JCB

Regulation of MT dynamics via direct binding of an Abl family kinase

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Review Timeline:

Submission Date:	2019-02-19
Editorial Decision:	2019-03-22
Revision Received:	2019-08-02
Editorial Decision:	2019-09-06
Revision Received:	2019-09-24

Monitoring Editor: Melissa Gardner

Scientific Editor: Melina Casadio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.201902123

March 22, 2019

Re: JCB manuscript #201812144

Dr. Anthony J Koleske
Yale University
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Dear Dr. Koleske,

Thank you for submitting your manuscript entitled "Regulation of MT dynamics via direct binding of an Abl family kinase". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Thank you very much for your patience with the review process. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

Indeed, while the reviewers all found the results interesting, they were not convinced yet by the data and shared some overlapping concerns about the degree to which the conclusions are supported by the data and approaches. Strengthening these core analyses and addressing the following points would be needed for the paper to reach the level of definitive and impactful advance needed for publication in JCB as a Report

1) The most serious issue in our view is the lack of sufficient evidence that (1) Alb2 kinase binds microtubules in a location on dynamic microtubules that would allow it to regulate plus-end microtubule dynamics (e.g., at the plus-end), and (2) Alb2 kinase can directly regulate microtubule dynamics at physiological Alb2 kinase concentrations. These two points provide some of the core novel conclusions and must be demonstrated convincingly. We recommend that you focus experimental efforts in the revision to address these two most important experimental issues (Rev#1, Fig 1 paragraph, last part, especially B comment; Rev#2 "now more problematic.." paragraph; Rev#3 #1).

2) None of the reviewers are convinced by the claim of two separate microtubule-binding elements (Rev#3 minor #1, Rev#2 #1, Rev#1 Fig 1 point). However, editorially we consider this claim a secondary point in the manuscript that could potentially be resolved by clarifying the experimental results that you currently have and by strongly toning down the conclusions in this regard. Alternatively, you could perform additional experiments if you want to make this claim more definitively, as suggested by the reviewers. We would leave this decision to you.

3) We are concerned about the lack of diffusive dynamics on the MT surface of single molecules, which seems unusual for a MAP that only binds the E-hooks, as echoed by referee comments raising a concern that this mode of binding has been incorrectly identified, since a MAP that only binds the E-hooks (via an electrostatic interaction) would indeed be predicted to diffuse on the microtubule. Therefore, the subtilisin experiments must be repeated to ensure that the microtubules are correctly treated, and subtilisin removed prior to the introduction of Abl2, as noted by Reviewer #3. The final methods used should also be clearly and carefully described.

4) We agree with Reviewer #3 that whether Abl1 also functions like Abl2 with regards to MTs is an obvious question raised by the work. We would not require experiments testing Abl1 function for publication in JCB but carefully discussing this point from your expert perspective on the Abl family proteins would be welcome, interesting, and relevant.

5) Rev#2 discussed concerns over the scope of the study and degree of novelty/advance for JCB. We have discussed these concerns editorially in depth and also in the context of Reviewer #1's comments that the cell experiments do not connect very strongly with the in vitro experiments, which we agree with. While a detailed mechanistic analysis would be beyond the scope of this Report, providing evidence for the physiological relevance of the cell-free experiments is important. To address these issues, we recommend that you make a stronger attempt to use the cell experiments to demonstrate the physiological relevance of the results, without attempting to connect the in vitro experiments and/or mechanism to detailed cell results. For instance, perhaps looking at Abl2's functions in cell morphology, migration, or adhesion may be an appropriate start.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. It may be necessary to extend your manuscript to a full Research Article. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000; a full Research Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: A Report may include up to 5 main text figures; a full Research Article may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures; a full Research Article may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Melissa Gardner, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Hu and colleagues have submitted a 'Report' claiming that non-kinase regions of the Abl2 kinase can bind to microtubules and regulate their dynamics. The advance rests mainly on in vitro experiments, but later figures provide some evidence that expression of the microtubule-binding region of Abl2 in cells also affects microtubule dynamics. At least in principle, the work seems like it could be appropriate for the report format because the claims seem novel but the underlying mechanisms are not very clear. I think a suitably revised version of the manuscript might become acceptable for JCB. The main issue for me is that I am not convinced that the data support the claim that there are two distinct microtubule binding elements - addressing this will requires re-writing or additional experiments. Overall the demonstration and analysis of binding could be strengthened/clarified. A lesser issue is that the in cell experiments, while apparently more or less consistent with the in vitro results, do not connect to them very strongly and were discussed in a way that does not seem to consider alternative models. This can probably be addressed exclusively with writing, but experimental data would be welcomed if the authors happen to have it (for example, can Abl2 constructs be seen on the microtubules?). Some more detailed comments follow.

Figure 1 uses various truncation constructs and microtubule co-sedimentation experiments to identify binding determinants. The authors conclude that The C-terminal portion of Abl2 (after the kinase domain) contains two microtubule-binding elements, and that microtubule binding relies on the negatively charged C-terminal E-hooks of alpha and beta tubulin. The E-hook conclusion seems well-supported, but I have some questions about the claim of two separate microtubule binding elements:
+ N-668 binding MTs with comparable affinity to full-length Abl2. Since N-668 lacks the MT2 region that the authors claim contributes to MT binding, it seems N-668 should not bind as tightly as full-length. How do the authors explain this? Can the authors rule out the possibility that the 557 boundary (or proximity of the MBP fusion partner in that construct) is responsibly for the observed decrease in affinity relative to N-668?
+ In general how the binding data were fit could be better described. How significant was the 'nonspecific' binding component (this seemed like an unusual model to use to this reviewer)? I presume that the errors on KD are fitting errors. Since the authors have n>5 trials, could they fit each trial separately and then examine how variable the fitted KDs are?

