Review COMMONS

JCB

Conserved actin machinery drives microtubule-independent motility and phagocytosis in Naegleria

Katrina Velle and Lillian Fritz-Laylin

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Revision 0

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary: In this study the Fritz-Laylin lab performed an analysis of the cytoskeleton of the amoeba Naegleria, focusing on the function of actin and its nucleators. Actin is one of the most conserved proteins in eukaryotes and fulfils multiple functions in the cell. While most of our knowledge comes from studies within the phylum ophistoconts (from yeast to human), extensive studies of actin functions in other eukaryotes is scarce. Here the authors demonstrate that actin in Naegleria forms an extensive F-actin cytoskeleton, while microtubular structures are limited. Using inhibitors for F-actin dynamics and nucleators (Formins and Arp2/3) the authors furthermore demonstrate that F-actin dynamics is required for the formation of lamellar protrusions, motility, and phagocytosis. **Own opinion and major concerns:** This study represents a nice comparative analysis of F-actin functions in an evolutionary distant eukaryote, demonstrating an ancient and conserved role for actin driven processes, such as motility and phagocytosis. The study uses a neat combination of imaging analysis, which has been well quantified to identify different behaviour of cells upon treatment with inhibitors for actin and/or nucleators, such as formins and the Arp2/3 complex. While the imaging analysis appears to be solid and justifies the conclusions drawn by the authors, there are some concerns regarding the specificity of the inhibitors: 1.CK-666 and SMIFH2: The authors should perform additional assays to validate the specificity of these inhibitors. For example, the authors performed a nice sequence analysis, demonstrating that the putative binding sites for Cytochalasin D (CD), Latrunculin B (LatB) and Jasplakinolide (Jas) are conserved in Naegleria actin. However, no similar analysis has been performed on Formins and or Arp2/3. Can the authors exclude off-target effects? This should at least be discussed accordingly in the manuscript. 2.Upon incubation of Naegleria with LatB, Jas and CD, only LatB showed significant effects, since no F-actin appears to be formed, as expected. However, previous studies (see (Walsh, 2007 and Han et al., 1997) observed specific effects upon treatment of Naegleria with CD, similar to what is observed here for LatB: "Addition of cytochalasin D at the beginning of differentiation inhibited

the differentiation in a dose-dependent manner. In 20 μg/ml of cytochalasin D, 60% of the cells formed flagella. In 50 or 100 μg/ml of cytochalasin D, the differentiation was strongly inhibited (Fig. 5,A). In these experiments, most of the cells changed their shape into spheres less than 30 min after the initiation of differentiation and remained as spheres until the end of the differentiation (Fig. 6)." Similarly, Walsh 2007 described specific and rapid effects upon incubation of cells with CD. How do the authors explain these discrepancies? 3.Did the authors perform a time and concentration course of cells treated with these inhibitors? It is mentioned that different concentrations have been used in the Material section. However, given the relatively small effects (with exception to LatB), it might be useful to analyse longer incubation times and higher concentrations and show a comparison of the results. 4.Previous work demonstrated that siRNA can be used to knock down genes in Naegleria (see Jung et al., 2008). Can the authors validate observations made with (potentially non-specific inhibitors) using an alternative approach? Alternatively, dominant-negative expression of Formin or Arp2/3 might be possible to validate the results. 5.The authors rely almost exclusively on fixed assays, when describing differences in F-actin distribution or formation. Given that several options are available for live imaging of F-actin, including membrane permeable dyes, such as sir-Act or, as described in Sohn et al., 2019, Naegleria expressing GFP-Act, the conclusions drawn by the authors could be strengthened by live imaging of F-actin dynamics. This would be especially informative, when analysing F-actin nucleation and accumulation in the presence of inhibitors. **Minor comments:** -Figure 1, while interesting the phylogenetic analysis of MyoII is unrelated to the whole study and could be removed (or showing in supplements). -Quantification in Fig.4B and FigS5 regarding F-actin content/intensity: This seems to be a rather rough method to measure F-actin formation. Did the author perform this experiment also in presence of CK666 and SMIFH2? In theory blocking both nucleators should result in a similar disruption of F/actin as seen for LatB. -Live imaging of F-actin dynamics in presence/absence of inhibitors would be helpful.

3. Significance:

Significance (Required)

This study demonstrates a conserved role of actin in an evolutionary distant (and understudied) eukaryote. It might also open up the field to future studies, since F-actin dynamics can be studied in the absence of cytosolic microtubules I suspect it will be interesting to a broader audience, working on actin in different organisms. Expertise: Actin dynamics, Motility, Imaging

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Velle et al explored the role of Arp2/3 and formin in Naegleria cell shape, motility and phagocytosis. They found that Arp2/3 has an important role in cell shape and motility whereas formins had a role in persistence of motility. The role of formins in persistence of direction is interesting since microtubules have a key role in cell polarity and persistence of direction in other alpha-motile cells. This finding indicates that Naegleria has evolved or become more reliant on a formin based polarity mechanism. LatB treatment demonstrated a clear role for actin in phagocytosis. Experiments with CK-666 indicated that Arp2/3 plays some role in phagocytosis; however, the authors were unable to measure a statistically significant role in their assay with GFP expressing bacteria and flow cytometry. Yet, images of cells from the assay suggest that LatB and CK-666 treated cells are able to adhere to bacteria but not engulf them. We are left wondering about the role of formin. Overall this manuscript is well written, the data is clearly presented and the quality of the analysis is very good. **Major comments:** The key conclusions are supported by the results. One point for improvement would be to address the role of formin in phagocytosis. Formins can play a role in phagocytosis (PMID 16303559, 27152864, 29663616, etc), yet the authors did not include SMIFH2 treatment in the phagocytosis experiments. Since CK-666 treatment alone does not impair bacterial engulfment to the same extent as LatB treatment, formins could be playing a significant role and this should be examined for completeness. The bacterial adherence without phagocytosis shown in Fig S8B is compelling evidence that the flow cytometry assay is under-reporting the phagocytosis defect and should be moved into Fig 6. The authors could further characterize the role of Arp2/3 in this process with live cell imaging of phagocytosis. Or perhaps SEM after a phagocytosis assay that includes a wash step to remove bacteria that has not already adhered to amoebae. If phagocytosis is impaired the expectation is that bacteria would be seen on the cell surface in the CK-666 treated cells but not the controls. This SEM experiment would also serve to show how the inhibitors change the shape of the cells to complement data from Fig 3 and 4. The methods section appears to be complete with all the necessary information to reproduce the results presented in this manuscript. There are no concerns about sample size or the number of replicates. Regarding statistical analysis, it is not clear why the authors used "ordinary ANOVA" to analyze the results from Fig 6. The histograms are clearly non-parametric, perhaps a Mann Whitney test would be more appropriate. Figure 6C reports mean values, since the data is non-parametric median values would typically be reported. **Minor comments:** Although it is well established that Naegleria builds basal bodies de novo, the point of cells completely lacking microtubules seems at odds with their use in closed mitosis. Figure 2B shows some microtubule cytoskeleton transcripts are upregulated in amoebae, what are they? Fritz-Laylin 2010 includes a western blot of actin and basal body components over time, but examines gamma-tubulin, a component of basal bodies, rather than alpha or beta tubulin. A western blot of actin and tubulin in amoebae and flagellates would complement the staining shown in Figure 2A. Similarly, a microtubule inhibitor control would complement the persistence assays in Figure 5. -Together these small experiments would

