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The CUL4B-based E3 ubiquitin ligase regulates mitosis and brain development by recruiting phospho-specific DCAFs

Anna Stier, Samuel Gilberto, Weaam Mohamed, Lars Royall, Jonne Helenius, Ivan Mikicic, Tatjana Sajic, Petra Beli, Daniel Müller, Sebastian Jessberger, and Matthias Peter
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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Prof. Matthias Peter
ETH-Zürich
Institute of Biochemistry
Otto-stern weg 3
Zurich, Zurich 8093
Switzerland

8th Nov 2022

Re: EMBOJ-2022-112847
The CRL4B E3 ligase regulates mitosis by recruiting phospho-specific DCAFs

Dear Matthias,

Thank you again for submitting your study on CUL4B-specific DCAF recruitment and functions for our editorial consideration. I have now received reports from three experts in cullin-ring ligase biology, biochemistry and structure, as well as in molecular/cellular biology of brain development, which I am copying below for your information. As you will see, the referees consider your work interesting and important from all these angles, and also appreciate the overall quality and conclusiveness of the data. Following revision to address a number of specific concerns raised in the comments, we would therefore be happy to consider this work further for publication in The EMBO Journal.

Since the referees' points are well-defined in the report, I will not go through them in detail here, but would invite you to get in contact with me during the early stages of the revision work in case you should want to discuss particular issues with responding to these requests; as our policy to allow only a single round of experimental revision makes it important to comprehensively answer to all referee queries at the time of resubmission. As always, I would also be open to offering an extension of the revision period, during which our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) would of course remain valid.

Further, detailed information on preparing and uploading a revised manuscript can be found below and in our Guide to Authors. Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to your revision!

With kind regards,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (6th Feb 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In the present manuscript Stier et al. investigate paralog-specific functions of the CRL4 ligase. Using RNAi- and CRISPR/Cas9 approaches the authors demonstrate that loss of CUL4B, but not CUL4A, increases the time of Hela cells to undergo mitosis. Through a series of elegant cell biological, biochemical, and mass spectrometry-based approaches, the authors then reveal that this CRL4B-specific mitotic function relies on the unstructured N-terminus of CUL4B, which is not conserved in CUL4A, and undergoes phosphorylation during mitosis with T49 being a major site. N-terminal phosphorylation is required to displace CUL4B from mitotic chromatin and promotes association to actin cytoskeleton regulators, including LIS1 and WDR1. The authors then provide several lines of evidence that CUL4B forms mitotic- and phosphorylation specific CRL4B complexes. Through in vitro assays, in particular fluorescence polarization experiments, the authors show that LIS1 and WDR1 bind both DDB1 and the phosphorylated N-terminus of CUL4B, providing a molecular rationale for the CUL4B- and mitosis-specific formation of CRL4B-LIS1 and CRL4B-WDR1 complexes. Combining cell biological, live cell imaging, and atomic force microscopy approaches, the authors reveal that the functions of CUL4B in promoting mitotic progression are likely mediated through CRL4B-LIS1 in regulating spindle positioning and CRL4B-WDR1 in stabilizing the mitotic actin cortex.

This is an elegant study that uses a variety of different in vitro and in cellulo approaches to identify novel CUL4B-specific E3 ligase complexes that control mitotic progression through distinct actin-dependent mechanisms. A particular strength of this work is its immediate relevance to human disease. Throughout the manuscript the authors include a XLID variant (CUL4p.P50L), which reduces phosphorylation CDK1-CyclinB-mediated phosphorylation of CUL4A at T49, fails to assemble CUL4B-WDR1/LIS1 complexes, and abrogates binding to other actin regulators, providing a mechanistic basis for how this variant could interfere with proper neurodevelopment. Another very interesting aspect about this study is the identification and characterization of CRL4B-sepcific complexes. These findings raise several intriguing questions, e.g. regarding the underlying structural basis and regarding the substrates of the newly discovered mitosis-specific CRL4B complexes. Answering these questions lays the

foundation for exciting future investigations, but would be far beyond the scope of this manuscript.

Overall, the manuscript is well written, the experiments are of high quality and support the author's claims. The only aspect of this study that requires strengthening are the in vitro binding assays (see major concern). In addition, I am raising some minor concerns, mostly regarding typos, inconsistencies, and clarifications that might help the reader to more readily understand the rationales for experiments. Finally, I am providing some suggestions for additional experiments to further improve this already very strong study (for the authors to consider).

Major concern:

1) The authors propose that LIS1 and WDR1 are able to specifically assemble into CRL4B complexes via interaction with DDB1 and with the phosphorylated N-terminus of CUL4B. While the FP assays support this model, the binding assays from SF9 cells and the binding assays using purified components are less convincing.

Fig 5A,C,D and ED Fig 5A are lacking specificity controls and the observed co-purifying proteins could just be sticking to the beads. Another concern for Fig 5A and ED Fig 5A is that with coommassie gels as read-out, the authors cannot be sure whether the bands they are observing are indeed their bait protein or just a contaminating protein from SF9 cells. I would suggest repeating these assays with appropriate controls (e.g. when using FLAG IPs or Strep pull downs, have one condition in which the bait protein is expressed but no FLAG-tagged or Strep-tagged prey) and use immunoblotting as read-out.

For the pull down experiments with recombinant proteins in Fig 5E,G and ED Fig 5F,G, similar to above, it would be desirable to have a specificity control added to these experiments (strep pull down with CRL4A/B in the absence of strep-LIS1/WDR1). In addition, the authors should chose a representative coommassie gel with more even loading of the strep pull down fraction. This is especially important for ED Fig 5F,G for which by eye it is really hard to see the differences quantified in Fig 5 J,L.

Would a phosphorylated N-terminus of CUL4B be sufficient to stabilize the WDR1-DDB1 or LIS1-DDB1 interaction? Could the authors test this using their FP assay and compare affinities measured in Fig 5B in the presence and absence of the phosphorylated N-terminus of CUL4B?

Minor concerns:

1) Figure 1G: it should say 3min between images on top of the figure, not 30 min
2) P5, L137: consider revising to "no obvious mitotic phosphorylation could be detected for CUL4A" as not every phosphorylation event will result in gel shifts
3) P6, L155: could the author elaborate why T49 emerged as major mitotic site? Was it the most abundant in the mass spec?
4) P6, L165 and Fig 2G: while there is a noticeable difference between phosphorylation of wildtype CUL4B and CUL4Bp.P50L, the difference is subtle. Could the authors provide quantifications?
5) Figure 4B/6A: Out of curiosity, what are the proteins most reduced in the P50L and NP1 mutants depicted in Fig 6A and why did the authors not follow up on these hits?
6) P9, L269 and following: there is something off about this paragraph, I think the first sentence was supposed to be later in the paragraph. Please revise this section.
7) P24, L587: it should be DDB1
8) Figure 4H: the model suggests that the phosphorylated N-terminus of CUL4B also interacts with DDB1, but no evidence for this is provided in the paper. Consider redrawing.

Additional suggestions:

1) To further strengthen the disease link, could the authors include CUL4p.P50L variant in the interaction studies shown in Figure 5J,L and ED Figure 5FG?
2) To further validate that the observed defects underlying loss of CRL4B-WDR1 are due to reduced cortical actin stability, could the authors rescue CUL4 or WDR1 depletion phenotypes by adding ConA to stiffen cortices from the extracellular side? (e.g. Kunda et al 2008 PMID: 18207738)?

Referee #2:

Stier et al. contribute an exciting study illuminating the longstanding conundrum why there are two paralogues of the Cullin4-RING E3 ligase (CRL4) scaffold subunit present in vertebrate genomes, CUL4A and CUL4B. Both are very similar, with the main difference being a unique CUL4B N-terminal extension (NTE). Importantly, previous work demonstrated distinct functions of CUL4A and CUL4B in various models, probably mediated by differences in substrate receptor preference, however the underlying mechanistic principles have not been understood so far.

