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Review COMMONS

Upregulated-flotillins and sphingosine kinase 2 derail AXL vesicular traffic promoting epithelial-mesenchymal transition

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Reviewer 1

Evidence, reproducibility and clarity

The paper by Genest et al. describes the effect of flotillins and sphingosine kinase 2 to stabilize AXL as a mechanism to promote epithelial-mesenchymal transition in breast (cancer) cells. The potential role of vesicles trafficking EMT-promoting proteins is of high interest in the field, also for exploring new opportunities of pharmacological targeting. However, the paper fails to convincingly demonstrate that the proposed mechanism is of real importance to support or promote EMT for the following main reasons:

1) The role of flotillins is studied only by overexpression and in the context of non-cancerous MCF10A cells, while breast cancer cells of epithelial-like origin are not analyzed. This is contrast with the purpose of the paper (see abstract, introduction, patients' data) which is to study tumors and EMT. Effect of shRNAs is also not reported, making it difficult to estimate the importance on the EMT phenotype. Then, alteration of EMT should be concluded also from other non-genetic functional parameters, not just by markers. For instance: was morphology of the cells changed? Was cell migration affected with F1F2?

2) AXL up-regulation is not very strong (2-fold). What is unclear is if the minimal AXL increase due to F1F2 really really provides a significant contribution to the EMT phenotype (as the authors conclude). The siRNA experiment knocks down all AXL, not just the F1F2- induced levels, making it difficult to estimate the real effect of the mechanism proposed. Why didn't the author focus on EphA4 (or to a lesser extent ALK), which showed better regulation?

3) The conclusions of the manuscript are contradicted by the reported clinical data. In Figure S4 the authors clearly observe co-expression of Flotillin 1 and AXL prevalently in luminal breast cancers, which is the subtype known to not be driven by EMT. This evidence already indicates that this (otherwise interesting) mechanism is not relevant to EMT in breast cancer. So, the conclusions are not supported by the data, and the experimental setup and model chosen are not appropriate to generalize the findings to cancer.

Minor (here the most important):

4) The point of the Figure 2 is not clear. Why this part should have such a central role in the story? The entire data presented are not followed up in the rest of the paper. Moreover, in some cases upregulations also questionably significant (like RAS and STAT3 are not even 2 fold). Moreover, the error bars are so small that it seems unrealistic that the plots indicate three independent experiments.

5) More robust statistical analysis should be provided in the Figure 1 to support that EMT is suppressed with F1F2 overexpression. For instance a more standard GSEA on hallmark signatures.

6) In Figure 3 E-Cadherin is rescued with siAXL in the IF but not in the western blot.

7) Some sentences require clarifications. The authors should be more clear on why ZEB2 antibody was not available or what they mean with "Unfortunately the available tools..".

8) Western blot from the CHX experiment should be shown, at least in the supplements. Again, the standard deviation in this experiment is minimal, was this really an average of three independent experiments (and not three western on the same lysates)?

9) All conclusions are derived from one single cells MCF10a. NMuMG cells are shown at the beginning but not used for the rest of the paper. Anyway, if this wants to be a cancer research paper, then cancer cells needs to be used.

10) The methods section contains inconsistent data about patients' samples (9 are indicated, but the Figure S4 features 37). Then, where those other 527 come from?

11) Some figures do not match with the legends or with the description in the text. It has not been easy to review this paper.

Significance

I am a cancer biologist working on EMT.

Referee Cross-commenting

I have nothing to comment on other's reviews.

Reviewer 2

Evidence, reproducibility and clarity

Genest and co-authors present in this paper new fascinating evidence on how intracellular trafficking can modulate oncogenic sigalling.

First of all, they show how overexpression of Flotillin1 and 2 in non-cancerous breast lines can induce a strong reprogramming towards a EMT phenotype. They analyse mRNA and protein expression, intracellular distribution of activated proteins, cell phenotypes to demonstrate a strong activation of oncogenic signalling pathways. They then identify AXL as a key player in this process and show how this protein is stabilised upon Flotillin expression. The authors use an amazing variety of approaches to study the endocytosis and the trafficking of endogenous, GFP-tagged, Halo-tagged and Myc-tagged AXL in different cell lines and their data are strong and very convincing, the images are of very high quality and the analysis rigorous. Their data strongly support the hypothesis that high Flotillin levels triggers AXL endocytosis and accumulation in non-degradative late endosomes where signalling remains active. The authors then show how SphK2 has a key role in AXL stabilisation, it colocalises with Flotillin, AXL and CD63 and its activity (which they block by using inhibitors or siRNA) is necessary for flotillin-induced AXL stabilisation and EMT induction.

The paper is extremely well written, the data flow logically and they are appropriately presented and analysed.

I don't have any major comment and I believe the paper is suitable for publication.

I have only some minor comments/questions:

1) did the authors try to colocalise AXL with endogenous Flotillin in MDA-MB-231 cells? They could use the antibodies used in Fig S1B. Of note, the authors have shown it in luminal tumours in Fig S4C.

2) In Fig6G, it appears that AXL-Flotillin colocalization is lost upon SphK2 inhibition. Is this the case? It could be that the correct lipids are necessary for the formation of Flotillin- positive internalisation domains and this could be very interesting and reinforce the model proposed in the paper.

3) I would remove the sentence on line 995-997 "to our knowledge this is the first report to describe ligand-independent AXL stabilization..." as the cells are not serum starved in all experiments and animal serum can contain variable amounts of the ligand GAS6.

Please note that the authors don't have to necessarily address comments 1-2, their paper is already very rich in convincing data.

Significance

AXL is a major oncogene that promotes EMT in a variety of tumour types. Understanding how its signalling can be triggered by endocytic pathways even in cells that are non- cancerous is very important and of high significance for the cancer field and the trafficking community.

Reviewer 3

Evidence, reproducibility and clarity

This is an interesting and well written paper describing that upregulated flotillin promotes an endocytic pathway called upregulated flotillins-induced trafficking (UFIT) that mediates AXL endocytosis and allows its stabilization. Consequently, stabilized AXL in these flotillin-positive late endosomes enhances activation of oncogenic signaling pathways that promotes EMT. The authors suggest that Flotillin upregulation-induced AXL stabilization requires the activity of SphK2. However, this latter point is not supported by the data and further studies are needed to support this important conclusion.

Major concerns:

1. Most of the conclusions are based on effects of high concentrations (50 uM) of an ill-defined SphK2 inhibitor. The experiment described in Figure 6C-H need to be confirmed by downregulation of SphK2.
2. Does overexpression of SphK2 reverse the effects of the SphK2 inhibitor? In a similar manner, does overexpression of SphK2 enhance stabilization of AXL?
2. Although the authors suggest recruitment of SphK2 and formation of S1P in UFIT, there are no measurements of S1P. Also, there is no indication that SphK2 is activated despite the fact that ERK and AKT are activated in UFIT and are known to phosphorylate and activate SphK2. Is SphK2 that is recruited to flotillin phosphorylated?
3. It should be determined whether the optogenetic system used to induce flotillin oligomerization also induces recruitment and activation of SphK2.
4. Most importantly, it has not been established that the effects are mediated by S1P. Does addition of S1P enhance stabilization of AXL? Are the effects of S1P mediated by a S1P receptor? If so, which S1P receptor? There are several specific agonists and antagonists of S1PRs that can be utilized to answer this question. It's also possible that the effects of S1P are mediated by intracellular actions as were suggested by the De Camilli group (Nat Cell Biol. 2014 Jul;16(7):652-62).

5. There is a commercial antibody for endogenous SphK2 that can be used to validate and substantiate the data with GFP-SphK2. (F1000Res . 2016 Dec 6;5:2825. doi: 10.12688/f1000research.10336.2. eCollection 2016. Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence)

Significance

This is an interesting paper. If the authors confirm the involvement of Sphk2 and mechanism of action of S1P, this would be an important contribution to the field.

Author response to reviewers' comments

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

The paper by Genest et al. describes the effect of flotillins and sphingosine kinase 2 to stabilize AXL as a mechanism to promote epithelial-mesenchymal transition in breast (cancer) cells. The potential role of vesicles trafficking EMT-promoting proteins is of high interest in the field, also for exploring new opportunities of pharmacological targeting. However, the paper fails to convincingly demonstrate that the proposed mechanism is of real importance to support or promote EMT for the following main reasons:

1-a) The role of flotillins is studied only by overexpression and in the context of non-cancerous MCF10A cells, while breast cancer cells of epithelial-like origin are not analyzed.

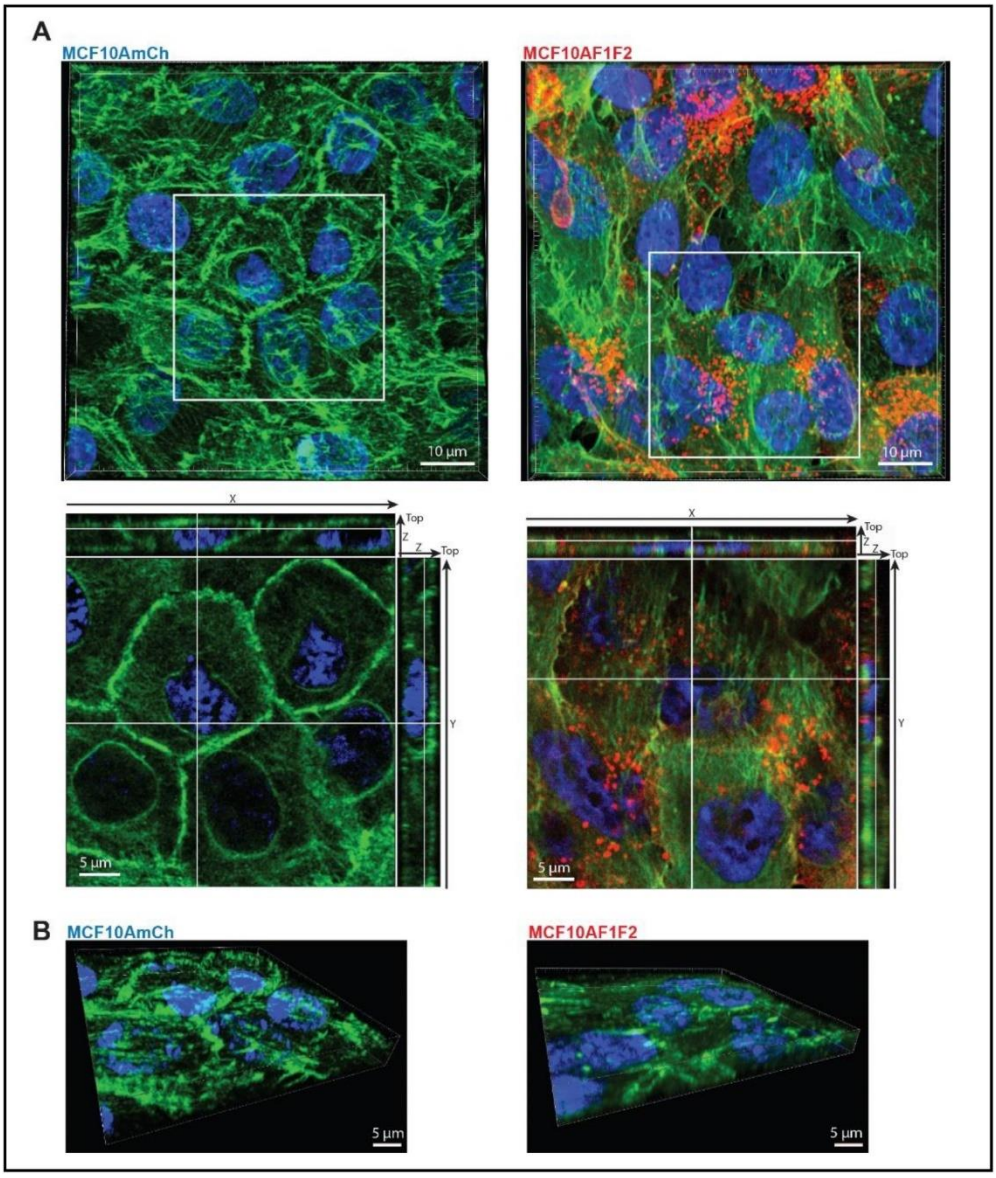
Regarding the first part of the point raised here, we are not sure to understand correctly the sentence "[…] while breast cancer cells of epithelial-like origin are not analyzed". Indeed, we used the breast cancer cell line MDA-MB-231 and a derived cell line that we generated by knocking down flotillin expression (MDA-MB-231shFlot2) in the second part of this study (Figure 6C, F and H and S7A, E and F). This previously characterized cell line allowed us to demonstrate that abolishing flotillin overexpression was sufficient to significantly inhibit the invasive properties of MDA-MB-231 cells (Planchon et al, J Cell Science 2018, https://doi.org/10.1242/jcs.218925
Although flotillin upregulation induces some major mechanisms of the EMT process in MCF10A cells, flotillin downregulation was not sufficient to reverse the EMT phenotype in MDA-MB-231 cells. This could be explained by the fact that EMT is a multifactorial process and that MDA-MB-231 cells went through too many irreversible changes leading to this process. By contrast, when we analyzed EMT markers after SphK2 inhibition or knock down in MCF10AF1F2 and in MDA-MB-231 cells (Figure 6A-C), we could observe a significant decrease in ZEB1 expression.

1-b) This is contrast with the purpose of the paper (see abstract, introduction, patients' data) which is to study tumors and EMT. Effect of shRNAs is also not reported, making it difficult to estimate the importance on the EMT phenotype.