strengthen support for the idea that tubulin has no role in persistence of motility in Naegleria. Figure 4D and the text indicate there is no difference in cell area. Yet, the image of the LatB treated cell in Figure 4C is roughly half the size of the other cells and it made this reader expect to see a significant difference in cell size in Fig 4D. This image should be replaced with a more representative one. The term "NEGM amoeba" is found in the legend of Fig 2 without any explanation in the text. A google search revealed that ATCC 30224 is NEG-M. Either introduce this term in the main text or just say amoeba. How does SMIFH2 impact persistence of motility in amoeba from other eukaryotic lineages?

3. Significance:

Significance (Required)

This work is foundational for understanding the role of actin in Naegleria cell shape, alpha-motility and phagocytosis. Naeglaria has many conserved actin cytoskeletal proteins so it not surprisingly that these components have similar roles in all eukaryotes. This indicates an ancient evolutionary origin for these proteins in motility and phagocytosis. According to the authors a major finding is that formins have a role in directing motility. However, SMIFH2 has also been shown to regulate directionality of Dictyostelium amoeba and this was not discussed (PMID 30808751). If the point of this work is to move toward a comparative understanding of cytoskeletal function and the mechanics of cell motility across eukaryotes, the authors should include a more inclusive discussion of what is known about formin's role in motility and phagocytosis. This work should be of interest to the broader cytoskeleton/cell biology readership as well as evolutionary cell biologists and parasitologists. Reviewer expertise: Cell biology and function of the cytoskeleton in microbial eukaryotes.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary: The authors use a range of actin probes and small molecule inhibitors to perturb and monitor actin dynamics in the heteroloboseans Naegleria gruberi and Naegleria fowlerii using a combination of light and electron microscopy. They show that Naegleria assembles Arp2/3-dependent actin structures such as lamellae; that Arp2/3 is required for migration and phagocytosis; that cells without Arp2/3 assemble filopodia instead. Overall, the Naegleria actin network exhibits some properties typical of other amoeboid cell types from various branches of the eukaryotic tree, with implications for the evolutionary origins of amoeboid motility. This is a well-executed study. A few additional experiments and analyses would help support the claims of the paper. **Major comments:** 1.The paper should look more closely at the actin-regulated genes that are up-regulated in the flagellated form. Authors should see whether these are unusual in anyway - especially the SCAR/WAVE homologue (WAVE_58655) and formins (Formin_81446; Formin_79644; Formin_78968). 2.Figure 1 focuses on Myosin II. This is surprising given that the authors have not looked at Myosin II or attempted Blebbistatin treatment. The authors could do this or they should re-think Figure 1. Given the paper is about the ensemble of genes that enable phagocytosis and Arp2/3-dependent movement, the authors should make clear in Figure 1 which actin-related genes have a distribution across evolution similar/different to that of Myosin II. 3.It is vital to test whether tubulin is up-regulated following treatments that affect actin, e.g. 5FG. If so, this would change the conclusions. Note that similar actin spikes are seen in RNAi screens in metazoan cells following the silencing of Arp2/3 components and are associated with actin spikes and tubulin. 4.Figure 5D should include data for CK666. 5.Figure S2B - does transcript abundance represent total abundance for duplicated genes? If not, this should be shown. In this Figure all individual actin regulators should be included, e.g. Cofilin, CAP, Capping protein. Each histogram should be associated with a gene, not a class of genes. **Minor comments:** 1.The genes should be marked in Figures 2C and S2B. Otherwise there is the possibility of confusion (i.e. which WAVE homologue is shown in 2C and S2B)? 2.In Figure S1, it would be better to normalise so we can see the extent to which each gene is up/down regulated independent of its levels (e.g. Myosin II heavy and light chains). 3.It would be good to reference work on Dictyostelium (Tunacliffe et al., 2018) where the role of multiple actin genes has been studied, and to discuss actin in plants/SAR as well as Metamonads and Opisthokonts. 4.There is a slight over-representation of references to own papers over contributions by others in the field. What would be great (but isn't essential): 1.Would be great to have TEM to image the actin network +/- CK666, but this is not be necessary for publication.

3. Significance:

Significance (Required)

The paper goes a long way towards establishing Naegleria as a sophisticated new model in which to study actin's role in cell shape control - and, uniquely, one that can be analysed in the absence of microtubules. Our understanding of the origins of amoeboid motility and phagocytosis has derived largely from studies in opisthokonts and amoebozoa (e.g. Dictyostelium) with little data from other parts of the tree. The demonstration here that Arp2/3-dependent actin-based protrusions are important for Heterolobosean motility and phagocytosis supports the notion of a deeply conserved, ancient origin for these features of the actin network - which in turn has implications for the origins of eukaryotes (Burns et al. Nat. Eco. Evol. 2018) Overall, this study

would be a welcome addition to the field, and of general interest to the broader cell and evolutionary biology communities.

