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Lats2 kinase potentiates Snail1 activity by promoting nuclear retention upon phosphorylation

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

1st Editorial Decision 02 February 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees express interest in your finding that Snail is a substrate for the Lats2 kinase, and are broadly supportive of publication, but a number of significant concerns are raised that will need to be addressed first.

The referees' reports are explicit, so I see no need to repeat all their points here. I would, however, draw your attention a couple of points. Firstly, referee 1 raises a large number of technical concerns - particularly with regard to the use of nocodazole to induce Lats2 activity, and the question as to whether Lats2 phosphorylates Snail in the absence of mitotic damage. Secondly, both referees 1 and 3 highlight the question of how these results fit with the known function of Lats2 in the Hippo pathway, which has been shown to regulate EMT via YAP and TAZ. Minimally, this needs to be discussed, but I would strongly encourage you to try to address this experimentally. Thirdly, referee 3 requests some better evidence for the relevance of this regulation in the cancer context - while I do recognise that analysis of tumour samples may not be possible (although if you do have access to such samples, I would encourage you to analyse them), the suggested experiments with cell lines would go some way towards addressing this concern.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For

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We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is a very interesting paper describing Snail1 as a new Lats2 kinase target substrate. Nevertheless, a number of caveats undermine overall enthusiasm at this juncture.

1. From this Reviewer's perspective, the peripatetic use of nocodazole is problematic. While it appears that all of the phosphorylation studies required the use of nocodazole to induce Lats2 kinase activation/trafficking into the nuclear compartment, experiments ranging from the kinome screen itself to the analyses of EMT which do not employ nocodazole do not explain how/if Lats2 kinase affected nuclear Snail1 directly. Indeed, it is not clear that T203 phophorylation can even be detected under these conditions (or for that matter, in vivo). While nocodazole can trigger Lats2-dependent Snail1 phosphorylation, I am wondering if Lats2 might not affect Snail1 activity by other, as yet undescribed, mechanisms in the kinome screen, during EMT or in vivo during development. Related to this point, a number of issues need to be addressed further:
a. The fact that GSK3 β inhibition did not enhance scoring as effectively as proteasome inhibition is certainly consistent with your model, but what about the fact that GSK3 α may also phosphorylate Snail1?
b. Can you detect pT203 in 293 cells under the conditions of the kinome screen?
c. Does nocodazole affect Snail1 transcript levels? How about GSK3 β protein levels?
d. Did depletion of Lats2 in HCT116/HT-1080 cells affect Snail1 mRNA levels?
e. The effect of nocodazole on pT203 in MEFs (Fig. 4A) is striking relative to the very small effect on Snail1 protein levels.
f. The UV experiments in Fig. 4B are not described in the text. Also, this is one of the few experiments where nocodazole is not used, but when pT203 levels were probed, the signal is barely detected. Could you IP with this antibody and determine the percentage of nuclear Snail1 that has actually undergone phosphorylation? I realize that nocodazole was not used in Fig. 4C, but Snail1 as well as Lats2 were exogenously introduced here.
g. A requirement for Lats2 kinase activity in Fig. 5 is intriguing given the fact that phosphorylation of T203 was not required. Is it possible that Lats2 is interacting with another molecule here?

2. The Snail1 nuclear retention experiments are interesting, but raise a number of issues. First, if T203 is not required for transcriptional activity, shouldn't the nuclear fraction still remain functional in your EMT experiments? Second, what is the effect of placing an NLS sequence on T203A? Third, can you determine the half-life of wt and T203A Snail1 in the absence or presence of Lats2? Fourth, what effect does nocodazole have on these trafficking experiments? Finally, as intimated above, if pT203 is critical to nuclear retention, I'm not sure I understand how Lats2 exerts such profound effects on Snail1 protein levels in 293, HT1080 or HCT116 cells in the absence of mitotic stress or DNA damage.

3. The ability of Lats2 to promote EMT-like effects is certainly striking. However, it is not clear that these effects can be linked directly to the phosphorylation of T203, i.e., Lats2 is certainly "functional" while T203A Snail1 is not "active" (assuming, of course, that the Ala residue substitution does not affect DNA binding), but unless it can be shown that substantive quantities of nuclear Snail1 really are phosphorylated, I am uncertain as to whether Lats2 exerts other effects on EMT. Indeed, though you mention anti-YAP antibodies in your Methods, I think many readers will wonder about the Lats2/YAP/TAZ network, particularly since these molecules can induce EMT (indeed, TAZ has been reported to induce Snail1 transcription, MCB 28:2426, 2008).

4. Data relevant to Fig. 7D should be shown. Also, you should note that EMT-like effects can be induced by simply altering cell density (e.g., JCB 163:847, 2003). As such, conference should be monitored carefully when manipulating Snail1 or Lats2 levels. It might also prove informative to determine whether the inability of Lats2-silenced cells to respond to TGFβ can be "rescued" with another EMT-inducing factor (e.g., ZEB1). It is also important to show that your cells remain responsive to TGFβ in the absence of Lats2 or Snail1.

5. Fig. 8A. Are Snail1 transcript levels normal? Is it possible to detect pT203? The enormous number of YAP/TAZ substrates, and impact on transcription, make this experiment somewhat difficult to interpret.

6. Fig. 8B. I am not certain that the zebrafish experiments are that helpful here. First, I was under the impression that both Lats 1 and 2 affect mesodermal elements in this model and there are no data to support directly the role of Lats2 in Snail1 phosphorylation or nuclear localization.

7. The text, in general, is not always well written, e.g.,

a. "Normal epithelia do not expression Snail1" (pg. 3) "Although normal epithelia do not expression Snail1" (pg.7).
b. Pg. 8. "For example, the colon cancer cell line HCT116." - This is a fragment and there is no reference.
c. Pg. 9. "Despite their identification long ago, and important tumor suppressive activity..." An awkward sentence and again, no reference citation.
d. "For Snail1 to induce EMT it needs to be in the nucleus... (Zhou et al, 2004" per page 10 versus "Cytoplasmic Snail1 is rapidly degraded while nuclea-Snail1 is stable (Yook et al, 2006)" pg. 12.
e. No introductory statement regarding the use of T203E-Snail1 on page 12.
f. The Discussion, particular regarding the role of Lats2 as a tumor suppressor, is confusing and many times, redundant. Also, the last section on p53-Mdm2 should be deleted.