As we mentioned in our manuscript, previous studies by other groups who downregulated flotillin expression in different cancer cell lines using siRNA approaches or re-expression of miRNAs that inhibit flotillin expression, already showed flotillin participation in EMT (for review please see, Gauthier-Rouvière et al, Cancer Metastasis Review, 2020, doi: 10.1007/s10555-020-09873-y).
In this context, the novelty and the first goal of our study was to investigate how strong is the contribution of flotillin upregulation to EMT induction. To achieve this goal, we chose on purpose to use non-tumoral epithelial cells that do not harbor the anomalies already favoring EMT, unlike the cancer cell lines used in previous studies. In these non-tumoral models (the human MCF10A and mouse NMuMG mammary epithelial cell lines), we ectopically overexpressed flotillins (MCF10AF1F2 and NMuMGF1F2) to levels similar to what observed in invasive breast cancer cells. Using this approach, we found that flotillin overexpression is enough to induce EMT.

1-c) Then, alteration of EMT should be concluded also from other non-genetic functional parameters, not just by markers. For instance: was morphology of the cells changed? Was cell migration affected with F1F2?
Our conclusion that flotillin upregulation is sufficient to induce EMT in MCF10AF1F2 and NMuMGF1F2 cells is not based only on genetic functional parameters or markers. For instance, Figure S1 (panels H and I) shows a strong modification of the cell morphology and of the actin cytoskeleton organization in NMuMG cells upon flotillin upregulation. NMuMGF1F2 cells became flat and lost their apical F-actin belt and exhibited an increase instress fibers.

As shown below (Additional Figure 1), similar modifications of the cell morphology and of theF-actin cytoskeleton organization occur also when flotillins are upregulated in MCF10A cells(see below the comparison of MCF10A and MCF10AF1F2 cells) (**these data could be added in the manuscript**).



Additional figure 1: Upregulation of flotillins in MCF10A cells leads to changes in the cell morphology and in F-actin cytoskeleton organization. Comparison of the morphology and of the actin cytoskeleton organization in MCF10AmCh and MCF10AF1F2 cells. Confluent cells were fixed and stained for F-actin (green) using Alexa488- conjugated-Phalloidin and for nuclei (blue) using Hoechst (in panel A flotillin2-mCherry signal is shown).
1) Upper panels show the maximum intensity projection images (MIP) of MCF10AmCh (control) and MCF10AF1F2 (flotillin overexpression) cells obtained from a stack of images acquired by confocal microscopy.

Lower panels show magnified images from the boxed areas, including one single plane and the x-z and y-zprojections along the indicated axes.
2) 3D reconstruction images obtained from the region in the boxed area from the MIP-images shown in A. *These data show that in MCF10AF1F2 cells the apical actin belt is lost and the height of the cellular monolayer islower compared with control MCF10AmCh cells.*

*We also analyzed the migration capacity of these cells (shown in Figure 3G of the submitted manuscript). Briefly, using a Boyden chamber assay, we showed that flotillin upregulation significantly increased migration of MCF10A cells (Figure 3G). We previously demonstrated that flotillin upregulation also promotes cell invasion in 3D using a spheroid assay (Planchon et al, J Cell Science, 2018, https://doi.org/10.1242/jcs.218925). As shown below (Additional Figure 2), using a wound healing assay, we also observed that cell velocity is higher in flotillin-overexpressing NMuMGF1F2 cells than in control NMuMG cells (**this could be added to the manuscript**).*

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

2) AXL up-regulation is not very strong (2-fold). What is unclear is if the minimal AXL increase due to F1F2 really provides a significant contribution to the EMT phenotype (as the authors conclude). The siRNA experiment knocks down all AXL, not just the F1F2-induced levels, making it difficult to estimate the real effect of the mechanism proposed.

As shown in figure 3A and D, in MCF10AF1F2 cells compared with MCF10AmCh cells, we measured a significant 2.5 ± 0.7-fold increase in the AXL protein level. We do not think that this can be considered as a minimal increase.
Considering that flotillin upregulation may affect simultaneously different receptors (Figure S2I, Figure S6A-F), we did not expect that downregulating a single receptor would have a major impact on the level of EMT markers and on cell migration. Yet, after knocking down AXLin MCF10AF1F2 cells, we observed a decrease in ZEB1 and N-cadherin expression and the re-expression of E-cadherin (Figure 3D-F) and the inhibition of cell migration (Figure 3G). The fact that we observed such an effect by downregulating AXL, which according to Reviewer#1 is minimally increased, might be explained by its well-known ability to act not alone but through cross-talk with other signaling receptors (Graham et al, Nature Reviews Cancer 2014;Halmos and Haura, Science Signaling 2016; Colavito et al, Journal of Oncology 2020).
As suggested by Reviewer #1, ideally, it would be interesting to bring back AXL to its level in MCF10AmCh cells to better evaluate only the contribution of its increase. However, adjustingso precisely the efficacy of AXL downregulation by siRNA seems quite difficult to achieve.

3) Why didn't the author focus on EphA4 (or to a lesser extent ALK), which showed better regulation ?
As we mentioned (page 18) "the available tools allowed us to validate this result only for AXL,but not for EphA4 and ALK".
Nevertheless, for EphA4, we showed in Figure S6 that it is located in flotillin-positive late endosomes (Figure S6 A and C, for MCF10AF1F2 and NMuMGF1F2 cells, respectively) in a phosphorylated form (using an antibody against P-Y588/Y596-EphA4 that works in NMuMG cells, Figure S6D). However, the signals obtained by western blotting using the same antibodywere too low to validate any significant variation of EphA4 Y-phosphorylation status, as suggested by the results from the phospho-RTK array.
Regarding ALK, the increase in its phosphorylation, suggested by the phospho-RTK array, remains puzzling to us. By western blotting of cell lysates and in the presence of positive controls, we did not detect any positive signal for phosphorylated ALK and even for total ALKin MCF10A and MCF10AF1F2 cells. In addition, to our knowledge, ALK expression in MCF10A cells has never been reported in the literature. These observations did not encourageus to pursue our investigations on ALK.
Moreover, several points led us to focus on AXL. Indeed, AXL expression is associated with the acquisition of a mesenchymal cell phenotype, invasive properties, and resistance to treatments

and AXL is an attractive therapeutic target against which several inhibitors are in preclinical and clinical development (Shen Y et al. Life Sciences 2018). Moreover, AXL expression in tumors is attributed to post-transcriptional regulation, but the mechanisms are totally unknown. Understanding how its stabilization and signaling can be triggered by flotillin- mediated endocytic pathways is new and of high significance for the cancer field and the trafficking community.

3) The conclusions of the manuscript are contradicted by the reported clinical data. In Figure S4 the authors clearly observe co-expression of Flotillin 1 and AXL prevalently in luminal breast cancers, which is the subtype known to not be driven by EMT. This evidence already indicates that this (otherwise interesting) mechanism is not relevant to EMT in breast cancer. So, the conclusions are not supported by the data, and the experimental setup and model chosen are not appropriate to generalize the findings to cancer.

We acknowledge that flotillin 1/AXL co-expression is highest in the luminal subtype. If this co-expression was observed only in this particular subtype, we would have agreed that it excluded that flotillins and AXL co-overexpression may participate in EMT in tumor cells. However, our results show that flotillin 1 and AXL are co-expressed also in other subtypes that have undergone EMT. Considering this observation and the influence of flotillin upregulation on AXL overexpression we reported here, we believe that the point raised by the Reviewer is not sufficient to exclude that the co-upregulation of flotillins and AXL can participate in EMT induction in breast cancer cells.

Minor (here the most important):
4) The point of the Figure 2 is not clear. Why this part should have such a central role in the story? The entire data presented are not followed up in the rest of the paper. Moreover, in some cases upregulations also questionably significant (like RAS and STAT3 are not even 2 fold).
Moreover, the error bars are so small that it seems unrealistic that the plots indicate three independent experiments.

Because the activation of oncogenic signaling pathways is crucial to promote EMT, we think that analyzing these pathways in the context of flotillin upregulation is coherent with the message of the paper.
To our knowledge, the amplitude of up- or down-regulation has nothing to do with its significance. The amplitude also depends strongly on the context (stimulation with an agonist, overexpression of GEF, etc). For instance, increases lower than 2-fold are frequently reported (Bodin and Welch, Mol Biol Cell, 2005; Miura SI et al, Arteriosclerosis, Thromb and Vasc Biology, 2003; Matsunaga-Udagawa R et al, J Bio Chem 2010) when assessing the activity of Ras or small GTPases, but they represent real upregulations. Furthermore, Ras activation is supported by the downstream 4-fold activation of ERK that we measured (Figure 2C).
In Figure 2, panels B, C, E, F and J, considering the amplitude of the mean increases shown, the error bars corresponding to SEM do not seem disproportionately small.
As the Reviewer seems to insinuate that we have not performed independent experiments, we are presenting in the table below the detailed results all obtained from independent experiments.

Panel	***Parameter measured***	***Number of independent experiments***	***Fold of increase value in MCF10AF1F2 cells compared with MCF10AmCh cells in each experiment***	***Mean***	***SEM***	***p-value***
B	*Ras-GTP*	*5*	*1.95 ; 1.96 ; 1.18 ; 1.67 ; 1.86*	*1.72*	*0.14*	*0.001*
C	*Phospho- ERK*	*5*	*1.24 ; 5.43 ; 3.22 ; 6.11 ; 3.52*	*3.71*	*0.73*	*0.0042*
E	*Phospho-AKT*	*4*	*2.29 ; 6.54 ; 3.76 ; 2.6*	*3.8*	*0.97*	*0.0276*
F	*Phospho-STAT3*	*4*	*1.63 ; 1.63 ; 2.42 ; 1.60*	*1.82*	*0.20*	*0.0066*
J	*Phospho-SMAD3*	*8*	*4.1 ; 5.12 ; 6.29 ; 1.82 ; 2.58 ; 6.66 ; 2.82 ; 5.40*	*4.35*	*0.64*	*0.0001*

In the legend to figure 2 panels C, E, F, J, "The histograms show […] with control MCF10AmCh cells calculated from 4 independent experiments" was corrected by "The histograms show […] with control MCF10AmCh cells calculated from <u>at least</u> 4 independent experiments" as data

shown in panel J were actually calculated from 8 independent experiments.

5) More robust statistical analysis should be provided in the Figure 1 to support that EMT is suppressed with F1F2 overexpression. For instance a more standard GSEA on hallmark signatures.
To avoid confusion, we understand that Reviewer #1 meant "… that EMT is induced with F1F2 overexpression" and not "… suppressed …".
As recommended by Reviewer #1, ***we performed a GSEA on the hallmark signature and the results are already included in the current revised version of our manuscript (figure 1C).***

6) In Figure 3 E-Cadherin is rescued with siAXL in the IF but not in the western blot.

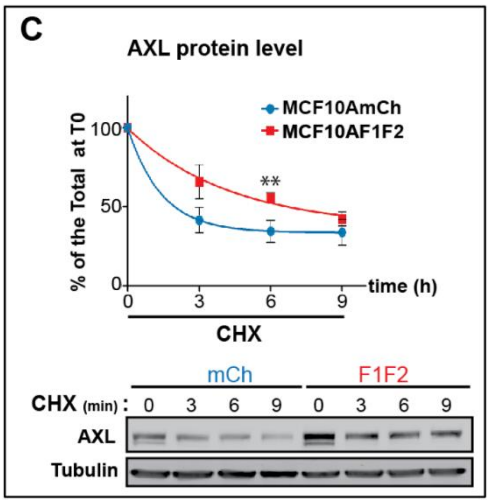
Using siRNA transfection, we can have a mosaic effect due to the fact that not all the cells of the sample are transfected and thus efficiently knocked down. This mosaicism was clear when

we analyzed E-cadherin by immunocytochemistry. Indeed, in some cells, probably the ones that have been more efficiently transfected with the AXL siRNA, E-cadherin expression is clearly seen. By western blotting, which provides a global analysis in which transfected and non-transfected cells are mixed, this was not significantly higher than in MCF10AF1F2 cells transfected with a control siRNA, although there was a trend towards increased E-cadherin expression in MCF10AF1F2 transfected with the AXL siRNA.
For the revised version of our manuscript we will try to improve the efficacy of the AXL siRNAand test whether we can fully rescue E-cadherin expression. ***The corresponding panel could be modified according to the data we will obtain.***

7) Some sentences require clarifications. The authors should be more clear on why ZEB2 antibody was not available or what they mean with "Unfortunately the available tools..". *Page 7: we wrote «no anti-Zeb2 antibody is available». We should have said: «none of the anti- Zeb2 antibodies tested worked in MCF10A cells». We decided to remove "no anti-Zeb2 antibody is available" from the sentence to avoid confusion in the revised version of our manuscript.*
Page 19: we wrote «unfortunately the available tools» to refer the available tools against EphA4 and ALK that did not allow us to validate the data obtained using the phospho-RTK array showing that the Y-phosphorylation of these two RTK is increased in cells with upregulated flotillins. (see also our answer to major point 2).

8) Western blot from the CHX experiment should be shown, at least in the supplements. Again, the standard deviation in this experiment is minimal, was this really an average of three independent experiments (and not three western on the same lysates)?

As asked, a representative western blot ***is now shown in Figure 3C in the current revised version of the manuscript.***



As indicated in the legend to the figure already in the initial version of our manuscript: "The results are the mean ± SEM of 6 to 8 independent experiments depending on the time point, and are expressed as the percentage of AXL level at T0". We wish to reassure Reviewer#1 that the results are really based on western blots performed on different lysates obtained in independent experiments. We can show the Reviewer these data obtained from independent experiments if necessary.

9) All conclusions are derived from one single cells MCF10a. NMuMG cells are shown at the beginning but not used for the rest of the paper. Anyway, if this wants to be a cancer research paper, then cancer cells needs to be used.

It is true that we did not use a cancer cell line at the beginning of the paper because, as expected, flotillin knock-down did not allow to revert the mesenchymal phenotype of MDA-MB-231 cells toward an epithelial one. If this had been obtained, we would have used these cells from the beginning of the paper. The lack of reversion of the mesenchymal phenotype after flotillin knock-down was expected. Indeed, the EMT process is multifactorial and the decrease of flotillins alone is obviously not sufficient to reverse it in a tumor cell line bearing multiple oncogenic mutations. Moreover, because we wanted to assess whether flotillin upregulation is sufficient in normal cells to acquire the properties of tumor cells and particularly to induce EMT, we used human MCF10A and murine NMuMG cells, two non-tumoral epithelial cell lines. Until now, the studies carried out on the effects of flotillin overexpression have used tumor cells that already harbor pro-oncogenic perturbations, preventing to show that flotillin overexpression alone activates oncogenic processes leading to EMT, and to identify the downstream mechanisms.