To address this, the authors characterised CUL4B knockdown and knockout cell lines, and found a growth delay phenotype, which was - unlike a growth defect of similar magnitude observed upon CUL4A knock down - not accompanied by accumulation of DNA damage. In contrast, CUL4B-deficient cells exhibited a lengthened mitotic duration, implying a CUL4B-specific role in mitosis. In turn, the authors examined expression levels of CUL4B throughout the cell cycle, and demonstrated slower electrophoretic mobility of CUL4B, but not CUL4A, specifically in cells arrested in mitosis. The band shift could be reverted by phosphatase treatment, indicative of phosphorylation. Indeed, phosphoproteomics, together with in vitro phosphorylation assays and mutagenesis, identified T49 in the CUL4B NTE as major mitotic CDK1 phosphorylation site. Importantly, the CUL4B P50L mutation, which has been previously identified in patients suffering from X-linked intellectual disability (XLID), is situated adjacent to T49 in the CDK1 consensus site. Again, using mutagenesis and in vitro phosphorylation, the authors showed that the P50L substitution diminishes, but not fully abrogates, CUL4B NTE phosphorylation. Subsequently, the CUL4B P50L variant, and a phospho-null mutant (NP1), alongside WT CUL4B, were expressed in CUL4B knockout cells, and failed to rescue the mitotic delay phenotype, demonstrating the importance of NTE phosphorylation for mitotic function. Further immunofluorescence studies indicated that the extent of CUL4B NTE phosphorylation was associated with exclusion of CUL4B from mitotic chromatin, with CUL4B WT and P50L strongly dissociated from chromatin, while a ΔNTE variant remains chromatin-bound, and NP1 is partially retained. Together with the finding that phosphorylation-defective CUL4B variants exhibited similar in vitro ubiquitylation activity as WT, it was suggested that phosphorylation-triggered mitotic chromatin exclusion does not regulate CUL4B catalytic activity per se, but enables association with additional factors important for mitotic progression. The authors then turned to quantitative proteomics, comparing IPs of WT to phosphorylation-defective variants, to identify factors that preferentially interact with phosphorylated CUL4B. Among WT-enriched hits, further analysis was focused on LIS1 and WDR1, because these contained WD40-repeat domains and putative DDB1-interacting sequence motifs, similar to canonical CRL4 substrate receptors (DCAFs). To validate these findings, co-IP experiments were conducted and demonstrated mitosis- and phosphorylation-specific co-purification of LIS1 and WDR1 with CUL4B. In addition, knockdown of WDR1 and partial knockdown of LIS1 fully and partially phenocopied the mitotic lengthening phenotype of CUL4B knockdown cells, respectively. In a series of follow-up in vitro reconstitution experiments, the authors corroborated CUL4B- and phospho-specificity of the interactions, and importantly detected not only direct interaction of the putative substrate receptors LIS1 and WDR1 with the CRL4 adaptor DDB1 (similar to canonical DCAFs), but also direct, phospho-dependent interaction with CUL4B in the absence of DDB1 (unlike canonical DCAFs). Together, these results establish LIS1 and WDR1 as phospho-specific DCAFs (pDCAFs), which, in addition to canonical DDB1-binding, are further stabilised by interaction with the phosphorylated CUL4B NTE. Lastly, the authors turn back to GO term analysis of the phospho-specific CUL4B interactome, revealing enrichment of cytoskeleton organisation and actin-related processes. Indeed, co-IP proved phospho- and mitosis-specific interaction of CUL4B with the actin-regulating Arp2/3 complex, with actin, and dynactin. In line with this, AFM experiments proved reduction of actin-dependent cell stiffness during mitosis upon CUL4B and LIS1 knockdown. Furthermore, live-cell microscopy showed off-centered mitotic spindles, and increased spindle motility upon CUL4B and LIS1 knockdown. Together, these data implicate that the pDCAF LIS1 is needed to anchor the mitotic spindle, thus explaining the mitotic lengthening phenotype of CUL4B and LIS1 knockdown cells, while the function of WDR1 potentially involves regulation of the actomyosin network.
Altogether, the paper is very well written and relevant, since it provides for the first time a mechanistic explanation for the differing substrate receptor preferences of CUL4B and CUL4A. It conclusively establishes cell-cycle dependent phosphorylation of the CUL4B NTE, which allows for recruitment of a subset of DCAFs (pDCAFs) in a bipartite interface involving the phosphorylated NTE alongside canonical DDB1 interactions. Two potential pDCAFs, LIS1 and WDR1 have been identified, and indications regarding their specific mitotic function have been obtained. Furthermore, a functional rationale for the XLID-associated P50L disease mutation has been provided, which hints at an involvement of pDCAF recruitment also in neuronal development. Accordingly, I recommend the paper for publication in the EMBO Journal, after certain minor issues detailed in the following have been addressed.

Minor comments

1. Line 115. EDF 1E is referenced before 1D, maybe rearrange?

2. Line 179 (Fig. 3). The authors state "The mutations neither altered expression levels nor the neddylation status of CUL4A or CUL4B". However, as mentioned in the sentence before, the P50L and NP1 expression levels are clearly higher than CUL4B WT. Furthermore, in the case of NP1, the CUL4A levels seem reduced. Could the authors clarify?

3. Line 218 (Fig. 3F). It is obvious that the phosphorylation-defective variants have similar in vitro ubiquitylation activity than WT. But has it been checked if the purified WT protein used in the assay was actually phosphorylated? The modification might not occur in the Sf9 expression system, or might have been lost during purification.

4. Line 245. "Both proteins possess WD40 domains followed by a putative helix-loop-helix motif ..." should read "Both proteins possess WD40 domains preceded by a putative helix-loop-helix motif ..."

5. Line 245/246 (EDF 4A). The authors show conservation of the putative HLH DDB1-binding motif in LIS1 and WDR1. This should be discussed in light of a high-confidence AlphaFold2 molecular model of WDR1 (https://alphafold.ebi.ac.uk/entry/O75083) which shows that the protein actually consists of two WD40-repeat domains, and that the putative HLH-box is integral part of WD40-repeat domain 2 and of the interface between the two domains.

6. Line 282: Fig. 5E is referenced before 5F, maybe rearrange? Likewise Figs. 5K and J.

7. Line 339/340. "Thus, phenotypic characterization indicates that CRL4BWDR1 regulates mitotic duration possibly by actin rearrangements..." While the relationship between LIS1 knockdown and spindle mobility seems clear from the data, on which observations is this statement based?

8. Fig. 4B. There are a lot more phospho-specific CULB binders visible in the upper right corner of the plot. Could the authors elaborate a bit more on this list? Do these hits correlate with CUL4B-specific DCAFs from Reichermeier et al., 2020? Maybe a point for the Discussion.

9. Fig. 4H. The schematic might be placed more appropriately in Fig. 5, because the in vitro reconstitution experiments substantiate the model further.

10. Fig. 5B. N=2 but error bars shown? Likewise EDF 5C. N=2 is stated in the text but three data points and error bar shown in Figure.

11. Fig. 6B/EDF 6E. What do the thin lines in the tension vs. time plots signify?

12. Fig. 6G. The model should be described more clearly in the text, and eventually picked up again in the Discussion. Also, the figure legend states there is a "left half" illustrating CRL4B bound to interphase chromatin. However, there is no CRL4B to be seen on the left half.

Referee #3:

The CRL4B E3 ligase regulates mitosis by recruiting phospho-specific DCAFs

The manuscript by Stier et al. reveals new aspects of CUL4B, a RING E3 ligases function, and sheds light on the possible etiology of X-linked intellectual disability caused by a point mutation in CUL4B-P50L.
Through a well-designed study exclusively done in human cell lines, the authors show that CUL4B has specific mitotic functions and that its loss influences cell cycle progression and, consequently, a substantial accumulation of DNA damage. The authors describe a phosphorylation defect in a residue adjacent to the P50L, XLID mutation in CULB4 and link it to CUL4B exclusion from mitotic chromatin but not E3 ligase activity. Two phospho-specific new DCAFs, LIS1 and WDR1, were identified, and both complexes were found to impact mitotic spindle organization and encore to the cell cortex.
The findings presented in the study uncover a novel aspect of LIS1, on which a wealth of data was accumulated in the last decades, and of WDR1, both were identified as CUL4B-specific DCAF. They also explain the cellular context that allows CUL4A and CUL4B to have unique functions despite their broad expression and a large common set of binding partners.
Overall, the paper is interesting and important. As this study points out a possible mechanism for human brain mitotic defects, it would have been excellent if a more relevant system had been included. Yet we understand that this is beyond the scope of the current study.