Referee #2 (Remarks to the Author):

The manuscript reports interesting findings that Lats2 phosphorylates and stabilizes Snail1 protein and induces EMT. Lats2 is activated by various stimuli, and involved in the Hippo pathway. The manuscript is thus important to understand the mechanisms of regulation of EMT.
Critiques
1. page 5. Most of this part is redundant. They should describe more about Lats2 here. In page 9, Lats2-/- cells were used; the phenotype of Lats2 mice should be described somewhere (maybe in Introduction). Lats1 knockdown was performed in page 9; the function of Lats1 should be described somewhere.
2. page 11 and Fig. 4. Why were the pLats2(S408) antibody and pLats2(S871) antibody used in Fig. 4B and 4C, respectively, but not in other experiments (Fig. 3D and 4A)?
3. page 14, the 3rd paragraph. Fig. 7D does not show any results. This is not acceptable.
4. Fig. 1C. What does "*" in the figure indicate? This should be explained in the figure legend.
5. Fig. 8. What does "1 through 5" mean?
6. Fig. S3D. This figure is discussed in page 18, after Fig. S4 was discussed in the text.
7. Fig. S4A. It should be shown in the figure that T203A.Snail is shown in triangle.
8. Throughout the manuscript, English is poor. Rewriting of the manuscript is required. Many grammatical errors are found. "Commas" are used in a strange way, which makes the manuscript very difficult to read.

Other minor points.
9. "Snail1" is used in the manuscript, but sometimes "Snail" is used. These should be corrected.
10. page 10, line 8. This sentence is incomplete.
11. page 17, line 20. I do not understand what this sentence means.
12. Methods section should be checked carefully. Since Dr. Nojima is a coauthor, it should not be stated that some of the reagents are provided by Dr. Nojima.

Referee #3 (Remarks to the Author):

The authors have used an unbiased RNAi screen to identify LATS2 kinase as a positive regulator of Snail1 protein levels and nuclear retention, and that LATS2 influences the ability of cells to undergo EMT through this regulatory pathway. The results suggest that the developmental functions of LATS2 involve induction of EMT, and that suggest unanticipated functions for LATS2 in facilitating the progression of established cancers-in contrast to previously defined only as a tumor suppressor. The experiments are largely convincing and clearly presented. However, the work leaves open some important questions regarding the relationship of the findings to previously defined functions of LATS2 in Hippo signaling and in EMT, and of the overall physiological significance of this LATS2-Snail1 pathway in both cancer and development.

1. Existing data has shown that Hippo pathway components including LATS2 negatively regulate EMT and sustain cellular polarity (e.g. Varelas, Dev Cell 2010, Lei MCB 2008) through regulation of Yap/Taz subcellular localization and control of Smad2/3 activity. The authors should include some basic evaluation of how Taz and Yap are regulated in the model systems. In addition, the authors should address whether the best-defined physiological activation of Hippo signaling, cell contact, can modulate Snail (e.g. this can be done in the LATS2 WT and KO MEFs).

2. The authors are careful not to over-interpret their data with respect to its relevance to cancer. However, as the potential capacity of LATS2 to promote EMT during the course of cancer progression is a major interest of this work, some studies are needed to address this more directly. Do carcinoma cell lines with mesenchymal features (Snail expression, low E-cadherin, mesenchymal marker expression) overexpress LATS2? Does knockdown of LATS2 affect Snail1 expression and tumorigenic growth in this context? Is LATS2 overexpressed in poorly differentiated epithelial cancer specimens?

Other points:

1. Does Lats1 also regulate Snail?

2. Does MG132 restore Snails levels in LATS2 KO MEFs and knockdown cell lines?

3. The LATS2 EMT data in MCF10A cells (Fig. 7D) should be shown directly

4. Do LATS2 and Snail co-localize during development.

1st Revision - authors' response | 10 May 2011

Below is a point-by-point response to the referees.

Referee #1

1. *From this Reviewer's perspective, the peripatetic use of nocodazole is problematic. ....*

In addition to nocodazole-induced mitotic dysfunction to activate Lats2, in new experiments we now show, in MEFs, that two other stimuli known to activate Lats2 kinase activity - oncogenic H-RasV12 and cell-cell contact growth inhibition (Hippo pathway) also result in phosphorylation of Snail1 at T203 and stabilization of Snail1 protein (Figures 4 and 5). These effects require the presence of Lats2. Moreover in HEK293.Snail1-CBG clone 8 cells (cell line used for the initial screen that identified Lats2 as a stabilizer of Snail1) we now show that Snail1-CBG is

phosphorylated, at T203, under basal conditions (confluent cells), and that RNAi-depletion of Lats2, but not Lats1, reduces pT203.Snail1 and Snail1 protein level (Figure 3). In summary Lats2, but not Lats1, phosphorylates Snail1 leading to its stabilization.

Of course we cannot exclude that Lats2 can affect Snail1 activity by mechanisms other than phosphorylation. Nonetheless, we believe the current data strongly supports the contention that Lats2 can affect Snail1 activity through phosphorylation at T203, in the nucleus.

a. The fact that GSK3 β inhibition did not enhance scoring as effectively as proteasome inhibition is certainly consistent with your model, but what about the fact that GSK3 α may also phosphorylate Snail1?

GSK3β activity influences Snail1 degradation and inhibition of GSK3b activity stabilizes Snail1. In new experiments we show that Nocodazole treatment of colon cancer HCT116 cells activates Lats2 and stabilizes Snail1, and occurs independent of GSK3 β inhibition (Figure 4A). Nocodazole treatment does not inhibit or activate GSK3b activity nor alter GSK3b protein level (Figure 4A).

b. Can you detect pT203 in 293 cells under the conditions of the kinome screen?

Yes. See new Figure 3D, and comments to 1, above.

c. Does nocodazole affect Snail1 transcript levels?

No. See new Figure 4B.

How about GSK3β protein levels?

No. See new Figure 4A

d. Did depletion of Lats2 in HCT116/HT-1080 cells affect Snail1 mRNA levels?

