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Microtubule motor transport in the delivery of melanosomes to the actin-rich apical domain of the retinal pigment epithelium

Mei Jiang, Antonio E. Paniagua, Stefanie Volland, Hongxing Wang, Adarsh Balaji, David G. Li, Vanda S. Lopes, Barry L. Burgess and David S. Williams

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First decision letter

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MS TITLE: Requirement of microtubule motor transport in the delivery of melanosomes to the actin-rich, apical domain of the retinal pigment epithelium

AUTHORS: Mei Jiang, Stefanie Volland, Hongxing Wang, Adarsh Balaji, David G. Li, Vanda S Lopes, and David S Williams
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Specifically I think that the nocodazole experiment suggested is simple and useful. Otherwise, the majority of changes are likely addressable by amendments to the text.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

In this manuscript Jiang and colleagues investigated the role of cytoskeleton motors and tracks in the transport of melanosomes in RPE cells. This follows up on previous work from the Williams group and here addresses the role of kinesin and dynein motors and microtubules in this process. This is an interesting cell biology topic that does advance knowledge in this area and will be of interest to the readership of JCS, but requires significant revision as indicated below.

Comments for the author

The main claim of this manuscript is that dynein deficiency in RPE cells phenocopies loss of myosin-VIIa in that i) fewer slow (less than or equal to 0.4um/sec speed) movements and ii) melanosomes are absent from apical domains of RPE cells. From these observations they conclude that microtubule-based movement is required for delivery of melanosomes to the actin-rich apical domain and that this supports a role for microtubules and actin motors in melanosome transport in RPE cells.
In support of this claim in figure 1 (and movies 1-3) they show evidence that the path of melanosome movements correlates with the path of actin filaments labelled with RFP-actin and GFP-Tractin actin probes in wild-type RPE cells and EB3-EGFP labelled microtubules in myosin-VIIa deficient shaker RPE cells. In figure 4 they show that melanosomes are reduced in the apical region of RPE cells in dynein shRNA injected retinas, consistent with requirement for dynein expression to deliver melanosomes to the apical region of RPE cells. In figure 5 they show that the proportion of slow moving melanosomes (less than or equal to 0.4um/sec speed) is decreased in cultured wild-type RPE cells transfected with dynein shRNA (control ~60% versus dynein shRNA 25%).

Major comments:
1) While these observations are consistent with their hypothesis and conclusion they do not directly show that microtubule–based movement is required for delivery of melanosomes to the actin-rich apical domain. This should be tested directly for instance by showing using nocodazole that depletion of microtubules prevents accumulation of melanosomes in apical processes.
2) They do not show directly that dynein, or subunits whose stability is known to be dependent upon dynein heavy chain expression, is depleted in dynein shRNA transfected RPE cells. This needs to be addressed e.g. by immunoEM.
3) Related to this it would also be very important, from the perspective of the interpretation of results, to know the dependence of the different classes of movements observed in figures 3 and 5 actin filaments and microtubules. The authors indicate throughout the manuscript that events </= 0.4um/sec speed are actin/myoVIIa dependent and faster movements are microtubule/dynein/kinesin dependent. However, they present no evidence that slower movements are actin dependent or faster movements are microtubule dependent. This needs to be addressed head-on e.g. using nocodazole and latruculin to deplete these filament networks.

Other comments:
1) Throughout the manuscript there is a lack of quantification of data/indication of the numbers of experiments conducted, cells/organelles measured and the time periods over which shRNA were allowed to deplete protein. Also statistical support for conclusions is lacking with one exception. These points need to be address.
2) For the non-specialist reader it would be extremely helpful if EM sections of retina shown in figures 2-4 were labelled to indicate more clearly the polarity of cells and the location of adjacent cell types within the retina so that the relationship between RPE apical processes and photoreceptors for instance is clearer.
3) Figure 1. It is not easy to see melanosomes. Can the melanosomes be false coloured to highlight more clearly their location?
4) Movies 1-3 – legends indicate a different period of time is shown compared with the counter in the corner of the images.
5) Figures 2 and 4. In the text the authors indicate that immunogold labelling for kinesin-1 and dynein is enriched 2.2 and 2.4 fold on melanosomes in the basal part of RPE cells compared with apical however they present no evidence of this or quantification for this e.g. how many cells/organelles counted. This needs to be addressed.