Specific comments:
Fig1B: an additional explanation of the visible Cul4 bands is required. Please, add arrows, sizes, etc. In Fig5E, the size differences between Cul4A and CUL4B are much more significant. What is the difference between the experiments?

Why is there a change in Cul4 bands in DDB1 RNAi? What does it mean?

Fig 1C - it's unclear what bands were measured for neddylated versus non-neddylated forms. From the gel in Fig 1B? If so - they should be labeled (as in Fig 2B). The verification that these bands are bands of neddylated protein is lacking.

Fig 1D - there is no SE in the growth versus time graph.

Minor comment, Fig 1D - in the legend n=3×3. In the graph, there are 12 dots. Please, change the legend.

Fig 1G - Cell cycle analysis is based only on brightfield analysis. If DNA stain or additional methods (FUCCI stains) had been used, a more detailed analysis of the progression through cell cycle phases could have been achieved.

In the text lines 106-109, there is a conclusion of redundant and non-redundant functions of CRL4A and CLR4B for cell growth. Not clear how a non-redundant function can be concluded in this experiment. Please, rephrase the explanation.

Minor comment, EDF2D: the size marker should be added to show that HA Abs recognize the same band as Cul4b.

For all figures: I recommend using an alternative way to show time-lapse images of cell proliferation. A 30min interval is not convincing and not a conventional way to be displayed. Not clear which points were used as a beginning and an end of the cell cycle.

Fig 2B, C- the labeling of the lane is a little confusing; in particular, the phosphatase treatment and the mitotic shake-off are indicated too far from the relevant lanes. (Fig 5C, D style is clearer, in my opinion)

Fig 3A and text 179-180. It's stated that mutations didn't alter expression levels of Cul4a. According to the figure, there is a reduction in the NP1 condition. Please, add quantifications to the statement.

Fig 5A, the control of His pull-down without overexpressing His-DDB1 is lacking. In Fig5C, D - controls Strep pull-down without the presence of SA-Cul4b and Flag pull-down without Flag-Lis1 or Flag-WDR1.

According to Fig 5E and 5G, the pull-down of Cul4A with both LIS1 and WDR1 worked. The EDF5B and C graphs could be moved to the main figure to confirm the differential binding.

Fig 5 JL- It is unclear how many repeats were used to quantify the data. In some cases, there is only one visible data point (L) and sometimes 2 points (J). Please clarify, and indicate n.

For the functional role of Lis1-Cul4b interaction in the spindle orientation experiment (Fig6F), it would be more elegant to add the NP1 condition when the protein is present but does not interact with LIS1.

ETH
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ibc
Institut für Biochemie
Institute of Biochemistry

Hartmut Vordermaier, PhD
Senior Editor
EMBO Journal

Zürich, April 16th, 2023

Dear Hartmut,

Please find attached to this letter the revised version of our manuscript EMBOJ-2022-112847, entitled "The CRL4B E3 ligase regulates mitosis by recruiting phospho-specific DCAFs". We were excited to learn that you and the reviewers considered the manuscript very interesting as it provides unexpected insights into the regulation and physiological function of CUL4B that together with unconventional DCAFs regulates cytoskeletal dynamics during mitosis.

Nevertheless, the reviewers raised some important points and asked to clarify several aspects of the manuscript with additional experiments. We have now carefully revised the manuscript and provide extensive new data that support and extend our original claims. The major changes of the manuscript are as follows: (i) We included new experiments showing that CUL4B is required for maintaining cortical units in developing brain organoids (Fig. 7 and Suppl. Fig. 7). (ii) We provide new control experiments confirming specificity of the *in vitro* measurements, including mutational analysis of the predicted HLH motifs. (iii) We purified and analyzed the P50L mutant and the unique N-terminal CUL4B domain and found that the N-terminal domain is not sufficient to bind LIS1 and WDR1 with reasonable affinity. (iv) We improved labeling and statistical analysis, and revised the result and discussion sections to expand the experimental description and clarify specific points mentioned by the reviewers.

Finally, we have followed the recommendations and checklist of your production team to adhere to the various formatting guidelines in Figures and text. The revised manuscript now comprises 7 main Figures and approximately 5400 words of main text. We

Prof. Dr. Matthias Peter, Institute of Biochemistry, ETH Zürich, HPM G8, CH-8093 Zürich
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wish to thank the reviewers for their thoughtful and detailed comments and hope that our revisions address their criticism. Please find below more detailed responses to all points raised by the reviewers. We confirm that none of the results presented have been published or submitted elsewhere and declare that the authors do not have any conflicting financial or commercial interest.

Thank you very much for taking the time to consider our revised manuscript, and we are looking forward to hearing from you soon.

With best regards,

Anna Samuel Weaam Matthias

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Point-by-point response to all reviewer comments:

Referee #1

Major concern:

Fig. 5A,C,D and ED Fig. 5A are lacking specificity controls and the observed co-purifying proteins could just be sticking to the beads. Another concern for Fig 5A and ED Fig 5A is that with coommassie gels as read-out, the authors cannot be sure whether the bands they are observing are indeed their bait protein or just a contaminating protein from SF9 cells. I would suggest repeating these assays with appropriate controls (e.g. when using FLAG IPs or Strep pull downs, have one condition in which the bait protein is expressed but no FLAG-tagged or Strep-tagged prey) and use immunoblotting as read-out.

We performed quantitative Fluorescence Polarization (FP) experiments to overcome shortcomings with Coomassie gels or pull-down experiments, and the results of these orthogonal assays are fully consistent. Nevertheless, to further address the criticism, we now included an additional specificity control, where we co-express Flag-DDB1 and Strep-LIS1 (EV Figure 5A, right panel). We incubated Flag-DDB1 with empty strep beads, and we don't observe unspecific binding in Coomassie gels. We also performed a second control experiment, where we deleted the N-terminal domains of LIS1 and WDR1 encompassing the predicted HLH motifs. Despite equal protein expression of LIS1-ΔN and WDR1-ΔN, only LIS1-ΔN interfered with DDB1 binding (EV Figure 5B). Beyond specificity, this experiment further demonstrates that the predicted HLH-motif of LIS1 likely mediates DDB1-binding, but another region is responsible for this in WDR1. We have thus discussed the implications in the revised text. See also point 5 of reviewer 2.

For the pull down experiments with recombinant proteins in Fig 5E,G and ED Fig 5F,G, similar to above, it would be desirable to have a specificity control added to these experiments (strep pull down with CRL4A/B in the absence of strep-LIS1/WDR1). In addition, the authors should choose a representative coomassie gel with more even loading of the strep pull down fraction. This is especially important for ED Fig 5F,G for which by eye it is really hard to see the differences quantified in Fig 5 J,L.

To address this comment, we expanded the Materials and Method section to better explain how we normalized the band intensities. Briefly, we always normalized the amount of pulled proteins (CUL4A or CUL4B WT/MUT) to the corresponding amount of LIS1 in the same lane. As described above, we believe that the quantitative FP experiments shown in Figure 5I and K strengthen the pull-down data.

Would a phosphorylated N-terminus of CUL4B be sufficient to stabilize the WDR1-DDB1 or LIS1-DDB1 interaction? Could the authors test this using their FP assay and compare affinities measured in Fig 5B in the presence and absence of the phosphorylated N-terminus of CUL4B?

