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Specialization of actin isoforms derived from the loss of key interactions with regulatory factors

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Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers find the topic of the study interesting. However, all reviewers indicate multiple substantial concerns that affect the core conclusions of your study and indicate that substantial further characterisation of the experimental system, e.g., regarding the expression levels of the various actin isoforms and actin binding proteins, F-actin/G-actin ratio and so on, would be needed. Furthermore, they point out that a more detailed analysis of the basis of the observed differences in actin network formation and their dependence on modulation of interactions with actin-binding proteins is required.

Based on the overall interest expressed by the reviewers, I would like to invite you to submit a revised version of your manuscript, in which you address the comments of all referees. Please note that a strong referee support will be required for the acceptance of the revised manuscript.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. Since extensive additional work would be needed to fulfill all referee requests, I would ask you to provide a preliminary revision plan at an early stage of the revision to assess the feasibility of the various aspects of the revision. Please also let me know if you find that particular issues will not be addressable in the revised version, in which case I would be happy to discuss alternative publication possibilities within EMBO Press journals.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Since a rather major revision would be need in this case, I would be happy to extend the revision time to 6 months at the first instance. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

REFEREE REPORTS

Referee #1:

The question of how multiple actin isoforms contribute to the complex arrangement and dynamics of the cytoskeleton is one of the most fascinating unresolved questions in actin biology. Even in the case of mammalian beta- and gamma-actin, which only differ by four amino acids and are (virtually) biochemically indistinct, there appears to be some specialization of the isoforms into how the incorporate and effect the cytoskeleton. However, almost every time a new result is discovered which differentiates the two, another is presented which repudiates or at least cofounds it. This has been true of isoform-specific reagents, isoform-specific gene knockouts, and more recently isoform specific gene editing to differentiate nucleotide vs. amino acid effects. Thus,

there is still a lot to learn about why different isoforms are evolutionarily conserved and how they co-exist in a cell.

Here Sanders et al. ambitiously tackle how different actin orthologs can control the cytoskeleton in a living system by creating a library of budding yeast strains that have been altered to express actins from different species and then using cell growth and analysis of the formation of different actin structures as readouts. There are also in vitro biochemical assays and structural predictions to help support some of the claims of the paper. The work that went in to creating this experimental system is extensive and it has exciting potential. However, while I appreciate the premise of the study, I fear that many of its conclusions can be called into question and significantly more work would be required to validate the model system and the conclusions that the authors are drawing from it. Further, I question how accurately the study yields what is promised in the title. The proof that effects of the different actin orthologs on the yeast cytoskeleton are due to a loss of interactions with regulatory factors is fairly weak and could be caused by one of the several concerns which are raised below. A substantial amount of work would be required to validate this claim. Finally, while this study will extensively characterize the effects of expressing on or two different actin orthologs will have on the yeast cytoskeleton, it won't shed a significant amount of light as to how co-expressed isoforms regulate the cytoskeleton in a natural settings, for example a mammalian cell that expresses beta, gamma, and alpha actin. Nor does it, at least at its present stage, yield universal rules that would apply to all systems. There is potential for this study to achieve this goal, or at least approach it, but that potential has not yet been realized.

Major concerns:
1. In order for these experiments to be interpretable, then every strain that is used must be comparable in their expression of actin-binding proteins. It is highly likely that forcing expression of another species actin ortholog, or even altering the amount of actin protein that is available, is causing differential gene expression. At the very least, the possibility that cytoskeletal genes are all expressed at the same level amongst the strains needs to be excluded, by RNA-seq for example, since this could have direct impact on the results and their interpretation.
2. Serial dilution and quantification of colony formation is highly inaccurate measure of cell fitness, as even the wild-type Sc cells have +/- 60% error in the measurement presented (Fig. 2C,D). The same could be said for the assay that is used almost exclusively to measure how the cytoskeleton is affected (network deviation index) Why is this important? Because the results of this assay are used to define a range of actin expression that is acceptable for comparison of the different strains, with the authors stating that even a ~65% reduction of actin protein does not appear to alter cell fitness or cytoskeletal architecture. I am not familiar if this has been done in budding yeast, but in fission yeast that have undergone molecular counting experiments, it has been estimated that 50-75% of the total actin molecules expressed are present as filaments in patches, contractile rings, and cables (I took these numbers from the Burke 2014 Current Biology paper that is heavily cited in this manuscript, there are citations there to the various papers from the Pollard, Cheng, Wu, and Vavylonis labs which calculate this). Based on these numbers, it seems highly unlikely that actin organization is unaffected by a 50% loss of available protein. Even if filaments levels were almost the same, the monomer pool would be greatly reduced. And as the authors state numerous times in the manuscript, the monomer pool is limited and can affect which types of actin structures assemble (Burke Current Biology 2014). Thus, a more sensitive assay to determine cell fitness (ex. growth rate) and a more extensive work up on how the cytoskeleton is or isn't affected by different levels of actin expression needs to be done before. For the latter, I would want to see a quantification of the individual types of actin structures, not just a ratio of the two, as well as a measurement of how much actin is present as monomers or filaments. This could be used to generate a more accurate range of what is acceptable in order to compare the results from strains expressing actin orthologs.
3. Having verified the experimental system, I would then request to see a similar quantification of actin (the #'s of different structures, the G/F actin ratio, etc.) in cells expressing different orthologs in order to better understand how the cytoskeleton is affected. The deviation and polarity indexes are just not enough to be able to draw general conclusions from.
4. The in vitro assays are interesting, but with everything done at only one concentration, it a relatively blunt measure of an ortholog's interaction with an actin binding protein.
5. The same could be said for the single concentration used to test for drug sensitivity.
6. Figure 6- what is the expression ratio of the different actin orthologs, and what is the concentration of total actin with respect to wild-type cells?
7. Finally, in order to lay claim to the title of the manuscript that the differences are truly do differences in interactions with other actin regulators, one would want to do a study that quantifies all of the interactions that the different orthologs have in the cell, perhaps using proximity-ligation and proteomics. Again here, the actual concentration of the actin ortholog in the cell is likely to be important unless proven otherwise.

Minor Concerns
1. Figure 2F- please zoom in on the cells more. It would also help to highlight the features that are changing the deviation index. The differences in these examples appear to be more subtle than the quantification in 2G-I would suggest. Actually, this goes for all of the figures. It is particularly difficult to see what is going on in Fig. 3J.
2. Figure 4B, C. The images do not appear to match the quantification. For example, the Cof/Actin ratio for branched actin is very low in the graph for Sc actin, however, the images show the opposite- the merged image is completely green. This is not isolated to those panels, either.
3. There is no discussion about the implication that this study has on "Frankenstein" experiments- either when actin binding proteins and actin from different species are used in biochemical assays or cell biology studies were actin orthologs from other species are introduced (for example, using rhodamine-labeled rabbit skeletal muscle actin in lower eukaryotes). Not essential, but I think this paper would speak well to that.

Referee #2:

In this manuscript by Dr. Michelot and colleagues, the authors make a clever use of the budding yeast system, to test an important hypothesis in the cytoskeleton field: the idea that small molecular differences between actin isoforms (or variants) can cause their segregation into different filament networks within the same cell. The principle that actin-binding proteins can distinguish actin orthologs is already accepted (for example, it is known that yeast cofilin binds and severs yeast actin filaments far more efficiently than rabbit alpha-skeletal actin filaments) but the authors go much further here, by showing that different actin variants preferably assemble into branched or linear filament networks, in cells and in vitro. I believe this work will be of great interest to the field, and to a broad readership.

