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SCF (Fbxl17) ubiquitylation of Sufu regulates Hedgehog signaling and medulloblastoma development

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Transaction Report:

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1st Editorial Decision 30 November 2015

Thank you for submitting your manuscript on Sufu regulation by Fbxl17 to The EMBO Journal. We have now received feedback from three expert referees, whose reports are copied below for your information. I am pleased to inform you that all referees consider your findings interesting and potentially important, and therefore in principle suitable for publication in our journal. Nevertheless, they do raise a number of major issues that would need to be satisfactorily addressed before eventual acceptance. As you will see, the majority of these issues, especially in the reports of referees 1 and 3, are of technical nature, referring mainly to the conclusiveness of the biochemical data and knockdown analyses, and so would appear overall straightforward to address.

However, there are also some more significant conceptual concerns that would also need to be taken into account, especially relating to the functional significance of Sufu degradation via Fbxl17. In this respect, it would be important to extend/validate at least some of the cellular assays in physiologically more relevant (cell culture) settings; as well as to better place Fbxl17 into the context of Smo-mediated Hh signaling (see referee 2, as well as ref 3 point 15), ideally by including at least some follow-up investigation on how Fbxl17 may be regulated by Smo activity and/or able to swiftly overcome the inhibitory effect of Sufu phosphorylation.

In this light, I would like to invite you to prepare a revised version of the manuscript, which - pending adequate answering of the discussed issues - we would be happy to consider further for publication in The EMBO Journal.

REFEREE REPORTS

Referee #1:

Through a well established and validated approach, Raducu et al. have identified the HH regulator Sufu as an interactor and a substrate of the SCF ubiquitin ligase subunit Fbxl17. The authors conduct a number of studies to confirm their findings and to also show that a mutation in Sufu identified in patients affected by medulloblastoma in Gorlin syndrome, increases Sufu turnover through Fbxl17-mediated ubiquitylation, leading to enhanced HH pathway activation. The authors also provide a convincing study correlating Fbxl17 expression with the Shh subtype of medulloblastoma.

Overall the work is of novel and of good technical quality, it would have been significantly strengthened had the authors pursued further validation in more relevant systems. There are a number of points that need to be experimentally addressed.
Much of the work is conducted in non-biologically relevant systems, although I acknowledge the inclusion of the one medulloblastoma line.

Overall immunoblotting quantification is hard to evaluate as control bands (e.g. GAPDH) are significantly overexposed. I often insist on the need to develop dilution curves to better quantify changes.

In fig. 1G first point evaluated is 6 hours, so half-life could be significantly shorter in this system
The essential role of Fbxl17 for Sufu ubiquitylation could be limited to the experimental system chosen (HEK-293, fig. 2)
Sufu phosphorylation in control vs. mutant not demonstrated (fig. 3)
Standard (rescue) controls for siRNAs and shRNAs are not being provided
Fbxl17 protein quantification upon silencing is not provided
Impact of Fbxl17 silencing in PTCH1-/- cells was quite modest (fig. 4C).
Standard representation for proliferation curves should be cell growth over time (fig 5C A, B).
Fbxl17 non-targetable cDNA rescue should have been supplied.

Referee #2:

In this paper, the authors describe the identification of the SuFu protein as an interacting partner with the E3 ubiquitin ligase Fbxl17 through LC-MS/MS analysis of proteins immunoprecipitated from HEK293T cells. Following up on this finding, they perform a number of studies to test the hypothesis that Fbxl17 controls SuFu protein levels in response to Hh signaling. They demonstrate that Fbxl17 binds directly to SuFu to promote its ubiquitylation and degradation and that this binding is inhibited by phosphorylation of SuFu and potentiated by Gli1. The data in support of these conclusions look quite good; however, quantitative analysis of the Western blots would be appropriate, along with an indication of the number of replicates performed for each assay.
The authors also investigate the functional consequences of SuFu regulation by Fbxl17; they present evidence that knock down of Fbxl17 abrogates Gli1 transcription factor activity in Ptch1 MEFs, in which the Hh pathway is constitutively activated, as well as in PC3 cells. In addition, they show that the response of medulloblastoma cells to Hh pathway activation can be abrogated by Fbxl17.
The authors suggest that Fbxl17 may play a similar role in regulating SuFu levels to that played by the Fused kinase in Drosophila (though they later suggest on page 15 that Fbxl17 may play an analogous role in Drosophila). However, while it is well established that Fused activity is regulated in response to Smo activation, the authors provide no indication as to whether or how Smo activity might regulate Fbxl17, save for ruling out an effect at the transcriptional level. It is quite possible that Fbxl17 acts passively, binding to SuFu in response to its dephosphorylation - the authors hint at this in the Discussion when they state that "upon Hh ligand binding to Ptch1, PKA and GSK3beta are inhibited" - though they provide no justification of this statement. A better discussion of this important issue is warranted. It is also not clear to me why simply overexpressing Fbxl17 is sufficient to inhibit SuFu - this implies that simply increasing the concentration of Fbxl17 is sufficient to overcome the inhibitory effect of SuFu phosphorylation, but this is could be explicitly investigated.