*Following the reviewer's suggestion, we expressed and purified the N-terminal domain of CUL4B in insect cells, and then tested its direct binding to LIS1 by FP measurements. As shown in the **Figure 1, below**, we observed only unspecific binding, where no saturation signal could be reached. Likewise, adding the N-terminal domain of CUL4B did not rescue the loss of binding of CRL4A to LIS1. We conclude from these results that the N-terminal domain is necessary but not sufficient for binding non-conventional DCAFs, consistent with*

the co-immunoprecipitation and in vitro binding assays shown in Figures 4 and 5. However, we do not know if the N-terminal domain is efficiently phosphorylated at the right sites in the absence of the rest of CUL4B, and we would thus prefer not including these results in the current manuscript.

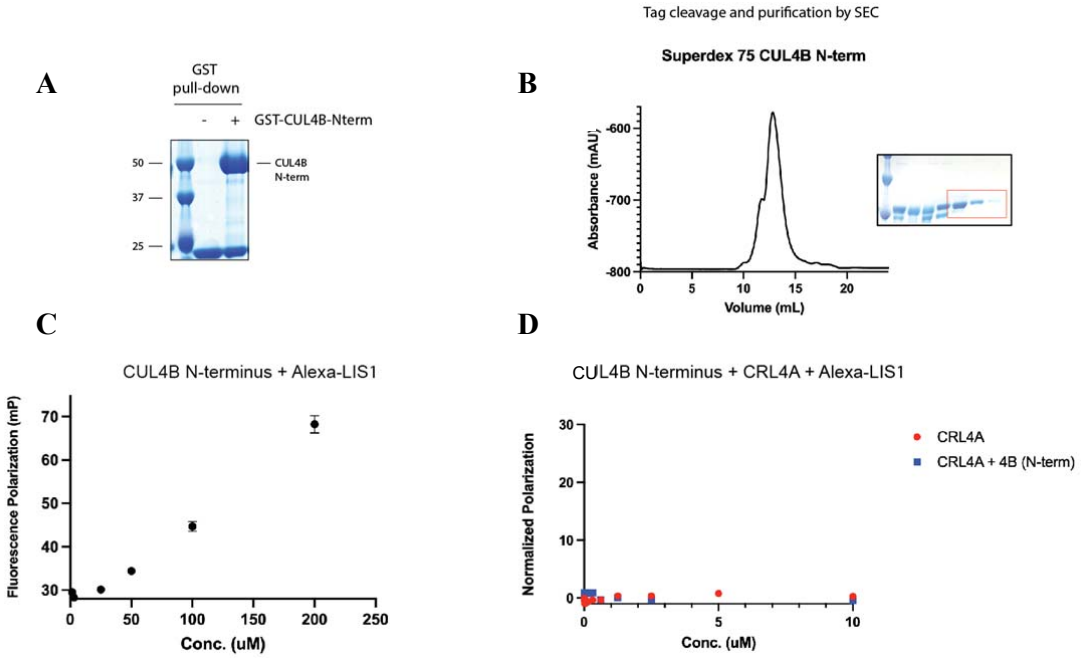

***Fig. 1**: **A**. Coomassie get showing GST pulldown of the GST-tagged N-terminal domain of CUL4B expressed in Sf9 insect cells. **B**. Superdex 75 column was used to separate the CUL4B N-terminal domain after cleaving its GST-tag. **C**. Direct binding was tested using fluorescence polarization (FP) measurements of the N-terminal domain of CUL4B and Alexa-labeled LIS1. **D**. Direct binding was tested using FP measurements of the N-terminal domain of CUL4B (N-term), CRL4A and Alexa-labeled LIS1.*

<u>Minor concerns</u>:

1) Figure 1G: it should say 3 min between images on top of the figure, not 30 min.

The cells were imaged every 3 min, however, the representative images are chosen every 30 min. We have changed the labeling of the images and now added the entire movies in the Extended View (EV Folder 1: Mitotic duration movies from Figure 1).

2) P5, L137: consider revising to "no obvious mitotic phosphorylation could be detected for CUL4A" as not every phosphorylation event will result in gel shifts

We agree with the reviewer, and changed the sentence as suggested to no obvious phosphorylation.

3) P6, L155: could the author elaborate why T49 emerged as major mitotic site? Was it the most abundant in the mass spec?

T49 was especially interesting to us because of the P50L disease mutation and the high occurrence of this phosphorylation site on phosphosite.org. However, it was not the most enriched phospho-site in our mass spectrometric measurements, and we thus changed the sentence in the revised text.

4) P6, L165 and Fig 2G: while there is a noticeable difference between phosphorylation of wildtype CUL4B and CUL4Bp.P50L, the difference is subtle. Could the authors provide quantifications?

*We agree with the reviewer that the reduced in vitro phosphorylation of the P50L mutant is subtle, consistent with the N-terminal domain harboring multiple phosphorylation sites. As suggested, we have quantified the defects from three different experiments, and normalized the phospho-signal to total CUL4B levels (**Fig. 2, below**). For comparison we also included the NP1 mutant control, which lacks additional mitotic phosphorylation sites. However, we did not include the quantification it in the paper.*

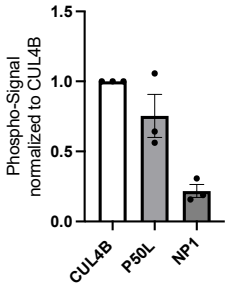

***Fig. 2:** Quantification of the in vitro kinase assays with purified CDK1-cyclinB and Sf9-purified CUL4B and mutant proteins in the presence of [γ-^{32}P]ATP. The autoradiogram signal was normalized to CUL4B and mutant protein levels (N=3).*

5) Figure 4B/6A: Out of curiosity, what are the proteins most reduced in the P50L and NP1 mutants depicted in Fig 6A and why did the authors not follow up on these hits?

In the initial validation experiments, we focused especially on WD40- and HLH-containing proteins, which allowed us to identify and characterize mitotic CUL4B-specific DCAFs (Figure 4). As requested by reviewer 2 (see below), we also include a new EV Figure showing a volcano blot with all detected DCAFs. In addition to the CUL4-related interactors, we then investigated other, more significantly phospho-dependent CUL4B interactors, which includes various proteins involved in cytoskeletal regulation (Figure 6). We indicated several top hits in Figure 6A and the GO analysis (Figure 6B) and further validated the Arp2/3 subunit ACTR3 by co-immunoprecipitation experiments (Figure 6C). A full list of all MS-data is provided in the EV. Table 2. Below, we extract the top ten phospho-dependent CUL4B interactors for the reviewer:

ANKRD31
KRT2
KATNAL2

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LIMA1; TRMT1
NSF
ACTC1; ACTA1
ACTG1
PPP1R18
ANXA2; ANXA2P2

6) P9, L269 and following: there is something off about this paragraph, I think the first sentence was supposed to be later in the paragraph. Please revise this section.

Thanks - we revised the paragraph accordingly.

7) P24, L587: it should be DDB1

We corrected the typo.

8) Figure 4H: the model suggests that the phosphorylated N-terminus of CUL4B also interacts with DDB1, but no evidence for this is provided in the paper. Consider redrawing.

As suggested by the reviewer, we have redrawn the model, as we do not know whether the phosphorylated N-terminal domain also interacts with DDB1.

Additional suggestions:

1) To further strengthen the disease link, could the authors include CUL4p.P50L variant in the interaction studies shown in Figure 5J,L and ED Figure 5FG?