I nonetheless have a few concerns, which should be addressed to make the paper acceptable for publication.

Main issues:

1. The incorporation of fluorescently labeled actin, in reconstituted assays (Fig 4), is unknown.

If I understand correctly the Methods section, the in vitro experiments are done with 1% fluo-labeled rabbit actin (none of the other actins are labeled). This should be said clearly in the main text. Simply writing "8 µM F-actin (1% Alexa-568-labeled)" in the caption of figure 4 is misleading.

The overall architecture of the filament networks is likely unaffected by this 1% labeled rabbit actin. However, the fact that all the results of Figure 4 rely on the fluorescence intensity as a readout of the total amount of actin (either to compare the amounts of actin in branched and linear networks, or to normalize the fluorescent signal of cofilin and Tpm) is potentially a problem. One can easily imagine that fluo-labeled rabbit actin may not copolymerize equally well with the different actin variants, and that the labeling fraction of the filaments and network will not be 1%, and will not be the same in the different experiments. Also, formin-elongated filaments are known to incorporate less labeled actin. This could lead to incorrect conclusions.

For the robustness of results obtained from the reconstituted assays, this point must be addressed. Ideally, with a dedicated experiment to measure the effective labeling fraction of filaments made with the different actin variants (for example, by running gels after spinning down the filaments, or by measuring the fluorescent intensity per unit length of single filaments). At the very least, it should be thoroughly discussed.

2. The way the fluorescence intensities are quantified in Figure 4 is unclear.

The actin networks shown in Figure 4, both branched an linear, have varying lengths and appear to be very inhomogeneous in intensity (of actin, cofilin, and Tpm). How and where are these intensities measured? For example, in Fig 4C, the Tpm-to-actin ratio strongly varies along the branched comet tail, and the result will be highly dependent on where the measurement is made. If the signal is averaged over the whole comet tail, then the overall length of the comet will have an impact (so will its age, and the concentration of the other proteins). Another example, the branched networks made of N2 actin all appear shorter than those made with Sc actin, indicating a slower rate of assembly. Shouldn't that be taken into account, somehow? These points need to be clarified.

The authors should explain better how they analyze these data. What does "average normalized intensity" mean (methods, line 704)? i.e., normalized by what? In Figure 4A, the "relative actin intensity" is plotted. Relative to what?

According to the caption of Fig 4, the images have different contrast settings, and this can be very misleading. For example, in the images of Fig 4A, N2 actin appears to incorporate more in the branched network than the linear network, compared to Sc actin, but the quantification tells us it is actually the opposite (deviation index for N2 is on the linear side). Perhaps the authors could provide images with the same settings.

3. To assess the impact of CK666 on different strains (Fig. 3K) we would need to see the same data (the distribution of the number of patches per cell) in the absence of CK666. It is currently missing.

4. It is not clear to me at all why cells expressing Act_N2 (Fig 3JK and Fig 6) manage to generate branched filament networks, better than wild type, in the presence of CK666. Since CK666 binds to the Arp2/3 complex (see, for example, Hetrick et al. Chem Biol 2013), how can a difference in actin offer protection? The idea put forward in the paper (in the title, the abstract, and throughout) is that defects in the interaction with some ABPs could increase the monomer pool's availability for incorporation into branched networks. I don't see how this could counter the effect of an Arp2/3-targeting drug. The authors should discuss this point, and propose explanations.

Other, more minor points:

5. In several figures, it would help to indicate which variant each data point corresponds to. In Fig 3E, in particular: there are 16 data points, with 14 values for identity, what do they correspond to? (only 10 strains are listen in previous panels).

6. Fig 7 is a bit hard to read. Differences in network densities are not clear enough, and could be amplified to help the reader get the message rapidly. Maybe it would be easier to represent networks by 'zooming out' a bit?

7. Line 187, I am not sure there is a threshold percentage, maybe a few key nucleotides are important, so perhaps the authors should be more cautious in their interpretation.

8. Regarding the number of repeats. For experiments in cells, are the 30 cells per strain taken from one or more experiments? Please clarify.

9. There are several typos and minor mistakes. For example,
Line 184, "...the level of expression levels..."
Line 246, "branched- and linear-actin network assembly, respectively from formin and WASp-coated beads" should be the other way around.
Line 279, "effect" instead of "affect"
Line 741, "pulled" instead of "pooled"
Line 1041, "quantifications on the left" instead of right.
Table 2, Candida albicans column, a couple of errors in the amino acid numbers.

Referee #3:

In this article, the authors examine the physiological consequence of expressing multiple actin isoforms. Starting from yeast that expresses only one actin isoform, the authors examine how expression of heterologous actin affects two types of actinous structures: actin patches and actin cables. These are generated by different actin nucleators and possess a different complement of actin binding proteins. They find that some actin isoforms favour assembly into patches, while others favour assembly into cables. In addition to more or less favourable interactions with nucleators, post-assembly, actin binding proteins show selective binding to filaments assembled by some isoforms. By co-expressing one isoform that favours patches and another favouring cables, the authors engineer yeast cells with patches and cables assembled by independent networks and that can be controlled independently. Overall, the topic of the study is very interesting and novel. It poses some intriguing questions. In general, the experiments are well executed and well controlled. This is a very interesting article that merits publication but prior to this a few issues need to be addressed.

Major issues:
-I find the resistance to CK-666 treatment merits further characterisation to really support the authors' message. The authors show that strains over assembling patches are more resistant to Arp2/3 perturbations such as Act_N2. Can the authors provide further mechanism about why resistance to treatment arises? There are several potential explanations: 1) because the isoform doesn't assemble cables, when you perturb Arp2/3 all G-actin does not get taken up by patches, 2) the amount of Arp2/3 in Act_N2 cells may be larger than in control cells - as a consequence, more patches would subsist. To test these hypotheses, the authors could quantify the amount of Arp2/3 subunits in control and Act_N2 strains. It may also be useful to show that the ratio of arp2/3 staining to F-actin staining stays to same in the patches even with different isoforms. This would provide the most basic illustration that the patches have roughly the same composition and are therefore comparable from strain to strain. For example, it is possible that in Act_N2 strains there may be a higher density of branching.
-The authors quantify the total actin in each strain but it would also be useful to systematically quantify the amount of F-actin. This would allow the reader to make sure that differences in total actin translate (or not) into differences in F-actin content. In particular providing a quantitation of the F-actin/G-actin ratio may allow the authors to make their main point more strongly: if you perturb patch formation in strains with one isoform, the F/G ratio doesn't change because the G-actin freed by Arp2/3 inhibition gets assembled into cables. Conversely, in a system with 2 actin isoforms, the F/G ratio would change because the isoform favouring patches cannot be used in cables.
-p11: Can the authors provide further detail as to why Act_Ca does not assemble into patches? Does the more favourable binding of tropomyosin impede branch formation? What happens in the in vitro assays if tropomyosin is omitted?
-p12: the authors state that Act_N2 does not incorporate into cables because an ABP that stabilises cables cannot bind to it. In vitro, they show that tropomyosin cannot bind Act_N2. However, for completeness, they should also confirm that knocking down tropomyosin in control strains impairs cable formation.
-p13: the authors state that Act_N2 has got substitutions at all interfaces with nucleators and ABPs used in the assays. Yet, the amount of F-actin that Act_N2 assembles appears to be similar to the control strains. Why is that the case?
-p14: the authors state that in cells expressing two actin isoforms it is difficult to localise each. Is it not possible to express very small tags like tetracysteine tags? Alternatively, in the in vitro assays, it should be possible to label each of the purified actin

isoforms with a different fluorophore. The in vitro assays could help to determine if isoforms co-assemble into filaments or if they segregate - which would make the authors main message stronger. One may expect co-assembly in branched structures because elongation takes place spontaneously at the barbed end but not in cables because formins (or profilin) may be isoform selective.