Also: A) have the authors examined whether Abl2 binds to unpolymerized tubulin? Their experiments and discussion strongly assume/imply that all the activity stems from microtubule binding. There are a variety of ways one could test for this, gel filtration chromatography being the most obvious. B) the concentrations of Abl2 fragements used is rather high relative to the concentration of tubulin. That can be perfectly acceptable, but it can also raise questions about specificity. Can the authors do/say anything more to establish that the in vitro effects they see are specific to Abl2? The MBP controls are a step in the right direction, maybe testing some other poly-cation would be nice?

Figure 2 is relatively clear although it might have been nice to show additional kymographs in the supplement, and I was somewhat surprised by the immobility on the microtubule. Figure 3 is also relatively clear (but could use additional kymographs in a supplement); is 'relative frequency' the best y-axis label for 3I? Fig. 4 is realtively straightforward but as mentioned above not as well-connected to the in vitro findings as it could be.

Minor/stylistic points:
+ In Fig 2 where the authors are using turbidity (=light scattering) to measure microtubule assembly, 'relative fluorescence unit' does not seem like the right axis label
+ There is no scale bar for the electron micrographs in Sup Fig 2
+ In the discussion, comparisons to tau seem premature and in danger of being misleading.

Reviewer #2 (Comments to the Authors (Required)):

Review of manuscript "Regulation of MT dynamics via direct binding of an Abl family kinase" by Hu and colleagues.

In this manuscript, the authors show that the tyrosine kinase Abl2 binds to microtubules and promotes MT growth by increasing growth rate, suppressing catastrophe, and decreasing depolymerization rate. The authors characterize the binding region, showing that it is separable from the kinase domain. To provide evidence that the MT binding site is physiologically relevant, they generate an Abl2 knockout, show that it has perturbed MT dynamics, and show that expressing the C-terminal domain can rescue this phenotype. The authors use these data to conclude that Abl has a previously unrecognized direct role in regulating MT dynamics.

First the good: This manuscript is clearly and carefully written, the experiments are well-controlled and well-performed, and (with some exceptions noted below) the conclusions are robust and well-supported. Recognizing that Abl binds MTs directly is important for understanding its function and seems likely to be relevant for Abl's role in cancer.

Now more problematic: I'm not convinced that this paper is "over the bar" for JCB. It would not be over the bar for a regular JCB paper, but it might be over the bar for a Report. Briefly, the authors do reveal something new and likely important about a well-studied protein, i.e., they show that Abl2 tyrosine kinase binds MTs and can stabilize them in vitro. However, the significance of this interaction in vivo is not yet clear. Most importantly, I'm not convinced that the effect of Abl on microtubule dynamics is direct. Instead, it seems equally if not more likely that it acts by regulating other MT binding proteins. In other words, it seems equally likely that the purpose of the MT binding region is to localize the kinase to MTs, not to regulate them directly. Yes, the authors do show that the kinase domain is not necessary for the rescue, and that is encouraging, one could imagine other

proteins being recruited to MTs via the C-terminal domain. One of the main reasons I'm concerned about this is that the level of many signaling proteins is quite low, much lower than typical structural proteins like lattice binding MT regulators. What is the level of Abl2? Some proteins do manage to regulate MT dynamics when present at low level, but these are typically either end binding proteins (which concentrate at MT tips) or proteins that contain a multitude of MT binding sites and so influence dynamics by creating a high local concentration of binding sites.

Thus, I'm on the fence. If the paper does proceed in JCB, I think that the following issues need to be addressed. In fact, these should be addressed regardless of where the paper is published; all can be addressed at the level of writing and/or minor additional non-experimental analysis.

1) My biggest concern is that I'm not convinced that the authors have enough data to justify the map presented in Table 1. In particular, what is the evidence for the domain boundaries shown? For example, as far as I can tell from the data presented, there could simply be one extended MT binding domain that starts somewhere between 557 and 688 and goes into ABD1. Thus, I think that the authors need to carefully re-examine this figure and the conclusion that there are two separable MT binding domains and revise as necessary to either clarify the explanation or alter the interpretations. Note that if there is only one domain, it really isn't a problem for the paper - it is just important to make sure that this map corresponds appropriately to the data presented from the sum of this paper and other publications.
2) A related issue is that the authors should comment on the sequence in the C-terminal half of Abl2. How do the activities mapped thus far relate to recognizable sequence motifs and/or sequence characteristics? How well conserved is this region in Abl relatives?
3) The authors need to carefully re-examine their paper and edit to remove overstatements. For example, on the sixth page of the Results, the text reads: "Together, these results indicate that Abl2 uses its C-terminal MT binding domains to stabilize MT structure by slowing MT shortening and reducing MT catastrophe frequency". This text is an overstatement because while the results do show that Abl2 CAN use its MT binding domain this way in vitro and under perturbed conditions in vivo, that is very different from showing that it DOES use this domain this way in vivo. Many MT binding proteins have similar activities when present at higher than normal concentration. The authors should carefully review the whole manuscript for similar cases of overstatement.
4) Please consult with the JCB editorial rules, but I do not think that SEM is appropriate for microtubule binding assays. SEM is used for situations where one is trying to use samples pulled from a normally distributed population to estimate the center of that normal distribution. Instead, what one needs to do here is represent the scatter in the data. That is typically done by standard deviation. I recognize that many people use SEM for data like this because SEM looks better (more datapoints always lead to smaller reported errors, which is not true with SD), but that doesn't make it correct.

Minor:
Figure 2: Panels D,E: the legend should say what the concentration of Abl2-557-C-GFP is.
I assume that it is 20nM, but the legend should still say this.

Figure 4: The legend should say what is being visualized. The text says that it is mCherry-MACF43, but it would still be good to have this in the legend or figure itself.

Reviewer #3 (Comments to the Authors (Required)):

Regulation of MT dynamics via direct binding of an Abl family kinase
Hu et al.