Following the reviewer's suggestion, we expressed and purified the CUL4B-P50L mutant and tested it's binding to LIS1. For better quantification, we used FP assays instead of the standard pull-down experiments (shown in Fig. 5J,L and EV Fig. 5F,G). As shown in ***Figure 3, below****, we do not observe a significant Kd change when we compare wild-type CUL4B and the P50L mutant. This new data is now shown in EV Fig. 5, panel I, and described in the revised text.*

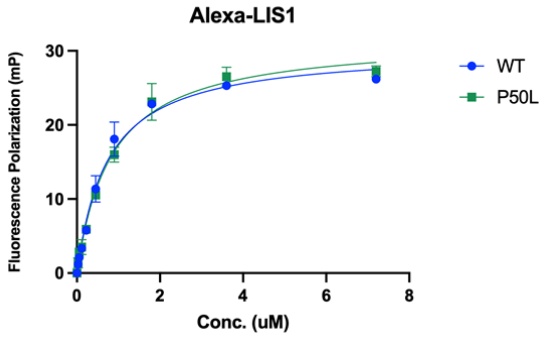

Fig. 3: *Fluorescence polarization assay comparing binding of wild type CRL4B (WT, blue circles) and the CRL4B-P50L mutant (P50L, green squares) to Alexa-labeled LIS1.*

2) To further validate that the observed defects underlying loss of CRL4B-WDR1 are due to reduced cortical actin stability, could the authors rescue CUL4 or WDR1 depletion phenotypes by adding ConA to stiffen cortices from the extracellular side? (e.g. Kunda et al 2008 PMID: 18207738)?

This is a good suggestion, and we indeed further investigate how mitotic CUL4B complexes regulate cortical stiffness and cytoskeletal dynamics. We hope to publish these experiments in a dedicated follow-up manuscript.

Referee #2:

Minor comments

1. Line 115. EDF 1E is referenced before 1D, maybe rearrange?

As suggested, we rearranged these panels.

2. Line 179 (Fig. 3). The authors state "The mutations neither altered expression levels nor the neddylation status of CUL4A or CUL4B". However, as mentioned in the sentence before, the P50L and NP1 expression levels are clearly higher than CUL4B WT. Furthermore, in the case of NP1, the CUL4A levels seem reduced. Could the authors clarify?

We agree with the reviewer that NP1 is always slightly higher expressed compared to wild-type controls, which makes its mitotic defects even more significant. With respect to compensatory changes in CUL4A levels, we do not observe a consistent and significant change of CUL4A levels in comparison to the different phospho-deficient CUL4B mutants. ***Figure 4, below****, shows a quantification of CUL4A levels normalized to GAPDH in wild type and CUL4B-deleted HeLa cells, compared to CUL4B-deleted cells expressing wild type CUL4B or the P50L or NP1 mutants.*

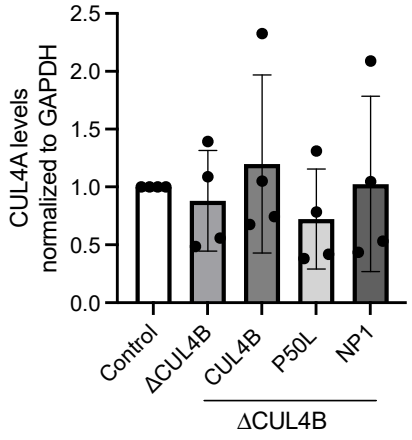

Fig. 4: *Quantification of CUL4A levels by immunoblotting in CUL4B knockout cells expressing either wild type CUL4B or the indicated P50L or NP1 mutants. The CUL4A signal was normalized to GAPDH (N=4). Note that while the experimental variations are rather large, we do not detect a consistent change in CUL4A levels.*

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3. Line 218 (Fig. 3F). It is obvious that the phosphorylation-defective variants have similar in vitro ubiquitylation activity than WT. But has it been checked if the purified WT protein used in the assay was actually phosphorylated? The modification might not occur in the Sf9 expression system or might have been lost during purification.

Mass spectrometric data shows that Sf9-expressed CUL4B is phosphorylated on many of the mitotic sites, including T49. Moreover, treating purified CUL4B with lambda-phosphatase leads to a faster migrating species (EV Figure 5, panel F), indicating that at least some sites are stoichiometrically phosphorylated. Nevertheless, we agree with the reviewer that we do not know which percentage of CUL4B is phosphorylated in insect cells, and thus the measured phosphorylation-dependent binding affinity of Sf9-purified CUL4B and its unconventional DCAFs LIS1 and WDR1 may be an underestimation. We have revised the text to better discuss this limitation.

4. Line 245. "Both proteins possess WD40 domains followed by a putative helix-loop-helix motif ..." should read "Both proteins possess WD40 domains preceded by a putative helix-loop-helix motif ..."

Thanks - we changed the wording as suggested.

5. Line 245/246 (EDF 4A). The authors show conservation of the putative HLH DDB1-binding motif in LIS1 and WDR1. This should be discussed in light of a high-confidence AlphaFold2 molecular model of WDR1 (https://alphafold.ebi.ac.uk/entry/O75083) which shows that the protein actually consists of two WD40-repeat domains, and that the putative HLH-box is integral part of WD40-repeat domain 2 and of the interface between the two domains.

We would like to thank the reviewer for this insightful comment. Following this suggestion, we deleted the N-terminal domains of LIS1 and WDR1, encompassing the predicted HLH motifs.
Despite equal protein expression of LIS1-ΔN and WDR1-ΔN, only LIS1-ΔN interfered with DDB1 binding (EV Figure 5B). Therefore, while the predicted HLH-motif of LIS1 likely mediates DDB1-binding, another still unknown region mediates this interaction in WDR1. We now discussed the implications of this results in the revised text.

6. Line 282: Fig. 5E is referenced before 5F, maybe rearrange? Likewise, Figs. 5K and J.

We have re-arranged the text and adjusted the revised Figure so that the order of panels now reflects the text. We show the binding comparison of LIS1 and WDR1 to wild type and lambda-phosphatase treated CUL4B both with quantitative FP-measurements as well as pull-down experiments. This allows a direct comparison to the NP1-mutant. As suggested by reviewer 1, we now also compared binding of wild type CUL4B and the P50L mutant by FP-measurements but did not detect a significant difference (Figure 3 above; EV Fig. 5, panel I).

7. Line 339/340. "Thus, phenotypic characterization indicates that CRL4BWDR1 regulates mitotic duration possibly by actin rearrangements..." While the relationship between LIS1 knockdown and spindle mobility seems clear from the data, on which observations is this statement based?

We agree with the reviewer that we provide no direct evidence to suggest that WDR1

regulates actin dynamics during mitosis. Nevertheless, since WDR1 is known to interact with several actin-regulators, we think it is warranted to keep this suggestion. However, we have weakened the statement and revised this section to avoid over-interpretation of the available data.

8. Fig. 4B. There are a lot more phospho-specific CULB binders visible in the upper right corner of the plot. Could the authors elaborate a bit more on this list? Do these hits correlate with CUL4B-specific DCAFs from Reichermeier et al., 2020? Maybe a point for the discussion.

*In the validation experiments, we initially focused on WD40- and HLH-containing proteins, which allowed us to identify and characterize the mitotic CUL4B-specific DCAFs LIS1 and WDR1 (Figure 4B). After this, we investigated other, more significantly phospho-dependent CUL4B interactors, which includes various proteins involved in cytoskeletal regulation (Figure 6A). As pointed out by the reviewer, we have compared our phospho-dependent CUL4B analysis with the CUL4B-specific dataset published by Reichermeier et al. (2020). It is important to note, however, that while their targeted MS-approach allows better quantification of known DCAFs, novel/unconventional DCAFs such as LIS1 or WDR1 are not part of the QconCAT Standard and were thus not analyzed. Interestingly, some CUL4B-specific DCAFs overlap with our phospho-dependent data set, such as PHIP and AMBRA, suggesting that CUL4B phosphorylation may also regulate interactions with conventional DCAFs binding CUL4B at cell cycle stages other than mitosis. We have now included an annotated volcano blot in EV Figure 4A (see also **Fig. 5, below**) and revised the text to discuss these implications.*