We thank the reviewers for their careful reading of our manuscript and for their helpful feedback that has strengthened our paper. We have outlined our response to each of the reviewers' comments inline below (reviewers' comments in black and our responses in blue). In addition to comments from the reviewers, we have been given feedback on our preprint from members of broader community, and have made additional minor changes to the text in response.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary:

In this study the Fritz-Laylin lab performed an analysis of the cytoskeleton of the amoeba Naegleria, focusing on the function of actin and its nucleators. Actin is one of the most conserved proteins in eukaryotes and fulfils multiple functions in the cell. While most of our knowledge comes from studies within the phylum ophistoconts (from yeast to human), extensive studies of actin functions in other eukaryotes is scarce. Here the authors demonstrate that actin in Naegleria forms an extensive F-actin cytoskeleton, while microtubular structures are limited. Using inhibitors for F-actin dynamics and nucleators (Formins and Arp2/3) the authors furthermore demonstrate that F-actin dynamics is required for the formation of lamellar protrusions, motility, and phagocytosis.

Own opinion and major concerns:

This study represents a nice comparative analysis of F-actin functions in an evolutionary distant eukaryote, demonstrating an ancient and conserved role for actin driven processes, such as motility and phagocytosis. The study uses a neat combination of imaging analysis, which has been well quantified to identify different behaviour of cells upon treatment with inhibitors for actin and/or nucleators, such as formins and the Arp2/3 complex. While the imaging analysis appears to be solid and justifies the conclusions drawn by the authors, there are some concerns regarding the specificity of the inhibitors:

1.CK-666 and SMIFH2: The authors should perform additional assays to validate the specificity of these inhibitors. For example, the authors performed a nice sequence analysis, demonstrating that the putative binding sites for Cytochalasin D (CD), Latrunculin B (LatB) and Jasplakinolide (Jas) are conserved in Naegleria actin. However, no similar analysis has been performed on Formins and or Arp2/3. Can the authors exclude off-target effects? This should at least be discussed accordingly in the manuscript.

Thank you for this suggestion. We have expanded our *Naegleria* sequence analyses to include the CK666 binding site and formin FH2 domains (please see the updated Figure S5, and corresponding text on page 5, line 216: "We verified that the target regions for both SMIFH2 and CK666 were conserved in *Naegleria* (**Fig S5C-E**)." Based on this new analysis, CK666 is likely to be an effective inhibitor of the Arp2/3 complex (Figure S5C). Similarly, our assessment of key residues within the FH2 domains of *Nageleria*'s formins reveals many conserved residues, and overall percent identities that are comparable to other SMIFH2-sensitive formins (Fig. S5D-E). Based on these new analyses, we expect these *Naegleria* formins to be sensitive to SMIFH2, consistent with the findings we present in Figures 4 and 5.

We now explicitly state in the revised manuscript, on page 11, line 554: "Although we cannot entirely rule out off-target effects, as is generally the case for inhibitor-based studies, we selected short time points (5-10 min) for most experiments to minimize the potential for off-target effects."

2.Upon incubation of Naegleria with LatB, Jas and CD, only LatB showed significant effects, since no F-actin appears to be formed, as expected. However, previous studies (see (Walsh, 2007 and Han et al., 1997) observed specific effects upon treatment of Naegleria with CD, similar to what is observed here for LatB: "Addition of cytochalasin D at the beginning of differentiation inhibited the differentiation in a dose-dependent manner. In 20 µg/ml of cytochalasin D, 60% of the cells formed

flagella. In 50 or 100 µg/ml of cytochalasin D, the differentiation was strongly inhibited (Fig. 5,A). In these experiments, most of the cells changed their shape into spheres less than 30 min after the initiation of differentiation and remained as spheres until the end of the differentiation (Fig. 6)." Similarly, Walsh 2007 described specific and rapid effects upon incubation of cells with CD. How do the authors explain these discrepancies?

We appreciate the amount of background reading that went into this comment. In designing this study, we were concerned about mitigating the potential for off-target effects. We therefore chose to test concentrations of inhibitors within the lower range of typical use (please see the table and associated references below), reasoning that we would rather risk missing a phenotype (false negative) than report an effect that is off-target (false positive). For CytoD specifically, we tested 0.1 µM, 1 µM, 10 µM, and 20 µM concentrations by live phase-contrast microscopy, and found no obvious phenotypic changes on a short time scale. We continued with 20 µM (the highest concentration we tested) for all experiments. The CytoD concentrations used by Han et al., 1997 and Walsh, 2007 were much greater than what we found in other systems; 50 µg/ml is approximately 100 µM, which is 100 times more concentrated than what is used in other organisms.

	LatB	**CytoD**	**CK666**	**SMIFH2**
Leukocytes (HL-60)	5 µM [1]	0.5-1 µM [2,3]	10-100 µM [3,4]	40 µM [3]
Epithelial or Fibroblast cells (3T3, HeLa, HT-29, or chick embryonic fibroblasts)	0.02-0.9 µM [5-7]	1-2 µM [6-8]	50 µM [9]	25 µM [9]
Amoebae (*D. discoideum* or *E. histolytica*)	0.1-10 µM [10-12]	0.02-20 µM [13,14]	40-60 µM [11,12]	5-30 µM [11,15]
Our study	5 µM	20 µM	50 µM	25 µM