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6) Figure 3A the blots are not calibrated for molecular mass.
7) Figure 3B the horizontal axis lacks units.
8) Figure 4C the numbers and shape of RPE melanosomes in scramble and dynein shRNA injected retinas appear different. How frequently is this observed? Is it possible that the difference in distribution reflects differences in the biogenesis of melanosomes rather than defects in their transport. This would not be entirely surprising given the role of dynein heavy chain in many different membrane trafficking steps. Perhaps short term treatment of RPE/cells with dynein inhibitors e.g. ciliobrevin might provide stronger for the role of dynein in transport of mature melanosomes than long term depletion of dynein and possible effects of this on a host of other trafficking processes. The authors need to explain this and/or present data more consistent with a transport defect rather than a possible biogenesis defect, if this indeed reflects the general picture in a large number of experiments. On the other hand if there really are systematic difference in the number shape of melanosomes in the 2 populations this needs to be addressed.
9) Related to figure 4 the authors need to present direct evidence that dynein is expressed in RPE cells and that its expression is affected by dynein specific shRNA. The data presented currently are from IMCD3 cells and show reduction on expression of an intermediate chain as a proxy for dynein.
10) Figure 5A shows that slow movements are reduced in shRNA transfected wild-type RPE cells but do not indicate the overall proportion of total melanosomes that were motile over the observation window. This would be very useful data to help interpret the results from WT and shaker mutant cells altogether.
11) The title of the manuscript needs revision to more accurately reflect the data shown or more convincing evidence needs to be presented to confirm the role of microtubule dependent transport in delivery of melanosomes to the apical processes of RPE cells.
Typos The manuscript is littered with typographical errors and needs to be thoroughly proofread and corrected for these e.g. in the abstract 'deficient cytoplasmic dynein' should be 'deficiency of/in cytoplasmic dynein'.

Reviewer 2

Advance summary and potential significance to field

In this study the authors investigate the role of microtubule motors in the transport of melanosomes in retinal pigment epithelial cells. This is timely as recent data in melanocytes opposes the traditionally held view that melanosomes move to the dendrites of melanocytes on microtubules and are then 'captured' in the periphery through interaction with the actin skeleton. The Hume lab have presented evidence that microtubules are required for melanosome movement to the cell centre in melanocytes (minus end-directed) whilst movement to the cell periphery is actin-based so that microtubule-based movement opposes, rather than co-operates with actin-based movement. In RPE cells, the apical distribution of melanosomes is equivalent to the peripheral distribution of melanosomes in melanocytes, in that both depend on myosin-dependent interaction with the actin cytoskeleton. However, microtubules in the RPE are primarily oriented with their minus ends apically whilst the plus ends are peripheral in melanocytes. In this manuscript the authors clearly show that the minus end directed motor, dynein, regulates melanosome motility on microtubules in cultured RPE cells and is required for the apical distribution of melanosomes in the RPE in vivo. They also show a minor role of the plus end directed motor, kinesin, in regulating melanosome motility in the RPE in vitro and no detectable role of kinesin in regulating melanosome distribution in vivo. This study advances our understanding of the regulation of melanosome movement in RPE cells and provides an interesting parallel with melanosome movement in melanocytes.

Comments for the author

This is a very well written manuscript. The studies on cultured RPE cells and in vivo models are carefully performed, well-presented and convincing. I have the following comments that should be addressed before publication:

In Figure 1A the authors investigate motility of melanosomes on actin filaments in wild type cells and in Figure 1B investigate motility of melanosomes on microtubules in Shaker 1 cells where interaction between melanosomes and actin filaments is prevented. What is the rationale for wild

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type versus shaker? It is possible that, in the absence of actin-based motility, microtubule-based motility compensates in a way that is not necessary in wild type cells. Indeed the authors state that fast movement on microtubules is less frequent and over shorter distances in wild type cells but don't show the data. I think this data should be shown and reasons for any differences in microtubule-based movement in the presence or absence of actin-based movement should be discussed.