Nevertheless, we have used the MDA-MB-231 cell line in several experiments to analyze: i) AXL distribution and internalization following the knock-down of flotillins (Figures 4 and S5), ii) SphK2 and flotillin 2 co-localization and co-endocytosis (Figures 5A and D and S7A), iii) the impact of SphK2 inhibition on AXL expression level distribution and endocytosis (Figure 6), iv) SphK2 expression level upon flotillin knock-down (Figure S7E) and AXL expression level upon SphK1 inhibition (Figure S7F). With these experiments performed in MDA-MB-231 cells, we showed that AXL and SphK2 colocalize in flotillin-positive late endosomes and are co- endocytosed from the plasma membrane containing flotillin-rich domains to flotillin-positive vesicles. We also demonstrated that flotillins and SphK2 control the rate of AXL endocytosis and its stabilization.
We recently obtained additional data with HS578T cells, another triple negative breast cancer cell line, on the co-trafficking of AXL and flotillins as well as the co-trafficking of SphK2 and flotillins (Additional Figure 3, ***this data could be added in the fully revised version of our manuscript)****.*
In addition, we observed that inhibiting SphK2 also decreased the level of AXL in HS578T cells. ***This data could be added in the revised version of the manuscript*** *(see data in our answer to Point #1 from Reviewer #3).*

NOTE*: We have removed unpublished data that had been provided for the referees in confidence.*

10) The methods section contains inconsistent data about patients' samples (9 are indicated, but the Figure S4 features 37). Then, where those other 527 come from?

We corrected the manuscript and added all characteristics regarding the 37 patients in the "Supplementary information" section.
The 527 patients are from another cohort and were used for the analysis of the correlation between the mRNA levels of FLOT1 and p63 in breast cancer biopsies from 527 patients (Figure 2I). This cohort was described in our previous study (Planchon et al. J Cell Science 2018, https://doi.org/10.1242/jcs.218925). In the revised version of our manuscript, we now refer to this previous article in the "Result" section and in the legend to figure 2I to explain the origin and characteristics of this cohort.

11) Some figures do not match with the legends or with the description in the text. It has not been easy to review this paper.

We apologize as we indeed made one mistake in figure 2 that was inserted into the manuscript and that was actually figure S2 (that appeared twice). However, the correct figure 2 was uploaded on the website of Review Commons and BioRxiv. Regarding the comments made in point 4, it seems that Reviewer #1 examined the correct figure 2 that was uploaded and that matches the legend indicated in the manuscript.
Besides this mistake, we do not see any other mismatch between figures and legends.

Reviewer #1 (Significance (Required)):
I am a cancer biologist working on EMT.

Referee Cross-commenting
I have nothing to comment on other's reviews.

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

Genest and co-authors present in this paper new fascinating evidence on how intracellular trafficking can modulate oncogenic signalling.

First of all, they show how overexpression of Flotillin1 and 2 in non-cancerous breast lines can induce a strong reprogramming towards an EMT phenotype. They analyse mRNA and protein expression, intracellular distribution of activated proteins, cell phenotypes to demonstrate a strong activation of oncogenic signalling pathways. They then identify AXL as a key player in this process and show how this protein is stabilised upon Flotillin expression. The authors use an amazing variety of approaches to study the endocytosis and the trafficking of endogenous, GFP-tagged, Halo-tagged and Myc-tagged AXL in different cell lines and their data are strong and very convincing, the images are of very high quality and the analysis rigorous. Their data strongly support the hypothesis that high Flotillin levels triggers AXL endocytosis and accumulation in non-degradative late endosomes where signalling remains active. The authors then show how SphK2 has a key role in AXL stabilisation, it colocalises with Flotillin, AXL and CD63 and its activity (which they block by using inhibitors or siRNA) is necessary for flotillin-induced AXL stabilisation and EMT induction.

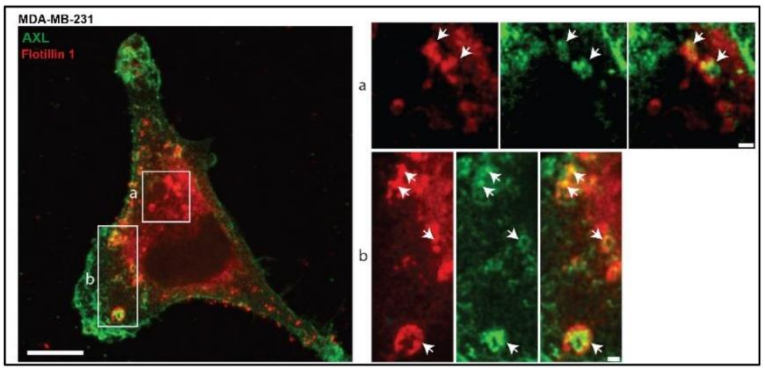
The paper is extremely well written, the data flow logically and they are appropriately presented and analysed. I don't have any major comment and I believe the paper is suitable for publication.

We thank the Reviewer for the positive appreciation on our manuscript.

I have only some minor comments/questions:

1. did the authors try to colocalise AXL with endogenous Flotillin in MDA-MB-231 cells? They could use the antibodies used in Fig S1B. Of note, the authors have shown it in luminal tumours in Fig S4C.

We performed co-immunofuorescence experiments to detect endogenous AXL with endogenous Flotillin in MDA-MB-231 cells. As shown below (Additional Figure 4), we could find AXL and Flotillin being present in the same intracellular endosomes. ***Images could be added in the revised version of the manuscript.***



Additional figure 4: Endogenous AXL and flotillin 1 are found in the same in intracellular vesicles in MDA-MB-231 cells*. MDA-MB-231 cells were fixed and labelled with relevant antibodies directed against Flotillin1 and AXL. Scale bar in the main image : 10 µm. Scale bars in the magnified images from the boxed area : 1 µm. Arrows indicate flotillin and AXL positives vesicles*

2. In Fig6G, it appears that AXL-Flotillin colocalization is lost upon SphK2 inhibition. Is this the case? It could be that the correct lipids are necessary for the formation of Flotillin-positive internalisation domains and this could be very interesting and reinforce the model proposed in the paper.
In figure 6G, cells were not permeabilized. Thus, only AXL at the cell surface was labelled using an antibody against the extracellular domain of AXL. Because flotillin 2 is tagged with mCherry, this allowed its visualization revealing its localization both at the cell surface and intracellularly in the inset of the lower pane l of figure 6G.
After 6 hours of treatment using the opaganib inhibitor, we did not notice any major change in AXL-flotillin colocalization at the cell surface. Somehow, this is expected because blocking the generation of S1P is more likely to inhibit the invagination of flotillin-rich membrane microdomains rather than their formation.

3. I would remove the sentence on line 995-997 "to our knowledge this is the first report to describe ligand-independent AXL stabilization..." as the cells are not serum starved in all experiments and animal serum can contain variable amounts of the ligand GAS6.

We understand and agree with Reviewer #2, this sentence has been modified by "To our knowledge this is the first report to describe AXL stabilization following its endocytosis"

Please note that the authors don't have to necessarily address comments 1-2, their paper is already very rich in convincing data.

Reviewer #2 (Significance (Required)):

AXL is a major oncogene that promotes EMT in a variety of tumour types. Understanding howits signalling can be triggered by endocytic pathways even in cells that are non-cancerous is very important and of high significance for the cancer field and the trafficking community.

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

This is an interesting and well written paper describing that upregulated flotillin promotes an endocytic pathway called upregulated flotillins-induced trafficking (UFIT) that mediates AXL endocytosis and allows its stabilization. Consequently, stabilized AXL in these flotillin-positive late endosomes enhances activation of oncogenic signaling pathways that promotes EMT. The authors suggest that Flotillin upregulation-induced AXL stabilization requires the activity of SphK2. However, this latter point is not supported by the data and further studies are needed to support this important conclusion.

Major concerns:

1. Most of the conclusions are based on effects of high concentrations (50 uM) of an ill-defined SphK2 inhibitor. The experiment described in Figure 6C-H need to be confirmed by downregulation of SphK2.

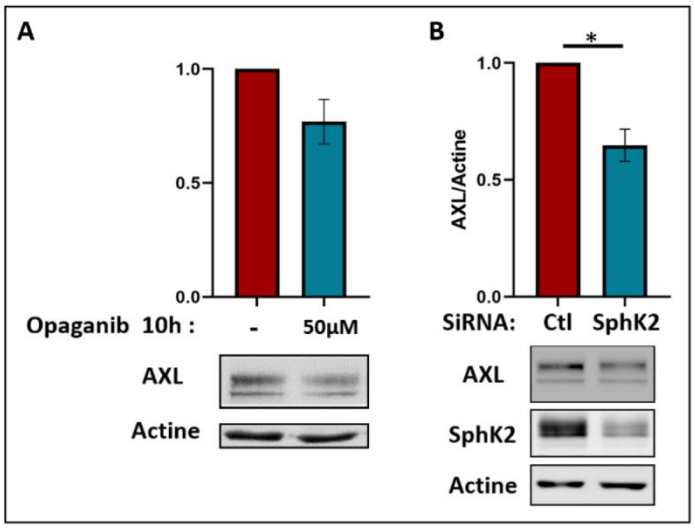
We understand that Reviewer #3 is concerned that in our experimental conditions, the effects we observed could be really explained by a specific inhibition of SphK2.
From the literature, among all the inhibitors described for SphK2, opaganib (ABC294640) is the most specific inhibitor available. It was shown to have no inhibitory effect on SphK1 up to100 µM (French et al, J Pharmacol Experimental Exp Ther 2010; Neubauer HA and Pitson SM, The FEBS Journal 2013). In agreement, we found that PF543, the most specific SphK1 inhibitor, had no effect on AXL level (Figure S7F), unlike incubation with opaganib (Figure 6Aand C), and that was confirmed in MCF10AF1F2 cells by the knock down of SphK2 with a specific siRNA (Figure 6B).
In the literature, depending on the cell lines, opaganib is used in vitro in the 10 to 60 µM range. Opaganib IC50 on recombinant SphK2 was established at 60 µM (French et al, J Pharmacol Experimental Exp Ther 2010). In our experiments, opaganib was used at a concentration of 50µM, below the IC50 value, as previously done by Nichols' group (Riento and al, PloS ONE, 2018). In most of our experiments (Figure 6, A, D, E-I, Figure S7D), opaganib was added for a maximum of 10 hours, which is shorter compared to what done in other studies (24-48 hours).Furthermore, it was shown that an opaganib concentration of 50 µM does not have any inhibitory effect in vitro on 20 protein kinases tested, including PKA, PKB, PKC, CDK, MAP-K, PDK1 and Src (French et al, J Pharmacol Experimental Exp Ther 2010).

In addition to inhibit SphK2, acting in a sphingosine-competitive manner, opaganib also was shown to act as an antagonist of estrogen receptor (ER), and inhibits ER-positive breast cancer tumor formation in vivo (Antoon JW et al, Endocrinology 2010). If Reviewer #3 is concerned about the possibility that the opaganib downstream effects we observed in our study might be explained by ER inhibition, we remind that we used cellular models that do not express ER. Indeed, the MDA-MB-231 cell line is a triple negative breast cancer cell line. MCF10A cells also do not express ER (Lane MA et al, Oncolgy Report, 1999,) and our transcriptomic analysis(Table S1) did not reveal any increase in the expression of ER genes in MCF10AF1F2 cells inwhich flotillins are upregulated, thus eliminating a possible non-specific effect of opaganib in these cells.

In conclusion, we hope that these arguments help to convince Reviewer #3 that our experiments were performed in conditions where we carefully limited the possibility of opaganib off-target effects, on the basis of the currently available opaganib-related data from the literature.

We totally agree with Reviewer #3 that complementary experiments by downregulating SphK2 must be used. In agreement, we already downregulated SphK2 by siRNA in MCF10AF1F2 cells. This led to a significant decrease in AXL and ZEB1 expression. ***In the current revised version of the manuscript we have added data*** *obtained with similar siRNA experiments performed in MDA-MB-231 cells (now Figure 6C). In agreement, we observed AXL and ZEB1downregulation.*

As shown below (Additional Figure 5) we recently obtained similar data in HS578T cells, showing that inhibiting SphK2 also affects AXL protein level in this triple negative breast cancer cell line ***(these data could be added in the manuscript)****.*



Additional figure 5: SphK2 inhibition decreases AXL level in HS578T cells. HS578T cells were incubated with opaganib (50µM, 10 hours) **(A)** or with siRNA Ctrl or siRNA SphK2 for 72 hours **(B)**. Cell lysates were blotted with relevant antibodies against AXL, SphK2 and actin. The histograms show AXL level (normalized to actin) expressed as fold-increase compared with the control condition, and data are the mean ± SEM of 3 (A)and 4 (B) independent experiments.

Reviewer #3 also asks to use the siRNA approach on experiments shown in previous panels D-H (now panels E-I) of figure 6.
In complement to Figure 6D (now Figure 6E), experiments using a siRNA against SphK2 to show that "AXL decrease upon SphK2 inhibition is not due to protein synthesis inhibition" ***areon-going and the obtained data could be added in the full revised version of our manuscript****.*

However, we are unfavorable to use a siRNA against SphK2, in addition to opaganib, in the experiments done to measure the effect of SphK2 inhibition on the rate of AXL internalization (previously in Figure 6E and F, now Figure 6F and G) and the level of AXL at the cell surface (previously in Figure 6G and H, now Figure 6H and I). Indeed, we carefully chose a short (4 hours) incubation with opaganib at the end of which the total cellular level of AXL was not yetdecreased, allowing to measure unambiguously a defect in AXL endocytosis or a change in thelevel of AXL at the cell surface. We believe that it would be very difficult to achieve similar experiments using a siRNA against SphK2. It would require to determine the exact time after siRNA transfection leading to a sufficient SphK2 level reduction but in conditions where AXL level is still maintained. We think that due to the mosaic transfection efficiency, being able to precisely synchronize the effect of a siRNA at its beginning is impossible.