Minor issues:
-Fig 2E and 3E would benefit from a legend. I realise that the colour code is the same as in 2D and 3D but the colours are not very different in 3E so it is difficult to distinguish which isoform is which.
-Fig 4B, C: The branched parts of the graphs are really difficult to read because the ratios are close to 0. I think it would be better to replot those on separate graphs with a more appropriate scale.
-Fig 7: It may be useful to also have a diagrammatic representation of the reservoir of G-actin.

Response to referees comments
(Manuscript EMBOJ-2021-107982 – Boiero Sanders et al.**)**

Please find below our response to the comments of the Referees. Their comments appear in black, and our responses in blue.

Referee #1 (Remarks to the Author):

The question of how multiple actin isoforms contribute to the complex arrangement and dynamics of the cytoskeleton is one of the most fascinating unresolved questions in actin biology. Even in the case of mammalian beta- and gamma-actin, which only differ by four amino acids and are (virtually) biochemically indistinct, there appears to be some specialization of the isoforms into how the incorporate and effect the cytoskeleton. However, almost every time a new result is discovered which differentiates the two, another is presented which repudiates or at least cofounds it. This has been true of isoform-specific reagents, isoform-specific gene knockouts, and more recently isoform specific gene editing to differentiate nucleotide vs. amino acid effects. Thus, there is still a lot to learn about why different isoforms are evolutionarily conserved and how they co-exist in a cell.

Here Sanders et al. ambitiously tackle how different actin orthologs can control the cytoskeleton in a living system by creating a library of budding yeast strains that have been altered to express actins from different species and then using cell growth and analysis of the formation of different actin structures as readouts. There are also in vitro biochemical assays and structural predictions to help support some of the claims of the paper. The work that went in to creating this experimental system is extensive and it has exciting potential. However, while I appreciate the premise of the study, I fear that many of its conclusions can be called into question and significantly more work would be required to validate the model system and the conclusions that the authors are drawing from it. Further, I question how accurately the study yields what is promised in the title. The proof that effects of the different actin orthologs on the yeast cytoskeleton are due to a loss of interactions with regulatory factors is fairly weak and could be caused by one of the several concerns which are raised below. A substantial amount of work would be required to validate this claim. Finally, while this study will extensively characterize the effects of expressing on or two different actin orthologs will have on the yeast cytoskeleton, it won't shed a significant amount of light as to how co-expressed isoforms regulate the cytoskeleton in a natural settings, for example a mammalian cell that expresses beta, gamma, and alpha actin. Nor does it, at least at its present stage, yield universal rules that would apply to all systems. There is potential for this study to achieve this goal, or at least approach it, but that potential has not yet been realized.

We thank Referee #1 for appreciating our efforts to resolve one of the most fascinating questions in actin biology. We took note that Referee #1 had a more critical opinion on our work than Referees #2 and #3. Despite this criticism, we think that Referee #1 brought up a number of valid points, and we thank him/her for that. We have performed a significant amount of work to improve this manuscript and we believe that all these results confirm and strengthen our claims, and justify the title of this manuscript.

We would also like to discuss Referee #1's concern about the generality of the mechanism and whether it applies in a natural setting. This is a very good question that needs to be clarified to fully understand the positioning of this study. Briefly, we think that this question of segregation of actin isoforms to different networks has been little addressed in the past for all the experimental difficulties described in the introduction. The aim of our work was to approach this question from a different angle, in order to determine for the first time a plausible and efficient mechanism to achieve this segregation. Although we do not determine

here whether this mechanism is generally used by all eukaryotes expressing various actin isoforms within the same cell type, we believe that our work provides a solid and unprecedented framework for all the future studies which will focus on particular cellular models.

Major concerns:

1. In order for these experiments to be interpretable, then every strain that is used must be comparable in their expression of actin-binding proteins. It is highly likely that forcing expression of another species actin ortholog, or even altering the amount of actin protein that is available, is causing differential gene expression. At the very least, the possibility that cytoskeletal genes are all expressed at the same level amongst the strains needs to be excluded, by RNA-seq for example, since this could have direct impact on the results and their interpretation.

We agree that mutating cells can generally affect protein expression levels (not only in yeast). We would like to highlight that it is potentially true for all type of mutations; for example, knocking-out genes coding for actin-binding proteins frequently leads to growth phenotypes and cytoskeletal disorganization. However, these mutants are used and described in countless publications, and we know of very few (if any) studies that systematically check, for each mutant generated, whether expression of other genes or proteins are affected. These analyses are costly and time consuming, and are usually performed only if there is a suspicion that the expression of a particular protein is different.

Nevertheless, we wanted to make this effort here to address the concerns of Referee #1. We performed RNA-seq for the 8 most important strains used in this study, results which are provided in the EV Figures. These results indicate that none of the actin regulators that we know of are expressed differentially beyond what is typically expected in this type of analysis.

2. Serial dilution and quantification of colony formation is highly inaccurate measure of cell fitness, as even the wild-type Sc cells have +/- 60% error in the measurement presented (Fig. 2C,D).

We based these measurements on previous papers doing similarly (e.g. Costanzo et al., *Science*, 2010). All the methods quantifying cell fitness that we know have pros and cons, and ideally it is better to use two different methods to validate results. Therefore, we also present in the revised manuscript cell doubling times (in the main Figures). Results are similar, although we found with this method a small growth difference between wild-type and Sc[Sp] cells, undetected by previous colony size measurements (which are now left in the Appendix).

Please note that throughout this paper, we represent standard deviations (SD) and not standard errors of the means (SEM), which could give a visual impression that dispersion of the measurements is larger than in other publications.

The same could be said for the assay that is used almost exclusively to measure how the cytoskeleton is affected (network deviation index) Why is this important?

We apologize for not explaining more clearly the logic behind calculating network deviation indexes to evaluate how the cytoskeleton is affected. Basically, 3 different analysis methods are possible to characterize the cytoskeleton of phalloidin-labeled fixed cells:

i/ either by counting numbers of actin patches and cables (which we now provide in the Appendix). However, these parameters are not always the most precise, as they cannot distinguish between cells assembling many "small" patches (or cables) and cells assembling few "large" patches (or cables).

ii/ or by measuring the intensity of actin patches and cables. These parameters are informative for many strains, and we now also provide these measurements in the Appendix. However, for strains Act_Yl and Act_Nc, the actin cytoskeleton could not be phalloidin-labeled. Similarly, for strains Act_Ca and Act_Op, we found that phalloidin labeling was much less efficient. On the contrary, phalloidin labeling was much more efficient for Act_N2 and Act_Hs strains. Because labeling affected in these 6 strains patches and cables equally, and because these 6 strains present different phalloidin-binding sites compared to *S. cerevisiae* (see Appendix Fig S3H and Mentes et al. *PNAS*, 2018), we suspect that differences in patch/cable intensities are only due to the fact that phalloidin binds to these actins with different affinities.

iii/ or by exploiting the fact that branched and linear actin networks in yeast do not assemble independently, but are in homeostasis in the cell (Kovar lab). Assembly defects in one actin assembly pathway are systematically compensated by over-assembly of the other pathway. Our previous publication (Antkowiak et al., *PLoS Biology*, 2019) has generalized this mechanism, by showing that the size of distinct actin networks is determined by their relative capacity to assemble in a common and competing environment. In this context, measuring a network deviation index based on patch and cable intensities is a reasonable way to evaluate how F-actin is shared between the 2 structures, even for strains which are expressing actins of lower or higher affinities for phalloidin.