No. See revised Figure 4B

e. The effect of nocodazole on pT203 in MEFs (Fig. 4A) is striking relative to the very small effect on Snail1 protein levels.

This is because the exposure of the pT203.Snail1 Western blot panel was much longer than other blots. This has been added to the figure legend. Regardless, the extent of Snail1 stabilization, in response to nocodazole, does differ between cell types and in response to different signals activating Lats2. This point has also been added to the text.

f. The UV experiments in Fig. 4B are not described in the text. Also, this is one of the few experiments where nocodazole is not used, but when pT203 levels were probed, the signal is barely detected. Could you IP with this antibody and determine the percentage of nuclear Snail1 that has actually undergone phosphorylation? I realize that nocodazole was not used in Fig. 4C, but Snail1 as well as Lats2 were exogenously introduced here.

We have now determined whether Snail1 is phosphorylated and stabilized following many different stimuli that activate Lats2 – nocodazole induced mitotic dysfunction, oncogenic H-RasV12 that involves a DNA damage response, Hippo pathway activation. The UV experiments have been removed and replaced with these new experiments.

g. A requirement for Lats2 kinase activity in Fig. 5 is intriguing given the fact that phosphorylation of T203 was not required. Is it possible that Lats2 is interacting with another molecule here?

Yes, in the absence of a demonstration that purified proteins interact in vitro, this is always possible in co-IP experiments. However, to address this we have performed new mapping experiments (Figure S3) and found that Lats2 interacts with the N-terminal 10-40 amino acid region of Snail1. This domain is well removed from T203, and likely explains why the Lats2-Snail1 association, in co-IP experiments, does not depend upon phosphorylation of T203.

That Lats2 and Snail1 associate in cells, and that this association is dependent upon active Lats2, is consistent with Snail1 being a Lats2 substrate. While we have not demonstrated, in vitro, that purified active Lats2 kinase (not available and not a trivial undertaking) phosphorylates purified Snail1 we have shown that Lats2 immunoprecipitated from cell extracts does phosphorylate purified GST-Snail1, specifically, at T203, in vitro (Figure 3B). Yes, this analysis cannot exclude that there could be another associated kinase in the Lats2 immunoprecipitate, however, when kinase dead

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Lats2 was immunoprecipitated from transfected cells, that immunoprecipitate does not phosphorylate GST-Snail1 (Figure 3B).

2. *The Snail1 nuclear retention experiments are interesting, but raise a number of issues. First, if T203 is not required for transcriptional activity, shouldn't the nuclear fraction still remain functional in your EMT experiments?*

Second, what is the effect of placing an NLS sequence on T203A?

We have done this experiment. Forced nuclear accumulation of T203A (NLS-T203A.Snail1) is functional – induces EMT in MCF10A cells (see new Figure 7). We speculate that T203A.Snail1 is not functional in MCF10A cells because the nuclear fraction of T203A is rapidly exported (i.e., not retained in the nucleus). This means that a non-phosphorylated Snail form can function, but with a much lower efficiency, and therefore, able to induce EMT in overexpression experiments with forced nuclear localization such as those in MCF10A cells. This is also compatible with in vivo data in zebrafish (Figure S6) in which we have injected the T203A.Snail mRNA and found that it is indeed able to rescue the Lats2 morphant (loss of function) embryos but with much lower efficiency that the WT form (partial rescue). Thus phosphorylation of Snail1 at T203 is likely not absolutely necessary for its activity but does potentiate its function in cells and in vivo.

Third, can you determine the half-life of wt and T203A Snail1 in the absence or presence of Lats2?

Yes. In the absence of Lats2 (wt vs Lats2-/- MEFs) the protein half-life of wt Snail1 is significantly reduced (Figure 2F). The protein half-life of T203A.Snail1, and T203E.Snail1, in transfected HEK293 cells, is also reduced compared to WT Snail1 transfected into HEK293 cells (Figure S2D and data not shown).

Fourth, what effect does nocodazole have on these trafficking experiments?

In new Figure 6 we show that when colon cancer HCT116 cells are exposed to nocodazole, Lats2 levels increase, as anticipated, and active Lats2 (pT1041.Lats2) is found exclusively in the nucleus. In addition, the correspondingly increased pT203.Snail1 and Snail1 protein are predominantly nuclear in nocodazole treated cells.

Finally, as intimated above, if pT203 is critical to nuclear retention, I'm not sure I understand how Lats2 exerts such profound effects on Snail1 protein levels in 293, HT1080 or HCT116 cells in the absence of mitotic stress or DNA damage.

There are many other signals, some known (oncogenic stress and cell-cell contact) and others not known, that affect Lats2 activity. In addition to Lats2, other kinases, enzymes and cellular signals affect Snail1 level and function (e.g., GSK3b, PAK, Lysyl Oxidase, NF-kB). We have also identified more candidates in our screen. Our future efforts are directed at determining the relative contributions of the various known Snail1 phosphorylation events and kinases to its cellular biology and function. This was the rationale for performing the unbiased screen at the outset. In this manuscript our intent was to demonstrate that Lats2, specifically identified in our screen, actually affects Snail1 level and function, and to determine whether this effect is biologically important.

3. *The ability of Lats2 to promote EMT-like effects is certainly striking. However, it is not clear that these effects can be linked directly to the phosphorylation of T203, i.e., Lats2 is certainly "functional" while T203A Snail1 is not "active" (assuming, of course, that the Ala residue substitution does not affect DNA binding), but unless it can be shown that substantive quantities of nuclear Snail1 really are phosphorylated, I am uncertain as to whether Lats2 exerts other effects on EMT. Indeed, though you mention anti-YAP antibodies in your Methods, I think many readers will wonder about the Lats2/YAP/TAZ network, particularly since these molecules can induce EMT (indeed, TAZ has been reported to induce Snail1 transcription, MCB 28:2426, 2008).*

We show that phosphorylation of endogenous Snail1 at T203, by Lats2 in response to nocodazole treatment, was predominantly nuclear (Figure 6A). Also cellular EMT induced by Lats2 requires Snail1 (Figure 7A,B). In more new data we show that Lats2 levels are increased in 2 aggressive breast cancer cell lines and that depletion of Lats2 in these cells inhibits their invasive ability, a Snail1-dependent function (Figure 8A,B). So Lats2 is a component of the Hippo pathway and the Hippo pathway has been shown to limit EMT, however, we now suggest that Lats2 can also positively influence EMT through its effects upon Snail1.

