EMBO reports

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# Src-dependent autophagic degradation of Ret in FAK-signaling defective cancer cells

Emma Sandilands, Bryan Serrels, Simon Wilkinson and Margaret C Frame

*Corresponding author: Margaret C Frame, University of Edinburgh*

**Review timeline:**

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|---|---|
| Submission date: | 17 December 2011 |
| Editorial Decision: | 10 January 2012 |
| Revision received: | 26 April 2012 |
| Editorial Decision: | 18 May 2012 |
| Revision received: | 31 May 2012 |
| Accepted: | 31 May 2012 |

## Transaction Report:

(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 10 January 2012

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. Referee 1 took part in a structured referee report trial, and you will notice that the format of this report is different from the other two. As you will see, all the referees find the topic of interest and in principle suitable for us, although all of them, and especially referees 2 and 3 have a number of concerns that would need to be addressed in a revised version before publication can be considered in EMBO reports.

I would like to invite you to revise your manuscript, with the understanding that the referee concerns will need to be addressed in full during revision. If they are adequately addressed -including a more thorough characterization of the activation, glycosylation status and isoform of the Ret receptor, the double mutant FAK experiment suggested by referee 1 and a conclusive demonstration that Ret is degraded by autophagy- we would be happy to accept your manuscript for publication. In addition, it would strengthen the message of a more general role for Src in targeting RTKs for degradation if some more information were available on other kinases, as referee 2 mentions in point 3, although this would not be strictly required for publication. Please note that it is EMBO reports policy to undergo only one round of revision and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I have also noted that you manuscript refers to and relies on results shown in your recent Nature Cell Biology numerous times. Although I appreciate this is an extension of that work, it should be an

 

independent, stand-alone study. Referee 3's comments go along the lines of strengthening this set of data, but the text also needs to be modified in this regard. Please also ensure that all figures and supplementary figures legends in which statistics are described include information on the number of independent experiments performed, the type of error bars used and statistical test applied to the data.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

1. Do the contents of this manuscript report a single key finding?

The study is focused and reports a single key finding. The authors show in their manuscript that the Ret tyrosine kinase is sequestred from FAs to autophagosomes in a FAK- and Src kinase-dependent manner. This report represents an extension of a recently published observation from the saem group showing that FAK loss routes Src to authophagosomes where it becomes degraded. This mechanism seems to protect cancer cells of elevated Src actvity and apoptosis.
The authors show in this paper that Src seems to have two functions in their cell model system; one function is through FAK phopshorylation of Y4Y9 and a second is FAK-indepdnent. The latter seems to be required for routing Src and Ret from FAs to authophagosomes. I suggest to more clearly state the two roles of Src in the last, discussion-like paragraph of the paper.

2. Is the main message supported by compelling experimental evidence?

Yes, the main message is supported by state-of the art experiments. They represent an extension of a recently published paper the same group in Nature Cell Biology. Despite this previous paper, the findings reported here are still novel. The paper solves important questions but at the same time raises new ones, such as how does Src-Y4/Y9 regulate Ret binding to FAK, is Src activity needed to maintain Ret in FAs of FAK-Y4E/Y9E expressing cells?
I suggest one more, easy to do experiment. If the findings of the authors are correct a double mutant FAK (Y4E/Y9E and Y397F) should be able to bind Ret and to restore Ret levels in FAK-deficient cells. If true, it could then be tested whether inhibtion of Src is changing this result.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)?

A role for FAK in Src routing into the lysosomes have been shown in a recent paper in Nature Cell Biology published by the same group. The new findings are novel and important.

4. Is the main finding of general interest to molecular biologists? YES

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

 

in EMBO reports

Referee #2:

The manuscript by Sandilands is an interesting follow-up of a recently published paper from the lab (Sandilands et al 2012 NCB). In that paper it was shown that disturbance of the FAK pathway by various means causes active Src to get sequestered in autophagosomes. When this process is inhibited active Src relocalizes to its usual localization in peripheral adhesions and the cancer cells die. The group speculated that this could be a mechanism for a cancer cell to remove activated oncogenes and thereby survive perturbations that could cause cancer cell death.