Fig 3B shows an increase in frequency of faster movements in the absence of Kif5a. Does this suggest the involvement of another motor that mediates more rapid movements with which Kif5a competes? This could be discussed more clearly in the text.

In the image shown in Figure 3C it appears that there are more melanosomes apically and in the apical processes in the absence of KIF5a compared with floxed control. I think that a relatively mild effect would make sense if kinesin 1-dependent movement towards the plus ends of microtubules opposes apical transport. Would it be possible to quantify changes in distribution of melanosomes in the RPE of wild type versus mutant mice?

First revision

Author response to reviewers' comments

We are very grateful to the reviewers for their thorough reviews, and the time they have taken to help us improve the paper. Our responses are in blue font, following each comment.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript Jiang and colleagues investigated the role of cytoskeleton motors and tracks in the transport of melanosomes in RPE cells. This follows up on previous work from the William's group and here addresses the role of kinesin and dynein motors and microtubules in this process. This is an interesting cell biology topic that does advance knowledge in this area and will be of interest to the readership of JCS, but requires significant revision as indicated below.

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In support of this claim in figure 1 (and movies 1-3) they show evidence that the path of melanosome movements correlates with the path of actin filaments labelled with RFP-actin and GFP-Tractin actin probes in wild-type RPE cells and EB3-EGFP labelled microtubules in myosin-VIIa deficient shaker RPE cells. In figure 4 they show that melanosomes are reduced in the apical region of RPE cells in dynein shRNA injected retinas, consistent with requirement for dynein expression to deliver melanosomes to the apical region of RPE cells. In figure 5 they show that the proportion of slow moving melanosomes (less than or equal to 0.4um/sec speed) is decreased in cultured wild-type RPE cells transfected with dynein shRNA (control ~60% versus dynein shRNA 25%).

Major comments:
1) While these observations are consistent with their hypothesis and conclusion they do not directly show that microtubule-based movement is required for delivery of melanosomes to the actin-rich apical domain. This should be tested directly for instance by showing using nocodazole that depletion of microtubules prevents accumulation of melanosomes in apical processes.

We appreciate the reviewer's point. However, nocodazole perturbs the polarity of RPE cells (which is dependent on the vertical microtubules), thus affecting the ability to assess the subcellular localization of melanosomes in polarized RPE. We have tested nocodazole treatment on primary

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RPE cell cultures by live-cell imaging to determine the effect on melanosome motility. The organization of RPE cells in culture is also affected by nocodazole, but we are able to assess melanosome motility, and the effect on motility is clear. We have now added a figure (new Fig. 2) that shows the disruption of microtubules caused by the nocodozole, and quantification of the effect of this disruption on melanosome speed.

2) They do not show directly that dynein, or subunits whose stability is known to be dependent upon dynein heavy chain expression, is depleted in dynein shRNA transfected RPE cells. This needs to be addressed e.g. by immunoEM.

We have now addressed this issue by showing knockdown in primary RPE cell cultures by western blot, after using lentiviral transduction to administer the shRNA, so that the majority of cells expressed the shRNA-mCherry plasmids (unlike with transfection). This western blot replaces the one performed with transfected IMCD cells in Fig. 5B (previously Fig. 4B).

3) Related to this it would also be very important, from the perspective of the interpretation of results, to know the dependence of the different classes of movements observed in figures 3 and 5 actin filaments and microtubules. The authors indicate throughout the manuscript that events microtubule/dynein/kinesin dependent. However, they present no evidence that slower movements are actin dependent or faster movements are microtubule dependent. This needs to be addressed head-on e.g. using nocodazole and latruculin to deplete these filament networks.