1 Wang, M. J., Artemenko, Y., Cai, W. J., Iglesias, P. A. & Devreotes, P. N. The directional response of chemotactic cells depends on a balance between cytoskeletal architecture and the external gradient. *Cell Rep* **9**, 1110-1121, doi:10.1016/j.celrep.2014.09.047 (2014).
2 Olins, A. L. & Olins, D. E. Cytoskeletal influences on nuclear shape in granulocytic HL-60 cells. *BMC Cell Biol* **5**, 30, doi:10.1186/1471-2121-5-30 (2004).
3 Wilson, K. *et al.* Mechanisms of leading edge protrusion in interstitial migration. *Nat Commun* **4**, 2896, doi:10.1038/ncomms3896 (2013).
4 Fritz-Laylin, L. K. *et al.* Actin-based protrusions of migrating neutrophils are intrinsically lamellar and facilitate direction changes. *Elife* **6**, doi:10.7554/eLife.26990 (2017).
5 Spector, I., Shochet, N. R., Kashman, Y. & Groweiss, A. Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science* **219**, 493-495, doi:10.1126/science.6681676 (1983).
6 Yang, D. H., Lee, J. W., Lee, J. & Moon, E. Y. Dynamic rearrangement of F-actin is required to maintain the antitumor effect of trichostatin A. *PLoS One* **9**, e97352, doi:10.1371/journal.pone.0097352 (2014).
7 Wakatsuki, T., Schwab, B., Thompson, N. C. & Elson, E. L. Effects of cytochalasin D and latrunculin B on mechanical properties of cells. *J Cell Sci* **114**, 1025-1036 (2001).
8 Wells, C. L., van de Westerlo, E. M., Jechorek, R. P., Haines, H. M. & Erlandsen, S. L. Cytochalasin-induced actin disruption of polarized enterocytes can augment internalization of bacteria. *Infect Immun* **66**, 2410-2419 (1998).
9 Velle, K. B. & Campellone, K. G. Enteropathogenic E. coli relies on collaboration between the formin mDia1 and the Arp2/3 complex for actin pedestal biogenesis and maintenance. *PLoS Pathog* **14**, e1007485, doi:10.1371/journal.ppat.1007485 (2018).
10 Jahan, M. G. S. & Yumura, S. Traction force and its regulation during cytokinesis in Dictyostelium cells. *Eur J Cell Biol* **96**, 515-528, doi:10.1016/j.ejcb.2017.06.004 (2017).
11 Williams, T. D. & Kay, R. R. The physiological regulation of macropinocytosis during Dictyostelium growth and development. *J Cell Sci* **131**, doi:10.1242/jcs.213736 (2018).
12 Manich, M. *et al.* Morphodynamics of the Actin-Rich Cytoskeleton in Entamoeba histolytica. *Front Cell Infect Microbiol* **8**, 179, doi:10.3389/fcimb.2018.00179 (2018).
13 Bettadapur, A. & Ralston, K. S. Direct and High-Throughput Assays for Human Cell Killing through Trogocytosis by Entamoeba histolytica. *Mol Biochem Parasitol*, 111301, doi:10.1016/j.molbiopara.2020.111301 (2020).
14 Ralston, K. S. *et al.* Trogocytosis by Entamoeba histolytica contributes to cell killing and tissue invasion. *Nature* **508**, 526-530, doi:10.1038/nature13242 (2014).
15 Jasnin, M., Ecke, M., Baumeister, W. & Gerisch, G. Actin Organization in Cells Responding to a Perforated Surface, Revealed by Live Imaging and Cryo-Electron Tomography. *Structure* **24**, 1031-1043, doi:10.1016/j.str.2016.05.004 (2016).

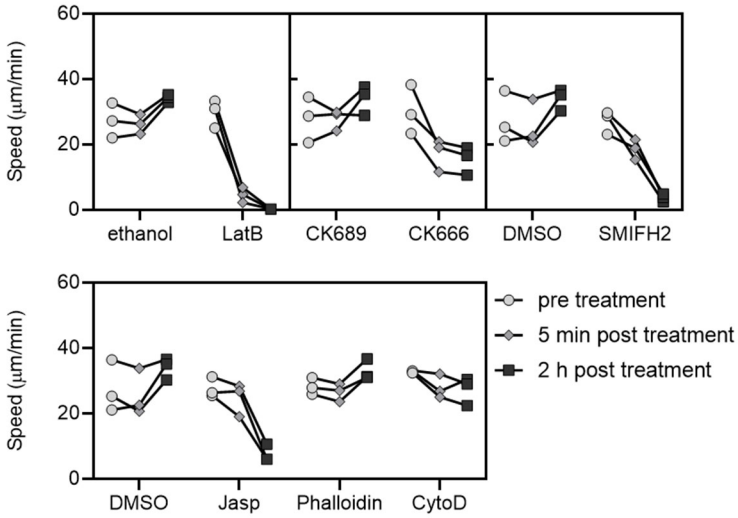
We now include a brief note of this on page 11, line 551: “While cytochalasin D has generated phenotypes in studies of *Naegleria’s* transformation into flagellates (Han et al., 1997; Walsh, 2007), the concentrations used in these studies (up to 200 µM) are ~100 times more concentrated than the amounts used in other systems, so we chose lower, more conservative concentrations.”

3.Did the authors perform a time and concentration course of cells treated with these inhibitors? It is mentioned that different concentrations have been used in the Material section. However, given

the relatively small effects (with exception to LatB), it might be useful to analyse longer incubation times and higher concentrations and show a comparison of the results.

We chose to be conservative with our application of inhibitors (see comments in point 2), and therefore purposely selected early time points to minimize off-target effects. While it is possible that later time points and/or higher concentrations of inhibitors would result in strong phenotypes, we specifically chose to risk missing such subtle effects rather than report phenotypes with questionable specificity.

For example, Jasplakinolide treatment shows no effect on motility within the first 5 min, but cells move slower after 2 h of incubation (see figure to the right). We suspect this 2 h result is off-target, because we know that the actin networks of *Naegleria* turn over rapidly; amoebae move entire cell lengths in under a minute (meaning actin used for motility must turnover within this timeframe). Supporting this rapid turnover, we see loss of virtually all actin networks within minutes of exposure to Latrunculin (Fig. on right, see also Fig 4-5). Therefore, if Jasplakinolide was stabilizing polymerized actin in *Naegleria* as expected, we would expect an early phenotype.



We also observe a striking difference in cell migration after two hours of exposure to SMIFH2. We have additional data that indicates this delayed phenotype is due to an obvious contractile vacuole defect, which we are following up on now and which we believe is outside the scope of this paper. Because this time course does not add new information (both of these long-term phenotypes are not likely to be informative and the other inhibitors give the same phenotypes at long and short timepoints), we believe adding this data to the paper will only add confusion. We therefore chose to exclude it from our original submission and this revision. If the reviewers and/or editors disagree and would rather we add this data to the paper, we would be happy to reconsider. We have, however, drawn more attention in the text to the cells in Fig 5, which exhibit spikes following CK666 at both early (~50 seconds) and late (50 min) timepoints after the addition of the drug; see page 7, line 329: “These actin-rich filopodia were observed both at early time points (90 sec post-treatment, **Fig 5G**, cell 2) and late time points (50 min after treatment, **Fig 5G**, cell 3).”

4.Previous work demonstrated that siRNA can be used to knock down genes in Naegleria (see Jung et al., 2008). Can the authors validate observations made with (potentially non-specific inhibitors) using an alternative approach? Alternatively, dominant-negative expression of Formin or Arp2/3 might be possible to validate the results.