In the current ms the group has studied the Ret receptor, which had previously been shown by another group to bind FAK leading to a reciprocal transactivation of both kinases. Thus, the problem studied in the current manuscript is what happens to Ret in the SCC model that the authors used in the NCB paper. This is an interesting model in which the cancer cells were originally isolated from a squamous cell tumor in mice that expressed a floxed fak plus a K-14Cre-ER transgene. Treatment of isolated SCC tumor cells with 4-HT causes loss of fak.

The experiments are well performed and presented and, despite the complexity of the system, the paper is well written and generally straightforward to follow. I have only a few specific comments and general suggestions.

1-All the work on Ret was performed with an antibody that seems to recognize only the non-glycosylated form of the receptor. The receptor bands on the western are small (Fig 1a-124kDa in the WT cells plus an 80kDa form present in the FAK null cells). Is the Ret detected by the antibody used in their work only detecting intracellular non-glycosylated Ret? The fully glycosylated Ret is larger (150-170 kDa) and can be activated, as seen my increased P-tyr content, upon ligand treatment. What is the status of the Ret receptor in the SCC model? Is it active? Can it be activated by a ligand?

I assume that the antibody used for the IF is the same as the western since only one Ret ab is mentioned in the methods. From the IF pictures (Fig 1c) they mention Ret is in peripheral adhesions. Are they detecting the full-length glycosylated form in the IF or the non-glycosylated smaller form? Whatever the form is - is it active in the cancer cells? This can easily be checked with available Ret specific P-tyr antibodies that recognize the mouse receptor. They should perform a western analysis on the SCC cells treated with a Ret ligand such as GDNF and also use the Ret P-Tyr ab in the IF assay.
These experiments are important the authors speculate that this autophagy mechanism might represent a "general mechanism that cancer cells use to deal with activated protein kinases" when FAK is missing. Thus it would be important to show whether or not Ret is active in these cancer cells or if it can activated by GDNF?

2- Since the Ret receptor has 3 different known isoforms (Ret9, Ret43 and Ret51) with different possibilities of glycosylation (150/170kDa) and phosphorylation, the use of the alternative antibodies in the western blots might shed more light on which Ret isoform is expressed in these cells.

3- Is this process selective for Ret? Do they have any data on other RTKs that are expressed in the cancer cells? This should be discussed a bit more in the ms.

4- Ret has not been implicated in SCC, but rather in thyroid cancer as a rearranged oncoprotein and in breast where the WT receptor is expressed. Have the authors examined any tyroid or breast cancer cell lines for their sensitivity to a FAK inhibitor and the consequnces on Ret localization?

5- The ms should be read carefully for "typos".

 

Referee #3:

This manuscript reports the autophagic degradation of the Ret tyrosine kinase when focal adhesion kinase (FAK) is lost in cancer cells. The targeting of Ret to the autophagic pathway is dependent on Src kinase activity. This study follows on from a recent publication from the same authors (Sandilands et al 2011 Nat. Cell Biol) showing that autophagy targets Src when the Src/FAK pathway is impaired. The present work is a straightforward demonstration that autophagy is a general mechanism for sequestering activated protein kinases that have been incorrectly located because of the absence of adaptor proteins.

There is still some doubt as to whether the concept proposed by the authors about the role of autophagy in restraining the activity of protein kinases in the absence of adaptors in cancer cells is a general mechanism, because Src and Ret act in the same signaling pathway. Nevertheless, the findings reported in the manuscript are convincing, and the manuscript is well written.
However, several control experiments are lacking that would totally convince this referee that Src and Ret are indeed sequestered and degraded by autophagy in the same pathway.
In order to substantiate the role of autophagy in Ret sequestration the authors need to show that Ret is co-localized with an autophagosomal marker.
The presence of the 80kDa form of Ret and the low, steady-state levels of Ret in FAK-deficient cells are assumed to be a consequence of the targeting of Ret to the autophagic pathway. This needs to be directly demonstrated by blocking autophagy.
In their previous work (Sandilands et al 2011 NCB), the authors showed that Src targeting to the autophagic pathway is mediated by the c-Cbl E3 ligase. Is c-Cbl required for Ret to be targeted to the autophagic pathway?

Other points
The Immuno EM shown in Supplementary Figure 2a does not say anything about the nature of the autophagic vacuole or the co-localization with Src. The authors should either simply remove this picture or provide a more informative figure. A figure showing the co-localization of Ret with endo/lysosomal markers in immunofluorescence images before and after inhibiting autophagy would certainly be more convincing.