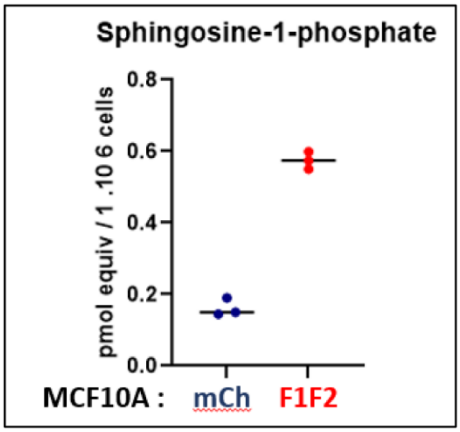
1. Does overexpression of SphK2 reverse the effects of the SphK2 inhibitor? In a similar manner, does overexpression of SphK2 enhance stabilization of AXL?

To answer the first question, it is not clear for us how to test whether SphK2 overexpression can reverse the effects of the SphK2 inhibitor because the ectopically expressed SphK2 would also be sensitive to the inhibitor. This would require to overexpress a SphK2 mutant that is catalytically active but insensitive to the inhibitor, and to our knowledge, such a mutant does not exist.
Regarding the second question, we are currently generating a retroviral DNA construct allowing to overexpress SphK2 homogeneously in the cell population. Then we will test whetherit further increases AXL level through its stabilization. This will be tested in cells upregulated for flotillin. As we showed in Figure 6 A and D (previously Figure 6 A and C) that AXL level depends on SphK2 activity only in cells that overexpress flotillins, we anticipate that there willbe no impact in a cell line with a moderate level of flotillin. ***Results could be added in the fully revised manuscript****.*

2. Although the authors suggest recruitment of SphK2 and formation of S1P in UFIT, there are no measurements of S1P. Also, there is no indication that SphK2 is activated despite thefact that ERK and AKT are activated in UFIT and are known to phosphorylate and activate SphK2. Is

SphK2 that is recruited to flotillin phosphorylated?

To answer the first point raised by Reviewer#3, we recently performed, in collaboration with a lipidomic platform, a comparative analysis by quantitative mass-spectrometry of S1P levels between MCF10AmCh and MCF10AF1F2 cells. ***As we anticipated, the results show a 3,5-fold increase in S1P in MCF10AF1F2 cells compared with MCF10AmCh*** *(Additional Figure 6). This data agrees with the fact that we found that the SphK2 catalytic activity is required for theUFIT pathway mediated AXL stabilization. This result is also in agreement with the study from the Nichols' group which detect a decrease in S1P in cells in which flotillins were knocked out(Riento et al, PloS ONE, 2018).* ***The results regarding the analysis of S1P level along with thecomplete methodology used will be added in the fully revised version of our manuscript****.*



Additional figure 6: Upregulation of flotillins in MCF10A cells promotes an increase in the level of Sphingosine- 1-phosphate. The level of sphingosine-1-phosphate was compared by quantitative mass-spectrometry analysis from three independent samples of MCF10AmCh and MCF10AF1F2 cells. The results are expressed in pmol equiv/ 1 . 10^6 cells. The graph shows the value for each sample and the bar horizontal bars indicate the mean value for each condition.

Regarding the second point, we would like to clarify that we do not think that SphK2 interacts directly or indirectly with flotillins because SphK2 did not co-immunoprecipitate with flotillins (not shown). Thus, investigating by western blotting SphK2 phosphorylation status in flotillin immunoprecipitates is pointless. In theory, we could investigate the activity-related phosphorylation status of SphK2 associated with flotillin rich-membranes and endosomes. Butthis seems difficult to achieve because unfortunately, the only two commercially available antibodies against phosphorylated SphK2 are not described to work for immunofluorescence staining. One is against the Thr578 residue (), identified as phosphorylated downstream of ERKby Sarah Spiegel's group (Hait et al, J Biol Chem, 2007). The second is designed to recognize specifically the phospho-Thr614 residue (https://www.abcam.com/sphk2-phospho-t614-antibody-ab111948.html), but this site has not been rigorously demonstrated to be phosphorylated downstream of AKT or ERK or to stimulate SphK2 activity. Thus, considering the lack of appropriate tools and considering that we already showed, using opaganib, that the catalytic activity of SphK2 is required for the UFIT pathway, we believe that investigating the phosphorylation status of SphK2 reflecting its activity in flotillin-positive vesicles will be complicated to achieve in a reasonable amount of time and we think that it will not bring a higher value to our present study.
To answer more broadly to the question "Is SphK2 recruited to flotillin phosphorylated?", we anticipate that it could be the case at least on the Ser419 and Ser420 residues because Nakamura's group demonstrated that the phosphorylation of these sites favors the nuclear export of SphK2 (Ding G et al, J Biol Chem, 2007). This group developed an antibody against these phospho-sites, potentially working by immunofluorescence. However, as it is unknown whether phosphorylation of these residues influences SphK2 activation status, we do not plan to perform immunofluorescence experiments with this tool (not available commercially) because the results would not address the Reviewer's question.

3. It should be determined whether the optogenetic system used to induce flotillin

oligomerization also induces recruitment and activation of SphK2.

As we already have all the available tools, optogenetic experiments will be performed to answer this point and ***the results could be added to the fully revised version of our manuscript****.*

4. Most importantly, it has not been established that the effects are mediated by S1P. Does addition of S1P enhance stabilization of AXL? Are the effects of S1P mediated by a S1P receptor? If so, which S1P receptor? There are several specific agonists and antagonists of S1PRs that can be utilized to answer this question. It's also possible that the effects of S1P are mediated by intracellular actions as were suggested by the De Camilli group (Nat Cell Biol. 2014 Jul;16(7):652-62).

As suggested, ***we plan to perform experiments in which exogenous S1P*** *will be added to cells with a moderate flotillin expression level to check whether it could recapitulate the effect of flotillin upregulation on AXL expression.* ***Results could be added to the fully revised version of the manuscript****.*
However, our current results on the localization and the involvement of SphK2 suggest that the generation of S1P involved in the UFIT pathway occurs at the plasma membrane and in late endosomes. Because the exogenous S1P that will be added in the culture medium will not go through the plasma membrane, we anticipate that it could be insufficient to mimic all the mechanisms of the UFIT pathway. Its effect will be limited to the plasma membrane. In addition, these mechanisms are very likely based on a local concentration of S1P in some microdomains(at the plasma membrane and in intracellular membranes) scaffolded by flotillins. It will be very difficult to mimic such local concentration of S1P just by adding S1P to the cells.

We agree that identifying the S1P receptors involved would be of valuable interest for a better characterization of the UFIT pathway. ***However, we think that this is beyond the scope of our present study****. Among the five known S1P receptors, we do not know if any could be involved in membrane remodeling at the plasma membrane to promote endocytosis. To our knowledge, involvement of S1P receptors in endocytosis has never been reported. However, based on the work by Nakamura's group (Kajimoto et al, Nat Comm, 2013 and Kajimoto et al, J Biol Chem, 2018), the S1P1 and S1P3 receptors are involved in membrane remodeling and cargo sorting from the outer membrane of late endosomes (where flotillins accumulate in our cell models). We could hypothesize that these receptors are influenced by flotillins and are involved in the UFIT pathway.* ***But we think that testing this hypothesis would be the subject of a distinct study****.*
At the plasma membrane, we totally agree that the effect of S1P could be mediated, as suggested by De Camilli's group (Shen et al, Nat Cell Biol 2014), by the formation of tubular endocytic structure rich in sphingosine after acute cholesterol extraction. Reciprocally, in our cell models, upregulated flotillins, thanks to their ability to bind to sphingosine (demonstrated by Nichols' group (Riento et al, PloS ONE, 2018)) and to oligomerize, could create sphingosine- rich membrane regions.

5. There is a commercial antibody for endogenous SphK2 that can be used to validate and substantiate the data with GFP-SphK2. (F1000Res. 2016 Dec 6;5:2825. doi: 10.12688/f1000research.10336.2. eCollection 2016. Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence)

We thank Reviewer #3 for this suggestion and advice. Being able to detect the localization of endogenous SphK2 in late endosome would be valuable for our study. We already tried with no success with antibodies from Sigma and Cell Signaling Technology (not described to work in immunofluorescence experiments).
We will follow the advice from Reviewer #3 and test the anti-SphK2 antibody from ECM-Biosciences mentioned in the article by Neubauer and Pitson F1000 research, 2016. ***If we obtain interesting results, they will be included in the revised version of our manuscript****.*

However, in experiments using SphK2-GFP, we noticed that in live cells, the signal in late endosomes was completely lost after fixation using paraformaldehyde. Similarly, we also

observed in live cells that NBD-Sphingosine, added in the culture medium, quickly accumulated in flotillin-positive late endosomes (Additional Figure 7, ***this data could be added in the fully revised version of the manuscript****), but this accumulation was no longer detectable after fixation. Based on these observations, we believe that SphK2 recruitment to flotillin-positive late endosomes is highly labile probably because it mainly involves its interaction with sphingosine molecules that are enriched in these intracellular compartments. This is supported by our observation that addition of opaganib, characterized as a sphingosine competitive inhibitor, displaces SphK2-GFP from flotillin-positive late endosomes in live cells (Figure S7D). In addition, we showed that SphK2-Halo is more recruited in CD63-positive late endosomes in cells overexpressing flotillins (Figure 5E). This could be due to a higher concentration of sphingosine promoted by flotillins (that bind to sphingosine) accumulating in these compartments.*
Thus, we will try the immunofluorescence staining of endogenous SphK2 using the recommended antibody, but it might be difficult to detect its presence in flotillin-rich late endosomes in fixed cells. ***The data could be added in the fully revised version of the manuscript****.*

NOTE*: We have removed unpublished data that had been provided for the referees in confidence.*

Reviewer #3 (Significance (Required)):

This is an interesting paper. If the authors confirm the involvement of Sphk2 and mechanism of action of S1P, this would be an important contribution to the field.

Modifications done in the initial revised-version of our manuscript (at the time of the initial response). A full revised version will be provided after all the additional experiments asked by all the Reviewers will be achieved.

Revisions are highlighted in grey in the initial revised-version of the manuscript

1) Figure 1 has been modified and now includes results from a GSEA analysis as recommended by Reviewer #1. The texts of the corresponding legend and of the "Results" and "Methods" sections have been modified accordingly.

1) The Figure 2 version that was inserted in the manuscript was wrong because it was a copy of Figure S2. However, the correct Figure 2 was uploaded to the Review Commons website and accessible for the Reviewers. The correct Figure 2 is now inserted in the manuscript.

2) In the legend to panels C, E, F, J of Figure 2, the sentence: "The histograms show […] with control MCF10AmCh cells calculated from 4 independent experiments" was corrected to "The histograms show […] with control MCF10AmCh cells calculated from <u>at least</u> 4 independent experiments" because data shown in panel J are actually calculated from 8 independent experiments.

3) Figure 6 has been modified with the addition of panel C showing the effect of SphK2 downregulation by siRNA on AXL and ZEB1 level in MDA-MB-231 cells. The text has been modified accordingly.
4) In Figure 3 C, representative western blots have been added as asked by Reviewer #1.

5) In the Supplementary information section, the full clinicopathological characteristics of only 9 patients were indicated, whereas Figure S4 mentioned 37 patients. We corrected this mistake and now provide the characteristics of all patients.

6) In the sentence "Conversely, it induced ZEB 1 and 2 mRNA expression (Figures 1H and S1K) and ZEB1 protein expression (Figures 1I and S1L) (no anti-ZEB2 antibody is available)", we removed "no anti-ZEB2 antibody is available".

7) The sentence previously on line 995-997 "to our knowledge this is the first report to describe ligand-independent AXL stabilization..." *has been modified to "To our knowledge this is the first report to describe AXL stabilization following its endocytosis"*

8) We are now referring to reference 18 (Planchon et al. J Cell Science, 2018) for the description of the cohort of 527 patients with breast cancer because this was missing.

Original submission

First decision letter

MS ID#: JOCES/2021/259178

MS TITLE: Upregulated-flotillins and sphingosine kinase 2 derail vesicular trafic to stabilize AXL and promote epithelial-mesenchymal transition

AUTHORS: Mallory Genest, Franck Comunale, Damien Planchon, Pauline Govindin, Sophie Vacher, Ivan Bieche, Bruno Robert, Himanshu Malhotra, Andreas Schoenit, Liubov Tashireva, Cecile Gauthier-Rouviere, and Stephane Bodin
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

I have looked over all the information you submitted to the Journal of Cell Science regarding your paper "Upregulated-flotillins and sphingosine kinase 2 derail vesicular traffic to stabilize AXL and promote epithelial-mesenchymal transition" (JOCES/2021/259178). I would like to invite a revised version of the paper that incorporates the data you outline in your "answer to reviewers" document, to the best of your ability.

I would also like you to consider further the comment from referee #1 concerning lack of correspondence between your conclusions and the reported clinical data. The referees point out that co-expression of flotillin 1 and AXL are prevalent in luminal breast cancers, which they state is the subtype known to not be driven by EMT. Your respond that you see flotillin 1 and AXL also co-expressed in the other subtypes. However, there is a lot of variability, and as you don't show statistics for the comparison, I'm assuming co-expression is not significant? Rather than pushing the point about EMT, you may want to consider other aspects of luminal cancers that might be affected by this association (e.g. invasion/mestastasis) and also further elaborate on the variability in the other cancers.

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 raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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.