This manuscript by Hu et al reports the discovery of microtubule binding and direct regulation by Abl2. Abl kinases are famous for their roles in regulating actin cytoskeletal dynamics during cell migration and shape changes. Although Abl2 had been previously shown to bind microtubules, details of the interaction and its cellular function have remained poorly understood. Here the authors revisit the interaction between Abl2 and microtubules. The results clearly demonstrate a direct interaction between domains of Abl2 and microtubules, and show that Abl2 acts to stabilize microtubules in vitro and in vivo by accelerating polymerization, slowing depolymerization and preventing catastrophes. Overall, that results are clear and have the potential to open new avenues for investigation into the regulation of the cytoskeleton by Abl kinase.

This study will be of interest to the readership of JCB. However, there are several major issues that should be addressed prior to publication. Addressing these issues would improve the clarity and mechanistic insight of the study. At that point, it would be suitable for publication in JCB

Major Points:
1. The study stops short of suggesting a mechanism through which Abl2 regulates microtubule dynamics. The results in Figures 3 and 4 convincingly demonstrate that C-terminal domains of Abl2 promote polymerization and inhibit catastrophe and depolymerization. These activities suggest that Abl2 likely regulates the microtubule plus end; however, the 2-color TIRF experiments that investigate the microtubule binding activity of Abl2 in Figure 2 are not designed to test for plus end localization. Instead, these experiments assess the localization of Abl2 on microtubules stabilized with either GMPCPP or taxol, which would not have dynamic plus ends analogous to those in Figure 3. This could simply be resolved by modifying the experiments in Figure 2 to instead assess the localization of Abl2 on dynamic microtubules. This is an important point because either result would be interesting and give deeper insight into the mechanism - either Abl2 selectively binds plus ends, which could then be related to its plus-end stabilizing activity; or Abl2 does not selectively bind plus ends and would somehow regulate plus end dynamics without accumulating at that site. This latter result would be particularly intriguing, given the interaction between Abl and CLASP proteins, which may regulate plus end dynamics by binding to the lattice.

2. The authors conclude that Abl2 contains two microtubule-binding domains, one with high affinity and another with low affinity, and suggest in the conclusions section that
We propose that Abl2 may use its two MT-binding regions to bridge adjacent protofilaments, providing longitudinal or lateral structural support to enhance MT rigidity and promote assembly.
The data do not convincingly support the conclusion that there are two domains, much less the provocative model that two domains could bridge tubulin subunits together. The 688-C has very weak, but still measurable binding, and apparently no effect on microtubule dynamics. Thus, it is not clear that this is truly a distinct domain of Abl2, rather than an adjacent region that is part of the same microtubule binding domain. The authors could reword this conclusion as speculation that requires further testing.

3. The results from the S-tubulin experiments in Figure 1E-F are somewhat difficult to interpret, and do not clearly support the conclusion that Abl2 requires the tubulin tails to bind to microtubules. This is compounded by the minimal description of these experiments in the materials and methods section. The authors should provide a more detailed description, as subtilisin digestion is a finicky

reaction and can easily produce artifacts.

The apparent partial proteolysis of Abl2 by subtilisin, as indicated by the mobility shift in Figure 1E, raise the possibility that the disruption of microtubule binding could be due to damage to Abl2, rather than the loss of tubulin tails. Indeed, the authors acknowledge this possibility on page 8. To prevent damage to Abl2, the authors should add subtilisin to tubulin first, and then inhibit the protease with PMSF before adding Abl2. As an additional step to remove subtilisin, the treated tubulin could be assembled and pelleted before adding Abl2. It's not clear from the methods whether these or other steps were taken.

The other important issue is whether subtilisin was added to pre-assembled microtubules, or to free tubulin that was then assembled after digestion. This is important because it is well documented that subtilisin digestion lowers the critical concentration for tubulin assembly and would therefore be expected to increase the amount of microtubule polymer in the pellet. The data in Figure S1K do not indicate that more tubulin is in the pellet, so perhaps the pre-assembled polymer was digested? Either way, this should be made clear in the materials and methods section, so that the reader can adequately interpret the binding data.

4. Is the Abl1 kinase also expected to bind microtubules and exhibit similar activity? Although it may not be necessary to perform parallel experiments with Abl1, it would be very interesting for the authors to add some discussion of whether they would expect Abl1 to exhibit similar behavior, based on its domain structure and sequence conservation compared to Abl2. The authors' expertise on Abl kinases would be greatly appreciated here.

Minor Points:
1. The authors state in the last paragraph of the introduction and again in the discussion that the microtubule-regulating activity of Abl2 is "kinase independent". It may be more clear to say this activity "does not require the kinase domain". While fragments lacking the kinase domain are sufficient to alter microtubule stability in vitro and in vivo, the authors have not tested a full length Abl2 that lacks kinase activity (e.g. a point mutant). Thus it is unknown whether the presence of an inactive kinase domain may modulate the activity of the C-terminal domains. Expressing a point mutant that disrupts kinase activity could convincingly address this point.

2. I was confused by the use of the term "growth length" on p11 of the results and in Figure 1J. It is not clear whether this means length at catastrophe or simply a sampling of microtubule lengths from "snapshot" data.

3. I found the organization of panels in Figures 1 and S1 to be confusing. The order of panels in Figure 1 does not match the order in which they are described in the text. This makes the figure difficult to navigate. The authors should consider re-ordering these panels for clarity. Similarly, Figure S1 contains 18 panels, only 4 of which are called out in the text, and these do not follow the order in which they are called out. While I applaud the authors for showing this data, the panels should be re-ordered to match the flow of the text, and called out alongside the appropriate panels in Figure 1.

Journal of Cell Biology Response: 08-02-2019
Manuscript Identifier: 201812144
Title: Regulation of MT dynamics via direct binding of an Abl family kinase

Dear Dr. Gardner and Dr. Casadio,

Please find enclosed our revised manuscript “Regulation of MT dynamics via direct binding of an Abl family kinase" (201812144) for consideration for publication as Report in Journal of Cell Biology. We thank the reviewers for their helpful comments and in response have performed many key additional experiments. We believe we have addressed each of the reviewers’ concerns satisfactorily and hope they will now find our paper suitable for publication. We provide a point-by-point response to each issue raised by the reviewers below.

