguous genes, several of which have been shown through comparative genetics to contribute to the severity of the lissencephaly phenotype [1, 2]. In addition to the PAFAH1B1 (LIS1) gene responsible for lissencephaly (tending to manifest as smooth cortex in the occipital pole with frontal pachygyria), genes deleted in the MDLS critical region such as MNT and 14-3-3-epsilon increase the extent of the agyria. If the intent were to compare with a classical lissencephaly, they might better have used cells with a point mutation in or targeted knockout of LIS1 [3]. Therefore, the effects of mTOR pathway function may have more to do with other genes in the MDLS critical region than lissencephaly per se.

Thank you for this comment. It is correct that the microdeletion region of chromosome 17p13.3 contains several genes that have been associated with lissencephaly. By extending our analyses beyond PIDD1-associated lissencephaly, we intended to investigate whether mTOR pathway dysregulation represents a converging molecular mechanism in lissencephaly spectrum, despite differences in the underlying genetic lesions.

We studied MDLS for the following reasons:

a) MDLS was the first identified genetic cause of severe lissencephaly (Dobyns et al., 1983; PMID: 6834189)
b) Abnormally thick cortex is a common characteristic of MDLS (Dobyns 1991 et al.; PMID 1671808; Dobyns et al., 2003; PMID 7607669) and PIDD1-lissencephaly (herein and previously reported patients with "PIDD1-lissencephaly" [please see our response to point 1 above for details])

c) We elected to utilize patient-derived iPSCs, rather than generating a point mutation in LIS1 by gene editing, because, in our view, patient material is preferable over genetic manipulation in a non-patient line, and, to the best of our knowledge, iPSCs (or somatic cells/fibroblasts) from patients with LIS1 (or other lissencephaly-associated genes) are not available in public repositories.
d) MDLS organoids (Iefremova et al., 2017; PMID: 28380362; Bershteyn et al., 2017; PMID: 28111201) and PIDD1 organoids (our study) share cellular abnormalities (e.g., increased horizontal divisions and enhanced neurogenesis). The observations reported in these earlier studies were critical, as they validated MDLS organoids as the optimal experimental system modeling lissencephaly in which to test the generality of our findings.

Our analyses of MDLS organoids demonstrated downregulation of mTOR signaling within the progenitor zones. In other words, MDLS and PIDD1-mutant organoids share not only cellular, but also molecular phenotypes. This finding suggested to us that despite their different genetic causes, MDLS and "PIDD1-lissencephaly" both converge to a common molecular mechanism.

4. Novelty. Leaving that aside, it has been recognized for many years now that lissencephaly associated with LIS1 deficiency is not a pure migration problem, but overlaps with dysregulated spindle orientation, cytokinesis, cell polarity and cortical progenitor division (for recent example [4]). Authors provide evidence that PIDD1 mutations similarly alter expected cleavage plane orientation of dividing progenitors. It is not surprising then, that several pathways, among them mTOR, may recapitulate cellular level aspects of a lissencephaly/cortical malformation phenotype.

We respectfully disagree with this assessment. Hypoactivity of mTOR signaling has not been associated with lissencephaly previously and is reported here for the first time. More importantly, though, the novelty of our study lies in the finding that mTOR pathway dysregulation is a converging molecular mechanism in lissencephaly spectrum of diverse genetic causation. We also provide a thorough and unbiased characterization of patient-derived lissencephaly organoids by single-cell transcriptomics and mass spectrometry, followed by orthogonal validation of the findings, as well as pharmacological manipulation of mTORC1 signaling to rescue the observed phenotypes, a comprehensive approach never undertaken for lissencephaly.

We believe that the identification of a common downstream pathway in lissencephaly significantly enhances our understanding of this heterogenous spectrum of severe cortical malformations and serves as a starting point for further mechanistic study. Moreover, our findings extend mTORopathies, classically considered to be malformations of cortical development underlain by <u>hyper</u>activation of mTOR signaling (Crino 2015; PMID: 25833943), to include the lissencephaly spectrum converging to <u>hypo</u>activation of mTOR signaling, thereby providing strong evidence that mTOR pathway dysregulation (hyper- or hypo-activity) is detrimental to human brain development.

5. Therapeutic role for mTOR activation. The comment made in several locations in the text that

pharmacologic activation of mTOR signaling may prove therapeutically useful for these patients is unduly optimistic. The critical window for significant intervention would be when cortical neurogenesis is going on in utero, and there are many events emerging in that period that are sensitive to alterations in mTOR pathway function. Indeed somatic mutational overactivity of mTOR signaling during corticogenesis is known to cause hemimegalencephaly or focal cortical dysplasia (FCD) [5, 6] and activating this pathway may do more harm than good. For couples carrying this recessively inherited PIDD1 mutation, preimplantation genetic diagnosis would be the most effective option. Authors should refrain from making statements regarding therapeutic potential for their mTOR link.

Thank you for this remark. We agree that the critical period for intervention would be in-utero; we are not suggesting that our findings can be directly and immediately translated to clinical trials. Rather, we posit that the identification of a molecular mechanism represents the first step for exploring a potential (and targeted) therapeutic intervention. Even though mTOR activation is known to disrupt cortical development (e.g., as the referee notes, mosaic somatic activation in hemimegalencephaly and focal cortical dysplasia), one could envision a scenario in which activation of mTOR pathway is carefully calibrated in the setting of an inherited recessive disorder, in which pathway hypoactivity is global. This aside, the words of caution by the referee are much appreciated, prompting us to moderate statements of immediate therapeutic application throughout the manuscript and in the discussion and to remove references to potential pre- or postnatal clinical intervention to treat lissencephaly of various genetic etiologies.

1. Leventer, R.J., et al., LIS1 missense mutations cause milder lissencephaly phenotypes including a child with normal IQ. Neurology, 2001. 57(3): p. 416-22.
2. Cardoso, C., et al., The location and type of mutation predict malformation severity in isolated lissencephaly caused by abnormalities within the LIS1 gene. Human Molecular Genetics, 2000. 9(20): p. 3019-3028.
3. Lo Nigro, C., et al., Point mutations and an intragenic deletion in LIS1, the lissencephaly causative gene in isolated lissencephaly sequence and Miller-Dieker syndrome. Hum Mol Genet, 1997. 6(2): p. 157-64.
4. Moon, H.M., et al., LIS1 determines cleavage plane positioning by regulating actomyosin-mediated cell membrane contractility. Elife, 2020. 9.
5. Lee, J.H., et al., De novo somatic mutations in components of the PI3K-AKT3-mTOR pathway cause hemimegalencephaly. Nat Genet, 2012. 44(8): p. 941-5.
6. Marin-Valencia, I., R. Guerrini, and J.G. Gleeson, Pathogenetic mechanisms of focal cortical dysplasia. Epilepsia, 2014. 55(7): p. 970-8.