First revision

Author response to reviewers' comments

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

The paper by Genest et al. describes the effect of flotillins and sphingosine kinase 2 to stabilize AXL as a mechanism to promote epithelial-mesenchymal transition in breast (cancer) cells. The potential role of vesicles trafficking EMT-promoting proteins is of high interest in the field, also for exploring new opportunities of pharmacological targeting. However, the paper fails to convincingly demonstrate that the proposed mechanism is of real importance to support or promote EMT for the following main reasons:

1-a) The role of flotillins is studied only by overexpression and in the context of non-cancerous MCF10A cells, while breast cancer cells of epithelial-like origin are not analyzed.

Regarding the first part of the point raised by the reviewer, we are not sure to understand correctly the sentence "[…] while breast cancer cells of epithelial-like origin are not analyzed". Indeed, we used the breast cancer cell line MDA-MB-231 and a cell line that we generated by knocking down flotillin expression (MDA-MB-231shFlot2) in the second part of this study (figures 4H, I, and K, 5B, C, D, and E, 6C, F and H, S5E, F and H and S7B, F and I). This previously characterized cell line allowed us to demonstrate that abolishing flotillin overexpression was sufficient to significantly inhibit the invasive properties of MDA-MB-231 cells (Planchon et al, J Cell Science 2018, https://doi.org/10.1242/jcs.218925). Moreover, we used a second breast cancer cell line (Hs 578T cells) in experiments performed during the revision process (figure S7A,E and H).
Although flotillin upregulation induces some major mechanisms of the EMT process in MCF10A cells, flotillin downregulation was not sufficient to reverse the EMT phenotype in MDA-MB-231 cells. This could be explained by the fact that EMT is a multifactorial process and that MDA-MB-231 cells went through too many irreversible changes leading to this process. Conversely, when we analyzed EMT markers after SPHK2 inhibition or knock down in MCF10AF1F2 and in MDA-MB-231 cells (figure 6A-D), we could observe a significant decrease in ZEB1 expression.

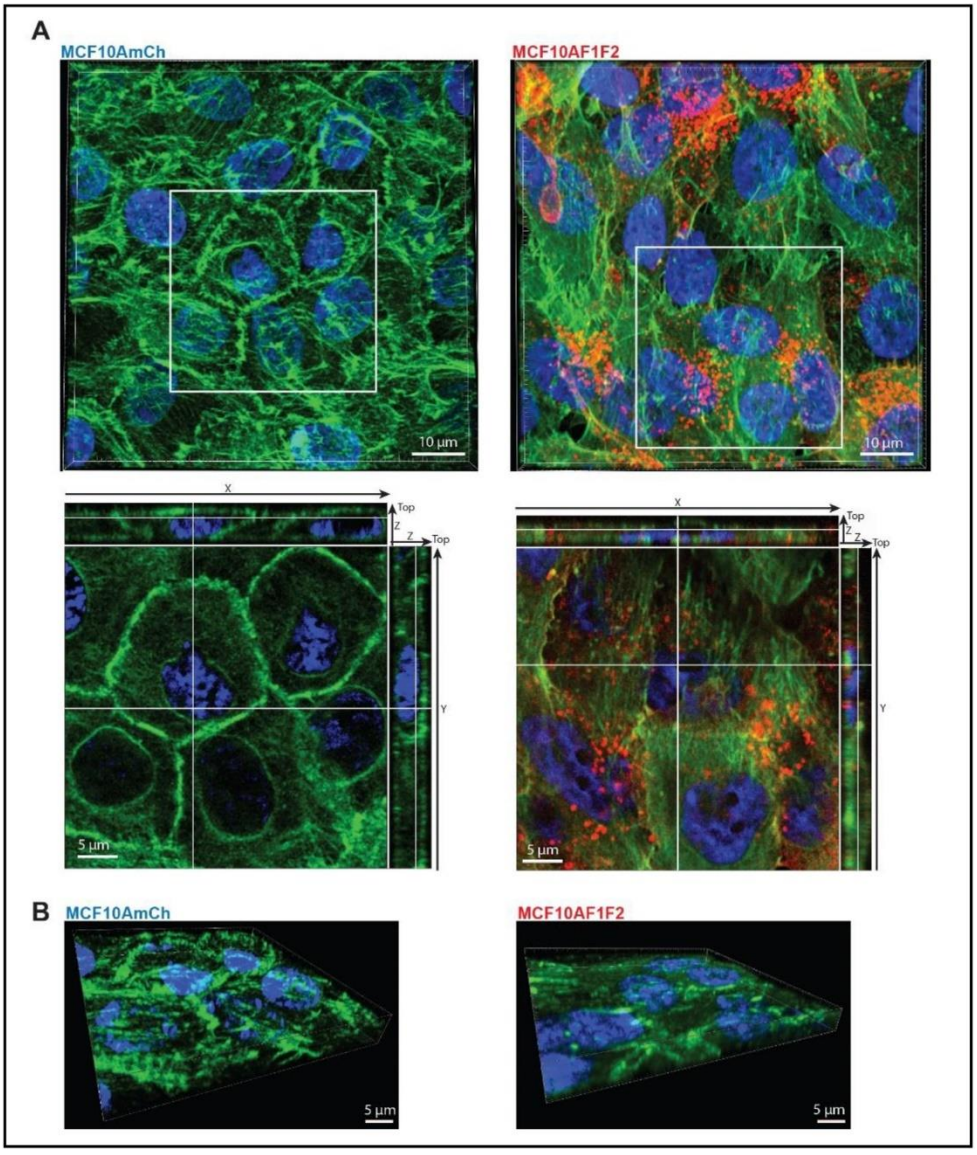
1-b) This is contrast with the purpose of the paper (see abstract, introduction, patients' data) which is to study tumors and EMT. Effect of shRNAs is also not reported, making it difficult to estimate the importance on the EMT phenotype.

As we mentioned in our manuscript, previous studies by other groups, who downregulated flotillin expression in different cancer cell lines using siRNA approaches or re-expression of miRNAs that inhibit flotillin expression, already showed flotillin participation in EMT (for review please see, Gauthier-Rouvière et al, Cancer Metastasis Review, 2020, doi: 10.1007/s10555-020-09873-y).
In this context, the novelty and the first goal of our study was to investigate how strong is the contribution of flotillin upregulation to EMT induction. To achieve this goal, we chose on purpose to use non-tumoral epithelial cells that do not harbor the anomalies that already favorEMT, unlike the cancer cell lines used in the previous studies. In these non-tumoral cell lines (the human MCF10A and mouse NMuMG mammary epithelial cell lines), we ectopically overexpressed flotillins (MCF10AF1F2 and NMuMGF1F2 cells) to levels similar to what observed in invasive breast cancer cells. Using this approach, we found that flotillin overexpression is enough to induce EMT.

1-c) Then, alteration of EMT should be concluded also from other non-genetic functional parameters, not just by markers. For instance: was morphology of the cells changed? Was cell migration affected with F1F2?

Our conclusion that flotillin upregulation is sufficient to induce EMT in MCF10AF1F2 and NMuMGF1F2 cells is not based only on genetic functional parameters or markers. For instance, figure S1 (panels H and I) shows a strong modification of the cell morphology and of the actin cytoskeleton organization in NMuMG cells upon flotillin upregulation. NMuMGF1F2 cells became flat, lost their apical F-actin belt, and exhibited an increase in stress fibers.

As shown below (additional figure 1), similar modifications of the cell morphology and of the F-actin cytoskeleton organization occur also when flotillins are upregulated in MCF10A cells(see below the comparison of MCF10A and MCF10AF1F2 cells) We have now added this datain figure S1J of the revised version of our manuscript.



Additional figure 1: Upregulation of flotillins in MCF10A cells leads to changes in the cell morphology and in the F-actin cytoskeleton organization*. Comparison of the morphology and of the actin cytoskeleton organization*

in MCF10AmCh and MCF10AF1F2 cells. Confluent cells were fixed and stained for F-actin (green) using Alexa488- conjugated-Phalloidin and with Hoechst (nuclei; blue); in panel A, flotillin2-mCherry signal (red) is shown.

(A) Upper panels show the maximum intensity projection images (MIP) of MCF10AmCh (control) and MCF10AF1F2 (flotillin overexpression) cells obtained from a stack of images acquired by confocal microscopy. Lower panels show magnified images from the boxed areas, including one single plane and the x-z and y-z projections along the indicated axes.
(B) 3D reconstruction images obtained from the region in the boxed area from the MIP-images shown in A.
These data show that in MCF10AF1F2 cells, the apical actin belt is lost and the height of the cellular monolayer is lower compared with control MCF10AmCh cells.

We also analyzed the migration capacity of these cells (shown in figure 3G of the submitted manuscript). Briefly, using a Boyden chamber assay, we showed that flotillin upregulation significantly increased migration of MCF10A cells (figure 3G). We previously demonstrated that flotillin upregulation also promotes cell invasion in 3D using a spheroid assay (Planchon et al, J Cell Science, 2018, https://doi.org/10.1242/jcs.218925). As shown below (additional figure 2), using a wound healing assay, we also observed that cell velocity is higher in flotillin-overexpressing NMuMGF1F2 cells than in control NMuMG cells (this result is not included in the revised version of our manuscript).

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

2) AXL up-regulation is not very strong (2-fold). What is unclear is if the minimal AXL increase due to F1F2 really provides a significant contribution to the EMT phenotype (as the authors conclude). The siRNA experiment knocks down all AXL, not just the F1F2-induced levels, making it difficult to estimate the real effect of the mechanism proposed.
As shown in figure 3A and D, in MCF10AF1F2 cells compared with MCF10AmCh cells, we measured a significant 2.5 ± 0.7-fold increase in the AXL protein level. We do not think that this can be considered as a minimal increase.

Considering that flotillin upregulation may affect simultaneously different receptors (figure S2H-I, Figure S6A-F), we did not expect that downregulating a single receptor would have a major impact on the level of EMT markers and on cell migration. Yet, after knocking down AXL in MCF10AF1F2 cells, we observed a decrease in ZEB1 and N-cadherin expression, the re- expression of E-cadherin (figure 3D-F), and the inhibition of cell migration (figure 3G). The fact that we observed such an effect by downregulating only AXL, which according to Reviewer#1 is minimally increased, might be explained by its well-known ability to act not alone but through cross-talk with other signaling receptors (Graham et al, Nature Reviews Cancer 2014; Halmos and Haura, Science Signaling 2016; Colavito et al, Journal of Oncology 2020).
As suggested by Reviewer #1, ideally, it would be interesting to bring back AXL to its level in MCF10AmCh cells to better evaluate only the contribution of its increase. However, adjusting so precisely the efficacy of AXL downregulation by siRNA seems quite difficult to achieve.

3) Why didn't the author focus on EphA4 (or to a lesser extent ALK), which showed better regulation ?

As we mentioned (page 9, in the initial version of our manuscript, now page 8) "the available tools allowed us to validate this result only for AXL, but not for EphA4 and ALK". Nevertheless, for EphA4, we showed in Figure S6 that it is located in flotillin-positive late endosomes (figure S6 A and B,C, for MCF10AF1F2 and NMuMGF1F2 cells, respectively) in a phosphorylated form (using an antibody against P-Y588/Y596-EphA4 that works in NMuMG cells, figure S6D). However, the signals obtained by western blotting using the same antibody were too low to validate any significant variation of EphA4 Y-phosphorylation status, as suggested by the results from the phospho-RTK array.
Regarding ALK, the increase in its phosphorylation, suggested by the phospho-RTK array, remains puzzling to us. By western blotting of cell lysates and in the presence of positive controls, we did not detect any positive signal for phosphorylated ALK and even for total ALK in MCF10A and MCF10AF1F2 cells. In addition, to our knowledge, ALK expression in MCF10A cells has never been reported in the literature. These observations did not encourage us to pursue our investigations

on ALK.
Moreover, several points led us to focus on AXL. Indeed, AXL expression is associated with the acquisition of a mesenchymal cell phenotype, invasive properties, and resistance to treatments and AXL is an attractive therapeutic target against which several inhibitors are in preclinical and clinical development (Shen Y et al. Life Sciences 2018). Moreover, AXL expression in tumors is attributed to post-transcriptional regulation, but the mechanisms are totally unknown. Understanding how its stabilization and signaling can be triggered by flotillin- mediated endocytic pathways is new and of high significance for the cancer field and the trafficking community.

3) The conclusions of the manuscript are contradicted by the reported clinical data. In Figure S4 the authors clearly observe co-expression of Flotillin 1 and AXL prevalently in luminal breast cancers, which is the subtype known to not be driven by EMT. This evidence already indicates that this (otherwise interesting) mechanism is not relevant to EMT in breast cancer. So, the conclusions are not supported by the data, and the experimental setup and model chosen are not appropriate to generalize the findings to cancer.

We acknowledge that flotillin 1/AXL co-expression is highest in the luminal subtype, but it is also found in the HER2-positive subtype that undergoes EMT (see figure S4). Adding new tumorsamples to this co-expression analysis allowed us to obtain significant results in these two subtypes. As reported by Goyette et al (Cell Reports 2018, DOI: 10.1016/j.celrep.2018.04.019),AXL is expressed in all breast cancer molecular subtypes. Moreover, they demonstrated that AXL promotes the mesenchymal phenotype of HER2-positive breast cancer. Considering this observation and the influence of flotillin upregulation on AXL overexpression we reported here,we believe that the co-upregulation of flotillins and AXL can participate in EMT induction in breast cancer cells. Considering the luminal subtype, the co-upregulation of flotillins and AXLmight be important to promote cancer cell migration and invasion.

Minor (here the most important):
4) The point of the Figure 2 is not clear. Why this part should have such a central role in the story? The entire data presented are not followed up in the rest of the paper. Moreover, in some cases upregulations also questionably significant (like RAS and STAT3 are not even 2 fold). Moreover, the error bars are so small that it seems unrealistic that the plots indicate three independent experiments.