Because the results of this assay are used to define a range of actin expression that is acceptable for comparison of the different strains, with the authors stating that even a ~65% reduction of actin protein does not appear to alter cell fitness or cytoskeletal architecture. I am not familiar if this has been done in budding yeast, but in fission yeast that have undergone molecular counting experiments, it has been estimated that 50-75% of the total actin molecules expressed are present as filaments in patches, contractile rings, and cables (I took these numbers from the Burke 2014 Current Biology paper that is heavily cited in this manuscript, there are citations there to the various papers from the Pollard, Cheng, Wu, and Vavylonis labs which calculate this). Based on these numbers, it seems highly unlikely that actin organization is unaffected by a 50% loss of available protein. Even if filaments levels were almost the same, the monomer pool would be greatly reduced. And as the authors state numerous times in the manuscript, the monomer pool is limited and can affect which types of actin structures assemble (Burke Current Biology 2014). Thus, a more sensitive assay to dsetermine cell fitness (ex. growth rate) and a more extensive work up on how the cytoskeleton is or isn't affected by different levels of actin expression needs to be done before. For the latter, I would want to see a quantification of the individual types of actin structures, not just a ratio of the two, as well as a measurement of how much actin is present as monomers or filaments. This could be used to generate a more accurate range of what is acceptable in order to compare the results from strains expressing actin orthologs.

We have followed this suggestion and measured these values (cell fitness, G/F actin ratio, intensity and number of actin structures) for all the yeast strains.

With cell fitness measurement of increased sensitivity, we were able to detect small growth defects for the Sc[Sp] strain of 35% reduced actin expression (Fig 2C). Though, we were still surprised to generate yeast strains of reduced actin expression (e.g. 46 % for Act_Sc[Ca] and 39 % for Act_N1) with no visible growth or actin organization defects. But this result is very solid and was verified multiple times.

About why budding yeast cells can survive with few defects despite the presence of less actin, we can only raise hypotheses at this point (1st paragraph of the Discussion). A direction that we would like to explore in the future is the role of the oligomeric actin reservoir present in the cells, which is impossible to evaluate from G/F actin ratio measurements. The presence of these oligomers is attested in multiple systems such as yeast (e.g. Okreglak et al., *J Cell. Biol.*, 2010) or mammalian cells (e.g. Raz-Ben Aroush et al., *Curr Biol*, 2017)

without their function being clearly determined. One possibility could be that this reservoir is useful to buffer variations of actin expression in cells, and that yeasts expressing less actin draw from this reservoir to compensate. A more complete study (which is beyond the scope of this paper) should be done to understand this.

About G/F actin ratios, we have always been hesitant in the past with this kind of measurements which, by lack of reference values, do not allow to know the accuracy of the measurements (we even created a Twitter feed to ask our community for the best way to measure this, and people who replied generally agreed that these measurements are difficult). Eventually, we performed these measurements with the following method. We worked with experimental conditions which enable to “freeze” and isolate actin pools in their G- and F- forms. Good experimental conditions which enable to block actin assembly at half polymerization *in vitro* are 20 µM Lat A + 20 µM phalloidin for 15 minutes at room temperature, followed by centrifugation at 240,000 x g for 1h. Therefore, we lysed yeast cells in these conditions and blotted actin from the supernatants and pellets. If not representing “exact” G/F actin ratios in the cell for the reasons explained above, we are nevertheless confident that our values can be meaningfully compared from strain-to-strain in this study. Our measurements do not reveal any significant difference in G/F ratios, except for the 3 haploid strains expressing the most divergent actins where monomeric actin is in excess (EV Figures). This result is discussed in the manuscript (line 419-424).

3. Having verified the experimental system, I would then request to see a similar quantification of actin (the #'s of different structures, the G/F actin ratio, etc.) in cells expressing different orthologs in order to better understand how the cytoskeleton is affected. The deviation and polarity indexes are just not enough to be able to draw general conclusions from.

This comment is related to comment 2. We have measured these parameters for these yeast strains too.

4. The in vitro assays are interesting, but with everything done at only one concentration, it a relatively blunt measure of an ortholog's interaction with an actin binding protein.

We thank Referee #1 for his/her enthusiasm about these interactions. We agree that weak loss-of-interactions, detailed biochemical analyses across concentrations are necessary to characterize thoroughly these effects. In our case, the effect is undeniable and for this manuscript we feel this analysis exceeds the requirements to demonstrate a defective interaction.

5. The same could be said for the single concentration used to test for drug sensitivity.

We tested additional drug concentrations (see Appendix). For haploid cells, we used 75, 150 and 300 µM CK-666. For diploid cells, 37.5 and 75 µM CK-666.

6. Figure 6- what is the expression ratio of the different actin orthologs, and what is the concentration of total actin with respect to wild-type cells?

We have quantified that. By western-blot, we show that total actin expression level in N2/Ca or Ca/N2 cells is similar to level in Sc/Sc cells. RNAseq provides information about the relative expression at the RNA level, which is of 24,192 counts for Sc/Sc cells, vs. 7,071 counts for Act_N2 and 11,168 counts for Act_Ca in N2/Ca cells (Fig EV3B).

7. Finally, in order to lay claim to the title of the manuscript that the differences are truly do differences in interactions with other actin regulators, one would want to do a study that quantifies all of the interactions that the different orthologs have in the cell, perhaps using

proximity-ligation and proteomics. Again here, the actual concentration of the actin ortholog in the cell is likely to be important unless proven otherwise.

We appreciate Referee #1's concern that point mutations in yeast can potentially have consequences for binding interactions with multiple regulators. In theory, all yeast genetics and cell biology studies where mutants are generated should adopt such approach and verify proteome-wide which interactions are modified or not.

In practice, this is not doable and we have to base our work on a certain number of very reasonable hypotheses. In the particular case of the actin cytoskeleton, decades of research with budding yeast have led to the identification of most actin-binding proteins (it is possible that few have missed our filter, but it wouldn't be the purpose of this study to identify them). We also know quite well about their roles in the generation of actin patches and cables, which give valuable information to use. We wanted to take advantage of this knowledge, in order to narrow down possibilities/explanations for defective patch or cable assembly rather than reviewing again all possible interactions with actin in the cell.

For this specific reason, we purposely adopted a reductionist approach and used a biomimetic assay that we had previously characterized (Antkowiak et al., *PLoS Biology*, 2019). In this assay, we use a large set of purified proteins which are all coming from budding yeast, and which includes most of the factors whose absence leads to a disorganization of the actin cytoskeleton in cells. Overall, we do not see why our final model would be unreasonable. We show beyond any doubt that one of the actins, Act_Ca, does not assemble branched-actin networks in vitro efficiently, which can only arise from a defective interaction with one of the proteins present in our assay. We also show beyond any doubt that the other actin, Act_N2, does not bind well to tropomyosin, which is a clear essential protein for cable assembly (Appendix Fig. S4C and Pruyne et al., *J Cell Biol*, 1998)

Minor Concerns:

1. Figure 2F- please zoom in on the cells more. It would also help to highlight the features that are changing the deviation index. The differences in these examples appear to be more subtle than the quantification in 2G-I would suggest. Actually, this goes for all of the figures. It is particularly difficult to see what is going on in Fig. 3J.

