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Membrane protrusion powers clathrin-independent endocytosis of interleukin-2 receptor

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(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 19 January 2015

Thank you for submitting your manuscript entitled 'Membrane protrusion powers clathrin-independent endocytosis of interleukin-2 receptor'. I have now received reports from all referees, which are enclosed below.

As you will see, the referees find your study interesting and support publication of a revised version in The EMBO Journal. They raise a number of concerns, which I will not specifically list here, as all reports are quite clear and constructive. Given the comments provided, I would like to invite you to submit a revised version of the manuscript. Some of the concerns raised can be addressed with appropriate text changes, while others need better quantification as well as additional data. As you can see below, referee #2 raises several different issues. You might already have data on hand to address some of these points, but I am available to discuss further what can be addressed within a reasonable time frame. Please also contact me in case of other questions regarding the revision of your manuscript.
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

The paper by Basquin et al. provides electron microscopic evidence for a novel mode of clathrin-independent endocytosis of IL2 receptors that is initiated by membrane protrusion rather than invagination. The authors show that uptake of IL2 receptors requires both the WAVE and WASP actin machineries and provide evidence for direct or indirection interaction between the WAVE complex and IL2R. While these should be considered as concurrent submissions, it is difficult not to compare the results in this paper with the recently published findings of McMahon and colleagues (Boucrot et al., Nature 2014) showing that IL2 receptors are internalized via a endophilin- and lamellipodin-dependent manner. These published reports provide additional support for the authors' conclusions, but also present some inconsistencies that the authors might want to resolve and/or address.

Although the EM tomographic studies presented here are of the highest quality, it would be difficult to draw conclusions regarding the dynamic behavior of these membranes and the resulting mechanism of endocytosis based only on these static images. The strongest evidence for such a mechanism lies in the sequential recruitment of the WAVE and WASP actin regulators both triggering Arp2/3 and actin assembly at sites of IL2 internalization. However, actin assembly can drive both membrane protrusion and invagination. Thus, there is little direct evidence for the protrusion mechanism and this must remain a speculation. The data also lack quantification or a negative control. For example, while coated pits are distributed differently with respect to protrusios, would TfnR-antibody/protein A gold particles also be detected at the bases of these protrusions?

Is this pathway dependent on endophilin and/or lamellipodin?

The Boucrot paper suggests that IL2 uptake occurs preferentially at the leading edge of cells, which might be consistent with a coincidence with cell protrusive activity, but the data presented here seems to show no such polarity.

The Boucrot paper suggested that this IL2 pathway was 'fast'. The TIRF data in this paper shows the lifetimes of the IL2 endocytic events to be of the same order of magnitude as clathrin-mediated endocytosis. Perhaps quantification of this could add to the discussion regarding the Boucrot claims of discovery of a "Fast endophilin-mediated endocytosis' pathway.

Minor comments:

The paper should be carefully read and edited by a native English speaker. There are many errors, e.g..
Abstract: "nutriment"
Pg 3 "an important issue concern the mechanism"; "a dozen of factors"; "N-WASP and cortactin are regulated differently upon the pathway"; "Beside this current model"
Pg 4: 'short' labeling vs 'brief' labeling.
These occur throughout the text.

Referee #2:

The manuscript by Basquin et al dissects in an elegant study using transmission electron microscopy, EM tomography, and live cell TIRF imaging the clathrin-independent endocytosis of the IL2-receptor. Clathrin-independent endocytosis has recently attracted much attention with the discovery of the FEME pathway (Boucrot et al., 2014, Nature) and several other proposed pathways. However, the mechanism and regulation of Clathrin-independent endocytosis is far from clear. Thus, this study is timely and of high interest to a wide audience.
The authors reveal using TEM and EM tomography that IL2-receptor containing endocytic pits localise close to the base of cell protrusions. Mechanistically, they provide evidence for a role of the Scar/WAVE complex in the uptake of the IL2-receptor, which so far has not been implicated in endocytosis. They suggest that the IL2-receptor is in a biochemical complex with the Scar/WAVE complex. Most convincingly, they show sequential recruitment of Abi1 and N-WASP and two concomitant peaks of recruitment of the Arp2/3 complex to IL2-receptor containing endocytic assemblies at the plasma membrane. The authors had implicated N-WASP and dynamin previously

in the uptake of the IL2-receptor. Furthermore, they suggest that dynamin acts as a "transition controller" for the recruitment of the two Arp2/3 activators Scar/WAVE and N-WASP.
In general the experiments have been performed with great care. However, it is not clear whether the authors analyse the same endocytic invaginations that they observe by EM at the base of protrusions, which appear to be located at the apical side of cells compared to the endocytic assemblies that they monitor using TIRF at the base of cells opposing the glass coverslip and where one would rarely observe protrusions. Furthermore, the biochemical data suggesting a link between the IL2-R and the Scar/WAVE complex is rather weak.
In general I would support a publication in the EMBO Journal if the authors repeat the TIRF experiments with another subunit of the Scar/WAVE complex besides GFP-Abi (which is not only a component of the Scar/WAVE complex but also binds N-WASP) and should address my mayor concerns. The authors should discuss their data in light of their recent publication that the IL2-R uptake is mediated by the FEME pathway. The authors should remove overstatements throughout the manuscript. Throughout the study, conclusions should not be drawn from experiments conducted with only one siRNA. Off-target effects cannot be excluded and it is standard in the field to use at least 2 siRNAs to control for these.
Furthermore, the following concerns needs to be addressed before I can recommend publication.