Editor, Issue 1: *The Editor and Reviewer 3 asked whether Abl2 binds dynamic microtubules in a location that could allow it to regulate plus-end microtubule dynamics?*

> We thank the editor for this key experimental suggestion. We addressed this question by monitoring the binding of single molecules of Abl2-557-C-GFP, which is sufficient to bind MTs in our cosedimentation assays and regulate MTs in our turbidity and imaging assays, to growing rhodamine-labeled MTs. In this imaging experiment we perfused the flow chamber with 50 nM Abl2-557-C-GFP and 7 μM rhodamine-tubulin with anchored GMPCPP-stabilized biotin-MT-seeds and monitored with images taken at 1 frame per second. Our new data identified numerous examples of Abl2-557-C-GFP binding to growing rhodamine-MTs. The majority (20/27) bound stably to the lattice of the growing MTs, while a smaller subset minority (5/27) associated with the growing MT tip. In rarer cases (2/27), Abl2-557-C-GFP bound the MT lattice and glided toward the MT growing end. These data are presented in new Figs. 2F and 2G and detailed in the text and the experimental procedures are documented in the Material and Methods section.

Editor, Issue 2: *The Editor and Reviewer 2 asked if Abl2 can regulate microtubule dynamics at physiological concentrations.*

> Using purified recombinant Abl2 as the blotting standard, we found that Abl2 represents 0.19% of total protein in lysates of mouse fibroblasts and COS-7 cells. Assuming that total cytoplasmic protein concentration to be 100 mg/ml (Finka and Goloubinoff, 2013; Albe et al., 1990) and that the molecular weight of Abl2 is 134 kD, we estimate the physiological concentration of Abl2 in these cell types to be 1.4 μM. These data are presented in Fig. S3A and detailed in the text. This is well above the concentration range at which Abl2 promotes tubulin assembly in our turbidity assay (0.5 μM Abl2) and regulates MT dynamics in our *in vitro* imaging assay (1 μM Abl2 increased MT growth rate, slowed MT shrinkage, and reduced catastrophe frequency).

Editor, Issue 3: *The Editor and all three reviewers asked us to clarify our results describing the portions of Abl2 that bind MTs and to tone down the conclusions. regarding this point.*

We are grateful for the opportunity to clarify this section, which in hindsight was confusing. In this revised manuscript, we identify Abl2 amino acids 557-688 as the primary MT-binding region, which is necessary and sufficient for saturable high-affinity MT-binding (K_d = 1.50 ± 0.30 μM). We provide data showing that regions flanking amino acid 557-688 increase MT binding affinity (Abl2-N-557: Not Binding, Abl2-N-688: K_d = 0.36 ± 0.08 μM). We also show that Abl2-557-C binds MTs with a K_d = 0.98 ± 0.1 μM and Abl2-688-C exhibits low affinity binding to MTs, but we could not achieve high enough MT concentrations for Abl2-688-C to reach saturation and hence could only put a lower limit on the affinity of this interaction (K_d > 3 μM). Hence, we toned down our conclusion as binding of Abl2-557-688 to MTs is augmented by determinants in the N-terminus, as Abl2-N-688 binds MTs with higher affinity than Abl2-557-688, and in Abl2-688-C, which mediates low affinity binding to MTs. These data are presented in Fig. 1 and Table 1 and detailed in the text.

Editor, Issue 4: *The Editor and Reviewer 3 asked that we repeat the subtilisin experiments to ensure that MTs are correctly treated and that subtilisin protease activity was properly removed.*

We repeated the experiment to investigate how proteolytic removal of the tubulin E-hook on Abl2:MT interaction. We found Abl2 bound subtilisin-treated MTs (s-MTs) only weakly and binding did not saturate at the highest s-MT concentration in our testing range, hence we can only put a lower limit on the affinity (K_d > 3 μM). These new data are presented in Figs. 1E, 1F, and S1J and the experimental results are detailed in the text. Our finding that subtilisin or KCl treatment weakens, but does not abolish Abl2:MT interaction suggests that in addition to tubulin E-hook, Abl2 may also bind MTs via one or more additional interfaces (Our findings detailed in the answer to *Issue 3* above is also consistent with this possibility).

As requested, we have also carefully expanded our description of the experimental procedure in the Material and Methods section. s-MTs were made by incubating subtilisin (10 μg/ml) with pre-assembled taxol-stabilized MTs at 37°C for 1 hour and the protease was deactivated with 4 mM PMSF and 100 μg/ml aprotinin. s-MTs were then pelleted and resuspended in BRB80. A portion of s-MTs was depolymerized in 4 ºC and the concentration of s-tubulin was reassessed using Bradford assay. We performed the cosedimentation experiment with 0.25 μM Abl2 and increasing concentrations of s-MTs (0 to 6 μM). Under these conditions, total Abl2 levels (in pellet + in supernatant) did not change as we increased the s-MT concentration, indicating there is no detectable residual protease activity.

Editor, Issue 5: *The Editor and Reviewer 3 asked that we carefully discuss the potential for other Abl family kinases to regulate MT elongation in cells.*

This is an excellent suggestion. We note that the primary interaction site of Abl2 with MTs, amino acids 557-668, share 43% sequence identity with the corresponding region in Abl1, which is also both basic (pI = 10.2) and proline-rich (12/122). We anticipate that Abl1 (or other Abl family kinases) might also directly bind to MTs and regulate MT dynamics. In fact, our preliminary experiments suggest that Abl1 indeed binds MTs using its C-terminal half (W. Lyu, unpublished data). In contrast, corresponding region in *Drosophila* and *C. elegans* Abl has much less sequence identity, proline content, and pKa (Drosophila Abl: sequence identity = 25%, proline content = 17/232, pI = 7.2; C. elegance Abl: sequence identity = 29%, proline-content (9/192), pI = 6.5). We are less certain as to whether these proteins impact MT dynamics, but plan to investigate this in future studies. These are detailed in the discussion section of the text.