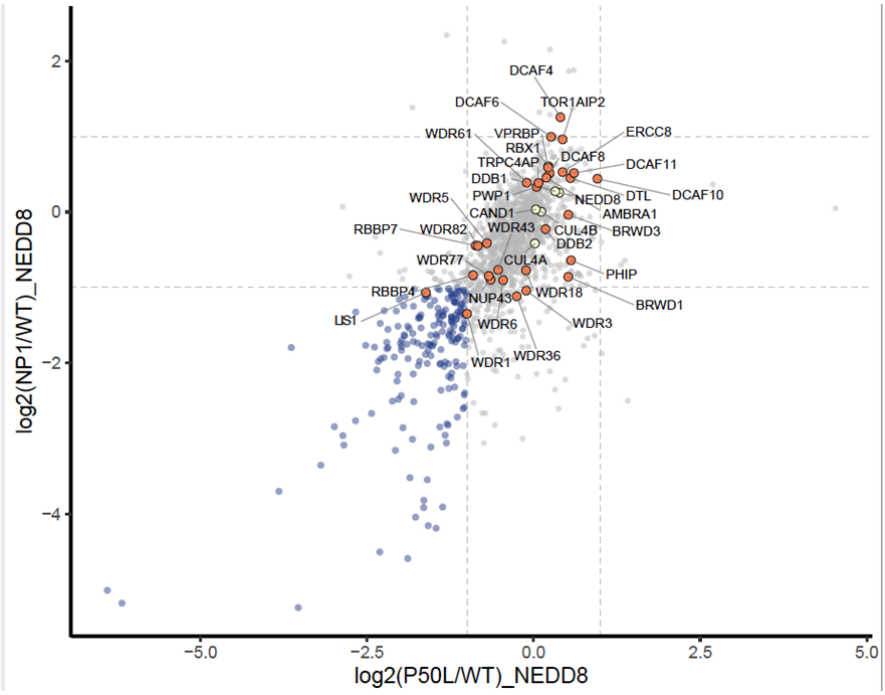

Fig. 5: *Scatter plot comparing mitotic immunoprecipitations (IP) of phosphorylated CUL4B with the phospho-mutants NP1 and P50L. The axes represent the log2-transformed fold*

change of wild type (WT) CUL4B vs mutant IPs, with dashed lines marking 2x fold change cutoffs.

9. Fig. 4H. The schematic might be placed more appropriately in Fig. 5, because the in vitro reconstitution experiments substantiate the model further.

As suggested, we have now moved the schematic of the mitotic CUL4B complexes to Figure 5 (new panel M). As requested by reviewer 1, we also adjusted the drawing, as we do not (yet) know how the phosphorylated N-terminal domain interacts with DDB1.

10. Fig. 5B. N=2 but error bars shown? Likewise, EDF 5C. N=2 is stated in the text but three data points and error bar shown in Figure.

Thank you for pointing this out. We corrected the number of repeats in the figure legends.

11. Fig. 6B/EDF 6E. What do the thin lines in the tension vs. time plots signify?

The thin lines indicate the standard deviation. We have now added this missing information in the revised Figure legend.

12. Fig. 6G. The model should be described more clearly in the text, and eventually picked up again in the Discussion. Also, the figure legend states there is a "left half" illustrating CRL4B bound to interphase chromatin. However, there is no CRL4B to be seen on the left half.

As suggested, we have corrected the model and expanded the Figure legend to better describe the illustration (now moved to Figure 7). Moreover, we now take advantage of the depicted roles of the mitotic CUL4B complexes in the revised discussion.

Referee #3:

Overall, the paper is interesting and important. As this study points out a possible mechanism for human brain mitotic defects, it would have been excellent if a more relevant system had been included. Yet we understand that this is beyond the scope of the current study.

As suggested by the reviewer, we now included new data in Figure 7 and EV Figure 7 demonstrating an essential role of CUL4B in the development of brain organoids. Interestingly, lack of CUL4B function prevents the formation of stable cortical units, despite the presence of CUL4A. This phenotype is reminiscent of previously published organoids lacking LIS1, and consistent with our findings that a CUL4B-LIS1 E3-ligase complex may regulate spindle dynamics. We now discuss these exciting findings in the revised discussion. Although further analysis is needed to substantiate the function of CUL4B phosphorylation in this process, the requirement of CUL4B in these developing brains greatly expands the physiologically relevance of our findings.

Specific comments:

Fig1B: an additional explanation of the visible Cul4 bands is required. Please, add arrows,

sizes, etc. In Fig5E, the size differences between Cul4A and CUL4B are much more significant. What is the difference between the experiments?

As suggested, we have now marked the bands corresponding to CUL4A and CUL4B and their neddylated variants. Depending on acrylamide percentage and the running time, migration of the different CUL4 species can vary.

Why is there a change in Cul4 bands in DDB1 RNAi? What does it mean?

The existence of the different CUL4 species is known to change in DDB1 depleted cells. In the absence of DDB1, CUL4A and CUL4B accumulate in their un-neddylated, inactive states, since DDB1 is required to recruit DCAFs among gels.

Fig 1C - it's unclear what bands were measured for neddylated versus non-neddylated forms. From the gel in Fig 1B? If so - they should be labeled (as in Fig 2B). The verification that these bands are bands of neddylated protein is lacking.

The quantification shown in Figure 1C was obtained by analyzing CUL4A and CUL4B immunoblots of extracts prepared from three independent experiments. To validate migration of the different CUL4A and CUL4B species, we have included a new experiment in EV Figure 1 (panel A). Specifically, we treated cells with MLN4924 or CSN5i-3 preventing neddylation or de-neddylation of cullins, respectively. Migration of the different CUL4A or CUL4B species was then followed by immunoblotting. GAPDH serves as a loading control.

Fig 1D - there is no SE in the growth versus time graph.

The left panel of Figure 1D compares growth of the indicated cells in a single, representative experiment, while the right graph quantifies the growth rate deduced from multiple experiments with standard deviation and statistical analysis. We adjusted the legend to avoid misunderstanding.

Minor comment, Fig 1D - in the legend n=3×3. In the graph, there are 12 dots. Please, change the legend.

As suggested, the legend was changed to n = 3x4.

Fig 1G - Cell cycle analysis is based only on brightfield analysis. If DNA stain or additional methods (FUCCI stains) had been used, a more detailed analysis of the progression through cell cycle phases could have been achieved.

We intentionally used brightfield live cell analysis to avoid artifacts induced by the fluorescence light. Moreover, we followed cell division with FACS analysis. However, we agree with the reviewer that an in-depth cell cycle analysis using FUCCI or other markers will be needed to further dissect the defects underlying the observed mitotic delay.

In the text lines 106-109, there is a conclusion of redundant and non-redundant functions of CRL4A and CLR4B for cell growth. Not clear how a non-redundant function can be concluded in this experiment. Please, rephrase the explanation.

We have revised the text describing the redundant and non-redundant functions of CUL4A and CUL4B.

Minor comment, EDF2D: the size marker should be added to show that HA Abs recognize the same band as Cul4b.

As suggested, a size marker was added. However, it is expected that HA-tagged CUL4B migrates slightly slower compared to endogenous, un-tagged CUL4B.

For all figures: I recommend using an alternative way to show time-lapse images of cell proliferation. A 30min interval is not convincing and not a conventional way to be displayed. Not clear which points were used as a beginning and an end of the cell cycle.

To address this point, we now include live cell movies (EV Folder 1: Mitotic duration movies from Figure 1, EV Folder 2: Mitotic duration movies from Figure 3 and EV Folder 3: Spindle positioning movies from Figure 6). We also changed the labeling of the still images (Figure 1, panel G). Note that in the right graph the time cells spend in mitosis was quantified and displayed with standard deviation and statistical analysis.

Fig 2B, C- the labeling of the lane is a little confusing; in particular, the phosphatase treatment and the mitotic shake-off are indicated too far from the relevant lanes. (Fig 5C, D style is clearer, in my opinion)

As suggested, we adapted the labeling of Figure 2, panels B and C.

Fig 3A and text 179-180. It's stated that mutations didn't alter expression levels of Cul4a. According to the figure, there is a reduction in the NP1 condition. Please, add quantifications to the statement.

See our response to reviewer 1. Figure 4 above shows a quantification of CUL4A levels normalized to GAPDH in wild type and CUL4B-deleted HeLa cells, compared to CUL4B-deleted cells expressing wild type CUL4B or the P50L or NP1 mutants. While there is some experimental variation, we do not observe a consistent and significant change of CUL4A levels in the different phospho-deficient CUL4B mutants.

