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How tropomyosin regulates lamellipodial actin-based motility: a combined biochemical and reconstituted motility approach

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Transaction Report:

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

1st Editorial Decision 30 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three reviewers, whose comments are attached below. As you will see, these referees in principle appreciate your careful in vitro approaches to investigate the role of tropomyosin in lamellipodial dynamics and cell migration, and - to varying degrees - also find your results potentially important for further understanding of the in vivo situation and its molecular basis. Nevertheless, they also raise a number of important points, regarding specific experimental issues as well as more general concerns with interpretations and the conclusiveness of the presented data for understanding the apparent complexity of the system. In this respect, the comments (in particular of referee 1) indicate that the manuscript would be significantly strengthened by at least some experiments testing the derived assumptions in vivo, especially given the possibly different roles of endogenous tropomyosin(s) compared to the in vitro employed skeletal muscle isoform.

Should you be able to adequately address these major points, we should be happy to consider a revised manuscript for publication. I would therefore like to invite you to prepare such a revision in the spirit of the reviewers' comments and suggestions. Please be however reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Understanding exactly how the polymerization, turnover and organization of the actin cytoskeleton promotes lamellipodium protrusion and cell migration still remains to be definitively established. Recent observations have demonstrated that excess tropomyosin inhibits lamellipodia formation. Curiously, however, excess tropomyosin also results in persistent leading edge protrusion and increased cell migration. To understand the molecular basis for the increased cell migration, Bugyi et al., have used in vitro reconstituted motility assays to explore the role of tropomyosin in Arp2/3 driven actin polymerization. Bugyi et al provide convincing data demonstrating that tropomyosin promotes increased velocity by decreasing Arp2/3 complex mediated actin branching. They also show tropomyosin inhibits the action of ADF, changes actin tail morphology and reduces the available actin monomer pool. Based on their in vitro observations the authors propose a model to explain how tropomyosin regulates leading edge protrusion. The paper and results are straightforward and I only have minor comments.

The authors should make it clear in their text that actin polymerization at the leading edge is largely driven by the WAVE complex and not N-WASP, as they have used in their experiments. Given this it would be nice to see that tropomyosin actually regulates an N-WASP-Arp2/3 dependent process in cells and not just in vitro.

The authors use N-WASP bound to beads to activate the Arp2/3 complex. However, within a cell N-WASP is unlikely to be permanently bound to a cellular compartment. The authors have recently used N-WASP functionalized GUV's, which provides a more "dynamic" situation as N-WASP is able to diffuse on the membrane (Delatour 2008). This is likely to be closer to the situation in vivo. The authors should provide a few key experiments using N-WASP functionalized GUV's to see if they get similar results.

The authors show the distribution of Arp2/3 (Fig. 2) and tropomyosin (Fig. 6) in their bead induced actin comets in separate images. I think it would be informative to show images and analysis of the levels of Arp2/3 and tropomyosin in the same rather than separate actin comets.

It would be nice to see a wider range of concentrations in some of the experiments.

Addition of ADF rescues the stabilizing effect of tropomyosin (Fig. 5A). Is the same true when extra G-actin is added to the motility assay?

Referee #2 (Remarks to the Author):

Manuscript # EMBOJ-2009-71828 "How tropomyosin regulates lamellipodial actin-based motility: a combined biochemical and reconstituted motility approach"

Bugyi et al. have explored the effects of skeletal muscle tropomyosin (skTm) on different types of actin-based motility. Most significant and new are the results obtained from the biomimetic motility assays, in which Arp2/3-complex-dependent bead motility is examined with a minimal set of 5 essential components, actin, Arp2/3, one of its activators, a capping protein and ADF/cofilin. Profilin is also helpful (Loisel et al, 1999) and used here. As observed previously (Blanchoin et al, 2001), tropomyosin inhibited Arp2/3-dependent actin assembly in pyrene assays. In the biomimetic motility assay, the authors observed tropomyosin to have essentially two effects: first, complete abolishment of tail disassembly, which was already observed at very low skTm concentrations, but could be counteracted by excess ADF. In addition, skTm appeared to slow down or accelerate bead velocities, but this depended on the packing density of the Arp2/3-complex activator on the bead, in