We agree that this point is important, but feel that previously published work addresses this point. What has been lacking is the direct visualization of melanosome movements along microtubules and actin filaments; this is what we focused on in the present study.
Loss of functional myosin-7a, an actin-based motor, has been shown to increase the proportion of faster movements of melanosomes by severely decreasing the slower movements (Gibbs et al., 2004). Experiments with cytochalasin D showed a similar effect to loss of MYO7A, and nocodazole abolished these faster movements (Lopes et al., 2007a). We have explored the nocodazole treatment further, and have added a quantitative analysis of the effect on melanosome speed to the revised ms; this is shown as new Fig. 2. Pharmacological treatments can have indirect effects, however. Importantly, in the present study, we have also performed observations of melanosomes moving along labeled actin filaments (with slower, short-range movements), and along microtubules (with faster, long-range movements speeds), to provide direct evidence of melanosome motility – and the nature of that motility - on actin filaments and microtubules.

Other comments:
1) Throughout the manuscript there is a lack of quantification of data/indication of the numbers of experiments conducted, cells/organelles measured and the time periods over which shRNA were allowed to deplete protein. Also statistical support for conclusions is lacking with one exception. These points need to be address.

Information has been added to the legends of the figures showing the data.
The time period over which shRNA was allowed to deplete protein had already been stated in the Methods: analysis was performed 3-4 days post transfection or transduction in all cases (second paragraph under Cell culture).

2) For the non-specialist reader it would be extremely helpful if EM sections of retina shown in figures 2-4 were labelled to indicate more clearly the polarity of cells and the location of adjacent cell types within the retina so that the relationship between RPE apical processes and photoreceptors for instance is clearer.

Thank you for the suggestion. Labeling of the figures and explanation in the legends have been added.

3) Figure 1. It is not easy to see melanosomes. Can the melanosomes be false coloured to highlight more clearly their location?

Thank you for the suggestion. This has been done.

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4) Movies 1-3 – legends indicate a different period of time is shown compared with the counter in the corner of the images.

Thank you for noticing this. The legend of video 2 has been corrected to reflect the correct playback rate (20fps rather than 10fps).

5) Figures 2 and 4. In the text the authors indicate that immunogold labelling for kinesin-1 and dynein is enriched 2.2 and 2.4 fold on melanosomes in the basal part of RPE cells compared with apical however they present no evidence of this or quantification for this e.g. how many cells/organelles counted. This needs to be addressed.

This information (# of fields of view from sections from 3 animals) has been added.

6) Figure 3A the blots are not calibrated for molecular mass.
Apparent molecular masses have been added (this figure is now Fig. 4A).

7) Figure 3B the horizontal axis lacks units.
Thank you for noticing this. Units (um/sec) have been added.

8) Figure 4C the numbers and shape of RPE melanosomes in scramble and dynein shRNA injected retinas appear different. How frequently is this observed? Is it possible that the difference in distribution reflects differences in the biogenesis of melanosomes rather than defects in their transport. This would not be entirely surprising given the role of dynein heavy chain in many different membrane trafficking steps. Perhaps short term treatment of RPE/cells with dynein inhibitors e.g. ciliobrevin might provide stronger for the role of dynein in transport of mature melanosomes than long term depletion of dynein and possible effects of this on a host of other trafficking processes. The authors need to explain this and/or present data more consistent with a transport defect rather than a possible biogenesis defect, if this indeed reflects the general picture in a large number of experiments. On the other hand if there really are systematic difference in the number shape of melanosomes in the 2 populations this needs to be addressed.