Referee #3:

Raducu et al., propose that the SCF(Fbxl17) E3 ligase complex targets Sufu for poly-ubiquitination and subsequent degradation in a Hh activation-dependent manner. They describe that phosphorylation events of Sufu regulate the interaction between Fbxl17 and Sufu, and the Fbxl17-mediated Sufu degradation leads to enhancement of Gli transcriptional activity. The authors further demonstrated that depletion of Fbxl17 results in Sufu accumulation, leading to attenuation of medulloblastoma tumor growth. Indeed, Fbxl17 and Gli mRNA expression levels are significantly elevated in clinical samples of the Shh subtype medulloblastoma. This study may provide a molecular link between Sufu alterations and cancer development/progression in various tumors, especially in medulloblastoma. The animal and clinical studies are convincing, however the biochemical data presented are somewhat premature and therefore further analyses should be carried out.

Specific comments:

1. Fig. 1B and 1C: WCL lane needs to be provided in the same panel with IP samples to show the MW of obtained Fbxl17 bands in WCL are equal to the IP bands. In addition, a WB panel of IgG bands needs to be provided to indicate the same amount of IgG were used in control and Fbxl17 IP reactions.

2. Fig 1E and 1G: The effects of Fbxl17 knockdown are not convincing. Adding Hh stimulation may be beneficial to demonstrate a significant Sufu stabilization following Fbxl17 depletion.

3. Fig 1E, 1G, 2A, 4A, 4E, 5A-5C and 6E: Fbxl17 blots should be provided to show relevant Fbxl17 knockdown at protein level. This should be relatively straight forward as the Fbxl17 antibody for WB analysis has been validated in Fig. 1B and 1C.

4. Fig. 1G: It would be helpful to present the data in a graph by quantifying the band intensities of three independent experiments.

5. Fig. 2A: WB panels of input (HA, Fbxl17 and GAPDH blots) should be included.

6. Fig. 2: In vitro ubiquitination assay needs to be performed to prove that SCF(Fbxl17) can directly transfer polyubiquitin chain to Sufu on K257.

7. Fig. 3A: The phosphorylation mimetic S to D mutant should be added in this analysis as performed in Fig. 2B.

8. Fig. 3B: It would be better to include Flag-Gli1 in the assay as performed in Fig. 2A.

9. Fig 3D: It is difficult to interpret the data as the panels are confusing and not clearly or appropriately labeled.

10. Fig 3E: The strong polyubiquitination band, which is observed in the last lane, is not supposed to appear, as Fbxl17 is absent in this lane. Is it a contaminating band derived from polyubiquitinated Gli1 protein? The authors need to exclude this possibility.

11. Fig. 5B: Sufu and Fbxl17 blots need to be provided.

12. Fig. 6: Did the authors examine the frequency of Sufu mutations at the S352, S342 and S346 phosphorylation sites in the cohorts of the 285 medulloblastoma clinical samples?

13. EV3A-3B: Sufu Δ350-425 mutants should be included for the mapping. As authors mention that Fbxl proteins recognize a larger surface rather than a linear degron motif on substrates, it is critical to use a minimal deletion mutant. Extensive deletion might cause mis-folding, leading to a nonspecific interaction.

14. EV3C: S342/346 mutant need to be included as a positive control to set the criteria of what extent of enhancement is significant.