this case N-WASP. In my understanding, the precise mechanism of how the latter skTm effects are brought about are not entirely clear, but the authors presume that it must somehow occur through competition with the Arp2/3-dependent branching reaction. The authors also concluded that skTm does not affect debranching nor Arp2/3-complex dissociation from the network. Finally, fluorescently-labelled skTm localized to the entire actin tail, but with higher intensity in more distal tail regions, which the authors propose may be due to higher affinity for debranched actin networks (Figure 7), although this could not be confirmed experimentally (Supplemental Figure 5).
This is an interesting and important study. However, I would like to request some minor additional experiments and controls, and suggest some changes in how the data are presented and interpreted (see also specific comments). I do not share the view that these data fully explain the complicated effects that had previously been observed upon tropomyosin injection into live cells (Gupton et al, 2005). As exemplified by the model and summary in Figure 7, I feel that the authors go a bit far in their attempt to extrapolate their in vitro observations to what has been observed in vivo. The model in Figure 7 contains significant amount of speculation, and should therefore probably be removed, or exchanged for a model or summary of how the authors think tropomyosin exerts its effects in their specific model system. Furthermore, it should be emphasized that Gupton et al. (2005) have proposed to have entirely removed lamellipodia - and thus Arp2/3-complex from the cell periphery - by tropomyosin injection, although the changes in bead motility observed here still all occur in the context of Arp2/3-complex-dependent movement. Interestingly, the authors' data unveil much more complexity in tropomyosin action, and suggest for instance that skTm can only partially block Arp2/3-complex-dependent motility. This should be emphasized more. In the discussion (page 16, top), the authors state that the "main in vivo effects of tropomyosin, including the inhibition of the lamellipodial array, and its replacement by a lamella-like network are recapitulated in the reconstituted assay". I do not agree with this, as (i) Arp2/3-dependent motility is not fully abolished (see above) and (ii) I do not see much indication for the tropomyosin-treated tails to have similarity with filaments corresponding to the lamella. Although this is an interesting concept, more data would be required for such a statement to be justified. In other words, I find it difficult that the authors propose the addition of one single component (skTm) to effect the generation of an additional, distinct self-organized network (top, page 17), like the lamella. Instead, I would fully agree with the authors' view that "the effects of tropomyosin on the lamellipodial array in itself appears sufficient to account for the observed protrusive behavior of cells in the presence of high levels of tropomyosin" (page 16, bottom). So I would favor a "modified lamellipodium" over its complete disappearance; neither the abolishment of the lamellipodium nor a distinct underlying lamella are needed.

Specific comments:

1.) Figure 1: Tropomyosin inhibits Arp2/3-dependent actin assembly. I would be nice though to include as control that tropomyosin does not affect Arp2/3-complex-independent actin assembly, which should be very easy to do.

2.) Figure 2: The authors show for low N-WASP density on the beads (d = 6nm) that skTm lowers Arp2/3 concentration in the tail relative to actin (Figure 2E, G). What happens with high N-WASP packing density? The authors propose skTm accelerates motility because it counteracts inefficient branching, so this should then also coincide with reduced Arp2/3-complex incorporation, although the velocity is increased upon skTm addition. Can this be shown?

3.) Figure 2: All bead motility assays were performed with gelsolin, which actually does appear to do the job, but I think most people would use heterodimeric capping protein instead. Can the authors comment on this? Since gelsolin has biochemical activities slightly distinct from CP (severs and then caps), it would be nice to see in one simple control experiment that skTm has the same effects when gelsolin is replaced by CP.

4.) I guess Figure 3B also shows that the presence of ADF significantly lowers the amount of incorporated Arp2/3-complex. The average reader will not be aware of this effect, so can the authors comment on this?

5.) Figure 5: The data nicely show that even low amounts of skTm fully block tail disassembly. Supplemental Figure 2 also suggests competitive binding between ADF and skTm, so does this mean that skTm removes bound ADF from tails? Can this be verified experimentally?