In our experiments on dynein knockdown, an effect on melanosome biogenesis seem very unlikely. First, the progression to mature melanosomes in the mouse RPE is largely complete before birth (Lopes et al., 2007b) – and so, before the treatment with shRNA. Second, if there were an effect, we would expect to see significant numbers of stage II and III melanosomes, as there are in biogenesis mutants (e.g. Lopes et al., 2007b). However, nearly all melanosomes appeared to be stage IV, as in normal RPE.
The apparent difference in melanosome shape is largely due their long oval shape and their different orientation according to whether they are in the apical processes (and therefore oriented in the plane of the section) or not in the apical processes (in which case, they are randomly oriented and mostly appear in oblique section); this was described in Gibbs et al. (2004).
The regions of the retina examined corresponded to that near the site of plasmid injection, and therefore their relative location varied somewhat from animal to animal. This resulted in some variation in melanosome numbers, since the number of melanosomes per RPE cell varies across a normal mouse retina. Even though melanosomes are still present in the apical processes of all regions, there is a greater abundance in the ventral retina vs the dorsal retina (Williams et al., 1985), and there is a gradient of increasing abundance from the center to the periphery (own observation in mouse; Howell et al., 1982, in rat). We have added this comment to the figure legend of the figure.

9) Related to figure 4 the authors need to present direct evidence that dynein is expressed in RPE cells and that its expression is affected by dynein specific shRNA. The data presented currently are from IMCD3 cells and show reduction on expression of an intermediate chain as a proxy for dynein.

We have now addressed this issue by showing knockdown in primary RPE cell cultures by western blot, after using lentiviral transduction, so that the majority of cells expressed the shRNA-mCherry plasmids (unlike with transfection). This western blot replaces the one performed with IMCD cells in the previous Fig. 4B (replacement blot is now Fig. 5B).

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10) Figure 5A shows that slow movements are reduced in shRNA transfected wild-type RPE cells but do not indicate the overall proportion of total melanosomes that were motile over the observation window. This would be very useful data to help interpret the results from WT and shaker mutant cells altogether.

The data have been added to the legend of Fig. 6A (previously Fig. 5A).

11) The title of the manuscript needs revision to more accurately reflect the data shown or more convincing evidence needs to be presented to confirm the role of microtubule dependent transport in delivery of melanosomes to the apical processes of RPE cells.

The title has been revised.

Typos
The manuscript is littered with typographical errors and needs to be thoroughly proofread and corrected for these e.g. in the abstract 'deficient cytoplasmic dynein' should be 'deficiency of/in cytoplasmic dynein'.

We have made the suggested change.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this study the authors investigate the role of microtubule motors in the transport of melanosomes in retinal pigment epithelial cells. This is timely as recent data in melanocytes opposes the traditionally held view that melanosomes move to the dendrites of melanocytes on microtubules and are then 'captured' in the periphery through interaction with the actin skeleton. The Hume lab have presented evidence that microtubules are required for melanosome movement to the cell centre in melanocytes (minus end-directed) whilst movement to the cell periphery is actin-based so that microtubule-based movement opposes, rather than co-operates with actin-based movement. In RPE cells, the apical distribution of melanosomes is equivalent to the peripheral distribution of melanosomes in melanocytes, in that both depend on myosin-dependent interaction with the actin cytoskeleton. However, microtubules in the RPE are primarily oriented with their minus ends apically whilst the plus ends are peripheral in melanocytes. In this manuscript the authors clearly show that the minus end directed motor, dynein, regulates melanosome motility on microtubules in cultured RPE cells and is required for the apical distribution of melanosomes in the RPE in vivo. They also show a minor role of the plus end directed motor, kinesin, in regulating melanosome motility in the RPE in vitro and no detectable role of kinesin in regulating melanosome distribution in vivo. This study advances our understanding of the regulation of melanosome movement in RPE cells and provides an interesting parallel with melanosome movement in melanocytes.

Reviewer 2 Comments for the Author:
This is a very well written manuscript. The studies on cultured RPE cells and in vivo models are carefully performed, well-presented and convincing. I have the following comments that should be addressed before publication:

In Figure 1A the authors investigate motility of melanosomes on actin filaments in wild type cells and in Figure 1B investigate motility of melanosomes on microtubules in Shaker 1 cells where interaction between melanosomes and actin filaments is prevented. What is the rationale for wild type versus shaker?