15. The authors propose the failsafe model through which PKA and GSK3beta phosphorylate Sufu to prevent unscheduled Sufu degradation by Fbxl17. However, upon Hh activation, the cells require a swift response to transmit the Hh signals into nucleus. Regarding the dephosphorylation of Sufu, which molecular mechanisms are involved in efficiently releasing the failsafe system?

1st Revision - authors' response 24 March 2016

Please find below our point-by-point answers to referees:

Referee #1:
Through a well established and validated approach, Raducu et al. have identified the HH regulator Sufu as an interactor and a substrate of the SCF ubiquitin ligase subunit Fbxl17. The authors conduct a number of studies to confirm their findings and to also show that a mutation in Sufu identified in patients affected by medulloblastoma in Gorlin syndrome, increases Sufu turnover through Fbxl17-mediated ubiquitylation, leading to enhanced HH pathway activation. The authors also provide a convincing study correlating Fbxl17 expression with the Shh subtype of medulloblastoma.

Overall the work is of novel and of good technical quality, it would have been significantly strengthened had the authors pursued further validation in more relevant systems. There are a number of points that need to be experimentally addressed.

1. Much of the work is conducted in non-biologically relevant systems, although I acknowledge the inclusion of the one medulloblastoma line.

Answer 1:
One of the main limitations in the Hedgehog signaling field is the poor availability of cell lines, which maintain Hedgehog pathway active or in which the pathway can be modulated by treatments with agonists/antagonists. There are controversial opinions about the cell systems suitable for studying molecular regulation of Hedgehog signaling. For this reason, we conducted an extensive validation of pathway activation in DAOY in Fig. EV7. In addition to this, we have confirmed the functional role of Fbxl17-Sufu axis in PC3 cell lines (Fig EV5 and 6), which do not rely on a ligand dependent mechanism of Hedgehog pathway activation but show regulation of Sufu by proteolysis and transcriptional regulation of Gli, in accordance to previous findings (Zhang et al, 2007). Mouse embryonic fibroblasts (MEFs) deficient in either Ptch1 (a major negative regulator of Hedgehog signaling) or Sufu have been extensively used in Hedgehog signaling to assess pathway activation. These have been used in our study in Figure 4 and 7F.

Most importantly, to establish a role for Fbxl17 in SHH medulloblastoma development, we show in Fig EV 10 A, B, C and D that Fbxl17 has an important role in the proliferation of Granule Cell Progenitors, the cells of origin of SHH medulloblastoma (Marino et al, 2000; Oliver et al, 2005; Schuller et al, 2008; Wechsler-Reya & Scott, 1999).

2. Overall immunoblotting quantification is hard to evaluate as control bands (e.g. GAPDH) are significantly overexposed. I often insist on the need to develop dilution curves to better quantify changes.

Answer 2:
We do agree that some of the immunoblots contained oversaturated loading control bands and we apologize for that. To overcome this issue, some of these immunoblots were repeated and new panels containing less exposed loading control bands were included in Fig 1B, 1C, 1E, 2B and 5A. Relative quantifications have been modified accordingly without substantial changes in the significance of findings.

3. In fig. 1G first point evaluated is 6 hours, so half-life could be significantly shorter in this system

Answer 3:
Fbxl17 levels could significantly change in different cell lines (http://www.broadinstitute.org/ccle/home) and affect Sufu half-life accordingly. Thus, it is difficult to make a general statement regarding Sufu half-life. Of note, in the experiments performed in Fig 1G and Fig7E siRNA of Fbxl17 with two different oligos induced a significant increase of Sufu half-life.
4. The essential role of Fbxl17 for Sufu ubiquitylation could be limited to the experimental system chosen (HEK-293, fig. 2)

Answer 4:
We have performed ubiquitylation assay of Sufu in a more relevant cell system (DAOY cells) and this is shown now in Figure 2A. Absent polyubiquitylated species of Sufu upon Fbxl17 depletion reinforce the essential role for Fbxl17 in Sufu ubiquitylation in DAOY medulloblastoma cancer cell lines.

5. Sufu phosphorylation in control vs. mutant not demonstrated (fig. 3)

Answer 5:
Sufu was found to be phosphorylated on S352 in the following previous publication using LC/MS (Hsu et al, 2011). We have performed LC/MS analysis of Sufu secondary modifications in FigEV4A, where it is shown that the corresponding peptide could contain numerous modifications. Furthermore, we raised an antibody against a peptide 347-360 of Sufu containing S352 and T353 phosphorylated. We detected Sufu phosphorylation on Sufu WT but not on a mutant of Sufu where S352 and T353 were substituted to alanine (Fig.3C). This shows that Sufu is phosphorylated *in vivo* on S352/T353.