As recommended by Referee #1, we have zoomed on our images to highlight the features that are changing the deviation index. We agree that especially for the images in the previous Fig 3J, it was difficult to appreciate the differences.

2. Figure 4B, C. The images do not appear to match the quantification. For example, the Cof/Actin ratio for branched actin is very low in the graph for Sc actin, however, the images show the opposite- the merged image is completely green. This is not isolated to those panels, either.

The problem that we faced is that fluorescence intensities on branched and linear actin networks are quite different in this assay. We adapted intensity levels in the Figures because we wanted the networks to appear nicely, but we agree with Referee #1 that Figure 4 ended up being a little bit confusing. The quantification of the data was correct of course. In the revised manuscript, we have:

1/ added precisions in the Legend of Fig 4 that branched and linear actin networks are shown for different intensity levels for the reason explained above.

2/ presented fluorescence signals for ADF/cofilin and tropomyosin at levels equivalent to those used to image actin in the same networks. Hence, images of Fig. 4B and C appear

coherent with the quantification, and as a consequence, ADF/cofilin and tropomyosin labeling of branched networks are not very visible anymore.

3. There is no discussion about the implication that this study has on "Frankenstein" experiments- either when actin binding proteins and actin from different species are used in biochemical assays or cell biology studies were actin orthologs from other species are introduced (for example, using rhodamine-labeled rabbit skeletal muscle actin in lower eukaryotes). Not essential, but I think this paper would speak well to that.

Thank you for bringing up that point! We took the opportunity of this comment to discuss that point in our paper (line 410-412).

We are indeed very careful with the choice of proteins in all our experiments, because most actin-binding proteins are optimized to work with actins from the same species. Our recent paper Antkowiak et al., for example, was made using only proteins from the same species (*S. cerevisiae*). We know many examples of actin-binding proteins which have a high affinity for actin from the same species (e.g. *S. cerevisiae* ABP – *S. cerevisiae* actin), but weak or no affinity for other actins (e.g. *S. cerevisiae* ABP – muscle actin).

Referee #2 (Remarks to the Author):

In this manuscript by Dr. Michelot and colleagues, the authors make a clever use of the budding yeast system, to test an important hypothesis in the cytoskeleton field: the idea that small molecular differences between actin isoforms (or variants) can cause their segregation into different filament networks within the same cell. The principle that actin-binding proteins can distinguish actin orthologs is already accepted (for example, it is known that yeast cofilin binds and severs yeast actin filaments far more efficiently than rabbit alpha-skeletal actin filaments) but the authors go much further here, by showing that different actin variants preferably assemble into branched or linear filament networks, in cells and in vitro. I believe this work will be of great interest to the field, and to a broad readership.

I nonetheless have a few concerns, which should be addressed to make the paper acceptable for publication.

We thank Referee 2 for this appreciation.

Main issues:

1. The incorporation of fluorescently labeled actin, in reconstituted assays (Fig 4), is unknown.

If I understand correctly the Methods section, the in vitro experiments are done with 1% fluo-labeled rabbit actin (none of the other actins are labeled). This should be said clearly in the main text. Simply writing "8 µM F-actin (1% Alexa-568-labeled)" in the caption of figure 4 is misleading.

We corrected this legend to avoid any confusion.

The overall architecture of the filament networks is likely unaffected by this 1% labeled rabbit actin. However, the fact that all the results of Figure 4 rely on the fluorescence intensity as a readout of the total amount of actin (either to compare the amounts of actin in branched and linear networks, or to normalize the fluorescent signal of cofilin and Tpm) is potentially a problem. One can easily imagine that fluo-labeled rabbit actin may not copolymerize equally well with the different actin variants, and that the labeling fraction of the filaments and

network will not be 1%, and will not be the same in the different experiments. Also, formin-elongated filaments are known to incorporate less labeled actin. This could lead to incorrect conclusions.

For the robustness of results obtained from the reconstituted assays, this point must be addressed. Ideally, with a dedicated experiment to measure the effective labeling fraction of filaments made with the different actin variants (for example, by running gels after spinning down the filaments, or by measuring the fluorescent intensity per unit length of single filaments). At the very least, it should be thoroughly discussed.

We thought about that point. We did not originally investigate that because our objective was to compare branched vs. linear actin network assembly from the same actin variants. If one actin was not copolymerizing well with rabbit muscle actin, this should have affected equally both structures.

Nevertheless, we agree that controlling this point is easy and avoids questioning. Our new results (Appendix Fig S4) show clearly that all these actins copolymerize similarly with 1% labeled-rabbit muscle actin. The dotted appearance of the actin filaments in this experiment comes from this low percentage of labeling.

2. The way the fluorescence intensities are quantified in Figure 4 is unclear.

The actin networks shown in Figure 4, both branched an linear, have varying lengths and appear to be very inhomogeneous in intensity (of actin, cofilin, and Tpm). How and where are these intensities measured? For example, in Fig 4C, the Tpm-to-actin ratio strongly varies along the branched comet tail, and the result will be highly dependent on where the measurement is made. If the signal is averaged over the whole comet tail, then the overall length of the comet will have an impact (so will its age, and the concentration of the other proteins). Another example, the branched networks made of N2 actin all appear shorter than those made with Sc actin, indicating a slower rate of assembly. Shouldn't that be taken into account, somehow? These points need to be clarified.

Binding of ADF/cofilin and tropomyosin to actin networks is indeed dependent of the architecture (including density) of actin networks. Binding of ADF/cofilin is dependent as well as of the ADP content of the filaments. These effects are well documented.

We indicated in the Methods section (line 821-823) that fluorescent intensities were measured all along actin networks, independently of their density, after 30 min. The first reason is that we do not know how the density of our networks compares with the density of actin networks present in cells, therefore such measurement represents an average of how an ABP binds to a type of network independently of its density. The second reason is that nucleotide hydrolysis and phosphate release occur at the minute-scale, therefore all these networks should be mostly ADP.

As requested by Referee #2, we now provide a more in depth description of our observations:
1/ Act_N2 indeed assembles noticeably shorter but brighter Arp2/3 comet tails, for an overall similar amount of assembled actin (line 287-289). We do not exactly know why, but it is possible that both Arp2/3 and CP binding are mildly affected, to levels at which the structure of the comet would be affected without changing the total amount of actin assembled.
2/ ADF/cofilin binds half as well to both Act_N2 and Act_Ca (line 297-298). One could think that weaker binding to branched-networks is due to the assembly of denser networks with the mutant actins. However, the fact that half weaker binding is observed on both branched and linear actin networks more likely indicates that the effect is due to differences in actin

itself. Moreover, both actins share a common mutation T350S compared to S. cerevisiae's actin which might impair ADF/cofilin binding (Fig 5A).
3/ Branched networks are almost not assembled anymore when Act_Ca actin is used. Not only comet tails are absent, but the amount of assembled actin is dramatically reduced.
4/ Tropomyosin binding is almost absent from networks assembled from Act_N2 actin. Again here, the fact that the effect is observed on both branched and linear actin networks, all along their length, makes us believe that the effect is actin-dependent and not geometry-dependent.

The authors should explain better how they analyze these data. What does "average normalized intensity" mean (methods, line 704)? i.e., normalized by what? In Figure 4A, the "relative actin intensity" is plotted. Relative to what?

We have modified these terms for more clarity.