Possible explanation for these opposing views could be that Snail1 is a nuclear target of Lats2 in contrast to the Hippo components YAP and TAZ, which are cytoplasmic targets. Hippo

pathway activation of Lats2 inhibits YAP and TAZ leading to a repression of EMT. At the same time, activation of Lats2 either by ectopic overexpression of upstream kinases or in response to cell-cell contact growth inhibition, also resulted in T203.Snail1 phosphorylation and increased Snail1 level, contributing not only to EMT but also to the arrest of cell proliferation in response to increasing cell density and to increased survival. Essentially, we propose that the same stimulus leading to Lats2 activation can generate two opposing responses depending on the compartmentalization of Lats2 kinase activity in the cytoplasm or in the nucleus.

Another possibility worth noting is that there may be distinct functions or substrates for Lats1 and Lats2. Lats1 does not phosphorylate Snail1 or affect Snail1 cellular protein levels. Recent studies in MCF10A cells have shown that Lats1, but not Lats2, suppresses YAP function (Zhang et al., 2008a), raising the intriguing possibility that Lats2 function in MCF10A cells is primarily via its phosphorylation of Snail1 and not through inhibition of YAP or TAZ. Moreover the ability of the two Lats kinases to suppress TGFb signals also differs with Lats1 being much more effective (Varelas et al., 2010). The phenotype of Lats1 and Lats2 deficient mice are distinct (McPherson et al., 2004) (Yabuta et al., 2007) (Visser and Yang, 2010), and only for Lats1 has in vivo tumor suppressive activity been observed (St John et al., 1999).

All this has been expanded in the revised discussion section.

4. Data relevant to Fig. 7D should be shown. Also, you should note that EMT-like effects can be induced by simply altering cell density (e.g., JCB 163:847, 2003). As such, conference should be monitored carefully when manipulating Snail1 or Lats2 levels.

We are aware that cell density can influence cellular EMT determinations, particularly in MCF10A cells. All our EMT analyses were initiated in confluent cells, and they are not split prior to biochemical and morphologic analysis. Data in original Fig. 7D table is now shown in new Figure 6 and Figure S4.

It might also prove informative to determine whether the inability of Lats2-silenced cells to respond to TGFβ can be "rescued" with another EMT-inducing factor (e.g., ZEB1).

Lats2 depleted MCF10A cells still respond to TGFb signals (see comment below) but are inhibited, but not blocked, in their capacity to undergo EMT. We have not done the suggested control experiment this as one needs to be careful with experiments of this nature. Twist overexpression can induce Snail expression (Casas et al, 2011).

It is also important to show that your cells remain responsive to TGFβ in the absence of Lats2 or Snail1.

In new Figure 7B we show that Snail1- and Lats2-depleted MCF10A are still responsive to TGFb. Compared to control untreated cells, pSmad2 is present and Smad2 is nuclear, when these cells are exposed to TGFb.

5. Fig. 8A. Are Snail1 transcript levels normal? Is it possible to detect pT203? The enormous number of YAP/TAZ substrates, and impact on transcription, make this experiment somewhat difficult to interpret.

Despite continuing effort our new pT203.Snail1 antibody has not been optimized for tissue IHC – there is much non-specific background. Snail1 mRNA levels were not performed on that set of embryos. All were used for protein determinations.

6. Fig. 8B. I am not certain that the zebrafish experiments are that helpful here. First, I was under the impression that both Lats 1 and 2 affect mesodermal elements in this model and there are no data to support directly the role of Lats2 in Snail1 phosphorylation or nuclear localization.

We respectfully disagree with this point, as the fish experiments are in vivo experiments that fully support the conclusions reached by the in vitro experiments. The referee is right when mentioning that both Lats kinases affect mesodermal elements but LOF experiments in the fish show that the main defect for Lats1 affecting the mesoderm is a convergence phenotype, whereas Lats2, as with Snail1b morphants, have a very similar phenotype - both show defects in extension of the axial mesoderm. This can be seen in Figure 3 of Chen et al., 2009. The arc of Dlx3 expression is much wider in Lats1MO than in the wt or Lats2MO and the retardation in migration of axial mesoderm is clearly evident in Lats2MO (Fig. 3J-L). The similarity between the phenotype of Lats2 and Snail1b is crucial. In addition, their expression patterns are also compatible with Lats2 and Snail1 interacting both in the fish and the mouse (see response to last point from reviewer 3). We have also carried out a series Lats1 morpholino injections and can confirm that only the *lats2*

morphant phenotype is similar to *snail1b* morphant (not shown). The fish model also supports the need for Snail1 to be in the nucleus to work efficiently, as when we inject the equivalent to the T203E.Snail1 version in zebrafish embryos (T196E), we cannot rescue the *lats2* morphant phenotype (Figure S6G). We know that this version does not enter the nucleus in cell lines (Figure 6). Yet when we inject an isoform of T196E that is forced to translocate to the nucleus (NLS-T196E.Snail1b), the rescue is very clear (Figure 8E).

7. The text, in general, is not always well written, e.g.,
a. "Normal epithelia do not expression Snail1" (pg. 3) "Although normal epithelia do not expression Snail1" (pg.7).
b. Pg. 8. "For example, the colon cancer cell line HCT116." - This is a fragment and there is no reference.
c. Pg. 9. "Despite their identification long ago, and important tumor suppressive activity..." An awkward sentence and again, no reference citation.
d. "For Snail1 to induce EMT it needs to be in the nucleus... (Zhou et al, 2004" per page 10 versus "Cytoplasmic Snail1 is rapidly degraded while nuclea-Snail1 is stable (Yook et al, 2006)" pg. 12.
e. No introductory statement regarding the use of T203E-Snail1 on page 12.
f. The Discussion, particular regarding the role of Lats2 as a tumor suppressor, is confusing and many times, redundant. Also, the last section on p53-Mdm2 should be deleted.
Text has been corrected and edited. The discussion has largely been rewritten.