Major concerns:
1. Fig. 2; Page 4: The results from Fig 2 I, O, need to be analysed for statistically significant differences. Fig 2 I: At the base of protrusions (0nm), there appears to be no significant difference between CCPs and IL2-R positive invaginations. Therefore, the interpretation is an overstatement. There appears to be an association of both CCPs and IL-2R invaginations at the base of (filopodial) protrusions. CCPs but not IL2-R invaginations are also found in flat membrane sections. Knockdown of clathrin heavy chain and analysis of IL2-R to base of protrusion would clarify whether these are clathrin independent invaginations.
2. Fig 2O: page 5: the association of IL2-R invaginations with protrusions appear to be not induced by IL2 stimulation which is in contrast to what has been proposed for the FEME pathway (that the FEME pathway is induced by the respective ligands).
3. Fig. 2J,K,L,P; page 5: The knockdown of dynamin needs to be done with two different siRNAs. Off-target effects cannot be excluded and it is standard in the field to use at least 2 siRNAs to control for these. The number of IL2-R in unconstricted/tubular pits compared to pinched-off vesicles should be quantified to make this statement. The results from Fig 2 P, need to be analysed for statistically significant differences.
4. Fig. 3: General concern with IL2-R and transferrin uptake assays: Most labs in the field quantify and present the ratio of surface bound to internalised transferrin (or equivalent IL2-R) since knockdown of proteins may affect the amount of receptors present at the cell surface. The authors need to repeat their experiments using this quantification or show that their knockdown does not affect surface localization of receptors.
5. Fig. 3D: Conclusions should not be drawn from experiments conducted with only one siRNA. Off-target effects cannot be excluded and it is standard in the field to use at least 2 siRNAs to control for these.
6. Fig. 3I: Abi knockdown reduces filopodia protrusions by 26%. If the IL2-Rs are distributed just by chance, wouldn't you expect a reduction of association already by 26%? Are the differences statistically significant? You observe a reduction in association by 50% in Abi KD cells. If you normalize for the reduction in filopodia number, is this a significant difference?
7. Fig. 4: Page 6: To make the statement that the IL2-R and the Scar/WAVE are in complex with each other, an endogenous coIP should be shown. The coIP's of the overexpressed proteins shown are not convincing since the appropriate controls are missing: The GFP-IL2-R control just controls for stickiness of GFP but not of stickiness of GFP-tagged subunits of the Scar/WAVE complex. An appropriate control would be to use a non-immune mouse IgG control for IP from the same lysate expressing GFP-tagged Scar/WAVE subunits and IL-2R. How often were the experiments repeated? The quantification of the blots should have been done from three blots from independent experiments and tested for statistically significance. Fig. 4B: The blots should not be stitched together from snippets. It is not clear whether these are from the same blot. The GFP lanes are clearly overexposed and cannot be used for quantifications.
8. Fig. 4 Does the IL2-R contains a "WIRS" motif that has been identified by the Rosen lab in several receptors to mediate direct interaction with the Scar/WAVE complex? If not, how is the interaction mediated?

9. Fig. 5: Very nice data and important result! However, it is not clear whether the authors analyse the same endocytic invaginations that they observe by EM at the base of protrusions which appear to be located at the apical side of cells compared to the endocytic assemblies that they monitor using TIRF at the base of cells opposing the glass coverslip and where one would rarely observe protrusions. For Fig 5a GFP-Abi was used. Since Abi can interact not only with the Scar/WAVE complex but also with N-WASP, this should be verified with another, more specific subunit of the Scar/WAVE complex.
10. Fig. 6: page 8: Dynamin should be knocked down with two independent siRNAs. The coIP's of the overexpressed proteins shown are not convincing since the appropriate controls are missing: The GFP-IL2-R control just controls for stickiness of GFP but not of stickiness of GFP-tagged subunits of the Scar/WAVE complex. An appropriate control would be to use a non-immune mouse IgG control for IP from the same lysate expressing GFP-tagged Scar/WAVE subunits and IL-2R. How often were the experiments repeated? The quantification of the blots should have been done from three blots from independent experiments and tested for statistically significance. The GFP lanes are clearly overexposed and cannot be used for quantifications.
11. Fig 5G and Fig 6E. This data should be in one graph together and the significance of the difference statistically analysed.
12. It is an overstatement that dynamin "controls" the departure of the Scar/WAVE complex and is necessary for N-WASP recruitment. Since you don't provide the molecular mechanism, this might just be a consequence of deficiency of scission.

Minor concerns:
1. Fig. 1; Page 4; Do these intracellular vesicles (early endosomes and MVBs) look different from CME derived early endosomes and MVBs? The last sentence that IL-2R containing intracellular vesicles are morphologically distinct from "all known endocytic carriers" is thus an overstatement.
2. Fig. 2; page 4: What type of protrusions are these? These appear to be filopodia, which are not thought to arise from Scar/WAVE mediated actin nucleation. Please clarify.
3. Fig. 5 G: Only 20% colocalization between IL2-R and Abi or N-WASP has been observed, was this analysis done from fixed images or from movies. If from movies, at which time point?
3. FigS3: It would be more convincing if not just an Arp2/3 inhibitor was used but if this would be verified by knockdown of Arp2/3.
4. Fig 3 C: quantifications of knockdowns: was this done just from one blot? This should be done from 3 independent experiments. For Sra1 and Brk1 the blots appear to have been stitched together. Are the individual snippets from the same film with same exposure?

Referee #3:

In this manuscript the authors describe a series of interesting findings about the mechanisms of clathrin independent endocytosis of interleukin-2 receptor. They first show that IL2R internalization is closely associated with plasma membrane protrusions. Then the authors show that WAVE complex is involved in IL2R internalization and show that the WAVE complex is recruited in a specific time window before N-WASP recruitment. The findings are interesting and the experiments mostly well done. I have some comments and suggestions to improve the manuscript.

- The authors could explain more clearly why the two cell lines were chosen for the studies.

- The meaning of the following sentence on page 3 is not clear. Please, rewrite. "...we performed immunogold labeling of IL-2Rbeta combined to a kinetic of endocytosis that we finally observed by transmission electron microscopy".

- On page 4 the authors write: "...protein A-gold alone showed very rarely gold particles...". It would be important to provide a more quantitative estimate of the background level. The quantitation that is presented later is somewhat meaningless if the background level is not defined.