Referee #3 (Remarks to the Author):

The authors have studied a new causative gene, PIDD1, in lissencephaly patients. Through the use of cerebral organoid models, they have demonstrated that PIDD1 deficiency leads to

increased cortical thickness and layer disorganization in comparison to patient-rescue and control organoids. Notably, the combination of transcriptomic and proteomic data strongly supports the role of the mTOR pathway in influencing protein translation not only in PIDD1-deficient organoids but also in MDS organoids. By employing the mTOR activator NV-5138, the authors were able to prevent cortical thickening in PIDD1-deficient and MDS organoids. This discovery of the mTOR pathway's involvement in PIDD1-related and MDS-related lissencephaly holds promise for potential therapeutic options for these patients.

Major points:
In my opinion, conducting scRNAseq experiments following treatment with mTOR activators, along with performing functional assays like MEA or calcium imaging, would enhance the clinical relevance of the model.

Thank you for the suggestion to conduct additional scRNA-seq experiments (was also requested by Referee #1). We now include transcriptomic data from organoids treated with NV-5138 in Extended Data Fig. 10. Please also see above our detailed response to Referee 1's major point 2.

[REDACTED]

Furthermore, I would recommend that several result panels be presented in a clearer way.

Specific Comments:
• With regard to the WES data and genetic investigation, it is advisable to provide a clearer distinction between what has previously been reported and what constitutes novel findings in this study. It appears that the genetic findings may have been previously published, which could warrant their relocation to the introduction section rather than the results section.

Thank you for this comment and we apologize for the confusing wording. We have modified the relevant paragraph to clarify that the WES analyses leading to the identification of recessive mutations in PIDD1 as the causative gene are reported in our manuscript for the first time. Two earlier studies preceded the WES analyses; the first (Guzel et al., 2007; PMID: 17343267) described a syndrome of pachygyria and mental retardation in a single family (NG8) in the Yale Neurogenetics cohort, and was followed by genome-wide linkage analyses in the NG8 family, establishing linkage to a single 10.6 million base pair area at chromosome band 11p15 with a maximum lod score of 2.5 (the theoretical maximum lod score attainable for the NG8 family (Bilguvar et al., 2009; PMID: 19876906); however, we failed to identify the disease-causing mutation. In our current work, we applied WES to NG8 and discovered the causative gene, PIDD1; WES also revealed PIDD1 mutations in two additional families (NG375 and NG1801) with lissencephaly in the Yale Neurogenetics cohort.

• In addition to the six patients with the described PIDD1 variants, have the authors explored other databases to identify additional cases with PIDD1 variants?

We have not been able to identify other PIDD1 variants in publicly available databases.
As mentioned in our manuscript, additional biallelic PIDD1 mutations have been reported in families with intellectual disability, pachygyria, and lissencephaly (Zaki et al., 2021 PMID: 34163010; Sheikh et al., 2021 PMID: 33414379).

• Is it possible that the R331X mutation is predicted to trigger NMD-mediated transcript degradation? This aspect should be better discussed.

Thank you for this insightful comment. We performed qRT-PCR to quantify PIDD1 transcript levels in organoids at baseline and following treatment with cycloheximide, an inhibitor of NMD, which is translation-dependent. These experiments indicated that the mutated (R331X) mRNA is being degraded by NMD and are reported in Extended Data Fig. 1h.

• Did the authors conclude on putative differences in genomic backgrounds and establish genotype-phenotype causality?

We addressed genotype-phenotype causality in two ways:

a) we corrected the patient mutation in patient-derived iPSCs, generating an isogenic, "patient-rescue" iPSC line
b) we introduced the patient mutation in both alleles of unrelated, control iPSCs, obtaining a knock-in iPSC line harboring the R331X homozygous patient mutation in a genetic background different from the patient's.

We then generated and analyzed organoids from all 4 genotypes (patient, isogenic patient rescue, control, and knock-in), demonstrating a concordance of cellular and molecular phenotypes between patient and knock-in organoids on one hand, and patient rescue and control organoids on the other. This enabled us to ascertain genotype-phenotype causality, and to attribute the phenotypes we report to the presence of the mutation, irrespective of genetic background.

• The predicted impact of mutations on various domains and protein forms (PIDD1, PIDD1-C, PIDD1-CC) is not straightforward (the figure should be clarified). It would be helpful to clarify the expected effects of increased cell repair versus apoptosis, particularly since the death domain is part of PIDD1-C. Is there a potential link to the enrichment of "DNA repair" clusters in the proteomics analyses, as shown in Extended Data Fig. 1c-d (please clarify in Supplementary Figure 1d)?

Thank you for this suggestion. We modified the schematic describing PIDD1 autoproteolysis leading to C-terminal fragments PIDD1-C and PIDD1-CC (Extended Data Fig. 1e). WB analyses (Extended Data Fig. 3i) demonstrate that endogenous PIDD1-CC fragments are detectable in lysates from control (C) and patient rescue (PR), but not from patient (P) or knock-in (KI) organoids. The endogenous C-terminal fragment PIDD1-C, unless sequestered in the NEMO-PIDDosome, undergoes rapid autoproteolysis to generate PIDD1-CC; we did not detect PIDD1-C in control or mutant organoids. Importantly, we note that PIDD1-CC (but not PIDD1-C) is involved in the CASP2- PIDDosome that is strongly associated with lissencephaly (mutations in each of its three components [CASP2, CRADD, and PIDD1] lead to lissencephaly). On the other hand, mutations in RIP1, a component of the NEMO-PIDDosome, lead to severe

immunodeficiency (Cuchet-Lourenco et al., 2018 PMID: 30026316). Furthermore, the expected effects of increased cell repair vs. apoptosis in PIDD1-C would be difficult to predict. We also note that PIDD1 is activated in response to DNA repair, and it is not surprising that loss of PIDD1 and hypoactive mTOR signaling leads to downregulation of DNA repair pathways in the proteomic analyses.

• In Fig.1/2b-d-e, there is a lack of statistical comparison between patients and patient rescue (also in general in the ms). This information should be added.

Thank you for this suggestion – this information is now provided.

• Fig.1a: The authors show a reduction in the number of Cleaved Caspase-3 (CC3)+ neural progenitor cells. At day 70, and even more so at day 50, this reduction seems to affect especially SVZ. Given that the SVZ houses oRG and IPCs, it would be valuable to determine which of these cell populations are impacted by the reduced apoptosis. To address this, conducting a co-labeling experiment that combines cleaved caspase with hopx, tbr2, and dapi could offer insights and help address this question.

Thank you for this suggestion; we have now performed co-labeling analyses to address the question raised. Please see Extended Data Fig. 4g,h, demonstrating reduced CC3 staining in the TBR2+ IPC population.

• The authors demonstrate a reduction in the number of oRG (hopx+ cells) and mTOR hypoactivity in the PIDD1 and MDS organoids. A prior study (Cell Stem Cell 20, 435–449) has shown the crucial role of oRG in MDS and their sensitivity to mTOR alterations. It would be particularly interesting to quantitatively assess mTORC1 activity in oRG and the number of oRG when treated with and without the mTOR activator.