Referee #2

1. page 5. Most of this part is redundant. They should describe more about Lats2 here. In page 9, Lats2-/- cells were used; the phenotype of Lats2 mice should be described somewhere (maybe in Introduction). Lats1 knockdown was performed in page 9; the function of Lats1 should be described somewhere.
Page 5 has been edited. We now show that depletion of Lats1 has no effect upon either T203 phosphorylation or Snail1 protein stability (see new Figure 3D)

2. page 11 and Fig. 4. Why were the pLats2(S408) antibody and pLats2(S871) antibody used in Fig. 4B and 4C, respectively, but not in other experiments (Fig. 3D and 4A)?
We have now used pT1041.Lats2 and pS871.Lats antibodies for all experiments. pT1041 is the upstream Mst (Hippo) kinase phosphorylation site, and pS871 is the autophosphorylation site. Both represent enzyme activation.

3. page 14, the 3rd paragraph. Fig. 7D does not show any results. This is not acceptable.
All of this data has now been added in new Figure 6 and Figure S$.

4. Fig. 1C. What does "" in the figure indicate? This should be explained in the figure legend.*
This has been added – it represents the difference in Snail1 stabilization (Snail1-CBG bioluminescence) following GSK3b inhibition and proteasome inhibition. This was the basis for our screen to identify novel post-translational regulators of Snail1.

5. Fig. 8. What does "1 through 5" mean?
Lanes 1-5 is extracts from different embryos – wt, Lats2-/- or Lats2+/-. This has been clarified in the figure legend.

6. Fig. S3D. This figure is discussed in page 18, after Fig. S4 was discussed in the text.
We have reordered figures including supplemental figures in the revised version. Hopefully they are all presented chronologically now.

7. Fig. S4A. It should be shown in the figure that T203A.Snail is shown in triangle.
Done.

8. Throughout the manuscript, English is poor. Rewriting of the manuscript is required. Many grammatical errors are found. "Commas" are used in a strange way, which makes the manuscript very difficult to read.

The revised manuscript has been rewritten.

Other minor points.
9. "Snail1" is used in the manuscript, but sometimes "Snail" is used. These should be corrected.
Done

10. page 10, line 8. This sentence is incomplete.
Corrected

11. page 17, line 20. I do not understand what this sentence means.
It has been re-written

12. Methods section should be checked carefully. Since Dr. Nojima is a coauthor, it should not be stated that some of the reagents are provided by Dr. Nojima.
It has been edited.

Referee #3

1. Existing data has shown that Hippo pathway components including LATS2 negatively regulate EMT and sustain cellular polarity (e.g. Varelas, Dev Cell 2010, Lei MCB 2008) through regulation of Yap/Taz subcellular localization and control of Smad2/3 activity. The authors should include some basic evaluation of how Taz and Yap are regulated in the model systems. In addition, the authors should address whether the best-defined physiological activation of Hippo signaling, cell contact, can modulate Snail (e.g. this can be done in the LATS2 WT and KO MEFs).

Possible explanation for the opposing views of Lats kinases (Hippo pathway) either inhibiting or facilitating EMT could be that Snail1 is a nuclear target of Lats2 in contrast to the Hippo components YAP and TAZ, which are cytoplasmic targets. Hippo pathway activation of Lats2 inhibits YAP and TAZ leading to a repression of EMT. At the same time, activation of Lats2 either by ectopic overexpression of upstream kinases or in response to cell-cell contact growth inhibition, also resulted in T203.Snail1 phosphorylation and increased Snail1 level, contributing not only to EMT but also to the arrest of cell proliferation in response to increasing cell density and to increased survival. Essentially, we propose that the same stimulus leading to Lats2 activation can generate two opposing responses depending on the compartmentalization of Lats2 kinase activity in the cytoplasm or in the nucleus. This may also be the case for Lats2 regulation of ASPP1 and p53 activity – see new discussion section.

Another possibility worth noting is that there may be distinct functions or substrates for Lats1 and Lats2. Lats1 does not phosphorylate Snail1 or affect Snail1 cellular protein levels. Recent studies in MCF10A cells have shown that Lats1, but not Lats2, suppresses YAP function (Zhang et al., 2008a), raising the intriguing possibility that Lats2 function in MCF10A cells is primarily via its phosphorylation of Snail1 and not through inhibition of YAP or TAZ. Moreover the ability of the two Lats kinases to suppress TGFb signals also differs with Lats1 being much more effective (Varelas et al., 2010). The phenotype of Lats1 and Lats2 deficient mice are distinct (McPherson et al., 2004) (Yabuta et al., 2007) (Visser and Yang, 2010), and only for Lats1 has in vivo tumor suppressive activity been observed (St John et al., 1999).

All this has been expanded in the revised discussion section.

2. The authors are careful not to over-interpret their data with respect to its relevance to cancer. However, as the potential capacity of LATS2 to promote EMT during the course of cancer progression is a major interest of this work, some studies are needed to address this more directly. Do carcinoma cell lines with mesenchymal features (Snail expression, low E-cadherin, mesenchymal marker expression) overexpress LATS2? Does knockdown of LATS2 affect Snail1 expression and tumorigenic growth in this context? Is LATS2 overexpressed in poorly differentiated epithelial cancer specimens?

First, despite our best attempts we have not been able to use either a Lats2 or pT203.Snail1 antibody for IHC on human tissues. We cannot establish convincing staining specificity for either. This has been added to the text. But in two different aggressive/metastatic human breast cancer cell lines with EMT features (BT549 and MDA-MB-231) we find that Lats2 protein level is increased, trace level of activated Lats2 is present (pT1041), pT203.Snail1 is present as well as Snail1 is present, compared to MCF10A cells (Figure 8A). Importantly when we shRNAi deplete Snail1 in

MDA-MB-231, invasion/migration in 3D collagen gels is dramatically reduced. ShRNAi depletion of Lats2 in MDA-MB-231 cells also inhibited invasion in 3D collagen I gels. These new data could suggest that Lats2 levels are increased in aggressive breast cancers and that Lats2 affects Snail1 function (invasion/migration) in human breast cancer cell metastasis.

1. *Does Lats1 also regulate Snail?*
   No. See new Figure 3D. See also response to point 6 from referee 1 regarding fish experiments.