6. Standard (rescue) controls for siRNAs and shRNAs are not being provided
Fbxl17 protein quantification upon silencing is not provided

Answer 6:
Rescue of cell proliferation and Sufu protein levels is observed after siRNA of Fbxl17 and rescue upon expressing Fbxl17 full length in DAOY cells (Fig 5A, 5C, Fig EV8A and B) and PC3 cells (Fig EV5C-E). Protein levels of Fbxl17 are presented in Fig 5C and Fig EV5D. Due to antibody limitation we measured the extent of Fbxl17 siRNA by QPCR in Fig 5B and E and Fig EV 5F.

7. Impact of Fbxl17 silencing in PTCH1-/- cells was quite modest (fig. 4C).

Answer 7:
Fbxl17 siRNA induces a reduction of Gli1 mRNA, which is highly significant ($p<0.0005$).

8. Standard representation for proliferation curves should be cell growth over time (fig 5C A, B).

Answer 8:
Given the nature of growth of DAOY cells we perform few determinations of cell numbers, which are not well represented using cell growth over time. This representation has been used in previous publications (McKee et al, 2012). The effect of Fbxl17 on cell proliferation has been followed with different methods, which confirm the validity of findings:

1. Measurements of relative cell proliferation
2. The use of an orthotopic rat model of medulloblastoma to monitor cell growth at different time points by MRI scan, which gives accurate determination of tumour volume and size superior to bioluminescence.
3. Using marker of cell proliferation such as Ki67.
4. Using BrdU incorporation in GCPs.

9. Fbxl17 non-targetable cDNA rescue should have been supplied.

Answer 9:
Rescue using cDNA for Fbxl17 is provided in: Fig 5A and C, Fig EV8A and B and in Fig EV5C-E.

Referee #2:

1. In this paper, the authors describe the identification of the SuFu protein as an interacting partner with the E3 ubiquitin ligase Fbxl17 through LC-MS/MS analysis of proteins immunoprecipitated from HEK293T cells. Following up on this finding, they perform a number of studies to test the hypothesis that Fbxl17 controls SuFu protein levels in response to Hh signaling. They demonstrate that Fbxl17 binds directly to SuFu to promote its ubiquitylation and degradation and that this binding is inhibited by phosphorylation of SuFu and potentiated by Gli1. The data in support of these conclusions look quite good; however, quantitative analysis of the Western blots would be appropriate, along with an indication of the number of replicates performed for each assay.

Answer 1:

Quantification of immunoblots was performed for Fig 1E (see Fig EV1A), Fig 1F (see Fig EV1B), Fig 1G (see Fig EV1E), Fig 5C (see Fig EV8A), Fig 5F (see Fig EV8B), Fig 6B (see Fig EV9A), Fig EV 5D (see Fig EV5E) and Fig EV6B (see Fig EV6C). The number of replicates performed for these experiments was added in the corresponding figure legend.

2. The authors also investigate the functional consequences of SuFu regulation by Fbxl17; they present evidence that knock down of Fbxl17 abrogates Gli1 transcription factor activity in Ptch1 MEFs, in which the Hh pathway is constitutively activated, as well as in PC3 cells. In addition, they show that the response of medulloblastoma cells to Hh pathway activation can be abrogated by Fbxl17.

The authors suggest that Fbxl17 may play a similar role in regulating SuFu levels to that played by the Fused kinase in Drosophila (though they later suggest on page 15 that Fbxl17 may play an analogous role in Drosophila). However, while it is well established that Fused activity is regulated in response to Smo activation, the authors provide no indication as to whether or how Smo activity might regulate Fbxl17, save for ruling out an effect at the transcriptional level. It is quite possible that Fbxl17 acts passively, binding to SuFu in response to its dephosphorylation - the authors hint at this in the Discussion when they state that "upon Hh ligand binding to Ptch1, PKA and GSK3beta are inhibited" - though they provide no justification of this statement. A better discussion of this important issue is warranted.