Because the activation of oncogenic signaling pathways is crucial to promote EMT, we think that analyzing these pathways in the context of flotillin upregulation is coherent with the message of the paper.
To our knowledge, the amplitude of up- or down-regulation has nothing to do with its significance. The amplitude also depends strongly on the context (e.g. stimulation with an agonist, overexpression of GEF). For instance, increases lower than 2-fold are frequently reported (Bodin and Welch, Mol Biol Cell, 2005; Miura SI et al, Arteriosclerosis, Thromb andVasc Biology, 2003; Matsunaga-Udagawa R et al, J Bio Chem 2010) when assessing the activity of Ras or small GTPases, but they represent real upregulations. Furthermore, in our study, Ras activation is supported by the downstream 4-fold activation of ERK (figure 2C).
In figure 2, panels B, C, E, F and J, considering the amplitude of the mean increases, the error bars corresponding to s.e.m. do not seem disproportionately small.
As the Reviewer seems to insinuate that we have not performed independent experiments, we present in the table below the detailed results we obtained from independent experiments.

Panel	*Parameter measured*	*Number of independent experiments*	*Fold-increase in MCF10AF1F2 cells compared with MCF10AmCh cells in each experiment*	*Mean*	*SEM*	*p-value*
B	*Ras-GTP*	*5*	*1.95 ; 1.96 ; 1.18 ; 1.67 ; 1.86*	*1.72*	*0.14*	*0.001*
C	*Phospho- ERK*	*5*	*1.24 ; 5.43 ; 3.22 ; 6.11 ; 3.52*	*3.71*	*0.73*	*0.0042*
E	*Phospho-AKT*	*4*	*2.29 ; 6.54 ; 3.76 ; 2.6*	*3.8*	*0.97*	*0.0276*
F	*Phospho-STAT3*	*4*	*1.63 ; 1.63 ; 2.42 ; 1.60*	*1.82*	*0.20*	*0.0066*
J	*Phospho-SMAD3*	*8*	*4.1 ; 5.12 ; 6.29 ; 1.82 ; 2.58 ; 6.66 ; 2.82 ; 5.40*	*4.35*	*0.64*	*0.0001*

In the legend to figure 2 panels C, E, F, J, "The histograms show […] with control MCF10AmCh cells calculated from 4 independent experiments" was corrected to "The histograms show […] with control MCF10AmCh cells calculated from at least 4 independent experiments" as data shown in panel J were actually calculated from 8 independent experiments.

5) More robust statistical analysis should be provided in the Figure 1 to support that EMT is suppressed with F1F2 overexpression. For instance, a more standard GSEA on hallmark signatures.

To avoid confusion, we understand that Reviewer #1 means "… that EMT is induced with F1F2 overexpression" and not "… suppressed …".
As recommended by Reviewer #1, we performed a GSEA on the hallmark signature and the results are now included in the new version of our manuscript (figure 1C).

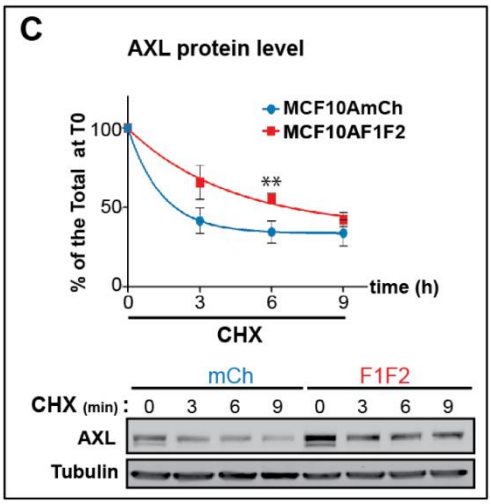
6) In Figure 3 E-Cadherin is rescued with siAXL in the IF but not in the western blot.

Using siRNA transfection, we can have a mosaic effect due to the fact that not all cells of the sample are transfected and thus the silencing effect can vary. This mosaicism was clear when we analyzed E-cadherin by immunocytochemistry. Indeed, in some cells, probably the ones that have been more efficiently transfected with the AXL siRNA, E-cadherin expression is clearly seen. By western blotting, which provides a global analysis in which transfected and non- transfected cells are mixed, this was not significantly higher than in MCF10AF1F2 cells transfected with a control siRNA, although there was a trend towards increased E-cadherin expression in MCF10AF1F2 transfected with the AXL siRNA.
In a recent article, Pier Paolo Di Fiore's group described a similar mosaicism for the loss of E-cadherin expression in MCF10A cells following Epsin 3 overexpression (Lomoriello et al., Nat Com, 2020, doi: 10.1038/s41467-020-16836-y)

7) Some sentences require clarifications. The authors should be more clear on why ZEB2 antibody was not available or what they mean with "Unfortunately the available tools.". *In Page 7 of the initial submitted version of our manuscript, we wrote «no anti-Zeb2 antibody is available». We should have said: «none of the anti-Zeb2 antibodies tested worked in MCF10A cells». We decided to remove "no anti-Zeb2 antibody is available" from the sentence to avoid confusion in the revised version of our manuscript.*
In Page 9 of the initial submitted version of our manuscript, we wrote «unfortunately the available tools» to refer the available tools against EphA4 and ALK that did not allow us to validate the data obtained using the phospho-RTK array showing that the Y-phosphorylation of these two RTK is increased in cells with upregulated flotillins (see also our answer to majorpoint 2).

8) Western blot from the CHX experiment should be shown, at least in the supplements. Again, the standard deviation in this experiment is minimal, was this really an average of three independent experiments (and not three western on the same lysates)?

As asked, a representative western blot is now shown in figure 3C of the revised version of the manuscript.



As indicated in the legend to the figure already in the initial version of our manuscript: "The results are the mean ± s.e.m. of 6 to 8 independent experiments depending on the time point, and are expressed as the percentage of AXL level at T0". We wish to reassure Reviewer #1 that the results are based on western blot experiments performed with different protein lysates obtained in independent experiments. As now indicated in the figure legend, we improved our statistical analysis by using the least square fit method (using GraphPad prism) that validated a significant difference in AXL decay (p=0.0033) upon CHX treatment between MCF10AmCh and MCF10AF1F2 cells.

9) All conclusions are derived from one single cells MCF10a. NMuMG cells are shown at the beginning but not used for the rest of the paper. Anyway, if this wants to be a cancer research paper, then cancer cells needs to be used.

It is true that we did not use a cancer cell line at the beginning of the paper because as expected, flotillin knock-down did not allow reverting the mesenchymal phenotype of MDA-MB-231 cells towards the epithelial phenotype. If this would have been possible, we would have used these cells from the beginning of the paper. However, the lack of reversion of the mesenchymal phenotype after flotillin knock-down was expected. Indeed, the EMT process is multifactorial and the decrease of flotillins alone is obviously not sufficient to reverse it in a tumor cell line that bears multiple oncogenic mutations. Moreover, as we wanted to assess whether flotillin upregulation is sufficient in normal cells to acquire the properties of tumor cells and particularly to induce EMT, we used human MCF10A and murine NMuMG cells, two non- tumoral epithelial cell lines. Until now, the studies on the effects of flotillin overexpression have been carried out using tumor cells that already harbor pro-oncogenic perturbations. This precluded the possibility to show that flotillin overexpression alone activates oncogenic processes leading to EMT, and to identify the downstream mechanisms.

Nevertheless, we used the MDA-MB-231 cell line in several experiments to analyze: i) AXL distribution and internalization following the knock-down of flotillins (figures 4 G, H and I and S5E, F and H), ii) SPHK2 and flotillin 2 co-localization and co-endocytosis (figures 5C and D and S7B), iii) the impact of SPHK2 inhibition on AXL expression level, AXL distribution, stability and endocytosis (figure 6C, D, E, F, H and J), iv) SPHK2 expression level upon flotillin knock-down (figure S7F) and AXL expression level upon SPHK1 inhibition (figure S7I). With these experiments performed in MDA-MB-231 cells, we showed that AXL and SPHK2 colocalize in flotillin-positive late endosomes and are co-endocytosed from the plasma membrane containing flotillin-rich domains to flotillin-positive vesicles. We also demonstrated that flotillins and SPHK2 control the rate of AXL endocytosis and its stabilization.

We recently obtained additional data in Hs 578T cells, another triple negative breast cancer cell line, on the co-trafficking of AXL and flotillins (not shown in the revised version of the manuscript but shown in the additional figure 3 below), as well as on the co-trafficking of SPHK2 and flotillins (shown in the figure below and also added in figure S7E).

In addition, we observed that SPHK2 inhibition decreased AXL level also in Hs 578T cells (added in figure S7H).

NOTE: *We have removed unpublished data that had been provided for the referees inconfidence.*

10) The methods section contains inconsistent data about patients' samples (9 are indicated, but the Figure S4 features 37). Then, where those other 527 come from?

We corrected the manuscript. Indeed, for Figure S4, at the initial time of submission, we used37 samples from patients with a breast tumor and 6 samples from patients with non-tumoral lesions. For the full revised version of our manuscript, we increased to 43 the number of breast tumor samples (Luminal n= 17, Triple Negative n =15, HER2^{+} =11) as indicated in the legendto figure S4. We added all patient characteristics in the "Material and methods" section. The 527 patients are from another cohort and were used for the analysis of the correlation between the mRNA levels of FLOT1 and p63 in breast cancer biopsies from 527 patients (figure2I). This cohort was described in our previous study (Planchon et al. J Cell Science 2018, https://doi.org/10.1242/jcs.218925). In the revised version of our manuscript, we now refer to this previous article in the "Results" section and in the legend to figure 2I to explain the origin and characteristics of this second cohort.

11) Some figures do not match with the legends or with the description in the text. It has not been easy to review this paper.

We apologize as we indeed made one mistake in figure 2 that was inserted into the manuscript and that was actually figure S2 (that appeared twice). However, the correct figure 2 was uploaded on the website of Review Commons and BioRxiv. Regarding the comments made in point 4, it seems that Reviewer #1 examined the correct figure 2 that was uploaded and that matches the legend indicated in the manuscript.
Besides this mistake, we do not see any other mismatch between figures and legends.

Reviewer #1 (Significance (Required)):
I am a cancer biologist working on EMT.

Referee Cross-commenting
I have nothing to comment on other's reviews.

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

Genest and co-authors present in this paper new fascinating evidence on how intracellular trafficking can modulate oncogenic signalling.

First of all, they show how overexpression of Flotillin1 and 2 in non-cancerous breast lines can induce a strong reprogramming towards an EMT phenotype. They analyse mRNA and protein expression, intracellular distribution of activated proteins, cell phenotypes to demonstrate a strong activation of oncogenic signalling pathways. They then identify AXL as a key player in this process and show how this protein is stabilised upon Flotillin expression. The authors use an amazing variety of approaches to study the endocytosis and the trafficking of endogenous, GFP-tagged, Halo-tagged and Myc-tagged AXL in different cell lines and their data are strong and very convincing, the images are of very high quality and the analysis rigorous. Their data strongly support the hypothesis that high Flotillin levels triggers AXL endocytosis and accumulation in non-degradative late endosomes where signalling remains active. The authors then show how SphK2 has a key role in AXL stabilisation, it colocalises with Flotillin, AXL and CD63 and its activity (which they block by using inhibitors or siRNA) is necessary for flotillin-induced AXL stabilisation and EMT induction.

The paper is extremely well written, the data flow logically and they are appropriately presented and analysed. I don't have any major comment and I believe the paper is suitable for publication.

We thank the Reviewer for the positive appreciation on our manuscript.

I have only some minor comments/questions:

1) did the authors try to colocalise AXL with endogenous Flotillin in MDA-MB-231 cells? They could use the antibodies used in Fig S1B. Of note, the authors have shown it in luminal tumours in Fig S4C.

We performed co-immunofluorescence experiments to detect endogenous AXL with endogenous flotillin 1 in MDA-MB-231 cells. One representative image that shows AXL and flotillin 1 colocalization in the same intracellular endosomes has been added in Figure S5F.

2) In Fig6G, it appears that AXL-Flotillin colocalization is lost upon SphK2 inhibition. Is this the case? It could be that the correct lipids are necessary for the formation of Flotillin-positive internalisation domains and this could be very interesting and reinforce the model proposed in the paper.

In figure 6G (now figure 6I), cells were not permeabilized. Thus, only AXL at the cell surface was labeled using an antibody against the extracellular domain of AXL. As flotillin 2 was tagged with mCherry, this allowed its visualization and revealing its localization both at the cell surface and intracellularly (see inset of the lower panel of figure 6G, now figure 6I).
After 6 hours of treatment with the opaganib inhibitor, we did not notice any major change in AXL-flotillin colocalization at the cell surface. Somehow, this is expected because blocking S1P generation is more likely to inhibit the invagination of flotillin-rich membrane microdomains rather than their formation.

3) I would remove the sentence on line 995-997 "to our knowledge this is the first report to describe ligand-independent AXL stabilization..." as the cells are not serum starved in all experiments and animal serum can contain variable amounts of the ligand GAS6.

We understand and agree with Reviewer 2. We modified this sentence into: "To our knowledge this is the first report to describe AXL stabilization following its endocytosis"

Please note that the authors don't have to necessarily address comments 1-2, their paper is already very rich in convincing data.

Reviewer #2 (Significance (Required)):

AXL is a major oncogene that promotes EMT in a variety of tumour types. Understanding how its signalling can be triggered by endocytic pathways even in cells that are non-cancerous is very important and of high significance for the cancer field and the trafficking community.

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

This is an interesting and well written paper describing that upregulated flotillin promotes an endocytic pathway called upregulated flotillins-induced trafficking (UFIT) that mediates AXL endocytosis and allows its stabilization. Consequently, stabilized AXL in these flotillin-positive late endosomes enhances activation of oncogenic signaling pathways that promotes EMT. The authors suggest that Flotillin upregulation-induced AXL stabilization requires the activity of SphK2. However, this latter point is not supported by the data and further studies are needed to support this important conclusion.