- What are the "various shapes" of the vesicles mentioned on page 4. Please, describe the shapes.

- How were the CCPs identified from the EM images? This should be explained in the text.

- I have some concerns about the quantification of the distance of gold particles from the protrusions. According to the figure 2I the membrane areas in the categories "On protrusion" and "Base" are larger that the other categories. Therefore even random beads would fall more frequently on those larger categories. I think it would be important to control for this effect, for example by calculating the membrane areas in different categories and calculating the true enrichment on protrusions and at their bases. In principle, randomly binding gold particles would be a better control than the CCPs, but I'm not sure how feasible that would be in practice.

- The claim (on page 5 and 8) that the EM results confirm the role of dynamin in scission seems over interpreted. No quantitative data is shown to support this claim. The U-shaped pit in 2L doesn't look different from the other normal pits shown in other panels of Figure 2. Also, it is difficult from a thin section to conclude that the tubule in 2K is truly an endocytic tubule and not just a furrow at the cell surface. I would recommend toning down this claim and focusing on the conclusions that are better supported by the data, i.e. that dynamin depletion blocks the uptake of IL2R but that it doesn't prevent it's association with the protrusions.

- On few places in the manuscript the term "filopodia" is used instead of "protrusion". The authors should use only one term or explain what is the difference between these two terms if there is some need to use both.

- Figures 5H and 6F. It wasn't clear to me how the intensity curves are aligned in time. This should be explained clearly. Also, what do the error bars represent? They are very small. For example, the errors in the IL2R curve are smaller than the fluctuations of the curve between ~50-70 s.

1st Revision - authors' response 20 April 2015

Referee #1:

The paper by Basquin et al. provides electron microscopic evidence for a novel mode of clathrin-independent endocytosis of IL2 receptors that is initiated by membrane protrusion rather than invagination. The authors show that uptake of IL2 receptors requires both the WAVE and WASP actin machineries and provide evidence for direct or indirection interaction between the WAVE complex and IL2R. While these should be considered as concurrent submissions, it is difficult not to compare the results in this paper with the recently published findings of McMahon and colleagues (Boucrot et al., Nature 2014) showing that IL2 receptors are internalized via a endophilin- and lamellipodin-dependent manner. These published reports provide additional support for the authors' conclusions, but also present some inconsistencies that the authors might want to resolve and/or address.

Although the EM tomographic studies presented here are of the highest quality, it would be difficult to draw conclusions regarding the dynamic behavior of these membranes and the resulting mechanism of endocytosis based only on these static images. The strongest evidence for such a mechanism lies in the sequential recruitment of the WAVE and WASP actin regulators both triggering Arp2/3 and actin assembly at sites of IL2 internalization. However, actin assembly can drive both membrane protrusion and invagination. Thus, there is little direct evidence for the protrusion mechanism and this must remain a speculation. The data also lack quantification or a negative control.
For example, while coated pits are distributed differently with respect to protrusions, would TfnRantibody/protein A gold particles also be detected at the bases of these protrusions?

To answer this question, we used the protocol described in Fig. 2 to label transferrin (Tf) using 10 nm-gold conjugated Tf and acquired TEM images. The results (Fig. S2A, B) show that Tf follows a distribution close to CCP. Around 40% of Tf and CCP were close to protrusions (< 300 nm) and 60% were at flat membranes (> 300 nm), in contrast to IL-2R distribution. These new data confirmed our previous result and strengthen our conclusion that the distribution of IL-2R close to membrane protrusions is specific. The supplementary figure and text have been inserted accordingly.

Is this pathway dependent on endophilin and/or lamellipodin?

Yes this pathway is endophilin-dependent. Since we are co-authors of the article (Boucrot et al, Nature 2015) we can easily confirm to the reviewer that endophilin is involved in IL-2R uptake. Furthermore, endophilin is involved in both IL-2-induced and constitutive endocytosis. We have recently tested the role of lamellipodin using a pool of siRNAs targeting 4 parts of lamellipodin mRNA and we did not obtain any strong effect on the constitutive uptake of IL-2R (see Figure 1 for reviewers). However, it should be noted that a previous article (Vehlow A et al, EMBO, 2013) described the importance of lamellipodin on EGFR uptake. The effect is rather low (20%), although EGFR uses the FEME pathway. Thus, lamellipodin function in this pathway is not fully understood, we will investigate further its function on IL-2R endocytosis in a future work.

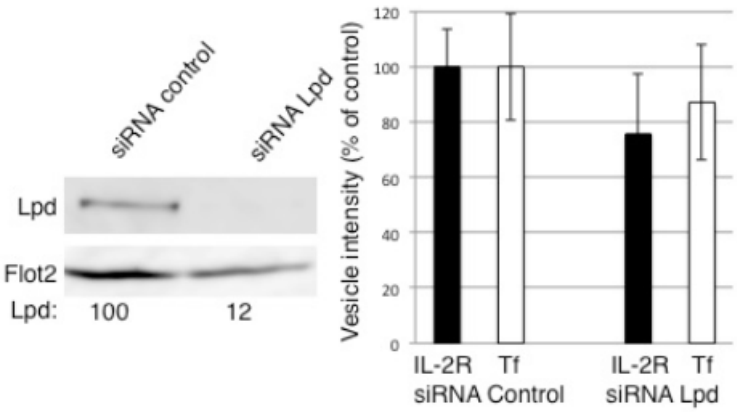
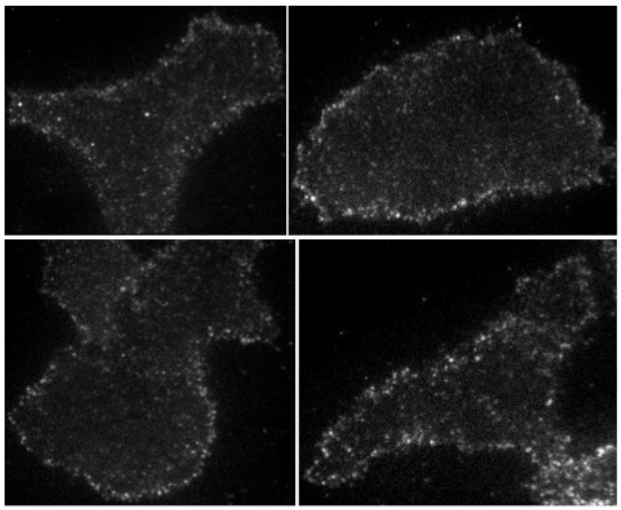