2. *Does MG132 restore Snails levels in LATS2 KO MEFs and knockdown cell lines?*
   Yes. See new Figure 2E.

3. *The LATS2 EMT data in MCF10A cells (Fig. 7D) should be shown directly*
   The data are now shown in new Figure 6 and Figure S4

4. *Do LATS2 and Snail co-localize during development.*
   As mentioned above our Lats2 and pT203.Snail1 antibodies are not clean enough for unambiguous interpretation of tissue IHC. This has been added to the text. Nevertheless, by in situ hybridization, the expression patterns both in mouse and fish indicate that Lats2 and Snail1 transcripts do co-localize in several tissues at the appropriate stages. In the fish, both *Lats1 and Lats2* are ubiquitously expressed at early stages and at stages equivalent to embryonic days 7-9 in the mouse, *Lats2,* but not *Lats1,* is expressed in the neural crest, a predominant place of Snail1 expression. In the mouse, *Lats2* co-localizes with *Snail1* in different tissues such as the branchial arches (neural crest derived) and the limb buds. At earlier stages, *Lats1* is mainly expressed in ectodemal tissues while *Lats2,* like *Snail1*, is mainly expressed in mesodermal tissues (McPherson et al, 2004, EMBO J).

2nd Editorial Decision 03 June 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-76841R. It has now been seen again by referees 1 and 2, whose comments are enclosed below. Referee 2 has only minor remaining concerns, but referee 1 still expresses many major concerns with the manuscript, and does not recommend publication. He/she has provided a lengthy review, and while I recognise that some of his/her comments may be somewhat harsh, I agree with much of what he/she has to say. It is therefore clear that we can not offer to publish your manuscript at this point, but since we - and the referee - continue to recognise the potential interest here, we are willing to permit an exceptional second round of major revisions here. I do need to stress that, if you choose to undertake these revisions, there can be no guarantee of a positive outcome, and I understand if you therefore wish to take the manuscript elsewhere at this point - in which case, please get in touch so we can withdraw your submission from our system. However, if you are willing to work on the manuscript further for potential publication here, I would like to summarise what we see to be the critical experimental points from the referee's comments.

- Concerns are raised throughout about the use of multiple cell lines. I do realise that you need to use more than one cell type here - to look at the effects in cells that endogenously express Snail (e.g. HCT116) or that do not without stimulus (e.g. MCF-10A) but it would be important to try and justify and rationalise the use of different cell lines, and to use a single line to confirm all key results.

- Related to this, I agree with the referee that the exclusive use of MEFs to look at the effects of Ras and Hippo pathway on Snail is not ideal. I also agree that your data do not directly show that Ras or Hippo signalling act on Snail via Lats2, and it would be critical to do this. Given the pleiotropic effects of all the Lats2 stimulators you use, detailed analyses to demonstrate that each stimulator has the same effect via the same pathway is essential.

- You provide evidence in figure 2 that silencing of Lats2 in HCT116 cells leads to decreased Snail protein levels. However, in figure 4, you show that - without nocodazole treatment - Lats2 is not phosphorylated (and therefore presumably inactive) in this cell line. It is therefore surprising that its

silencing should have any effect on Snail. This critical issue needs to be resolved: is Lats2 active in these cells, and if not, how do you account for the knock-down phenotype?

A couple of places where I do not fully agree with the referee:
- Point 8 - the referee argues that the T203A mutant is not relevant here. I disagree with this, but I do agree that, in the absence of direct manipulation of Lats2, it is hard to draw strong conclusions from these experiments.
- Point 11 - while I agree that the zebrafish data do not provide biochemical evidence for the stabilisation of Snail by Lats2, I do find these data valuable. There is, however, one critical experiment missing here - which would be to show that the phosphomutant Snail can not rescue the lats2 morphant phenotype.

Regarding the text, I also noted numerous grammatical errors throughout that hinder understanding, and a major re-write of the text is essential.

If you are able to address these concerns, we would be willing to reconsider a further revised version of the manuscript, but as I said above, their can be no guarantee as to the outcome here. If you have any questions or comments about this potential revision, please don't hesitate to get in touch and I am happy to discuss this with you.

With best wishes,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have, no doubt, made an important series of observations. But, the impact of the findings is almost completely undermined by a host of deficiencies. First, what are readers to make of a revised text where grammatical/editorial errors riddle the manuscript? In the Introduction alone, almost every required comma is missing (e.g., "in invasive tumors Snail1 mRNA...", "Commonly during tumor invasion expression of Snail1 and its transcriptional target E-cadherin are reciprocally...") or misplaced ("Snail1 phosphorylation, by GSK3β regulates..."). This oversight alone rendered the text very difficult to read and I found myself inserting commas as I read to make certain that I had the flow pinned down correctly. In other places, the grammar is problematic (e.g., "...the levels of Snail1 protein are decreased and those of E-cadherin increased, a direct target of Snail1 repression." Or "To do so isoform containg a strong nuclear localization sign (NLS-T203E/A.Snail1) were expressed in MCF10A cells.") or the text highly repetitive (e.g., pg. 9-10, you mention that nocodazole induces mitotic dysfunction no few than 3 times). Making the review task all the more difficult, the entire text is riddled with long passages that are bereft of a single reference citation. For example, the entire second paragraph of the Introduction (pg. 3) and the first sentence of the third paragraph have no reference citations - similar problems were noted throughout the Results and Discussion. Of course, these problems alone - though irritating - are not cause for rejecting a given work - especially one of such interest and novelty. However, by emphasizing breadth (from HCT116 and HT-1080 cells to TGFβ-treated MCF10A cells, mouse embryos and zebrafish to fibroblasts, oncogene Ras, nocodazole, Hippo pathway and beyond) over depth (see below), the impact of the observations are, in this Reviewer's opinion, lessened rather than strengthened. In the interest of constructive criticism, I took the time to provide a rather lengthy set of criticisms that might prove helpful in preparing a more compelling story.

1. The screen and the discovery of Lats2 as a Snail1 modulator comprise the most compelling data of the text. However, as before, the statement that "...however, inhibition of GSK3β alone was not equivalent to proteosome inhibition (Fig. 1C), suggesting that cellular pathways or signaling pathways, other than GSK3β, likely also influence Snail1 protein level(s)." remains problematic. Wouldn't this conclusion be altered if you inhibited both GSK3β and GSK3α, and found that Snail1 levels increased to levels similar to those observed with MG132?