Thank you for this suggestion, we have now performed these analyses. Please see Extended Data Fig. 10 b-d. We found that the mTOR activator increases the number of oRG and can increase mTORC1 activity in this cell population. We also found that oRG cells display decreased mTORC1 activity in P and MDLS organoids.

• In Extended Data Fig. 5c,d, the authors observe an increase in horizontal (asymmetric) divisions and a decrease in vertical divisions. Does this data suggest a premature increase in neurogenesis at the expense of progenitor cell numbers?
(1) Quantification should be done in the same way in all cell types (either DAPI+ counting or per mm2).
(2) If an increase in neurogenesis is anticipated, quantifying the total number of neurons, for instance, using Tuj1 staining, should be conducted. Furthermore, the authors note that the number of CTIP2 cells remains unaffected while the number of SATB2 cells increases. Since the latter cells are formed at a later stage, how does this align with the concept of premature neurogenesis?

Increase in neurogenic (asymmetric) divisions indeed suggests premature neurogenesis, however we also observe neuronal differentiation defects, as implied by the differences in the number of neurons expressing SATB2, CTIP2, or both markers in control vs. patient organoids (Extended Data Fig. 6f), further confirming transcriptomic analyses (Fig. 3).

To the specific points:

(1) Thank you for this suggestion, we have now performed the quantification in a uniform manner within figures.

(2) Thank you for this suggestion; we performed MAP2 staining and quantification (please see Extended Data Fig. 5g,h). Regarding the number of CTIP2+ and SATB2+ neurons, we report the following observations:

a. D50: increased CTIP2+ cells (Extended data Fig. 6)
b. D70: increased CTIP2+/SATB2+ double positive cells, but fewer single-positive (CTIP2+ or SATB2+) cells (Figure 2 and Extended Data Fig. 6)
c. D120: increased SATB2+ cells, a major phenotype in P and KI organoids (Figure 2).

These observations suggest premature neurogenesis, as well as neuronal differentiation defects. Supporting premature neurogenesis, we also documented a reduction in the SOX2-expressing progenitor population at D70 (Extended Data Fig. 5).

• The specific persistence of PIDD1 expression in the patient's radial glia (RG) requires a more clearly articulated interpretation. The data presentation should be improved for better clarity.

Thank you for this comment, which we interpret to pertain to scRNA-Seq data presented in Figure 3. Even if they might undergo NMD in the cytoplasm during translation, PIDD1 transcripts are still generated in patient organoids, and the transcriptomic data suggest reduced expression of PIDD1 in neural progenitor cell clusters.
We interpret the referee's comment regarding data clarity to concern the color scheme used in the dot plot (Fig. 3d), which has been modified in response.

• In Fig. 2c-e, the authors mention that the "layering" of the cortical plate is disrupted. Visually, it is evident that CTIP2 and SATB2 staining appears more dispersed, with SATB2 cells seemingly forming a second layer at D120. This suggests the possibility of migration defects. Is this phenomenon rescued by the mTOR activator NV-5138?

Thank you for this observation and suggestion – we performed these experiments and report the findings in Fig. 6e,g,h. Briefly, we found that delayed-onset and sustained treatment with NV-5138 (from D50 to analysis at D120) rescues the "second layer" of SATB2-expressing cells at D120.

• In Supp Fig. 6e, the authors mention that a smaller proportion of CTIP2+ and SATB2+ cells is present in Patient and KI organoids, but the graph appears to show the opposite trend. Additionally, there is a missing legend for the X-axis.

We are grateful for this comment – we inadvertently made an error when generating the graph in the original figure. The correct graph is now included, which indicates fewer SATB2 and fewer CTIP2 (single-positive for either marker), but increased number of CTIP2/SATB2 double-positive cells in patient and knock-in organoids.
Thank you for pointing out the missing legend for the X-axis, which has been added.

• Cortical thickness and abnormal cortical folding are pivotal characteristics of lissencephaly and MDS. Recent advancements in the field of brain organoids have highlighted the ability to induce folding in cerebral organoids (doi: 10.4103/1673-5374.335789). To enhance their study, the authors might consider incorporating one of these engineering techniques to visually illustrate alterations in cerebral folding, thereby adding a valuable dimension to their research or at least referencing this development in the discussion.

Thank you for this comment. Our study is centered on elucidating the molecular mechanisms underlying the pathogenesis of lissencephaly spectrum using organoid modeling. As such, understanding the mechanisms of human brain gyrification is beyond the scope of our work. We would also argue that a much-improved organoid model that can be maintained in culture long-term and recapitulates late stages of human cortical development would need to be developed before attempting to model human brain gyrification in 3D cultures. The current brain organoid protocols do not support complete maturation of the cortical plate, or folding in a manner that mimics human brain gyrification. For example, a recent study (Qian et al., 2020; PMID: 32142682) demonstrated that laminar structure is not maintained in whole organoids beyond 120 days in culture. Even though through physical or mechanical confinement organoids can be forced to "fold" and to display increased "wrinkling index" at day 20, at this stage organoids consist of only neural progenitor cells. Forced "folding" at day 20 would be a non-representative model of the natural/physiological folding of the developing human cerebral cortex, which at mid-gestation is still lissencephalic, except for the presence of the interhemispheric and Sylvian fissures and central sulcus, with all primary, secondary, and tertiary gyri forming between mid-gestation and birth (Chi 1997; PMID 560818).
This comment is well taken, however, prompting us to include this sentence in the discussion: "It will be important to develop to cerebral organoid models with a more mature CP that can undergo gyrification to link our findings to cortical folding".

• It is greatly appreciated that the authors generated appropriate control organoids, including a mutation-corrected version of the patient-derived hiPSC and a KI introduced in the control iPSC. However, in the manuscript, there is a tendency to compare the patient organoids with the control organoids rather than with the patient-rescue organoids. Even though significant differences in cellular analyses between the control and patient rescue organoids are not observed, it would be sensible to include these comparisons. Interestingly, in the sc-RNA sequencing analysis (Fig. 3d, GOBP analysis of upregulated DEGs in RG and oRG), the enrichment of terms related to oxidative phosphorylation appears to be specific to comparisons

with the control organoids and absent in the comparison of patient vs. patient rescue organoids, so it may not warrant significant emphasis.

We thank the referee for commending our efforts. The point raised is well-taken; comparisons of patient organoids to patient-rescue counterparts are now provided. We also thank the referee for suggesting we do not emphasize enrichment of terms related to oxidative phosphorylation, and followed their recommendation in the revised manuscript.

• In Fig. 3, the authors have primarily focused on the analysis of oRG and RG scRNA-seq data. However, Fig. 3d suggests potentially interesting differences in IPs as well. It may be worthwhile to include this analysis in the manuscript or provide it as supplementary data.