Figure 1 for reviewers

The Boucrot paper suggests that IL2 uptake occurs preferentially at the leading edge of cells, which might be consistent with a coincidence with cell protrusive activity, but the data presented here seems to show no such polarity.

I agree with the referee that the edge of the cell coincides with cell protrusive activity. Thus, the Boucrot paper and our story are in agreement. Indeed, very often we saw in TIRF images that IL-2R is enriched at the edge of the cell like it is shown in the figure 2 for reviewers below. Thanks to the referee's suggestion, we decided to add a comment about this point in the discussion.

Examples of IL-2R distribution at the plasma membrane using TIRF



IL-2R is enriched at the edge of the cell

Figure 2 for reviewers

The Boucrot paper suggested that this IL2 pathway was 'fast'. The TIRF data in this paper shows the lifetimes of the IL2 endocytic events to be of the same order of magnitude as clathrin-mediated endocytosis. Perhaps quantification of this could add to the discussion regarding the Boucrot claims of discovery of a "Fast endophilin-mediated endocytosis' pathway.

In Boucrot et al. they looked at endophillin recruitment to IL-2R containing vesicles after 10 min of IL-2 incubation using confocal microscopy. This work looked at a late stage: when the vesicle is detached from the plasma membrane. Moreover, cells were incubated during 10 min (a long time) with IL-2 to observe endophilin- IL-2-positive assemblies. In our work, we are looking at an earlier stage since we observed IL-2R at the PM using TIRF, allowing us to analyse the endocytosis from the beginning. Thus, our two studies did not perform the same experiment and thus cannot be compared.
In the FEME pathway the word **fast** can only be applied to some of cargoes like β1-adrenergic receptor for which the recruitment of endophilin is indeed very rapid "*Very shortly (t1/2 = 7 s) after addition of β1-AR but not β2- AR agonists, numerous endophilin–RFP-positive assemblies (EPAs) emanated from the cell edges and rapidly (1.62 ± 0.29 μm s−1) travelled to the perinuclear area » (*cited from Boucrot *et al*, Nature, 2015*).* Therefore, the speed of this FEME pathway might depend on the cargo and on the cell type.

Minor comments:
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Abstract: "nutriment"
Pg 3 "an important issue concern the mechanism"; "a dozen of factors"; "N-WASP and cortactin are regulated
differently upon the pathway"; "Beside this current model"
Pg 4: 'short' labeling vs 'brief' labeling.
These occur throughout the text.

We tried to do our best to correct these mistakes.

Referee #2

The manuscript by Basquin et al dissects in an elegant study using transmission electron microscopy, EM tomography, and live cell TIRF imaging the clathrin-independent endocytosis of the IL2-receptor. Clathrin-independent endocytosis has recently attracted much attention with the discovery of the FEME pathway (Boucrot et al., 2014, Nature) and several other proposed pathways. However, the mechanism and regulation of Clathrin-independent endocytosis is far from clear. Thus, this study is timely and of high interest to a wide audience. The authors reveal using TEM and EM tomography that IL2-receptor containing endocytic pits localise close to the base of cell protrusions. Mechanistically, they provide evidence for a role of the Scar/WAVE complex in the uptake of the IL2-receptor, which so far has not been implicated in endocytosis. They suggest that the IL2-receptor is in a biochemical complex with the Scar/WAVE complex. Most convincingly, they show sequential recruitment of Abi1 and N-WASP and two concomitant peaks of recruitment of the Arp2/3 complex to IL2-receptor containing endocytic assemblies at the plasma membrane. The authors had implicated N-WASP and dynamin previously in the uptake of the IL2-receptor. Furthermore, they suggest that dynamin acts as a "transition controller" for the recruitment of the two Arp2/3 activators Scar/WAVE and N-WASP. In general the experiments have been performed with great care.

However, it is not clear whether the authors analyse the same endocytic invaginations that they observe by EM at the base of protrusions, which appear to be located at the apical side of cells compared to the endocytic assemblies that they monitor using TIRF at the base of cells opposing the glass coverslip and where one would rarely observe protrusions.

I understand the point made by the referee but our data and previous works are not in agreement with it:
- All our EM images have been done from the basal side of the cells. The ultrathin sections were

done from the coverslip and up to maximum 800 nm. Therefore, all EM data showing protrusions were coming from the basal or the lateral side of the cell and never from the apical plasma membrane. To convince the referee about this, below there are some TEM images in figure 3 for reviewers, showing the first slice of the cell from the coverslip (ventral/basal side) where the referee can see many protrusions. Therefore, our TEM images show clearly that ventral sides of the cell have many protrusions.