1st Revision - authors' response 26 April 2012

**Many thanks for the helpful comments. In response to the general editorial comments, we have removed as many references to our previous paper as possible. We have also added statistical detail to each figure legend, and changed the format of the references to the new EMBO Reports style. Regarding the specific points of the reviewers, we respond as described below:**

**Referee #1:**

**1. Do the contents of this manuscript report a single key finding?**

The study is focused and reports a single key finding. The authors show in their manuscript that the Ret tyrosine kinase is sequestred from FAs to autophagosomes in a FAK- and Src kinase-dependent manner. This report represents an extension of a recently published observation from the saem group showing that FAK loss routes Src to authophagosomes where it becomes degraded. This mechanism seems to protect cancer cells of elevated Src actvity and apoptosis.
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authophagosomes. I suggest to more clearly state the two roles of Src in the last, discussion-like paragraph of the paper.

**We have now added the following statement to page 10: "Hence, we have shown that Src tyrosine kinase has two functions in this process; firstly, normal flux through the Src/FAK pathway (i.e. Src-dependent phosphorylation of FAK) is necessary to keep Ret from being sequestered to autophagosomes; secondly, Src kinase activity has a FAK-independent role that orchestrates not only its own sequestration into autophagosomes, but also the sequestration of other binding partners, as we show here for Ret."**

**2. Is the main message supported by compelling experimental evidence?**

Yes, the main message is supported by state-of the art experiments. They represent an extension of a recently published paper the same group in Nature Cell Biology. Despite this previous paper, the findings reported here are still novel. The paper solves important questions but at the same time raises new ones, such as how does Src-Y4/Y9 regulate Ret binding to FAK, is Src activity needed to maintain Ret in FAs of FAK-Y4E/Y9E expressing cells?
I suggest one more, easy to do experiment. If the findings of the authors are correct a double mutant FAK (Y4E/Y9E and Y397F) should be able to bind Ret and to restore Ret levels in FAK-deficient cells. If true, it could then be tested whether inhibtion of Src is changing this result.

The mutant FAK protein the reviewer suggests (with substitution of six tyrosine residues, five of them charged glutamic acid residues) has perturbed structural/functional properties and so we do not believe results with this complex mutant are simple to interpret. On re-reading, we think that we had not explained clearly enough our interpretation of the experiments with our existing FAK phosphor-acceptor mutant proteins, which is a little complicated. We have now made this much clearer in the text (page 8), and think the results with the existing mutants are conclusive as we describe now in revised text.

**3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)?**

A role for FAK in Src routing into the lysosomes have been shown in a recent paper in Nature Cell Biology published by the same group. The new findings are novel and important.

**4. Is the main finding of general interest to molecular biologists?**
YES

**5. After appropriate revision, would a resubmitted manuscript be most suited for publication:** in EMBO reports

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**The manuscript by Sandilands is an interesting follow-up of a recently published paper from the lab (Sandilands et al 2012 NCB). In that paper it was shown that disturbance of the FAK pathway by various means causes active Src to get sequestered in autophagosomes. When this process is inhibited active Src relocalizes to its usual localization in peripheral adhesions and**

 

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**The experiments are well performed and presented and, despite the complexity of the system, the paper is well written and generally straightforward to follow. I have only a few specific comments and general suggestions.**

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I assume that the antibody used for the IF is the same as the western since only one Ret ab is mentioned in the methods. From the IF pictures (Fig 1c) they mention Ret is in peripheral adhesions. Are they detecting the full-length glycosylated form in the IF or the non-glycosylated smaller form? Whatever the form is - is it active in the cancer cells? This can easily be checked with available Ret specific P-tyr antibodies that recognize the mouse receptor. They should perform a western analysis on the SCC cells treated with a Ret ligand such as GDNF and also use the Ret P-Tyr ab in the IF assay.

These experiments are important the authors speculate that this autophagy mechanism might represent a "general mechanism that cancer cells use to deal with activated protein kinases" when FAK is missing. Thus it would be important to show whether or not Ret is active in these cancer cells or if it can activated by GDNF?