Thank you for this recommendation; this analysis is now provided. We extended Figure 3c to include GO analyses for IPCs and dividing IPCs.

• Fig. 3d: Given that the mutation is a stop gain, it is expected that the PIDD1 transcript would be subject to degradation by NMD. However, some cell types express it. Is there a predicted cell-type-specific action of NMD? Please discuss this point.

Thank you for raising this very interesting point. NMD-mediated degradation of PIDD1 mRNA would not result in complete elimination of the transcript, as the effect of NMD on transcript levels is dynamic. The average expression of PIDD1 (dot plot) is lower in the patient cell clusters, even though the expression levels in the RG cluster appears only mildly affected. This is consistent with in situ hybridization findings (Extended Data Fig. 7b), where PIDD1 transcripts are detected in progenitor cells in patient (and control) organoids. This could mean that NMD, even though triggered, may not be as efficient.

To the second point: to our knowledge, cell-type-specific actions of NMD have not been investigated in the developing central nervous system in vivo; yet this is an intriguing possibility. A just-published study (https://doi.org/10.1016/j.neuron.2024.04.006; PMID 38697111) reports differential effects in neural progenitors vs. immature neurons upon conditional inactivation of the core NMD factor Upf2 in the developing mouse brain. It appears, therefore, that inhibition of NMD may have different consequences for cell types of the neural lineage along their developmental trajectory. This observation is reminiscent of previous reports indicating differences in tissue and cell-type specificity of NMD. For example, an early study demonstrated that a patient mutation in COL10A1 leads to complete NMD in cartilage, whereas the mutant transcripts escape NMD in lymphoblasts and bone cells (Bateman et al., 2003; PMID 12554676). More recently, genome-wide studies reported heterogenous effects across tissues and significant NMD escape (Rivas 2015, PMID 25954003), and in vitro assays demonstrated a broad range of NMD efficiency within a cell population, from complete elimination to almost complete escape (Sato and Singer, 2021; PMID 34893608).

• In Fig. 3f, the comparison presented lacks clarity. It is unclear which PIDD1-expressing versus

non-expressing cell types were compared and how the data are presented (e.g., pseudo-bulk). What is the rationale for this approach, and what are the potential interpretations or biases?

Thank you for this comment. We computationally isolated and compared all PIDD1-expressing cells in patient and control organoids. We wanted to understand how the patient mutation impacts normal cellular function in cells that express PIDD1, and therefore we compared the transcriptome of PIDD1-expressing cells in control and patient organoids. We show that PIDD1 in patient organoids (Extended Data Fig. 7d) have top upregulated/downregulated GO terms similar to those found in the progenitor clusters (RG, oRG, IPC, dividing IPC) vs. control. We reasoned that pseudobulk analysis was a valid approach since progenitors (our main population of interest) are the cells that mostly express PIDD1 in 70D organoids, which we provide GO analyses for. The main bias is the analyses would be skewed towards progenitors, and so would not be appropriate to understand for the impact of the PIDD1 mutation in neurons (lack of PIDD1 expression). We also amended the figures to indicate what each comparison entails.

• In Fig. 3f, the authors state that "downregulated GO biological processes for mutant PIDD1-expressing cells were related to neurogenesis, neuron development, and neuron differentiation," but Fig. 3f seems to show terms related to metabolic processes. Please discuss this subtle inconsistency.

Thank you for this comment. We have rephrased the relevant passage (please note that the pseudobulk analysis is now presented in Figure 3e):
"We also compared PIDD1-expressing cells in P vs. C organoids to determine the impact of the patient mutation on the normal function of PIDD1 (Fig. 3e); metabolic processes and translation ontologies were upregulated and generation of neurons was downregulated in PIDD1-expressing cells in P organoids, consistent with what was observed in the PIDD1-expressing progenitor cell clusters, suggesting that the patient mutation might lead to dysregulation of translation, metabolic, and neurogenic pathways, and contrasting the normal stress and cell death function of PIDD1."

• Regarding the dysregulated mTOR signaling, the comparisons are not clearly explained.
(1) In the cortical plate (CP) of the brain organoids, the pS6 staining appears to be less intense in the patients and MDS organoids. Could the authors also quantify this observation?

Thank you for this comment. We followed the referee's recommendation to quantify p-S6 expressing cells in the CP as well (please see Fig. 6f,g).

(2) Have the authors found any evidence supporting the same phenomenon in human patients with lissencephaly/MDS, such as previously published RNA-seq data or postmortem brain samples of MDS patients?

To our knowledge, RNA-seq datasets from patients with lissencephaly (of any genetic causation) or from post-mortem brain samples of MDLS patients are not available. Additionally, to our

knowledge, there is no published report on the expression of mTORC1 pathway elements in post-mortem analyses of MDLS brains.

• Regarding the MTOR activator as a potential therapeutic target for lissencephaly patients, the authors have demonstrated the drug's ability to prevent cortical thickening. Do they have data that supports a curative effect of the drug, for example, by administering treatment at D50–70 and assessing cortical thickness at D120? Moreover, since epilepsy is a major clinical manifestation and mTOR overactivity is known to play a role in epilepsy, please discuss what is the predicted effect on seizures? In vitro, testing with functional readouts such as MEA or calcium imaging would be needed to assess potential effects on neuronal hyperexcitability.

Thank you for this suggestion; the results of "delayed" treatment of organoids with the mTOR activator are included in the revised manuscript (Fig. 6e,g,h). Briefly, we observed that delayed treatment onset at D50 resulted in increased p-S6 intensity and reversed the abnormal neuronal distribution in the CP of P and KI organoids at D120.
Likewise, thank you for the suggestion regarding predicted effects of drug treatment on seizures; please see our response above detailing our MEA findings.

• Important experiments appear to be missing: scRNA-seq data after NV-5138 rescue.

Thank you for this suggestion; this experiment was also requested by Referee #1. We have performed these experiments and present our findings in Extended Data Fig. 10.

Minor comments:
• The term "genetic causation" is used multiple times in the text. I believe this term may not be commonly utilized.

We respectfully disagree; the term genetic causation is not uncommon; please see, for example, "Distinguishing genetic correlation from genetic causation across 52 diseases and complex traits" (Nature Genetics, PMID: 30374074); "Genetic Causation in Complex Regulatory Systems: An Integrative Dynamic Perspective" (BioEssays; PMID: 32449193); "Genetic determinism, essentialism and reductionism: semantic clarity for contested science" (Nature Reviews Genetics PMID: 36316396) for some recent examples in the Genetics literature.

• Text on page 12: Add "PIDD1-expressing" to the sentence, "Strikingly, lissencephaly was the second most significant disease process enriched for differentially expressed genes (DEGs) that were downregulated in patients vs. control organoids."

The suggested text has been added to the sentence in question.

• Text p13: "Furthermore, we identified 848 DEGs in patient vs. control organoids in scRNA-seq (oRG cell-type) (…) mass spectrometry datasets.". Please include a brief sentence explaining why selecting the oRGs sc-RNA seq dataset.