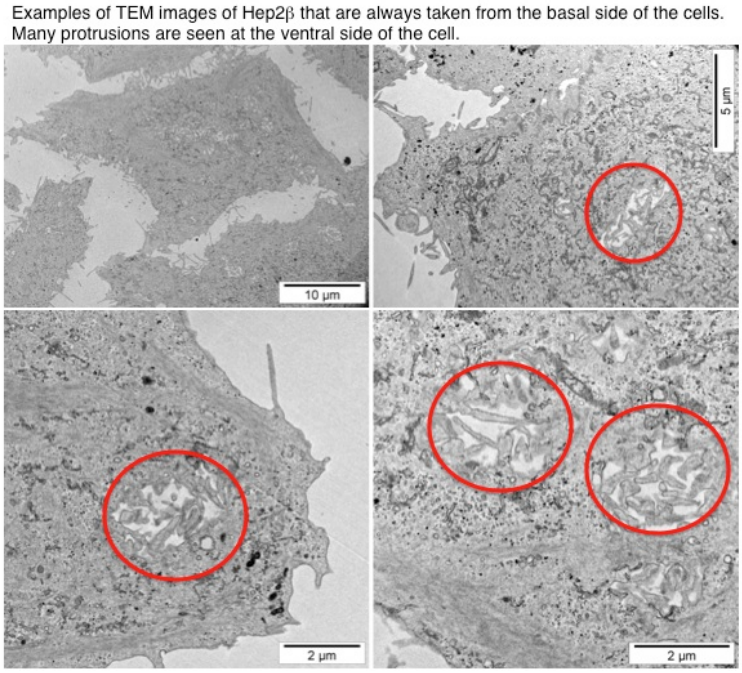

Figure 3 for reviewers

- About the use of TIRF microscopy to observe protrusions, many reports describe this technique to show these structures. In particular, TIRF has been used to observe invadopodia that are defined as protrusions from the basal side of the cell (Xu X, Johnson P, Mueller SC (2009) Breast cancer cell movement: imaging invadopodia by TIRF and IRM microscopy. *Methods in molecular biology (Clifton, NJ)* 571: 209-225).
Therefore, our TIRF and EM data came from the same side (basal) of the cell where protrusions can be seen. We add more details about the sectioning and EM observations in our revised manuscript.

Furthermore, the biochemical data suggesting a link between the IL2-R and the Scar/WAVE complex is rather weak. In general I would support a publication in the EMBO Journal if the authors repeat the TIRF experiments with another subunit of the Scar/WAVE complex besides GFP-Abi (which is not only a component of the Scar/WAVE complex but also binds N-WASP) and should address my mayor concerns. The authors should discuss their data in light of their recent publication that the IL2-R uptake is mediated by the FEME pathway. The authors should remove overstatements throughout the manuscript. Throughout the study, conclusions should not be drawn from experiments conducted with only one siRNA. Off-target effects cannot be excluded and it is standard in the field to use at least 2 siRNAs to control for these. Furthermore, the following concerns needs to be addressed before I can recommend publication.

Major concerns:
1. Fig. 2; Page 4: The results from Fig 2 I, O, need to be analysed for statistically significant differences.
Fig 2 I: At the base of protrusions (0nm), there appears to be no significant difference between CCPs and IL2-R positive invaginations. Therefore, the interpretation is an overstatement. There appears to be an association of both CCPs and IL-2R invaginations at the base of (filopodial) protrusions. CCPs but not IL2-R invaginations are also found in flat membrane sections.

Knockdown of clathrin heavy chain and analysis of IL2-R to base of protrusion would clarify whether these are clathrin independent invaginations.

Yes we did it, we statistically analysed (paired or unpaired t-test) our data from Fig. 2I, O, P. The difference between CCPs and IL2-R distribution at the base of protrusions is significant. It should be noted that Fig. 2I has been changed to answer one of reviewer 3's comments. In addition, we performed the immunogold labelling of IL-2R and TEM in a context where clathrin was depleted (using CHC siRNA). Our result shows that the depletion of clathrin does not affect IL-2R distribution close to protrusion (see Fig. S2C, D). Moreover, the referee can read our answer to reviewer 1 about our additional negative control using gold conjugate transferrin (Fig. S2A, B).

2. Fig 2O: page 5: the association of IL2-R invaginations with protrusions appear to be not induced by IL2 stimulation which is in contrast to what has been proposed for the FEME pathway (that the FEME pathway is induced by the respective ligands).

Yes our results do not show any effect of IL-2 on protrusion association of IL-2R. However, Boucrot et al. (Boucrot et al Nature 2015) showed a completely different set of experiments than our present study and thus they cannot be compared. Indeed, in this previous work they looked at endophilin recruitment to IL-2R in internalized vesicles after 10 min of IL-2. This is a late stage, when the vesicle is already detached from the plasma membrane. From our present study, we know that the WAVE complex is only recruited at an early stage of the process. The protrusion association of IL-2R is involved at an early step, during the pit initiation and not for the scission of the vesicle. However, the vesicle scission might be enhanced by IL-2 as we have already proposed in a previous publication (Basquin et al, JCS 2013). Our hypothesis is that IL-2 is not involved at an early stage of the endocytic process (the pit initiation) but probably at the end, during the scission of the vesicle.

3. Fig. 2J,K,L,P; page 5: The knockdown of dynamin needs to be done with two different siRNAs. Offtarget effects cannot be excluded and it is standard in the field to use at least 2 siRNAs to control for these. The number of IL2-R in unconstricted/tubular pits compared to pinched-off vesicles should be quantified to make this statement. The results from Fig 2 P, need to be analysed for statistically significant differences.

The referee is right about the putative off target effect of siRNA. Thus, we used another siRNA targeting Dnm2 (Dnm2.2) and we verified that IL-2R endocytosis was strongly affected by this treatment (Fig. S3) as it was the case for the another siRNA Dnm2.1 (Fig. S3). In addition, we also used this siRNA Dnm2.2 to immunoprecipitate the WAVE complex and we confirmed that the interaction IL-2R-WAVE complex was increased by the depletion (Fig. 6A). Since our 2 siRNAs targeting Dnm2 gave the same result on two kind of experiments, we can be confident in our conclusion and exclude an off target effect.

Fig. 3: General concern with IL2-R and transferrin uptake assays: Most labs in the field quantify and present the ratio of surface bound to internalised transferrin (or equivalent IL2-R) since knockdown of proteins may affect the amount of receptors present at the cell surface. The authors need to repeat their experiments using this quantification or show that their knockdown does not affect surface localization of receptors.