Editor, Issue 6: *The Editor and Reviewer 1 asked that we attempt to perform more cell-based experiments to address the physiological relevance of Abl2:MT interactions.*

Using dual-color TIRF microscopy, we observed MT plus-tip grow adjacent to Abl2 puncta at the cell edge region in *abl2-/-* COS-7 cells re-expressing Abl2-RFP and MT plus-tip tracker, EB3-GFP.

We also conducted cell migration assay to assess the impact of Abl2-557-C, which is sufficient to promote MT elongation, on cell migrating pattern and speed. When plated on fibronectin *abl2-/-* fibroblast travelled at a significant faster speed than WT (WT: 11.88 ± 4.501 μm/h; *abl2-/-*: 22.18 ± 8.137 μm/h). We report that re-expressing Abl2-557-C-GFP in *abl2-/-* 3T3 cells significantly decreased their cell migration speed as compared to *abl2-/-* 3T3 cells (17.19 ± 9.01 μm/h), but did not rescue to WT levels. Re-expressing GFP in *abl2-/-* 3T3 did not rescue the cell migration speed (20.00 ± 7.56 μm/h). This observation agreed with our previous finding (Peacock et al., 2007) that *abl2-/-* cells showed significantly higher migration speed compared to WT, and the re-expression of Abl2-GFP or Abl2-N-557-GFP in *abl2-/-* cells significantly slowed migration. These observations in combination suggest that Abl2 C-terminus is a part of Abl2-mediated cell migration regulation, while likely acts in concert with determinants in the N-terminus for full proper regulation of cell migration.

These new data are presented in the Figs. S3E and S3F and detailed in the text.

Reviewer 1, Issue 7: *Reviewer 1 asked whether the 557 boundary or proximity of the MBP might interfere with the decrease in Abl2-557-C affinity relative to full-length Abl2 and N-688.*

We addressed this by swapping the MBP-tag to a much smaller his-tag on Abl2-557-C and measured MT-binding affinity. We found his-tagged Abl2-557-C bound MTs with a K_d = 0.82 ± 0.18 (mean ± SD), similar to MBP-tagged Abl2-557-C (K_d = 0.98 ± 0.10 μM), suggesting that MBP does not interfere with Abl2-557-C binding to MTs. We also toned down our statement as we identified Abl2-557-688 as the primary MT-binding region, which is necessary and sufficient for saturable high-affinity MT-binding, and we discuss that other regions in the Abl2 N- or C-terminal half may facilitate MT binding. Please see also *Issue 3.*

Reviewer 1, Issue 8: *Reviewer 1 requested that we better describe how quantify and fit the MT binding data.*

We have added details of this method to the Materials and Methods section. We stained the SDS-PAGE gels with Coomassie G-250 (Bio-Rad Laboratories, Hercules, CA) and destained in water, then scanned with Cannon LiDE-120 scanner. The linear range of signal intensity of G-250 staining vs. amount of protein loaded was determined using 0.025 - 8 μg BSA and obtained the R^2 value = 0.97 for the linear fit. This is within the concentration range (0.05-0.5 mM or 0.75-7.5 μg) of Abl2 or Abl2 fragments in our cosedimentation experiments. We used the binding curve equation $y = B_{max} \times x/ (K_d + x)$ to fit the curve, where y is specific binding, x is the ligand concentration, B_{max} is the maximal binding in the same units as y, and K_d is the binding affinity in the same units as x. K_d are expressed as mean ± SD, error bars of fitting curve represent standard deviation. The R^2 value for Abl2:MT binding curve fitting is 0.88, for 557-C is 0.94, for 688-C is 0.85, N-688 is 0.85, 557-688 is 0.89. These data are presented in Fig. 1 legend.

Reviewer 1, Issue 9: *Reviewer 1 asked whether Abl2 binds to tubulin dimers?*

Mapping this interaction and characterizing its role in regulation of MTs is part of an ongoing thesis project in the lab and we respectfully request that the investigator performing this work be allowed to pursue it independently of this paper.

Reviewer 1, Issue 10: *Reviewer 1 asked about the specificity of Abl2 binding to MTs in vitro and the effects on MT dynamics.*

> We provide analyses of several control proteins or Abl2 fragments that do not bind MTs, including MBP (control), Abl2-N-557, Abl2-924-1090 (pI = 9.85; proline content = 17%, 28/166 amino acids), and Abl2-688-1090. We observed Abl2 or Abl2 fragments that bind MTs with strong or reduced affinity: Abl2 (strong affinity), Abl2-557-C (strong affinity), Abl2-N-688 (strong affinity), Abl2-688-C (reduced affinity). Thus, we think Abl2:MT interaction is specific and Abl2-557-688 is necessary and sufficient for high-affinity saturable MT-binding.
>
> In our in vitro experiments, we also find that MBP (control) does not influence tubulin assembly or MT dynamics (elongation, shortening, or catastrophe). Abl2-557-688 or Abl2-688-C did not promote MT elongation. Thus, we think the regulation of MT dynamics by Abl2 or Abl2 C-terminal half is specific and direct.

Reviewer 1, Issue 11: *Reviewer 1 asked for additional kymographs for the effects of MBP, Abl2-557-C, Abl2-557-688, Abl2-688-C on MT elongation*

> Kymographs of MT dynamics for MBP, Abl2-557-C, Abl2-557-688, Abl2-688-C were added in Fig. S2F as per reviewer request

Reviewer 1, Issue 12: *The Reviewer suggested we come up with a better label for* Figure 3I y-axis.