Thank you for this suggestion, the following text was added: Furthermore, we identified 848 DEGs in patient vs. control organoids in scRNA-seq (oRG cell-type, which are preferentially affected in patient organoids; Fig. 1c,d) …

• Fig. 2b: Could the authors provide a more detailed explanation of what is meant by "relative thickness (CTIP2)"? It is clear for the CP, but it is not evident how CTIP2 staining helps quantify the thickness of the SVZ and VZ. The description in the methods section lacks clarity..

Thank you for noticing this oversight, now clarified in the Methods; the Y axis label in Fig. 2b has been amended (CTIP2 was erroneously placed in the label – the VZ, SVZ, and CP regions were delineated based on DAPI staining and cell density, as well as CTIP2 and SOX2 staining; please see methods).

• Fig. 3: For the scRNA-seq data, a color scale with a stronger contrast between positive and negative average expression would improve the figure's interpretability.

Thank you for this suggestion – the color scheme was replaced to improve contrast and facilitate interpretation of the findings.

• Fig. 3: Consider placing figure 3e after figure 3f.

Thank you, Fig. 3 has been reorganized.

• Fig. 6c: In the MDLS organoids in the SVZ, the color is brown instead of grey.

Thank you for the sharp eye! This has been corrected.

• Some of the images and graphs could be transitioned from the supplementary figures to the main figures. For example, none of the information about the patients or PIDD1 expression is found in the main figures.

Thank you for this remark – we are limited by the number of display items and had to prioritize what to include in each main vs. extended data figure. As our analyses were all performed on organoids, we elected to provide the patient-related information as extended data.

• Supp Fig. 3j: The method description for this heterologous expression experiment appears to be missing. It would be helpful to explain the significance of V5/6His.

Thank you for this observation; we removed this panel, which involves a heterologous overexpression experiment, because the antibody we used has been previously validated to detect PIDD1-CC (Ref 19; Sheikh et al., 2021 PMID: 33414379).

• Supp figure 3k: Please provide better annotations for the figure (PIDD1-FL, PIDD1-C, PIDD1-

CC, and other bands). Alternatively, consider semi-quantitative analysis and include a bar plot for clearer interpretation.

The annotation of Extended Data Fig. 3k (3i in the revised manuscript) has been updated.

• Supplementary Figure 6e-f: The "+" and "-" symbols are small, and the y-axis legend is missing in Supplementary Figure 6e. In Figure 6f, simplifying the representation of double-stained cells might facilitate interpretation.

Thank you. We added the missing Y axis label, and changed the labels to SATB2+, CTIP2+, and SATB2+/CTIP2+.

• Supplementary Figure 7a: Write out the abbreviations in full in the figure legends.

Abbreviations are now provided.

• Supplementary Figure 7c: Consider capturing a subpart of the fetal cortical tissue with higher magnification and adding it to this figure for improved visualization of the in situ hybridization result.

Thank you for this suggestion, we now include a high-magnification view of the fetal cortical wall.

• Supplementary Figure 9b: Include the quantification of pS6+/Hopx+ and pS6+/Tbr2+ cells.

Quantification now provided in Extended Data Fig. 9c.

Referee #1 (Remarks to the Author):

Appropriately revised to address all the major concerns with additional new data.

One minor point: regarding the same pathways that are both up and down regulated in the bioinformatic analyses in Figure 4- the authors may want to annotate the figure legend to indicate that different members of the same pathway are up or down regulated, thus a given pathway can appear both up- and down-regulated in the bioinformatic analysis.

The legend has been annotated as suggested.

Referee #2 (Remarks to the Author):

In their revised manuscript, the authors present additional data and further clarification of their rationale for interpretations is provided. On the whole, it continues to be an interesting manuscript, though there are several points of interpretation that should be further modified as, in this reviewer's opinion, they are overstated.

Reviewer point 1. Is the phenotype really lissencephaly. The 3 MR images in Supplementary figure 1 of patients with PIDD1 R331X, W589X, and c.2042-2A>G variants do not appear to manifest a classical lissencephaly phenotype, but are more in line with a simplified gyral pattern. The frontal gyri in patient R331X may have a thickened cortical grey (elsewhere it looks pretty normal), but this is often misleading in frontal gyri. It would take 1mm slices and several cuts above and below that shown as well as additional imaging sequences to ensure it is not apparent thickening due to polymicrogyria (PMG) or simply the imaging sequence used. It is all the more suspicious, when the W589X patient appears to have a patch of PMG in the left fronto-parietal cortex. With the diffuse white matter abnormality in the splice defect patient, it is unclear whether there is grey matter thickening or not. Thus, it is debatable whether this is a lissencephaly condition. It would be prudent to call it a brain malformation phenotype or cortical dysplasia.

Author response. Thank you for this comment. We revised Extended Data Fig. 1 to include additional images of the index patient (NG375-1) of the NG375 family, who donated somatic cells from which the iPSCs and organoids analyzed in our study were derived. The patient presented with bilateral diffuse pachygyria and enlarged ventricles; the upper and middle gyri in the temporal lobes are diffusely and symmetrically thickened and smooth; no microgyria was observed. The sister of the index patient also displayed a similar, albeit more subtle, pattern of bilateral fronto-temporal pachygyria. Extended Data Fig. 2 contains additional imaging sequences from the patients in our families.

Respectfully, we note that the lissencephaly spectrum comprises a continuum of structural brain abnormalities. Indeed, the patterns of malformations seen with the

multiple (>19) genes causally associated with lissencephaly spectrum are increasingly diverse, prompting an update in the imaging and clinical classification of lissencephaly, which also includes patients with malformations intermediate between lissencephaly and polymicrogyria (dysgyria) (Di Donato 2017, PMID: 28440899).

Consistent with the appreciated phenotypic diversity of lissencephaly spectrum, previously reported neuroradiological characteristics of patients with validated PIDD1 mutations (Zaki et al., 2021 PMID: 34163010; Sheikh et al., 2021 PMID: 33414379) include predominant anterior pachygyria, dysgyria/simplified gyral pattern (Zaki et. al, 2021), and subtle smooth gyration and shallow sulcation affecting the frontal and temporal lobes, compatible with lissencephaly (Sheikh et al., 2021). Reports of patients harboring mutations in CRADD, an established direct interactor of PIDD1 in the tripartite CASP2-PIDDosome protein complex, describe a “thin lissencephaly” variant (TLIS) of relatively mild lissencephaly characterized by megalencephaly, anterior-predominant pachygyria with shallow and unusually wide sulci and mildly thick cortex especially in the frontal regions (Di Donato et al., 2016; PMID: 27773430), fronto-temporal predominant pachygyria (Polla et al., 2019; PMID: 30914828), hypogyria with frontal predominance, and delayed cortical sulcation suggestive of lissencephaly (Harel et al., 2017; PMID: 28686357). Finally, a recent study (published when our manuscript was under review) reports recessive truncating variants in CASP2 (the third member of the CASP2-PIDDosome) in individuals with lissencephaly, with neuroimaging revealing bilateral pachygyria and mild cortical thickening in the frontotemporal lobes (Uctepe et al., 2023; PMID: 37880421). For these reasons, the patients identified in our study are within the diverse lissencephaly spectrum.