We totally agree with the reviewer. Each time, we normalise our endocytic quantification data (total vesicle intensity) obtained by immunofluorescence microscopy and ICY software with the results of the surface expression of the receptor assayed by immunostaining at 4°C and FACS. We have more precisely described this in the Material and Methods and we have provided all additional data: FACS and endocytosis with and without normalisation in Fig. S5.

Fig. 3D: Conclusions should not be drawn from experiments conducted with only one siRNA. Off-target effects cannot be excluded and it is standard in the field to use at least 2 siRNAs to control for these.

Again I agree with the referee but he can read in the Material and Methods that we have used 2 siRNAs for Abi1: UAAUAGCACCUGCGAAUAU and GGACGGAAUACUCCUUAUA, for Sra1: GAUAAACGGUUACGAUCAG and GAGUACGGCUCUCCUGGUA, and for Brk1: GGGCUAACCGGGAGUACAU and GGAGAAUAGAGUACAUUGA.

For Wave2 we used another siRNA depleting it (Wave2.2) and it has the same effect on IL-2R endocytosis than Wave2.1 (Fig. S5). Therefore, our data on the requirement of the WAVE complex during IL-2R uptake are very solid.

4. Fig. 3I: Abi knockdown reduces filopodia protrusions by 26%. If the IL2-Rs are distributed just by chance, wouldn't you expect a reduction of association already by 26%? Are the differences statistically significant? You observe a reduction in association by 50% in Abi KD cells. If you normalize for the reduction in filopodia number, is this a significant difference?

Here, I must apologize since there was an error in the text of the manuscript but not in the figure S3 (now named Fig. S4). The depletion of Abi1 led to a reduction of 18% (less than 20%) of total protrusion number (and not 26% as it is written in the text) and in this context 42% of IL-2R is nearby protrusions. In normal condition around 80% of IL-2R is close to protrusions, in contrast, around 40% of CCP and transferrin-gold conjugate are nearby these structures (Fig. S2). Thus, IL-2R cannot be distributed by chance close to protrusions when our two negative controls are not. A 50% difference of IL-2R distribution close to protrusions upon Abi1 depletion is clearly significant (with or without normalization). This figure S3 (now Fig. S4) was shown to explain the reader that Abi1 depletion did not affect drastically the total number of protrusions. However, the reviewer can also read in our manuscript at the end of this part that we could not distinguish whether WAVE is required for the formation of these protrusions or to drive IL-2R close to it *"This indicates that WAVE is required to drive the receptors to protrusions and/or is only involved in the formation of such protrusions».*

5. Fig. 4: Page 6: To make the statement that the IL2-R and the Scar/WAVE are in complex with each other, an endogenous coIP should be shown. The coIP's of the overexpressed proteins shown are not convincing since the appropriate controls are missing: The GFP-IL2-R control just controls for stickiness of GFP but not of stickiness of GFP-tagged subunits of the Scar/WAVE complex. An appropriate control would be to use a non-immune mouse IgG control for IP from the same lysate expressing GFP-tagged Scar/WAVE subunits and IL-2R. How often were the experiments repeated? The quantification of the blots should have been done from three blots from independent experiments and tested for statistically significance. Fig. 4B: The blots should not be stitched together from snippets. It is not clear whether these are from the same blot. The GFP lanes are clearly overexposed and cannot be used for quantifications.

I understand the comments of the reviewer.
First, all co-IP experiments have been done more than three times. Since it is not usual to read quantification of co-IP in publications, we initially decided to present them below each blot. To answer to the reviewer's point we now present the quantification of co-IPs in histograms with SD and performed t-test for statistical analysis (see Fig. S6), normalization was done using non-overexposed GFP signal.
Second, we also did new experiments of co-IPs with another negative control asked by the referee using an anti mouse IgG (see new Fig. 4B, Fig. 6A, Fig. S6). In addition, to show that the effect of siRNAs directed against Dnm2 (Dnm2.1) and Wave2 (Wave2.1) do not have off target effect, we have also used two additional siRNAs, Dnm2.2 and Wave2.2, and performed new co- IPs (Fig. 4B, 6A).
Finally, the original IP from Fig. 4B was presented in full (not stitched) in Fig. S6E showing the experiment performed using siRNA Wave2.1 and Dnm2.1 and now Fig. 4B represents a new IP experiment using the siRNA Wave2.2.

6. Fig. 4 Does the IL2-R contains a "WIRS" motif that has been identified by the Rosen lab in several receptors to mediate direct interaction with the Scar/WAVE complex? If not, how is the interaction mediated?

Yes, I thank the referee for asking this question. Based on the definition of the WIRS consensus motif, Φ-x-T/SF- x-x (Φ = preference for bulky hydrophobic residues; x = any residue, (Chen et al Cell, 2014)), we found that the IL-2Rβ cytoplasmic tail contains a potential WIRS (motif Y-c-T-F-p-s) that is conserved throughout the mammals except rodents (see the new Fig. 4C). We have mutated the two conserved amino acids of the WIRS, T420 and F421 and replaced them by alanines to generate a mutant of IL-2Rβ named TFAA. This mutant was stably transfected into Hep2 cells. Normalised endocytosis results for this mutant show a 50% reduction when compared to IL-2Rβ

WT (see the new Fig. 4D). Moreover, we could not co-immunoprecipitate this mutant with the WAVE complex (see the new Fig. 4C, D, E). Thus, we conclude that IL-2Rβ-WAVE interaction is mediated by a WIRS motif within the receptor polypeptide.

7. Fig. 5: Very nice data and important result! However, it is not clear whether the authors analyse the same endocytic invaginations that they observe by EM at the base of protrusions which appear to be located at the apical side of cells compared to the endocytic assemblies that they monitor using TIRF at the base of cells opposing the glass coverslip and where one would rarely observe protrusions. For Fig 5a GFP-Abi was used. Since Abi can interact not only with the Scar/WAVE complex but also with N-WASP, this should be verified with another, more specific subunit of the Scar/WAVE complex.