6.) Figure 6: On page 12, the authors propose that tropomyosin "remained stationary during bead propulsion, demonstrating that branched filament assembly and tropomyosin binding are linked by a reaction that follows network assembly and kinetically limits the binding of tropomyosin". I don't understand this statement. Do the authors want to imply that tropomyosin does not show any turnover once it is associated with the tails? If this is the case, I think the authors should do a simple FRAP experiment to confirm this, and explore the on/off kinetics of tropomyosin in different tail locations, close to and away from the bead.

7.) Supplemental Figure 2B, legend: I think the sentence should read: "To verify that the increase in the fluorescence signal upon skTm addition is ...NOT...due to an increased amount of polymerized actin..."

References:

Blanchoin L, Pollard TD, Hitchcock-DeGregori SE (2001) Inhibition of the Arp2/3 complex-nucleated actin polymerization and branch formation by tropomyosin. Curr Biol 11(16): 1300-1304

Gupton SL, Anderson KL, Kole TP, Fischer RS, Ponti A, Hitchcock-DeGregori SE, Danuser G, Fowler VM, Wirtz D, Hanein D, Waterman-Storer CM (2005) Cell migration without a lamellipodium: translation of actin dynamics into cell movement mediated by tropomyosin. J Cell Biol 168(4): 619-631

Loisel TP, Boujemaa R, Pantaloni D, Carlier MF (1999) Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401(6753): 613-616

Referee #3 (Remarks to the Author):

In this work Bugyi et al. have used a model system for actin polymerization-driven motility to attempt to understand the observed distribution of tropomyosin in the lamellipodium (low, better not to say absent) versus the lamella (high) at the leading edge of migrating cells, and the effect of microinjected skeletal muscle tropomyosin on cellular motility. The work addresses a significant question of general interest to cell biologists.

In previous work Carlier and her colleagues developed a reconsituted a motility system that includes just five proteins that are enriched in the lamellipodium of cells, that they present as a model for the cellular compartment (in the absence of tropomyosin). Dendritic actin networks are nucleated from N-WASP-functionalized beads in the presence of Arp2/3 complex, F-actin,profilin, ADF, and gelsolin. By adding tropomyosin into the system they aim to mimic the lamella, and the transition between the two compartments.

Strong points
1. Addition of tropomyosin increases or decreases the rate of motility, depending on the density of N-WASP on the beads, and stabilizes the actin tails. They convincingly showed the polar distribution of tropomyosin in actin tails, increasing with distance from the coated bead. The plausible explanation is that tropomyosin competes for Arp2/3 complex binding, and doesn't then bind to the dendritic filament until the branches dissociate. Following branch dissociation the tropomyosin can bind to give "linear" filaments.

2. The system is complex, and the authors extensively investigate certain parameters in order to attempt to understand the how tropomyosin inhibits lamellipodium formation while promoting migration. Much of the experimental work is confirmatory of previously published findings.

3. Tropomyosin does affect propulsion in a way that is influenced by the density of N-WASP on the beads, the ADF concentration, and the tropomyosin concentration. The opposing effects of tropomyosin on propulsion depending on the N-WASP density on the beads is complex, but the authors' explanation seems reasonable.

4. Although confirmatory, the experiment showing that tropomyosin does not debranch filaments

the result helps understand the polar distribution of tropomyosin in actin tails,
increasing with distance from the coated bead. (Primary data for this conclusion in Figure 3 would be helpful.)

Weak points
1. The complexity of the system makes it difficult to understand what is actually happening, especially since tropomyosin affects the function of at least four of the five components. While the emphasis is on Arp2/3 complex and to some extent ADF, the assay system includes gelsolin, used as a barbed end capping protein (not a CapZ-family capping protein). Gelsolin has been reported to bind to tropomyosin. Finally, there is profilin, that has been recently reported to affect actin dynamics with tropomyosin (in yeast). There is also the question of the effect of tropomyosin itself on actin polymerization dynamics (both filament ends), on annealing of short actin filaments, and its inhibition of filament severing by ADF, not just pointed-end depolymerization-are there other possible explanations of the results?.