Answer 2:

We have now extended the discussion to clarify this point. We do agree that Fbxl17 acts passively after Sufu dephosphorylation. It has been reported that Sufu phosphorylation promotes its retention within the cilium (Chen et al, 2011) thus establishing a spatio-temporal determinant of Sufu dephosphorylation and ubiquitylation after pathway activation.

However, the regulation of Fbxl17-Sufu axis is likely to be complex and different in the diverse tissue and models analyzed. For instance it is tantalizing to speculate that cells with a functional cilium could also regulate Fbxl17. These studies could not be undertaken in the current work, which focuses on Sufu regulation by Fbxl17.

We don't think that Fbxl17 is playing an analogue role in Drosophila in Sufu degradation since the role of Sufu is substantially different in this model system and Fbxl17 has a little to poor sequence conservation to CG31633 (the postulated homologue). However, the essential role of CG31633 in Drosophila development emphasizes the importance of Fbxl17 during embryogenesis. We hope that our comments will stimulate studies on CG31633, which will clarify its role in this fascinating model system. We have changed the discussion to clarify our statement.

3. It is also not clear to me why simply overexpressing Fbxl17 is sufficient to inhibit SuFu - this implies that simply increasing the concentration of Fbxl17 is sufficient to overcome the inhibitory effect of SuFu phosphorylation, but this is could be explicitly investigated.

Answer 3:

From previous literature it is clear that Sufu dephosphorylation facilitate its relocalization outside the cilium (Chen et al, 2011). Our data are in accordance to a passive model whereby the dephosphorylated fraction of Sufu is polyubiquitylated by Fbxl17. This mechanism is similar but opposite to the regulation of Gli2 and Gli3 operated by SCF^{bTrcp} (Bhatia et al, 2006; Wang & Li, 2006). In both cases proper activation of Hh signaling needs reversal of phosphorylation mediated by PKA and GSK3b. Of note, while dephosphorylation is a central mechanism to proper Hh signaling (Eisner et al, 2015), the players mediating Sufu dephosphorylation are unknown.

Sufu can inhibit Hedgehog signaling also in the absence of cilia (Jia et al, 2009). In cancer cell lines, lacking cilia, a pool of Sufu, which is not phosphorylated could be present, due to constitutive pathway activation. In PC3 and DAOY activating mutation in Hedgehog signaling components could generate a pool of Sufu readily degradable by Fbxl17. This could explain the effect of Fbxl17 overexpression on Sufu levels. Importantly, in MEFs with two copies of *Ptch1*, in which the pathway is inactive, siRNA of Fbxl17 does not induce alteration in Sufu levels.

Referee #3:
Raducu et al., propose that the SCF(Fbxl17) E3 ligase complex targets Sufu for poly-ubiquitination and subsequent degradation in a Hh activation-dependent manner. They describe that phosphorylation events of Sufu regulate the interaction between Fbxl17 and Sufu, and the Fbxl17-mediated Sufu degradation leads to enhancement of Gli transcriptional activity. The authors further demonstrated that depletion of Fbxl17 results in Sufu accumulation, leading to attenuation of medulloblastoma tumor growth. Indeed, Fbxl17 and Gli mRNA expression levels are significantly elevated in clinical samples of the Shh subtype medulloblastoma. This study may provide a molecular link between Sufu alterations and cancer development/progression in various tumors, especially in medulloblastoma. The animal and clinical studies are convincing, however the biochemical data presented are somewhat premature and therefore further analyses should be carried out.

Specific comments:
1. Fig. 1B and 1C: WCL lane needs to be provided in the same panel with IP samples to show the MW of obtained Fbxl17 bands in WCL are equal to the IP bands. In addition, a WB panel of IgG bands needs to be provided to indicate the same amount of IgG were used in control and Fbxl17 IP reactions.

Answer 1:
This has been provided as requested in the new Fig 2B and C. It is important to note that the commercially available antibody that we use recognize Fbxl17 only after enriching its levels by immunoprecipitation of Sufu. As a reference we have immunoprecipitated exogenous Fbxl17 which migrates at the same molecular weight as the endogenous. A panel containing IgG bands was also introduced to indicate the amount of IgG used for each immunoprecipitation.

2. Fig 1E and 1G: The effects of Fbxl17 knockdown are not convincing. Adding Hh stimulation may be beneficial to demonstrate a significant Sufu stabilization following Fbxl17 depletion.