We agree that siRNAs would be very exciting and such an approach would certainly add to our paper. However, the knockdowns achieved in Jung et al., 2008 reduced protein levels by ~50% (see Figure 4 from Jung et al., copied below), and our work and work from other labs has shown that even small amounts of residual Arp2/3 complex in mammalian cells are sufficient for some phenotypes (e.g. Velle and Campellone, 2018, PLOS Pathogens; DiNardo et al., 2005, PNAS). Lastly, there are 14 *Nageleria* formins; we believe knocking down each individually would not be a wise use of time and resources at this point, especially without a robust protocol for gene

knockdown. We are currently working on developing robust methods for gene expression and knockdown, but believe this to be beyond the scope of this paper.

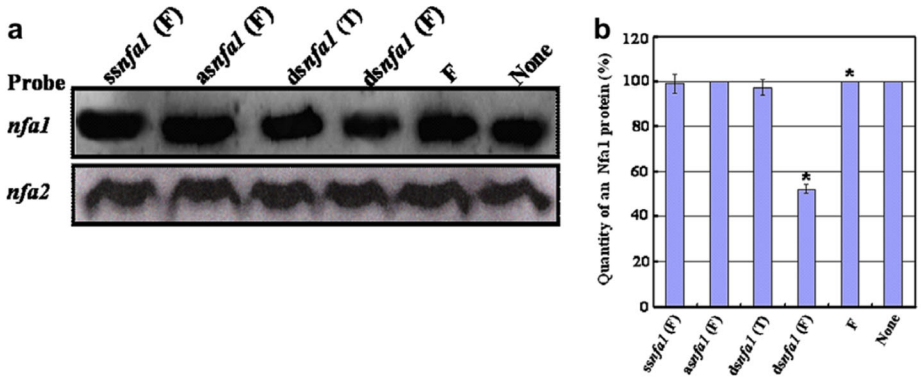


Fig. 4. Western blotting and quantitative analysis of the Nfa1 protein from transfected *N. fowleri* trophozoites. (a) Western blotting of the Nfa1 or Nfa2 protein detected with the respective polyclonal antibody. Equal amounts of protein (12 μg/lane) were electrophoresed through the gel. (b) Quantitative analysis of (a). F, RNAiFect reagent. Results are representative of three independent experiments performed in triplicate and presented as means ± standard error. Asterisks indicate a significant difference, i.e., $P < 0.05$, using paired *t*-test, one-tail distribution.

5.The authors rely almost exclusively on fixed assays, when describing differences in F-actin distribution or formation. Given that several options are available for live imaging of F-actin, including membrane permeable dyes, such as sir-Act or, as described in Sohn et al., 2019, Naegleria expressing GFP-Act, the conclusions drawn by the authors could be strengthened by live imaging of F-actin dynamics. This would be especially informative, when analysing F-actin nucleation and accumulation in the presence of inhibitors.

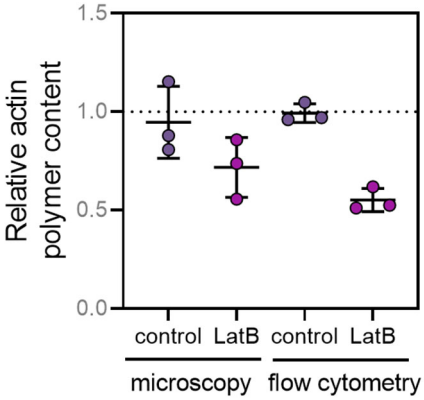
We agree this will be an exciting avenue for future research. However, multiple researchers in our lab and in other labs have tried to label *Naegleria*'s actin using SiR-Actin without success. This could be due to clearing by efflux pumps, or because SiR-Actin is based on Jasplakinolide (Lukinavičius et al., 2014, Nature Methods), which may not effectively bind *Naegleria* actin based on the data we show in this paper. Further, we have not been able to replicate the findings of Sohn et al., and while a promising new protocol for *Naegleria* transformation was just published in April of this year (Faktorová et al., 2020, Nature Methods), it will take time to verify and adapt this protocol.

Minor comments:

-Figure 1, while interesting the phylogenetic analysis of MyoII is unrelated to the whole study and could be removed (or showing in supplements).

We removed the references to MyoII in Figure 1, and emphasise in the legend that the tree represents relationships between various eukaryotic organisms, rather than a phylogenetic analysis of any particular protein family.

-Quantification in Fig.4B and FigS5 regarding F-actin content/intensity: This seems to be a rather rough method to measure F-actin formation.



While the flow cytometry data is rough in the sense that it produces only one value for each cell, the ability to measure *tens of thousands* of cells gives a robust estimate of the total actin polymer content (also see Kakley et al., 2018, BioProtocol). Perhaps due to differences in thickness between cells, or to the immensely lower number of cells analyzed, using microscopy to estimate total actin content was less reproducible than using flow cytometry (see graph on right). We therefore chose the more robust analysis for the data presented in Fig. 4B and S6 (previously S5). As the information in the graph on the right is tangential to our paper, we did not include it in the original or the revised manuscript. We would be happy to include text describing this choice and this data plot if the reviewer thinks it is necessary.

Did the author perform this experiment also in presence of CK666 and SMIFH2? In theory blocking both nucleators should result in a similar disruption of F/actin as seen for LatB.

Although we did not include the inhibitor combination in our cytometry analysis, it seems unlikely that the combination of CK666 and SMIFH2 would reduce the total actin polymer content in these cells, as they look morphologically distinct from LatB-treated cells (Fig 4). While we aren't sure how these SMIFH2+CK666 cells retain the capacity to form filopodia, we speculate this could be due to the activity of an additional (yet to be identified) *Naegleria* actin nucleator, possibly with a mechanism similar to a tandem actin monomer binding protein.

-Live imaging of F-actin dynamics in presence/absence of inhibitors would be helpful.

We agree, and are currently developing new protocols to try to make this a reality. Unfortunately, it is currently beyond the scope of this study (see response to major point 5, above).

Reviewer #1 (Significance (Required)):

This study demonstrates a conserved role of actin in an evolutionary distant (and understudied) eukaryote. It might also open up the field to future studies, since F-actin dynamics can be studied in the absence of cytosolic microtubules

I suspect it will be interesting to a broader audience, working on actin in different organisms.