We studied shaker1 cells in order to isolate microtubule motility from myosin-7a-based motility on actin filaments. Thus, we avoided confusing microtubule motility with myosin-7a-based motility on any (unlabeled) actin filaments that were close to the microtubules. Use of shaker1 RPE also increased the number of melanosomes that would be available for microtubule movements (Gibbs et al., 2004). We have added this comment to the Results section (bottom page 6).

It is possible that, in the absence of actin-based motility, microtubule-based motility compensates in a way that is not necessary in wild type cells. Indeed the authors state that fast movement on microtubules is less frequent and over shorter distances in wild type cells but don't show the data.

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I think this data should be shown and reasons for any differences in microtubule-based movement in the presence or absence of actin-based movement should be discussed.

This effect of mutant *Myo7a* was shown and discussed by Gibbs et al. (2004) and Lopes et al. (2007a). The data are also evident by comparing the control (scrambled shRNA) bars between Fig. 6A and 6C. We have now cited these publications, and made this point about the data, drawing a reader's attention to this comparison, in last paragraph of the Results.

Fig 3B shows an increase in frequency of faster movements in the absence of Kif5a. Does this suggest the involvement of another motor that mediates more rapid movements with which Kif5a competes? This could be discussed more clearly in the text.

We have revised this part of the Discussion to make this valid point more clearly. We have also added discussion of an alternative possibility that the absence of kinesin-1 results in more dynein motility. This would be consistent with the reviewer's next suggestion. However, the in vivo speed of cytoplasmic dynein has been indicated to be slower than that of kinesin-1.

In the image shown in Figure 3C it appears that there are more melanosomes apically and in the apical processes in the absence of KIF5a compared with floxed control. I think that a relatively mild effect would make sense if kinesin 1-dependent movement towards the plus ends of microtubules opposes apical transport. Would it be possible to quantify changes in distribution of melanosomes in the RPE of wild type versus mutant mice?

This is an interesting suggestion. However, we did not detect a significant difference in the proportion of apical melanosomes. This result is now discussed in the text.

Second decision letter

MS ID#: JOCES/2019/242214

MS TITLE: Microtubule motor transport in the delivery of melanosomes to the actin-rich, apical domain of the retinal pigment epithelium

AUTHORS: Mei Jiang, ANTONIO E ESCUDERO PANIAGUA, Stefanie Volland, HONGXING WANG, Adarsh Balaji, David G. Li, Barry L. Burgess, Vanda S Lopes, and David S Williams
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed all the points that I previously raised. I therefore support publication of this manuscript.

Comments for the author

None

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Reviewer 2

Advance summary and potential significance to field

As in my previous review:
In this study the authors investigate the role of microtubule motors in the transport of melanosomes in retinal pigment epithelial cells. This is timely as recent data in melanocytes opposes the traditionally held view that melanosomes move to the dendrites of melanocytes on microtubules and are then 'captured' in the periphery through interaction with the actin skeleton. The Hume lab have presented evidence that microtubules are required for melanosome movement to the cell centre in melanocytes (minus end-directed) whilst movement to the cell periphery is actin-based so that microtubule-based movement opposes, rather than co-operates with actin-based movement. In RPE cells, the apical distribution of melanosomes is equivalent to the peripheral distribution of melanosomes in melanocytes, in that both depend on myosin-dependent interaction with the actin cytoskeleton. However, microtubules in the RPE are primarily oriented with their minus ends apically whilst the plus ends are peripheral in melanocytes. In this manuscript the authors clearly show that the minus end directed motor, dynein, regulates melanosome motility on microtubules in cultured RPE cells and is required for the apical distribution of melanosomes in the RPE in vivo. They also show a minor role of the plus end directed motor, kinesin, in regulating melanosome motility in the RPE in vitro and no detectable role of kinesin in regulating melanosome distribution in vivo. This study advances our understanding of the regulation of melanosome movement in RPE cells and provides an interesting parallel with melanosome movement in melanocytes.

Comments for the author

There is a typo in the title: "of" or "in" the retinal pigment epithelium (not both). Otherwise I recommend acceptance of the manuscript without further revision.

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