According to the caption of Fig 4, the images have different contrast settings, and this can be very misleading. For example, in the images of Fig 4A, N2 actin appears to incorporate more in the branched network than the linear network, compared to Sc actin, but the quantification tells us it is actually the opposite (deviation index for N2 is on the linear side). Perhaps the authors could provide images with the same settings.

Please read our response to Referee #1 Minor point 2. Providing images with the same settings for branched and linear networks is difficult, but we modified the Figures so that ADF/cofilin and Tropomyosin signals could be compared between branched and linear networks. We made sure that Legends explain clearly how images should be interpreted (line 1242-1246).

3. To assess the impact of CK666 on different strains (Fig. 3K) we would need to see the same data (the distribution of the number of patches per cell) in the absence of CK666. It is currently missing.

We have followed this recommendation.

4. It is not clear to me at all why cells expressing Act_N2 (Fig 3JK and Fig 6) manage to generate branched filament networks, better than wild type, in the presence of CK666. Since CK666 binds to the Arp2/3 complex (see, for example, Hetrick et al. Chem Biol 2013), how can a difference in actin offer protection? The idea put forward in the paper (in the title, the abstract, and throughout) is that defects in the interaction with some ABPs could increase the monomer pool's availability for incorporation into branched networks. I don't see how this could counter the effect of an Arp2/3-targeting drug. The authors should discuss this point, and propose explanations.

Please read our response to Referee #3 who has a similar comment (first major issue).

Other, more minor points:

5. In several figures, it would help to indicate which variant each data point corresponds to. In Fig 3E, in particular: there are 16 data points, with 14 values for identity, what do they correspond to? (only 10 strains are listen in previous panels).

We have modified the figures so that each actin variant is assigned a dot of different shape.

There are 16 points in the graph of the new Fig. 3 D and H because it also includes some strains that we tried to generate but which were not viable (and therefore growth constant =

0). Some data points have the same X-axis value because they have the same degree of identity compared to *S. cerevisiae*'s actin, although corresponding to different actin variants.

6. Fig 7 is a bit hard to read. Differences in network densities are not clear enough, and could be amplified to help the reader get the message rapidly. Maybe it would be easier to represent networks by 'zooming out' a bit?

Done.

7. Line 187, I am not sure there is a threshold percentage, maybe a few key nucleotides are important, so perhaps the authors should be more cautious in their interpretation.

We are not sure to understand this comment and try to reply as precisely as we can. The idea of threshold is simply expressed to define a level of actin protein expression above which we do not detect (at the precision of our experiments) growth or cytoskeletal organization defects. We agree that this is not an exact threshold, as more precise measurements than ours would surely detect minimal differences between these mutants and wild type cells. This threshold actually changes slightly in the revised manuscript as we detected a small growth defect with Act_Sc[Sp] strain by using a different method to characterize growth phenotypes.

At the nucleotide level, we agree that few nucleotides could be crucial for protein expression. We were careful to not put any strong claim about the correlation between nucleotide conservation and protein expression level (New Fig. EV1A). In fact, we would like to highlight the case of Act_N1 mutant, which is *S. cerevisiae* coded at the nucleotide level (99.3% nucleotide identity) and which is 98.4% identical at the protein level. Despite this high similarity at both nucleotide and amino acid levels, Act_N1 is surprisingly only expressed at 39% compared to WT actin, but mutant cells behave like wild-type cells (Fig 3A-C). This strain demonstrates very well that very few changes at the nucleotide level can have important consequences for protein synthesis.

8. Regarding the number of repeats. For experiments in cells, are the 30 cells per strain taken from one or more experiments? Please clarify.

When we generate new yeast mutants, we usually analyze a minimum of 3 different clones in different experiments (to verify that they behave similarly). Data presented here correspond to the analysis of 30 cells picked from one of these clones (which we keep in our collection after publication). We clarified this point in the text (see paragraph on Data Reproducibility).

9. There are several typos and minor mistakes. For example,
Line 184, "...the level of expression levels..."
Line 246, "branched- and linear-actin network assembly, respectively from formin and WASp-coated beads" should be the other way around.
Line 279, "effect" instead of "affect"
Line 741, "pulled" instead of "pooled"
Line 1041, "quantifications on the left" instead of right.
Table 2, Candida albicans column, a couple of errors in the amino acid numbers.

Thank you very much for such careful reading. All corrected.

Referee #3 (Remarks to the Author):

In this article, the authors examine the physiological consequence of expressing multiple actin isoforms. Starting from yeast that expresses only one actin isoform, the authors examine how expression of heterologous actin affects two types of actinous structures: actin patches and actin cables. These are generated by different actin nucleators and possess a different complement of actin binding proteins. They find that some actin isoforms favour assembly into patches, while others favour assembly into cables. In addition to more or less favourable interactions with nucleators, post-assembly, actin binding proteins show selective binding to filaments assembled by some isoforms. By co-expressing one isoform that favours patches and another favouring cables, the authors engineer yeast cells with patches and cables assembled by independent networks and that can be controlled independently. Overall, the topic of the study is very interesting and novel. It poses some intriguing questions. In general, the experiments are well executed and well controlled. This is a very interesting article that merits publication but prior to this a few issues need to be addressed.

We thank Referee 3 for this appreciation.

Major issues:

-I find the resistance to CK-666 treatment merits further characterisation to really support the authors' message. The authors show that strains over assembling patches are more resistant to Arp2/3 perturbations such as Act_N2. Can the authors provide further mechanism about why resistance to treatment arises? There are several potential explanations: 1) because the isoform doesn't assemble cables, when you perturb Arp2/3 all G-actin does not get taken up by patches, 2) the amount of Arp2/3 in Act_N2 cells may be larger than in control cells - as a consequence, more patches would subsist. To test these hypotheses, the authors could quantify the amount of Arp2/3 subunits in control and Act_N2 strains. It may also be useful to show that the ratio of arp2/3 staining to F-actin staining stays to same in the patches even with different isoforms. This would provide the most basic illustration that the patches have roughly the same composition and are therefore comparable from strain to strain. For example, it is possible that in Act_N2 strains there may be a higher density of branching.

We agree with Referees #2 and #3 that our explanation of the sensitivity of these strains to CK-666 was insufficient. In the revised manuscript, we followed their recommendation and analyzed thoroughly actin patches assembled by wild-type (Sc) and N2 strains. This analysis was complicated, as mentioned above, by the fact the Act_N2 and Act_Sc have different phalloidin-binding sites, which does not allow to quantify the amount of actin assembled per patch directly and as precisely as we wished.

About the amount of actin assembled per patch:
Our results indicate that all patches assembled by N2 cells are in total 6 times brighter than all patches assembled by Sc cells, while all cables assembled by N2 cells are in total twice brighter than all cables assembled by Sc cells (Appendix Fig. S3F). As for the number of structures, we observe that N2 cells assemble 3 times more patches as Sc cells, but a similar number of cables (Appendix Fig. S3G). Moreover, the total amount of actin expressed in N2 cells is similar (Fig 3A,B) and F/G actin ratios are not significantly different compared to Sc cells (Fig EV2B). At the single patch scale, we measure that actin patches are 1.6 fold brighter in N2 cells as in Sc cells (Appendix Fig S3M).

Taken together, these observations suggest that phalloidin binds with a 1.5-2 fold stronger affinity to Act_N2. N2 cells assemble on average 3 times more patches, but individual patches probably assemble similar or slightly higher amounts of actin than Sc cells.

About the amount of Arp2/3 assembled per patch:

Arp2/3 expression in N2 cells and Sc cells does not seem very different by RNA seq nor by western-blot (Appendix Fig. S3K). Arc15-GFP signal per patch is on average 1.6-fold brighter for N2 cells compared to Sc cells (Appendix Fig. S3L,M).