Expertise: Actin dynamics, Motility, Imaging

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Velle et al explored the role of Arp2/3 and formin in Naegleria cell shape, motility and phagocytosis. They found that Arp2/3 has an important role in cell shape and motility whereas formins had a role in persistence of motility. The role of formins in persistence of direction is interesting since microtubules have a key role in cell polarity and persistence of direction in other alpha-motile cells. This finding indicates that Naegleria has evolved or become more reliant on a formin based polarity mechanism. LatB treatment demonstrated a clear role for actin in phagocytosis. Experiments with CK-666 indicated that Arp2/3 plays some role in phagocytosis; however, the authors were unable to measure a statistically significant role in their assay with GFP

expressing bacteria and flow cytometry. Yet, images of cells from the assay suggest that LatB and CK-666 treated cells are able to adhere to bacteria but not engulf them. We are left wondering about the role of formin. Overall this manuscript is well written, the data is clearly presented and the quality of the analysis is very good.

Major comments:

The key conclusions are supported by the results. One point for improvement would be to address the role of formin in phagocytosis. Formins can play a role in phagocytosis (PMID 16303559, 27152864, 29663616, etc), yet the authors did not include SMIFH2 treatment in the phagocytosis experiments. Since CK-666 treatment alone does not impair bacterial engulfment to the same extent as LatB treatment, formins could be playing a significant role and this should be examined for completeness.

Although we made several attempts to study phagocytosis following SMIFH2 treatment, an obvious contractile vacuole phenotype interfered with our ability to accurately measure phagocytosis. We are currently exploring this phenotype for a future study specifically about *Naegleria* contractile vacuoles. For the purposes of this paper, we have added text to the results section on phagocytosis, and including additional references on this topic (see page 7, line 342).

“While it remains possible that formins contribute to phagocytosis, as in other systems (Bharadwaj et al., 2018; Colucci-Guyon et al., 2005; Naj et al., 2013; Rengarajan et al., 2016), we were unable to directly assess this using SMIFH2, as a strong contractile vacuole phenotype prevented an accurate assessment of phagocytosis.”

The bacterial adherence without phagocytosis shown in Fig S8B is compelling evidence that the flow cytometry assay is under-reporting the phagocytosis defect and should be moved into Fig 6. The authors could further characterize the role of Arp2/3 in this process with live cell imaging of phagocytosis. Or perhaps SEM after a phagocytosis assay that includes a wash step to remove bacteria that has not already adhered to amoebae. If phagocytosis is impaired the expectation is that bacteria would be seen on the cell surface in the CK-666 treated cells but not the controls. This SEM experiment would also serve to show how the inhibitors change the shape of the cells to complement data from Fig 3 and 4.

Thank you for this suggestion; we have moved Figure S8B into the main figure 6. While we are not able to perform new electron microscopy at this time due to COVID-19 related limitations, we have included a new figure with SEM of CK666 treated cells, which show an obvious lack of ruffles (new Fig S3).

The methods section appears to be complete with all the necessary information to reproduce the results presented in this manuscript.

Excellent; we strive to ensure reproducibility of our work and are pleased that the reviewer found our methods to have acceptable levels of detail.

There are no concerns about sample size or the number of replicates. Regarding statistical analysis, it is not clear why the authors used "ordinary ANOVA" to analyze the results from Fig 6. The histograms are clearly non-parametric, perhaps a Mann Whitney test would be more appropriate. Figure 6C reports mean values, since the data is non-parametric median values would typically be reported.

We sincerely appreciate this level of detail/care in evaluating our analyses. We have updated these graphs to show medians instead of mean values (see Fig 6). We chose an ordinary ANOVA because the statistical analysis is based only on the sample-level means (now medians), and not

the entire dataset, which circumvents the problems with using ordinary ANOVAs on nonparametric data (Lord et al., 2020, JCB). We have updated the text to better explain this method. Please see the text edits on page 13, line 664; “Whenever applicable, SuperPlots (Lord et al., 2020) were employed to show data on each individual cell (smaller, gray symbols), while also displaying averages (or medians, as in Fig 6C where the underlying data was nonparametric) from each experimental replicate (larger, colorful symbols). The experimental replicates were used to determine the mean, standard deviation, and statistical significance (with a one-way ordinary ANOVA and Tukey’s multiple comparison test). We used ordinary ANOVAs because it is a straightforward method to determine significance with a low chance of false positives (Lord et al., 2020) and because cell-to-cell variability was higher than the run-to-run variation.”

Minor comments:

Although it is well established that Naegleria builds basal bodies de novo, the point of cells completely lacking microtubules seems at odds with their use in closed mitosis. Figure 2B shows some microtubule cytoskeleton transcripts are upregulated in amoebae, what are they? Fritz-Laylin 2010 includes a western blot of actin and basal body components over time, but examines gamma-tubulin, a component of basal bodies, rather than alpha or beta tubulin. A western blot of actin and tubulin in amoebae and flagellates would complement the staining shown in Figure 2A.

One fascinating aspect of *Naegleria* cytoskeletal biology that we did not discuss in the previous version of this manuscript is the difference between *Naegleria* mitotic and flagellar tubulins. The flagellar tubulins, whose sequences look very much like tubulins of other eukaryotes, are only expressed in flagellates (Fritz-Laylin and Cande, 2010, JCS; Lai et al., 1988, JCB; Fulton, 1983, J of Protozoology; Fulton and Kowit, 1975, Annals of the New York Academy of Sciences). The flagellate state is transient, and after de-differentiation to amoebae, the flagellate tubulin is degraded (Levy et al., 1998, Cell Motil Cytoskeleton).

However, we were careful to state in the original paper that: “The only microtubules found to date in *Naegleria* amoebae are found in the mitotic spindle during closed mitosis (Fulton and Dingle, 1971; Gonzalez-Robles et al., 2009; Walsh, 1984).” What we did not explain in the original paper is that these mitotic microtubules are built from divergent tubulin that is expressed specifically during mitosis (Chung et al., 2002, Gene).

To clarify these points, we have:

1. Added text to the introduction of the manuscript (page 2, line 63): “These mitotic microtubules are built from divergent tubulin that is expressed specifically during mitosis (Chung et al., 2002). Microtubules have not been observed outside of the nucleus in amoebae as visualized by immunofluorescence (Walsh, 2007; Walsh, 2012) and electron microscopy (Fulton and Dingle, 1971).”

2. Edited the discussion from (page 8, line 378) “These proteins are primarily expressed in the microtubule-free, amoeboid cell state.” To “These proteins are primarily expressed in the microtubule-free, amoeboid cell cytoplasm.”

3. We also discuss the microtubule genes expressed in amoebae in the legend for Fig S1: “The microtubule cytoskeletal genes expressed in amoebae include the mitotic tubulins, as well as putative spindle components.”