Fig 5A, the control of His pull-down without overexpressing His-DDB1 is lacking. In Fig5C, D - controls Strep pull-down without the presence of SA-Cul4b and Flag pull-down without Flag-Lis1 or Flag-WDR1.

See also response to Reviewer 1 above. We performed quantitative Fluorescence Polarization (FP) experiments to overcome shortcomings with Coomassie gels or pull-down experiments, and the results of these orthogonal assays are fully consistent. Nevertheless, we now included an additional specificity control, where we co-express Flag-DDB1 and Strep-LIS1 (EV Figure 5A, right panel). We incubated Flag-DDB1 with empty strep beads, and we don't observe unspecific binding. We have also performed a second control experiment, where we deleted the N-terminal domains of LIS1 and WDR1 encompassing the predicted HLH motifs. Despite equal protein expression of LIS1-ΔN and WDR1-ΔN, only LIS1-ΔN interfered with DDB1 binding (new EV Figure 5B). Beyond specificity, this experiment further demonstrates that the predicted HLH-motif of Lis1 likely mediates DDB1-binding, another region is responsible for this in WDR1. We have thus discussed the implications in the revised text.

According to Fig 5E and 5G, the pull-down of Cul4A with both LIS1 and WDR1 worked. The EDF5B and C graphs could be moved to the main figure to confirm the differential binding.

Thanks for this suggestion. Indeed, the affinity of CRL4A binding to LIS1 is approx. 13 µM (EV Figure 5E). We believe that FP assays provide more convincing quantification of binding of CRL4A and CRL4B to LIS1 and WDR1, respectively, and thus prefer to keep the quantification of the pull-down experiment in the EV Figures 5C and D. We describe both assays in the revised text and direct the reader to the appropriate Figures.

Fig 5 JL- It is unclear how many repeats were used to quantify the data. In some cases, there is only one visible data point (L) and sometimes 2 points (J). Please clarify, and indicate n.

Thank you for pointing this out. We now indicate the number of repeats in the revised Figure legends.

For the functional role of Lis1-Cul4b interaction in the spindle orientation experiment (Fig 6F), it would be more elegant to add the NP1 condition when the protein is present but does not interact with LIS1.

Unfortunately, we do not have the necessary fluorescent reporters to monitor spindle positioning in the CUL4B knockout HeLa cells complemented by the NP1-mutant. Moreover, the HeLa$_{FLP}$ cell line used for mutant expression are more mobile, which makes it more difficult to assess spindle positioning. However, in the brightfield images used to assess mitotic duration, we can also observe enhanced spindle mobility in NP1 expressing cells. We now highlight this result in the included live cell imaging movies (EV Folder 3: Spindle positioning movies from Figure 6). The corresponding stills are shown in ***Figure 6, below****.*

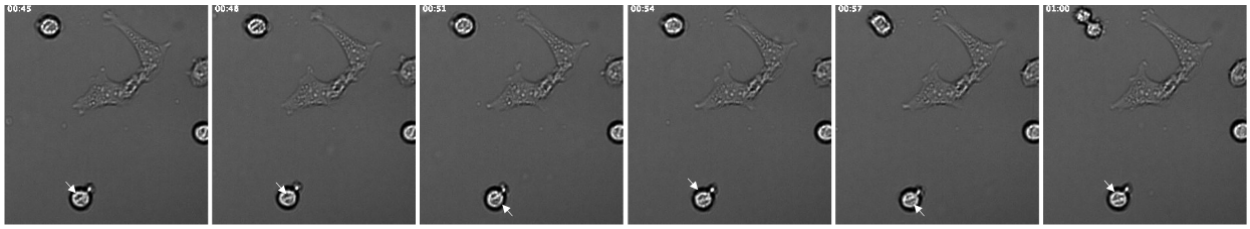

Fig. 6: *Brightfield imaging of HeLa$_{FLP}$ cells expression the NP1 mutant. The white arrow highlights the aberrant spindle movement during mitosis.*

Dr. Matthias Peter
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24th May 2023

Re: EMBOJ-2022-112847R
The CRL4B E3 ligase regulates mitosis and brain development by recruiting phospho-specific DCAFs

Dear Matthias,

Thank you again for submitting your revised manuscript for our consideration. It has now been re-reviewed by referee 1, and I am happy to say that this referee retains only a few presentational concerns (see below), which I would kindly invite you to incorporate into a final version of the text during a final round of minor revision. Please make sure to make your revisions in the uploaded (and attached) manuscript text file, as I already introduced several other corrections and stylistic edits into it.

At this point, I additionally need to ask to fully address several important editorial points:

- In the attached file, I made some minor changes to the title and abstract, mainly to make them somewhat more explicit and appealing to a broad readership - please check and approve.

- Please double-check to make sure to all relevant funding information in the manuscript is congruent with the info entered into our submission system: Boehringer Ingelheim Fonds are still not mentioned in the submission system, only in the manuscript. Furthermore, ETH Zürich is only inserted in the comments box in the submission system, but not formally entered.

- In the Data Availability section, please include a direct hyperlink to the deposited dataset at the respective repository.

- None of the 14 EV Movie ZIP files appear to be correct - each of these ZIPs should contain (a) a universally accessible movie file and (b) a text file with the according label (Movie EV1/2/3...) and the corresponding legend for the respective movie.

- Please correct the wrong label ("S5") in the figure EV5 file, and provide a higher-quality image for figure EV4B.

- It appears that several Coomassie gel images in Figure EV5F are duplicated in other panels (Fig 5E, Fig EV5G),without any indication/clarification. Any such re-display needs to be explicitly mentioned in all respective figure legends, including clear explanations as to why certain lanes were re-displayed in multiple locations.

- In Figure 5B, it appears as if there are still error bars displayed on the WDR1 curve despite N<3, which does not warrant any calculation of error bars (as explained in detail in our Guide to Authors), but would instead warrant plotting of the 2 individual data points per x-axis value.

- Finally, I am not convinced that equally mixing data points from both biological and technical replicates (as e.g. in Figures 1DFG, 3BE, 4D, 6EG, EV1DFH, EV6BC) is the correct procedure - it would appear more reasonable to (a) first calculate mean values from all technical replicates underlying one biological replicate, and then (b) to display the mean value + error bars of all these biological replicate means (or, if only N<3 biological replicates, to display the individual data points for these biological means). E.g., in Fig 1D, for each of the N=3 biological replicates, generate one mean from the N=4 technical replicates; then, display those 3 biological replicates incl. error bars as e.g. in Fig 1C. Please, carefully revisit all the above-mentioned figure panels, as well as any others to which similar inappropriate statistical analyses have been applied.

I am therefore returning the manuscript to you for a final round of minor revision, to allow you to make these adjustments and upload all modified files. Once we will have received them, we should be ready to swiftly proceed with formal acceptance and production of the manuscript.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD

Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

*** PLEASE NOTE: All revised manuscript are subject to initial checks for completeness and adherence to our formatting guidelines. Revisions may be returned to the authors and delayed in their editorial re-evaluation if they fail to comply to the following requirements (see also our Guide to Authors for further information):

1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed. Further information: embopress.org/page/journal/14602075/authorguide#dataavailability

2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps, .tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: http://bit.ly/EMBOPressFigurePreparationGuideline

5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File. A download link is found at the top of our Guide to Authors: embopress.org/page/journal/14602075/authorguide

7) All authors listed as (co-)corresponding need to deposit, in their respective author profiles in our submission system, a unique ORCiD identifier linked to their name. Please see our Guide to Authors for detailed instructions.