To answer to the referee, we have tested another subunit of the Scar/WAVE complex using GFP-Brk1. Although GFP-Brk1 was less easy to observe by TIRF microscopy than Abi1, due to a weaker signal at the plasma membrane, we saw a comparable recruitment of GFP-Brk1 than GFP-Abi1, during IL-2R endocytosis (Fig. S7). In summary, Brk1 appears before IL-2R and stays much longer associated when dynamin is depleted.

8. Fig. 6: page 8: Dynamin should be knocked down with two independent siRNAs. The coIP's of the overexpressed proteins shown are not convincing since the appropriate controls are missing: The GFPIL2- R control just controls for stickiness of GFP but not of stickiness of GFP-tagged subunits of the Scar/WAVE complex. An appropriate control would be to use a non-immune mouse IgG control for IP from the same lysate expressing GFP-tagged Scar/WAVE subunits and IL-2R. How often were the experiments repeated? The quantification of the blots should have been done from three blots from independent experiments and tested for statistically significance. The GFP lanes are clearly overexposed and cannot be used for quantifications.

See my comment to point 5 and new Fig. 6A and Fig. S6

9. Fig 5G and Fig 6E. This data should be in one graph together and the significance of the difference statistically analysed.

This has been done and put in Fig. 6E.

10. It is an overstatement that dynamin "controls" the departure of the Scar/WAVE complex and is necessary for N-WASP recruitment. Since you don't provide the molecular mechanism, this might just be a consequence of deficiency of scission.

Ok we tried to be milder in our conclusions.

Minor concerns:
1. Fig. 1; Page 4; Do these intracellular vesicles (early endosomes and MVBs) look different from CME derived early endosomes and MVBs? The last sentence that IL-2R containing intracellular vesicles are morphologically distinct from "all known endocytic carriers" is thus an overstatement.

No, early endosomes and MVBs are not different than regular ones. We are talking about the new-born endocytic vesicles containing IL-2R before their fusion with EE (Fig.1A, B, E) that do not have a complete spherical shape (as clathrin vesicles and caveolae), nor having a tubulo-ring shape structure like in GEEC-CLIC pathway. I tried to clarify this point in the revised manuscript.

2. Fig. 2; page 4: What type of protrusions are these? These appear to be filopodia, which are not thought to arise from Scar/WAVE mediated actin nucleation. Please clarify.

Sometimes IL-2R is close to filopodia but often it is associated to other membrane extensions, therefore we called them protrusions.

3. Fig. 5 G: Only 20% colocalization between IL2-R and Abi or N-WASP has been observed, was this analysis done from fixed images or from movies. If from movies at which time point?

The analysis was done on whole movies. The co-localization rate is a mean from all time points of

the movies.

3. FigS3: It would be more convincing if not just an Arp2/3 inhibitor was used but if this would be verified by knockdown of Arp2/3.

As this supplementary Fig.S3 (now FiG S4) was only a verification, we did not perform this additional experiment asked by the reviewer.

4. Fig 3 C: quantifications of knockdowns: was this done just from one blot? This should be done from 3 independent experiments. For Sra1 and Brk1 the blots appear to have been stitched together. Are the individual snippets from the same film with same exposure?

See my comment to point 5

Referee #3:

In this manuscript the authors describe a series of interesting findings about the mechanisms of clathrin independent endocytosis of interleukin-2 receptor. They first show that IL2R internalization is closely associated with plasma membrane protrusions. Then the authors show that WAVE complex is involved in IL2R internalization and show that the WAVE complex is recruited in a specific time window before N-WASP recruitment. The findings are interesting and the experiments mostly well done. I have some comments and suggestions to improve the manuscript.
- The authors could explain more clearly why the two cell lines were chosen for the studies.

Here we modified the text of the manuscript to clarify this:
« As IL-2R internalization occurs in several cell types, constitutively or ligand-induced, we decided to use Hep2β cells, a human epithelial cell line stably expressing IL-2Rβ (Grassart et al, 2008) to address the constitutive endocytosis and Kit225, a human T cell line expressing the endogenous high affinity IL-2R, to study the IL-2-induced endocytosis »

- The meaning of the following sentence on page 3 is not clear. Please, rewrite. "...we performed immunogold labeling of IL-2Rbeta combined to a kinetic of endocytosis that we finally observed by transmission electron microscopy".

We changed it for *"To characterize the morphology of membrane and intracellular carriers during all the stages of endocytosis, we performed a cell surface immunogold labelling of IL-2R. We incubated cells at 37°C for several time points to allow for endocytosis, fixed them, and observed them by transmission electron microscopy (TEM).»*

- On page 4 the authors write: "...protein A-gold alone showed very rarely gold particles...". It would be important to provide a more quantitative estimate of the background level. The quantitation that is presented later is somewhat meaningless if the background level is not defined.

In Fig. S1 the reviewer can see the number of beads/sections in negative and positive controls that is a quantitative estimate of the background level. More precisely, we quantified for the negative control (proteinAgold) 7 beads among 260 sections (2.6%) in contrast for the positive control (antibody+ proteinA-gold) we observed 398 beads among 464 sections analysed (85.8%). I do not see another way to perform this estimate.

- What are the "various shapes" of the vesicles mentioned on page 4. Please, describe the shapes.

I agree with the reviewer that it was not clear. We changed for *« in vesicles of ovoid shapes having sizes from 40 to 180 nm »*

- How were the CCPs identified from the EM images? This should be explained in the text.

CCPs are identified visually by the presence of the electron-dense coating of the membrane having a regular structure of about 100 nm in diameter.