> We corrected the y-axis label to “cumulative distribution” for Figs. 3I, S2H, and S2I as per reviewer request.

Reviewer 1, Issue 13: *The Reviewer felt Figure 4 was not well connect to the in vitro findings.*

> As detailed above in the response to *Issue 5*, we have performed extensive new experiments detailing the observation of MT tip going through Abl2 puncta in cells and explored the impact of the Abl2 C-terminus (Abl2-557-C) on cell migration.

Reviewer 1, Issue 14: *The Reviewer suggested we change the y-axis for the turbidity measurements in Figures 2H and I to Absorbance at 350 nm (Abs. 350nm).*

> We modified the y-axis label for Figs. 2H, 2I, S2C, and S2D as per the reviewer's request.

Reviewer 1, Issue 15: *The Reviewer requested we add a scale bar for the EM images in Supplemental Figure 2.*

We did this for Fig. S2E.

Reviewer 1, Issue 16: *The Reviewer requested we tone down or eliminate the* comparison of Abl2 and Tau in the discussion.

We did this in the discussion section.

Reviewer 2, Issue 17: *The Reviewer asked use about the concentration of Abl2 in cells.*

Please see the response to *Issue 2.*

Reviewer 2, Issue 18: *The Reviewer asks us to clarify our description of the mapping of MT on Table 1 and in the text.*

Please see our response to *Issue 3*, *Issue 7, and Issue 8*.

Reviewer 2, Issue 19: *The Reviewer asks to speculate on potential MT regulation by other Abl family kinases.*

We thank the reviewer for the opportunity to add this to the discussion section of the text. Please see our response to *Issue 5*.

Reviewer 2, Issue 20: *The Reviewer asked us to remove overstatement.*

We believe this comment focused mostly on the mapping of the MT binding regions of Abl2. We have edited this section to carefully describe our observations and to remove any over-interpretations about this or potential mechanisms of regulation. We also carefully reviewed and edited the manuscript for statements that we made for Abl2's function in our in vitro and in vivo experiments. For MT-binding sites mapping, please see our response to *Issue 3*.

Reviewer 2, Issue 21: *The Reviewer requested that we correct our data presentation of MT-binding affinity of Abl2 or Abl2 fragments to include the mean ± SD for each independently derived data point.*

We replotted these data using mean ± SD for error bars of binding data and values of dissociation constants (K_d) as per reviewer request. We also expanded our description of the methods by which we obtained dissociation constants as outlined in our responses to *Issue 3 and Issue 8*.

Reviewer 2, Issue 22: *The Reviewer suggested edits to Figure legends.*

We added the concentration of Abl2-557-C-GFP in the legend of Figs. 2B and 2C. We added mCherry-MACF43 to legend of Figs. 4A, 4B, 4E, and S3D.

Reviewer 3, Issue 23: *The Reviewer asked us to analyze Abl2 binding and movement on growing MTs*

We thank the reviewer for this key experimental suggestion. We performed the experiment as detailed in the response to *Issue 1*.

Reviewer 3, Issue 24: *The Reviewer asked us to clarify Abl2's MT-binding regions*

We thank the reviewer for this key suggestion on experimental result clarification. We rewrote and better described our findings, please see *Issue 3, Issue 7,* and *Issue 8*.

Reviewer 3, Issue 25: *The Reviewer asked us to repeat Abl2 and s-MT binding experiment and document the experimental procedure*

We thank the reviewer for this key experimental suggestion. We performed the experiments again. We documented the experimental procedure carefully in the material method section as per reviewer request. Please see *Issue 4*.

Reviewer 3, Issue 26: *The Reviewer asked us to* discuss about Abl1 and other Abl family kinases with regard to MT-interaction

We thank the reviewer for this key comment on discussion. We compared Abl2 with other Abl family kinase and discussed about MT-binding or MT dynamic regulation. Please see *Issue 5.*

Reviewer 3, Issue 27: *The Reviewer asked us to edit the wording to "does not require the kinase domain"*

We thank the reviewer for suggestion on word editing. We edited it in the text.

Reviewer 3, Issue 28: *The Reviewer asked us to specify the term "growth length"*

We thank the reviewer for this suggestion. We modified in the text and figure legend as "length at catastrophe".

Reviewer 3, Issue 29: *The Reviewer asked us to reorganize Supplemental Figure 1*

We specified the Fig. S1 content in the text and in Fig. 1 legend for better matching, as per reviewer suggested.

References:

Albe, K.R., M.H. Butler, and B.E. Wright. 1990. Cellular concentrations of enzymes and their substrates. *J. Theor. Biol.* doi:10.1016/S0022-5193(05)80266-8.

Finka, A., and P. Goloubinoff. 2013. Proteomic data from human cell cultures refine mechanisms of chaperone-mediated protein homeostasis. *Cell Stress Chaperones*. doi:10.1007/s12192-013-0413-3.

Peacock, J.G., A.L. Miller, W.D. Bradley, O.C. Rodriguez, D.J. Webb, and A.J. Koleske. 2007. The Abl-related Gene Tyrosine Kinase Acts through p190RhoGAP to Inhibit Actomyosin Contractility and Regulate Focal Adhesion Dynamics upon Adhesion to. *Mol. Biol. Cell*. doi:10.1091/mbc.E07-01-0075.

September 6, 2019

RE: JCB Manuscript #201812144R

Dr. Anthony J Koleske
Yale University
Department of Molecular Biochemistry and Biophysics Yale University 333 Cedar Street
SHM CE31
New Haven, CT 06420

Dear Dr. Koleske,

Thank you for submitting your revised manuscript entitled "Regulation of MT dynamics via direct binding of an Abl family kinase". It has been re-reviewed by all original reviewers. Thank you for your patience with the re-review process. All referees are now supportive of moving forward with publication pending some final edits/changes that can be done in the text (Revs#1/2) or experimentally (Revs#2/3); however, the revs (#2/3) both state that, in their view, experiments are not absolutely required for publication. We discussed these suggestions editorially and agree that, consistent with JCB policy, the new experiments suggested by the reviewers are not required for publication. However, we would encourage you to consider performing them if it is straightforward and possible for you and your colleagues, as they will strengthen the manuscript.