Considered together, the neuroradiological findings in patients with recessive mutations in the three genes encoding the core proteins of the CASP2-PIDDosome (PIDD1, CRADD, and CASP2) support their classification in the lissencephaly spectrum.

Reviewer 2 response: The authors are thanked for their detailed review, in which they reinforce the point of the original comment. To describe a phenotype simply as “lissencephaly” tends to undercut the highly heterogeneous nature of the term and implies the more classical appearance of highly thickened cortex—whether agyric or pachygyric. The images provided in this manuscript are quite variable and include multiple forms of dysgyria, with either thickened or thinned cortical gray matter, asymmetric ventricles, areas of polymicrogyria, etc. It would be better for the phenotype to be described as dysgyria, but since earlier manuscripts have used the term lissencephaly for mutations encompassing the PDD1 region, the authors for this paper should refer to the phenotype as a “lissencephaly spectrum disorder” or “cortical dysplasia in the lissencephaly spectrum”.
In addition, the abstract acknowledges that lissencephaly spectrum comprises a group of genetically heterogeneous congenital brain malformations. In the next sentence (starting line 96) they state incorrectly that the cellular pathogenesis is thought to be defective neuronal migration. Substantial literature includes mechanisms such as progenitor proliferation defects, extracellular signaling and matrix abnormalities. In fact, this manuscript does not touch on migration abnormalities at all. It is recommended that

authors either omit the sentence or replace with something like: “The cellular pathogenesis is multi-factorial and may encompass abnormalities of progenitor proliferation and survival, displacement of proliferation zones, neuronal migration and guidance mechanisms.”

Author response: Thank you for this comment. We followed the referee’s advice and used “lissencephaly spectrum disorders” whenever possible in the text. We no longer refer to neuronal migration in the manuscript; as the referee correctly points out, our study does not address migration abnormalities in the organoids.

Reviewer point 2. Interpretation of cortical thickening. The comparable time frame of observation in these models is short relative to cortical development. While the increase in neurogenesis along with reduction in apoptosis observed may well account for a relative thickening of the later generated SATB2+ neurons, the organoids only go to d120. Especially since the SVZ is depleted of progenitors more quickly, one would expect that the overall upper layer neuron numbers would eventually be lower than controls. And what does the morphology of the neurons look like?

Author response. Thank you for this comment. We respectfully note that 120 days in culture, even though short relative to fetal cortical development, represents a significant period for organoid modeling of structural brain abnormalities.

Relevant previous studies employing organoid models to investigate structural brain disorders performed analyses at significantly earlier stages of organoid differentiation:

a) day 22 (Lancaster et al., 2013; PMID: 23995685; CDK5RAP2-associated microcephaly)
b) day 22 (Krenn et al., 2021; PMID: 33838105; virus-induced microcephaly)
c) day 25 (Iefremova et al., 2017; PMID: 28380362; Miller-Dieker lissencephaly syndrome)
d) day 30 (Dell’Amico et al., 2023; PMID: 37272619; WDR62-associated microcephaly)
e) day 35 (Bershteyn et al., 2017; PMID: 28111201; Miller-Dieker lissencephaly syndrome).

The studies yielded insight into the cellular mechanisms underlying these structural brain malformations, even though the analyses were confined to early stages of organoid differentiation. Considering these previous studies, day 70 and day 120 represent significantly more advanced timepoints of analysis. In this context, it is also worth noting that a recent study demonstrated no further utility in extending time in culture beyond 120 days (i.e., to 150 days) when whole organoids are used as structural brain disease models, as cortical organization does not improve after the 120-day timepoint (Qian et al., 2020; PMID: 32142682). Considering current practice in the field and these findings, we performed most of our analyses at days 70 and 120. Cortical thickening was obvious already at day 70 and remained a striking feature of day 120 organoids.

The question of whether there would be more upper layer neurons in control is indeed interesting but would require another advancement in cerebral organoid technology (e.g., organoids with expanded oSVZ) to test experimentally. To answer the question of neuronal morphology, we performed Golgi-Cox staining of organoids at D120. These qualitative results are presented in Extended Data Fig. 6g.

Reviewer 2 response: The authors are thanked for their defense of 3-D organoids as an investigative system. There is no doubt that this in vitro method is relevant to pursue mechanisms that impact progenitor proliferation/survival and early neural specification. It is these aspects of cell biology that their observations relating to mTOR signaling appears to influence. The appropriate use of cortical organoids in mechanistic investigations is certainly supported by the 5 studies that the authors list in response to the comment, each of which interrogates aspects of radial glial cell, oRG, and intermediate progenitor proliferation and differentiation. Nevertheless, now relatively standard organoid protocols do not adequately model other aspects of cortical neogenesis, including neuronal migration, neurite elaboration, generation of multiple cell types including but not limited to interneurons and microglia. Therefore, the contribution of mTOR signaling in the clinical syndromes associated with lissencephaly spectrum disorders is far from settled and will likely vary among the disorders associated with now nearly 2 dozen different genes. Furthermore, the study has investigated only 2 genetic associations with a lissencephaly spectrum phenotype, when there are at least 17 more. The manuscript should avoid over-generalization of the mTOR pathway importance to dysgyria in the clinical picture and should avoid language that implies that mTOR is now established as a final common pathway to lissencephaly.
The inclusion of Golgi-cox stains in the extended data figure 6g is appreciated, but it is far too limited to draw an inference much less a conclusion about the contribution of neuritogenesis in the variably observed cortical thickening associated with PIDD1-lissencephaly spectrum

Author response: In the revised manuscript, prompted by a major point raised by Referee 1, we discussed the restricted cell repertoire of the organoids as a limitation of the model and the need for further investigation of mTOR signaling in other human lissencephaly spectrum genes. We do not state that mTOR is a final common pathway to lissencephaly; instead, we suggest that mTOR dysregulation is a converging point in lissencephaly and discuss the need to investigate whether this is also true in other genetic causes of lissencephaly spectrum disorders. We included the Golgi-Cox staining of neurons in the cortical plate in response to a previous comment by Referee 2 (please see above) and noted its qualitative nature. We do not (and cannot) draw any conclusions on neuritogenesis, which is beyond the scope of our study, based on a snapshot of neuronal morphology.