Answer 2:
After Hedgehog stimulation using SAG (Fig 1E, and quantified in Fig EV1A) the effects were more significant and panels have been changed accordingly. We do agree that a proper activation of Hedgehog signaling is a limiting factor for Sufu stabilization upon Fbxl17 depletion. A better effect can be observed in Fig 4A, where Ptch1-depleted cells, with a constitutive Hh signaling activation, were used.

3. Fig 1E, 1G, 2A, 4A, 4E, 5A-5C and 6E: Fbxl17 blots should be provided to show relevant Fbxl17 knockdown at protein level. This should be relatively straight forward as the Fbxl17 antibody for WB analysis has been validated in Fig. 1B and 1C.

Answer 3:
Please note (Fig 1B and C) that the antibody against Fbxl17 did not work on endogenous protein but only after enriching by immunoprecipitation of Sufu. We have performed qPCR of Fbxl17 for the experiments in Fig 1E (see Fig 1D), 1G (see Fig EV1F), 2A and B (see Fig 2C), 4A (see Fig 4B), 4E (see EV 4B), 5A (see Fig 5B), Fig 5D (see Fig 5E), Fig 6B (see Fig 6A), Fig EV5A (see Fig EV5F) and Fig EV6A (see Fig EV 6D). All the qPCR analysis show a drastic reduction of Fbxl17 mRNA in all cases.

4. Fig. 1G: It would be helpful to present the data in a graph by quantifying the band intensities of three independent experiments.

Answer 4:
This has been presented in figure EV1E.

5. Fig. 2A: WB panels of input (HA, Fbxl17 and GAPDH blots) should be included.

Answer 5:
In the new figure 2B, Western blot of Sufu and GAPDH have been included, and also QPCR of Fbxl17 has been reported in Fig 2C.

6. Fig. 2: In vitro ubiquitination assay needs to be performed to prove that SCF(Fbxl17) can directly transfer polyubiquitin chain to Sufu on K257.

Answer 6:
In vitro ubiquitylation assay of Sufu WT and Sufu K257R mutant was introduced in Fig EV2C.

7. Fig. 3A: The phosphorylation mimetic S to D mutant should be added in this analysis as performed in Fig. 2B.

Answer 7:
This has been added in Figure EV3D

8. Fig. 3B: It would be better to include Flag-Gli1 in the assay as performed in Fig. 2A.

Answer 8:
A new western blot has been introduced in Fig EV3E.

9. Fig 3D: It is difficult to interpret the data as the panels are confusing and not clearly or appropriately labeled.

Answer 9:
We apologize for this and have modified the figure for improved clarity.

10. Fig 3E: The strong polyubiquitination band, which is observed in the last lane, is not supposed to appear, as Fbxl17 is absent in this lane. Is it a contaminating band derived from polyubiquitinated Gli1 protein? The authors need to exclude this possibility.

Answer 10:
This band derives from polyubiquitylation of Sufu mediated by endogenous Fbxl17. Sufu polyubiqutylation by endogenous Fbxl17 can also be observed in Fig 2A and B.

11. Fig. 5B: Sufu and Fbxl17 blots need to be provided.

Answer 11:
As we mentioned earlier, due to antibody limitations, levels of endogenous Fbxl17 could not be assessed by Western blot. For Fig 5B (now Fig 5D), qPCR showing downregulation in Fbxl17 mRNA levels has been added in Fig 5E. Also, a representative image of three independent experiments along with the corresponding relative quantification has been added for Sufu protein in Fig EV8A and Fig EV8B.

12. Fig. 6: Did the authors examine the frequency of Sufu mutations at the S352, S342 and S346 phosphorylation sites in the cohorts of the 285 medulloblastoma clinical samples?

Answer 12:
This cohort does not contain information on Sufu mutations.

13. EV3A-3B: Sufu Δ350-425 mutants should be included for the mapping. As authors mention that Fbxl proteins recognize a larger surface rather than a linear degron motif on substrates, it is critical to use a minimal deletion mutant. Extensive deletion might cause mis-folding, leading to a nonspecific interaction.

Answer 13:
We have included the mutant in Figure EV3C, which binds Fbxl17.

14. EV3C: S342/346 mutant need to be included as a positive control to set the criteria of what extent of enhancement is significant.