Because the expression patterns of actin and tubulin in amoebae and flagellates have been published—actin protein levels remain constant between amoebae and flagellates (Fritz-Laylin et al., 2016, Cytoskeleton), while non-mitotic tubulin is expressed only in flagellates (Fritz-Laylin and Cande, 2010, JCS; Lai et al., 1988, JCB; Fulton, 1983, J of Protozoology; Fulton and Kowit, 1975,

Annals of the New York Academy of Sciences), we did not include new Western blots showing these results in the revised manuscript. However, if the reviewer would like us to repeat these experiments for this paper, we could do so.

Similarly, a microtubule inhibitor control would complement the persistence assays in Figure 5. - Together these small experiments would strengthen support for the idea that tubulin has no role in persistence of motility in Naegleria.

Because untreated interphase *Naegleria* amoebae do not express tubulin (see references above), to have the SMIFH2-dependent phenotype be caused by microtubules, the cells would need to transcribe, translate, and polymerize tubulin on the time scales of our experiments. Because motility phenotypes were observed from the point of drug addition for a total of five minutes, we think it is unlikely that the cells could accomplish this feat within this time frame. However, if the reviewer remains concerned about this, we would be happy to perform a few additional motility trials in the presence of a microtubule inhibitor(s) such as nocodazole or oryzalin.

Figure 4D and the text indicate there is no difference in cell area. Yet, the image of the LatB treated cell in Figure 4C is roughly half the size of the other cells and it made this reader expect to see a significant difference in cell size in Fig 4D. This image should be replaced with a more representative one.

While the difference in size did not reach our threshold for statistical significance, the data displayed in the graph (Fig 4D) show a vast majority of the LatB-treated cells fall below the average control cell size (indicated by dashed line). This cell was chosen as representative of the cells on the coverslip as its phenotype was the most common we observed based on a number of parameters (cell shape, protrusions, and actin puncta).

The term "NEGM amoeba" is found in the legend of Fig 2 without any explanation in the text. A google search revealed that ATCC 30224 is NEG-M. Either introduce this term in the main text or just say amoeba.

We have removed this from the legends, and add an explanation in the methods (see page 10, line 489: "*Naegleria gruberi* cells (strain NEGM; a gift from Dr. Chan Fulton, Brandeis) were axenically cultured…").

How does SMIFH2 impact persistence of motility in amoeba from other eukaryotic lineages?

The role of formins in motility has been best explored in *Dictyostelium*. We have added the following text to the revised manuscript:

In the results, we have added text on page 6, line 309; "Further, impairment of either formins or Arp2/3 complex in *D. discoideum* disrupted protrusions associated with navigating complex environments (Jasnin et al., 2016)."
In the discussion, we have added text on page 9, line 456: "Similarly, *D. discoideum* cells lacking three diaphanous formins have an impaired actin cortex and are unable to polarize myosin II or migrate in 2D confinement. Unlike SMIFH2-treated *Naegleria*, however, unconfined mutant *D. discoideum* cells appeared more directionally persistent, and crawled using a mechanism reminiscent of keratocytes (Litschko et al., 2019). "

Reviewer #2 (Significance (Required)):

This work is foundational for understanding the role of actin in Naegleria cell shape, alpha-motility and phagocytosis. Naeglaria has many conserved actin cytoskeletal proteins so it not surprisingly that these components have similar roles in all eukaryotes. This indicates an ancient evolutionary

origin for these proteins in motility and phagocytosis. According to the authors a major finding is that formins have a role in directing motility. However, SMIFH2 has also been shown to regulate directionality of Dictyostelium amoeba and this was not discussed (PMID 30808751). If the point of this work is to move toward a comparative understanding of cytoskeletal function and the mechanics of cell motility across eukaryotes, the authors should include a more inclusive discussion of what is known about formin's role in motility and phagocytosis. This work should be of interest to the broader cytoskeleton/cell biology readership as well as evolutionary cell biologists and parasitologists.

Reviewer expertise: Cell biology and function of the cytoskeleton in microbial eukaryotes.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

The authors use a range of actin probes and small molecule inhibitors to perturb and monitor actin dynamics in the heteroloboseans Naegleria gruberi and Naegleria fowlerii using a combination of light and electron microscopy. They show that Naegleria assembles Arp2/3-dependent actin structures such as lamellae; that Arp2/3 is required for migration and phagocytosis; that cells without Arp2/3 assemble filopodia instead. Overall, the Naegleria actin network exhibits some properties typical of other amoeboid cell types from various branches of the eukaryotic tree, with implications for the evolutionary origins of amoeboid motility.

This is a well-executed study. A few additional experiments and analyses would help support the claims of the paper.

Major comments:

1.The paper should look more closely at the actin-regulated genes that are up-regulated in the flagellated form. Authors should see whether these are unusual in anyway - especially the SCAR/WAVE homologue (WAVE_58655) and formins (Formin_81446; Formin_79644; Formin_78968).

The SCAR/WAVE homolog upregulated in flagellates has a WCA domain that is likely non-functional. This could mean that SCAR/WAVE complexes that incorporate this protein would reduce the complex's capacity to activate Arp2/3. In turn, this could suppress the formation of actin-filled pseudopods in swimming flagellates. We hope to explore this idea further in the future, and now include a brief discussion in the text (page 4 line 167: "Alternatively, because these SCAR/WAVE genes are not identical, their differential expression may modulate the Arp2/3 activating capacity of the SCAR/WAVE complex.").

For formins, we now include the percent identity of *Naegleria* formin FH2 domains compared to some well-studied formins, and also examine key residues from a consensus sequence (see updated Fig S5). Using this analysis, Formin_79644 has the least conserved FH2 domain, followed by Formin_81446. It is unclear if these differences impact the ability to polymerize actin, and it would make sense for flagellates to employ at least some actin cytoskeletal proteins to carry on normal cellular functions like intracellular trafficking during this transient life stage.

2.Figure 1 focuses on Myosin II. This is surprising given that the authors have not looked at Myosin II or attempted Blebbistatin treatment. The authors could do this or they should re-think Figure 1. Given the paper is about the ensemble of genes that enable phagocytosis and Arp2/3-dependent movement, the authors should make clear in Figure 1 which actin-related genes have a distribution across evolution similar/different to that of Myosin II.