- I have some concerns about the quantification of the distance of gold particles from the

protrusions. According to the figure 2I the membrane areas in the categories "On protrusion" and "Base" are larger that the other categories. Therefore even random beads would fall more frequently on those larger categories. I think it would be important to control for this effect, for example by calculating the membrane areas in different categories and calculating the true enrichment on protrusions and at their bases. In principle, randomly binding gold particles would be a better control than the CCPs, but I'm not sure how feasible that would be in practice.

I understand the referee's comment. It is true that in our initial Fig. 2I the "Base" category had a membrane area of 2 times larger than the others (except "> 500nm" and "On" categories). Therefore, we changed Fig. 2I in this revised version where we defined 5 classes of location with respect to protrusions (Fig. 2I): on protrusion, at its base, 100-300 nm, 300-500 nm and >500 nm from the next protrusion.
In addition, to strengthen our data we added another negative control using transferrin (Tf) gold conjugate. The results (Fig. S2) show that Tf follows a distribution close to CCP: 40% of Tf was close to protrusions (< 300 nm) and 60% were in flat membrane (> 300 nm). Thus, Tf distribution is in marked contrast to IL-2R distribution. These new data strengthen our conclusion that the distribution of IL-2R close to membrane protrusions is specific.

-The claim (on page 5 and 8) that the EM results confirm the role of dynamin in scission seems over interpreted. No quantitative data is shown to support this claim. The U-shaped pit in 2L doesn't look different from the other normal pits shown in other panels of Figure 2. Also, it is difficult from a thin section to conclude that the tubule in 2K is truly an endocytic tubule and not just a furrow at the cell surface. I would recommend toning down this claim and focusing on the conclusions that are better supported by the data, i.e. that dynamin depletion blocks the uptake of IL2R but that it doesn't prevent it's association with the protrusions.

I agree that we do not have quantified data about this, but rather qualitative ones. We have thus toned down this claim.

- On few places in the manuscript the term "filopodia" is used instead of "protrusion". The authors should use only one term or explain what is the difference between these two terms if there is some need to use both.

We deleted filopodia in the revised manuscript.

- Figures 5H and 6F. It wasn't clear to me how the intensity curves are aligned in time. This should be explained clearly. Also, what do the error bars represent? They are very small. For example, the errors in the IL2R curve are smaller than the fluctuations of the curve between ~50-70 s.

We tried to describe it better in the revised manuscript. The analysis was done on at least 1000 IL-2R tracks per condition coming from 10 movies (except for GFP-Brk1, 5 movies). Then, we searched for co-localised tracks with GFP-Abi1/Brk1 (WAVE), GFP-N-WASP or GFP-16 (Arp2/3) and determined a rate of co-localisation (Fig 6E). We used the data from all co-localised tracks to obtain
the mean time of recruitment (appearance/departure) of WAVE, N-WASP or Arp2/3 with respect to IL-2R tracks (time=0 represents the initiation of IL-2R track (mean +/- S.E.).
Finally, we quantified the fluorescence 5 frames before and 5 frames after the appearance/departure of each track, normalized the data upon max intensity of each track and exported into Excel file to draw the curve of intensities for WAVE, N-WASP, Arp2/3 and IL-2R. We compared IL-2R tracks of similar duration with the mean fluorescence intensity profiles of WAVE, N-WASP and Arp2/3; time 0 being arbitrarily determined by the first protein recruited (for Fig. 5G, 6F mean of 10 tracks, for Fig. S7C, D mean of 5 tracks, +/- S.E.).

2nd Editorial Decision 26 May 2015

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are all in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #1 and #2's remaining concerns and to provide a final version of your manuscript.

A few editorial points need to be taken care of at this stage as well:

- please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.
- please suggest (in a cover letter) a one-sentence summary 'blurb' of your paper, as well as 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper; this will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- please add references and slightly change the wording to avoid self-plagiarism/plagiarism at the beginning of the introduction and in the methods description (please see attached screenshots)
- as you might know, we encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE COMMENTS

Referee #1:

The authors have done a good job in responding to the previous review. My apologies for not connecting them with the Boucrot et al paper, but this is in part due to the confusion in re-naming this pathway. I personally do not like the FEME name, as the authors here show that it is not necessarily 'fast'. Also, in contrast to the claims of Boucrot et al and those made in the first paragraph of the discussion it is not a 'new mechanism', rather these authors and Boucrot et al., are adding important new insight into a pathway originally described by Lamaze et al, 2001. It would be helpful to be explicit about this in the Introduction so as not to confuse. Likewise in the abstract the authors refer to this as "The clathrin and caveolae-independent pathway" rather than "A clathrin and caveolae-independent pathway".

I would also urge caution as to the interpretation of the dynasore and dyng4a experiments as De Camilli has shown that these compounds inhibit clathrin and caveolin-independent endocytosis even in dynamin triple knock out cells. Clearly their effects are off target.

Changes in the text, rather than any additional experimentation would address my concerns .

Referee #2:

In the revision of the manuscript by Basquin et al the authors have addressed all my major concerns. The new data directly linking the IL2-receptor to the Scar/WAVE complex is exciting. This is now a very well done study and I can recommend publication in the EMBO Journal and to highlight this paper since its findings on clathrin independent endocytosis should be of interest for a wide cell biology audience.

Minor comment: In Fig S3 the labelling of the western blot appears mislabelled: dynamin and WAVE should be swapped.

Referee #3:

The authors have answered all my concerns in their revised manuscript.

2nd Revision - authors' response 27 May 2015

We would like to submit our final version of our manuscript "Membrane protrusion powers clathrin-independent endocytosis of interleukin-2 receptor" [Paper #EMBOJ-2014-90788].

We answered to the reviewers 1 and 2 and avoid self-plagiarism and thus we changed the text, Fig S3 and uploaded all figure files in adequate resolution and quality for production (only for main figures not for supplemental ones).

Thank you for your interest in this contribution and we are very much looking forward to reading your comments about this revised version of our manuscript.