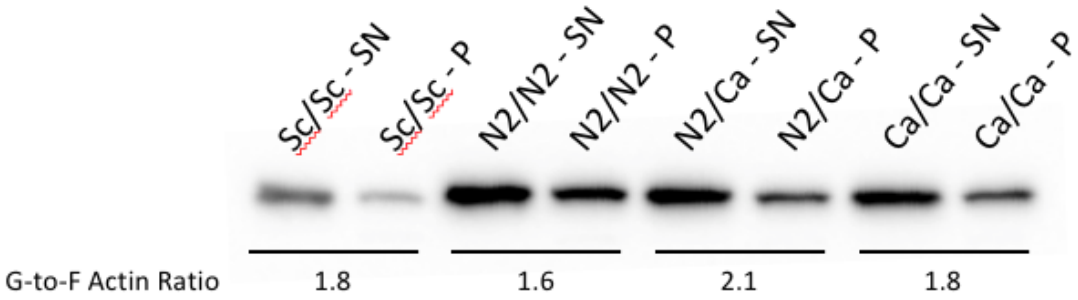
In the end, these measurements allow us to provide a rough estimate. Individual actin patches in N2 cells have an Arp2/3:actin ratio which is most probably higher in N2 cells if the amount of branched actin is overestimated by phalloidin staining. This slight densification of branched networks assembled by Act_N2 is consistent with our biomimetic assays, where we also observe the formation of denser comets with Act_N2.

As discussed above, it is difficult to explain at this stage why Act_N2 branched networks are denser. It could come from a higher affinity for the Arp2/3 complex of from a higher nucleation rate as the flux of Act_N2 monomers available for branched-actin network assembly is more important in N2 cells. However, this result explains why an inhibition of the Arp2/3 complex by CK-666 has a lesser effect on N2 cells.

-The authors quantify the total actin in each strain but it would also be useful to systematically quantify the amount of F-actin. This would allow the reader to make sure that differences in total actin translate (or not) into differences in F-actin content. In particular providing a quantitation of the F-actin/G-actin ratio may allow the authors to make their main point more strongly: if you perturb patch formation in strains with one isoform, the F/G ratio doesn't change because the G-actin freed by Arp2/3 inhibition gets assembled into cables. Conversely, in a system with 2 actin isoforms, the F/G ratio would change because the isoform favouring patches cannot be used in cables.

As requested by Referee #1 and #3, we have measured F/G actin ratios in all strains generated for this study (EV Figures). In fact, our measurements in haploid strains do not detect truly significant differences in F/G ratios, except for the 3 haploid strains expressing the most divergent actins (which are the ones showing the strongest phenotypes) (Fig EV2B). For these strains, it suggests that divergent actins have difficulties in assembling both into cables and patches, therefore leading to this significant increase of G-actin relative to F-actin.

In the case of the diploid strains expressing Act_N2 and Act_Ca, we did not detect a clear difference in F/G ratios when we performed these measurements in the presence of CK-666 (75 µM for 15 min before sample analysis). We believe that this result is due to the fact the assembly of Act_N2 into cables and Act_Ca into patches are not completely impossible (otherwise N2 and Ca strains would probably not be viable), but simply less favorable. Therefore we expect that differences in F/G actin ratios in the presence of CK-666 would be too minor to be detected by typically imprecise measurements of F/G actin ratios. We present below one of our western-blots below for information.



-p11: Can the authors provide further detail as to why Act_Ca does not assemble into patches? Does the more favourable binding of tropomyosin impede branch formation? What happens in the in vitro assays if tropomyosin is omitted?

Experiments of Fig 4A are performed in the absence of tropomyosin, so this is a clear defect of actin nucleation.

-p12: the authors state that Act_N2 does not incorporate into cables because an ABP that stabilises cables cannot bind to it. In vitro, they show that tropomyosin cannot bind Act_N2. However, for completeness, they should also confirm that knocking down tropomyosin in control strains impairs cable formation.

This result was published previously and we cited the paper (Pruyne et al., *J Cell Biol*, 1998). As requested by Referee #3, we took new images to confirm this finding and show them in Fig. S4C.

-p13: the authors state that Act_N2 has got substitutions at all interfaces with nucleators and ABPs used in the assays. Yet, the amount of F-actin that Act_N2 assembles appears to be similar to the control strains. Why is that the case?

We have decided to include this structural analysis in the manuscript because we think that careful structural analyses could help predict in the future which molecular interactions are disrupted for each actin isoform expressed in eukaryotes. Such success would open the possibility to anticipate the probabilities of assembly of each actin isoform in different actin networks. For this particular study, however, we wanted to use careful words, so as not to overinterpret the results of this preliminary analysis (particularly in the case of Act_N2).

In the case of Act_N2, a first possibility would be that despite bearing several substitutions at interfaces with all nucleators and ABPs used in this study, most of them are silent and do not change the strength of these interactions. However, as discussed above (Referee #2 point 2.), the actin networks that Act_N2 assemble are not quite similar to control ones suggesting that more likely there are differences. Particularly, Arp2/3 comet tails are noticeably shorter and brighter than control ones, for an overall similar amount of assembled actin. It is therefore possible that both Arp2/3 and CP binding are affected, to levels at which the structure of the comet would be affected without changing the amount of actin. Other interesting observation is that both Act_Ca and Act_N2 actins bind S. cerevisiae's ADF/cofilin less well (Fig 4B), which is coherent with some substitutions present at their binding interface.

-p14: the authors state that in cells expressing two actin isoforms it is difficult to localise each. Is it not possible to express very small tags like tetracysteine tags?

Labeling actin directly in cells leads generally to many artefacts. Even small tetracysteine tags have been shown to impair actin assembly in yeast (Melak et al., *J. Cell Science*, 2017).

Alternatively, in the in vitro assays, it should be possible to label each of the purified actin isoforms with a different fluorophore. The in vitro assays could help to determine if isoforms co-assemble into filaments or if they segregate - which would make the authors main message stronger. One may expect co-assembly in branched structures because elongation takes place spontaneously at the barbed end but not in cables because formins (or profilin) may be isoform selective.

We thought of that experiment and agree that performing segregation of actin isoforms *in vitro* would eventually be a wonderful achievement. Unfortunately, we believe that at this time, several factors make our biomimetic assay too simple to lead to such segregation in vitro.

1/ First, our data show clearly that the formin Bni1 (and profilin) are not isoform selective, because both Act_N2 and Act_Ca assemble well on formin beads. Rather, our data show that Act_N2 does not bind to the tropomyosin Tpm1, so cells are very likely to assemble Act_N2 cables bare of tropomyosin which must be disassembled instantaneously. Therefore, any reconstitution should also include an efficient actin disassembly machinery (including perhaps factors such as Aip1) which we do not have at the moment.

2/ Second, as Referee #3 mentions, we cannot exclude in our assay partial assembly of Act_Ca into branched networks when Act_N2 is present for nucleation of new filaments (our model takes into consideration this possibility in Figure 7). Our prediction is that in a cellular context, where the actin monomer pool is limiting, extra disassembly of Act_N2 will increase the monomeric Act_N2 flux towards actin patches, therefore favoring Act_N2 assembly over Act_Ca in actin patches. On the contrary, as Act_Ca can stably assemble into cables, it will comparatively be depleted compared to Act_Ca for its assembly into patches. Thus, an efficient reconstitution of this process *in vitro* would also require conditions closer to that of the cell, where the reservoir of monomeric actin is limiting and where an efficient redistribution of G-actin between patch and cables is possible.