Thank you for pointing this out; we now realize that the focus on Myosin II in this figure probably felt like a bait and switch to the reader. While we remain very interested in exploring the functional role(s) of myosin II in *Naegleria*, such experiments, in our opinion, are outside the scope of this paper. We have therefore removed the references to MyoII in Fig 1.

3.It is vital to test whether tubulin is up-regulated following treatments that affect actin, e.g. 5FG. If so, this would change the conclusions. Note that similar actin spikes are seen in RNAi screens in metazoan cells following the silencing of Arp2/3 components and are associated with actin spikes and tubulin.

Because the actin spikes occur as quickly as 90 seconds after CK666 treatment (**Fig. 5G**), and because amoebae do not normally express tubulin mRNA or protein (Fritz-Laylin and Cande, 2010, JCS; Lai et al., 1988, JCB; Fulton, 1983, J of Protozoology; Fulton and Kowit, 1975, Annals of the New York Academy of Sciences; Levy et al., 1998, Cell Motil Cytoskeleton), we believe it is unlikely that the cells could transcribe, translate, and assemble microtubule-based spikes in less than two minutes. We have added text to emphasize the time scale of these spikes to the manuscript (page 7, line 329: “These actin-rich filopodia were observed both at early time points (90 sec post-treatment, **Fig 5G**, cell 2) and late time points (50 min after treatment, **Fig 5G**, cell 3).). If the reviewer remains concerned that the actin spikes are caused by induction and polymerization of tubulin, we could perform an additional experiment to confirm the spikes are made of only actin by treating cells with CK666 and performing immunofluorescence using an anti-tubulin antibody and co-staining with phalloidin.

4.Figure 5D should include data for CK666.

We have updated Fig S8 to include CK666 data and CK689 data (with SMIFH2 and DMSO tracks for comparison).

5.Figure S2B - does transcript abundance represent total abundance for duplicated genes? If not, this should be shown. In this Figure all individual actin regulators should be included, e.g. Cofilin, CAP, Capping protein. Each histogram should be associated with a gene, not a class of genes.

Thank you for this suggestion. Each bar of Figure S2B represents a single gene; but we used only one representative actin in the original figure S2B. We appreciate that presenting this data in this way was not as clear as we had intended. To help the reader understand our data, we have added labels to the graph, and included expression data for all of the actin genes.

Minor comments:

1.The genes should be marked in Figures 2C and S2B. Otherwise there is the possibility of confusion (i.e. which WAVE homologue is shown in 2C and S2B)?

We have added extensive labels to Fig S2, and added text to the legend of Fig 2 to clarify the relationship between Fig 2, Fig S1, and Fig S2.

2.In Figure S1, it would be better to normalise so we can see the extent to which each gene is up/down regulated independent of its levels (e.g. Myosin II heavy and light chains).

We have included additional panels set to a log scale to display genes expressed at low levels in both amoebae and flagellates. Please see the updated figure S1C.

3.It would be good to reference work on Dictyostelium (Tunacliffe et al., 2018) where the role of multiple actin genes has been studied, and to discuss actin in plants/SAR as well as Metamonads and Opisthokonts.

Because the manuscript is already quite long, we have decided to retain our original focus on literature and organisms that explore mechanisms of actin-dependent cell migration and/or phagocytosis, as these are the phenotypes we investigate in this manuscript. If the editor agrees, however, we would be delighted to add additional paragraphs to either the introduction or discussion sections of the manuscript discussing actin functions in these other lineages. We ave, however, added the additional reference and half sentence to our discussion on *Dictyostelium*'s many actins; see page 8, line 407, "This abundance of actin genes likely indicates the importance of actin to an amoeboid lifestyle, and in *D. discoideum*, different promoters allow for transcriptional bursts to fine-tune cellular actin concentrations (Tunnacliffe et al., 2018)."

4.There is a slight over-representation of references to own papers over contributions by others in the field.

Respectfully, there are not many labs studying the cytoskeleton of *Naegleria*. Our papers represent >20% of the *Naegleria* cytoskeleton literature over the past 10 years (based on a pubmed search for "Naegleria Cytoskeleton"), and represented only ~8% of our references in the original submission. We did our best to cite the most relevant papers on work from amoebozoa, mammalian systems, and metamonads, and are happy to include the additional citations brought up in this review process. If there are additional specific references the Reviewer would like us to cite, we would be happy to do so.

What would be great (but isn't essential):

1.Would be great to have TEM to image the actin network +/- CK666, but this is not be necessary for publication.

While we agree this (or even platinum replica electron microscopy) would be a great way to visualize changes in the structure of the actin cytoskeleton, we are unable to provide this at this time. However, we have included additional SEM images +/- CK666, which show a clear difference in ruffling (please see new Fig S3).

Reviewer #3 (Significance (Required)):

The paper goes a long way towards establishing Naegleria as a sophisticated new model in which to study actin's role in cell shape control - and, uniquely, one that can be analysed in the absence of microtubules.

Our understanding of the origins of amoeboid motility and phagocytosis has derived largely from studies in opisthokonts and amoebozoa (e.g. Dictyostelium) with little data from other parts of the tree. The demonstration here that Arp2/3-dependent actin-based protrusions are important for Heterolobosean motility and phagocytosis supports the notion of a deeply conserved, ancient origin for these features of the actin network - which in turn has implications for the origins of eukaryotes (Burns et al. Nat. Eco. Evol. 2018)

Overall, this study would be a welcome addition to the field, and of general interest to the broader cell and evolutionary biology communities.

August 5, 2020

RE: JCB Manuscript #202007158T

Dr. Lillian Fritz-Laylin
University of Massachusetts Amherst
611 N. Pleasant St., Morrill 323
Amherst, MA 01003

Dear Dr. Fritz-Laylin:

Thank you for submitting your manuscript entitled "Arp2/3 complex-mediated actin assembly drives microtubule-independent motility and phagocytosis in the evolutionarily divergent amoeba Naegleria" from Review Commons. We find that your paper provides fundamental evolutionary insight and establishes a new model system in an underrepresented eukaryotic lineage with detailed quantified cell biology data that will be of interest to a broad audience. Therefore we would be happy to publish your paper in JCB as a Report pending final revisions necessary to meet our formatting guidelines (see details below).

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9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: as we editorially find that your study works best as a Report, we can allow an extension of our limits of SI figures, however please try to condense them to as few figures as possible without sacrificing the visibility. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

Magdalena Bezanilla, PhD
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