Regardless of these experimental suggestions, we feel that all of the textual changes and the recalculations described by Rev#2 should be made prior to publication. When resubmitting, please detail how these points were addressed and note the corresponding changes in the manuscript.

We would be happy to publish your paper in JCB pending these revisions as well as final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Text limits: Character count for Reports is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) The Report format is divided into an "Introduction" section and a "Results and Discussion" combined section. Please remove the "Conclusion" header (the text can stay as is).

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 2DE, 3F, 4A (magnifications), S3E (right panels)

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

Please indicate n/sample size/how many experiments the data are representative of: 1BDFH, 2HI, 3DEFGHIJ, 4CDFG, S1R, S2CDGHI, S3E

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- More information about tubulin purification and labeling, MT dynamic imaging assays, cell migration tracking data analysis, even if described in other published work.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Melissa Gardner, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this revised submission, the authors have largely addressed the concerns I originally had. In my opinion the paper would benefit from some speculation/commentary about how Abl2 is doing what it appears to be doing (or at least that there is not an obvious mechanistic explanation for what it is doing), but I recognize that this is a matter of taste. Two minor stylistic comments:
+ The text about 'length at catastrophe' did not really make sense to me and seemed redundant with that they had already said in terms of faster growth rates and decreased frequency of catastrophe. If there is some deeper reason to emphasize this point, the authors should try to clarify.
+ I thought that 'propose' in the conclusion section was too strong for what they are addressing, 'speculate' might be more appropriate.

Reviewer #2 (Comments to the Authors (Required)):

Review of revised manuscript "Regulation of MT dynamics via direct binding of an Abl family kinase."

The authors have done a good job of responding to the reviewer comments, and with all the additional data, I'm now convinced that the paper belongs in JCB after a few additional changes.

1. The newly provided methods on the microtubule binding assays make it apparent that there is a problem with the present analysis, but thankfully one that is easy to fix. Briefly, from the information provided, it appears that the authors extracted their Kd measurements by fitting the binding data against [total polymer]. This is valid only under conditions where it is reasonable to assume that [total polymer] ~= [free polymer], i.e., under conditions where [total Abl] < Kd. If [total Abl] is similar to or greater than the Kd (as it is in some trials in these experiments), a significant amount (perhaps most) of the polymer will be occupied by Abl, and [free polymer] ≠ [total polymer]. The result of fitting a standard binding curve against [total polymer] under these conditions will be to overestimate the Kd, i.e., underestimate the strength of the reaction.

What one needs to do in this situation is compensate for the fact that some of the [polymer] is occupied. One straightforward way to do this is to estimate the [free polymer] by making an assumption about the Abl-MT binding ratio, calculating the [free polymer] at each point, and then plotting against this estimated [free polymer] using the standard binding equation. Alternatively, one can use an altered form of the binding equation that has as inputs [total polymer] instead of [free polymer] (different versions of this equation exist for different binding ratios). For more information on these points, see Pollard MBOC 2010 "A Guide to Simple and Informative Binding Assays," text on p. 4605.

Thus, the authors need to recalculate their Kd values from their binding data and report the corrected method in the paper.

2. The authors interpreting differences in the light scattering data with/without various Abl fragments as differences in the amount of polymerization. However, light scattering amounts depend both on the amount of polymer and its arrangement: bundled microtubules scatter a lot more light than single microtubules. It seems very unlikely that adding 2µM Abl causes 3x the amount of polymer seen in with tubulin alone (see Figure 2I). Instead, these observations are more consistent with the idea that 2µM Abl is causing MT bundling.

If Abl is indeed bundling the MTs when it is present at high concentration, it wouldn't change much in terms of the conclusions, but it is worth pointing out for two reasons. First, the authors should probably tell readers that the strong absorbance signals in the presence of 2µM Abl could potentially be related to bundling, since otherwise these results misleadingly imply that there is a ~3x increase in the amount of polymer. Second, the authors might want to directly test whether Abl is bundling the MTs. I realize that the paper already has a lot of work, and so do not want to require this experiments, but doing so is relatively easy: one can simply adding 2µM Abl to some labeled MTs, putting a drop on a microscope, and seeing what is there.

3. I'm worried that in some places the text still overstates the results. In particular, I'm uncomfortable with statements like this, from the abstract: "In cells, knockout of Abl2 significantly impairs MT growth and this defect can be rescued via reexpression of Abl2 or an Abl2 fragment containing the MT-binding domain." The statement that "this defect can be rescued..." is very strong, and I'm not sure that it is justified by the data, especially given that the rescues involved overexpression. Moreover, this statement that the C-terminal domain is sufficient to rescue the phenotype implies that the kinase domain has no role in the MT defects observed in the Abl2 knockout. That would be very surprising. I think that the authors should tune down this statement, but if they decide to keep it, I think that they should explore it more fully.

Reviewer #3 (Comments to the Authors (Required)):

The revisions sufficiently address my concerns and strengthen the manuscript, which is now suitable for publication in JCB.

I do have one additional suggestion for the authors. The new results in Figure 2F-G describe the recruitment of Abl2-557-C to dynamic microtubules in vitro, and indicate that the majority of association events are "static" binding to the microtubule lattice. I suggest that the authors take this analysis a step further, specifically by distinguishing where the "static" associations are

happening - on the seed MT or the dynamic MT. This is important because the seed MT regions in this experiment are stabilized with GMPCPP, while the dynamic MT regions are presumably GDP-lattice assembled from free tubulin during the experiment. Therefore, the nucleotide state in the seed MT region is expected to resemble that of the growing plus end. If Abl2-557-C tends to exhibit static association with the seed MT, then this would be evidence of selective binding based on the nucleotide state of tubulin. Indeed, the "Static" panel in 2G appears to show Abl2-557-C binding to the GMPCPP seed. Putting numbers on this would be helpful in determining whether this a common behavior of Abl2-557-C. I do not view this as necessary for publication, but it may add deeper mechanistic insight.