**We have now clarified this. In answer to all of the above comments on Ret status, we have included the following information:**

**1) Anti-Ret H300 (sees both Ret9 and Ret51) recognises a doublet at 150-170kDa from lysates of FAK-expressing and FAK-deficient cells (Supplementary Figure 1b). Upon immunoprecipitation, these Ret species appear not to be bound to FAK.**

**2) Figure 1b now shows immunofluorescence using a phospho-tyrosine-specific Ret antibody, namely PY1062 Ret, which shows that there is indeed activated Ret in cytoplasmic puncta. The immunoblot (Figure 1b, right panel) shows that both Ret (124kDa) and the lower molecular weight form species (80kDa) are both tyrosine phosphorylated. This is consistent with active Ret being degraded. This was confirmed by immunoprecipitation using anti-p-Tyr, probed with anti-Ret (Supplementary Figure 1c). We confirmed that the Ret-selective inhibitor RPI-1 reduces the PY1062 Ret signal (Supplementary Figure 1d).**

 

**3) We have shown that while Ret (124kDa) is modestly up-regulated (around 1.3 fold) in SCC cancer cells used here when compared to their normal keratinocyte counterparts, tyrosine phosphorylation at Y1062 is greatly enhanced (around 50 fold), indicating that Ret is already hugely activated during the development of the cancers from which these cells were derived. At this point we do not know the molecular basis for this apparent activation (subject of on-going sequencing work). However, upon addition of GDNF there was no further activation, confirming that Ret is constitutively activated in these cancer cells. This is discussed in the text (page 5).**

2- Since the Ret receptor has 3 different known isoforms (Ret9, Ret43 and Ret51) with different possibilities of glycosylation (150/170kDa) and phosphorylation, the use of the alternative antibodies in the western blots might shed more light on which Ret isoform is expressed in these cells.

**We show by RT-PCR that Ret9 and Ret51 are expressed in the SCC cells (Supplementary Figure 1a) and, as mentioned above, that there is a protein doublet at 150-170kDa recognised by anti-Ret H300 (Supplementary Figure 1b). Neither is obviously affected by FAK loss, and these forms are not found in complex with FAK in the cancer cells.**

**All of the new information regarding Ret status is discussed in the text, on page 4.**

3- Is this process selective for Ret? Do they have any data on other RTKs that are expressed in the cancer cells? This should be discussed a bit more in the ms.

**We have included the following statement (page 11): More work is needed to establish the full range of tyrosine kinases that are targeted to autophagosomes upon adhesion stress. What we can say is that Src and Ret are highly activated FAK binding partners in SCC cells, and both of these are targeted to the autophagy pathway upon adhesion stress (such as when FAK is deleted or cells detached from matrix). Thus far, we have not found other TKs present in autophagosomes, although the list of those tested has not been exhaustive. A more complete study (beyond the scope here) may permit identification of a range of TK inhibitors that could be of therapeutic value when used in conjunction with autophagy inhibitors.**

**4- Ret has not been implicated in SCC, but rather in thyroid cancer as a rearranged oncoprotein and in breast where the WT receptor is expressed. Have the authors examined any tyroid or breast cancer cell lines for their sensitivity to a FAK inhibitor and the consequnces on Ret localization?**

**In our experience, FAK inhibitors are not always robustly effective at blocking FAK activity in cancer cells in which flux through the Src/FAK pathway is elevated. Hence, we instead chose to examine Ret localisation after induction of adhesion stress in MCF7 breast cancer cells by detachment. Under these conditions, Ret was targeted to cytoplasmic puncta (as we had reported previously for Src in SCC cells upon detachment-induced FAK de-phosphory;ation). We have added this data as Supplementary Figure 1f and added appropriate legends and text (Page 6).**

**5- The ms should be read carefully for "typos".**

**Apologies for the "typos" – hopefully we have proof read these out now.**

**Referee #3:**

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**on Src kinase activity. This study follows on from a recent publication from the same authors (Sandilands et al 2011 Nat. Cell Biol) showing that autophagy targets Src when the Src/FAK pathway is impaired. The present work is a straightforward demonstration that autophagy is a general mechanism for sequestering activated protein kinases that have been incorrectly located because of the absence of adaptor proteins.**

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In order to substantiate the role of autophagy in Ret sequestration the authors need to show that Ret is co-localized with an autophagosomal marker.