2. The morphology of the tails in the presence of tropomyosin-uneven density, discontinuous rate of motility, presence of "spurs", and the unusual fan-shaped tails need further explanation,possibly ultrastructural analysis, and localization of other components in the mix. There are so many potential variables, it is difficult for this reviewer to suggest how to proceed.

3. The premise for the present work is the report by Gupton et al. (2005) that microinjection of excess skeletal muscle tropomyosin into fibroblasts on increased the rate of migration at the leading edge--hence the choice of skeletal tropomyosin rather than a cytosolic form. Myosin II was recruited to the cell edge by tropomyosin, consistent with its established cooperative function in vitro; the observed migration was myosin II-actin-dependent. While certainly what is actually going on in the cell is not fully known (tropomyosin could also promote formin-dependent actin polymerization), the increase rate of migration would not seem to be "puzzling" or the mechanism "elusive" as the authors present as the premise for the current work.

In summary, there are some valid new findings to help solve the puzzle of actin dynamics in the lamellipodium and lamellum. The results may contribute to our understanding of the transit of an actin filament from the lamellipodium to the lamellum, regions with different actin dynamics, and protein populations. The system is complex, making it difficult to exclude alternative explanations for the results. The discussion may be more relevant if the authors would weigh in with their ideas about how their results give insights into the role of endogenous tropomyosins in understanding and defining the relationship between the lamellipodium and lamella.

1st Revision - authors' response 15 September 2009

We are pleased to hear that our manuscript EMBOJ-2009-71828, entitled ´ How tropomyosin regulates lamellipodial actin-based motility : a combined biochemical and reconstituted motility approach ª was found to be ´ an interesting and important study ª (referee 2), presenting ´ convincing data ª (referee 1), ´ addressing a significant question of general interest to cell biologists ª (referee 3). Although the referees expressed generally positive views for publication, they raised a number of comments that we found worth considering and we set out to improve the manuscript by addition of most of the experiments that were needed, and providing all needed clarification in the text. The details are fully given in the response to reviewers.
Briefly, the revised manuscript contains additional data in Figure 2, Figure 3 and Figure 6, and two supplemental figures. These new data bring answers to overlapping comments from reviewers 1 and 3 concerning the possible effects of tropomyosin on profilin or gelsolin, they illustrate the absence of effect of tropomyosin on debranching and they straigthen our point concerning the competition between Arp2/3 and tropomyosin.
One referee would have liked to see supplementary assays performed with functionalized GUVs in addition to the present bead assays. This is certainly an excellent idea but for technical reasons linked to the difficult handling of liposomes, it would be impossible to carry out the complete quantitative analysis and statistics that has been done here with beads, hence our conclusions would be much less strong and quantitatively documented with GUVs. So, we have been reluctant to start

this new study in the present paper.
Following are the list of changes and point by point answers to each reviewer.

Reviewer 1.
1. We have made clear in the text that the lamellipodial actin meshwork is driven by WAVE, while our assay examines the effect of tropomyosin on N-WASP coated beads, however tropomyosin inhibits the filament branching activity of the constitutively active VCA domain, which is conserved among members of the WASP/WAVE family (page 8 , line 10)
2. As discussed above, testing the effects of tropomyosin on the N-WASP-functionalized GUVs would be closer to the in vivo situation, but technically would not provide the quantitative results obtained with solid beads.
3. The distribution of Arp2/3 and tropomyosin is now shown on the same actin tails, and analyzed, these new data are added in Figure 6B, D, E.
4. Addition of ADF rescues the stabilizing effect of tropomyosin. The reviewer suggests to add extra G-actin mimicking the increased level of G-actin by ADF in the motility medium to see the same effect. This experiment cannot be done, since in the motility assay the concentration of G-actin is maintained stationary by the composition of the medium. If we add more G-actin, added monomers will immediately polymerize until the initial stationary level of free G-actin is restored.