8) Please note that supplementary information at EMBO Press has been superseded by the 'Expanded View' for inclusion of additional figures, tables, movies or datasets; with up to five EV Figures being typeset and directly accessible in the HTML version of the article. For details and guidance, please refer to:
embopress.org/page/journal/14602075/authorguide#expandedview

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (22nd Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In their revised manuscript, Stier et al. provide additional data that supports their original study, which identifies novel CUL4B-specific E3 ligase complexes that control mitotic progression through distinct actin-dependent mechanisms. In particular, they have included additional specificity controls for the pull-down assays that satisfies my major concern. Furthermore, they present new data showing that CRISPR-mediated deletion of CUL4B leads to aberrant forebrain development in an organoid model (through IF analysis and quantification of relevant markers), adding further physiological relevance to their proposed mechanistic studies on how the XLID variant (CUL4p.P50L) variant interferes with proper neurodevelopment.

As mentioned in my original review, this is an exciting study with important implications for principles of CRL4 assembly and molecular mechanisms of normal and aberrant neurodevelopment. I recommend it for publication.

Below I am suggesting minor points for the authors to consider:

• I completely agree with the authors that they show direct interactions via the FP assays and via the pull down assays performed with purified components (Fig 5E,F). However, a direct interaction cannot be claimed from a pulldown of proteins upon co-infection in insect cells. I would therefore suggest the following textual changes:
o Line 293 - line 296: Indeed, both LIS1 and WDR1 specifically interact with DDB1 under these conditions, although with weaker affinity compared to the conventional DCAF8 (Fig. 5A, EV Fig. 5A). FP assays confirmed that these interaction are direct and revealed that LIS1 and WDR1 bind DDB1 with a Kd of approx. 5 µM (Fig. 5B).
o Line 1256: change "directly" to "specifically"
o Line 1463: change "directly" to "specifically"
• Fig 5A and S5A,B: it took me a bit of time to figure out that F, H and S are used in the figure label to indicate that a FLAG, His or Strep pull down was performed. It might be helpful for the reader to explicitly mention this in the figure legend.
• Line 1469 and 1470: the figure legend refers to (C,D) but (B) and (C) are mentioned in the legend. Change to (C) and (D).
• Line 844: could the authors please specify in the material and method which hESC line was used for their studies?
• Line 1339: the panel label (A) should be moved to this line right after the figure title

Point-by-point response to the remaining comments:

Referee #1

We are happy that this reviewer recommends publication and agrees that we present an exciting study with important implications for principles of CRL4 assembly and molecular mechanisms of normal and aberrant neurodevelopment. We have now addressed the remaining minor points as follows:

I completely agree with the authors that they show direct interactions via the FP assays and via the pull down assays performed with purified components (Fig 5E,F). However, a direct interaction cannot be claimed from a pulldown of proteins upon co-infection in insect cells. I would therefore suggest the following textual changes:

line 294: Indeed, both LIS1 and WDR1 specifically interact with DDB1 under these conditions, although with weaker affinity compared to the conventional DCAF8 (Fig. 5A, EV Fig. 5A). FP assays confirm that these interaction are direct and revealed that LIS1 and WDR1 bind DDB1 with a Kd of approx. 5 µM (Fig. 5B). As suggested by the reviewer, we have now revised the text and changed "directly" to "specifically":
o New line 1340: changed "directly" to "specifically"
o New line 1626: changed "directly" to "specifically"

Fig 5A and S5A,B: it took me a bit of time to figure out that F, H and S are used in the figure label to indicate that a FLAG, His or Strep pull down was performed. It might be helpful for the reader to explicitly mention this in the figure legend.

As suggested, we now explicitly state in the revised Figure legends that "F" stands for "FLAG", "S" for "Strep", and "H" for "His".

Line 844: could the authors please specify in the material and method which hESC line was used for their studies?

We used the H9 hESC cell line, which is now specified in the revised Material and Method section as requested.

Line 1469 and 1470: the figure legend refers to (C,D) but (B) and (C) are mentioned in the legend. Change to (C) and (D).
Line 1339: the panel label (A) should be moved to this line right after the figure title

These labeling/formatting errors have now been corrected.

Editorial points:

In the attached file, I made some minor changes to the title and abstract, mainly to make them somewhat more explicit and appealing to a broad readership - please check and approve.

Prof. Dr. Matthias Peter, Institute of Biochemistry, ETH Zürich, HPM G8, CH-8093 Zürich
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Thank you for your edits. We have used your text file as a template for our revisions and approved your corrections accordingly.

Please double-check to make sure to all relevant funding information in the manuscript is congruent with the info entered into our submission system: Boehringer Ingelheim Fonds are still not mentioned in the submission system, only in the manuscript. Furthermore, ETH Zürich is only inserted in the comments box in the submission system, but not formally entered.

As suggested, we checked the funding information and now added the Boehringer Ingelheim Fonds in the submission system.

In the Data Availability section, please include a direct hyperlink to the deposited dataset at the respective repository.

We added a direct link to the MS dataset. Please note that this link will only be publically released upon final acceptance of the manuscript.

None of the 14 EV Movie ZIP files appear to be correct - each of these ZIPs should contain (a) a universally accessible movie file and (b) a text file with the according label (Movie EV1/2/3...) and the corresponding legend for the respective movie.

We apologize for this error. We have now generated new ZIP files, which include text files with Movie names and legends.

Please correct the wrong label ("S5") in the figure EV5 file, and provide a higher-quality image for figure EV4B.

We have corrected the label and included a higher quality image for EV4B.

It appears that several Coomassie gel images in Figure EV5F are duplicated in other panels (Fig 5E, Fig EV5G), without any indication/clarification. Any such re-display needs to be explicitly mentioned in all respective figure legends, including clear explanations as to why certain lanes were re-displayed in multiple locations.

The three panels Fig. 5E, EV5G and F originate from the same Coomassie gel, but each cropped group of lanes conveys a different message. Overlapping lanes were used as controls. As requested, we now clearly state this in the final figure legends.

In Figure 5B, it appears as if there are still error bars displayed on the WDR1 curve despite N<3, which does not warrant any calculation of error bars (as explained in detail in our Guide to Authors), but would instead warrant plotting of the 2 individual data points per x-axis value.

We have now adjusted Fig. 5B panel without error bars for the WDR1 dataset, and plotted the individual data points for each replicate.

*I am not convinced that equally mixing data points from both biological and technical replicates (as e.g. in Figures 1**DF**G, 3BE, 4D, 6EG, EV**1DFH**, EV6**B**C) is the correct procedure - it would appear more reasonable to (a) first calculate mean values from all technical replicates underlying one biological*

replicate, and then (b) to display the mean value + error bars of all these biological replicate means (or, if only N<3 biological replicates, to display the individual data points for these biological means). E.g., in Fig 1D, for each of the N=3 biological replicates, generate one mean from the N=4 technical replicates; then, display those 3 biological replicates incl. error bars as e.g. in Fig 1C. Please, carefully revisit all the above-mentioned figure panels, as well as any others to which similar inappropriate statistical analyses have been applied.

We agree that mixing data points of technical and biological replicas for the statistical analysis is not ideal. As suggested, we have thus adjusted panels D and F of Figure 1 and EV1, and calculated the mean of the biological replicates which are now represented in the revised graphs. We believe however that the situation is different for the single cell measurements, were it is relevant to compare the cell cycle length or position of the metaphase plate of individual cells. In this case, it is important to measure sufficient number of cells in different experiments, and then assess the spread of the relevant cell cycle times between single cells. We would thus like to show all measured cells as individual data points to visualize and statistically analyze the effect, as the spread of the data points provides valuable information. In fact, our previously labeled technical replicate is not really a technical replicate, as each cell is a biological experiment and should be considered as an individual data point. To avoid misunderstanding, we have thus changed the legend of these Figure panels and display the total number of analyzed cells obtained from “n” experiments.

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13th Jun 2023

Re: EMBOJ-2022-112847R1
The CUL4B-based E3 ubiquitin ligase regulates mitosis and brain development by recruiting phospho-specific DCAFs

Dear Dr. Peter,

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Yours sincerely,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods, and Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	No blinding was necessary
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	For MTT assays, wells with obvious technical problems were eliminated. Otherwise, no data points were excluded in the analysis
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures, Material and Methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
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Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Figures