The presence of the 80kDa form of Ret and the low, steady-state levels of Ret in FAK-deficient cells are assumed to be a consequence of the targeting of Ret to the autophagic pathway. This needs to be directly demonstrated by blocking autophagy.

**Direct co-staining between LC3B and Ret is unfortunately not possible due to species constraints of the good available antibodies (we cannot get mouse anti-LC3 to work well in IF). We have however been able to make a strong case by showing direct co-staining of active Src and Ret (Figure 2a), and similar direct co-staining between active Src and LC3B (Figure 2b) in quite distinctive cytoplasmic puncta. Moreover, Atg5 and Atg7 siRNA, as well as 3MA, cause loss of Ret-containing puncta (Figure 2c and 2d) and suppression of Ret cleavage product (80kDa) (Supplementary Figure 3c). In addition, Ret is accumulated upon chloroquine treatment (Figure 2e), implicating a requirement for lysosome fusion in Ret turnover. Taken together, these data support the conclusion that active Ret is targeted to the autophagy/lysosomal pathway for turnover.**

**In their previous work (Sandilands et al 2011 NCB), the authors showed that Src targeting to the autophagic pathway is mediated by the c-Cbl E3 ligase. Is c-Cbl required for Ret to be targeted to the autophagic pathway?**

**This is a very interesting question. In response to the reviewer's point, we have addressed this and now provide clear evidence that while c-Cbl is required for Src targeting to the autophagy pathway, it is not similarly required for Ret targeting (Supplementary Figure 4a, b and c). This implies that the biochemical interaction we now show between LC3B and Ret (Supplementary Figure 4d) is mediated by an alternative cargo receptor mechanism. This observation suggests that different protein tyrosine kinases use distinct autophagy cargo receptors after induction of adhesion stress.**

**Other points**
**The Immuno EM shown in Supplementary Figure 2a does not say anything about the nature of the autophagic vacuole or the co-localization with Src. The authors should either simply remove this picture or provide a more informative figure. A figure showing the co-localization of Ret with endo/lysosomal markers in immunofluorescence images before and after inhibiting autophagy would certainly be more convincing.**

 

**The immune-EM image has been removed from the Figures. As mentioned above, we have shown that chloroquine causes Ret accumulation (Figure 2e).**

2nd Editorial Decision 18 May 2012

Thank you for your patience while we have reviewed your revised manuscript. As I mentioned and you will see below, all referees are now supportive of publication in EMBO reports and have no further comments. I am therefore writing an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

I have noticed that, at almost 33,000 characters (excluding the supplementary information) your manuscript somewhat exceeds the length that we can accommodate. I must therefore ask you to go through the text once again with the aim of shortening it in approximately 3,000 characters. Your efforts at shortening might be aided by the fact that, although basic materials and methods essential to the understanding of the experiments must be described in the main body of the manuscript, more detailed explanations necessary to reproduce them may be presented as supplementary information. In addition, when the P values are indicated in the figures, they need not be repeated in the legends (although the legends must maintain descriptions of "n", identity of the error bars and statistical test used).

In addition, our screening software has identified a noticeable amount of textual overlap between some sentences of your manuscript and your recent NCB paper, as well as between the Material and Methods section and both the NCB and your 2010 Curr Biol.

I appreciate that both are your own publications and most overlapping sentences are heavily methodological. Nevertheless, in order to avoid potential future accusations of self-plagiarism by third parties, it would be necessary to rephrase the sentences with most extensive overlap in the introduction and results sections, and reference the mentioned studies in the Material and Methods section while making the descriptions here more succinct. This rewriting would also help your shortening of the text.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Many thanks you for your contribution to EMBO reports.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors responded appropriately to my earlier criticisms. This is a nice and well done paper!

Referee #2:

 

The authors have answered all my questions. I have no further comments and recommend that the revised manuscript be published.

Referee #3:

The authors have responded comprehensively to the reviews. Proper controls for experiments were included to strengthen the authors' data and provide a strong and succinct argument.

2nd Revision - authors' response 31 May 2012

All remaining issues have been resolved in this final version.

3rd Editorial Decision 31 May 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editorial Assistant
EMBO Reports

 