Answer 14:
Provided in figure EV3E.

15. The authors propose the failsafe model through which PKA and GSK3beta phosphorylate Sufu to prevent unscheduled Sufu degradation by Fbxl17. However, upon Hh activation, the cells require a swift response to transmit the Hh signals into nucleus. Regarding the dephosphorylation of Sufu, which molecular mechanisms are involved in efficiently releasing the failsafe system?

Answer 15:
From previous literature it is clear that Sufu dephosphorylation facilitate its relocalization outside the cilium(Chen et al, 2011). Our data are in accordance to a passive model whereby the dephosphorylated fraction of Sufu is polyubiquitylated by Fbxl17. This mechanism is similar but opposite to the regulation of Gli2 and Gli3 operated by SCF^{bTrcp}. In both cases proper activation of Hh signaling needs reversal of phosphorylation mediated by PKA and GSK3b. Of note, while dephosphorylation is central to proper Hh signaling (Eisner et al, 2015), the mechanisms underlying Sufu dephosphorylation are unknown.

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2nd Editorial Decision 29 April 2016

Thank you for submitting your revised manuscript for our consideration by the EMBO Journal. It has now been seen again by two of the original referee (see comments below). I am please to inform you that both of them are satisfied with the responses and revisions and have no further objections towards publication. We are therefore happy to accept your manuscript in principle at this stage.

REFEREE REPORTS

Referee #2:

The authors have addressed the points raised in my original review adequately.

Referee #3:

The authors have improved the manuscript. This is suitable for publication.

THE EMBO JOURNAL

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

Corresponding Author Name: Vincenzo D'Angiolella
Manuscript Number: EMBOJ-2015-93374

Reporting Checklist For Life Sciences Articles

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript (see link list at top right).

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars only for independent experiments and sample sizes where the application of statistical tests is warranted (error bars should not be shown for technical replicates)
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation (see link list at top right).

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://emboj.embopress.org/authorguide
http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-biosciences

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recomm

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	On previous and similar study. Serres et al 2014 Int J of Cancer
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal work, we based our sample size on a previous and similar study. Serres et al Int J of Cancer.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	Animal studies were randomised as it follows and this order: - 3 animals injected with DAOY cells transfected with shRNA against Fbxl17 - 2 animals injected with DAOY cells transfected with scrambled shRNA - 2 animals injected with DAOY cells transfected with shRNA against Fbxl17 - 3 animals injected with DAOY cells transfected with scrambled shRNA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	The individual doing the measurement did not know the identity of the samples
5. For every figure, are statistical tests justified as appropriate?	Yes, if we compare two unpaired groups we used unpaired t-test. When we have two variables with one independent and one repeated measures factor, two-way ANOVA followed by post hoc t test was used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, normality test was performed (e.g. The Kolmogorov-Smirnov test)
Is there an estimate of variation within each group of data?	Yes using F test
Is the variance similar between the groups that are being statistically compared?	Yes and there are not significantly different

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Please note all antibodies used in the study have been described in the relevant material and method section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Please note all cell lines used in the study have been described in the relevant material and method section. Cell lines are routinely tested for mycoplasma presence using LookOut® mycoplasma qPCR detection kit.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male nude rats (Harlan, France), 3-4 weeks old at start of the study. They were housed in IVC cages in an enriched environment.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures used in this study were approved by the United Kingdom Home Office.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines' (see link list at top right). See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes, our project licence that is approved by the United Kingdom Home Office complies with ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines' (see link list at top right).	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines' (see link list at top right).	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition' (see link list at top right). Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Proteomic data will be deposited in
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section: Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Affymetrix Human Gene 1.1 ST Array profiling of 285 primary medulloblastoma samples (Northcott et al., 2012) was obtained from Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo, accession: GSE37382). Normalized, logged base 2, gene expression determined using Affymetrix Expression Console (1.1) as previously described (Northcott et al., 2012) was considered. One-way ANOVA and Kruskal-Wallis tests were both used to test equality of expression values between groups. Spearman's rank correlation test was used to test gene expression association. Data analysis was performed using R.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	One-way ANOVA and Kruskal-Wallis tests were both used to test equality of expression values between groups. Spearman's rank correlation test was used to test gene expression association. Data analysis was performed using R.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A