Major concerns:

1. Most of the conclusions are based on effects of high concentrations (50 uM) of an ill-defined SphK2 inhibitor.

From the literature, among all the inhibitors described for SPHK2, Opaganib (ABC294640) is the most specific inhibitor available. It has been shown that it does not have any inhibitory effect on SPHK1 up to 100 µM (French et al, J Pharmacol Experimental Exp. Ther. 2010; Neubauer HA and Pitson SM, The FEBS Journal 2013). In agreement, we found that PF543, the most specific SPHK1 inhibitor, had no effect on AXL level (Figure S7I). In contrast, incubation with opaganib

decreased AXL level (figure 6A and previously 6C, now 6D), as observed upon knock-down of SPHK2 with a specific siRNA in MCF10AF1F2 cells (figure 6B)and in MDA-MB-231 cells (now Figure 6C).
In the literature, depending on the cell line, Opaganib is used in vitro in the 10 to 60 µM range. Opaganib IC_{50} with recombinant SPHK2 was established at 60 µM (French et al, J Pharmacol Experimental Exp Ther 2010). In our experiments, opaganib was used at a concentration of 50µM, below the IC_{50} value, as previously done by Nichols' group (Riento and al, PloS ONE, 2018). In most of our experiments (figures 6A, D, E, G, H, I, J, previously figure S7D now S7J),opaganib was added for a maximum of 10 hours, which is shorter compared to other studies (24-48 hours). Furthermore, it was shown that an opaganib concentration of 50 µM does not have any inhibitory effect in vitro on the 20 protein kinases tested, including PKA, PKB, PKC, CDK, MAP-K, PDK1 and Src (French et al, J Pharmacol Experimental Exp Ther 2010).

Besides inhibiting SPHK2, by acting in a sphingosine-competitive manner, opaganib also actsas an estrogen receptor (ER) antagonist, and inhibits ER-positive breast cancer tumor formation in vivo (Antoon JW et al, Endocrinology 2010). If Reviewer 3 is concerned about thepossibility that the opaganib downstream effects we observed in our study might be explained by ER inhibition, we remind that we used cell lines that do not express ER. Indeed, the MDA- MB-231 cell line is a triple negative breast cancer cell line. MCF10A cells also do not express ER (Lane MA et al, Oncology Report, 1999,) and our transcriptomic analysis (Table S1) did not reveal any increase in the expression of ER-responsive genes in MCF10AF1F2 cells in which flotillins are upregulated, thus eliminating a possible non-specific effect of opaganib in these cells.

In conclusion, we hope that these arguments help to convince Reviewer 3 that our experiments were performed in conditions where we carefully limited the possibility of opaganib off-target effects, on the basis of the currently available literature data on opaganib.

The experiment described in Figure 6C-H need to be confirmed by downregulation of SphK2.

We fully agree with Reviewer 3 that complementary experiments by downregulating SPHK2 must be used in addition of the opaganib treatment to inhibit SPHK2. In the previous version of the manuscript, we downregulated SPHK2 by siRNA in MCF10AF1F2 cells and showed thatthis led to a significant decrease in AXL and ZEB1 expression (figure 6B), as did opaganib treatment (figure 6A). In the current revised version of the manuscript, we added data obtained following SphK2 downregulation in MDA-MB-231 cells using siRNAs that show AXL and ZEB1 downregulation (figure 6C), as previously observed upon SPHK2 inhibition using opaganib (figure 6D).
Moreover, in the current revised version of the manuscript we added data we obtained in Hs 578T cells, another triple negative breast cancer cell line we used during the revision process. Again, SPHK2 knock down using a specific siRNA decreased AXL protein level, as did opaganib treatment of Hs 578T cells. This has now been added in figure S7H of the revised version of the manuscript.

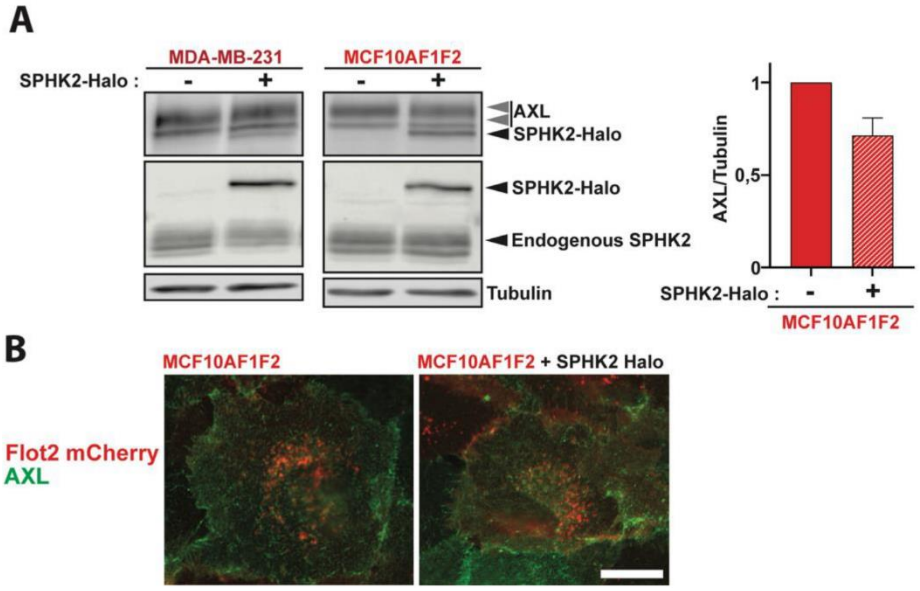
Concerning the experiments that use cycloheximide to remove the contribution of protein synthesis on AXL expression level (figure 6E), we could show that the siRNA-mediated knock down of SPHK2 has a similar effect as incubation with opaganib. By comparing AXL levels during incubation with cycloheximide in control siRNA and SPHK2 siRNA-treated MCF10AF1F2 cells and also in control siRNA and SPHK2 siRNA-treated MDA-MB-231 cells,we found that in both cell lines, AXL decay was significantly faster upon SPHK2 downregulation (figure 6F). We did not observe any significant change in CD71 decay (that is not a cargo of the UFIT-pathway (figure S3A, C, D and E) in SPHK2 downregulated cells (figure S3F).

However, we are unfavorable to use a siRNA against SPHK2, in addition to opaganibtreatment, in the experiments done to measure the effect of SPHK2 inhibition on the rate of AXLinternalization (previously in figure 6E and F, now figure 6F and G) and the level of AXL at the cell surface (previously in figure 6G and H, now figure 6H and I). Indeed, we carefully chose a short (4 hours) incubation with opaganib at the end of which the total cellular level of AXL was not decreased yet (see figure 6A and D). This allowed us to measure unambiguously a defect in AXL endocytosis or a change in the level of AXL at the cell surface (figure 6G, H, Iand J). We believe that it would be very difficult to do these experiments using a siRNA against SPHK2. This would require to

obtain a sufficient SPHK2 level reduction but in conditions where AXL level is still maintained. We think that due to the mosaic transfection efficiency, being able to precisely synchronize the effect of a siRNA at its beginning is impossible.

1. Does overexpression of SphK2 reverse the effects of the SphK2 inhibitor? In a similar manner, does overexpression of SphK2 enhance stabilization of AXL?

To answer the first question, it is not clear to us how to test whether SPHK2 overexpression can reverse the effects of the SPHK2 inhibitor because the ectopically expressed SPHK2 wouldalso be sensitive to the inhibitor. This would require to overexpress a SPHK2 mutant that is catalytically active but insensitive to the inhibitor, and to our knowledge, such a mutant does not exist. Regarding the second question, we generated a retroviral DNA construct that expresses SPHK2and stable cell lines that overexpress SPHK2. We did not detect any increase in AXL level or localization in cells that express or not SPHK2 (see below additional figure 4)



Additional figure 4: Overexpression of SPHK2 does not increase AXL level. MDA-MB-231 cells and MCF10AF1F2 cells that stably express SPHK2-Halo were generated by retroviral infection. **A.** AXL level was assessed by western blotting. In the western blot panels, the upper panels show the membrane after simultaneously probing with anti-AXL and -SPHK2 antibodies. SPHK2-Halo is detectable as a single band below the bands corresponding to AXL. The middle panels show a western blot of the same lysates probed only with the anti-SphK2 antibody on a separate membrane allowing to visualize both endogenous SPHK2 and exogenous SPHK2-Halo. Histograms show the comparative quantification of AXL level in MCF10AF1F2 cells and MCF10AF1F2 cells that express SPHK2-Halo. Data are the mean ± s.e.m. of 3 independent experiments (obtained from 3 independent lysates). **B.** Fluorescence microscopy images of MCF10AF1F2 cells and MCF10AF1F2 cells that express SPHK2-Halo after staining for AXL. The AXL and Flotillin 2-mCherry signals are merged. Two representative images are shown. No detectable change in AXL distribution was detected in cells that overexpress SPHK2-Halo.

2. Although the authors suggest recruitment of SphK2 and formation of S1P in UFIT, there areno measurements of S1P.

To answer the point raised by Reviewer 3 we recently compared, in collaboration with a lipidomic facility, S1P levels in MCF10AmCh and MCF10AF1F2 cells by quantitative mass-spectrometry. The results showed a 3.5-fold increase in S1P in MCF10AF1F2 cells comparedwith MCF10AmCh (shown in the revised Figure 5H). This result agrees with our finding that SphK2 catalytic activity is required for the UFIT pathway-mediated AXL stabilization. This result is also in agreement with the study by Nichols' group showing a decrease in S1P level inflotillins knock-out cells (Riento et al, PloS ONE, 2018).

Also, there is no indication that SphK2 is activated despite the fact that ERK and AKT are activated in UFIT and are known to phosphorylate and activate SphK2. Is SphK2 that is recruited to flotillin phosphorylated?

As recommended by Reviewer #3, because SphK2 was reported to be activated by phosphorylation on Thr578 (Hait et al, J Biol Chem, 2007), we checked by western blot the phosphorylation status of SphK2 on Thr578 (using Anti-phospho-Thr578-SphK2 antibody fromECM Biosciences). We detected a strong signal indicating that at least a fraction of SphK2 is activated by phosphorylation on Thr578 in MCF10AF1F2.
In theory, we could investigate the activity-related phosphorylation status of SPHK2 associatedwith flotillin-rich membranes and endosomes. However, this seems difficult to achieve because unfortunately, the only two commercially available antibodies against phosphorylated SPHK2do not work for immunofluorescence. The first one (mentioned above and used in western blot) is against the Thr578 residue identified as phosphorylated downstream of ERK by Sarah Spiegel's group (Hait et al, J Biol Chem, 2007). The second antibody was designed to recognizespecifically phosphorylated Thr614 (https://www.abcam.com/sphk2-phospho-t614-antibody- ab111948.html), but this residue has not been rigorously demonstrated to be phosphorylated downstream of AKT or ERK or to stimulate SPHK2 activity. Thus, considering the lack of appropriate tools and because we already showed, using opaganib, that SPHK2 catalytic activity is required for the UFIT pathway, we believe that investigating SPHK2 phosphorylationstatus reflecting its activity in flotillin-positive vesicles would be difficult to achieve in a reasonable amount of time. Moreover, we think that it will not bring a higher value to our present study.
To answer more broadly to the question "Is SPHK2 recruited to flotillin phosphorylated?", we anticipate that it could be the case, at least on the Ser419 and Ser420 residues, because Nakamura's group demonstrated that Ser419 and Ser420 phosphorylation favors the nuclear export of SPHK2 (Ding G et al, J Biol Chem, 2007). However, as it is unknown whether phosphorylation of these residues influences SPHK2 activation status, we do not plan to perform immunofluorescence experiments with this antibody (not available commercially) because the results would not address the Reviewer's question.

3. It should be determined whether the optogenetic system used to induce flotillin oligomerization also induces recruitment and activation of SphK2.

We thank Reviewer 3 for suggesting to perform this interesting experiment. Nevertheless, we would like to highlight that with our optogenetic system we can monitor only SPHK2 recruitment, but not its activation in flotillin-rich microdomains (because no anti-phosphorylated SPHK2 antibody is available for immunocytochemistry). We performed this experiment in Hs 578T cells that express CRY2-mCitrin, Flot2-CIBN-mCherry and SPHK2- Halo labeled with Janelia 646 conjugated Halo-Tag-ligand. We induced the formation of flotillin microdomains by 488nm-light illumination and collected images every 2 seconds by TIRF-microscopy to visualize the potential recruitment of fluorescent SPHK2-Halo in the flotillin-rich microdomains formed at the ventral surface of the cells. In some cases, as expected, we could observe SPHK2-Halo accumulation that lasted from 20 to 70 seconds (seeadditional figure 5 below). However, we could not visualize SPHK2-Halo accumulation in all the flotillin-microdomains that were formed probably because of the rapid recruitment and turnover of SPHK2 molecules.