Reviewer point 3. Comparison to lissencephaly. The authors compare their findings in cortical organoids with a more widely recognized classical lissencephaly, MDLS. While technically a microdeletion syndrome, MDLS results from deletion of a substantial number of contiguous genes, several of which have been shown through comparative genetics to contribute to the severity of the lissencephaly phenotype [1, 2]. In addition to

the PAFAH1B1 (LIS1) gene responsible for lissencephaly (tending to manifest as smooth cortex in the occipital pole with frontal pachygyria), genes deleted in the MDLS critical region such as MNT and 14-3-3-epsilon increase the extent of the agyria. If the intent were to compare with a classical lissencephaly, they might better have used cells with a point mutation in or targeted knockout of LIS1 [3]. Therefore, the effects of mTOR pathway function may have more to do with other genes in the MDLS critical region than lissencephaly per se.

Author response. Thank you for this comment. It is correct that the microdeletion region of chromosome 17p13.3 contains several genes that have been associated with lissencephaly. By extending our analyses beyond PIDD1-associated lissencephaly, we intended to investigate whether mTOR pathway dysregulation represents a converging molecular mechanism in lissencephaly spectrum, despite differences in the underlying genetic lesions.

We studied MDLS for the following reasons:

a) MDLS was the first identified genetic cause of severe lissencephaly (Dobyns et al., 1983; PMID: 6834189)
b) Abnormally thick cortex is a common characteristic of MDLS (Dobyns 1991 et al.; PMID 1671808; Dobyns et al., 2003; PMID 7607669) and PIDD1-lissencephaly (herein and previously reported patients with “PIDD1-lissencephaly” [please see our response to point 1 above for details])
c) We elected to utilize patient-derived iPSCs, rather than generating a point mutation in LIS1 by gene editing, because, in our view, patient material is preferable over genetic manipulation in a non-patient line, and, to the best of our knowledge, iPSCs (or somatic cells/fibroblasts) from patients with LIS1 (or other lissencephaly-associated genes) are not available in public repositories.
d) MDLS organoids (Iefremova et al., 2017; PMID: 28380362; Bershteyn et al., 2017; PMID: 28111201) and PIDD1 organoids (our study) share cellular abnormalities (e.g., increased horizontal divisions and enhanced neurogenesis). The observations reported in these earlier studies were critical, as they validated MDLS organoids as the optimal experimental system modeling lissencephaly in which to test the generality of our findings.

Our analyses of MDLS organoids demonstrated downregulation of mTOR signaling within the progenitor zones. In other words, MDLS and PIDD1-mutant organoids share not only cellular, but also molecular phenotypes. This finding suggested to us that despite their different genetic causes, MDLS and “PIDD1-lissencephaly” both converge to a common molecular mechanism.

Reviewer 2 response. Somewhat related to point 2 above, it is well established that LIS1 deficiency alters cortical progenitor proliferation and radial glial cleavage plane orientation and that mTOR signaling impacts progenitor proliferation in the setting of LIS1 deficiency. Previous studies, including a recent investigation in iPSCs (Moon et al., 2020, doi: 10.7554/eLife.51512) indicate the LIS1 effect is mediated through

perturbation of its role in microtubule stability, dynein protein motor function or localization, mitotic spindle orientation, chromosomal segregation, and nuclear migration. Use of proliferation outcomes is a relatively crude way of examining molecular mechanisms and any manipulation (such as of the mTOR pathway) that can enhance or suppress progenitor proliferation may mimic or compensate for a mutation-associated phenotype such as cell division. However, there are published observations that mTOR dysregulation has consequences for oRG proliferation in an organoid model of MDLS (the Bershteyn et al., 2017article authors mention above). The present work indicates an impact of PDD1 mutation on mitotic cleavage plane and oRG proliferation. Thus, demonstration of similar sensitivity of their PIDD1 and MDLS organoids to NV-5138 mTOR activator is not particularly indicative of the "generalizability" of the mTOR pathway to lissencephaly. The claim of generalizability and of a final common pathway to lissencephaly is not proven in this study.

Author response: Please see our response to related point 2 above. We do not state in the paper that mTOR pathway dysregulation is a final common pathway to lissencephaly. The cellular defects (mitotic cleavage plane defects, oRG defects, increased neurogenicity) we established in the PIDD1-mutant organoids and their similarity to previously described MDLS organoid phenotypes, in conjunction with the abnormal expansion of the cortical plate in organoids of both genotypes (which we documented in our study), warranted our undertaking unbiased molecular analyses to interrogate (and then validate orthogonally) potential commonalities in the underlying molecular mechanisms.

The reviewer has cited two elegant studies by Bershteyn et al. (PMID: 28111201) and Moon et al. (PMID: 32159512).

We respectfully note that Bershteyn and coauthors did not investigate, or make any reference to, mTOR dysregulation in the MDLS organoid model.

On the other hand, Moon and co-authors interrogated the role of Pafah1b1 (encoding LIS1) in cytokinesis in mouse neocortical neural progenitor cells (NPCs) and mouse embryonic fibroblasts (MEFs), but not in iPSCs. In murine NPCs and MEFS, the authors demonstrate defects in cleavage plane positioning and dysregulation of cell membrane contractility, concluding that "LIS1 is a key molecular link connecting MTs/dynein and actomyosin". The authors do not question a role for LIS1 in regulation of the cleavage plane of NPCs and acknowledge as a limitation the sparsity of oRGs in mice ("in our current mouse model, we could not investigate oRG phenotypes due to lower number of oRGs in the neocortices of mice"). Because of inherent differences between apical NPCs in the mouse and oRGs in primates, findings in murine apical NPCs (or MEFs) cannot be extrapolated to human oRGs, which can be better studied in organoids harboring HOPX+ oRGs, a model which, relative to the mouse, is more representative of early human cortical development. Furthermore, the study by Moon and coauthors does not preclude that LIS1 deficiency/haploinsufficiency dysregulates other molecular pathways such as the mTOR pathway.

In regard to the referee's comments on our own findings of mTOR pathway involvement in the pathogenesis of lissencephaly spectrum disorders, we would like to note that aside from the pharmacological rescue, the transcriptomic, proteomic, immunostaining, and analytical protein analyses data, all point to shared mTOR dysregulation/hypoactivity in PIDD1-mutant and MDLS organoids, which suggests a converging molecular mechanism in these organoid models of lissencephaly spectrum disorders. We believe that the use of an mTOR activator drug to rescue mTOR hypoactivity is a key experiment in our study, demonstrating the link between mTOR hypoactivity and the cellular phenotypes; testing drugs for their ability to modulate phenotypic and molecular outcomes of perturbed gene function represents an additional advantage of using patient iPSC-derived organoids to model early stages of malformations of cortical development.

Lastly, the reviewer mentions progenitor manipulation by the drug. We would like to point out our major finding is that the mTOR activator rescued CP thickening/lamination defects in PIDD1-mutant as well as MDLS organoids at two endpoints (D70 and D120), notably both in preventative and delayed settings (Figure 6). This provides strong evidence that mTOR hypoactivity accounts for the neuronal defects observed.