Minor issues:

-Fig 2E and 3E would benefit from a legend. I realise that the colour code is the same as in 2D and 3D but the colours are not very different in 3E so it is difficult to distinguish which isoform is which.

Thank you. We corrected that.

-Fig 4B, C: The branched parts of the graphs are really difficult to read because the ratios are close to 0. I think it would be better to replot those on separate graphs with a more appropriate scale.

We improved these plots.

-Fig 7: It may be useful to also have a diagrammatic representation of the reservoir of G-actin.

We attempted to include a reservoir to the figure, but all feedback was unsupportive in that it created noise and confused the representation. We feel that the addition of the new G/F actin ratio data in the manuscript now well illustrates this concept throughout the manuscript and this addition is unnecessary.

Thank you for submitting your revised manuscript to The EMBO Journal. The three original reviewers have now assessed it once more, and I am pleased to say that they all found the previously-raised points satisfactorily addressed. We shall therefore be happy to accept the study for publication in our journal, following a final minor revision of the remaining presentational issues noted by reviewers 2 an 3 (see below), and addressing of the following editorial points.

REFEREE REPORTS

Referee #1:

The authors have sufficiently addressed my concerns from the first round of review and I now enthusiastically recommend this manuscript for publication in The EMBO Journal. The new experiments greatly enhance the interpretability of the results. I predict that this manuscript will be an inspiration for future studies investigating the effect that expression of different actin orthologs has on the cytoskeleton- an extremely important question about how actin functions in cells/organisms that is still largely unresolved.

Referee #2:

The manuscript has been improved, and my concerns have been properly addressed, except for the following points. They can easily be taken care of, allowing me to recommend publication in EMBO Journal.

- Regarding my point 2, about the contrast settings in Fig 4, the authors replied "We made sure that Legends explain clearly how images should be interpreted (line 1242-1246)." I could not find such explanations at, or around, the indicated lines.

- Perhaps I missed it, but I could not find any modification/clarification of the term 'average normalized intensity' (in methods, now lines 830-833).

Other comments:

- Lines 485-486: Writing 'over-assembling' rather than 'over assembling' would be clearer.
- Reviewing the revised manuscript would be much easier if changes were highlighted.

Referee #3:

The authors have answered all of my queries. Congratulations on this nice work.

I have only one minor point that has not been addressed:
-Figs 2D, 2G, 3D, 3H need a figure legend. I realise the markers are the same as in other panels of Fig 2 and Fig 3 respectively. However, it will make the readers' life much easier to have that legend.

Referee #1:

The authors have sufficiently addressed my concerns from the first round of review and I now enthusiastically recommend this manuscript for publication in The EMBO Journal. The new experiments greatly enhance the interpretability of the results. I predict that this manuscript will be an inspiration for future studies investigating the effect that expression of different actin orthologs has on the cytoskeleton- an extremely important question about how actin functions in cells/organisms that is still largely unresolved.

We thank Referee #1 for the valuable input to improve the quality of this study and for his/her enthusiasm.

Referee #2:

The manuscript has been improved, and my concerns have been properly addressed, except for the following points. They can easily be taken care of, allowing me to recommend publication in EMBO Journal.

We thank Referee #2 for his/her appreciation and careful reading of the manuscript.

- Regarding my point 2, about the contrast settings in Fig 4, the authors replied "We made sure that Legends explain clearly how images should be interpreted (line 1242-1246)." I could not find such explanations at, or around, the indicated lines.

We apologize as line numbers were shifted at the time of the final edition of the manuscript, which made it difficult in places to identify the modified sections.
This information was written down in the paragraph named “Data information” of Figure 4. It reads as: “For all microscopy images, intensity levels were adapted for images of branched- and linear-actin networks separately as their brightness is different. To obtain a correct representation of the amount of ADF/cofilin and tropomyosin bound to the two networks, the intensity levels of ADF/cofilin and tropomyosin were matched similarly to those of the corresponding actin networks.”

- Perhaps I missed it, but I could not find any modification/clarification of the term 'average normalized intensity' (in methods, now lines 830-833).

Thank you for noticing that. We had modified the corresponding axis titles in the Figures, but we forgot to do so in the text. The word “normalized” was unnecessary in these lines (because we calculate a ratio) and was removed.

- Other comments:

- Lines 485-486: Writing 'over-assembling' rather than 'over assembling' would be clearer. We modified it.

- Reviewing the revised manuscript would be much easier if changes were highlighted.
We apologize for that. We should have uploaded both versions (with and without tracked changes).

Referee #3:

The authors have answered all of my queries. Congratulations on this nice work.

We thank Referee #3 for his/her kind words.

I have only one minor point that has not been addressed:

-Figs 2D, 2G, 3D, 3H need a figure legend. I realise the markers are the same as in other panels of Fig 2 and Fig 3 respectively. However, it will make the readers' life much easier to have that legend.

We have modified the Legends of Figure 2 and 3 to make the readers' life easier. Before the description of the panels, we have added the following notes that should allow readers to follow the shape and color codes.

For Figure 2, it mentions:
"In this figure, the shape of the dots allows to identify the strains on the different graphs (circles for Sc, squares for ScNI, triangles for Sc[Ca], inversed triangles for Sc[Sp] and diamonds for Sc[At]). The color of the dots indicates the percentage of identity of the nucleotide sequences to the actin gene of *S. cerevisiae*, ranging from 100% (blue) to 76% (orange)."

For Figure 3, it mentions:
"In this figure, the shape of the dots allows to identify the strains on the different graphs (closed circles for Sc, closed squares for ScNI, closed triangles for N1, inversed closed triangles for Kl, closed diamonds for N2, closed pentagons for Op, stars for Ca, open circles for Nc, half-open triangles for Yl, half-open inversed triangles for Hs and crosses for non-viable strains). The color of the dots indicates the percentage of identity of the amino acid sequences to *S. cerevisiae*'s actin, ranging from 100% (green) to 84% (magenta)."

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

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Alphée Michelot
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For experiments that could be carried out on large number of samples and objects (cell and bead motility measurements), we determined the number of samples and objects to be analyzed beyond which mean values and standard deviations were not gaining in precision. The statistical analysis for these experiments takes into account the number of sample analyzed per condition, in order to calculate p-values precisely. For experiments that could not be carried out on large number of samples (western-blots, RNAseq), we based sample numbers according to standards in the field, and sample size is similar for all conditions analyzed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All treatments were applied similarly to samples and control conditions. Cells were coming from the cell culture and were split just before drug or DMSO treatment.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For all experiments, data were quantified blindly, i.e. without knowing which sample it was, in order to avoid any bias in the result.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Brown-Forsythe and Welch ANOVA tests were used when the data followed a Gaussian distributions (under a Shapiro Wilk or Kolmogorov Smirnov test), and non-parametric tests were used to compare non-Gaussian distributions.
Is there an estimate of variation within each group of data?	Yes, standard deviations are indicated

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	The variance of each sample was taken into account for the statistical analysis

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibody references are provided in the manuscript. They include anti-Actin C4 antibody (Fisher Scientific ref. 08691002), anti-alpha tubulin antibody (Abcam ref. ab184970), anti-mouse HRP (Jackson Immunoresearch ref. 115-035-146) and anti-rabbit HRP (Abcam ref. ab205718).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Data availability section is present in the manuscript.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNAseq data, which are the only large datasets coming from this study, are deposited in GEO (accession number GSE189213).
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No