2. Pg. 8/Fig. 2. Later in the text, you introduce the notion that Lats2 phosphorylates Snail1 after Lats2 itself has been activated by nocodazole, oncogenic ras or Hippo pathway activation. However, I do not believe that you ever show - or discuss - if i) Lats2 is actually activated in HCT116, HT1080 or MEFs under baseline conditions where Snail1 is apparently stabilized by a Lats2-dependent process or ii) how Lats2 might have been activated in these various cell types. That is, in Fig. 2A and 2D, you show dramatic effects on Sanil1 protein levels by silencing Lats2 expression. As such, one would presume that Lats2 must be present in its activated/phosphorylated state (at least based on the presented story). However, in Fig. 4A/C, neither HCT116 nor MEFs appear to express pT104.Lats2 or pT203.Snail1. While I am certain that the authors can explain these results (i.e., agents such as nocodazole may simply increased the low but functionally active, levels of activated Lats2 found in "resting" cells), these discrepancies make it difficult to evaluate the veracity of the outlined claims, and leave careful readers with an impression of a scattershot, rather than cohesive, "story line". This caveat aside, if activation of Lats2 does play a (patho)physiologically relevant role in regulating Snail1 in cancer cells such as HCT116 or HT1080 cells, we return to the original criticism that the only proven Lats2 "activator" used in these cells is nocodazole. No role for oncogenic Ras or Hippo pathway activation were shown for epithelial cells/carcinomas. Indeed, experiments with Ras or Hippo activation (actually, Hippo activation was never shown nor was the pathway ever targeted - only cell density was altered) were confined to MEFs, a cell type that does not undergo EMT. I understand that the Lats2-Snail1 interactions are operative here, but the "ideal" cell type would be one in which the effect of Ras or Hippo could be evaluated from the perspective of EMT.

3. Pg. 11. No direct evidence has been presented to support the contention that H-Ras stabilizes Snail1 in MEFs via a Lats2-dependent process. For example, your data also show that RasV12 activated pErk1/2, a pathway frequently implicated in EMT-like processes (e.g., Cancer Res 69:3228, 2009; Mol Cell 38:114, 2010). I realize that pT203 is increased here, but from a rigorous perspective, unless you silence Lats2 in the ras-transfected cells, it cannot be claimed that Ras increased Snail1 via the Lats2 pathway. Likewise, in Fig. 5, varying cell density - not Hippo - is shown to affect lats2-Snail1 activity.

4. Fig. 6A. Having shown that "resting" HCT116 cells use Lats2 to stabilize Snail1 (Fig. 2) and that Snail1 and active Lats2 form complexes in both HCT116 and HT1080 cells (Fig. 5C), many readers will wonder why nuclear Lats2 or pT1041.Lats2 are only detected in nocodazole-treated cells. In addition, what is pT203.Snail1 doing in the nucleus in the absence of pT1041.Lats2? Again, I am certain that the authors could present a cogent argument that would explain these results - indeed, they frequently do so in their "Comments to Reviewers" - but the text itself does not display this insight or rigor. The manuscript - as presented - simply leaves readers to "connect the dots" as best they can.

5. Pg. 12/13 and Fig. 6C/D. I have little doubt that the results obtained with T203E will confuse virtually every reader until they reach page 14 and digest the results obtained with the NLS chimera.

6. Fig. 7. Does TGFβ affect Lats2 activation/phosphorylation? Was Snail1 phosphorylated at T203? Also, looking at Fig. 6B, I think it is difficult to convince someone that shLats2 did not almost completely block EMT.

7. Pg. 19, 3rd paragraph. As T203E Snail1 does not access the nuclear compartment, many will question why the MCF10A or MDCK cells were used here. (let alone the need to introduce another cell type).

8. Pg. 15, 2nd paragraph. The key issue here is not whether Snail1 regulates all of these different cell functions (this is already known). The issue is whether Lats2 participates with Snail1 in these processes - a question left unaddressed. Further, as noted above, the T203A.Snail1 mutant has little relevance here and provides no additional insight.

9. Pg. 16. Fig. 8. The data are not properly organized here. First, readers will want to know whether Lats2 stabilizes Snail1 in BT549 or MDA-MB-231 cells. You do have data with regard to Lats2 silencing in MDA-MB-2331 cells (though only a simple shRNA was used here), but this is presented at the end of the paragraph in the Supplementary Data. Further, the effects of Lats2 silencing on E-cad and vimentin expression should be shown prior to introducing the invasion

studies. Further, if you are going to include your zebrafish data where NLS-TE-Snail1 is used, you might show that the construct similarly "rescues" the phenotype observed in the shLats2-transduced cells used in Fig. 8B.

10. Pg. 16. As indicated before, there is no evidence that Snail1 mRNA levels are normal in Lats2-/- embryos. Without these data, no claim can be made with regard to Lats2-dependent Snail1 protein stabilization during early development.

11. I continue to hold that while the zebrafish data are consistent with the working hypothesis, no hard data have been presented to prove that Lats2 stabilizes Snail1 protein/levels in this system.

12. The long discussion of YPA/TAZ, p53 has little to do with the presented results and should be dramatically condensed. The authors would be better advised to focus on what they have actually shown with their data rather than to spend the bulk of their efforts on conjecture.

Referee #2 (Remarks to the Author):

The manuscript has improved after revision. However, there are some minor points which should be modified.
1. page 6, line 3 from the bottom. LiCl treatment is not shown in this manuscript.
2. I believe that "data not shown" and "unpublished data" are not allowed in EMBO Journal. All data should be presented or should not be discussed in the manuscript.
3. I am still concerned that English is not good enough, and I hope that all authors of the manuscript try to improve the writing of the manuscript. I do not think that "comma" is needed before "in vivo" or "in vitro".