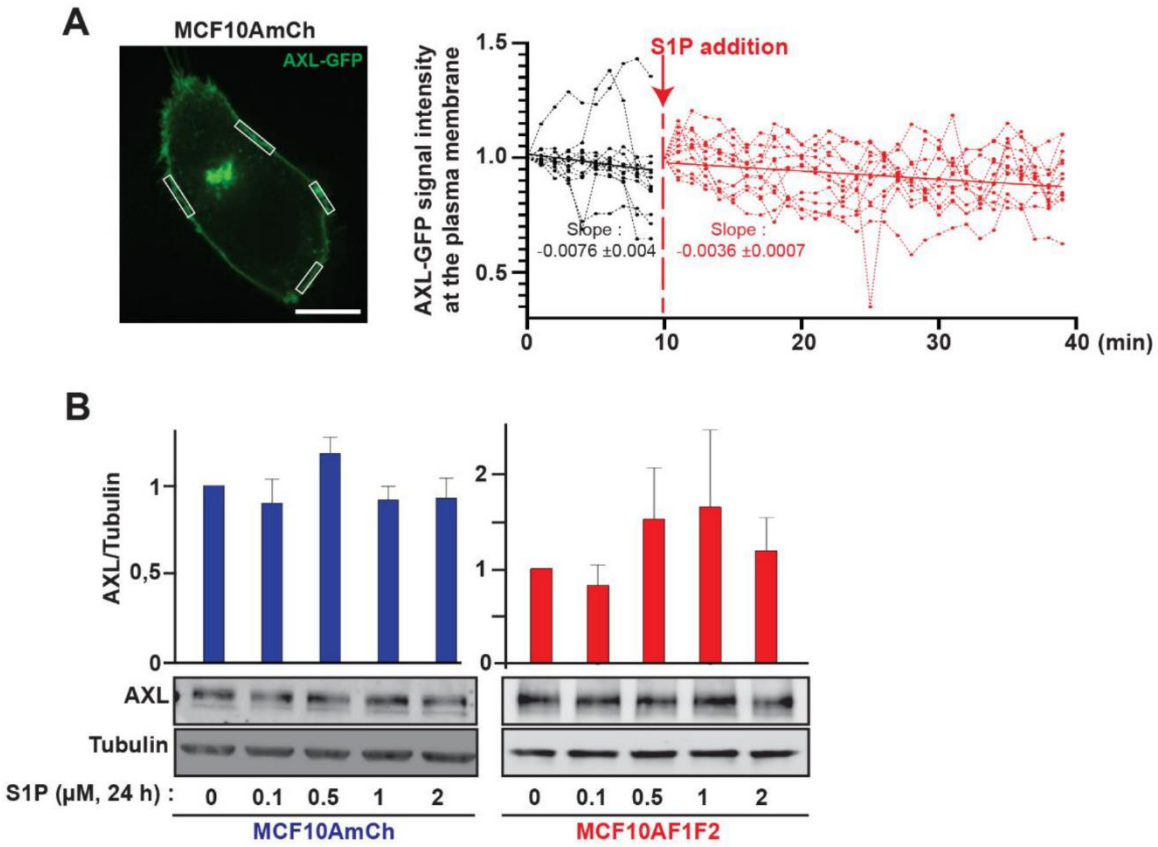
***NOTE:** We have removed unpublished data that had been provided for the referees inconfidence.*

4. Most importantly, it has not been established that the effects are mediated by S1P. Does addition of S1P enhance stabilization of AXL? Are the effects of S1P mediated by a S1P receptor? If so, which S1P receptor? There are several specific agonists and antagonists of S1PRs that can be utilized to answer this question. It's also possible that the effects of S1P are mediated by intracellular actions as were suggested by the De Camilli group (Nat Cell Biol. 2014 Jul;16(7):652-62).

As suggested, we performed experiments in which exogenous S1P was added to cells and analyzed whether this affects AXL level at the plasma membrane and its expression (see

additional figure 6 below). Using live imaging of MCF10AmCherry cells that express AXL- GFP we could measure a decrease in AXL level from the plasma membrane following addition of exogenous S1P (0. 5 µM) (additional figure 6A). This suggested that addition of exogenous S1P was not sufficient to trigger AXL internalization from the plasma membrane. We also measured the impact on AXL level of S1P added at different concentrations (from 0.1 µM up to2 µM) for 16 hours and 24 hours. Both in MCF10AmCherry and in MCCF10F1F2 cells, we did not observe any increase in AXL level. Because exogenous S1P was added in the culture medium, we anticipate that its effect is mainly limited to the external face of the plasma membrane where it can bind to specific receptors. However, our current results on the localization and the involvement of SPHK2 suggest that S1P involved in the UFIT pathway is generated at the internal face of the plasma membrane and in late endosomes. In addition, the mechanisms of the UFIT pathway are very likely based on a local concentration of S1P in somemicrodomains (at the internal face of the plasma membrane and in intracellular membranes) scaffolded by flotillins that concentrate its precursor, sphingosine, in the inner leaflet. It is thus very difficult to mimic such local concentration of S1P just by adding S1P to the cells.

We agree that identifying the S1P receptors involved would be of valuable interest for a better characterization of the UFIT pathway. However, we think that this is beyond the scope of our present study. Among the five known S1P receptors, we do not know if any could be involved in membrane remodeling at the plasma membrane to promote endocytosis. To our knowledge, involvement of S1P receptors in endocytosis has never been reported. However, based on the work by Nakamura's group (Kajimoto et al, Nat Comm, 2013 and Kajimoto et al, J Biol Chem, 2018), the S1P1 and S1P3 receptors are involved in membrane remodeling and cargo sorting from the outer membrane of late endosomes (where flotillins accumulate in our cell models). We could hypothesize that activation of these receptors are influenced by flotillins and are involved in the UFIT pathway. However, we think that testing this hypothesis would be the subject of a distinct study.
At the plasma membrane, we totally agree that the effect of S1P could be mediated, as suggestedby De Camilli's group (Shen et al, Nat Cell Biol 2014), by the formation of tubular endocytic structures rich in sphingosines after acute cholesterol extraction. Reciprocally, in our cell models, upregulated flotillins, thanks to their ability to bind to sphingosine (demonstrated by Nichols' group (Riento et al, PloS ONE, 2018) and to oligomerize, could create sphingosine- rich membrane regions.



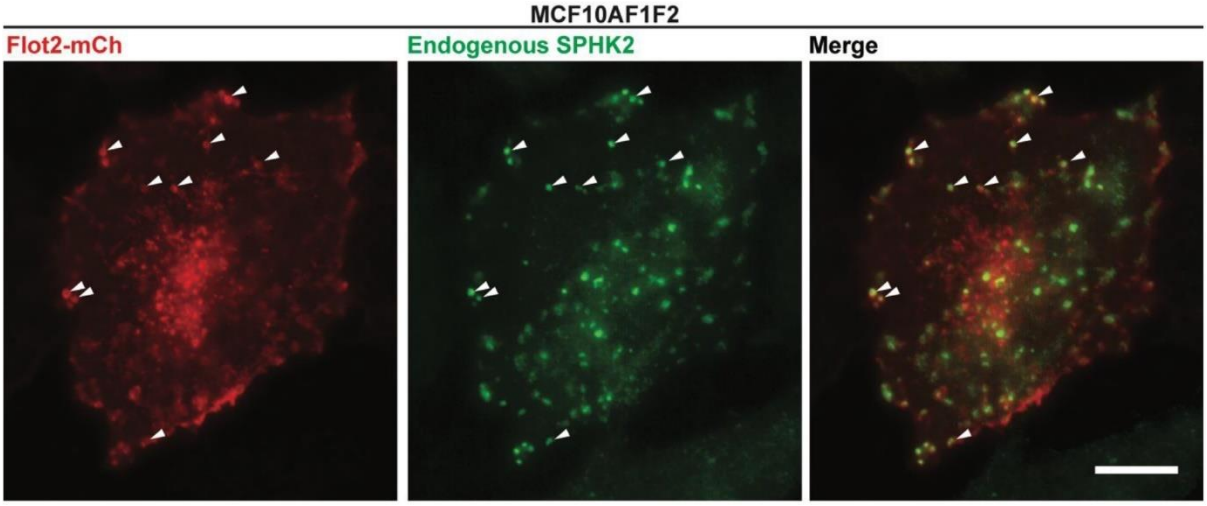
Additional figure 6: Analysis of exogenous Sphingosine 1-Phosphate addition on AXL distribution at the plasma membrane and AXL expression level.

A. Exogenous extracellular addition of Sphingosine 1-Phosphate (S1P) does not promote a decrease in AXL- GFP from the cell surface. MCF10AmCh cells that express AXL-GFP were monitored by spinning disk confocal microscopy (1 frame / min). A first 10-min length movie before S1P addition was made. Image acquisition was stopped for 1 min when S1P was added in the medium (final concentration: 0.5 µM). Then, a second 30-min movie after S1P addition was made in the same conditions and following the same cells. The mean fluorescence intensities of the GFP signal, in four different regions of interest (ROI) located at the plasma membrane, were measured along time in each movie. The experiment was performed by monitoring three different cells. One representative cell is shown with the white rectangles corresponding to the four ROIs. The graph shows all the mean intensity values from all the ROIs normalized to the initial value at the beginning of each movie. A simple linear regression (considering each replicate Y value as an individual point) was performed with GraphPad Prism for each condition (before and after S1P addition) and indicates that the two slopes are not significantly different. The minor decrease observed in both conditions might be due to photobleaching. We obtained similar results in MCF10AF1F2 cells that express AXL-GFP (not shown). **B. Exogenous extracellular addition of Sphingosine 1- Phosphate is not sufficient to increase AXL level.** MCF10AmCh and MCF10F1F2 cells were incubated with the indicated concentrations of S1P added to the medium for 24 hours. Cell lysates were then probed by western blotting using antibodies against AXL and tubulin. Data show the AXL/tubulin ratio and are the mean ± s.e.m. of three independent experiments. We did not observe any major modification of AXL levels. We obtained similar results after a shorter incubation (16 hours) with the same concentrations of S1P (not shown).

In A and B, the S1P solution was prepared as follows: S1P (purchased from Avanti Polar lipids) was resuspended in methanol/H2O (95/5), dried in glass tubes, and stored at -20°C. The day of the experiment, S1P was resuspended (10 µM) in DMEM/BSA (4 mg/ml) at 37°C by sonication and straightaway added to the cell medium at the indicated final concentrations.

5. There is a commercial antibody for endogenous SphK2 that can be used to validate and substantiate the data with GFP-SphK2. (F1000Res . 2016 Dec 6;5:2825. doi: 10.12688/f1000research.10336.2. eCollection 2016. Validation of commercially available sphingosine kinase 2 antibodies for use in *immunoblotting, immunoprecipitation and immunofluorescence)*

We thank Reviewer 3 for this suggestion and advice. We already tried, but without success, two antibodies from Sigma and Cell Signaling Technology (not described to work in immunofluorescence experiments). We tested in MCF10AF1F2 cells and MDA-MB-231 cells the anti-SPHK2 antibody from ECM-Biosciences mentioned in the article by Neubauer and Pitson F1000 research, 2016. The labeling of endogenous SPHK2 in MDA-MB-231 cells was weak and diffuse in the cytoplasm. In MCF10AF1F2 cells, we had the same problem althoughin some cases, we could observe cells with flotillin2-positive vesicles labeled with the anti- SPHK2 antibody (see additional figure 7 below



Additional figure 7: Endogenous SPHK2 can be detected in flotillin 2-positive vesicles in MCF10AF1F2 cells. MCF10AF1F2 cells that express Flot2-mCherry were fixed, permeabilized and immunolabeled for SPHK2 using the EC-SP4621 antibody and an Alexa488-conjugated secondary antibody. The maximum intensity projection image made from a stack of 6 images acquired every 2 µm, is shown. Arrows show examples of flotillin2-mCherrypositive vesicles labelled for SPHK2.

We believe that the difficulty to visualize SPHK2 in flotillin-positive vesicles in fixed cells comesfrom the fixation / permeabilization steps that affects lipids and also from the labile SPHK2 interaction with the membrane. This later point is supported by our observation that the vesicular localization of SPHK2-GFP in live cells is completely lost after fixation using paraformaldehyde. SPHK2 localization in flotillin-positive late endosomes might rely on its interaction with sphingosine molecules that are enriched in these intracellular compartments.In agreement, we observed that addition of opaganib, characterized as a sphingosine competitive inhibitor, displaces SPHK2-GFP from flotillin-positive late endosomes in live cells(previously figure S7D, now figure S7G). In addition, we showed that SPHK2-Halo is recruitedmore in CD63-positive late endosomes in cells that overexpress flotillins (previously figure 5E,now figure 5G). This could be due to the presence of a higher concentration of sphingosine promoted by flotillins (that bind to sphingosine) accumulating in these compartments. Moreover, we performed experiments in which we added fluorescent NBD-sphingosine to the cell culture medium. We observed by spinning disk confocal microscopy of live MCF10AF1F2 cells, MDA-MB-231 cells and Hs 578T cells that NBD-sphingosine quickly accumulated in flotillin-positive late endosomes. These data are shown in the additional figure 8 below, and representative images have been added in the revised version of the manuscript (figure 5A andB for MCF10AF1F2 cells and MDA-MB-231 cells, respectively, and figure S7A for Hs 578T cells). We did not observe this accumulation in flotillin-positive late endosomes using anotherfluorescent lipid (Oregon-Phosphatidylethanolamine) (figure 5A). Related to the loss of SPHK2signal in fixed cells, the accumulation of NBD-sphingosine in flotillin-positive late endosome was no longer observed after fixation (not shown).

NOTE: *We have removed unpublished data that had been provided for the referees in confidence.*

Reviewer #3 (Significance (Required)):

This is an interesting paper. If the authors confirm the involvement of Sphk2 and mechanismof action of S1P, this would be an important contribution to the field.

Second decision letter

MS ID#: JOCES/2021/259178

MS TITLE: Upregulated-flotillins and sphingosine kinase 2 derail AXL vesicular traffic promoting epithelial-mesenchymal transition

AUTHORS: Mallory Genest, Franck Comunale, Damien Planchon, Pauline Govindin, Sophie Vacher, Ivan Bieche, Bruno Robert, Himanshu Malhotra, Andreas Schoenit, Liubov Tashireva, Cecile Gauthier-Rouviere, Stephane Bodin, Dune Noly, and Josefina Casas
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

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

OK for publication

Comments for the author

OK for publication

Reviewer 2

Advance summary and potential significance to field

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Comments for the author

The authors adequately addressed all previous concerns. I recommend accepting the paper.

Reviewer 3

Advance summary and potential significance to field

AXL is a major oncogene that promotes EMT in a variety of tumour types. Understanding how its signalling can be triggered by endocytic pathways even in cells that are non-cancerous is very important and of high significance for the cancer field and the trafficking community.

Thanks for addressing my points of concern, the manuscript is now suitable for publication

Comments for the author

All the points have been addressed, the manuscript is suitable for publication