Journal of Cell Biology Response: September-9-2019
Manuscript Identifier: 201812144
Title: Regulation of MT dynamics via direct binding of an Abl family kinase

Dear Dr. Gardner and Dr. Casadio,

Please find enclosed a revision of our manuscript “Regulation of MT dynamics via direct binding of an Abl family kinase" (201812144R) for which you requested additional textual revisions and experiments. We thank the reviewers for their helpful comments. We also believe that the additional experimental concerns were addressed in our revised manuscript, but perhaps were missed by reviewers and we clarify these points below. We hope they will now find our paper suitable for publication.

Reviewer 1, Issue 1: The reviewer asked that we clarify the text regarding the length at catastrophe.

We use this measurement of total length of MT growth as a secondary measure to integrate how the effects of Abl2 on growth rate and catastrophe frequency impact net MT length. We explain this in the text.

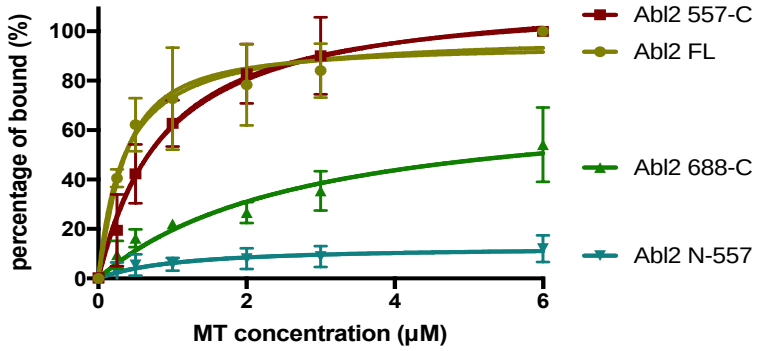
Reviewer 1, Issue 2: The reviewer asked change the work 'propose' in the conclusion to 'speculate'.

We did this in the text.

Reviewer 2, Issue 3: The reviewer asked that we reanalyze the MT binding data, taking into account the fact that some of the polymer is occupied.

We thank the reviewer for this suggestion. We have reanalyzed the data using the following equation: $Y=B_{max}*(c+K_d+X-sqrt((c+K_d+X)*(c+K_d+X)-4*c*X))/(2*c)$, where $c=[Abl2]_{total}$, $X=[MT]_{total}$, $Y=[Abl2]_{bound}$, K_d is the dissociation constant of the Abl2-MT complex, and B_{max} is the saturation percentage (%) of Abl2 that can bind to microtubule. We have reported the new values in the text and Table 1.

The figure below showed the comparison between curves from the previous fitting and the current fitting. The curves from the same experimental group has shown mostly overlapping results and we can only observe slight differences in Abl2:MTs group when very closely examine the figure.



Reviewer 2, Issue 4: The reviewer asked whether Abl2 bundles MTs, which might explain the increase in MT turbidity.

Using electron microscopy, we showed (in original Fig S2E) that MTs grown in the presence of Abl2 are not significantly bundled, which the reviewer may have missed. Also, we did not observe Abl2 bunding MTs in previous work (Miller et al., 2004).

Reviewer 2, Issue 5: The reviewer is concerned about overstating the ability of the Abl2 C-terminus to rescue the MT elongation defects, considering the over-expression of Abl2 proteins in COS-7 cells.

We have toned down this claim in the abstract: *"In cells, knockout of Abl2 significantly impairs MT growth and this defect can be rescued via re-expression of Abl2. Stable re-expression of an Abl2 fragment containing the MT-binding domain alone was sufficient to restore MT growth at the cell edge. "*

We point out to the reviewer that Abl2/Abl2-557-C were expressed in 3T3 cells at 0.25-fold and 0.34-fold endogenous levels, not overexpressed.

Reviewer 3, Issue 6: The reviewer asked us to discriminate between Abl2-557-C molecules binding to the seed vs dynamic MTs in vitro.

Unlabeled/non-fluorescent dark tubulin seeds were used in these single molecule imaging experiments, and hence all of the single Abl2-557-C-GFP molecules that we observed were binding to the dynamic rhodamine-MTs. We have elaborated on this point in the text. "*To visualize how Abl2-557-C-GFP bind to growing MTs, 50 nM Abl2-557-C-GFP and 7 μM rhodamine-tubulin were incubated with unlabeled GMPCPP-stabilized biotin-MTs. Abl2-557-C-GFP molecules that bound to the rhodamine-labeled growing part of the MT were imaged at 1 FPS (Fig. 2F)."*

Editor, Issue 7: Microscopy images need to have scale bars on every image and need magnification on insets. (2DE, 3F, 4A (magnifications), S3E (right panels)).

We thank the editor for this key suggestion. We have added these. For 3F the total distance traveled was marked on the left side of the graph (top and bottom graph shared the same distance, no scale bar necessary).

Editor, Issue 8: Figure legends need to include "s.d." and also data points n. on Figures 1BDFH, 2HI, 3DEFGHIJ, 4CDFG, S1R, S2CDGHI, S3E.

We have added these.

Editor, Issue 9: Methods sections need some additional details.

To the Methods Section, we have added:

- new description of how we calculated the K_d (see also ***Issue 3***)
- detailed information about tubulin purification and labeling, MT dynamic imaging assays, cell migration tracking data analysis, and referenced the sources accordingly.
- microscope and image acquisition details including, make and model of microscopy, type magnification and NA of lenses, temperature of acquisition, imaging medium, fluorochromes uses, acquisition software, and details regarding any software operations performed.