2nd Revision - authors' response 05 August 2011

Thank-you for the opportunity to respond to the new set of reviewer's concerns. With respect to general comments raised we have rewritten the manuscript. We have tried to better explain the rationale for changing cell lines for different experiments. HEK293 cells were used for the initial screen and for subsequent biochemical studies confirming Lats2 as a post-translational regulator of Snail1 and to show phosphorylation of Snail1 by Lats2. In these experiments cells were transfected with indicated plasmids. However, HEK293 cells are not a particularly useful epithelial cell line for epithelial cell biology studies. HCT116 and HT1080 were chosen as they express endogenous Snail1. HCT116 cells are an epithelial cancer cell line (colon) that has undergone partial EMT: Snail1 +ve, E-cadherin +ve, Vimentin +ve. HT1080 cells are a mesenchymal cell line (fibrosarcoma). Normal epithelial cells, such as MCF10A, cannot be used for such signaling experiments, as they do not express Snail1 until EMT is induced. Finally MDA-MB-231 cells are an invasive, highly metastatic breast cancer cell lines that has undergone a complete EMT: Snail1 +ve, E-cadherin –ve, Vimentin +ve. Thus MCF10A and MDA-MB-231 cells were chosen for Snail1 cell functional studies: EMT and tumor cell invasion, respectively.

In new experiments we show that a low level of active Lats2 is present in unstimulated cells including HEK293, HCT116, and HT1080. This would explain why RNAi-depletion in these cells affects Snail1 protein level. In new experiments we show that oncogenic stress (H-RasV12 overexpression) of HCT116 cells activates Lats2, phosphorylates Snail1 and increases total cellular Snail1 protein level. It should be noted that parental HCT116 already contain oncogenic mutations in Ras. Finally in new experiments we have we have further mapped the requirement for Lats2 and Snail1 T203 phosphorylation in the regulation of MD-MB-231 tumor cell invasion.

Point-by Point response

Reviewer 1.

The manuscript has been rewritten with an emphasis on presenting a better rationale for the use of different cell lines for different experiments.

1. Possibly, but this doesn't change the fact that we discovered a novel regulator of Snail1 (Lats2) in the screen.

2. By overexposing blots we show that there is a low basal activity of Lats2 in HEK293 (Fig. 2), HCT116 (Fig. 2, 4), HT1080 (Fig. 2), and MEFs (Fig. S4). Mitotic and oncogenic stress (Fig. 4), Hippo pathway (Fig. 5), and TGFb-induced EMT (Fig. 7) activate Lats2 further. This is the first direct demonstration that any of these signals actually activate Lats2 kinase. With the exception of the Hippo pathway, prior studies only showed that total cellular Lats2 protein level changed and translocated into the nucleus in response to mitotic or oncogenic stress.

3. In new experiments we show that oncogenic Ras phosphorylates Snail1 at T203 and increases total cellular Snail1 protein level in a Lats2 dependent manner in HCT116 cells (Fig. 4) and MEFs (Fig. S4).

4. See new experiments detailed above showing low level Lats2 activity in basal cells. The results section has been rewritten.

5. Other reviewers did not have difficulty with the T203E and NLS-T203E mutants.

6. In new experiments we show that TGFb-induced EMT of MCF10A cells results in activation of Lats2 (pT1041.Lats2 antibody), phosphorylation of Snail1 at T203, and stabilization of Snail1 protein (Fig. 7E).

7. As described in the revised and rewritten results section, a central function of Snail1 during development and pathologic processes, such as cancer metastasis, is to induce epithelial to mesenchymal transitions (EMT). The breast epithelial cells MCF10A cells were used as they have been extensively utilized for studies of cellular EMT. MCF10A cells undergo EMT, in response to TGFb1, by activating expression of Snail1. MCF10A cells also undergo TGFb-independent EMT when EMT inducers, such as Snail1, are overexpressed. MCF10A cells do not express Snail1 until induced to undergo EMT. HCT116 colon cancer cells have undergone partial EMT and MDA-MB-231 breast cancer cells have undergone full EMT.

8. This experiment simply tests whether T203 phosphorylation is important for other documented Snail1 cellular functions. As pointed out by the reviewer it did not test whether Lats2 is required for these same functions. We make no claim to this effect in the text. We do show, however, that Lats2 does affect EMT in a Snail1-dependent manner (Fig. 7) and that Snail1 can rescue Lats2 depletion phenotypes in zebrafish (Fig. 9).

9. This set of experiments has been repeated and an expanded use of the MDA-MB-231 to explore the role of T203 phosphorylation in affecting tumor cell invasion through collagen gels has been added. See new Figure 8.

10. This has been added and when normalized to GAPDH mRNA level there was no change in Smail1 mRNA level in E7.5 embryos. This is now shown in new Suppl. Figure 7.

11. We did not assess Snail1 protein level in zebrafish embryo development, in the presence or absence of Lats2. Rather the zebrafish experiments were performed to see if Snail1 and Lats2 morpholinos shared overlapping phenotypes (yes) and whether Snail was genetically downstream of Lats2 (yes – Snail1 could rescue Lats2 MO). In mouse embryos we show that Snail1 protein level is dependent upon the presence or absence of Lats2.

12. The discussion has been condensed. We don't believe that the discussion was largely spent on conjecture. Rather it is our belief that there was extensive discussion about why certain conclusions were reached and their potential implications.

Reviewer 2

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“Data not shown” or “unpublished” has been removed. The manuscript has been extensively rewritten.

Preacceptance Letter 05 September 2011

I'm really sorry for the delay in getting back to you: I thought I had replied to your email last week, but it appears I hadn't - apologies. When I received your R1 version, I wanted to get one of the referees (not the critical referee 1) to take a quick look at it, since you had added additional data, and I wanted this to be seen by an expert. I initially asked referee 3, but he/she never got back to me, and so then I went to referee 2. This explains the delay. I have now heard back from referee 2, whose comments are enclosed below. As you will see, he/she finds the manuscript further improved and acceptable for publication without further changes.

I am therefore pleased to tell you that we can now accept the paper here, without the need for anything else from your side. Many thanks for all your hard work on this study: I think it's improved greatly over the course of the review process, and I'm happy to be publishing it!

REFEREE REPORT

Referee comments:

I have found that the manuscript has been improved. The authors explained the reason for use of different cell types in their experiments. They have also shown that the oncogenic Ras signal, Hippo pathway, and TGF-b-induced EMT activate Lats2. I believe that the manuscript is now acceptable for publication.

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