Reviewer 2.
We agree with this referee that our simple system cannot reconstitute the full complexity of the live cell, in which tropomyosin may bind to other actin structures. Our goal was to test the consequences of the effect of tropomyosin on the sole dendritic array and compare with its effects in vivo, this is a primary step. So we have tempered our views by clearly discussing the limitations of our conclusions (page 8 ,line 8). On the other hand, we point to the power of the in vitro assay in exploring a broader range of concentrations of tropomyosin than what is possible to achieve in vivo. In fact in Figure 2 of Gupton et al., 2005, at variance with the refereeís view, the lamellipodium is not totally abolished and some Arp2/3 is still visible, which is also expressed in the text ´ We cannot rule out that lamellipodia, which are very transient or not resolvable by our criteria, still exist ª. This may be why motility is not totally abolished, in contrast with the total arrest that we observe in vitro above 3 μM tropomyosin.
We have amended the discussion to state the similarities between the morphologies of lamellipodial array and actin tails upon addition of tropomyosin, and we present our model as a working hypothesis, attempting to comprehensively account for the known properties of N-WASP and the present data on tropomyosin function. We therefore wished to keep the Figure 7 in, because it clearly illustrates this point.
Specific comments :
1. We have added new data which confirm that tropomyosin does not affect barbed end assembly of either G-actin or profilin-actin, see Supplemental Figure 1.
2. We have added new data showing that at high N-WASP density, tropomyosin lowers the amount of actin and Arp2/3 in the tail. The analysis is shown in Figure 2H.
3. We have added new data showing that as expected, the effects of tropomyosin are quantitatively identical when gelsolin is replaced by Capping Protein, see Supplemental Figure 2. The reason why this is so is fully explained in the text (page 9, line 18).
4. It is right that Figure 3B also shows that the amount of Arp2/3 is lowered by ADF (like the amount of actin), due to the depolymerizing effect of ADF (see also Figure 2G). This is pointed out in the legend.
5. We have no probe allowing to measure displacement of ADF from the actin tails by addition of tropomyosin. However the competition is established by in vitro sedimentation assays. In the motility assays, we clearly see immediate arrest of depolymerization of the tails upon addition of tropomyosin, or the abolishment of the comet stabilising effect of tropomyosin by adding sufficient amount of ADF (see Figure 5A), which strongly suggests that this competitive binding also takes place.
6. What we write here is that the spatial pattern of tropomyosin binding preferentially at the rear of the actin tail is stationary during propulsion. Tropomyosin binds in rapid equilibrium, but its binding to filaments of the actin array generated by N-WASP-Arp2/3 complex is kinetically limited by a ´ clock ª which appears to have the time constant of filament debranching.
7. Supplemental Figure 2B, legend : We confirm (as stated) that ADF quenches pyrenyl-actin fluorescence, hence addition of tropomyosin first relieves the quenching by displacing ADF, then causes an increase in polymerization.

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Reviewer 3.
Primary data showing the absence of effect of tropomyosin on the debranching is added in Figure 3A.
Answers to weak points
1. We have added new data showing that replacing gelsolin by Capping Protein 1 2 does not change the effects of tropomyosin (see answer 3 to reviewer 2). This rules out the possibility that the effects are mediated by binding of tropomyosin to gelsolin. New data (Supplemental Figure 1) show that tropomyosin has no effect on barbed end growth from profilin-actin.
As discused in the text, we do not believe that ADF (nor gelsolin) exerts a stationary constant severing activity in the actin tails (page 9, line 24).
2. The fan-shaped tails are the result of the inhibition of ADF-promoted pointed end depolymerization by tropomyosin, which leaves in place the dense ´ cloud ª of actin that precedes the break of symmetry and propulsion of the bead.
3. We have added a discussion of the possible additional effects of tropomyosin on other actin structures in cells (page 18, line 8)

We hope the above amendments will have improved our manuscript and we look forward to your editorial decision,

2nd Additional Correspondence 06 October 2009

Thank you for submitting your revised manuscript for our consideration. We have now heard back from referees 1 and 2, who have reevaluated the study (see comments below), and I am pleased to inform you that both of them now consider the study suitable for publication. We shall therefore be happy to proceed with its acceptance and production.

You will receive a formal acceptance letter shortly.

Yours sincerely

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REFEREE REPORTS

Referee 1:

Although the authors did not address all my or the other reviewers questions I still feel the revised paper is worthy of publication in EMBO J.

Referee 2:

I am satisfied with the changes, experimental additions and clarifications.