Reviewer point 4. Novelty. Leaving that aside, it has been recognized for many years now that lissencephaly associated with LIS1 deficiency is not a pure migration problem, but overlaps with dysregulated spindle orientation, cytokinesis, cell polarity and cortical progenitor division (for recent example [4]). Authors provide evidence that PIDD1 mutations similarly alter expected cleavage plane orientation of dividing progenitors. It is not surprising then, that several pathways, among them mTOR, may recapitulate cellular level aspects of a lissencephaly/cortical malformation phenotype.

Author response. We respectfully disagree with this assessment. Hypoactivity of mTOR signaling has not been associated with lissencephaly previously and is reported here for the first time. More importantly, though, the novelty of our study lies in the finding that mTOR pathway dysregulation is a converging molecular mechanism in lissencephaly spectrum of diverse genetic causation. We also provide a thorough and unbiased characterization of patient-derived lissencephaly organoids by single-cell transcriptomics and mass spectrometry, followed by orthogonal validation of the findings, as well as pharmacological manipulation of mTORC1 signaling to rescue the observed phenotypes, a comprehensive approach never undertaken for lissencephaly.

We believe that the identification of a common downstream pathway in lissencephaly significantly enhances our understanding of this heterogenous spectrum of severe cortical malformations and serves as a starting point for further mechanistic study. Moreover, our findings extend mTORopathies, classically considered to be malformations of cortical development underlain by hyperactivation of mTOR signaling (Crino 2015; PMID: 25833943), to include the lissencephaly spectrum converging to hypoactivation of mTOR signaling, thereby providing strong evidence that mTOR pathway dysregulation (hyper- or hypo-activity) is detrimental to human brain development.

Reviewer 2 response. The authors themselves make the point that critical molecular signaling pathways often have a bimodal relationship to important developmental events. Thus, just as hyperactivity of a crucial pathway may result in brain malformation, reduced activity of the same pathway may be expected to have deleterious consequences. This appears to be the case in their examples of PIDD mutations. It is not a compelling insight to provide additional evidence that mTOR dysregulation is detrimental to brain development. The value of the present manuscript is in the multi-omic investigation of transcript and protein expression changes in the wake of these mutations.
I take exception to authors' comment here that "… the novelty of our study lies in the finding that mTOR pathway dysregulation is a converging molecular mechanism in lissencephaly spectrum of diverse genetic causation." The finding that pharmacologic manipulation of mTOR activity can mitigate progenitor proliferation abnormalities in two genetic conditions—the second comparative example having been selected precisely because of previously demonstrated mTOR dysfunction sensitivity of proliferation in mutant progenitors—does not make this a unifying mechanism in lissencephaly spectrum disorders any more than downstream consequences for GTPase dysregulation or HIPPO pathway dysfunction constitutes a final common pathway for this malformation spectrum.

I recommend that the language in the abstract be modified to replace the last sentence with something like: "These findings illuminate an unexpected molecular mechanism contributing to several genetically caused cortical malformations in the lissencephaly spectrum."

This also would require a modification of the title of the article replacing "Dysregulation of mTOR signaling is a converging mechanism in lissencephaly" with something like "Dysreguation of mTOR signaling contributes to several genetically caused malformations in the lissencephaly spectrum"

Autor response: We thank the reviewer for pointing out the value of our multi-tiered investigation of transcript and protein expression. It was not expected that mTOR pathway hypoactivation would be a molecular mechanism in structural brain disorders; activating mutations leading to hyperactivation of the pathway have been well documented, but to our knowledge, this is the first documentation that reduced mTOR activity is implicated in lissencephaly spectrum disorders. The ability to prevent and reverse CP expansion in the organoids by application of a brain-selective activator of mTORC1, causally implicates the pathway in two organoid models of lissencephaly spectrum disorders. This finding, along with our transcriptomic, proteomic (mass spec), and analytical (IF, western blotting) data point to a converging molecular mechanism in PIDD1-mutant and MDLS lissencephaly spectrum organoids that provides new research avenues for approaching these disorders.

We would also point out that the MDLS paradigm was not selected "because of previously demonstrated mTOR dysfunction sensitivity of proliferation in mutant progenitors". One of the published studies (Iefremova et al., PMID: 28380362) contains absolutely no reference (or evidence) of mTOR pathway dysregulation in MDLS organoids. The authors did observe a decrease in phosphorylated AKT in MDLS-derived high-density 2D cortical cultures, which they connected to defective Wnt signaling. The other published study of MDLS organoids (Bershteyn et al., 2017; PMID: 28111201) contains no reference to AKT, or mTOR.

As stated above, and in agreement with the reviewer's suggestion, we are using "lissencephaly spectrum disorders" whenever possible in the manuscript, but not in the title, which cannot exceed 75 characters. We also stand by our findings that there is a shared mTOR hypoactivity mechanism in PIDD1-mutant and MDLS organoids and thus the title of it being a converging mechanism for lissencephaly spectrum disorder is appropriate. In addition, we modified the last line of the abstract to state that "Our findings unveil an unexpected converging molecular mechanism contributing to genetically heterogeneous lissencephaly spectrum disorders," to be in line with the statement by the reviewer the mTOR signaling may not be a final, unifying pathway for lissencephaly.

5. Therapeutic role for mTOR activation. The comment made in several locations in the text that pharmacologic activation of mTOR signaling may prove therapeutically useful for these patients is unduly optimistic. The critical window for significant intervention would be when cortical neurogenesis is going on in utero, and there are many events emerging in that period that are sensitive to alterations in mTOR pathway function. Indeed somatic mutational overactivity of mTOR signaling during corticogenesis is known to cause hemimegalencephaly or focal cortical dysplasia (FCD) [5, 6] and activating this pathway may do more harm than good. For couples carrying this recessively inherited PIDD1 mutation, preimplantation genetic diagnosis would be the most effective option. Authors should refrain from making statements regarding therapeutic potential for their mTOR link.

Thank you for this remark. We agree that the critical period for intervention would be in-utero; we are not suggesting that our findings can be directly and immediately translated to clinical trials. Rather, we posit that the identification of a molecular mechanism represents the first step for exploring a potential (and targeted) therapeutic intervention. Even though mTOR activation is known to disrupt cortical development (e.g., as the referee notes, mosaic somatic activation in hemimegalencephaly and focal cortical dysplasia), one could envision a scenario in which activation of mTOR pathway is carefully calibrated in the setting of an inherited recessive disorder, in which pathway hypoactivity is global. This aside, the words of caution by the referee are much appreciated, prompting us to moderate statements of immediate therapeutic application throughout the manuscript and in the discussion and to remove references to potential pre- or postnatal clinical intervention to treat lissencephaly of various genetic etiologies.

Reviewer 2 response. The authors are thanked for their moderation of statements implying immediate therapeutic application of mTOR activation as a treatment strategy in these lissencephaly spectrum disorders. This should include modification of the abstract last sentence as recommended in point 4 above.

Author response: The summary paragraph was edited to comply with journal style and the last sentence was modified in response to the reviewer's comments (please also see response to point 4 above).